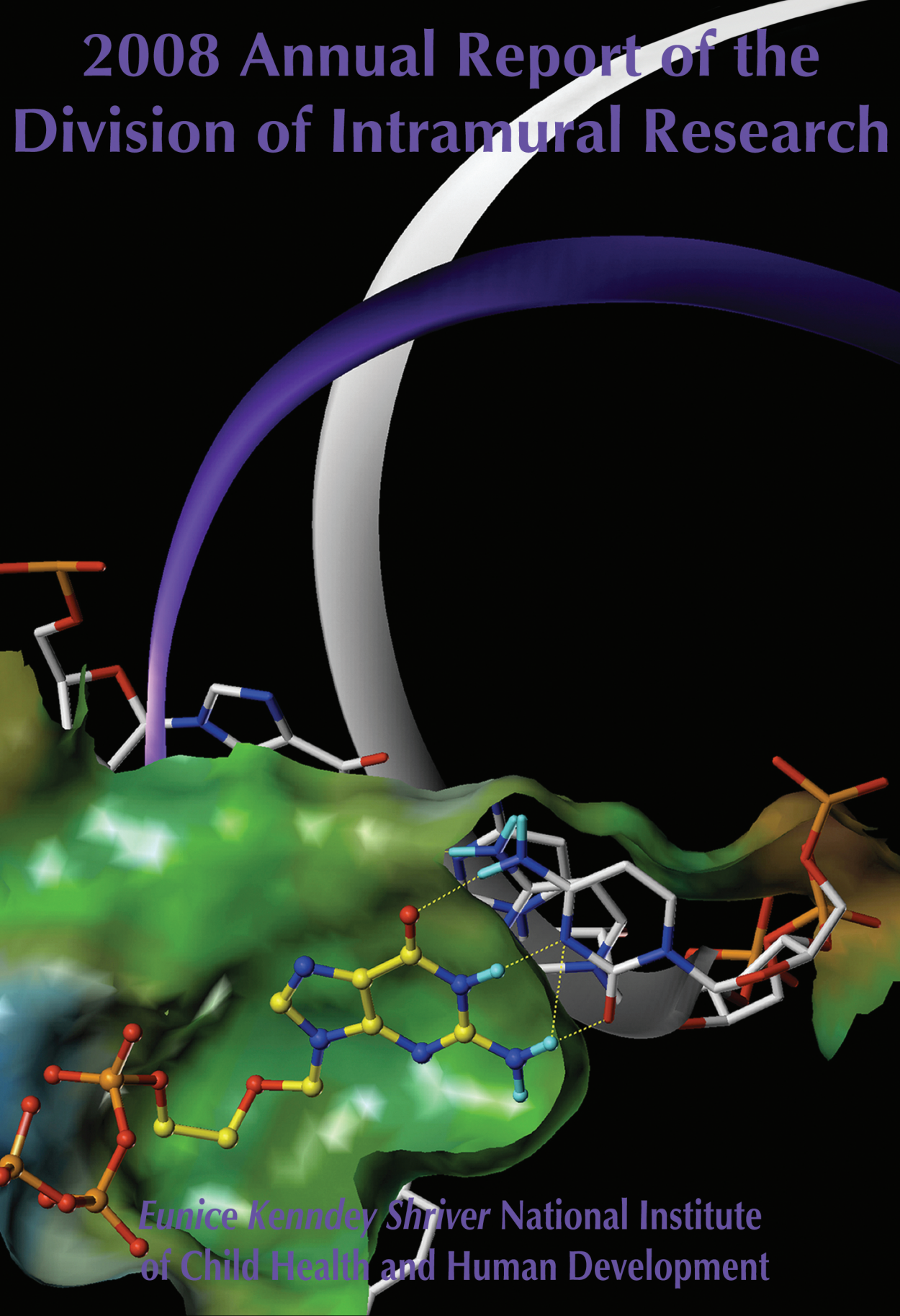
2008 Annual Report of the Division of Intramural Research



Eunice Kennedy Shriver National Institute of Child Health and Human Development

COVER LEGEND

Members of the Section on Intercellular Interactions reported the suppression of HIV replication by acyclovir (ACV) in human tissues co-infected with herpesviruses as a result of the inhibition of HIV reverse transcriptase (RT). The molecular model developed by colleages James Nettles and Raymond Schinazi (Emory University/ VA Medical Center) illustrates ACV triphosphate (foreground, with yellow carbons) interacting (dotted lines) with HIV RNA (white ribbon) in the binding site of HIV-RT (green). ACV triphosphate is positioned for incorporation into HIV viral DNA (purple ribbon) and functions as a chain terminator, as described in this publication. (See p. 341 and the following reference: Lisco A, Vanpouille C, Tchesnokov EP, Grivel J-C, Biancotto A, Brichacek B, Elliott J, Fromentin E, Shattock R, Anton P, Gorelick R, Balzarini J, McGuigan C, Derudas M, Götte M, Schinazi RF, Margolis L. Acyclovir is activated into a HIV-1 reverse transcriptase inhibitor in herpesvirus-infected human tissues. *Cell Host Microbe* 2008;4:260-270.)

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EUNICE KENNEDY SHRIVER NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT

DIVISION OF INTRAMURAL RESEARCH

ANNUAL REPORT

2008

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SCIENTIFIC DIRECTOR'S PREFACE

"Adaptability is an ancestral distinction of human intelligence, but today's instant variations in rhythm call for something stronger: elasticity. The by-product of adaptability and acceleration, elasticity means being able to negotiate change and innovation without letting them interfere excessively with one's own rhythms and goals. It means being able to embrace progress, understanding how to make it our own."

Paola Antonelli, quoted in Longhito S, "The Elastic Mind,"

FASEB Journal 2008;22:3753

Several weeks ago, I attended a lecture by Walter Isaacson in which he discussed two of his books—*Albert Einstein: His Life and Universe* and *Benjamin Franklin: An American Life*. He highlighted parallels between these two giants and lessons that might be learned from them. He stressed their passions to understand the nature of events and their refusal to be inhibited in their search for knowledge by the dogma of the times. What parallels exist now?

The world is in a state of conflict. The once ever-growing economy, which spurred and supported growth in all spheres of activity including science, is contracting. Castles that were built on "margins" are like the proverbial castles built from a deck of cards. Society mandates the need for stricter oversight, ways in which to measure the consequences of investments that allow one to define tangible outcomes as results of industry (in Latin "industria" means diligence, skill, or a department or branch of a craft, art, business, or manufacture). As I review the 2008 research highlights for the NICHD's Division of Intramural Research, I also focus on the outcome of the presidential election and wonder about its impact on biomedical science for the next four years and how our science and its impact will be assessed.

Two months ago, I had the privilege to present a talk to the NICHD Advisory Council, highlighting the nature of our intramural research program, its contributions to the field of biological science, and its special relevance to our mission of furthering women and children's health and reproductive medicine. It underscored the privilege I have enjoyed in serving as the Scientific Director of the Division of Intramural Research of this Institute. As a primary pathway for discovery about human development, health, and an understanding of disease, our research efforts have been organized around the theme of the orchestration of life by molecular messengers. Scientists within the division have made ground-breaking advances such as:

- Co-discovery of the phenomenon of RNA splicing
- Identification of the role of corticotropin-releasing hormone in the hypothalamic-pituitary axis
- Cloning and identification of the major histocompatibility complex (MHC) genes
- Development of conjugate vaccines for *Hemophilus influenzae* type B (HiB), typhoid, shigella, pertussis toxoid, anthrax, and malaria
- Eradication of HiB as a cause of bacterial meningitis and its subsequent causation of mental retardation
- Discovery of the basic mechanisms of excitatory synaptic transmission

- Development of neural tissue culture to study synapse formation
- Elucidation of the molecular basis of cell membrane fusion
- Co-invention of Diffusion Tensor Imaging
- Invention of Laser Capture Microscopy
- Pioneering use of fluorescent probes to study the process and kinetics of cell organelle formation and trafficking

These are but a few of the important consequences and outcomes of research by scientists within our Division of Intramural Research. Our scientists also have made a major commitment to training future scientists and educators. There are 283 postdoctoral and 90 predoctoral trainees in our research laboratories. Graduates from our program hold prominent academic positions in universities and research foundations across the country and around the world.

The successes of our recent and past investigative efforts give us optimism for the future. Research scientists within the Division of Intramural Research exemplify the combination of creativity and convergence of fundamental and applied science: neuro-electrophysiology combined with structural biology, cell biology coupled with technology development, genomic technology coupled with developmental and reproductive endocrinology, genetic methodology applied to behavioral science, and physical biology applied to the study of regulation of cell division and differentiation. We are committed to increasing our understanding of developmental and reproductive science so that we can maintain the health and treat the diseases of women and children.

"People like you and I, though mortal of course like everyone else, do not grow old no matter how long we live. . . . [We] never cease to stand like curious children before the great mystery into which we were born."

Albert Einstein

Owen M. Rennert, M.D.
Scientific Director
Eunice Kennedy Shriver National Institute
of Child Health and Human Development

October 2008

INTRODUCTION

RESEARCH FOCUS OF NICHD'S INTRAMURAL RESEARCH PROGRAMS

1. CELL BIOLOGY AND METABOLISM PROGRAM

Juan S. Bonifacino, PhD, Program Director

The Cell Biology and Metabolism Program (CBMP) conducts studies in various areas of molecular cell biology, including the mechanisms of intracellular protein trafficking and organelle biogenesis, the adaptive responses to environmental stresses, the regulation of the cell cycle during oogenesis, liver cell physiology, and the biology of small non-coding RNAs and small proteins. A salient feature of the CBMP is its outstanding capabilities in state-of-the-art fluorescence microscopy techniques, including the imaging of cells in real time, photobleaching, fluorescence resonance energy transfer, and fluorescence correlation spectroscopy. The CBMP's imaging capabilities have been greatly enhanced with the development of photoactivated localization microscopy (PALM). In addition, the CBMP maintains facilities for working with many model organisms, including bacteria, yeast, *Drosophila,* mice, and mammalian cells. The knowledge gained from the study of basic cellular processes is applied to the elucidation of the causes of human diseases, including disorders of protein trafficking and bile acid secretion as well as neurodegeneration and viral pathogenesis.

The *Unit on Cell Polarity*, headed by **Irwin Arias**, uses live-cell fluorescent imaging, biochemical, genetic, and molecular techniques to study mechanisms responsible for trafficking of proteins selectively to the apical domain of hepatocytes and other polarized cells. The Unit's goal is to identify the components and regulation of these trafficking processes, their role in creating and maintaining cellular polarity, and the molecular defects responsible for inheritable and acquired bile secretory failure (cholestasis).

The *Section on Intracellular Protein Trafficking*, led by **Juan Bonifacino**, has continued its work on the molecular machinery involved in protein sorting to endosomes and lysosomes. Research over the past year has shed light on the mechanisms used by human immunodeficiency virus-1 (HIV-1) to exploit the endocytic and lysosomal targeting machinery of host cells for optimal replication. In particular, the research demonstrated that the Nef protein of HIV-1 downregulates the CD4 co-receptor from the cell surface of host cells through interaction with the adaptor protein-2 (AP-2) complex at the plasma membrane and the multivesicular body machinery on endosomes. Other studies revealed the atomic structure of another sorting complex named retromer and showed that it is recruited to membranes through interaction with the small GTPase Rab7. The Section showed that an additional sorting complex named GARP participates in the sorting of mannose-6-phosphate receptors from endosomes to the Golgi complex.

The *Protein Biogenesis Section*, led by **Ramanujan Hegde**, has continued its investigations into the trafficking of newly synthesized secretory and membrane proteins. Notable advances this past year include the discovery of a new pathway for neurodegeneration caused by mislocalization of prion protein (PrP), identification of a novel interacting partner for mislocalized PrP, and identification of an altered conformation associated with a disease-causing form of PrP. In addition, the Section is studying the pathways of normal PrP trafficking and degradation and how disease-causing mutations in PrP influence such events. The Section continues its progress in identifying additional factors in a novel membrane protein insertion pathway for tail-anchored proteins which was discovered during the previous year.

Headed by **Mary Lilly**, the *Unit on Cell Cycle Regulation* studies the developmental regulation of the cell cycle. The Unit continued its examination of the cell-cycle forces that define developmentally programmed endocycles, a common variant cell cycle in which DNA replication occurs without an intervening mitosis. Highly metabolically active cells, such as *Drosophila* ovarian nurse cells and the giant trophoblast of the mammalian placenta, often grow via endoreplication. The Unit demonstrated that the cyclic activation of the anaphase-promoting complex/cyclosome (APC/C) facilitates the licensing of DNA replication origins and cell-cycle progression during the endocycle. Based on these studies, the Unit formulated and tested a model for the minimum cell-cycle inputs needed to construct a G-/S-phase oscillator. In addition, the Unit has worked to define the pathways that coordinate meiotic progression with gamete differentiation during oogenesis and identified a novel role for components of the nup107-133 nucleoporin complex in the maintenance of the meiotic cycle and oocyte identity in developing ovarian cysts.

Jennifer Lippincott-Schwartz's *Section on Organelle Biology* has continued to investigate new features of numerous cellular processes by using novel fluorescence imaging approaches combined with quantitative analysis and mathematical modeling. Among the areas of investigation are (1) membrane partitioning and its role in protein sorting and transport in the Golgi apparatus, (2) biogenesis and turnover of peroxisomes, (3) mitochondrial morphology and its regulation of cell-cycle progression, (4) control of primary cilia dynamics, (5) intercellular transfer between stem and niche cells, (6) origin of autophagosomes, (7) live-cell photoactivation localization microscopy (PALM) for single-particle tracking, and (8) cytoskeletal and endomembrane cross-talk in three-dimensional polarized cells and developing *Drosophila* syncytial blastoderm embryos.

The *Section on Environmental Gene Regulation*, headed by **Gisela Storz**, studies small, non-coding RNAs in *E. coli*. Many of these bacterial RNAs act analogously to eukaryotic miRNA and siRNAs to regulate mRNA stability and translation. Along with identifying additional non-coding RNAs and characterizing their functions, the Section has helped develop general tools for the study of these regulators. The Section also initiated a project to identify and characterize a category of largely overlooked genes that encode small proteins of less than 50 amino acids, along with their regulatory RNAs. Systematic screens for growth conditions that lead to increased expression and for phenotypes associated with null mutations are yielding insights into the physiological roles of these small proteins.

2. PROGRAM IN CELLULAR REGULATION AND METABOLISM

Alan G. Hinnebusch, PhD, Program Director

The Program in Cellular Regulation and Metabolism brings together two Laboratories whose investigators apply a combination of genetics, biochemistry, and structural biology to elucidate the molecular basis of processes that are fundamentally important in cell biology or animal development. Four of the eight groups in the *Laboratory of Gene Regulation and Development* (LGRD), which is headed by Alan Hinnebusch, study transcriptional and translational control of gene regulation, chromosome condensation and segregation, and the transposition of retroelements in budding or fission yeast. Two LGRD groups employ the fruit fly *Drosophila* to investigate molecular mechanisms in development, including the molecular basis of neuronal connection specificity in the visual system and of cell signaling by TGF-beta factors. The aneuran *Xenopus laevis* serves as a model system for the work of the other two groups, which focus on chromosome segregation and cell-cycle control and transcriptional programming of organ development during metamorphosis. The *Laboratory of Genomic Integrity,* headed by **Roger Woodgate**, studies translesion DNA replication.

Mary Dasso's *Section on Cell Cycle Regulation* uses a variety of metazoan systems, including *Xenopus laevis* egg extracts, to study mitotic roles of the Ran GTPase, the nuclear pore complex

(NPC), and the SUMO family of ubiquitin-like proteins. During the past year, analysis of the Ulp/SENP family of SUMO proteases demonstrated that these enzymes play essential roles at mitotic kinetochores as well as in the process of ribosome biogenesis. The Section also uncovered novel roles for NPC components in kinetochore function and spindle assembly.

The *Section on Protein Biosynthesis*, headed by **Thomas Dever**, is characterizing the structure and function of several translation initiation factors and the molecular principles of kinase-substrate recognition by the stress-responsive eIF2a kinases. The Section recently characterized the dimerization-dependent activation of the dual kinase-endoribonuclease IRE1 that catalyzes the splicing of the mRNA encoding the transcription factor HAC1/Xbp1, a key modulator of ER stress and the unfolded protein response.

The *Section on Nutrient Control of Gene Expression*, headed by **Alan Hinnebusch**, studies the transcriptional and translational control of amino acid–biosynthetic genes. Recently, the Section identified specific residues of 18S rRNA in the P site of the small (40S) ribosomal subunit involved in recruiting initiator tRNA and AUG recognition, implicating a specific amino acid in the large (60S) subunit protein L33 in subunit joining and gene-specific translational repression of *GCN4*. The Section also demonstrated that the a/TIF32 subunit of initiation factor 3 (eIF3) interacts with sequences in *GCN4* mRNA to stimulate re-initiation by post-termination 40S subunits. In addition, members of the Section provided evidence that SUS1, a subunit of transcriptional co-activator (SAGA) and nuclear export (TREX-2) complexes, plays a pivotal role in transcription elongation and mRNA export.

The *Unit on Neuronal Connectivity*, headed by **Chi-Hon Lee**, investigates the structure and development of color-vision circuitry in *Drosophila*. Recently, the Unit identified the neural substrate of spectral preference. The data indicate that Dm8, a specific amacrine neuron subtype, relays signals from several UV-sensing R7 photoreceptors to projection neurons, thus sacrificing spatial resolution for sensitivity. The Unit also investigated the roles of TGF-beta/Activin signaling in regulating synaptogenesis between R7 photoreceptors and their target neurons.

Henry Levin heads the *Section on Eukaryotic Transposable Elements*, which analyzes long term repeat retrotransposons and the integration of their cDNA into the chromosomes of host cells. Recently, the Section demonstrated that the preferential insertion of Tf1 into the 5′ regions of genes results from the recognition of sequences within the promoter. In the case of the *fbp1* promoter, the Section found that integration occurred at the binding site for the transcription factor Atf1p and that the binding of Atf1p was required for integration. In addition, the Section's recent study of Tf1 integrase revealed that the C-terminal chromodomain plays an important role in directing integration to pol II promoters.

The *Unit on Cellular Communication*, headed by **Mihaela Serpe**, uses the *Drosophila* system to investigate molecular mechanisms that regulate cellular signaling during development. The Unit focuses primarily on signaling by TGF-beta factors and on genes that modulate the function and distribution of the signaling ligands in time and space. The Unit's studies have shown that secreted BMP-binding proteins such as Short gastrulation and Crossveinless-2 (Cv-2) have versatile effects on signaling and, depending on context, promote or inhibit signaling. Cv-2 binds to cell surfaces and forms a transient complex with BMP and the type I receptor. Through this complex, Cv-2 at moderate levels stimulates signaling by recruiting BMPs from the extracellular space onto receptors while Cv-2 at higher levels antagonizes signaling by sequestering BMPs.

The *Section on Molecular Morphogenesis*, headed by **Yun-Bo Shi**, studies the gene-regulatory mechanisms controlled by the thyroid hormone receptor (TR) that establish the developmental program of metamorphosis. The Section has demonstrated that TR is necessary and sufficient for metamorphosis; unliganded TR recruits co-repressors to control metamorphic timing, and liganded TR recruits co-activators for metamorphosis progression. The Section has also studied the

roles of TR-regulated matrix metalloproteinases in extracellular matrix remodeling and cell-fate determination during metamorphosis.

Alexander Strunnikov's *Unit on Chromatin Structure and Function* is studying SMC protein complexes, particularly the role of the condensin complex in mitotic chromosome condensation and segregation. Recently, the Unit characterized mitosis-specific sumoylation of condensin in yeast and demonstrated that it cooperates with sumoylated topoisomerases in rDNA and nucleolar chromatin. Using human cells, the Unit analyzed the structure of condensin-defective centromeres and kinetochores biochemically, genetically, and cytologically. The loss of condensin resulted in both depletion of CENP-A and a high incidence of merotelic attachments. The studies indicate that human condensins play a pivotal role in the proper spatial positioning of the kinetochore relative to the microtubule-regulating protein complexes in the inner centromere.

Scientists in the *Laboratory of Genomic Integrity*, headed by **Roger Woodgate**, are studying the mechanisms by which mutations are introduced into damaged DNA. Many of the proteins long implicated in the mutagenic process are now known to be low-fidelity DNA polymerases that can replicate past damaged DNA in a process termed translesion DNA synthesis (TLS). In the past year, experiments aimed at understanding TLS have spanned the evolutionary spectrum and include characterization of DNA polymerase switching in *E. coli*; interactions between archaeal Dpo4 and PCNA; and the in vivo and in vitro characterization of the eukaryotic TLS DNA polymerases eta, iota, and kappa.

3. PROGRAM IN DEVELOPMENTAL AND MOLECULAR IMMUNITY

John B. Robbins, MD; Rachel Schneerson, MD, Joint Program Directors

The Program in Developmental and Molecular Immunity (PDMI) pioneered the development of a new generation of vaccines in which capsular polysaccharides (CP) are chemically bound to immunogenic carrier proteins to form conjugates. Such conjugates, conferring T-cell dependence and booster responses to polysaccharide antigens, have proven successful as exemplified by the *H. influenzae* type b conjugate vaccine such that *H. influenzae* type b meningitis has been virtually eliminated wherever the vaccine has been used. The PDMI's methods have now yielded conjugate vaccines against pneumococci, *Salmonella typhi*, non-typhoidal Salmonella, *Shigella*, *Vibrio cholerae*, anthrax, and malaria.

The Vi CP conjugate was over 90 percent protective against typhoid fever in 2- to 5-year-olds. Found to be protective and of long duration in 2- to 5-year-olds based on IgG anti–Vi levels, Vi-rEPA, administered concurrently with DTP, was evaluated by antibody level measurement in infants, with no serious adverse events observed in any of the infants.

By producing a non-toxic mutant Shigella toxin and conjugating it to the *O*-specific polysaccharide (O-SP) of *E. coli* O157, PDMI researchers produced an investigational vaccine against this pathogen, which causes the often fatal hemolytic uremic syndrome, especially in small children. The vaccine was safe and immunogenic in infants; clinical efficacy trials are planned. Following Phase 1 and Phase 2 studies that showed safety and immunogenicity, a double-blinded randomized, vaccine-controlled Phase 3 evaluation of *Shigella sonnei* and *Shigella flexneri* 2a O-SP–protein conjugates, injected twice into healthy 1- to 4-year-olds in Israel, was completed. No serious adverse reactions related to vaccination occurred. Both the antibody levels and efficacy were age-related. The efficacy of the *S. sonnei* conjugate in the 3- to 4-year-olds was about 70 percent after two years of follow-up, with a similar trend for *S. flexneri* 2a. Accordingly, the PDMI has developed methods to increase the conjugates' immunogenicity by using the KDO residue at the reducing end of the O-SP and oxime chemistry—a method that could have general applicability to lipopolysaccharides (LPS) considered for vaccines.

In an effort to produce an anthrax vaccine with fewer side effects than those associated with the licensed vaccine (AVA), the PDMI, in collaboration with researchers at the NIDDK and NIAID, produced a recombinant Protective Antigen (rPA) of the *Bacillus anthracis* toxin on an industrial scale. In mice, rPA elicited neutralizing antibody levels comparable to those of AVA; in a Phase 1 trial in adults, rPA caused no serious adverse reactions. The spore coat of *B. anthracis* contains a saccharide with anthrose (immunodominant) at its non-reducing terminus. A cross-reactive saccharide has been detected in the CP of the marine bacterium *Shewanella* and in the pili of *Pseudomonas syringae*. The PDMI conjugated *Shewanella* CP to proteins; the conjugate-induced antibodies reacted with *B. anthracis* spores. Such conjugates could provide yet another antibody against *B. anthracis* to be added to the arsenal of immunologic defense against bioterrorism.

Despite high rates of immunization with a safe acellular pertussis vaccine, pertussis continues to occur in infants too young to be fully immunized and in young adults. The PDMI genetically engineered a *Bordetella bronchiseptica* strain to produce a mutant pertussis toxin and devised culture conditions to increase its yield; such a mutant pertussis toxin would replace the licensed, chemically inactivated toxin and other pertussis components that constitute the current vaccine. The PDMI published a method for synthesizing LPS conjugates for prevention of *B. pertussis* and other Bordetellae; the conjugates are undergoing pre-clinical testing.

In other projects, the PDMI is developing micro-methods for measuring lipid components of group B streptococcus (GBS) in order to permit evaluation of the relationship among maternal colonization with GBS, treatment with penicillin, and newborn respiratory distress. To complement its study of autoimmune disease following systemic Group B *Neisseria* meningitis (GBM) infection, the PDMI has coordinated long-term clinical studies in Denmark and Iceland on the effect of maternal GBM infection on pregnancy outcome. Production of clinical lots of the program's GBM polysaccharide and the cross-reacting *Escherichia coli* K92 conjugates is ongoing. Work is also in progress to produce a conjugate vaccine against *Borrelia burgdorferi*, the causative agent of Lyme disease.

4. PROGRAM IN DEVELOPMENTAL ENDOCRINOLOGY AND GENETICS

Constantine A. Stratakis, MD, D(med)Sci, Program Director

The mission of the Program in Developmental Endocrinology and Genetics (PDEG) is to carry out translational research that addresses a wide spectrum of issues that converge in endocrine and genetic disorders, with particular emphasis on disorders of development, growth, and puberty. PDEG investigators have equally diverse backgrounds that range from biochemistry to molecular endocrinology and genetics to clinical obesity research.

Greti Aguilera's *Section on Endocrine Physiology* focuses on the molecular mechanisms of the hypothalamic stress response. Research during the past year has yielded novel information on the transcriptional regulation of corticotropin releasing hormone (CRH) and the physiological actions of vasopressin (VP) during chronic stress adaptation. Contrary to the conventional view, Aguilera found that phosphorylation of the transcription factor CREB is not sufficient for activation of CRH transcription. Concerning the effects of VP, new findings demonstrate novel roles for the prominent increases in vasopressinergic activity during chronic stress. In addition to minor effects modulating pituitary adrenocorticotropic hormone secretion, the Section found that vasopressin mediates mitogenic responses in the pituitary and anti-apoptotic actions in the brain.

Jeffrey Baron's *Section on Growth and Development* investigates the cellular and molecular mechanisms governing longitudinal bone growth in childhood. Baron has developed a novel technique that allows quantitative assessment of gene expression within individual zones of the growth plate. This approach, combined with functional studies, has provided evidence that a bone-morphogenetic signaling gradient across the growth plate provides a key mechanism for chondrocyte

differentiation. Analogous studies have provided insight into the roles of fibroblast growth factor and insulin-like growth factor signaling in the spatial and temporal regulation of growth plate chondrogenesis.

Carolyn Bondy's *Section on Women's Health* primarily studies Turner syndrome and its cardiovascular effects in late age. Pioneering the use of high-resolution magnetic resonance angiography (MRA) in this syndrome, Bondy demonstrated cardiovascular anomalies in about half of study subjects. Whereas congenital heart defects in Turner syndrome were thought to be limited to left-sided, outflow-tract defects, MRA revealed a high prevalence of major venous malformations, including partial anomalous pulmonary venous return and persistent left superior vena cava affecting over 20 percent of the study population. Bondy also described a new aortic abnormality that seems to be predictive of aortic complications in Turner syndrome. Bondy is considered the world's leading expert in adult complications of Turner syndrome.

Kevin Catt's *Section on Hormonal Regulation* investigates the molecular mechanisms of activation, signaling, and function of G protein–coupled receptors, particularly those for angiotensin II (AT_1R and AT_2R) and gonadotropin-releasing hormone (GnRHR), and the receptors' interactions with the epidermal growth factor receptor (EGFR). The Section identified specific and shared signaling pathways and interactions between the AT_1R and EGFR and their dependence on caveolin. In addition, the Section identified Invariant Chain (Ii/CD74) as a novel interacting protein of the AT_1R and as a negative regulator of its expression. Pulsatile neurosecretion by GnRH neurons appears to depend on the neurons' endogenous GIRK channels, activation of which prevents the firing of action potentials and inhibits episodic GnRH release. GPR54, the receptor that mediates kisspeptin-induced activation of GnRH secretion, and GnRHR appear to form heterodimers in GnRH neurons. The Section also investigates the mechanisms by which GnRH activates several G proteins in the GnRH neuron. Current studies on GFP-expressing GnRH neurons address the regulatory roles of endogenous estrogen receptor subtypes in the control of GnRH secretion.

Janice Chou's *Section on Cellular Differentiation* studies glycogen storage disease type I (GSD-I), which is caused by deficiencies in the glucose-6-phosphatase-alpha (G6Pase-alpha)–glucose-6-phosphate transporter (G6PT) complex. The deficiencies result in disturbed glucose homeostasis; GSD-Ib also presents with myeloid dysfunctions. Chou showed that an increase in cellular cholesterol efflux and antioxidant capacity in the sera of GSD-Ia patients may protect against premature atherosclerosis. Using $G6PT^{-/-}$ mice, the Section showed that myeloid dysfunctions in GSD-Ib are intrinsically linked to G6PT deficiency in the bone marrow and neutrophils and that an adenoviral vector–mediated gene transfer improved metabolic and myeloid functions. The Section has recently characterized a G6Pase-alpha isoform, G6Pase-beta, that is expressed ubiquitously, as is G6PT. The Section showed that *G6Pase-beta*–null mice manifest myeloid dysfunctions mimicking GSD-Ib and that G6Pase-beta–deficient neutrophils undergo ER stress and an enhanced rate of apoptosis. The results demonstrate that a functional G6Pase-beta–G6PT complex is critical for neutrophil function, defining a molecular pathway to neutropenia and neutrophil dysfunction of unknown etiology.

Maria Dufau's *Section on Molecular Endocrinology* investigates the molecular basis of hormonal regulation of gonadal function, focusing on (1) modes of transcriptional repression and derepression of receptors for human luteinizing hormone (LHR) and prolactin (PRLR) and (2) the functions of novel short PRLR inhibitory forms, identified by the Section, in physiological regulation and cancer. The Section also investigates mechanisms involved in Leydig cell function and the progression of spermatogenesis. Recent studies revealed cell-specific contributions of phosphatases (PP2A and PP1) in activation of LHR transcription. Release of a phosphatase by changes in chromatin structure favors phosphorylation of Sp1 by PI3K/PKCζ, causing release of p107 inhibitor and transcriptional activation of the LHR. Other studies demonstrated conformational determinants required for the inhibitory action of the PRLR short form on prolactin-induced signaling through the long form.

Studies on the gonadotropin-regulated testicular helicase (GRTH/Ddx25), a protein discovered by this Section and shown to be essential for completion of spermatogenesis, demonstrated the protein's multifunctionality in nuclear transport, storage/degradation of messages, and translation. A missense mutation (Arg242H) of GRTH was found in infertile patients, and its expression revealed lack of the phosphorylated form, which could be relevant to functional aspects of this protein that affect germ cell development and/or function.

David Klein's *Section on Neuroendocrinology* has played a dominant role in the circadian and pineal fields, maintaining a focus on the key regulatory enzyme arylalkylamine *N*-acetyltransferase. Recently, the Section's interests broadened to characterize the pineal transcriptome. Studies use cDNA microarrays to investigate the regulatory cascades that determine cell fate and maintain phenotype, in addition to the cascades dedicated to control of the daily global changes in gene expression. The daily changes involve nearly 1,000 genes, some of which exhibit 100-fold night/day differences in expression. Klein's Section uses a variety of approaches to analyze the cascades, including conditional knockouts and RNAi strategies. An initiative recently undertaken by the Section is characterizing the microRNAs expressed in the pineal gland in order to determine their role in controlling the pineal transcriptome. The Section has initiated collaborations with other PDEG researchers in an effort to extend the PDEG's analysis of melatonin to clinical studies, with the ultimate goal of facilitating the analysis of circadian function.

Anil Mukherjee's *Section on Developmental Genetics* conducts both laboratory and clinical investigations to understand the molecular mechanism(s) of heritable childhood neurodegenerative diseases and inflammatory/autoimmune disorders. Investigations focus primarily on two genes: palmitoyl-protein thioesterase-1, mutation of which causes infantile Batten disease (IBD), and uteroglobin (UG), an anti-inflammatory protein. UG-knockout mice develop IgA-nephropathy, allergic airway inflammation, and tumor susceptibility. During the past year, the Section showed that (1) in PPT1-KO mice, a model for IBD, increased cystolic phospholipase A_2–catalyzed production of lysophosphatidylcholine in the brain mediated microglial recruitment and activation; (2) oxidative and ER stress are common manifestations of both neurodegenerative and non-neurodegenerative storage disorders; and (3) mice lacking UG are highly susceptible to pulmonary fibrosis. The Section is continuing a clinical trial to determine if a combined regimen of Cystagon™ and *N*-acetylcysteine (Mucomyst®) is beneficial for patients with IBD.

Ida Owen's *Section on Genetic Disorders of Drug Metabolism* studies UDP-glucuronosyltransferase (UGT) isozymes that detoxify numerous endogenous and exogenous chemical toxins/mutagens found in our daily diet and environment. Extending its earlier discovery that all UGTs require phosphorylation, the Section determined that UGT1A7 and UGT1A10 phosphorylations are ongoing processes, each mediated by PKCε and PKCα/δ signal transduction, respectively. Owens has shown that the UGT2B family isozymes UGT2B7 and UGT2B15, which detoxify endogenous genotoxic catechol estrogens and dysfunctional prostate–causing dihydrotestosterone (DHT), respectively, require tyrosine phosphorylation, with UGT2B15 also requiring serine/threonine phosphorylation. Src–tyrosine kinase phosphorylation of UGT2B7 is lost in breast tumors; UGT2B7 capacity to detoxify genotoxic catechol estrogens is dependent on active Src in these tissues. The Section found that curcumin reversibly downregulated UGT phosphorylation in antigen-stimulated mice such that, when treated with the immunosuppressant mycophenolic acid (MPA), which is widely used for renal transplant patients, the mice exhibited a substantial increase in both free circulating MPA and immunosuppression. Thus, glucuronidation can markedly compromise drug efficacy while transient downregulation of UGT phosphorylation can have a significant effect on the efficacy of glucuronidatable drugs.

Forbes Porter's *Section on Molecular Dysmorphology* studies a group of human and mouse malformation syndromes attributable to inborn errors of cholesterol synthesis. The most common of

these disorders is the Smith-Lemli-Opitz syndrome (SLOS). The Section studies both basic science and clinical aspects of SLOS, with the goal of developing and testing therapeutic interventions for SLOS. A clinical trial evaluating the safety and efficacy of simvastatin therapy in SLOS has completed enrollment. This past year, the Section received a Bench-to-Bedside award to investigate the role of impaired glycosphingolipid transport in SLOS. The Section also continues to study other inborn errors of cholesterol synthesis and recently described the biochemical and phenotypic consequences of disrupting sterol Δ^{14}-reductase. Over the past year, the Section initiated a clinical study of patients with Niemann-Pick disease, type C (NPC), a neurodegenerative disorder caused by impaired intracellular transport of cholesterol and glycosphingolipids. The protocol is designed to investigate biochemical markers and clinical aspects of NPC that could potentially be used as outcome measures in a future clinical trial.

Constantine Stratakis' *Section on Endocrinology and Genetics* investigates the genetic and molecular mechanisms leading to disorders affecting the adrenal cortex, with emphasis on those that are developmental, hereditary, and associated with adrenal hypoplasia or hyperplasia, multiple tumors, and abnormalities in other endocrine glands (especially the pituitary gland and, to a lesser extent, the thyroid gland). The Section has studied congenital adrenal hypoplasia caused by triple A syndrome; several endocrine deficiencies; familial hyperaldosteronism; adrenocortical and thyroid cancer; pituitary tumors; multiple endocrine neoplasia (MEN) syndromes affecting the pituitary, thyroid, and adrenal glands; and Carney complex (CNC), an autosomal dominant disease. Stratakis and colleagues first identified the regulatory subunit type 1-α (RIα) of protein kinase A (PKA) (the *PRKAR1A* gene), which is mutated in most CNC patients. Most recently, the Section found phosphodiesterase-11A (*PDE11A*) mutations in patients with isolated adrenal hyperplasia and Cushing syndrome. A significant part of the Section's work focuses on cyclic AMP (cAMP)/PKA–stimulated signaling pathways, PKA effects on tumor suppression, and/or development and the cell cycle. The Section's various studies take advantage of *prkar1a* and *pde11a* gene mouse models, in which the respective genes have been knocked out. Genome-wide searches for other genes responsible for CNC and related diseases of the adrenal and pituitary glands are ongoing.

Charles Strott's *Section on Steroid Regulation* explores the molecular mechanisms and biologic implications of substances modified by sulfonation. The Section cloned human and animal sulfotransferase genes to examine gene products, tissue expression, transcriptional regulation, and biological significance.

Jack Yanovski's *Unit on Growth and Obesity* studies metabolic and behavioral factors involved in body weight regulation and body composition during childhood. The Unit is investigating the effects of several genetic mutations in human pediatric obesity and murine models. The Unit has also investigated the role played by brain-derived neurotrophic factor (BDNF) in overweight children. Ongoing studies attempt to identify genetic abnormalities that predispose children to binge eating and related disorders.

5. PROGRAM IN DEVELOPMENTAL NEUROSCIENCE
Chris J. McBain, PhD, Program Director

The Program in Developmental Neuroscience (PDN) takes a comprehensive approach to the study of developmental neuroscience by using techniques of neurophysiology, molecular and cellular biology, crystallography, imaging, and biobehavioral analysis in a variety of animal models as well as in humans and non-human primates. Together, the research focuses on the development, physiology, and pathophysiology of the mammalian central nervous system. Researchers study receptors, ion channels, and signaling mechanisms in preparations that range from isolated proteins and cells to highly ordered neural networks in both physiological and pathophysiological conditions observed in both wild-type and numerous transgenic animals. Basic biobehavioral research aims

to understand cognitive, social-emotional, and biological development in humans and non-human primates. The PDN studies both genetic and environmental factors and their interactions from a comparative perspective in order to characterize the developmental trajectories of individuals across a broad range of species, populations, and settings.

Laboratory of Cellular and Synaptic Neurophysiology

Tamás Balla's *Section on Molecular Signal Transduction* investigates the role of phosphoinositide-derived messengers in mediating the actions of hormones, growth factors, and neurotransmitters in mammalian cells. The Section's studies focus on the cellular functions of phosphatidylinositol 4-kinase (PI4K) enzymes that regulate the first committed step in phosphoinositide synthesis. Over the last year, the Section characterized the developmental defects caused by the elimination of one of these enzymes, PI4KIIIalpha, from zebrafish embryos. The Section found that downregulation of the enzyme resulted in the loss of pectoral fins as a result of a defect in the Fgf signaling cascade. The defect is attributable to the inability of PI 3-kinases to activate their downstream targets because of an insufficient supply of phosphoinositides. The studies are the first to identify the role of a PI4K enzyme in the developmental process of a vertebrate organism.

Using a combination of molecular, cellular, electrophysiological, and behavioral approaches, **Andres Buonanno** and colleagues in the *Section on Molecular Neurobiology* study how experience (activity) modulates neuronal plasticity during development. The Section has focused on the neurotrophic factor Neuregulin-1 and signaling via its receptor ErbB4, recognizing that this signaling pathway uniquely reverses long-term potentiation (LTP) at glutamatergic synapses in an activity-dependent fashion and modulates gamma oscillatory network activity in the hippocampus. The Section recently uncovered a novel, potentially important functional link among NRG-1/ErbB4, dopamine, and glutamate signaling pathways—important because the three pathways are genetically and pharmacologically implicated in cognitive functions and psychiatric disorders.

Dax Hoffman's *Unit on Molecular Neurophysiology and Biophysics* continues to investigate the role of the voltage-gated K^+ channel subunit Kv4.2 in the regulation of dendritic excitability and synaptic integration of CA1 pyramidal neurons of the hippocampus. Over the past year, the Unit demonstrated bidirectional remodeling of CA1 synapses by Kv4.2. Neurons exhibiting enhanced A-type K^+ current showed a reduced synaptic fraction of the NMDA receptor subunit NR2B while genetic reduction of A-currents led to an increased fraction of synaptic NR2B. Bidirectional synaptic remodeling was dependent on spontaneous activation of NMDA receptors and active CaMKII. The data suggest that A-type K^+ channels are an integral part of a synaptic complex that regulates Ca^{2+} signaling through spontaneous NMDAR activation to control synaptic NMDAR expression and plasticity.

Kuo-Ping Huang's *Section on Metabolic Regulation* investigates the signaling mechanisms involved in synaptic plasticity. Using neurogranin (Ng) knockout mice, the Section demonstrated the protein's critical role in the expression of LTP and normal cognitive function. Environmental enrichment and treatment with Ritalin® ameliorated the behavioral impairments of young adult Ng-knockout mice. Immunohistochemical studies demonstrated that Ng localized in neuronal cell bodies and dendrites in the hippocampus, whereas calmodulin (CaM) concentrated in the nucleus. High-frequency stimulation resulted in the co-localization of Ng and CaM in the dendritic spines, which may function as tags for directing the stimulated neuronal network to increase synaptic efficacy.

Peng Loh's *Section on Cellular Neurobiology* explores the mechanisms of intracellular trafficking and secretion of peptide hormones, neuropeptides, and neurotrophins in endocrine cells and neurons as well as the role of the prohormone-processing enzyme carboxypeptidase E in tumorogenesis. Major

accomplishments over the past year include (1) elucidation of the carboxypeptidase E–dependent mechanisms for microtubule-based transport and tethering of BDNF and ACTH vesicles at specific sites on the plasma membrane for exocytosis in hippocampal neurons and pituitary cells and (2) identification of a novel splice isoform of carboxypeptidase E that promotes and predicts metastasis in various human cancers.

Bai Lu's *Section on Neuronal Development* made a discovery regarding BDNF that may have important implications for synaptic plasticity, neurodevelopment, and psychiatric disorders. Brain produces two BDNF mRNAs coding for the same protein: one mRNA with a short and one with a long 3′-untranslated region (3′UTR). The short 3′UTR mRNAs are restricted to neuronal somas, whereas the long 3′UTR mRNAs localize in both soma and dendrites. Truncation of the long 3′UTR in mice decreased the BDNF level only in dendrites. In addition, the mutant exhibited deficits in spine pruning as well as long-term potentiation in the dendrites, but not in the soma, of CA1 pyramidal neurons. The results reveal a novel mechanism for differential regulation of subcellular functions of proteins.

Chris McBain's *Section on Cellular and Synaptic Physiology* investigates the development and regulation of cortical excitability, in particular glutamatergic and GABAergic synaptic transmission and plasticity in hippocampal formation. Work over the past year focused on the differential regulation of transmitter release at functionally divergent presynaptic terminals along a common axon, the roles of ionotropic and metabotropic glutamatergic and cholinergic receptors in controlling cell excitability, and bidirectional synaptic plasticity at both inhibitory and excitatory axon terminals. The Section also investigated the embryonic neurogenesis, migration, and development of specific cohorts of local circuit inhibitory interneurons of the hippocampus.

James Russell's *Section on Cell Biology and Signal Transduction* studies signaling between neurons and glia in the nervous system. To enable direct measurement of glial cell Ca^{2+} signals, the Section developed transgenic mouse lines expressing a fluorescent Ca^{2+} indicator photoprotein in astrocytes and Schwann cells, using the S-100β promoter. The Section uses two-photon microscopy to image (1) labeled cells deep (500 to 700 μm) within brains in the transgenic mice and (2) glial cell signals in isolated brain slice and isolated nerve preparations. In a collaborative study with an NINDS intramural laboratory, the Section is investigating the role of astrocytes in regulating cerebral blood flow and is developing new transgenic lines by using an improved indicator protein together with other cell-specific promoters that target astrocytes, oligodendrocyte progenitor cells, and Schwann cells.

Stanko Stojilkovic and colleagues in the *Section on Cellular Signaling* investigate receptors and channels expressed in pituitary cells. The Section has identified a novel pathway by which dopamine 2 receptors control prolactin release downstream of voltage-gated calcium influx. The Section also showed that hypotonicity induces hormone discharge from readily releasable prolactin-containing vesicles in a "kiss-and-run" mode. In related studies, the Section characterized molecular, pharmacological, and functional properties of GABA-A receptors in normal and immortalized pituitary cells and demonstrated that activation of the receptors results in depolarization of cell membranes and modulation of spontaneous electrical activity. The Section's work on purinergic P2X4 channels cloned from pituitary lactotrophs revealed the helical topology of the two transmembrane regions of the receptor in the activated state.

Mark Stopfer's group, the *Unit on Sensory Coding and Neural Ensembles*, is interested in understanding how brain mechanisms gather and organize sensory information to build transient and sometimes enduring internal representations of an animal's surroundings; the animal actively collects information, which is then processed and dramatically transformed in myriad ways. The Unit's goal is to understand the fundamental mechanisms by which sensory information is collected, transformed, stabilized, and compared as it makes its way through the nervous system. Using relatively simple

animals and focusing primarily on olfaction, the Unit combines electrophysiological, anatomical, genetic, behavioral, computational, and other strategies to examine how fully intact neural circuits, driven by real sensory stimuli, process information.

Laboratory of Cellular and Molecular Neurophysiology

Mark Mayer's *Section on Neurophysiology and Biophysics* investigates ionotropic glutamate receptors (iGluRs)—the molecular pores that mediate signal transmission at the majority of excitatory synapses in the mammalian central nervous system. Given the receptors' essential role in normal brain function and development and increasing evidence that dysfunction of GluR activity mediates several central nervous system diseases and damage during stroke, the Section studies receptor mechanisms by combining atomic-resolution structural data obtained by X-ray diffraction and functional data collected from rapid perfusion techniques and patch clamp recording. The Section recently identified the binding site for allosteric ions and solved structures for NR3 subtype NMDA receptors.

Laboratory of Comparative Ethology

Marc Bornstein's *Child and Family Research Section* investigates dispositional, experiential, and environmental factors that contribute to physical, mental, emotional, and social development in human beings across the first two decades of life course. The Section's overall goals are to describe, analyze, and assess the capabilities and proclivities of developing human beings, including their physiological functioning; perceptual and cognitive abilities; and emotional, social, and interactional styles. At the same time, the Section describes and analyzes the nature and consequences for children and parents of family development and children's exposure to and interactions with their designed and natural surroundings. Project designs are experimental, longitudinal, and cross-sectional as well as intra- and cross-cultural.

John Newman's *Unit on Developmental Neuroethology* studies the mechanisms of primate vocal communication during development. This year, the Unit found that, in the brains of marmosets exposed to infant cries, Fos labeling increased in cells in all components of the limbic system as well as in the superior temporal gyrus (auditory cortex), insular cortex, and ventro-medial frontal cortex. In crying infant marmosets, more cortical cells were positive for Fos labeling at one month than at two months of age.

Stephen Suomi and colleagues in the *Comparative Behavior Genetics Section* conduct broad-based investigations of primate biological and behavioral development through comparative longitudinal studies of rhesus monkeys and other primates. Their primary goals are to characterize various distinctive biobehavioral phenotypes in their rhesus monkey colony, to determine how genetic and environmental factors interact to shape the developmental trajectories of each phenotype, and to assess the long-term behavioral and biological consequences, for monkeys from various genetic backgrounds, of rearing in different physical and social environments. A second major program of research investigates how rhesus monkeys and other non-human primate species born and raised under different laboratory conditions adapt to placement into environments that model specific features of their natural habitat.

6. PROGRAM IN GENOMICS OF DIFFERENTIATION

Igor B.H. Dawid, PhD, Program Director

The Program in Genomics of Differentiation comprises three laboratories. The Laboratory of Mammalian Genes and Development generates gene-altered mice to study embryonic and adult stem cells, pattern formation, T cell development, and genomic imprinting. The Laboratory of Molecular Genetics studies the regulation of gene expression and the genetic control of developmental and physiological processes in model organisms from bacteria to vertebrate animals. The Laboratory of Molecular Growth Regulation

conducts research on the control of cell proliferation, DNA replication, epigenetic gene regulation, regulation of the immune system, gene expression during early embryogenesis, chromatin-mediated gene silencing, and transcription of small RNA–encoding genes.

Laboratory of Mammalian Genes and Development

Using advanced mouse genetics, **Sohyun Ahn** and colleagues in the *Unit on Developmental Neurogenetics* study the molecular and genetic mechanisms by which neural stem cells (NSC) are specified and regulated. They investigate the role of Sonic hedgehog (Shh) signaling and its downstream effectors in the developing and mature nervous system. The Unit uses microarray gene expression analysis to identify genes specific to Shh-responding NSCs in adult mouse brain. The Unit recently generated novel transgenic mouse lines, first, to study how NSC-generated newborn granule neurons in the dentate gyrus of the hippocampus form functional circuits and, second, to understand the elusive role of adult neurogenesis.

Paul Love and the *Section on Cellular and Developmental Biology* focus on T lymphocyte development, particularly signal transduction molecules and pathways that regulate T cell maturation in the thymus. The Section discovered an important difference in the subunit composition and signaling potential of the antigen receptors (TCR) expressed on two distinct classes of T cells. In studying molecules that control T cell migration and trafficking, the researchers found a critical function for the chemokine receptor CCR9 in regulating the migration of developing T cells to and within the thymus. Additional studies have focused on the role of LIM-domain proteins and the partner LIM-domain binding protein in the regulation of T cells.

Karl Pfeifer and the *Section on Genomic Imprinting* examine the regulated expression and biological function of a cluster of imprinted genes on the distal end of mouse chromosome 7. In particular, the Section focuses on the *Igf2/H19* gene cluster, for which it has characterized a 2.4 kb DNA element called the *H19ICR* that is both necessary and sufficient to imprint the locus. The Section recently described the *ICR*'s role in organizing higher-order chromosome structures and long-range DNA interactions that determine the mono-allelic expression patterns of both *Igf2* and *H19*. Current work emphasizes the identification of proteins that interact with the *ICR*. The Section also continues to develop and analyze mouse models carrying ion channel mutations that phenocopy cardiac disorders associated with this gene cluster in humans.

Heiner Westphal and colleagues in the *Section on Mammalian Molecular Genetics* use a loss-of-function approach to study the function of LIM-homeodomain (LIM-HD) proteins and associated co-factors. They investigate the regulation of target gene transcription by oligomeric complexes of LIM-HD, Ldb, and Ssdp proteins and the role of these processes in tissue patterning and organ formation in the mouse embryo, specifically in brain, limb, and heart development. A novel aspect of the work concerns the role of the LIM-HD transcriptional machinery in embryonic stem (ES) cell activation, the reprogramming of somatic cells to an induced pluripotent stem (iPS) cell state, and maintenance and activation of stem cells in the adult organism.

Laboratory of Molecular Genetics

Mike Cashel and the *Section on Molecular Regulation* have described the guanine nucleotide analogue ppGpp as a regulator of RNA synthesis in *E. coli*. Throughout the microbial and plant kingdoms, ppGpp acts as a regulator of global gene expression in response to stress. Regulation by ppGpp does not operate on transcription initiation but rather interacts with secondary channel proteins that non-covalently modify RNA polymerase to alter transcription kinetics. The Section studied new ppGpp regulatory roles in terms of (1) transcription factors GreA and GreB, (2) the small RNA GraL emerging from the GreA leader transcript, (3) an adenine nucleotide analogue (AppppA) with known functions in eukaryotic DNA replication, (4) TraR encoded by the episome governing conjugational recombination, and (5) intermediary metabolism.

Ajay Chitnis and colleagues in the *Section on Neural Developmental Dynamics* study the formation of the nervous system in zebrafish, particularly neuronal versus non-neuronal fate determination and how different regions assume different structural and functional identities. The Section examined proteins interacting with Mindbomb (Mib), a known regulator of Notch signaling, which is required for proper specification of neuronal differentiation. The study revealed that the protein Mosaic eyes (Moe) is a Mib-interacting protein; the Section is now investigating Moe's role in modulating Notch signaling.

Robert Crouch, who leads the *Section on Formation of RNA*, studies RNases H, which are enzymes that degrade RNA in RNA/DNA hybrids. Type I RNase H is structurally and functionally related to an essential RNase H of the HIV/AIDS virus. A collaborative effort determined the structure of the RNase H domain of human RNase H1 in complex with an RNA/DNA hybrid, yielding significant insight into the mechanism of replication of the HIV viral genome. Translation initiation of *Rnaseh1* mRNA occurs from two distinct sites, leading to synthesis of mitochondrial and nuclear isoforms of RNase H1. Studies on type II RNase H, an enzyme consisting of three subunits, provide information on Aicardi-Goutières syndrome, an encephalopathy that mimics in utero viral infection.

Igor Dawid in the *Section on Developmental Biology* studies early development in the frog and zebrafish. In one project, the Section identified a guanine nucleotide exchange factor named WGEF that is a component of the Wnt-PCP signaling pathway involved in the regulation of cell movements in frog gastrulation. Another project identified Lrig3, a transmembrane protein that plays a role in the formation of the neural crest by modulating the Fgf signaling pathway. The Section showed that Lrig3 keeps Fgf signaling within an optimal range required for normal neural crest development.

Judith Kassis, who heads the *Section on Gene Expression,* studies the mechanism of gene silencing by the Polycomb group genes (PcG) in *Drosophila* as well as the nature of DNA elements responsible for such silencing. Several proteins, most notably an Sp1/Klf-type protein and the Pho and Pho-like proteins, are required for functional silencing through sequences called Polycomb Response Elements (PRE). The Section has characterized the sequences responsible for Sp1/KLF binding in a particular PRE. Investigators studied recruitment of the silencing protein complex to the PRE, showing that Pho and Pho-like play largely redundant roles in the recruitment process. In addition, studies analyzed the transcriptional regulation of the *Engrailed* gene and the role played by the *Engrailed* PRE in such regulation.

Jim Kennison, who heads the *Section on Drosophila Gene Regulation*, studies the genomics of pattern differentiation in *Drosophila*. The Section showed that the previously identified *verthandi* gene encodes a subunit of the cohesin complex, which is required for proper segregation of sister chromatids during anaphase. The finding that *verthandi* is required for transcription of homeotic genes suggests that cohesion may also play a fundamental role in developmental gene regulation. The Section has defined *cis*-acting regulatory sequences in the *Sex combs reduced* gene, indicating that, after the end of embryogenesis, two genetic elements about 70 kb apart in the *Sex combs reduced* gene must be in *cis* to maintain proper repression.

Judith Levin and colleagues in the *Section on Viral Gene Regulation* study the molecular aspects of HIV-1 replication and reverse transcription. Nucleocapsid protein (NC) is a nucleic acid chaperone that destabilizes highly structured nucleic acid reverse transcription intermediates. NC's chaperone activity, together with RNase H cleavage, ensures the specificity of plus-strand DNA synthesis. The Section demonstrated that human APOBEC3G, an anti–HIV cytidine deaminase, inhibits all reverse transcriptase–catalyzed DNA elongation reactions. Inhibition is deaminase-independent and is determined by differences among the nucleic acid–binding properties of A3G, NC, and reverse transcriptase. Studies on the HIV-1 capsid protein demonstrated that mutations in the linker region result in abnormal core structure and loss of infectivity.

Tom Sargent and colleagues in the *Section on Vertebrate Development* investigate TFAP2-regulated genes in the cranial neural crest (CNC), focusing on those encoding MyosinX, which is a non-muscle motor protein, and Inca. The Section has shown that both genes are necessary for normal craniofacial development in *Xenopus* and, in the case of Inca, in zebrafish. Inca, a novel protein that promotes actin stress-fiber formation and inhibits microtubule acetylation, is required for gastrulation. Inca inhibits p21-activated kinase 4 (PAK4), which is a Rho-GTPase signaling factor. Under certain circumstances, Inca can regulate signal transduction through the ERK/MAPK. Loss of *MyosinX* expression inhibits CNC cell migration in *Xenopus*. Live confocal imaging revealed co-localization of MyosinX and beta3 integrin and disruption of filopodia in *MyosinX*-knockdown embryos.

Brant Weinstein in the *Section on Vertebrate Organogenesis* studies blood and lymphatic vessel formation during vertebrate embryogenesis. Vessel formation is of intense clinical interest because of the roles played by blood and lymphatic vessels in cancer and ischemia. Using the zebrafish, the Section developed a widely used confocal microangiography method, compiled an atlas of zebrafish vasculature, developed numerous vascular-specific transgenic lines, and pioneered methods for high-resolution in vivo imaging of blood vessels. The Section discovered a novel pathway of artery specification, a role for neuronal guidance factors in vascular patterning, and a mechanism for vascular tube formation in vivo; it also identified the zebrafish's lymphatic vascular system. Current studies use genetic screening and imaging to examine cues directing vascular patterning and morphogenesis, regulation of vascular integrity, and assembly of the lymphatic system.

Robert Weisberg and colleagues in the *Section on Microbial Genetics* explore the control of gene expression in bacteria and bacterial viruses, with a focus on the regulation of transcript elongation. The Section showed that newly synthesized transcripts can control transcriptional pausing by binding to RNA polymerase molecules. Given that control of pausing is an important component of many regulatory networks and permits targeting of individual pauses, the Section's discovery may have widespread application. In a separate project, the Section determined and annotated the genome of bacteriophage B40-8. The host of this phage is *Bacteroides fragilis*, a human commensal bacterium that is frequently pathogenic. Phylogenetic comparisons and experimental data suggest that the distribution of promoters and terminators and the mechanism of translation initiation in this virus do not resemble those of other viruses.

Laboratory of Molecular Growth Regulation

David Clark and colleagues in the *Unit on Chromatin and Gene Expression* study gene activation resulting in the formation of an initiation complex by RNA polymerase II and transcription. These events must occur in the presence of nucleosomes, which are compact structures capable of blocking transcription at every step. To circumvent the chromatin block, eukaryotic cells possess chromatin-remodeling and nucleosome-modifying complexes. The Unit has developed a model system involving native plasmid chromatin purified from the yeast *Saccharomyces cerevisiae* to investigate the role of chromatin structure in gene activation. The studies revealed that activation correlates with large-scale movements of nucleosomes and conformational changes within nucleosomes over entire genes.

Melvin DePamphilis in the *Section on Eukaryotic Gene Regulation* studies the control of gene expression and DNA replication during early mouse embryogenesis, particularly the differentiation of trophoblast stem (TS) cells and ES cells. One project investigates cell-cycle control and the role of the origin recognition complex (ORC) in mammalian cell DNA replication. Orc1 is activated by ubiquitination and phosphorylation during the M- to G1-phase transition. The bromo-adjacent homology (BAH) domain in human Orc1 promotes association of the ORC(2–5) core complex with chromatin and is thus involved in origin recognition. In an additional project, the Section and collaborators showed that the transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development.

A major effort of the *Human Genetics Section*, led by **Bruce Howard**, focuses on higher-order chromatin structure, particularly how defects in the maintenance of such structures (or failures in programmed transitions, especially in the perinatal period) may underlie common developmental disorders and age-related diseases. Random genome sampling, customized search algorithms for comparisons of annotated genomes, and genomics-style high-throughput approaches facilitate the detection and mapping of age-related areas of chromatin remodeling.

Richard Maraia, who heads the *Section on Molecular and Cell Biology*, studies the biogenesis pathways for tRNAs and other small RNAs and how they affect cell proliferation, growth, and development. Efforts focus on transcription termination by RNA polymerase III and the RNA-binding phosphoprotein La. The La protein is an autoantigen found in patients with Sjögren's syndrome, systemic lupus, and neonatal lupus. It functions in tRNA production and in the translation of mRNAs encoding ribosome subunits and growth-related factors. The Section uses genetics, cell and structural biology, and biochemistry techniques in model systems that include yeast, human tissue culture cells, and gene-altered mice.

Keiko Ozato and colleagues in the *Section on Molecular Genetics of Immunity* investigate transcription factors and chromatin binding proteins that control the development of innate immunity. The Section showed that IRF8 drives the development of plasmacytoid dendritic cells (DC) and $CD8\alpha^+$ DCs, subsets that produce type I interferons (IFN) and IL-12. These cytokines confer anti-microbial activities. IRF8 contributes to high IFN production in DCs by amplifying the feedback phase of transcription. The Section also showed that the bromodomain protein Brd4 binds to acetylated chromatin and is implicated in the maintenance of transcriptional memory. Further, Brd4 regulates cell-cycle progression by binding to the promoters of many G1 genes to recruit P-TEFb, a kinase that triggers transcriptional elongation.

7. PROGRAM IN MOLECULAR MEDICINE

Tracey Rouault, MD, Program Director

Researchers in the Program in Molecular Medicine seek to understand mechanisms of disease through analysis of mouse models and studies of human patients. Abnormalities of metal homeostasis are under investigation, including the role of iron misregulation in the pathogenesis of neurodegeneration and refractory anemias and the role of copper transport abnormalities in Menkes disease and related conditions.

Stephen Kaler's *Unit on Pediatric Genetics* investigates the copper transport disorders Menkes disease and its allelic variant, occipital horn syndrome—in particular the effects of early diagnosis and treatment. In an ongoing clinical trial, the Unit evaluates patient materials with cellular and molecular bench methods. This year, the Unit published a study demonstrating that initiation of copper replacement therapy in neonatal Menkes disease patients shortly after birth greatly improves neurological outcomes. In a separate project, the Unit documented skewed X-chromosome inactivation in the family of a female child with PHACES syndrome—a disorder manifesting midline developmental abnormalities. In conjunction with the pronounced female gender bias in this condition, the results suggest inheritance of the syndrome as an X-linked lethal trait. With regard to the platelet glycoprotein adhesion complex GPIbalpha-GPIbbeta-GPIX, the Unit continues its efforts to express and purify the platelet glycoprotein GP1b beta for crystallography and for generating neutralizing antibodies that might modulate the platelet adhesion response in patients at risk for thromboembolic events.

Using mouse models and tissue culture, **Tracey Rouault**'s *Section on Human Iron Metabolism* studies mammalian iron metabolism. Rouault previously identified and characterized two major cytosolic iron-regulatory proteins (IRP). Targeted deletion of each IRP in mice revealed that misregulation of iron metabolism due to loss of IRP2 causes functional iron deficiency, erythropoietic

protoporphyria, anemia, and neurodegeneration. The Section also focuses on mammalian iron-sulfur cluster assembly because of its relevance to IRP1 regulation. Researchers characterized numerous mammalian genes involved in iron-sulfur cluster synthesis and developed in vitro and in vivo methods to assess cluster biogenesis. The Section's discoveries may promote understanding and treatment of neurodegenerative diseases, especially Parkinson's disease and Friedreich ataxia, and hematologic disorders such as refractory anemias and erythropoietic protoporphyria. The Section recently discovered that use of Tempol, a stable nitroxide, prevents neurodegeneration in a mouse model.

8. PROGRAM IN PERINATAL RESEARCH AND OBSTETRICS

Roberto Romero, MD, Program Director

The Program in Perinatal Research and Obstetrics (PPRO) encompasses clinical and laboratory research in maternal and fetal diseases responsible for excessive infant mortality in the United States. It focuses on the mechanisms of disease responsible for premature labor and delivery, with particular emphasis on the role of subclinical intrauterine infection and inflammation. The prenatal diagnosis of congenital anomalies is also a major area of interest.

The NICHD awarded a contract and lease to establish a facility and support the operations of the PPRO in Michigan. The facility, housed at Hutzel Women's Hospital, was delivered to NICHD in February 2005, and work to establish the Program is well advanced. It has access to a patient population with a high rate of pregnancy complications and brings to bear the academic resources of three major universities: Wayne State University, the University of Michigan, and Michigan State University. The PPRO has developed strong collaborative relationships with these universities, which are all part of the Life Science Corridor established by the state of Michigan to attract and support research and development in the biomedical field. The PPRO has been and will continue focusing on development of the physical plan, organization, and recruitment of support staff.

Premature birth is the leading cause of perinatal mortality and morbidity worldwide. The PPRO has defined preterm labor as a syndrome and determined that at least 25 percent of preterm neonates are born to women with subclinical intrauterine infection. The PPRO has also provided evidence that many premature neonates are critically ill before birth and proposed that, in the context of intrauterine infection, the onset of premature labor has survival value.

The PPRO also strives to improve the diagnosis and treatment of fetal disease and congenital anomalies, focusing last year on congenital heart disease, the leading cause of infant death among newborns with congenital anomalies. The PPRO is also exploring sonographic and biochemical indicators of the risk factors for preeclampsia.

9. PROGRAM IN PHYSICAL BIOLOGY

Joshua Zimmerberg, MD, PhD, Program Director

The Program in Physical Biology (PPB) uses systems ranging in complexity from van der Waals interactions to the physics of imaging human tissue to investigate the physicochemical basis of molecular, physiological, and pathological processes and interactions. The PBB develops novel, non-invasive technologies to probe the processes' physical and chemical parameters. The research focuses on the physical chemistry of surface forces, DNA-protein interactions, polymer organic chemistry, membrane biochemistry, pore-forming antibiotics, electrophysiology, cell biology, parasitology, immunology, tissue culture, laser micro-dissection, cancer imaging in vivo, virology, macular degeneration, and HIV pathogenesis. This year's breakthroughs include a new explanation for the stability of the influenza virus in winter and a new anti-viral agent against HIV.

Peter Basser heads the *Section on Tissue Biophysics and Biomimetics*, which strives to understand fundamental relationships between function and structure in soft tissues, in "engineered" tissue

constructs, and in tissue analogues (e.g., polymer gels). The Section produced a method based on anomalous X-ray scattering to measure the ion distribution around charged biopolymer molecules and built a tissue micro-osmometer that permits continuous monitoring of water uptake of small specimens. It also developed an experimental method to map the elastic properties of tissues and cells and designed and constructed calibration standards for MRI imaging. The Section designs and implements novel quantitative in vivo neuroimaging methods to scan children and adults non-invasively, combined with effective strategies for correcting distortion in diffusion-tensor imaging scans of the human brain. The Section elaborates new quantitative MRI methodologies to probe the microstructure and architectural organization of tissue, with an emphasis on the brain, including a method to measure the axon diameter distribution within nerve fascicles. Another MRI-based approach uses concepts from the theory of porous media to characterize features of gray matter microstructure in order to perform Brodmann parcellation of the cerebral cortex.

The *Section on Molecular Transport*, led by **Sergey Bezrukov**, studies channel-facilitated transport of metabolites across cellular and organelle membranes by reconstituting channel-forming proteins into planar phospholipid bilayers. The Section discovered a functional relationship between the cytoskeleton and the voltage-dependent anion channel (VDAC) of the outer membrane of mitochondria. Nanomolar concentrations of dimeric tubulin induced highly voltage-sensitive reversible blockade of mammalian VDAC channels that were reconstituted into planar phospholipid membranes. Experiments with isolated mitochondria confirmed the Section's findings. Tubulin added to isolated mitochondria dramatically decreased the availability of ADP to adenine nucleotide translocase and restored the low permeability of the mitochondrial outer membrane found in permeabilized cells. This newly revealed evidence of critical cytoskeletal involvement in cellular metabolism regulation may shed light on the mechanism of action of a group of anti-tumor drugs, such as paclitaxel.

The *Section on Medical Biophysics*, led by **Robert Bonner**, develops and applies new optical technologies to critical problems in medicine. To map the distribution of several intrinsic photochemicals implicated in health preservation and early disease, the Section is currently developing multispectral, non-invasive clinical retinal autofluorescence imaging with automated image analysis. The Section's broader goals are to quantify earlier stages of local molecular imbalance throughout the retina and to develop reliable means to quantify early disease and the effectiveness of strategies to prevent its progression. The Section is further developing laser capture micro-dissection, which it invented, and its newer variant, expression micro-dissection, to improve integration with multiplex molecular analyses of specific cells and organelles extracted from complex tissue.

Recent studies performed by the *Section on Membrane Biology*, led by **Leonid Chernomordik**, have concentrated on late stages of cell-to-cell fusion and the mechanisms of drug delivery by cationic cell-penetrating peptides. The work on cell fusion initiated by viral protein fusogens has established that expansion of nascent fusion pores into an open lumen of cell-size diameter requires cell metabolism. The actin cytoskeleton restricts rather than drives pore expansion. The work on cell-penetrating peptides has emphasized the importance of endosomal escape as a critical stage in delivery of the peptide into cytosol.

The *Section on Analytical and Functional Biophotonics*, led by **Amir Gandjbakhche**, devises quantitative biophotonics methodologies and associated instrumentation to study biological phenomena at different length scales, from nanoscopic to microscopic. During the past year, the Section (1) began characterizing various types of breast carcinoma with a near-infrared, scanning, time-resolved imaging system, specifically targeting HER-2 receptors; (2) developed theoretical models of photon migration needed to monitor tumor status by pH deep inside tissues; (3) designed an optical camera with liquid crystal retarder for active polarization imaging of biological texture while improving statistical tools to enhance visualization of structures hidden in biological tissue;

(4) developed new algorithms to assess vascularity in AIDS-associated Kaposi's sarcoma; (5) developed numeric models to assess quantitatively the effects of multiple light scattering on two-photon microscopy and fluorescence correlation microscopy; and (6) developed a mathematical model of diffuse optical tomography for brain imaging of war veterans whose heads retain metallic shrapnel.

The goal of the *Section on Intercellular Interactions*, led by **Leonid Margolis**, is to understand the mechanisms of HIV pathogenesis in the context of human tissues, particularly the role of non–HIV-microbes ("co-pathogens") associated with HIV infection. The Section developed a new anti–HIV strategy based on the unique ability of herpesvirus (HHV) kinases to phosphorylate acyclovir (ACV) to yield an inhibitor of herpesviruses DNA polymerases. We showed that HIV-1 reverse transcriptase incorporates ACV-triphosphate into the nascent HIV-1 DNA chain, leading to the complete termination of reverse transcription. Accordingly, HIV-l proliferation was inhibited in human tissues co-infected with HHVs, which are capable of phosphorylating ACV. The results suggest that ACV may be therapeutically beneficial for various HIV-1–infected patients, given that the majority of humans are already infected with various HHVs, including the low-pathogenic HHV-6 and HHV-7. Clinical trials have been launched to test the therapeutic benefits in vivo.

The *Section on Cell Biophysics*, led by **Ralph Nossal**, aims to understand the physical basis for various basic cell processes. During the past year, the Section (1) studied how HIV penetrates cervical mucus; (2) determined the properties of clathrin triskelions in solution, showing that the lowest-energy conformation has an intrinsic pucker but that the triskelion is sufficiently flexible to change conformation when in a polyhedral clathrin cage; (3) used experimental vibro-fluidized granular models of spatially constrained "molecular" mixtures to reveal that the pressure resulting from the presence of spherical crowders (akin to "depletion forces") drives a collection of rod-like objects confined to narrow spaces to form aggregates; (4) identified several molecules that preferentially bind to *Leishmania* tubulin over mammalian tubulin, thus preventing parasite multiplication inside human macrophage cells; and (5) developed an improved technique for the morphological analysis of bacterial biofilms, which reveals new details about biofilm architecture and cellular adhesion.

The *Section on Molecular Biophysics*, led by **Adrian Parsegian**, collaborated on a series of measurements of van der Waals dispersion forces and analyzed the pressures required to pack flexible polymers into confined spaces. The Section aims to derive a quantitative measure of molecule assembly as such assembly drives cellular processes. To see the connections between DNA pressure and ejection, the Section has been growing viruses of different lengths for introduction into the same size capsid and then plan to use X-ray diffraction to follow the packing and pressure of DNA within the capsid. The Section also succeeded in following the condensation of triple-helical DNA, which is implicated in the behavior of telomeres. The response to several divalent ions in various tsDNA constructs revealed differences in packing and liquid crystalline tendencies. The Section also extended the Lifshitz theory of van der Waals interactions in lipid multilamellar systems in order to compute forces between bodies with extended interfaces. Of particular importance is the ability to measure repulsive van der Waals forces in systems in which the properties of the medium are intermediate between those of the interacting bodies, thus creating switchable systems.

The *Section on Macromolecular Recognition and Assembly*, headed by **Donald Rau**, focuses on elucidating the coupling of the forces, structure, and dynamics of biologically important assemblies. Interacting macromolecules tenaciously retain their hydration waters unless their surfaces are complementary. To investigate the role of water in binding, the Section measures and correlates changes in binding energies and hydration that accompany recognition reactions of biologically important macromolecules, particularly sequence-specific DNA-protein complexes. The Section observed a strong correlation between retained water and binding energy. The Section also devised a novel method for measuring one-dimensional sliding rates and applied it to the linear diffusion

of EcoRI, which moves at a rate about one-thousandth of that predicted by simple hydrodynamics, suggesting that DNA-protein charge-charge interactions limit the diffusion rate. DNA-DNA force measurements confirm that arginine and lysine peptides package DNA by employing the same attractive hydration interactions as previously seen for metal ions and the biogenic alkylamines spermidine and spermine.

The *Section on Membrane and Cellular Biophysics*, led by **Joshua Zimmerberg**, studies membrane mechanics, intracellular molecules, membranes, viruses, organelles, and cells in order to understand viral and parasitic infection, exocytosis, and apoptosis. For malaria, the Section studies the mechanisms by which proteases release parasitic cells from erythrocytes, suggesting a protease inhibitor therapy for malaria. Second, the Section discovered that, in enveloped viral disease, lipids forming the envelope of the influenza virus gel at cool temperatures, suggesting a new hypothesis to explain the winter-time occurrence of flu epidemics. Third, the Section found that cholesterol plays double role in viral fusion mediated by the hemagglutinin of influenza: as a lipid-curvature agent that helps hemifusion and as a specific widener of the fusion pore. Fourth, the Section investigates the theoretical effect of protein wetting to determine its role in protein domain formation on cell membranes.

10. PROGRAM IN REPRODUCTIVE AND ADULT ENDOCRINOLOGY

Alan DeCherney, MD, Program Director

The Program in Reproductive and Adult Endocrinology (PRAE) can point to a broad, comprehensive, and exceptional list of publications and activities in the areas of endometriosis, endocrine aspects of disease, both basic and clinical, fibroids, and infertility. The Program has earned recognition as a national leader in the field; in fact, 10 percent of the papers accepted for presentation at a recent annual meeting of the American Society for Reproductive Medicine came from the PRAE.

In the past year, the *Section on Developmental Genomics*, led by **Wai-Yee Chan**, established an expression database of mouse male germ cells and developing embryonic gonads through the use of Serial Analysis of Gene Expression (SAGE) and microarrays; the database revealed the prominent presence of antisense transcripts. Of particular interest is the existence of anti-parallel transcription unit pairs. Mapping the transcriptome of murine male embryonic gonads has allowed the creation of a chromosome heatmap of gonad development that permits researchers to highlight chromosomal "hot spots" with significant gene activities at any time of development; the heatmap will be useful for identifying the importance of different regions at specific time points during the development process and for determining the potential consequences of chromosome duplication or deletion. Related to these studies is a study of the role of luteinizing hormone/choriogonadotropin receptor (hLHR/hCG) in human development and diseases. Studies in cell models uncovered a novel role of hCG in neuronal development. Expression profiling revealed distinct genes and hence physiological changes triggered by two mutations of the same amino acid in hLHR.

Lawrence Nelson's *Unit on Gynecologic Endocrinology* focuses on the pathophysiology and clinical management of spontaneous premature ovarian failure. The Unit demonstrated a highly significant association between circulating adrenal cortex auto-antibodies and histologically confirmed autoimmune oophoritis, which is a mechanism of 46,XX spontaneous premature ovarian failure. Nelson's Unit is also investigating the physiology of the normal ovarian aging process, showing that, of several markers of ovarian aging, age correlated most strongly with follicle-stimulating hormone–stimulated serum inhibin B levels. Investigating the psychological response to the diagnosis of spontaneous premature ovarian failure, the Unit found that women with the condition perceive a need for clinicians to spend more time with them after making the diagnosis and to provide more information about the disorder.

The *Section on Reproductive Medicine*, headed by **Lynnette Nieman,** works on the hypothalamic pituitary adrenal axis in pregnancy, which presents a challenge for detecting disease and assessing

the relative merits of immunometric and radioimmunoassay measurement of ACTH for differential diagnosis of Cushing's syndrome. The Section continues its work on the diagnosis and management of patients with hypercortisolism, recently focusing on ectopic ACTH-secreting tumors and their localization. The Section continues to focus on endometrial physiology and endometriosis pathologies. It has published studies on the endometrium, describing the localization of lesions in women with endometriosis and characterizing the estrogen alpha receptor and use of raloxifene as an adjunctive treatment for endometriosis. The Section found that staining of CD10, a cell-surface metalloendopeptidase present in normal endometrium, is helpful in diagnosing endometriosis.

The *Section on Medical Neuroendocrinology*, under the direction of **Karel Pacak**, continues to focus on neuroendocrine disorders, particularly pheochromocytoma. The Section used micro computed tomography for early diagnosis of ectopic pheochromocytoma tumor in the liver of the nude mouse, a technique that may be applied to the human model and that would be an excellent way to diagnose early small metastases. In addition, members of the Section noted that mutations of the *SDHB* gene are associated with one-half of all malignancies originating from extra-adrenal paragangliomas. The high frequency of SDHB germline mutations among patients with malignant disease, particularly when originating from adrenal paraganglioma, may justify a high priority for SDHB germline-mutation testing in such patients.

The *Section on Clinical Genomics*, led by **Owen Rennert**, applies information gained from biochemical and genomic studies to clinical investigations. Using the database generated by the Section on Developmental Genomics, members of the Section on Clinical Genomics cloned several genes, including the novel *mArd2*, which demonstrates testis specificity and elevated expression in pachytene spermatocytes. Subsequent studies showed that Ard2 has acetyltransferase activity and may be responsible for compensating for the loss of X-linked transacetylation activity during meiosis and post-meiotic differentiation in germ cells. The Section also applied genomic approaches to the study of human diseases, designing two hybridization-based high-throughput methods for screening risk factors for thrombophilia and age-related macular degeneration. The methods will provide a more accurate tool for risk calculation and disease prediction for these two complex disorders.

James Segars and the *Unit on Reproductive Endocrinology and Infertility* addressed disparities in access to reproductive services and treatment. Collaborative work established a complex relationship between the *BRX* gene complex and fibroid generation, and the Unit is expanding its work on several aspects of fibroids, including gene regulation and growth factors. With regard to fertility, the Walter Reed Army Medical Center's Assisted Reproductive Technologies Program continues to provide material for clinical studies. The Oocyte Physician Program, involving investigators at the NIH and the laboratory of Jacques Cohen in Livingston, New Jersey, looked at the effect of leiomyomas on early pregnancy, anti-Müllerin hormone pre- and post-myomectomy, and ovarian reserves in patients with fibroids. A new area of investigation has been the assessment and preservation of ovarian function in women and girls undergoing cancer treatment.

The *Section on Pediatric Endocrinology* continues to investigate the glucocorticoid signaling system and the clinical implications of changes in tissue sensitivity to glucocorticoids. It also serves as the world's referral center for analyzing pathologic mutations of the glucocorticoid receptor (GR). This past year, the Section continued to describe mutations in glucocorticoid-resistant patients and to elucidate the molecular mechanisms of target-tissue glucocorticoid resistance. The Section also discovered that the brain-specific cyclin-dependent kinase 5 (CDK5) that is essential for brain development has implications for the pathogenesis of Alzheimer's disease. It phoshorylates the GR at several serine residues and regulates its transcriptional activity in a target gene–specific fashion. The Section continued to examine the biologic activity of 3-*O*-formyl-20*R*,21-epoxyresibufogenin and 3-*O*-formyl-20*S*,21-epoxyresibufogenin, known isolates from the Chinese toad skin extract

drug Ch'an Su, and discovered that these compounds act as inhibitors of IL-6–type cytokine action by attenuating the function of the common receptor subunit gp130. The results suggest that this class of compounds may be employed in the treatment of disorders in which excessive circulation/production/action of IL-6 type–cytokines play important pathologic roles.

11. BONE AND EXTRACELLULAR MATRIX BRANCH

Joan C. Marini, MD, PhD, Chief

Several years ago, the Bone and Extracellular Matrix Branch (BEMB) made a series of discoveries that shifted the paradigm for collagen-related bone disorders—identifying the genes for recessive osteogenesis imperfecta (OI) that encode proteins that modify type I collagen. Forming a complex in the endoplasmic reticulum (ER), the two proteins CRTAP and P3H1 3-hydroxylate a single proline residue (Pro986) on the α1(I) chain. Null mutations in either *CRTAP* or *P3H1* cause deficiency of their respective transcripts and protein, lack of 3-hydroxylation of type I collagen, and delay in folding of the collagen helix, causing overmodification of the entire alpha chains by lysyl hydroxylase and prolyl 4-hydroxylase. Clinically, OI patients display severe or lethal osteochondrodystrophy, which has been designated as type VII (CRTAP deficiency) or type VIII (P3H1 deficiency) OI. For type VIII OI, the BEMB has been investigating an unusual recessive founder mutation in LEPRE1: IVS5+G→T. The mutation occurs only in African Americans and contemporary West Africans; homozygosity for the mutation is lethal in infancy. The BEMB studied the molecular anthropology of the mutation. The carrier frequency among African Americans in Pennsylvania, Maryland, and the District of Columbia is 1 in 200 to 300 individuals. Among contemporary West Africans, the BEMB found a carrier frequency of over 1 percent. This very high incidence predicts that the rate of recessive OI in West Africa will equal the incidence of dominant OI. BEMB researchers are currently investigating the mechanism that would maintain such an unusually high carrier frequency for a lethal disorder and the age of the founder mutation, which presumably reached the Americas via the Atlantic slave trade.

BEMB researchers generated the Brtl mouse, a non-lethal knockin model for OI. Recent investigations into the cellular aspects of Brtl have yielded novel insights into OI pathology. Brtl osteoblast matrix production decreases to half of control values after puberty, but osteoclasts remain significantly elevated. The RANKL/OPG signaling pathway was shown not to be involved in this cellular uncoupling. Surprisingly, $TRAP^+$ precursors are markedly elevated in Brtl and form both more and larger, intensely staining osteoclasts. These exciting data suggest that OI pathology may be attributable to combined deficiency of bone formation and increased osteoclasts triggered by an immune response to abnormal bone matrix.

The BEMB continues to lead the Consortium for Mutations in Osteogenesis Imperfecta, an international group of eight laboratories. Genotype-phenotype analyses identified different patterns for the alpha1 and alpha2 chains. The Consortium's database supports the Regional Model previously proposed by the BEMB for α2(I) mutations, in which eight regularly spaced clusters of lethal mutations coincide with binding sites for matrix proteoglycans on the collagen fibril. A collaboration with the laboratory of Sergey Leikin at NICHD defined discrete structural regions along the α1(I) chain. The BEMB also collaborated in efforts to map (1) major and minor osteonectin binding sites on collagen and (2) functional interaction domains on the collagen fibril, supporting an important role for the interaction of integrin and the cell interaction domain.

The BEMB's clinical investigations continue to play an important role in the diagnosis and treatment of OI. Previous controlled trials with the bisphosphonate pamidronate demonstrated both beneficial and detrimental effects on Brtl mice and children with OI. Children with types III and IV OI experienced significant increases in vertebral bone mineral density z-scores, mid-vertebral height, and vertebral area after 1 to 2 years of treatment. However, treated patients did not experience

improved ambulation level, lower-extremity strength, or amelioration of pain, which appear to have been placebo effects in uncontrolled trials. The BEBM is currently engaged in a dose comparison trial, working from the hypothesis that a reduced cumulative dose of bisphosphonate can result in the same improvements as the higher dose with lower detrimental effects. The BEMB also completed a retrospective radiographic study demonstrating that popcorn calcifications occur in both dominant OI caused by mutations in collagen chains and in recessive OI.

WORKSHOPS AND CONFERENCES ORGANIZED BY NICHD INTRAMURAL RESEARCH PROGRAM STAFF IN 2008

Irwin Arias organized, chaired, and participated in the 18th Annual Irwin M. Arias MD Symposium entitled "Bridging Basic Science and Liver Disease," sponsored by the American Liver Foundation. The one-day symposium was held in Boston on October 29, 2008. The Arias symposia are educational programs that focus on applying advances in basic science to improve the diagnosis, treatment, and prevention of liver disease. Dr. Arias also organized, directed, and participated in the 7th Annual NIH Course on "Demystifying Medicine for PhD Students, Fellows and Staff," held weekly from January to May at NIH. The professionally prepared and distributed course is used as a major teaching aid worldwide in over 20 institutions.

NICHD, in conjunction with the Office of Rare Diseases, supported the XIII International Adrenal Conference, which was organized by **Maria Dufau**, NICHD; Bernard Schimmer, University of Toronto; and Alexander C. Brownie, University of Buffalo. The meeting, held in San Francisco from June 11 through 14, drew 101 participants, including several trainees and young scientists at the beginning of their independent careers, who benefited from discussing their work with expert and seasoned scientists. The meeting brought together many of the leading research groups from the United States and abroad and featured presentations by prominent investigators describing new and important developments in basic and clinical research on the adrenal cortex. Topics included rare genetic disorders that affect adrenal steroidogenesis and other endocrine and non-endocrine functions as well as familial glucocorticoid deficiency, congenital lipoid adrenal hyperplasia, adrenocortical carcinoma, and adrenal insufficiency (P450 oxidoreductase deficiency, cortisone reductase deficiency, and congenital adrenal hyperplasia). Speakers provided new insights into the role of adrenal stem cells in the differentiation and development of the adrenal cortex and the transcriptional regulation of adreno-primordia development. Others described post-transcriptional modifications that modulate the activity of Ad4BP/SF1, a member of the nuclear receptor family that regulates genes essential for steroidogenesis. Researchers also described new mechanisms for tissue-specific actions of glucorticoids resulting from multiple receptor isoforms.

Alan Hinnebusch is co-organizer of the recurring Cold Spring Harbor Laboratory Meeting on Translational Control, which this year was held in September 2008 at Cold Spring Harbor, New York. He was also co-organizer of the Keystone Symposium on Translational Regulatory Mechanisms held in January, 2008, in Coeur d'Alene, Idaho.

The XVII International AIDS Conference, Mexico City, August 2008, was the central event of the year in the field of HIV/AIDS research, prevention, and treatment. The meeting attracted more than 22,000 delegates from around the world. Within the framework of the meeting, the International AIDS Society invited **Leonid Margolis** to organize and chair a special session entitled "War and Peace between Microbes: Co-pathogens as Determinants of HIV." During the session, scientists from the United States, Brazil, India, and Peru presented new data on co-pathogens' interactions with HIV and discussed the possibility of preventing or containing HIV infection with the help of such interactions. The 17th Annual International Conference on AIDS, Cancer and Public Health took place in St. Petersburg (Russia) in May 2008. The conference always attracts many internationally acclaimed scientists from the United States and Europe. As before, the organizers invited Dr. Margolis to organize and chair a mini-symposium on "Microbicides/Local Immunity," at which scientists from the United States, Holland, and Belgium presented their latest data and theoretical considerations on how to prevent local HIV transmission.

The proceedings of an NICHD Workshop held in Potomac, Maryland, in October 2007, were published in 2008 in the Annals of the New York Academy of Sciences as volume 1135, entitled "The Menstrual Cycle and Adolescent Health" and edited by Catherine M. Gordon, Corrine Welt, Robert W. Rebar, Paula J.A. Hillard, Martin M. Matzuk, and **Lawrence M. Nelson**. Sponsors of the two-day symposium included the U.S. Department of Health and Human Services (DHHS), the National Institutes of Health (NIH), the NICHD, the American Society for Reproductive Medicine, the NIH Office of Research on Women's Health, the NIH Office of Rare Diseases, the U.S. Food and Drug Administration Office of Women's Health, the DHHS Office of Women's Health, and Rachel's Well, Inc. Patients, patient advocates, and experts from a variety of fields and disciplines attended the workshop, which identified areas for which the scarcity of data hampers the issuance of evidence-based recommendations for the management of menstrual problems in young adolescents. Accordingly, participants developed a manifesto on research on the menstrual cycle in adolescents, emphasizing the need for (1) a new research model that integrates grass-roots community passion for participatory research with research planning and regulatory oversight and (2) a coordinated research effort on the menstrual cycle and its disorders in adolescents. The effort could initially take the form of a Study of Puberty Across the Nation (SPAN), similar to the Study of Women Across the Nation (SWAN), which addressed the normal menopausal process.

In line with the spirit of his program, which emphasizes teamwork, collaboration with outside medical centers, and patient participation, **Karel Pacak** helped organize the Second International Symposium on Pheochromocytoma held in Cambridge, UK, in September 2008. The symposium provided an international forum for over 150 investigators and health care professionals from around the world, all with a common interest in pheochromocytoma. The symposium facilitated the forging of new and improved collaborations, interdisciplinary studies, and concerted, comprehensive approaches to resolving the outstanding scientific and medical problems associated with pheochromocytoma.

Yun-Bo Shi, together with Zahra Zakeri (Queens College, CUNY), Richard Lockshin (St. Johns University, New York), Junying Yuan (Harvard Medical School, Boston), Jiarui Wu (Shanghai Institutes for Biological Sciences, China), and Dengxi Zheng (Institute of Basic Medical Sciences, China), organized The Seventh International Cell Death Society Symposium on targeting cell death pathways for human diseases. Held in Shanghai in June 2008, the meeting covered most of the important topics in current cell death research, including genetic control of programmed cell death, cell death in the immune system, autophagy, cell-death pathways and their regulation, and cell death in disease and therapeutics.

REPORTS ON NICHD'S INTRAMURAL RESEARCH PROGRAMS

MOLECULAR AND CELLULAR MECHANISMS OF HEPATOCELLULAR POLARITY AND BILIARY SECRETION IN HEALTH AND CHOLESTASIS

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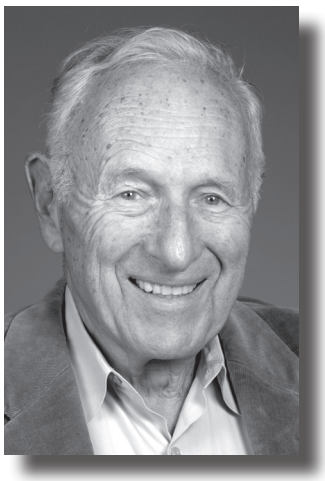
Dong Fu, PhD, *Postdoctoral Fellow*
Malte Renz, MD, *Postdoctoral Fellow*
Divny Narendra, *Summer Program College Student*



In collaboration with Jennifer Lippincott-Schwartz's laboratory, we use biochemical, genetic, molecular, and live-cell imaging techniques to study mechanisms responsible for selective trafficking of proteins to the apical domain of hepatocytes and other polarized cells. Our goal is to identify the components and regulation of these trafficking processes, their role in creating and maintaining cellular polarity, and the molecular defects responsible for inheritable and acquired bile secretory failure (cholestasis).

Intracellular pathways for trafficking ATP-binding cassette (ABC) transporters to the bile canalicular domain

Wakabayashi, Fu, Narendra; in collaboration with Bhatia, Brauer, Calgano, Gottesman, Kagawa, Wolkoff

The lack of a stable primary culture system previously limited studies on intracellular trafficking in hepatocytes. However, a recent advance in the development of two systems (a "collagen sandwich" and a platform bioengineered by the Massachusetts Institute of Technology) now permits long-term (10–60 day) cultures of primary rat and human hepatocytes. In collaboration with the pioneers of these systems (Brauer and Bhatia), we used live-cell imaging and immunocytochemistry to characterize such cultured hepatocytes maintained with respect to the expression profiles of ABC transporters and intracellular trafficking. We established the time-dependent pattern of formation of tight junctions, canalicular structure, and trafficking of ABC transporters to the canalicular membrane. Sequential live-cell FRAP (fluorescence recovery after photobleaching) and FLIP (fluorescence loss in photobleaching) studies confirmed previous findings obtained in WIFB9 cells and revealed the direct Golgi-to-canalicular trafficking pathway for ATP cassette transporters and rab11a-myosin Vb–dependent cycling to the apical membrane.

Previously, we discovered two pathways by which apical membrane proteins traffic from the Golgi to the bile canaliculus in mammalian hepatocytes and polarized WIFB9 cells (the latter cells are a hybrid of rat hepatoma and human fibroblasts). Canalicular ABC proteins, such as ABCB11 (bile acid transporter), ABC1 (non–bile acid organic anion transporter), and ABCB1 (organic cation transporter), enter a large intracellular rab 11a–enriched endosomal pool from which they cycle to the apical plasma membrane. In contrast, single transmembrane proteins, such as cCAM 105 and 5′ nucleotidase, traffic from Golgi to the basolateral plasma membrane domain, from which they undergo transcytosis to the apical membrane. We identified critical roles for dynamic and stable

microtubules, actin HAX1, N-linked glycans, myosin Vb, PI 3-kinase, and rab 11a in the direct trafficking pathway. Live-cell imaging of ABCB11-YFP constructs revealed downstream docking sites in the canalicular membrane; we are now in the process of identifying those sites.

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The metabolic sensor AMPK and its upstream activator LKB1 participate in bile canalicular and tight junction formation and maintenance and apical content of ABCB11

Fu, Wakabayashi, Renz, Arias; in collaboration with Cantley, Ruderman, Brauer, Cal

An increased AMP:ATP ratio activates AMPK, which increases ATP synthesis (glucose uptake, mitochondria and fatty acid oxidation) and downregulates ATP consumption (protein, lipid synthesis). AMPK is also activated by LKB1-dependent phosphorylation, which responds to stress and growth factor–dependent signals. Studies in *Drosophila* and mammalian cell lines demonstrate a role for LKB1 and AMPK in polarization.

In sandwich cultures of rat hepatocytes, agonists of AMPK phosphorylation enhanced apical polarization, whereas inhibitors delayed maturation and prevented development of apical polarization. Removal of calcium from the medium prompted depolarization; however, co-incubation with AMPK agonists prevented depolarization. Our discovery links metabolism (AMP:ATP ratio) to polarization and ABC transporter trafficking. As such, it provides a potential link with bile secretory failure (cholestasis) in hyperalimentation, starvation, diabetes, pregnancy, and liver regeneration, a link that we are currently exploring. Our goal is to determine the pathway, components, and mechanism whereby the AMPK-LKB pathway is linked to tight junction structure and function, apical polarization, and the direct pathway for trafficking of ABC transporters to the canalicular membrane.

Role of dynamic microtubules in apical trafficking of the bile acid transporter ABCB11 in WIFB9 cells

Wakabayashi, Arias; in collaboration with Lippincott-Schwartz

Microtubules are required for ABCB11 trafficking from rab11a-positive endosomes to the bile canaliculus. However, the specific contributions of microtubule subsets remain unknown. Using polarized WIF-B9 cells, we investigated the role of dynamic microtubules in canalicular targeting of ABCB11. At the steady state, ABCB11 traffics to the canalicular membrane. After specifically disassembling dynamic microtubules by using a marine sponge–derived quinone, we observed that ABCB11-containing endosomal movement continued along stable microtubules; however, canalicular targeting was abolished. Immunostaining of alpha-tubulin and EB1, a dynamic microtubular plus-end tracking protein, revealed a pericanalicular web that includes the plus end of dynamic microtubules. To explore how dynamic microtubules regulate canalicular targeting of ABCB11, we performed immunostaining of IQGAP, Rac, APC, and EB1, which link the dynamic microtubular plus end and actin. IQGAP, Rac, and APC surrounded the bile canaliculus in association with actin and EB1. Our results demonstrate that canalicular targeting of ABCB11 depends on dynamic microtubules whose plus ends may establish the long-sought bridge between microtubules and actin, which is required for endosome trafficking to the bile canaliculus. Our goal is to determine whether depolymerization of dynamic microtubules is the mechanism whereby various quinone-

based drugs and chemicals cause cholestasis in humans and/or animals. We are also exploring the mechanism and sites for microtubular assembly after depolymerization.

The role of rab 1a, myosin Vb, and other proteins in canalicular polarity

Wakabayashi, Fu, Narendra; in collaboration with Cantley, Wolkoff, Bhatia, Brauer

While studying mechanisms of apical targeting in WIFB9 cells, we observed that rab 11a and myosin Vb are required for canalicular formation. Expression of dominant negative constructs or RNAi prevented polarization and resulted in trafficking patterns found in non-polarization cells. Our observations prompted a revision of current polarity concepts and suggest that polarization is initiated upon delivery of rab 11a/myosin Vb–containing vesicles to the surface, causing the plasma membrane at the site of delivery to differentiate into the apical domain (bile canaliculus).

Our goal is to confirm preliminary results in long-term non-dividing rat hepatocyte cultures by using similar constructs and quantitative live-cell imaging of ABC transporter movement. We are exploring molecular mechanisms of polarization and the specific role of the direct ATB transporter pathway in the polarization process.

Wakabayashi Y, Dutt P, Lippincott-Schwartz J, Arias IM. Rab 11a and myosin Vb are required for polarization in WIFB Cells. *Proc Natl Acad Sci USA* 2005;102:15087-15092.

Biology and pathobiology of fenestrae in hepatic endothelial cells

Arias; in collaboration with Cogger

Our previous studies revealed that the fenestrae of hepatic endothelial cells require an actin-myosin–based cytoskeleton and that contraction of the fenestrae may be regulated physiologically. Given the absence of a basement membrane in hepatic sinusoids, fenestrae constitute the only barrier between the circulation and plasma membrane of hepatocytes, regulating transfer of many substances between the plasma and hepatocytes. Using scanning electron microscopy of hepatic endothelial cells from mice in which caveolin I was deleted, we observed reduced numbers and abnormal shapes of cell fenestrae. In a collaborative study, we are exploring the relation between caveolin 1 and fenestra biology. Additional studies in SK Hep1 cells revealed fenestrae that are structurally and functionally similar to those in hepatic endothelial cells associated with caveolin-1. Our immediate goal is to measure the functional effects of caveolin-1 deletion on throughput kinetics in rat liver by using a multiple isotope technique.

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Function of MRP6 in pseudoxanthomatosis elasticum

Arias; in collaboration with Rojkind

MRP6, an ABC transporter restricted to the basolateral plasma membrane of hepatocytes, is mutated in patients with pseudoxanthomatosis elasticum, a disease of impaired elastic tissue in blood vessels, eye, and skin. We are exploring the possibility that the hepatocyte normally secretes an elastase inhibitor into the serum. A sensitive, specific assay for elastase activity provides a screen for candidate small peptides that may serve as biologic substrates for MRP6 and elastase inhibitors in vivo. To date, we have identified no such molecules.

Thus far, we have tested a large number of candidate peptides, none of which is an elastase inhibitor. A more recent proposal is that MRP6 transports vitamin K components, which are secreted as GLA-modified peptides that function as regulators of peripheral elastases. Our goal is to test this hypothesis in *MRP6*$^{-/-}$ mice by determining GLA peptides in serum, urine, and bile.

Gene expression in cholestasis associated with hyperalimentation

Arias; in collaboration with Calgano, Gottesman, Fishbein, Teitelbaum

We are exploring gene expression patterns in clinical and experimental cholestasis associated with hyperalimentation. In particular, we are quantifying expression of canalicular ABC transporters and

transcriptional regulators. Our working hypothesis posits that hyperalimentation alters intracellular trafficking of canalicular ABC transporters, particularly the bile salt export pump (BSEP), resulting in cholestasis.

Molecular pathogenesis of progressive familial intrahepatic cholestasis, type 1
Arias; in collaboration with Shneider, Paulusma, Perez-Victoria

Previously, we showed that *FIC1* encodes a P-type ATPase, which functions as an aminophospholipid flippase in the basolateral plasma membrane of hepatocytes and small intestinal cells. FIC1 regulates FXR, a nuclear transcription factor, which in turn regulates the activity of the BSEP and other apical ABC transporters. In a collaborative study, we are seeking to elucidate how the P-ATPase and its lipid traffic regulate bile acid secretion.

In collaboration with Shneider, Paulusma, and Perez-Victoria, we seek to identify cytosolic factors for vesicle formation. Thus far, we have demonstrated only that several yeast P4-ATPases are involved in membrane trafficking. Of interest is that one P-type ATPase, Drs2P, regulates retrograde trafficking from the plasma membrane and endosomes to the trans-Golgi network. We propose that the retrograde route, which is controlled by several recently identified proteins and is critical for membrane homeostasis, may be linked to FIC1 function. Our goal is to determine the possible role of FIC1 in retrograde trafficking in hepatocytes and, more specifically, whether FIC1 regulates membrane protein sorting at the trans-Golgi network, regulates recycling from the plasma membrane, and/or interacts with members of the retrograde machinery.

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Listowsky I, Arias IM. Ligandin and glutathione S-transferases: historical milestones. In: Awasthi Y, ed. *Toxicology of Glutathione Transferases.* CRC Press, 2006;1-10.

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PROTEIN SORTING IN THE ENDOSOMAL-LYSOSOMAL SYSTEM

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We investigate the molecular mechanisms by which transmembrane proteins are sorted to intracellular compartments such as endosomes, lysosomes, and a group of cell-type–specific organelles known as lysosome-related organelles (e.g., melanosomes and platelet dense bodies). Sorting to these compartments is mediated by adaptor proteins' recognition of signals present in the cytosolic domains of the transmembrane proteins; the adaptor proteins are components of membrane coats. Among the adaptor proteins are the heterotetrameric AP-1, AP-2, AP-3, and AP-4 complexes; the monomeric GGA1, GGA2, and GGA3 proteins (GGAs) (Figure 1.1); and the heteropentameric "retromer" complex. Proper sorting requires, in addition to these adaptors, the function of other components of the trafficking machinery that mediate vesicle tethering and fusion. Current work in our laboratory aims to elucidate the structure, regulation, and physiological roles of coat proteins and vesicle tethering factors and to investigate human diseases that result from genetic defects (Hermansky-Pudlak syndrome) or pathogens' (HIV-1) exploitation of these proteins.

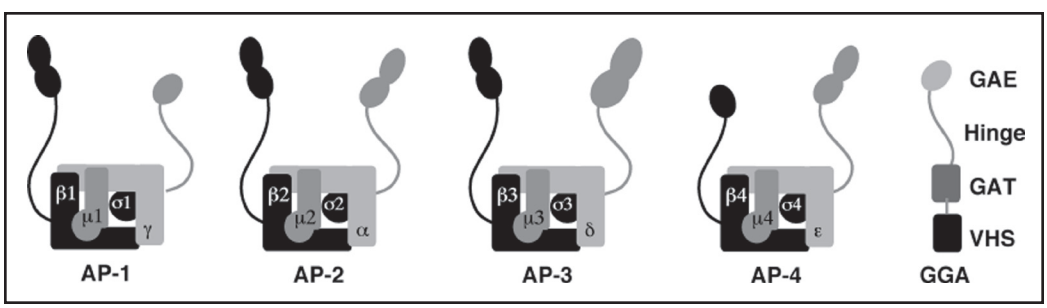

FIGURE 1.1
Structure of AP complexes and GGAs

Exploitation of adaptor proteins by HIV-1

Lindwasser, Smith, Chaudhuri, Yang[1]; in collaboration with Freed, Guatelli, Hurley

The AP-2 complex comprises four subunits (alpha, beta2, mu2, and sigma2) and is a component of plasma membrane clathrin coats. Its primary function is to mediate clathrin-dependent endocytosis through recognition of endocytic signals in the cytosolic tails of transmembrane proteins. In previous work, we showed that the Nef protein of HIV-1 requires AP-2 for the downregulation of the CD4 co-receptor from the surface of host cells. Formation of a CD4–Nef–AP-2 complex promotes CD4 endocytosis via plasma membrane clathrin-coated pits. In addition, we found that a "dileucine" signal in Nef interacts with a combination of the alpha and sigma2 subunits of AP-2. We have now discovered that a second, "diacidic" motif in Nef interacts with a different binding site on the alpha-sigma2 hemicomplex. Both interactions are required for Nef to downregulate CD4. We are currently conducting further analyses of the tripartite interactions among CD4, Nef, and AP-2 and are examining the mechanism by which internalized CD4 is targeted to lysosomal degradation upon expression of Nef.

Another effect of HIV-1 Nef on host cells is the upregulation of immature class II molecules of the major histocompatibility complex (MHC-II) in association with the invariant chain (Ii). In collaboration with John Guatelli and colleagues, we have found that such upregulation is caused by competition between dileucine signals in Nef and in the cytosolic tail of Ii for binding to the alpha-sigma2 hemicomplex. Overexpression of Nef during infection saturates the dileucine binding site on AP-2, thus interfering with dileucine- and AP-2–mediated endocytosis of MHC-II-Ii from the cell surface. The trapping of MHC-II-Ii at the cell surface prevents MHC-II from acquiring viral peptides in endosomes, thus interfering with immune surveillance.

In collaboration with Eric Freed and colleagues, we have also examined the role of adaptor proteins in HIV-1 budding from host cells. We found that the GGA proteins, which are monomeric adaptors associated with trans-Golgi network clathrin coats, modulate HIV-1 release into the extracellular space by acting on the small GTP-binding protein Arf.

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Cleft palate caused by a defect in AP-2

Puertollano[2]; in collaboration with Everett, Overbeek, Gahl

Mutant organisms have previously aided in investigations of the physiological roles of AP complexes. For example, in collaboration with William Gahl (NHGRI), we showed that mutations in one of the subunits of the AP-3 complex in humans cause the pigmentation and bleeding disorder Hermansky-Pudlak syndrome type 2. To investigate the role of AP-2, we previously ablated the gene encoding the mu2 subunit in mice. This approach yielded little information because defective expression of *mu2* resulted in early embryonic lethality. In collaboration with Eric Everett and Paul Overbeek, we have now characterized a mutant mouse deficient in expression of the beta2 subunit of AP-2. Unlike the *mu2* mutant mouse, the *beta2* mutant mouse survives until birth but dies shortly thereafter. The viability of *beta2* mutant embryos is attributable to substitution of the beta1 subunit of AP-1 for beta2 in the AP-2 complex. Most interestingly, the newborn *beta2* mutant mouse exhibits a single

visible developmental defect: cleft palate. Thus, endocytosis must be critical for the development of the palate, probably by promoting the internalization of a key signaling receptor. Our finding identifies a possible cause for cleft palate, which is one of the most frequently occurring congenital deformities in humans.

Structure and regulation of the retromer complex

Rojas, Prabhu, Murthy, Mardones; in collaboration with Hurley, Steven, van der Sluijs

The retromer complex is another sorting device that mediates "retrograde" transport from endosomes to the trans-Golgi network. This function is essential for many important physiological processes in higher eukaryotes, including lysosomal enzyme sorting, processing of the Alzheimer's disease amyloid precursor protein, and formation of morphogen gradients during development. The retromer comprises a membrane-binding subcomplex made up of two "sorting nexin" (SNX) subunits and a cargo-recognition subcomplex composed of Vps26, Vps29, and Vps35. We collaborated with James Hurley and Alasdair Steven to solve the atomic structure of the Vps26-Vps29-Vps35 subcomplex. The structure revealed that Vps35 is a rod-shaped, alpha-helical solenoid onto which Vps26 and Vps29 assemble at either end of the rod. Vps26 resembles a clathrin adaptor known as arrestin, whereas Vps29 belongs to a family of metallophosphoesterases. The structure has several sites for binding to SNX proteins and cargo and can flex to conform to the curved shape of transport vesicles and tubules.

We recently found that the recruitment of the Vps26-Vps29-Vps35 subcomplex to membranes involves, in addition to the SNX subcomplex, the small GTP-binding protein Rab7. Perturbation of either the SNX subcomplex or Rab7 results in dissociation of the Vps26-Vps29-Vps35 subcomplex from membranes. Loss of retromer function leads to missorting of lysosomal enzymes and consequent accumulation of undegraded materials in lysosomes, a phenotype characteristic of lysosomal storage disorders.

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Role of the GARP complex in transport from endosomes to the Golgi complex

Pérez-Victoria, Mardones

Endosomal transport carriers formed by the action of the retromer must dock at and fuse with the trans-Golgi network in order to deliver their cargo. We recently found that GARP, a multiprotein complex originally described in yeast, plays such a role in mammalian cells. Interference with GARP blocks the delivery of cargos such as mannose 6-phosphate receptors and Shiga toxin from endosomes to the trans-Golgi network, indicating that GARP plays a general role in retrograde transport. A mutation in Vps54, one of the GARP subunits, was recently identified in the Wobbler mouse mutant, an animal model of amyotrophic lateral sclerosis. We found that the Wobbler mutation does not prevent the function of GARP in retrograde transport, suggesting that the disease is likely attributable to a more subtle defect in transport or some other function of GARP.

Pérez-Victoria FJ, Mardones GA, Bonifacino JS. Requirement of the human GARP complex for mannose 6-phosphate-receptor-dependent sorting of cathepsin D to lysosomes. *Mol Biol Cell* 2008;19:2350-2362.

Ubiquitin binding and conjugation regulate protein recruitment to endosomes

Mattera

Rab GTPases and ubiquitination are additional factors that regulate transmembrane cargo sorting in endocytic and lysosomal targeting pathways. The endosomal protein Rabex-5 intersects these two

layers of regulation by functioning as both a guanine-nucleotide exchange factor (GEF) for Rab5 and a substrate for ubiquitin (Ub) binding and conjugation. This past year, we demonstrated that the ability of Rabex-5 to bind to Ub is essential for its recruitment from the cytosol to endosomes, independently of its GEF activity and of Rab5. We also showed that monoubiquitinated Rabex-5 is enriched in the cytosol. These observations are consistent with a model whereby a cycle of Ub binding and monoubiquitination regulates the association of Rabex-5 with endosomes.

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REGULATION OF SECRETORY AND MEMBRANE PROTEIN BIOGENESIS

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Sichen Shao, *Rotation Student*

We seek to understand how newly synthesized secretory and membrane proteins are made, matured, sorted, and metabolized in cells. This class of proteins are essential to all intercellular and intracellular communication, and the proteins' precise locations and abundances are tightly regulated to maintain normal cellular and organismal physiology. Indeed, most current drugs target secreted and membrane proteins, underscoring their central role in human biology. Our goal is to develop a molecular-level understanding of the pathways of secretory and membrane protein biosynthesis and metabolism. We are especially interested in the regulatory machinery controlling protein entry and protein insertion into the endoplasmic reticulum (ER), the site at which nearly all secreted and membrane proteins are first made. We use biochemical approaches to purify, identify, and functionally reconstitute the machinery underlying these basic cellular pathways. In parallel, we are analyzing, in cellular and whole-animal models, the physiologic importance of regulating the metabolism of secretory and membrane proteins. We hope that, by developing a greater understanding of the basic cellular pathways, we can gain insight into the ways they are perturbed in various diseases of protein misfolding and processing.

Prion protein cell biology and neurodegeneration

Chakrabarti, Ashok, Emerman, Bhat S,[1] Appathurai, Shao; in collaboration with Feigenbaum, Lippincott-Schwartz

The prion protein (PrP) is a widely expressed and highly conserved cell surface glycoprotein of uncertain function. Aberrant metabolism of PrP is responsible for a variety of neurodegenerative diseases in both humans and animals. These diseases include the transmissible "prion diseases" such as bovine spongiform encephalopathy as well as inheritable neurodegenerative diseases caused by mutations in the *PrP* gene. In neither case is the pathway (or pathways) leading to cell death and neuronal damage understood. The overall goal of this project is to define the pathways of PrP-mediated neurodegeneration. To that end, we are studying the molecular pathways of PrP biosynthesis, intracellular trafficking, metabolism, and degradation. A quantitative analysis of these events is expected to provide insight into how the various inherited mutations in PrP influence its biosynthesis or metabolism in a manner that leads to the cellular dysfunction.

Our analysis suggests that at least two cytotoxic forms of PrP (termed CtmPrP and cyPrP) are made during the initial translocation of PrP into the ER. We have created transgenic mice to determine

whether CtmPrP-mediated and cyPrP-mediated neurodegeneration may be averted in vivo by modulating this newly discovered step during PrP biogenesis. To determine whether changes in these pathways are involved in the progression of neurodegeneration, we are also investigating how the various forms of PrP are normally metabolized by the cell. We hypothesize that a combination of defects in biosynthesis and/or clearance of certain forms of PrP collaborate to cause eventual neuronal dysfunction and death. Conversely, manipulation of these events may be able to slow or reverse the neurodegenerative process in prion diseases.

In parallel, we are performing a systematic analysis of the biosynthesis, trafficking, and metabolism of disease-associated PrP mutants. We aim to identify the precise cellular locale and mechanism of PrP misfolding that initiates the disease process. We discovered that, for a large number of mutants, misfolded PrP is found in a post–ER location, an observation that is notable because it suggests that the misfolded PrP species have escaped the normal cellular quality control mechanisms in the ER. In still other parallel studies, we are investigating the downstream consequences of PrP misfolding and aggregation to identify the mechanism by which these events lead to cellular dysfunction. We have found that the aggregates recruit various cellular factors, thereby depleting the factors' functional availability. One such factor is of particular importance because its disruption in mice leads directly to a neurodegenerative phenotype reminiscent of diseases caused by PrP.

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Regulation of protein biogenesis and function by signal sequences

Kang, Sharma; in collaboration with Sharan, Tessarollo

Our discovery that the N-terminal signal sequence of PrP regulates Prp's topology established a new function for this domain independent of its well-studied role in protein targeting. We subsequently developed and used a novel assay for signal-mediated translocon gating to demonstrate that signal sequences display a remarkable degree of variation in initiating nascent chain access to the lumenal environment. We found such substrate-specific properties of signals to be evolutionarily conserved, functionally matched to their respective mature domains, and important for the proper biogenesis of some proteins. A recent analysis of several naturally occurring disease-associated mutants in signal sequences revealed that, contrary to previous assumptions, many are altered in their gating rather than targeting function. Thus, we have discovered that the long-observed sequence variations in signals do not simply represent functional degeneracy but instead encode differences in translocon gating that are critical to the proper biogenesis of the attached substrate. In a particularly dramatic example, we showed that conditions of ER stress attenuate the translocation of some but not other substrates in a signal sequence–dependent manner. This "pre-emptive" quality control (pQC) pathway is part of the adaptive cellular stress response, and it minimizes protein misfolding in the ER lumen. We are currently investigating whether the cell exploits the substrate-specific properties of signal sequences in other ways in order to regulate the subcellular localization of certain proteins, such as calreticulin, that have been shown to be present in several compartments. Our ongoing studies focus on developing a molecular understanding of the critical interaction between signal sequences and the translocon.

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Cryo-electron microscopy of the protein translocon

Hegde; in collaboration with Akey

In collaboration with Christopher Akey's group, we are using cryo-electronmicroscopy (EM) to compare the structures of ribosome-translocon complexes prepared under different conditions. Conditions include a quiescent translocon, translocons engaged with a translocating substrate, and translocons lacking or containing individual components. The topography of these cryo-EM structures will be fitted with atomic models of individual translocon structures obtained from existing and forthcoming crystal structures to develop an understanding of the architecture of the protein translocation apparatus.

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Degradation machinery for mislocalized secretory and membrane proteins

Hessa, Sharma

We are interested in the fate of copies of secretory and membrane proteins that fail to be properly segregated into the ER. We know that they are degraded, but a more delineation of the pathway of their selective recognition, ubiquitination, and degradation is lacking. Because these proteins are often hydrophobic and/or contain unprocessed hydrophobic elements, they are at high risk for aggregation and inappropriate interactions. Thus, the pathway for the selective recognition and degradation of non-translocated secretory and membrane proteins is likely to be of importance not only during pQC but also for normal cellular homeostasis. We are addressing this issue by using an in vitro system in which secretory and membrane proteins may be synthesized in their "non-translocated" state simply by omitting ER-derived rough microsomes from the reaction (or, if they are included, by inhibiting translocation with Cotransin). With either of these manipulations, non-translocated proteins are rapidly ubiquitinated in preparation for their degradation. We are employing both classical fractionation and affinity-purification approaches to identify the machinery for the selective recognition and degradation of these non-translocated proteins.

Small-molecule inhibitors of protein translocation

Hegde; in collaboration with Taunton

To develop new methods and probes for protein translocation, we have been developing pharmacologic methods of modulating this process in vivo. We recently synthesized and characterized a novel small-molecule inhibitor of co-translational protein translocation and demonstrated that, both in vitro and in cultured cells, the compound inhibits the translocation of some but not other proteins. Remarkably, we found this substrate specificity to be encoded in the signal sequence. Thus, the sensitivity or resistance to the inhibitor may be conferred to any protein of interest simply by choice of signal. We have identified the likely target of this inhibitor as the Sec61 complex, which is the central component of the protein translocation channel. These tools and findings open the way for selectively and potently modulating the translocation of individual substrates in live cells, an approach that we are applying to study the role of protein translocation in various cellular events such a protein aggregation and toxicity, protein degradation, and the cellular response to ER stress.

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Mechanism of tail-anchored membrane protein insertion

Mariappam, Li; in collaboration with Keenan

Insertion of proteins into biological membranes is a fundamental process vital to all organisms. Most membrane proteins use the classical co-translational translocation pathway. The essential and universally conserved machinery for substrate recognition, targeting, and insertion by this pathway is well established and extensively characterized. By contrast, little is known about post-translational membrane protein insertion pathways. The main clients for this pathway are tail-anchored (TA) membrane proteins defined by a cytosolic-facing N-terminal domain followed by a single C-terminal transmembrane domain (TMD). Examples of TA proteins are found on essentially all cellular membranes in every organism; the proteins have diverse functions ranging from intracellular trafficking to regulation of cell death. Despite the proteins' widespread importance, the machinery and mechanisms underlying the recognition, targeting, and insertion of TA proteins into the correct organellar membrane remain largely unknown. Recently, we discovered a cytosolic TMD recognition complex (TRC) that selectively interacts with TA proteins destined for the ER membrane. We identified a central component of TRC as a highly conserved 40 kD ATPase that represents the first molecular factor in this widely used membrane protein insertion pathway. Still unknown are other components that collaborate with TRC40 to mediate selective recognition, targeting, and insertion of TA proteins into the ER. We are now taking various approaches to identify these additional factors and reconstitute the targeting reaction for TA proteins with defined components. Achieving these goals will define the core machinery and functions for a fundamental protein trafficking pathway and pave the way for future mechanistic and structural analyses.

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CELL CYCLE REGULATION IN OOGENESIS

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We use *Drosophila* oogenesis as a model to explore the developmental regulation of the cell cycle. In *Drosophila*, the oocyte develops within the context of a 16-cell germline cyst. Individual cells within the cyst are referred to as cystocytes and are connected by actin-rich ring canals. While all 16 cystocytes enter premeiotic S phase, only a single cell remains in the meiotic cycle and becomes the oocyte. The other 15 cells enter the endocycle and develop as highly polyploid nurse cells. Currently, we are working to understand how cells within the ovarian cyst enter and maintain either the meiotic cycle or the endocycle. In addition, we are examining how this cell cycle choice drives development of the mature egg.

Regulation of developmentally programmed endocycles

Narbonne, Senger; in collaboration with Asano, Deák, Richardson, Aladjem, Riesgo-Escovar

The endocycle is a commonly observed variant cell cycle in which cells undergo repeated rounds of DNA replication with no intervening mitosis. Cells that are highly metabolically active, such as the giant trophoblast of the mammalian placenta and *Drosophila* ovarian nurse cells, often grow via endoreplication. How the cell cycle machinery is modified to transform a mitotic cycle into endocycle has long been a question of interest. In both plants and animals, the transition from the mitotic cycle to the endocycle requires Fzr/Cdh1, a positive regulator of the anaphase-promoting complex/cyclosome (APC/C). However, with many of APC/C's targets transcriptionally downregulated upon entry into the endocycle, it remained unclear whether the APC/C functioned beyond the mitotic/endocycle boundary. We have now shown that APC/$C^{Fzr/Cdh1}$ activity is required to promote G/S oscillation of the *Drosophila* endocycle. Compromised APC/C activity following cell entry into the endocycle inhibits DNA replication and results in the accumulation of several APC/C targets, including the mitotic cyclins and geminin. Notably, our data suggest that the activity of APC/$C^{Fzr/Cdh1}$ during the endocycle is not continuous but rather cyclic, as demonstrated by the APC/C-dependent oscillation of the prereplication complex component ORC1. Taken together, our data suggest a model in which the cyclic activity of APC/$C^{Fzr/Cdh1}$ during the *Drosophila* endocycle is driven by the periodic inhibition of Fzr/Cdh1 by cyclin E/Cdk2. Thus, we propose that, as observed in mitotic cycles, APC/$C^{Fzr/Cdh1}$ functions during endocycles to reduce levels of the mitotic cyclins and geminin in order to facilitate the relicensing of DNA replication origins and cell cycle progression. Our work has provided important new insights into the basic cell requirements of the endoreplicative cycle.

In *Drosophila*, the oscillations of cyclin E/Cdk2 activity drive the endocycle. How the periodicity of cyclin E/Cdk2 activity is achieved during endocycles is poorly understood. We have determined

that the $p21^{cip1}$/$p27^{kip1}$/$p57^{kip2}$-like cyclin-dependent kinase inhibitor (CKI) Dacapo (Dap) promotes replication licensing during *Drosophila* endocycles by reinforcing low Cdk activity during the endocycle Gap phase. In *dap* mutants, cells in the endocycle exhibit reduced levels of the licensing factor double-parked/Cdt1 (Dup/Cdt1) as well as decreased levels of chromatin-bound MCM2-7 complex. In addition, mutations in *dup/cdt1* dominantly enhance the dap phenotype in several polyploid cell types. Consistent with their reduced ability to complete genomic replication, *dap* mutants accumulate increased levels of DNA damage during the endocycle S phase. Intriguingly, we find that Dap also promotes replication licensing and genomic stability during premeiotic S phase. Our data suggest a model in which dap inhibits cyclin E/Cdk2 activity during the Gap phase and thus promotes the efficient licensing of DNA replication origins. Our work represents the first example of a CKI acting to promote replication licensing in a metazoan.

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Meiotic progression and oocyte differentiation in early ovarian cysts

Senger, Decotto

The pathways that control entry into the meiotic cycle and early meiotic progression are poorly understood in metazoans. We previously identified a gene, *missing oocyte* (*mio*), that regulates nuclear architecture and meiotic progression in early ovarian cysts. In *mio* mutants, the oocyte enters the meiotic cycle and progresses to pachytene; however, it does not maintain the meiotic state and ultimately withdraws from meiosis, enters the endocycle, and becomes polyploid. The *mio* gene is predicted to encode a protein of 867 amino acids that is highly conserved from yeast to humans. Conservation of the Mio protein can be observed in two main blocks. First, the N-terminal block contains four to six WD-40 repeats, which often function as protein-protein interaction domains. Second, the C-terminal block contains a putative U box, which is structurally similar to the RING finger domain and has been implicated in ubiquitylation. The Mio protein accumulates in the oocyte nucleus in early prophase of meiosis I. Moreover, *mio* mutants display some of the earliest meiotic defects reported in *Drosophila*. Therefore, *mio* provides an excellent entry point for exploring the unique cell biology of the early ovarian cyst and how the establishment of the meiotic program influences the downstream events of oocyte differentiation and meiotic progression.

We are using both biochemical and genetic strategies to identify additional genes that act in the *mio* pathway. To identify proteins that physically interact with Mio, we used tandem affinity purification (TAP) and mass spectrometry. From these studies, we determined that Mio strongly associates with the nucleoporin Nup44A. In support of the biological relevance of the Mio/Nup44A interaction, *nup44A* mutations act as strong dominant suppressors of the mio 16 nurse cell phenotype. Moreover, we determined that mutations in *Nup44A* specifically disrupt meiotic progression and female sterility. Our work provides the framework for future studies on how nuclear pore components influence meiotic progression and maintenance of oocyte identity.

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DYNAMICS OF MEMBRANE TRAFFICKING, SORTING, AND COMPARTMENTALIZATION WITHIN EUKARYOTIC CELLS

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We investigate the global principles underlying secretory membrane trafficking, sorting, and compartmentalization within eukaryotic cells. We use live-cell imaging of green fluorescent protein (GFP) fusion proteins in combination with photobleaching and photoactivation techniques to investigate the subcellular localization, mobility, transport routes, and turnover of a variety of proteins with important roles in the organization and regulation of membrane trafficking and compartmentalization. To test mechanistic hypotheses about protein and organelle dynamics, we use quantitative measurements of these protein characteristics in kinetic modeling and simulation experiments. Among the topics under investigation are (1) membrane partitioning and its role in protein sorting and transport in the Golgi apparatus; (2) biogenesis and dynamics of unconventional organelles; (3) mitochondrial morphology and its regulation of cell cycle progression; and (4) cytoskeletal and endomembrane cross-talk in polarized epithelial cells, hematopoietic niche cells, and the developing *Drosophila* syncytial blastoderm embryo. We have also devoted considerable effort to developing new fluorescence microscopy for imaging fluorescently tagged proteins at near-molecular resolution.

Development of green fluorescent protein technology
Patterson, Manley, Gillette, Sougrat; in collaboration with Hess, Betzig

Super-resolution techniques such as photoactivated localization microscopy (PALM) permit the imaging of fluorescent protein chimeras, revealing the organization of genetically expressed proteins on the nanoscale with a density of molecules high enough to provide structural context. The PALM method involves serial photoactivation and subsequent bleaching of numerous sparse subsets of photoactivated fluorescent protein molecules. A statistical fit of the point-spread function of individual molecules' centers of fluorescent emission then localizes the molecules at near-molecular resolution. The aggregate position information from all subsets is then assembled into a super-resolution image that isolates individual fluorescent molecules at high molecular densities (up to 105 molecules/μm^2). We have previously demonstrated PALM imaging of intracellular structures (including lysosome, Golgi apparatus, and mitochondria) in cryo-prepared thin sections as well

as imaging of vinculin and actin in fixed cells with TIRF (total internal reflection fluorescence) excitation along with correlative PALM/transmission electron microscopy of a mitochondrial marker protein.

We helped develop a dual-label PALM assay system that uses two different photo-activatable molecules expressed within cells. In addition, using PALM, we have developed a system for single-particle tracking in living cells, allowing protein diffusion and immobilization to be characterized at the single-molecule level. Called single-particle tracking PALM (sptPALM), the technique involves activating, localizing, and bleaching many subsets of photoactivated fluorescent protein chimeras in live cells. With sptPALM, we are able to image membrane proteins and obtain spatially resolved maps of single-molecule motions, providing several orders-of-magnitude more trajectories per cell than with traditional single-particle tracking. By probing distinct subsets of molecules, including Gag and vesicular stomatitis virus G, we demonstrated that sptPALM provides a powerful means for exploring the origin of spatial and temporal heterogeneities in membranes.

Another fluorescent protein technique developed in our laboratory allows determination of a protein's topology in living cells. Termed fluorescence protease protection (FPP), the assay provides a fluorescent readout in response to trypsin-induced destruction of GFP attached to a protein of interest before and after plasma membrane permeabilization. The FPP assay involves the attachment of a fluorescent protein to the N- or C-terminus of a protein of interest. Subsequently, cells expressing the fusion protein are exposed to trypsin either before or after plasma membrane permeabilization by digitonin. If the fluorescent protein moiety on the expressed protein faces the environment exposed to trypsin (that is, the cytoplasm), it loses its fluorescent signal. Conversely, if the fluorescent protein moiety on the expressed protein faces the environment protected from trypsin (that is, the lumen of a compartment), its fluorescence persists. Given these outcomes and the fluorescent protein's known engineered position within the protein, we are able to deduce the orientation of the protein across the lipid bilayer. We demonstrated the broad applicability of FPP by using it to define the topology of proteins localized to several organelles, including the endoplasmic reticulum (ER), Golgi apparatus, mitochondria, peroxisomes, and autophagosomes.

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Membrane partitioning and its role in protein sorting and transport within the Golgi apparatus

Patterson, Sougrat, Sengupta, Elsner; in collaboration with Phair, Hirschberg, Gerlich, Polishchuk

The Golgi apparatus processes and filters newly synthesized protein and lipid moving through the secretory pathway, but how it transfers secretory cargo through its 6 to 8 flattened cisternae has remained unclear. The most widely accepted model for intra-Golgi transport is cisternal progression. This classical model postulates that the stack of cisternae in the Golgi apparatus constitute a historical record of progression from entry at the *cis* face to exit at the *trans* face. Recently arrived cargo molecules are confined in the *cis*-most cisterna, undergo initial processing there, and await the arrival of enzymes delivered by retrograde vesicles from more distal cisternae for subsequent processing. Cargo molecules remain within a given cisterna as it passes, conveyor belt–like, through an average of seven locations within the Golgi stack on their way to the *trans* face; the molecules then exit from the Golgi through transport carriers. A key prediction of the model is that newly arrived cargo

exhibits a discrete lag or transit time before export. To test such a prediction, we analyzed various classes of cargo molecules within the Golgi stack after they were fluorescently pulse-labeled and quantitatively visualized as they transited from the Golgi. Our results revealed an exponential loss of cargo from the entire Golgi rather than the linear pattern predicted by the classical model. Moreover, when transmembrane cargo entered the Golgi apparatus, it differentially partitioned between two membrane environments—processing domains enriched in Golgi enzymes and export domains capable of budding transport intermediates. Based on the results of our experiments, we constructed and tested a new model of intra-Golgi trafficking in which cargo molecules continuously partition between processing and export domains defined by different lipid compositions as they move up and down the Golgi stack. Cargo can be exported to the plasma membrane from within the export domain found within every cisterna. Simulation and experimental testing of this rapid partitioning model produced all the key characteristics of the Golgi apparatus, including polarized lipid and protein gradients, exponential cargo export kinetics, and cargo waves, thus representing a viable description of the mechanism of intra-Golgi transport.

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Biogenesis and dynamics of unconventional organelles

Kim, Hailey, Ott

We explored the origin and dynamics of unconventional organelles, including autophagosomes, primary cilia, and peroxisomes. Autophagosomes form during autophagy, which is a highly conserved, bulk degradation pathway that is also involved in turnover of large aggregates and organelles within cells. In the initial step of autophagy, an isolation membrane forms in the cytoplasm through the activation of specific autophagy effectors. The membrane wraps around the protein aggregate or organelle to form a double membrane–bounded structure called the autophagosome. The autophagosome then targets to and fuses with the lysosome, where the sequestered materials are degraded by various hydrolytic enzymes and recycled as amino acids for macromolecule synthesis and energy production. While emerging results have revealed the importance of autophagy in various biological and pathological processes, such as cellular remodeling, tumorigenesis, and neurodegeneration, how this pathway operates is far from clear. We used various live cell–imaging and molecular-genetic approaches to investigate the membrane origin of autophagosomes and the signals that recruit substrates to this organelle. Our data revealed that the outer membrane of mitochondria serves as the membrane source during starvation-induced autophagy formation and maturation. Furthermore, ubiquitin modification acts as a targeting signal for delivery of small cytosolic proteins and larger organelles to autophagosomes.

The primary cilium is a chemosensory and mechanosensory organelle. The axoneme of a cilium is composed of nine doublets of microtubules that extend out from the triplet microtubules of the centrosome. Primary cilia have been described as antennae because they often project away from the cell surface and are able to receive signals (both chemical and mechanical) from the extracellular environment. Imaging this important organelle is essential to developing a better understanding of how it functions. To that end, we imaged live polarized epithelial cells and found that cilia of one cell can make direct contact (bridges) with cilia of adjacent or nearby cells. In imaging live cells expressing different cilia-localized fluorophores, we observed that the bridges are composed of cilia from individual cells; that is, they are not a continuation from one cell to the next. Cilia bridges can be stable over many hours. Trypsin and other protein-disrupting treatments did not disrupt cilia-cilia adhesion. Our findings suggest that cilia may do more than just passively sense the environment; they may be able to initiate and mediate cell-cell communication through direct contact.

The peroxisome is involved in the oxidation of fatty acids, bile salts, and cholesterol and converts hydrogen peroxide to nontoxic forms. Using a photolabeling, pulse-chase strategy in living cells, we

demonstrated that peroxisomal membranes originate from the ER. To investigate the mechanism for peroxisome turnover, we attached monoubiquitin to peroxisomal membrane components facing the cytosol and observed that the peroxisome containing these components was a substrate for autophagy; the peroxisome was engulfed by autophagosomal membranes and, upon autophagosome-lysosome fusion, was degraded. The process was dependent on the ubiquitin-binding protein p62. Our results suggest that peroxisomal turnover occurs by autophagy through a pathway involving ubiquitination of peroxisomal membranes and p62-mediated autophagosome targeting.

Mitochondrial morphology and its regulation of cell cycle progression
Mitra, Wunder, Sougrat

Mitochondria are important energy-producing organelles within cells. Their morphology, including fragmented elements and tubular networks, results from a balance between fission and fusion events. To determine whether changes occur in mitochondrial dynamics at different stages of the cell cycle, we carried out live-cell imaging experiments in cells stably expressing RFP (red fluorescent protein) targeted to the mitochondrial matrix. We found that mitochondria exhibit distinct morphological and physiological states at different stages of the cell cycle. In mitosis, mitochondria fragmented into hundreds of small units for partitioning into daughter cells at cytokinesis. Strikingly, at G1/S, mitochondria fused into a single huge, dynamic filamentous system, unlike at any other cell cycle stage. Photobleaching of an area across the filamentous system revealed that the mitochondrial matrix was continuous. In addition, the mitochondrial network was electrically coupled and had a higher membrane potential than mitochondria at all other stages of the cell cycle. When the filamentous network or its membrane potential was disrupted or its dynamics perturbed, cell cycle progression from G1 into S phase was arrested in a p53-dependent manner. Moreover, p21-overexpression, which induces a G1/S arrest, resulted in filamentous mitochondria with reduced matrix continuity and loss of electrical coupling. The data revealed that, during the cell cycle, mitochondrial dynamism and morphology undergo critical changes that are sensed by the cell at G1/S to control cell cycle progression.

Cytoskeletal and endomembrane cross-talk in polarized epithelial cells, hematopoietic niche cells, and the developing *Drosophila* syncytial blastoderm embryo
Chua, Gillette, Rikhy, Mavrakis

We studied cytoskeletal and endomembrane cross-talk between cells by using three cellular systems: polarized epithelial cells, hematopoietic niche cells, and developing *Drosophila* syncytial blastoderm embryos. In polarized MDCK-cell epithelial monolayers, we employed live-cell imaging approaches to probe dynamin's role in integrating membranes and the cytoskeleton. Dynamin is a large GTPase, a regulatory mechanoenzyme that participates in nascent vesicle formation and actin cytoskeletal regulation. Expression of the nucleotide-free form of dynamin led to dramatic apical constriction of expressing cells in the epithelial monolayer. The phenotype was dependent on cortactin, actin, and myosin II but independent of conditions that affect Arp2/3 activity. General endocytic inhibitors and GTP-bound dynamin mutants did not cause apical constriction. Rather, the response was mediated by nucleotide-free/GDP-bound dynamin interacting with cortactin in an Erk kinase signaling pathway. Thus, nucleotide-free/GDP-bound dynamin modulates the actomyosin contractile system through cortactin and Erk kinase activation to induce apical constriction, a process that underlies epithelial sheet folding and invagination during development as well as the shaping of tubular organs such as kidney and lung.

Hematopoietic stem/progenitor cells (HSPC) reside in the bone marrow niche, where adhesive interactions with osteoblasts provide essential cues for the cells' proliferation and survival. We used live-cell imaging approaches to characterize the site of contact between osteoblasts and hematopoietic progenitor cells (HPC) as well as events at the contact site that result in downstream signaling responses important for niche maintenance. HPCs made prolonged contact with the

osteoblast surface via a specialized membrane domain. At the contact site, the osteoblast took up portions of the specialized domain and internalized them into long-lived, SARA (Smad anchor for receptor activation)-positive, signaling endosomes. This caused the osteoblasts to downregulate Smad signaling and to increase their production of stromal-derived factor-1 (SDF-1), a chemokine responsible for HSPC homing to bone marrow. Targeted regulation of signaling and remodeling events within the osteoblastic niche microenvironment thus involves intercellular transfer—from HSPC to signaling endosomes within osteoblasts.

Patterning in the *Drosophila* embryo requires local activation and dynamics of proteins in the plasma membrane (PM). How these events are coordinated before cellularization—in the absence of PM barriers—remains unclear. We used in vivo fluorescence imaging to characterize the organization and diffusional properties of the PM in embryos expressing various PM proteins. Before cellularization, the PM was polarized into discrete domains with epithelial-like characteristics. One domain resided above individual nuclei and exhibited apical-like characteristics while the other domain was lateral to nuclei and contained markers associated with basolateral membranes and junctions. Pulse-chase photoconversion experiments showed that molecules can diffuse in each domain but do not exchange between PM regions above adjacent nuclei. Drug-induced F-actin depolymerization disrupted the localization of PM polarity markers and abolished the restricted diffusion pattern in the PM. Our findings suggest a new model of PM organization in the syncytial embryo, in which epithelial-like properties and an intact F-actin network compartmentalize the PM and shape morphogen gradients.

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SMALL NONCODING RNAS AND SMALL ORFS

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Currently, we have two main interests: (1) identification and characterization of small, noncoding RNAs and (2) identification and characterization of small ORFs. For several reasons, researchers have tended to overlook small RNAs and small proteins. Small, noncoding RNAs and small ORFs have been overlooked because they do not lend themselves to detection in biochemical assays. Most genome annotation misses the corresponding genes, which are poor targets for genetic approaches.

Identification of small, noncoding RNAs
Zhang, Storz; in collaboration with Gottesman

We have carried out several systematic screens for small, noncoding RNA genes in *Escherichia coli*. The screens are all applicable to other organisms. One approach—based on computer searches of intergenic regions for extended regions of conservation among closely related species—led to the identification of 17 conserved small RNAs. Another screen for small RNAs that co-immunoprecipitate with the RNA-binding protein Hfq allowed us to detect six less well-conserved RNAs. A third approach—size fractionation of total RNA followed by linker ligation and cDNA synthesis—resulted in the identification of still other small RNAs. We recently obtained tiled microarrays that provide coverage of the entire *E. coli* genome and are now using the arrays to extend our identification of small RNAs.

Storz G, Haas D. A guide to small RNAs in microorganisms. *Curr Opin Microbiol* 2007;10:93-95.

Characterization of specific small, noncoding RNAs
Durand, Fontaine, Fozo, Mendieta, Opdyke, Stamper, Waters, Zhang, Storz; in collaboration with Aravind, Kawano, Rudd

Increasingly, we have been focusing on elucidating the functions of the small RNAs in *E. coli*. We previously showed that the OxyS RNA, whose expression is induced in response to oxidative stress, represses translation by basepairing with target mRNAs. OxyS RNA action is dependent on the Sm-like Hfq protein, which functions as a chaperone to facilitate OxyS RNA basepairing with its target mRNAs. We also discovered that the abundant 6S RNA binds to and modifies RNA polymerase. In addition, we elucidated the functions of the MicC RNA and GadY RNA, which also bind to Hfq and act by basepairing. We found that the MicC RNA represses translation of the OmpC outer membrane porin. Interestingly, under most conditions, the MicC RNA shows expression opposite that of the MicF RNA, which represses expression of the OmpF porin. Basepairing between the GadY

RNA and the 3′-untranslated region (3′ UTR) of the *gadX* mRNA encoded opposite *gadY* leads to an increase in levels of the *gadX* mRNA and GadX protein. Increased GadX levels in turn result in increased expression of the acid-response genes controlled by the GadX transcription factor.

In one recent study, we characterized a small RNA (SymR). It is encoded in *cis* to an SOS-induced gene whose product shows homology to the antitoxin MazE (SymE). We demonstrated that synthesis of the SymE protein is tightly repressed at several levels by the LexA repressor, the SymR RNA, and the Lon protease. SymE co-purifies with ribosomes, and overproduction of the protein leads to cell growth inhibition, decreased protein synthesis, and increased RNA degradation. These properties are shared with several RNA endonuclease toxins of the toxin-antitoxin modules, and we reported that the SymE protein represents evolution of a toxin from AbrB fold proteins, which are typically antitoxins. We suggest that SymE promotion of RNA cleavage may be important for the recycling of RNAs damaged under SOS-inducing conditions.

In another recent study, we characterized the Sib RNAs, which are encoded by five repeats in *E. coli* K-12, though the number of repeats varies among *E. coli* strains. All five Sib RNAs in *E. coli* K-12 are expressed, and we observed no phenotype for a five-*sib* deletion strain. However, we observed a phenotype reminiscent of plasmid addiction for overexpression of the Sib RNAs. Further examination of the SIB repeat sequences revealed conserved open reading frames encoding highly hydrophobic 18–19 amino acid proteins (Ibs) opposite each *sib* gene. The Ibs proteins were toxic when overexpressed, although the toxicity could be prevented by co-expression of the corresponding Sib RNA. Two other RNAs encoded divergently in the *yfhL-acpS* intergenic region were similarly found to encode a small hydrophobic protein (ShoB) and an antisense RNA regulator (OhsC). Overexpression of both IbsC and ShoB led to immediate changes in membrane potential, suggesting that both proteins affect the cell envelope. Whole-genome expression analysis showed that overexpression of IbsC and ShoB, as well as of the small hydrophobic LdrD and TisB proteins, has both overlapping and unique consequences for the cell. We are currently engaged in studies characterizing the OxyS, GadY, and Sib RNAs and elucidating the roles of other newly discovered small RNAs.

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Identification and characterization of small ORFs

Astarita, Fontaine, Hemm, Hobbs, Storz; in collaboration with Paul, Rudd, Schneider

In our genome-wide screens for small RNAs, we found that several short RNAs actually encode small proteins. The correct annotation of the smallest proteins is one of the greatest challenges of genome annotation. Perhaps more important, few annotated short ORFs have been confirmed to correspond to synthesized proteins. Although small proteins have been largely overlooked, the few small proteins subjected to detailed study in bacterial and mammalian cells have revealed important functions in signaling and in cellular defenses. Thus, we initiated a project to identify *E. coli* proteins of less than 50 amino acids and elucidate their functions by using many of the approaches with which our laboratory has characterized the functions of small, noncoding RNAs.

We used sequence conservation and ribosome binding site models to predict genes encoding small proteins, which are defined as proteins with 16–50 amino acids, in the intergenic regions of the *E. coli* genome. We tested expression of the predicted and previously annotated small proteins by integrating the sequential peptide affinity tag directly upstream of the stop codon on the chromosome and then using immunoblot assays to assay for synthesis. Our approach confirmed the synthesis of 20 previously annotated and 18 newly discovered proteins of 16–50 amino acids. Remarkably, more than half of the newly discovered proteins are predicted to be single transmembrane proteins,

of which nine co-fractionate with cell membranes. Systematic screens for (1) growth conditions that lead to increased expression and (2) phenotypes associated with null mutations are beginning to provide insights into the physiological roles of these small proteins.

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Characterization of the OxyR and Fur transcription regulators

Storz; in collaboration with Schneider

Previously, a major focus of our laboratory was the characterization of the OxyR transcription regulator, particularly its sensitivity to oxidation and its binding to DNA. We concluded our work in this area with a study of OxyR mutants that defined a region where OxyR contacts RNA polymerase. In collaboration with Thomas Schneider, we also completed a computational analysis of DNA sites that bind to the iron repressor protein Fur.

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MITOTIC REGULATION IN HIGHER EUKARYOTES BY RAN AND SUMO-1

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Our studies focus on two closely linked biochemical pathways that have been implicated in both mitotic regulation and nuclear-cytoplasmic trafficking: the SUMO pathway and the Ran pathway. SUMO proteins constitute a family of ubiquitin-like proteins that become covalently conjugated to cellular targets. Our studies on SUMOylation investigate the physiological roles of ubiquitin-like protease/sentrin-specific protease (Ulp/SENP) family members. Ulps/SENPs play a pivotal role in determination of the spectrum of conjugated species by directly regulating the production of free, conjugatable SUMO proteins and the half-life of conjugated species. The Ran GTPase controls many cellular functions, including nucleocytoplasmic trafficking, spindle assembly, nuclear assembly, and cell cycle progression. We are particularly interested in the role(s) of Ran at mitotic kinetochores, where it is essential for regulation of the spindle assembly checkpoint and for assembly of microtubule fibers that attach kinetochores to spindle poles. We are currently focusing on mechanisms that target Ran pathway components to the kinetochore as well as on interactions of Ran pathway components at kinetochores with other proteins that are structural or functional components of the interphase nuclear pore.

SUMO family small ubiquitin-like modifiers in higher eukaryotes

Mukhopadhyay, Ouspenskaia, Wang, Yun, Choi; in collaboration with Yergey, Wilkinson

SUMO proteins are ubiquitin-like modifiers that become covalently attached (conjugated) to other proteins within cells, thereby regulating the behavior of their targets. While budding yeast has a single SUMO, called Smt3p, there are three commonly expressed mammalian SUMO paralogues called SUMO-1, SUMO-2, and SUMO-3. SUMO-2 and SUMO-3 are 96 percent identical while SUMO-1 is approximately 45 percent identical to either SUMO-2 or SUMO-3. (Here, SUMO-2 and SUMO-3 will be collectively called SUMO-2/3 when they cannot be distinguished from each other.) SUMO conjugation targets play essential roles in many processes, including gene expression, cell cycle progression, synthesis and repair of DNA, response to extracellular stimuli, and nucleocytoplasmic transport. Through these targets, SUMO proteins have been implicated in many human diseases, including cancers, Alzheimer's disease, Huntington's disease, Parkinson's disease, and type I diabetes.

The conjugation pathway for SUMO proteins is similar to the ubiquitin conjugation pathway: SUMO proteins are processed by Ulps/SENPs to reveal a diglycine motif at their C-termini. After

processing, SUMO proteins undergo ATP-dependent formation of a thioester bond to their activating (E1) enzyme, Aos1/Uba2. The activated SUMO proteins are transferred to form a thioester linkage with their conjugating (E2) enzyme, Ubc9. Finally, an isopeptide bond forms between SUMO proteins and substrates through the cooperative action of Ubc9 and protein ligases (E3). Ulps/SENPs can sever the linkage of SUMO proteins to their substrates; therefore, it is likely that SUMO modification is highly dynamic in vivo.

There are six members of the Ulp/SENP family in mammals and five in amphibians such as *Xenopus laevis*. We are systematically evaluating the physiological roles and regulation of these enzymes and are especially interested in the role of vertebrate Ulps/SENPs in the cell cycle and nuclear transport. In budding yeast, Ulps/SENPs are both cell cycle–regulated and required for mitotic events, particularly chromosome segregation. Similarly, yeast data suggest that Ulps/SENPs are controlled through localization to the nuclear pore complex (NPC) and that the SUMO pathway plays a significant role in nuclear trafficking.

We have shown that SENP6 (also called SUSP1) localizes within the nucleoplasm, where it plays a specialized role in dismantling highly conjugated SUMO-2 and SUMO-3 species. The function is similar to the chain-editing activity of SENP6's closest relative in budding yeast, Ulp2. We examined the role of SENP6 in chromosome segregation, a process that, in yeast, requires Ulp2p, and found that siRNA-mediated knockdown of SENP6 in HeLa cells leads to chromosome misalignment. This phenotype is accompanied by defects in spindle assembly and mitotic progression. To understand the underlying molecular defect, we systematically evaluated the behavior of kinetochore-associated proteins, particularly those that are SUMOylated in other contexts. We found both microtubule-dependent and microtubule-independent changes in kinetochore composition and are currently working to establish whether the alterations may be directly linked to the modification of individual kinetochore proteins.

We have found that two Ulp/SENP family members, SENP3 and SENP5, localize within the granular component of the nucleolus, a subnucleolar compartment that contains B23/Nucleophosmin. B23/Nucleophosmin is an abundant shuttling phosphoprotein that plays important roles in ribosome biogenesis and has been strongly implicated in hematopoietic malignancies. Moreover, we found that B23/Nucleophosmin binds to SENP3 and SENP5 in *Xenopus* egg extracts and promotes the stability of SENP3 and SENP5 in mammalian tissue culture cells. After either co-depletion of SENP3 and SENP5 or depletion of B23/Nucleophosmin, we observed accumulation of SUMO proteins within nucleoli. Finally, depletion of these Ulps/SENPs caused defects in ribosome biogenesis and export reminiscent of phenotypes observed in the absence of B23/Nucleophosmin. Together, our results suggest that regulation of SUMO deconjugation may be a major facet of B23/Nucleophosmin function in vivo. In an effort to understand how SUMOylation contributes to ribosome biogenesis, we are currently examining SENP3 and SENP5 regulation and identifying ribosomal SUMOylation targets.

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Regulation of mitotic kinetochores by the Ran GTPase

Arnaoutov, Boyarchuk, Furuta, Ouspenskaia, Lukasiewicz; in collaboration with Yergey

The Ran GTPase is required for many cellular functions, including nucleocytoplasmic trafficking, spindle assembly, nuclear assembly, and cell cycle control. The sole nucleotide exchange factor for Ran, RCC1, binds to chromatin throughout the cell cycle. RanGAP1, the GTPase-activating protein

for Ran, localizes to the cytosolic face of the nuclear pore complex (NPC) during interphase through association with RanBP2, a large nucleoporin. The interphase distribution of Ran regulators leads to a high concentration of Ran-GTP in nuclei and low Ran-GTP in cytosol. The major effectors for Ran are a family of Ran-GTP–binding proteins called karyopherins, which were discovered as nuclear transport receptors. Karyopherins transit the NPC in a Ran- and cargo-independent fashion. Karyopherins that mediate import are called importins, which bind to their cargo in the cytoplasm. Import complexes traverse the NPC and dissociate upon Ran-GTP-importin binding. Karyopherins that mediate export are called exportins, which bind to their cargo inside nuclei in complexes that contain Ran-GTP. After passage through the NPC, export complexes dissociate upon Ran-GTP hydrolysis. To date, two nuclear transport receptors have been shown to act as Ran effectors during mitosis, importin-beta and the exportin Crm1.

Our studies have focused on Ran functions at kinetochores, which are proteinaceous structures that assemble at the centromere of each sister chromatid during mitosis and serve as sites of spindle microtubule attachment. The kinetochore fibers (k-fibers) that link mammalian kinetochores to spindle poles contain microtubules that are directly attached to the kinetochores at their plus ends (kMTs) and microtubules that are not so attached. Kinetochore attachment is monitored through the spindle assembly checkpoint (SAC), which prevents mitotic exit by blocking anaphase-promoting complex/cyclosome (APC/C) activation until all chromosomes are attached and aligned onto the metaphase plate. The APC/C is a ubiquitin ligase that regulates the destruction of key mitotic regulatory proteins. Components of the SAC include Mad1, Mad2, Mps1, Bub1, Bub3, BubR1, and CENP-E. During mitosis, RanGAP1 and its binding partner RanBP2 associate with kinetochores in a microtubule-dependent fashion.

We showed that Crm1 localizes to kintochores and that inhibition of Crm1 ternary complex formation blocks recruitment of RanGAP1/RanBP2. Crm1 itself requires neither ternary complex assembly nor microtubules for kinetochore binding. We observed that, in the absence of Crm1 function, centromeres of mammalian tissue culture cells were under increased tension and that their kinetochores dramatically failed to maintain discrete attachments to single k-fibers. These defects caused increased chromosome mis-segregation. Our findings have several implications. First, they link RanGAP1/RanBP2 to correct k-fiber assembly. Second, they suggest that Ran has a kinetochore-associated effector pathway through Crm1. Third, they show that several karyopherins act as Ran effectors during mitosis; in principal, it is possible that other members of this family may also act during mitosis. The component(s) at kinetochores that is directly involved in Crm1 recruitment is a major focus of our current studies.

In addition, we have examined the function of SAC components within mitotic cells, particularly the Bub1 kinase's role at the inner centromeres (IC) and its relationship with the chromosomal passenger complex (CPC). Protein complexes of the IC regulate sister chromatid cohesion and modulate MT attachment. The CPC consists of the Aurora B kinase, INCENP, Survivin, and Dasra/Borealin. Our findings suggest that Bub1 regulates localization of IC components through mechanisms that are both CPC-dependent and -independent. Remarkably, Bub1's kinase activity is essential for IC assembly, in contrast to its roles at outer kinetochores.

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Mitotic roles of nuclear pore complex proteins

Mishra, Ouspenskaia, Yoon; in collaboration with Yergey, Fontoura, Joseph

Vertebrate nuclear pore complexes (NPC) are large structures comprising about 30 distinct resident polypeptides (nucleoporins). During interphase, NPCs are conduits for transport of large substrates into the nucleus, allowing the passive diffusion of small molecules. Mutations in many nucleoporins are associated with human disease, including triple A syndrome, leukemias, and hematological malignancies as well as other cancers. During mitosis, metazoan NPCs disassemble into approximately a dozen subunits, at least two of which are targeted to mitotic kinetochores. The RanBP2 complex associates with kinetochores in a microtubule-dependent manner and consists of RanBP2 (a large nucleoporin also known as Nup358), SUMO-1–conjugated RanGAP1 (the activating protein for the Ran GTPase), and Ubc9 (the conjugating enzyme for the SUMO family of ubiquitin-like modifiers). The vertebrate Nup107–160 complex associates with kinetochores throughout mitosis in a microtubule-independent manner and includes Nup160, Nup133, Nup107, Nup96, Nup85, Nup43, Nup37, Sec13, and Seh1.

Kinetochores are the primary site of attachment for chromosomes to the microtubules of the mitotic spindle and thus mediate the correct segregation of genetic material into daughter cells at anaphase. The SAC, which monitors kinetochore attachment, is a cell cycle–regulatory pathway that prevents the onset of anaphase until all chromosomes are properly attached to the mitotic spindle and aligned on the metaphase plate. Failure of the SAC is associated with chromosomal instability (CIN) leading to an abnormal chromosome number (aneuploidy). Such genetic alteration appears to be an important event in the origin of many human cancers. Our laboratory and others have shown that kinetochore-bound nucleoporins play essential roles in mitosis. We wish to understand these roles and to elucidate nucleoporin interactions with other kinetochore proteins. We use both in vivo (tissue culture cells) and in vitro (*Xenopus laevis* egg extracts) approaches to study these issues at a biochemical level, with an eventual goal of applying the knowledge gained to understanding the roles of NPC components in human disease.

During the past year, we demonstrated an interaction of the RanBP2 complex with the interphase cytoskeleton and found that a fraction of endogenous RanBP2 interacts with interphase microtubules through its N-terminal region (BPN). Overexpression of the BPN domain of RanBP2 caused dramatic increases in microtubule bundling and stability. Ectopic expression of BPN and full-length RanBP2 resulted in elevated levels of acetylated and detyrosinated alpha-tubulin, two markers for stable microtubules. Under the same circumstances, microtubules showed resistance to nocodazole, a depolymerizing agent. Depletion of RanBP2 disrupted polarized stabilization of microtubules during directed cell migration, showing that RanBP2 regulates interphase microtubules under physiological conditions. We are currently examining novel RanBP2 binding components and their role in regulation of RanBP2's activity in microtubule dynamics during both interphase and mitosis.

Nup107–160 remains bound to the kinetochore throughout mitosis and shows enhanced accumulation on unattached kinetochores. We recently found that Nup107–160 interacts with an active form of the gamma-tubulin ring complex (gamma-TuRC), an essential and conserved microtubule nucleator. Like Nup107–160, gamma-TuRC localized to unattached mitotic kinetochores, prompting us to test whether Nup107–160 and gamma-TuRC might function in a coordinated manner to promote nucleation of microtubules near mitotic chromosomes and at kinetochores. *Xenopus* egg extracts lacking the Nup107–160 complex or gamma-TuRC failed to assemble spindles around sperm chromatin or DNA beads. Moreover, HeLa cells lacking Nup107–160 or gamma-TuRC were profoundly deficient in kinetochore-associated microtubule nucleation. Our findings indicate that Nup107–160 promotes spindle assembly through the regulated nucleation of microtubules by gamma-TuRC at kinetochores and perhaps other sites within mitotic spindles. These observations suggest an important and novel relationship between the NPC and the microtubule cytoskeleton.

Our future studies will focus on several issues, including the components at kinetochores involved in recruitment of the Nup107–160 and RanBP2 complexes, the relationship between these complexes during mitosis, and how the complexes combine to regulate the attachment of microtubules to kinteochores and the spindle assembly checkpoint. We are also examining the mitotic localization and function of other NPC components.

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MECHANISM AND REGULATION OF EUKARYOTIC PROTEIN SYNTHESIS

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We study the mechanism and regulation of protein synthesis, focusing on GTPases and protein kinases that control this fundamental cellular process. We use molecular-genetic and biochemical studies to dissect the structure-function properties of the translation initiation factors eIF2—a GTPase that binds methionyl-tRNA to the ribosome—and eIF5B—a second GTPase that catalyzes ribosomal subunit joining in the final step of translation initiation. Our studies have revealed a critical role for eIF2 in start codon selection and have defined the important role of GTP hydrolysis in governing the release of eIF5B and eIF1A from the ribosome following subunit joining. We also study stress-responsive protein kinases that phosphorylate eIF2alpha. Recent studies on the antiviral kinase PKR revealed the importance of dimerization for kinase activation, and we are currently studying viral regulation of PKR by poxvirus inhibitors of the kinase. Finally, in related studies, we have characterized the structure-function properties of IRE1, an endoplasmic reticulum (ER) stress–responsive dual kinase-endonuclease.

Structure-function analysis of eIF2gamma, the GTP- and Met-tRNA–binding subunit of the eIF2 complex

Alone,[1] Cao, Dever; in collaboration with Lorsch

The translation initiation factor eIF2 is composed of three polypeptide chains that assemble to form a stable complex. The gamma subunit of eIF2 contains a consensus GTP-binding (G) domain, and the factor must bind to GTP to form a stable eIF2•GTP•Met-tRNA ternary complex. We previously demonstrated that the GTPase-activating protein (GAP) eIF5, which promotes GTP hydrolysis by eIF2, and the guanine-nucleotide exchange factor (GEF) eIF2B, responsible for exchanging GTP for GDP on eIF2, directly bind to the G domain of eIF2gamma (Alone and Dever, 2006).

To gain a deeper understanding of the role of GTP binding and hydrolysis by eIF2, we mutated the conserved Asn135 residue in the eIF2gamma Switch I element to Asp. The N135D mutation impaired Met-tRNA binding to eIF2 and caused a Sui$^-$ phenotype, enhancing initiation from a non-canonical UUG codon. Previous studies in the Donahue laboratory correlated a Sui$^-$ phenotype with decreased Met-tRNA binding affinity, suggesting that premature release of Met-tRNA from eIF2 led to initiation at the UUG codon. Consistently, an A208V mutation restored Met-tRNA binding affinity and suppressed the slow-growth and Sui$^-$ phenotypes of the eIF2gamma-N135D mutant. In contrast, an A382V mutation restored Met-tRNA binding and suppressed the slow-growth, but not the Sui$^-$, phenotype. Moreover, an eIF2gamma-A219T mutation impaired Met-tRNA binding

but unexpectedly enhanced the fidelity of initiation, suppressing the Sui⁻ phenotype associated with the eIF2gamma-N135D,A382V mutant. This uncoupling of start codon selection and Met-tRNA binding affinity to eIF2 indicates a more direct role for eIF2 in start site recognition. Interestingly, overexpression of eIF1, which is thought to monitor codon-anticodon interaction during translation initiation, likewise suppressed the Sui⁻ phenotype of the eIF2gamma mutants. We propose that structural alterations in eIF2gamma subtly alter the conformation of Met-tRNA on the 40S subunit and thereby affect the fidelity of start codon recognition independently of Met-tRNA binding affinity.

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Structure-function analysis of the universally conserved translational GTPase eIF5B/IF2
Shin, Fringer, Cao, Kim, Dever; in collaboration with Lorsch

In the final step of translation initiation, the large 60S ribosomal subunit joins with the 40S subunit, already bound to an mRNA, to form an 80S ribosome competent for protein synthesis. We previously discovered the translation initiation factor eIF5B, an orthologue of the bacterial translation factor IF2, and showed that it catalyzes ribosomal subunit joining. The GTPase binds to GTP and hydrolyzes the nucleotide in the presence of 80S ribosomes. Our current efforts aim to elucidate eIF5B's structure-function properties and to understand the role played by eIF5B in GTP binding and hydrolysis.

Our previous studies on an eIF5B mutant that was unable to hydrolyze GTP revealed that GTP hydrolysis by eIF5B activates a regulatory switch required for eIF5B release from the ribosome following subunit joining (Shin et al., *Cell* 2002;111:1015). Consistently, mutation of the conserved Gly479 residue in the D-x-x-G G-3 sequence motif, whose movement is thought to be critical for structural transitions of the G domain during GTP binding and hydrolysis, impaired yeast cell growth, eIF5B guanine-nucleotide binding, GTPase, and ribosomal subunit joining activities (Shin et al., 2007). Intragenic suppressor mutations in the G domain (A444V) and in a residue in domain 2 of eIF5B (D740R) that interacts with the G domain restored yeast cell growth and eIF5B nucleotide binding, GTP hydrolysis, and subunit joining activities. We propose that the Gly479 mutation distorted the geometry of the GTP-binding active site, impairing nucleotide binding and the eIF5B domain movements associated with GTP binding. Accordingly, the two suppressor mutations induce the D-x-x-G motif to adopt a conformation favorable for nucleotide binding and hydrolysis and thereby re-establish coupling between GTP binding and eIF5B domain movements (Shin et al., 2007).

In collaboration with Jon Lorsch, we showed that interaction between the C-termini of eIF5A and eIF1A is critical for eIF5B association with 40S ribosomes in vivo and for ribosomal subunit joining and translation-coupled eIF5B GTPase activity in vitro (Fringer et al., 2007; Acker et al., 2006). Moreover, blocking eIF5B GTP hydrolysis led to the accumulation of both eIF1A and eIF5B on the 80S products of subunit joining both in vivo and in vitro. Based on these findings, we propose that eIF1A facilitates the binding of eIF5B to the 40S subunit to promote subunit joining and that subsequent release of eIF1A is dependent on GTP hydrolysis and release of eIF5B from the 80S ribosome (Fringer et al., 2007).

The function of both eIF5B and the translational GTPases that promote elongation and termination of protein synthesis relies on proper interaction with the ribosome. While cryo-EM studies have revealed binding sites for the GTPases on the ribosome, in vivo data supporting these sites have not been reported. As mentioned above, mutations that impair GTP hydrolysis by the eIF5B impair yeast cell growth due to failure to dissociate from the ribosome following subunit joining. After identifying intragenic suppressor mutations of eIF5B GTPase-deficient mutants that restore cell growth by lowering the ribosome binding affinity of eIF5B, we reasoned that it should be

possible to obtain mutations in the ribosome that likewise reduce eIF5B binding and suppress the toxic affects associated with expression of GTPase-defective mutants of eIF5B. An eIF5B-H480I mutation abolishes GTPase activity and causes a severe growth defect in yeast. We identified a mutation in helix h5 of the 18S rRNA in the 40S ribosomal subunit and intragenic mutations in domain II of eIF5B that suppress the toxic effects associated with expression of the eIF5B-H480I mutant in yeast. Both the rRNA and intragenic mutations lowered the ribosome binding affinity of eIF5B, indicating that the mutations enable eIF5B release from the ribosome in the absence of GTP hydrolysis. Linking the hydroxyl radical generator BABE at the sites of the domain II suppressors in eIF5B, we mapped the region of the ribosome contacted by domain II of eIF5B. Interestingly, the domain II suppressors contacted the body of the 40S subunit in the vicinity of helix h5. Thus, the rRNA and domain II suppressors affect the same contact surface between eIF5B and the 40S ribosomal subunit. Given that the helix h5 mutation also impairs translation elongation factor function, we propose that the rRNA and eIF5B suppressor mutations provide in vivo evidence supporting a functionally important docking of domain II of the translational GTPases on the body of the small ribosomal subunit.

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Activation and substrate recognition by the eIF2alpha protein kinases PKR and GCN2
Dey, Cao, Rothenburg, Dever; in collaboration with Sicheri

Phosphorylation of eIF2alpha is a common mechanism for downregulating protein synthesis under stress conditions. Four kinases phosphorylate eIF2alpha on Ser51 under different cellular stress conditions. GCN2 responds to amino acid limitation, HRI to heme deprivation, PERK to ER stress, and PKR to viral infection. Consistent with their common activity to phosphorylate eIF2alpha on Ser51, the kinases show strong sequence similarity in their kinase domains. Phosphorylation of eIF2alpha converts eIF2 from a substrate to an inhibitor of its guanine-nucleotide exchange factor eIF2B. The inhibition of eIF2B impairs general translation, slowing the growth of yeast cells and, paradoxically, enhancing the translation of the *GCN4* mRNA required for yeast cells to grow under amino acid starvation conditions. We used structural, molecular, and biochemical studies to define how the eIF2alpha kinases recognize their substrate.

In collaboration with Frank Sicheri, we obtained the X-ray structure of eIF2alpha bound to the catalytic domain of PKR (Dar et al., *Cell* 2005;122:887). Back-to-back dimerization enables each PKR protomer to engage a molecule of eIF2alpha in the crystal structure. Given that all four eIF2alpha kinases share the PKR residues mediating kinase domain dimerization and eIF2alpha recognition, we propose that all four kinases similarly dimerize and recognize eIF2alpha. Consistent with this hypothesis, mutations designed to disrupt and then restore an intermolecular salt bridge in the PKR dimer interface had the expected impact on PKR, PERK, and GCN2 kinase activity (Dey et al., *Cell* 2005;122:901; Dey et al., 2007). We conclude that the back-to-back dimer orientation observed in the PKR crystal structure is critical for the activity of PKR, GCN2, and PERK and that the PKR structure represents the active state of the eIF2alpha kinase domain.

In a systematic screen of residues conserved among the eIF2alpha kinase domains, we identified mutations in helix alphaG of PKR that blocked phosphorylation of eIF2alpha but not phosphorylation

of the non-specific substrate histone, consistent with the docking of eIF2alpha on helix alphaG in the PKR•eIF2alpha co-crystal structure. Based on our results, we propose an ordered mechanism of PKR activation in which catalytic domain dimerization triggers autophosphorylation on Thr446, which in turn is required for specific eIF2alpha substrate recognition (Dey et al., *Cell* 2005;122:901).

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Poxvirus regulation of protein kinase PKR

Seo, Cao, Rothenburg, Dever; in collaboration with Sicheri

As part of the mammalian cell innate immune response, the double-stranded RNA–activated protein kinase PKR phosphorylates the translation initiation factor eIF2α to inhibit protein synthesis and thus block viral replication. To subvert this host cell defense mechanism, viruses produce inhibitors of PKR. Several members of the poxvirus family express two types of PKR inhibitor: a pseudosubstrate inhibitor and a double-stranded RNA–binding protein called E3L. The vaccinia virus K3L protein resembles the N-terminal third of eIF2alpha, with both proteins containing a beta-barrel fold of the OB-fold family. Whereas high-level expression of human PKR was toxic in yeast, co-expression of the vaccinia virus K3L protein or the related variola (smallpox) virus C3L protein suppressed such growth inhibition. We used this yeast assay to screen for PKR mutants resistant to K3L inhibition and identified 12 mutations mapping to the C-terminal lobe of the PKR kinase domain in the vicinity of the eIF2alpha binding site. The PKR mutations specifically conferred resistance to the K3L protein, but not to the E3L protein, both in yeast and in vitro. In vitro studies revealed that wild-type PKR and the PKR mutants phosphorylated eIF2alpha with the same kinetics; however, the mutant kinase was less sensitive to inhibition by K3L. Consistently, the PKR-D486V mutation led to a nearly 15-fold decrease in K3L binding affinity. Our results support the identification of the eIF2alpha binding site on an extensive face of the C-terminal lobe of the kinase domain and indicate that subtle changes to the PKR kinase domain can drastically affect pseudosubstrate inhibition while leaving substrate phosphorylation intact. We propose that these paradoxical effects of the PKR mutations on pseudosubstrate versus substrate interactions reflect differences between the rigid K3L protein and the plastic nature of eIF2alpha around the Ser51 phosphorylation site.

In related studies, we are identifying and characterizing PKR mutants resistant to inhibition by the E3L protein. These PKR mutations map to both the kinase domain dimerization interface and the N-terminal regulatory [double-stranded RNA– (dsRNA) binding] domain of the protein. Consistent with the different natures of the two poxvirus inhibitors of PKR, the PKR mutations that confer resistance to E3L do not confer resistance to K3L. The identification of E3L-resistant mutations in the PKR dimerization interface is consistent with the notion that E3L inhibits PKR by forming inactive heterodimers with the kinase. Future in vitro studies will directly examine the impact of the mutations on PKR dimerization and E3L inhibition.

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Structural and molecular analysis of the ER stress–responsive kinase-endonuclease IRE1
Dey, Cao, Dever; in collaboration with Sicheri

Accumulation of misfolded proteins in the ER under stress conditions activates a stress response pathway referred to as the unfolded protein response (UPR). The primary sensor of ER stress in the UPR is the protein IRE1. IRE1 is a transmembrane protein with an N-terminal domain situated in the ER lumen and a cytoplasmic domain consisting of a protein kinase domain and C-terminal kinase-associated nuclease (KEN) domain. Dimerization of IRE1 under ER stress conditions activates kinase autophosphorylation and nuclease activity. The KEN domain then splices, in a spliceosome-independent manner, mRNAs encoding transcriptional regulators of the UPR—*HAC1* mRNA in yeast and *Xbp1* mRNA in mammals. In collaboration with Frank Sicheri, we obtained the crystal structure of the cytoplasmic catalytic domain of IRE1, which revealed the structure of both the kinase and KEN domain (Lee et al., 2008). Back-to-back dimerization of the kinase domain in the IRE1 crystal structure juxtaposes the KEN domains and activates the ribonuclease. We identified four autophosphorylation sites in the IRE1 kinase domain, and mutational and biochemical studies revealed that autophosphorylation facilitates ATP binding and the accompanying dimerization of the kinase domain. Yeast cells expressing IRE1 mutants with mutations in the dimer-contact residues were unable to grow in medium containing tunicamycin, an inhibitor of protein glycosylation and inducer of ER stress. Likewise, the dimerization mutants were unable to splice the Xbp1 mRNA in vitro. Comparison of the structure of the IRE1 KEN domain with the structure of the structurally distinct tRNA endonuclease identified putative nuclease active site-residues. Mutation of these residues blocked Xbp1 mRNA splicing in vitro and yeast cell growth under ER stress conditions. Taken together, the data reveal an unexpected convergent evolution of the tRNA endonuclease and IRE1 KEN-domain catalytic mechanism (Lee et al., 2008). We propose that dimerization of IRE1 lumenal domains in response to ER stress promotes kinase domain trans-autophosphorylation, which in turn facilitates nucleotide binding and back-to-back dimerization of the kinase domains. Dimerization of the KEN domains in the resultant structure enables recognition and splicing of the *HAC1*/Xbp1 mRNA.

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TRANSCRIPTIONAL AND TRANSLATIONAL REGULATORY MECHANISMS IN NUTRIENT CONTROL OF GENE EXPRESSION

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We study transcriptional and translational mechanisms that control the expression of amino acid–biosynthetic genes by nutrients in the yeast *Saccharomyces cerevisiae*. We analyze the physical and functional interactions of the of translation initiation factor eIF2 with other initiation factors (eIF-1, eIF -1A, eIF-3, and eIF-5) and the 40S ribosome that promote the eIF2-GTP-$tRNA_i^{Met}$ ternary complex (TC) recruitment and ribosomal scanning during general translation and translation that is specific for the transcriptional activator GCN4. We also study the conformational changes that both occur in the scanning pre-initiation complex and govern stringent selection of AUG as start codon. Regarding transcriptional control, we analyze co-activators required for gene activation by GCN4 to define the molecular program for recruitment of chromatin-remodeling enzymes and adaptor proteins that deliver TATA-binding protein, other general factors, and RNA polymerase II to the promoter. We also analyze association of co-activators with transcribed coding sequences and their roles in histone eviction, transcription elongation, and mRNA export from the nucleus.

Genetic identification of yeast 18S rRNA residues required for recruitment of initiator $tRNA^{Met}$ and AUG selection

Dong, Rahman,[1] Pruitt,[2] Wong,[3] Hinnebusch; in collaboration with Nanda, Lorsch, Shin

Translation of the key transcriptional activator GCN4 is stimulated in amino acid–starved cells by a mechanism involving short open reading frames (uORF) in the mRNA leader and by phosphorylation of translation initiation factor 2 (eIF2), which, bound to GTP, delivers initiator $tRNA_i^{Met}$ to the 40S ribosome. Phosphorylation of eIF2 by the kinase GCN2 inhibits formation of the eIF2-GTP-$tRNA_i^{Met}$ ternary complex (TC), reducing general protein synthesis but derepressing translation of GCN4, with attendant transcriptional activation of amino acid biosynthetic–genes (the Gcd^- phenotype).

In translation initiation, the AUG start codon is decoded in the peptidyl (P) site of the ribosome by initiator Met-$tRNA_i^{Met}$. However, given the near absence of information on the importance of particular residues in 18S rRNA for efficient P-site binding of $tRNA_i^{Met}$ and AUG selection during initiation in eukaryotes, researchers are illuminating the ribosomal determinants of tRNA binding to the P-site during elongation in bacteria using high-resolution crystal structures of 70S ribosomes

bound to mRNA with cognate tRNA in the P-site. While crystal structures of eukaryotic ribosomes do not exist, the three-dimensional structure of the eukaryotic 80S ribosome appears to be similar to that of bacterial 70S ribosomes. Hence, it is possible to make good predictions from the bacterial crystal structures about the locations of specific residues in yeast 18S rRNA that reside in the conserved core of the 40S subunit. Translation of *GCN4* mRNA is a sensitive indicator of the recruitment of eIF2•GTP•Met-tRNA$_i^{Met}$ TC to 40S subunits in yeast cells, a property that we have exploited to identify mutations in eIFs that reduce TC assembly or impair TC recruitment and permit constitutive derepression of *GCN4* translation (Gcd$^-$ phenotype). We used the Gcd$^-$ selection to obtain the first functional evidence for specific residues in 18S rRNA required for efficient TC binding and AUG selection by 40S subunits in vivo. By random mutagenesis of rDNA, we obtained mutations in the residue corresponding to bacterial A928 in conserved helix 28 (h28) that appear to derepress *GCN4* translation by reducing the rate of TC loading on post-termination 40S subunits scanning downstream from uORF1. Interestingly, given that such mutations greatly increase the bypass, or "leaky scanning," of AUG codons at uORFs during primary initiation events, it is possible to distinguish these mutations from existing Gcd$^-$ mutations in various eIFs. The A928 substitutions could allow TC to dissociate from the PIC or disturb the orientation of Met-tRNA$_i^{Met}$ in the P-site in a way that allows AUG to be ignored during the scanning process. Indeed, our biochemical studies reveal defects in both the association rate and stability of TC binding to 40S subunits conferred by the nonlethal A928U substitution (Dong et al., 2008).

The A928:U1389 base pair in helix 28 is not conserved in bacteria and was not previously implicated in P-site binding of tRNA; however, h28 contains a "bulge" residue, G926, that directly contacts

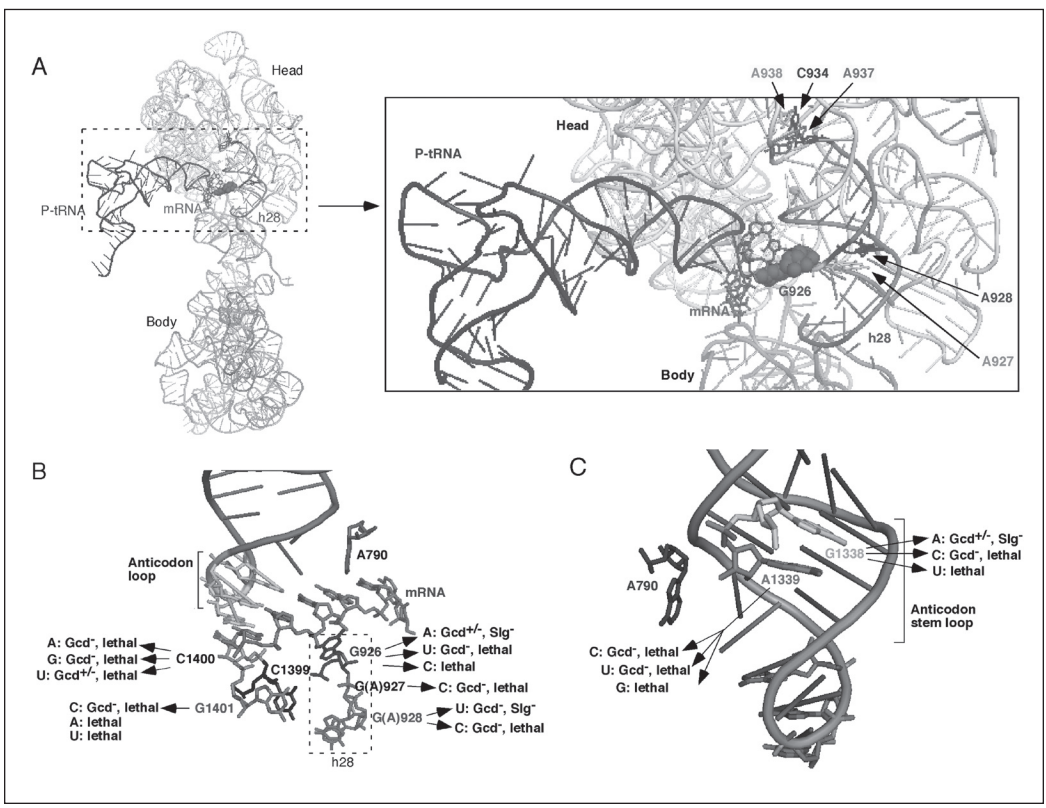

FIGURE 2.1

Substituting nucleotides predicted to contact initiator tRNA in the 40S ribosomal P-site derepress translation of GCN4 mRNA in yeast cells.

the P-site codon in the crystal structures of bacterial 70S elongation complexes. We mutated G926 and other residues that contact the P-site tRNA anticodon (C1400) or ASL (G1338, A1339) in the bacterial 70S crystal structures and found that many such mutations also confer Gcd⁻ phenotypes and increased leaky scanning. Together, our results implicate a novel residue of h28 and a subset of residues making direct contacts with P-site tRNA in bacterial 70S elongation complexes in the stable anchoring of Met-tRNA$_i^{Met}$ to the P-site and efficient AUG recognition during scanning by eukaryotic PICs (Figure 2.1) (Dong et al., 2008).

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Ribosomal protein L33 is required for ribosome biogenesis, subunit joining, and repression of *GCN4* translation

Hinnebusch; in collaboration with Martín-Marcos, Tamame

We identified a new complementation group of Gcd⁻ mutants defined by the *gcd17-1* mutation, which we showed to be an allele of *RPL33A*, one of the two genes encoding the essential 60S ribosomal subunit protein L33. The *gcd17-1* mutation, which substitutes Gly-76 with Arg in rpL33A (*a-G76R*), impedes accumulation of 60S ribosomal subunits at the restrictive growth temperature. The impeded accumulation engenders halfmer polysomes, indicating a decreased rate of 60S–40S subunit joining at the final step of initiation and a strong reduction in general protein synthesis. Interestingly, at permissive temperature, a-G76R derepresses *GCN4* translation and produces a Gcd⁻ phenotype stronger than that produced by deleting *RPL33A* (Δ*A*). This outcome is remarkable because *a-G76R* is less severe than Δ*A* in reducing 60S subunits at the permissive temperature. Analysis of *GCN4-lacZ* reporters suggests that both *a-G76R* and Δ*A* mutations derepress *GCN4* translation in the presence of high TC levels (i.e., in the *gcn2* background) by impairing 60S–40S subunit joining at uORF4 of the *GCN4* mRNA leader. Thus, *a-G76R* might impair an important intersubunit bridge required for efficient 80S initiation complex formation. We propose that inefficient subunit joining at uORF4 allows 40S subunits to abort initiation at the uORF4 start site, to resume scanning, and to reinitiate downstream at *GCN4*. Our finding that the Gcd⁻ phenotype of the *a-G76R* mutation is suppressed by overexpressing tRNA$_i^{Met}$ further suggests that dissociation of tRNA$_i^{Met}$ from the 40S subunit is responsible for the postulated abortive initiation events at uORF4. Our data indicate that rpL33 plays a critical role in the ribosome biogenesis pathway required for efficient production of ribosomal subunits and a second role in 40S–60S subunit joining. Both activities contribute to repression of *GCN4* translation under nonstarvation conditions and, hence, proper functioning of the general amino acid control (Martín-Marcos et al., 2007).

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eIF3a cooperates with sequences 5′ of uORF1 to promote resumption of scanning by post-termination ribosomes for re-initiation on *GCN4* mRNA

Hinnebusch; in collaboration with Szamecz, Valášek

A crucial but vaguely understood feature of *GCN4* translational control is the highly disparate capacities of uORF1 and uORF4 to permit efficient resumption of scanning after translation termination. AU-rich sequences surrounding the stop codon of uORF1 favor resumption of scanning and re-initiation, whereas GC-rich sequences flanking the uORF4 stop codon likely trigger ribosome release. We demonstrated that sequences 5′ of uORF1 are also critical for re-initiation. However, little was known about the *trans*-acting factors crucial for the retention of post-termination 40S subunits at the uORF1 stop codon and for the resumption of scanning required for re-initiation. We

previously found that deleting the N-terminal domain (NTD) and C-terminal domain (CTD) of the a/TIF32 subunit of eIF3 impaired association of otherwise intact eIF3 complexes with the 40S and that the NTD can interact with the 40S protein RPS0A. Here, we found that a partial deletion of the RPS0A-binding site in the tif32-NTD (*Δ8*) is not lethal but reduces the amount of 40S-bound eIFs in vivo, consistent with the idea that the eIF3a-NTD forms a crucial bridge between eIF3 and the 40S. Strikingly, the *Δ8* truncation provokes a severe Gcn$^-$ phenotype with the novel characteristic of impairing retention of post-termination 40S ribosomes at the uORF1 stop codon. Genetic epistasis interactions between mutations in stimulatory sequences upstream of uORF1 and *Δ8* indicate that eIF3a interacts with the re-initiation–enhancing element located 5′ of uORF1. Thus, we propose that interaction between eIF3a-NTD and sequences 5′ of uORF1 at or near the mRNA exit channel of the 40S subunit stabilizes its association with mRNA and promotes the resumption of scanning for downstream re-initiation (Szamecz et al., 2008).

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SUS1 is recruited to coding regions and functions during transcription elongation in association with SAGA and TREX2

Govind, Hinnebusch; in collaboration with Pascual García, Rodríguez-Navarro

Gene expression in eukaryotes depends on the coordinated action of multiprotein complexes that regulate transcription, mRNA biogenesis, or export of mature mRNA from the nucleus. The identification of SUS1 revealed an important link between transcription and mRNA export. SUS1 interacts with the transcriptional co-activator SAGA and the nuclear pore–associated complex TREX2, which is composed of SAC3, THP1, and CDC31. SUS1, SAC3, and THP1 mediate the post-transcriptional tethering of active genes to the nuclear rim for transcription-coupled mRNA export. In addition to the HAT (histone acetyl transferase) subunit GCN5, SAGA contains the ubiquitin protease UBP8, which, together with SUS1 and SGF11, forms a module in SAGA for deubiquitinylation of H2B. Given that binding of SUS1 to SAGA depends on UBP8 and SGF11, the deubiquitinylation module works at the junction between SAGA-dependent transcription and nuclear mRNA export. We showed previously that SAGA localizes to transcribed coding sequences and that GCN5-dependent acetylation promotes nucleosome eviction and processivity of RNA polymerase II (RNAP II) during elongation (Govind et al., 2007). The association of SAGA with coding sequences is dependent on phosphorylation of the CTD of the RNAP II subunit RPB1, indicating that SAGA could interact with actively transcribing RNAP II during elongation.

We have investigated the mechanisms underlying SUS1's role in coupling transcription and mRNA export. We found that SUS1 is required for gene transcription in a length-dependent fashion, indicating an active role in mRNA biogenesis during transcription elongation. Consistently, SUS1 co-purifies with the elongating form of RNAP II phosphorylated on Ser5 and Ser2 of the CTD, the mRNA adaptor YRA1, and the export factor MEX67. We also provide evidence that SUS1 is associated at high levels with *ARG1* coding sequences and that SUS1 occupancy of the ORF requires transcription and Ser5 CTD phosphorylation. Loss of the SAGA subunit SGF73 prevents SUS1 binding to SAGA and, unexpectedly, partially disrupts SUS1-TREX2 association. Whereas UBP8 and SAC3 both promote association of SUS1 with the *ARG1* UAS and coding region, SGF73 is more critical for these interactions. Our data suggest a mechanism by which SUS1 plays a pivotal role in transcription during elongation mediated by both SAGA and TREX2 complexes (Pascual-García et al., 2008).

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Disrupting vesicular trafficking at the endosome attenuates transcriptional activation by GCN4
Zhang, Gaur, Kim,[4] Qiu, Swanson,[5] Hinnebusch; in collaboration with Hašek

We and others have previously identified numerous mutants defective in factors required for translational induction of *GCN4* mRNA or lacking co-activators required for transcriptional activation by GCN4 on the basis of their sensitivity to sulfometuron methyl (SM) or other inhibitors of amino acid biosynthesis. To identify novel factors involved in general amino acid control, we screened a library of haploid deletion mutants for SM sensitivity (SM). Surprisingly, we identified several SM^S/Gcn^- strains with deletions of genes involved in vesicular protein trafficking at the late endosome/multivesicular body (MVB). The late endosome/MVB plays a key role in coordinating vesicular transport of proteins among Golgi, vacuole/lysosome, and plasma membrane. The *vps* mutants are defective for an array of molecules required for producing vesicles with the appropriate cargo proteins or for the tethering and fusion of vesicles at the correct target membranes. We found that GCN4 function is impaired to the greatest extent in class C and D *vps* mutants, which are defective for various aspects of vesicle fusion at the endosome. Mutations in these factors impair activation of GCN4 target promoters and reduce pre-initiation complex (PIC) assembly at *ARG1* without reducing the amount of GCN4 bound to the UAS_{GCRE} in vivo. We also observed SM^S phenotypes for class E *vps* mutants, which lack factors needed to sort cargo proteins into intralumenal vesicles (ILV) at the MVB for subsequent transport to the vacuole lumen. The heteromeric protein complexes ESCRT-I, ESCRT-II, and ESCRT-III, which bind ubiquitinated cargo to the MVB outer membrane, carry out the sorting function. The AAA-ATPase VPS4 then recycles E-III and segregates the cargo into ILVs. Class E *vps* mutants accumulate cargo proteins in aberrant structures lacking ILVs, called class E compartments. The missorted proteins include vacuolar hydrolyases, which are improperly matured and capable of proteolyzing other cargoes that accumulate in the class E compartment. Our detailed analysis of two class E mutants lacking a key component of ESCRT complex E-II (*snf8*Δ) or E-III (*snf7*Δ) revealed significant reductions in activation by nuclear-localized GCN4.

Interestingly, we found class E mutant *vps4*Δ, which is unable to generate ILVs or transport MVB cargo correctly to the vacuole, to have a considerably weaker Gcn^- phenotype than does the *snf7*Δ mutant lacking an E-III subunit. Moreover, the stronger Gcn^- phenotype of *snf7*Δ cells was diminished by deletion of vacuolar protease PrA (*pep4*Δ). These genetic findings suggest that transcriptional attenuation in *snf7*Δ cells results, at least partly, from PrA-dependent proteolysis of cargo proteins in the class E compartment rather than from the inability to transport cargo via ILVs to the vacuole. Combining these results with our finding that defects in endocytosis alone do not confer strong Gcn^- phenotypes, we propose that either impaired delivery of MVB cargo originating in the Golgi (class C/D mutants) or rapid proteolysis of the cargo in the aberrant class E compartment (*snf7*Δ and *snf8*Δ mutants) is a key condition of MVB dysfunction that leads to a strong reduction in transcriptional activation by GCN4.

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DEVELOPMENT AND FUNCTION OF *DROSOPHILA* VISUAL CIRCUITS

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Using the *Drosophila* color-vision circuitry as a model, we are studying two neurobiology issues: (1) how neurons form complex interconnections during development and (2) how the assembled neural circuits function to guide animal behaviors. The fly retina contains three types of photoreceptors, R1-6, R7, and R8, each responding to a specific spectrum of light and connecting in a retinotopic fashion to a specific layer in the brain. Our studies focus on the connections made by the ultraviolet-sensitive R7 neurons. In a forward genetic screen, we identified Activin-signaling components by their requirement for establishing a precise retinotopic map of R7 termini. We further uncovered an extrinsic mechanism that is mediated by the protocadherin Flamingo and functions redundantly to the intrinsic mechanism by Activin signaling. To understand the structure and function of color-vision circuits, we are using molecular genetics and serial electronmicroscope (EM) reconstruction to determine the connection patterns of the first-order interneurons. Using quantitative behavioral assays and genetic methods to inactivate specific types of neurons, we are studying the function of the amacrine cell Dm8, which relays inputs from several R7s to projection neurons, thus integrating a large field of columnar elements.

Molecular mechanisms regulating synaptic target selection of R7 photoreceptor axons
Ting, Yang, Li, Lee

In both vertebrates and invertebrates, interneuronal connections are often organized into columns and layers that facilitate information processing and propagating. We use the *Drosophila* visual system as a model to study circuit assembly and focus on the mechanisms guiding R7 axons into specific layers and columns during development. In a large genetic screen based on R7-dependent behavior, we identified two loci, *baboon* and *importin-alpha3*, that are required for the establishment of a precise R7 retinotopic map. *Baboon* encodes a type I Activin receptor and *importin-alpha3* a component of nuclear import machinery. Removal of Baboon or Importin-alpha3 in single R7s caused the cells' axons to invade neighboring columns, indicating that Baboon and Importin-alpha3 are required cell-autonomously in R7s to restrict their growth cones to retinotopically appropriate columns. In addition, the synaptic boutons of *baboon*- or *importin-alph3*–mutant R7s appeared to be smaller and more irregular than those of the wild type, suggesting that these two gene products are involved in synaptogenesis. An examination of other known components of the Activin pathways, including the ligand Activin and the downstream transcription factor Smad2, revealed that the canonical Activin signaling pathway is required for restricting R7 growth cones to their retinotopically appropriate

columns. Interestingly, Activin is functionally required in R7s, suggesting that it serves as an autocrine ligand; that is, it is secreted from and acts on R7 growth cones.

Several lines of evidence indicate that Importin-alph3 is a new component of the Activin signaling pathway. First, Smad2 and Importin-alph3 form a physical complex in the growth cones and axons. Second, nuclear accumulation of Smad2 depends on Importin-alpha3. Most important, these observations raise the intriguing possibility that Importin-alpha3 plays a role in the retrograde axonal transport of Smad2. A recent proposal posited a similar role for Importins in transporting transcription factors from axons/dendrites to nuclei, suggesting that Importins' transport function is conserved in both flies and vertebrates. In summary, our results support a novel model for Activin signaling in R7s, by which autocrine Activin activates Baboon on R7 growth cones, resulting in the phosphorylation of the downstream transcription factor Smad2, which, together with Importin-alpha3, is transported from the growth cones back to the nuclei to regulate transcription. This model is further supported by our observation that blocking retrograde axonal transport in R7s pheno-copies importin-alpha3/baboon phenotypes.

The removal of Importin-alpha3 or Baboon resulted in incomplete penetrance of R7 phenotypes, with only 12 to 30 percent of mutant R7 axons invading their neighboring columns. The outcome suggests an additional mechanism that functions redundantly to the Activin signaling pathway in restricting R7 growth cones to their retinotopically appropriate columns. To test whether repulsive interactions among neighboring R7s play a role in restricting R7 termini to appropriate columns, we genetically ablated most of the R7s and examined the targeting of the remaining R7s. We found that wild-type R7 axons form normal synaptic boutons in retinotopically correct columns even in a largely empty R7 terminal field. By contrast, removing neighboring R7s greatly increased the tendency of *importin-alpha3–* or *baboon*-mutant R7s to invade adjacent columns. The results suggested that *importin-alpha3–* and *baboon*-mutant R7 remained responsive to repulsion by neighboring R7s and that the repulsive interactions accounted for the mutations' incomplete phenotypic penetrance. To determine the molecular nature of the R7-R7 interactions, we examined several candidate genes whose products are known to mediate repulsive interactions. Among them, we identified the gene encoding the protocadherin Flamingo. Removing Flamingo alone in single R7s did not cause any obvious phenotype, although the invasiveness of *baboon* mutant R7s was greatly enhanced by the removal of Flamingo in the neighboring R7s. In summary, at least two redundant mechanisms restrict R7 termini to the correct columns: (1) an intrinsic mechanism mediated by autocrine Activin signaling and (2) an extrinsic mechanism involving Flamingo-mediated repulsion among R7s.

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Mapping color-vision circuits

Gao, Huang, Ting, Lee; in collaboration with Meinertzhagen, Wang

To understand how color information is processed in the fly brain, we set out to (1) identify the neurons constituting the chromatic circuits and their synaptic connections and (2) determine the neurons' functions by using various behavioral paradigms. To achieve the first objective, we identified the first-order interneurons in the medulla ganglion, which receive direct synaptic inputs from the chromatic channels R7 and R8. We exploited the knowledge that the photoreceptor neurons are histaminergic and therefore assumed that their synaptic target must express histamine receptors, which are histamine-gated chloride channels (encoded by the *ort* and *HisCl1* genes). Using an *ort-Gal4* driver, we identified the first-order interneurons, which include two projection neurons, Tm5 and Tm9, as well as an amacrine neuron, Dm8. Using serial EM reconstruction, we determined

the synaptic circuits of these neurons and found that (1) the amacrine neuron Dm8 receives inputs from several R7s and relays the information to the projection neurons and (2) the projection neurons Tm5 and Tm9 receive direct synaptic inputs from the chromatic channels' UV-sensing R7s and from the blue/green-sensing R8s, respectively, as well as hitherto unsuspected indirect input from the achromatic channel via L3 (R1–R6). Thus, the projection neurons integrate both chromatic and achromatic channels and relay the information to the higher visual center, the lobula. The integration of several channels at the level of first- or second-order interneurons in flies is similar to that observed in the primate system of color opponency, suggesting a convergent color-coding solution to a common problem in both visual systems.

To determine the function of the first-order interneurons for the second objective, we used a behavioral assay called the spectral preference test. This behavioral paradigm tests a few flies for their innate preference for UV light over green light, a behavior that has been known to depend on the UV-sensing R7s. We found that mutant flies lacking Ort, but not those lacking the other histamine receptor HisCl1, exhibited an aberrant preference for green light. The ort phenotype could be phenocopied by inactivating Ort-expressing neurons, indicating that Ort-expressing neurons are required for UV preference. To determine the specific neurons that mediate UV preference, we subdivided the Ort-expressing neurons into several categories based on their use of neurotransmitters. Using a combinatorial gene expression system called the split-Gal4 system, we found that the Tm5 projection neurons and the Dm8 amacrine neurons are glutaminergic while the Tm9 projection neurons are cholinergic. By restoring Ort expression in specific neuron subsets, we found that the glutaminergic Tm5 and Dm8 neurons are sufficient to drive UV preference, whereas the cholinergic Ort-expression neurons, including Tm9, are sufficient to drive green preference. In addition, inactivating glutaminergic Ort-expressing neurons abolished UV preference, indicating that they are required for UV preference. The results suggest that Tm5 and Dm8 relay the information from the UV-sensing R7s to the lobula and that Tm9 relays blue/green-sensing R8s to the lobula. We further refined the Gal4 expression pattern by dividing the *ort* promoter into three highly conserved regions: C1, C2, and C3. We found that the glutaminergic Ort-C2 subset, which includes primarily Dm8 neurons, is both required and sufficient for conferring a fly's preference for UV. Similar to the vertebrate amacrine cells, which link bipolar to retina ganglion cells, *Drosophila* Dm8 neurons receive their main synaptic input from R7s and provide input to the projection neuron Tm5. The importance of these connections is underscored by the finding that the indirect connections, from R7 to Dm8 and then to Tm5, are both necessary and sufficient to drive UV preferences. We propose that Dm8 sacrifices spatial resolution for sensitivity by relaying signals from many R7s to small-field projection neurons. We note that Ort-expressing neurons do not include any Dm8-like wide-field neurons for R8s and that restoring activity in Tm9 projection neurons is sufficient to confer stronger green preference in *ort* mutants. Thus, we speculate that Dm8 evolved uniquely to meet the ecological need to detect dim UV in the background of ample visible light.

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RETROTRANSPOSONS AS MODELS FOR THE REPLICATION OF RETROVIRUSES

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The increasing prevalence of retroviral diseases such as AIDS and leukemia has intensified the need to understand the mechanisms of retrovirus replication. Our primary objective is to investigate how retroviral cDNAs are integrated into the genome of infected cells. Given their similarities to retroviruses, long terminal repeat (LTR) retrotransposons are important models for retrovirus replication. The retrotransposon under study in our laboratory is the Tf1 element of the fission yeast *Schizosaccharomyces pombe*. We are particularly interested in Tf1 because of its strong preference for integrating into pol II promoters. This choice of target site is similar to the strong preference of human immunodeficiency virus 1 (HIV-1) and murine leukemia virus (MLV) for integration into pol II transcription units. Little is known about how these viruses recognize their target sites. We therefore study the integration of Tf1 as a model system from which we hope to uncover mechanisms general to the selection of target sites. Understanding the mechanisms responsible for targeted integration may lead to new approaches for blocking the replication of HIV-1.

GPY/F is a highly conserved domain of integrase that mediates multimerization and catalytic activity

Ebina, Chatterjee, Judson,[1] Levin

The C-terminal domains in the integrases (IN) of LTR-retrotransposons and retroviruses are not well conserved. However, close examination of C-termini revealed one motif that is present in a wide variety of INs. This module, termed the GPY/F motif, is found in the INs of a diverse set of LTR-retrotransposons in the *Metaviridae* family (formally Ty3/gypsy) and in the gamma class of retroviruses. The function of the motif has not been studied. Given that the IN of Tf1, as a recombinant protein, is highly soluble and possesses robust catalytic activity, we decided to use it as a model for studying the function of the GPY/F domain.

In solution, the INs of HIV-1, MLV, and avian sarcoma virus (ASV) form a dimer-tetramer equilibrium. To measure the ability of Tf1 IN to multimerize, we tested full-length IN for interactions with the individual portions of the protein. Using a precipitation procedure and recombinant proteins, we found that the N-terminal domain, the central core, and a 71–amino acid fragment containing the GPY/F domain all bound to the full-length IN. To test directly for stable multimers, we performed gel filtration with Superdex™ 200. IN eluted as a single peak with an observed molecular weight of 126.5 kDa, a size consistent with a dimer. We also observed that the central core by itself formed

a stable dimer. The ability of the full-length proteins and the central core to dimerize was typical of other INs.

To investigate the contribution of the C-terminal domain to the multimerization of IN, we subjected the GPY/F fragment to gel filtration with Superdex™ 75. The profile produced three major peaks with apparent sizes of monomer, dimer, and trimer. To test whether the GPF residues that define the motif contributed to multimerization, we generated alanine substitutions. Both substitutions G364A and P365A completely disrupted multimerization of the GPY/F fragment. The data indicate that the GPY/F residues play a role in promoting multimerization. In separate experiments to test for multimers, we subjected the GPY/F fragment to the chemical cross-linker dithiobis-(succinimidyl propionate). The results indicated that the protein was in an equilibrium of monomers, dimers, trimers, and tetramers. We propose that the full-length IN in its synaptic complex with the donor and target DNA may form a tetramer. Our hypothesis is supported by the recent finding of R. Craigie and colleagues that HIV-1 IN does form a tetramer when purified in a synaptic complex.

The C-terminal domains of INs are known to bind to DNA without sequence specificity. To map which sections of Tf1 IN interact with DNA, we assayed each of the individual domains for DNA binding by mixing labeled oligonucleotides with the individual domains and cross-linking the mixtures with UV. The full-length IN, core, and GPY/F fragment exhibited substantial DNA binding activity, which corresponded well to what has been described for other INs. In additional experiments, the G364A and P365A substitutions in the GPF residues of the GPY/F fragment did not reduce DNA binding, indicating that other sequences in the GPY/F fragment mediated the DNA-binding activity and that the GPY/F residues appear to be specific for promoting multimerization. Our finding that the G364A and P365A mutations did not reduce DNA binding also indicated that multimerization was not required for DNA binding.

We tested the contribution to catalysis of the GPY/F residues by generating recombinant IN with the substitution G364A. This mutation significantly reduced strand transfer activity—a result consistent with the model that tetramer formation is required for strand transfer activity. The widespread conservation of the GPY/F motif suggests that the motif may also promote multimerization and strand transfer activity in other INs.

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Integration preference of Tf1 for Pol II promoters

Leem, Ripmaster, Kelly,[2] Ebina, Heincelman,[3] Levin; in collaboration with Grewal, Hoffman, Zhang

Our analysis of the genome sequence of *S. pombe* revealed large numbers of pre-existing Tf transposons that are positioned 200 to 400 bp upstream of open reading frames (ORFs). The production of new integration events revealed that the position of Tf1 upstream of ORFs results from integration preference. The similarity of Tf1's integration pattern to HIV-1's preference for integration into pol II transcription units and the MLV's preference for transcription start sites motivated us to study Tf1 as a model for retrovirus integration. To define the determinants of the target sites recognized by Tf1, we developed an in vivo assay for integration, using a plasmid that contained potential targets of integration. In addition to the target plasmids, the assay strains of *S. pombe* contained a plasmid with an inducible copy of Tf1. We expressed a version of Tf1 that contained *neo,* a gene that causes cells with integration events to be resistant to the antibiotic G418.

To determine which type of sequence would function as targets of integration, we placed five genes on target plasmids and introduced each into the *S. pombe* strain expressing Tf1. In each target

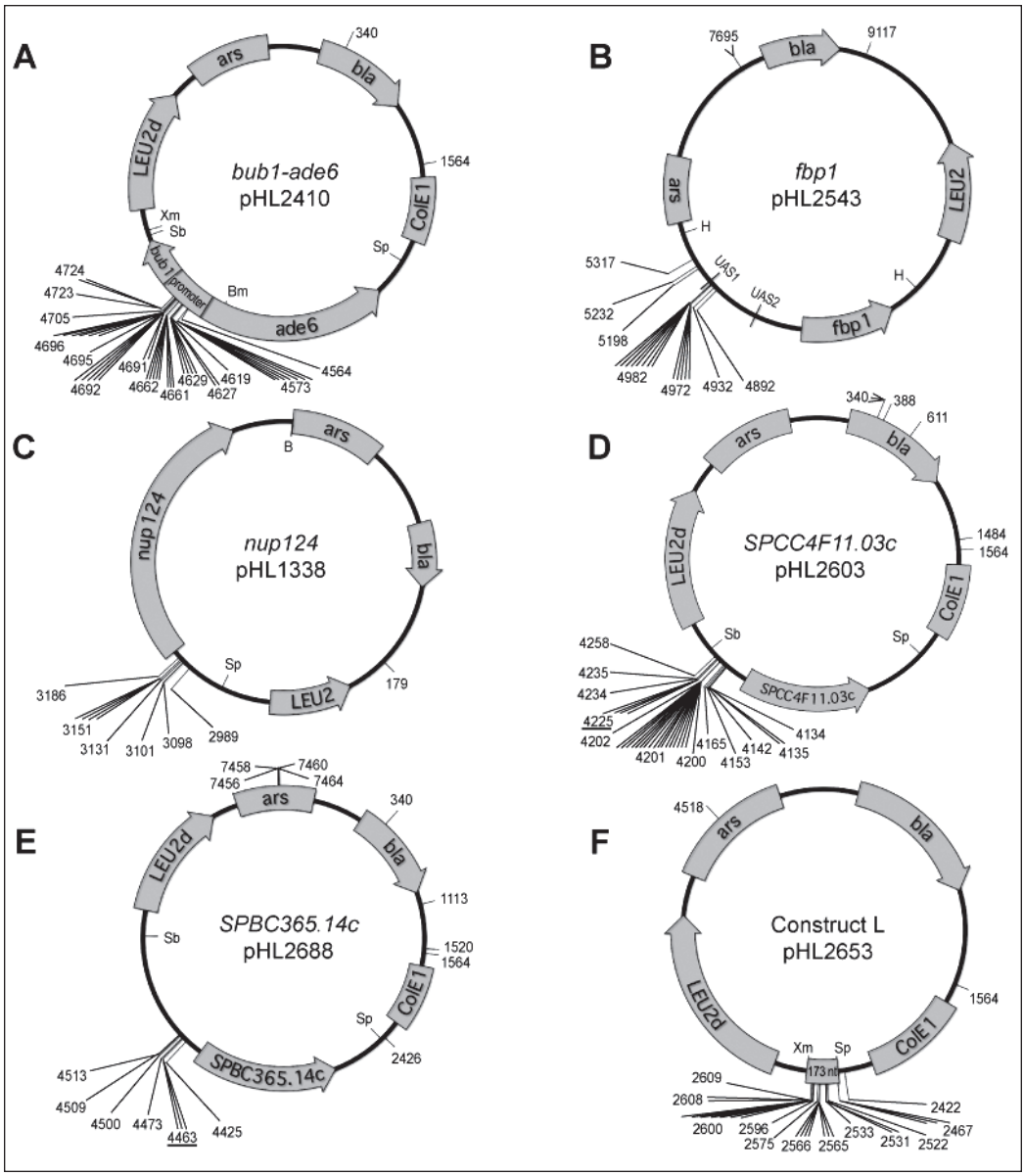

FIGURE 2.2

The positions of insertions in target plasmids are shown with the plasmid coordinates. The plasmids contained the genes *bub1-ade6* (A), *fbp1* (B), *nup124* (C), *SPCC4F11.03c* (D), *SPBC365.14c* (E), and the 173 bp insertion window of *bub1-ade6* (F).

plasmid, the insertions of Tf1 clustered at specific positions upstream of the ORFs (Figure 2.2). For example, 50 insertions were isolated in a plasmid containing *ade6*, with 95 percent of these insertions occurring within a 160 nt window upstream of the ORF. To determine which sequences of *ade6* were required for efficient integration, we created a series of deletions within the target plasmid. Our analysis revealed that the 160 nt region was the only sequence required for efficient integration. To determine whether promoter activity was essential for integration, we measured transcript levels of *ade6.* Deletions of sequence on either side of the 160 nt region caused 5- to 10-fold reductions in *ade6* mRNA. However, the deletions caused no reduction in integration

efficiency. Our results indicate that the transcription activity of the promoters per se did not play an important role in targeted integration.

Another plasmid tested for integration activity contained the gene *fbp1*. Eighty-six percent of the insertions in the plasmid occurred in sequence upstream of the ORF (Figure 2.2B). Of the 18 inserts upstream of the ORF, 15 clustered within a 90 bp window adjacent to UAS1, the binding site for the transcription activator Atf1p. The proximity of these insertion sites to UAS1 suggested that Tf1 recognized the promoters upstream of the ORFs. To test whether the integration upstream of *fbp1* required the function of UAS1, we mutated six nucleotides in UAS1 and observed that they caused a nine-fold reduction in integration in the promoter of *fbp1*. To test whether Atf1p, the protein that binds to UAS1, contributed to integration, we deleted the *atf1* gene. Strikingly, the absence of Atf1p caused a 15.5-fold reduction in integration at UAS1, indicating that Atf1p was required to position integration at the promoter of *fbp1*. One model for the specific role of Atf1p in positioning integration at the promoter of *fbp1* holds that Atf1p bound to UAS1 mediates integration thereby forming a complex with IN. We tested such a possibility by immunoprecipitating Atf1p from cell extracts and showed that FLAG-Atf1p precipitated a substantial amount of IN, indicating that the IN formed a specific interaction with Atf1p. The results support the model that Atf1p mediates integration at the promoter of *fbp1*. Interestingly, integration in the promoter of *ade6* was independent of Atf1p. Results of chromatin immunoprecipitation experiments confirmed the binding of Atf1p upstream of *fbp1* but revealed that Atf1p did not bind to the sequences upstream of *ade6*. As a result, a distinct factor or factors likely mediate integration upstream of *ade6*. Nevertheless, the role of a DNA-bound transcription factor in the targeting of integration is strong evidence that Tf1 recognizes the pol II promoters. This finding is significant in that previous work demonstrated only that integration occurred upstream of ORFs.

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The integration of retrotransposons into heterochromatin

Ebina, Levin; in collaboration with Gao, Voytas

Mobile elements constitute a major portion of the genetic material in eukaryotic cells. To protect cellular genes from the disruption caused by transposition, cells have evolved strategies, such as RNA interference (RNAi), to restrict the amplification of mobile elements. These surveillance systems lead to epigenetic inactivation of transposable elements through post-transcriptional histone silencing and DNA modifications. The genetic and epigenetic impact of mobile elements is largely determined by the elements' choice of integration sites. Many mobile elements target integration to specific chromosomal sites by forming interactions between integrase and chromatin factors. Such is the case for the Ty5 transposon that relies on a specific interaction between the chromatin factor SIR4p and the C-terminal end of its integrase to direct integration to regions of heterochromatin. An interesting lineage of retrotransposons referred to as the chromoviruses (*Metaviridae* family) possess C-terminal regions of integrase with a chromodomain (CHD)—a smaller than 40– to 50–amino acid sequence motif that can interact with diverse targets, including proteins, RNA, and DNA. The best-characterized CHD partners are methylated histone residues. The heterochromatin protein 1 (HP1) possesses a CHD that interacts with histone H3 methyl-K9. Consequently, when CHDs were identified in the retrotransposon integrases, it was proposed that they play a role in target specificity. In a collaborative study, we sought to understand the role of the CHD in the integrases of these chromovirus retrotransposons. We described three groups of chromoviruses based on amino acid–sequence relationships of their integrase C-termini. Genome sequence analysis

indicated that representative chromoviruses from each group are enriched in gene-poor regions of the genome relative to other retrotransposons, and, when fused to fluorescent marker proteins, the chromodomains target proteins to specific subnuclear foci coincident with heterochromatin. The chromodomain of the fungal element MAGGY interacts with histone H3 dimethyl- and trimethyl-K9, and, when the MAGGY chromodomain is fused to integrase of the *Schizosaccharomyces pombe* Tf1 retrotransposon, new Tf1 insertions are directed to sites of H3 K9 methylation. Repetitive sequences such as transposable elements trigger the RNAi pathway resulting in the elements' epigenetic modification. The results suggest a dynamic interplay between retrotransposons and heterochromatin, whereby mobile elements recognize heterochromatin at the time of integration and then perpetuate the heterochromatic mark by triggering epigenetic modification.

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Host genome surveillance of retrotransposons

Ebina, Levin; in collaboration with Grewal, Cam, Noma

Transposable elements and their fragments constitute a substantial portion of eukaryotic genomes. Host genomes have evolved defense mechanisms, including the use of chromatin modifications and RNA interference, to inhibit transposable elements. In a collaborative study, we identified a genome surveillance mechanism for retrotransposons that is mediated by the transposase-derived centromeric protein CENP-B homologues of *S. pombe*. CENP-B homologues of *S. pombe* silence TP2 retrotransposons by localizing and recruiting histone acetylases to the transposon sequences. CENP-Bs also repressed the transcription of solo LTRs and LTR-associated genes. Tf2 elements were clustered into "Tf" bodies, the organization of which depended on CENP-Bs and displayed discrete nuclear structures. Furthermore, CENP-Bs inactivate the Tf1 retrotransposon by blocking its recombination with pre-existing copies of Tf2; CENP-Bs silenced and immobilized a Tf1 integrant that became sequestered into Tf bodies. The results reveal a probable ancient retrotransposon surveillance pathway important for host genome integrity and highlight potential conflicts between DNA transposons and retrotransposons.

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The activity of the hermes transposon in *S. pombe*

Evertts, Guo, Levin

Tf1 integrates primarily into pol II promoters. Although we currently believe that such preference results from a mechanism that actively targets Tf1, it is possible that the insertion bias is attributable to greater accessibility at the promoter sequences. We are currently testing such a possibility by studying the integration pattern in *S. pombe* cells of hermes, a "cut and paste" transposon that was isolated from the house fly. Given that hermes propagates in a host that is evolutionarily distant from *S. pombe*, it is unlikely that an existing mechanism would actively position insertion sites. Thus, any integration of hermes in *S. pombe* would likely occur at positions accessible to the transposase. In addition, unbiased activity of a transposon in *S. pombe* could be widely adapted as a tool for random mutagenesis. As no methods currently exist for transposon mutagenesis of *S. pombe*, a method for insertional mutagenesis would be a significant contribution to the field.

We expressed the transposase of hermes in *S. pombe* by fusing its gene to the promoter of *nmt1*, using three versions of the *nmt1* promoter to express various levels of the transposase. Immunoblots of cell extracts demonstrated that the transposase was expressed in a stable form and in amounts corresponding to the strength of the promoter. To permit measurement of transposition activity, the cells expressing the transposase also contained a plasmid encoded copy of *neo* flanked by the terminal inverted repeats (TIR) of hermes. We tested the ability of the transposase to cut out *neo* with the TIRs and insert this plasmid DNA into the *pombe* genome. Once the transposase was

expressed, we grew cells on medium containing 5-fluoro-orotic acid, a treatment that removed the plasmid carrying the hermes TIRs and *neo.* We then grew the strains on agar plates containing G418 to select for cells with transposed copies of hermes. The strains expressing the transposase generated surprisingly high levels of resistance to G418. We analyzed for potential insertion events 26 independent strains that became G418-resistant. It was significant that each strain had acquired a copy of hermes as the result of a bona fide integration event. Analysis of the inserted copies revealed that 54 percent of them disrupted ORFs. Given that 60 percent of the *pombe* genome is coding sequence, our results indicate that the insertion of hermes did not discriminate between coding and noncoding sequences, a finding that stands in strong contrast to the integration of Tf1, where virtually none of the inserts occurs in ORFs.

To measure accurately the level of transposition of the hermes system, we conducted a quantitative assay that allowed us to calculate the number of transposition events per generation. We observed similar transposition frequencies in three experiments. After approximately 25 cell generations, 1.5 to 2.75 percent of cells contained insertions. Such a rate of transposition is ideal for generating mutation libraries because it produces a substantial number of cells with insertions while keeping the frequency of double insertions to a minimum.

In pilot experiments, we screened cultures of mutagenized cells for mutations in two genes, *ade6* and *ade7.* Of 106,000 cells screened, seven produced red colonies, the phenotype expected for mutations in either *ade7* or *ade6.* This number of *ade*$^-$ colonies was consistent with randomly distributed integration. Together, the data indicate that hermes can readily be used as a tool for the random disruption of *pombe* genes.

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BIOCHEMICAL GENETICS OF SIGNAL TRANSDUCTION

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We investigate molecular mechanisms that regulate cell-cell communication during development and homeostasis. Cells communicate with each other by relying on a handful of conserved families of signaling molecules. The TGF-β superfamily of growth and differentiation factors, including TGF-βs/Activins and the bone morphogenetic proteins (BMP), is one of the largest of these families. TGF-β signaling factors have the ability to function as morphogens, that is, to specify cell fate in a concentration-dependent manner. In addition, TGF-β–type factors provide a mechanism for coupling a cell to its environment, endowing the cell with the necessary plasticity to respond appropriately to changes in its environment or even its own state. We use the *Drosophila* model system and a variety of molecular and biochemical approaches as a paradigm for cell-cell signaling mechanisms to study genes that modulate the function of TGF-β–type signals. We are interested in how the extracellular availability of TGF-β factors is regulated in time and space as well as in the role of the cell surface in modulating local activation of TGF-β–type signaling. We are working toward a genetic and biochemical description of how signaling is modulated and how it has been adapted for different roles during development and evolution.

The role of enzyme-substrate interactions in formation of BMP morphogen gradients
Nguyen, Serpe; in collaboration with Umulis, O'Connor

In the early *Drosophila* embryo, *decapentaplegic (dpp)*, the gene encoding a BMP-type ligand, is transcribed uniformly throughout the dorsal domain, yet only about 10 percent of cells along the dorsal midline receive high levels of signal. In the pupal wing, Dpp diffuses from the longitudinal veins into the posterior cross-vein–competent zone and creates a corridor of peak signaling that is perpendicular to the source of morphogen. In both instances, the formation of the Dpp gradient occurs at a post-transcriptional level and involves modulation by additional secreted gene products.

In the early embryo, Dpp is bound in a complex containing Short gastrulation (Sog). The complex inhibits binding of Dpp to its receptors in lateral regions and facilitates long-range ligand diffusion, shuttling Dpp from the lateral domain toward the midline. An important component that helps create flux is the processing of Sog by Tolloid (Tld), a metalloprotease of the BMP-1 family. The net movement of Dpp dorsally is generated by reiterated cycles of complex formation, diffusion, and destruction of Sog by Tld.

Drosophila has two highly homologous proteases of the BMP-1/Tld family, Tld and Tolloid-related (Tlr), which are both expressed in similar patterns in the early embryo and process Sog.

However, mutations in *tlr* do not affect early patterning while mutations in *tld* cause a ventralized phenotype. In contrast, in the wing Sog needs to be processed by Tlr to enable proper Dpp signaling for posterior cross-vein development—Tld cannot take its place. We previously demonstrated that enzyme kinetics regulate developmental specificity, at least in part. Tlr processes Sog at least an order-of-magnitude more slowly than does Tld. It appears that, for both developmental processes, a balance between Sog's inhibitory and positive activities is crucial for proper patterning. The Tlr catalytic properties fulfill the temporal constraints of pupal wing development while the more rapid kinetics of Tld are matched to the rapid development of the embryo.

In both the embryo and posterior cross-vein, Sog plays both positive and negative roles in regulating BMP activity, a phenomenon previously referred to as the Sog paradox. The negative role derives from Sog's ability to block ligands' access to receptors. The positive effect derives from Sog's ability to facilitate Dpp diffusion. Interestingly, Chordin, the vertebrate orthologue of Sog, acts as an inhibitor only when expressed in *Drosophila* and cannot promote long-range Dpp signaling. At the molecular level, Sog and Chordin differ in that processing of Sog by Tld requires the BMP ligand as an obligatory co-substrate while Chordin does not. To determine the source of this difference, we modeled the Tld catalytic domain by using the crystal structure of crayfish Astacin, the founder member of this metalloprotease family. We purified and sequenced the Sog cleavage fragments, derived a consensus cleavage recognition sequence, and used the peptide to study the enzyme-substrate interactions in Sog for comparison with Chordin sequences. From this modeling, we hypothesized that several residues at the processing site might be responsible for making one, but not the other substrate dependent on BMP binding for processing. We successfully generated Sog variants that are BMP-independent Tld substrates in vitro. We are now characterizing their in vivo effect on BMP signaling.

Our working hypothesis is that Sog's ability to function in a transport process as a long-range BMP agonist resides, in molecular terms, in the BMP's co-substrate requirements for Tld-mediated Sog degradation. Computational modeling by our collaborator David Umulis supported this hypothesis, indicating that the co-substrate requirement for Sog processing by Tld is critical for proper Dpp gradient formation. Computations that relax this constraint and allow for Sog degradation when not complexed with Dpp show a great reduction in Dpp flux toward the dorsal midline.

Shaping BMP gradients through ligand-dependent modulation and positive feedback
Serpe; in collaboration with Blair, Umulis, O'Connor

BMP gradients in the early embryo and the pupal wing share similar features. They both form sharp boundaries and require two BMP-type ligands, one of which is Dpp. They differ in their spatial constraints and in their requirement for another BMP binding molecule, Crossveinless-2 (Cv-2). Cv-2 is crucial for BMP signaling in the posterior cross-vein; however, the null *cv-2* alleles we created by targeted mutagenesis exhibit normal early embryonic patterning. Loss of Cv-2 causes loss of BMP signaling in the developing cross-veins while high levels of Cv-2 sequester ligands. As with Sog, Cv-2 exhibits both BMP agonistic and antagonist activities. In contrast to *sog* mutants, null *cv-2* early embryos retain peak BMP signaling dorsally; in fact, at low temperatures, a slight enhancement of the signaling level occurs. Thus, Cv-2 appears to have an antagonistic effect on Dpp. Further analysis confirmed that Cv-2 has Dpp-antagonistic activity for the large range of Dpp and Cv-2 concentrations tested. Yet, in the pupal wing, BMP signaling shows a biphasic response to changes in Cv-2 levels: low signaling without Cv-2, maximum signaling with moderate levels of Cv-2, and then decreased signaling with elevated levels of Cv-2. Our studies indicate that Cv-2 behaviors are in fact ligand-dependent activities.

The cross-vein specification requires two BMP ligands, Dpp and Glass Bottom Boat (Gbb). Peak BMP signaling in the future cross-vein region could be attributable to Dpp and Gbb homo- and/or heterodimers. Cv-2 binds to BMP ligands but, unlike Sog, does not mediate the ligands' long-range

transport. We have shown that Cv-2 binds to the cell surface and heparan sulfate proteoglycans. In collaboration with Seth Blair, we demonstrated that Cv-2 acts locally within the cross-vein itself. It forms a transient complex with the BMP signaling molecule and the type I BMP receptor. Through this complex, Cv-2 can antagonize BMP ligands with high affinity for the receptor (such as Dpp) while facilitating the recruitment of low-affinity ligands (i.e., Gbb) in close proximity to the receptors, thereby potentiating their signaling. Consequently, Cv-2 can distinguish between various BMP ligands and differentially modulate their signaling. Our model of Cv-2 function shows how a cell-surface ligand-binding molecule can act locally to promote or inhibit signaling.

In addition, Cv-2 is a transcriptional target of BMP signaling. This positive feedback appears to play a role in refining the initially broad region of BMP signaling observed at early stages of posterior cross-vein development to the more tightly focused signaling observed at later stages. The formation of sharp boundaries is termed spatial bistability. In the case of Cv-2, mathematical modeling demonstrated that coupling the extracellular effects with positive feedback on the production of Cv-2 itself can lead to bistable signaling wherein a very sharp transition can be generated between cells that receive high versus low levels of signal. Therefore, the positive feedback of a cell-surface BMP-binding protein provides a mechanism for spatial refinement of signaling.

Together, our data indicate that, depending on the particular context in which it acts, the combined properties of Cv-2 enable the protein to have remarkably versatile effects on signaling and thus to tune BMP signaling exquisitely.

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MOLECULAR MECHANISM OF FROG METAMORPHOSIS

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We investigate the molecular mechanisms of amphibian metamorphosis. The control of this developmental process by thyroid hormone (TH) offers a unique paradigm in which to study gene function in post-embryonic organ development. During metamorphosis, distinct organs undergo vastly different changes. Some, such as the tail, undergo complete resorption while others, such as the limb, develop de novo. In the frog, most larval organs persist through metamorphosis but are dramatically remodeled. For example, tadpole intestine in *Xenopus laevis* is a simple tubular structure consisting primarily of a single layer of larval epithelial cells. During metamorphosis, it is transformed, through specific cell death and selective cell proliferation and differentiation, into an organ with a multiply folded adult epithelium surrounded by elaborate connective tissue and muscles. The wealth of knowledge from past research and the ability to manipulate amphibian metamorphosis both in vivo, by using transgenesis or hormone treatment of whole animals, and in vitro, by using organ cultures, offer excellent opportunities to study in vivo the developmental function of TH receptors (TRs) and their underlying mechanisms and to identify and characterize the function of genes critical for post-embryonic organ development in vertebrates.

Mechanism and function of TR during development

Buchholz,[1] Das, Heimeier, Matsuda, Oetting, Sato,[2] Wang, Washington,[3] Shi

We have proposed a dual function model for TR during frog development, that is, the heterodimers between TRs and RXRs (9-*cis* retinoic acid receptors) bind to target genes constitutively in vivo. In premetamorphic tadpoles, the heterodimers repress gene expression in the absence of TH to prevent metamorphosis, thus ensuring a proper tadpole growth period. When TH is present either from endogenous synthesis during development or is added exogenously to the rearing water of premetamorphic tadpoles, TR/RXR heterodimers activate TH-inducible genes to initiate metamorphosis. By using a transgenic approach, we previously showed that TR is both necessary and sufficient for the metamorphic effects of TH, whereas the non-genomic action of TH, while it exists, plays a minor role, if any, during this post-embryonic process. Thus, metamorphosis provides the first example of TR mediating directly and sufficiently the developmental effects of TH in individual organs by regulating target gene expression in these organs.

More recently, we designed dnN-CoR, a dominant negative form of the TR-binding co-repressor N-CoR (nuclear receptor co-repressor); dnN-CoR contains only the receptor-interacting domain

of N-CoR. We overexpressed dnN-CoR under the control of a heat shock–inducible promoter in tadpoles through transgenesis and observed significant derepression of TH-response genes in transgenic animals. More important, transgenic tadpoles developed faster than wild-type siblings, with an acceleration of as much as 7 days in the 30-day experiment. By the end of the experiment, the animals reached early stages of metamorphosis. The data suggest that unliganded TR recruits co-repressors to control metamorphic timing, as predicted by our model.

In addition, the complexity of metamorphic changes in different organs argues for the presence of distinct gene regulation programs regulated by TR. Knowledge of systematic gene regulation will help identify not only molecular markers but also important cellular pathways or critical genes for future mechanistic studies. Thus, we have begun to use the recently developed *Xenopus laevis* cDNA array to analyze genome-wide gene expression changes associated with TH-induced intestinal remodeling. Our initial analysis of animals treated with TH for varying numbers of days has provided a molecular description of the gene-regulation pathways associated with various metamorphic processes in the intestine. However, the analysis cannot determine whether TRs directly or indirectly regulate the TH-response genes thus identified. Given that directly regulated genes act earlier in the metamorphic process and are more likely to be important regulators of metamorphosis, we are currently employing cDNA array analysis to identify such genes by using RNA from animals treated with TH in the presence or absence of protein synthesis inhibitors. By determining tissue-specific direct target genes of TR, we should be able to gain insight into how TH controls tissue-specific changes during metamorphosis.

While the above studies using *Xenopus laevis* as a model have led to several important in vivo findings about the function and mechanisms of TR action during development, the lack of genomic sequence information and the frog's tetraploid genome and lengthy developmental cycle make it difficult for further analyses of TR functions in this species. However, the closely related species *Xenopus tropicalis* offers many advantages. Toward exploiting *X. tropicalis* for genome-wide and genetic studies of TR function, we analyzed the expression profiles of TRs and RXRs during *X. tropicalis* development. We showed that their expression correlates with organ transformations and that TR/RXR heterodimers are capable of repressing and activating gene expression in vivo in the absence and presence of TH, respectively. Furthermore, TRs are bound to endogenous target genes in *X. tropicalis* tadpoles. Our results support a role for TRs in mediating the metamorphic effects of TH in *X. tropicalis*. More important, the similarities in the expression and function between *X. tropicalis* and *X. laevis* TRs and RXRs, as demonstrated by our study, pave the way for taking advantage of existing morphological, molecular, and cellular knowledge of *X. laevis* development and the greater genetic and sequence suitability of *X. tropicalis* to dissect the molecular pathways governing tissue- and organ-specific transformations during vertebrate post-embryonic development.

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Roles of co-factors in gene regulation by TR

Choi,[4] Fu, Heimeier, Hsia,[5] Matsuda, Paul,[6] Shi

TR regulates gene transcription by recruiting co-factors to target genes. In the presence of TH, TR can bind to co-activators while unliganded TR binds to co-repressors. Many biochemical and molecular studies have been conducted on such co-factors. Much less is known, however, about whether and how they participate in gene regulation by TR in various biological processes in vivo. We investigate how TR uses different co-factors in the context of development in various organs.

Among co-repressors, we have studied the role of N-CoR and SMRT (silencing mediator of retinoid and thyroid receptors) in gene repression by TR in premetamorphic tadpoles. We previously showed that both are expressed and, more important, bound to TH-response genes in such tadpoles. Furthermore, the repressors are released from the target genes upon TH treatment of premetamorphic tadpoles or during natural metamorphosis. Our studies with the dnN-CoR, as summarized above, have demonstrated in vivo a role of co-repressor complexes in gene repression by unliganded TR during premetamorphic tadpole development. On the activator side, we used the chromatin immunoprecipitation (ChIP) assay to show that *Xenopus* co-activators SRC3 (steroid receptor co-activator 3) and p300 are both recruited to TH-responsive promoters. More important, by using transgenesis to overexpress dominant negative forms of SRC3 and p300, we showed that co-activators, particularly the SRC3-p300 family of co-activator complexes, are required for gene regulation by liganded TR and for TH-dependent metamorphosis. These findings represent the first known example of specific co-activator complexes playing critical roles in the developmental function of a nuclear receptor in vertebrates.

The SRC/p300 complexes also contain the methyltransferases CARM1 and PRMT1, which have both been implicated in TR function in mammalian cell culture studies. Thus, to investigate further the role and mechanisms of the SRC/p300 complexes in development, we cloned and characterized *Xenopus* CARM1 and PRMT1. We obtained two alternative splicing forms of Carm1, CARM1a and CARM1b. Both isoforms are expressed throughout metamorphosis, suggesting that they play a role during the process. Surprisingly, transcriptional analysis in the *Xenopus* oocyte revealed that overexpression of CARM1b had little effect on TR-mediated transcription, although CARM1a expectedly enhanced gene activation by liganded TR. ChIP assays showed that liganded TR recruited both endogenous CARM1a and overexpressed CARM1a and CARM1b to a target promoter. However, the binding of liganded TR to the target promoter was lower when CARM1b was overexpressed and was accompanied by a slight reduction in histone methylation at the promoter. The results suggest that CARM1 plays a role in TR-mediated transcriptional regulation during frog development and that alternative splicing regulates the enzyme's function. Likewise, our studies in the frog oocyte model system have shown that liganded TR recruits PRMT1 to enhance transcriptional activation by TH. We are currently investigating the in vivo function of these methyltransferases during metamorphosis.

In addition to histone modifications involving complexes such as SRC-p300, transcriptional regulation by TR involves chromatin remodeling. In particular, the BRG1-containing, ATP-dependent chromatin remodeling complexes have been implicated in gene regulation by nuclear receptors. To investigate their potential involvement in metamorphosis, we showed that the expression of BRG1, a chromatin-remodeling enzyme, is upregulated at the climax of *Xenopus laevis* metamorphosis while BAF57, a BRG1-binding protein in BRG1-containing chromatin remodeling complexes, is constitutively expressed during development. Consistent with this finding, TH treatment of premetamorphic tadpoles led to upregulation of the expression of BRG1 but not of BAF57. Studies using a reconstituted TH-dependent *Xenopus* oocyte transcription system, in which we can study TR function in the context of chromatin, revealed that BRG1 enhances the transcriptional activation by ligand-bound TRs in a dose-dependent manner while a remodeling-defective *BRG1* mutant

inhibited the activation, suggesting that the process relies on chromatin remodeling. Further studies showed that BAF57 interacted with BRG1 in oocytes and cooperatively enhanced gene activation by TR with BRG1 in vivo. Chromatin immunoprecipitation revealed that BAF57 was recruited to the TR-regulated promoter in the presence of TR and TH. Together, the findings suggest a role for BRG1-/BAF57-containing chromatin remodeling complexes in TR-regulated gene expression during metamorphosis.

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Regulation and function of the matrix metalloproteinases during TH-induced tissue remodeling
Fiorentino, Fu, Hasebe,[7] Mathew, Shi; in collaboration with Ishizuya-Oka

We previously identified several TH-response genes encoding matrix metalloproteinases (MMPs) during intestinal metamorphosis. MMPs are Zn^{2+}-dependent proteases capable of cleaving various proteins of the ECM (extracellular matrix). The ECM not only provides essential physical support within an organism but also influences cell fate during developmental and pathological processes. Upregulation of MMP genes has been observed in many developmental processes such as limb morphogenesis and in diverse pathological conditions such as arthritis, wound healing, and cancer metastasis. However, it has been difficult to investigate the roles of MMPs in mammals due to the lack of good models and the relatively subtle phenotypes in limited tissues in knockout mice. The activation of MMP genes by TH together with the observed ECM remodeling during metamorphosis suggests that MMPs participate in this process through ECM remodeling. Frog metamorphosis affects essentially all organs in a tadpole within a short developmental period and thus offers an excellent opportunity to investigate the in vivo functions of MMPs and associated mechanisms during development.

Expression and function studies in organ cultures have shown that the MMP stromelysin-3 (ST3) is directly regulated by TR at the transcription level and is required for ECM remodeling and TH-induced cell death in the animal intestine. Furthermore, in premetamorphic tadpoles, transgenic overexpression of ST3, but not of a catalytically inactive mutant, is sufficient to cause ECM remodeling and apoptosis in the intestinal epithelium, demonstrating that ST3 is necessary and sufficient for larval epithelial cell death. Toward understanding the mechanism by which ST3 affects tissue remodeling, we have also identified the 37 kDa laminin receptor (LR), a cell surface receptor for the ECM protein laminin, as an in vivo substrate of ST3. LR binds to ST3 and can be cleaved by ST3 at two sites in the extracellular domain between the transmembrane domain and laminin binding sequence, separating the cells from laminin. Interestingly, ST3 cleavage sites in LR are conserved in human LR, and high levels of LR are known to be present in tumor cells, which are often surrounded by fibroblasts expressing ST3. Thus, LR may be a conserved substrate of ST3, and its cleavage by ST3 may alter cell-ECM interactions, thereby mediating the effects of ST3 on cell fate and behavior during development and pathogenesis. To investigate the functional importance of LR cleavage by ST3 during development, we carried out a series of mutational analyses on the two cleavage sites in LR. Our findings revealed that, in addition to primary sequence at the cleavage site (positions P3 through P3′, with the cleavage occurring between P1 and P1′), flanking sequences/conformation influenced the cleavage of LR by ST3. Moreover, alanine substitution studies led to the surprising finding that the surrounding sequence and/or conformation dictated the site of cleavage in LR by ST3. The results have important implications for our understanding of

substrate recognition and cleavage by ST3 and underscore the importance of studying ST3 cleavage in the context of full-length substrates. More important, we plan to use the LR cleavage mutants in transgenic studies to investigate the role of LR cleavage by ST3 during metamorphosis.

In addition to ST3, several other MMPs are regulated by TH during metamorphosis, consistent with the complex nature of the ECM, which would require several enzymes for its remodeling and degradation during metamorphosis. Our earlier studies showed that the MMPs GelA and MT1-MMP (a membrane-type MMP) are coordinately regulated during metamorphosis. Using *Xenopus* embryogenesis as a model, we showed that *Xenopus* GelA and MT1-MMP are co-expressed during embryogenesis and associated with each other in vivo. Furthermore, we showed through overexpression of both MMPs in developing embryos that GelA and MT1-MMP play cooperative roles during development, at least in part through the activation of pro-GelA by MT1-MMP. We are now analyzing the role of these MMPs during metamorphosis by using transgenic overexpression in a manner similar to our ST3 overexpression experiments.

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Thyroid hormone regulation of adult intestinal epithelial stem cell development during metamorphosis

Hasebe,[7] Shi; in collaboration with Ishizuya-Oka

As the larval epithelial cells undergo apoptosis during intestinal metamorphosis, the adult epithelium develops. Currently, the origin of the adult epithelial stem cells is unknown, although some evidence supports the notion that they derive from dedifferentiated larval epithelial cells. We previously isolated sonic hedgehog (Shh), a cell-cell signaling molecule, as a direct TR target gene during intestinal remodeling. Furthermore, Shh is highly expressed in proliferating adult epithelial cells during metamorphosis. These results suggest that Shh is involved in the larval-to-adult remodeling of the *Xenopus laevis* intestine and is likely to play a role in the development and/or proliferation of adult epithelial stem cells. Indeed, organ culture studies have allowed us to show that exogenous Shh enhances epithelial cell proliferation. The Shh signal is known to be regulated at post-translational levels in other animal species. In particular, the pan-hedgehog inhibitor hedgehog-interacting protein (Hip) has been implicated in the regulation of Shh signaling. Using real-time RT-PCR and in situ hybridization, we showed that *Hip* expression is transiently upregulated during both natural and TH-induced metamorphosis and that Hip mRNA is localized in the connective tissue adjacent to the adult epithelial primordia expressing Shh. Interestingly, the expression of bone morphogenetic protein-4 *(BMP-4)*, an Shh target gene, is remarkably low where *Hip* is strongly expressed, suggesting that Hip regulates Shh signaling. Finally, we demonstrated that Hip binds to the N-terminal fragment of processed Shh in vivo, indicating that Hip may suppress Shh signaling by sequestering Shh and thus might play a role in regulating the spatial and temporal signaling of Shh during intestinal stem cell development and proliferation.

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SMC FAMILY PROTEINS AND ASSOCIATED FACTORS IN MITOTIC CHROMOSOME SEGREGATION

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Our main goal is to understand the biological roles and mechanisms of higher-order chromosome structure, which facilitates accurate chromosome segregation during cell division. The eukaryotic ATP-ases of the SMC family (Structural Maintenance of Chromosomes) form several essential protein complexes that determine higher-order chromosome structure and chromatin dynamics in eukaryotic cells. Currently, we focus primarily on mitotic chromosome condensation. The SMC complexes termed *condensins* play a pivotal role in chromosome condensation and are essential for chromosome segregation. Fission and budding yeast have only one condensin complex (condensin I) while multicellular organisms usually have two. The two condensin complexes both consist of five subunits: two shared SMC subunits SMC2/CAP-E and SMC4/CAP-C, which form the enzymatic (ATPase) and structural core of the complex, and three complex-specific non–SMC subunits. Condensins bind to DNA/chromatin and change its superhelical state, introducing positive DNA supercoiling. Our work has three major objectives: (1) characterizing regulatory pathways that target condensin to specific chromosomal sites in mitosis; (2) examining functional condensin's requirements for distinctive chromosomal domains, such as nucleolar organizer and centromeres; and (3) quantitatively defining structural changes introduced into DNA during chromosome condensation in vivo. We identified several regulatory circuits and cellular requirements for condensin functions.

Regulation and specificity of condensin targeting to nucleolar chromatin
Takahashi, Dulev, Strunnikov

Characterization of the authentic binding sites for condensin activity in vivo is an essential step toward elucidating the molecular mechanisms of chromosome condensation. Our previous characterization of the *S. cerevisiae* rDNA (ribosomal RNA gene cluster) as a main target of mitotic condensin activity may have identified one of the major applications of condensin activity in all eukaryotes. To find out how condensin recognizes particular DNA sequences on a chromosome, we need to understand the molecular roles of post-translational condensin modifications.

Our previous work identified a promising candidate for a condensin regulator: Smt4p, which is the SUMO isopeptidase (Smt3p in budding yeast). New data suggest that balanced sumoylation regulates condensin; that is, both the addition and timely removal of SUMO moiety have specific effects on condensin targeting. To investigate whether Smt3p directly modifies condensin itself and/

or some other proteins cooperating with condensin, we developed an unbiased screening procedure that permits the identification of the authentic Smt3p conjugates in vivo with differential-length SUMO tags. Using this "SUMO footprint" assay, we detected SUMO modification of four condensin subunits (Smc2p, Smc4p, Ycs4p, and Ycs5p). However, assigning a specific biological role to the modification proved to be a challenge because the mere discovery of the condensin subunits' modification did not provide insight into the biological function of the modification. Eventually, we achieved a breakthrough by constructing GFP-Smt3p fusion expressed from the native *SMT3* promoter and showing that SUMO conjugates are significantly enriched in the nucleolus—the essential hub of activities of condensin and both topoisomerases I and II. Further investigation of the biological role of condensin's sumoylation led to the hypothesis that SUMO modification of condensin facilitates its targeting to the nucleolus, promoting condensin cooperativity with nucleolar pools of sumoylated topoisomerases I and II.

We tested our hypothesis with series of experiments. First, we mutated lysine residues to arginine (KR mutations) in all 16 of the sumoylation consensus sites (spread across the four condensin subunits) and assessed whether the generated SUMO-less condensin had any defects in its targeting to the nucleolus, chromosome condensation, chromosome segregation, and cell viability. Second, we combined the condensin KR mutants with topoisomerase mutations, which reduced the nucleolar presence of topoisomerases. Third, we identified the SUMO E3 enzyme for condensin—the Mms21 protein—and showed that Mms21p E3's most significant activity is to modify condensin and cohesin complexes. We established that (1) SUMO is prominently enriched in the nucleolus; (2) triple SUMO E3 mutants are defective in rDNA segregation and maintenance; (3) genetic interactions among *mms21-CH*, *top1*, and *top2* mutations signify a common/redundant pathway in rDNA maintenance; (4) condensin and cohesin binding to rDNA are directly regulated by Mms21-mediated sumoylation; and (5) condensin sumoylation is essential in the absence of Top1 and sumoylated Top2. These findings substantiated the paradigm that the SUMO "code" plays a role in the subnuclear compartmentalization of chromatin proteins, suggesting that SUMO-mediated co-targeting of individual proteins to a specific chromosomal compartment can induce the proteins' functional cooperation.

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The role of active condensin in the functionality of the critical chromosomal sites—centromere and kinetochore

Samoshkin, Dulev; in collaboration with Cleveland

We previously conducted a ChIP-chip analysis of the genome-wide condensin binding pattern and showed that the peri-centromeric regions are enriched in condensin binding. Such enrichment suggested that condensin activity might facilitate centromere function. In addition, our earlier studies in budding yeast confirmed that condensin plays some role at centromeres; that is, ChIP-chip analysis revealed a notable increase in interphase enrichment of condensin near centromeres in mitosis. Furthermore, the mitotic checkpoint (spindle assembly checkpoint, or SAC) is the factor responsible for condensin mutant arrest, and both Dsn1 (a part of the MIND complex). We also found that Cse4 (the orthologue of the CENP-A centromeric histone) proteins were partially delocalized from centromeres in condensin mutants.

While our analyses uncovered a putative molecular interface between condensin and centromeres in yeast, they did not explain why checkpoints did not prevent chromosome non-disjunction and aneuploidy upon condensin dysfunction in humans. Therefore, we generated tools for efficient RNAi knockout of condensins in human cells and undertook biochemical, genetic, and cytological analyses of the structure of condensin-defective centromeres and kinetochores in human cells. The data showed that the centromere structure was abnormal and that the inter-kinetochore distance increased almost 2-fold in SMC2-depleted metaphase chromosomes. Furthermore, upon depletion of condensins I and II in both human and amphibian cells, the resulting cumulative centromeric structural defects activated the mitotic checkpoint; however, chromosome mis-segregation in anaphase still occurred. While we were investigating the molecular nature of such a slippage through the mitotic checkpoint in human cells, we found that depletion of condensin activity results in a significant loss of CENP-A from the centromere and a SAC-mediated metaphase delay. Given that CENP-A is loaded onto centromeres in the previous cell cycle, we analyzed, in collaboration with Don Cleveland, CENP-A enrichment during several cell divisions, using covalent fluorescent pulse-chase labeling (SNAP tagging).

The extreme stretching of centromeric chromatin by spindle forces led to the deformation of both inner kinetochores and the microtubule-capturing module (HEC1 complex), a configuration that strongly resembled a merotelic orientation. As a result, Aurora B was mis-localized and partially lost activity in condensin-depleted centromeres, suggesting that the correction of merotelic attachments before anaphase onset was severely impaired without condensin. Monastrol recovery experiments showed that the phenotype of the high incidence of merotelic attachment was nearly identical and epistatic in the cases of condensin depletion and Aurora B inactivation, indicating that condensin plays a pivotal role in the proper spatial positioning of the kinetochore relative to the microtubule-regulating protein complexes in the inner centromere (Figure 2.3).

The results allow us to conclude that condensin dysfunction both compromises an important controlling mechanism (merotelic attachment correction) and leads to chromosome damage (breaks) and non-disjunction. Genome stability mechanisms are compromised in many cancer cell types and are thus believed to contribute to multi-step oncogenic transformations. The link of condensin to

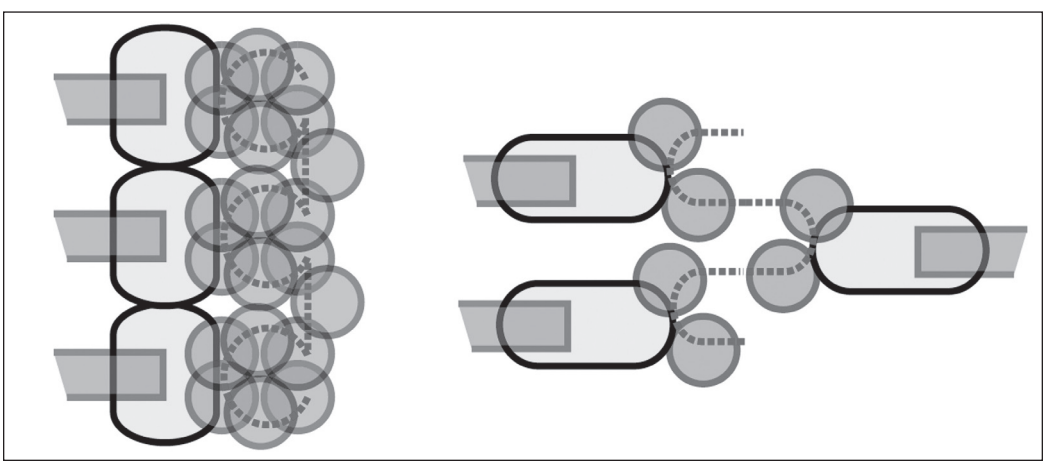

FIGURE 2.3

Putative decondensation of centromeric chromatin resulting from the loss of condensin and CENP-A. The single sister centromere/kinetochore is shown. Left: wild-type condensed centromere attached to microtubules emanating from a single pole. Right side: condensin dysfunction. Once the centromeric chromatin (gray circles) becomes depleted and disorganized, the individual microtubule-attachment sites in the kinetochore (ovals) become semi-independent and acquire more flexibility to attach to a microtubule (truncated rectangles) for the incorrect pole. Dotted lines: DNA.

cancer was recently proposed for at least one cancer case. Our work shows that, in the instance of condensin dysfunction, chromosomal damage appears to result directly from centromere defects rather than from an independent non-disjoining defect at the chromosomal arms. Thus, a single hypomorphic condensin mutation can potentially generate a wide spectrum of genome instability signatures, as seen in cancers.

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[1]*Graduate Partnerships Program*

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STUDIES ON DNA REPLICATION, REPAIR, AND MUTAGENESIS IN EUKARYOTIC AND PROKARYOTIC CELLS

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Under optimal conditions, the fidelity of DNA replication is extremely high. Indeed, it is estimated that, on average, only one error occurs for every 10 billion bases replicated. However, given that living organisms are continually subjected to a variety of endogenous and exogenous DNA-damaging agents, optimal conditions rarely prevail in vivo. While all organisms have evolved elaborate repair pathways to deal with such damage, the pathways rarely operate with 100 percent efficiency. Thus, the persisting DNA lesions are replicated, but with much lower fidelity than is undamaged DNA. Our aim is to understand the molecular mechanisms by which mutations are introduced into damaged DNA. The process, commonly referred to as translesion synthesis (TLS) or translesion replication (TR), is facilitated by one or more members of the Y-family of DNA polymerases that are conserved from bacteria to humans. Based on phylogenetic relationships, Y-family polymerases may be broadly classified into five subfamilies; DinB-like (polIV/pol kappa-like) proteins are ubiquitous and found in all domains of life; in contrast, the Rev1-like, Rad30A (pol eta)-like, and Rad30B (pol iota)-like polymerases are found only in eukaryotes and the UmuC (polV)-like polymerases only in prokaryotes. We continue to investigate TLS in all three domains of life: bacteria, archaea, and eukaryotes.

Mutagenesis in prokaryotes

Curti, Howard, Karata, McDonald, Theisen, Vaisman, Woodgate

Escherichia coli possesses five known DNA polymerases (pols); given their multiplicity, the pols require exquisite regulation of their access to genomic DNA. The cell's main replicase is the pol III holoenzyme while pol I is responsible for the maturation of Okazaki fragments and for filling gaps generated during nucleotide excision repair. As part of the cell's global SOS response to DNA damage, pol II, pol IV, and pol V are significantly upregulated; under these conditions, they may alter the fidelity of DNA replication by potentially interfering with the ability of pol I and pol III to complete their cellular functions. To gain insight into the interplay among *E. coli*'s five DNA polymerases, we exploited the fact that each polymerase leaves a distinct genetic fingerprint when copying DNA. We analyzed the spectrum of *rpoB* mutants generated in a set of 13 isogenic strains carrying a mutation in one or more of *E. coli*'s chromosomally encoded DNA polymerases as well as in three related strains that moderately overexpressed polV-like polymerases. The key to our study was that all strains carried a defect in mismatch repair, thereby allowing us to monitor polymerase-specific misincorporation events rather than assaying mutations that escape repair. Indeed, it is

well known that mismatch repair largely protects an organism against transition mutations and has little effect on transversions; therefore, it was no surprise that, in our *rpoB* assays, the spectrum of mutations was dominated by AT→GC and CG→TA transitions at four well-defined hot spots. The mutations were not dependent on pol II, pol IV, or pol V, as we recovered a considerable number of mutants at the hot spots in the absence of each polymerase. However, given that the magnitude of mutagenesis at certain positions varied considerably in the polymerase-deficient strains, it appears that all three polymerases can modulate the extent of mutagenesis at the hot spots. In contrast, in a strain lacking *polA*, we observed a striking reduction in transition mutagenesis, suggesting that such mutagenic events are largely dependent on pol I. The observation that pol I is responsible for most transition mutagenesis is remarkable in that pol I possesses both 3′→5′ and 5′→3′ exonuclease activities and is generally thought to be an accurate polymerase, with misincorporations occurring with a frequency of less than 1 in a million bases replicated.

Our study also revealed that transversion mutations are largely dependent on the combined actions of pol IV and pol V and that the cellular levels of pol V limit transversion mutagenesis. Furthermore and in contrast to pol I, pol IV, and pol V, which promote mutagenesis, pol II appears to play a largely anti-mutagenic role by suppressing mutations at certain hot spots.

Until now, it has tacitly been assumed that spontaneous mutations occurring in the *E. coli* genome arise largely during genome duplication performed by the cell's replicase pol III. However, our observations led us to conclude that, in fact, considerable switching occurs among *E. coli*'s five DNA polymerases in the absence of exogenous DNA damage and that such interplay modulates the extent of spontaneous mutagenesis occurring on the *E. coli* chromosome.

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Characterization of archaeal Dpo4

McDonald, Vaisman, Woodgate; in collaboration with Bell

Orthologues of *E. coli* pol IV are found in all domains of life, including archaea. The best-characterized archaeal orthologue is Dpo4, which originates from the hyperthermophilic crenarchaeota *Sulfolobus solfataricus*. We previously showed that Dpo4 is a low-fidelity polymerase; it can facilitate the bypass of a number of lesions that block cellular replicases. Interestingly, the crystal structure of Dpo4 revealed that, although the enzyme possesses structural domains similar to replicases, its active site is large and solvent-exposed, explaining its ability to accommodate bulky adducts in that site. While Dpo4 is moderately processive, the polymerase possesses a putative "PIP-box," which is thought to facilitate an interaction between the polymerases and PCNA (proliferating cell nuclear antigen), the cell's sliding-clamp. In a recent collaborative study with Stephen Bell, we assayed the effect of PCNA on the processivity of Dpo4 when replicating an undamaged DNA template and investigated the mechanism whereby circular PCNA is loaded onto DNA.

Unlike the case of eukaryotic PCNA, which is composed of a homotrimer of PCNA protomers, previous studies on *S. solfataricus* PCNA demonstrated that the sliding clamp in fact consists of a heterotrimer of three discrete PCNA subunits (PCNA1, PCNA2, and PCNA3). We first confirmed that the processivity of Dpo4 is greatly stimulated in the presence of heterotrimeric PCNA1/2/3. Given that changing the two phenylalanines to alanines in the consensus PIP-box of Dpo4 abrogated the polymerase's ability to be stimulated by PCNA, we concluded that the increased processivity occurred through a physical interaction between PCNA and Dpo4. Interestingly, yeast two-hybrid analyses revealed that Dpo4 specifically interacts with the PCNA1 subunit and shows little to no affinity for PCNA2 or PCNA3 subunits.

We next investigated the mechanism of clamp loading. While molecular dynamics simulations suggest that only a single interface within PCNA may be opened during loading, we have yet to test biochemically the number of inter-subunit interfaces—one, two, or even three—that are opened in PCNA during the loading process in order to facilitate the entry of DNA during loading. Accordingly, we exploited the unique asymmetry of *Sulfolobus solfataricus* PCNA. Our strategy was to fuse PCNA subunits covalently by using linker peptide sequences to join the N-terminus of one PCNA subunit to the C-terminus of its neighbor, thereby covalently sealing inter-subunit interfaces. Employing such constructs in clamp-loading assays allowed us to determine the number and identity of interfaces important for loading. All combinations of PCNA1, PCNA2, and PCNA3, which reconstituted the heterotrimeric PCNA, stimulated Dpo4 activity. With the dimer fusion constructs, we could detect replication factor C (RFC)–dependent stimulation in reactions containing the PCNA1/2 fusion plus PCNA3 and when the PCNA2/3 fusion plus PCNA1 was present. Significantly, the PCNA3/1 fusion plus PCNA2 could not support RFC-dependent stimulation of Dpo4 activity. Our data therefore indicate that productive loading of PCNA requires the opening of only a single inter-subunit interface—specifically, that between PCNA3 and PCNA1.

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Characterization of eukaryotic Y-family polymerases

Chumakov, Kuban, McDonald, Ohashi, Woodgate; in collaboration with Yang, Wilson

DNA is subject to a variety of chemical modifications that alter its structure. Such alterations can block basic cellular functions such as transcription and/or replication and can lead to cell death, mutagenesis, and, in higher eukaryotes, cancer. One such modification is DNA methylation, which can be caused by endogenous chemicals, products of metabolism, environmental exposure, or treatment with several cancer chemotherapeutics. Not surprisingly, cells have developed several evolutionarily conserved mechanisms for repairing or tolerating this type of DNA damage, including base excision repair (BER), nucleotide excision repair (NER), recombination, and TLS. The major products in DNA exposed to S_N2-methylating agents are *N*7-methylguanine and *N*3-methyladenine (3MeA). 3MeA accounts for approximately 20 percent of the base damage formed by S_N2-methylating agents and is considered the major cytotoxic lesion produced by such chemicals. However, it has been extremely difficult to prove that 3MeA directly blocks replication, as 3MeA's half-life in vitro is estimated at between 12 and 24 hours, thereby precluding biochemical analysis. To circumvent these problems, we synthesized a stable 3-deaza analogue of the nucleoside 3-methyl-2′-deoxyadenosine (3dMeA) that we incorporated into synthetic oligonucleotides and used as templates for DNA replication in vitro. As expected, the 3dMeA lesion blocked both human DNA polymerases alpha and delta. In contrast, human polymerases eta, iota, and kappa as well as *S. cerevisiae* pol eta were able to bypass the lesion, albeit with varying efficiencies and accuracy. To confirm the physiological relevance of our findings, we demonstrated that, in *S. cerevisiae* lacking 3MeA repair, human pol eta, pol iota, and especially pol kappa are capable of restoring methyl methanesulfonate (MMS) resistance to the normally MMS-sensitive strain.

In a collaborative study with Samuel Wilson, we investigated the role of DNA polymerase iota in BER, which plays a major role in maintaining genomic integrity. For example, the BER pathway predominantly repairs alkylated DNA bases in higher eukaryotes. During single-nucleotide BER, a damage-specific glycosylase removes an alkylated base, and apurinic/apyrimidinic endonuclease cleaves the resulting abasic site. The outcome is a nick with a 5′-dRP group that can be removed by the dRP lyase activity of pol beta; the same polymerase then fills the single-nucleotide gap. Finally, a DNA ligase seals the nick, thereby completing repair.

Mouse embryonic fibroblasts (MEF) deficient in pol beta demonstrate increased sensitivity to alkylating agents such as MMS, yet cellular extracts from pol beta–null cells evidence some residual BER activity, suggesting that other polymerases could substitute for pol beta in BER. Previous studies indicated that reversal of MMS hypersensitivity requires the dRP lyase activity of pol beta; therefore, the polymerase that substitutes for pol beta in BER must also possess dRP lyase activity. Interestingly, several years ago, we demonstrated that human pol iota has intrinsic dRP lyase activity and, accordingly, hypothesized that pol iota might function as a back-up BER enzyme in pol beta–null cells. To test this hypothesis directly, we determined the MMS sensitivity of MEFs deficient in pol beta and pol iota. We found that MEFs doubly deficient in pol beta and pol iota were more sensitive to the killing effects of MMS than MEFs deficient in pol beta alone, suggesting that pol iota may indeed participate in the BER of MMS-induced lesions.

However, the pol beta–deficient MEFs used in our study had different origins. To minimize the effect of genetic background variability, we expressed mouse pol iota in the pol beta–deficient and pol iota–deficient cells at a level equal to or greater than wild-type pol iota. Unfortunately, analysis of growth inhibition revealed that expression of pol iota did not affect the cells' resistance to the alkylating agents MMS or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. Thus, we concluded that the difference in the MMS sensitivity of the pol iota–deficient and –proficient cell lines is unlikely to result from the difference in pol iota expression but rather is attributable to uncharacterized differences between the two cell lines. As a consequence, we concluded that, although pol iota possesses dRP lyase activity, expression of the polymerase has negligible impact on the alkylation damage sensitivity of cells lacking pol beta.

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Role of DNA polymerase iota in humans with the xeroderma pigmentosum variant (XP-V) syndrome

McLenigan, Vaisman, Woodgate; in collaboration with Kraemer

Xeroderma pigmentosum variant (XPV) patients have normal DNA excision repair yet are predisposed to develop sunlight-induced cancer. They exhibit a 20-fold higher-than-normal frequency of UV-induced mutations with a highly unusual spectrum. The primary defect in XP-V cells is a lack of functional pol eta, which normally inserts adenine nucleotides opposite photoproducts involving thymine. Both the high frequency and striking difference in spectrum of UV-induced mutations in XP-V cells strongly suggest that, in the absence of pol eta, translesion replication of UV-induced DNA lesions is catalyzed by an alternate DNA polymerase that is much more error-prone than pol eta. Indeed, we previously presented data suggesting that pol iota may be responsible for the hypermutability observed in patients with XP-V.

As part of a collaborative study with Kenneth Kraemer, we surveyed the molecular basis of XP-V worldwide by measuring levels of pol eta protein in skin fibroblasts from putative XP-V patients (age 8–66 years) from 10 families in North America, Turkey, Israel, Germany, and Korea. DNA sequencing identified 10 distinct *POLH* mutations, suggesting that the mutations arose independently. They included two splicing mutations, one nonsense mutation, five frameshift mutations (three deletion and two insertion), and two mis-sense mutations. Nine of the mutations were located in the

catalytic domain of the polymerase and one in the C-terminus of the protein involved in mediating protein-protein interactions between pol eta and its partners.

We also examined the level of pol eta protein in 16 cell strains from other UV-sensitive, cancer-prone patients. As expected, the cells exhibited normal post–UV cell survival, host cell reactivation, or DNA repair. However, unlike true XP-V cells, the cells had normal levels of pol eta protein, and we were unable to detect any mutations in *POLH*. We therefore considered the possibility that these clinical phenotypes may have arisen because of mutations in another polymerase involved in the translesion synthesis of UV photoproducts. An obvious candidate was pol iota, given that our previous studies had demonstrated that it interacts physically with pol eta and that both polymerases co-localize to sites of UV-induced DNA damage. We therefore measured the level of pol iota protein in the cells of interest and sequenced the entire *POLI* gene. However, all cells exhibited normal levels of pol iota protein and no mutations in *POLI*. The molecular defect causing the phenotype resembling XP-V in these cells thus remains to be determined.

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DEVELOPMENT OF VACCINES AGAINST BACTERIAL DISEASES ESPECIALLY IN CHILDREN

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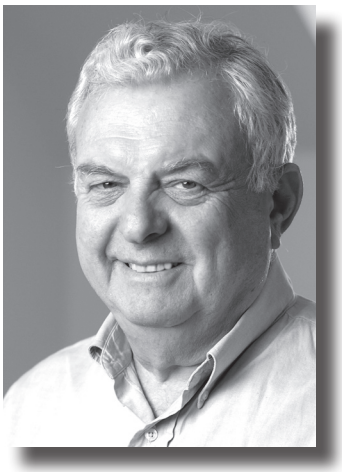

To achieve our overall objective of developing vaccines for infectious diseases, especially those of children, we study, discover, and re-evaluate clinical, epidemiologic, and immunologic data. We evaluate investigational vaccines suitable for clinical investigation, first, in experimental animals and then for submission to the Institutional Review Board and the FDA for evaluation of safety and immunogenicity in adults, children, and infants, and, finally, of efficacy.

Cross-reacting polysaccharides (*H. influenzae* types a and b and *B. pumilus*)

Robbins, Kubler-Kielb, Schneerson, Pozsgay, Chu; in collaboration with Carlone, Miller M, Romero-Steiner, Shiloach

Herd immunity followed widespread use of the Hib conjugate vaccine, and the near-elimination of Hib led to speculation that other *Haemophilus influenzae* (Hi) types may emerge as causes of meningitis. For example, in Brazil, the incidence of Hib meningitis decreased by 69 percent during the first year after initiation of Hib conjugate immunization while the incidence of Hia meningitis increased 8-fold. The Netherlands Reference Laboratory reported that type a was observed only in children younger than 4 years old. Our goal is to provide a vaccine against Hi type a, which is the second most prevalent type of Hi causing invasive diseases; it is especially common among certain populations of children. A second goal is to investigate the feasibility of providing a single vaccine to several pathogens. We have elucidated the structures of the six capsular polysaccharide (CP) types, a, b, c, d, e, and f of Hi (systemic infections were attributable mostly to *H. influenzae* type b [Hib]). A critical level of serum IgG anti–Hib CP confers type-specific immunity by complement-mediated bacteriolysis.

Properties of Hia and Hib CP. Hi may be classified into three virulence groups of two that are related to their CP structures. Types a and b, which are the most virulent, are composed of a neutral sugar, an alcohol (ribitol), and a phosphodiester; types c and f are composed of an *N*-acetylated aminosugar, a monosaccharide, and a phosphodiester; and (3) types d and e have a repeat unit of an *N*-acetylglucosamine and *N*-acetylmannosamineuronic acid. As compared with the other four types, Hib and Hia exhibit greater resistance to the bactericidal effects of complement alone. Challenge of infant rats showed that the 50 percent infective dose (ID50) for bacteremia of both Hib and Hia was several logs lower than that of the other types. Intranasal challenge of infant rats with type b or type a resulted in 55 to 90 percent bacteremia with type b and 35 percent with type a . The other types were not invasive.

Invasive diseases caused by Hia. Rates of Hia disease have remained constant in the United States despite Hib vaccination. From 1998 to 2002, the Emerging Infections Program of the CDC conducted active laboratory- and population-based surveillance for Hi disease in data from nine sites with approximately 35 million people. Seventeen of 1,743 invasive isolates were Hia, which is an important cause of meningitis in certain populations such as White Mountain Apache Indian children—with an annual incidence of 254 cases/100,000 children of Hia meningitis. Hammitt et al. (*Pediatr Infect Dis J* 2005;24:453) reported an outbreak of invasive Hia disease among Native Alaska infants. During a 6-month period in two proximate villages, five Hia cases were documented. In Brazil, an incidence of Hia meningitis of 0.16 cases/100,000 person-years was reported. South Africa and native populations in Manitoba Canada also report Hia meningitis in children.

Hia vaccine. The number of Hia cases is too low for a randomized, double-blinded, controlled trial. Yet, precedent exists for adding types within a species to a vaccine without evidence for efficacy. For example, the licensing of several pneumococcal types, including meningococcal groups Y and W135 and poliovirus type 2, was based on their safety and immunogenicity. The structural, experimental, and clinical properties of Hia CP closely resemble those of type b, and the increasing number of reports of Hia-invasive disease suggests that development of an Hia conjugate is warranted. Methods for conjugating type b CP to a protein are applicable to Hia. D-1,5-ribitolphosphate is a constituent of the CPs of Hia and Hib. We reported that the cell wall polysaccharide (PS) of *B. pumilus* Sh18 contains a poly-1,5-ribitolphosphate as a major component and that antibodies induced in mice by such conjugate cross-reacted with both Hia and Hib. We synthesized polyribitolphosphate chains containing either 8 or 12 repeat units, with the terminal keto groups used for conjugation to aminooxylated bovine serum albumin (BSA or tetanus toxoid. We injected the conjugates into mice, three times at 2-week intervals at 2.5 g/mouse and obtained sera a week later. ELISA demonstrated

antibodies to both Hia and Hib, with the octamer conjugate a better immunogen than the dodecamer conjugate. Some of the tested sera showed bactericidal activity against both type a and type b as roughly correlated with their ELISA values.

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Shigellae

Schneerson, Robbins, Chu, Majadly, Mocca, Kubler-Kielb, Guo, Trinh; in collaboration with Ashkenazi, Chowers, Shiloach

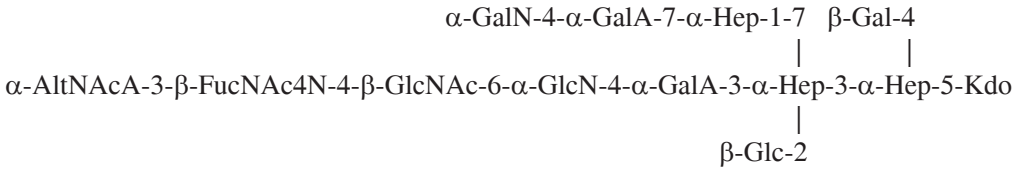
Shigellosis is endemic throughout the world and hyperendemic in developing countries; its estimated annual incidence is about 200 million cases, with a mortality of about 650,000. Shigellosis is common among military recruits in field conditions, refugees, and patients in institutions. *Shigella* spp. are the most common cause of dysentery in children, leading to stunted growth. Vaccine development has been hindered because *Shigella* spp. are pathogens for and inhabitants of humans only. More than 100 years since the discovery of the causative organism, there is still no licensed vaccine against shigellosis. The WHO has declared the prevention of shigellosis a top priority, and it is the goal of our project.

Surface polysaccharides of pathogenic bacteria, including CPs or the *O*-specific polysaccharide (O-SP) of lipopolysaccharides (LPS), function as both essential virulence factors and protective antigens. Covalent binding of these saccharides to medically useful proteins to form conjugates increases their immunogenicity and confers on them T cell dependence, making them suitable vaccines for infants and children. The O-SP of *Shigella sonnei* bound to recombinant non-toxic *P. aeruginosa* exoprotein A (rEPA) exhibited an efficacy of over 70 percent in young adults exposed to 6 to 14 percent attack rates. This conjugate and that of *S. flexneri* 2a bound to the succinylated exoprotein A (rEPA-succ) were safe and induced IgG antibodies to the homologous LPS in 1- to 4-year-olds. A randomized, blinded, Phase 3 study of the conjugates in 1- to 4-year-olds, with each conjugate serving as a control for the other, showed the vaccines to be safe. Immunogenicity and efficacy were age-related with little efficacy in 1- to 2-year-olds but with about 70 percent in 3- to 4-year-olds. As before, percentage increases in antibody levels were similar to those for adults, but the actual achieved levels were lower. Using Caco2 and HeLa cells, we studied the effect of the children's (ages 1-4) immune sera and the IgG isolated from them on shigella invasion into epithelial intestinal cells. The sera inhibited shigella invasion in a type-specific manner. Pretreatment of the sera or of Caco-2 cells with O-SP abrogated these effects in a type-specific and dose-dependent manner.

To enhance the immunogenicity of the conjugates, we bound *S. sonnei* and *S. flexneri* 2a O-SPs to additional carrier proteins—tetanus toxoid (TT) and recombinant protective antigen (rPA)—and injected mice twice, with the same or a different carrier the second time. For *S. flexneri* 2a, the twice-injected TT conjugate induced the highest (over 4-fold) antibody levels. For *S. sonnei*, the TT conjugate followed by the rPA conjugate induced significantly higher levels than the other combinations.

We have shown that synthetic oligosaccharides of *S. dysenteriae* type 1 O-SP, bound by their reducing end to a carrier protein (sun configuration), induced significantly higher antibody levels than conjugates of the native O-SP bound to protein by several point attachments (lattice configuration). Synthesis of *S. sonnei* oligosaccharides was not successful. Therefore, we isolated low–molecular mass O-SP-core (O-SPC) fragments of the native O-SP and used them to bind to proteins by oxime linkages between the terminal Kdo residues of the reducing end core and the aminoxy linkers bound to either BSA or a non-toxic diphtheria toxin mutant. The coupling reaction was performed at a neutral pH, at room temperature, and in a short time. Levels of anti–*S. sonnei* LPS IgG induced by these conjugates in young outbred mice were significantly higher than those induced by the full-length O-SP conjugates.

The O-SP of *Plesiomonas shigelloides* O17 is structurally identical to that of *S. sonnei*, but only the core structure of *S. sonnei* has been published. We investigated the core structure of *P. shigelloides* O17, including its linkage to the O-SP, by nuclear magnetic resonance (NMR) and mass spectroscopy. On the basis of our studies, we assigned the following structure of the repeat unit:



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***Bordetellae* spp.**

Kubler-Kielb, Ginzberg, Schneerson, Guo, Mocca; in collaboration with Lagergard, Vinogradov

The major goal of this project is to investigate the structural characteristics of LPS and lipo-oligosaccharides (LOS) of human pathogens and use the information gained to bind the detoxified O-SP to carrier proteins in order to develop experimental vaccines against such pathogens. To this end, we are developing conjugation methods of wide applicability; our secondary goal is to develop potential veterinary vaccines.

Bordetellae are Gram-negative bacilli that cause respiratory tract infections in mammals and birds. *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* are all clinically important. *B. pertussis* vaccines have succeeded in preventing pertussis in infants and children. Veterinary vaccines against *B. bronchiseptica* are available, but their efficacy and mode of action have not been established; there is no vaccine against *B. parapertussis*. Working from the concept that immunity to non-capsulated Gram-negative bacteria may be conferred by serum IgG anti–LPS, we studied chemical, serological, and immunological properties of the O-SP, obtained by different degradation procedures, from *B. bronchiseptica* and *B. parapertussis* obtained by different degradation procedures. Based on their non-reducing end saccharide structure, we identified one type of *B. parapertussis* O-SP and two types of *B. bronchiseptica* O-SP with no cross-reaction between the two *B. bronchiseptica* types. Competitive inhibition assays showed the immunodominance of the non-reducing end of these O-SPs. For purposes of binding to an amino-oxylated protein, we prepared conjugates of *B. bronchiseptica* and *B. parapertussis* O-SP by using either the Kdo residue exposed by mild acid hydrolysis of the LPS or the core glucosamine residue exposed by deamination of the LPS. We carried out both coupling methods at a neutral pH, at room temperature, and in a short time. All conjugates injected into mice as saline solutions at a fraction of an estimated human dose induced antibodies to the homologous O-SP. These methods are applicable to the preparation of LPS-based vaccines against other Gram-negative bacteria.

Probably owing to the absence of the direct bactericidal effect of anti-toxin antibodies, the protection afforded by licensed subunit pertussis vaccines is incomplete on an individual basis. However, herd immunity, which occurs with wide vaccine usage, provides almost complete protection. Nonetheless, an additional vaccine component inducing bactericidal antibodies such as anti-LPS could increase vaccine efficacy on an individual basis. To that end, we isolated and analyzed *B. pertussis* and *B. bronchiseptica* LPS. *B. pertussis* expresses only the core region saccharide (OS), which is composed of 12 sugars. *B. bronchiseptica*, which is easier to culture and produces higher yields, expresses LPS with a core structure almost identical to that of *B. pertussis*, although we observed

small variations; (1) the methylation of Fuc4Nme—100 percent in *B. pertussis*—is only 50 percent in *B. bronchiseptica*, and (2) Hep is phosphorylated at about 30 percent in *B. bronchiseptica* but not in *B. pertussis*. *B. bronchiseptica* LPS is mostly further substituted by *O*-specific chains. For this study, we used only a free core fraction with no O-SP. We prepared conjugates of both *B. pertussis* OS and *B. bronchiseptica* core by reacting their reducing-end Kdo moieties with an amino-oxy linker bound to BSA. Both conjugates incorporated an average of 10 saccharide chains per BSA molecule, both reacted with anti–*B. pertussis* and anti–BSA sera with a line of identity, and both were immunogenic in mice, inducing similar antibody levels as those measured by ELISA.

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Vaccine development for Group B *Neisseria meningitidis* and *Escherichia coli* K1

Robbins, Schneerson, Lin; in collaboration with Gottfredsson, Grove Krause, Howitz, Miller M, Mølbak

Vaccine development against Group B *Neisseria meningitidis* (GBM) meningitis is complicated by the pathogen's capsular polysaccharide (PSA), an alpha 2-8-linked polysialic acid that is identical to the surface saccharide of the K1 capsular polysaccharide of *E. coli* as well as to host structures, especially during the host's development. Despite effective antibiotic and supportive therapy, the mortality and morbidity of systemic infections, especially as a result of meningitis caused by GBM and *Escherichia coli* K1, remain unacceptably high. GBM continues to cause epidemics and outbreaks throughout the world, and *E. coli* K1 is a major cause of neonatal meningitis and kidney infections. With the goal of eventually providing a vaccine against these organisms, we examined evidence or the lack thereof for an association between PSA antibodies and autoimmune disease or effects on fetuses.

Meningococci are classified into serogroups according to their CPs. Of the 13 reported CP groups, 5 (A, B, C, W135, and Y) cause almost all meningococcal disease. As essential virulence factors, the five groups inhibit the protective actions of complement; in addition, they function as protective antigens as evidenced by the fact that a critical level of serum IgG CP antibodies specifically induces both complement-mediated lysis of groups A, C, W135, and Y and opsonophagocytic killing of group GBM. Effective CP-based vaccines exist for groups A, C, W135, and Y, but none exists for GBM or *E. coli* K1. GBM causes more infections in infants and young children than do menigococci of groups A, C, W135, or Y.

Although PSA antibodies bind to many fetal and adult tissues in vitro, there is no evidence for in vivo binding or associated pathology. We have therefore focused on developing vaccines that use non-capsular antigens, including outer membrane proteins, lipopolysaccharides, iron-binding proteins, and other antigens identified by examining the organism's DNA. Many of these antigens are polymorphic, heterogeneous, and subject to antigenic variation and may not be representative of all GBM. Furthermore, none will be useful against *E. coli* K1. Based on the performance of the *Haemophilus influenzae* type b, *Salmonella typhi* (Vi), pneumococcal, and group C meningococcal (GCM) vaccines, we developed a PSA conjugate that induced protective levels of serum IgG anti–PSA. It is simple to produce and easy to standardize and should be close to 100 percent effective at all ages. We have confirmed its performance in laboratory mice.

Few studies have compared severity of infection and outcome among meningococcal serogroups, and published studies of the sequelae of meningococcal meningitis patients make no mention of autoimmune diseases, such as Guillain-Barré syndrome, multiple sclerosis, and so forth. For

mortality or nearly every category of sequela associated with GBM meningitis, our review shows rates that are equal to or lower than those associated with other meningococcal serogroups. In the absence of epidemiological or clinical evidence to associate pathology with PSA antibodies, we conducted a retrospective cohort study of meningococcal patients to examine evidence for autoimmunity. The entire Danish population constituted our study cohort of 7,467,001 individuals followed for autoimmune disease between 1977 and 2004. GBM meningitis was diagnosed in 2,984 individuals, and the control population was 914 patients with GCM meningitis. Ratios of incidence rates of autoimmune diseases provided a measure of relative risk. Patients with GBM meningitis, either in comparison with people with group C meningococcal meningitis or those with no history of meningococcal meningitis, exhibited no increased risk of autoimmune diseases. We also studied this cohort for possible increased risk of preterm or stillbirth to women with previous GBM disease and whether their first-born children were at increased risk for birth defects. We found no such associations. Our findings suggest that systemic infection with GBM is not associated with autoimmune diseases or with immunoreactive diseases that may affect the health of offspring for up to 31 years after meningococcal disease.

In a Phase 1 study, 100 healthy adults will be injected with our PSA conjugate starting in early 2009. We expect to complete the study by the end of 2009.

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Peptide-protein conjugate vaccines

Schneerson, Robbins, Kubler-Kielb, Keith, Biesova, Lin, Liu, Wu, Majadly, Mocca, Guo; in collaboration with Bellanti, Leppla, Miller L, Miller M, Shiloach, Singer

Bacillus anthracis. *B. anthracis*, a cause of lethal human infection with potential for bioterrorism, has two essential virulence factors without either of which it is not pathogenic for humans: the anthrax toxin and the capsule. The toxin is composed of three peptides: Lethal Factor, Edema Factor, and Protective Antigen (PA). The capsule is composed of poly-D-gamma-glutamic acid (PGA). By itself, the capsule is non-immunogenic and its protective effect is not clear. Anthrax is transmitted indirectly, via spores, of different structure and composition than the vegetative organism. The PA-based licensed vaccine is safe and protective under normal conditions, but enhancement of its immunogenicity may be needed in the event of bioterrorism. We sought to induce capsular and anti-spore antibodies as a potential means to expand the immunity conferred by the available anthrax vaccines. We isolated a recombinant PA from an uncapsulated strain, several formaldehyde-treated and/or alum-adsorbed formulations of which were immunogenic in mice. The formulations were safe in adult volunteers, and local and systemic reactions were rare and minor. We are now analyzing serum IgG anti–PA levels.

For comparison, we measured serum IgG anti–PA in 246 sera of recruits injected with the Anthrax Vaccine Adsorbed (AVA) and then analyzed paired sera by ELISA; the sera are stored in the U.S. Department of Defense Serum Repository. Serum conversion rates of greater than or equal to a 4-fold increase in antibody levels were pre-3rd–post-3rd 85.3 percent, pre-4th–post-4th 67.9 percent, and pre-6th–post-6th 45 percent. Geometric mean (GM) levels of all individuals were 59.9 µg per mL following the third injection, 157.4 µg per mL following the fourth injection, and 277 µg per mL following the sixth injection.

We isolated the capsule from a non-toxic strain and bound it or corresponding synthetic peptides to BSA, rEPA, rPA, or tetanus toxoid. Thioether, hydrazone, and oxime linkages between the

PGA and the proteins with active groups at the C- or N-termini yielded conjugates immunogenic in mice, with no statistically different responses to the conjugates. The induced antibodies were opsonophagocytic. Peptides 10- to 20-mer long and 10 to 15 mole PGA per mole protein were the most immunogenic. Dose-response experiments of an rPA-PGA conjugate, using between 0.31 and 20 μg PGA per mouse, showed 1.25 μg to be optimal for a PGA response while PA antibody levels increased with higher immunizing dosages. The use of alum increased PA antibody levels with little effect on anti–PGA levels. We also immunized two chimpanzees, one with PA-PGA and the other with TT-PGA, with the goal of preparing humanized monoclonal antibodies to PGA. Both responded with antibodies to both vaccine components. The chimpanzee injected with TT-PGA had higher anti–PGA levels. We prepared PGA-specific IgG1 and IgG3.

The glycosyl part of the glycoprotein BclA (collagen-like protein of *B. anthracis*) is an oligosaccharide composed of 2-*O*-methyl-4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-D-glucose (referred to as anthrose) and three rhamnose residues. We found structures similar to anthrose in the side-chain of the CP of *Shewanella* spp. MR-4: 4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-D-glucose. Under certain growth conditions, the bacteria produce a variant CP lacking one methyl group on the hydroxybutyrate: 4-(3-hydroxybutanamido)-4,6-dideoxy-D-glucose. In contrast to anthrose, neither of the *Shewanella* CPs is 2-*O*-methylated. Both *Shewanella* CP variants reacted with anti–*B. anthracis* spore sera. We also found structures similar to anthrose in the flagellae of *Pseudomonas syringae*, which are reportedly glycosylated with a similar terminal saccharide: 4-(3-hydroxybutanamido)-4,6-dideoxy-2-*O*-methyl-D-glucose. In a fluorescence microscopy assay, sera produced by immunization with *Shewanella* or *P. syringae* cells bound to *B. anthracis* spores but not to *B. cereus* spores. Protein conjugates of the two variants of *Shewanella* CP induced antibodies that bound to both *Shewanella* CP variants, as judged by ELISA, and to *B. anthracis* spores, as detected by fluorescence microscopy. We propose the use of *Shewanella* CP conjugates as a component of an anthrax vaccine. We also elucidated the structure of the *Shewanella* MR-4 LPS carbohydrate backbone but found no anthrose-like sugar, confirming the presence of such a sugar in the CP only.

Plasmodium falciparum. Malaria, a leading cause of morbidity and mortality globally, especially in children, is estimated to cause over a million childhood deaths annually. *P. falciparum* causes the most severe form of the disease. Experimental vaccines have been described and some tested clinically, but no licensed vaccine is available. We therefore set out to develop vaccine candidates. The most studied experimental malaria vaccines are the circumsporozoite protein (CSP), expressed extracellularly on the sporozoite, and various forms of its synthesized repeat unit, NANP. The vaccines have been shown to be safe and immunogenic, but their protection is poor and of limited duration even when administered with adjuvants. We used two approaches to provide experimental malaria vaccines. The first was directed to the asexual, human-host stage of the parasite; we cloned the CSP into *E. coli* and, after isolation, evaluated several formulations, including adsorption onto alum, in young outbred mice. Based on findings from our studies with peptides of the *B. anthracis* capsule, we synthesized peptides of four to five repeat units of the four amino acids NANP (i.e., the four amino acids of the repeat unit frame shifted by one amino acid, yielding four configurations) and bound them to carrier proteins at different molar ratios and end groups. Injected into general purpose mice, all tested preparations induced high levels of antibodies that bound to circumsporozoites as demonstrated by immunofluorescence assay. Conjugates of NANP with the ookinete surface protein Pfs25 induced long-lasting antibodies to both vaccine components; 3-month serum levels were higher than at 1 week after the last injection. Alum-adsorbed CSP induced higher antibody levels than the non-adsorbed protein. We found that the terminal amino acid of the CSP-derived peptides was an important determinant and that NPNA-protein was the best immunogen.

The second approach was directed to the sexual, mosquito parasite stage in order to provide a transmission-blocking vaccine. We bound Pfs25—a low–molecular-mass protein that is non-

immunogenic by itself—to itself, or to carrier proteins by amide, hydrazone, or thioether linkages. Injected into mice, all conjugates were immunogenic with booster responses upon reinjection. Remarkably, the serum antibody levels at 3 and 7 months after immunization were higher than at 1 week after the last injection. The best immunogens used adipic acid dihydrazide as the linker. Adsorption of the conjugates onto alum further increased antibody levels. As measured by ELISA, transmission-blocking activity of immune sera correlated with antibody levels.

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Protein and polysaccharide conjugate vaccines to enteric diseases

Szu, Schneerson, Lin, Ftacek, Hunt, Li, Nelson, Wu; in collaboration with Ahmed, Clements, Griffin, Kopecko, Moore, Robertson, Shiloach, Cao, Hoshino, Kapikian, Osterholm

Diarrheal diseases account for more than 3 million deaths annually. Diseases such as cholera are common in developing countries and are underreported. Few vaccines for enteric infections are licensed, and all have limitations. For example, the newly licensed rotavirus vaccines elicit only low levels of antibody in infants and may cause adverse reactions. Whole-cell vaccines for enteric fevers and for cholera were withdrawn from the market because of adverse reactions. Our goal is to prepare vaccines against enteric infections suitable for routine administration to infants and young children. We study investigational vaccines against bacteria such as *Salmonella typhi*, non-typhoidal Salmonellae, *Escherichia coli* (in particular *E. coli* O157), *Vibrio cholerae*, and *Campylobacter jejuni* and viruses such as rotavirus.

Our vaccines are designed to induce serum IgG that inactivates the inoculum. The protective antigens are proteins, such as toxins or capsid proteins, and polysaccharides, such as capsules or lipopolysaccharides. To enhance antigen immunogenicity, we conjugated polysaccharides and polypeptides to proteins. Surface polysaccharides of Gram-negative pathogens—capsules or LPSs—are essential virulence factors and protective antigens. Covalent binding to carrier proteins enhances the immunogenicity of polysaccharides. To serve as vaccines, LPSs must be detoxified and their O-SPs isolated and conjugated to proteins. Bacterial toxins or toxoids and viral capsid proteins may be protective antigens and serve as carrier proteins.

The Vi capsular polysaccharide of *Salmonella typhi* (Vi) is a licensed vaccine with limited efficacy in children under 5 years old. To provide a vaccine for younger children, we conjugated Vi to recombinant exoprotein A of *Pseudomonas aeruginosa* (rEPA). A Phase 3 trial of Vi-rEPA in 11,600 Vietnamese 2- to 5-year-olds showed an efficacy of 89 percent at 47 months. In a Phase 2 trial, 301 infants were injected with Vi-rEPA concurrently with DPT at 2, 4, 6, and 12 months. Controls received Hib-TT plus DTP or DTP. No serious adverse reactions occurred. We will assay IgG anti–Vi levels in the infants and compare them with those elicited in 2- to 5-year-olds. We evaluated antibody persistence after vaccination with Vi-rEPA in adults and children and found that the GM IgG anti–Vi level of 18 adults 10 years after a single injection was 20-fold higher than the proposed protective level (3.52 EU), whereas the GM antibody level of 97 children injected twice at ages 2 to 5 years, 9 years after immunization, was only 2.2-fold higher than the proposed

protective level. We observed no significant difference between Vi antibody levels in the latter group and in those that had only one injection (7.04 EU) or no injection (8.14 EU), reflecting a waning of anti–Vi in young children on one hand and the development of natural antibodies on the other. Four years after a single injection into 5- to 8-year-olds, the GM antibody level was 15.9 EU, significantly higher than in age-matched controls.

Salmonella paratyphi A (SPA) is the second most common cause of enteric fever in developing countries. Our Phase 1 and 2 studies showed that SPA O-SP conjugated to TT was safe and immunogenic in adults, teenagers, and toddlers. To improve conjugate immunogenicity, our collaborator Dennis Kopecko replaced the SPA LPS chain-length elongation regulator gene *wzz* with that of *E. coli* K12. Several TT conjugates of the elongated O-SP elicited significantly higher levels of anti–LPS than those prepared with O-SP from the wild type.

Enterotoxigenic *E. coli* (ETEC) is the most common cause of diarrhea in developing countries and in travelers to such countries. ETEC disease is mediated by two exotoxins: heat-labile toxin (LT) and heat stable toxin (ST). LT is immunogenic in humans. ST is a polypeptide of 19 amino acids and is not immunogenic because of its small size. The exotoxins' efficacy against infection has not been demonstrated in humans. LT192, a mutant of reduced toxicity (provided by John Clements), elicited high levels of IgG anti–LT in mice. However, dosage-dependent swelling occurred at the injection site even after formalin detoxification, an adverse reaction that could be reduced by using a double mutant LT (mutation at amino acids 192 and 211) as an immunogen. The wild-type LT and the double mutant elicited similar IgG anti–LT levels in mice. We plan to undertake clinical studies with the double mutant. Several attempts at chemical synthesis of ST have failed owing to ST's high number of disulfide bonds (three pairs). In collaboration with Donald Robertson, we purified ST from *E. coli*. We have prepared a conjugate of ST linked to bovine serum albumin and will evaluate its immunogenicity in mice.

Vibrio cholerae O1 remains a major health problem in the Indian subcontinent and in Africa. Field studies showed that serum vibriocidal activity is directed toward the bacterium's LPS. In a Phase 1 trial, O-SP conjugates elicited IgG anti–LPS with vibriocidal activity. To enhance immunogenicity, we devised a new scheme of conjugation by thiolating both the carrier protein and the O-SP with heterobifunctional reagents. Antibody levels induced in mice by these conjugates were significantly higher than those induced by the conjugate used in our Phase 1 study. We also synthesized a hexamer of *V. cholerae* O-SP. Conjugated to TT, the hexamer was antigenic. We plan to evaluate the immunogenicity of both the new bacterial O-SP conjugates and the synthetic saccharide conjugates.

E. coli O157 is a major cause of hemolytic uremic syndrome, especially in young children. An O157 O-SP-rEPA conjugate, injected into 55 2- to 5-year-old children once or twice, was safe and immunogenic; 6 months after injection, 98 percent of the children had a greater than 4-fold increase in IgG anti–LPS over levels before immunization. The second injection did not induce a booster response. In collaboration with Patricia Griffin and Michael Osterholm, we developed a proposal to immunize children between 2 and 10 years of age in States with the highest incidence of *E. coli* O157 infection.

Rotavirus is the most common cause of infantile diarrhea worldwide. Two rotavirus vaccines are licensed—a live attenuated vaccine and a reassortant vaccine, both orally administered. We are designing a parenteral vaccine based on capsid proteins VP8 and VP7. We purified VP8 from *baculovirus*-infected insect cells (from Yasutaka Hoshino) and observed that mice injected with VP8 produced neutralizing antibodies against rotavirus of both homologous (P4) and heterologous (P8) serotypes. To increase the yield of VP8, we expressed its cDNA in *E. coli*. The recombinant VP8 elicited neutralizing antibodies to both P4 and P8 rotavirus serotypes.

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Synthetic vaccines against human pathogenic bacteria

Pozsgay, Schneerson, Beyene, Claude, Dulcey, Ekborg, Santacroce

Using innovative glycochemical technologies, we design and synthesize oligosaccharide- and glycolipid-based conjugate vaccines for the prevention of human diseases. We develop high-yielding, mild, and experimentally simple methods for covalent conjugation of glycans to proteins. Human bacterial pathogens may have surface-exposed saccharides that serve as both virulence factors and protective antigens. Such saccharides include CPs, LPSs, and cell-wall polysaccharides varying in size and complexity. We are studying synthetic chemical approaches to bacterial surface–exposed saccharides that can be used to elicit serum IgG antibodies against such pathogens.

SYNTHETIC GLYCOCONJUGATES AS EXPERIMENTAL VACCINES. *Shigella dysenteriae* type 1 is the causative organism of endemic and epidemic dysentery in many parts of the world. It has acquired resistance to most available antibiotics. Evidence suggests that antibodies to the O-SP of the LPS of this bacterium may be protective. The O-SP consists of a tetrasaccharide repeating unit containing l-rhamnose, d-galactose, and *N*-acetyl-d-glucosamine units with the →3)-alpha-l-Rhap-(1→2)-alpha-d-Galp-(1→3)-alpha-d-GlNAcp-(1→3)-alpha-l-Rhap-(1→ linear tetrasaccharide structure. We have shown that covalent conjugates of bovine serum albumin to oligosaccharides corresponding to the O-SP in the 8- to 16-mer range elicit O-SP–specific antibodies in mice. We achieved the highest antibody levels when the non-reducing terminus was occupied by either a d-galactose or an *N*-acetyl-d-glucosamine residue, whereas oligosaccharides that contained an l-rhamnose residue at that terminus produced low levels of anti-saccharide antibodies. With this finding, we have redesigned our previous synthetic sequence and prepared a decamer and an undecamer containing the GlcNAc-Rha-Rha-Gal-GlcNAc-Rha-Rha-Gal-GlcNAc-Rha and the Gal-GlcNAc-Rha-Rha-Gal-GlcNAc-Rha-Rha-Gal-GlcNAc-Rha structures, in amounts exceeding 300 mg. When using our oxime-based conjugation method, these oligosaccharides contain a linker at their reducing end for covalent attachment to a medically acceptable protein. We plan to evaluate the conjugate-induced anti–O-SP antibody levels in volunteers.

It has been proposed that Shigellae are descendants of *E. coli* and that *E. coli* O-148 is the precursor of *S. dysenteriae* type 1. Similar to *S. dysenteriae* type 1, *E. coli* O-148 causes diarrhea in children, soldiers, and travelers. The repeating unit of the O-SP in *E. coli* O-148 is almost identical to that of *S. dysenteriae* type 1; the only difference is that D-glucose replaces the galactose residue in the latter. To establish cross-reactivity between the two bacteria, we chemically synthesized oligosaccharide fragments of the O-SP of *E. coli* 148. These probes are composed of 1, 2, and 3 consecutive repeating units containing 4, 8, and 12 monosaccharide residues. The saccharide constructs also contain a spacer equipped with a keto moiety. Using our oxime-based conjugation technique, we have prepared human serum albumin conjugates containing up to an average of 28 oligosaccharide chains per protein molecule. We will investigate the immunogenicity of these conjugates to study cross-reactivity with the *S. dysenteriae* type 1 O-SP.

SYNTHETIC VACCINE AGAINST *BORRELIA BURGDORFERI*. *Borrelia burgdorferi,* the etiological agent of Lyme disease, expresses two major glycolipids on its surface. Termed BBGL-1 and BBGL-2, both constructs contain a galactose residue in addition to palmitoyl and oleoyl moieties. BBGL-1 also has a cholesterol unit, whereas BBGL-2 has a glycerol residue acylated by the palmitic and oleoic acids. The location of the two glycolipids on the bacterial surface makes them a suitable target for vaccine development. We have chemically synthesized BBGL-1 in both its native and bioconjugatable forms. Native BBGL-1 or its protein conjugate elicited low levels of anti–BBGL-1 antibodies in mice. We are now examining the immunogenicity of BBGL-2. First, we undertook

its chemical synthesis. Starting from a commercially available, optically active glycerol derivative, we attached a fully protected galactose residue stereospecifically to the free hydroxyl of the glycerol moiety. Next, we removed the original protecting groups on the glycerol. We acylated the resulting diol regioselectively with palmitic acid followed by placement of the oleoyl moiety at the secondary hydroxyl group of the glycerol residue. Chemoselective removal of the protecting groups on the galactose moiety yielded BBGL-2. We will adapt this methodology for the synthesis of a conjugatable derivative of BBGL-2, which we will link to a protein in order to evaluate the feasibility of enhancing immunogenicity by conjugation to an immunogenic protein.

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Neonatal respiratory distress related to colonization with group B streptococci

Lin, Ogbonna, Turner; in collaboration with Curtis, Levine, Troendle, Yergey

Despite the decrease in the incidence of early-onset group B streptococcal (GBS) disease as a consequence of the widespread use of antibiotic prophylaxis during labor, GBS remains a leading cause of neonatal sepsis. We hypothesized that newborns colonized with GBS, especially newborns of penicillin-treated mothers, acquire bacterial phospholipids as a result of prophylaxis during labor, leading to pulmonary hypertension and respiratory distress. It has been shown that infusion of live or heat-killed GBS into sheep promptly induced pulmonary hypertension. The goal of our study was to examine clinical and epidemiologic data related to this potential association. Clinical features of early-onset GBS disease are similar to those of endotoxic shock, and a prominent symptom is respiratory distress, which may occur in newborns without a known GBS infection. Jerri Curtis and colleagues purified and identified phospholipids (cardiolipin and phosphatidylglycerol) from the GBS cell wall as pulmonary hypertensive compounds and observed that infusion of either cardiolipin or phosphatidylglycerol into neonatal lambs caused pulmonary hypertension. Others have demonstrated that exposure of *Streptococcus mutans* to penicillin induces a 15-fold increase in phospholipid release from the organism. Penicillin is one of the beta-lactam antibiotics, which inhibit the normal assembly of peptidoglycan of bacterial cell wall. Given the recommendation to treat newborns infected with GBS or their GBS-carrying mothers with penicillin or its derivatives, our findings raised the question of whether exposure of GBS to penicillin or to other lactam antibiotics in newborns and mothers induces a release of GBS phospholipids causing pulmonary hypertension respiratory distress in newborns.

Analysis of a data file from the NICHD multicenter GBS study found a possible association among GBS colonization, penicillin treatment, and respiratory distress in neonates, namely that nearly 9 percent of GBS-colonized newborns who did not develop early-onset GBS disease experienced respiratory distress within 48 hours of birth compared with 1 to 3 percent of non-colonized newborns. Furthermore, colonized newborns of penicillin-treated mothers were more than twice as likely to develop respiratory distress as GBS-carrying but untreated mothers.

Although the effect of cardiolipin on pulmonary hypertension and of penicillin on phospholipid release from *S. mutans* supports the role of GBS colonization and penicillin in respiratory distress in newborns, proof of these associations requires assay of GBS phospholipids in serum samples

of newborns—with and without respiratory distress—born to GBS-colonized mothers treated or untreated with penicillin or other beta-lactam antibiotics during labor. We are developing such an assay in collaboration with Alfred Yergey. A prospective study aimed at relating serum GBS phospholipid levels to the occurrence of respiratory distress in newborns of GBS-colonized mothers is under way at two U.S. academic centers: Baylor College of Medicine, Houston, Texas; and Children's Hospital and Research Center, Oakland, California. Our sample size estimate suggests a need for 1,600 colonized mothers who are at least 32 weeks' gestation at delivery. We use a semiquantitative culture method to determine the degree of colonization in the mothers. We are assembling three groups of GBS-colonized mothers: untreated, beta-lactam antibiotic–treated, and treated with non–beta-lactam antibiotics. Maternal and cord blood samples are being collected for the cardiolipin assay, and all newborns are monitored for signs of respiratory distress. We are assembling two groups of newborns—with and without respiratory distress.

Design, synthesis, and testing of recombinant proteins for influenza vaccines
Keith, Robbins, Biesova, Schneerson, Trinh; in collaboration with Miller M, Shiloach

Highly pathogenic H5N1 avian influenza A virus has the potential to cause the next human influenza pandemic. To avert a world health crisis, innovative methods of rapidly producing sufficient quantities of human vaccine must be explored. Early each year, circulating human influenza virus strains are selected to be included in the vaccine formulation for the upcoming season. During the next 9 months, vaccine manufacturers produce the vaccine by growing the selected virus strains in embryonated chicken eggs. This vaccine production platform, with a maximum capacity of 300 million doses, lacks the capacity for rapid production of the 2 to 3 billion vaccine doses that would be required to prevent a worldwide avian flu pandemic. The essential immunogen in all influenza vaccines is the immunodominant hemagglutinin protein (HA). FDA mandates a minimum of 15 μg HA per dose. Based on the immunological diversity of HA, the WHO classified avian influenza viruses into three clades and several subclades and has encouraged vaccine developers to produce human vaccines effective against these virus clades. Our approach to this challenge is to use recombinant DNA technology, cellular protein expression systems, and chemical conjugation methods to produce unique protein antigens as influenza vaccine candidates.

We have developed a protocol for rapid production of the HA of H5N1 avian pandemic influenza viruses. The procedure does not require access to the pandemic influenza virus strains—select agent regulations, USDA control measures, and biosafety level constraints restrict our ability to work with the pathogens. Using only DNA sequences from the CDC/WHO pandemic influenza virus database, we chemically synthesized genes de novo in a procedure optimized for protein expression in *E. coli*. The procedure can reduce the response time required to produce a vaccine to less than 1 month compared with the 6 to 8 months needed for the traditional egg-based preparation of influenza vaccines. In addition to this new de novo gene protocol, we have developed cloning protocols that use influenza viruses as templates for reverse transcription-polymerase chain reaction gene amplification. With these procedures and protocols, we have constructed bacterial seed clones/cultures, used them to produce pilot plant quantities of recombinant HA proteins, and have now developed standardized downstream processing protocols for protein extraction, purification, refolding, and vaccine formulation. We have produced recombinant HA immunogens from four representative influenza A viruses: H3N2 A/California/7/2004 (standard control from human 2006 influenza vaccine); H5N1 A/Vietnam/1203/2004 Clade 1; H5N1 A/Indonesia/5/2005 Clade 2, subclade 1; and H5N1 A/Bar-headed Goose/Qinghai/1A/2005 Clade 2, subclade 2.

We designed the recombinant protein constructs to represent the mature configuration of HA, with the amino acid domain spanning the viral membrane deleted from the carboxyl terminal. We then replaced the domain with a Gly3X-His6X tag to facilitate purification of the expressed protein by Ni-ion chelating chromatography. We produced recombinant HA (rHA) from four representative

virus strains in either BL21(DE3) or Rosetta 2(DE3) strains of *E. coli* and purified it from isolated inclusion bodies by urea solubilization of the inclusion body protein and by Ni-ion column chromatography. Using rapid dilution into refolding buffer, extensive dialysis, and spin-filter concentration, we further processed the purified rHA protein derived from the A/Vietnam/1203/2004 and A/Indonesia/5/2005 H5N1 influenza viruses for use in vaccine formulation and immunization studies. We formulated various vaccine candidates by adsorbing the rHA onto alum, treating the protein with formalin, or both. Injected into young mice three times (2.5 to 5µg per mouse), rHA induced antibodies with hemagglutination inhibition titers of 40 and 80 or higher, suggesting that rHA could induce protective immune responses against influenza virus infection (FDA guidelines require a minimum titer of 40). Our preliminary data suggest that the alum-absorbed rHA vaccine, produced in just 4 weeks, can fulfill FDA requirements. We plan to conduct antibody neutralization experiments based on plaque reduction analysis and inhibition of viral replication within a unique tissue culture cell line.

Recent reports in the literature suggest that antibodies to the exposed N-terminal of 23 amino acids (M2e) of the mature matrix 2 protein (M2) may ameliorate disease symptoms. The M2 protein provides an ion-channel through the viral membrane and is recognized as a target for prophylaxis and treatment with the antiviral drug amantadine. Unlike the virion's surface proteins HA and neuraminidase, which are subject to constant genetic drift and shift, the M2 protein is highly conserved probably because its protected location within the viral membrane prevents a strong host immune response. However, recent studies showed that when the exposed 23–amino acid M2e peptide was genetically fused to the N-terminus of hepatitis B virus core particles as a carrier, the chimeric protein conferred complete protection against a lethal heterologous influenza virus challenge in a mouse model. We bound a synthetic M2e peptide to a genetically detoxified diphtheria toxin (DT-H21G) via thioether linkages. MALDI-MS (matrix-assisted laser desorption/ionization mass spectrometry) analyses showed an average of seven chains of M2e per DT molecule. We injected the conjugate, in aqueous form or alum-adsorbed, into mice at 2.5 µg peptide, two or three times, 2 weeks apart and collected sera 1 week after the last injection. ELISA showed high anti–M2e levels after the third injection, with or without alum. We also observed an antibody response against the DT(H21G) carrier. Our preliminary results suggest that this candidate vaccine may induce immunity against heterologous strains of influenza A virus.

Modulation of protein and cell functions by heparin/heparan sulfate and mimetics
Stone, Chen, Kazanina; in collaboration with McMahon, Horne, Lewis, Longas

The rate of HIV infection is rising, now reaching over 1 million cases in the United States and 50 times that globally, with long-term control threatened by drug toxicity, the emergence of multidrug resistant strains, and treatment options limited mostly to protease and reverse transcriptase inhibitors. Thus, inhibitors against viral components that play other roles in HIV-1 attack are critically needed.

Found in cell membranes, heparin and heparan sulfates (H/HS) constitute a class of highly sulfated glycocaminoglycan biopolymers based on the specificity of their optically active structures (Stone, *Biopolymers* 1963;2 and 1964;3). These polysaccharide polymers occur as modulatory receptor systems throughout the body. Their diverse sulfation provides H/HS with unique sugar sequences (S-oligoS), accounting for their ability to modulate diverse proteins and biological systems in cell growth, inflammation, development, and microbial infections. Current structural considerations indicate that the S-OligoS structure contains a tetrasaccharide motif of three xyloses and a glucuronic acid as a branch on the xylan chain, i.e., -D-glucuronyl-alpha 1,2 beta 1,4 D-(xylyl)3 with up to one-third of the GlcA *O*-methylated. The sugars are about 90 percent sulfated, yielding a high negative charge density. Such a motif could accommodate the subtle variations in sulfate geometries, potentially providing for multifunctional mimicry of the heparins.

Owing to the similarity of the physicochemical properties of H/HS chains and absent cognate ligands, libraries of unique S-oligoS are not obtainable for research. We obtained a heparin-mimetic library that is based on the structure-function model for the antithrombin-dependent anticoagulant heparin (Stone et al., *Proc Natl Acad Sci USA* 1982;79:7190) and that comprises S-oligoS that mimic unfractionated heparin in its biological actions. We demonstrated that in vitro inhibition of both HIV-1 cytotpathology and syncytium formation are governed by structural specificity and separable from the anticoagulant S-oligoS—essential indicators for useful drug development (Stone et al., *Glycoconjugate J* 1998;15:697). We devised a procedure to obtain a clinical preparation of a highly active inhibitor of HIV-1 virus fusion free from antithrombin toxicity (SOLIS). Our clinical preparation of SOLIS for a Phase I safety trial is ongoing. Heparin has long been known to affect conformation of proteins, thus modulating their biological function. Given that conformational changes in bound gp41 are required for fusion progression, inhibition of this process is a possible mechanism of action of heparin and SOLIS.

Abu-Raddad et al. (*Science* 2006;314:1603) "estimated that, since 1980, the disease interaction may have been responsible for 8,500 excess HIV infections and 980,000 excess malaria episodes" in a selected Kenyan population. Co-treatment of malaria and HIV/AIDS is feasible because of the similarity of the respective S-oligoS inhibitors. We identified another component (Cp11) as a potential antimalaria drug (see below). We posited that SOLIS and Cp11 would be physicochemically compatible for combined treatment formulation and thus studied the effect of co-treatment in vitro. We investigated whether the addition of increasing amounts of Cp11 (relatively inactive against HIV-1) to the standard SOLIS doses would affect the capacity of SOLIS to protect cells in a standard formazan cytotoxicity protection assay. Results indicated that the presence of Cp11, the antimalarial S-oligoS, diminished SOLIS's inhibitory effect in vitro, which would cause a significant mutual enhancement of vulnerability to the other pathogen in patients with either disease and a consequent increase in the incidence and spread of AIDS. However, the mechanism of the process is not fully understood. The reduction of inhibitory capacity of our anti–HIV drug in vitro may reflect a common mechanism for production of the endogenous H/HS receptors for malaria and HIV-1, such as the specific expression of enzyme isoforms in the biosynthesis of anticoagulant H/HS. We will study this interpathogen effect on disease and antipathogen activity in an animal model.

Further preparation of Cp11 and CpC (smallest-mass component retaining high activity against cytopathological effects of HIV-1 in vitro) is ongoing. We will use CpC to prepare conjugates for immunological studies on SOLIS, e.g., to obtain a potent immunogen to produce an antibody for direct measurement of SOLIS concentration in the blood after intravenous administration. We plan to purify further CpC 8b regions to be used for advanced Fourier transform infrared spectral analysis and Heteronuclear Multiple Quantum Coherence correlation spectra to determine the relation of alternative chair structure to inhibition of HIV-1 gp120 and gp41.

Heparin-mimetic sulfated oligoxylan inhibitors of malaria parasites

Stone, Chen; in collaboration with Milhous, Sacci

Malaria is one of three deadliest diseases globally (more than 300 million cases annually). *P. falciparum* causes cerebral malaria and the death of about 2 million people annually (90 percent of whom are young children). There is no vaccine against malaria, which is becoming increasingly resistant to drugs. We are studying the antimalaria components of our S-OligoS (1) as in vitro inhibitors against primary invasion of hepatocytes and by (2) identifying S-oligoS component(s) against cerebral malaria in young children in a rosetting assay and (3) elucidating potential combined therapy for HIV infection and malaria (see above).

The initial infective process of malaria takes place in hepatocytes. The portal for invasion implicates the H/HS receptor systems. Heparin inhibits the rosetting of and cytoadhesion of parasitized *P. falciparum* erythrocytes (PfRBC) to normal RBC and the endothelium and clears blockage of the

microcirculation, which ameliorates cerebral malaria in children. To explore the antimalarial potential of our heparin-mimetic S-oligoS, produced as anti–HIV drugs, we characterized its inhibition of malaria. We found that structural specificity and concentration governed the ability of S-OligoS to inhibit the invasion of hepatocytes by freshly isolated *P. yoelii* sporozoites; highest potency resided in two S-OligoS with mass of about 7,200 (Cp6) and 3,700 (Cp11). Cp11 has negligible antithrombin capacity, and we are preparing more of this S-oligoS. In contrast to sporozoite invasion of hepatocytes, components with mass less than 4,500 exhibited low inhibitory capacity toward the erythrocyte invasion stage of malaria while high potency was associated with a mass equal to or greater than 10,000, suggesting that inhibition of the two parasite stages differ. Preparation of additional Cp11 and expansion of our library in the mass range about 3,000–4,000 is under way, and we will continue to study inhibition of sporozoites, parasitized RBC, and rosetting as a method for stable, inexpensive, heparin-based antimalarials against infection, pathologies, and/or acute cerebral malaria.

Definition of angular dependence of ^{1}H-^{15}N coupling constants in amino sugars

Coxon

Our goal is to define the stereochemical dependence of nitrogen-15–proton coupling constants in amino sugars by high-resolution NMR measurements of such constants in model compounds. Our current experimental method of choice is the Carr-Purcell-Meiboom-Gill heteronuclear single quantum multiple bond correlation (CPMG-HSQMBC) NMR procedure. Using data we obtained for amino sugar derivatives during the past year, we expanded our project from defining the dihedral angular dependence of the coupling constants to include the general stereochemical dependence of these couplings, which also involves dependence on atomic electronegativities and their orientation. We plan to apply the nitrogen-15 NMR data to determine or verify the structures of nitrogen-containing bacterial polysaccharides and aminoglycoside antibiotics with examples from the fortimicin group.

We have completely analyzed the one-dimensional (1D) ^{1}H spectra and 1D ^{1}H–^{15}N CPMG-HSQMBC NMR spectra of the subject amino sugars (with ^{15}N at natural abundance) and correlated the resulting $^{3}J_{HCCN}$ coupling constants with molecular geometry. Commonly present in bacterial polysaccharides, the amino sugars we are analyzing include D-glucosamine, D-mannosamine, and D-galactosamine; for this study we are using the sugars' *N*-acetyl and hydrochloride derivatives. In a molecular modeling study using molecular dynamics (MD) calculations with energy minimization by molecular mechanics (MM) and implicit solvent, we defined the geometries of the alpha and beta pyranose anomers of *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, and *N*-acetyl-D-galactosamine. The study yielded values of the vicinal nitrogen-hydrogen dihedral angles (ϕ) that we have correlated with measured values of the $^{3}J_{HCCN}$ coupling constant.

Using MD/MM, we found that *gauche* arrangements of the hydrogen and nitrogen-15 nuclei in the common *N*-acetyl amino sugars had ϕ values in the range of 52 to 66, which corresponded to $^{3}J_{HCCN}$ values of 0.8 to 2.5 Hz. *Trans* orientations of the hydrogen and nitrogen-15 nuclei displayed ϕ values in the range of 175 to 178 in MD/MM, which correlated with larger $^{3}J_{HCCN}$ values in the range of 3.2 to 4.0 Hz. However, if the coupling involves the anomeric proton ^{1}H, then the magnitude of the *trans* coupling constant is reduced by the electronegativity of the pyranose ring oxygen to 2.7 to 2.8 Hz. Thus, it appears that the magnitude of the $^{3}J_{HCCN}$ coupling constant could be used to distinguish *gauche* and *trans* orientations of vicinal nitrogen-15–proton pairs in amino saccharides, offering the possibility of defining the stereochemical configuration (axial or equatorial) of the nitrogen atom in unknown structures. Even though these common amino sugar models contain only limited ranges of ^{15}N–^{1}H dihedral angles, the results for their $^{3}J_{HCCN}$ coupling constants generally agree with those measured for the organic-soluble amino-sugar derivatives from which we previously formulated a Karplus-type equation: $^{3}J_{HCCN}$ = 3.1 cos2 phi - 0.6 cos phi + 0.4.

Coxon B. A Karplus equation for $^{3}J_{HCCN}$ in amino sugar derivatives. *Carbohydr Res* 2007;342:1044-1054.

NMR verification of structures of bacterial saccharide precursors for vaccines
Coxon, Schneerson, Kubler-Kielb, Mocca, Nelson, Szu

High-resolution NMR spectroscopy is a powerful method for analysis of the conformations, molecular structures, purity, and stereochemistry of carbohydrates, including monosaccharide, oligosaccharide, and polysaccharide types that are of prime interest in bacterial vaccine development. Part of our function is to provide analytical NMR support as needed for current vaccine projects in the laboratory that involve the characterization of saccharide structure and purity. We investigate new vaccine-related substrates as they are developed.

Using 1D ^{1}H and 1D ^{13}C NMR, we have analyzed about 40 monosaccharide and oligosaccharide intermediates for the preparation of a hexasaccharide-protein conjugate by high-resolution NMR at 500 MHz. When we needed additional information on spectral assignments, we used two-dimensional (2D) methods such as correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and heteronuclear single quantum correlation spectroscopy (HSQC). We based validation of the chemical structures on integration of the 1D ^{1}H NMR spectra and on the counting of carbon resonances in the ^{13}C spectra, especially in well-separated spectral regions, such as those of the C = O, aromatic C, C-1, C-N, and C-CH_3 moieties. We also used 1D ^{13}C distortionless enhancement by polarization transfer (DEPT) to differentiate $^{13}CH_2$ resonances from those of ^{13}CH and $^{13}CH_3$. This simple spectral editing technique yields a $^{13}CH_2$ count in the form of ^{13}C resonances of inverted phase and was particularly applicable to these intermediates because of the mixed CH, CH_2, and CH_3 resonances of the amide and linker side-chains in the structures.

We provided NMR service spectra measured at 500 MHz for the identification of 20 polysaccharide fragments isolated from *Shigella sonnei*, including products of nitrous acid deamination that are used in structural studies and spectra that facilitate the detection of core polysaccharide in the samples. The techniques include 1D ^{1}H and ^{13}C NMR but sometimes 2D COSY, TOCSY, and HSQC as well. We acquired the spectra at an elevated sample temperature of 50°C to reduce the viscosity of the solutions, thus permitting the measurement of sharper spectra. The techniques provide complex fingerprint spectra that lend themselves to comparison with data in the literature in order to verify identity. We also measured service spectra for nine fractions isolated from *Klebsiella pneumoniae* type 2, including a series of three samples of the polysaccharide that had been subjected to sonication for 5, 15, or 30 minutes to test a molecular weight–reduction procedure. We identified one sample of a polysaccharide found in *S. flexneri* type 2a by 1D ^{1}H and ^{13}C NMR.

Isolation and NMR elucidation of three saponins from *Blighia sapida*
Coxon; in collaboration with Mazzola, Kennelly, Parkinson, Freedberg

Saponins are steroid glycosides, steroid alkaloids (steroids with a nitrogen function), or triterpenes that are found especially in plant skins, where they form a waxy, protective coating. They may be useful in the human diet for controlling cholesterol, but some are poisonous or may cause urticaria. We set out to isolate three saponins from *Blighia sapida* and to determine their structures by high-resolution NMR spectroscopy and mass spectrometry.

QS-21 is a complex saponin that has been used as a vaccine adjuvant, thereby stimulating interest in this general class of compounds. In a collaborative study, we isolated three complex saponins—Blighosides A, B, and C—from the tree *Blighia sapida*, determined their structures by high-resolution NMR, and obtained their molecular weights by mass spectrometry. Our results show that Blighoside A (MW 1086 Daltons) has an unbranched tetrasaccharide containing arabinose, rhamnose, glucose, and a second arabinose linked through one of the arabinose moieties to C-3 of a 30-carbon triterpene aglycone hederagenin. The rhamnose is linked to C-2 of the first arabinose, the glucose to C-3 of the rhamnose, and the second arabinose to C-4 of the glucose, which is acetylated at O-3. Blighoside B (MW 1,070 Daltons) contains the same tetrasaccharide moiety as Blighoside A but is linked to a slightly different triterpene, oleanolic acid, in which C-23 of hederagenin is a methyl

group instead of a hydroxymethyl group. Blighoside C (MW 1,504 Daltons) has six monosaccharide units linked to oleanolic acid. Attached to C-3 of this triterpene is a linear hexasaccharide consisting of two xyloses, rhamnose, glucose, a second rhamnose, and another glucose. The second xylose is linked to C-3 of the first xylose, the first rhamnose to C-2 of the second xylose, the first glucose to C-3 of the preceding rhamnose, the second rhamnose to C-4 of the preceding glucose, and the final glucose to C-3 of the preceding rhamnose. In addition, the first glucose is acetylated at O-3 and O-6 and the terminal glucose at O-4 and O-6.

The NMR methods used for determining structure included 1D slices of 2D TOCSY data sets to generate separate sub-spectra for the individual monosaccharide residues, which allowed identification of sugar type by measurement of vicinal $^{1}H–^{1}H$ coupling constants. We also used 2D HMBC experiments that gave $^{1}H–^{13}C$ through-bond connectivities that defined nuclear neighbors in the structures as well as the sugar linkages and positions of acetyl substitution. 2D ROESY (rotating-frame NOE spectroscopy) experiments indicated $^{1}H–^{1}H$ through-space interactions, especially for proximal protons in adjacent sugar residues. We achieved additional resolution of the NMR signals of similar sugar residues with 2D ^{1}H-coupled HSQC spectra, which also allowed identification of equatorial or axial protons in the sugars and determination of their anomeric configuration by measurement of $^{1}J_{C\text{-}1,H\text{-}1}$ coupling constants.

NMR analysis of synthetic oligosaccharide fragments of the OSP of *E. coli* O-148
Coxon, Pozsgay

In efforts related to preparation of a potential vaccine against *Shigella dysenteriae* type 1, we acquired 1D and 2D high-resolution NMR spectra at 500 MHz from synthetic oligosaccharide fragments of the O-SP of *E. coli* O-148, which cross-reacts with *S. dysenteriae* type 1. The repeating units of the O-SPs are closely related; the main difference is the presence of D-glucose units in the *E. coli* OSP instead of D-galactose in *S. dysenteriae* type 1. Studies of a tetrasaccharide (4-mer), octasaccharide (8-mer), and dodecasaccharide (12-mer) are under way, including one, two, or three repeating units of the O-SP of *E. coli* O148. We derivatized these oligosaccharides as their 5-methoxycarbonylpentyl glycosides in advance of coupling to a carrier protein that would be used to prepare a protein-saccharide conjugate.

We employed several NMR techniques, including 1D ^{1}H, 1D ^{13}C, 2D $^{1}H–^{1}H$ COSY, 2D $^{1}H–^{1}H$ TOCSY, 2D $^{13}C–^{1}H$ HSQC, 2D $^{13}C–^{1}H$ heteronuclear multiple bond correlation (HMBC), 2D ^{1}H-coupled $^{13}C–^{1}H$ HSQC, and 1D ^{1}H-coupled ^{13}C NMR. COSY yields information about ^{1}H spectral assignments, TOCSY provides separate ^{1}H sub-spectra for individual sugar residues, HSQC provides ^{13}C spectral assignments by correlating the ^{1}H and ^{13}C chemical shifts, and HMBC pulse sequence delineates the positions of substituents by correlating their ^{1}H or ^{13}C chemical shifts with those of nuclei in the sugar rings. The ^{1}H-coupled 1D ^{13}C and ^{1}H-coupled 2D HSQC techniques measure $^{13}C–^{1}H$ coupling constants of the oligosaccharides. The latter method is particularly useful when the ^{1}H-coupled, 1D ^{13}C NMR spectra are insufficiently dispersed for a facile analysis. We measured $^{1}J_{C\text{-}1,H\text{-}1}$ values, which typically yielded information on the configuration (alpha or beta) of all the anomeric linkages in the oligosaccharide. Confirmatory information is often available from the $^{3}J_{H\text{-}1,H\text{-}2}$ values measured from the ^{1}H NMR spectra, with the exception of mannopyranose and rhamnopyranose, for which the values of the alpha and beta anomers are similar.

We encountered a problem with broadness of the ^{1}H spectra of the 8-mer and 12-mer in D_2O solutions, which could inhibit the analysis and assignment of the spectra. Surmising that such broadness might be attributable to the viscosity of the solutions of the 8-mer and 12-mer, we acquired ^{1}H and ^{13}C spectra of the 12-mer at elevated temperatures of 50°C and 60°C but discovered that the higher temperatures did not resolve the issue. We are investigating the possibility that the unexpected broadness of the spectra results from paramagnetic metal ion contaminants in the 8-mer and 12-mer. Carbohydrates are often excellent metal-complexing agents, and the occasional spectral broadening

observed in the presence of scavenged paramagnetic metal ions is a well-known phenomenon. In an effort to remove any polyvalent, paramagnetic ions, we have therefore treated the 8-mer with Chelex 100 cation exchange resin (sodium form). Following treatment, the 8-mer showed improved ^{1}H spectral resolution.

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NEUROENDOCRINOLOGY OF STRESS

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The goal of the laboratory is to understand the neuroendocrine mechanisms underlying the stress response, with emphasis on the regulation of the hypothalamic pituitary adrenal (HPA). Not only during early development but also during adult life, the ability of the organism to adapt to acute and chronic stress situations is determined by genetic constitution and life experiences. The organism's degree of adaptability may lead to long-term consequences for the responsiveness of the HPA axis, with altered expression of hypothalamic corticotrophin releasing hormone (CRH) and circulating levels of glucocorticoids—hormones implicated in the pathogenesis of several psychiatric and metabolic disorders. Our laboratory studies the mechanisms of positive and negative regulation of expression of the hypothalamic hormones CRH and vasopressin (VP) and their receptors under different stress situations and the impact of such stress situations on HPA axis regulation. The influence of life experiences, especially during early development, on neuroendocrine regulation and the expression of genes involved in the stress response are important aspects of our research program. Elucidation of the effects of life experiences on the stress response and its regulation is critical for understanding the mechanisms leading to HPA axis dysregulation and for developing diagnostic, preventive, and therapeutic tools for stress-related disorders.

Regulation of hypothalamic CRH expression
Liu, Kim, Aguilera

Appropriate responses of the HPA axis to stress, with adequate control of adrenal glucocorticoid secretion, is essential for homeostasis. Our studies have played a critical role in understanding the interaction between the peptides CRH and VP in the regulation of pituitary adrenocorticotropic hormone (ACTH) and the regulation of the expression of these peptides in the paraventricular nucleus (PVN) during stress and other alterations of the HPA axis. Co-expressed in the same parvocellular neuron of the PVN, both peptides are differentially regulated during stress or exposure to glucocorticoids.

CRH coordinates behavioral, autonomic, and hormonal responses to stress and is the main regulator of ACTH secretion in acute and chronic conditions. Following CRH release, activation of CRH transcription is required to restore mRNA and peptide levels, but termination of the response is essential to prevent pathology associated with chronic elevation of CRH and glucocorticoid production. Our laboratory has furthered the understanding of the mechanisms of negative and positive transcriptional regulation of CRH. We demonstrated that CRH transcription is under

positive control by cAMP/phospho–cyclic AMP response element binding protein (CREB) signaling and negatively regulated by glucocorticoid feedback. Our research led to the demonstration that, in addition to glucocorticoids, intracellular feedback mechanisms in the CRH neuron, which involve induction of repressor forms of cAMP response element modulator (CREM), limit CRF transcriptional responses by competing with the positive regulator phospho-CREB. Rapid repression of CRH transcription following stress-induced activation is likely to help limit the stress response and work to prevent disorders associated with excessive CRH production.

With respect to positive regulation, the conventional view holds that activation of CRH transcription is initiated by binding of phosphorylated CREB to the CRH promoter. During the past year, our laboratory challenged this view by reporting that cAMP/phospho-CREB signaling is essential but not sufficient to activate CRH transcription. Our finding strongly suggests that transcriptional activation of the CRH gene requires a co-activator of CREB. In a number of systems, it has been shown that CREB-mediated transcription involves the co-activator TORC (transducer of regulated CREB activity), which could be the missing link required for CREB-induced regulation of CRH transcription. We examined the ability of TORC to regulate CRH transcription in a hypothalamic cell line 4B transfected with a CRH promoter–driven luciferase reporter gene. Consistent with the above observations, at threshold concentrations for cAMP production and CREB phosphorylation, forskolin induced marked CRH promoter activation while phorbol-12-myristate-13-acetate (PMA) failed to activate the CRH promoter despite PMA's ability to phosphorylate CREB. However, in cells co-transfected with an expression vector for TORC2, PMA significantly activated the CRH promoter compared with baseline activity in TORC-transfected cells or PMA administration to cells transfected with the empty vector. TORC2 transfection also potentiated the stimulatory effect of submaximal, but not that of maximal, concentrations of forskolin. We examined the involvement of endogenous TORC in cAMP-dependent activation of CRH transcription by using Western blot and blockade of endogenous TORC production by siRNA. Under basal conditions, Western blot analysis revealed, in cytosolic but not nuclear fractions, a 200 kDa band that corresponded to the phosphorylated form of TORC2. Incubation with forskolin resulted in transient nuclear translocation (maximal at 30 minutes) of a lower–molecular weight band corresponding to dephosphorylated TORC. In contrast, PMA slightly delayed the migration of the cytosolic band (suggesting hyperphosphorylation) and had no effect on nuclear TORC levels, suggesting that the inability of phorbolesters to stimulate transcription is attributable to the lack of nuclear translocation of TORC. Transfection of TORC1 and TORC2 siRNA oligonucleotides 24 hours before incubation with forskolin abolished the immunoreactive TORC bands in the Western blot and prevented activation of the CRH promoter by forskolin. Our study provides strong evidence that the co-activator TORC is required for activation of CRH transcription. Our current research focuses on the interactions of TORC with the CRH promoter and on the importance of the co-activator TORC during physiological regulation of CRH transcription in vivo.

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Anti-apoptotic actions of VP

Chen, Aguilera

VP secreted within the brain modulates neuronal function by acting as a neurotransmitter. We previously showed that the expression of VP in parvocellular neurons of the hypothalamic paraventricular nucleus increases markedly during prolonged stress, but that VP plays a relatively minor role in the regulation of the hypothalamic pituitary adrenal axis under chronic conditions. These observations led to the search for new roles of vasopressin during stress adaptation. Based on the observation that VP prevents serum deprivation–induced cell death in the neuronal cell line H32, which expresses endogenous V1 receptors, we tested the hypothesis that VP has anti-apoptotic properties. Flow cytometry experiments showed that 10nM VP prevented serum deprivation–induced

cell death and annexin V binding. Serum deprivation increased caspase-3 activity in a time- and serum concentration–dependent manner while VP prevented these effects through interaction with receptors of the V1 subtype. The signaling pathways mediating the anti-apoptotic effect of VP involve mitogen-activated protein (MAP) kinase and extracellular signal–regulated kinases (ERK), Ca^{2+}/calmodulin-dependent kinase (CaMK), and protein kinase C (PKC). Western blot analyses revealed time-dependent decreases of Bad phosphorylation and increases in cytosolic levels of cytochrome c following serum deprivation, effects prevented by 10nM VP. These data demonstrate that activation of endogenous V1 VP receptors prevents serum deprivation–induced apoptosis through phosphorylation inactivation of the pro-apoptotic protein Bad and consequent decreases in cytosolic cytochrome c and caspase-3 activation. The data suggest that VP has anti-apoptotic activity in neurons and may act as a neuroprotective agent in the brain.

Using the cell line H32, we performed additional studies on the role of protein kinase C signaling in the anti-apoptotic effects of VP in neurons. Serum deprivation for 6 hours induced caspase-3 activity and dephosphorylation of Bad while co-incubation with VP reversed these changes. The selective PKC α/β subtype inhibitor Gö 6976 and PKCα and PKCβ dominant negatives reduced the effect of VP in caspase activation while PCKδ inhibition or a PCKδ dominant negative reduced serum deprivation–induced caspase-3 activity without affecting the protective action of VP. Consistently, serum deprivation increased PKCδ but not PKC α or PKCβ activity, and VP increased PKCα and PKCβ activity without affecting PKCδ activity. VP-induced Bad phosphorylation was reduced by PKCα and PKCβ or MEK inhibitors but was completely blocked by both inhibitors combined. However, total inhibition of caspase-3 activation required additional inhibition of the PI3K/Akt pathway. The data demonstrate distinct roles of PKC subtypes, with PKCδ mediating the apoptotic effect of serum deprivation and PCKα and PCKβ together with the MAP kinase and PI3K/Akt pathways mediating the antiapoptotic actions of VP.

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REGULATION OF CHILDHOOD GROWTH

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We investigate the cellular and molecular mechanisms governing childhood growth and development, focusing particularly on the skeletal system. We are especially interested in the mechanisms that allow rapid proliferation in the young organism and then suppress proliferation later in life. One goal of our work is to improve the medical treatment of childhood growth disorders. In addition, we seek to uncover general principles of developmental biology, given that the cellular processes underlying bone growth, such as cell proliferation, terminal differentiation, angiogenesis, and cell migration, are also essential for development of other tissues.

Spatial organization of the growth plate
Barnes, Baron

Longitudinal bone growth occurs at the growth plate, which consists of three principal layers: the resting zone, the proliferative zone, and the hypertrophic zone. Studies in our laboratory indicate that stem-like cells in the resting zone differentiate into rapidly dividing chondrocytes of the proliferative zone. The proliferative chondrocytes then terminally differentiate into the nondividing chondrocytes of the hypertrophic zone.

We have investigated the molecular mechanisms responsible for spatial organization of the growth plate. Microarray analysis followed by real-time PCR analysis identified genes whose expression changed dramatically during the differentiation program, including several genes functionally related to bone morphogenetic proteins (BMPs). Additional investigation suggests the existence of a BMP signaling gradient across the growth plate, which is established by differential expression of several BMPs and BMP inhibitors in specific zones. Previous functional studies suggest that the BMP signaling gradient may be a key mechanism responsible for spatial regulation of chondrocyte proliferation and differentiation in the growth plate. Using a similar approach that combined expression and functional studies, we explored the role of fibroblast growth factor (FGF) signaling in the postnatal growth plate. The studies revealed a complex pattern of spatial regulation of FGFs and FGR receptors (FGFRs) in the different zones of the growth plate that appears to help regulate proliferation and differentiation. Understanding the FGF system is of particular importance because abnormalities in FGF signaling cause human skeletal dysplasias, including achondroplasia.

More recently, we analyzed the role of Wnt signaling in the spatial organization of the growth plate. We used microdissection and real-time PCR to study mRNA expression of *Wnt* genes in

the mouse growth plate. Of the 19 known members of the Wnt family, only 6 were expressed in postnatal growth plate. Of these, Wnt-2b, Wnt-4, and Wnt-10b signal through the canonical β-catenin pathway, whereas Wnt-5a, Wnt-5b, and Wnt-11 signal through the noncanonical calcium pathway. The spatial expression for these six Wnts was remarkably similar, showing low mRNA expression in the resting zone, increasing expression as the chondrocytes differentiated into the proliferative and prehypertrophic state, and then (except for Wnt-2b) declining expression as the chondrocytes underwent hypertrophic differentiation. We also found that mRNA expression of these Wnt genes persisted at similar levels at 4 weeks, when longitudinal bone growth is waning. Thus, we identified for the first time the specific *Wnt* genes that are expressed in the postnatal mammalian growth plate. The six identified *Wnt* genes showed a similar pattern of expression during chondrocyte differentiation, suggesting overlapping or interacting roles in postnatal endochondral bone formation.

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Temporal regulation of growth plate chondrogenesis

Lui, Barnes, Baron

With age, growth plate chondrocyte proliferation slows, causing longitudinal bone growth to slow and eventually stop. This functional change in the growth plate is accompanied by structural changes; with age, the number of resting, proliferative, and hypertrophic chondrocytes declines, as does the size of individual hypertrophic cells. The chondrocyte columns also become more widely spaced. We have termed this developmental program growth plate senescence. It appears to be caused by a mechanism intrinsic to the growth plate.

The developmental program of growth plate senescence could be a function of time per se. Thus, a biological timing mechanism could drive the change in chondrocyte physiology, including the decline in proliferation rate. Alternatively, growth plate senescence could be a function of growth itself. In this case, the system might be driven by a cell-cycle counter, that is, a cellular mechanism that progressively changes with each cell cycle, instead of by a biological timing mechanism.

To distinguish between the two alternative hypotheses, we treated newborn rats with propylthiouracil (PTU) for 8 weeks to induce hypothyroidism and thus inhibit growth at the growth plate. After discontinuation of the PTU, we studied several functional, structural, and molecular markers in both treated animals and untreated controls to determine whether the previous period of hypothyroidism had delayed the programmed senescence of the growth plate.

Control animals showed the normal senescent decline in tibial growth rate, chondrocyte proliferation rate, growth plate height, resting zone height, number of resting zone chondrocytes, number of proliferative zone chondrocytes per column, number of hypertrophic chondrocytes per column, terminal hypertrophic cell height, and column density. In the previously hypothyroid animals, the senescent decline in every one of these variables was delayed. We also studied molecular markers of growth plate senescence—genes whose mRNA expression in growth plate chondrocytes changed markedly during growth plate senescence. These genes included those encoding chondroadherin, osteoprotegerin, secreted frizzled-related protein 4, reelin, and nuclear protein 1. In control animals, mRNA levels of all of these genes increased markedly with age. Previously hypothyroid

animals expressed chondroadherin, osteoprotegerin, secreted frizzled-related protein 4, and nuclear protein 1 mRNAs at levels similar to those in younger control animals. Thus, molecular markers of senescence, like the structural and functional markers of senescence, also appeared to be delayed by the previous hypothyroidism.

The observed delay in several functional, structural, and molecular markers of growth plate senescence in the previously hypothyroid animals strongly supports the hypothesis that hypothyroidism slows the developmental program of growth plate senescence. We previously found evidence, though less extensive, that glucocorticoid excess in the rabbit also slows growth plate senescence. The combined finding, that growth plate senescence is slowed by two growth-inhibiting conditions in two species, supports our model that growth plate senescence is not a function of time per se but rather of growth; therefore, inhibition of growth slows this developmental process.

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Temporal regulation of growth in non-skeletal tissues

Barnes, Finkielstain, Lui, Chang, Forcinito, Baron

The rate of cell proliferation and consequent somatic growth slows with age and ceases not only in the growth plate but also in several other tissues. To investigate the underlying changes in cell-cycle kinetics, we used [methyl-^{3}H]thymidine and 5′-bromo-2′deoxyuridine to double-label proliferating cells in 1-, 2-, and 3-week-old mice for 4 weeks. Proliferation of renal tubular epithelial cells and hepatocytes declined with age. The average cell-cycle time did not increase in liver and increased only 1.7-fold in kidney. The fraction of cells in S-phase that divided again declined approximately 10-fold with age. Concurrently, average cell area increased approximately 2-fold. The findings suggest that somatic growth deceleration primarily results not from a rise in cell-cycle time but from a reduced growth fraction (fraction of cells that continue to proliferate). During the deceleration phase, cells appear to reach a proliferative limit and undergo their final cell divisions, staggered over time. Concomitantly, cell volume increases, perhaps because cells are relieved of the size constraint imposed by cell division. In summary, a decline in growth fraction with age causes somatic growth deceleration and thus sets a fundamental limit on adult body size.

The rate of cell proliferation that causes somatic growth to decline simultaneously in several organs does not appear to be coordinated by a systemic mechanism. We therefore hypothesized that growth deceleration results from a growth-limiting genetic program that is common to several tissues. To test this hypothesis, we performed microarray analysis to identify changes in gene expression in mice during early postnatal life. We focused on genes that were up- or downregulated in several organs and thus are more likely to contribute to the putative common program of growth deceleration. We noticed that some of the genes showing the greatest changes in expression with age are imprinted; that is, genes show differential expression from the maternal and paternal alleles. Earlier research demonstrated that some imprinted genes positively regulate fetal growth. Our microarray observations raised the possibility that expression of these genes persists into early postnatal life and that their subsequent downregulation contributes to the dramatic decline in proliferation and somatic growth that determines adult body size.

We therefore systematically assessed the age-related changes in gene expression of all known imprinted genes by using microarray analysis for kidney, lung, and heart. We identified a set of 11 imprinted genes that show downregulation of mRNA expression with age in several organs. For

these genes—*Igf2*, *H19*, *Plagl1*, *Mest*, *Peg3*, *Dlk1*, *Gtl2*, *Grb10*, *Ndn*, *Cdkn1c*, and *SLC38a4*—the declines show a temporal pattern similar to the decline in growth rate. All 11 genes have been implicated in the control of cell proliferation or somatic growth. Thus, our findings suggest that the declining expression of the genes contributes to coordinate growth deceleration in several tissues. We next hypothesized that the coordinate decline in expression of these imprinted genes is caused by altered methylation and consequent silencing of the expressed allele. However, the methylation status of the promoter regions of *Mest*, *Peg3*, and *Plagl1* did not change with age. In summary, our findings suggest that a set of growth-regulating imprinted genes are expressed at high levels in several tissues in early postnatal life, contributing to rapid somatic growth, but that these genes are subsequently and simultaneously downregulated in several tissues, contributing to coordinate growth deceleration and cessation, thus imposing a fundamental limit on adult body size.

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THE X-CHROMOSOME AND GENDER EFFECTS IN PHYSIOLOGY, PATHOPHYSIOLOGY, AND LONGEVITY

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Many obvious phenotypic differences between normal women and men result from differential exposure to sex steroids. However, exposure to sex steroids does not adequately explain important differences in gender-specific susceptibility to disease and longevity. We propose that differential X-chromosome gene dosage contributes to fundamental biological differences between females and males. While researchers had considered the second X-chromosome in female cells totally inert as a result of random X-inactivation, the distinct phenotype in 45,X females with Turner syndrome (TS) indicates that the second X-chromosome is important for normal female development. Epigenetic mechanisms are involved in regional X-inactivation as well as in genomic imprinting and thus contribute to sex differences by X-chromosome gene-dosage effects. Our research aims to identify and define the function of X-chromosome genes involved in the differential development and function of brain, reproductive, metabolic, and immune systems in women and men. Studying girls and women with TS provides a unique opportunity to elucidate X-chromosome gene-dosage effects and deepen our understanding of TS, which affects approximately 1 in 2,000 females. Our research will improve our ability to care for girls and women with TS and will enhance our understanding of women's increased susceptibility to autoimmune disease and men's increased risk for coronary disease.

X-chromosome, genomic imprinting, and longevity
Bakalov, Wooten,[1] Cheng, Zhou, Bondy

Genomic imprinting involves the selective expression, determined by parental origin, of certain genes, often associated with DNA methylation of imprinted, or silenced, alleles. Genomic imprinting of X-linked genes causes different gene expression in females and males, given that normal women are mosaic for maternally and paternally inherited active X-chromosomes (X^M and X^P) while men are monosomic for X^M. Imprinted X^M genes would still be expressed in about 50 percent of female cells but not in male cells.

Women enjoy greater longevity than men mainly because of their lower risk, across all age groups, for ischemic heart disease. Women's primary advantage in this regard is their salutary "gynoid" fat distribution, meaning adipose tissue preferentially concentrated subcutaneously in the hips and thighs. In contrast, normal men tend to concentrate fat in the abdominal area, especially intraperitoneal visceral fat, which has many adverse metabolic effects, including an atherogenic lipid profile and

elevated mediators of inflammation, which cause coronary and cerebral artery atherosclerosis. To test the hypothesis that X-chromosome genomic imprinting contributes to the regional fat and metabolic differences between normal women and men, we studied the risk factors in groups of women monosomic for X^M versus X^P. The X^M and X^P groups had a similar BMI and total body fat, but women with X^M had greater abdominal and intra-abdominal visceral fat, which were associated with higher LDL-cholesterol and triglycerides than in the X^P group. Our finding of a male-type fat distribution and lipid profile in X^M women supports the view that differential X-chromosome gene dosage, determined by genomic imprinting, contributes to the excess mortality from ischemic heart disease in 46, X^MY men.

Given the different parental origins of the X-chromosome in males and females, genomic imprinting is predictably associated with differential effects depending on the sex of the offspring (in contrast to autosomal imprinting). Thus, X-linked imprinting is not expected to regulate traits that are not sexually dimorphic, such as renal or cardiac development. We proved our prediction in a recent study showing equal prevalence of renal and cardiovascular defects in X^M versus X^P groups with TS. In contrast, somatic size is sexually dimorphic, typically with men considerably larger than women; in fact, we have shown that size is related to X origin.

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Congenital cardiovascular defects in Turner syndrome

Matura, Bakalov, Bondy; in collaboration with Rosing, Sachdev, Ho

Congenital cardiovascular disease may be the most serious medical problem in monosomy X or TS. Pioneering the use of high-resolution magnetic resonance angiography (MRA) for TS, we demonstrated cardiovascular anomalies in about 50 percent of study subjects, in contrast to the previously accepted 20–30 percent prevalence based on echocardiographic studies. Whereas congenital heart defects in TS were thought to be limited to left-sided, outflow tract defects, MRA revealed a high prevalence of major venous malformations, including partial anomalous pulmonary venous return and persistent left superior vena cava affecting over 20 percent of the study population. The most common anomaly disclosed by our study was a distinctive aortic deformation affecting about 50 percent of women with TS, termed elongated transverse arch of the aorta (ETA). This newly described aortic abnormality seems to be predictive of aortic complications and hence mandates vigilant surveillance of aorta dimensions in affected yet asymptomatic patients.

In recent years, we have seen frequent case reports of fatal aortic dissections occurring in relatively young women with TS. The actual incidence was unclear, and it was unknown if dilatation of the ascending aorta predicted aortic dissection in TS and, if so, what specific aortic diameters should provoke concern. In other patient groups at risk for dissection such as Marfan syndrome, dilatation of the ascending aorta is a predictor of an acute aortic event. Thresholds of 5.5–6 cm for the general population and 5 cm for those with Marfan syndrome guide "prophylactic" intervention to replace or stabilize the aneurysmal segment. Under this "one size fits all" prescription, however, women (who are smaller than men, for whom the guidelines were developed) have a higher likelihood of dissection/rupture and death. Accordingly, it has been suggested that intervention at a smaller diameter would save more female lives. Given that aortic diameter is closely correlated with somatic size, the aorta should be smaller than "normal" in very petite women with TS; such women might actually have significant aortic dilatation at diameters considered in the normal range.

We recently reported the first prospective measure of the incidence of aortic dissection in TS and proposed new guidelines to identify high-risk patients (Matura et al., 2007). We evaluated aortic diameters and other parameters in a large group of asymptomatic, unselected women with TS and followed the women for an average of three years. During the period, we recorded three cases of aortic dissection among 158 patients, translating into an incidence of about 618 cases per 100,000 TS years compared with about 1 per 100,000 non–TS women years. The women who dissected were in their 40s and had aortic diameters from 3.7–4.8 cm. They were under cardiologist care in their home area, but, because their aortic diameter was not less than 5 cm, they were not considered candidates for prophylactic intervention. Hence, it is inadvisable to wait for aortic diameter to reach 5 cm in TS patients. We investigated different methods of normalizing MRI-measured aortic diameters to individual body size and found that aortic diameter/body surface area (Aortic Size Index, ASI) provided the highest correlation and greatest accuracy in identifying those at risk for dissection. In summary, 25 percent of women with absolute ascending aortic diameter greater 3.5 cm and 33 percent of women with ASI greater than 2.5 cm/m^2 experienced aortic dissection within approximately three years of follow-up.

Thus, for screening purposes, we suggest use of the ASI 95th percentile of 2 cm/m^2 for the ascending aorta determined by MRI, CT, or cardiac echo. This measure takes into account the considerable size variation of patients and identifies about 30 percent of women with TS who require close monitoring. If the ASI is equal to or greater than 2.5 cm/m^2, the patient should be evaluated for prophylactic intervention. Clearly, further study is needed to determine if beta blocker or renin-angiotensin system blockade may prevent or retard aortic dilatation in TS patients and if prophylactic surgery may reduce the incidence of aortic dissection and rupture.

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Growth hormone treatment of girls with Turner syndrome

Bondy, Wooten,[1] Bakalov

Short stature is the most common, readily recognizable clinical feature of TS. The deficit in height is caused by haploinsufficiency of the short-stature homeobox-containing pseudoautosomal gene (SHOX). It affects virtually all individuals with TS and results in an average adult height about 20 cm shorter than that predicted by parental height. Although girls with TS are not usually growth hormone (GH) deficient, pharmacological GH treatment increases growth velocity and final adult stature; in 1986, the Food and Drug Administration (FDA) approved the use of GH to enhance stature in TS. Since FDA approval made GH treatment readily available to all TS girls, researchers have faced difficulties in conducting studies of GH effects in TS with essential untreated TS control groups. Our NIH study recruits girls and women with TS from all over the United States and includes mostly girls who were treated with GH in private endocrinology practice settings, along with a smaller number never treated with GH because of a very recent diagnosis or diagnosis at an age deemed too late to start GH therapy. Thus, we have the rare and valuable opportunity to evaluate GH effects in girls with TS compared to contemporaneous, age-matched controls.

In addition to promoting longitudinal bone growth, GH affects bone and body composition. To determine how GH treatment affects these other important aspects of growth and development, we compared body composition and bone density by DXA and phalangeal cortical thickness by hand radiography in girls with TS who had never received GH and girls treated with GH for at least one year. After adjusting for size and bone age, we observed no significant differences in trabecular or cortical bone density at the lumbar spine or wrist. However, in the group treated with GH, lean body mass percent was higher, and total body fat percent was lower. These effects were independent of estrogen exposure and persisted in girls who finished GH treatment a year or more before the study. To summarize, our study showed that GH treatment has little effect on cortical or trabecular bone in girls with TS but is associated with increased lean body mass, reduced adiposity, and improved glucose tolerance (Wooten et al., 2008).

GH also has effects on the cardiovascular system, with GH excess in acromegaly causing cardiac hypertrophy and eventually cardiomyopathy. Given the high prevalence of congenital heart defects, including problems of the aortic valve and aorta, we were concerned about potential adverse effects of GH treatment on the cardiovascular system in TS. Therefore, we compared the aortic diameters and left ventricular dimensions and function of girls with TS who received GH treatment with those of girls with TS who did not receive GH treatment. The two groups were similar in age and weight, but subjects treated with GH were on average 8 cm taller. The diameter of the ascending aorta increased by 7.3 percent and the descending aorta by 8.9 percent in the group treated with GH. However, after correction for age, height, and weight by using multiple regression, neither history nor length of GH treatment affected aortic diameter. Likewise, cardiac dimensions corrected for age and body size did not differ significantly in the two groups, and LV function assessed by the fractional shortening index was similar in the two groups. Thus, it seems that GH treatment of girls with TS does not affect cardiac or aortic dimensions above or beyond the proportionate increase related to larger body size, at least over intermediate-term (5–10 years) follow-up. More long-term follow-up is necessary to be certain of no residual effects on aortic and LV dimensions or function.

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The X-chromosome, ovarian failure, and psychosocial function

Bondy; in collaboration with Biesecker, Schmidt, Cardoso, Ross, Sutton

Previous small surveys and anecdotal reports indicated that many girls and women with TS suffer from shyness and social anxiety. This "shyness trait" was traditionally attributed to stigmatization because of short stature (Cardoso et al., *Gynecol Endocrinol* 2004;19:313). More recently, researchers have suggested that social difficulties could be neurobiologically based, caused by genomic imprinting of X-linked genes involved in "social cognition." We showed that women with TS experience a rate of life-time depression similar to women from gynecologic clinic samples, with no "autistic spectrum" diagnoses. In open-ended interviews, girls and women identified ovarian failure, infertility, or "gender identity" as major issues related to their experience of TS. Thus, our findings suggest that the experience of ovarian failure is a major hurdle for older girls and women with TS while major psychological diagnoses and autistic-type disorders are uncommon.

To investigate the behavioral impact of early ovarian failure, we compared psychosocial functioning in women with TS and women with 46,XX premature ovarian failure. We reasoned that, if the experience of premature ovarian failure per se leads to specific difficulties with social interactions in young women, then the two groups should demonstrate similar responses on tests of psychosocial function. Women with normal ovarian function served as contemporaneous controls. Our two ovarian failure groups reported similar increases in shyness and social anxiety and decreased self-esteem levels compared with women with normal ovarian function. The findings suggest that the shyness and poor self-esteem observed in women with TS reflect the experience of premature ovarian failure. These feelings of social inadequacy are associated with social isolation, difficulty in partnering, and very limited sexual functioning. Current studies target the role of infertility, childlessness, sex steroid effects, or altered body image in impaired social functioning in women with TS. We will also address the issue of how best to inform a young woman and her family about the diagnosis of premature ovarian failure so as to minimize the psychological trauma. In addition, we plan to extend our studies to girls and young women who have survived cancer treatments with compromised ovarian function.

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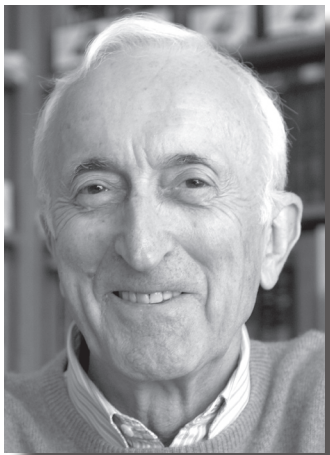
PEPTIDE HORMONE RECEPTORS AND SIGNAL TRANSDUCTION



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We study the receptors and signaling mechanisms by which peptide hormones activate functional responses in their target cells. In particular, we characterize structure-function properties, signal transduction, and cellular processing of specific G protein–coupled receptors (GPCR). Our research program focuses on GPCRs for gonadotropin-releasing hormone (GnRH) and angiotensin II (Ang II) and on the intracellular signaling pathways that mediate the receptors' cellular actions. The GnRH decapeptide mediates the neural control of the pituitary gland and gonadotropin secretion; it also exerts autocrine actions on the GnRH neurons and is essential for normal reproductive function. GnRH is a primary regulatory factor in the neuroendocrine control of reproduction and is released in an episodic manner from hypothalamic GnRH neurons, which, as we previously found, express GnRH receptors. The angiotensin octapeptide exerts regulatory actions on aldosterone secretion, sodium balance, and blood pressure and has been implicated in the etiology of cardiac, vascular, and renal disease as well as in diabetes mellitus. We study the regulation and functions of these receptors in normal and immortalized hypothalamic neurons, pituitary gonadotropes, adrenal glomerulosa cells, hepatic cells, and transfected cell lines in order to elucidate their signaling pathways and the mechanisms by which they regulate metabolic, secretory, and growth responses.

Expression and regulatory functions of estrogen receptor-α and -β subtypes in hypothalamic GnRH neurons

Hu, Gustofson,[1] Feng, Leung, Krsmanovic, Catt; in collaboration with Mores

Estradiol (E_2) acts as a potent feedback molecule between the ovary and hypothalamic GnRH neurons, exerting both positive and negative regulatory actions on GnRH synthesis and secretion. However, the extent to which these actions are mediated by estrogen receptors expressed in GnRH neurons has been controversial. In our study, single-cell RT-PCR revealed the expression of both estrogen receptor alpha (ERα) and beta (ERβ) isoforms in cultured fetal and adult rat hypothalamic GnRH neurons. Both ERα and ERβ or individual ERs were expressed in 94 percent of cultured fetal GnRH neurons. In adult female rats at diestrous, 68 percent of GnRH neurons expressed ERs, followed by 54 percent in estrous and 19 percent in proestrous. In addition, we found expression of individual ERs in 24 percent of adult male GnRH neurons. ERα exerts marked G_i-mediated inhibitory effects on spontaneous AP firing, cAMP production, and pulsatile GnRH secretion, indicating its capacity for negative regulation of GnRH neuronal function. In contrast, increased E_2 concentrations and ERβ agonists raised the rate of AP firing, GnRH secretion, and cAMP production, consistent with ERβ-dependent positive regulation of GnRH secretion. Consonant with the coupling of ERα

to pertussis toxin (PTX)–sensitive $G_{i/o}$ proteins, E_2 also activated G protein–activated inwardly rectifying potassium (GIRK) channels, thereby decreasing membrane excitability and slowing the firing of spontaneous APs in hypothalamic GnRH neurons. Our findings demonstrate that the dual actions of E_2 on GnRH neuronal membrane excitability, cAMP production, and GnRH secretion are mediated by the dose-dependent activation of ERα and ERβ expressed in hypothalamic GnRH neurons.

Role of tyrosine^{324} of the GnRH receptor in ligand binding and cAMP signaling

Krsmanovic, Hu, Feng, Leung, Wada,[2] Catt

The Asn-Pro-any amino acid-Tyr ($NPX_{2-3}Y$) sequence of the seven-transmembrane (TM) GPCRs, in which X represents an aliphatic amino acid, mediates interactions with intracellular effector molecules. In the GnRH receptor, the sequence is modified to DPXXY by exchange of the Asp and Asn residues normally present in TM II and TM VII, respectively, but retains the Tyr residue that is conserved in the NPXZY sequence motif of most GPCRs. In the mouse GnRH-R, Y^{324} is outside the DPLIY motif and is one of only two tyrosine residues that face the side of the receptor. We mutated Tyr^{324} to phenylalanine to preserve the original aromatic component of the tyrosine residue. We analyzed binding properties and cAMP signaling in cells expressing endogenous GnRH-R and in COS-7 cells transfected with mouse GnRH-R. In cultured hypothalamic cells and immortalized GnRH neurons (GT1-7 cells), activation of the endogenous GnRH-R caused a biphasic cAMP response for which inhibition was G_i-dependent. Agonist stimulation of GnRH-R expressed in COS-7 cells caused a monotonic dose-dependent increase in cAMP production. In contrast to the case of neuronal cells, the stimulatory action of GnRH on cAMP in HEK-293 cells expressing the GnRH-R was PTX-sensitive, suggesting cell specificity in $G_{i/o}$ activation. In COS-7 cells expressing the $Tyr^{324}F$ mutant receptor, maximal binding of a ^{125}I-labeled GnRH agonist analogue was reduced by only 10 percent. We noted that cAMP production remained monotonic and dose-dependent and that the ED_{50} of the $Tyr^{324}F$ mutant receptor declined about 200 fold. In contrast to the wild-type (WT) GnRH-R, PTX pretreatment of COS-7 cells expressing the $Tyr^{324}F$ mutant receptor had no effect on cAMP production. Substitution of the aromatic-hydrophobic Tyr^{234} of GnRH-R with neutral, hydrophilic serine caused a 70 percent decrease in ^{125}I-labeled binding and loss of cAMP signaling. In summary, our data indicate that Tyr^{234} plays an important role in ligand binding, cAMP signaling, and GnRH-R–$G_{i/o}$ interaction.

BRET analysis of constitutive and ligand-induced homo- and hetero-oligomerization of GnRH and GPR54 receptors

Krsmanovic, Hu, Feng, Leung, Quaynor, Wada,[2] Catt

Kisspeptins, a family of peptides encoded by the *KiSS-1* gene, promote GnRH secretion and are endogenous ligands for the GPCR GPR54. Both *KiSS-1* and *GPR54* are expressed in the mammalian hypothalamus, consistent with observations that kisspeptins and GPR54 are involved in activation and regulation of the hypothalamic-pituitary-gonadal axis. Disrupted GPR54 signaling results in hypogonadotrophic hypogonadism in rodents and humans. Central or peripheral administration of kisspeptin potently stimulates the hypothalamic-pituitary-gonadal axis and increases circulating gonadotrophin concentrations in animal models. *GPR54* is widely expressed in many tissues related to reproductive function, and evidence suggests that kisspeptin acts predominantly within the central nervous system to regulate GnRH secretion.

The GnRH receptor (GnRH-R) and GPR54 and their specific ligands, GnRH and kisspeptin, are essential regulators of the hypothalamic-pituitary-gonadal axis. Bioluminescence resonance energy transfer (BRET) analysis revealed constitutive and GnRH-activated $BRET^2$ signals in HEK-293 cells transfected with DNAs for GnRH-R-Rluc and GnRH-R-GFP^2. $BRET^2$ signaling reached a maximum at a 1:12 GFP^2/Rluc ratio and was significantly increased by the GnRH-R agonist analogue [D-Ala^6]Ag. In contrast, treatment of HEK-293 cells expressing GnRH-R homo-

oligomers with kisspeptin-10, a GPR54 agonist, had no significant effect on the BRET^2 signal. We also observed formation of GPR54 homo-oligomers in cells transiently transfected with GPR54-Rluc cDNA and increasing amounts of GPR54-GFP2 cDNA. Similarly, treatment of HEK-293 cells expressing GPR54 homo-oligomers with [D-Ala^6]Ag reduced the BRET^2 signal, suggesting that direct activation of GPR54 by kisspeptin-10 or co-activation of GPR54 by [D-Ala^6]Ag causes inhibition of homo-oligomer formation. Transfection of HEK-293 cells with GPR54-Rluc cDNA and increasing amounts of GnRHR-GFP2 led to formation of GPR54/GnRH-R hetero-oligomers. Treatment of such complexes with kisspeptin-10 or GnRH-R activation by [D-Ala^6]Ag significantly increased the BRET^2 signal. Constitutive and GnRH-activated increases in BRET signals occurred in HEK-293 cells expressing GnRH-R homo-oligomers. Previous treatment with GnRH antagonist prevented the GnRH-induced increase in the BRET signal. We observed GPR54 homo-oligomers when we transfected HEK-293 cells with a constant amount of GPR54-Rluc cDNA and increasing amounts of GPR54-GFP^2 cDNA. BRET^2 signal intensity decreased significantly during treatment with kisspeptin-10. Transfection of HEK-293 cells with GPR54-Rluc and increasing amounts of GnRHR-GFP2 caused formation of GPR54/GnRH-R hetero-oligomers. Treatment of such complexes with kisspeptin-10, a GPR54 agonist, or selective GnRH-R activation by GnRH significantly increased the BRET^2 signal. Our results suggest that constitutive and agonist-mediated formation of GnRH-R and GPR54 homo- and hetero-oligomers could play a significant role in the regulation of receptor activation in hypothalamic GnRH neurons.

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Functional interactions between the AT_1 receptor and the Ii protein

Chen H-D, Baukal, Catt; in collaboration with Chen H-C, Szaszák, Hunyady

Site-directed mutagenesis studies have defined the membrane-proximal region of the cytoplasmic tail of the AT_1 receptor (AT_1R) as a site required for normal AT_1R folding and surface expression. Using yeast two-hybrid screening of a human embryonic kidney cDNA library with the AT_1R carboxyl-terminal tail as bait, we identified the Invariant chain (Ii protein) or CD74 as a novel interacting protein. We confirmed such association with co-immunoprecipitation and co-localization studies. We localized the binding site for Ii on the AT_1R carboxyl-terminal tail to a region that is important for exit of AT_1R from the endoplasmic reticulum (ER) and that is conserved in many G protein–coupled receptors. Transient co-expression of Ii with AT_1-R in CHO (Chinese hamster ovary) cells consistently reduced AT_1R density at the cell surface. Furthermore, the interaction of Ii with the carboxy-terminal tail of AT_1R increased the receptor's retention in the ER and promoted its proteasomal degradation. These observations indicate that Ii and AT_1R become associated in the early biosynthetic pathway and demonstrate that the Ii protein is a negative regulator of AT_1R expression.

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Role of angiotensin II in prostate cancer

Catt; in collaboration with Louis S, Louis W

Angiotensin II (Ang II) promotes cell growth and proliferation and has been implicated in several forms of tumorigenesis. It is present in the basal cell layer of the normal prostate gland and in benign prostatic hyperplasia (BPH) and stimulates cell growth via AT_1 receptors. Furthermore, AT_1 receptor blockers have been shown to reduce prostate-specific antigen and to inhibit prostate cancer cell growth. An analysis of Ang II expression in BPH and prostate cancer, including high-grade prostatic intraepithelial neoplasia (HGPIN), showed Ang II's presence not only in basal epithelial cells in BPH but also in proliferating malignant cells in prostate cancer (Gleason grades 2–5) and in the cytoplasm of LNCaP, DU145, and PC3 prostate cancer cell lines. The data demonstrate the presence of Ang II staining in malignant cells in all grades of prostate cancer and indicate that Ang II expression in non-basal epithelial cells is an early indication of pre-malignant and malignant changes. In view of its known mitogenic activity, Ang II likely contributes to the growth and infiltration of malignant epithelial cells in the prostate. Furthermore, based on the recent observation by Baker et al. (*Am J Physiol Cell Physiol* 2006;291:C995) that elevated levels of cytoplasmic Ang II can increase cell proliferation via a non–AT_1R mechanism, it is possible that angiotensin-converting enzyme (ACE) inhibitors might also be of value in the treatment of prostate cancer by reducing intracellular Ang II formation.

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Role of caveolin in receptor signaling

Yin, Chen H-D, Catt

Caveolin1 (Cav1) is an important component of the plasma-membrane microdomains, such as caveolae and lipid rafts, that are associated with AT_1 and epidermal growth factor (EGF) receptors in certain cell types. An analysis of the interactions between Cav1 and other signaling molecules that mediate AT_1R function in Ang II– and EGF-stimulated hepatic C9 cells demonstrated that cholesterol-rich domains mediate the actions of early upstream signaling molecules such as Src and intracellular Ca^{2+} in cells stimulated by Ang II, but not by EGF, and that Cav1 plays a scaffolding role in the process of MAPK (mitogen-activated protein kinase) activation. Furthermore, intracellular Ca^{2+} and Src regulate Cav1 phosphorylation by Ang II and epidermal growth factor (EGF). Phosphorylation of Cav1 and epidermal growth factor receptor (EGFR) by Ang II, but not extracellular signal-regulated kinase (ERK)1/2 activation, is dependent on intracellular Ca^{2+}. The PI 3-kinase inhibitors LY294002 and wortmannin differentially modulate both Cav1 and EGFR activation by Ang II through intracellular Ca^{2+}. Our findings further demonstrate the importance of Cav1 in conjunction with receptor-mediated signaling pathways involved in cell proliferation and survival. It is clear that differential signaling pathways operate in Ang II– and EGF-stimulated C9 cells and that cholesterol-enriched microdomains are essential components in cellular signaling processes that are dependent on specific agonists and/or cell types.

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Role of Ang II in insulin resistance

Catt; in collaboration with the Olivares-Reyes research group

Insulin resistance is an important factor in the development of Type 2 diabetes mellitus (DM2). At the molecular level, insulin resistance is linked to reduced tyrosine phosphorylation of insulin-receptor substrate-1 (IRS-1). The mechanisms of this process have not been completely identified, but the role of serine/threonine phosphorylation of IRS-1 in desensitization of insulin action is well established. Studies performed in collaboration with Alberto Olivares-Reyes revealed that blockade of the renin-angiotensin system by inhibiting either ACE or the Ang II AT1 receptor reduces insulin resistance and may decrease DM2 risk. We examined the interactions between the Ang II and insulin signaling systems in hepatocytes as well as the regulation of IRS-1 phosphorylation and insulin-Akt activation by Ang II in clone 9 (C9) hepatocytes. In C9 cells, Ang II specifically desensitized insulin-induced Akt Thr308 activation. The inhibition was associated with increased IRS-1 phosphorylation on Ser636/Ser639 that was prevented by inhibition of EGFR tyrosine kinase activity. Insulin-induced phosphorylation of IRS-1 on Ser636/639 is mediated mainly by PI3K/mTOR/S6K-1. Inhibition of PI3K (by wortmannin) and mTor (by rapamycin) revealed that Ang II stimulates IRS-1 phosphorylation on Ser636/639 via the PI3K/mTOR/S6K-1 pathway. Both agents also blocked the inhibitory effect of Ang II on insulin-induced activation of Akt. Our studies suggested that MAPK/S6K-1 is a secondary pathway involved in the Ang II–induced inactivation of insulin signaling. In summary, Ang II inhibits insulin signaling by increasing ser636/ser639 IRS phosphorylation via a mechanism dependent on EGFR transactivation that leads to MAPK/S6K1 and PI3-K/Akt/mTor/S6K-1 activation. Our findings indicate that Ang II plays a significant role in the development of insulin resistance via the EGFR.

Kisspeptin/GPR54 in the endocrine pancreas

Catt; in collaboration with the Martínez-Fuentes research group

As discussed above, the KiSS-1/GPR54 system plays a major role in the regulation of the gonadotropic axis by stimulating GnRH secretion at the hypothalamic level and activating gonadotropin release. The KiSS-1/GPR54 system is expressed in both human and mouse pancreas, indicating that the neuroendocrine actions of KiSS-1 and GPR54 are not restricted to the hypothalamic/pituitary system and might participate in the regulation of pancreatic islet function. Studies conducted with Antonio Martínez-Fuentes and colleagues confirmed the expression of KiSS-1 and GPR54 transcripts in the rat pancreas and Ins-1 beta cells. Analysis of the direct effects of kisspeptin-10 on calcium kinetics in cultured beta cells in a microfluorimetric system revealed that 70 percent of insulin-producing cells responded with a prominent calcium increase. Given the coupling between calcium increase and exocytosis, the data suggested that kisspeptin directly induces insulin release in pancreatic beta cells. Furthermore, kisspeptin-10 administration caused a significant increase in FM5-95 incorporation in single beta cells, similar to that elicited by glucose administration, thus supporting a role for kisspeptins in insulin secretion. Analysis of KiSS-1 and GPR54 expression under high glucose conditions revealed a significant reduction in the number of KiSS-1 transcripts while transcripts for GPR54 remained unaltered. In summary, the presence of KiSS-1 and GPR54 transcripts suggests that endogenous kisspeptins may regulate insulin production through an autocrine mechanism in pancreatic beta cells. Furthermore, regulation of their expression by one of the main stimuli of insulin secretion supports the participation of the KiSS-1/GPR54 system in the pathophysiology of the endocrine pancreas.

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Glycogen storage disease type I (GSD-I) comprises a group of autosomal recessive disorders consisting of GSD-Ia, which are caused by a deficiency in the liver-/kidney-/intestine-restricted glucose-6-phosphatase-alpha (G6Pase-alpha), and GSD-Ib, which are caused by a deficiency in a ubiquitously expressed glucose-6-phosphate transporter (G6PT).Normally, G6Pase-alpha hydrolyzes G6P to glucose, and G6PT translocates G6P from the cytoplasm to the lumen of the endoplasmic reticulum (ER). Both diseases manifest a phenotype of disturbed glucose homeostasis, although GSD-Ib patients also suffer from neutropenia and neutrophil dysfunctions. There is no cure for GSD-I, and the current dietary therapy cannot prevent the development of long-term complications. The recent development of animal disease models presents an opportunity to delineate the disease more precisely and develop therapies that target the underlying disease process. Recently, we reported a second ubiquitously expressed G6P hydrolase, G6Pase-beta (G6PC3). Neutrophils express both G6Pase-beta and G6PT, suggesting that neutrophil homeostasis and function may require endogenous glucose production in the ER via the concerted action of G6Pase-beta and G6PT. To examine this hypothesis, we generated mouse lines deficient in either G6Pase-beta or G6PT and showed that both G6Pase-beta–deficient (*G6pc3*$^{-/-}$) and G6PT-deficient mice manifest neutropenia and neutrophil dysfunctions that mimic the symptoms of human GSD-Ib patients.

Mice lacking G6Pase-beta manifest neutropenia and neutrophil dysfunctions
Cheung, Kim,[1], Yiu, Pan, Jun, Ruef,[2] Mansfield, Chou; in collaboration with Lee, Westphal

Previous views held that a single ER enzyme, G6Pase-alpha, limited to the liver, kidney, and intestine, was solely responsible for hydrolysis of G6P to glucose for release into the blood. Recently, we characterized a second ubiquitously expressed G6Pase enzyme, G6Pase-beta (G6PC3), that shares kinetic properties and topological and active site structure with G6Pase-alpha and couples functionally with the G6PT to form an active G6Pase complex that hydrolyzes G6P to glucose. The G6Pase-beta-G6PT complex might be functional in neutrophils, and the myeloid defects in GSD-Ib might be attributable to the loss of activity of the G6Pase complex. To test such a hypothesis, we generated G6Pase-beta–deficient (*G6pc3*$^{-/-}$) mice and showed that the mice manifest neutropenia and neutrophil dysfunctions mimicking GSD-Ib. We further showed that neutrophils from *G6pc3*$^{-/-}$ mice undergo ER stress and an enhanced rate of apoptosis. The results demonstrate that G6P translocation and metabolism in the ER are critical for normal neutrophil function and define a molecular pathway to neutropenia and neutrophil dysfunction of previously unknown etiology.

Cheung YY, Kim SY, Yiu WH, Pan CJ, Jun HS, Ruef RA, Lee EJ, Westphal H, Mansfield BC, Chou JY. Impaired neutrophil activity and increased susceptibility to bacterial infection in mice lacking glucose-6-phosphatase-beta. *J Clin Invest* 2007;117:784-793.

Neutrophil stress and apoptosis underlie myeloid dysfunction in GSD-Ib

Kim,[1] Jun, Mead, Mansfield, Chou

GSD-Ib patients and mice manifest neutropenia and neutrophil dysfunctions of unknown mechanism. Neutrophils express both G6PT and G6Pase-beta that together transport G6P into the ER lumen and hydrolyze it to glucose. Given that G6PT-deficient neutrophils should be unable to produce endogenous glucose, we hypothesized that such an inability would lead to ER stress and increased apoptosis. Using GSD-Ib mice, we showed that GSD-Ib neutrophils exhibit increased production of ER chaperones and oxidative stress, consistent with ER stress, and increased Annexin V binding and caspase-3 activation, consistent with an increased rate of apoptosis. Bax activation, mitochondrial release of pro-apoptotic effectors, and caspase-9 activation demonstrate the involvement of the intrinsic mitochondrial pathway in these processes. The results show that G6P translocation and hydrolysis are required for normal neutrophil function, supporting the hypothesis that neutrophil dysfunction in GSD-Ib is attributable, at least in part, to ER stress and increased apoptosis.

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The angiotensin system mediates renal fibrosis in GSD-Ia nephropathy

Yiu, Pan, Ruef, Peng, Mansfield, Chou; in collaboration with Starost

GSD-Ia patients develop renal disease of unknown etiology despite intensive dietary therapies. We showed that the expression of angiotensinogen (Agt), angiotensin type 1 receptor, transforming growth factor-beta1 (TGF-beta1), and connective tissue growth factor (CTGF) was higher in the kidneys of GSD-Ia mice than in those of the controls. The increase in renal expression of Agt occurred one week earlier than the increase in TGF-beta1 and CTGF, consistent with the upregulation of TGF-beta1 and CTGF by angiotensin II. Renal fibrosis was characterized by a marked increase in the synthesis and deposition of extracellular matrix proteins in the renal cortex and histological abnormalities, including tubular basement membrane thickening, atrophy, dilation, and multifocal interstitial fibrosis. Our results suggest that activation of the angiotensin system plays an important role in the pathophysiology of renal disease in GSD-Ia.

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Yiu WH, Pan C-J, Ruef RA, Peng WT, Starost MF, Mansfield BC, Chou JY. The angiotensin system mediates renal fibrosis in glycogen storage disease type Ia nephropathy. *Kid Internal* 2008;73:716-723.

Hepatic injury correlates with increased neutrophil infiltration of the liver in GSD-Ia

Kim,[2] Mansfield, Chou; in collaboration with Weinstein, Starost

GSD-Ia patients manifest hepatocellular adenoma (HCA) of unknown etiology. We showed that both GSD-Ia patients and mice have an underlying immune abnormality characterized by subclinical neutrophilia and elevated serum chemokine IL-8 or KC. We further showed that the elevation in serum IL-8 was more prominent in HCA-bearing GSD-Ia patients. Correlated with this elevation, in the mouse model of GSD-Ia, we observed hepatic injury characterized by necrotic foci and increased chemokines KC and MIP-2 as well as elevated neutrophil infiltration of the liver, suggesting one mechanism by which adenoma may arise.

Kim SY, Chen L-Y, Weinstein DA, Chou JY. Neutrophilia and elevated serum cytokines are implicated in glycogen storage disease type Ia. *FEBS Lett* 2007;581:3833-3838.

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The G6PT is a phosphate-linked antiporter deficient in GSD-Ib and GSD-Ic

Chen, Pan, Mansfield, Chou; in collaboration with Nandigama, Ambudkar

It is well established that a deficiency in G6PT causes GSD-Ib. Interestingly, deleterious mutations in the *G6PT* gene were identified in clinical cases of GSD-Ic that were thought to result from a deficiency in an inorganic phosphate (P_i) transporter. We hypothesized that G6PT is both the G6P and P_i transporter. Using reconstituted proteoliposomes, we showed that both G6P and P_i were taken up efficiently into P_i-loaded G6PT-proteoliposomes but were not detectable in P_i-loaded proteoliposomes containing the p.R28H G6PT null mutant. The specific G6PT inhibitors chlorogenic acid and vanadate inhibited the G6PT-proteoliposome–mediated G6P or P_i uptake. Taken together, our results indicate that G6PT plays a dual role as a G6P and a P_i transporter and that GSD-Ib and GSD-Ic are attributable to a deficiency in the same *G6PT* gene.

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RECEPTORS AND ACTIONS OF PEPTIDE HORMONES AND REGULATORY PROTEINS IN ENDOCRINE MECHANISMS

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We investigate the molecular basis of peptide hormone control of gonadal function, with particular emphasis on the structure and regulation of the luteinizing hormone receptor (*LHR*) and prolactin receptor (*PRLR*) genes. We also investigate the regulatory mechanism(s) involved in the progress of spermatogenesis and the control of Leydig cell function. Our studies focus on the regulation of human *LHR* transcription (nuclear orphan receptors, epigenetics, DNA methylation, and second messengers) as well as on the multiple-promoter control of *hPRLR* gene transcription. We are elucidating the function of two inhibitory short forms of the prolactin receptors and their relevance to physiological regulation and breast cancer. We also investigate novel gonadotropin-regulated genes of relevance to the progression of testicular gametogenesis, Leydig cell function, and other endocrine processes.

Epigenetic control of *LHR* transcription
Liao, Zhang, Dufau

Our studies have revealed that transcription of *LHR* is subject to repression and derepression through complex modulation at both genetic and epigenetic levels. In breast (MCF-7) and placenta (JAR) cancer cells that express *LHR*, we have identified several modes of regulation centered on the Sp1-/Sp3-binding domains of the *LHR* promoter. Furthermore, our studies have determined signal transduction pathways involved in the regulation of *LHR*.

LHR transcription is subject to an epigenetic regulatory mode whereby the proximal Sp1 site acts as an anchor to recruit HDAC 1/2 and the mSin3A co-repressor complex, resulting in promoter-localized histone hypoacetylation and limited Pol II recruitment, which partly contribute to the gene silencing observed in MCF-7 and JAR cells. Site-specific methylation of histones H3 and H4 acts in concert with changes in histone acetylation levels to regulate the expression of *LHR*. The methylation status of the *LHR* promoter provides another layer of modulation in the control of *LHR* expression in a cell-specific manner. Maximal derepression of *LHR* is dependent on complete DNA demethylation of the promoter in conjunction with histone hyperacetylation. We have shown that the phosphadildyl inositol 3-kinase pathway (PI3K), protein kinase C-zeta (PKC-zeta) signaling pathways, and Ser/Thr phosphatases PP1 and PP2A are key participants in derepression of *LHR* in cancer cells induced by Trichostatin A (TSA). Phosphorylation of Sp1 at Ser 641 by PI3K/PKC-zeta causes dissociation of repressor protein p107 from the *LHR* promoter, inducing gene activation. On the other hand,

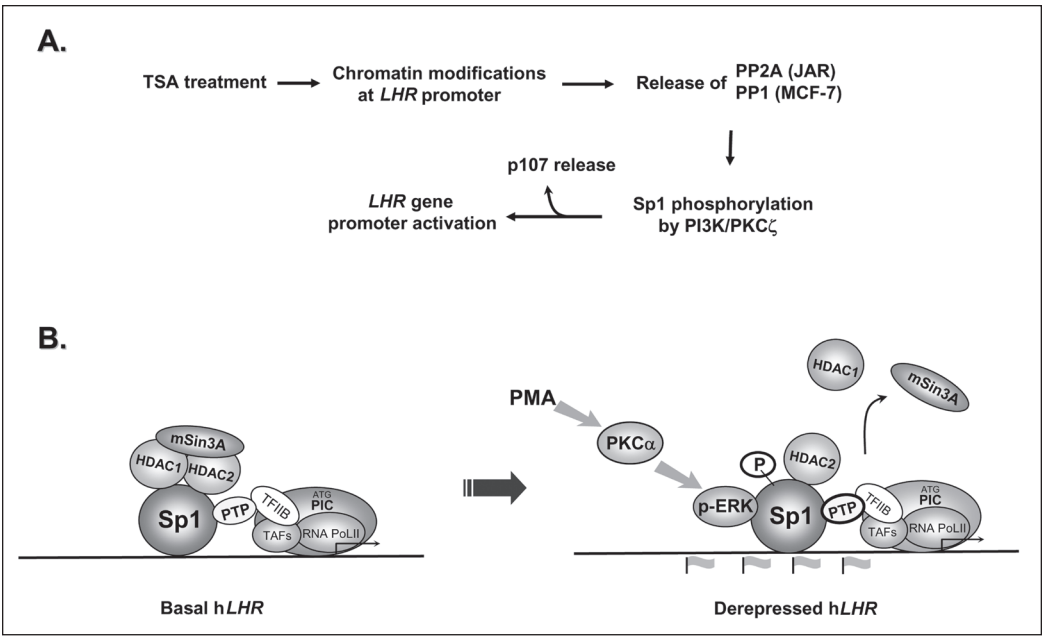

FIGURE 4.1 TRANSCRIPTIONAL REGULATION OF THE LH RECEPTOR

A. Schematic representation of functional roles of PP1 and PP2A and signaling events participating (PI3K/PKCζ) in TSA-induced *LHR* activation

The TSA-induced changes of the chromatin modification status at the *LHR* promoter favor cell-specific release of PP1 (MCF-7 cells) or PP2A (JAR cells) from the promoter. This in turn facilitates Sp1 phosphorylation by PI3K/PKCζ, which triggers the dissociation of p107 from the promoter and *LHR* activation.

B. Participation of PKCα/ERK pathway in transcriptional activation of the LH receptor

The transcriptional activator Sp1 is the anchor site of the HDAC1/2/mSin3A repressor complex within the *LHR* promoter. Endogenous PKCα activation by PMA (phorbol-12-myristate-13-acetate) raises *LHR* promoter activity and transcript levels through the ERK downstream pathway. Phospho-ERK (pERK) interacts with and phosphorylates Sp1 at serine residue(s), causing the release of the HDAC1/mSin3A repressor complex from the promoter. Such release facilitates histone acetylation, recruitment of TFIIB and Pol II, and derepression of *LHR*. mSin3A functions as a co-repressor for HDAC1 but not for HDAC2 in the regulation of *LHR* expression.

TSA-mediated chromatin changes cause PP1 and PP2A release from the promoter and function as an "on" mechanism for Sp1 phosphorylation by PI3K/PKC-zeta, leading to p107 derecruitment and *LHR* activation. The coordinated balance between PI3K-PKC-zeta (constitutive or induced activity) and phosphatase(s) is critical for up- or downregulation of *LHR* expression through their effects on Sp1 phosphorylation status (Figure 4.1A). We have also demonstrated the participation of the PKC-alpha/Erk pathway, where its activation by phorbol esters enhances *LHR* expression by inducing Sp1 phosphorylation by PKC-alpha/Erk at serine residue(s), thus causing dissociation of the HDAC1/mSin3A complex from the promoter and transcriptional activation (Figure 4.1B). Dissociation was independent of promoter-associated chromatin changes. Collectively, our findings indicate that complex and diverse networks regulate *LHR* expression at the transcriptional level and that coordination and interaction among diverse regulatory effectors are crucial for silencing/activation of *LHR* expression.

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Gonadotropin-regulated testicular genes

Tsai-Morris, Gutti, Sato, Fukushima, Maeda, Dufau

We previously identified a novel gonadotropin-regulated testicular helicase (GRTH/Ddx25) present in the nucleus and cytoplasm of pachytene spermatocytes and round spermatids (Sheng Y, et al., *J Biol Chem* 2006;281:35048). GRTH is a component of messenger ribonucleoprotein particles, which transport target mRNAs for storage in chromatoid bodies in the cytoplasm of spermatids. These

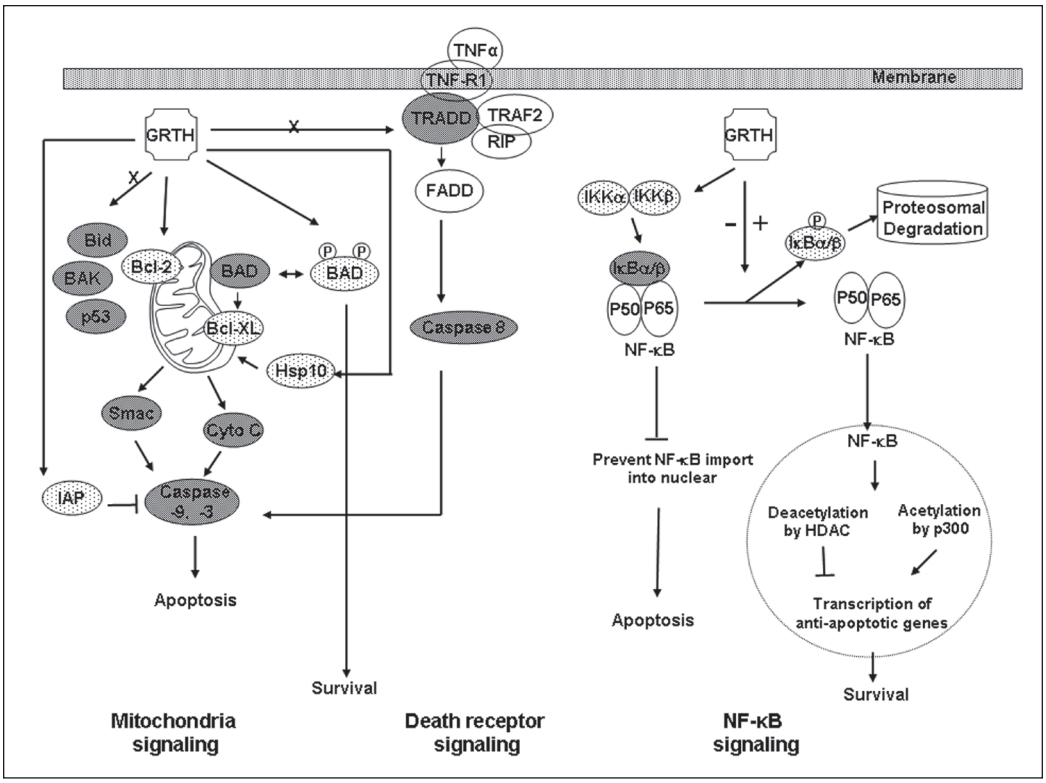

FIGURE 4.2 SUMMARY OF GRTH IMPACT IN APOPTOTIC PATHWAYS IN GERM CELLS

GRTH regulates subsets of pro-apoptotic and anti-apoptotic factors involved in mitochondria, death receptor, and NF-κB signaling pathways in germ cell apoptosis. *Left*, GRTH acts as a negative regulator to prevent anti-apoptotic factor expression (x) during spermatogenesis. In the absence of GRTH, expression of anti-apoptotic factors increased (*solid oval*) while that of pro-apoptotic factors was suppressed (*stippled oval*) in the mitochondria-controlled apoptotic pathway (and the subsequent cytochrome *c* release) and downstream caspase-3 and caspase-9 were activated (*middle*). Regarding the TNFα/TNF-R1 signaling pathway, the lack of GRTH increased expression of the TNF-R1–associated adaptor protein TRADD, which could cause downstream caspase-8 activation (*solid oval*). Other cytoplasmic adaptor proteins are apparently not involved in GRTH's regulatory action (*open oval*). *Right*, NF-κB pathway. GRTH could have an impact on NF-κB action at both the cytoplasmic and nuclear levels. In the cytoplasm, lack of GRTH (–) caused a decrease in IKKα/β kinase complex expression and IκB phosphorylation; consequently IκB's proteasomal-dependent degradation is curtailed. This favors non-phosphorylated IκBα/β association with NF-κB, which reduces NF-κB's import into the nucleus and presumably the transcriptional activation of anti-apoptotic genes. Lack of GRTH could also alter the acetylation status of NF-κB in the nucleus by increasing HDAC and decreasing acetylase p300 expression, resulting in a reduction in anti-apoptotic gene transcription and apoptosis. The presence of GRTH (+) promotes cell survival. P denotes phosphorylation.

messages are released for translation during spermatogenesis. GRTH is also found in polyribosomes, where it regulates the translation of mRNAs encoding spermatogenic factors. *GRTH*$^{-/-}$ male mice are sterile owing to spermatid arrest (step 8 of spermatogenesis), with marked diminution of chromatoid bodies and failure of spermatids to elongate. We found that the transcription of messages in spermatids of these mice was not altered but that their translation was selectively abrogated. We identified two GRTH species: 56 kDa and phosphorylated 61 kDa forms. The 56-kDa nuclear species interacted with CRM1 and participated in nuclear export of specific messages. The phosphorylated cytoplasmic 61-kDa species was associated with polyribosomes and regulated the translation of genes that govern spermatogenesis. GRTH/Ddx25 is thus a multifunctional RNA helicase that is an essential regulator of sperm maturation.

Apoptosis is a major event in prepubertal testis but occurs only sporadically in adult testis. In *GRTH*$^{-/-}$ mice, major apoptosis occurred in spermatocytes entering the metaphase of meiosis before the appearance of round spermatids and was dose-related, with apoptotic cells observed in *GRTH*$^{+/-}$ mice. In *GRTH*$^{-/-}$ mice, activation of survival mechanisms and/or the participation of other helicases might permit progression of the remaining viable meiotic cells to the point of arrest in haploid germ cells with no evidence of ultrastructural changes. The striking apoptosis observed in spermatocytes of adult *GRTH*$^{-/-}$ mice underscored the important role of GRTH in determining the survival and apoptotic fate of adult germ cells. In comparative studies of spermatocytes from wild-type and *GRTH*$^{-/-}$ mice, we found pro- and anti-apoptotic factors to be under GRTH regulation. Knockout mice displayed lower levels of Bcl-2 and Bcl-xL (anti-apoptotic factors), and phospho-Bad, higher levels of Bid, Bak, Bad (pro-apoptotic), as well as release of cytochrome *c*. We also observed an increase in Smac, a competitor of inhibitor apoptotic proteins that release caspases. These changes caused more cleavage of caspases-9 and caspase-3, activation of caspase-3, and increases in cleavage products of PARP [poly (ADP-ribose) polymerase]. The half-life of caspase-3 transcripts was markedly elevated in *GRTH*$^{-/-}$, indicating that GRTH had a negative effect on the caspase's mRNA stability. GRTH-mediated apoptotic regulation was further supported by its selective association with mRNAs of certain pro- and anti-apoptotic factors. GRTH also associated with caspases-9 and -3 and PARP mRNAs, the NF-κB cytoplasmic inhibitor IκBα, and the nuclear regulators p300 and HDAC1. IκBα, which sequesters NF-κB from its transcriptional activation of pro-apoptotic genes, was highly elevated in *GRTH*$^{-/-}$ mice, and its phospho-form, which promotes its dissociation, was reduced. The increase of HDAC1 and abolition of p300 expression in *GRTH*$^{-/-}$ indicated a nuclear action of GRTH on the NF-κB–mediated transcription of anti-apoptotic genes. GRTH also regulated the associated death-domain pathway and caspase-8–mediated events. Our studies demonstrate that, as a component of mRNP particles, GRTH acts as a negative regulator of the tumor necrosis factor receptor 1 and caspase pathways and promotes NF-κB function to control apoptosis in spermatocytes of adult mice (see Figure 4.2).

Given that GRTH is essential for spermatogenesis in mice, we are investigating its potential role in male infertility. In a Japanese population, an SNP- (single-nucleotide polymorphism) scanning study identified a polymorphic mis-sense mutation at exon 8 (nt 725G→C, aa242, R→H) and a silent mutation at exon 11 (nt 1245, C→G, aa 415, L→L) that might be associated with non-obstructive azoospermia. The unique heterozygous mis-sense mutation Arg^{242}His in exon 8 could affect the post-translational modification of the expressed protein as it cannot undergo phosphorylation and consequently could affect translational regulation of essential genes during germ cell development. In a population from Western China, infertility has been associated with a silent mutation (nt 1194 C→T) of the GRTH gene that might alter RNA splicing and increase the risk of impaired spermatogenesis. In contrast to this finding, we found no statistically significant change in this allele in Japanese men with non-obstructive azoospermia. Interestingly, we also noted that the SNPs of exons 8 and 11 in Japanese patients were not evident in Chinese patients.

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Prolactin receptor

Xie, Qazi, Dong, Kang, Tsai-Morris, Dufau; in collaboration with Hassan

In earlier studies, we demonstrated that short forms (SF) of the prolactin (PRL) receptor—S1a and S1b—that have an abbreviated cytoplasmic domain, can silence PRL-induced activation of gene transcription by the long form (LF). This results from LF/SF heterodimerization and the absence of cytoplasmic (C) sequences in the SF partner, which are essential for STAT (signal transducer and activator of transcription) activation. Compared with the LF, S1b does not contain the conserved cytoplasmic motif structure beyond the Box1 JAK2 site. Our studies also demonstrated that hetero- and homodimerization of hPRLR can occur independently of ligand and that PRL is a conformational modifier inducing JAK (Janus kinase) /STAT signaling. In recent studies, we provided evidence, derived from both functional analysis and computer simulations, that the four conserved Cys residues in the D1 subdomain of the extracellular domain (EC) of the hPRLR are essential for maintaining the correct conformation of dimerized PRLR and for the dominant negative action of the short form (S1b) on ligand-induced long-form–mediated STAT5-dependent transcriptional activation. Our studies have demonstrated that the inhibitory action of wild-type S1b on LF function was abolished upon disruption of these intramolecular disulfide bridges. The lack of inhibitory action of mutated S1b forms might result from their higher affinity to form homodimers than the wild type and from a significant reduction in their affinity to form heterodimers with the LF. The differential preference of mutated S1b forms to form homodimers rather than heterodimers contrasts with the wild type's S1b propensity to form heterodimers with LF. This in turn facilitates the formation of LF homodimers competent to mediate PRL-induced downstream signaling. Thus, the EC Cys mutant lacks the inhibitory action normally observed in the wild-type SF.

We recently studied the impact of the Cys mutation on JAK2 phosphorylation. In the absence of hPRL, we observed a basal level of JAK2 phosphorylation in cells transiently transfected only with wild-type S1b but not with the S1b Cys mutant (S1b4x). Following hPRL treatment, we observed significantly higher JAK2 phosphorylation than control in cells transiently transfected with wild-type S1b but not with mutant S1b4x. Similarly, cells stably transfected with S1b displayed JAK2 basal phosphorylation that was stimulated (2–3 fold) upon exposure to PRL. In addition, we observed a significant amount of basal JAK2 phosphorylation in cells stably expressing LF transfected with S1b. In contrast, JAK phosphorylation was not present in control cells stably expressing LF or upon transfection of S1b4x. Co-immunoprecipitation (Co-IP) studies of cells transiently co-transfected with either S1b or S1b 4x with JAK2 followed by Western analysis showed loss of JAK2 association with S1b4x compared with wild-type S1b. It was of interest to observe basal levels of JAK2 phosphorylation with S1b in the absence of PRL stimulation in cells both transiently and stably expressing the short-form S1b. Thus, in the absence of hormone, the abbreviated length of the cytoplasmic domain seems to favor basal JAK2 phosphorylation, presumably via auto- and/or

transphosphorylation. Alternatively, other associated kinase modules may be involved. The apparent loss of JAK2 binding to S1b4x compared with the S1b wild type observed in the Co-IP study revealed a functional link between the EC domain conformation and the JAK2 association with its docking site at Box 1. Our findings indicate an effect of the mutation (S1b4x) on the conformation of the SF homodimer and heterodimer with LF that compromised the JAK2 basal phosphorylation activity and on the JAK2 association observed in wild-type S1b. Overall, our studies demonstrate the relevance of the intramolecular disulfide bridges of the prolactin receptor for S1b-inhibitory action on PRL-induced LF-mediated STAT5-dependent action and for cytoplasmic events related to JAK2 association/activity.

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PINEAL GLAND AND CHRONOBIOLOGY

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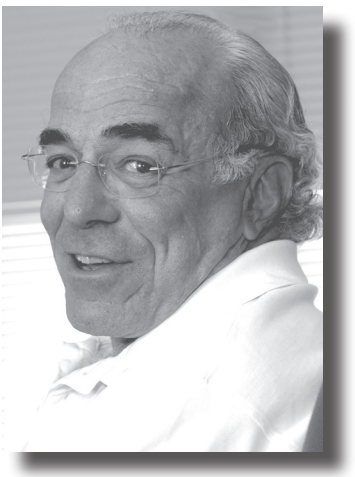

We focus primarily on the pineal gland and regulation of the production of melatonin, which is the pineal hormone. Our work has broad implications for vertebrate biology and is of special interest to clinical scientists studying human diseases relating to circadian rhythms, including endocrine pathologies, sleep and mood disorders, and deficiencies in alertness. We also address the factors controlling remarkable global changes in gene expression that occur in the pineal gland on a 24-hour basis in addition to factors associated with establishment of the pineal phenotype.

The timezyme: arylalkylamine *N*-acetyltransferase

Pavlicek, Coon, Namboodiri, Pollard, Weller, Clokie, Shi, Ganguly, Klein; in collaboration with Aitken, Chemineau, Cole, Dawid, Dyda, Falcón, Gothilf, Hickman, Hwang, Iuvone, Jaffe, Koonin, Malpaux, Obsil, Sackett, Schuck, Szewczuk, Toyama, Zhen

A pivotal advance in our investigations into arylalkylamine *N*-acetyltransferase (AANAT), the second enzyme in the pathway for melatonin synthesis from serotonin, was our finding that the enzyme is critical to the control of the rhythm in melatonin synthesis. In all species examined to date, the large increase in melatonin synthesis at night causes an increase in the production of melatonin, which is a highly conserved feature of vertebrate physiology.

Transcriptional mechanisms. The transcriptional mechanisms that control expression of the *AANAT* gene include interactions of cyclic AMP–response elements in the promoter region; other response elements in the promoter, including the E-box and photoreceptor conserved elements, appear to determine the marked tissue-specific pattern of expression of the gene, which is limited to the pineal gland and retina. We determined that, in rodents, expression of *AANAT* rises about 100-fold at night; however, this feature of regulation is not highly conserved. We discovered little or no nocturnal increase in *AANAT* expression in ungulates and primates. Thus, transcriptional control mechanisms are not essential in regulating melatonin synthesis in all vertebrates.

Post-translational control. Sequence analysis revealed that all vertebrate AANATs contain C- and N-terminal sites for phosphorylation by cyclic AMP–dependent protein kinase. We determined that the sites control the stability and biological half-life of the enzyme. Phosphorylation of the sites controls binding of AANAT to 14-3-3 protein, which protects the enzyme from destruction; when the enzyme is released, it can be destroyed rapidly. The phosphorylation state of AANAT and binding to 14-3-3 appear to govern the balance between destruction and protection. In collaboration with Fred Dyda and Alison Hickman, we determined the molecular basis of this interaction by analyzing

the crystal structure formed by AANAT and 14-3-3- proteins. Surajit Ganguly and Joan Weller, in collaboration with Benoit Malpaux and Philippe Chemineau, demonstrated that the interaction is critical for regulation of the enzyme in vivo (Ganguly et al., *Proc Natl Acad Sci USA* 2005;102:1222). In collaboration with Alastair Aitken and based on both mRNA and protein analysis, Qiong Shi used a comparative approach to analyze and establish the developmental appearance of each 14-3-3 isoform in the pineal gland. He found marked differences in the relative abundance of each isoform and in the binding and activation capacities of the 14-3-3 isoforms.

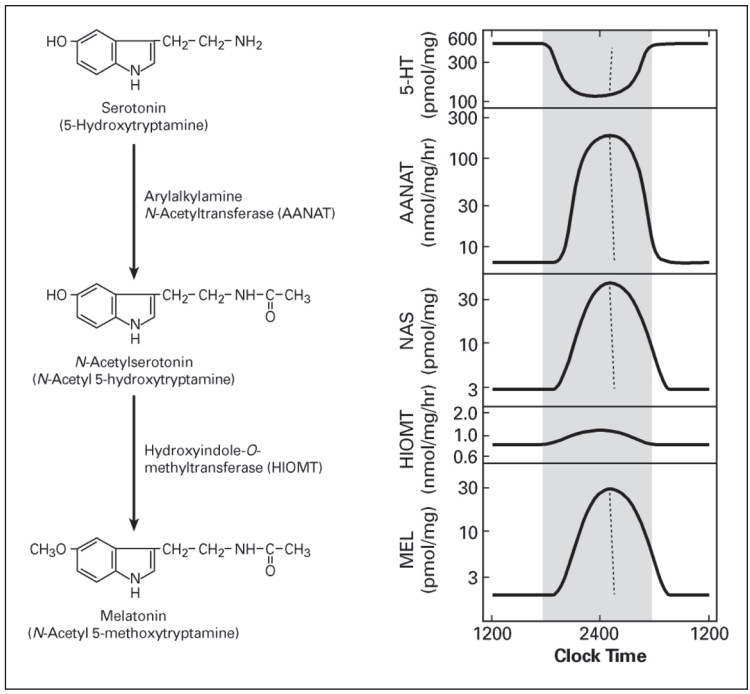

FIGURE 4.3 REGULATION OF PINEAL FUNCTION

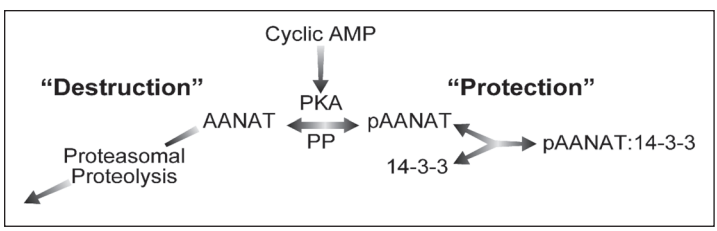

FIGURE 4.4

Recent research has focused on one of the phosphorylation sites of AANAT located in the C-terminal region. Joan Weller developed highly specific antisera to determine the phosphorylation state of the enzyme and revealed that the C-terminal site is phosphorylated at night and that the phosphorylation state of the enzyme decreases immediately after animals are exposed to light, most likely leading to destruction of the enzyme. Surajit Ganguly established that both the C- and N-terminal PKA sites are important for binding to 14-3-3 proteins. A remarkable on/off mechanism centered around the C-terminal PKA site swiches the enzyme from highly active to highly inactive. In collaboration with P. Michael Iuvone, we found that the same mechanism operates in the retina to control both the activity of AANAT and melatonin production. In the retina, light acts directly on photoreceptor cells to control cyclic AMP, thereby controlling AANAT. We suspect that light acts through a similar mechanism in

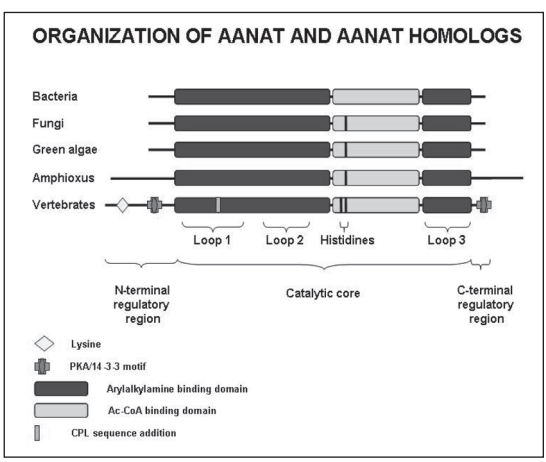

FIGURE 4.5

photosensitive pinealocytes. In collaboration with Philip Cole, Larry Szewcuk, Yousang Hwang, and Weiping Zhen, we used novel synthetic approaches to examine the role of phosphorylation in controlling AANAT. We made AANATs that contain non-hydrolyzable phosphate at the N-terminal PKA site, injected them into cells, and measured their biological half-life by using antisera. The results indicate that the non-hydrolyzable phosphate at the N-terminal PKA site prevents destruction of AANAT. We have also pursued new approaches to the development of AANAT inihibitors in order to identify lead compounds for drug development.

Steven Coon established that, in the monkey pineal, large changes occur in the abundance of AANAT protein and activity, even though *AANAT* mRNA, i.e., gene expression, does not change, underscoring the importance of post-translational regulatory mechanisms in primate biology.

Humphries A, Wells T, Baler R, Klein DC, Carter DA. Rodent *Aanat*: intronic E-box sequences control tissue specificity but not rhythmic expression in the pineal gland. *Mol Cell Endocrinol* 2007;270:43-49.

Hwang Y, Ganguly S, Ho AK, Klein DC, Cole PA. Enzymatic and cellular study of a serotonin *N*-acetyltransferase phosphopantetheine-based prodrug. *Bioorg Med Chem* 2007;15:2147-2155.

Klein DC. Arylalkylamine *N*-acetyltransferase: "the Timezyme." *J Biol Chem* 2007;282:4233-4237.

Pozdeyev N, Taylor C, Haque R, Chaurasia SS, Visser A, Thazyeen A, Du Y, Fu H, Weller J, Klein DC, Iuvone PM. Photic regulation of arylalkylamine *N*-acetyltransferase binding to 14-3-3 proteins in retinal photoreceptor cells. *J Neurosci* 2006;26:9153-9161.

Zilberman-Peled B, Appelbaum L, Vallone D, Foulkes NS, Anava S, Anzulovich A, Coon SL, Klein DC, Falcón J, Ron B, Gothilf Y. Transcriptional regulation of arylalkylamine-*N*-acetyltransferase-2 gene in the pineal gland of the gilthead seabream. *J Neuroendocrinol* 2007;19:46-53.

Molecular evolution of AANAT

Pavlicek, Coon, Ganguly, Weller, Klein; in collaboration with Sackett, Hassan, Gaildrat, Falcón

In a line of research spearheaded by Steven Coon and subsequently led by Jiri Pavlicek, Pavlicek focused on an important element of the AANAT molecule, a floppy loop described as Loop 1, which forms a portion of the arylamine-binding pocket. Loop 1 has undergone notable changes during the course of the enzyme's evolution. With Daniel Sackett and Sergio Hassan, Pavlicek evaluated the importance of specific residues in the floppy loop to determine their contribution to the greater than 1,000-fold increase in specific activity of AANAT that occurred during chordate evolution. With Pascaline Gaildrat and Jack Falcón, he analyzed a primitive form of AANAT in the cephalochorate *Amphioxus*. During the course of chordate evolution, Loop 1 acquired the tripeptide CPL65, which is responsible for increased enzyme activity. Removal of the CPL tripeptide markedly reduced activity. Moreover, increasing the local flexibility of the tripeptide region by P64G and P64A mutations had a counterintuitive effect, reducing both activity and the overall movement of Loop 1, as estimated from Langevin dynamics simulations. Binding studies indicate that the mutations increased the off-rate constant of a model substrate without altering the dissociation constant. The structural kink and local rigidity imposed by Pro-64 may enhance activity by favoring configurations of Loop 1 that facilitate catalysis without becoming immobilized by intramolecular interactions.

Pavlicek J, Coon SL, Ganguly S, Weller JL, Hassan SA, Sackett DL, Klein DC. Evidence that proline focuses movement of the floppy loop of arylalkylamine *N*-acetyltransferase (EC 2.3.1.87). *J Biol Chem* 2008;283:14552-14558.

Pineal/retinal evolution, AANAT, and the formation of conjugates of arylalkylamine and retinaldehyde

Klein, Coon; in collaboration with Kirk, Falcón, Iyer, Koonin

According to our theory of the evolution of the pineal gland, both the pineal gland and retina evolved from the same primitive photoreceptor cell after that cell acquired AANAT and HIOMT, the enzymes required to make melatonin. We believe that, originally, the two enzymes were important only in the detoxification of arylalkylamines, which can be dangerous in all tissues because of the

amine's reactivity and the aldehyde that arises from the amine's oxidation. Detoxification would have led to the production of melatonin and eventually to the development of the rhythm in melatonin as a day/night signal. However, our theory proposes that high levels of melatonin would have been destructive to the primitive photorecetor by requiring high levels of the melatonin precursor serotonin; serotonin would have been especially toxic to photoreceptor function because it can react with and thus remove retinaldehyde, the key photodetection molecule. A2S, the theoretical product formed by this reaction, would contain two molecules of retinaldehyde and one molelcule of serotonin; homologous compounds would be formed from other arylalkylamines. Such products belong to a larger family of *N*-bis-retinyl compounds, including bis-retinal-ethanolamine (A2E), which is thought to be toxic to the retina through effects of the retinal side chains. The formation of A2S in the primitive photoreceptor would reduce photosensitivity by removing retinal; in addition, the product would be toxic. Segregation of these processes to the pinealocyte and retinal photoreceptor would have resolved the conflict, permitting melatonin production and photodetection to evolve and improve.

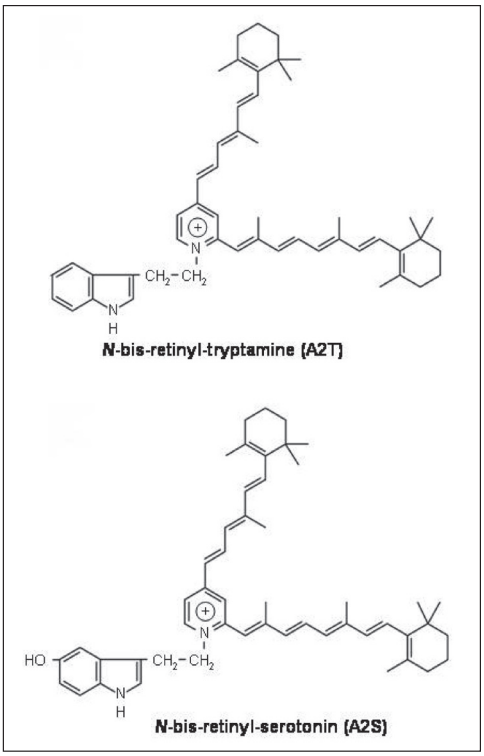

FIGURE 4.6

In collaboratin with Ken Kirk, we have synthesized A2S and related compounds and are monitoring their formation in order to test the hypothesis that their formation in the retina is a function of AANAT activity. In addition, we are examining whether the compounds might play a role in human retinal disease, specifically macular degeneration.

Coon SL, Klein DC. Evolution of arylalkylamine *N*-acetyltransferase: emergence and divergence. *Mol Cell Endocrinol* 2006;252:2-10.

Klein DC. Evolution of the vertebrate pineal gland: the AANAT hypothesis. *Chronobiol Int* 2006;23:5-20.

Global analysis of gene expression

Coon, Ganguly, Weller, Rath, Klein; in collaboration with Dawid, Gaildrat, Gothilf, Morin, Kim, Toyama, Baler, Blackshaw, Carter, Hogenesch, Humphries, Møller, Munson, Sugden

We have initiated several projects aimed at (1) obtaining a global picture of differences in gene expression that occur on a night/day basis and (2) identifying genes that are highly enriched in the pineal gland. We began our work by analyzing a relatively small number of genes. Now, with microarrys, we can examine tens of thousands of genes and ESTs (expressed sequence tags). Together with Igor Dawid, we faciliated the NICHD-sponsered commercialization of an Affymetrix chip for analysis of gene expression in zebrafish and *Xenopus*. In collaboration with Dawid, Yoav Gothilf, and Reiko Toyama, we are characterizing pineal gene expression in the zebrafish and other species as a function of time of day and of development. We have already identified a set of genes highly expressed in the pineal gland. Steven Coon and Michael Bailey are coordinating the work, which involves a collaboration with Peter Munson, David Carter, and Ruben Baler and is providing a basis for analysis of the control of gene expression in second-messenger cascades. The work has triggered several investigations that analyze the expression of genes that previously had not appeared in the pineal literature, including those encoding methionine adenosyl transferase,

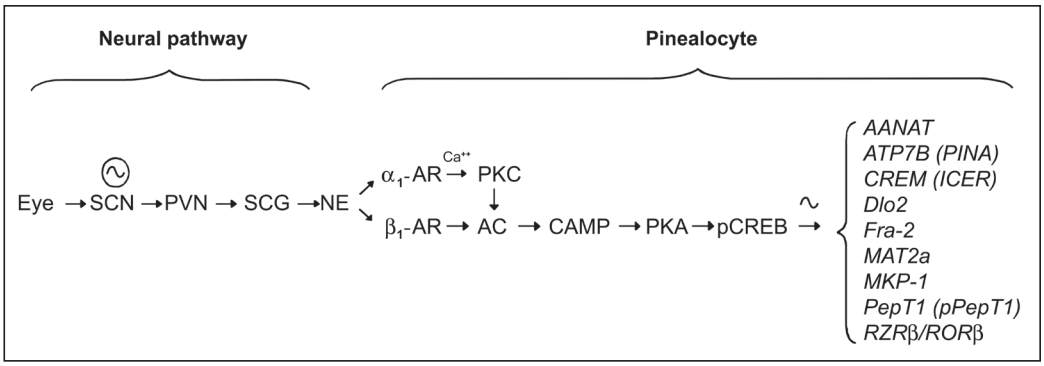

FIGURE 4.7

MAP kinase phosphatase-1, PepT1, phoshodiesterase 4D2, dopamine D4R receptor, and a subunit of the IgE receptor.

The group of pineal-specific genes expressed in the pineal gland of several vertebartes includes genes known to be associated with melatonin production and visual signal transduction. In addition, we have identified many genes (over 600), new to the pineal literature, that exhibit more than 2-fold night/day differences in expression and another set of genes that are exceptionally highly expressed in the pineal gland as compared with other tissues. This work is leading to a rapid increase in knowledge of the biochemical profile, conserved across species, of the pineal gland. It is also pointing to new transcriptional pathways controlled by previously unrecognized transcription factors; by analyzing these transcription factors and the promoters of genes that are either upregulated at night or highly expressed in the pineal gland, we will be able to construct a regulatory network that describes the cascade of transcription factors underlying the control of pineal gene expression. We have also identified potentially new and important pathways involved in cell-cell communication and signal transduction in the pineal gland; expression of some genes points to new functions for the pineal gland.

Using quantitative RT-PCR, our collaborator David Sugden has confirmed the results of our microarray studies and has provided a more detailed and quantitative description of the 24-hour patterns of gene expression, revealing that the patterns are not all similar and in phase but rather exhibit novel features. Work by Morten Møller and Martin Rath, using radiochemical in situ hybridization histology, has also confirmed the 24-hour patterns of rhythmic gene expression as well as the marked tissue specificity of genes of interest.

Our results permit a full-scale biochemical and physiological analysis of the control of genes in the pineal gland. Results from organ culture work revealed that the vast majority of genes that exhibit night/day changes in expression are also regulated by norepinephrine acting through a cyclic AMP mechanism that may involve cyclic AMP–response element binding sites in regulated genes and less specific epigenetic mechanisms.

Regulation of *S*-adenosyl methionine synthesis

Kim,[2] Klein; in collaboration with Charlton, Møller, Cepko, Blackshaw, Zhao

S-adenosylmethionine is the co-factor of the last enzyme in the melatonin biosynthesic pathway. Jong-So Kim determined that the expression of the enzyme that catalyzes the synthesis of this co-factor in the pineal gland—methionine adenosyl transferase 2a (MAT)—increases at night and is accompanied by increased enzyme activity and enzyme protein. The molecular basis of the increase involves neural stimulation of the pineal gland by norepinephrine, which results in the elevation of cyclic AMP (Kim et al., *J Biol Chem* 2005;280:677).

The nocturnal rise in *S*-adenosylmethine synthesis is obviously linked to the increased requirement for this methyl donor. While the literature has yet to describe regulation of the synthesis of *S*-adenosylmethionine by neural mechanisms, the co-factor does play a central role in the synthesis and metabolism of many transmitters (catecholamines, indoles, histamine, and so forth). Accordingly, evidence from the pineal gland indicating that MAT 2a activity can be regulated by a neural circuit via a cyclic AMP mechanism suggests that activityof MAT 2a might be regulated by transmitters in other brain regions and that the levels of *S*-adenosyl methionine might be controlled through pharmacological manipulation of MAT 2a expression. Our work provides evidence of strong and rapid control of melatonin production downstream of the enzyme commonly thought to be the only regulated element of the serotonin-to-melatonin pathway. Accordingly, it appears that adrenergic cyclic AMP control of melatonin production from serotonin involves at least two targets: AANAT and *S*-adenosymethionine production.

Regulation of phosphodiesterase 4B2

Kim,[2] Klein; in collaboration with Baler, Carter, Munson, Møller

The microarray analysis of the pineal suggested that expression of the gene encoding a cyclic AMP–selective phosphodiesterase, termed *PDE4B2*, is elevated at night. Jong-So Kim found that the level of mRNA encoding this isoform is 5 times higher at night than during the day and that expression in the pineal gland is higher than in other tissues, as confirmed by Morten Møller with in situ hybridization. Kim found that the increase in *PDE4B2* mRNA is associated with increased protein and activity, which influence the accumulation of cyclic AMP. Expression of the *PDE4B2* gene is thus controlled by the same neural pathway that regulates AANAT and MAT; in addition, cyclic AMP controls expression of *PDE4B2*, representing a negative feedback mechanism. Our work provides the first demonstration of a negative feedback mechanism involving cyclic AMP destruction in the pineal gland. It also represents a means of telling time—an internal interval timer.

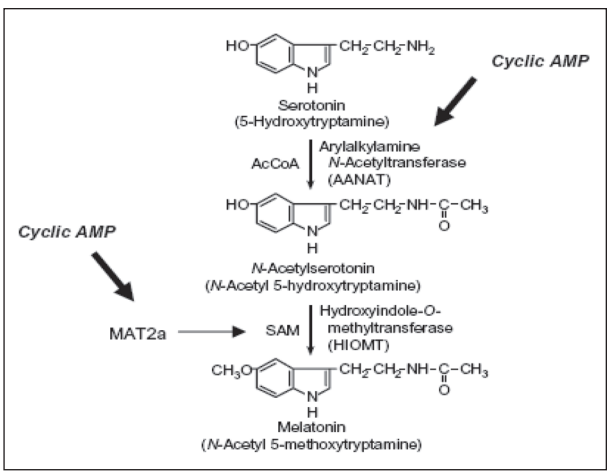

FIGURE 4.8

Kim JS, Bailey MJ, Ho AK, Møller M, Gaildrat P, Klein DC. Daily rhythm in pineal phosphodiesterase activity reflects adrenergic/cAMP induction of the PDE4B2 variant. *Endocrinology* 2007;148:1475-1485.

Induction of the oligopeptide transporter PEPT1

Gaildrat,[3] Klein; in collaboration with Ganapathy, Inui

One outcome of our microarray studies was the finding that expression of the oligopeptide transporter (*PEPT1*) gene increases markedly at night (about 100-fold). In collaboration with Vadivel Ganapathy, Pascaline Gaildrat discovered that expression of the gene at night in the pineal gland truncates the gene. Regulation reflects neural cyclic AMP activation of the pineal gland and large changes in both the mRNA encoding the protein and the protein itself. PEPT1 is relatively unstable and disappears rapidly. Gaildrat also found that the expression of *PEPT1* is highly restricted to the pineal gland and identified a section of the gene that appears to be responsible for the restricted pattern of tissue distribution and for the night/day pattern of expression, namely, cyclic AMP–response elements and putative sites for the binding of CRX/OTX transcription factors, features shared with the *AANAT* gene and located in an internal promoter (Gaildrat et al., *J Biol Chem* 2005;280:16851). It appears that we have uncovered a mechanism that selectively provides the pinealocyte with a membrane-linked function and that may involve a regulatory role of the *PEPT1* product in melatonin production.

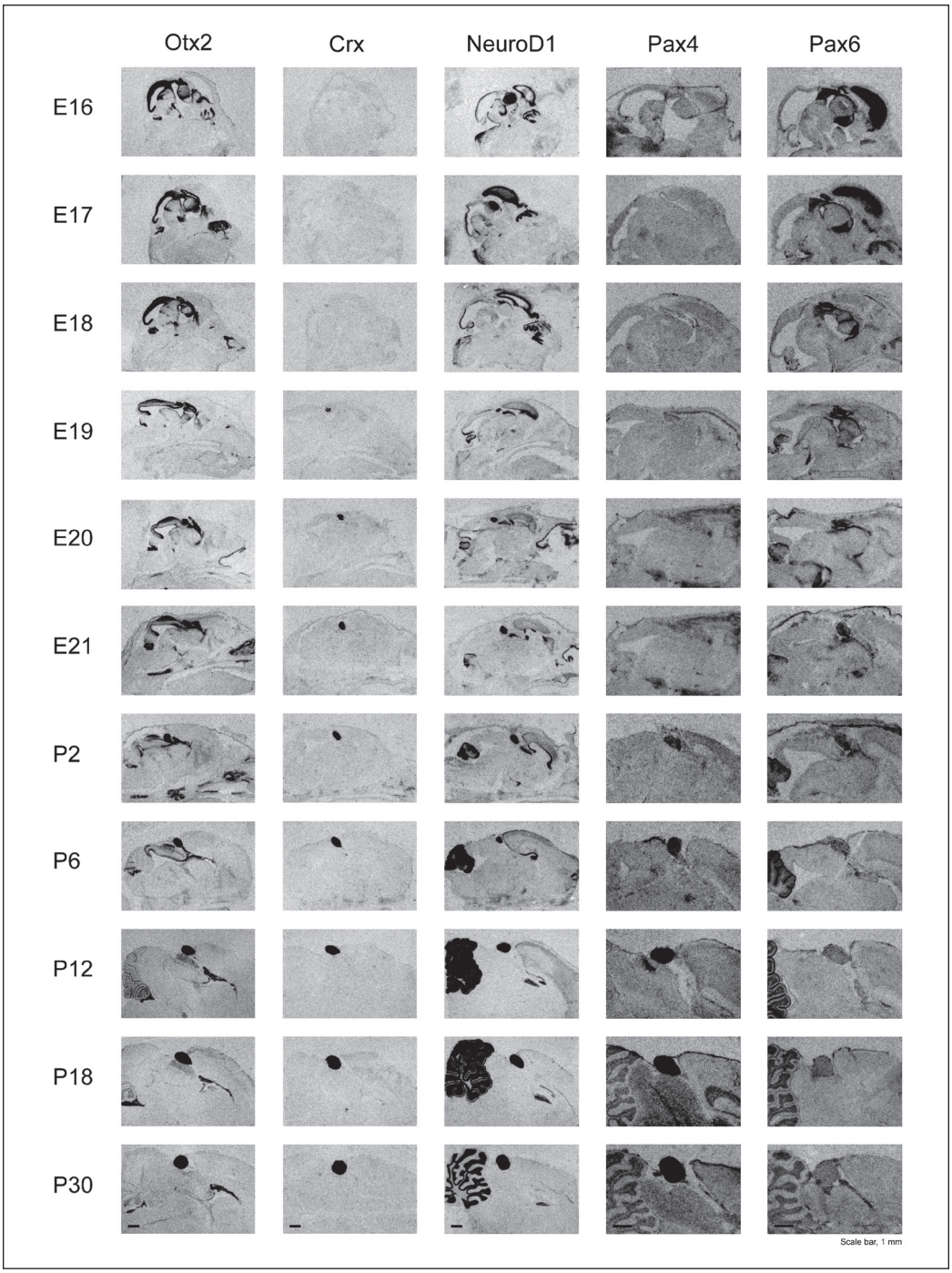

FIGURE 4.9

Developmental appearance of genes encoding the transcription factors Crx, Otx2, NeuroD1, Pax4, and Pax6 in the rat brain. The pineal gland is the heavily stained body most clearly visible in the Crx and Pax6 series. The method used to detect these transcripts is radiochemical *in situ* hybridization histology. These images are available in high resolution at *http://science.nichd.nih.gov/confluence/display/sne/Developmental+Gallery.*

NeuroD-1, *CRX*, *OTX*, *Pax4*, and *Pax6* expression in the pineal gland

Bustos, Coon, Ochocinska, Rath, Klein; in collaboration with Carter, Munson, Baler, Møller, Muñoz

Through our microarray analyses and as confirmed with independent techniques, we revealed that the pineal gland expresses high levels of several transcription factors, including *NeuroD1*, *CRX OTX*, *Pax4*, and *Pax6*, that play a role in the development of the eye and other tissues. With collaborators under the direction of Morten Møller, we undertook a detailed description of the developmental appearance of these transcripts (Figure 4.9); the long-term plan is to determine the transcripts' role in developmental and adult expression of genes in the pineal gland.

Otx2 is a vertebrate homeobox gene essential for the development of rostral brain regions. It also appears to play a role in the development of retinal photoreceptor cells and pinealocytes. Work led by Martin Rath revealed the temporal expression pattern of *Otx2* in the rat brain, with exceptionally strong expression in the pineal gland throughout late embryonic and postnatal stages. Widespread high expression of *Otx2* in the embryonic brain becomes progressively restricted in the adult to the pineal gland. *Crx* (cone-rod homeobox), a downstream target gene of Otx2, showed a pineal expression pattern similar to that of *Otx2*, despite a distinct lag in time of onset. We identified the Otx2 protein in pineal extracts and found it to be localized in pinealocytes. Total pineal *Otx2* mRNA neither showed night/day variation nor was influenced by the removal of sympathetic input, suggesting that the level of *Otx2* mRNA is independent of the photoneural input to the gland. Our observations are consistent with the view that pineal expression of *Otx2* is required for development and plays a role in the adult by controlling the expression of the cluster of genes associated with phototransduction and melatonin synthesis.

NeuroD1/BETA2, a member of the bHLH transcription factor family, influences the fate of specific neuronal, endocrine, and retinal cells. In collaboration with Estela Muñoz, we found that *NeuroD1* mRNA is highly abundant in the developing and adult rat pineal gland. Pineal expression begins in the 17-day embryo, at which time it is also detectable in other brain regions. Expression in the pineal gland increases during the embryonic period and is maintained thereafter at levels equivalent to those found in the cerebellum and retina. In contrast, *NeuroD1* mRNA decreases markedly in non-cerebellar brain regions during development. Pineal NeuroD1 levels are similar during night and day and do not appear to be influenced by sympathetic neural input. Gene expression analysis of the pineal glands from neonatal *NeuroD1* knockout mice identified 127 transcripts that are more than 2-fold downregulated and 16 that are more than 2-fold upregulated. According to quantitative RT-PCR, the most dramatically downregulated gene is *KIF5C* (kinesin-family member 5C*)* (about 100-fold); the most dramatically upregulated gene is that encoding glutamic acid decarboxylase 1 (about 4-fold). Other affected transcripts encode proteins involved in differentiation, development, signal transduction, and trafficking. To elucidate the role of NeuroD1 in the rodent pinealocyte, Margaret Ochocinska is using a Cre-Lox conditional knockout stategy to block expression of *NeuroD1* in both the pineal gland and retina.

Studies by Martin Rath and other laboratory members revealed that *Pax4* is strongly expressed in the rat pineal gland and retina. The levels of pineal *Pax4* transcripts are low in the fetus and increase postnatally; *Pax6*, on the other hand, exhibits an inverse pattern of expression and is more strongly expressed in the fetus. In the adult, the abundance of *Pax4* mRNA exhibits a diurnal rhythm in the pineal gland, with maximal levels occurring late during the light period. Sympathetic denervation of the pineal gland by superior cervical ganglionectomy prevents the nocturnal decrease in pineal *Pax4* mRNA. At night, the release of norepinephrine by sympathetic innervation adrenergically stimulates the pineal gland. We found that treatment with adrenergic agonists suppresses pineal *Pax4* expression both in vivo and in vitro. As indicated by the observation that treatment with a cyclic AMP reduces pineal *Pax4* mRNA levels, suppression of pineal *Pax4* appears to be mediated

by cyclic AMP, a second messenger of norepinephrine in the pineal gland. These findings suggest that the nocturnal decrease in pineal *Pax4* mRNA is controlled by the sympathetic neural pathway that, in turn, controls pineal function via an adrenergic-cyclic AMP mechanism. The daily changes in *Pax4* expression may influence gene expression in the pineal gland.

Muñoz E, Bailey MJ, Rath MF, Shi Q, Morin F, Coon SL, Møller M, Klein DC. *NeuroD1*: developmental expression and regulated genes in the rodent pineal gland. *J Neurochem* 2007;102:887-899.

Rath M, Bailey MJ, Kim J-S, Ho AK, Gaildrat P, Coon SL, Møller M, Klein DC. Developmental and diurnal dynamics of Pax4 expression in the mammalian pineal gland: nocturnal down-regulation is mediated by adrenergic-cyclic AMP signaling. *Endocrinology* 2008; [E-pub ahead of print].

Rath MF, Morin F, Shi Q, Klein DC, Møller M. Ontogenetic expression of the *Otx2* and *Crx* homeobox genes in the retina of the rat. *Exp Eye Res* 2007;85:65-73.

Rath MF, Muñoz E, Ganguly S, Morin F, Shi Q, Klein DC, Møller M. Expression of the *Otx2* homeobox gene in the developing mammalian brain: embryonic and adult expression in the pineal gland. *J Neurochem* 2006;97:556-566.

Studies on the pineal-immune link

Rath, Ganguly, Klein; in collaboration with Møller

Analysis of gene expression in the pineal gland also revealed a large daily rhythm in the expression of the gene encoding a subunit of the IgE receptor. We confirmed expression of the gene with several techniques and established that such expression reflects adrenergic/cyclic AMP signaling in pinealocytes. Our current work aims to determine the functional role played by such expression in physiology and to examine the hypothesis that the pinealocyte is a sentinel that modulates the immune response on a circadian schedule. In addition, we found that perivascular phagocytes in the vertebrate pineal gland serve as antigen-presenting cells. These findings, together with evidence of expression of the peptide transporter PEPT1, provide the initial molecular building blocks of a system mediating the relationship between antigens and the pineal gland. Microarray technology studies have revealed that the pineal gland expresses a set of genes known to be associated with immune/inflammation responses in other tissues. Accordingly, our goal is to determine how the pineal gland may be involved in these responses.

Ganguly S, Grodzki C, Sugden D, Møller M, Odom S, Gaildrat P, Gery I, Siraganian RP, Rivera J, Klein DC. Neural adrenergic-cyclic AMP regulation of the IgE receptor alpha-subunit expression in the mammalian pinealocyte: a neuroendocrine-immune response link? *J Biol Chem* 2007;282:32758-32764.

Møller M, Rath MF, Klein DC. The perivascular phagocyte of the mouse pineal gland: an antigen-presenting cell. *Chronobiol Int* 2006;23:393-401.

Regulation of dopamine D4R receptor: cAMP + T3 "AND" gate regulation

Kim,[2] Bailey, Klitten,[4] Klein; in collaboration with Møller, Celi

Preliminary cDNA microarray studies suggested that D4R expression increases at night. Morten Møller confirmed such expression by in situ analysis and Jong-So Kim and Michael Bailey by Northern blot and quantitative PCR. Other than trace levels, we could not find expression of other dopamine receptors in the pineal gland. *D4R* expression in the pineal gland is many-fold higher than in most other tissues, suggesting that the D4R receptor plays an important role in pineal signal transduction, perhaps as an inhibitor of adrenergic stimulation of adenylate cyclase. Regulation of the expression of this gene is unusual; in the case of other genes studied to date, including *AANAT*, *MAT2*, *MKP-1*, *Type II deiodinase*, *ICER*, and *FRA-2*, the nocturnal increase in mRNA can be reproduced in organ culture by treatment with either norepinephrine or a cyclic AMP agonist. However, in the case of the D4R receptor, *D4R* mRNA does not increase following treatment with either norepinephrine or a cyclic AMP agonist. Rather, co-treatment with the thyroid hormone triiodothyronine (T3) and either norepinphrine or cyclic AMP is required to increase *D4R* mRNA, thereby establishing that expression of the D4R receptor is regulated by an "AND" gate operated

by cAMP and T3 and providing the first evidence of a role for the thyroid gland in regulating a specific gene in the pineal gland. This finding is consistent with the knowledge of an increase in the capacity to convert thyroxine (T4) to T3 through induction of type II deiodinase at night in the pineal gland. In indicating that melatonin synthesis might be a function of thyroid status, our study has broad implications for dopamine signal transduction in other tissues.

As part of a broader effort to understand the similarities between the retina and pineal gland, Laura Klitten led a semiquantitative in situ hybridization study that revealed prominent night/day variation in *Drd4* expression in the retina of the rat, with a peak during the night. *Drd4* expression is seen in all retinal layers, but the nocturnal increase is confined to the photoreceptors. Removal of sympathetic input to the eye does not affect retinal *Drd4* expression, but triiodothyronine treatment induces *Drd4* expression in the photoreceptors. In a developmental series, we found that expression of *Drd4* is restricted to postnatal stages with a peak at postnatal day 12. The high *Drd4* expression in the rat retinal photoreceptors during the night supports physiological and pharmacologic evidence that the Drd4 receptor is involved in the dopaminergic inhibition of melatonin synthesis upon light stimulation. The sharp increase of *Drd4* expression at a specific postnatal time suggests that dopamine is also involved in retinal development.

Klitten LL, Rath MF, Coon SL, Kim JS, Klein DC, Møller M. Localization and regulation of dopamine receptor D4 expression in the adult and developing rat retina. *Exp Eye Res* 2008; [E-pub ahead of print].

Role of islet antigen 2 and islet antigen 2beta in circadian biology

Klein; in collaboration with Notkins, Schnerman, Constance, Weaver, Piggins

We discovered that the elimination of two proteins, islet antigen 2 and islet antigen 2beta, removes daily rhythms in activity, cardiovascular function, and temperature. The proteins are found in secretory vesicles and are best recognized by their association with type 1 diabetes; autoantibodies against the proteins are a predictor of the disease. Our studies suggest that the absence of daily rhythms may be attributable to a combination of effects on neurotransmission and downstream effects.

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HERITABLE NEURODEGENERATIVE AND AUTOIMMUNE DISODERS

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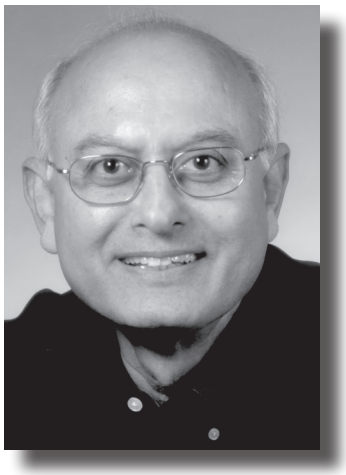

We conduct laboratory and clinical investigations into the molecular mechanisms underlying genetic disorders of neurodegeneration, inflammation, and autoimmunity for the purpose of developing therapeutic approaches to diseases for which no effective treatment exists. We focus on the regulation and physiological functions of two genes: (1) palmitoyl-protein thioesterase-1 (*PPT1*), mutations of which result in infantile neuronal ceroid lipofuscinosis, a devastating neurodegenerative storage disease of childhood; and (2) uteroglobin (*UG*), also known as Clara cell 10 kDa protein (CC10), which manifests potent anti-inflammatory and immunomodulatory properties. UG deficiency in mice causes IgA-nephropathy and focal pulmonary fibrosis and predisposes the animals to invasive cancers. Our investigations into both genes have led to ongoing clinical trials. Using *PPT1*-knockout mice, which recapitulate virtually all clinical and pathological features of INCL, we discovered that deficiency in this enzyme leads to endoplasmic reticulum stress, which in part mediates neuronal apoptosis, a discovery that provides insight into the complex mechanism of neurodegeneration in INCL and identifies several potential therapeutic targets. We also delineated the mechanism by which PPT1 deficiency leads to disrupted recycling of synaptic vesicle (SV) components and prevents the regeneration of SVs required for maintaining a steady SV pool size at neuronal synapses; SVs are essential for uninterrupted neurotransmission.

Synaptic vesicle recycling at the nerve terminals contributes to INCL neuropathology
Kim, Zhang, Sarkar, Tsai,[1] Lee,[2] Mukherjee; in collaboration with Dye

Neuronal ceroid lipofuscinoses (NCL) are the most common (1 in 12,500 births) neurodegenerative storage disorders of childhood. The infantile form of NCL, or INCL, is caused by palmitoyl protein thioesterase-1 (PPT1) deficiency. We previously reported that PPT1 deficiency causes endoplasmic reticulum (ER) stress, which at least in part mediates neuronal apoptosis and contributes to neurodegeneration in INCL. Although INCL patients show signs of abnormal neurotransmission as manifested by myoclonus and seizures, the molecular mechanisms by which PPT1 deficiency causes this abnormality remain obscure. Neurotransmission relies on repeated cycles of exo- and endocytosis of SVs in which several palmitoylated proteins play critical roles. These proteins facilitate membrane fusion, which is required for neurotransmitter exocytosis, recycling of the fused SV membrane components, and regeneration of fresh vesicles. However, palmitoylated proteins require depalmitoylation for recycling. Using postmortem brain tissues from an INCL patient and tissue from *PPT1*-knockout (PPT1-KO) mice that mimic INCL, we found that PPT1 deficiency caused persistent membrane anchorage of the palmitoylated SV proteins, hindering the recycling

of the vesicle components that normally fuse with the presynaptic plasma membrane during SV exocytosis. Thus, the regeneration of fresh SVs, essential for maintaining the SV pool size at the synapses, was impaired, leading to a progressive loss of readily releasable SVs and abnormal neurotransmission. This abnormality may contribute to INCL neuropathology.

Kim SJ, Zhang Z, Sarkar C, Tsai PC, Lee YC, Dye L, Mukherjee AB. Palmitoyl protein thioesterase-1 deficiency impairs synaptic vesicle recycling at nerve terminals, contributing to neuropathology in humans and mice. *J Clin Invest* 2008;118:3075-3086.

Zhang Z, Lee Y-C, Kim S-J, Choi MS, Tsai PC, Xu Y, Xiao YJ, Zhang P, Heffer A, Mukherjee AB. Palmitoyl-protein thioesterase-1 deficiency mediates the activation of the unfolded protein response and neuronal apoptosis in INCL. *Hum Mol Genet* 2006;15:337-346.

ER and oxidative stresses are common mediators of apoptosis in lysosomal storage disorders and are alleviated by chemical chaperones

Wei, Kim, Zhang, Tsai,[1] Mukherjee; in collaboration with Wisniewski

It is estimated that over 40 lysosomal storage disorders (LSD) cumulatively affect 1 in 5,000 live births; indeed, neurodegeneration is a prominent feature of the majority of such disorders. As a group, NCLs represent one of the most common (1 in 12,500 births) neurodegenerative LSDs. INCL is the most devastating neurodegenerative LSD. We previously reported that ER and oxidative stresses at least in part cause neuronal death by apoptosis in both INCL and PPT1-KO mice that mimic INCL. Over the past year, we unexpectedly found that ER and oxidative stresses are not unique manifestations of INCL but are common to both neurodegenerative and non-neurodegenerative LSDs. Moreover, all LSD cells studied in our laboratory show extraordinary sensitivity to brefeldin A–induced apoptosis, suggesting pre-existing ER stress conditions. Further, we found that chemical disruption of lysosomal homeostasis in normal cells causes ER stress, suggesting cross-talk between lysosomes and the ER. Most important, we found chemical chaperones that alleviate ER and oxidative stresses to be also cytoprotective in all forms of LSDs studied. We propose that ER and oxidative stresses are common mediators of apoptosis in both neurodegenerative and non-neurodegenerative LSDs and posit that, at least in part, alleviation of stress conditions permits chemical/pharmacological chaperones to exert their beneficial effects.

Kim SJ, Zhang Z, Lee YC, Mukherjee AB. Palmitoyl-protein thioesterase-1 deficiency leads to the activation of caspase-9 and contributes to rapid neurodegeneration in INCL. *Hum Mol Genet* 2006;15:1580-1586.

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Cytosolic PLA_2 activation in the brain of mice lacking PPT1 mediates phagocyte infiltration

Zhang, Lee,[2] Kim, Choi,[3] Tsai,[1] Saha, Wei, Zhang, Heffer,[4] Mukherjee; in collaboration with Xiao, Xu

In the majority of neurodegenerative storage disorders, neuronal death in the brain is followed by infiltration of phagocytic cells (e.g., activated microglia, astroglia, and macrophages) for the efficient removal of dead cells. However, it is increasingly evident that the phagocytes may also cause death of adjoining viable neurons, thus contributing to rapid progression of neurodegeneration. PPT1 catalyzes the cleavage of thioester linkages in *S*-acylated (palmitoylated) proteins while a deficiency in the enzyme leads to abnormal accumulation of thioesterified polypeptides (ceroid) in lysosomes, thereby causing INCL pathogenesis. As noted, PPT1-KO mice mimic the clinical and pathological features of human INCL, including rapid neuronal death by apoptosis and phagocyte infiltration. We previously reported that, in PPT1-KO mice, neurons undergo ER stress, activating the unfolded protein response, which mediates caspase-12 activation and apoptosis. However, the molecular mechanism(s) by which the phagocytic cells are recruited in the PPT1-KO mouse brain remains poorly understood. We found that increased production of lysophosphatidylcholine (LPC), catalyzed by the activation of cytosolic phospholipase A_2 ($cPLA_2$) in the PPT1-KO mouse

brain, is a "lipid signal" for phagocyte recruitment. We also report that an age-dependent increase in LPC levels in the PPT1-KO mouse brain positively correlates with elevated expression of the genes characteristically associated with phagocytes. We propose that increased $cPLA_2$-catalyzed LPC production in the brain is at least one of the mechanisms that mediates phagocyte infiltration contributing to INCL neuropathology.

Zhang Z, Lee YC, Kim SJ, Choi MS, Tsai PC, Saha A, Wei H, Xu Y, Xiao YJ, Zhang P, Heffer A, Mukherjee AB. Production of lysophosphatidylcholine by cPLA2 in the brain of mice lacking PPT1 is a signal for phagocyte infiltration. *Hum Mol Genet* 2007;16:837-847.

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Clinical trial of a combination of Cystagon™ and Mucomyst® for INCL patients

Levin, Zhang, Mukherjee; in collaboration with Baker, Caruso, Quezado, Miao, Gropman

Thioester-linkaged *S*-acylated polypeptides are susceptible to nucleophilic attack. Thus drugs with nucleophilic property have therapeutic potential for INCL. Several such compounds (e.g., cysteamine, phosphocysteamine, cysteamine bitartrate [Cystagon™], and N-acetylcysteine [Mucomyst®]) disrupt thioester linkages in the model PPT1-substrate [^{14}C] palmitoyl~CoA, releasing [^{14}C] palmitic acid. Among the drugs tested, we previously characterized phosphocysteamine, Cystagon™, and Mucomyst® in detail and found that they do indeed cleave thioester linkages and deplete ceroid in cultured cells from INCL patients (Zhang et al., *Nat Med* 2002;7:478). It has also been reported that cysteamine is lysosomotrophic, crosses the blood-brain barrier, and is relatively non-toxic. Thus, we have been conducting a clinical trial involving 20 INCL patients to determine whether a combination of Cystagon™ and Mucomyst® is beneficial. Given that INCL is a fatal disease, that Mucomyst® has anti-apoptotic effects, and that both drugs are neuroprotective and have a proven record of safety, we received approval for combination therapy of those patients who were originally enrolled in the Cystagon™ monotherapy protocol.

Uteroglobin is the founder of the Secretoglobin superfamily of proteins

Mukherjee, Zhang; in collaboration with Chilton

UG, also known as blastokinin, is a steroid-inducible, evolutionarily conserved, secreted protein whose structure and molecular biology have undergone extensive study. However, the physiological function(s) of UG remains elusive. Isolated from the uterus of rabbits during early pregnancy, UG is the founding member of a growing superfamily of proteins called Secretoglobins (Scgb). Numerous studies demonstrated that UG is a multifunctional protein with anti-inflammatory/immunomodulatory properties. It inhibits soluble phospholipase A_2 activity and binds to and perhaps sequesters hydrophobic ligands such as progesterone, retinols, polychlorinated biphenyls, phospholipids, and prostaglandins. In addition, UG manifests anti-chemotactic, anti-allergic, anti-tumorigenic, and embryonic growth–stimulatory activities. Several steroidal hormones regulate the tissue-specific expression of the *UG* gene, although a non-steroid hormone, prolactin, further augments the gene's expression in the uterus. The mucosal epithelia of virtually all organs that communicate with the external environment express UG, which is present in blood, urine, and other body fluids. Although UG's physiological functions are still under investigation, a single nucleotide polymorphism in the *UG* gene appears to be associated with several inflammatory/autoimmune diseases. Investigations with *UG*-knockout mice revealed that the absence of the protein leads to phenotypes suggestive of the protein's critical homeostatic role(s) against oxidative damage, inflammation, autoimmunity, and cancer. Recent studies on UG-binding proteins (receptors) provide further insight into the protein's multifunctional nature. Given its anti-inflammatory and anti-allergic properties, UG is a potential drug target.

Mukherjee AB, Zhang Z, Chilton BS. Uteroglobin: a steroid-inducible immuno-modulatory protein that founded the Secretoglobin superfamily. *Endocr Rev* 2007;28:707-725.

UG prevents fibronectin-IgA complex formation found in IgA-nephropathy

Zhang, Mukherjee; in collaboration with Chowdhury

Immunoglobulin A (IgA) nephropathy (IgAN) is the most common primary renal glomerular disease in the world without effective treatment. High levels of circulating IgA-fibronectin (Fn) complexes characteristic of IgAN patients are thought to cause abnormal deposition of IgA and Fn in the renal glomeruli of these patients, causing renal failure. We previously reported that binding of Fn to UG inhibits Fn-IgA heteromerization (Zheng et al., *Nat Med* 1999;5:1018). However, the specific site of Fn-UG interaction was unknown until now. We report that UG interacts with the heparin-binding site of Fn and propose that small molecules competing for interaction with this site may reduce the level of circulating Fn-IgA complexes in IgAN.

Chowdhury B, Zhang Z, Mukherjee AB. Uteroglobin interacts with the heparin-binding site of fibronectin and prevents fibronectin-IgA complex formation found in IgA-nephropathy. *FEBS Lett* 2008;582:611-615.

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MOLECULAR BIOLOGY, REGULATION, AND BIOCHEMISTRY OF UDP-GLUCURONOSYLTRANSFERASE ISOZYMES

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UDP-glucuronosyltransferase (UGT) isozymes, distributed primarily in liver, kidney, and the gastrointestinal tract, carry out the essential role of converting to glucuronides innumerable, frequently encountered, structurally diverse lipophilic chemicals, including toxic metabolites, dietary constituents, environmental carcinogens, and therapeutics, whose conversion hastens excretion and prevents their accumulation in tissue and their toxic effects in the body. Neurotoxic bilirubin is the most important endogenous substrate. Given that the UGT isozymes prevent bilirubin neurotoxicities in children and inactivate common mutagens and carcinogens but prematurely clear therapeutic chemicals, it is important to understand the mechanism of glucuronidation in order to develop methods and therapeutics for accelerating the removal of toxic chemicals while retaining medications to achieve maximal therapeutic benefits. Moreover, the enzymatic mechanism(s) and properties that enable a limited number of endoplasmic reticulum–bound UGTs to convert numerous structurally diverse lipophiles to innocuous glucuronides remain unknown. We discovered and characterized *UGT1A*, the novel complex locus encoding 13 *UGT* genes, that is organized to share a common carboxyl terminus. We also cloned *UGT2B7* and *UGT2B15* and characterized catalysis. Thus, an important research goal is to determine the properties, mechanism(s), and molecular events controlling these isozymes in order to understand how exogenous as well as endogenous chemicals lead to the disease process.

Control of the UGT1 family of isozymes by PKC phosphorylation
Basu N, Banerjee, Garza[1]; in collaboration with Rivera

As noted, the special properties and enzymatic mechanism(s) that enable endoplasmic reticulum (ER)–bound UGT isozymes to convert innumerable structurally diverse lipophiles to excretable glucuronides remain unknown. Inhibition of cellular UGT1A7 and UGT1A10 activities and of time- and concentration-dependent [^{33}P]orthophosphate incorporation into immunoprecipitable proteins following exposure to curcumin or calphostin-C indicated that the isozymes are phosphorylated (Basu et al., *Proc Natl Acad Sci USA* 2005;102:6285). Furthermore, inhibition of UGT phosphorylation and activity by treatment with PKCε-specific inhibitor peptide supported PKC involvement. Co-immunoprecipitation, co-localization via immunofluorescence, and cross-linking studies of PKCε and UGT1A7His revealed that the proteins reside within 11.4 Å of each other. Moreover, mutation of three PKC sites in each UGT isozyme demonstrated that T73A/G and T202A/G caused null activity, whereas S432G-UGT1A7 caused a major shift of the isoenzyme's pH optimum from

8.5 to 6.4 as well as changes in substrate specificity to include 17β-estradiol. S432G-UGT1A10 exhibited a minor pH shift without changing substrate specificity. We confirmed PKCε involvement by demonstrating, first, that PKCε overexpression enhanced the activity of UGT1A7 but not that of its S432 mutant and, second, that S432G-1A7 but not UGT 1A7 glucuronidates 17β-[^{14}C]estradiol. Consistent with these observations, treatment of UGT1A7-transfected cells with PKCε-specific inhibitor peptide or general PKC inhibitors increased 17β-estradiol catalysis between 5- and 11-fold with parallel decreases in the level of phospho-serine-432. We report a novel mechanism involving protein kinase C–mediated phosphorylation of UGT such that phospho-serine/threonine regulates substrate specificity in response to chemical exposures, possibly conferring survival benefit.

Expansion of the phosphorylation requirement of UGT1 family members

Basu N, Mbas-Jones,[2] Chang[3]

Inhibition by curcumin or calphostin-C of recombinant UGT1A6 and UGT1A9 expressed in COS-1 cells provides further evidence that each UGT isozyme requires phosphorylation. Time-dependent inhibition of UGT1A6 by curcumin showed a modest reversal by 5 hours. The equivalent mutation, S434G/A/D/R/K, in *UGT1A6* caused a shift from a single broad pH optimum to two pH optima. For *UGT1A9*, the equivalent mutation, S432G/A/D/E, caused progressive diminution of activity for mycophenolic acid. Similarly, mutations inserted into the *UGT1A9*-His construct resulted in reduced activity as assessed by Western blot. Curcumin and calphostin-C inhibited UGT1A9 activity in a dose-dependent manner. Whereas reversal of 95 percent inhibition by curcumin occurred in 5 hours for UGT1A9 as a consequence of the residual 5 percent metabolism, reversal was much slower for UGT1A6, which did not metabolize the agent. The evidence indicates that phospho-serine/threonine in UGT1A6 and UGT1A9 directly or indirectly controls activity. Thus, our cumulative evidence indicates that each UGT requires phosphorylation.

Dependence of UGT2B7 activity on phosphorylation by c-Src kinase

Chakraborty K, Basu N, Mitra,[4] Garza[1]

Earlier, we cloned UGT2B7 and demonstrated that it metabolizes genotoxic catechol estrogens produced by the mammary gland and other estrogen target tissues and that are associated with initiation of breast cancer. UGT2B7 is the putative relevant isozyme that detoxifies these endogenous estrogen metabolites in the mammary gland. For UGT2B7, tyrosine kinase (TK) phosphorylation sites, the enzyme's incorporation of immunoprecipitable [^{33}P]orthophosphate, and inhibition by TK inhibitors are all consistent with tyrosine phosphorylation, a claim further supported by the following observations: mutational studies have shown that phosphorylation of only Y236 and Y438 is required; PP2 [(4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine)], a potent and selective inhibitor of the Src-family tyrosine kinase, specifically inhibits Src with loss of phospho-Y438-UGT2B7 content; and overexpression of Src/*active* Src, but not of dominant-negative Src, significantly increases UGT2B7 activity and the level of phospho-Y438. PP2 disrupts co-localization of Src/*active* Src and UGT2B7, and UGT2B7 and Src/*active* Src can be cross-linked, demonstrating that UGT2B7 and Src/*active* Src interact in situ. AKAP12-siRNA–dependent depletion of SrcTK organization, involving the ER-scaffold protein AKAP12, in *UGT2B7*-transfected COS-1 cells caused 70 percent destruction of AKAP12, 80 percent loss of UGT2B7 activity, and 90 percent loss of phospho-Y438-UGT2B7 without affecting the UGT2B7 protein level. This evidence supports an AKAP12-dependent signaling network. Expression of *UGT2B7* in SYF$^{+/-}$ or SYF$^{+/+}$ fibroblast cells led to 2- to 4-fold higher activity than that in SYF$^{-/-}$ cells. In vitro treatment of solubilized UGT2B7-containing microsomes from SYF$^{-/-}$ cells with SrcTK and [γ^{33}P]ATP caused the microsomes to incorporate 12 times more label with markedly higher phospho-Y438-UGT2B7 content than after [γ^{33}P]ATP treatment alone; similarly microsomes treated with unlabeled ATP showed a 5-fold increase in UGT2B7 activity. Importantly, dramatic losses of Src activity and Src/*active* Src content, which paralleled loss of UGT2B7 activity and phospho-Y438-UGT2B7 in breast

carcinomas compared with control tissues, are consistent with Src phosphorylation of UGT2B7. Src phosphorylation of UGT2B7, which detoxifies genotoxic catechol estrogens associated with DNA depurination and tumor initiation, indicates that UGT2B7 effectively behaves as an Src-dependent tumor suppressor.

Dependence of UGT2B15 on PKC phosphorylation but independent downregulation by Src kinase phosphorylation

Chakraborty K, Banerjee, Basu N

Earlier, we cloned prostate- and testis-distributed UGT2B15 and demonstrated that it metabolizes several androgen intermediates as well as dihydrotestosterone, which, at elevated concentrations, is associated with benign prostate hyperplasia and prostate cancer. Treatment of UGT2B15-transfected COS-1 cells revealed that both PKC- and tyrosine kinase inhibitors downregulated activity. Appropriate concentrations of the PKC-specific inhibitor calphostin-C inhibited activity by 97 percent. Computer analysis showed that UGT2B15 contains three PKC and two tyrosine phosphorylation sites. Mutation of phosphorylation sites in UGT2B15 demonstrated that a single PKC site is required for activity. Unlike trivial levels of catechol-estrogen–metabolizing UGT2B7 activity in transfected $SYF^{-/-}$ cells, UGT2B15 expressed its highest activity in $SYF^{-/-}$ cells; such activity was inhibited by 64 and 88 percent when expressed in $SYF^{+/-}$ and $SYF^{+/+}$ cells, respectively. As a broad screen to determine other pathways involved in controlling UGT2B15 activity, we carried out rescue experiments following expression in COS-1 cells. The Src-specific inhibitor PP2, the p38MAP kinase–specific inhibitor SB230580, and the JNK kinase–specific inhibitor DATS (diallyl trisulfide) increased activity between 2- and 4-fold, indicating that the p38MAP kinase and JNK kinase pathways are likely upstream of Src kinase, which downregulates UGT2B15. Our evidence indicates that c-Src inhibits UGT2B15.

Dependence of UGT2B17 on PKC phosphorylation

Park, Basu N

Like UGT2B15, prostate- and testis-distributed UGT2B17 metabolizes several androgen-intermediates and dihydrotestosterone, but its activity is 5-fold higher than UGT2B15. Thus, UGT2B17 is also associated with benign prostate hyperplasia and prostate cancer and is likely as important as UGT2B15. The two proteins are differentially distributed in luminal (UGT2B15) and basal (UGT2B17) cells. Using UGT2B17-transfected COS-1 cells, we demonstrated that PKC inhibitors downregulated activity; appropriate concentrations of the PKC-specific inhibitor calphostin-C inhibited PKC activity by 97 percent. Computer analysis revealed that UGT2B17 contains two PKC- and two tyrosine-phosphorylation sites. Mutation analysis of UGT2B17 phosphorylation sites demonstrated that, parallel to UGT2B15, only S172 alterations caused null activity, whereas S422A, Y99F, and Y237F lost, respectively, 20, 66, and 75 percent activity. Moreover, UGT2B17, like UGT2B15, has higher activity in $SYF^{-/-}$ cells than when expressed in COS-1 cells. Currently, we are further characterizing UGT2B17.

Regulation of UGT phosphorylation via signaling

Basu N, Kole,[5] Garza,[1] Mitra[4]

Catalase or herbimycin-A inhibition of constitutive or hydrogen peroxide–activated UGTs demonstrated that ROS-related oxidants behave as second messengers in maintaining constitutive PKC-dependent signaling, evidently sustaining UGT phosphorylation and activity. Given that cells use signal transduction collectively to detect and respond appropriately to a changing environment, our study, combined with our earlier demonstration of phospho-dependent substrate selections by UGT, suggests that, to function efficiently, regulated phosphorylation allows adaptations via differential phosphate utilization by UGTs.

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Tertiary structure of UGT(s)

Garza,[1] Basu N, Basu M

To obtain further details of the requirements of phosphorylation of ER-bound UGTs, we attempted to purify a catalytically active UGT protein for structural analysis. UGT1A7- and UGT2B7-cDNAs, adapted with thrombin/his/myc affinity ligands, not only establish a highly effective UGT-solubilizing system that retains activity but also permit us to isolate UGT-containing complexes involved in PKC and/or TK signaling pathways similar to those described for other cellular processes. The complex contained one 58 kDa UGT1A7His and one 110 kDa β-COP to two 29 kDa 14-3-3 phosphoserine chaperone proteins. For PKC-dependent signaling, we found that UGT1A7 associated with RACKε in a 225 kDa adapter complex that includes the phosphoserine-dependent 14-3-3 protein. Mutation of UGT1A7His at its 14-3-3 binding sites led to marked lability of solubilized 1A7His. Like all UGTs, UGT1A7 has two 14-3-3 binding sites: S162 and T403. Mutations at these sites indicate that 14-3-3 also stabilizes UGT stored at 4°C. These important findings should enable us to isolate a UGT and carry out analysis of its tertiary structure as well as identify events and components surrounding phosphorylation-required signaling. It is notable that 14-3-3 binding sites exist in all UGTs. We confirmed binding of UGTs to phosphoserine-14-3-3 by co-immunoprecipitation and/or co-localization with UGT1A1 His, UGT1A6His, UGT1A7His, UGT1A10, or UGT2B7His. In summary, our studies indicate that UGT1A7 exists as a cellular complex(es) that sustains activity via protein kinase(s) signaling. Our findings lay the foundation for further studies concerning this critical endogenous chemical defense enzyme system.

Structural analysis and identification of the common donor-substrate binding site in UGT1A10

Banerjee, Pennington[6]

Given that UGT isozymes generate inactive chemical glucuronides by linking glucuronic acid donated by UDP-glucuronic acid to lipophilic acceptor substrates, thereby enhancing their excretion, it is important to determine the binding properties and sites for the donor substrate. It would be difficult to purify ER-bound UGTs for structural studies, so we carried out homology-based computer modeling to aid analysis. Consistent with predicted similarities involving the common UDP moiety in substrates UDP-glucose and UDP-glucuronic acid, the model identified structural homology in *Escherichia coli* UDP-galactose 4-epimerase and UGT1A10; the model predicted binding sites at N292, K314, K315, and K404 in 1A10. Two informative sets of UGT1A10 mutants, K314R/Q/A/E /G and K404R/E, had null activities and 2.7-fold higher activity, respectively. Scatchard analysis of the binding of the affinity ligand 5-azido-uridine-[β-^{32}P]-diphosphoglucuronic acid to UGT1A10-His or UGT1A7-His revealed high- and low-affinity binding sites. Discontinuous SDS-PAGE of 2-nitro-5-thiocyanobenzoic acid–digested UGT1A10-His bound to radiolabeled affinity ligand revealed 11.3 and 14.3 kDa peptides associated with K314 and K404, respectively, with both peptides undetectable in UGT1A10-K314 mutants. Increased basicity of residue 404 led to increased 14.3 kDa peptide content and high-affinity binding sites and Kd, accounting for increased UGT activity. Notably, K314 and K404 are strictly conserved in 70 aligned UGTs, except for S321—which is equivalent to K314—in UGT2B15 and UGT2B17 and for I321 in the inactive UGT8, suggesting that UGT2B15 and UGT2B17 are suboptimally active. Hence, our data strongly support UDPglcA binding to K314 and K404 in UGT1A10.

Banerjee R, Pennington MW, Garza A, Owens IS. Mapping the UDP glucuronic acid binding site in UDP-glucuronosyltransferase-1A10 by homology-based modeling: confirmation with biochemical evidence. *Biochemistry* 2008;47:7385-7392.

Marked improvement in drug efficacy by targeted inhibition of glucuronidation in mice
Basu N, Kole,[5] Basu M; in collaboration with McDonagh

The finding that UGTs require protein kinase C–mediated phosphorylation is important information that allows manipulation of this critical glucuronidating system. Given that UGTs may be inactivated by downregulating PKCs with reversibly acting dietary curcumin, we determined the impact of gastrointestinal (GI) glucuronidation on free drug uptake and efficacy by using the immunosuppressant mycophenolic acid (MPA) in mice. Expressed in COS-1 cells, mouse GI-distributed Ugt1a1 glucuronidates curcumin and MPA and undergoes irreversible and reversible dephosphorylation by the PKC-specific inhibitor calphostin-C and the general kinase inhibitor curcumin, respectively, with parallel effects on activity. Moreover, oral administration of curcumin to mice reversibly inhibits glucuronidation in GI tissues. Finally, successive oral administration of curcumin and MPA to antigen-treated mice raises free MPA in serum and causes 9-fold immunosuppression. Using MPA as a model, our results indicate that targeted inhibition of GI glucuronidation in mice markedly improved free chemical uptake and efficacy.

Basu NK, Kole L, Basu M, McDonagh AF, Owens IS. Targeted inhibition of glucuronidation markedly improves drug efficacy in mice—a model. *Biochem Biophy Res Commun* 2007;360:7-13.

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CHOLESTEROL DEFICIENCY AND GENETIC SYNDROMES

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We study the molecular, biochemical, and cellular processes that underlie dysmorphic syndromes and birth defects attributable to inborn errors of cholesterol synthesis. Human malformation/mental retardation syndromes caused by inborn errors of cholesterol synthesis include Smith-Lemli-Opitz syndrome (SLOS), lathosterolosis, desmosterolosis, X-linked dominant chondrodysplasia punctata type 2 (CDPX2), and CHILD syndrome. We conduct both basic and clinical research. Our basic research uses mouse models of these disorders to understand the biochemical, molecular, cellular, and developmental processes that underlie the birth defects and clinical problems found in the various disorders. Our clinical research focuses on characterization and treatment of patients with SLOS. Our emphasis on both basic and clinical research allows us to integrate laboratory and clinical data in order to increase our understanding of the pathological mechanisms underlying SLOS and to improve the clinical care of SLOS patients.

Smith-Lemli-Opitz syndrome

Goodwin, Sparks, Tierney, Wassif, Porter; in collaboration with Bailey-Wilson, Javitt, Platt, Shackleton

SLOS is an autosomal recessive, multiple-malformation syndrome characterized by dysmorphic facial features, mental retardation, hypotonia, poor growth, and variable structural anomalies of the heart, lungs, brain, limbs, gastrointestinal tract, and genitalia. The SLOS phenotype is extremely variable. At the severe end of the phenotypic spectrum, infants often die as result of multiple major malformations. At the mild end of the phenotypic spectrum, SLOS combines minor physical malformations with behavioral and learning problems. The syndrome is attributable to an inborn error of cholesterol biosynthesis that blocks the conversion of 7-dehydrocholesterol (7-DHC) to cholesterol. Our laboratory initially cloned the human 3beta-hydroxysterol delta 7-reductase gene (*DHCR7*) and demonstrated mutations of the gene in SLOS patients (Correa-Cerro et al., *J Med Genet* 2005;42:350). To date, together with others we have identified over 100 mutations of *DHCR7*. To further our understanding of the mechanisms underlying the broad phenotypic spectrum in SLOS, we used deuterium oxide labeling to measure residual DHCR7 activity in fibroblasts from patients with known genotypes and well-characterized phenotypes. In collaboration with other laboratories, we have been identifying and characterizing the biological activity of aberrant oxysterols, steroids, and neuroactive steroids.

In addition to our basic science work focusing on understanding the pathophysiological processes underling SLOS, we are actively recruiting and following SLOS patients in three clinical protocols.

Given that SLOS patients have a cholesterol deficiency, they may be treated with dietary cholesterol supplementation. To date, we have evaluated over 60 SLOS patients and continue to follow many of them in a longitudinal natural history study. Parents frequently report improved behavior in SLOS children on dietary cholesterol supplementation. However, we had yet to conduct a blinded protocol studying the efficacy of dietary cholesterol therapy to ameliorate behavioral problems associated with SLOS. Thus, we designed and implemented a protocol to study the short-term efficacy of dietary cholesterol therapy to improve behavioral problems in 40 SLOS children. Basic laboratory experiments using patient cells and our SLOS mouse model suggested that simvastatin may be efficacious in improving the sterol abnormality in SLOS (Wassif et al., *Mol Genet Metab* 2005;85:96; Correo-Cerro et al. 2006). Accordingly, we initiated a controlled, blinded cross-over protocol to study the safety and efficacy of simvastatin therapy in SLOS. Previously, we showed that SLOS fibroblasts have a secondary defect in intracellular cholesterol transport. As part of a Bench-to-Bedside proposal, we continue, in collaboration with Fran Platt's laboratory, to study impaired cholesterol and glycosphingolipid transport in SLOS and to investigate novel therapeutic interventions.

One of the most interesting facets of SLOS is its distinct behavioral phenotype. Most patients with SLOS exhibit autistic characteristics. We are currently working in collaboration with groups from both NHGRI and the Kennedy Krieger Institute in Baltimore to analyze this association further. We have also received funding from Autism Speaks to initiate a multicenter double-masked placebo-controlled trial of cholesterol in hypocholesterolemic autism spectrum disorder.

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Mouse models of SLOS

Cupp, Jiang, Song, Lindegaard, Merkel, Wassif, Wasmuth, Porter; in collaboration with Fliesler, Javitt, Remaley, Shackleton, Watson, Yergey

Using gene targeting in murine embryonic stem cells, we produced three SLOS mouse models: a null mutation, a hypomorphic point mutation, and a conditional mutation. Mouse pups homozygous for the null mutation (*Dhcr7*$^{-/-}$) exhibit variable craniofacial anomalies, are growth-retarded, feed poorly, and appear weak. *Dhcr7*$^{-/-}$ pups die during the first day of life because they fail to feed. Biochemical characterization showed that the mutant pups had markedly elevated serum and tissue 7-DHC levels as well as reduced serum and tissue cholesterol levels. Cleft palate was present in 9 percent of the *Dhcr7*$^{-/-}$ pups and is found in approximately one-third of all SLOS patients. Further investigation of the neurological abnormalities in these mice showed that cortical neurons had an impaired response to glutamate. A decreased glutamate response is consistent with the phenotypic observation of poor feeding by the mutant animals. Neurological dysfunctions, including poor feeding, hypotonia, mental retardation, and behavioral dysfunction, are major clinical problems in SLOS. The impaired glutamate response observed in our mouse model may yield insight into the etiology of some of the neurological dysfunction in SLOS.

Given that the *Dhcr7*$^{-/-}$ pups die during the first day of life, we are not able to study postnatal brain development, myelination, and behavior or to test therapeutic interventions. For this reason, we developed a mis-sense allele (*Dhcr7*^{T93M}) and a conditional *Dhcr7* mutant allele (*Dhcr7*$^{loxP\Delta3\text{-}5loxP}$).

The T93M mutation is the second most common mutation found in human patients. *Dhcr7*$^{T93M/T93M}$ and *Dhcr7*$^{T93M/\Delta3\text{-}5}$ mice are viable and demonstrate SLOS with a gradient of biochemical severity (*Dhcr7*$^{\Delta3\text{-}5/\Delta3\text{-}5}$ > *Dhcr7*$^{T93M/\Delta3\text{-}5}$ > *Dhcr7*$^{T93M/T93M}$). We used *Dhcr7*$^{T93M/\Delta3\text{-}5}$ mice to test the efficacy of therapeutic interventions on tissue sterol profiles. As expected, dietary cholesterol therapy improved the sterol composition in peripheral tissues but not in the central nervous system. Treatment of mice with simvastatin improved the biochemical defect in both peripheral and central nervous system tissue, suggesting that simvastatin therapy may be used to treat some of the behavioral and learning problems in children with SLOS. In collaboration with Cedric Shackleton and Gordon Watson, we are using our hypomorphic mouse model to investigate adeno-associated viral gene therapy for SLOS.

As part of our clinical studies on SLOS, we previously identified a novel oxysterol, 27-hydroxy-7-dehydrocholesterol (27-7DHC), derived from 7-DHC in SLOS patients. We thus investigated whether 27-7DHC contributes to the pathology of SLOS and found a strong negative correlation between plasma 27-7DHC and cholesterol levels in SLOS patients. In addition, previous work showed that low cholesterol levels impair hedgehog signaling. Therefore, we hypothesized that increased 27-7DHC levels would have detrimental effects during development in response to suppression of cholesterol levels. To test our hypothesis, we produced SLOS mice (*Dhcr7*$^{-/-}$) expressing a *CYP27* transgene. *CYP27Tg* mice have elevated *CYP27* expression and 27-hydroxycholesterol levels but normal cholesterol levels. *Dhcr7*$^{-/-}$ mice are growth-retarded, exhibit a low incidence of cleft palate (9 percent), and die during the first day of life. *Dhcr7*$^{-/-}$*:CYP27Tg* embryos are stillborn and have multiple malformations, including growth retardation, micrognathia, cleft palate (77 percent), lingual and dental hypoplasia, ankyloglossia, umbilical hernia, cardiac defects, cloacae, curled tails, and limb defects. We observed autopod defects (polydactyly, syndactyly, and oligodactyly) in 77 percent of the mice. Consistent with our hypothesis, sterol levels were halved in the liver and were 20-fold lower in brain tissue of *Dhcr7*$^{-/-}$*:CYP27Tg* than of *Dhcr7*$^{-/-}$ embryos. Recognition of the role of 27-7DHC in SLOS may explain some of the phenotypic variability and may lead to the development of a therapeutic intervention.

Recognizing its potential impact on prenatal therapy, we have been characterizing maternal-fetal cholesterol transport in *Abca1*, *Abcg1*, and *Sr-b1* mutant mice. We found that disruption of *Abca1* decreased maternal-fetal cholesterol transport and conversely the elevated expression of *Abca1* caused an increased in maternal-fetal cholesterol transport. In our SLOS mouse model, we were able to show, as a proof of principle, that increased expression of Abca1 increased cholesterol content in SLOS mutant embryos.

We continue to use the SLOS mouse models to understand the pathophysiological processes that underlie the birth defects and clinical problems in SLOS and are using various biochemical, molecular, and proteomic approaches to investigate these issues. We are also using the mouse models to test various prenatal and postnatal therapeutic interventions.

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Lathosterolosis, desmosterolosis, and HEM dysplasia

Jiang, Wassif, Porter

Lathosterol 5-desaturase catalyzes the conversion of lathosterol to 7-dehydrocholesterol, representing the enzymatic step immediately preceding the defect in SLOS. Thus, to understand further the roles of decreased cholesterol versus increased 7-dehydrocholesterol in SLOS, we disrupted the mouse lathosterol 5-desaturase gene (*Sc5d*) by using targeted homologous recombination in embryonic stem cells. The *Sc5d*$^{-/-}$ pups are stillborn, present with micrognathia and cleft palate, and exhibit limb-patterning defects. Many of the malformations in these mutant mice resemble malformations in SLOS and are consistent with impaired hedgehog signaling during development. Biochemically, the mice have markedly elevated serum and tissue lathosterol levels and reduced cholesterol levels.

One goal of producing a lathosterolosis mouse model was to gain phenotypic insight for the purpose of identifying a corresponding human malformation syndrome. We have now identified a human infant patient with lathosterolosis, a human malformation syndrome that has not yet been described. Biochemically, fibroblasts from the patient show decreased cholesterol and increased lathosterol levels. Mutation analysis showed that the patient is homozygous for a single A→C nucleotide change at position 137 in *SC5D*, resulting in a mutant enzyme in which the amino acid serine is substituted for tyrosine at position 46. Both parents were heterozygous for the mutation. The infant's phenotype resembled severe SLOS. Malformations found in both the human patient and mouse model include growth failure, abnormal nasal structure, abnormal palate, micrognathia, and postaxial polydactyly. A unique feature of lathosterolosis is the clinical finding of mucolipidosis in the affected infant. This clinical presentation is not reported in SLOS and may help clinically distinguish SLOS from lathosterolosis. This lysosomal storage disorder can be replicated in embryonic fibroblasts from the *Sc5d* mutant mouse model.

Desmosterolosis is another inborn error of cholesterol synthesis that resembles SLOS. It results from a mutation of the 3beta-hydroxysterol delta 24-reductase gene (*DHCR24*). DHCR24 catalyzes the reduction of desmosterol to cholesterol. We disrupted the mouse *Dhcr24* gene by using targeted homologous recombination in embryonic stem cells. Surprisingly, although most *Dhcr24* mutant mice die at birth, the pups are phenotypically normal.

Others have shown that mutations of the lamin B receptor (LBR) cause HEM dysplasia in humans and ichthyosis in mice. LBR has both lamin B–binding and sterol Δ14-reductase domains. Although only a minor sterol abnormality has been reported, it has been proposed that LBR is the primary sterolΔ14-reductase and that impaired sterol Δ14-reduction underlies HEM dysplasia. However, *DHCR14* also encodes a sterol Δ14-reductase. To test the hypothesis that LBR and DHCR14 are redundant sterol Δ14-reductases, we obtained ichthyosis mice (*Lbr*$^{-/-}$) and disrupted *Dhcr14*. *Dhcr14*$^{-/-}$ mice are phenotypically normal. We found no sterol abnormalities in either *Lbr*$^{-/-}$ or *Dhcr14*$^{-/-}$ tissues at 1 and 21 days of age. We then bred the mice to obtain compound mutant mice. *Lbr*$^{-/-}$*:Dhcr14*$^{-/-}$ and *Lbr*$^{-/-}$*:Dhcr14*$^{+/-}$ died in utero. *Lbr*$^{+/-}$*:Dhcr14*$^{-/-}$ mice appeared normal at birth but, by 10 days of age, were growth-retarded and neurologically abnormal (ataxia and tremors) and, consistent with a demyelinating process, evidenced vacuolation and swelling of the myelin sheaths in the spinal cord upon pathological evaluation. We observed neither vacuolation nor swelling of the myelin sheaths in either *Lbr*$^{-/-}$ or *Dhcr14*$^{-/-}$ mice. In contrast to *Lbr*$^{-/-}$ mice, *Lbr*$^{+/-}$*:Dhcr14*$^{-/-}$ mice had normal skin and did not display the Pelger-Huët anomaly. Peripheral tissue sterols were normal in all three mutant mice, although we found significantly elevated levels (50 percent of total sterols) of cholesta-8,14-dien-3β-ol and cholesta-8,14,24-trien-3β-ol in brain tissue from 10-day-old *Lbr*$^{+/-}$*:Dhcr14*$^{-/-}$ mice. In contrast, we observed relatively small transient elevations of Δ^{14}-sterols in

Lbr$^{-/-}$ and *Dhcr14*$^{\Delta 4\text{-}7/\Delta 4\text{-}7}$ brain tissue. Our data support the idea that HEM dysplasia and ichthyosis result from impaired lamin B receptor function rather than from impaired sterol Δ14-reduction. Impaired sterol Δ14-reduction gives rise to a novel murine phenotype for which a corresponding human disorder has yet to be identified.

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Niemann-Pick type C

Jiang, Wassif, Yanjanin, Porter; in collaboration with Ioannou, Patterson, Pavan, Platt, Yergey

Niemann-Pick type C (NPC) is a neurodegenerative disorder caused by a defect in intracellular lipid and cholesterol transport. As part of a Bench-to-Bedside initiative, we initiated a clinical protocol to identify and characterize biomarkers that could be used in a subsequent therapeutic trial. The project continues to receive support from the Ara Parsegian Medical Research Foundation and Dana's Angels Research Trust. To date, we have enrolled 26 NPC patients. To complement the clinical work, we have begun to apply proteomic approaches to both mouse and human biomaterials to identify biological pathways disrupted in NPC.

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MOLECULAR GENETICS OF ADRENOCORTICAL TUMORS AND RELATED DISORDERS

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We aim to understand the genetic and molecular mechanisms leading to disorders that affect the adrenal cortex, with emphasis on those disorders that are developmental, hereditary, and associated with adrenal hypoplasia or hyperplasia, multiple tumors, and abnormalities in other endocrine glands. We study congenital adrenal hypoplasia caused by triple A syndrome and several endocrine deficiencies; familial hyperaldosteronism; adrenocortical and thyroid cancer; pituitary tumors; multiple endocrine neoplasia (MEN) syndromes affecting the pituitary, thyroid, and adrenal glands; and Carney complex (CNC), an autosomal dominant disease. We focus on cyclic AMP (cAMP)/protein kinase A (PKA) stimulated signaling pathways and PKA effects on tumor suppression, development, and the cell cycle. The *prkar1a* and *pde11a* gene mouse models, in which we have knocked out the respective genes, facilitate our research. Genome-wide searches for other genes responsible for CNC and related diseases of the adrenal and pituitary glands are ongoing. We described a new disease (Carney-Stratakis syndrome, or CSS) and observed adrenocortical tumors in association with tumors of the peripheral nervous system and gastrointestinal system (Carney Triad, or CT). The laboratory identified mutations in the succinate dehydrogenase subunits B, C, and D in CSS and, in collaboration with other investigators and the NCI, is currently looking for gene(s) responsible for CT and related tumors.

Carney complex genetics

Horvath, Patronas, Tsang, Boikos, Giatzakis,[6] Levine, Stratakis; in collaboration with Carney, Kirschner, Bertherat

We have collected families with CNC and related syndromes from several collaborating institutions worldwide. Through genetic linkage analysis, we identified loci harboring genes for CNC on chromosomes 2 (2p16) and 17 (17q22–24) and are currently searching for other possible loci for this genetically heterogeneous condition. With the application of state-of-the-art molecular cytogenetic techniques, we are investigating the participation of these currently identified genomic loci in the

expression of the disease and have constructed a comprehensive genetic and physical map of the 2p16 chromosomal region for cloning the CNC-associated sequences from this region. Studies in cultured primary tumor cell lines (established from our patients) identified a region of genomic amplification in CNC tumors in the center of the map. The *PRKAR1A* gene on 17q22–24, which is the gene responsible for CNC in most cases of the disease, appears to undergo loss of heterozygosity in at least some CNC tumors. *PRKAR1A* is also the main regulatory subunit (subunit type 1-α) of PKA, a central signaling pathway for many cellular functions and hormonal responses. We have increased the number of CNC patients in genotype-phenotype correlation studies, which are expected to provide insight into the complex biochemical and molecular pathways regulated by *PRKAR1A* and PKA. We expect to identify new genes by ongoing genome-wide searches for patients and families who do not carry *PRKAR1A* mutations.

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PRKAR1A, protein kinase A activity, and endocrine and other tumor development

Levine, Horvath, Boikos, Muchow, Robinson-White, Nesterova, Stratakis; in collaboration with Bertherat, Grimberg

We are investigating the functional and genetic consequences of *PRKAR1A* mutations in cell lines established from CNC patients and their tumors. We measure both cAMP and PKA activity in these cell lines, along with the expression of the other subunits of the PKA tetramer. In addition, we are seeking mutations of the *PRKAR1A* gene in sporadic endocrine and non-endocrine tumors (thyroid adenomas and carcinomas, adrenocortical adenomas and carcinomas, ovarian carcinomas, melanomas and other benign and malignant pigmented lesions, and myxomas in the heart and other sites)—mutations that would further establish the gene's role as a general tumor suppressor. Many investigators within the NIH and around the world provide specimens on a collaborative basis.

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Prkar1a$^{+/-}$ and antisense (AS) *Prkar1a* transgenic animal models

Tsang, Muchow, Bauer, Boikos, Nesterova, Stratakis; in collaboration with Griffin, Westphal, Kirschner, Batista

In collaboration with Heiner Westphal, Lawrence Kirschner, while in our laboratory, developed a *Prkar1a* knockout mouse floxed by a lox-P system for the purpose of generating, first, a novel *Prkar1a*$^{+/-}$ and, second, knockouts of the *Prkar1a* gene in a tissue-specific manner after crossing the new mouse model with mice expressing the *cre* protein in the adrenal cortex, anterior lobe of the pituitary, and thyroid gland (Kirschner et al., *Cancer Res* 2005;65:4506). The heterozygote mouse develops several tumors reminiscent of the equivalent human disease. Ongoing crosses with mice such as the transgenic GHRH–expressing mouse attempt to identify tissue-specific effects (in the case of the GHRH-expressing mouse, the pituitary) or specific signaling events (such as involvement of the p53 and Rb proteins in *Prkara1a*-related tumorigenesis). We also created a transgenic mouse carrying an antisense transgene for exon 2 of the mouse *Prkar1a* gene (X2AS) under the control of a regulable promoter. As in human CNC tumors, tissues from mice with the X2AS transgene showed elevated cAMP-stimulated kinase activity. The mice had several CNC-compatible histologic and clinical changes, including obesity attributed to subclinical Cushing syndrome.

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PRKAR1A, the cell cycle, and other signaling pathways

Robinson-White, Hsiao, Patronas, Levine, Nesterova, Stratakis; in collaboration with Lippincott-Schwartz

We work to identify *PRKAR1A*-interacting mitogenic and other growth-signaling pathways in cell lines expressing *PRKAR1A* constructs and/or mutations. Several genes that regulate PKA function and increase cAMP-dependent proliferation and related signals may be altered in the process of endocrine tumorigenesis initiated by a mutant *PRKAR1A*, a gene with important functions in the cell cycle and in chromosomal stability. Recently, we found an interaction with the mTOR pathway in both human and mouse cells with altered PKA function.

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Phosphodiesterase (PDE) genes in endocrine and other tumors

Horvath, Tsang, Patronas, Giatzakis,[6] Boikos, Levine, Stratakis; in collaboration with Bertherat

In patients who did not exhibit CNC or *PRKAR1A* mutations but presented with bilateral adrenal tumors similar to those in CNC, we found inactivating mutations of the *PDE11A* gene, which encodes phosphodiesterase-11A and regulates PKA in the normal physiologic state. Phosphodiesterase 11A is a member of a 22 gene–encoded family of proteins that break down cyclic nucleotides that control PKA. PDE11A appears to act as a tumor suppressor such that tumors develop when its action is abolished. In fact, in what proved to be the first time that mutated PDE was observed

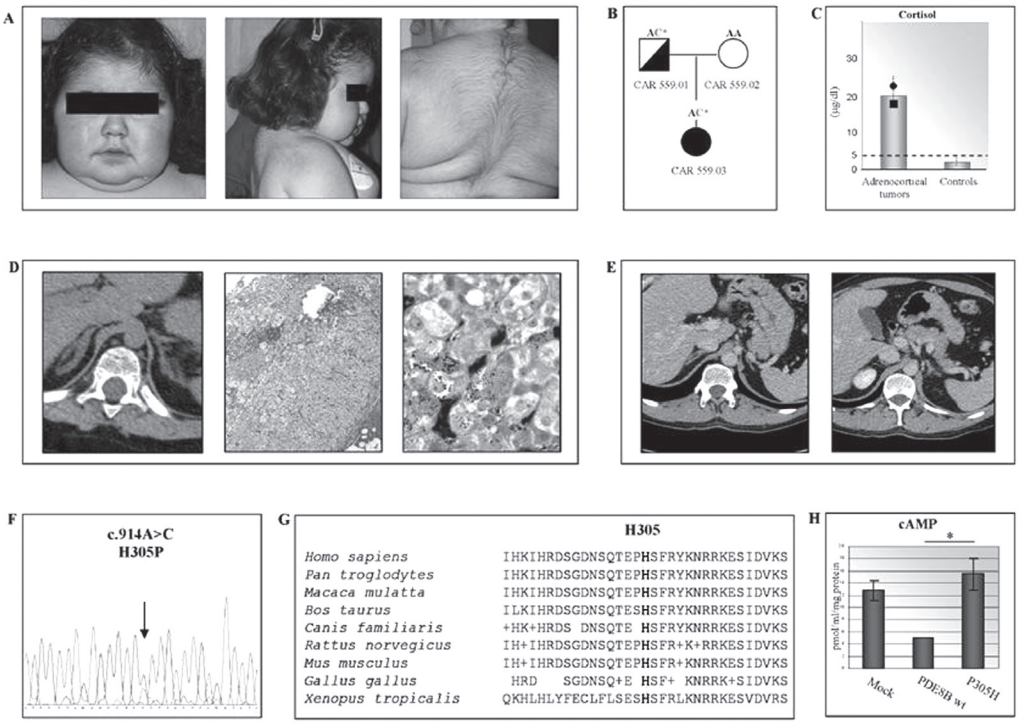

FIGURE 4.10 A GENETIC DEFECT IN PDE8B

A. Female patient CAR 559.03 with Cushing syndrome: (1) characteristic facial plethora and roundness ("moon" facies); (2) body fat in the upper back, known as "buffalo" hump, and supraclavicular fat pads and lipomastia; and (3) hirsutism. B. Family CAR 559: the mutant allele (C) of the proband (CAR559.03) was inherited from mildly affected father (CAR559.01) while unaffected mother (CAR550.02) had normal PDE8B alleles. C. Midnight cortisol levels (in mg/dl) in patients with adrenocortical tumors (N = 23) and control individuals (N = 7) recently studied at the NIH and levels of the proband (CAR 559.03) and patient's father (CAR 559.01) as indicated by a full circle and a full square, respectively. D. Imaging and pathological findings in the proband: (left panel) adrenal computed tomography of CAR 559.03, with mild enlargement of both the left and right adrenal glands; (center panel) hematoxylin and eosin staining (5× magnification) of the adrenal gland of the proband, with disturbed adrenocortical zonation pattern and both nodular tissue and hyperplasia of the surrounding cortex visible; and (right panel) pigment (lipofuscin) in adrenocortical nodules (visible at 40x magnification. E. Computed tomography of the adrenal glands of patient's father (CAR 559.01): (left panel) right and (right panel) left adrenal gland. F. Electrophoregram showing the A→T substitution in patient CAR 559.03 with the arrow pointing to the position of the mutation. G. Conservation of histidine (H) at position 305 of PDE8B across species. H. In vitro expression of constructs employing the normal (wild-type; wt) and mutant (H305P) coding sequence of the PDE8B gene in HEK293 cells, leading to significant decrease of cyclic AMP levels or no change, respectively, compared with the "mock" transfection; thus, the H305P mutation abolishes the ability of PDE8B to degrade cyclic AMP.

in a genetic disorder predisposing to tumors, we found pediatric and adult patients with bilateral adrenal tumors. Recent data indicate that *PDE11A* sequence polymorphisms may be present in the general population. The finding that genetic alterations of such a major biochemical pathway may be associated with tumors in humans raises the reasonable hope that drugs that modify PKA and/or PDE activity may eventually undergo development for use in both CNC and patients with other, non-genetic, adrenal tumors—and perhaps other endocrine tumors. Most recently, we identified a patient with a *PDE8B* mutation and Cushing syndrome (Figure 4.10), with the PDE8B transcript and protein seemingly expressed widely in the endocrine system (Figure 4.11).

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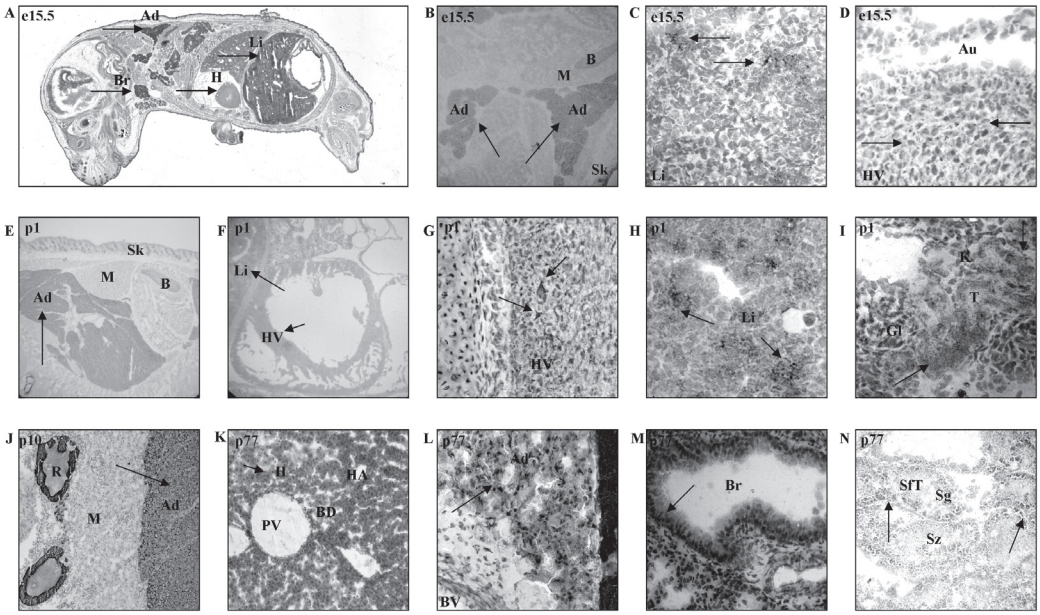

FIGURE 4.11 MOUSE EXPRESSION OF PDE8B

(A) PDE8B immunostaining (arrows) in the mouse embryo on day 15.5 seen in a whole-body section (Br = brain; H = heart; Li = liver; Ad = adipose tissue, with upper arrow indicating the hibernating gland); (B) PDE8B immunostaining (arrows) in the adipose tissue, with arrows indicating the hibernating gland (B = bone; M = skeletal muscle; Sk = skin); (C) Immunostaining in embryonic liver showing labeling forming the islets in between the liver cells (arrows); (D) PDE8B in the heart ventricle (HV) forming small spots between muscle fibers (arrows) while the atria (Au) do not show any labeling; (E) PDE8B immunostaining in the adipose tissue of the hibernating gland of a newborn mouse, with arrow indicating the hibernating gland; (F) PDE8B immunostaining (arrows) in the heart ventricle and liver of a newborn mouse; (G) The wall of the heart ventricle, with PDE8B labeling (arrow) forming a network around the muscle fibers; (H) Liver, with PDE8B labeling (arrow) as a heterogeneous distribution throughout the liver parenchyma; (I) Kidney tubules, most likely distal tubules, but not glomeruli, showing PDE8B labeling (arrow) (Gl = glomerulus; K = kidney; T = tubules); (J) Intense PDE8B immunostaining (arrow) in adipose tissue (R = rib); (K) PDE8B immunostaining (arrow) in the liver parenchyma with blood vessels, including branches of the portal vein and hepatic artery, and bile duct remaining unlabeled (BD = bile duct; H = hepatocyte; HA = hepatic artery; PV = portal vein branch); (L) PDE8B immunostaining (arrow) in the adipose tissue surrounding the thymus, with thymus and blood vessels remaining unstained (BV = blood vessel; Th = thymus); (M) Light PDE8B immunostaining (arrow) in the epithelial cells of the bronchioles (Br = bronchiole); (N) PDE8B immunostaining (arrow) in the seminiferous tubules of the adult mouse testis, showing spermatogonia to be slightly labeled and spermatozoa seeming to be unstained (SfT = seminiferous tubule; Sg = spermatogonia; Sz = spermatozoa).

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Genetic investigations into other adrenocortical diseases and related tumors

Boikos, Hsiao, Horvath, Muchow, Nesterova, Stratakis; in collaboration with Libutti, Chan, Voutetakis, Carney, Hammer, Stowasser, Lacroix, Bertherat

Through collaborations, we (1) apply general and pathway-specific microarrays to a variety of adrenocortical tumors, including single adenomas and massive macronodular adrenocortical disease (MMAD), to identify genes with important functions in adrenal oncogenetics; (2) examine candidate genes for their roles in adrenocortical tumors and development; and (3) identify additional genes that play a role in inherited adrenocortical and related diseases, such as Allgrove syndrome.

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Genetic investigations into pituitary tumors, other endocrine neoplasias, and related syndromes

Horvath, Boikos, Stratakis; in collaboration with Marx, Carney

In collaboration with several other investigators at the NIH and elsewhere, we are investigating the genetics of CNC- and adrenal-related endocrine tumors, including childhood pituitary tumors, related or unrelated to *PRKAR1A* mutations. As part of this work, we have identified novel genetic abnormalities in other endocrine glands.

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Genetic investigations into other endocrine neoplasias and related syndromes; hereditary paragangliomas and related conditions

Muchow, Boikos, Stratakis; in collaboration with Carney

As part of a collaboration with other investigators at the NIH and elsewhere (including an international consortium organized by our laboratory), we are studying the genetics of a rare syndrome that predisposes to adrenal and other tumors, the Carney Triad, and related conditions (associated with gastrointestinal stromal tumors, or GIST). In the course of our work, we identified a patient with a

new syndrome, known as the paraganglioma and gastrointestinal stromal tumor syndrome (or Carney-Stratakis syndrome), for which we found mutations in the genes encoding succinate dehydrogenase (SDH) subunits B, C, and D. In another patient, we found a novel germline mutation of the *PDFGRA* gene (Figure 4.12).

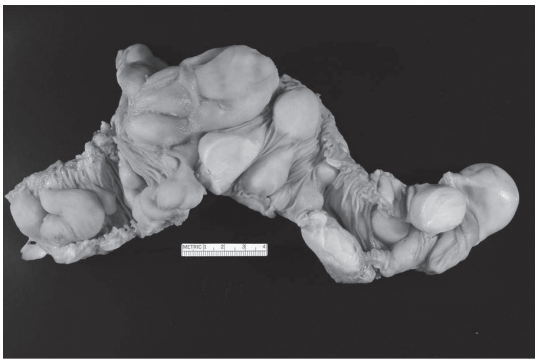

FIGURE 4.12

Several small intestinal tumors of a patient studied in the context of cases of multiple endocrine tumors: formalin-fixed specimens of small intestine showed several polypoid tumors covered by intact mucosa; the tumors were generally hemispherical and protruded into the bowel lumen while some were confluent. The cut surface was white and homogeneous. The patient had a germline mutation in the *PDFGRA* gene; the mutation was V561D (GTC→GAC) in exon 12. Both the normal gastric mucosa and the tumor cells exhibited the mutation (*J Clin Endocrinol Metab* 2007;92:3728).

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Clinical investigations into the diagnosis and treatment of adrenal and pituitary tumors

Verma, Hsiao, Stratakis; in collaboration with Keil, Patronas, Batista

Patients with adrenal tumors and other types of Cushing syndrome (and occasionally other pituitary tumors) come to the NIH Clinical Center for diagnosis and treatment. Ongoing investigations focus on (1) the prevalence of ectopic hormone receptor expression in adrenal adenomas and massive macronodular adrenocortical disease; (2) the diagnostic use of high-sensitivity magnetic resonance imaging for the earlier detection of pituitary tumors; and (3) the diagnosis, management, and post-operative care of children with Cushing syndrome and other pituitary tumors.

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Clinical and molecular investigations into other pediatric genetic syndromes

Kotz, Lodish, Verma, Horvath, Hsiao, Boikos, Stratakis; in collaboration with Keil, Raygada, Rennert

Largely in collaboration with a number of other investigators at the NIH and elsewhere, we are conducting work on pediatric genetic syndromes seen in our clinics and wards.

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[4]*Special Volunteer from Israel*
[5]*Special Volunteer from Taiwan*
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THE BIOTRANSFORMATION OF ENDOBIOTICS BY SULFONATION

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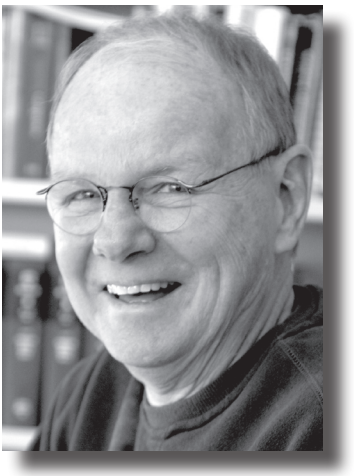

We investigate the molecular mechanisms and biologic implications of modifying substances by sulfonation, a fundamental process in the biotransformation of endobiotics and of drugs and xenobiotics. Sulfonation, the transfer of an SO3-1 group from the universal sulfonate donor molecule 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to an acceptor compound, is essential for normal growth and development as well as for maintenance of the internal milieu. We investigate enzymes that transfer the sulfonate moiety, i.e., sulfotransferases as well as the bifunctional PAPS synthetases producing the sulfonate donor. There are two classes of sulfotransferases: one class is tightly associated with membranes, especially the Golgi complex, while the other is composed of a superfamily of soluble or cytosolic enzymes (designated SULT). The former class sulfonates macromolecules such as glycosaminoglycans and proteoglycans, whereas the SULT class sulfonates low molecular weight compounds, such as hormones and neurotransmitters. We study the SULT2 family that sulfonates steroids and sterols. By modulating the availability of biologically active substances, sulfonation can influence biologic activity regardless of whether compounds act (1) in their unconjugated or sulfoconjugated form or (2) via a genomic or nongenomic mechanism. Thus, sulfotransferases play an essential role in specific physiologic systems and their associated disorders.

Cholesterol sulfate regulation of profilaggrin mRNA expression: studies involving siRNA for SULT2B1b and RORalpha

Hanyu, Nakae, Fuda, Strott; in collaboration with Higashi

Cholesterol sulfate, which is produced by the action of the sterol/steroid sulfotransferase SULT2B1b, is a ubiquitous sulfolipid that has emerged as a multifaceted molecule with regulatory capabilities. Cholesterol sulfate is present in the epidermis, where it accumulates in the stratum granulosum; in addition, epidermal keratinocytes amass cholesterol sulfate during squamous differentiation. It is notable that expression of SULT2B1b localizes to the granular layer of the epidermis and thus coincides with the epidermal area containing the highest content of cholesterol sulfate. Furthermore, both SULT2B1b and the barrier protein filaggrin are localized in the epidermis; filaggrin is an acknowledged late marker of keratinocyte terminal differentiation and, in contrast to involucrin, an early marker of differentiation, which is expressed throughout the suprabasal region.

Interestingly, others have reported that, based on crystallographic data, cholesterol sulfate binds with high affinity to the retinoic acid receptor–related orphan receptor alpha (RORalpha); moreover, the high-affinity binding of cholesterol sulfate was found to translate into increased transcriptional

activity of RORalpha. RORalpha mRNA is widely expressed in the mouse, although RORalpha protein is expressed in the periphery at detectable levels only in skin and testis. While RORalpha's expression in mouse skin provides the basis for assuming similar expression in human skin, researchers had not yet conclusively demonstrated expression in human skin. The observation that, in the human epidermis, the granular layer contains the highest concentration of cholesterol sulfate, coupled with the observation that SULT2B1b and filaggrin co-localize in the granular layer, suggests an important functional association. The association is strongly supported by the finding that mouse keratinocytes in primary culture respond to administration of cholesterol sulfate with a dose-dependent increase in filaggrin formation. The possibility of association is even more intriguing when the above findings are considered in terms of the discovery that cholesterol sulfate binds to the nuclear receptor RORalpha with high affinity and that the latter is similarly expressed in the epidermis. Therefore, we raised several questions with respect to the physiological significance of SULT2B1b expression in the epidermal granular layer vis-à-vis filaggrin expression: whether cholesterol sulfate has a direct influence on the expression of filaggrin; the location of RORalpha expression in the skin of humans (if indeed RORalpha is expressed in human skin), particularly in relation to the expression of SULT2B1b and filaggrin; which of RORalpha's four subtypes (a_1–a_4) are expressed in human skin; and whether RORalpha participates in the molecular action of cholesterol sulfate in the human epidermis.

We have now demonstrated that RORalpha is in fact expressed in human skin and localizes to the outer reaches of the epidermis. When we stained sections of normal human skin with an antibody against RORalpha, we observed intense staining of nuclei located primarily in the granular layer of the epidermis. Furthermore, it appears that only the a_1 and a_4 subspecies are expressed in human skin; a_2 and a_3 were not detectable. The pattern of RORalpha subspecies expression in human skin was similar to that found in the human cerebellum. In contrast to human skin and cerebellum, however, primary cultures of normal human epidermal keratinocytes (NHEK) clearly expressed the a_4 species, whereas the a_1 species was not demonstrable.

Cholesterol sulfate added to NHEK cultures induced expression of profilaggrin mRNA (profilaggrin is a large precursor protein that contains 10 to 12 identical copies of the mature filaggrin protein arranged in tandem) in a dose-dependent manner, suggesting a new regulatory role for this sulfolipid. That the nuclear receptor RORalpha has been shown to bind tightly to cholesterol sulfate further suggested that induction of profilaggrin by cholesterol sulfate might occur via an interaction with RORalpha. RORalpha expression in normal human skin is similar to that of filaggrin and SULT2B1b. We carried out knockdown experiments with siRNA directed against mRNAs for SULT2B1b and RORalpha, with NHEK undergoing Ca^{2+}-induced differentiation. Concomitant with siRNA-induced reduction in SULT2B1b and RORalpha expression, we observed a decrease in profilaggrin mRNA expression of up to 80 percent. Furthermore, supplementing cultures with cholesterol sulfate during knockdown of SULT2B1b resulted in partial recovery of profilaggrin expression, whereas supplementing cultures with cholesterol sulfate during RORalpha knockdown did not. Our studies thus suggest that cholesterol sulfate may regulate profilaggrin expression via the receptor's ability to interact with the nuclear receptor RORalpha.

Direct interaction of cholesterol sulfate and RORalpha in the regulation of expression of the profilaggrin gene

Nakae, Fuda, Endo, Strott

To obtain evidence for a direct interaction of cholesterol sulfate with RORalpha, we cotransfected HeLa cells, which express neither cholesterol sulfotransferase (SULT2B1b) nor RORalpha, with RORalpha and the 2.1 kb 5′-flanking region of the profilaggrin promoter, which contains an RORalpha response element fused to a reporter gene. Interestingly, we noted a significant induction of profilaggrin gene expression in the presence of either cholesterol sulfate or RORalpha while

the combination of cholesterol sulfate and RORalpha led to a striking enhancement of induction. Cholesterol acetate, on the other hand, had no effect on promoter activity in the presence or absence of RORalpha. In addition, either mutation or deletion of the RORalpha response element resulted in loss of induction by RORalpha with or without cholesterol sulfate. These studies strongly suggest that cholesterol sulfate can modulate gene expression by directly interacting with a nuclear receptor (RORa lpha), an action reminiscent of a classical hormone.

RORalpha and cholesterol sulfate binding parameters

Endo, Hanyu, Fuda, Strott; in collaboration with Gorshkova, Schuck

Recent structural and functional studies have led to the hypothesis that either cholesterol or a cholesterol derivative is the natural ligand of RORalpha, an orphan member of the subfamily of nuclear hormone receptors. Following a ligand exchange experiment, we solved the X-ray crystal structure of the ligand-binding domain of RORalpha in complex with cholesterol sulfate. In contrast to the 3-hydroxyl of cholesterol, the 3-O-sulfate group makes additional direct hydrogen bonds with three residues of the RORalpha ligand–binding domain. Compared with the complex with cholesterol, seven well-ordered water molecules are displaced, and the ligand is slightly shifted toward the hydrophilic part of the ligand-binding pocket, which is ideally suited for interactions with a sulfate group. The additional ligand-protein interactions result in a higher affinity of cholesterol sulfate than of cholesterol, as shown by mass spectrometry analysis under native conditions and by differential scanning calorimetry. Moreover, mutational studies have shown that cholesterol sulfate's higher binding affinity translates into increased transcriptional activity of RORalpha. These findings suggest that, at least in part, the molecular action ascribed to cholesterol sulfate involves interaction with the RORalpha nuclear receptor. Nevertheless, we have yet to determine binding parameters for this important interaction. Thus, in an effort to generate the parameters, we attempted to overexpress the RORalpha protein in bacteria; however, success remains elusive owing to an inability to obtain sufficient amounts of soluble protein, undoubtedly because of its high content of cystine, particularly in its N-terminal region. We have now shifted to the baculovirus expression system and are in the process of expressing the sterol-binding domain of RORalpha, which is located in the C-terminal portion of the protein. With a sufficient amount of pure protein and by using Biocore T100 technology, we hope to determine RORalpha–cholesterol sulfate binding parameters.

SULT2B1b in oxysterol metabolism: role in cytotoxicity and cell signaling

Fuda, Javitt, Strott; in collaboration with Ikegawa

In contrast to the many studies that have established the deleterious effects of oxysterols on biologic processes, few studies have focused on metabolic pathways for oxysterol disposal. Of particular interest are the high levels of oxysterols in atheromas, which are associated with instability and rupture, a prelude to myocardial infarction. A major oxysterol found in atheromas as well as in other tissues is 7-ketocholesterol (7-KC), which is known from cell culture studies to induce cell injury at concentrations present in vivo. For this reason, researchers are investigating metabolic pathways that can lead to a reduction in 7-KC's toxicity. For example, it has been shown that 7-KC is a substrate for 27-hydroxylation, thus forming a more water-soluble triol that lowers the intracellular concentration of 7-KC in macrophages. We recently reported that, in addition to accelerating transport, 27-hydroxylation prevents loss in cell viability and, in co-culture, nullifies the toxicity of 7-KC, findings consonant with the differential effects of oxysterols when used in combination. Another potential metabolic pathway for the metabolism of oxysterols emerged with the discovery that one member of the SULT2 family of cytosolic sulfotransferases, SULT2B1b, has a particular affinity for cholesterol. Although it is well recognized that sulfonation of steroid hormones affects the hormones' biologic activity and can influence their disposal, the concept of an analogous pathway for C_{27} sterols has received limited attention.

Oxysterols constitute a class of cholesterol derivatives that exhibit broad biological effects, ranging from cytotoxicity to regulation of cell signaling; for example, the liver X nuclear receptor (LXR) regulates expression of genes engaged in fatty acid and cholesterol metabolism. The role of oxysterols such as 7-KC in the development of retinal macular degeneration and atheromatous lesions is of particular interest, but little is known about their metabolic fate. We established that the steroid/sterol sulfotransferase SULT2B1b, which is known to sulfonate cholesterol efficiently, effectively sulfonates a variety of oxysterols, including 7-KC. We observed that the cytotoxic effect of 7-KC on 293T cells was attenuated when we transfected the cells, which do not express SULT2B1b, with SULT2B1b cDNA. Importantly, protection by transfection from 7-KC–induced loss of cell viability correlated with the synthesis of SULT2B1b protein and production of the 7-KC sulfoconjugate (7-KCS). Moreover, adding 7-KCS to the culture medium of 293T cells in amounts equimolar to 7-KC protected against loss of cell viability. In addition, MCF-7 cells, which highly express SULT2B1b, were significantly more resistant to the cytotoxic effect of 7-KC. We found that oxysterol substrates for SULT2B1b extend to 7alpha/7beta-hydroxycholesterol and 5alpha,6alpha/5beta,6beta-epoxycholesterol as well as the 7alpha-hydroperoxide derivative of cholesterol. Thus, by acting on a variety of oxysterols, SULT2B1b offers a potential pathway for modulating the injurious effects of these compounds in vivo. Employing LC-ESI/MS (liquid chromatography–electrospray ionization/mass spectrometry) analysis, a technique commonly used to identify a variety of biologically important substances, we also found that the sulfoconjugate of 7-KC does indeed occur in vivo as demonstrated for human atheromatous tissue. This is an important finding, given the high levels of oxysterols, particularly 7-KC in atheromas. In contrast to 7-KC's toxic effects, as we report here, the sulfoconjugate of 7-KC is completely non-toxic to cells that are sensitive to the adverse effects 7-KC. Thus, SULT2B1b, which is expressed in human macrophages, offers an important in vivo metabolic route for the efficient detoxification of 7-KC and other cytotoxic oxysterols. In summary, it now appears that oxysterols have a broad range of biological effects and that SULT2B1b plays a significant role throughout the spectrum. Not only does SULT2B1b inactivate classes of oxysterols that are cytotoxic, but it also inactivates classes of oxysterols involved in cell signaling.

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PHYSIOLOGY, PSYCHOLOGY, AND GENETICS OF OBESITY

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The prevalence of overweight and obesity in children and adults has tripled during the past 30 years. The alarming rise in body weight has likely occurred because the current environment affords easy access to calorie-dense foods and requires less energy expenditure. However, the same environment leads to obesity only in those individuals whose body weight–regulatory systems are not able to control body adiposity with sufficient precision in our high-calorie/low-activity environment, thus suggesting that some subgroups in the United States have a uniquely high susceptibility to weight gain. Our goal is to elucidate the genetic underpinnings of the metabolic and behavioral endophenotypes that contribute to the development of obesity in children. Using our unique longitudinal cohort of children at risk for adult obesity, we examine genetic and phenotypic factors predictive of progression to adult obesity in children in the "pre-obese" state, allowing characterization of phenotypes unconfounded by the impact of obesity itself. We focus on genetic variants linked to obesity that impair gene function. We expect our approaches to improve our ability to predict which children are at greatest risk for obesity and its co-morbid conditions and to lead to more targeted, etiology-based prevention and treatment strategies for pediatric obesity.

Molecular studies of factors important for childhood body weight regulation
Adler-Wailes, Gustafson, Han, Levinn, Ranzenhofer, Sanford, Savastano, Yanovski J; in collaboration with Aguilera, Farooqi, Gavrilova, Gordon, Leibel, Liu, Lu, Kleinman, O'Rahilly, Tanofsky-Kraff, Tessarollo, Uhl, Westphal

To identify gene variants affecting body composition, we have been examining polymorphisms in genes involved in the leptin signaling pathway. Genes include proopiomelanocortin (*POMC*), the melanocortin 3 receptor (*MC3R*), brain-derived neurotrophic factor (*BDNF*), and the type 2 neurotrophin receptor *TrkB*. We previously studied genes important for energy expenditure, such as the mitochondrial uncoupling proteins, and genes potentially involved in cortisol metabolism that may affect intra-abdominal adipose tissue, such as 11-beta-hydroxysteroid dehydrogenase. We are currently studying a variant *MC3R* that is associated with adiposity in children and appears to have functional significance for MC3R signal transduction (Feng et al., *Diabetes* 2005;54:2663). Children who were homozygous-variant for both the polymorphisms Thr6Lys and Val81Ile had significantly greater BMI (body mass index)-SD score, fat mass, and body circumference measurements and

higher plasma levels of insulin and leptin than children who either did not bear the variant or were heterozygous. In vitro studies subsequently found that *MC3R* expression was significantly lower for the double mutant. Ongoing studies are attempting to understand the mechanisms by which these sequence alterations may affect body weight. In collaboration with Heiner Westphal, we developed transgenic knock-in mice expressing the human non-mutant and human double mutant *MC3R* and will study them over the next two years.

We have also recently investigated the BDNF-TrkB pathway as it relates to body mass in children. We measured serum BDNF in 328 children age 3 to 19, many of whom suffered from extreme obesity. BDNF was significantly lower in overweight children; multiple regression analyses with log-BDNF as the dependent variable showed that BMI, BMI Z-score, and body fat were all negatively associated with BDNF. The data suggest that some obese individuals with low serum BDNF for age and platelet count may have mutations that alter BDNF function. In collaboration with Sadaf Farooqi and Stephen O'Rahilly, we found that individuals with heterozygous *TrkB* mutations do not have unusually high circulating BDNF, and we identified a child with hyperphagia and severe obesity associated with functional loss of one copy of the *BDNF* gene. The child had a de novo chromosomal inversion, 46,XX,inv(11)(p13p15.3), a region encompassing the *BDNF* gene. The patient's genomic DNA was heterozygous for a common coding polymorphism in *BDNF*, but we observed monoallelic expression in peripheral lymphocytes. The child's serum BDNF was markedly lower than in age- and BMI-matched control subjects. Functional haploinsufficiency for BDNF was thus associated with increased ad libitum food intake and severe early-onset obesity. Our findings provide evidence for the role of BDNF in human energy homeostasis.

A recent initiative assessed the role of BDNF haploinsufficiency as a cause of obesity in patients with syndromes attributable to deletions in the vicinity of 11p14.1, which is where the human *BDNF* gene is located. Using a comparative genomic hybridization approach verified with PCR-based assays, we examined genotype-phenotype relationships in patients with 11p deletion syndromes such as WAGR (Wilms tumor, aniridia, genitourinary, and renal abnormalities) syndrome. In 33 subjects with heterozygous 11p deletions ranging in size from 1.0 to 26.5 Mb, 19 had regions of deletion that involved the *BDNF* gene ($BDNF^{+/-}$). Compared with those with intact *BDNF* ($BDNF^{+/+}$), $BDNF^{+/-}$ individuals had significantly greater body mass during childhood, starting at age 2, and 100 percent of $BDNF^{+/-}$ individuals were overweight by age 10 compared with only 20 percent of $BDNF^{+/+}$. Parent-completed hyperphagia questionnaires suggested significantly greater hyperphagic behavior, drive, and severity for $BDNF^{+/-}$ than for $BDNF^{+/+}$. Mean serum BDNF was approximately 50 percent lower among $BDNF^{+/-}$. The result remained statistically significant after adjustments for sex, current age, BMI, and platelet count. We then examined the centromeric and telomeric boundaries of each individual's deletion and observed no association between the extent of subjects' centromeric deletion and childhood overweight. However, analysis of the telomeric deletion boundaries indicated the presence of a critical region for pediatric-onset overweight within 80 kb of the *BDNF* exon. One overweight subject had deletion of *BDNF* exons I through III but preservation of subsequent *BDNF* exons. Each subject who maintained normal weight during childhood had a deletion that did not involve *BDNF*. These findings suggest that BDNF haploinsufficiency in patients with WAGR syndrome leads to pediatric-onset obesity. In the coming year, we plan to undertake a full characterization of the energy intake and expenditure of subjects with WAGR syndrome and other 11p deletion syndromes.

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Physiology, metabolism, and psychology of childhood body weight regulation

Han, Roberts, Savastano, Brady, Gustafson, Sorg, Checchi, Yanovski J; in collaboration with Troendle, Malley, Chen K, Yanovski S, Gorbach, Hubbard, Reynolds, Kozlosky, Sebring, Salaita, Denkinger, Drinkard, Faith, Levine, Cox, Rolls, Schoeller, Tanofsky-Kraff, Walsh, Wilfley

Our studies focus on understanding the physiological, psychological, and metabolic factors that place children at risk for undue weight gain. As part of these studies, we have examined how best to measure psychopathology, insulin sensitivity, changes in body composition, energy intake, and energy expenditure in children. We have found that leptin is an important predictor of weight gain in children. In fact, children with high leptin whom we have followed longitudinally experience accelerated weight gain. Recent investigations documented a greater prevalence of fractures and musculoskeletal complaints in significantly overweight children as well as a greater degree of valgus misalignment of the lower extremities that may contribute both to difficulties with exercise and further weight gain in overweight youth. We have also found marked effects of obesity on quality of life in adolescents.

Our evaluations concentrating on binge-eating behaviors in children suggest that such behaviors also are associated with adiposity in children. We found that binge-eating and dieting behaviors may predict future weight gain in children at risk for overweight. Over an observation period of about 4 years, children reporting binge-eating behaviors gained, on average, an additional 15 percent more fat mass than non–binge-eating children. Our recent data also suggest that children endorsing binge eating consume more energy during meals. Actual intake during buffet meals averaged 400 kcal more in children indulging in binge eating, but despite their greater intake, such children reported shorter-lived satiety than children without binge-eating episodes. The ability to consume large quantities of palatable foods, especially when coupled with a decrease in subsequent satiety, may play a role in the greater weight gain observed in binge-eating children. The data also suggest that interventions targeting disordered eating behaviors may potentially be useful in preventing excessive fat gain in children prone to obesity.

In two ongoing protocols, we study normal-weight children and adolescents, children who are already obese, and the non-obese children of obese parents in order to determine the factors most important for development of the complications of obesity in youth. We examine body composition, metabolic rate, insulin sensitivity, glucose disposal, energy intake at buffet meals, and genetic factors believed to regulate metabolic rate and body composition. We also study psychological and behavioral factors, such as propensity to engage in binge-eating behavior. In two longitudinal protocols, we study actual food consumption of children during meals in order to elucidate differences in the calorie and macronutrient content of meals and the circulating hormones related to hunger and satiety in those who either endorse binge-eating behaviors or report no such behaviors. We hypothesize that differences in these factors predict the development of obesity in the populations studied and may be of great importance in developing rational approaches for the prevention and treatment of obesity in the diverse U.S. population. A new clinical protocol based on a successful pilot study will examine the effects of a targeted interpersonal therapy intervention on body weight change in adolescents who endorse binge-eating behaviors.

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Treatment of obesity and the co-morbid conditions associated with obesity

Yanoff, Roberts, Savastano, Brady, Adler-Wailes, Glasofer, Sorg, Gustafson, Checchi, Yanovski J; in collaboration with Hubbard, Krakoff, Reynolds, Calis, Kozlosky, Sebring, Salaita, Denkinger, Drinkard, Booth, Levine, Rolls, Schoeller, Tanofsky-Kraff

Given the rapid increase in the prevalence of obesity, the development of treatments for obesity in childhood is urgently needed. In three ongoing clinical protocols, we are studying approaches for the control of body weight in children. We completed a pilot study demonstrating that severely overweight adolescents can lose weight when enrolled in a comprehensive weight management program that includes the gastrointestinal lipase inhibitor orlistat as an adjunct to a behavior-modification program. We recently completed a placebo-controlled randomized trial to determine whether the use of orlistat 120 mg t.i.d. improved the weight loss of African American and Caucasian children and adolescents with obesity-related co-morbidities. Subjects participated in a 12-week weight reduction program. We compared body weight and body composition (by DXA and air displacement plethysmography), glucose homeostasis by the frequently sampled intravenous glucose tolerance test (FSIGT), fasting lipids, pulse, and blood pressure before and after treatment. The study group consisted of 200 adolescents, 65 percent female, 61 percent African American, mean age 14.6 years, and BMI 41.7 kg/m^2 (range 27–87 kg/m^2). At baseline, subjects randomized to orlistat or placebo did not differ significantly in age, sex, race, BMI, or fat mass. Adolescents treated with orlistat lost more weight, BMI units, and fat mass. Although subjects' pulse and blood pressure decreased during the trial, orlistat treatment played no role in the reduction. Similarly, HOMA-IR (homeostasis model assessment of insulin resistance), SI (insulin sensitivity index) by FSIGT, Apo B, total and LDL cholesterol, and triglycerides decreased in proportion to weight loss, but orlistat use was not associated with significant reductions in any of these obesity-related laboratory co-morbidities. Orlistat treatment did not significantly alter concentrations of the fat-soluble vitamins 25OH vitamin D, vitamin A, and vitamin E. Both AST (aspartate aminotransferase) and ALT (alanine amino transferase) unexpectedly increased significantly with orlistat treatment. We concluded that a behavior-modification program that includes orlistat significantly improved weight loss over a 6-month interval but had little impact on obesity-related co-morbid conditions in overweight adolescents.

A second study examined the mechanism by which metformin may affect the body weight of younger children who have hyperinsulinemia and therefore are at risk for later development of type 2 diabetes. We conducted a single-center, 6-month, randomized, double-blind, placebo-controlled trial of the effects of metformin, 1,000 mg b.i.d. administered with meals, in severely overweight children (age 6 to 12 years) who manifested hyperinsulinemia and insulin resistance. Subjects participated in a monthly dietitian-administered weight reduction program. We measured BMI and body composition (by air displacement plethysmography), glucose homeostasis (by HOMA-IR), and lipids before and after 6 months' treatment. We enrolled 100 overweight children (60 percent female, 11 percent Hispanic, 3 percent Asian, 40 percent African American), mean age 10.2±1.5 years, with mean BMI 34.6±6.6 kg/m^2 (range 23–58 kg/m^2) between October 2000 and April 2007. Compared with placebo-treated children, those randomized to metformin evidenced a decrease in

BMI, BMI Z-score, and body fat mass to a significantly greater extent. Serum glucose, HOMA-IR, and total cholesterol also declined more in metformin-treated than in placebo-treated children. We concluded that metformin, added to a monthly behavior-modification program, significantly improved weight loss, insulin resistance, and cholesterol over a 6-month interval in severely overweight, insulin-resistant children.

A third ongoing study examines the role played by central nervous system histamine in controlling food intake at meals. Subjects are randomized to take placebo or one of several doses of betahistine, and we measure food intake at meals.

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Our group investigates signal transduction pathways that mediate the actions of hormones, growth factors, and neurotransmitters in mammalian cells, with special emphasis on the role of phosphoinositide-derived messengers. Phosphoinositides are a small fraction of the cellular phospholipids, but they play critical roles in regulating many signaling protein complexes that assemble on the surface of cell membranes. Phosphoinositides regulate protein kinases and GTP-binding proteins as well as membrane transporters and ion channels, thereby controlling cellular processes such as proliferation, apoptosis, metabolism, and cell migration and differentiation. Our group focuses on the phosphatidylinositol 4-kinases (PI4Ks), which catalyze the first committed step in phosphoinositide synthesis. Current studies aim to (1) understand the function and regulation of several phosphatidylinositol (PI) 4-kinases that control the synthesis of hormone-sensitive phosphoinositide pools; (2) characterize the structural features that determine the catalytic specificity and inhibitor sensitivity of PI 3- and PI 4-kinases; (3) define the molecular basis of protein-phosphoinositide interactions via the pleckstrin homology and other domains of selected regulatory proteins; (4) develop tools to analyze inositol lipid dynamics in live cells; and (5) determine the importance of the lipid-protein interactions in the activation of cellular responses by G protein–coupled receptors and receptor tyrosine kinases.

Identification of mammalian Sfk1 proteins as regulators of complex glyco-sphingolipid metabolism

Tóth, Kim, Balla A, Tuymetova,[1] Balla T

PI4Ks catalyze the first step in the synthesis of phosphoinositides, a small but uniquely important class of phospholipids that regulate almost every aspect of a cell's life. These enzymes have remained highly conserved during evolution; two of them, Pik1 and Stt4, assume non-redundant and essential functions in *S. cerevisiae*. Pik1 is a Golgi-localized enzyme that regulates Golgi-to-cell surface secretion while Stt4 plays several roles in cell wall biogenesis, lysosomal functions, and lipid transport, apparently by maintaining the signaling pool of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] in the plasma membrane. The mammalian homologues of the two enzymes, PI4KIIIbeta and PI4KIIIalpha (for Pik1 and Stt4, respectively), have been identified as type-III PI4Ks; however, progress in understanding their roles in mammalian cell physiology or finding their regulatory partners and mechanisms has been generally slow. The only known regulator of the Stt4 PI4K identified so far is the yeast Sfk1 protein (suppressor of four kinase), which rescues the temperature-sensitive allele of *Stt4* in *S. cerevisiae*. Sfk1 is a non-essential gene encoding a

protein with 6-membrane-spanning domains and of unknown function with no easily recognizable homologues in higher organisms.

Using multiple sequence alignments of Sfk1 homologues identified in various yeast and fungal genomes, we were able to determine the most conserved regions of these proteins and find their homologues in higher eukaryotes, including five genes in the mouse and human genomes. None of these proteins had any known or assigned functions or showed similarity to any proteins that could provide clues to their cellular roles. However, a recent report identified one of the proteins as the product of a gene sharply upregulated by p53 and as a lysosomal protein important for autophagy. A subsequent study recognized three additional human homologues, but neither study found the homology of the proteins to yeast Sfk1 or assigned any biochemical function to them. We characterized the five mammalian homologues of Sfk1 (designated as hSfk1–5) and found that the proteins are localized in distinct cellular compartments, with two of them located in lysosomes. RNAi-mediated knockdown of the individual proteins showed minor effects on phospholipid metabolism but a prominent effect on the level of certain complex glyco-sphingolipids, such as GM3. Overexpression as well as downregulation of hSfk1, one of the lysosome-associated forms, drastically changed lysosomal morphology. The data indicated that Sfk1 proteins play an important role in the metabolism of complex glyco-sphingolipids. We hypothesize that the proteins recognize and associate with glyco-sphingolipids within the membranes and regulate their trafficking and hence their metabolic fate within the cell. The functional connection between phosphoinositides and glycolipid metabolism rests in the ability of phosphoinositides to regulate the trafficking of these transmembrane domain proteins. Our studies reveal a novel connection between glyco-sphingolipid metabolism, lysosomal functions, and autophagy. Further studies will focus on identifying the molecular steps controlled by the Sfk proteins.

PI 4-kinase IIIalpha and FgF signaling in zebrafish development
Ma, Balla T; in collaboration with Blake, Liu

Previously, we identified four isoforms of PI4Ks and characterized their expression patterns during embryonic development in zebrafish. Even though these enzymes catalyze the same biochemical reaction, their intracellular localization and presumably their regulation are different. Studies in yeast show that the enzymes assume non-redundant functions. To identify developmental processes and signal transduction pathways in which specific PI4Ks play pivotal roles, we downregulated the expression of these enzymes by injection of morpholinos targeting the splicing of exons that coding the catalytic domains of the individual enzymes.

Last year, we reported that downregulation of PI4KIIIalpha with morpholino oligonucleotides resulted in several developmental abnormalities, with the most severe effects observed in the hindbrain and the branchial arches in zebrafish. These changes were associated with highly increased apoptotic activity and were partially mimicked by treatment of the embryos with the PI 3-kinase inhibitor LY294002. The most striking phenotype of PI4KIIIalpha downregulation was the lack of pectoral fin development. Downstream targets of the FGF8 signaling pathway, such as MKP3 (a MAP kinase phosphatase) and Sef, were strongly inhibited in the morphant embryos, especially in the branchial arches and pectoral finbuds. To evaluate further the exact site of defect in the cascade of FgF signaling, we determined the expression pattern of other *Fgf* genes known to be upstream of Fgf8 induction in the pectoral fin bud. Our studies showed that the earliest detectable defects were in the movement of Fgf24-positive cells from the mesenchyme to the apical ectodermal ridge (AER) in the developing fin buds at the 32 hpf[2] stage, leading to the loss of Fgf10 induction in the underlying mesenchyme. We observed a similar defect after treatment of the embryos with LY294002, consistent with a role of PI 3-kinases in regulating the migration of the Fgf24-positive cells. The data suggest that PI4KIIIalpha is a part of the major pathway by which PI(4,5)P2 is synthesized in the plasma membrane to be used by PI 3-kinases for their signaling roles. The studies are the first to identify

the role of a PI4K enzyme in the developmental process of a vertebrate organism and to reveal a functional connection between PI4KIIIalpha and the PI 3-kinases. Current studies aim to elucidate the role of PI4KIIIalpha enzyme in the production of PI(4,5)P2 in a zebrafish cell line.

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Cell biology of the STIM1 and Orai1 proteins regulating store-operated calcium entry
Korzeniowski, Várnai, Szentpetery, Balla T; in collaboration with Hunyady

STIM1, a recently described endoplasmic reticulum (ER) protein, rapidly translocates to the ER compartment adjacent to the plasma membrane (PM) upon depletion of the ER calcium stores. At that location, it activates the newly identified calcium channel Orai1, forming the molecular basis of the so-called store-operated calcium entry (SOCE) phenomenon. This calcium entry pathway is significant for two reasons. First, mutations in Orai1 have been linked to severe inborn human immunodeficiencies, and, second, this route of calcium entry is pivotal to the calcium-regulated activation of regulatory T-cells mediated by the NFAT transcription factors. Studying both the molecular mechanism of STIM1 translocation to the PM-adjacent ER compartment and the molecular definition of this compartment could expedite the identification of novel molecular targets for immunosuppression.

We tagged STIM1 and Orai1 with fluorescent proteins to follow their movements in live cells. Given that the mechanism of Ca^{2+}-regulated formation of the ER-PM junctional contact sites between the two proteins is not understood, we generated new research tools for the visualization and manipulation of these contact sites. We based our approach on the inducible heterodimerization of the FRB fragment of mTOR and the FKBP12 protein targeted to the cytoplasmic leaflet of the ER and PM, respectively. In cells expressing these constructs, addition of rapamycin generates contact areas between the PM and ER that are highly reminiscent of those generated by expressed STIM1 after store depletion, yet the contact zones do not activate Ca^{2+} influx even though STIM1 is also present within them. Curiously, increasing STIM1 expression alone also produces contact zones between the PM and ER that coalesce into large sheets of membranes where the ER and PM are juxtaposed, again without activation of Orai1 channels. Our most recent data suggest that the binding partner of the ER-localized STIM1 in the PM at the large sheets is neither Orai1 nor the PM-localized endogenous STIM1 protein and that the structural requirements for ER-PM sheet formation differ from those that trigger STIM1-Orai1 interaction in the Ca^{2+}-depleted state. The carboxy-terminal polybasic tail of STIM1 is required for generation of both the constitutive contact zones and the large ER sheets as well as for microtubule association. However, this segment is dispensable for the store-operated calcium influx process. The data point to additional molecular players in the STIM1-Orai1 partnership. As a result, our current studies aim to identify these putative plasma membrane components.

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Translocation of C-met to the nucleus is necessary for induction of a Ca^{2+} signal
Várnai, Balla T; in collaboration with Nathanson

Hepatocyte growth factor (HGF) is important for cell proliferation, differentiation, and related activities. HGF acts through its receptor c-Met, which activates downstream signaling pathways. HGF binds to c-Met at the plasma membrane, where c-Met signaling is generally believed to be initiated.

In collaboration with Michael Nathanson's laboratory, we found that c-Met rapidly translocates to the nucleus upon stimulation with HGF. Intriguingly, the calcium signals induced by HGF result from PI(4,5)P2 hydrolysis and inositol 1,4,5-trisphosphate (InsP3) formation within the nucleus rather than within the cytoplasm. We based this conclusion on the observation that an InsP3-binding domain targeted to the nucleus was able to inhibit HGF- but not vasopressin-induced Ca^{2+} signals while the same domain expressed in the cytosol and excluded from the nucleus had a reverse effect in that it was inhibitory on vasopressin- but not HGF-induced Ca^{2+} signaling. Therefore, while vasopressin generated InsP3 at the plasma membrane, c-Met receptor stimulation appears to bypass the cytoplasm and generate the messenger in the nucleus. We found that translocation of c-Met to the nucleus depends on the adaptor protein Gab1 and importin 1 and that formation of Ca^{2+} signals in turn depends upon this translocation. Our studies suggest a unique mechanism of Ca^{2+} signaling well suited to affecting primarily nuclear processes.

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CHILD AND FAMILY DEVELOPMENT ACROSS THE FIRST TWO DECADES OF LIFE

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We investigate dispositional, experiential, and environmental factors that contribute to physical, mental, emotional, and social development in humans across the first two decades of life. Our goals are to describe, analyze, and assess the capabilities and proclivities of developing human beings, including their physiological functioning; perceptual and cognitive abilities; emotional, social, and interactional styles; and the nature and consequences for children and parents of family development and children's exposure to and interactions with their natural and designed surroundings. To meet this multifaceted charge, we pursue four integrated multi-age, multi-variate, multi-cultural research programs. One is a prospective longitudinal study designed to explore several aspects of human development in the context of major sociodemographic comparisons. The second encompasses cultural influences on development within that longitudinal framework. The third consists of basic neuroscience research and the fourth of applied extensions to behavioral pediatrics. Project designs are experimental, observational, longitudinal, and cross-sectional as well as intra- and cross-cultural. Sociodemographic comparisons include family socioeconomic status, maternal age and employment status, and child parity and day care experience. Study sites include Argentina, Belgium, Brazil, Cameroon, Chile, England, France, India, Israel, Italy, Japan, Kenya, Peru, South Korea, and the United States.

Child development and parenting

Bornstein, Hahn, Haynes, Hendricks, Putnick, Suwalsky

Three studies illustrate the range of our work in the area of child development and parenting. One study examined whether fetal cardiac patterns are stable indicators of individual differences and prognostic of variation in postnatal development. We measured fetal heart rate and variability longitudinally from 20 through 38 weeks' gestation and again at postnatal age 2. We demonstrated significant within-individual stability during the prenatal period and into childhood. Fetal heart rate variability at or after 28 weeks' gestation and steeper developmental trajectories were significantly associated with mental and psychomotor development at 2 years and language ability at 2½ years. The data suggest that the foundations of individual differences in autonomic control originate during gestation and that the developmental momentum of the fetal period continues after birth.

Parenting constitutes an all-encompassing ecology of child development. Parents play central roles in children's physical survival, social growth, emotional maturation, and cognitive development.

We are broadly concerned with analyzing and understanding the roles of parenting in human development. To that end, a second study looked at several parenting cognitions and practices in mothers ranging in age from 15 to 47 years. All were first-time parents of 20-month-old children. Some age effects were zero; others were linear or nonlinear. Nonlinear age effects determined by spline regression showed significant associations to a "knot" age (about 30 years) with little or no association afterward. For age-sensitive parenting cognitions and practices, we propose a two-phase model of parental development. Our findings stress the importance of considering maternal chronological age as a factor in the study of human development.

The role of adoptive family dynamics in the etiology of both resilient and adverse developmental outcomes is not well understood. In a third study, we examined socioemotional aspects of the mother-infant relationship in families by adoption and by birth by observing matched groups of mothers and their 5-month-old first babies in the home setting. Dyads in the two groups were comparable in the frequency and ranking of a full array of age-appropriate behaviors. Group differences emerged for selected infant and maternal behaviors; infants by birth were in an alert state and smiled more often than infants by adoption while adoptive mothers nourished their infants more than did mothers by birth. Although the structure of the infants' behavior repertoire was similar for both groups, we observed twice as many significant correlations among maternal behaviors for the birth group as for the adoptive group. We also observed more correlations between maternal and infant behaviors for dyads by birth than for dyads by adoption, and the nature of the correlations differed for the two groups. We argue that both groups of mothers and babies were functioning in the adaptive, healthy range and that observed differences between them reflect subtle differences in behavioral emphasis, possibly related to the unique paths to parenthood represented by adoption and birth. Adopted children are more likely to develop learning and school adjustment problems than are their non-adopted peers, even though learning potential appears to be comparable in the two groups. In an effort to explain this phenomenon, the present study also examined infants' cognitive behavior repertoires. We examined two areas of functioning: vocal/verbal communication and exploration. Infants and mothers in both groups were similar in the frequency and ranking of a full array of age-appropriate cognitive behaviors. Both groups of babies experienced rich and comparable opportunities for the development of language competence. In the exploratory realm, group differences emerged for some infant measures; infants by birth were in an alert state and mouthed objects more than infants by adoption. Examination of the linkages among infant behaviors and between mothers and infants suggested that, while mothers by birth and adoption provided comparable opportunities for exploration, infants by birth were engaging in exploratory behavior to a somewhat greater extent.

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Behavior problems in adolescence

Bornstein, Barker, Hendricks, Putnick, Suwalsky

Adolescence is a period well recognized for major transitions in self and self vis-à-vis others. We undertook analyses of several facets of this multivariate transition. For example, self-esteem, satisfaction with one's appearance, and dieting are three constructs closely bound together in the life and mind of the young adolescent. Global self-esteem is the evaluative component of self-knowledge. Generally, high self-esteem refers to favorable global evaluations of the self, and low self-esteem refers to unfavorable evaluations of the self. Measures of appearance satisfaction tap

general aspects of body satisfaction, the affective component of body image. Body dissatisfaction generally refers to negative subjective evaluations of one's body. Self-report measures of dieting often include questions about the desire to lose weight, specific weight-loss behaviors, or attempts to maintain current weight or prevent weight gain. We asked about the temporal ordering of global self-esteem and appearance satisfaction across the early adolescence transition; the independent associations of self-esteem and appearance satisfaction on self-reported dieting; and gender differences in these processes. Investigations of agreement between child and parent reports of child emotional and behavior problems showed that children and their parents often do not agree on the number or severity of problems children display. Understanding the nature of these discrepancies has important implications for help seeking and intervention and, ultimately, the well-being of the child because, for example, parents are more likely to seek help for their children than children are for themselves.

Understanding informant disagreement also has implications for research on childhood disorders. Depending on the reporter, different samples of children will be identified as facing problems. Indeed, children sometimes report more problems than parents and vice versa. Despite acknowledgment that the direction of discrepancies warrants attention in an examination of congruence between adolescent and parent reports, the literature has devoted little attention to such discrepancies. We addressed this gap in the literature by examining agreement between adolescent and maternal reports of adolescent problem behaviors.

Experiences with age mates are believed to affect adjustment in several domains of functioning in childhood and adolescence. Ample evidence points to effects on behavior and affect, and research has identified the pathways and mechanisms thought to account for the effects. For example, we already know that interaction with antisocial peers increases a child's level of aggression and that friendship experiences can both protect children against depressed affect, through opportunities for support and closeness, and put them at risk for it via opportunities for co-rumination. Despite the large database showing the effects of peer experiences, some basic questions about the effects remain unaddressed. Typically, research on the protective effects of peer relationships has examined an important but limited range of potential stressors and outcomes, particularly those related to other social experiences. As a result, evidence that peer experiences can protect children from the negative effects of stress is drawn from a narrow set of circumstances. Moreover, most studies conceive of the effects of peer relationship as either producing direct/additive effects or moderating other effects. We examined the claims that positive relationships with peers not only protect against the negative effects of a potentially stressful event but also mediate between children's current affective state and their response to a subsequent tragic event, such as the 9/11 attacks on Washington, DC.

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Acculturation in children and parents in contemporary America

Bornstein, Cote

Culture defines the ways in which a collection of people process and make sense of their experiences and influences a wide array of family processes, including family roles, decision-making patterns, and cognitions and practices about childrearing and child development. Thus, acculturation (which

may be defined as the dual processes of cultural and psychological change that take place as a result of contact between two or more cultural groups and their individual members) causes major transformations in family life, parenting, child health, and human development. Immigrants face several challenges in acculturating within the dominant or existing society—including deciding which cultural cognitions or practices to retain from their culture of origin and which to adopt from their culture of destination. Yet, acculturation as a scientific phenomenon remains poorly understood. Acculturation entails learning each others' languages, sharing each others' food preferences, and adopting forms of dress and social interactions that are characteristic of each group. Sometimes these mutual adaptations take place easily, but they may also create culture conflict and acculturative stress during intercultural interactions.

Cross-cultural comparisons show that virtually all aspects of human development—cognitions and practices alike—are informed by their cultural framework. Today, 25 percent of children under the age of 18 in the United States, for example, are either immigrants themselves or the children of immigrants. Despite increasing numbers of migrants in the 20th and 21st centuries, research on the influences of acculturation on child development has been relatively scarce. To redress the information void, we study how cultural context influences human development among immigrants.

We examined cultural variation in the durations, relations, and contingencies of mother-infant person-directed and object-directed interactions in non-migrant Latino mother-infant dyads living in South America, Latina immigrant mothers from South America and their infants living in the United States, and European American mother-infant dyads living in the United States. Non-migrant Latina mothers and their infants engaged in person-directed behavior longer than both immigrant and European American mothers and infants in the United States; results for object-directed behaviors were not as consistent. Mother and infant person-directed behaviors were positively related in all groups; mother and infant object-related behaviors were positively related for dyads living in the United States and unrelated for non-migrant Latino dyads. Nonetheless, nearly all mother and infant behaviors (person- and object-directed) were mutually contingent in dyads in all three cultural groups. Mothers were more responsive to infants' behaviors than infants were to mothers in each cultural group and for both person- and object-directed behaviors. Latina immigrant mothers tended to be the least responsive for person-directed behaviors, and Latino immigrant infants the least responsive for object-directed behaviors. Immigrant status plays a differentiated role in mother-infant interactions.

Parents often worry that their child is lagging behind in the "achievement" of one or more developmental milestones. For example, parents frequently express concern if their child is not yet walking when other children of the same age are walking or is not talking as soon or as well as they would like or expect. Comparing notes with other parents at the neighborhood playground may be a source of stress rather than a source of solace and support if parents find themselves negatively comparing their child's development to other children's development. Today's young adults are more isolated from children than ever before, and many miss out on the opportunity to observe and engage with young children until they have a child of their own. Although new parents know more about children's development than those who are not yet parents, their knowledge of child development is not always accurate. It is important that parents have accurate information about and knowledge of child development because research indicates that parents' knowledge of child development influences their parenting practices. A theory chapter (Cote and Bornstein, in press) explored issues in immigration and acculturation, and a handbook chapter (Bornstein and Cote) advises parents and practitioners on reputable sources of information about normative child development.

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DEVELOPMENTAL REGULATION OF NEURONAL AND MUSCLE PLASTICITY

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Genetic (nature) and epigenetic (nurture) interactions influence the functional properties of neurons and skeletal muscles during development. We are investigating how Neuregulin-1 (NRG-1), a growth and differentiation factor, regulates synaptic formation and plasticity during development. We found that NRG-1, signaling via its receptor ErbB4, functions to repress or reverse hippocampal long-term potentiation (LTP)—a cellular mechanism implicated in learning, memory, and cognition. We identified a novel interrelationship among NRG-1, glutamate, and dopamine signaling pathways that regulate LTP at central synapses and found that NRG-1 regulates gamma-rhythm network activity in the brain. Given that these three signaling pathways and alterations in gamma oscillations are implicated both genetically and pharmacologically in schizophrenia, our findings could have important implications for understanding biological mechanisms affected by psychiatric disorders. In another series of studies, we investigated how developmental factors and motoneuron activity work to differentially regulate the contractile properties of slow-twitch (red) and fast-twitch (white) muscles. We identified DNA-regulatory elements and transcription factors that confine contractile protein expression to either slow- or fast-twitch fibers in response to "slow-" and "fast-"patterned motoneuron electrical stimulation. The studies emphasize the importance of both nature and nurture in modulating the plastic properties of excitable tissues.

Isolation and characterization of the neuregulin-1 type IV isoform
Shamir, Buonanno

Accumulating evidence supports the involvement of NRG-1 and ErbB-4 receptors in the etiology of schizophrenia. The NRG-1 gene generates numerous transcripts by using different transcriptional promoters and alternative splicing. Interestingly, a single nucleotide polymorphism (SNP8NRG243177) is located close to a region proposed to function as a promoter for the novel NRG-1 isoform denoted as Type IV. The SNP8NRG243177 [T/T] polymorphism, which maps within the previously identified "schizophrenia at-risk haplotype," is associated with higher levels of Type IV transcripts in postmortem tissue from persons diagnosed with schizophrenia and with lower prefrontal activation and the development of psychotic symptoms.

NRG-1 Type IV transcripts were originally identified by RT-PCR as partial RNA fragments. Therefore, it currently is not known whether these partial transcripts originated from full-length

NRG-1 mRNAs and whether the mRNAs encode pro-NRG-1 proteins that are post-translationally processed to produce a biologically active form of NRG-1. Toward understanding a possible role of Type IV NRG-1 in the human brain, we isolated two full-length mRNAs encoding Type IV proteins. We found that the transcripts are translated to generate the pro–NRG-1 Type IV protein, which is post-translationally processed, released from cells, and capable of activating the ErbB receptor and its downstream signaling pathways. This study provides the first evidence for the existence of the NRG-1 Type IV protein. Experiments are in progress to determine whether (and how) expression of the NRG-1 Type IV protein is altered in schizophrenia.

Neuregulin-1 regulates LTP at CA1 hippocampal synapses through activation of dopamine D4 receptors: possible implications for schizophrenia

Kwon, Paredes, Gonzalez, Neddens, Vullhorst, Buonanno; in collaboration with Hernández

Although NRG-1 function has undergone extensive study in the developing peripheral nervous system, its role in the developing and adult brain remained, until recently, largely unknown. Our hypothesis that the NRG/ErbB signaling pathway regulates activity-dependent synaptic plasticity was based on the fact that ErbB-4 and NMDA receptors (NMDAR) co-localize at glutamatergic postsynaptic densities, where they interact directly with scaffolding proteins that integrate synaptic signaling. Our recent work supports this hypothesis.

We previously reported that, by selectively reducing AMPA-type glutamate receptor currents, NRG-1 reverses (depotentiates) LTP at hippocampal CA1 glutamatergic synapses in an activity-dependent fashion. We have now demonstrated that NRG-1 stimulates dopamine release in the hippocampus and reverses early-phase LTP via activation of D4 dopamine receptors (D4R). NRG-1 fails to depotentiate LTP in hippocampal slices treated with the antipsychotic clozapine and other more selective D4R antagonists. Moreover, LTP is not depotentiated in D4R knockout mice by either NRG-1 or theta-pulse stimuli. Conversely, direct D4R activation mimics NRG-1 and acts by reducing AMPAR currents and increasing receptor internalization. This novel functional link among NRG-1, dopamine, and glutamate is consistent with animal studies reporting behavior deficits in NRG-1 and ErbB receptor hypomorphic mice that are ameliorated by treatment with the antipsychotic clozapine. The link also has important implications for understanding how imbalances in Neuregulin-ErbB signaling can impinge on dopaminergic and glutamatergic function—neurotransmitter pathways associated with schizophrenia synapses.

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Neuregulin-1 modulates hippocampal gamma oscillations

Neddens, Yan,[1] Buonanno; in collaboration with Fisahn

Alterations in gamma-frequency oscillations are implicated in psychiatric disorders, and their amplitude (power) has been reported to increase selectively during psychotic episodes. In collaboration with Andre Fisahn, we found that NRG-1 dramatically increases the power of kainate-induced gamma oscillations in acute hippocampal slices. NRG-1 effects are blocked by PD158780, a pan-specific antagonist of ErbB receptors, and are absent from slices prepared from ErbB4-null mice. Moreover, we demonstrated that 50 percent of GABAergic parvalbumin-positive interneurons, which heavily contribute to the generation of gamma oscillations, express ErbB4 receptors. Importantly, both the number of parvalbumin-immunoreactive interneurons and the power of kainate-induced gamma oscillations are reduced in ErbB4 knockout mice. Our investigation provides the first plausible link between NRG-1/ErbB4 signaling and rhythmic network activity that may be altered in persons with schizophrenia.

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ErbB-4 surface clustering by PSD-95 at inhibitory hippocampal neurons

Chatani-Hinze,[2] Longart,[3] Gonzalez, Vullhorst, Yan,[1] Buonanno

To extend our earlier work showing that ErbB-4 directly interacts with the postsynaptic density protein PSD-95 at glutamatergic synapses, we investigated the developmental expression and trafficking of the receptor. These interactions may be of special interest because they appear altered in postmortem brain tissue isolated from persons diagnosed with schizophrenia. Using immunofluorescence analysis in hippocampal slices and dissociated neurons in culture, we found that ErbB-4 receptors are expressed predominantly at glutamatergic synapses in GABAergic interneurons. We investigated the trafficking of ErbB-4 in cultured hippocampal neurons by using surface protein biotinylation and antibody labeling of receptors in live cells and found that ErbB-4 immunoreactivity in developing neurons precedes PSD-95 expression, with ErbB-4 cluster initially forming in the absence of, but later associating with, PSD-95-positive puncta. The surface fraction of dendritic ErbB-4 increases from 30 percent at 6 days in culture to 65 percent by day 16. Interestingly, despite the increased association of ErbB-4 with PSD-95, receptor activation by NRG-1 triggers significant internalization in young and mature neurons. PSD-95 primarily seems to control receptor clustering. These findings enhance our understanding of the role of ErbB-4/PSD-95 protein interaction for NRG-mediated signaling at glutamatergic synapses.

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Expression and function of NMDA NR2C receptor in beta-galactosidase knockin mice

Karavanov, Buonanno; in collaboration with Lu, Logan, Vicini, Wolfe

We previously reported that, in cultured organotypic slices from cerebellum, expression of the NR2C subunit of NMDAR is regulated by NRG-1. NR2C has generally been considered a "cerebellar subunit" because its expression is strikingly higher in the cerebellum than in other brain areas. To study the precise expression and function of the NR2C subunit in the developing brain, we developed knockin mice. Using homologous recombination, we replaced DNA sequences encoding most of the NR2C protein with the *E. coli nlacZ* gene that encodes the beta-galactosidase (*B-gal*) reporter. We histologically identified cells that express the B-gal reporter, under control of NR2C transcriptional regulatory elements, by staining whole brains and sections with *X-gal*. Using these knockin mice, we found that NR2C is more dynamically and broadly expressed in brain than previously reported. In the cerebellum, NR2C is expressed in a caudal-rostral gradient and in a series of parasagittal bands in subsets of cerebellar granule cells. We also found NR2C expression in previously unappreciated areas, such as the retrosplenial and cerebral cortex, hippocampus, and basal ganglia. Using cell type–specific antibodies, we unexpectedly found that NR2C is expressed by glial cells, not neurons, dispersed in the hippocampus, striatum, olfactory bulb, and cerebral cortex. We confirmed all novel sites of expression identified in the NR2C*tg-nlacZ* knockin mice by in situ hybridization using ^{33}P-labeled NR2C cRNA probes. The expression of NR2C in discrete brain areas outside the cerebellum and in glia suggests that NMDARs with different subunit compositions may serve distinct functions.

In collaboration with the laboratories of Stefano Vicini and Barry Wolfe, we studied the NMDAR excitatory postsynaptic currents (EPSC) in solitary cerebellar neurons cultured in microislands from wild-type (WT) and NR2C*tg-nlacZ* knockin mice and in NR2A subunit knockout mice. NR2C*tg-nlacZ* granule neurons have larger NMDA-EPSCs than WT cells. The decay times of these currents were all unexpectedly fast, and the quantal content was elevated in the mutant mice. The most striking result was a significant increase in the NMDA-EPSC peak amplitude and charge transfer in NR2C*tg-nlacZ* knockin mice, which is mostly attributable to an increase in quantal size, as estimated from miniature NMDA-EPSCs.

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Transcription factors that differentially regulate transcription of muscle genes

Rana,[4] Vullhorst, Buonanno; in collaboration with Gundersen

Genetic background and energetic demands from the environment determine the contractile properties of adult slow- and fast-twitch skeletal muscles. Lineage is important during early development for determining the fate of muscles as either slow- or fast-twitch, but the properties of myofibers remain plastic and are later modified by activity (i.e., exercise). Transcription is the major mechanism determining the fiber type–specific properties of muscles; it regulates the expression of genes encoding contractile proteins and metabolic enzymes characteristic of slow and fast muscles. The troponin I slow (*TnIs*) and fast (*TnIf*) genes are selectively expressed in slow and fast muscles during development and are later regulated by distinct patterns of electrical impulses elicited by motor neurons. Using the *TnI* genes as a model system, we identified a slow (SURE) and fast (FIRE) enhancer that regulate the genes' fiber type–specific transcription. We used reporter constructs driving the expression of either luciferase or the green fluorescent protein (GFP) to map the transcription-regulatory elements in SURE and FIRE. Our initial studies showed that General Transcription Factor 3 (GTF3) binds to a region of the *TnIs* SURE necessary for slow-specific transcription during early development. Our studies using ectopically transfected GTF3 constructs in adult muscles and GTF3 knockout mice support a role for GTF3 in regulating muscle contractile properties during development.

ACTIVITY-DEPENDENT REPRESSION OF FAST MUSCLE GENES BY NFAT. Activity presumably is coupled to distinct programs of transcription by modulating the levels of intracellular calcium and metabolites. The NFAT family of calcium-dependent transcription factors has been implicated in the upregulation of genes encoding slow contractile proteins in response to slow-patterned motoneuron depolarization. We uncovered a novel and unexpected function of NFATc1 in slow-twitch muscles. Using the *TnIf* intronic regulatory element FIRE, we identified sequences that downregulate FIRE's function selectively in response to patterns of electrical activity that mimic slow motoneuron firing. We identified a bona fide NFAT-binding site in the *TnIf* FIRE by site-directed mutations and EMSAs and showed that NFAT mediated the activity-dependent transcriptional repression of FIRE. Knockdown, mediated by siRNA, of NFATc1 in adult muscles resulted in ectopic activation of FIRE in the slow soleus without affecting enhancer activity in the fast EDL (extensor digitorum longus) muscle. Our findings demonstrate a novel function of NFAT as a repressor of transcription of fast contractile genes in slow muscles and underscore how muscles can modify their adult contractile properties in response to distinct types of exercise.

PPARδ EXPRESSION IS INFLUENCED BY MUSCLE ACTIVITY AND INDUCES SLOW MUSCLE PROPERTIES. Metabolites such as free fatty acids (FFA) that are generated during exercise could activate transcriptional pathways that regulate fiber types. In collaboration with Kristian Gundersen, we found that mRNA levels for peroxisome proliferator–activated receptor (PPAR)-δ were three times higher in the slow/oxidative soleus than in fast/glycolytic EDL muscle. PPARδ mRNA levels were more than 50 percent lower in solei stimulated with fast-patterned activity while levels increased three-fold in EDLs stimulated with slow-patterned activity. Overexpression of a constitutively active form of PPARδ in normally active adult fibers tripled the number of I/IIa hybrids in fast EDL muscle and increased the activity of the oxidative enzyme succinate dehydrogenase. Therefore, PPARδ provides an additional important link between motoneuron activity and transcriptional changes in slow muscles.

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TEF3/Tead4 knockout mice

Karavanov, Vullhorst, Buonanno; in collaboration with Yagi, Kohn, Kaneko, DePamphilis

Based on the analysis of *TnI* SURE regulatory elements, we analyzed another transcription factor Tead4 (also known as TEF3) for its potential role in regulating early muscle development and regeneration in the adult. To this end, we generated *Tead4*-floxed mice by homologous recombination. We could not, however, study a possible role of *Tead4* in muscle development because null mice were not born. Developmental studies indicated that ablation of the gene resulted in a pre-implantation failure. *Tead4*$^{-/-}$ embryos do not express trophectoderm-specific genes, and the morulae do not produce trophoblast stem cells, trophectoderm, or blastocoel cavities and therefore fail to implant. *Tead4*$^{-/-}$ embryos can produce embryonic stem cells, and the embryos can complete development if the gene is deleted after the implantation period, as shown by crossing the *Tead4*-floxed mice with *Meox2-Cre* recombinase mice. Consequently, to determine if *Tead4* plays any role in regulating fiber type properties during development, we would need to undertake additional experiments using *cre*-recombinase to delete *Tead4* later in development.

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POTASSIUM CHANNELS AND DENDRITIC FUNCTION IN HIPPOCAMPAL PYRAMIDAL NEURONS

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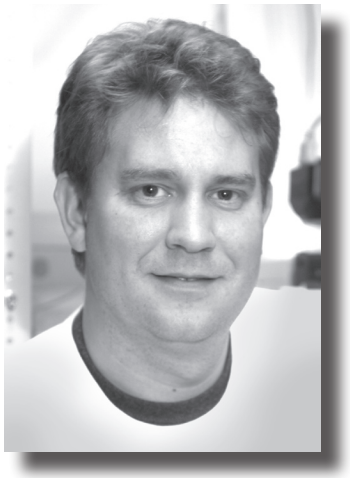

The central nervous system underlies all our experiences, actions, emotions, knowledge, and memories. With billions of neurons each firing hundreds of times per second, the complexity of the brain is stunning. To pare down such complexity, our research approach calls for studying the workings of a single central neuron—the pyramidal neuron from the CA1 region of the hippocampus, a region of the brain important for learning and memory and among the first affected in Alzheimer's disease and epilepsy. In the dendrites of hippocampal CA1 pyramidal neurons, a nonuniform density of subthreshold, rapidly inactivating potassium channels regulates signal propagation. With higher expression in the dendrites than in the soma, this distribution means that the electrical properties of the dendrites differ markedly from those of the soma. Incoming synaptic signals are shaped by the activity of these channels, and, once initiated in the axon, action potentials (AP) progressively decrease in amplitude as they propagate back into the dendrites. Combining patch clamp recording with molecular biology and imaging, we investigate the electrophysiological properties and molecular nature of the voltage-gated channels expressed in CA1 dendrites, how their expression is regulated, and their role in learning and memory.

Kv4.2 control of firing patterns in hippocampal CA1 pyramidal neurons

Kim, Hoffman, Hahm

Although recent molecular cloning studies have found several families of voltage-gated K^+ channel genes expressed in the mammalian brain, information about the relationship between the protein products of these genes and their various neuronal functions is currently lacking. We have used a combination of molecular, electrophysiological, and imaging techniques to show that the voltage-gated potassium channel subunit Kv4.2 controls AP half-width, frequency-dependent AP broadening, and dendritic AP propagation. To visualize Kv4.2, we fused the enhanced green fluorescence protein (EGFP) to the cytoplasmic C-terminus (Kv4.2g). Using a modified Sindbis virus, which reduces cytotoxicity and improves the neuro-specificity of infection, we expressed either Kv4.2g or an EGFP-tagged dominant negative mutant of Kv4.2 [$Kv4.2g^{(W362F)}$] in dissociated hippocampal neurons and in CA1 pyramidal neurons of organotypic slice cultures. Others have shown that $Kv4.2g^{(W362F)}$ acts as a subfamily-specific dominant negative (i.e., blocking only Kv4.x or *shal*-containing channels after heterologous expression).

Nucleated and outside-out patch clamp recordings from CA1 hippocampal neurons expressing Kv4.2g showed a two-fold–higher transient outward current density than in non-expressing control neurons without an effect on the sustained component density (Kim et al., *J Physiol* 2005;569:41). Expression of the dominant negative Kv4 mutation knocked down the transient outward current density to 63 percent of control, again without affecting the sustained outward current density. Kv4.2g and Kv4.2g$^{(W362F)}$ expression did not alter transient current steady-state activation and inactivation. A comparison of Kv4.2g properties expressed in HEK-293 cells and in CA1 neurons suggests that an auxiliary subunit modulates Kv4.2g channel properties in neurons. In CA1 neurons, all measured kinetics (time-to-peak, inactivation rate, and rate of recovery from inactivation) are greater than those recorded in HEK-293 cells. These differences can be attributed to the auxiliary protein DPPX. We have started to characterize the functional role of DPPX in CA1 neurons (below).

To assess the contribution of Kv4 family subunits to the electrical properties and firing patterns of CA1 pyramidal neurons, we performed whole-cell current-clamp experiments in each of the three experimental groups (EGFP, Kv4.2g, and Kv4.2g$^{(W362F)}$). Suprathreshold current injections in Kv4.2g- and Kv4.2g$^{(W362F)}$-expressing neurons showed that Kv4.2 is engaged throughout the physiologically relevant voltage range, contrary to the established view of somatic A-type currents acting solely subthreshold. In response to depolarization, Kv4.2 prevented AP initiation by delaying AP onset and increasing the threshold for initiation. We found that, subsequent to initiation, Kv4.2 aids in repolarization and contributes to the fast after-hyperpolarization potential. Single AP half-width was greatly reduced in Kv4.2g-expressing neurons, and somatic Ca^{2+} influx was barely half that of control neurons. Accumulating Kv4.2 inactivation during an AP train contributed to frequency-dependent AP broadening and subsequent somatodendritic Ca^{2+} influx. Finally, Kv4.2g limited backpropagation, as measured with Ca^{2+} imaging, whereas Kv4.2g$^{(W362F)}$ enhanced propagation.

The results show a prominent role for Kv4.2 in regulating AP shape and dendritic signaling. As Ca^{2+} influx occurs primarily during AP repolarization, Kv4.2 activity can regulate cellular processes involving Ca^{2+}-dependent second messenger cascades such as gene expression and synaptic plasticity. We plan to extend our studies to include dendritic recordings of back-propagating APs in acute slice from adult mice after in vivo viral injections.

Kv4.2 trafficking in CA1 pyramidal neuron dendrites

Kim, Jung, Lin, Hammond; in collaboration with Petralia

Using a modified Sindbis virus system to overexpress EGFP-labeled Kv4.2 (Kv4.2g) in cultured hippocampal neurons, we found that the EGFP fluorescence in dendritic spines of Kv4.2g-expressing neurons appeared brighter than that from the adjacent dendritic shaft. The ratio of spine-head to dendritic-shaft fluorescence in Kv4.2g-expressing neurons was approximately two-fold greater than in neurons expressing EGFP. In collaboration with Ron Petralia, we further demonstrated Kv4.2 expression in spines with electronmicroscopy.

We found that stimulation (AMPA) resulted in an activity-dependent redistribution of Kv4.2g away from spines to the dendritic shaft and a punctate accumulation of Kv4.2g within the soma. This AMPA-induced redistribution of Kv4.2g occurred within 15 minutes of stimulation and was reversible, indicating that the treatment was not excitotoxic. Co-expression with pre- and postsynaptic markers showed that Kv4.2 undergoes activity-induced redistribution without a gross change in synaptic architecture or number. We confirmed these findings with live imaging of Kv4.2g removal from the spine in response to AMPA stimulation. We directly measured the effect of internalization as a decrease in the endogenous whole-cell transient K^+ current from uninfected hippocampal neurons without a change in sustained or non-inactivating delayed rectifier-type voltage-gated K^+ current amplitudes. Thus, activity-dependent Kv4.2 internalization occurs natively and is not an artifact of overexpression.

More recently, we examined the role of protein kinase A (PKA) in Kv4.2 activity-dependent trafficking. In hippocampal neurons, PKA activation with forskolin or 8-Br-cAMP induced Kv4.2 internalization from dendritic spines, whereas PKA inhibition prevented AMPA-induced internalization. Furthermore, introduction of a point mutation at the C-terminal PKA phosphorylation site of Kv4.2 (S552A) prevented the AMPA-induced internalization of Kv4.2. Together, the data demonstrate that activity-dependent internalization of Kv4.2 requires PKA phosphorylation of Kv4.2 at S522. We also examined the role of the Kv4.2 C-terminal dileucine internalization motif in PKA-mediated Kv4.2 internalization. The dileucine motif on Kv4.2 is important for dendritic targeting of Kv4.2 but does not affect the basal rate of Kv4.2 endocytosis. To determine whether the Kv4.2 dileucine motif mediates activity-dependent Kv4.2 internalization, we mutated the Kv4.2 C-terminal dileucine residues 481to 482 to alanines (Kv4.2LLAA). When expressed in COS7 cells, Kv4.2g but not Kv4.2LLAA is internalized after forskolin application. The residues are therefore likely to mediate activity-dependent internalization of Kv4.2. We continue to investigate the mechanisms of Kv4.2 expression and trafficking.

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Role of voltage-gated potassium channels in synaptic and intrinsic plasticity

Jung, Hoffman

For several decades, synaptic plasticity has been considered the best candidate mechanism for the formation and storage of memories. However, efficacy in driving a neuron to fire is dependent not only on the size of synaptic input but also on its location and timing, which is subsequently shaped by types and distributions of voltage- and calcium-gated conductances in dendrites. Several studies, including the original description of long-term potentiation (LTP) (Bliss and Lomo, *J. Physiol* 1973;232:331), have reported that the induction of synaptic plasticity is accompanied by changes in the intrinsic excitability of the neuron, indicating a potential concurrent change in voltage-gated channel activity. Activity-dependent regulation of intrinsic excitability (intrinsic plasticity), which is induced by learning, has been observed in several preparations. Plasticity in voltage- and/or calcium-gated channel function may then contribute to the memory process.

Using the Sindbis virus system to infect organotypic slice cultures with Kv4.2g and Kv4.2g$^{(W362F)}$, we have begun investigating the role of Kv4.2 in synaptic LTP, a potential mechanism for learning and memory. For the first 10 minutes after virus-mediated induction, potentiation is similar in all three groups—control, Kv4.2g and Kv4.2g$^{(W362F)}$—achieving about a 100 percent increase in excitatory postsynaptic current (EPSC) size. After this period, however, Kv4.2-overexpressing neurons fail to maintain potentiation, such that EPSC size returns to baseline after about 25 minutes. Conversely, expression of Kv4.2g$^{(W362F)}$ results in a potentiation that reaches a greater level 40 to 50 minutes after initiation than do controls . The data indicate that the functional expression level of Kv4.2 influences the degree of potentiation.

We found that altering the functional Kv4.2 expression level also leads to a rapid, bidirectional remodeling of CA1 synapses. Neurons exhibiting enhanced A-type K^+ current (I_A) showed a decrease in relative synaptic NR2B/NR2A subunit composition and, as noted, did not exhibit LTP. Conversely, reducing I_A by expression of a Kv4.2 dominant negative or through genomic knockout of Kv4.2 led to an increased fraction of synaptic NR2B/NR2A and enhanced LTP. Bidirectional synaptic remodeling was mimicked in experiments manipulating intracellular Ca^{2+} and was dependent on spontaneous activation of NMDA receptors and active CaMKII. Our data suggest that A-type K^+ channels are an integral part of a synaptic complex that regulates Ca^{2+} signaling through spontaneous NMDAR activation to control synaptic NMDAR expression and plasticity. We are currently characterizing the mechanisms of Kv4.2's effect on NMDARs and LTP.

We have found that LTP induction results in a rapid, long-lasting increase in intrinsic excitability of CA1 pyramidal neurons from organotypic slice cultures. While several channels may be modulated by protocols used to induce synaptic plasticity and thus lead to enhanced intrinsic excitability, A-type K^+ channels, with a subthreshold activation range, are among the first channels activated upon depolarization, thereby determining AP onset time, threshold, and interspike intervals (Storm, *Prog Brain Res* 1990;83:161). The significant decreases in AP threshold, onset time, and AHP after LTP are all consistent with A-current downregulation (Kim et al., *J Physiol* 2005;569:41).

To measure changes in A-current density and properties after LTP, Sung-Cherl Jung pulled nucleated patches from CA1 somata in organotypic slice cultures before and then every 10 minutes after LTP induction by a depolarization-pairing protocol. We then compared the current density values obtained with those found for neurons undergoing depolarization but no pairing (unpaired). Preliminary results show a clear, substantial (about 15 mV) negative inactivation curve shift in A-current density10 minutes after pairing with a return to control values by 30 minutes after induction. In addition, our results indicate that Kv4.2 may be internalized upon LTP induction. We observed no lasting change in sustained current density. The time course of the decrease in A-current matches well that shown previously after chemical LTP induction. We propose that the long-lasting increase in excitability induced with LTP occurs through the two-phase decrease in A-current activity: an immediate but short-lived (about 10 minutes) hyperpolarized shift in the voltage dependence of the steady-state inactivation curve of A-type K^+ currents followed by a progressive, long-term decrease in peak A-type K^+ current amplitude that outlasts the observed A-channel inactivation curve shift.

Jung SC, Kim J, Hoffman DA. Rapid, bidirectional remodeling of synaptic NMDA receptor subunit composition by A-type K^+ channel activity in hippocampal CA1 pyramidal neurons. *Neuron* 2008;60:657-671.

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Creation and characterization of Kv4.2 transgenic mice

Hoffman; in collaboration with Anderson

We are characterizing a transgenic mouse that expresses a dominant negative pore mutation in the voltage-gated potassium channel subunit Kv4.2—the likely molecular identity of transient currents recorded in CA1 dendrites. The mouse expresses the mutant Kv4.2 channel along with GFP under control of a tetracycline transactivator (tTA)–responsive promoter. Expression is spatially controlled in a new line of tTA-expressing mice that limit tTA activity to the CA1 and dentate gyrus regions of the hippocampus. Expression may be controlled temporally by administration of doxycycline. We will use experiments in acute hippocampal slices from these mice to investigate Kv4.2's role in regulating AP backpropagation into CA1 dendrites and in synaptic integration and plasticity. In collaboration with Anne Anderson, we are investigating seizure susceptibility in these mice. In addition, we are using the mice to investigate Kv4.2's role in hippocampus-dependent learning and memory in the Morris water maze, which is a well-established task for examining hippocampal-dependent learning and memory in rodents.

Role of auxiliary proteins in regulating Kv4.2 expression and function

Kim, Lin, Wikenheiser, Hoffman; in collaboration with Rudy

A-type K^+ currents have unique kinetic and voltage-dependent properties that allow them to fine-tune synaptic events, APs, and neuronal firing. However, Kv4 currents in heterologous cells display slower kinetics of inactivation and recovery from inactivation than that typically recorded in neurons. Moreover, Kv4.2 channels likely make up the majority of the A-type current in several cell types, including hippocampal CA1 pyramidal neurons, layer 5 cortical neurons, and granule cells of the olfactory bulb, yet A-current properties and distributions vary between these cell types. Reconciliation of these properties came with the finding of neuron-specific auxiliary subunit expression of KChIPs and DPLs. In hippocampal CA1 pyramidal neurons, DPPX (also called

DPP6) is the prominent DPL family member while two distinct KChIPs (KChIP2 and KChIP4) are expressed. When Kv4 subunits are co-expressed with DPPX, many properties of native CA1 A-type currents are restored.

To investigate the physiological role of DPPX in CA1 neurons, we developed, in collaboration with Bernardo Rudy's laboratory, short interfering RNAs (siRNAs) to suppress the expression of all DPPX variants. We tested the efficacy of the siRNAs in CHO cells co-expressing Kv4.2 and DPPX-S. The reduction of DPPX proteins in CHO cells transfected with DPPX siRNA (siDPPX) was more than 95 percent complete as quantified by immunoblotting. To investigate whether DPPX alters the kinetics of A-type currents in a native system, we conducted voltage-clamp experiments in outside-out patches from CA1 pyramidal neurons in hippocampal organotypic slices infected with siDPPX, using the Sindbis virus system. After allowing 2 to 3 days post-infection for DPPX knockdown, we found, in accordance with heterologous studies, that siDPPX results in a delayed recovery from inactivation, a slowed time-to-peak, and a rightward shift in the steady-state inactivation and activation curves for A-type currents.

To determine the physiological effect of the A-type current kinetic modifications by siDPPX, we carried out current-clamp experiments in siDPPX-expressing cells. Compared with negative control siRNA-expressing neurons, siDPPX-infected neurons exhibited lower input resistance, delayed time to AP onset, higher AP threshold, lower firing frequency, greater AP half-width, and lower fast AHP amplitudes. Thus, siDPPX had contrasting effects; it decreased the excitability subthreshold and increased the excitability suprathreshold. We used computer modeling to determine which of these sub- and suprathreshold effects may be explained by the shift in inactivation and activation curves. In addition, we are investigating the role of DPPX in synaptic integration and plasticity in CA1 neurons and have prepared siRNAs against other auxiliary subunits for similar analysis.

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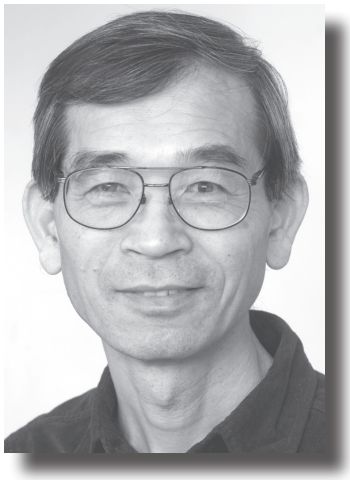
SIGNAL TRANSDUCTION IN SYNAPTIC TRANSMISSION AND PLASTICITY



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We investigate the signal transduction mechanisms involved in synaptic transmission and plasticity. Studies of these neural processes are essential to understanding the complex problems related to cognition and behavioral disturbances. Our approach is to generate genetically modified mice by deletion or expression of a gene specifically expressed in the brain. Our efforts have led us to generate a strain of mice devoid of neurogranin (Ng), a neural-specific protein. Ng is normally expressed at a high level in specific neurons in the forebrain and has been implicated in the regulation of Ca^{2+}/calmodulin (CaM)–dependent reactions. Ng levels in the neuronal soma and dendrites are very high, and the protein sequesters apoCaM at basal physiological Ca^{2+}. Upon synaptic stimulation, influxed Ca^{2+} displaces Ng from the Ng/apoCaM complex to form Ca^{2+}/CaM and free Ng. The buffering of CaM by Ng functions as a mechanism to regulate neuronal free Ca^{2+} and Ca^{2+}/CaM concentrations. We aim to define the regulatory functions of Ng in neuronal signaling and to design therapeutic approaches to treat cognitive deficits and behavioral disturbances.

Regulation of synaptic plasticity by neurogranin
Huang K-P, Huang F

Ng binds to apoCaM under basal physiological conditions and dissociates from CaM upon synaptic stimulation that raises intracellular Ca^{2+}. It has been suggested that Ng modulates synaptic responses by buffering and sequestering CaM to regulate the level of free Ca^{2+} and Ca^{2+}/CaM complexes. The binding affinity of Ng for CaM is reduced by increasing Ca^{2+}, phosphorylation by phosphokinase C (PKC), or oxidation by oxidants. The Ng concentration in the hippocampus of adult mice is one of the highest among all neuronal CaM-binding proteins. Among $Ng^{+/-}$ mice, but less so in $Ng^{+/+}$ mice, a significant relationship exists between the hippocampal levels of Ng and performance in several cognitive tasks. $Ng^{-/-}$ mice perform poorly in all such tasks; they also display deficits in high-frequency stimulation (HFS)–induced long-term potentiation (LTP) in area CA1 of hippocampal slices but enhanced low frequency–induced long-term depression (LTD). Measurements of Ca^{2+} transients in CA1 pyramidal neurons following weak and strong tetanic stimulations revealed a significantly greater $[Ca^{2+}]_i$ response in $Ng^{+/+}$ than in $Ng^{-/-}$ mice. The diminished Ca^{2+} dynamics in $Ng^{-/-}$ mice is a likely cause of the animals' decreased propensity to undergo LTP and of their deficits in cognitive function.

An immunohistochemical study showed that, in the wild-type mice, Ng was localized in the cell bodies and dendrites of the principal neurons in the hippocampus, whereas CaM concentrated in the nucleus, with a relatively low level in the dendrites. However, following HFS of the Schaffer/collateral fibers of the hippocampal CA1 region, CaM became concentrated in the dendritic spines where it co-localized with Ng. The accumulation of Ng and CaM in the spines may serve as tags for the stimulated neuronal network to increase synaptic efficacy. Given that the HFS-induced LTP is initiated by Ca^{2+} influx through NMDA receptors, we reasoned that stimulation of postsynaptic downstream signaling components or increasing presynaptic transmitter release may rescue the deficits of $Ng^{-/-}$ mice. We focused on the PKC and PKA signaling pathways as well as on gene transcription. Treatment of hippocampal slices of $Ng^{-/-}$ mice with phorbol ester, which enhances presynaptic responses by increasing neurotransmitter release, induced robust LTP in $Ng^{-/-}$ mice. Short-term (5-minute) application of forskolin (an adenylate cyclase activator), rolipram (a phosphodiesterase inhibitor), and picrotoxin (a $GABA_A$ inhibitor) to $Ng^{-/-}$ hippocampal slices also effectively induced LTP. By stimulating cAMP/PKA signaling and suppressing the $GABA_A$-mediated inhibitory pathway, these drugs could bypass the requirement of Ng to induce LTP. Histone deacetylase (HDAC) inhibitors are known to enhance both memory and synaptic plasticity at transcriptional level. Bath application of an HDAC inhibitor, trichostatin A, could also augment the HFS-induced LTP in the $Ng^{-/-}$ hippocampal slices. Using these chemically induced LTP protocols, we have established a potential drug treatment regimen to ameliorate the behavioral deficits of $Ng^{-/-}$ mice.

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Effect of environmental enrichment and drug treatment on the cognitive behaviors of neurogranin knockout mice

Huang F, Huang K-P

Synaptic responses triggering LTP or LTD depend on the amplitude of calcium influx and the sensitivity of the transduction machinery to amplify the signal. Ng knockout (KO) mice performed poorly in cognitive behavior tasks and exhibited deficits in LTP and activation of CaMKII (calcium/calmodulin-dependent protein kinase II) by autophosphorylation. More specifically, deletion of Ng in mice caused severe deficits in learning and memory of spatial tasks, in induction and maintenance of LTP, and in amplification of Ca^{2+}-mediated signaling in the hippocampus. However, environmental enrichment (EE) can increase neurogenesis, alter neural plasticity, and improve cognitive performance. To that end, we attempted to employ EE and drug treatment to improve the cognition and behavioral abnormalities of the mutant mice.

To determine if an increase in the physical activities of the mutant mice can improve their performance of memory tasks, we transferred groups of mutant mice and their wild-type littermates of different age groups to roomier cages, each with several toys and a running wheel for voluntary exploration and exercise. The control groups were housed in their regular home cages without any toys. Provision of short-term environmental enrichment (SEE) (3 weeks for the mutant mice) did not improve their performance but did benefit the wild-type and heterozygous mice, whose hippocampal Ng levels and LTP were higher under SEE than in control mice. Interestingly, for Ng KO mice, SEE caused a negligible effect on their LTP even though other important signaling components for synaptic plasticity, including CaMKII and cAMP-responsive element–binding protein (CREB), were elevated to the same levels as in the wild-type and heterozygous mice. In contrast, a long-term environmental enrichment (LEE) for the aging mice benefited the Ng KO as well as the wild-type and heterozygous mice by preventing age-related cognitive decline. LEE also caused an increase in the hippocampal CREB level of all three genotypes and the Ng level of wild-type and heterozygous mice but not the levels of CaMKII or ERK (extracellular signal-regulated kinase). Hippocampal slices from the enriched aging Ng KO mice, unlike those from the wild-type

and heterozygous mice, did not show enhancement in LTP. It appears that the learning and memory processes in the enriched aging Ng KO mice do not correlate with LTP, which is facilitated by Ng. The results indicate that LEE for the aging Ng KO mice may improve their cognitive function through an Ng-independent plasticity pathway. To improve the behavioral deficits of young adult Ng KO mice, we treated the animals with Ritalin®, which has proven beneficial for human patients suffering from ADHD. Short-term treatment (up to 1 month) of the mutant mice with Ritalin® in combination with EE appeared to improve their cognitive function and reduced their anxiety and hyperactivity behaviors. These Ng mutant mice may serve as a useful model for investigating the cause and treatment strategy for ADHD.

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Ischemia-elicited oxidative modulation of CaMKII in the brain

Shetty, Huang F, Huang K-P

CaMKII is one of the major Ca^{2+}-sensing enzymes involved in transducing neuronal, hormonal, and electrical signals in brain, heart, and other tissues. In the central nervous system, CaMKII plays a pivotal role in facilitating synaptic plasticity, learning and memory, and activity-dependent developmental processes. The CaMKII holoenzyme is a dodecamer composed of two stacked hexameric rings, and the kinase undergoes inter-subunit autophosphorylation in the presence of Ca^{2+}/CaM that converts the kinase into an activator-independent autonomous enzyme. The autophosphorylation also leads to increased affinity of the kinase for several proteins near the sites of elevated Ca^{2+}, with functional consequences. In the brain, translocation and aggregation of CaMKII have been implicated in NMDA receptor–dependent enhancement of synaptic plasticity as well as in neurological disorders associated with ischemic injury and seizure. The mechanism for this activity-dependent aggregation of CaMKII is not entirely clear. We hypothesize that excessive oxidants generated during ischemic and excitotoxic stress may trigger the aggregation of CaMKII.

Previously, we showed that activation of NMDA receptors or direct administration of oxidants to brain slices could trigger thionylation (oxidation) of neurogranin and formation of intramolecular disulfide. We hypothesized that the oxidant-induced modification of protein resulted from oxidation by the endogenously produced glutathione disulfide S-oxides (GS-DSO). To investigate the mechanism and physiological response of CaMKII oxidation, we treated mouse brain synaptosomes with H_2O_2, diamide, and sodium nitroprusside. These oxidants caused aggregation of CaMKII through formation of disulfide and non-disulfide linkages and partial inhibition of the kinase activity. We found that CaMKII aggregates associated with the postsynaptic density. However, treatment of purified CaMKII with the oxidants did not replicate the effects observed in the synaptosomes. Using two previously identified potential mediators of oxidants in the brain, glutathione disulfide *S*-monoxide (GS-DSMO) and glutathione disulfide *S*-dioxide (GS-DSDO), we showed that they oxidized and inhibited CaMKII in a manner similar to the oxidant-treated synaptosomes—reminiscent of oxidative stress of acutely prepared hippocampal slices elicited by ischemia. Interestingly, the autophosphorylated and activated CaMKII was relatively refractory to GS-DSMO– and GS-DSDO–mediated aggregation. Short-term ischemia (10 minutes) caused a depression of basal synaptic response of the hippocampal slices, and re-oxygenation (after 10 minutes) reversed the depression. However, oxidation of CaMKII remained above the pre-ischemic level throughout the treatment. Oxidation of CaMKII also prevented full recovery of CaMKII autophosphorylation after re-oxygenation. Subsequently, the HFS-mediated synaptic potentiation in the hippocampal CA1 region was significantly lower than in the control without ischemia. Thus,

ischemia-evoked oxidation of CaMKII, probably via the action of glutathione disulfide *S*-oxides or their analogues, may be involved in the suppression of synaptic plasticity.

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SECRETORY PROTEIN TRAFFICKING AND GRANULE BIOGENESIS IN NEUROENDOCRINE CELLS AND ROLE OF CARBOXYPEPTIDASE E SPLICE ISOFORM IN TUMOR GROWTH AND METASTASIS

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We study the cell biology of endocrine and neuroendocrine cells, focusing on the mechanisms of biosynthesis and intracellular trafficking of peptide hormones and neuropeptides and their processing enzymes, the regulation of dense-core secretory granule biogenesis, and the physiological roles of the prohormone processing enzyme carboxypeptidase E. We discovered novel molecular mechanisms of protein trafficking to the regulated secretory pathway and identified players and mechanisms that control secretory granule biogenesis and transport in endocrine and neuroendocrine cells and neurons. Using cell lines, primary cell cultures, and mouse models, we have gained a better understanding of diseases related to defects in hormone and neuropeptide targeting, synaptic transmission, memory, and learning and of diabetes and obesity. Recently, we discovered a splice isoform of carboxypeptidase E (CPE-ΔN) that is highly expressed in metastatic tumor cells. We are investigating the mechanism by which CPE-ΔN promotes tumor growth and metastasis as well as the use of CPE-ΔN as a biomarker for metastatic potential of primary tumors and a therapeutic target for malignant tumors. Studies in human tumor cell lines, primary and metastatic tumors, and mouse models have provided new insights into the mechanism of tumor metastasis, facilitating the identification of a potential therapeutic target for suppressing tumor metastasis.

Mechanism of sorting pro-neuropeptides, neurotrophins, and their processing enzymes to the regulated secretory pathway

Cawley, Lou, Rathod, Young, Loh; in collaboration with Baum

The intracellular sorting of pro-neuropeptides and neurotrophins to the regulated secretory pathway (RSP) is essential for the processing, storage, and release of active proteins and peptides in the neuroendocrine cell. We investigated the sorting of pro-opiomelanocortin (POMC, pro-ACTH/endorphin), proinsulin, and brain-derived neurotrophic factor (BDNF) to the RSP. We showed that, in a concentration step, these pro-proteins undergo homotypic oligomerization as they traverse the cell from the site of synthesis in the endoplasmic reticulum to the trans-Golgi network (TGN), where they are sorted into dense-core granules of the RSP for processing by prohormone convertases (PC) and carboxypeptidase E (CPE) and then secreted. Site-directed mutagenesis studies identified a consensus sorting motif consisting of two acidic residues 12 to 15Å apart exposed on the surface of the pro-proteins molecules and two hydrophobic residues at a distance of 5 to 7Å from the acidic

residues, with both residue types needed for sorting to the RSP. BDNF, which is secreted in an activity-dependent manner, has such a consensus sorting motif, but constitutively secreted nerve growth factor (NGF) lacks one amino acid residue for completion of the motif. Introduction of the missing residue by mutagenesis (Val20Glu) redirected NGF to the RSP, further confirming the importance of the sorting motif in targeting to the RSP.

We identified an RSP sorting receptor specific for the sorting signal of POMC, proinsulin, and BDNF as the transmembrane form of CPE. The two acidic residues in the prohormone/pro–BDNF sorting motif specifically interact with two basic residues, R255 and K260, of the sorting receptor CPE to bring about sorting to the RSP. Using a CPE knockout (KO) mouse model, we showed that BDNF was not sorted to the RSP but rather was secreted constitutively in cortical and hippocampal neurons of the CPE KO mice. We observed that, in these mice, constitutive secretion of proinsulin from isolated pancreatic islets was elevated and plasma levels of proinsulin were significantly higher, indicating a role of CPE in the intracellular trafficking of proinsulin. Recent studies of POMC sorting in primary anterior pituitary cultures from CPE KO mice showed a defect in the regulated secretion of ACTH, consistent with CPE's role as a sorting receptor in vivo; however, we did observe some stimulated secretion of POMC.

Further investigation of the pituitaries revealed elevated expression of secretogranin III (SgIII), a lipid raft–associated molecule found in the TGN. Previous work reported in the literature has shown that SgIII binds to POMC, albeit with lower affinity than to CPE, and can potentially act as a sorting receptor for POMC. Thus, SgIII appears to be expressed at higher levels as a compensatory sorting receptor for POMC in some CPE KO mice. Our studies provide evidence of a sorting signal/receptor–mediated mechanism for targeting prohormones, pro-neuropeptides, and the neurotrophin BDNF to the RSP in endocrine cells and neurons.

In collaboration with Bruce Baum, we investigated the secretory behavior of peptide hormones in the exocrine salivary gland. The salivary gland is a target tissue for the expression of proteins for gene therapy as it secretes proteins into the upper GI tract via the RSP and into the circulation via the constitutive secretory pathway. Manipulation of sorting signals may be useful in directing therapeutic proteins into a specific pathway. Previously, we generated a bioactive mutant form of human growth hormone (hGH) and demonstrated that we could partially redirect it into the constitutive pathway and thus to the circulation of rats. More recently, the fusing of hGH to erythropoietin (Epo), a protein secreted constitutively from salivary gland cells, allowed the Epo to be targeted to the saliva via the RSP in both mouse and rat model systems, demonstrating a proof of concept that a therapeutic protein without an RSP sorting signal can be directed into the RSP by coupling it with hGH. We took a similar approach with proinsulin and found that a mutant proinsulin, which does not sort efficiently to the RSP in endocrine cells but is secreted constitutively, is secreted into the circulation and produces glucose-lowering effects in mice.

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The CPE knockout mouse exhibits neurodegeneration, behavioral deficits, and aberrant synaptic transmission

Woronowicz, Koshimizu, Cawley, Lou, Murthy, Loh; in collaboration with Wetsel

By deleting exons 4 and 5 from the *CPE* gene, we generated CPE KO mice and characterized their phenotype. The KO mice became obese by 10 to 12 weeks of age and weighed 60 to 80 g by 40 weeks. The null animals consumed more food, were less physically active during the light phase of

the light-dark cycle, and burned fewer calories as fat than wild-type (WT) littermates. The animals' fasting levels of glucose and insulin-like immunoreactivity (IR) in plasma were elevated at around 20 weeks; males recovered from the elevated levels by 32 weeks while the females did not. At 32 weeks, the plasma insulin-like IR, which consists of primarily proinsulin, was 50 to 100 times higher in the KO mice than in WT animals. The KO mice showed impaired glucose clearance and were insulin-resistant. They also exhibited high levels of leptin in plasma but showed no circulating fully processed CART, a peptide responsive to leptin-induced feedback inhibition of feeding. Besides the obesity and diabetes phenotypes, the KO mice were subfertile and showed deficits in GnRH processing in the hypothalamus.

Behavioral analyses revealed that adult KO mice had diminished reactivity to stimuli and reduced toe-pinch reflexes, muscle strength, coordination, and visual placing. Moreover, they showed delayed learning and poor memory consolidation in the water-maze and object-preference tests. Electrophysiological studies indicated an inability to generate long-term potentiation when the CA1 region of the hippocampus was stimulated. Analysis of brain sections revealed that, at 4 weeks of age and older, the KO mice had an abnormal hippocampal structure, including the absence of the CA3 region. Interestingly, postnatal KO animals at 3 weeks and younger showed a normal hippocampus. Thus, 3 weeks is a critical age at which the CA3 region undergoes degeneration in the absence of CPE. Moreover, the mossy fibers terminated prematurely before reaching the CA1 region. Interestingly, Sholl analysis of hippocampal CA1 region neurons showed that the complexity of dendritic arborizations of neuronal cells is more pronounced in CPE-KO mice than in WT littermates, suggesting enhanced dendrite formation coupled with lack of dendritic pruning. Our observations indicated that, in the adult animal, CPE plays an essential role in maintaining neuronal survival and the structure of the hippocampus. We further demonstrated the neuroprotective role of CPE in ex vivo experiments. Overexpression of CPE in hippocampal neurons resulted in protection of the neurons from apoptosis after hydrogen peroxide–induced oxidative stress.

Electron microscopic studies of the hypothalamus revealed additional morphological abnormalities in the CPE-KO mouse. Forty percent of the synapses in the hippocampus of the CPE KO mice showed an absence of presynaptic docked synaptic vesicles. In addition stimulated release of glutamate from embryonic hypothalamic neurons and adult hypothalamic synaptosomes was impaired. The mice also exhibited abnormal glutamate-mediated neurotransmission from the photoreceptors to the inner retina, showing a loss of the b wave in their retinogram. Thus, CPE appears to play a critical role in synaptic transmission, possibly by facilitating synaptic vesicle docking and exocytosis at the synapse (see preliminary evidence below). Absence of CPE in the KO mice therefore leads to failure in neurotransmission, deficits in learning and memory, and abnormal behavior and neurodegeneration.

Woronowicz A, Koshimizu H, Chang S-Y, Cawley N, Hill J, Rodriguiz RM, Abebe D, Dorfman C, Senatorov V, Zhou A, Xiong Z-G, Wetsel WC, Loh YP. Absence of carboxypeptidase E leads to adult hippocampal neuronal degeneration and memory deficits. *Hippocampus* 2008;18:1051-1063.

Role of CPE in vesicle transport, docking, and secretion in endocrine cells and neurons
Park, Lou, Phillips, Cawley, Loh

Post-Golgi transport of hormone and BDNF vesicles for activity-dependent secretion is important in mediating endocrine function and synaptic plasticity. We examined the role of the cytoplasmic tail of vesicular transmembrane CPE in the transport of POMC/ACTH and BDNF vesicles in the endocrine corticotrophic cell line AtT-20 and hippocampal neurons, respectively. Overexpression of the CPE cytoplasmic tail diminished localization of endogenous POMC and BDNF in the processes of AtT-20 cells and hippocampal neurons. Furthermore, live-cell imaging showed that overexpression of the CPE tail decreased both the velocity and processivity of POMC and BDNF vesicle movement in live primary anterior pituitary cells and hippocampal neurons. Our findings demonstrated that

the CPE tail is involved in the processive trafficking of POMC and BDNF vesicles to the plasma membrane for secretion. We then performed pulldown experiments using both AtT-20 cell and mouse brain cytosol in vitro and showed that the cytoplasmic tail of CPE interacted with the motor adaptor protein dynactin and microtubule motors kinesin-2, kinesin-3, and cytoplasmic dynein. Moreover, competition assays using a CPE tail peptide verified specific interaction between the CPE tail and dynactin. Thus, the mechanism for the transport of POMC and BDNF vesicles to the release site for activity-dependent secretion in endocrine cells and neurons requires the interaction of vesicular CPE cytoplasmic tail to anchor these organelles to the microtubule motors.

Recently, we demonstrated in CPE KO mice that high K^+–stimulated secretion of glutamate was absent from cultured primary embryonic hypothalamic neurons (E16) and adult mouse synaptosomes, suggesting the presence of CPE in WT glutamate vesicles and CPE's involvement in mediating the release of glutamate. Indeed, subcellular fractionation studies revealed that a synaptophysin-enriched synaptic vesicle fraction from mouse brain contained both CPE and the vesicular glutamate transporter Vglut2, but not the dense-core granule–specific protein marker chromogranin A (CgA). Moreover, intact synaptic vesicles could be labeled with extreme C-terminal CPE IgGs but not with non-specific IgGs. As in the case of peptidergic dense-core vesicles, our findings pointed to the existence of a population of synaptic vesicles that contain transmembrane CPE with a cytoplasmic tail. In GST (glutathione-*S*-transferase) pulldown assays of mouse brain cytosol, we found that GST tagged the CPE cytoplasmic tail CPE_{C10} and bound to Rab27A and Rim1, proteins necessary for vesicle docking to the plasma membrane. Thus, our findings suggest a function of the vesicular CPE cytoplasmic tail in the recruitment of Rab27A and Rim1 for docking of glutaminergic vesicles to the plasma membrane for exocytosis in the hypothalamus.

Yeast two-hybrid studies uncovered additional functions of the CPE cytoplasmic tail and showed that the CPE tail interacted with γ-adducin, as confirmed in precipitation studies in vitro. It is known that γ-adducin is primarily a component of the cytoskeleton that binds to and stabilizes F-actin and that it mediates actin binding to fodrin. To assess the significance of the CPE tail–γ-adducin interaction in a functional system, we overexpressed the last 25 amino acids of the CPE C-terminal domain (CPE25) in the cytoplasm of AtT-20 cells. Overexpression of the CPE_{C25} peptide reduced localization of γ-adducin to the processes of AtT-20 cells, suggesting that the interaction with the CPE cytoplasmic tail targets γ-adducin to the cells' processes. It is possible that γ-adducin binds to one of the vacant CPE cytoplasmic tails on the vesicles that are transported to the processes by microtubule-based motors. We hypothesize that, linked to the CPE tail, γ-adducin participates in the tethering of ACTH vesicles to cortical actin just below the plasma membrane in readiness for priming, docking, and release upon stimulation.

In addition, overexpression of the full-length γ-adducin induced formation of long processes in a non-neuronal cell line COS-7. By inducing intracellular cytoskeleton rearrangement, γ-adducin may play an important role in promoting neurite outgrowth in the nervous system.

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Regulation of secretory granule biogenesis by a chromogranin A–derived peptide
Koshimizu, Cawley, Loh; in collaboration with Yergey

Formation of large dense-core granules (LDCG) at the TGN is essential for regulated secretion of hormones and neuropeptides from neuroendocrine cells. Our previous studies uncovered an on/off switch, chromogranin A (CgA), that controls the formation of LDCGs in neuroendocrine cells.

Depletion of CgA in rat PC12 cells using antisense technology resulted in the loss of both LDCGs and regulated secretion and in degradation of granule proteins, including CgB and synaptotagmin. Overexpression of bovine CgA in these cells rescued the WT phenotype. In 6T3, a mutant endocrine cell line lacking CgA, LDCGs, and regulated hormone secretion, transfection of CgA resulted in the rescue of granule biogenesis and the RSP.

The importance of CgA in LDCG biogenesis was evident not only in cell lines but also in vivo. In the adrenal medulla of an antisense mRNA transgenic mouse model deficient in CgA, we observed both quantitative and qualitative severely aberrant granule formation as well as a correlation between the amount of depletion of CgA and the reduction in secretory granule biogenesis. Reduction in granule proteins accompanied the reduction in secretory granule biogenesis in both the adrenal medulla of the CgA-deficient transgenic animals and 6T3 cells lacking CgA. Using 6T3 cells lacking secretory granules as a model, we showed that the reduced granule protein levels were attributable to degradation occurring at the Golgi apparatus.

We therefore proposed that regulation of the stability of granule proteins at the Golgi apparatus by CgA is a focal point for control of granule biogenesis in neuroendocrine cells. In support of our hypothesis, we have found in the Golgi, protease nexin-1 (PN-1), a protease inhibitor that is transcriptionally activated by CgA and upregulated in cells actively forming LDCGs; however, PN-1 is downregulated in cells minimally expressing CgA and exhibiting low levels of LDCG biogenesis. Moreover, transfection of PN-1 into 6T3 cells lacking CgA prevented LDCG protein degradation and rescued granule biogenesis. Furthermore, downregulation of expression of PN-1 by antisense RNA in 6T3 cells transfected with CgA resulted in enhanced degradation of granule proteins and reduced secretory granule formation. Recently, we showed that 6T3 cells incubated with conditioned medium from 6T3-bCgA cells or AtT-20 cells exhibited an increase in PN-1 mRNA and granule biogenesis. Likewise, stimulation of AtT-20 cells with high potassium also resulted in an increase in PN-1 mRNA, which was blocked by actinomycin D. We hypothesized that secreted CgA, or a fragment of CgA, might signal the cell to increase levels of PN-1 in response to reduced LDCGs after stimulated secretion. We have now identified a 26–amino acid C-terminal fragment of CgA in the secretion medium of AtT-20 cells, which we named serpinin, that enhanced PN-1 transcription and granule biogenesis in 6T3 cells. Serpinin was elevated in the medium after high K^+ stimulation of AtT-20 cells. Thus, we have discovered that serpinin, a new CgA-derived peptide, is co-secreted with POMC-derived hormones upon stimulation of pituitary corticotrophs and signals replenishment of LDCGs by transcriptionally upregulating the expression of PN-1. PN-1 then stabilizes granule proteins, leading to increased LDCG biogenesis.

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Role of Aquaporin 1 in hormone secretion and secretory granule biogenesis
Arnaoutova, Cawley, Rathod, Kim, Loh

We found that the water channel protein aquaporin 1 (AQP1), which is normally present in the plasma membrane, is also localized in secretory granules of endocrine cells. Moreover, when we transfected 6T3 cells lacking CgA with CgA, which rescues granule biogenesis in these cells, the expression of AQP1 mRNA and protein rose significantly. To investigate the role of AQP1 in hormone sequestration and granule biogenesis, we stably transfected AtT-20 cells with an antisense construct of AQP1 to downregulate expression of the protein. AQP1-deficient AtT-20 cells showed a dramatic reduction of secretory granules. Pulse-chase studies demonstrated a defect in regulated

secretion of the endogenous ACTH hormone as well as increased degradation of newly synthesized granule proteins such as POMC and CPE at the Golgi apparatus. However, the deficiency of AQP1 in these cells did not affect the transcription and translation of the proteins. AQP1 appears to be critical for the mechanism of secretory granule exocytosis. A defect in secretion leads to a feedback to down-regulate secretory granule biogenesis. We used AQP1-knockout mice in vivo in cellular studies to verify our conclusions. Examination of the animals' pituitary content and serum revealed lower levels of ACTH in tissue and the circulation than in control mice, consistent with deficits in secretion and storage of ACTH in granules of the pituitary gland. Thus, AQP1 is necessary for maintaining hormone secretion and granule biogenesis in endocrine cells.

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Carboxypeptidase E regulates growth and metastasis in tumor cell lines
Murthy, Cawley, Lou, Loh; in collaboration with Lee, Poon, Ng, Hewitt, Pacak

Elucidation of molecules that control cancer cell growth and invasion will greatly facilitate the identification of biomarkers that can predict impending tumor metastasis while providing targets for therapy. Despite the huge repertoire of biomarkers reported to be useful in identifying aggressive tumors and predicting prognosis, for most cancers there is a dearth of reliable markers for predicting future metastasis from biopsies or resected primary tumors. Recently, we identified a new molecule, an alternatively spliced isoform of carboxypeptidase E (CPE-ΔN) that lacks the N-terminus present in the WT CPE. Human tumor cell lines from liver, colon, breast, prostate, colon, head, and neck that are highly metastatic showed higher expression of CPE-ΔN than did matched tumor lines with low metastatic potential. We also demonstrated that CPE-ΔN plays a role in promoting growth and cell invasion in human cancer cells. Overexpression of CPE-ΔN in the low-metastatic human hepatocellular carcinoma cells (HCC) MHCC97L significantly increased cell proliferation and migration by upregulating expression of a metastasis gene. When si-RNA downregulated CPE-ΔN expression by 56 to 85 percent in highly metastatic cell lines from breast (MDA-MB-23), prostate (DU145), head and neck (MDA 1986), colon (HT29), and liver (MHCC97M3), the si–CPE treated cells showed 56 to 85 percent inhibition. We used the matrigel invasion assay to assess the invasion capability of the tumor cell lines transduced with CPE si-RNA. In all the tumor lines, suppression of CPE expression led to a 70 to 85 percent inhibition of invasion. To complement these observations, we performed in vivo animal studies. In one study, we injected nude mice subcutaneously with MHCCLM3 cells transduced with either si-CPE-ΔN or si-scr. Thirty days after cell inoculation, control mice bearing the si-scr MHCCLM3 cells had much larger liver tumors than did mice injected with si-CPE-ΔN cells. Using a metastatic orthotopic nude mouse model, we subcutaneously injected into the animals' flank MHCCLM3 cells transduced with either si-scr or si-CPE-ΔN. We then removed the subcutaneous tumor, cut it into 1 to 2 mm cubes, and implanted it into the liver of nude mice. Thirty-five days post-implantation, mice with the si-scr-MHCCLM3–derived tumors developed intrahepatic metastasis and extrahepatic metastasis to the lung while mice inoculated with si-CPE-ΔN-MHCCLM3–derived tumors had smaller tumors and failed to demonstrate metastasis. Our in vitro and in vivo results demonstrated that CPE-ΔN is a newly discovered mediator of metastasis of several human tumor cells.

Clinical use of CPE-ΔN as a prognostic biomarker for metastasis and a therapeutic target
Murthy, Loh; in collaboration with Hewitt, Pacak, Lee, Poon, Ng

To determine if CPE-ΔN is a useful biomarker for predicting recurrence and metastasis, we analyzed two retrospective cohorts of patients in blinded studies. Using quantitative RT-PCR and Western blots, we measured CPE-ΔN mRNA and protein levels in primary tumors from HCC and colon cancer patients. RT-PCR verified that only CPE-ΔN mRNA but not WT CPE mRNA was expressed in primary HCC or colonic tumors. Using qRT-PCR, we compared CPE-ΔN mRNA in the primary

tumor (T) with that in surrounding non-tumor (N) tissue and determined the ratio (T/N). In 89.8 percent of HCC patients who were disease-free one year after surgery, their CPE-ΔN mRNA ratio was less than or equal to 2, whereas 92 percent of patients with extra- or intrahepatic metastasis/recurrence exhibited a T/N ratio greater than 2. The Kaplan-Meier plot of disease-free survival of 99 HCC patients showed shorter survival times when CPE-ΔN mRNA T/N was greater than 2 in the primary tumor compared with patients with T/N less than or equal to 2. We obtained similar results for 68 colon cancer patients. Western blots also showed significantly higher CPE-ΔN levels in HCC patients with recurrence than in the disease-free group. Immunohistochemistry of CPE-ΔN in HCC tumors from 37 HCC patients revealed immunostaining primarily in the nuclei of tumor cells in patients who subsequently developed recurrence; the immunostaining was absent in cell nuclei of patients who remained disease-free. We found the intensity of immunostaining, as determined by image analysis, to differ statistically between the groups. Thus, CPE-ΔN is a powerful prognostic biomarker for predicting future metastasis not only for HCC or colonic but potentially for other types of cancer as well.

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NEUROTROPHIC REGULATION OF SYNAPSE DEVELOPMENT AND PLASTICITY

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Our laboratory focuses on the role of neurotrophins, a class of secretory proteins critical for synapse development and plasticity. As one of the first laboratories to elucidate the synaptic functions of neurotrophins, we discovered that (1) brain-derived neurotrophic factor (BDNF) promotes the development of early-phase long-term potentiation (E-LTP) in the hippocampus mediated by an enhanced response to tetanic stimulation through vesicle docking; (2) BDNF elicits synapse-specific modulation by regulating TrkB receptor trafficking through activity-dependent insertion, endocytosis, synaptic localization, and so forth; (3) neurotrophic regulation occurs via two modes: *acute* modulation of synaptic transmission and plasticity and *long-term* alteration of the structure and function of synapses; (4) a single-nucleotide polymorphism (SNP) in the pro-domain of BDNF affects activity-dependent BDNF secretion, resulting in impairment in hippocampal function and short-term memory in humans; (5) extracellular conversion of proBDNF to mature BDNF by protease tPA/plasmin is essential for late-phase LTP (L-LTP), a cellular model for long-term memory; and (6) proBDNF, if uncleaved, facilitates hippocampal long-term depression (LTD) by activating the $p75^{NTR}$ receptor. Over the past year, we made significant progress in revealing several new aspects of neurotrophin functions.

Distinct role of long 3′UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal pyramidal neurons

Woo,[1] Lu; in collaboration with An, Gharami, Liao, Vanevski

A common and yet puzzling phenomenon in cell biology is the existence of transcripts that code for exactly the same protein but bear different 3′ untranslated regions (3′UTR). One such example is the messenger RNA for BDNF. There are two pools of BDNF mRNA in neurons with similar abundance: one with a short 3′UTR and the other with a long 3′UTR. It was not known why a neuron would need two mRNAs of different length encoding the same BDNF protein. We have now provided evidence suggesting that mRNAs of different 3′UTR confer different functions because of their distinct subcellular localization. We show that the short 3′UTR BDNF mRNAs are restricted to neuronal cell bodies, whereas the long 3′UTR BDNF mRNAs are also localized in dendrites. In a mouse mutant in which the long 3′UTR is truncated, dendritic targeting of BDNF mRNAs is impaired. We observed little BDNF in hippocampal dendrites despite normal levels of total BDNF protein. The mutant exhibits enlargement of dendritic spines and deficits in pruning as well as

selective impairment in long-term potentiation in dendrites, but not in somata, of hippocampal neurons. These results are significant for several reasons. First, they suggest a cellular mechanism whereby BDNF, an important regulator for brain development and plasticity, might elicit diverse cellular functions ranging from cell survival to synaptic transmission. Second, the results provide clear experimental evidence that dendritically localized mRNAs play crucial roles in regulating spine morphology, a long-sought result in the field of local protein synthesis and synaptic plasticity. Finally, to our knowledge, we have for the first time demonstrated that different transcripts with the same coding sequence play distinct roles in a cell.

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Activity-dependent BDNF transcription and secretion

Sakata,[2] Woo,[1] Nagappan, Zaitsev,[3] Lu; in collaboration with Martinowich, Shen, Enfors

In one study, we address the role of activity-dependent BDNF gene expression. Transcription of the BDNF gene is controlled by several promoters that drive the expression of several transcripts encoding the same protein. Promoter-IV contributes significantly to activity-dependent BDNF transcription. We generated promoter-IV–mutant mice (BDNF-KIV) by inserting the *GFP* gene followed by a stop codon into exon IV. This genetic manipulation resulted in disruption of promoter-IV–mediated BDNF expression, particularly in the prefrontal cortex (PFC), an area involved in working memory and other executive functions. Interestingly, the BDNF-KIV exhibited a selective impairment in parvalbumin-positive GABAergic neurons and inhibitory but not excitatory postsynaptic currents in the PFC, leading to an aberrant spike-timing–dependent synaptic potentiation (STDP). Behaviorally, BDNF-KIV mice are significantly impaired in PFC-mediated reversal learning and fear memory extinction, but not in working memory. Our results demonstrated the importance of promoter-IV–dependent BDNF transcription in GABAergic function and revealed an unexpected role of BDNF in behavioral perseverance. Our study may shed light on the pathogenesis of several cognitive disorders in which perseverance is prominent, including schizophrenia and post-traumatic stress disorder (PTSD).

In another study, we addressed the role of activity-dependent BDNF gene expression. We previously showed that pro- and mature BDNF often elicit opposing biological effects. For instance, mature BDNF (mBDNF) is critical for LTP induced by high-frequency stimulation (HFS), whereas proBDNF facilitates LTD induced by low-frequency stimulation (LFS). Given that mBDNF is derived from proBDNF by endoproteolytic cleavage, mechanisms regulating the cleavage of proBDNF may control the direction of BDNF regulation. Using methods that selectively detect proBDNF or mBDNF, we showed that LFS induced predominantly proBDNF secretion in cultured hippocampal neurons. In contrast, HFS preferentially increased extracellular mBDNF. Inhibition of extracellular, but not intracellular, cleavage of proBDNF greatly reduced HFS-induced extracellular mBDNF. Moreover, HFS, but not LFS, selectively induced the secretion of tPA, an important protease involved in extracellular conversion of proBDNF to mBDNF. Thus, high-frequency neuronal activity controls the ratio of extracellular proBDNF to mBDNF by regulating the secretion of extracellular

proteases. Our study demonstrated activity-dependent control of extracellular proteolytic cleavage of a secretory protein and revealed an important mechanism that controls diametrically opposed functions of BDNF isoforms. In addition to revealing for first time how neuronal activity can control the cleavage of a secreted protein, our work yielded useful tools for studying the distinct functions of proBDNF and mBDNF.

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Studies of genes involved in schizophrenia

Yang, Lu; in collaboration with Chang, Chen, Huffaker, Sambataro, Lipska, Kleinman, Weinberger

Conventional genetic linkage and association studies have confirmed that complex human diseases, such as diabetes and schizophrenia, involve several genes and that each gene elicits very small effects across populations. Moreover, given the complexity of genetic factors and their interaction with the environment, it is often difficult to link genetic findings with important biological or mechanistic aspects of an illness. To overcome these obstacles, we took a powerful translational approach that dissects the complex phenomenology of psychosis into several neural system and molecular components and maps genetic association onto several levels of analysis. Using this approach, we identified what may constitute a new class of schizophrenia susceptibility genes—*KCNH2,* which encodes a potassium channel and, more important, a novel isoform of this type of potassium channel that is primate- and brain-specific and whose levels are 2.5-fold elevated in schizophrenia brain tissue. Expression of this novel isoform is predicted by risk-associated SNPs in the gene, even in normal brain tissue; moreover, risk SNPs predict cognition and related brain physiology in normal subjects. Postmortem expression analysis shows a 2.5-fold increase in Isoform 3.1 over *KCNH2-1A* in the schizophrenic hippocampus. Structurally, Isoform 3.1 lacks most of the PAS (Per-ARNT-Sim) domain critical for slow channel deactivation. Electrophysiological characterization in primary cortical neurons revealed that overexpression of Isoform 3.1 results in a rapidly deactivating K^+ current and a high-frequency, non-adapting firing pattern. These results demonstrated a novel KCNH2 channel involved in cortical physiology, cognition, and psychosis, providing a potential new target for psychotherapeutic drugs**.**

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GLUTAMATE RECEPTOR STRUCTURAL BIOLOGY

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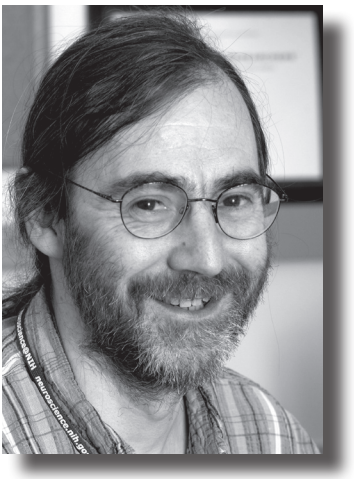

Ionotropic glutamate receptors (iGluR) are membrane proteins that act as molecular pores and mediate signal transmission at the majority of excitatory synapses in the mammalian nervous system. The seven gene families iGluRs in humans encode 18 subunits, which assemble to form three major functional families named after the ligands that were first used to identify iGluR subtypes in the late 1970s: AMPA, kainite, and NMDA. Given iGluRs' essential role in normal brain function and development and mounting evidence that dysfunction of iGluR activity mediates several neurological and psychiatric diseases and damage during stroke, we devote substantial effort to analyzing iGluR function at the molecular level. Atomic resolution structures solved by protein crystallization and X-ray diffraction provide a framework for designing electrophysiological and biochemical experiments aimed at defining the mechanisms underlying ligand recognition, the gating of ion channel activity, and the action of allosteric modulators. Information derived from these experiments will permit the development of subtype-selective antagonists and allosteric modulators with novel therapeutic applications and reveal the inner workings of a complicated protein machine that plays a key role in brain function.

Crystallographic and functional analysis of an allosteric binding site for sodium
Plested, Mayer; in collaboration with Biggin

Kainate-subtype glutamate receptors are strongly modulated by monovalent anions and cations. In the absence of either chloride or sodium, the receptors become nonfunctional. We used a combined experimental approach based on crystallography, patch-clamp recording, and all-atom molecular dynamics (MD) simulations to identify the binding site for sodium and the mechanism by which sodium modulates kainate receptor activity. We solved structures for the GluR5 ligand–binding domain dimer complex with lithium, sodium, potassium, rubidium, cesium, and ammonium ions in the cation-binding site. We observed two sodium binding sites in a dimer assembly (one per subunit); they flank the previously identified anion-binding site that lies on the molecular two-fold axis of symmetry. Sodium stabilizes the dimer assembly in its active conformation, which is required for ion channel gating; in the absence of sodium, the receptors desensitize much faster. Sodium selectivity is conferred by a high electric field strength in the cation-binding site, but larger cations can bind with lower affinity. Functional studies show that the cation-binding site is allosterically coupled to the anion-binding site. All-atom MD simulations and free energy calculations reveal that the binding of chloride is favored by 3 to 5 kcal per mole when the cation-binding site is occupied by sodium. Mutational analysis and molecular modeling demonstrated that it is possible to convert

the sodium-binding site to a site with micromolar affinity for the divalent cations calcium and magnesium, namely by substituting an aspartate residue for a hydrophobic amino acid that caps the sodium-binding site in kainate receptors. AMPA receptors, which are insensitive to allosteric modulation by either sodium or calcium, harbor a lysine at this site. Amino acid sequence analysis indicated that the divergence between iGluRs with and without allosteric binding sites for sodium arose early in evolution. We were unable to crystallize a kainate receptor with the aspartate mutation and therefore made molecular models of the binding site. The model reveals a unique geometry, with three closely apposed carboxylate groups together with two backbone carbonyl oxygen atoms that provide ligands for binding to calcium similar to those found in the protein data bank for a diverse range of proteins with calcium binding sites.

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Molecular biophysical studies on kainate receptor dimer assembly

Chaudhry, Mayer; in collaboration with Rosenmund, Schuck

Direct measurement of the effects of allosteric ions on dimer assembly by kainate subtype iGluRs is not possible with electrophysiological techniques. To obtain proof that allosteric ions regulate dimer formation, we developed a series of GluR6 dimer interface mutations remote from the ion-binding sites and set out to develop a preparation amenable to analysis by analytical ultracentrifugation (AUC). We designed the mutants on the basis of crystal structures for wild-type GluR5 dimers, using electrophysiological analysis to search for a phenotype with slowed kinetics of desensitization. We prepared the isolated ligand-binding domains of the same mutants and measured their affinity for dimer formation by sedimentation velocity analysis over a range of protein concentrations and by sedimentation equilibrium. The most stable mutants increased dimer stability by at least 3 kcal per mole, with excellent agreement between the Kd for dimer assembly and the rate of desensitization measured in functional experiments. To explore the molecular basis for control of dimer assembly, we crystallized the wt GluR6 dimer together with a series of six mutants at resolutions of 1.2 to 1.5 Å. Analysis of these structures and of the effects of allosteric ions on dimer assembly measured by AUC is in progress.

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Structural studies on the amino-terminal domain of iGluRs

Kumar, Mayer

Glutamate receptor ion channels are multidomain membrane proteins that assemble from tetramers of approximately 440 kD. Numerous crystal structures have been solved for the ligand-binding domains, which have a molecular weight of approximately 30 kD per subunit—approximately one-quarter of the mass of an intact receptor. Extensive trials with bacterial expression systems, which with one exception have been used for all published ligand-binding domain structures, failed to produce monodisperse soluble protein for other iGluR domains. The amino terminal domain (ATD) is an important structural target because it controls subtype-selective assembly in native iGluRs, limiting assembly to members of the same functional family. Protein expression at levels sufficient for structural biology in mammalian cells is much more difficult than expression in *E. coli*, but has the advantages that several check points select for correctly folded proteins and add sugars and other post-translational modifications required for normal function. Although a variety of cell-biological and biochemical techniques are required to subsequently trim the sugar chains, in order to obtain

proteins that crystallize and diffract to high resolution, and the yields are lower than for prokaryotic expression, mammalian cell culture is the only current approach likely to succeed for studies of the ATD. In ongoing work, we have screened the ATDs from several iGluR subtypes for expression in mammalian cells. We have performed crystallization trials using a nanoliter pipetting robot and have obtained, for one diffraction, data to a resolution of 2.65 Å for a complete data set at APS. Structure solution and refinement is in progress.

Structural analysis of NR3 ligand binding selectivity

Yao, Mayer; in collaboration with Schulten

NR3-subtype glutamate receptors have a unique developmental expression profile but are the least characterized members of the NMDA receptor gene family; those members play key roles in synaptic plasticity and brain development. Using ligand-binding assays, crystallographic analysis, and all-atom MD simulations, we investigated mechanisms underlying the binding of NR3A and NR3B to glycine and D-serine, which are candidate neurotransmitters for NMDA receptors containing NR3 subunits. The ligand-binding domains of both NR3 subunits adopt a similar extent of domain closure to that found in the corresponding NR1 complexes but have a unique loop 1 structure distinct from those in all other glutamate receptor ion channels. Within their ligand-binding pockets, NR3A and NR3B have strikingly different hydrogen bonding networks and solvent structures from those in NR1 and fail to undergo a conformational rearrangement observed in NR1 upon binding to the partial agonist ACPC. Replica exchange MD simulations of 650 ns duration revealed numerous interdomain contacts that stabilize the agonist-bound closed-cleft conformation as well as a novel twisting motion for the loop 1 helix that is unique in NR3 subunits. Mutation of these sites destabilized ligand binding as measured by titration assays that use quenching of endogenous tryptophan fluorescence.

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HIPPOCAMPAL INTERNEURONS AND THEIR ROLE IN THE CONTROL OF NETWORK EXCITABILITY

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GABAergic inhibitory interneurons constitute a population of hippocampal neurons whose high degree of anatomical and functional divergence make them suitable candidates for controlling the activity of large populations of principal neurons. Interneurons (INT) receive strong excitatory glutamatergic innervation via numerous anatomically distinct afferent projections; recent evidence has demonstrated that the molecular composition of the AMPA-preferring class of glutamate receptors expressed at INT synapses is often distinct from that of receptors found at principal cell synapses. Our goal is to identify the types of ligand-gated ion- and voltage-gated ion channels—and the roles they play—found on neurons of the mammalian hippocampus and cortex. Specifically, we strive to understand the functions of these receptors in regulating synaptic and neuronal excitability, short- and long-term plasticity, and the development and pathophysiology of the mammalian cortical structure. Through this research, we envision a better understanding of the specific mechanisms of the regulation of excitability in precise cohorts of central nervous system cells and thus more targeted and valid methods of treatment of a wide array of central nervous system disease states.

State-dependent cAMP sensitivity of presynaptic function underlies metaplasticity in a hippocampal feedforward inhibitory circuit
Pelkey; in collaboration with Lacaille, Topolnik

Synapses may reside along a continuum of discrete plastic competency states dictated by their activation history; in turn, the states govern the polarity, magnitude, duration, and cell-signaling cascades invoked during future plasticity episodes. Metaplasticity is the term describing such higher-order regulation of the ability to induce synaptic plasticity; it may preserve homeostasis by preventing neural connections from accumulating within saturated states of potentiation or depression while enabling synaptic integration across prolonged temporal domains. Metaplasticity is well documented for postsynaptically expressed NMDAR-dependent forms of long-term plasticity; however, metaplasticity of presynaptically expressed forms of long-term plasticity has received less attention. Recently, we discovered a striking form of presynaptic metaplasticity at mossy fiber–stratum lucidum INT (MF-SLIN) synapses of the hippocampus (Pelkey et al., *Neuron* 2005;46:89). At naive MF-SLIN terminals, presynaptic mGluR7 activation during high-frequency stimulation (HFS) yields long-term depression (LTD). However, following agonist-induced internalization of mGluR7, the same HFS protocol results in a presynaptically expressed form of long-term potentiation (LTP). Thus, at these synapses, mGluR7 functions as a metaplastic switch whose surface expression and

activation govern bidirectional plasticity. Surprisingly, our recent findings indicate that mGluR7 activation/internalization controls the polarity of MF-SLIN plasticity by gating the cAMP sensitivity of MF-SLIN presynaptic release. While naive surface mGluR7–expressing MF-SLIN synapses are completely insensitive to cAMP elevation, synapses that have internalized mGluR7 robustly potentiate following cAMP increases. Moreover, HFS-induced LTP at MF-SLIN synapses that have internalized mGluR7 requires adenylate cyclase (AC) activity. Intriguingly, we also discovered an in vivo association between mGluR7 and RIM1α—a PKA substrate required for presynaptic LTP; the association is regulated by mGluR7 activation/internalization. These findings reveal that a state-dependent cAMP sensitivity controlled by mGluR7-RIM1α interactions underlies presynaptic MF-SLIN metaplasticity. This mGluR7-controlled reversal in polarity of MF-SLIN plasticity has dramatic consequences for hippocampal information propagation, as the MF-SLIN pathway provides a highly efficient feedforward inhibitory circuit controlling dentate granule cell–mediated recruitment of the auto-associative CA3 pyramidal cell network.

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Co-requirements of PICK1 binding and PKC phosphorylation for stable surface expression of mGluR7

Pelkey; in collaboration with Roche, Huganir

As described above, the presynaptic metabotropic glutamate receptor (mGluR) mGluR7 modulates excitatory neurotransmission by regulating neurotransmitter release and plays a critical role in certain forms of synaptic plasticity at the mossy fiber synapse. Although the dynamic regulation of mGluR7 surface expression governs a form of metaplasticity in the hippocampus, little is known about the molecular mechanisms regulating mGluR7 trafficking. We showed that mGluR7 cell-surface expression is stabilized by both protein kinase C phosphorylation and receptor binding to the PDZ domain–containing protein PICK1. Phosphorylation of mGluR7 on serine 862 (S862) inhibits CaM binding, thereby increasing mGluR7 surface expression and receptor binding to PICK1. Furthermore, in mice lacking PICK1, PKC-dependent increases in mGluR7 phosphorylation and surface expression are diminished, and mGluR7-dependent plasticity at mossy fiber-INT hippocampal synapses is impaired. These data support a model in which PICK1 binding and PKC phosphorylation act together to stabilize mGluR7 on the cell surface in vivo.

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Depolarization-induced LTD (DiLTD) at immature mossy fiber–CA3 pyramid synapses produced by postsynaptic burst-firing–like protocols

Ho, Pelkey; in collaboration with Huganir

Elucidation of the cellular mechanisms of synapse maturation is crucial to understanding central nervous system development. Mossy fiber–CA3 pyramid (MF–PYR) synapses develop postnatally, providing a tractable model of synapse maturation in a defined circuit. Recently, we demonstrated that, in early postnatal development, AMPARs supporting MF-PYR transmission undergo a subunit switch: immature synapses contain mixed populations of GluR2-lacking and GluR2-containing AMPARs and then mature to a state dominated by GluR2-containing AMPARs. A similar subunit

switch occurs during DiLTD, a postsynaptic form of plasticity observed only at immature MF-PYR synapses. Sustained PYR depolarization (3 to 5 minutes) triggers DiLTD to promote Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels (L-VGCCs). Interestingly, the developmental window of DiLTD competence coincides with the emergence of action potential (AP) burst firing (BF) in PYRs, suggesting an attractive physiological mechanism to induce DiLTD. Using BF-like protocols in acute hippocampal slices, we therefore investigated whether phasic AP-dependent L-VGCC activation can also trigger DiLTD. To elicit PYR BF, we repetitively injected a depolarizing current pulse (125 ms, 0.5 nA) at 1 Hz for 5 minutes in current clamp mode. The protocol reliably produced AP bursts (4 to 5 APs per pulse) and persistently depressed MF-PYR synapses. However, AP blockade by intracellular QX-314 did not prevent this LTD, suggesting that the protocol directly activates L-VGCCs independently of APs. Indeed, in the absence of APs, the current step typically depolarized PYRs sufficiently to activate L-VGCCs while the L-VGCC blocker nifedipine prevented LTD. To avoid direct L-VGCC activation, we delivered brief current pulses (5 pulses of 0.8 nA, 5 ms duration, 25 ms apart) at 1 Hz for 5 minutes. This paradigm produced robust LTD that was prevented by either intracellular QX-314 or the L-VGCC blocker nifedipine. Like DiLTD, AP-induced LTD exhibited postsynaptic expression. We conclude that DiLTD is induced by phasic L-VGCC activation driven by trains of PYR APs, suggesting a previously unsuspected role for PYR BF in MF-PYR synapse plasticity and maturation.

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The balance of excitatory and inhibitory synaptic currents is maintained during short-term plasticity in the CA3 hippocampus

Torborg

Strong facilitation during repetitive stimulation is a defining feature of the synapse between the excitatory granule cell (GC) and CA3 pyramidal cell (PC) (Lawrence et al., *J Physiol* 2004;554.1:175). However, such a strong excitatory drive makes the network susceptible to hyperexcitability. Inhibition is one mechanism for tempering this potentially catastrophic circuit arrangement. PCs receive both GC-driven feedforward and PC-driven feedback inhibition from local inhibitory INTs. Using whole-cell voltage-clamp techniques, we tested the hypothesis that feedforward and feedback inhibition temporally overlap to regulate circuit excitability during short-term plasticity. Low-intensity extracellular stimulation of the GC layer elicited both monosynaptic EPSCs (excitatory postsynaptic currents) and polysynaptic IPSCs (inhibitory postsynaptic currents) in PCs. Surprisingly, the total inhibitory charge transfer progressively increased during a 20 Hz train of stimuli, balancing the increase in excitatory charge transfer and maintaining a constant excitation:inhibition (E:I). Given that the IPSC onset was delayed relative to the EPSC, the latter's peak amplitude was able to increase progressively during the train. Thus, excitation can facilitate an increase in spike probability even as the constant E:I ratio maintains a narrow temporal window and prevents hyperexcitability. Next, we sought to determine where within the inhibitory circuit such facilitation occurred. Consistent with previous results, we found that GC-INT synapses were either facilitating or depressing in response to the short train of stimuli. Post hoc morphological analysis revealed no correlation between the type of short-term plasticity and INT morphology. We then examined the INT-PC synapse and observed that the total inhibitory charge transfer was either depressed or did not change during the train for stimulation in all layers, suggesting that depression is the predominant form of short-term plasticity at that synapse. Taken together, our results suggest that INTs that receive facilitating synapses from GCs provide a larger contribution to polysynaptic inhibition than those receiving depressing synapses; moreover, such facilitation is sufficient to mask any depression at the INT-PC synapse. However, given the high degree of facilitation onto PC, feedback inhibition is likely to contribute to the facilitation of inhibition during repetitive stimulation.

A transgenic approach to elucidating hippocampal mossy fiber function

Pelkey; in collaboration with Tonegawa

As the primary connection between dentate gyrus granule cells and the CA3 region of the hippocampus, mossy fibers (MF) provide a critical pathway linking cortical activity with intrinsic hippocampal circuits. Thus, MF activity dramatically affects overall hippocampal excitability and information processing. In collaboration with Susumo Tonegawa, we are attempting to determine the role of MF transmission in hippocampal-dependent learning paradigms; we are using transgenic mice that conditionally lack synaptic transmission specifically in the MF pathway. The mice were genetically engineered to express tetanus toxin light chain (TeTX) specifically in dentate gyrus granule cells under the control of a doxycycline-sensitive promoter region. As tetanus toxin effectively blocks presynaptic release of transmitter by cleaving the vesicle-associated protein VAMP, the mice should allow us to functionally knock out the mossy fiber pathway in a temporally controlled and reversible manner while leaving all other hippocampal circuitry intact. Indeed, our preliminary in vitro electrophysiological characterization of the mice confirms that MF-CA3 pyramidal cell transmission is efficiently blocked in mice induced to express TeTX and that the blockade is fully reversible upon treatment of the animals with a diet to repress TeTX expression. Moreover, synaptic transmission in all other hippocampal pathways (perforant path, Schaffer collateral, and associational commissural pathways) remains indistinguishable between MF-TeTX–expressing and wild-type control animals. Future behavioral work with the mice will greatly expand our understanding of MF function in hippocampus-related learning and disease.

Varying proportions of asynchronous transmitter release from identified hippocampal GABAergic INTs

Daw

Chemical synaptic transmission classically occurs when neurotransmitter is released from a presynaptic terminal in response to an AP in a tightly time-locked fashion. Previously, it has been shown at several central synapses that, in addition to synchronous release, some transmitter may be released in a longer and less precise time window such that release is asynchronous and not locked to individual presynaptic triggers. A variety of classes of local GABAergic INTs mediate inhibitory synaptic transmission in the hippocampus. In the hippocampal dentate gyrus, basket cells (BC) that contain cholecystokinin (CCK) show a high proportion of asynchronous release onto principal cells in response to long presynaptic trains while those that contain parvalbumin (PV) exhibit highly synchronous release. Using paired INT-INT and INT-PC recordings, we further studied this cell-type specificity in terms of both pre- and postsynaptic cell type in the CA1 and CA3 regions of the hippocampus. We identified non–fast-spiking INTs by expression of GFP in a mouse line that expresses GFP under the control of the GABA-synthetic enzyme GAD-65 (GAD-65 GFP) promoter. We recorded fast-spiking INTs in either wild-type mice or mice expressing GFP under the promoter for PV (B-13 line).

As in the dentate gyrus, presumed CCK-containing, non–fast-spiking BCs showed a much higher proportion of asynchronous release than that shown by presumed PV-containing, fast-spiking BCs in a manner unaffected by the identity of the postsynaptic cell (INT or pyramid). We studied two other classes of INT: bistratified and trilaminar cells. Both cell types showed an even higher degree of asynchronous release than either BC class, again regardless of postsynaptic cell type. To study the mechanisms of the two modes of release, we lowered the release probability by decreasing the extracellular Ca^{2+} concentration. This manipulation reduced each mode of release equally, suggesting a common mechanism. Our observation that the N-type calcium channel blocker ω-conotoxin GVIA completely blocked both modes of release supports a common mechanism.

Cell type–specific kainate receptor modulation of GABAergic transmission in the hippocampus

Daw

A variety of classes of local GABAergic INTs mediate inhibitory synaptic transmission in the hippocampus. Activation of the kainate (KA) class of ionotropic glutamate receptors reduces inhibitory transmission onto hippocampal PCs but augments inhibitory transmission onto INTs. However, the classes of INT affected by KA receptor activation have yet to be investigated. Therefore, we used paired INT-INT and INT-PC recordings to explore the INTs affected by KA receptor activation.

KA application had no effect on unitary inhibitory postsynaptic current (uIPSC) amplitude between fast-spiking BC INTs and PCs while uIPSC amplitude in PCs mediated by non–fast-spiking BCs and bistratified cells was halved. Changes in the paired-pulse ratio and coefficient of variation suggest that the reduction was presynaptic in origin; a similar depression of uIPSC amplitude by KA in the presence of CGP 55845 (30 percent) showed that the reduction was not attributable to indirect activation of GABAB receptors, as previously reported.

The effects of KA synaptic transmission between non–fast-spiking INTs were more complex but appeared to be related to the short-term plasticity displayed by the synapse irrespective of INT class. Kainate application inhibited synapses that showed pronounced facilitation throughout long bursts of presynaptic stimulation (25 APs) while synapses that depressed during bursts either did not respond to kainate or did not potentiate. As with PCs, changes in the paired-pulse ratio were inversely correlated with changes in the first IPSC amplitude, suggesting a presynaptic site of action. Surprisingly, however, plasticity throughout longer bursts did not change. Experiments in which presynaptic release probability was directly reduced by lowering the extracellular calcium concentration showed that only large (greater than 60 percent) reductions in the amplitude of the first IPSC produced changes in plasticity during bursts. The results suggest that, in many experiments, the kainate-induced depression was too small to affect plasticity in bursts. Our experiments show that activation of presynaptic KA receptors on hippocampal INTs can modulate inhibitory transmission in a manner that is dependent on both pre- and postsynaptic cell type.

Hippocampal INT subtypes are associated with distinct muscarinic receptor mRNA expression profiles

Lawrence, Tricoire, Cea del Rio

INTs in neocortex and hippocampus differentially undergo muscarinic receptor (mAChR) activation, but it is not clear whether muscarinic phenotypes of INTs arise from differences in the expression or localization of mAChRs, intrinsic voltage-gated ion channels, calcium-buffering capacity, or some combination of these factors. Using single-cell PCR, biocytin labeling, and transgenic mice expressing GFP in parvalbumin- (PV, line B13) or GAD65-expressing INTs, we determined mAChR mRNA expression profiles (M1R–M5R) in morphologically, electrophysiologically, and neurochemically identified INT populations. We based neurochemical profiles for each INT on nine neurochemical markers: GAD67, GAD65, calretinin, PV, calbindin (CB), somatostatin (SOM), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), and CCK. In all CA1 PV-GFP cells identified as fast-spiking BCs, we detected PV mRNA but not CB, CCK, VIP, or SOM mRNA. PV^+ BCs expressed M1 (100 percent), M2 (40 percent), M3 (20 percent), and M4 (60 percent) muscarinic receptor mRNA. In contrast, CA1 GAD65 GFP^+ INTs expressed abundant CCK (92 percent), and CB (69 percent) mRNA, as did non–fast-spiking Schaeffer collateral–associated and CCK BC populations. We probed a subset of GAD65 GFP^+ cells for mAChR mRNAs. Similar to PV^+ BCs, M1 mRNA was present in all GAD65 GFP^+ cells. In contrast, M2 mRNA was absent while M3 mRNA was expressed in higher relative abundance (71 percent) than in PV-GFP^+ cells. The data are consistent with M1 antagonist sensitivity to mAChR responses in PV-GFP and GAD65 cell types. Immunocytochemical

evidence for M2Rs on PV^+ boutons prompted us to examine the functional consequence of M2Rs on PV-GFP INTs. In PV^+ BC–pyramidal cell pairs, bath application of the muscarinic receptor agonist muscarine reduced the unitary IPSC amplitude in wild-type, but not in PV-GFPxM2 KO, mice. Thus, our data provide evidence not only of the differential expression of mAChR subtypes between INT subtypes but also for differential targeting within an INT subtype.

Neurochemical identity governs cholinergic phenotype across hippocampal BC networks
Cea del Rio, Lawrence, Tricoire

Inhibitory INT BCs release GABA perisomatically onto PCs in order to synchronize PCs during gamma (γ; 40 Hz) oscillations. Two major BC subtypes are present in the hippocampus: CCK- and PV-BCs. In the neocortex, CCK- and PV-BCs possess distinct cholinergic phenotypes; however, the cholinergic neuromodulation of hippocampal CCK- and PV-BCs remains poorly understood. To examine differential cholinergic modulation in BC subtypes, we performed whole-cell recordings in hippocampal slices from GAD65- or PV-GFP transgenic mice and morphologically identified CCK- and PV-BCs, respectively. To determine neurochemical profiles and muscarinic-receptor (M1–M5) content, we also performed single-cell RT-PCR experiments. First, upon application of muscarine, CCK-BCs underwent a transient hyperpolarization while PV-BCs underwent depolarization. Second, mAChR activation induced an increase in firing frequency in response to depolarizing current steps in both CCK- and PV-BCs. However, the offset of the current stimulus elicited a larger mAChR-induced afterdepolarization (ADP) in CCK-BCs. To determine whether cholinergic neuromodulation tunes CCK- and PV-BCs differently, we systematically varied the frequency (1to 100 Hz) and number of APs (1 to 100 APs at 20 Hz) elicited in both BC subtypes. In CCK-BCs, the mAChR-induced ADP (mADP) was largest at 20 Hz and could be generated with as few as 10 APs. In contrast, the mADP of PV-BCs exhibited no frequency or AP preference.

The specific M1-mAChR antagonist telenzepine prevented the mADP but not the rise in firing frequency, suggesting the presence of both M1 and non–M1 mAChR subtypes. In contrast, in PV-BCs, telenzepine completely blocked both the mADP and an increase in firing. In addition, GAD65 x M3KO transgenic mice demonstrated that the mAChR-induced change in firing frequency and mAChR-induced ADP were lower than in wild-type animals. Finally, consistent with pharmacological data, single-cell RT-PCR confirmed that M1 receptor transcripts were present in all CCK- and PV-BCs and revealed that M3 receptors were more abundant in CCK-BCs cells than in PV-BCs. The data suggest that M1 and M3 mAChRs play synergistic roles in mAChR-induced changes in firing and the generation of ADP.

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Genetic fate mapping of hippocampal inhibitory INTs
Tricoire; in collaboration with Fishell, Cauli

Hippocampal INTs arise from progenitor cells of the caudal and medial ganglionic eminences (CGE and MGE), which are located in the ventral part of the telencephalon. Given the INTs' diversity, the precise birth date of INT subtypes remains unknown. Indeed, few data exist on the respective postnatal fate of the cohorts of INTs generated during embryogenesis. Olig2 is a basic helix-loop-helix transcription factor highly expressed in the MGE. To perform an inducible genetic fate mapping of INT precursors, we used transgenic mice that expressed a tamoxifen-inducible form of Cre recombinase under the control of the Olig2 promoter. After a single administration of tamoxifen, a Z/EG reporter allele allowed the expression of GFP upon the Cre-mediated removal of a stop cassette

as well as the irreversible labeling of precursors expressing Olig2. This configuration permitted cell labeling in a temporally and spatially precise manner and allowed for subsequent study of the cells in their mature state after birth and long after the Olig2 promoter switched off.

We examined hippocampal fate-mapped INTs in 3- to 4-week-old mouse pups after administering tamoxifen to pregnant females between E9.5 and E15.5. Early data revealed that hippocampal INTs are generated earlier than their cortical INT counterparts. The vast majority of hippocampal GFP^+ INTs localized to the CA1–3 subfields. Using immunofluorescence, we observed that the occurrence of INT markers such as PV, SOM, and NPY in GFP^+ cells was dependent on the time of tamoxifen administration. Few GFP^+ cells expressed calretinin or VIP, suggesting a different origin for hippocampal INTs expressing these markers. We then used patch clamp recording in combination with single-cell RT-PCR to study the electrophysiological, molecular, and morphological properties of the fate-mapped cells in acute hippocampal slices. Single-cell RT-PCR revealed the presence of GAD65 and GAD67, confirming the GABAergic phenotype of fate-mapped cells as well as the prevalence of PV, SOM, and NPY in these cells. Our observations suggest that select cohorts of hippocampal INTs are generated within the MGE at specific times throughout embryonic development. Importantly, the genetic approach used here permits the indelible marking of INT subtypes and links early developmental events to the physiological profiles of mature cells.

Developmental origin of hippocampal neuronal nitric oxide synthase–expressing INTs
Tricoire; in collaboration with Fishell

The hippocampus widely expresses the neuronal isoform of nitric oxide synthase (nNOS), an enzyme frequently found in NPY-expressing GABAergic INTs. So far, we have identified two distinct cell types in the mammalian cortex that are positive for nNOS/NPY: the Ivy cell and neurogliaform cell. While Ivy cells are located mainly in the stratum oriens and stratum radiatum, neurogliaform cells are typically found in the stratum lacunosum moleculare. Both cell types have a dense axonal arbor and are involved in slow synaptic transmission. The axon of neurogliaform cells overlaps with the glutamatergic input from the entorhinal cortex while the axonal field of Ivy cells is aligned with the CA3 input to stratum oriens and stratum radiatum.

Although Ivy cells have been described as one of the most abundant INT subtypes, it is unclear whether they represent a population of cells distinct from neurogliaform cells. To resolve this issue, we employed a genetic fate-mapping approach, using the Olig2CreER:Z/EG mouse (described above) to examine the origin of nNOS-expressing INTs. We found that a large portion of fate-mapped cells that expressed nNOS were located in both the stratum oriens and stratum pyramidale, but we observed few cells in the stratum lacunosum-moleculare. The occurrence of nNOS in fate-mapped cells shows maxima when tamoxifen is administered at E9.5 and E13.5, with a trough at E11.5 suggesting two successive waves of $nNOS^+$ INT precursors. Electrophysiological recordings, combined with morphological reconstruction and single-cell RT-PCR, allowed positive identification of Ivy cells in fate-mapped cells in Olig2CreER:Z/EG, suggesting an MGE origin for Ivy cells. We will extend these initial studies to include transgenic mice that express GFP in INTs exclusively derived from either the MGE or CGE. These experiments will determine where and when Ivy and neurogliaform cells are generated and whether they share a common or divergent developmental and genetic program.

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NEUROETHOLOGY OF CRYING

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Crying, a universal mammalian behavior in infancy, is an essential signal that activates care-giving behavior. Little is known about the neural basis of crying or about why crying can be such a compelling stimulus to the listener. Our goals are (1) to determine the neural pathways that underlie cry production and cry perception; (2) to track the developmental course of crying in infancy and, in particular, to determine the roles of inheritance and experience in individual variability in crying behavior; and (3) to examine the interplay of cry acoustics and the hormonal and experiential status of care-givers in regulating the motivation to respond to a crying infant. We undertake behavioral experiments in non-human primates aimed at defining the critical features of infant crying that promote care-giving; perform acoustic analysis of cry sounds in the search for acoustic markers of developmental status, familial traits, environmental influences, and neurological risk factors; and conduct functional neuroanatomical studies aimed at defining the neural populations activated during crying and cry perception.

The cry circuit in the brain

Newman, Bernhards, Garnett, Fuhr

A necessary step in understanding how the brain regulates crying and cry responding is to identify the neural circuits that mediate crying and cry-response behaviors. Using an approach never before applied to the brains of infant monkeys, we studied the brains of infant common marmosets by using immunocytochemical identification of Fos as a marker for functional activity of neurons after an extended bout of crying; Fos is the protein product of the immediate-early gene *c-fos*. Using a Leica microscope with a motorized stage and the Life Science program from Bioquant, we are systematically digitizing thousands of images at 100x magnification from sections throughout the brains of infant monkeys at 1, 2, 3, and 4 months of age. We subsequently collect the images into montages of each section, subject them to quantification of the number of Fos-expressing neurons per image, and map the distribution of regions within each montage with the greatest number of Fos-expressing neurons. We stain adjacent sections for Nissl granules to permit detailed anatomical identification of the regions of greatest expression. Reference to a brain atlas produced in our laboratory assists in the construction of a "wiring diagram" of the structures making up the circuit underlying cry production at different ages during development.

Our latest findings are based on the digitization and measurement of Fos-labeled cells from eight infant brains, four at 1 month of age, two at 2 months of age, and two at 4 months of age (including

one control brain). We digitized approximately 330 to 620 images (depending on the size of the original section) from each brain section and counted the labeled cells in each image. We created a grid overlay for each montage and entered the number of labeled cells from each image into the appropriate grid space. The result is an XY map of the distribution of counts for each montaged section. Examination of the map for each section clearly shows a differential distribution of subregions with high numbers of labeled cells, including periventricular grey in the diencephalon, septum, preoptic area, amygdala, and hippocampus in the rostral forebrain; anterior cingulate gyrus and gyrus rectus in the frontal cortical regions; superior temporal gyrus and periaqueductal grey of the midbrain. Studies of adult non-human primates indicate that most of these subregions are involved in auditory communication, but we demonstrated for the first time that the vocal production system is widely activated in such young animals. High counts in the superior temporal gyrus presumably reflect neurons in the auditory cortex activated during the infant's vocalization. We are currently counting additional brain sections from the group of eight infants. In addition, we have developed a new method that overlays the actual counts (number of neurons containing Fos) on each frame of a software-created montage of the entire brain section. By color-coding the frames with the highest counts, we can immediately see where the greatest activity (hot spots) is located on the sections. Of particular interest, we note that the anterior cingulate gyrus and adjacent gyrus rectus of the youngest infants in the group contain large numbers of labeled neurons. This finding contradicts the prevailing model of infant crying, which posits a "midbrain model" and proposes that crying in early infancy is elaborated in the midbrain.

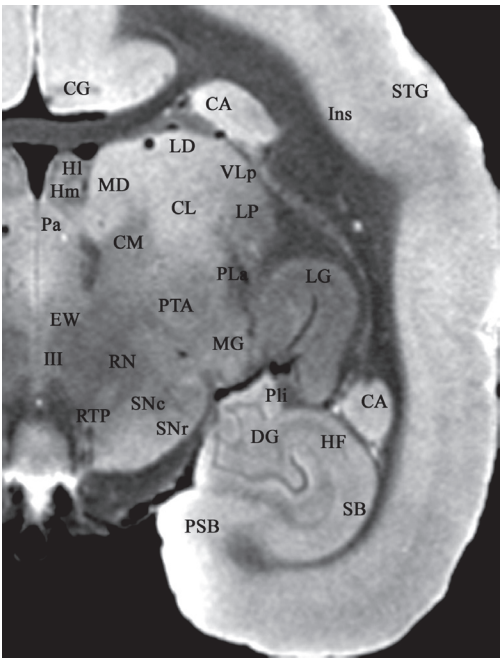

FIGURE 5.1

MRI IMAGE FROM FEMALE MARMOSET, WITH LABELED BRAIN STRUCTURES.

Key: CA = caudate nucleus; CG = cingulate gyrus; CL = central lateral nucleus; CM = center median nucleus; DG = dentate gyrus; EW = Edinger-Westphal nucleus; HF = hippocampal formation; HI = lateral habenular nucleus; HM = medial habenular nucleus; III = oculomotor nucleus; Ins = insula; LD = lateral dorsal nucleus; LG = lateral geniculate nucleus; MD = mediodorsal nucleus; Pa = paraventricular nucleus; Pla = anterior nucleus of the pulvinar; Pli = inferior nucleus of the pulvinar; PSB = presubiculum; RN = red nucleus; RTP = reticulotegmental pontine nucleus; SB = subiculum; SNc = pars compacta of substantia nigra; SNr = pars reticulate of substantia nigra; STG = superior temporal gyrus; VLp = ventral lateral posterior nucleus. MRI image provided by Nicholas Bock.

This year, we continued a study aimed at identifying neurons in the brains of individuals listening to infant cries. We exposed five adult marmosets to 30 minutes of recorded infant cries and then euthanized the animals in order to process their brains for Fos immunocytochemistry. Analysis of three brains from males demonstrated, for the first time in a non-human primate, that infant cries activate several defined areas of the brain, including the anterior cingulate gyrus and midline frontal cortex rostral to this area and the hippocampus, in addition to the expected temporal lobe auditory areas. All structures outside the temporal lobe auditory areas fall within the limbic system, as defined by Paul MacLean, suggesting that the limbic system may be a functionally integrated system for responding to cries, as originally proposed by MacLean for the thalamo-cingulate division of the limbic system.

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Marmoset brain atlas

Newman, Bernhards, Zametkin, Winslow, Bock, Silva

Any effort to describe the neuroanatomy of the cry circuit requires identification of the appropriate structures for examination. A brain atlas aids in such identification. Over the past year, we used a brain atlas (created from the brain of an adult female) to identify structures in MRI images from a female marmoset. We selected MRI images that match as closely as possible the plates for the histological atlas created by our laboratory. We applied the same labels used for the histological atlas to the corresponding regions of the MRI images, thereby providing the equivalent of a histological atlas for MRI studies for use in identifying structures of interest in planned fMRI experiments (Figure 5.1).

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CALCIUM-BASED EXCITABILITY IN GLIAL CELLS

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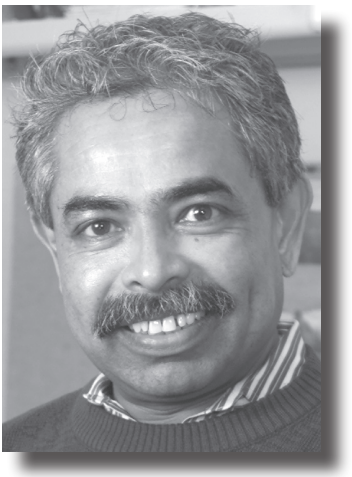

We investigate signaling between neurons and glial cells in the central and peripheral nervous systems. The intimate communication between glial cells and neurons is crucial for normal brain development and seems to play a critical role in the plastic functions of the brain. Glial cells monitor and respond to neural activity by conditioning the extracellular milieu, signaling within glial cell networks, and sending signals back to neurons. In the brain, glial cell responses to neural activity take the form of propagated Ca^{2+} waves that spread over long distances in response to synaptic activity. Similarly, the myelinating glial cells (oligodendrocytes and Schwann cells) receive signals from the axons they myelinate, and such signals are essential for the maintenance of the myelin sheath. One of our objectives is to understand different modalities of cell-cell signaling and the processes that support temporal and spatial characteristics of Ca^{2+} signals within and between cells. A second objective is to probe the nature of glial cell signals in response to neuronal activity and the consequence of such signals to central nervous system (CNS) function.

Ca^{2+} signaling between axons and myelinating glia
Patel

We are investigating aspects of signaling between axons and their myelinating glia (Schwann cells and oligodendrocytes) in the peripheral nervous system (PNS) and CNS. The myelin sheath serves as an electrical insulator; it reduces current flow across the axonal membrane in the internode by lowering capacitance and increasing resistance, thereby facilitating saltatory conduction. Myelin is formed by a highly specialized extension of myelinating glial cell (oligodendrocytes in the CNS and Schwann cells in the PNS) membranes that are intimately associated with axons.

Beyond the concept of saltatory conduction, several studies support the view that the axon/myelin/glial cell ensemble or "nodal complex" operates in an integrated manner during conduction of the nerve impulse. Over the last two decades, numerous anatomical and physiological studies have shown that an intricate set of signals might exist between the components of the nodal complex, although details of signaling between axons and the myelinating glia are not available.

We initiated a study aimed at describing the distribution of proteins involved in Ca^{2+} signaling in the axoglial apparatus. We believed that the study would point us to the spatial discreteness, if any, in the signaling between axons and Schwann cells. Initially, we hypothesized that the signaling is

localized to specific contact sites between the two cell types where the insulating layer of compact myelin gives way to non-compact myelin and Schwann cell membrane. We first characterized the distribution of Ca^{2+} signaling proteins in the axoglial apparatus of the nodes of Ranvier.

We performed immunohistochemical analysis with antibodies against major proteins expressed by Schwann cells and axons in a cell-specific manner to determine the anatomical arrangements of the two cell types in the nodes of Ranvier. Using specific antibodies against IP3Rs, RyRs, $P2Y_1$, M_1, and G_q, we then examined the distribution of proteins involved in Ca^{2+} signaling within the nodes. In support of our original hypothesis, we found a highly concentrated distribution of Ca^{2+}-signaling proteins in the paranodal loop regions on either side of the nodes of Ranvier (Toews et al., 2007). We are planning a higher-resolution electron microscopic analysis, to be conducted in collaboration with Mark Ellisman, that uses immunocytochemical techniques to describe the precise co-localization of various proteins in the axoglial apparatus.

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In situ imaging of Schwann cell calcium signals

Atkin,[1] Patel, Holtzclaw; in collaboration with Miyawaki, Pickel

To measure directly Schwann cell Ca^{2+} signals associated with action potential traffic along the axons they myelinate, we developed transgenic mouse lines expressing a fluorescent Ca^{2+}-indicator photoprotein within Schwann cells. We used the S100β promoter to target a mutant chameleon protein, YC3.60, to Schwann cells in peripheral nerves and astrocytes in the brain. We obtained a YC3.60 construct from Atsushi Miyawaki and engineered a plasmid construct containing the S100β promoter in tandem. YC 3.60 is designed to express a protein containing the structures of cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and calmodulin, a Ca^{2+} sensor. When the ambient Ca^{2+} concentration increases, the efficiency of fluorescent energy transfer (FRET) between the CFP and YFP increases significantly and is readily measurable. Transfection of such a plasmid in C6 glioma cell lines and HEK 293 cell lines showed that YC3.60 was expressed not by HEK293 cells but only by C6 glioma cells, which express S100β.

In collaboration with James Pickel, we used the plasmid to generate transgenic mouse founders. We established four founder lines expressing YC3.60 fluorescence in glial cells and bred the mice to homozygosity. We used two-photon confocal microscopy to image brain slice preparations obtained from two of the lines and readily visualized individual astrocytes brightly fluorescent with the YC3.60 chameleon. We observed cells with large spongiform process arbors, as well as smaller cells, containing YC3.60. Many of these cells—large and small—exhibited elongated cell somas with several large branches radiating parallel or perpendicular to those of their neighboring glia. Numerous YC3.60–positive astrocytic processes extended to wrap small blood vessels. We found YC3.60 expression in the cell soma, extending into all processes in astrocytes. Dual staining with anti–S100β and anti–GFP antibodies in the cerebellum showed that 78 percent of S100β-positive Bergmann glial cells expressed YC 3.60.

YC3.60 expressed by glial cells in transgenic mice responded to glial cell Ca^{2+} signals in isolated brain slice preparations and isolated, teased peripheral nerve preparations. The average stimulus-induced YFP/CFP ratio change was 65 percent. In most experiments, one or two cells showed very large YFP/CFP ratio changes (over 100 percent) whereas some cells did not respond at all. The largest response was over 300 percent change, or direct stimulation of neural pathways.

Stimulation of Schaffer collaterals in hippocampal slices evoked robust Ca^{2+} signals in astrocytes in the stratum radiatum and stratum moleculare-lacunosum, as judged by YC3.60 fluorescence change. In six slice preparations, the stimulus-evoked YFP/CFP ratio increase occurred in about 80 percent of 74 cells; in each case, we recorded extracellular field potentials. When we measured

Ca^{2+} signals in spongiform protoplasmic astrocytes, stimulus-evoked fluorescence changes were apparent within discrete local regions of the cell. Many of these regions represent glial microdomains within the amorphous spongiform morphology of the cell. The cellular Ca^{2+} response elicited by neural stimulation spreads as a wave through the cell. The glial microdomains of high activity are reminiscent of previously described small (less than 2μm) astrocytic terminal sheaths that enwrap single synapses or groups of synapses.

One of the transgenic mouse lines showed abundant YC3.60 fluorescence within all Schwann cells in the peripheral nerves while astrocytes in the CNS did not contain appreciable fluorescence. We used mice derived from the same transgenic line to investigate action potential–dependent Ca^{2+} signals in Schwann cells. We imaged sciatic nerves isolated from these mice with 2-photon confocal microscopy, stimulated the nerve bundles with a suction electrode, and recorded compound action potentials during stimulation. However, in numerous trials, we found no stimulus-associated Ca^{2+} signals in Schwann cells that myelinate the axons. Given that we cannot state with any certainty that the recorded compound action potential represents stimulation of all axons in the bundle, we are unable to draw clear conclusions without further experimentation. One previously published study showed that, in isolated frog nerve bundles, action potential generation resulted in Schwann cell Ca^{2+} signals, albeit only with stimulation at 50 Hz for many minutes. Similar experiments have not been replicated in mammalian axons.

While action potential–dependent Ca^{2+} signals were not detectable in isolated sciatic nerve bundles, application of exogenous purinergic agonists readily elicited Ca^{2+} signals as revealed by YC 3.60 fluorescence changes. The rank order of potency of purinergic agonists was ATP>UTP>2MeSATP, suggesting the presence of P2Y-subtype purinergic receptor on Schwann cells. Furthermore, these purinergic agonist–elicited Ca^{2+} signals persisted in the complete absence of Ca^{2+} ions in the extracellular medium, suggesting that P2X-type purinergic receptors did not contribute to the signal. In support of this conclusion, prolonged exposure to UTP to deplete intracellular Ca^{2+} stores while abolishing a response to ATP did not evoke a response to a P2X-selective purinergic agonist. Our experiments showed that Schwann cells in situ express a functional p2Y-subtype of purinergic receptor. It is likely that this receptor system might be involved in Schwann cell responses to acute nerve injury. It is well known that, following injury, Schwann cells retract, leading to initial demyelination followed by regeneration.

The strategy of directing expression of Ca^{2+} indicator photoproteins in a cell-specific manner has proven extremely valuable for investigating glial cells' physiological responses during nervous system function both in isolated preparations and in situ. One drawback in the design of Ca^{2+} indicator proteins has been the use of calmodulin, a ubiquitous nervous system Ca^{2+} sensor found abundantly in all cells. In addition, all nervous system cells have a number of calmodulin-binding proteins that can bind to the indicator in a Ca^{2+}-dependent manner and thus interfere with the indicator's responses. Moreover, the photoproteins CFP and YFP, which were used in YC 3.60, are relatively dim compared with more modern mutant proteins with brighter fluorescent yields. Two such proteins are cerulean (cyan) and venus (yellow). Oliver Griesbeck of the Max Planck Institute in Germany designed a novel indicator protein (called CerTn-L15) in which he replaced the calcium sensor with the Ca^{2+}-binding region of troponin-C of chicken muscle. While calmodulin and troponin-C are similar in their Ca^{2+}-binding affinity, troponin C does not bind to the proteins to which calmodulin binds in a Ca^{2+}-dependent manner. Hence, the troponin C–based sensor CerTn-L15 is superior to YC3.60 in this regard. We are now generating transgenic mice expressing CerTn-L15 in astrocytes, Schwann cells, and oligodendrocyte progenitors (OP cells). We plan to use the glial fibrillary acidic protein (GFAP) promoter to target to astrocytes and the cyclic nucleotide phosphodiesterase (CNP) promoter to target to Schwann cells and OP cells. Once we have generated such transgenic mice, we should have the tools we need to investigate signaling in all three types of glial cells. We are

already using the ROSA-26 locus to target the photoprotein, which should yield robust expression in cells, and are ensuring cell-specific expression by using cre recombinase–containing constructs that target cell-specific promoters.

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SIGNALING AND SECRETION IN NEUROENDOCRINE CELLS

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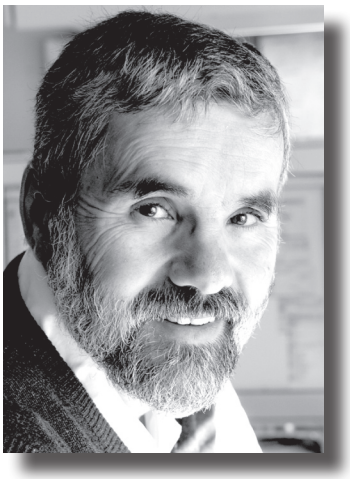

We investigate cellular signaling cascades and secretion in pituitary cells, with special emphasis on the interactions between plasma-membrane electrical events and receptor-controlled pathways. Pituitary cells exhibit spontaneous firing of action potentials and spontaneous calcium transients while hormonal stimulation leads to up- or downregulation of electrical activity and voltage-gated calcium influx–dependent secretion through a complex cascade of events. Our main objective is to elucidate the channels and receptors involved in calcium signaling and the role of calcium ions as messengers in controlling intracellular signaling and secretion. We are currently studying the biophysical basis of pituitary cell type–specific calcium signaling–secretion coupling, the metabolism and roles of cyclic nucleotides in the regulation of electrical activity and calcium signaling, the role of G protein–coupled receptors in the control of electrical activity, and the molecular properties of calcium-conducting purinergic receptor channels in pituitary functions.

Cyclic nucleotides and electrical signaling pathways

Andric,[1] Kretschmannova, Kucka, Murano, Tomić, Stojilkovic; in collaboration with Sherman, Schechter

Numerous plasma membrane channels have been characterized in pituitary cells, but the mechanism underlying their pace-making activity remains unknown. We observed that, in all pituitary cell types, the removal of sodium reversibly hyperpolarized membrane potential and suppressed calcium oscillations, followed by a reduction in the intracellular calcium concentration to near steady-state levels. In contrast, blockade by tetrodotoxin of voltage-dependent sodium channels, which are expressed in all secretory cell types, was ineffective. We also found that inhibition of electrical activity by removal of bath sodium was accompanied by an instantaneous blockade of cyclic nucleotide efflux transporter, suggesting a coupling between electrical activity and cyclic nucleotide efflux. In addition, our experiments indicated that (1) a sodium-conducting and tetrodotoxin-insensitive channel plays a role in controlling pace-making stimulated by growth hormone–releasing hormone in a cAMP-dependent manner and (2) spontaneously active inwardly rectifying potassium (K_{ir}) channels and G protein–regulated K_{ir} channels play an opposing role in somatostatin-mediated inhibition of pace-making. We further described a role for voltage-gated calcium channels in spiking and for large-conductance (BK-type) calcium-activated potassium channels in plateau bursting.

In collaboration with Arthur Sherman and colleagues, we combined experiments and theory and used pituitary somatotrophs as a cell model to clarify the mechanisms underlying spontaneous and receptor-controlled electrical activity. Our mathematical model is compatible with a wide variety of experimental data involving pharmacology and extracellular ion substitution and points to the importance of constitutively active tetrodotoxin-insensitive sodium and K_{ir} channels in maintaining spontaneous pace-making in pituitary somatotrophs. The model also suggests that these channels are involved in the up- and downregulation of electrical activity by growth hormone–releasing hormone and somatostatin. In the model, two functional populations of BK channels, characterized by the distance from the voltage-gated calcium channels, control plateau bursting. The rapid activation of the proximal BK channels is critical for the establishment of the plateau, whereas slow recruitment of the distal BK channels terminates the plateau. Our ongoing work focuses on identifying the channels responsible for the background sodium conductance and the relationship between spontaneous and receptor-controlled electrical activity and cyclic nucleotide efflux.

In collaboration with Alan Schechter and colleagues, we studied the effects of hydroxyurea on cyclic nucleotide signaling in human erythroid progenitor cells. Hydroxyurea, a drug widely used to treat myeloproliferative diseases, has been approved for the treatment of sickle cell disease because it raises fetal hemoglobin. Our earlier findings indicated that nitric oxide and the soluble guanylyl cyclase pathways are involved in hydroxyurea induction of fetal hemoglobin levels in erythroid progenitor cells. More recently, we showed that, during erythroid differentiation, endothelial nitric oxide synthase mRNA and protein levels decline steadily, as do the production of nitric oxide derivatives and cAMP levels, although cGMP levels remain stable. Hydroxyurea raised intracellular cGMP and cAMP levels in erythroid progenitor cells. The nitric oxide donor DEANONOate induced even higher cGMP levels but reduced cAMP levels. Hydroxyurea induced production of approximately 45 pM cGMP per minute per ng of purified soluble guanylyl cyclase, similar to induction by 1 μM DEANONOate. We found that hydroxyurea and ProliNONOate produced iron-nitrosyl derivatives of soluble guanylyl cyclase. Thus, we confirmed that hydroxyurea can directly interact with the deoxy-heme of soluble guanylyl cyclase, presumably by a free-radical nitroxide pathway, and activate cGMP production. These data add to an expanding appreciation of the role of hydroxyurea as an inducer of the nitric oxide/cGMP pathway in erythroid progenitor cells. Similar mechanisms may also be involved in the cytostatic effects of hydroxyurea as well as in the induction of fetal hemoglobin.

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Receptors and pathways controlling prolactin secretion

Gonzalez-Iglesias,[2] Hatae,[3] Kretschmannova, Kucka, Murano, Tomić, Stojilkovic; in collaboration with Ortmann, Zorec

Our recent work has focused on the secretion of prolactin by pituitary lactotrophs and the role of dopamine D2 and endothelin-A receptors, testosterone, and hypotonicity in the secretion process. Dopamine secreted from hypophysial hypothalamic neurons is a principal inhibitory regulator of prolactin (PRL) release caused by pituitary lactotrophs' activation of dopamine-2 receptors. Earlier work with dopamine D2 receptors revealed that the receptors' activation in pituitary lactotrophs leads to inhibition of PRL release. Other researchers have suggested that such inhibition occurs through the $G_{i/o}$-alpha protein–mediated inhibition of cAMP production and/or $G_{i/o}$-beta/gamma

dimer–mediated activation of K_{ir} channels and inhibition of voltage-gated calcium channels. We showed that the dopamine agonist–induced inhibition of spontaneous calcium influx and release of prestored PRL was preserved when we elevated cAMP levels by forskolin treatment. We further observed that dopamine agonists inhibited both spontaneous and depolarization-induced calcium influx in untreated, but not in pertussis toxin–treated, cells. We observed a similar inhibition in cells with blocked K_{ir} channels, suggesting that the dopamine effect on voltage-gated calcium channel gating is sufficient to inhibit spontaneous calcium influx. However, agonist-induced inhibition of PRL release was only partially relieved in pertussis toxin–treated cells, indicating that dopamine receptors also inhibit exocytosis downstream of voltage-gated calcium influx. The pertussis toxin–insensitive step in agonist-induced inhibition of PRL release was not affected by the addition of wortmannin, an inhibitor of phosphatidylinositol 3-kinase, and lithium, an inhibitor of glycogen synthase kinase-3, but it was attenuated in the presence of phorbol 12-myristate 13-acetate, which inhibits Gz signaling pathway in a protein kinase C–dependent manner. Our results indicate for the first time that dopamine inhibits basal PRL release by (1) blocking voltage-gated calcium influx through the pertussis toxin–sensitive signaling pathway and (2) desensitizing calcium-secretion coupling through the pertussis toxin–insensitive and protein kinase C–sensitive signaling pathway.

In collaboration with Robert Zorec and colleagues, we studied the release by hypotonicity of the pituitary hormone PRL, which also contributes to osmoregulation. In perifused rat lactotrophs, hypotonicity resulted in a transient increase followed by a sustained depression of PRL release, as monitored by radioimmunoassay. In single cells imaged by confocal microscopy, hypotonicity elicited discharge of the fluorescently labeled atrial natriuretic peptide cargo from about 2 percent of vesicles per cell, which synchronously loaded the styryl dye FM 4-64 through secretory fusion pores. In contrast, high potassium–induced depolarization resulted in a response by about 10 percent of vesicles per cell, with a different unloading/loading time course for the two fluorescent probes. In cell-attached studies, we recorded discrete changes in membrane capacitance in both unstimulated and stimulated conditions, reflecting single vesicle fusions/fissions with the plasma membrane. In stimulated cells, the probability of full fusion events was low and unchanged, whereas over 95 percent of fusion events were transient, with increased open fusion–pore probability, average pore-dwell time, frequency of occurrence, and fusion-pore conductance. Hypotonicity only rarely elicited new fusion events in silent membrane patches. The results indicate that, in hypotonicity-stimulated lactotrophs, rapidly releasable vesicles appear prefused and release hormone in a "kiss-and-run" mode. Ongoing experiments focus on comparing single-cell exocytotic events in lactotrophs and somatotrophs.

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Molecular, pharmacological, and functional properties of GABA-A receptors in anterior pituitary cells

Weber-Zemkova,[4] Tomić, Stojilkovic; in collaboration with Zemkova

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. It acts through three structurally and pharmacologically distinct classes of receptors: $GABA_A$ and $GABA_C$ ligand–gated Cl^- channels and G protein–coupled $GABA_B$ receptors. Earlier studies

suggested that GABA also inhibits prolactin release in vitro, but the receptors involved in such release have not been characterized. During the past year, we studied the expression of GABA-A receptor channels in pituitary cells, their distribution within secretory anterior pituitary cell types, and the nature (stimulatory or inhibitory) of the channels' actions. Our results show that mRNAs for all $GABA_A$ receptor subunits are expressed in pituitary cells and that alpha1/beta1 subunit proteins are present in all secretory cells. In voltage-clamped gramicidin-perforated cells, GABA induced dose-dependent increases in current amplitude that were inhibited by bicuculline and picrotoxin and facilitated by diazepam and zolpidem in a concentration-dependent manner. In intact cells, GABA and the GABA-A receptor agonist muscimol caused a rapid and transient increase in intracellular calcium, whereas the GABA-B receptor agonist baclofen was ineffective, suggesting that chloride-mediated depolarization activates voltage-gated calcium channels. Consistent with this finding, RT-PCR analysis indicated a high level of NKCC1 expression in pituitary cells but not of KCC2 cation/chloride transporter mRNAs. Furthermore, the GABA-A channel reversal potential for chloride ions was positive to the baseline membrane potential in most cells, and the activation of ion channels by GABA resulted in depolarization of cells and modulation of spontaneous electrical activity. Thus, secretory pituitary cells express functional, depolarizing GABA-A receptor channels. These results raise the possibility that GABA might be a releasing factor in the pituitary, but the physiological relevance of its excitatory effects are yet to be fully understood (see Perspectives: *J Physiol* 2008;586:3023).

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Structural and functional characterization of purinergic receptor channels

Yan, Li, Tomić, Stojilkovic; in collaboration with Zemkova

P2X receptors are a family of ligand-gated cation channels composed of two transmembrane domains, with N- and C-termini located intracellularly and a large extracellular loop containing the ATP binding domain. The P2X4 receptor, but not other members of this family of receptors, is sensitive to ivermectin (IVM), a high–molecular weight lipophilic compound used as an antiparasitic agent in human and veterinary medicine. Our initial whole-cell current recordings showed that IVM raises the sensitivity of the P2X4 receptor to agonists, 2-fold enhances the current amplitude in response to supramaximal agonist concentrations, and greatly prolongs the deactivation of current. An increase in the sensitivity of the wild-type receptor for ATP in the presence of IVM could provide a valuable pharmacological tool for evaluating the response of P2X4 receptor mutants to increased concentrations of ATP. We therefore studied the current responses of previously generated K190A, K190R, F230A, F230W, R278A, R278K, D280A, D280E, K313A, and K313R-$P2X_4$ mutants in the presence and absence of IVM. We also generated novel K67A, K67R, F185A, F185W, F294A, F294W, R295A, and R295K mutations of the P2X4 receptor because of the potential importance of these residues for ATP binding. To identify cells expressing receptors for electrophysiological recordings and to visualize the subcellular distribution of receptors by confocal microscopy, we used receptors tagged with enhanced green fluorescent protein at their C-termini. In the presence of IVM, all low or non-responsive mutants responded to ATP in a dose-dependent manner, with EC50 values for ATP of about 1, 2, 4, 20, 60, 125, 270, 420, 1,000, and 2,300 μM at D280A, R278A, F185A, K190A, R295K, K313R, R295A, K313A, K67A, and K67R mutants, respectively. The results indicate that lysines 67 and 313 and arginine 295 play a critical role in forming the proper three-dimensional structure of the P2X4 receptor for agonist binding and/or channel gating.

In further work on the structural-functional characterization of recombinant P2X4 receptor, we focused on the identification of residues contributing to allosteric regulation of the channels by IVM. Initial experiments demonstrated that IVM affects P2X4 receptor function when applied extracellularly but not intracellularly, suggesting that IVM could bind at the ectodomain. However, we further showed that transfer of the P2X4 ectodomain sequence to the backbone of the P2X2

receptor did not transfer the sensitivity for IVM. These initial observations led us to examine the potential relevance of transmembrane helices for IVM binding. To that end, we used cysteine-scanning mutagenesis of rat $P2X_4$ transmembrane regions. Mutations in 29 residues did not change the receptor function; among the residues, IVM effects were altered in Gln36, Leu40, Val43, Val47, Trp50, Asn338, Gly342, Leu346, Ala349, and Ile356 mutants. In addition, the substitution-sensitive Arg33 and Cys353 mutants could be considered IVM-sensitive hits. The pattern of these 12 residues was consistent with the helical topology of both transmembrane regions, with every third or fourth amino acid affected by substitution. These predominantly hydrophobic, non-polar residues are also present in the IVM-sensitive *Schistosoma mansoni* P2X subunit. The residues lie on the same side of their helices and could conceivably face lipids in the open conformation state and provide the binding pocket for IVM. In contrast, the IVM-independent hits Met31, Tyr42, Gly45, Val49, Gly340, Leu343, Ala344, Gly347, Thr350, Asp354, and Val357 map on the opposite side of their helices, probably facing the pore of the receptor or protein and playing important roles in gating. Once the receptor architecture becomes available from crystal studies, we will know the precise topology of these functionally important residues.

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OLFACTORY CODING AND DECODING BY ENSEMBLES OF NEURONS

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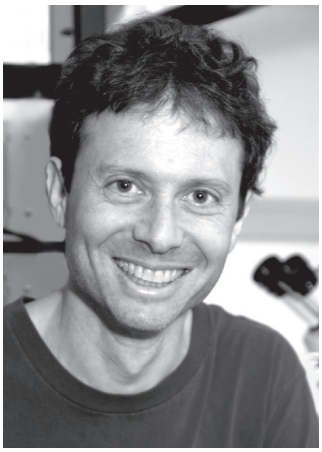

All animals need to know what is going on in the world around them; thus, brain mechanisms have evolved to gather and organize sensory information in order to build transient and sometimes enduring internal representations of the environment. Using relatively simple animals and focusing primarily on olfaction, we combine electrophysiological, anatomical, behavioral, and other techniques to examine the ways in which intact neural circuits, driven by sensory stimuli, process information. In the past year, we investigated mechanisms, including transient oscillatory synchronization and slow temporal firing patterns of ensembles of neurons, that underlie information coding and decoding, how spontaneous activity arises in a sensory system, how it is regulated, and how innate sensory preferences are determined. Our work reveals basic mechanisms by which sensory information is transformed, stabilized, and compared as it makes its way through the nervous system.

Sparse odor representation and olfactory learning
Ito I, Ong, Raman

In moths, as in other animals, learning experiences readily adjust the meanings of odors. Needless to say, within the brain, the odorants themselves are not matched with conditioning reinforcements; rather, neural representations of odors, presumably spiking activity in olfactory neurons, must undergo the matching process. The mushroom bodies have long been linked with associative learning and memory. In many insects, the bodies are sites of multimodal convergence that include olfactory and gustatory inputs. Many studies indicate that Kenyon cells, the intrinsic neurons of the mushroom bodies, play a critical role in olfactory learning. To understand how neural representations of odors become associated with reinforcement stimuli, we first characterized physiological responses of neurons along the olfactory pathway to odor pulses within the context of an associative learning procedure. The moth *Manduca sexta* has proved accessible for intracellular recording and is capable of performing the proboscis extension reflex (PER) conditioning, an appetitive olfactory learning task. Thus, in the moth, we examined neural representations of odor and performed PER training under identical conditions.

We used lengthy odor pulses (4–18s) in our investigations; such pulses, often used in studies of olfactory conditioning, reflect the odor exposures encountered by moths while feeding on flowers. With intracellular and multiunit recordings, we found that Kenyon cells were almost silent at rest and that odor responses typically consisted of single spikes in a small population of the cells.

Interestingly, spiking in Kenyon cells occurred almost entirely at an odor pulse's onset and sometimes offset, with few spikes in between.

After characterizing the responses of Kenyon cells to the odor stimuli, we used a set of behavioral studies to test a key requirement of a form of Hebbian learning called spike-timing–dependent plasticity (STDP), in which pre- and postsynaptic neurons must both fire spikes nearly simultaneously. To test the relationship between odor-evoked spikes and olfactory learning in Kenyon cells, we used several behavioral procedures with different intervals between odor and reward. Our results indicate that reinforcement delivered many seconds after the conclusion of spiking responses was able to support the formation and recall of associative memory. Thus, the acquisition of short-term memory does not require the concurrence of spikes in Kenyon cells with activation of a reward pathway in the moth. Further, we found that reinforcement delivered after the off-response did not support associative learning.

Our physiological and behavioral studies indicate that spikes in Kenyon cells cannot in and of themselves constitute the odor representation that coincides with appetitive reinforcement. Further, our results reveal that appetitive associative conditioning cannot occur by a Hebbian spike timing–dependent mechanism alone within Kenyon cells. We suggest instead that the odor representation in Kenyon cells that is paired with reward may be a sustained biochemical, perhaps second messenger, response triggered by highly transient spiking or perhaps even subthreshold activity.

Ito I, Ong RC, Raman B, Stopfer M. Sparse odor representation and olfactory learning. *Nat Neurosci* 2008;11:1177-1184.

Synaptic learning rules and sparse coding in a model sensory system

Stopfer; in collaboration with Finelli, Bazhenov, Sejnowski

Understanding how the brain encodes, processes, transforms, and stores sensory information is a fundamental issue in systems neuroscience. An important question is how several mechanisms such as neural oscillations, synchrony, population coding, and sparseness interact in the process of transforming and transferring information. Another question is how synaptic plasticity interacts efficiently with the various coding strategies to support learning and information storage. We approached these questions, which are rarely accessible to direct experimental investigation, by combining electrophysiological recordings with computational modeling of the olfactory system of the locust.

The transformation of odor responses from the antennal lobe to the mushroom body requires a number of coordinated processes, including an encoding function (the synchronization of groups of projection neurons through oscillatory dynamics) and a decoding function (the ability of Kenyon cells to respond as coincidence detectors, activated only by correlated input from the antennal lobe). For the transformation of odor responses to operate efficiently, encoding and decoding processes must be properly matched across a broad range of conditions. We are investigating the mechanisms that underlie the match and permit the appropriate selection of coherent signals.

We developed a biologically plausible model to test whether synaptic plasticity could control and tune synaptic weights of inputs to the mushroom body. Further, the model tested the relative advantages of various learning rules based on either spike rate– or spike timing–dependent induction of synaptic plasticity. We found that plasticity at the input afferents to the mushroom body can efficiently mediate the tuning necessary for selectively filtering intense sensory input arriving at the mushroom body. More specifically, we found that spike timing–dependent plasticity was more efficient than rate-dependent plasticity. Our results suggest a general mechanism for how plasticity could efficiently promote sparse representations in olfactory and other sensory systems, such as the visual system. More broadly, our findings illustrate a potential central role for plasticity in the efficient transfer of information between brain areas employing different coding strategies within neural systems.

Finelli LA, Haney S, Bazhenov M, Stopfer M, Sejnowski TJ. Synaptic learning rules and sparse coding in a model sensory system. *PLoS Comput Biol* 2008;4:e1000062.

Odor-evoked neural oscillations in *Drosophila* are mediated by widely branching interneurons

Tanaka; in collaboration with Ito K

Stimulus-evoked neural oscillatory synchronization is commonly observed in a wide and diverse range of species. However, important questions about how oscillations are generated, and what functions they serve, remain unanswered. *Drosophila* offers a great and growing variety of powerful, experimental genetic tools that permit the labeling and functional manipulation of specific classes of neurons while providing many advantages for analyzing the structure and functions of olfactory circuitry. In addition, given that *Drosophila* has become an important model system worldwide for studying olfaction and mechanisms underlying neural function, we investigated odor-elicited oscillations in *Drosophila*.

We found that odors evoked periodic oscillations similar to those we previously observed in locusts, honeybees, cockroaches, wasps, and moths. A wide assortment of odorants could elicit oscillatory responses at around 10Hz local field potential (LFP), including dilute monomolecular chemicals such as hexanol, natural fly attractants such as ripe banana, and the yeast paste that serves as fly food—all delivered at normal concentrations. Puffs of clean air elicited no oscillations.

To determine the origins of the LFP oscillations that we had recorded in the calyx of the mushroom body, we made paired recordings from the brains of intact, healthy flies and simultaneously monitored the odor-elicited LFP from the mushroom body calyx and the intracellular responses from the PNs that supply most of the olfactory input to the mushroom body. Our recordings routinely revealed subthreshold membrane potential oscillations correlated with the LFP recorded simultaneously in the mushroom body. We also noted that odor-elicited spikes in both projection neurons and inhibitory local neurons were tightly phase-locked to LFP, supporting the idea that the oscillations were generated in the antennal lobe and then transferred to the mushroom body calyx by projection neurons. Consistent with this observation, the oscillations could be reversibly abolished by applying the $GABA_A$ blocker picrotoxin, which interferes with the output of local neurons.

To examine the roles of the local neurons with unprecedented precision, we used genetic tools to target specifically and manipulate different types of local neurons. We found two classes of local neuron. One class has widely branching processes and is identified with the GAL4 strain NP2426; the other has narrow branch patterns and is identified with the GAL4 strain NP1227. We next analyzed the extent to which the two classes of local neuron contribute to the generation of odor-elicited oscillations. To gain control over the output of the neurons, we generated lines of flies expressing the *shibire*^{ts1} (*shi*) gene in each type of local neuron. The *shi* gene encodes a temperature-sensitive dynamin mutant protein that can conditionally and reversibly block chemical synaptic transmission at a restrictive temperature.

We found that flies expressing *shi* either in widely branching neurons alone or in both wide- and narrow-branch neurons showed a significantly greater temperature-sensitive decrease in oscillatory power than that in the wild type; we observed no significant differences between the narrow- and combined narrow- and wide-branching strains. The results indicate that only the widely branching local neurons contribute to the generation of odor-elicited oscillations.

Together, our results establish that *Drosophila* uses an oscillatory synchronization mechanism as part of its responses to odors. Further, the results reveal, with unprecedented precision, the mechanism underlying the oscillations. It will now be of great interest to use these new insights and tools to examine the functions of neural oscillations in odor coding and decision making.

Temporally diverse firing patterns in olfactory receptor neurons underlie neural codes for odors

Raman, Joseph, Tang[1]

Odorants are represented as spatio-temporal patterns of spiking in the antennal lobes of insects and the olfactory bulbs of vertebrates. These odor-evoked ensemble responses, which contain information about odor identity, intensity, and timing, are reliable over repeated trials and are sometimes remarkably elaborate, consisting of sequences of excitation and inhibition that together can outlast the eliciting odor stimulus. These patterns change most rapidly during the odor's onset and offset. During the middle portions of lengthy odor presentations, the firings of populations of principal neurons tend to settle into stable patterns that have been described, in the language of dynamical systems analysis, as a "fixed point." These activity patterns provide all the information that the brain receives about odors in the environment. The question is how the neural codes for odors are generated.

We hypothesized that the codes depend on a diversity of output from olfactory receptor neurons (ORN). To examine the characteristics of the input from the antenna to the antennal lobe, we first produced electroantennograms (EAG) from isolated locust antennae; the EAG provides an assay of total ORN output. EAG responses evoked by different odorants all showed relatively similar time courses. Earlier models of antennal lobe function were based on input similar to these EAG patterns. However, given that the EAG sums population activity, it obscures any odor-specific temporal structure contributed by individual receptor neurons. To characterize the responses of individual ORNs that underlie the EAG response (and that provide olfactory input to the antennal lobe), we made the first systematic recordings from ORNs in the locust. We found that the responses of individual ORNs showed a surprising diversity of temporal structure, including periods of inhibition, and sequences of excitation and inhibition. Viewed as a population, the response characteristics of the ORNs were in many ways remarkably similar to those observed downstream in the responses of the well-described projection neurons. Thus, many of the characteristics of odor codes thought to arise in the antennal lobe actually arise earlier in the periphery and include the odor-elicited spatio-temporal patterning of the principal neuron activity, the decoupling of odor identity from intensity, and the formation of fixed points for long odor pulses. The question arises as to the roles these complex peripheral responses might play in establishing neural codes for odors in the antennal lobe.

To examine systematically the significance of the diverse odor responses in the ORNs, we constructed a two-part computational model. The first part simulated a population of ORN odor responses while the second part realistically modeled the responses of the antennal lobe circuitry to the diversity of input. We found that our combined model generated responses that accurately matched the complexity, duration, and fixed-point characteristics of spatiotemporal odor codes recorded from the antennal lobe.

Further, given the surprising diversity and complexity of input provided by the receptor neurons, we considered what additional information processing roles the antennal lobe might play. With our combined computational model, we found that the network dynamics of the antennal lobe impose oscillatory synchronization, transform the spatio-temporal input from ORNs into a higher-dimensional representation, and evenly redistribute odor codes to make better use of coding capacity. In several instances, our results demonstrated that apparently complex forms of information processing described in other systems may be explained by simple interactions between receptor neurons and their followers.

In view of the strong structural and functional parallels between insect and vertebrate olfactory systems, it seems likely that similar mechanisms operate across species. We therefore look forward to determining how ORNs generate diverse temporal responses to odors.

Spontaneous odor receptor neuron activity determines follower cell response properties
Joseph, Dunn

Noisy or spontaneous activity poses a challenge to neural systems. Activity in the absence of obvious stimuli occurs throughout the central and peripheral nervous systems. Such spontaneous activity has been shown to play several useful roles; in some situations, however, it may not obviously benefit the organism and instead imposes limits on perception and behavior. We used the locust olfactory system to investigate fundamental properties of spontaneous activity at points along the sensory pathway. In insects, ORNs are distributed along the antenna. Our recordings from these neurons and their immediate and more distant followers revealed high levels of spontaneous activity in the ORNs themselves, local neurons, and projection neurons, but little spontaneous activity in Kenyon cells. Indeed, in a variety of species, spontaneous activity has been observed in ORNs and their immediate follower neurons (e.g., mitral cells in vertebrates); however, neurons one synapse farther (e.g., cortical neurons in vertebrates), such as Kenyon cells in insects, are typically almost silent at rest.

Where and how does this spontaneous activity originate? What rules govern its propagation from one group of neurons to the next, and how and why is such activity sharply limited two steps removed from the ORNs? What effects does spontaneous activity exert on olfactory coding? The locust olfactory system, where sensilla-containing ORNs are located both externally and accessibly, provided us with the opportunity to answer these questions directly.

We found that we could reversibly silence the ORNs by cooling them and that such silencing nearly abolished spontaneous and odor-elicited spiking in the projection neurons as well. Our investigation resolved a long-standing issue: spontaneous activity does not arise within the circuitry of the antennal lobe but rather is inherited entirely from the output of the ORNs. With a series of whole-cell patch-clamp recordings, we found that silencing the ORNs also significantly decreased the resting membrane potentials of projection neurons, local neurons, and Kenyon cells, indicating that spontaneous activity originating in the odor receptors exerts a constant influence on the response thresholds of follower cells.

To investigate the causes of spontaneous activity in ORNs, we controlled the delivery of odorant and the purity of air surrounding the antenna. In insects, as in vertebrates, absorbent fluid lymph surrounds ORNs. We found that isolating the antenna from its environment by coating it with an oily substance such as Vaseline® had little impact on spontaneous ORN output. However, manipulations designed to accelerate the removal of odorants from the sensillar lymph (surrounding the antenna with ultra-purified bottled air or directing high-speed streams of room air toward the antenna) significantly reduced the amount of output from the ORNs. Taken together, our results show that odorants or other ligands lingering in the sensillar lymph trigger spontaneous activity in the olfactory system. Environmental odorants continually presented to the antenna appear to play only a small role. Events within the ORNs' transduction machinery may make additional contributions to spontaneous activity.

To understand why spontaneous activity originating in ORNs is passed to the secondary projection neurons but is then sharply attenuated before reaching tertiary Kenyon cells, we developed a simple receiving operator characteristic (ROC) model to simulate the success of Kenyon cells in discriminating signal from noise, when presented with varying degrees of input convergence and ORN signal strength and various threshold set points. We found that, given the ongoing barrage of activity from ORNs, odor detection is optimal when projection neurons have a low response threshold; Kenyon cells, however, have a high response threshold. Our exploration of noise sources in the locust olfactory system provides a specific example of how a sensory system, bombarded with noise at the first stage of processing, balances the competing challenges of maintaining sensitivity to a wide range of stimuli and setting thresholds to eliminate noise and sparsen neural codes. We

expect that such strategies apply to other sensory systems that employ several stages of processing and circuitry convergence to achieve optimal detection.

Sensory neuron responses determine innate olfactory behaviors in the locust
Sun, Vislay-Meltzer

How do the sensory capacities of animals develop? Through their innate sensory preferences, animals often demonstrate the existence of inborn information. We are investigating how such information is encoded and how it differs from information acquired through direct experience. To conduct our studies, we use hatchling locusts, which offer several experimental advantages.

We found that newly hatched locusts, literally just crawling out of their egg hatching cups, immediately move toward fresh grass. In a series of behavioral studies using thousands of locusts, tested individually or in groups, we established that the hatchlings choose real grass over visually similar but odorless plastic grass; paper rubbed with fresh grass over clean paper of the same color; and paper dabbed with colorless monomolecular odorants that are components of grass odor over paper with other colorless odorants (even when the odorants were diluted to have identical vapor pressures). The results indicate that naive locusts, which had never eaten, touched, or otherwise encountered their natural food source, have a built-in preference for its odor.

Such preference could be attributed to a peripheral mechanism; for example, hatchling locusts could have a surplus of odor receptors for grass odors. We tested such a hypothesis by making electroantennograms from hatchlings and found that the antennae generate especially strong signals when stimulated by hexanol and octanol, which are volatile chemicals released by many green plants. Other odorants generated weaker responses. The same result obtained when the various odors were all diluted to provide equal vapor pressure, indicating either that the hatchling and adult antennae contain a surfeit of hexanol and octanol receptors or that each hexanol and octanol receptor provides a particularly strong output. In addition, we found that, in hatchling antennae, sensory adaptation occurs for grass odors with the same timing and extent as for non-plant odors. Our results strongly suggest that the locust's innate sensory preference for grass odors is encoded peripherally, in the antenna. We also found that the response profiles of adult locust antennae are virtually identical to those of the new hatchlings, indicating that no experience or "tuning" is required to prepare locusts to detect grass odors preferentially.

Frequency shifts reveal basic mechanisms for odor-evoked neural oscillations
Ito I, Ong, Raman; in collaboration with Bazhenov

Accumulating evidence suggests that in many animals, from insects to mammals, olfactory information is represented by the temporally structured, synchronized firing of a spatially distributed population of neurons in the olfactory systems. We found that a wide range of odorants and stimulus pulse durations all evoked clear oscillatory activity in the moth *Manduca sexta*. Interestingly, relatively brief, plume-like pulses (shorter than 750 ms) produced only fast oscillations (30–40 Hz), whereas long pulses (longer than 1 s), such as those that effectively induce associative learning, produced an oscillatory burst that was initially fast and then slow (10–20 Hz). These results provide the first clear evidence that moths, like locusts, produce oscillatory responses to odors. These findings also provide the first indication that (1) brief and lengthy presentations of the same odorant elicit somewhat different types of neural responses and (2) the oscillatory mechanism can operate stably in fast and slow modes.

What determines the oscillation frequency of a circuit, and how can a single neural circuit operate stably under two frequency regimes? We found that, in the moth, a lengthy odor pulse elicits an EAG deflection that, over the response, decreases in amplitude because of adaptation in ORNs. We also found that oscillation frequency roughly tracked EAG amplitude, suggesting that frequency may depend on the intensity of input to the circuitry of the antennal lobe.

In addition, however, we discovered that, over a wide range of odor concentrations, the initial odor-elicited oscillation frequency was invariant, whereas EAG amplitudes varied greatly with concentration. Thus, some evidence suggested that oscillation frequency was dependent on input intensity, but other evidence suggested the opposite. To explore this apparent contradiction, we developed a computational model of the moth antennal lobe that mimics the sharp transition between discrete fast and slow oscillatory states when input intensity gradually decreases. The recruitment of additional but less well-tuned ORNs to simulate responses to higher concentrations did not affect oscillation frequency. Our recordings from ORNs showed that long odor pulses caused most individual ORNs to adapt their firing rates rapidly; the firing-rate change closely matched the time course of the shift in oscillation frequency. Our results suggest that oscillation frequency can shift between two stable states, depending on the varying output intensity of adapting receptors rather than on odor concentration. Our model indicates that such a shift in oscillation frequency is possible if the ORNs that are highly tuned for a given odor fire at near-saturating rates even when presented with low odor concentrations; higher concentrations recruit additional but less tuned ORNs. The model also revealed that oscillation frequency is regulated flexibly by the intensity of input rather than rigidly by the duration of the inhibitory post-synaptic potential from inhibitory neurons, as is often assumed.

Several mechanisms extract features from natural odor stimuli
Joseph, Daffron, Stopfer

As information moves through the brain, it undergoes dramatic transformation in myriad ways. Our previous work suggested that one of the general mechanisms responsible for such transformation is neural plasticity. To investigate mechanisms underlying the transformations, we delivered rapid, repeated pulses of odors with timing designed to mimic features of natural plumes and monitored, in intact animals, neural activity in several locations: olfactory receptor neurons, ensembles of projection and local first-order interneurons of the antennal lobe (analogous to the olfactory bulb), and the second-order Kenyon cells of the mushroom body (analogous to the pyriform cortex). At each location, we sought to understand responses in terms of the interactions of plasticity occurring at earlier sites. We also sought to understand the transformations' potential value to the animal.

We found that interneuronal responses to natural odor stimuli are shaped by at least two plastic mechanisms: rapid adaptation in the receptors and relatively enduring facilitation of inhibition within the receptors' downstream targets. Peripheral adaptation renders the olfactory system relatively insensitive to stimuli that repeat very rapidly. Central facilitation of inhibition increases the reliability and sparseness of stimuli that are encountered repeatedly but are separated by long intervals. Further, these mechanisms constrain the projection neuron ensemble in order to provide relatively stable output to its downstream followers, thereby allowing the encoding of information about odor identity and concentration with firing patterns that are not confounded by the timing patterns of the stimulus.

How do the follower neurons decode this time-varying ensemble activity? Intracellular and extracellular recordings from Kenyon cells showed that the cells' firing rates change dramatically throughout trains of odor pulses in a timing-dependent manner; for brief inter-pulse intervals, the great majority of action potentials fire at the beginning of the train and again following the train's conclusion. We found that the Kenyon cells' firing threshold can be met when projection neurons fire at rates that are relatively low but with spikes that are highly synchronized across the population by the oscillatory mechanism of the antennal lobe (as occurs during the onset of the pulse train). On the other hand, the threshold can be met when the instantaneous firing rate of the projection neuron ensemble is high in the absence of pronounced synchronization (as occurs following the offset of the train).

Together, our work suggests that the non-associative plasticity elicited by odor plumes leads to responses in the projection neuron ensemble that combine an instantaneous report of sensory input with a record of recent input, allowing the extraction of high-level features.

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GENETIC AND ENVIRONMENTAL DETERMINANTS OF PRIMATE BIOBEHAVIORAL DEVELOPMENT

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Our research involves broad-based investigations of primate biological and behavioral development through comparative longitudinal studies of rhesus monkeys and other primates. Our primary goals are to characterize distinctive biobehavioral phenotypes in our rhesus monkey colony, to determine how genetic and environmental factors interact to shape the developmental trajectories of each phenotype, and to assess the long-term behavioral and biological consequences for monkeys from different genetic backgrounds when they are reared in different physical and social environments. A second major program of research investigates how rhesus monkeys and other non-human primate species born and raised under different laboratory conditions adapt to placement into environments that model specific features of their natural habitat.

Developmental continuity of individual differences in rhesus monkey biobehavioral development

Darcey, Hipp, Knappe, Ruggiero, Suomi; in collaboration with Adler, Alleva, Barr, Bennett, Chefer, Cirulli, Cole, Epel, Goldman, Heckman, Heilig, Herman, Higley, Lesch, Meaney, Nelson, Schwandt, Shoaf, Spinelli, Stein, Szyf, Winslow

This past year, we characterized, by assaying plasma BDNF and NGF, developmental changes in peripheral measures of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in rhesus monkeys with different early social rearing backgrounds reared either by their biological mother (MR) or in the neonatal nursery with subsequent continuous access to peers (PR). We replicated our previous findings that BDNF levels decrease dramatically over the first two months and then more gradually over the rest of the animals' first year, except for PR females, whose levels remain high throughout their first two months. In contrast, NGF levels remain relatively stable for the first two months and then increase sharply from two months to one year of age, achieving adult levels at that point, except for PR males whose increases in NGF levels occur much earlier. Among PR (but not MR) infants, plasma NGF and cortisol levels are significantly correlated. In addition, within each rearing group, individual differences in both plasma BDNF and NGF values remain largely stable throughout development, suggesting possible genetic influences. One potential candidate gene is the *BDNF* gene, for which functional polymorphisms have been characterized in

both humans and rodents. We identified a functionally similar BDNF polymorphism in our rhesus monkey colony and are determining whether allelic differences in the rhesus monkey *BDNF* gene are associated with individual differences in plasma BDNF values and other measures of biobehavioral functioning throughout development.

We carried out two brain neuroimaging studies on 2-year-old MR and PR rhesus monkey juveniles. Structural MRI revealed that PR juveniles had a significantly greater volume of cerebellar vermis, medial prefrontal cortex, and dorsal anterior cingulated cortex than MR juveniles. In addition, PR females had significantly lower hippocampal volume than PR males and both MR males and females. PET neuroimaging of the same monkeys revealed both gender and rearing-condition effects; females had significantly lower 5-HT_{1A} receptor densities throughout the brain than males, and both female and male PR juveniles had significantly lower 5-HT_{1A} receptor densities than their MR counterparts. We found significantly greater 5-HT_{1A} binding potential (BP) in the prefrontal cortex of female PR monkeys had than of MR females, whereas PR males had significantly lower BP than MR males in the medial cingulated cortex. We demonstrated that differences in early rearing history resulted in differences in laterality; as adolescents, MR monkeys had a greater right-handed behavioral bias than did PR monkeys. Finally, throughout their first year of life PR monkeys had chronically higher levels of cortisol than MR monkeys.

We continued data collection and analysis on two other projects that compared MR and PR rhesus monkeys on additional measures of biological functioning. In collaboration with colleagues from McGill University, we are assessing methylation patterns in glucocorticoid receptor genes in hippocampus and prefrontal cortex obtained from young adult MR and PR subjects as well as in buccal samples and lymphocytes obtained longitudinally from MR and PR monkeys throughout development. In collaboration with colleagues from the University of California Los Angeles and University of Chicago, we are performing whole-genome scanning of lymphocyte samples obtained longitudinally from MR and PR subjects from infancy onward in order to identify possible rearing-condition differences in patterns of gene expression in the initial week of life and how those patterns might change differentially throughout development.

We are also characterizing interactions between differential early social rearing and polymorphisms in several candidate genes (G x E interactions), most notably the serotonin transporter gene (*5-HTT*) and *MAO-A* gene, for a variety of measures of behavioral and biological functioning throughout development in MR and PR rhesus monkeys. This past year, we identified significant G x E interactions involving the *5-HTT* polymorphism among MR infants whose mothers differed significantly in their care-giving patterns. Infants whose mothers exhibited low levels of ventral contact and grooming vocalized and explored less and were more passive in an open field test, but only if they carried the "short" *5-HTT* allele. In addition, in collaboration with colleagues from NIAAA, we identified additional functional polymorphisms in the corticotrophin-releasing factor ($CRH)_{2A}$ gene and *mu* opioid receptor gene and characterized G x E interactions with respect to (1) behavioral responses to social separation by juvenile rhesus monkeys and (2) several measures of alcohol preference and consumption among young adult monkeys.

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Adaptation of laboratory-reared monkeys to field environments

Abbott, Dettmer, Ionica, Kershner, Mallott, Novak MFX, Paukner, Robbins, O'Neill Wagner, Suomi; in collaboration with Byrne, Ferrari, Fox, Huntsberry, Murray, Novak MA, Silberberg, Thierry, Timme, Visalberghi

We assess adaptation by examining behavioral repertoires and monitoring a variety of physiological systems in monkeys throughout the lifespan, developing broad-based indices of relative physical and psychological well-being. We also assess the responses of subjects to experimental manipulations of their environments. Whenever possible, we collect field data for appropriate comparisons. In addition, we investigate the cognitive, behavioral, and social processes involved in adaptation to new settings and circumstances.

We expanded our study of rhesus monkey infants' capacity to imitate facial expressions directed toward them by a human model throughout their initial week of life. Such early imitative capabilities have been reported for human neonates and are thought to be mediated reflexively by mirror neurons. We found that approximately 60 percent of tested NR newborns were able to mimic facial expressions involving differential mouth and tongue movements, whereas over 90 percent of MR neonates were successful imitators. Follow-up behavioral observations revealed that, compared with infants who failed to imitate during their first week of life, NR infants who exhibited imitative behavior during their first week subsequently exhibited significantly greater sensory-motor coordination throughout their first month and higher levels of social play during peer interaction sessions from four months onward. Moreover, when NR monkeys were subsequently moved permanently into large groups of same-age peers, those individuals who had failed to imitate during their initial days of life developed much higher rates of self-directed and autistic-like repetitive stereotypic behavior than NR monkeys who demonstrated imitative capabilities as neonates.

In collaboration with colleagues from the University of Maryland, we monitored EEG activity in the monkey neonates in their first postnatal week during imitative test sessions and during appropriate non-imitative control periods. Preliminary analyses revealed, concomitant with imitative behavior, patterns of slow-wave EEG alpha that are not seen in infants who fail to imitate in the same setting.

We intensified our study of rhesus monkey mother-infant interactions during the initial postnatal days. In marked contrast to previous reports concerning the normative development of attachment relationships in this species, we found that rhesus mother-infant dyads engaged in frequent and intensive face-to-face interactions throughout the infants' first three weeks of life, after which those behaviors largely disappear. We demonstrated that rhesus infants can differentiate pictures of monkey faces from non-social stimuli from day 1 onward and that, by day 10, they show a significant preference for pictures of adult female monkey faces over those of adult males, without any postnatal exposure to adults of either gender.

Another collaboration involves colleagues from the University of California San Francisco (UCSF) who had previously demonstrated differences in telomere length in adult humans as a function of differences in social status and cumulative social stress. Telomere length is thought to be a marker of relative cellular age. The UCSF researchers hypothesized that differences in telomere length may have their origin in differential experiences with social stress during the childhood years, a period when telomeres shorten rapidly. Accordingly we have been providing our UCSF

colleagues with DNA extracted from rhesus monkeys living outdoors in a physically and socially naturalistic setting. Initial analyses revealed both age and status differences in telomere length; younger monkeys had longer telomeres than older ones, and members of high-ranking families had longer telomeres than members of low-ranking families. In collaboration with colleagues from the University of Massachusetts, we assayed cortisol concentrations in hair samples as a potential index of chronic stress. We found significant differences in age, gender, and social status. Younger, female, and low-ranking monkeys had higher hair cortisol concentrations than older, male, and high-ranking individuals. We also compared these values with hair cortisol samples obtained from a captive colony of Tonkean macaques living in an outdoor enclosure similar to that in which the rhesus monkeys were living. The Tonkean macaques exhibited the same pattern of age, gender, and status differences, but their overall cortisol levels were significantly higher than those of their rhesus counterparts.

We completed several studies with our colony of tufted capuchin monkeys. One study demonstrated that capuchin monkeys are capable of recognizing when a human observer is imitating them and revealed that such imitation influences their behavioral preferences. Another study, in contrast, found that capuchin monkeys fail to exhibit the same pattern of memory awareness previously demonstrated in rhesus monkeys, apes, and humans. We found that (1) the animals consistently used visual but not auditory information when locating hidden food items; (2) when faced with choices offering differential potential reward, capuchin monkeys did not demonstrate loss aversion but instead chose on the basis of alternatives in delay of reinforcement; and (3) fur-rubbing behavior, previously thought to have largely medicinal functions, instead serves social purposes, especially with respect to affiliative and aggressive activities.

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In mammals, new neurons are continuously generated in the mature nervous system through the regulated proliferation and differentiation of quiescent neural stem cells (NSCs). One of the signaling molecules that influences the behavior of NSCs is sonic hedgehog (Shh). Using Genetic Inducible Fate Mapping (GIFM), we have shown that Shh-responsive NSCs self-renew and generate several cell types in the nervous system. Using conditional ablation of major effectors of the Shh signaling pathway,we are continuing to investigate the mechanisms by which Shh signaling maintains and regulates proliferation and differentiation of quiescent NSCs. Furthermore, we are pursuing the identification of novel downstream target genes of Shh signaling in NSCs in order to develop a deeper understanding of stem cell behavior. We have also undertaken novel genetic approaches to study the biological role of newly generated neurons in the adult mouse forebrain by analyzing the neural circuits formed by these newborn neurons. Our studies will provide the foundation needed for stem cell biology to develop therapeutic methods for treating various neurodegenerative diseases.

Molecular mechanism by which Shh acts on neural stem cells
Ge, Wang, Ahn

Shh signaling is mediated by the Smo receptor and by the Gli2 (activator) and Gli3 (repressor) transcription factors in responding cells. As in the developing neural tube and limb, the relative levels of Gli2 and Gli3R may play a critical role in determining whether quiescent NSCs exit the cell cycle to generate proliferative precursors. Therefore, we are dissecting the distinct contribution of each effector in NSC biology by using conditional genetic ablation approaches both in vitro and in vivo. First, to investigate the early developmental requirements of Gli2 or Gli3 specifically in neuronal populations, we used *Nestin-Cre* mice to delete *Gli2* or *Gli3* from all the neuronal progenitors. Unlike *Gli2* null mice, *Nestin-Cre;Gli2* conditional mutant mice survive to adulthood. As previously reported, the size and complexity of midbrain and cerebellum are greatly reduced in this mutant allele. However, the forebrain structure appears largely intact, as evidenced by histological analysis. Interestingly, *Nestin-Cre;Gli3* conditional mutant mice exhibit various forebrain phenotypes, including thinner cortical layers, enlarged lateral ventricles, and reduced hippocampal formation. We are currently characterizing the changes in the proliferation and/or specification of progenitors at different developmental stages and the integrity of the ependymal wall of the ventricles in the mutant mice. Second, we are administering Tamoxifen™ to adult *Gli1-CreER* mice to delete conditionally *Gli2* or *Gli3* in Shh-responding NSCs. Using immunohistochemistry,

we are characterizing the consequences of *Gli2* or *Gli3* deletion in the mice with desired genotypes at both anatomical and molecular levels. Drawing on combinatorial approaches in vivo and in vitro, we will be able to determine how Shh signaling maintains NSCs in their quiescent state or instructs the quiescent NSCs to proliferate and differentiate based on the intracellular balance of Gli activators and repressors.

Downstream target genes of Shh signaling in neural stem cells

Ralls, Ahn

By identifying downstream target genes, we will gain insight into the role played by Shh in NSC maintenance and/or proliferation. We are therefore isolating NSCs from the neurogenic regions of adult mouse forebrain based on their responsiveness to Shh signaling (Gli1-positive) and their expression of the putative stem cell marker GFAP. Specifically, we are labeling Shh-responding NSCs with Tamoxifen™ in *Gli1-CreER^{+};Z/EG* mice, which express the EGFP reporter protein in the desired cell population. Using FACS (fluorescence-activated cell sorting), we have successfully isolated *Gli1^{+};GFAP^{+}* neural stem cells from two neurogenic regions of the forebrain: the subventricular zone (SVZ) and hippocampus. We are currently using an Affymetrix microarray approach to identify genes expressed only in the stem cells of the SVZ and hippocampus. Identification of downstream target genes will shed light on Shh's role in neural stem cell maintenance and/or proliferation.

Neural circuit formation by newly generated neurons in the dentate gyrus of the hippocampus

Barrett, Chan,[3] Ahn

In the hippocampus, dentate gyrus (DG) granule neurons are continuously generated from NSCs located in the subgranular layer of the DG. By marking and following the projections of newborn granule cells with *Tau^{mGFP}* reporter mice (generated in the Arber laboratory) (see Figure 6.1), we are investigating how NSC-derived newborn neurons integrate into existing circuits. To study the

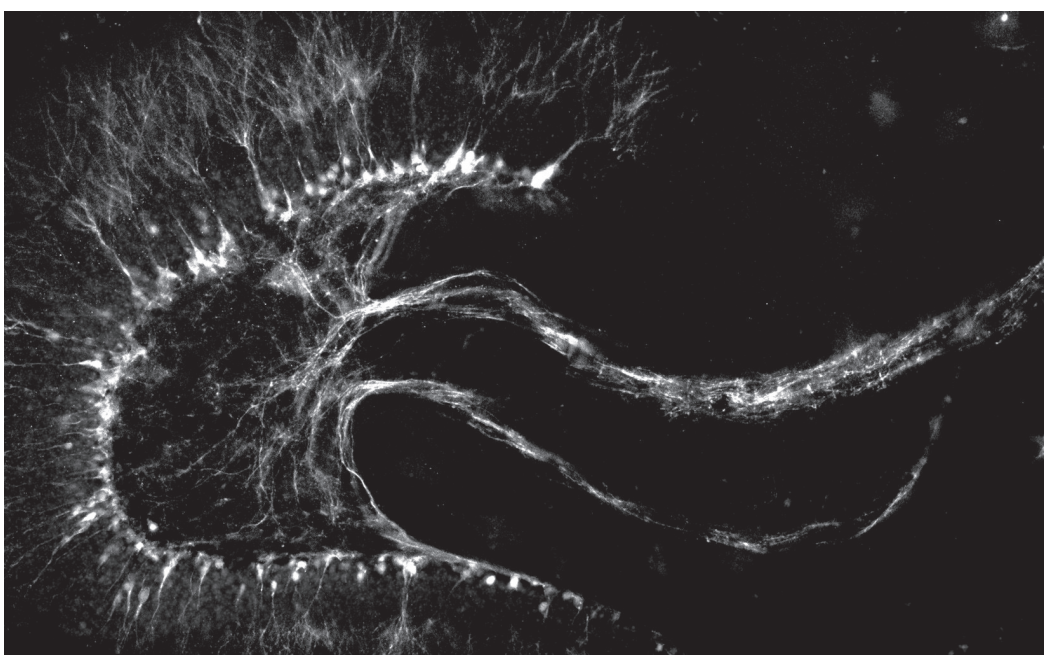

FIGURE 6.1

New granule neurons are generated from Shh-responding NSCs in the dentate gyrus of hippocampus and send out projections toward the CA3 region.

formation of the hippocampal trisynaptic circuits from the DG to CA3 to CA1, we generated novel *WGA* (wheat germ agglutinin) reporter mice in which a trans-synaptically transferable fluorescent protein is expressed in the Shh-responding NSCs and their progeny. By uncovering neural circuit formation by the newly generated neurons, we will be able to gain insights into the function and purpose of continued neurogenesis in normal mice. A parallel study using mutant mice or mouse models of neurological disease will help us understand the consequences of malformed neural circuits for learning and memory.

Genetic lineage of midbrain dopaminergic neurons

Hayes, Ahn; in collaboration with Zervas

Dopaminergic (DA) neurons in the ventral midbrain are involved in various neurological processes, including the control of voluntary movements and the regulation of emotion. Midbrain DA neurons may be segregated into several clusters that include the substantia nigra pars compacta and the ventral tegmental area. DA neurons in these regions exhibit distinct patterns of axonal projections and innervate specific target areas and their depletion or aberrant projections result in neurological disorders such as Parkinson's disease or schizophrenia. It has been also shown that Shh signaling is critical in specifying DA neural progenitors during early neural development. Using GIFM, we are testing our hypothesis that specific subclasses of DA neurons are specified by the genetic lineage at distinct developmental stages of their precursors in the ventral midbrain. In particular, we are addressing whether Shh-responding and Shh-expressing cells at different stages contribute to distinct DA neuron subpopulations that ultimately innervate functionally segregated target regions.

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INTEGRATION OF BACTERIAL NUTRITIONAL RESPONSES

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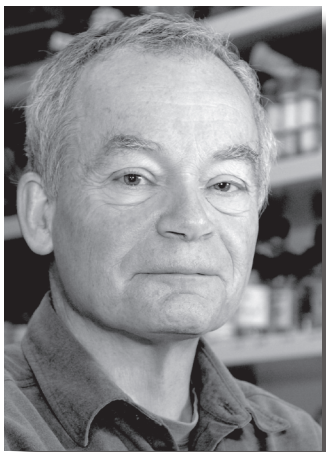

Remarkably, slowing bacterial growth by limiting almost any nutrient (nitrogen, carbon, amino acids, many vitamins, phosphate, iron, or oxygen) produces elevated levels of a regulatory nucleotide called ppGpp. The nucleotide signal in turn leads to positive or negative regulation of the expression of about one-third of the *E. coli* genome. We are interested in understanding this fundamental microbial regulatory mechanism. Although regulation by ppGpp occurs largely at the level of gene transcription by RNA polymerase, few molecular details are known. Many physiological changes accompanying ppGpp regulation may be viewed as coordinated to ensure cellular survival and adaptation to nutritional stress. Coordinated cellular responses are thought to be attributable in part to direct effects on promoter activity, in part to transcription regulation by alternative sigma factor activities, and in part to regulatory effects on metabolism. Our understanding of these phenomena is just beginning to take shape. It is also important to recognize that ppGpp may be correlated with virulence in pathogens. Nuclear genes for ppGpp synthesis from plants are functional in bacteria, chloroplasts being targeted for ppGpp production. The nucleotide mediates embryonic development and responses to environmental stress ranging from pathogens to wounding and nutrient stress.

DksA, GreA, GreB, and TraR proteins unexpectedly affect ppGpp transcription regulation
Potrykus, Vinella, Herman, Harinarayanan, Murphy, Cashel; in collaboration with Herman, Vinella

Regulation of transcription by ppGpp at the molecular level is unusual (for prokaryotes) in that it does not involve the typical interplay of sequence-specific DNA-binding repressor or inducer proteins. Instead, ppGpp is thought to modify RNA polymerase initiation kinetics in ways that inhibit or activate specific classes of promoters—ppGpp and RNA polymerase together targeting core sequences. Regulation occurs by a synergistic interaction between ppGpp and some member of a group of structurally similar proteins whose structural similarity parallels the group's ability to probe the secondary channel of RNA polymerase; however, it remains unclear how secondary-channel binding triggers regulation. DksA was the first regulatory protein of this type to be discovered (in another laboratory). Over the past few years, we found that, in some respects, two proteins (GreA and mutant GreB) behave similarly to DksA. For example, wild-type GreA or a GreB mutant can alter phenotypes produced simply by the absence of DksA. When both DksA and ppGpp are absent, many phenotypic features are found in addition to those dependent on DksA alone. GreA as well as the same GreB mutant can also reverse a subset these additional changes. Both GreA and GreB also have a well-known function that is distinct from mimicking ppGpp and DksA regulation, namely, the ability to reverse transcription elongation arrest. We do not know why GreA and GreB mimic

some features of regulation by DksA and ppGpp. A plausible explanation is that DksA can reverse arrest of transcription elongation, although researchers long ago ruled out such an explanation. One working hypothesis holds that the proteins induce a conformational change in RNA polymerase that leads to shared kinetic features for promoter recognition.

This year, in collaboration with Christophe Herman's group, we discovered that TraR should be added to the list of DksA-, GreA-, and GreB-like proteins. The *traR* gene is found on the F′ episomal element that mediates conjugation. While the function of TraR was previously unknown, TraR was named for its position between genes *traQ* and *traS*. Even small amounts of TraR complement the amino acid requirements for growth in the absence of ppGpp, demonstrating an ability similar to that of GreA and GreB mutants. Unlike GreA and GreB but like ppGpp, TraR is an inhibitor of ribosomal RNA transcription. TraR shares the coiled-coil structural feature with DksA, GreA, and GreB that has been postulated to be associated with secondary-channel penetration. However, researchers have yet to demonstrate secondary-channel binding of TraR or solve TraR's structure. Even so, a potentially important difference between regulation by ppGpp and by TraR is that inhibition of growth by even large amounts of TraR takes hours, whereas growth stops within minutes when ppGpp is increased. We believe that this difference indicates an additional regulatory activity for ppGpp that would be important to identify.

We discovered AppppA, another surprising candidate for a regulatory substitute for ppGpp. The compound has been implicated in DNA replication in eukaryotes. We discovered AppppA with multicopy library screens in a search for genes that can complement the growth defect of ppGpp^0 *dksA* double mutants. With preliminary evidence implicating AppppA as a substitute regulator for ppGpp and DksA, we are attempting to manipulate the activity of an AppppA hydrolase encoded by the *apaH* gene to control AppppA independently of synthesis, as occurs with our library isolates.

Synthetic ppGpp^0 lethal mutants of *tktA*, *aceE*, and *proB* genes reveal that ppGpp regulates enzymes of intermediary metabolism

Harinarayanan, Murphy, Cashel

We have documented the basis for ppGpp regulation of intermediary metabolism by isolating *tktA*, *aceE*, and *proB*—mutants that grow poorly or not at all only when ppGpp is missing. Surprisingly, all encode enzymes of intermediary metabolism. The first example involves transketolases, key enzymes that form erythrose-3-phosphate in the pentose phosphate shunt. Erythrose-3-phosphate is an intermediate for the biosynthesis of aromatic amino acids and pyridoxine-derived vitamins. A *ΔtktA*/Δ*tktB* double mutant is completely deficient in transketolase. The synthetic lethal *tktA* ppGpp^0 strain is only marginally less severe than a complete transketolase deficiency. Using a series of *tktB*::lacZ transcriptional fusions to localize the *tktB* promoter, we discovered that *tktB* expression was largely abolished in a ppGpp^0 strain owing to an indirect effect—*tktB* expression depends on the presence of RpoS, an alternative sigma factor coordinating expression of stationary phase genes. Deletion of *rpoS* in a ppGpp$^+$ background gives an almost complete loss of *tktB*::lacZ activity. The regulatory pathway deduced to relate ppGpp to *tktB* expression is ppGpp→*rpoS*→*tktB*. Weak *tktB* promoter activity is noticeable when *rpoS* is deleted but ppGpp is present. We proposed that this is a direct effect of ppGpp occurring at the level of RNA polymerase itself.

The second synthetic lethal is an *aceE* mutant. The *aceE* gene encodes a subunit of pyruvate dehydrogenase that functions in the pathway for acetyl CoA formation. We believe that a deficiency of acetyl CoA occurs in *aceE* ppGpp^0 double mutants—we demonstrated that supplementation with acetate restores viability and that mutants blocking complementation of growth by acetate occur in genes needed to form acetyl CoA from acetate. A redundant pathway catalyzed by *poxB* can generate acetyl CoA when pyruvate dehydrogenase is inactive in *aceE* mutants. Again, the explanation for why *aceE* mutants are ppGpp^0 synthetic lethal mutants lies in an *rpoS* requirement. Thus, *aceE* mutants become lethal in the absence of ppGpp because, similar to the synthetic lethality of *tktA* mutants

described above, the mutants unveil a ppGpp requirement for *poxB* transcription that is exerted indirectly through RpoS. With isozymes quite common for glycolytic and hexose shunt pathways, we can surmise from these two examples that ppGpp^0 synthetic lethals might occur more generally. Indeed, in solving the *tktA/B* puzzle, we discovered that a mutant of *zwf* is also a ppGpp^0 synthetic lethal; zwf is a key enzyme in the branch point linking glycolysis and the pentose shunt.

The third ppGpp^0 synthetic lethal mutant that we discovered does not involve intermediary carbon metabolism. This is a *proB* mutant that fails to grow at 42ºC on LB media in ppGpp^0 strains. Such behavior seemed especially odd because the peptide-rich LB medium contains enough proline to allow the *proA*, *proB*, or *proC* mutants to grow when ppGpp is present. Raising proline concentrations in LB does cure the ppGpp^0 growth defect, indicating that proline availability is likely to be the problem. As LB has proline-containing peptides but little free proline, we guessed that ppGpp might be needed in one of three ways: (1) to activate proteases for liberating free proline from peptides: (2) to facilitate chaperone functions already known to occur at very high intracellular proline levels; or (3) to transport low amounts of extracellular proline to achieve high intracellular pools. We then screened a multicopy random gene library for isolates that restored LB growth of *proAB* mutants. We did not find complementation by overexpression of genes encoding either proteases or chaperonins, thus arguing against the first or second explanation. We did find an isolate that suggests the third explanation—complementation by a multicopy gene, *nhaA*. NhaA is known to maintain the membrane proton gradient through a sodium-proton pump. Characterization of *nhaA* phenotypes for ppGpp^0 and wild-type strains revealed that ppGpp is partly needed for a wild-type phenotype. Our working hypothesis posits that a partially defective sodium-proton pump in ppGpp^0 cells creates a need for high external proline concentrations to achieve enough intracellular proline to allow growth. We will test the prediction that additional transport defects for compounds other than proline should also be apparent in the absence of ppGpp.

Discovery of GraL small RNA in the *greA* leader transcript arising from dual promoters
Potrykus, Cashel

Our studies surveying regulation of *greA* expression have led to the finding that the *greA* leader transcript generates small RNA molecules (which we call GraL) arising from two strong promoters. The upstream P1 promoter uses a housekeeping Sigma-70 promoter, yielding a start site 149 nt away from the AUG of the *greA* orf. The downstream P2 promoter begins only 11 nt beyond the P1 start and requires the alternative sigma factor SigmaE, which is known to activate a class of promoters in response to cell-wall and cell-membrane stress. The activation of SigmaE itself requires ppGpp by pathways that involve direct interaction of ppGpp with RNA polymerase as well as an indirect mechanism that controls an inactive SigmaE precursor complex to release an active protein. From both P1- and P2-promoted RNA chains, GraL is generated by a shared terminator that releases 50- to 60-nt-long transcripts with two unusual features. The first such feature is that the end of the transcript chain length is variable, giving frayed ends with size differences ranging from 1–10 nt rather than precise stops at one or two positions. We have genetic evidence suggesting that the frayed ends do not result from ribonuclease cleavage, as supported by evidence from an array of antisense oligonucleotides showing that the frayed ends reflect actual termination sites. The second unusual feature is a (weak) P3 *greA* promoter that is found within the run of uridylic acid residues comprising the termination site for GraL. We have demonstrated the resulting GraL with in vitro transcripts as well as by Northern blots with samples isolated from cells. LacZ reporter activities somewhat surprisingly revealed that GraL had no effect on cellular *greA* expression. We therefore assessed the more general biological importance of this small RNA by determining whether GraL conferred a selective advantage over cells not overexpressing GraL during repeated cycles of overnight growth in LB media. We inoculated cultures with equal admixtures of cells containing a GraL-overproducing plasmid (or vector) with one or the other marked with a *malT* mutant. In one admixture the cell with the plasmid was marked; in the other admixture the cell with the vector

was marked; in this way, we could be sure that the marker itself (a *malT* mutant) was not the cause of the fitness advantage. We could score the proportion of maltose-fermenting cells at any time of culture as the portion of red and white colonies on maltose indicator plates. We found that cells overexpressing GraL reproducibly and systematically outcompete wild-type cells. After four cycles of overnight growth, 95 percent of the culture consisted of GraL overexpressors whether or not the *malT* marker mutation was present together with the GraL plasmid.

We have also performed microarray analyses. When GraL was overproduced for 15 minutes, we found no effect in wild-type strains. However, in $ppGpp^0$ over 100 genes were affected. Specifically, many genes affected by GraL are known to be repressed by Fur, a major regulator of iron metabolism. Our finding is intriguing given that we long ago showed that ppGpp is also involved in iron regulation. On the other hand, flagellar genes comprise a general class inhibited by GraL—ppGpp and DksA have been implicated in regulation of flagellar genes. Computer searches relying on RNA-RNA hybrids as mediators of small RNA regulation did not yield meaningful results. Genetic evidence also excluded RNaseIII and Hfq involvement in regulation exerted by this small RNA. GraL apparently functions in a manner dramatically different from more typical small RNA species.

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DYNAMIC REGULATION OF NOTCH SIGNALING DURING ZEBRAFISH NEUROGENESIS

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Our goal is to understand how, during early development, the architecture of the mature nervous system emerges as a consequence of local interactions between cells. We use a combination of cellular, molecular, genetic, and computational tools to understand how neurons differentiate in distinct patterns in the various compartments of the zebrafish nervous system. We analyze zebrafish mutants and embryos microinjected with morpholinos or mRNA for the purpose of altering gene function, allowing us (1) to examine mechanisms involved in the division of the prospective neural tissue into compartments with distinct fate and (2) to investigate how neuronal differentiation is regulated within each compartment. Genetic analysis in zebrafish leads to identification of regulatory networks essential for specific aspects of neural patterning while cell-biological experiments identify trafficking events essential for regulating signaling. Delta-Notch signaling regulates the number of neurons. Our recent studies have focused on the role of Delta endocytosis in activating its receptor Notch in neighboring cells and revealed that interactions with Notch play a critical role in regulating Delta endocytosis.

Interaction with Notch determines endocytosis of specific Delta ligands in zebrafish neural tissue

Matsuda, Chitnis

Neurons in zebrafish embryos are generated in discrete neurogenic domains where expression of basic helix-loop-helix transcription factors, related to the proneural genes in *Drosophila*, gives cells the potential to differentiate as neurons. However, activation of the Notch receptor in progenitors by its ligands Delta and Jagged, expressed on the surface of neighboring cells, prevents early differentiation of some progenitors within these domains and allows them to be maintained or to differentiate later with other fates.

Both Notch and its ligands are single-pass membrane proteins expressed on the cell surface; their interactions allow the ligand-expressing cell to regulate cell fate in a neighboring Notch-expressing cell. The Notch receptor has an intracellular domain (NICD) that can function as part of a transcriptional activator complex. Removal of an extracellular fragment of Notch (NECD) regulates release of the NICD domain and its translocation to the nucleus. The NECD fragment is cleaved during synthesis of the Notch receptor but remains bound to the extracellular stub of the remaining

membrane-bound fragment of Notch, where it prevents access to critical proteolytic cleavage sites. Removal of the NECD fragment allows proteases to cleave the Notch receptor sequentially—first in the extracellular stub and then in the intramembranous domain. The result is the release of the NICD fragment, which can then translocate to the nucleus and activate target genes after forming a complex with co-factors that bind to regulatory sites in target genes.

Removal of the NECD fragment and subsequent activation of Notch is facilitated by the former's binding to ligands on the surface of neighboring cells. Recent studies have suggested that, once Notch ligands such as Delta and Jagged/Serrate bind to Notch in a neighboring cell, the ligand-expressing cell internalizes the ligand bound to the NECD fragment. The result is the separation of the NECD fragment from the remaining membrane-bound Notch receptor, thereby allowing proteolytic cleavages that activate Notch by releasing the NICD fragment into the cell. Previously, we showed that endocytosis of Delta is triggered by Mind bomb (Mib), a RING ubiquitin ligase that facilitates ubiquitylation of Delta. The process results in the covalent linkage of a single or several molecules of the 76–amino acid protein ubiquitin to lysines in Delta's intracellular domain. Ubiquitin recruits components of the endocytic machinery, which, in turn, facilitate endocytosis of ubiquitylated Delta.

In the context of the above studies, we were unable to determine whether ubiquitylation and endocytosis of Notch ligands were triggered by the ligands' interaction with Notch in a neighboring cell or whether Mib-mediated endocytosis of Notch ligands such as Delta is a constitutive process that occurs independently of the ligands' interaction with Notch. We have now shown that interactions with Notch play an important role in determining the endocytosis of two Delta homologues, DeltaD and DeltaA, but not of a third homologue, DeltaC. First, we showed that, in the central nervous system, most of the Notch ligand DeltaD is located in cytoplasmic vesicles. However, in *mib* mutants, DeltaD and DeltaA accumulate on the cell surface, suggesting that Mib-mediated DeltaD endocytosis plays an important role in determining the cellular distribution of Delta. We then showed that, in Notch morphants in which Notch receptors are reduced by injecting embryos with anti-sense morpholinos, DeltaD and DeltaA accumulate on the cell surface just as they do in *mib* mutants, suggesting that interaction with Notch is also an important determinant of DeltaA and DeltaD endocytosis but not of DeltaC endocytosis.

Delta has the potential to interact with Notch both on the surface of a neighboring cell (in *trans*) and on the surface of the same cell (in *cis*). We thus investigated whether interactions either in *cis* or *trans* are critical for Delta endocytosis. To that end, we transplanted cells from an embryo in which Notch had been knocked down with morpholinos into wild-type embryos with Notch and, conversely, we transplanted wild-type cells with Notch into embryos without Notch. Changes in the cellular distribution of Delta in the transplanted cells and their neighbors helped us determine whether interactions with Notch within the same cell (in *cis*) or with Notch in a neighboring cell (in *trans*) were critical for Delta endocytosis. Interestingly, our experiments demonstrated that interactions with Notch in both *cis* and *trans* contribute to DeltaD endocytosis.

As discussed, interactions with Notch in *trans* help activate Notch in a neighboring cell. In contrast, it is thought that interaction of Delta with Notch in *cis* interferes with Notch activation in that same cell. Our observation that Delta-Notch interactions in *cis* contribute to Delta endocytosis suggests that, following an interaction in *cis*, endocytosis of the ligand-receptor complex reduces the availability of both ligand and receptor at the cell surface. Together, our observations show how interactions between Delta and Notch within the same cell and between adjacent cells play a critical role in the dynamic regulation of Notch signaling during development.

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CHROMATIN REMODELING AND GENE ACTIVATION

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Gene activation involves the regulated recruitment of factors to a promoter in response to appropriate signals, ultimately resulting in the formation of an initiation complex by RNA polymerase II and transcription. These events must occur in the presence of nucleosomes, which are compact structures capable of blocking transcription at every step. To circumvent the chromatin block, eukaryotic cells possess chromatin-remodeling and nucleosome-modifying complexes. The former (e.g., SWI/SNF complexes) use ATP to drive conformational changes in nucleosomes and to slide nucleosomes along DNA. The latter contain enzymatic activities (e.g., histone acetylases) that modify the histones post-translationally to mark them for recognition by other complexes. Geneticists have described many interesting connections between chromatin components and transcription, but they have lacked a system to investigate the structural basis of the connections. We have developed such a model system, involving native plasmid chromatin purified from the yeast *Saccharomyces cerevisiae*, to perform high-resolution studies of the chromatin structures of active and inactive genes. Remarkably, our work reveals that activation correlates with large-scale movements of nucleosomes and conformational changes within nucleosomes over entire genes. Our current work focuses on the roles of chromatin remodeling and histone acetylation in gene regulation.

Transcriptional activation and SWI-/SNF-dependent nucleosome mobilization
Kim,[1] McLaughlin; in collaboration with Tsukiyama

We chose budding yeast as a model organism because biochemical studies of chromatin structure can be combined with molecular genetics. Current models for the role of the SWI/SNF ATP-dependent chromatin remodeling complex in gene regulation focus on promoters, which is where the most obvious changes in chromatin structure occur. However, using our plasmid model system with *HIS3*, a SWI-/SNF-regulated gene, we discovered that transcriptional activation creates a domain of remodeled chromatin structure that extends far beyond the promoter to include the entire gene. We addressed the effects of transcriptional activation on the chromatin structure of *HIS3* by mapping the precise positions of nucleosomes in basal-expressing and transcriptionally activated chromatin. In the absence of the Gcn4p activator, the *HIS3* gene is organized into a dominant nucleosomal array. In wild-type chromatin, the array is disrupted, and several alternative, overlapping, nucleosomal arrays form. Disruption of the dominant array also requires the SWI/SNF remodeling machine, indicating that the SWI/SNF complex plays an important role in nucleosome mobilization. The Isw1 remodeling complex plays a more subtle role in determining nucleosome positions on *HIS3*, favoring different positions from those preferred by the SWI/SNF complex. We propose that Gcn4p

stimulates nucleosome mobilization over the entire *HIS3* gene by the SWI/SNF complex. We suggest that the net effect of interplay among remodeling machines at *HIS3* is to create a highly dynamic chromatin structure (Kim et al., 2006). Our work on *HIS3* and our earlier work on *CUP1* indicate that, at least for these two genes, the target of remodeling complexes is a domain rather than just the promoter. This important finding suggests that remodeling complexes act on chromatin domains. As to the function of domain remodeling, we speculate that remodeling entire genes might facilitate elongation through nucleosomes by RNA polymerase II. The aims of our current work are (1) to elucidate the structure of the remodeled nucleosome; there are at least two possibilities—unstable nucleosomes (remodeled such that they fall apart easily) and nucleosomes with a dramatically altered conformation—and (2) to map remodeled nucleosomes on a genome-wide scale and determine which domains are remodeled by the SWI/SNF complex.

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The yeast Spt10 protein contains a DNA-binding domain fused to a putative histone acetylase domain

Mendiratta,[2] Eriksson, McLaughlin, Kotomura,[3] Vivekananthan

We have shown previously that induction of *CUP1* by copper results in targeted acetylation of nucleosomes at the *CUP1* promoter. The acetylation is dependent on *SPT10*, which encodes a putative histone acetylase (HAT). *SPT10* has been implicated as a global regulator of core promoter activity. After confirming by expression microarray analysis that Spt10p has global effects on transcription, we addressed the mechanism of global regulation. Surprisingly, using the chromatin immunoprecipitation (ChIP) assay we were unable to detect Spt10p at any of the most strongly affected genes in vivo. However, we confirmed that Spt10p is present at the core histone gene promoters, which it activates. We presented evidence that a defective chromatin structure forms in the absence of Spt10p, with consequent activation of basal promoters. Furthermore, we found that Spt10p binds specifically and highly cooperatively to pairs of upstream activating sequences (UAS elements) in the core histone promoters [consensus: (G/A)TTCCN6TTCNC], consistent with a direct role in histone gene regulation. No other high-affinity sites are predicted in the yeast genome. Our observations are consistent with the idea that the global changes in gene expression observed in spt10Δ cells are the indirect effects of defective regulation of the core histone genes. Thus, Spt10p is a sequence-specific activator of the histone genes, possessing a DNA-binding domain fused to a likely HAT domain rather than to a classical activation domain. Spt10p is therefore a highly unusual transactivator in which the HAT domain, normally recruited as a co-activator to promoters through an activation domain, is attached directly to a sequence-specific DNA-binding domain.

We have identified the DNA-binding domain of Spt10p: it contains an unusual zinc finger (His2-Cys2) with homology to the DNA integrase of foamy retroviruses. We proposed that this integrase might also be a sequence-specific DNA-binding protein. To test our hypothesis, we initiated a new project that used a SELEX approach to determine whether human foamy virus (HFV) integrase is indeed a sequence-specific DNA-binding protein.

We addressed the mechanism through which Spt10p simultaneously recognizes two UAS elements (Mendiratta et al., 2007). We demonstrated that Spt10p is an elongated dimer and that its N-terminal domain is necessary for dimer formation (Mendiratta et al., 2007). Unlike the full-length protein dimer, which requires two UAS elements, the isolated DNA-binding domain is a monomer and binds tightly to a single UAS element. We propose that the Spt10p dimer undergoes a major conformational change in order to recognize two UAS elements at the same time.

Our current work aims to (1) demonstrate the putative histone/protein acetylase activity of Spt10p by using a proteomics approach; (2) understand the role of Spt10p in the cell cycle–dependent

regulation of the core histone genes; and (3) identify negative regulatory proteins at the histone promoters, which counteract activation by Spt10p.

Mendiratta G, Eriksson PR, Clark DJ. Cooperative binding of the yeast Spt10p activator to the histone UAS elements is mediated through an N-terminal dimerization domain. *Nucleic Acids Res* 2007;35:812-821.

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PHYSIOLOGICAL, BIOCHEMICAL, AND MOLECULAR GENETIC EVENTS OF RECOGNITION AND RESOLUTION OF RNA/DNA HYBRIDS

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The aim of our research is to understand processes involved in cellular DNA replication and the relationship of HIV replication to cellular DNA replication events and how to use the resultant information for therapeutic purposes. We are examining the formation and resolution of RNA/DNA hybrids that form during DNA replication or transcription. Ribonucleases H participate in the removal of the RNA of the RNA/DNA hybrids and are intimately related to DNA replication in cells and in HIV replication during conversion of the virus's RNA genome to DNA. With similar protein architectures, RNases H of cells and HIV share common enzymatic mechanisms for cleaving RNA. Drugs that alter the levels of specific disease-related genes are in development to take advantage of RNases H within the cell. Regulated expression of RNases H could enhance the efficacy of the drugs. We employ molecular-genetic and biochemical tools and mouse animal models in our efforts.

Structure-function of ribonucleases H: RNase H1
Cerritelli, Crouch; in collaboration with Nowotny, Yang

RNA/DNA hybrids are essential intermediates in the replication of HIV's RNA genome. In addition, the hybrids are believed to be necessary for mitochondrial DNA replication and important for switching immunoglobulin isotypes (e.g., from IgM to IgA). How these enzymes recognize and cleave the RNA is important in understanding the biology of these diverse events and for possible regulation of RNase H activity. Bacterial RNases HI are generally small proteins (150 amino acids) that share significant similarity with their eukaryotic counterparts in structure, interaction with their substrates, and the mechanism of cleavage. The protein structure of *E. coli* RNase HI unbound to a substrate is similar to that of the structure of human RNase H1 in complex with an RNA/DNA hybrid. The enzyme recognizes at least four hydroxyl groups of the ribose, with the DNA significantly distorted so that one phosphate is able to bind in a pocket on the enzyme (Nowotny et al., *Cell* 2005;121:1005). Both properties contribute to the specificities of the enzyme. The "basic protrusion" of the enzyme has extensive interactions with the hybrid, adding to the stability of the complex. Eukaroytic RNases H have an N-terminal domain (absent from the bacterial enzyme) that binds to RNA/DNA, conferring processivity to the enzyme (Gaidamakov et al., *Nucleic Acids Res* 2005;33:2166). This N-terminal domain (HBD) interacts with RNA/DNA through contacts with both the DNA and RNA strands and may provide the initial contact between the enzyme and hybrid.

The N- and C-terminal domains are connected by 65 amino acids in the human enzyme and by 64 in the mouse enzyme. The length and sequence of the connection domain are extremely variable in other species, suggesting that RNase H activity may not depend on any particular amino acid number or sequence. Our studies indicate that the connection domain is important for flexibility, allowing the protein to bind and cleave more effectively. Our current model of RNase H1 action posits that binding of the enzyme occurs mainly via the HBD and that the RNase H domain searches for an appropriate cleavage site and cleaves; following release of the RNase H domain from the hydrolyzed substrate, the HBD anchors the protein while the RNase H domain recognizes another site and cleaves; the process continues until either the HBD releases from the hybrid or no more RNase H cleavage sites are available to be attacked.

Nowotny M, Cerritelli SM, Ghirlando R, Gaidamakov SA, Crouch RJ, Yang W. Specific recognition of RNA/DNA hybrid and enhancement of human RNase H1 activity by HBD. *EMBO J* 2008;27:1172-1181.

Nowotny M, Gaidamakov SA, Ghirlando R, Cerritelli SM, Crouch RJ, Yang W. Structure of human RNase H1 complexed with an RNA/DNA hybrid: insight into HIV reverse transcription. *Mol Cell* 2007;28:264-276.

Functions of RNase H1 in mitochondria and nuclei

Cerritelli, Sakhuja, Suzuki, Holmes, Crouch; in collaboration with Holt

One of the major challenges we face in understanding the roles of RNase H1 in cells is to determine how the translation of a single mRNA can produce both nuclear and mitochondrial forms of the enzyme. Knocking out the *Rnaseh1* gene in mouse results in embryonic lethality at embryonic day 8.5 due to a failure to replicate mitochondrial DNA. The outcome indicates that (1) the enzyme is essential for the maintenance of mtDNA and (2) there is no need for newly synthesized RNase H1 for replication/repair of nuclear DNA. We have generated a conditional knockout that permits us to determine whether RNase H1 is necessary in adult animals either for mtDNA or nuclear DNA replication and repair. It may be that RNase H1 is important for mtDNA during embryogenesis only when, after implantation, rapid mtDNA synthesis occurs, at which time it is suddenly activated. In addition, we will be able to generate organ-/tissue-specific knockouts of the *Rnaseh1* gene by using the Cre-lox system, including Tamoxifen®-inducible Cres for general ablation and others (e.g., heart-specific Cre expressers). However, the problem of inactivating the synthesis of both forms remains unresolved. Accordingly, we have generated transgenic mice that produce either both isoforms of RNase H1 or only the nuclear form in T- and B-cells. We are currently using the mice to examine effects on class switch recombination.

In collaboration with Ian Holt, we are searching for possible roles of RNase H1 in mitochondrial DNA replication by using, among other techniques, analysis of intermediates on two-dimensional gels. Our findings thus far indicate that elevated expression of RNase H1 in mitochondria alters mtDNA replication. In addition, data obtained in Holt's laboratory by John Holmes supporting the existence of RNA/DNA hybrids as replication intermediates indicate a bi-directional mode of DNA replication for this organelle.

Structure-function of ribonucleases: RNase H2

Chon, Cerritelli, Crouch; in collaboration with Kanaya, Burgers

We previously found that RNase H2, the second type of RNase H, in *Saccharomyces cerevisiae* is composed of three subunits (Jeong et al., *Nucleic Acids Res* 2004;32:407), two of which we did not find in higher eukaryotes when using a BLAST search. We determined that there are three subunits of RNase H1 in human cells and were able to find similar RNases H2 in other mammals, in particular the mouse. A recent report in *Nature Genetics* demonstrated that mutations in any one of these proteins leads to the rare Aicardi-Goutières syndrome (AGS). Our studies on the human and yeast enzymes indicate a connection between RNase H2 and PCNA (proliferating cell nuclear antigen)—a clamp-loader protein involved in recruiting proteins to DNA for repair and replication. A PCNA-Interacting-Peptide (PIP) is present in the RNase H2B subunit of yeast, mouse, and human

RNases H2. Using several types of analyses, we demonstrated that the PIP of the RNase H2B is indeed able to interact with PCNA, a finding whose biological significance we are continuing to explore. We also examined the effects of several of the AGS-causing mutations on recombinant RNase H2 activity. Only two of the mutant enzymes have shown decreased activity. The other altered proteins are most likely defective in vivo for complex formation and/or stability.

Rohman MS, Koga Y, Takano K, Chon H, Crouch RJ, Kanaya S. Effect of the disease-causing mutations identified in human RNase H2 on the activities and stabilities of yeast RNase H2 and archaeal RNase HII. *FEBS J* 2008;275:4836-4849.

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MOLECULAR GENETICS OF EMBRYOGENESIS IN *XENOPUS* AND ZEBRAFISH

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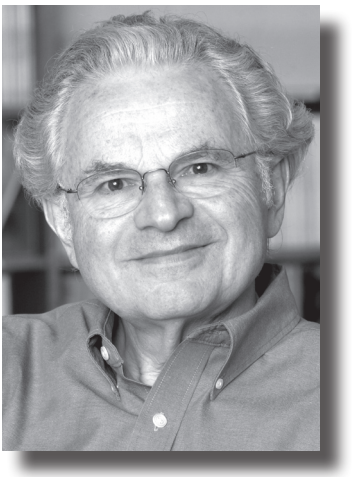

The laboratory uses the frog *Xenopus laevis* and the zebrafish *Danio rerio* as experimental systems to study molecular-genetic mechanisms of early vertebrate development. Recently, we focused on gene discovery by forward genetics, DNA microarray technology, and expression profiling in order to identify genes that play a role in the regulation of embryonic development. These approaches have led to studies on mechanisms of gastrulation, neural crest specification, and brain patterning during early embryogenesis. We are particularly interested in the development of the neural crest and its important derivatives, the pharyngeal arches.

WGEF is a component of the WNT-PCP pathway involved in regulating convergent extension during *Xenopus* gastrulation

Tanegashima, Zhao, Dawid

The Wnt-PCP pathway regulates cell polarity and convergent extension movements during axis formation in *Xenopus*. Given that Wnt stimulation in the embryo activates Rho and Rac, the involvement of at least one GEF[2] in Wnt-PCP signaling during convergent extension has been predicted. Using a microarray-based screen for notochord-enriched genes, we identified a GEF with sequence similarity to human WGEF[3]. We found that *Xenopus* WGEF is involved in Wnt-regulated convergent extension movements, which are critical for the progress of gastrulation. The expression of WGEF begins at the onset of gastrulation and becomes predominant in the notochord during subsequent stages. WGEF protein co-localized with Dishevelled in cells of the *Xenopus* gastrula, and overexpression of WGEF activated RhoA, but not Rac1 or Cdc42, in mammalian cells and frog embryos. Depletion of WGEF led to suppression of convergent extension in explants from *Xenopus* embryos, which could be rescued by Rho activation. WGEF protein can bind to Dishevelled and Daam-1, components of the Wnt-PCP pathway. Epistatic analysis placed WGEF below Wnt and Dishevelled in the pathway. Binding studies identified the Dishevelled-binding domain in WGEF as an auto-inhibitory domain, suggesting a mechanism for signal transduction. Our results indicate that WGEF is a component of the Wnt-PCP pathway that connects Rho activation with signal transduction through Dishevelled.

Tanegashima K, Zhao H, Dawid IB. WGEF activates Rho in the Wnt-PCP pathway and controls convergent extension in *Xenopus* gastrulation. *EMBO J* 2008;27:606-617.

Xenopus *Lrig3*, a novel organizer-specific gene, is required for the differentiation of neural crest

Zhao, Tanegashima, Ro, Dawid

We identified Lrig3, a member of the leucine-rich repeats and immunoglobulin-like domains family, within the microarray analysis described above by comparing RNA from different regions of the gastrula embryo. We focused on Lrig3, a predicted transmembrane protein that is expressed maternally. Later, Lrig3 is expressed in the entire neural plate during neurulation and becomes localized in the developing neural crest during tailbud stages. Knockdown of *lrig3* by an antisense morpholino impairs neural crest formation, as visualized by several marker genes. In the neural crest induction assay involving Chordin plus Wnt3a-injected animal caps, Lrig3 morpholino inhibited expression of *slug*, *sox9*, and *foxD3* but not of *pax3* and *zic1*. In line with this observation, *lrig3* knockdown prevented marker induction by *pax3* and *zic1*, suggesting that Lrig3 acts downstream of these two genes in neural crest formation. Injection of Lrig3 and Wnt3a led to low-level induction of neural crest markers and enhanced induction of Fgf3, Fgf4, and Fgf8 in animal caps, suggesting a positive role for Lrig3 in Wnt signaling. Lrig3 could attenuate Fgf signaling in animal caps, interacted with Fgf receptor 1 in cultured cells, and, according to context, reduced or augmented the induction of neural crest markers by Fgf. We suggest that Lrig3 functions in neural crest formation in *Xenopus* by modulating the Wnt and Fgf signaling pathways.

Zhao H, Tanegashima K, Ro H, Dawid IB. Lrig3 regulates neural crest formation in *Xenopus* by modulating Fgf and Wnt signaling pathways. *Development* 2008;135:1283-1293.

The *mych* gene is required for neural crest survival during zebrafish development

Hong and Dawid; in collaboration with Tsang

Among Myc family genes, *c-Myc* plays a role in neural crest specification in *Xenopus* and in craniofacial development in the mouse. However, there is no information on either the function of other Myc genes in neural crest development or any developmental role of zebrafish Myc genes. We isolated the zebrafish *mych* (myc homologue) gene. Knockdown of *mych* leads to severe defects in craniofacial development and in certain other tissues, including the eye. These phenotypes appear to be caused by cell death in the neural crest and in the eye field in the anterior brain. Thus, Mych is a novel factor required for neural crest cell survival in zebrafish.

Hong SK, Tsang M, Dawid IB. The *mych* gene is required for neural crest survival during zebrafish development. *PLoS One* 2008;3:e2029.

Zebrafish *barx1* is required for pharyngeal arch patterning

Sperber, Dawid

The Barx1 transcription factor has previously been shown to modulate cellular adhesion molecules and participate in specification of tooth type. To determine the role of *barx1* in zebrafish head formation, we examined the gene's expression during embryogenesis and performed a functional analysis by microinjecting targeted antisense morpholino oligonucleotides to attenuate its translation. *Barx1* is expressed in the cranial neural crest, the pharyngeal arches, the anterior aspect of the pectoral fin buds, and the gut wall. By 2.5 days after fertilization, *barx1* morpholino–injected embryos exhibit developmental delay exemplified by poor facial outgrowth and micrognathia. Histological analysis and labeling of cell membranes revealed reductions in differentiation and chondrocyte condensation within the arches. Affected larvae stained with Alcian blue exhibit small and dysmorphic arch cartilage elements, and expression of chondrogenic markers such as *dlx2a* and *col2a1* is perturbed. As seen in bead implantation experiments, the expression of *barx1* is controlled by bone morphogenetic protein (BMP). Our results suggest a role for *barx1* at early stages of chondrogenesis within the pharyngeal arches during zebrafish development.

Sperber SM, Dawid IB. *barx1* is necessary for ectomesenchyme proliferation and osteochondroprogenitor condensation in the zebrafish pharyngeal arches. *Dev Biol* 2008;321:101-110.

Protocadherin 18 plays a role in cell behavior during zebrafish embryogenesis
Aamar, Dawid

Protocadherin-18 (Pcdh18) belongs to the δ2-protocadherins, which constitute the largest subgroup within the cadherin superfamily. We isolated a full-length zebrafish cDNA for zebrafish *pcdh18*, which is expressed in a complex and dynamic pattern in the nervous system from gastrula stages onward, with lesser expression in mesodermal derivatives. Overexpression of *pcdh18* in embryos caused cyclopia, mislocation of hatching gland tissue, and duplication or splitting of the neural tube. Reduction of Pcdh18 expression by use of a morpholino led to delayed epiboly and shortened axis. Using cell transplantation, we showed that overexpression of *pcdh18* causes diminished cell migration and reduced cell protrusions, resulting in a tendency of cells to stay more firmly aggregated, probably because of increased cell adhesion. We suggest a role for *pcdh18* in cell adhesion, migration, and behavior, but not in cell specification, during gastrula and segmentation stages of development.

Aamar E, Dawid IB. Protocadherin-18a has a role in cell adhesion, behavior and migration in zebrafish development. *Dev Biol* 2008;318:335-346.

Foxj1 and foxj1.2 are expressed in overlapping but distinct patterns in the zebrafish embryo
Aamar, Dawid

The forkhead-related or Fox family of transcription factors play several important roles in development. Foxj1 factors are involved in ciliogenesis in various systems. We isolated two related genes, *foxj1* and *foxj1.2*, from zebrafish and analyzed their patterns through embryogenesis. The patterns suggest functions for the two genes, which we are currently exploring.

Aamar E, Dawid IB. Isolation and expression analysis of *foxj1* and *foxj1.2* in zebrafish embryos. *Int J Dev Biol* 2008;52:985-991.

The behavior of the chromatin protein Brd4 in early zebrafish development
Toyama, Rebbert, Dawid; in collaboration with Ozato

Brd4 is a member of the BET subfamily of bromodomain proteins that includes chromatin-modifying proteins and transcriptional regulators. Brd4 plays a role in cell-cycle progression, making it indispensable in mouse embryos and cultured cells. Further, the N-terminal domain of Brd4 participates in a fusion oncogene. Brd4 associates with acetylated histones in chromatin, and the association persists during mitosis, implicating Brd4 in epigenetic memory. We have shown that Brd4 is expressed and localized on mitotic chromosomes in early zebrafish embryos before and after the midblastula transition (MBT), indicating that the Brd4-chromosome association is a conserved property that is maintained even before zygotic transcription. The association of Brd4 with acetylated histones may also be conserved in early embryos, as we found that histones H3 and H4 are already acetylated during pre–MBT stages.

Toyama R, Rebbert ML, Dey A, Ozato K, Dawid IB. Brd4 associates with mitotic chromosomes throughout early zebrafish embryogenesis. *Dev Dyn* 2008;237:1636-1644.

Pineal-specific gene expression in zebrafish
Toyama, Dawid; in collaboration with Epstein, Klein, Gothilf

The role of the pineal gland in biological rhythm is an important topic that is discussed more fully by David Klein elsewhere in this volume. We are involved in a collaborative study of gene expression in the pineal gland of the zebrafish. Taking advantage of the specific expression pattern of the gene encoding the enzyme Aanat2, we drew on the analysis of the genomic control regions responsible for this gene and generated transgenic zebrafish in which green fluorescent protein (GFP) is expressed with high specificity in the pineal gland. We used the transgenic zebrafish line to guide dissection of the pineal gland from embryonic, larval, and adult zebrafish to analyze, by the DNA microarray

method, global transcription patterns at various stages of development. We used RNA from brain from which the pineal gland had been removed for comparison and compared RNA samples from 3-, 5-, and 10-day embryos and 3-month young adult and 1- to 2-year mature adult zebrafish. We obtained day and night samples at each time point at least in triplicate. The resulting experimental findings permitted evaluation of developmental changes in the pineal gland, evaluation of differences in gene expression between the pineal gland and brain, and comparison of diurnal differences. Our results indicate that a large set of genes is preferentially expressed in the pineal gland during development. The data support the known role of the fish pineal as a photoreceptor organ, as genes involved in photoreception and transduction were prominent among the pineal-enriched genes. Frequently, distinct isoforms of certain proteins were expressed in the pineal gland as compared with other organs, e.g., the eye. Developmental changes visualized in these studies suggest that the embryonic pineal gland is enriched for regulatory factors such as transcription factors and signal transducers, whereas adult pineal glands express genes involved in physiological functions such as enzymes, photo-transduction factors, and structural components.

[1]Left the group during the reporting period.
[2]Guanine nucleotide exchange factor.
[3]weak-similarity GEF

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REGULATION OF MAMMALIAN CELL PROLIFERATION AND DEVELOPMENT

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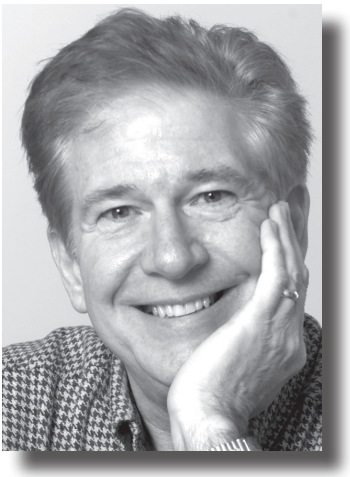

DNA replication origins are determined by genetic as well as epigenetic features. Genetic features are embedded in the DNA sequence itself and are generally protein binding sites or genes that encode the structure for specific proteins and RNAs. Epigenetic features are not determined by DNA sequence but are nevertheless heritable from one generation to the next. Moreover, epigenetic features are reversible and include chromatin structure, nuclear organization, transcription, transcription factors, deoxyribonucleotide pools, DNA topology, and DNA methylation. Consequently, the number and locations of replication origins in the cells of multicellular organisms can change from an average of one every 10 to 20 kb in the rapidly cleaving embryos of frogs, flies, and fish to one every 50 to 300 kb in the differentiated cells of adult organisms. Developmental changes in origin density also occur during specific stages in animal development. Thus, metazoan genomes contain many potential initiation sites for DNA replication, but, during development, some of these sites are selectively activated while others are suppressed. When we introduced the initiation/suppression concept many years ago, we termed it the "Jesuit Model" because the Jesuits remind us that while many are called, few are chosen.

The significance of genome duplication

Ghosh, Stehle, Vassilev A, Zhu, Zhang; in collaboration with Zhao, Vassilev L

Nothing is more fundamental to living organisms than the ability to reproduce. Each time a human cell divides, it must duplicate its genome once and only once; otherwise, the cell is programmed to commit suicide, an event termed apoptosis. For reproduction to occur, each cell selects from 20,000 to 40,000 sites throughout its 46 chromosomes at which it will initiate DNA replication. Selection of initiation sites is determined by the interaction between a DNA replication origin and a protein complex called the origin recognition complex (ORC). In addition, other proteins may facilitate this interaction and thereby contribute to initiation site selection.

Our goal is to discover how human cells determine where and when to initiate DNA replication and how the initiation process is regulated. During normal animal development, some cells are programmed to arrest mitosis and to duplicate their genomes many times in order to produce a large number of copies of critical genes without undergoing apoptosis. As we age, however, the ability to regulate DNA replication diminishes and results in genome instability and promiscuous cell division, the primary hallmarks of cancer. At least 122 human diseases result from aberrant

DNA replication, from mutation in DNA replication or repair genes, or from infection by DNA viruses (DePamphilis, 2006).

Several years ago, we discovered that the behavior of ORC in mammalian cells differs significantly from that in single-cell eukaryotes such as yeast. Yeast ORCs consist of a stable complex of six subunits that remain bound to chromatin throughout cell division and that target specific DNA sequences. In contrast, mammalian ORCs consist of a stable core complex of Orc2 through Orc5 that interacts weakly with Orc1 and Orc6. Nevertheless, the association of Orc1 with ORC(2-5) is essential for prereplication complex assembly and DNA replication. In vitro, however, metazoan ORCs exhibit little affinity for specific DNA sequences other than for asymmetric A:T-rich regions, whereas, in the differentiated cells of mammals and flies, ORCs localize at specific genomic sites that are coincident with DNA replication origins. Thus, the ability of ORC to activate a particular replication origin appears to depend on its ability to interact with DNA as it exists within the nucleus, an interaction that appears to be regulated by Orc1 (Figure 6.2).

During the past year, we established the basic features of the ORC cycle, which is a regulatory pathway that we previously proposed; it restricts initiation of DNA replication events so that genomes are duplicated once and only once each time a cell divides. Through its BAH domain, the largest subunit (Orc1) regulates association of the stable ORC(2-5) core complex with replication origins in vivo. The BAH domain interacts with another, as yet unidentified, protein that is required for ORC binding to chromosomes as cells exit mitosis and begin a new cell division cycle. Moreover, if Orc1 is not bound to ORC(2-5), it can induce apoptosis. However, if the unbound Orc1 is either monoubiquitinated or phosphorylated (two normal cell cycle–dependent modifications of Orc1), the modified Orc1 is localized in the cytoplasm where it cannot initiate replication, thus accounting for the fact that Orc1 is modified and in some cells degraded as the cells enter S phase (period of DNA replication). The modified forms of Orc1 do not induce apoptosis. Thus, cell cycle modifications regulate Orc1 activity, and Orc1 activity regulates ORC activity, which regulates initiation of DNA replication.

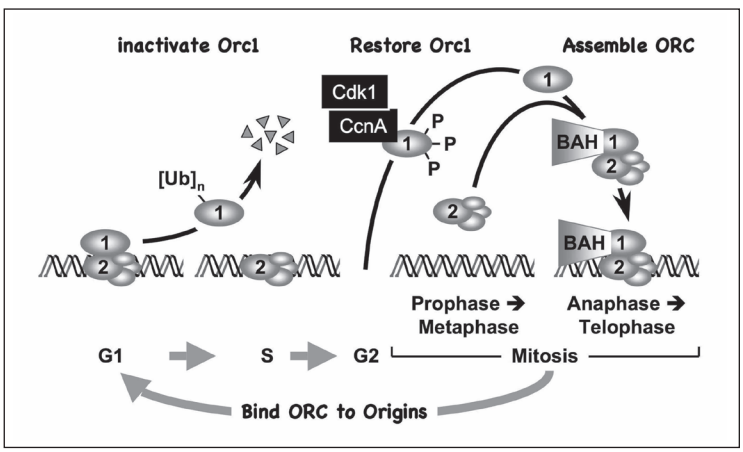

FIGURE 6.2

The ORC cycle in mammalian cells (reviewed in DePamphilis et al., *Curr Opin Cell Biol* 2006;18:231). ORC is bound to chromatin during the G1 phase of the cell cycle, at which time it is part of a prereplication complex. When S phase begins, human Orc1 is selectively degraded by the 26S proteasome via a ubiquitin-dependent mechanism and reappears during the M- to G1-phase transition. During metaphase, Orc1 is hyperphosphorylated, an event that prevents ORC assembly. As cells exit metaphase, Orc1 is dephosphorylated and, together with other ORC subunits, binds to chromatin, an event facilitated by the Orc1 BAH domain.

During mammalian development genome endoreduplication is a rare event and its mechanism is unknown. It first appears when FGF4 deprivation induces differentiation of trophoblast stem (TS) cells into the nonproliferating trophoblast giant (TG) cells required for embryo implantation. We discovered that selective inhibition of CDK1, the enzyme required to enter mitosis, by p57/Kip2 (a CDK-specific inhibitor in mammals) induced differentiation of TS cells into TG cells but

induced abortive endoreduplication and apoptosis in embryonic stem cells. Therefore, inactivation of CDK1 triggers endoreduplication only in cells programmed to differentiate into polyploid cells. TS cell mutants revealed that p57 was required to trigger endoreduplication by inhibiting CDK1 while p21 (another CDK-specific inhibitor) suppressed expression of the checkpoint protein kinase CHK1, thereby preventing induction of apoptosis. Furthermore, *Cdk2*$^{-/-}$ TS cells revealed that CDK2 is required for endoreduplication when CDK1 is inhibited. Expression of p57 in TG cells was restricted to G-phase nuclei to allow CDK activation of S phase. Thus, endoreduplication in TS cells is triggered by p57 inhibition of CDK1 with concomitant suppression of the DNA damage response by p21.

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Ullah Z, Kohn MJ, Yagi R, Vassilev LT, DePamphilis ML. Differentiation of trophoblast stem cells into giant cells is triggered by p57/Kip2 inhibition of CDK1 activity. *Genes Dev* 2008, in press.

The significance of early mammalian development

Kaneko, Kohn, Ullah, Yagi, Zhang; in collaboration with Buonanno, Karavanova, Liu, Maraia

Mammalian development begins when an egg is fertilized by a sperm. This event activates the first round of genome duplication, after which the fertilized egg cleaves into a two-cell embryo. In mice (a laboratory model for human development), the two-cell embryo then activates the expression of about 300 genes that are required to continue development of the organism. Initially, each of the cells (blastomeres) produced by the cleavage events are "totipotent"; that is, they are capable of giving rise to the entire organism. But within five rounds of cell cleavage, a blastocyst appears, marking the beginning of cell differentiation (the process whereby cells become specialized). Blastocysts consist of a spherical monolayer of cells called the trophectoderm that gives rise to TS cells and eventually to the placenta. The trophectoderm layer encompasses a group of cells called the inner cell mass that gives rise to embryonic stem (ES) cells and eventually the embryo. Life begins after the blastocyst implants into the uterine endometrium and initiates formation of a nervous system.

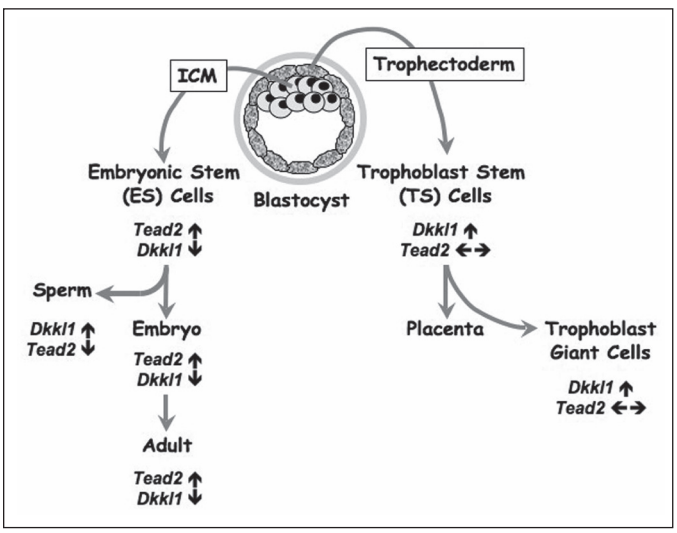

FIGURE 6.3

Differential expression of *Tead2* and *Dkkl1* mark divergent pathways to embryonic and placental tissues. The blastocyst is composed of two cell types—an outer layer of trophectodermal cells and the intermediate cell mass (ICM). The trophectoderm forms most of the extra-embryonic tissues and the placenta. Trophoblast stem (TS) cells may be derived from the trophectoderm cells that overlie the ICM, which gives rise to the embryo. Embryonic stem (ES) cells may be derived from the ICM. Northern blotting-hybridization, RT-PCR, and EST database analyses reveal that the trophectoderm and its derivatives strongly express *Dkkl1* (↑), whereas *Tead2* expression remains unchanged (↔). In contrast, the ICM and its derivatives strongly express *Tead2* (↑) and suppress expression of *Dkkl1* (↓). One notable exception occurs during the production of male germ cells. Developing spermatocytes strongly express *Dkkl1* in the absence of *Tead2* expression such that this Dkkl1 is eventually localized to the acrosome of mature sperm.

Our goal is to identify genes whose expression is required for development to begin and then to identify the role played by these genes in establishing a living organism. We expect that such genes are essential for genome duplication, cell proliferation, and differentiation of the totipotent blastomeres into placental and embryonic cell lineages.

Genome duplication undergoes two dramatic changes during animal development. First, the number and location of replication origins can and do change at specific times during development. Second, the several concerted pathways that restrict genome duplication to once per cell division are inactivated at a few specific stages during development in order to allow several cycles of genome duplication to occur without an intervening mitosis. This process is called endoreduplication, and it occurs in mammals when TS cells differentiate into trophoblast giant cells during the process of embryo implantation.

During the past year, we identified the biological roles of three genes that are unique to vertebrates and that are expressed at the very beginning of mammalian development (Figure 6.3). We discovered all three genes and note that one, *DKKL1*, is unique to mammals. We observed that *DKKL1* is expressed specifically during implantation of the embryo and development of spermatocytes into sperm. Moreover, genetic inactivation of DKKL1 in mice resulted in production of sperm that are defective in fertilization.

The second gene, *TEAD2*, is one of a highly conserved family of four transcription factors that share a common DNA binding domain. Surprisingly, TEAD2 is not required until after implantation has occurred and the nervous system begins to form. When we genetically inactivated TEAD2, mice had difficulty in forming a neural tube. In developing vertebrates, the neural tube is the embryo's precursor to the central nervous system, which comprises the brain and spinal cord. Failure to close the neural tube in mice is called exencephaly, which is related to anencephaly, a common human birth defect that can be prevented by folic acid.

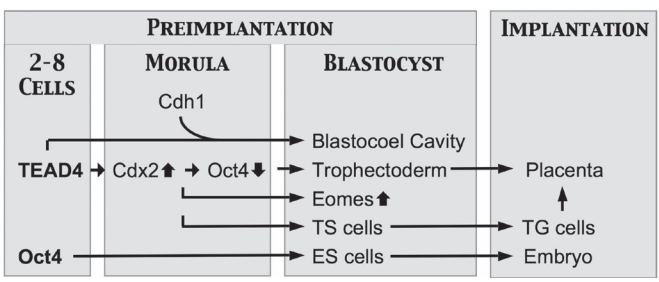

FIGURE 6.4

Genes encoding transcription factors Tead4 and Oct4 appear to function as master genes in that they mark the beginning of a chain of events that specifies a unique cell lineage. *Oct4* is the first gene whose expression is required for specification of the embryonic cell lineage (ICM and ES cells) that produces the embryo. *Tead4* is the first gene required for specification of the trophectoderm cell lineage that produces TS cells, trophoblast giant (TG) cells, and the placenta. In the absence of *Tead4* expression, all totipotent blastomeres produce OCT4 protein. Expression of *Tead4* induces expression of *Cdx2*, which suppresses expression of *Oct4* and thus causes the blastomere to differentiate into trophectoderm instead of ICM.

The third gene is *TEAD4*. We discovered that *TEAD2* and *TEAD4* are the only *TEAD* genes that are expressed in pre-implantation mouse embryos and that genetic inactivation of TEAD4 (but not of TEAD2) arrests development before formation of a blastocyst. In fact, we discovered that *TEAD4* is required for specification of the trophectoderm lineage, thus making it the earliest gene known so far on the pathway to formation of the placenta. It now appears that the totipotent blastomeres in mouse morula that express the transcription factor TEAD4 become trophectoderm and eventually placenta, whereas those that express the transcription factor OCT4 become the inner cell mass and eventually the embryo. Thus, *TEAD4* appears to be a master gene that sets into motion the first round of cell differentiation during mammalian development (Figure 6.4).

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THE CHROMATIN-BASED EPIGENOME IN DEVELOPMENT AND AGING

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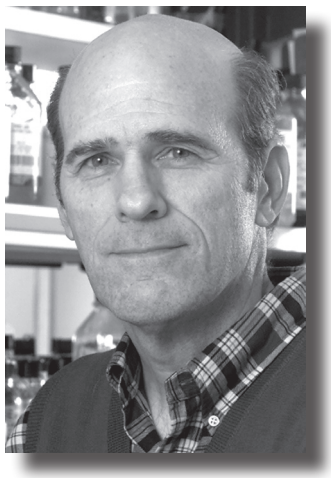

The normal human lifespan is marked by a complex series of developmental transitions, including relative stability during adulthood and, ultimately, a gradual decline in viability. Clock-like mechanisms presumably underlie the developmental events that occur through childhood and adolescence. Further, instabilities in such mechanisms are likely to be an integral part of the aging process and to contribute to many common degenerative diseases of later life. A breakthrough in the area of developmental transitions would have remarkable implications; thus, there is widespread interest in the prospect that epigenetics—and the rapidly evolving field of epigenomics—holds the key to further progress.

Linking gene expression to *cis*-acting epigenetic states

Russanova, Howard, Porrás,[1] Dimitropoulos, Kozar; in collaboratin with Romero

The past year has seen increasing refinement in studies that use human tissues from several clinical sources. We obtain peripheral blood monocytes from newborns (cord blood) through a collaboration with the Perinatology Branch, NICHD, while monocytes from adults are available through the NIH Department of Transfusion Medicine. Using the cytokines IL4 and GM-CSF, we induce these cells to differentiate in vitro into antigen-presenting dendritic cells. We procure human skin fibroblasts from newborns and adults, as needed, under a protocol approved by the NICHD Institutional Review Board. We induce the latter cells to enter a quiescent state by serum deprivation for six days and then examine them with respect to gene expression and chromatin structure upon serum stimulation.

With these monocyte- and fibroblast-based experimental systems, we employed RNA expression microarrays to identify several instances of developmental and age-related changes in gene regulation. Our work is thus shifting to studies on the mechanism(s) underlying such changes. Recognizing that *cis*-acting epigenetic states have several distinctive properties, we use several strategies to evaluate their presence or absence: (1) heterocellularity (variegation) in expression patterns—we apply RNA FISH and cytohistochemistry techniques; (2) allele independence—we use single-nucleotide polymorphisms (SNP) to search for examples of allelic skewing; and (3) memory of expression-state settings—we use heterokaryons between cells from newborns and adults or from young and old adults to determine the independence of development- and age-specific expression levels.

Complementary to these strategies are chromatin immunoprecipitation (ChIP) studies; we use both automated HPLC/fluorescence-based assays and ChIP-on-chip platforms. For the latter, we have

developed bioinformatics tools for the custom design of large deoxynucleotide probe arrays and genome annotation, pattern recognition, and pattern comparison algorithms in order to analyze data and generate new hypotheses. Further, in the coming year, we plan to use single-nucleotide sequencing (ChIP-Seq), if possible. Of special interest will be results obtained from monozygotic (identical) twins. As part of an NICHD epigenomics initiative, we have received the first paired peripheral blood samples from such twins.

Results to date indicate that genes subject to both differentiation and developmental controls operate at least in part through the remodeling of higher-order chromatin structures. We will focus on large domains over which acetylation patterns are altered as well as on the boundary elements that limit these domains. The emerging goal is to generalize our paradigm to address a range of current problems in pediatrics and medicine. Based on the genes currently under study, we will most likely investigate deficiencies in the innate immune systems of newborns, peripheral insulin resistance and diabetes in adolescents and young adults, and a spectrum of neurodegenerative processes, including Parkinson's and Alzheimer's diseases, in the elderly.

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CONTROL OF GENE EXPRESSION DURING DEVELOPMENT

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During development and differentiation, genes either become competent to be expressed or are stably silenced in an epigenetically heritable manner. This selective activation/repression of genes leads to the differentiation of tissue types. Recent evidence suggests that modifications of histones in chromatin contribute substantially to determining whether a gene will or will not be expressed. Our group is interested in understanding how chromatin-modifying protein complexes are recruited to DNA. In *Drosophila*, two groups of genes, the Polycomb group (PcG) and Trithorax group (TrxG), are important for inheritance of the silenced and active chromatin state, respectively. Regulatory elements called Polycomb group response elements (PRE) are *cis*-acting sequences required for the recruitment of chromatin-modifying PcG protein complexes. TrxG proteins may act through either the same or overlapping *cis*-acting sequences. Our group aims to understand how PcG and TrxG proteins are recruited to DNA.

DNA sequences that constitute a PRE
Brown, Durant, Kassis

PREs are DNA elements through which the PcG protein transcriptional repressors act. Many of the PcG proteins are associated in two protein complexes that repress gene expression by modifying chromatin. Both these complexes specifically associate with PREs in vivo; however, it is not known how they are recruited or held at the PRE. PREs are complex elements made up of binding sites for many proteins. Our laboratory has been working to define all the sequences and DNA-binding proteins required for the activity of a 181-bp PRE from the *Drosophila engrailed* gene. At least seven DNA-binding sites contribute to the activity of this 181-bp PRE. One of the required binding sites is for the Polycomb-group proteins Pleiohomeotic (Pho) and Pleiohomeotic-like (Phol). Binding sites for the proteins GAGA factor, Pipsqueak, Zeste, and Dsp1 also are present within the *engrailed* PRE. Proteins that bind to two other sites remain unidentified. Our laboratory found that members of the Sp1/KLF family of zinc-finger proteins bind to another required binding site (Brown et al., *Nucleic Acid Res* 2005;33:5181). This family of proteins encodes transcription factors and has undergone extensive study in mammals—there are 20 Sp1/KLF family members in mammals. *Drosophila* accounts for 10 Sp1/KLF family members, of which nine bind to the *engrailed* PRE. We derived a consensus-binding site for the Sp1/KLF *Drosophila* family members and showed that the consensus sequence is present in most of the molecularly characterized PREs. The data suggest that one or more Sp1/KLF family members play a role in PRE function in *Drosophila*.

We have been working to determine which of the Sp1/KLF family members in *Drosophila* may be involved in PcG function. We made antibodies to the three most likely candidates. Our analysis is now focusing on one candidate that is ubiquitously expressed in embryos. We are working to generate a mutant in the gene that encodes this protein to determine if it plays a role in PcG repression.

Although much is known about the protein-binding sites required for PRE function, we are not able to predict the location of a PRE based on the presence of binding sites alone. To help us identify either other protein-binding sites required for PRE function or other important characteristics of PREs (such as the number or spacing of binding sites), we have begun to analyze other PREs from the *engrailed* region of the genome. Our work should lead to a better understanding of the protein-binding sites required for PRE function.

Müller J, Kassis JA. Polycomb response elements and targeting of Polycomb group proteins in *Drosophila*. *Curr Opin Genet Dev* 2006;16:476-484.

Understanding the role of PREs and flanking sequences at the *engrailed* gene

Brown J, Cheng, Kwon,[1] Stefaniuk,[2] DeVido,[3] Kremer,[4] Brown A,[5] Kassis

The *Drosophila engrailed* gene encodes a homeodomain protein that plays an important role in the development of many parts of the embryo, including formation of the segments, nervous system, head, and gut. It also plays a particularly significant role in the development of the adult, specifying the posterior compartment of each imaginal disk. Accordingly, *engrailed* is expressed in a highly specific and complex manner in the developing organism. We have been studying the 181-bp *engrailed* PRE, which is located near the *engrailed* promoter from −576 to −395 upstream of the transcription start site. We were interested in determining the role of this PRE in the control of *engrailed* expression. One of our first findings demonstrated that this PRE is redundant with other flanking PREs in the endogenous *engrailed* gene; another strong PRE is located from −1100 to −1500, and probably other weak PREs are located nearby. In fact, when we examined the location of Ph and Pho proteins on *engrailed* DNA by chromatin immunoprecipitation (ChIP), we found that they are bound to a 2.5 kb region extending from the *engrailed* promoter to about −2.5kb upstream. Therefore, it is perhaps not too surprising that a 500-bp deletion that includes the 181-bp PRE and flanking sequence did not lead to ectopic *engrailed* expression. The remaining PREs were apparently sufficient to recruit PcG proteins. However, we were surprised that loss of the DNA led to a loss-of-function phenotype, suggesting that the DNA must also play a positive role in the expression of *engrailed*. In other experiments, we were able to show that PREs can either activate or repress transcription in a context-dependent manner. Further, our data suggest that PREs mediate looping between distant enhancers and the *engrailed* promoter. Our experiments suggest activities of PREs not foreseen by others in the field.

The regulatory sequences for the *engrailed* gene extend over a 70-kb region. Our laboratory has used reporter constructs to find sequences important for expression in stripes, the nervous system, the head, among others. Discrete regulatory elements are located throughout the 70-kb region. We also find at least seven additional potential PREs located throughout the region. Others have shown that PcG protein complexes bring together DNA fragments in vitro, and it is also possible that the complexes cause looping in vivo. We are interested in learning whether the additional PREs are involved in mediating interactions between distant enhancers and the *engrailed* promoter.

DeVido SK, Kwon D, Brown JL, Kassis JA. The role of Polycomb-group response elements in regulation of *engrailed* transcription in *Drosophila*. *Development* 2008;135:669-676.

A genetic screen identifies new members of the Trithorax and Polycomb groups

Stefaniuk,[2] Gordon,[6] Durant, Kassis; in collaboration with Kennison

We performed a genetic screen to identify new members of the Trithorax group and Polycomb group. The generation of transgenic *Drosophila* relies on an eye color gene, *white*, to detect

transgenic flies. Eye color is dependent on the expression level of the *white* gene; more expression of *white* causes a darker eye color whereas less expression causes a lighter eye color. PREs linked to *white* (a PRE–*white* transgene) cause less *white* expression, leading to a lighter eye color. We performed a genetic screen to identify mutations that will darken the eye color of transgenic flies with a PRE–*white* transgene. We reasoned that mutation of a PcG gene, which encodes a repressor, might lead to a darkening of the eye color. Increasing the activity of an activator protein (a potential Trithorax group gene) might also darken the eye color through competition with the PcG repressors. We screened over 60,000 flies and obtained nine mutants. We have now characterized two of the mutants. Interestingly, one of them has a PcG phenotype; the other has a TrxG phenotype. We are currently completing our analyses of these two genes and are beginning to characterize other mutants isolated in the screen. We hope to understand the molecular function of these newly identified PcG and TrxG proteins.

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REGULATION OF HOMEOTIC GENE FUNCTION IN *DROSOPHILA*

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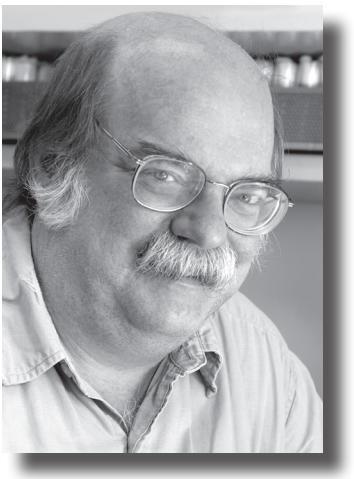

Our goal is to understand how genes control cell fates during development. The homeotic genes in *Drosophila* specify cell identities at both the embryonic and adult stages. They encode homeodomain-containing transcription factors that control cell fates by regulating the transcription of downstream target genes. The homeotic genes are expressed in precise spatial patterns that are crucial for the proper determination of cell fate. Both loss of expression and ectopic expression in the wrong tissues lead to changes in cell fate. These changes provide powerful assays for identifying the *trans*-acting factors that regulate the homeotic genes and the *cis*-acting sequences through which they act. Both the homeotic genes and the *trans*-acting factors that regulate them are conserved between *Drosophila* and human. In addition to many conserved developmental genes, at least half of the disease- and cancer-causing genes in man are conserved in *Drosophila*, making *Drosophila* a particularly important model system for the study of human development and disease.

Cis-acting sequences for transcriptional regulation of the *Sex combs reduced* homeotic gene

Kennison, Cooper, Jameel

Assays in transgenes in *Drosophila* previously identified *cis*-acting transcriptional regulatory elements from the homeotic genes. The assays identified tissue-specific enhancer elements as well as *cis*-regulatory elements that are required for the maintenance of activation or repression throughout development. While these transgenic assays have been important in defining the structure of the *cis*-regulatory elements and identifying *trans*-acting factors that bind to them, their functions within the context of the endogenous genes remain poorly understood. We used a large number of existing chromosomal rearrangements in the *Sex combs reduced* homeotic gene to investigate the functions of the *cis*-acting elements within the endogenous gene. These rearrangements identified an imaginal leg enhancer about 35 kb upstream of the *Sex combs reduced* promoter. This enhancer is not only able to activate transcription of the *Sex combs reduced* promoter that is 35 kb distant, but it can also activate transcription of the *Sex combs reduced* promoter on the homologous chromosome. Although the imaginal leg enhancer can activate transcription in all three pairs of legs, it is normally silenced in the second and third pairs of legs. The silencing requires the Polycomb group proteins. We are currently trying to identify the *cis*-regulatory DNA sequences in the *Sex combs reduced* gene that are required for transcriptional activation in the first leg and for *Polycomb* group silencing in the second and third legs. Characterization of the chromosomal rearrangements also revealed that two genetic elements about 70 kb apart in the *Sex combs reduced* gene must be in *cis* to maintain proper repression. When not physically linked to each other, these elements interact with elements on the

homologous chromosome and cause derepression of its wild-type *Sex combs reduced* gene. We tested DNA fragments from the *Sex combs reduced* gene in transgenic assays to identify endogenous *cis*-regulatory elements that could interact. From the regions that include the regulatory elements required for the maintenance of silencing, we have identified two clusters of DNA fragments that promote pairing-sensitive silencing (an assay for interaction of *cis*-regulatory DNA fragments). We are using targeted gene replacement to delete these clusters of elements in the endogenous gene to assay their role in transcriptional regulation.

Trans-acting activators and repressors of homeotic genes

Kennison, Mortin, Cooper; in collaboration with Vázquez, Zurita, Huang, Dorsett, Brock, Honda

The initial domains of homeotic gene repression are set by the segmentation proteins, which also divide the embryo into segments. Genetic studies have identified the *trithorax* group of genes that are required for expression or function of the homeotic genes, including the maintenance of transcriptional activation. Maintenance of transcriptional repression requires the proteins encoded by the Polycomb group genes. To identify new trithorax group activators and Polycomb group repressors, we are screening for new mutations that mimic the phenotypes: loss of function or ectopic expression of the homeotic genes. We have generated over 4,000 lethal mutants and, among those that die late in development, have identified two dozen mutants with homeotic phenotypes. These mutants identify genes required for expression or function of the homeotic genes (Kennison, *Methods Enzymol* 2004;377:61).

Reduced function of the *trithorax* group genes mimics loss of function of the homeotic genes. We previously identified several trithorax group genes that encode subunits of chromatin-remodeling complexes. The *brahma*, *moira*, and *osa* genes encode subunits of the Brahma chromatin-remodeling complex, which is conserved from yeast to human. To understand further the function of the Brahma complex, we have been isolating and characterizing mutations that interact with *brahma*. One of the interacting genes, *female-sterile homeotic*, encodes a bromodomain protein that interacts with regulatory elements near the *Ultrabithorax* homeotic gene promoter. The interaction appears to be important for rendering the homeotic promoter accessible to interactions with proteins bound to distant regulatory elements. The characterization of two other *brahma*-interacting genes, *verthandi* and *Pearl*, suggests that two protein complexes known to be important for chromosome segregation during mitosis also play important roles in transcriptional regulation. We have shown that *verthandi* encodes the RAD21 subunit of the cohesin complex. The cohesin complex is required for proper segregation of sister chromatids during anaphase. We have also shown that *Pearl* encodes one of the two gamma-tubulin isoforms in *Drosophila*. During the characterization of *Pearl*, we identified a recessive suppressor mutation that rescues the zygotic lethality associated with loss of gamma-tubulin function. This recessive suppressor mutation is in a gene that encodes a protein required for formation of the gamma-tubulin ring complex. Our results demonstrate that gamma-tubulin and microtubule organizer complexes are required not only for chromosome segregation during mitosis but may also play a role in transcriptional regulation.

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[j]Left the laboratory in May 2008.

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MOLECULAR GENETICS OF MAMMALIAN RETROVIRUS REPLICATION

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The goal our research is to define the molecular mechanisms responsible for the replication of HIV and related retroviruses and to investigate the role of host proteins that block virus infection. Our studies are critical for developing new strategies to combat the AIDS epidemic, which continues to pose a global threat to human health. We have developed reconstituted model systems to investigate the individual steps in HIV-1 reverse transcription, a major target of HIV therapy. Much of our work focuses on the viral nucleocapsid protein (NC), which is a nucleic acid chaperone; that is, NC facilitates nucleic acid conformational rearrangements that lead to formation of the most thermodynamically stable structures. This activity is critical for highly efficient and specific viral DNA synthesis. We are also studying the mechanism of antiviral activity of two host cytidine deaminases, APOBEC3G (A3G) and APOBEC3A (A3A). In other studies, we are working to understand the function of the viral capsid protein (CA) in HIV-1 assembly and early post-entry events during the course of virus replication in vivo.

Role of nucleocapsid protein in HIV-1 reverse transcription
Wu, Post, Levin; in collaboration with Gorelick, Musier-Forsyth, Rein

HIV-1 NC is a small basic protein with two zinc fingers, each containing the invariant CCHC zinc-coordinating residues. Its nucleic acid chaperone function consists of two independent activities: (1) nucleic acid aggregation (N-terminal basic residues) and (2) weak helix destabilizing activity (zinc fingers) (Levin et al., *Prog Nucleic Acids Res Mol Biol* 2005;80:217). NC plays a critical role in the two strand-transfer steps that occur during viral DNA synthesis. In minus-strand transfer, the first product of reverse transcription, which is (–) strong-stop DNA [(–) SSDNA], is annealed to the RNA sequence at the 3′ end of the genome (acceptor RNA) in a reaction facilitated by base-pairing of the complementary repeat regions in the nucleic acid substrates. This reaction is followed by reverse transcriptase– (RT) catalyzed elongation of minus-strand DNA. Our recent work on minus-strand transfer demonstrated that, instead of the overall structure, the local structure of the nucleic acid substrates at the nucleation site for annealing is a major determinant of NC's chaperone function.

Current studies focus on a comparison of the nucleic acid chaperone activities of HIV-1 Gag and its proteolytic cleavage products, including NC (also known as NC7) with its immediate precursors, NC9 and NC15. We use the minus-strand transfer assay as the read-out for chaperone function, as the assay system represents the most stringent biochemical test for the chaperone activity. We performed the

initial assays with Gag and the GagW-M mutant, which, unlike Gag, has weak dimerization activity. We found for the first time that both Gag and GagW-M stimulate minus-strand transfer and block the dead-end self-priming reaction (GagW-M stimulates slightly better than Gag), although not as efficiently as NC. The results showed that the two Gag proteins have sufficient helix destabilizing activity to unfold the highly stable TAR structures in acceptor RNA and (–) SSDNA. The data also indicate that our assay does not require Gag dimerization. Initial characterization of the chaperone activities of the NC precursors indicated that the order of activity in minus-strand transfer reactions is NC>NC9>NC15. We are now characterizing other Gag-related proteins.

In other work, we are investigating initiation of HIV-1 plus-strand DNA synthesis by the RNA polypurine tract (PPT) primer, along with the roles of HIV-1 NC and RNase H activity in ensuring the specificity of the synthesis. During the synthesis of minus-strand DNA, the RNase H activity of RT degrades viral genomic RNA sequences after their transcription into DNA. The degradation results in the release of several RNA fragments that have the potential to prime plus-strand DNA synthesis. We call such activity "mispriming." If mispriming were to occur, viral DNA would not have the correct ends and would not be competent for integration. We have compared priming by the authentic PPT primer with the activities of other purine-rich, non–PPT RNAs, which have sequences from the region near the 3′ PPT. The PPT is the most efficient primer; the other primers show a range of activities (e.g., from 50 to less than 1 percent of PPT activity). Significant RNase H cleavage occurs only in the non–PPT reactions, suggesting that RNase H plays a major role in blocking mispriming. Interestingly, the addition of NC further reduces priming by all non–PPT primers to essentially baseline level but does not affect the PPT. Modest inhibition of mispriming by NC also occurs in the absence of RNase H. Collaborative experiments show that the biochemical data correlate with the biophysical properties of the PPT and non–PPT duplexes, as determined by circular dichroism spectroscopy, temperature-dependent UV absorption, and fluorescence anisotropy. These assays indicate that the PPT duplex is distinct from the non–PPT hybrids and help explain the inability of NC to affect PPT priming. Zinc coordination by NC is also required for inhibition of mispriming, implying that NC functions by destabilizing the non–PPT duplexes, leading to dissociation of the RNA strand. Collectively, our findings demonstrated a previously unrecognized role for NC nucleic acid chaperone activity, which, together with RNase H cleavage, dramatically increases the fidelity of plus-strand initiation.

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Molecular analysis of the functional activities of APOBEC proteins

Hercik, Mitra, Wu, Iwatani,[4] Levin; in collaboration with Gronenborn, Musier-Forsyth, Rouzina, Strebel, Williams

A3G is a cellular cytidine deaminase with two zinc-finger domains. It acts as a potent inhibitor of HIV-1 reverse transcription and replication in the absence of the viral protein Vif. Most studies on human A3G have been performed primarily in cell-based systems or with unfractionated enzyme from viral lysates, but our interest lies in the host factors that can influence reverse transcription. We succeeded in producing highly purified, catalytically active protein expressed in a baculovirus system, permitting us for the first time to provide a detailed molecular analysis of A3G's deaminase and nucleic acid–binding activities.

Interestingly, in the course of our work, we found that A3G does not interfere with NC binding to single-stranded RNA (and vice versa), thereby suggesting that inhibition of reverse transcription by A3G is unlikely to be related to an effect on NC chaperon function. To test such a hypothesis and probe the mechanism that might be involved, we took advantage of defined biochemical assay systems that we have developed over the years for studies of viral DNA synthesis. Using our

highly purified A3G as well as purified NC and RT, we investigated the effect of A3G on a series of reconstituted reactions that occur during reverse transcription. We were thus able to perform an independent analysis of individual steps in the pathway, which is not possible with cell-based systems. We found that A3G inhibited *all* RT-catalyzed DNA elongation reactions but *not* RNase H activity or NC's ability to promote annealing. Inhibition of RT polymerase function was independent of A3G's deaminase activity—consistent with in vivo studies showing that deamination alone does not fully account for antiviral activity. The interpretation of our results is based on collaborative studies of the nucleic acid–binding properties of NC, A3G, and RT as measured by single-molecule DNA stretching and fluorescence anisotropy. The biophysical data suggest that A3G competes effectively with RT for binding to a single-stranded nucleic acid template but that it is not effective in displacing NC. Thus, our results support a novel mechanism for deaminase-independent inhibition of reverse transcription that is determined by critical differences in the nucleic acid binding properties of A3G, NC, and RT.

We have initiated studies on another human APOBEC protein, A3A, a cytidine deaminase with only one zinc-finger domain. A3A does not affect HIV replication but does inhibit retrotransposition of non–LTR (long-terminal repeat) elements representing 17 percent (LINE-1) and 11 percent (Alu) of the human genome, respectively. We expressed this protein in soluble form in *E. coli* and are now in the process of obtaining large amounts of purified protein for biochemical and structural analysis. Initial results showed that the partially purified protein has deaminase activity with a target sequence specificity that differs from that of A3G. Future work will focus on further characterization of A3A's functional activities.

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Function of HIV-1 capsid protein in virus assembly and early post-entry events

Jiang, Derebail,[4] Levin; in collaboration with Freed, Tang

Our laboratory has been investigating the role of the HIV-1 capsid protein (CA) in early post-entry events, a stage in the infection process that is still not completely understood. In our initial study, we used genetic, molecular, and ultrastructural approaches to describe the unusual phenotype associated with single alanine substitution mutations in a group of N-terminal conserved hydrophobic residues (including Trp23 and Phe40). We found that mutant virions are not infectious, lack a cone-shaped core, and, despite their functional RT enzyme, are blocked in the initiation of viral DNA synthesis in infected cells. Mutant viral cores display a marked deficiency in RT and have substantially increased amounts of CA, suggesting a defect in core disassembly. Taken together, our findings demonstrated the intimate connection among infectivity, proper core morphology, structural integrity of the CA protein, and ability to undergo reverse transcription.

More recently, we performed a study to provide new information on the plasticity of CA, i.e., its ability to tolerate changes in residues crucial for CA structure without total abrogation of biological activity. We constructed and tested mutants to determine whether they might retain the ability to replicate and thus offer an opportunity to isolate second-site suppressors. One mutant, W23F, exhibited infectivity in a single-cycle assay, albeit at a very low level. W23F has a phenotype that is intermediate between wild-type virus and the original W23A mutant. The W23F mutant (unlike W23A) is able to replicate during long-term culture in MT-4 cells, but with delayed replication kinetics. With continued passage, we were eventually able to isolate virions with a second-site

suppressor mutation (W23F/V26I). The suppressor mutation partially restores the wild-type phenotype, including production of particles with conical cores and normal CA content, as well as wild-type replication kinetics in MT-4 cells. A structural model that accommodates the spatial changes induced by the W23F and V26I mutations shows that hydrophobic interactions between Phe23 and Ile26 are possible and can explain the suppressor phenotype. Our novel findings demonstrated that, despite the limits imposed on assembly of CA structure, HIV-1 is able to adapt partially to severe structural distortions in a major viral protein.

The past year saw a major advance with a report of the three-dimensional structure of the hexameric arrays of full-length HIV-1 CA at 9 Å resolution. Nonetheless, there is still little information on the nature of the short linker peptide (residues 146–150; SPTSI) that connects the two domains. In current work, we have made alanine-scanning mutations in all of the residues in the linker region as well as in the two residues (Y145 and L151) that flank the linker. All mutants except P147A produce virus particles; we have therefore substituted P147L in this series. Interestingly, of the five linker mutants, only two (S146A and T148A) have substantial infectivity in a single-cycle assay; Y145A and L151A display almost no infectivity. The mutants all have a normal protein profile, but, in the electronmicroscope, mutants with low infectivity exhibit aberrant core structures. Experiments to examine the properties of isolated cores are now in progress. In addition, we have made mutations in two interacting lysine residues, N-terminal (K70) and C-terminal (K182). Substitution of alanine or aspartic acid resulted in loss of infectivity, but a conservative change to arginine resulted in a substantial level of infectivity. Transmission electronmicroscopy showed that only mutants K70R and K182R have conical cores. Further characterization of these mutants is under way.

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GENES AND SIGNALS REGULATING MAMMALIAN HEMATOPOIESIS

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We aim to elucidate the cellular and molecular events that regulate mammalian hematopoiesis by (1) characterizing the role of T cell antigen receptor (TCR) signals, particularly individual TCR signal–transducing subunits and signal-transducing motifs in T cell development—we employ several genetically altered mouse strains generated by gene targeting and transgenic technology; (2) identifying and analyzing signal-transducing molecules that function downstream of the TCR or inhibit TCR signaling—we aim to understand how these molecules participate in TCR-mediated signaling and to determine the roles played by the molecules and associated signaling pathways in regulating T cell maturation and T cell activation; (3) examining the function of chemokine receptor signaling in developing T cells—chemokine receptors are cell surface proteins that mediate chemotaxis in response to specific ligands that are expressed in discrete regions of the thymus and are candidates for regulating the homing of progenitor cells to the thymus and the intrathymic migration of thymocytes; (4) studying hematopoietic stem cells (HSCs) by characterizing the genes important for the generation, maintenance, and self-renewal of HSCs—we have uncovered a critical function for one protein (Ldb1) in controlling the self-renewal/differentiation cell fate decision in HSCs.

T cell antigen receptor (TCR) signaling in thymocyte development
Song, Lee, El-Khoury, Love; in collaboration with Hayes

Much of our research focuses on investigating the role of TCR signal transduction in thymocyte development. Signal transduction sequences (termed Immunoreceptor Tyrosine-based Activation Motifs; ITAMs) are contained within four distinct subunits of the multimeric TCR complex (zeta, CD3-gamma, CD3-delta, and CD3-epsilon). Di-tyrosine residues within ITAMs are phosphorylated upon TCR engagement, and their function is to recruit signaling molecules, such as protein tyrosine kinases, to the TCR complex, thereby initiating the T cell activation cascade. Though conserved, ITAM sequences are nonidentical, raising the possibility that the diverse developmental and functional responses controlled by the TCR may be partly regulated by distinct ITAMs. We previously generated zeta-deficient and CD3-epsilon–deficient mice by gene targeting. We genetically reconstituted the mice with transgenes encoding wild-type or signaling-deficient (ITAM-mutant) forms of zeta and CD3-epsilon and characterized the developmental and functional consequences of these alterations for TCR signaling. We found that TCR-ITAMs are functionally equivalent but act in concert to amplify TCR signals. TCR signal amplification was critical for thymocyte selection, the process by which potentially useful immature T cells are instructed to survive and differentiate further (positive selection) and by which potentially auto-reactive cells that may cause auto-immune disease are

deleted in the thymus (negative selection). Unexpectedly, we found that multiple TCR-ITAMs were not required for mature T cell effector functions. One possible explanation is that ITAM-mediated signal amplification is not required for mature T cell activation. Another possible explanation is that T cells in ITAM-mutant mice exhibit normal functional responsiveness because of compensatory mechanisms imposed during selection. To resolve this issue, we recently generated a TCR-zeta chain conditional knockin mouse in which T cell development and selection can occur without attenuation of TCR signaling (i.e., in the presence of wild-type 3 ITAM zeta chain), but in which mature, post-selection T cells may be induced to express TCRs containing signaling-defective (0 ITAM) zeta chains in lieu of wild-type zeta chain. Thus, mature T cell signaling should not be influenced by potential compensatory mechanisms that operate during T cell maturation such that T cells in these mice should be faithful indicators of the role of multiple TCR ITAMs in mediating specific, mature T cell responses. Preliminary experiments with the mice have confirmed that the knockin zeta locus functions as predicted. We are currently using this model system to evaluate the role of ITAM multiplicity in mature T cells.

A separate line of investigation centers on the importance of TCR signaling for gamma/delta T cell development. Most vertebrate species, including humans and mice, contain two separate lineages of T cells that are distinguished by the antigen-binding clonotype-specific chains contained within their TCRs: alpha/beta-T cells and gamma/delta-T cells. Although the more abundant alpha/beta TCR has been extensively characterized, much less is known about the structure or function of the gamma/delta TCR, which is expressed on the smaller subset of gamma-delta T cells. We found that the subunit composition of the gamma/delta TCR differs from that of the alpha/beta TCR in that a component of the alpha/beta TCR, the CD3delta chain, is not present in gamma/delta TCRs. These results revealed a major difference in the subunit structure of the alpha/beta and gamma/delta TCRs. Interestingly, we found signal transduction by the gamma/delta TCR to be superior to that by the alpha/betaTCR as assessed by several criteria. The data suggest that the structural difference between alpha/beta and gamma/delta TCRs may influence the signaling potential of the TCR complex and may have important functional consequences on T cell activation. Indeed, in a study performed in the past year, we showed that TCR signal intensity plays a critical role in regulating alpha/beta versus gamma/delta lineage choice in developing thymocytes. Current studies involve further analysis of the effect of TCR subunit structure on signaling responses.

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Identification and characterization of proteins important for TCR "fine tuning" and TCR signaling

Lesourne, Pinkhasov, Love

We have extended our analysis of TCR signaling subunits to other molecules that participate in or influence the TCR signaling response. The cell-surface protein CD5 negatively regulates TCR signaling and functions in thymocyte selection. Examination of CD5 expression during T cell development revealed that surface levels of CD5 are regulated by TCR signal intensity and by the affinity of the TCR for self-peptide ligands in the thymus that mediate selection. To determine if the ability to regulate CD5 expression is important for thymocyte selection, we generated transgenic mice that constitutively express high levels of CD5 throughout development. Overexpression of CD5 significantly impaired positive selection of some thymocytes (those that would normally express

low levels of CD5) but not of others (those that would normally express high levels of CD5). These findings support a role for CD5 in modulating TCR signal transduction and thereby influencing the outcome of thymocyte selection. The ability of individual thymocytes to regulate CD5 expression represents a mechanism for "fine tuning" the TCR signaling response during development. Reasoning that other molecules besides CD5 participate in TCR tuning, we initiated microarray-based screening for genes differentially expressed in developing T cells under conditions of high- or low-affinity TCR interactions and have identified several candidate genes for further study.

Using a subtractive cDNA library–screening approach, we recently identified a novel T cell–specific protein, T Lymphocyte Adaptor Protein (TLAP). We have cloned this protein, which we renamed AGAPE (Another Grb-Associated Protein Enigma), and generated AGAPE knockdown cell lines, AGAPE knockout mice, and AGAPE transgenic mice. Analysis of the effects of modulating AGAPE expression revealed a function for AGAPE in the TCR signaling pathway. In addition, we discovered an important role for in T cell development.

Role of the chemokine receptor CCR9 in T cell development

Uehara,[1] Love; in collaboration with Farber, Takahama, Butcher, Bhandoola

T cell development continues into adulthood and requires the periodic migration of T-progenitor cells from the bone marrow to the thymus. The ordered progression of thymocytes through distinct stages of development is also associated with migration into and between different thymus microenvironments, where they are exposed to different growth factors and signals. Chemokines are a group of small, structurally related molecules that regulate trafficking of leukocytes through interactions with a subset of seven-transmembrane, G protein–coupled receptors. The chemokine CCL25 is highly expressed in the thymus and small intestine, the two known sites of T lymphopoesis. CCR9, the receptor for CCL25, is expressed on the majority of thymocytes, raising the possibility that CCR9 and its ligand play an important role in thymocyte development. To investigate the role of CCR9 during lymphocyte development, we generated CCR9-deficient ($CCR9^{-/-}$) and CCR9-transgenic mice. Surprisingly, both T cell and B cell development appeared normal in $CCR9^{-/-}$ mice. However, bone marrow transplantation experiments demonstrated that lymphocyte progenitors from $CCR9^{-/-}$ mice had a markedly reduced capacity to repopulate the thymus when forced to compete with progenitor cells from $CCR9^{+/+}$ mice. In other experiments, overexpression of CCR9 in transgenic mice inhibited early thymocyte development and blocked the normal migration of immature thymocytes within the thymus. These results indicate that CCR9 participates in regulating both the migration of progenitor cells to the thymus and the migration of developing thymocytes within the thymus. However, CCR9 is not essential for normal T cell development, suggesting functional redundancy. We are currently testing such a hypothesis by generating mice deficient in several chemokine receptors (e.g., CXCR4 and CCR7).

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Role of Ldb1 in T cell development

Li, Lee, Love; in collaboration with Westphal

Lim domain binding protein-1 (Ldb1) is a ubiquitously expressed nuclear protein that contains a LIM-zinc-finger–protein interaction motif and a dimerization domain (see report by Westphal).

In hematopoietic cells, Ldb1 functions by interacting with and/or recruiting specific partners (including the LIM-only protein LMO2 and the transcription factors SCL and GATA-1) to form multimolecular transcription complexes. Within the hematopoietic lineage, expression of Ldb1 is highest in lineage-negative, $Sca1^{+}c\text{-}kit^{+}$ multipotent progenitors, which include HSCs, but gradually decreases as cells commit to and mature within specific lineages. $Ldb1^{-/-}$ mice die between day 9 and day 10 of gestation, preventing us from directly studying the impact of loss of Ldb1 on fetal or adult hematopoiesis. We investigated the role of Ldb1 in hematopoiesis by following the fate of Ldb1-null embryonic stem (ES) cells in mouse blastocyst chimeras and by conditional, stage-specific deletion of Ldb1. Significantly, Ldb1-null ES cells were capable of generating HSCs, which could give rise to both myeloid and lymphoid lineage cells; however, the number of $Ldb1^{-/-}$ HSCs gradually diminished at later stages of development. Following adoptive transfer of fetal liver cells, Ldb1-null LSKs were rapidly lost over a period of three months, indicating a failure of self-renewal or survival. More recent data indicate that the loss of Ldb1-null HSCs results from accelerated HSC differentiation. Although expressed in embryonic stem cells (ESC), Ldb1 is not required for ESC maintenance, indicating a selective requirement in adult stem cell populations. Our results provide the first example of a gene that directly regulates HSCs' cell-fate decision whether to differentiate or self-renew.

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LA ANTIGEN, RNA POLYMERASE III, AND ASSOCIATED RNA METABOLISM IN CELL BIOLOGY, GROWTH, AND DEVELOPMENT

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We are interested in how the pathways of tRNA and other RNA biogenesis interact with pathways that control cell proliferation, growth, and development. We focus on RNA polymerase (Pol) III and the post-transcriptional handling of its transcripts by the RNA-binding protein La, which, together with La-related protein-4 (LARP4), contributes to ribosome production, translational control, and the cell's growth capacity. In addition to its major products (tRNAs and 5S rRNA), Pol III synthesizes other non-coding RNAs. Tumor suppressors and oncogenes mediate deregulation of Pol III transcript production, contributing to increased capacity for proliferation of cancer cells. La protein is a target of autoantibodies prevalent (and diagnostic) in patients with Sjögren's syndrome, systemic lupus, and neonatal lupus. La contains several nucleic acid–binding motifs as well as several subcellular trafficking signals and associates with non-coding and messenger RNAs to coordinate activities in the nucleus and cytoplasm. We strive to understand the structure-function relationship and cell biology of La's and LARP4's contribution to growth and development. We use genetics, cell and structural biology, and biochemistry in model systems that include yeast, human tissue culture cells, and gene-altered mice.

Functions of the La antigen in RNA expression
Maraia, Bayfield

Recent paradigm-shaking findings such as nucleolar localization, cytoplasmic splicing, and retrograde transport indicate that the tRNA production pathway is more complex in its biochemistry, spatial organization, and sequential order than previously thought. By binding to UUU-3′OH, the La protein shields newly transcribed pre–tRNAs from 3′-end digestion and functions as a chaperone for misfolded or otherwise imperfect pre–tRNAs. Thus, it has become clear that La serves the tRNA pathway at several levels, including protection of pre–tRNAs from 3′ exonucleases; nuclear retention of pre–tRNAs, thereby preventing pre–tRNAs' premature export; and promotion of a newly identified processing step distinct from 3′-end protection.

To study Pol III- and La-dependent tRNA biogenesis, we had developed a red-white reporter system in the fission yeast *S. pombe*. That model system generally appears more similar to the human organism than does *S. cerevisiae* with respect to cell-cycle control, gene promoter structure, and the complexity of pre–mRNA splicing. From sequence analysis of Pol III–transcribed genes, we

predicted and then confirmed that Pol III termination signal recognition in *S. pombe* would be more similar to human Pol III than it is in S. *cerevisiae* Pol III. Our system is based on tRNA-mediated suppression of a nonsense codon in *ade6-704* and affords the benefits of fission yeast biology while lending itself to certain aspects of "humanization." We have been able to study the tRNA processing–associated function of the human La protein (hLa) because it is so highly conserved that it can replace the processing function of the *S. pombe* La protein Sla1p in vivo.

Briefly, we found that (1) the human pattern of phosphorylation of hLa on the CK2 target site serine-366 occurs faithfully in *S. pombe* and promotes tRNA production; (2) various conserved subcellular trafficking signals in La proteins can be positive or negative determinants of tRNA processing; (3) La can protect pre–tRNAs from the nuclear surveillance 3′ exonuclease Rrp6p; (4) the 3′ exonuclease that processes pre–tRNAs in the absence of Sla1p is distinct from Rrp6p; (5) Sla1p is limiting in *S. pombe* cells, and the extent to which it influences the use of alternative tRNA maturation pathways is balanced by the RNA 3′–5′ cleavage activity of the Pol III termination-associated Pol III subunit Rpc11p; and (6) La proteins use distinct RNA-binding surfaces, one on the La motif (LM) and the other on RNA recognition motif-1 (RRM1), to promote distinct steps in tRNA maturation.

Our recent work suggests that La can use several surfaces, perhaps combinatorially, to engage different classes of RNAs, e.g., pre–tRNAs versus mRNAs, or to perform different functions (Huang et al., *Nat Struct Mol Biol* 2006;13:611; Maraia and Bayfield, *Mol Cell* 2006;21:149). Consistent with this notion, some pre–tRNAs require only the UUU-3′OH binding activity while others depend on a second activity in addition to 3′-end protection that requires an intact RRM surface to promote a previously unknown step in tRNA maturation. One of our objectives is to identify cellular genes other than La that contribute to this "second" activity. Toward this goal, we have isolated and begun to characterize *S. pombe* revertant mutants that overcome a defect in the second activity.

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Activities of RNA polymerase III and associated factors

Maraia, Huang, Intine; in collaboration with Pack, White, Grewal

The Pol III enzyme consists of 17 subunits, several with strong homology to subunits of Pol I and Pol II. In addition, TFIIIC, composed of 6 subunits, binds to the A- and B-box promoters and recruits TFIIIB to direct Pol III to the correct start site. Pol III complexes are highly stable and demonstrate great productivity in supporting many cycles of initiation, termination, and re-initiation. For example, each of the 5S rRNA genes in human cells must produce approximately 10^4 to 10^5 transcripts per cell division to provide sufficient 5S rRNA for ribosomes. While Pol I, Pol II, and Pol III are homologous, their properties are distinct in accordance with unique functions related to the different types of genes they transcribe. Given that some mRNA genes can be hundreds of kilobasepairs long, Pol II must be highly processive and avoid premature termination. Pol II terminates in response to complex termination–RNA processing signals that require endonucleolytic cleavage of RNA upstream of the elongating polymerase. By contrast, formation of the UUU-3′OH

terminus of nascent Pol III transcripts appears to occur in the Pol III active center. The dT(n) tracts at the ends of class III genes directly signal pausing and release by Pol III such that termination and RNA 3′ end formation are coincident and efficient.

Rpc11p is an integral Pol III subunit that mediates conserved exoribonucleolytic cleavage of the nascent RNA 3′ end within the Pol III transcription complex. Accumulated data suggest that Rpc11p is involved in termination and efficient recycling of Pol III. We showed that mutations impairing Rpc11p's RNA 3′ cleavage activity alter RNA 3′ end formation in vivo with consequences for tRNA production. Given that Rpc11p has homologues in Pol II (Rpb9p and TFIIS) and Pol I (Rpa11p), we suspect that understanding Rpc11p's mechanism of action may be applicable to these homologues.

Our collaborator Michael Pack made an exciting finding related to Rpc11p. Impairment of a conserved interaction between Rpc11p and the core—Rpc2p (second-largest Pol III subunit)—leads to tissue-specific defects in zebrafish development. A random genetic screen uncovered a small deletion in Rpc2p that impairs interaction with Rpc11p. The homologous deletion engineered in *S. pombe* Rpc2p impaired recruitment of Rpc11p by *S. pombe* Pol III. The zebrafish developmental defects manifest in highly proliferative cells while sparing the less proliferative cells of the developing embryo. Remarkably, the mutant phenotype caused by the small deletion in Rpc1p can be rescued by overexpression of Rpc11p in zebrafish embryos. The precise role of Rpc11p in tissue-specific development remains to be determined.

A collaboration with Shiv Grewal focusing on the Sfc3p and Sfc6p subunits of *S. pombe* TFIIIC has provided insight into genome organization, as these B box–associated Pol III transcription factors were found to contribute to the boundary elements that partition silenced heterochromatin domains to the nuclear periphery.

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La-related protein-4 (LARP4) in translational control

Maraia, Yang

The eukaryote-ubiquitous La proteins are involved in two broad functions: (1) metabolism of a wide variety of precursor-tRNAs and other small nuclear RNAs by association with the RNAs' common UUU-3′OH terminal elements and (2) translation of specific subsets of mRNAs, such as those containing IRES and other motifs, by unknown mechanisms. The La-related protein LARP7/PIP7S exhibits a specialized UUU-3′OH–related function in its specific interaction with 7SK snRNA. Another La-related protein, LARP4, is conserved in metazoa and, in accordance with experimental criteria that we obtained, appears to be a translation factor. Unlike La and LARP7, LARP4 localizes to the cytoplasm as demonstrated by immunofluorescence and contains a highly conserved sequence similar to but a variant of the poly-A binding protein (PABP) interaction motif-2 (PAM2) consensus found in other translation factors, including Paip1 and Paip2. PABP co-immunoprecipitates with Flag-LARP4 (F-LARP4) from human cells in an RNase-insensitive manner while substitution of two key residues in the variant-PAM2 consensus reduces PABP co-immunoprecipitation. F-LARP4 specifically co-immunoprecipitates two other translation factors that we examined, elF4G and RACK1, although the interactions are sensitive to RNase. Antibodies to LARP4 showed that native endogenous LARP4 is cytoplasmic, co-immunoprecipitates PABP in

an RNase-insensitive manner, and co-sediments with the 40S subunit peak and polysomes; however, the peak shifts upon puromycin treatment to one indicating a smaller size than the 40S mRNP. Luciferase translation reporter assays in control and siRNA LARP4 knockdown cells provided evidence that LARP4 promotes general translation.

LARP4 immunoprecipitation followed by microarray analysis identified target mRNAs, which we verified by RT-PCR for the best two candidates. LARP4 levels declined by about 90 percent in these cells, but not in the control siRNA cells, without lowering actin or GAPDH. Preliminary data indicate that the LARP4-associated mRNAs peak on heavy polysomes in the control siRNA cells but shift off heavy polysomes and overaccumulate in the 80S peak in the siRNA-LARP4 cells. Attempts to reproduce and expand on these studies and identify common sequences in the LARP4-associated mRNAs are under way.

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DEVELOPMENTAL GENE REGULATION OF THE IMMUNE SYSTEM

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We study transcription factors and chromatin-binding proteins that control the development of innate immunity. Our aim is to study the activity of IRF8 in the development of dendritic cells (DC)—cells that play essential roles in innate immunity. We showed that IRF8 drives, among other DC subsets, the development of plasmacytoid DCs (pDC) and CD8α⁺ DCs, which produce type I interferons (IFNs) and IL-12, respectively. These cytokines are important for the establishment of an anti-microbial state. Our studies yielded clues to the mechanism of IRF8 action, namely, that (1) IRF8 contributes to high IFN production in DCs by amplifying the feedback phase of transcription and that (2) the naturally occurring IRF8 point mutation alters IRF8's interaction with partner proteins, thereby altering DC developmental pathways for a specific DC subset. We also work on the bromodomain protein Brd4. Brd4 binds to acetylated chromatin, which is implicated in the maintenance of transcriptional memory. By extending our earlier study showing that Brd4 regulates cell-cycle progression, we found that Brd4 binds to the promoters of many G1 genes and recruits P-TEFb, a kinase that triggers transcriptional elongation. These studies have provided a mechanistic basis for Brd4 action.

Role of IRF8 in type I IFN transcription in DCs
Tailor, Ramakrishna, Atsumi, Chang

The observation that DCs from *IRF8* knockout mice produce little type I IFNs led us to study the molecular basis of IFN transcription in DCs. We found that type I IFN mRNA induction follows two-phase kinetics. Induction of the early transcripts, seen within one hour of Newcastle disease virus (NDV) stimulation, was intact in IRF8 knockout DCs. Transcription in the second phase, which peaked at seven hours, produced much higher levels of IFN mRNA. The second transcript induction was completely missing in *Irf8* knockout DCs. Both pDCs and conventional DCs (cDCs) induced IFN transcripts in response to NDV infection. The second-phase induction represented an IFN feedback response in that it was also missing in DCs from *Ifnar1* knockout mice. IRF3 and IRF7 were normally expressed and translocated into the nucleus upon NDV stimulation in *Irf8* knockout DCs. Supporting the view that IRF8 plays a role in transcription, introduction of IRF8 into NIH3T3 fibroblasts led to a large amplification of the otherwise weak second phase of IFN induction. Using chromatin immunoprecipitation (ChIP) analysis, we showed that IRF8 is recruited to the promoters of the *Ifnb, Ifna4*, and *Ifna5* genes (which encode IFNβ, IFNα4, and IFNα5). These promoters have several virus response elements similar to IFN-stimulated response elements (ISREs). Consistent

with its role in the second phase, we observed recruitment of IRF8 following that of IRF7 and pol II—IRF7 and pol II were both bound to the promoters at an earlier time. Similarly, IRF8 bound to the IFN promoter after stimulation by TLR (Toll-like receptor) ligands such as CpG DNA and poly IC. We have thus provided a mechanistic basis of IRF8 action by showing that binding of pol II to the IFN promoters declined rapidly after initial recruitment in *Irf8* knockout DCs, while pol II binding continued for an extended period in *Irf8*^{wt} DCs. We expect that IRF8 supports re-recruitment of pol II, thereby boosting second-phase transcription.

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Mechanism of IRF8 action as revealed by the BXH2 mutation

Tailor, Ramakrishna, Atsumi, Chang, Smith

The BXH2 recombinant inbred mouse strain displays a phenotype similar to *IRF8* knockout mice. BXH2 mice are defective in IL12p40 production and hence highly susceptible to *Mycobacterium bovis* (BCG). They carry a point mutation in the *Irf8 /Icsbp* gene that changes arginine (R) to cysteine (C) in position 294. The mutation, designated *Irf8*^{R294C}, is within the IAD (IRF association domain), which is important for the interaction of IRF8 with partner proteins. We found that CD8α$^+$ DCs are selectively missing in these mice, although the mice have normal numbers of pDCs. Consistent with the selective inability to generate CD8α$^+$ DCs, FMS-like tyrosine kinase 3 ligand (Flt3L)–based bone marrow (BM) cultures from *Irf8*^{R294C} mutants failed to produce this DC subset but generated abundant pDCs. Accordingly, DCs generated from the *Irf8*^{R294C} mutants were defective in IL-20p40 production while intact in type I IFN production. This defect could be rescued when the *Irf8*^{wt} gene was transferred into the *Irf8*^{R294C} BM cells. Electrophoretic mobility shift assays and ChIP experiments showed that IRF8^{R294C} did not interact with its partners PU.1, SpiB, and IRF1,2 and was not recruited to the promoter of the *IL-12p40* and *Csc3* (*cystatin C*) genes, which are expressed mostly in CD8α$^+$ DCs. The results demonstrated that IRF8 drives the development of the two DC subsets by two distinct mechanisms and that the one requiring interactions with these partners is essential for CD8α$^+$ DCs. Our study demonstrates the molecular mechanisms of the developmental pathways that demarcate pDCs and CD8α$^+$DCs.

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Co-recruitment of Brd4, P-TEFb, and pol II drives G1 gene transcription and cell-cycle progression

Dey, Kanno, Ghosh, Smith

Brd4 interacts with P-TEFb and the RNA polymerase II mediator complex. P-TEFb is a kinase composed of Cdk9 and cyclinT and is important for transcriptional elongation of RNA polymerase II. The multiprotein complex mediator associates with polymerase II and contributes to transcription. This interaction pattern suggests that Brd4 is involved in transcriptional regulation.

On the other hand, mounting evidence points to a role for Brd4 in cell growth, including that of cancer cells. Thus, we investigated cell growth by using NIH3T3 cells in which Brd4 was knocked down by short hairpin RNA (shRNA). The use of shRNA was a practical alternative to studying *Brd4* knockout mice because the mice die early without allowing for cell culture. Brd4 knockdown cells, when synchronized to G0 by serum starvation, failed to progress through G1 after release and did not enter S-phase. Microarray analysis of synchronized Brd4 knockdown cells showed that, while numerous genes were induced in control shRNA cells during G1 progression, the majority of these genes remained uninduced in Brd4 knockdown cells, indicating that Brd4 regulates cell growth partly through the regulation of G1 genes. We confirmed the role of Brd4 in G1 progression by the rescue of G1 gene expression and restoration of S-phase entry following reintroduction of Brd4. We performed ChIP analysis for G1 genes downregulated in Brd4 knockdown cells, including those encoding cyclin D1, cyclin D2, Orc2, and Mcm2. We found that Brd4 was bound to these and other G1 genes at the transcription start sites when cells progressed from G0 to G1. However, Brd4 did not bind to *Tlr9*, a gene not expressed in NIH3T3 cells, validating the specificity of our ChIP data. By extending our ChIP experiments, we showed that P-TEFb and pol II were co-recruited along with Brd4 to these genes during G1. The results lend credence to the role of Brd4 in G1 gene regulation.

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Intracellular delivery of acetyl histone peptides inhibits native bromodomain-chromatin interactions and impairs mitotic progression

Dey, Ghosh, Smith

Post-translational modification of histone tails regulates the chromatin environment and influences gene expression and cell growth. The bromodomains in Brd4 and other chromatin-binding proteins bind to acetylated histone tails and modulate transcription. Changes in bromodomain-histone interactions are thought to affect epigenetic memory and cellular functions. With the aim of altering Brd4-chromatin interactions in vivo, we took a protein transduction approach to deliver acetylated histone tail peptides into cultured cells. NIH3T3 and P19 cells efficiently incorporated unacetylated and acetylated histone H3 and H4 peptides when fused to the protein transduction domain (PTD) derived from the Tat protein of the human immunodeficiency virus (HIV). We found that acetyl H4 peptides, but not their unacetylated counterparts, increased the real-time mobility of Brd4, as assessed by fluorescence recovery after photobleaching. Increased Brd4 mobility indicates that the acetylated H4 peptides specifically inhibited Brd4-chromatin interactions in living cells, consistent with previous in vitro observations that the same peptides bind to Brd4. The acetyl-H4 peptides also inhibited the interaction of Brd4 with mitotic chromosomes and compromised subsequent cell growth. In summary, delivery of PTD-mediated histone tail peptides offers a novel means both to manipulate bromodomain-chromatin interactions in vivo and to study their biological significance.

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MOLECULAR GENETICS OF AN IMPRINTED GENE CLUSTER ON MOUSE DISTAL CHROMOSOME 7

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Genomic imprinting is an unusual form of gene regulation by which an allele's parental origin restricts allele expression. Imprinted genes are not randomly scattered throughout the chromosome but rather are localized in discrete clusters. One cluster of imprinted genes is located on the distal end of mouse chromosome 7. The syntenic region in humans (11p15.5) is highly conserved in gene organization and expression patterns. Mutations disrupting the normal patterns of imprinting at the human locus are associated with developmental disorders and many types of tumors. In addition, inherited cardiac arrhythmia is associated with mutations in the maternal-specific *Kcnq1* gene. We use mouse models to address the molecular basis for allele-specific expression in this distal 7 cluster. We hope to use imprinting as a tool to understand the fundamental features of epigenetic regulation of gene expression. We also generate mouse models for the several inherited disorders of humans. We have generated models to study defects in cardiac repolarization associated with loss of *Kcnq1* function and, more recently, characterized the phenotype associated with loss of *Calsequestrin2* gene function. These cardiac studies all focus on the role of beta-adrenergic stimulation in cardiac arrhythmias.

Molecular basis for allele-specific expression of the mouse *H19* and *Igf2* genes
Gebert, Rong, Bennett, Blatch, Pfeifer

Our studies on the mechanisms of genomic imprinting focus on the *H19* and *Igf2* genes, which lie at one end of the distal 7 imprinted cluster. Paternally expressed *Igf2* lies about 70 kb upstream of the maternal-specific *H19* gene. Using cell culture systems as well as transgene and knockout experiments in vivo, we have identified the enhancer elements responsible for activation of these two genes. The elements are largely shared and located downstream of the *H19* gene. Parent-of-origin–specific expression of both genes is dependent on a shared element (called the *H19ICR* for H19 imprinting control region) located just upstream of the *H19* promoter and thus juxtaposed between the *Igf2* gene and the shared enhancers. The CpG sequences within this element are methylated specifically on the paternally inherited chromosome. Our conditional ablation of this element in vivo demonstrates that the non-methylated *H19DMR* (i.e., the copy on the maternal chromosome) is continually required for silencing of the maternal *Igf2* allele. Knockin experiments demonstrated that the *H19DMR* contains a methylation-sensitive transcriptional insulator. Thus, on the non-methylated maternal chromosome, the active insulator within the *H19DMR* prevents activation of *Igf2* by the downstream enhancers. Methylation of the paternal chromosome inactivates the insulator and

permits *Igf2* expression. Unexplained by this model is the effect of several small DMRs proximal to the *Igf2* transcription unit. Current studies are investigating the mechanistic significance of these elements. Imprinting of *H19* occurs via a distinct genetic mechanism. The conditional ablation of the *H19DMR* indicates that it is not continuously required to silence the paternal allele. Rather, the *H19DMR* is required early in development to establish a novel epigenetic state at the *H19* promoter. Once in this novel epigenetic configuration, the *H19* promoter is stably refractory to transcriptional activation. Current studies indicate that the epigenetic program includes but is not solely limited to the hypermethylation of the *H19* promoter.

To determine which elements are necessary and sufficient for imprinting at the locus, we moved the *H19DMR* and its mutated derivatives to heterologous loci. Our results demonstrated that the ICR alone is sufficient to imprint a normally non-imprinted chromosome. Moreover, such activity does not depend on germline differences in *DMR* methylation. Thus, the *DMR* likely marks its parental origin by a mechanism that is independent of DNA methylation. To determine the epigenetic signals that constitute the genomic imprint, we are now conducting genetic and molecular analyses of embryonic stem cells derived from mutant mice.

We are also continuing a series of experiments to elucidate the molecular mechanisms by which the *H19DMR* can act as a transcriptional insulator. Several groups have demonstrated the presence of four CTCF-binding sites within the *H19DMR*. CTCF (CCCTC-binding factor—a zinc finger protein) is a DNA-binding protein previously demonstrated to interact with the chicken beta-globin insulator. The ability of CTCF to recognize DNA is sensitive to methylation, i.e., CTCF cannot bind to the methylated paternally inherited *DMR*, thus explaining the activation of the paternal *Igf2* allele. To understand the molecular basis for insulator function, we have completed a series of experiments to characterize the three-dimensional organization of the *Igf2/H19* locus, comparing maternal and paternal and wild-type and mutant chromosomes. We demonstrated that transcriptional activation is invariably associated with physical interaction of the promoter and enhancer elements. Insertion of the ICR/insulator abrogates these interactions and induces alternative long-range chromosomal interactions between it and the regulated promoter elements. Curiously, induction of these alternative interactions with the insulator is enhancer-dependent.

Our research goals for the next year call for (1) identifying the original gametic imprint that distinguishes the parental origin of chromosome 7; (2) determining the molecular mechanisms that convert the gametic imprint into parent-of-origin–specific patterns of DNA methylation; (3) identifying key transcription factors associated with the *Igf2* and *H19* promoters; and (4) comparing promoter structure on wild-type and mutant chromosomes, both maternal and paternal, in order to determine the molecular role of enhancer and insulator elements.

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Mouse models for inherited long QT syndrome

Rong, Pfeifer; in collaboration with Ebert, Knollmann

Inherited long QT syndrome (LQTS) is characterized by an abnormal electrocardiogram indicative of repolarization defects; it can result in syncope or sudden death. Romano-Ward syndrome (RWS) patients generally inherit the LQTS disorder as a dominant phenotype and show no other traits. Jervell and Lange-Nielsen syndrome (JLNS) patients display profound congenital deafness in addition to LQTS. Both phenotypes are recessive. We have generated several mutations in the mouse *Kcnq1* gene to model the human diseases. Ablation of the gene results in vestibular and auditory defects. Histological analyses suggested that the defects are attributable to deficiency in the K^+ recycling pathway that is crucial for generating endolymph, the specialized fluid bathing the inner hair cells.

ECG tracings of mutant mice indicate profound defects in cardiac repolarization when measured in vivo. However, the defects are not evident in isolated hearts ex vivo, indicating that the Kcnq1 protein plays a key role in mediating critical extracardiac signals. Further analyses demonstrated that Kcnq1 function is specifically required to modulate cardiac function in the presence of beta-adrenergic stimulation.

We have also generated three point mutations to model RWS. We analyzed the mutations in the central pore region and in the sixth membrane-spanning domain. We observed that the phenotypes of the mutations are each a distinct subset of those seen in the null mutation, thereby demonstrating that the Kcnq1 protein plays distinct roles in the heart versus inner ear and in various aspects of cardiac function. While inherited LQTS is relatively rare, our genetic models represent excellent paradigms for addressing mechanisms for acquired LQTS, the single largest cause of death in Western societies.

Biochemical and pharmacological studies both predicted that the key biological function of the Kcnq1 protein is to associate with the helper protein Kcne1 to form the I_{KS} potassium channel. One of the most novel results of our studies is the discovery that that ablation of the *Kcnq1* gene leads to cardiac defects in addition to those noted in *Kcne1*-deficient mice. These results demonstrated a novel role for Kcnq1 in heart development and/or function. We have used our mutant mice as tools to detect a previously unappreciated potassium channel that was dependent on Kcnq1 but not on Kcne1. We are now investigating the role of this channel in mouse and human hearts.

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The role of calsequestrin2 in regulating cardiac function

Shirali, Van Druff,[1] Pfeifer; in collaboration with Knollmann

Mutations in the *CASQ2* gene, which encodes cardiac calsequestrin (Casq2), are associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) and sudden death. The survival of individuals homozygous for loss-of-function mutations in *CASQ2* is actually surprising, given the central role of Ca^{2+} ions in excitation-contraction (EC) coupling and the presumed critical roles of Casq2 in regulating Ca^{2+} release from the sarcoplasmic reticulum (SR) into the cytoplasm. To address this paradox, we have generated a mouse model for loss of *Casq2* gene activity. Comprehensive analysis of cardiac function and structure generated several important insights into Casq2 function. First, Casq2 is not essential for providing sufficient Ca^{2+} storage in the SR of the cardiomyocyte. Rather, a compensatory increase in SR volume and surface area in mutant mice appears to maintain normal Ca^{2+} storage capacity. Second, Casq2 is not required for the rapid, triggered release of Ca^{2+} from the SR during cardiomyocyte contraction. Rather, the RyR receptor opens appropriately, resulting in the normal, rapid flow of Ca^{2+} into the cytoplasm and thus allowing normal contraction of the cardiomyocyte. Third, Casq2 is required for normal function of the RyR during cardiomyocyte relaxation. In the absence of Casq2, significant Ca^{2+} leaks occur through the RyR and lead to premature contractions and cardiac arrhythmias. Fourth, Casq2 function is required to maintain normal junctin and triadin levels. We do not yet understand what role, if any, the compensatory changes in these two SR proteins plays in modulating the loss of Casq2 phenotype. To address these issues and to model cardiac disorders associated with late-onset (not congenital) loss of *Casq2* activity, we are beginning to analyze mouse models in which loss of *Casq2* function is induced in adult animals by hormone treatment. We have also established a mouse model in which *Casq2* function can be restored to adult mice that develop without any *Casq2* gene function. In the coming year, our primary goal will be to identify hormonal treatments that efficiently induce the genetic changes of interest but without toxic side effects.

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CONTROL OF ECTODERMAL DEVELOPMENT IN VERTEBRATE EMBRYOS

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We identify factors and mechanisms that are responsible for controlling early vertebrate development, focusing on the cranial neural crest (CNC). We use the frog *Xenopus laevis* and zebrafish as the primary experimental model organisms. Our earlier research revealed a central role in epidermal and neural crest (NC) development for the transcription activator TFAP2. We have built on that research by identifying downstream regulatory targets of TFAP2 and, in the past year, have concentrated on two genes shown to be essential for proper craniofacial development. The genes encode Inca, a novel p21-activated kinase-associated protein involved in cytoskeletal regulation, and MyosinX, a non-muscle motor protein expressed in NC, sensory placodes, and other tissues. The functions of the genes encoding these proteins are being analyzed by inhibiting expression with gene-specific anti-sense oligonucleotides and by overexpression strategies in embryos and cultured cells. We have discovered that, in addition to its function in CNC, Inca plays an important role in controlling cell movements during gastrulation. We are pursuing the molecular basis of this role in both frog and fish embryos.

Inca: a novel regulator of cytoskeletal dynamics
Luo, Xu, Hwang, Sargent; in collaboration with Williams

Inca (Induced in Neural Crest by AP2) is expressed strongly in the neural crest, beginning after gastrulation and continuing throughout development. As shown by gene knockdown experiments and standard genetics, maintenance of this expression is dependent on TFAP2 activity in the frog and zebrafish. *Inca* is also expressed in mesoderm during gastrulation and in additional tissues, such as heart, in tadpole and in later stages of frog development. Homologues of Inca exist in all vertebrates, including mouse, human, and zebrafish, but Inca genes are not found in invertebrates. The Inca protein sequence is novel with no distinguishing features enabling its assignment to existing protein families. The early expression pattern of *Inca* is conserved in fish and mouse embryos, and we have shown by anti-sense loss-of-function experiments that Inca is required for normal craniofacial development. A collaborative project to target the mouse *Inca* gene is currently under way in the Williams laboratory. Recently born *Inca* null mice do not appear to be grossly abnormal, suggesting that other genes in the mouse, in contrast to fish and frogs, might at least partially compensate for Inca function. Longer-term and more detailed analysis of the *Inca* null mice is in progress.

Using yeast two-hybrid analysis, we identified a p21-activated kinase (PAK4) as an interaction partner for Inca. PAKs function in the transduction of cell-cell signals mediated by the Rho class GTPases Rac and Cdc42 and have been implicated in the regulation of cytoskeletal dynamics as well as in other cell processes. Interestingly, PAK4 is upregulated in many tumors. We found that overexpression of *Inca* in NIH 3T3 cells leads to increased levels of active, GTP-bound Rho, resulting in alterations in the actin cytoskeleton, microtubule acetylation, and cell-migratory behavior. PAK4 is known to regulate Rho activation via repression of RhoGEF; we thus hypothesize that Inca affects Rho signaling via the same route. In frog embryos, loss of Inca function inhibits convergent extension (CE) movements in dorsal mesoderm, delaying or preventing the completion of gastrulation. In experiments with overexpression of a deletion mutation of *Inca*, we removed the conserved 35-residue "inca box" and observed complex effects on both CE and MAPK signaling, apparently through separate mechanisms. In the zebrafish, two *Inca* genes are equally similar to *Xenopus Inca*. One is expressed mainly in neural crest, and its knockdown yields a phenotype similar to what we observed in the frog. The second zebrafish *Inca*, *Inca2*, is most prominently expressed in the mesoderm, particularly in notochord (where *Xenopus Inca* is also expressed). Knockdown of zebrafish *Inca2* causes dramatic changes in the expression of marker genes for dorsoventral polarity, indicating a role of this Inca in basic embryonic axis formation, including notochord formation. We observed similarities between mesoderm movements during gastrulation and the extension of cranial cartilage following NC migration, suggesting that both processes might require Inca in a fundamentally equivalent manner.

Luo T, Xu Y, Hoffman TL, Zhang T, Schilling T, Sargent TD. Inca: a novel p21-activated kinase-associated protein required for cranial neural crest development. *Development* 2007;134:1279-1289.

Myosin10: a cytoskeletal motor protein required in CNC development

Hwang, Luo, Sargent

Myosin10 (Myo10) is a member of the large superfamily of non-muscle myosins. It has been recently shown to interact with both microtubules and F-actin filaments. The gene encoding Myo10 was strongly induced by TFAP2a in our microarray screen and is expressed in NC, placodes, and paraxial mesoderm. Myo10 is encoded by a maternal mRNA in *Xenopus*; suppression of this mRNA translation causes mitotic defects and arrest during early cleavage stages. To assess Myo10 function later in development, we designed two anti-sense morpholino oligonucleotides that inhibit processing of Myo10 precursor RNA. Loss-of-function of Myo10 results in inhibition of migration in vivo. NC cells migrate most efficiently on fibronectin, using integrin alpha5beta1 as the receptor. In vitro migration assays on fibronectin using explanted CNC cells showed significant inhibition of cell attachment as well as spreading and migration resulting from MyoX knockdown. Rhodamin-phalloidin staining revealed malformation of membrane processes, including filopodia. It has been reported that the FERM-domain of MyoX physically interacts with a conserved NPXY motif present in the cytoplasmic domain of most beta integrins. To test for such an interaction in the NC, we co-expressed beta3 integrin labeled with mCherry and MyoX labeled with GFP in frog embryos. We then excised NC tissue and used live confocal imaging after the NC cells had spread on a fibronectin substrate. We observed co-localization, particularly at the tips of filopodia, which was disrupted by MyoX knockdown, supporting our hypothesis that MyoX is required for integrin transport and/or activation in migrating NC. Our work is the first demonstration of such a function for MyoX in embryonic cells.

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ORGANOGENESIS OF THE ZEBRAFISH VASCULATURE

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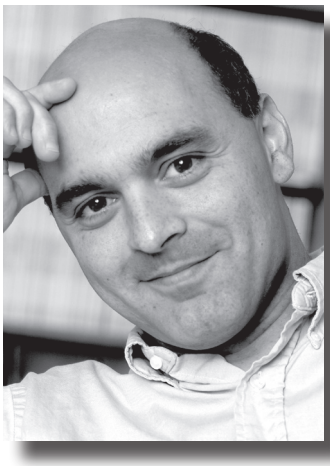

We aim to understand how the elaborate networks of blood and lymphatic vessels arise during vertebrate embryogenesis. Blood vessels supply every tissue and organ with oxygen, nutrients, and cellular and humoral factors. Lymphatic vessels drain fluids and macromolecules from the interstitial spaces of tissues and then return them to the blood circulation; in addition, lymphatic vessels are essential participants in immune responses. Understanding the formation of blood and lymphatic vessels has become a subject of intense clinical interest because of the important roles played by both types of vessels in cancer and ischemia. The zebrafish, a small tropical freshwater fish, possesses a unique combination of features that makes it particularly well suited for studying vessel formation. The fish is a genetically tractable vertebrate with a physically accessible, optically clear embryo. These features are highly advantageous for studying vascular development, permitting observation of every vessel in the living animal and simple, rapid screening for even subtle vascular-specific mutants. Major aims of our laboratory include (1) developing new tools for experimental analysis of vascular development in zebrafish, (2) forward-genetic analysis of vascular development, (3) analysis of vascular morphogenesis, (4) analysis of vascular patterning, and (5) analysis of lymphatic development.

Developing tools for experimental analysis of vascular development in the zebrafish
Cha, Fujita, Gore, McKinney, Swift, Yaniv; in collaboration with Davis, Dye

One important aim of our laboratory has been the development of new tools to facilitate vascular studies in the zebrafish. We have (1) established a microangiographic method for imaging patent blood vessels in the zebrafish and used the method to compile a comprehensive staged atlas of the vascular anatomy of the developing fish (http://eclipse.nichd.nih.gov/nichd/lmg/redirect.html); (2) generated a variety of transgenic zebrafish lines expressing various fluorescent proteins within vascular or lymphatic endothelial cells, allowing us to visualize vessel formation in intact, living embryos; and (3) developed methodologies for long-term multiphoton confocal time-lapse imaging of vascular development in transgenic fish. Recent technical advances have greatly facilitated the generation of new transgenic lines in the zebrafish. We are currently developing many new lines useful for in vivo vascular imaging as well as for in vivo endothelial-specific functional manipulation of signaling pathways involved in vascular specification, patterning, and morphogenesis.

Cha Y-R, Weinstein BM. Visualization and experimental analysis of blood vessel formation using transgenic zebrafish. *Birth Defects Res C Embryo Today* 2007;81:286-296.

Gore AV, Lampugnani MG, Dye L, Dejana E, Weinstein BM. Combinatorial interaction between CCM pathway genes precipitates hemorrhagic stroke. *Dis Model Mech* 2008;1:275-281.

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Genetic analysis of vascular development

Castranova, Cha, Gore, Lo, McKinney, Melani, Pham, Swift, Ungos, Yaniv; in collaboration with Dawid, Dye, Kennedy, Lawson, Liu, Roman

We use forward-genetic approaches to identify and characterize new zebrafish mutants that affect the formation of the developing vasculature. Using transgenic zebrafish expressing green fluorescent protein (GPF) in blood vessels, we are carrying out an ongoing large-scale genetic screen for mutants induced by *N*-ethyl *N*-nitrosourea (ENU). To date, we have screened well over 2,000 genomes and have identified over 100 new vascular mutants with phenotypes that include loss of most vessels or subsets of vessels, increased sprouting/branching, and vessel mispatterning. A bulked segregant mapping pipeline is in place to determine rapidly the rough position of newly identified mutants on the zebrafish genetic map. Fine mapping and molecular cloning are in progress for many mutants. We previously positionally cloned the defective genes from several vascular-specific mutants, including *violet beauregarde* (defective in Alk1/acvrl1), *plcg1* (defective in phospholipase C-gamma 1), *kurzschluss* (defective in a novel chaperonin), *beamter* (defective in trunk somite and vascular patterning), and *etsrp* (defective in an ETS[2]-related transcription factor). We are currently focusing on several mutants affecting VEGF[3]-dependent and VEGF-independent vascular signaling pathways. Our ongoing mutant screens and positional cloning projects continue to yield a rich harvest of novel vascular mutants and genes, bringing to light new pathways regulating the formation of the developing vertebrate vasculature.

Alvarez Y, Cederlund ML, Cottell DC, Bill B, Ekker SC, Torres-Vazquez J, Weinstein BM, Hyde DR, Vihtelic T, Kennedy B. Genetic determinants of hyaloid and retinal vasculature in zebrafish. *BMC Dev Biol* 2007;7:114-131.

Anderson MJ, Pham VN, Vogel AM, Weinstein BM, Roman BL. Loss of unc45a precipitates arteriovenous shunting in the aortic arches. *Dev Biol* 2008;318:258-267.

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Covassin LD, Villefranc JA, Kacergis MC, Weinstein BM, Lawson ND. Distinct genetic interactions between multiple Vegf receptors are required for development of different blood vessel types in zebrafish. *Proc Natl Acad Sci USA* 2006;103:6554-6559.

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Analysis of vascular morphogenesis

Gore; in collaboration with Davis, Dejana, Dye, Ginsburg, Kennedy, Lyons

In previous studies, we used high-resolution time-lapse two-photon imaging to show that the formation and intra- and intercellular fusion of endothelial vacuoles drive vascular lumen formation in vivo. We are currently examining the formation and maintenance of vascular junctions, which are important in stroke. To begin dissecting the molecular regulatory mechanisms controlling vascular morphogenesis and the maintenance of vascular integrity, we are studying a variety of genes required for vascular morphogenesis and vascular integrity, including the *pak2a* and *rap1b* genes, about which we recently reported. We are also developing transgenic lines that permit us to visualize the dynamics of endothelial cell-cell junctions and intracellular cytoskeletal structures in order to examine their role in the cellular rearrangements that occur during vascular sprouting and growth and vascular tube formation.

Buchner DA, Su F, Yamaoka JS, Kamei M, Shavit JA, McGee B, Hanosh AW, Kim S, Jagadeeswaran P, Weinstein BM, Ginsburg D, Lyons SE. *pak2a* mutations cause cerebral hemorrhage in *redhead* zebrafish. *Proc Natl Acad Sci USA* 2007;104:13996-14001.

Gore AV, Lampugnani MG, Dye L, Dejana E, Weinstein BM. Combinatorial interaction between CCM pathway genes precipitates hemorrhagic stroke. *Dis Model Mech* 2008;1:275-281.

Analysis of vascular patterning

Cha, Fujita, Isogai, McKinney, Swift; in collaboration with Childs, Epstein, Fishman, Lawson, Stemple

We have used multiphoton time-lapse imaging to characterize patterns of vessel assembly throughout the developing zebrafish. Ongoing studies in the laboratory aim to understand how the patterns arise and what cues guide vascular network assembly during development. We previously demonstrated that known neuronal guidance factors play an important, previously unknown role in vascular guidance and vascular patterning, showing that Semaphorin signaling is an essential determinant of trunk vessel patterning. Current studies are further elucidating our understanding of the role of additional factors that guide the patterning of developing vascular networks in vivo, both in the trunk and in vascular beds in the eye, aortic arches, hindbrain, and other anatomical locales.

Alvarez Y, Cederlund ML, Cottell DC, Bill B, Ekker SC, Torres-Vazquez J, Weinstein BM, Hyde DR, Vihtelic T, Kennedy B. Genetic determinants of hyaloid and retinal vasculature in zebrafish. *BMC Dev Biol* 2007;7:114-131.

Anderson MJ, Pham VN, Vogel AM, Weinstein BM, Roman BL. Loss of *unc45a* precipitates arteriovenous shunting in the aortic arches. *Dev Biol* 2008;318:258-267.

Analysis of lymphatic development

Cha, Isogai, Yaniv; in collaboration with Dye

The lymphatic system has become the subject of great interest in recent years because of its important role in normal and pathological processes, but progress in understanding the origins and early development of the system has been hampered by difficulties in observing lymphatic cells in vivo and performing defined genetic and experimental manipulation of the lymphatic system in currently available model organisms. Recently, we demonstrated for the first time that the zebrafish possesses a lymphatic system that shares many of the morphological, molecular, and functional characteristics of the lymphatic vessels found in other vertebrates, providing a powerful new model for imaging and studying lymphatic development. As we continue to examine the origins and assembly of the lymphatic system of the zebrafish, we are developing new transgenic tools for imaging the development of the lymphatic system and for forward-genetic screening for lymphatic mutants. Our genetic analysis has already identified several novel genes involved in lymphatic development. We are also studying the functional role of several previously identified genes implicated in lymphatic development. Our ongoing studies will provide new insights into the molecular regulation of lymphatic development.

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Additional Publications

Ungos JM, Weinstein BM. Vascular development in the zebrafish. In: Bodmer R, ed. *Cardiovascular Development*. Elsevier Press, 2007;301-332.

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[1]Arrived during 2008.
[2]E26 transformation-specific
[3]Vascular endothelial growth factor

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MECHANISMS FOR CONTROLLING GENE EXPRESSION IN BACTERIA AND BACTERIOPHAGE

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Temperate bacteriophages can establish lysogeny, a long-term and mutually beneficial association between phage and bacterial host. Lysogeny is a major mechanism of horizontal gene transfer between bacterial families, and temperate bacteriophages frequently contain genes that allow bacteria both to survive environmental challenges and to cause disease in mammalian hosts. To maintain lysogeny, temperate phages control the expression of their own genes so that host viability is unimpaired. Many phages control gene expression by regulating the elongation step of transcription, which is catalyzed by DNA-dependent RNA polymerase (RNAP). After initiating polymerization on a DNA template, the transcript elongates until RNAP reaches a terminator, where the enzyme, template, and transcript (the elongation complex or EC) dissociate from each other. Anti-terminators can control the efficiency of termination and hence the expression of downstream genes. The molecular mechanisms of termination and anti-termination are poorly understood. We aim to understand these mechanisms by studying transcription directed by an *Escherichia coli* phage, HK022, which uses a robust yet relatively simple anti-termination pathway to control gene expression. We recently expanded our studies to transcriptional regulatory mechanisms in a different temperate phage, B40-8, which parasitizes *Bacteroides fragilis*, a very distantly related bacterium.

Inhibition of a transcriptional pause by RNA anchoring to RNA polymerase
Komissarova, Velikodvorskaya, Sen, King

HK022 uses a novel, RNA-based anti-termination mechanism to express many essential genes. A nascent transcript of a viral sequence called *put* binds to the enzyme that catalyzed *put*'s synthesis and remains associated with the EC as the transcript continues to elongate. *put* RNA modifies the EC to which it is bound so that transcription no longer stops at terminators. No other protein factor is absolutely required, other ECs in the same cell are unaffected, and no obvious terminator specificity is involved. The apparent simplicity of this anti-termination pathway makes it an attractive target for deeper analysis. Modification of the EC by *put* RNA also suppresses transcriptional pausing at a U-rich sequence located very close to the *put* site. ECs have a high probability of pausing for variable amounts of time at particular template positions, and such pauses can have important physiological functions. Moreover, pausing is thought to precede termination, and intrinsic terminators contain U tracts at the termination point. Therefore, we decided to investigate the mechanism of *put*-mediated anti-pausing at this site.

We showed that the U-rich sequence promotes "backtracking" of the EC. Backtracking is a retrograde movement during which the nucleotides at the 3′ end of the transcript are melted from the template DNA strand and extruded from RNAP while compensating amounts of upstream DNA and nascent RNA re-enter the EC. The length of the RNA:DNA hybrid remains constant. Backtracking is favored by the replacement of thermodynamically weak with thermodynamically strong RNA:DNA hybrid (e.g., rU:dA with rC:dG). In vitro transcription and footprinting assays revealed that *put* RNA suppresses the U-rich pause by restricting re-entry of the nascent transcript into the RNA exit channel, thus limiting backtracking. The restriction is local and relaxes as the transcript elongates. The number of nucleotides over which the restriction operates allows us to use RNA as a "molecular tape measure" to locate the *put* binding site. Our results indicate that *put* RNA binds to the surface of polymerase within 10 to 28 Å of the end of the RNA exit channel, a region that includes amino acid residues known to be important for anti-termination and RNA binding. Even though binding is essential for anti-pausing and anti-termination, these two activities of *put* differ; anti-pausing is limited to the immediate vicinity of the *put* site, but anti-termination is not. We know that *put* RNA remains stably bound as the EC translocates and presumably ensures the persistence of anti-termination. While the mechanism of anti-termination remains unknown, our current work argues against the possibility that *put* acts by strengthening thermodynamically weak RNA:DNA hybrid.

Transcriptional pausing is an important component of many regulatory networks, and our knowledge of the networks is growing. For example, in a large number of *Drosophila* embryo genes, ECs stall near the transcription start site, possibly waiting for activation at later developmental stages. Backtracking is a mechanism that accounts for many known pauses, and RNA anchoring to RNAP is potentially a general mechanism that regulates backtracking-associated pauses. Moreover, given that the effect of anchoring is local, individual pauses can be targeted. Examination of the properties of several well-studied pause sites leads us to propose that RNA anchoring to RNAP is a widespread mechanism of pause regulation.

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Sloan S, Rutkai E, King RA, Velikodvorskaya T, Weisberg RA. Protection of antiterminator RNA by the transcript elongation complex. *Mol Microbiol* 2007;63:1197-1208.

Analysis of a bacteriophage that parasitizes a commensal anaerobe

Read

We have sequenced and annotated the genome of bacteriophage B40-8, whose host is *Bacteroides fragilis*, a human commensal anaerobe that is frequently pathogenic. B40-8, a member of the *Siphoviridae*, has a circular double-stranded DNA genome of 45,805 basepairs. The genome encodes 64 real or potential proteins, of which only 18 can be assigned functions based on homology, structural predictions, or N-terminal sequencing of capsid proteins. The predicted open reading frames are oriented in the same direction and generally conform to the temporal cassette organization of terminase-head-tail-lysis, except that the major tail gene is located after the terminase and before the head genes. The distribution of numerous predicted promoters, terminators, and conserved sequence motifs within the intergenic regions suggests a complex regulatory network of several overlapping transcripts similar to that of *Streptomyces* phage phiC31. A paucity of discernible Shine-Dalgarno sequences and the presence of a conserved $YA_{3\text{-}7}Y$ element in their place suggest a different mechanism of translational initiation than that usually found in prokaryotes. The presence of submolar restriction fragments of the virus chromosome, alignment of other terminase large-subunit coding sequences with CDS2, and other evidence strongly support a processive headful DNA-packaging mechanism. Sequencing of the chromosomal ends places the first cut at basepair 12013 and the second cut between bases 17491 and 17753, generating a first headful of approximately

51,400 base pairs and 10.9 percent terminal redundancy. Thus, B40-8 packaging is distinct from that of other well-studied phages; the first cut site is not within but rather almost 10 kb downstream of the putative terminase genes. PCR analysis of purified phage demonstrates that host DNA is also packaged into capsids, and sequencing of genomic clones suggests that about 1 percent of the phage particles contain host DNA.

Action of a sequence-specific transcript termination factor

Sloan, Gottesman

The Nun protein of phage HK022 protects HK022 lysogens against superinfection by phage lambda. Nun binds to and terminates ECs that transcribe lambda DNA, and nascent transcripts of the lambda *nutL* and *nutR* sites promote binding. Nun recognizes these transcripts with high specificity, effectively limiting termination to the lambda transcripts. In vitro, Nun arrests transcript elongation but does not by itself dissociate the arrested ECs. However, in vivo, Nun dissociates ECs that have transcribed the *nutR* site, presumably with the help of cellular termination factors such as Mfd and/or Rho, which are known to act on arrested ECs.

Our laboratory is identifying the requirements for Nun-dependent dissociation of the EC in vivo by using Northern blots to measure the abundance and stability of Nun-terminated transcripts containing the *nutL* site and initiated at the lambda P_L promoter. We reasoned that dissociation of the EC should allow cellular 3′–5′ exonucleases, the major catalysts of mRNA degradation in *E. coli*, access to the 3′ end of the terminated transcript. By contrast, simple arrest leads to a transcript with a protected 3′ end (it is inside the EC), likely increasing transcript stability. In addition, the arrested EC will sterically block transcription by ECs that follow, which should back up on the template and eventually occlude the promoter ("constipation"). Therefore, the factors required for dissociation of arrested ECs are likely to reduce the stability of Nun-terminated transcripts while increasing their rate of synthesis. We are currently determining the effects of inactivating Mfd, Rho, and cellular 3′–5′ exonucleases on the abundance and stability of such transcripts. Given that other work has suggested that dissociation is favored with increased distance, we will determine the effect of changing the distance between the promoter and the *nutL* site.

Our initial experiments show that, in cells with the wild-type P_L–*nutL* distance (32 bp), Nun increases the half-life of P_L mRNA more than 5-fold while decreasing its abundance about 2-fold. As expected, transcripts produced in the presence of Nun have 3′ ends that are dispersed over about 100 nt downstream of *nutL* while transcripts produced in its absence are considerably longer. This result argues that undissociated, Nun-arrested ECs protect at least some transcripts from degradation and that "constipation" caused by Nun-mediated arrest reduces promoter activity. Inactivation of Mfd increases transcript stability about another 5-fold in the presence of Nun, but has no effect in its absence. Therefore, Mfd appears to promote dissociation of a fraction of the arrested ECs. We suggest that Mfd dissociates only those ECs that have lost the initiation factor sigma when arrest occurs. Work from other laboratories suggests that sigma release occurs stochastically after an EC leaves the promoter and that sigma-containing ECs are resistant to the action of Mfd.

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GENES THAT CONTROL MOUSE DEVELOPMENT

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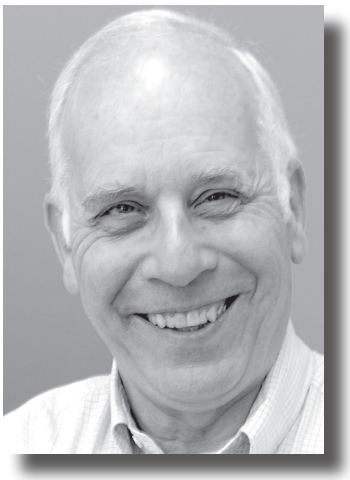

Our group uses a loss-of-function approach to study fundamental controls exerted by transcriptional regulators of the LIM-homeodomain (LIM-HD) proteins and associated co-factors. Oligomeric complexes involving individual members of the LIM-HD, Ldb, and Ssdp families together with additional transcriptional regulators regulate transcription of target genes, thereby allowing for a multifaceted temporal and spatial control of gene activity. Over the years, we have gained insights into the individual or combined action of individual LIM-HD factors in setting the premises for tissue patterning and organ formation in the developing mouse embryo. We have continued our work with a detailed analysis of LIM-HD involvement in the development of the mouse embryo. A novel aspect of our work concerns the role of the LIM-HD transcriptional machinery in embryonic stem (ES) cell activation, in the reprogramming of somatic cells to an induced pluripotent stem (iPS) cell state, and in the maintenance and activation of stem cells in the adult organism.

Functions of the LIM-homeobox genes *Lhx6* and *Lhx8*, and their transcriptional co-regulator *Ldb1* in mouse brain development

De la Cuesta,[1] Westphal, Zhao; in collaboration with Rubenstein

Lhx6 and *Lhx8* share high sequence homology and overlapping expression patterns in the developing mouse ventral telencephalon. Our previous analysis of the *Lhx8* knockout mutant revealed a defect in the generation of several major groups of cholinergic neurons in the telencephalon. Subsequently, our analysis of the *Lhx6* mutant showed that this gene is required for the differentiation of cortical and hippocampal GABA-ergic interneurons and their migration from the medial ganglionic eminence (MGE) to their ultimate positions in the neocortex and hippocampus. Given that the structures MGE and globus pallidus—the latter is a major component of the basal ganglia derived from the MGE—show no noticeable defects in single *Lhx6* or *Lhx8* mutants, the two genes may conceivably share redundant functions in the development of these two structures. To address this issue, we generated and analyzed mutants lacking the function of both *Lhx6* and *Lhx8*. For our initial analysis of the E18.5 double mutants, we used Er81, a marker specific for the developing MGE and globus pallidus, as well as the *PLAP* reporter gene, which expresses placental alkaline phosphatase and is present in the *Lhx6* mutant locus. We are now examining cell proliferation and apoptosis over a broader developmental time course in an effort to study details of the control exerted by *Lhx6* and *Lhx8* on interneuron patterning in the ventral telencephalon.

In a previously published experiment, we demonstrated that a conditional inactivation of *Ldb1* leads to defects in development of the cerebellum similar to those observed in the *Lhx1/Lhx5* double mutant. The experiment also identified Ldb1 as an obligatory co-factor of *Lhx* genes in the development of the cerebellum. To show that the function of the transcriptional regulators encoded by *Lhx6* and *Lhx8* mutants is equally dependent on Ldb1, we crossed the *Ldb1*-floxed mice with a transgenic line that expresses Cre recombinase under the control of regulatory elements of the *Nkx2.1* gene. *Nkx2.1* acts as an upstream regulator for both *Lhx6* and *Lhx8* in the ventral telencephalon. The *Ldb1/Nkx2.1-Cre* conditional mutants showed a loss of Ldb1 immunostaining in the GE. We are analyzing the mutants by using the various markers for the MGE and for cholinergic and GABAergic neurons, similar to those used in the analysis of *Lhx6* and *Lhx8* mutants.

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Reprogramming of somatic cells to a pluripotent state

Davidhi,[2] Tzchori, Westphal; in collaboration with Casellas, Meshorer

Our recent experiments have shown that somatic nuclei can be reprogrammed to a pluripotent state when fused with ES cells, giving rise to pluripotent hybrids. However, the resulting fusion and reprogramming efficiencies have thus far been particularly low. We examined the ability of undifferentiated ES cell lines to reprogram the nuclei of murine embryonic fibroblasts (MEF) through the cell-cell fusion method. Activated *baculovirus* induced fusion events in 70 to 85 percent of the cells, causing efficient reprogramming. The resulting ES/MEF mouse hybrids, although nearly tetraploid, exhibited characteristics of normal ES cells. We further showed by reverse transcriptase-PCR (RT-PCR) that the MEF/ES hybrids express ES markers while losing MEF markers. Comparing the potency of four ES cell lines, we found that the E14 line was significantly less potent than the R1, J1, and C57BL/6 lines in its ability to reprogram MEFs. This low reprogramming potency correlated with reduced H3 lysine-9 acetylation (H3K9ac) levels. Treatment of E14 cells with the histone deacetylase (HDAC) inhibitors trichostatin A (TSA) and sodium butyrate resulted in a statistically significant increase of H3K9 acetylation levels and raised the cells' reprogramming capacity to a level observed in the R1 ES cell line. Furthermore, we found that induced pluripotent stem (iPS) cells can also reprogram MEFs, albeit at low efficiency. However, addition of TSA did not increase the number of reprogrammed colonies. In line with these findings, we observed that iPS incubation with TSA did not increase the acetylation levels of the resulting hybrids. *Baculovirus* induction can substantially enhance fusion and subsequent reprogramming of somatic cells while the acetylation level of pluripotent stem cells directly correlates with their reprogramming efficiency.

Embryonic and adult stem cells

Dey-Guha, Geum,[3] Gorivodsky,[4] Makarev, Mukhopadhyay,[5] Narkis, Westphal; in collaboration with Niehrs, Tam

Lhx and *Ldb* genes are known to play important roles in stem cell maintenance and differentiation. ES cells are well suited for investigating the targets of *Ldb1*-mediated transcriptional events because their differentiation under controlled in vitro conditions is clearly established. In this scenario, ES cells give rise to a well-documented arsenal of transcripts, including all known *Lh*x transcription factors. To determine specific targets of *Ldb*-mediated transcription events, we have implemented neuronal differentiation protocols. We work with ES cells that contain a null deletion of the *Ldb2* gene and a floxed *Ldb1* gene; the cells are comparable to wild-type ES cells, as mice corresponding to this genotype are fully viable. Cre-mediated conditional ablation of *Ldb1* in the mutant ES cells enables us to study the cells' transcriptional profiles and differentiation potentials in the presence or absence of Ldb1 activity. We are establishing the validity of the approach by using RT-PCR or qRT-PCR to compare predicted marker gene expression, such as the standard neuronal markers

and *GATA* and *Lhx* genes. Subsequently, we will use whole-genome expression analysis to identify batteries of downstream targets that are positively or negatively regulated by *Ldb1*. Furthermore, using an Ldb1 antibody, we will perform chromatin immunoprecipitation assays to identify regulatory elements in select candidate genes.

To determine the role of the *Ldb1* gene in stem cell niches in adult tissues, we used our floxed mutant allele to interfere with Ldb1-mediated transcriptional activity in the small intestine and skin. Using tissue-specific differentiation markers and a set of tools that allow us to measure cell proliferation and cell apoptosis, we noticed that *Ldb1* ablation had a profound effect on the maintenance of stem cell niches of the gut and skin. We are investigating the mechanism by which the Ldb-mediated transcriptional apparatus controls stem cell maintenance in these tissues, with special emphasis on *Dkk* and *Kremen* genes and their function in regulating Wnt activity in the stem cell niches.

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TRANSLATIONAL RESEARCH ON INHERITED PEDIATRIC DISEASES

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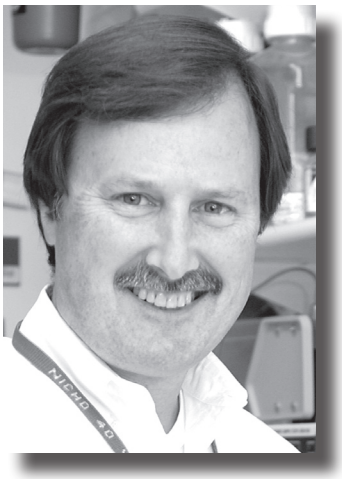

We research problems for which we believe patient-oriented studies can provide a springboard to advancing understanding in a broader area and for which novel treatment approaches are needed. Our overarching goal is to improve the understanding, diagnosis, and treatment of inherited pediatric diseases. We focus heavily on Menkes disease and occipital horn syndrome, allelic X-linked recessive disorders of copper transport, and maintain interest in two other areas: (1) platelet biology and hemostasis and (2) the clinical and genetic delineation of PHACES, a syndrome of midline developmental defects with strong female predilection. All three projects are associated with clinical conditions that affect infants and children and for which clinical, biochemical, or molecular knowledge is incomplete.

Disorders of copper transport

Desai, Donsante, Tang, Kaler; in collaboration with Goldstein, Holmes, Liew, Patronas, Sato, Steinbach

Menkes disease is an X-linked recessive disorder of copper transport caused by defects in a gene that encodes an evolutionarily conserved copper-transporting ATPase. In mammals, this gene product functions as an intracellular pump to transport copper into trans-Golgi spaces for incorporation into copper-requiring enzymes; it also mediates copper exodus from cells. The disorder presents in infancy with delayed development, failure to thrive, neurodegeneration, and premature death (typically by age 3). Our work on this disorder includes development of rapid and reliable neurochemical and molecular techniques for very early diagnosis, efforts that dovetail with a clinical trial of very early copper histidine treatment for affected infants. We use cell-biological, molecular, and biochemical approaches to characterize enrolled patients and to correlate the results with neurodevelopmental outcomes. We assess quantity and localization of mutant Menkes gene products with confocal imaging of patient fibroblasts. The blood-brain barrier poses a challenging treatment obstacle in many Menkes disease patients, and we hypothesized a molecular basis for treatment responsivity in the minority of patients (about one in five) who respond successfully (normal neurodevelopmental outcomes) to early copper histidine. These patients have mutations that permit at least some residual copper transport to the developing brain. Consequently, we are developing alternative therapeutic approaches, including gene therapy, that bypass the blood-brain barrier.

As above, although mean survival is significantly enhanced, only about 30 percent of patients with Menkes disease show good or excellent neurological outcomes when treated with copper injections beginning very early in life. The main reason for the disparate outcomes appears to be the amount of residual Atp7a function in these individuals. Those with less Atp7a function are unlikely to respond optimally to copper injection treatment. Therefore, new therapeutic strategies are need for the large percentage of patients who have little or no residual Atp7a function and currently have no ideal treatment options.

In a murine model of classical Menkes disease (*Atp7a$^{Mo\text{-}br}$*), which cannot be rescued by intraperitoneal or intravenous copper, we are studying two brain-directed therapies: (1) early intracerebroventricular (ICV) injection of copper chloride and (2) early ICV injection of a recombinant adeno-associated virus serotype 5 (AAV5) vector expressing *rsATP7A-AAV*, a reduced-size human *ATP7A*. We developed a genotyping assay that identifies mutant pups before clinical signs appear and administered 50 ng copper chloride to affected mice by ICV injection within three days of birth. We extrapolated the dose from a maximum tolerated dose previously determined in adult rats (Lem et al., *Molec Genet Metab* 2007;91:30). Treatment modestly extended survival in mutant males and increased mean brain cortex copper levels.

In an alternative approach, we are studying the expression and effects of *rsATP7A-AAV* delivered by ICV injection. The transgene product begins at methionine 461 of the Menkes ATP7A and includes the fifth and sixth copper-binding domains. In mice receiving 5×10^8 particles of vector, we documented preliminary evidence of improved brain catechol ratios, reflecting enhanced activity of dopamine-beta-hydroxylase, a copper-dependent enzyme.

Our preliminary data suggest that intracerebroventricular delivery of copper and *rsATP7A-AAV* results in distribution throughout the *Atp7a$^{Mo\text{-}br}$* brain. Whereas both treatments appeared to improve catecholamine biosynthesis in mutant brains, the effect of copper was more dramatic. Therefore, we may need to refine doses or employ combination therapy in our mouse model to optimize biochemical and clinical outcomes. Brain-directed therapies may ultimately provide a useful approach for treatment of Menkes patients with mutations that cause severe phenotypes.

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Hemostasis mediated by the platelet glycoprotein (GP)Ib alpha-Ib beta-IX complex

Kaler, Tang, Donsante; in collaboration with Steinbach

The platelet membrane glycoprotein (GP)Ib-V-IX complex is the receptor for von Willebrand factor and is composed of four polypeptides: GPIb alpha, GPIb beta, GPIX, and GPV, which all feature leucine-rich repeat motifs. A qualitative or quantitative deficiency in this complex causes the rare human bleeding diathesis Bernard-Soulier syndrome (BSS). BSS is an autosomal recessive trait presenting in infancy with thrombocytopenia, circulating "giant" platelets, and bleeding tendency. Bleeding in BSS is more severe than predicted by platelet count and is explained by a defect in primary hemostasis. We identified a novel mutation (P96S) at the GPIb beta locus in an infant haploinsufficient for this gene, with the haploinsufficiency attributable to heterozygous deletion of

chromosome 22q11 (velocardiofacial syndrome). Using flow cytometry and confocal imaging of transfected Chinese hamster ovary cells that stably surface-express human GPIb alpha and GPIX (CHO alpha-IX) when transfected with wild-type GPIb beta, we demonstrated that P96S GPIb beta abrogates surface assembly of the platelet vWF receptor complex. Based on amino acid homology to the nogo-66 neuronal receptor (also a leucine-rich repeat protein, whose crystal structure has been characterized), we proposed a model of GPIb beta protein structure that both supports the importance of P96 and other residues previously reported as mis-sense mutations in the conformation of GPIb beta and demonstrates P96's interaction with GPIX. GPIb beta represents the most crucial component of this recently characterized platelet adhesion complex. Further study of GPIb beta and its critical role in platelet adhesion and hemostasis is in progress to illuminate more precisely GPIb beta's interaction with GPIX and GPIb alpha and to develop novel therapeutic approaches for BSS patients. Our current efforts involve expression of a FLAG-tagged GPIb beta cDNA in HEK-293 cells in order to purify the native protein for crystallographic analysis and generate specific antibodies against it for future studies of the (GP)Ib-V-IX complex.

Clinical and molecular characterization of PHACES syndrome
Kaler

The acronym PHACES describes the association of posterior fossa malformations, hemangiomas, arterial anomalies (cardiovascular or cerebrovascular), coarctation of the aorta and cardiac defects, eye abnormalities, and sternal or ventral defects. We studied a female patient with an uncommon variant of this neurocutaneous disorder who manifested micrognathia; hemangiomas of the face, chest, and extremities; cerebrovascular anomalies; supra-umbilical raphe; and sternal cleft. A literature review of PHACES patients with both supra-umbilical raphe and sternal cleft revealed a marked female predilection. Taken together with cases of facial hemangiomas, supra-umbilical raphe, and sternal cleft reported in the literature, 91 percent (40/44) of patients are female. One affected male died shortly after birth. We hypothesized that the gender bias in PHACES results from mutation in an X-linked dominant gene that is often lethal in males; we performed X-inactivation analysis of the polymorphic androgen receptor locus in the family of the affected infant. We documented consistently skewed X-inactivation (80 percent/20 percent in two independent analyses) in the unaffected mother and consistently random X-inactivation (47:53 and 61:39 in independent analyses) in the proband. Our findings are consistent with favorably skewed X-inactivation producing a normal maternal phenotype, a phenomenon documented in X-linked dominant Rett syndrome. In searching for submicroscopic copy number changes in PHACES patients, our future efforts will depend on (1) ascertainment of other PHACES families in which maternal X-inactivation studies may be pursued and (2) application of X-chromosome–specific array-comparative genomic hybridization (array-CGH) experiments.

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REGULATION OF INTRACELLULAR IRON METABOLISM

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We aim to understand how mammals regulate iron metabolism. Iron-regulatory proteins 1 and 2 (IRP1 and IRP2) regulate expression of numerous proteins of iron metabolism. In iron-depleted cells, the proteins bind to RNA stem-loops in transcripts known as iron-responsive elements (IRE). IRP binding stabilizes the mRNA that encodes the transferrin receptor and represses the translation of transcripts that contain IREs near the 5′ end of ferritin H and L chains. IRP1 is an iron-sulfur protein that functions as an aconitase in iron-replete cells. IRP2 is homologous to IRP1 but undergoes iron-dependent degradation in iron-replete cells. In mouse models, loss of IRP2 results in mild anemia, erythropoietic protoporphyria, and adult-onset neurodegeneration—all the likely the result of functional iron deficiency. Biochemically and with expression arrays, we study the mechanisms that lead to anemia and neurodegeneration in IRP2$^{-/-}$ mice and are using our mouse model of neurodegeneration to identify compounds that can prevent neurodegeneration; we found that the antioxidant tempol works by activating the latent IRE-binding activity of IRP1. We are evaluating the possibility that loss of IRP2 in humans may cause mild refractory anemia and adult-onset neurodegeneration characterized by limb weakness, which might be diagnosed as amyotrophic lateral sclerosis in humans.

The molecular basis for regulation of intracellular iron metabolism in mammals
Rouault, Cooperman, Ghosh, Jeong, Ollivierre-Wilson, Singh, Zhang

In previous years, our laboratory identified and characterized the *cis* and *trans* elements mediating the iron-dependent alterations in the abundance of ferritin and the transferrin receptor. IREs are RNA stem-loops found in the 5′ end of ferritin mRNA and the 3′ end of transferrin receptor mRNA. We have cloned, expressed, and characterized two essential iron-sensing proteins, IRP1 and IRP2. IRPs bind to IREs when iron levels are depleted, resulting in either (1) inhibition of translation of ferritin mRNA and other transcripts that contain an IRE in the 5′-untranslated regions (UTR) or (2) stabilization of the transferrin receptor mRNA and possibly other transcripts that contain IREs in the 3′ UTR. The IRE-binding activity of IRP1 depends on the presence of an iron-sulfur cluster (see "Mammalian iron-sulfur cluster biogenesis" below). IRP2 also binds to IREs in iron-depleted cells, but, unlike

IRP1, is degraded in iron-replete cells. Experimental evidence indicates that IRP2 binds to iron and undergoes iron-catalyzed oxidation. In iron-replete cells, IRP2 is selectively ubiquitinated and then degraded by the proteasome. To approach questions about the physiology of iron metabolism, we generated loss-of-function mutations of IRP1 and IRP2 in mice through homologous recombination in embryonic cell lines. In the absence of provocative stimuli, we observed no abnormalities in iron metabolism associated with loss of IRP1 function. IRP2$^{-/-}$ mice develop a progressive neurologic syndrome characterized by gait abnormalities and axonal degeneration. Ferritin overexpression occurs in affected neurons and in protrusions of oligodendrocytes into the space created by axonal degeneration. IRP2$^{-/-}$ animals develop iron-insufficiency anemia and erythropoietic protoporphyria. In animals that lack IRP1, IRP 2 compensates for loss of IRP1's regulatory activity. Animals that lack both IRP1 and IRP2 die as early embryos. The adult-onset neurodegeneration of adult IRP2$^{-/-}$ mice is exacerbated when one copy of IRP1 is also deleted. IRP2$^{-/-}$ mice offer a unique example of spontaneous adult-onset, slowly progressive neurodegeneration; analyses of gene expression and iron status at various stages of disease are ongoing. At the same time, small-molecule treatments to prevent neurodegeneration have yielded promising results.

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Mammalian iron-sulfur cluster biogenesis

Rouault, Li, Shi, Tong, Uhrigshardt, Ye, Ha, Patel; in collaboration Camaschella, Fischbeck, Haller, Holm, Huynh, Singleton

Our goals in studying mammalian iron-sulfur biogenesis are to understand how iron-sulfur prosthetic groups are assembled and delivered to target proteins in the various compartments of mammalian cells, including mitochondria, cytosol, and nucleus. In addition, we seek to understand the role of iron-sulfur cluster assembly in the regulation of mitochondrial iron homeostasis and the pathogenesis of diseases such as Friedreich's ataxia and sideroblastic anemia, which are both characterized by incorrect regulation of mitochondrial iron homeostasis.

IRP1 is an iron-sulfur protein related to mitochondrial aconitase, which is a citric acid–cycle enzyme that functions as a cytosolic aconitase in iron-replete cells. Regulation of RNA-binding activity of IRP1 involves a transition from a form of IRP1 in which a 4Fe-4S cluster is bound to a form that loses both iron and aconitase activity. The 4Fe-4S–containing protein does not bind to IREs. Controlled degradation of the iron-sulfur cluster and mutagenesis reveal that the physiologically relevant form of the RNA-binding protein in iron-depleted cells is an apoprotein. The status of the cluster appears to determine whether IRP1 will bind to RNA. Recently, we identified mammalian enzymes of iron-sulfur cluster assembly that are homologous to those encoded by the *NifS*, *ISCU*, and *NifU* genes implicated in bacterial iron-sulfur cluster assembly and showed that the gene products facilitate assembly of the iron-sulfur cluster of IRP1.

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MECHANISMS OF DISEASE IN PRETERM LABOR AND COMPLICATIONS OF PREMATURITY; PRENATAL DIAGNOSIS OF CONGENITAL ANOMALIES

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The current taxonomy of disease in obstetrics is largely based on the clinical presentation of the mother rather than on the mechanism of disease responsible for clinical manifestations. Thus, the term "preterm labor" does not convey whether the condition is caused by infection, vascular insult, uterine overdistension, stress, or some other pathologic process. The same applies to other pregnancy complications such as preeclampsia, small for gestational age, and fetal death. We proposed that obstetrical disorders responsible for maternal death and perinatal morbidity and mortality are syndromes (hence, the designation the Great Obstetrical Syndromes) whose key features are (1) multiple etiology, (2) long preclinical stage, (3) frequent fetal involvement, (4) clinical manifestation that is often adaptive in nature, and (5) predisposition to a particular syndrome influenced by gene-environment interaction. Congenital anomalies continue to be a leading cause of perinatal mortality in the United States, many of which we can now detect prenatally with ultrasound. We use three- and four-dimensional ultrasound and develop novel approaches and algorithms aimed at improving the prenatal diagnosis of congenital anomalies, particularly congenital heart disease. We also combine sonographic and biochemical parameters to identify patients at increased risk of preeclampsia.

Proteomic analysis of amniotic fluid to identify women with preterm labor and intra-amniotic inflammation/infection: novel computational method to analyze mass-spectrometric profiling

Romero, Gotsch, Edwin; in collaboration with Espinoza, Rogers, Moser, Nien, Kusanovic, Erez, Gomez, Hassan

High-dimensional biology techniques are expected to improve our understanding of the physiology or mechanisms of disease of various biological processes and to help develop new diagnostic, prognostic, and therapeutic tools in medicine. These new techniques include genomics, transcriptomics, proteomics, and metabolomics, which allow simultaneous examination of changes in the genome (DNA), transcriptome (mRNA), proteome (proteins), or metabolome (metabolites). Examination of the amniotic fluid proteome has been used to identify biomarkers for intra-amniotic inflammation and those that may be useful in predicting the outcome of patients with spontaneous preterm labor. In a cross-sectional study, we combined a novel computational method of pattern

discovery with mass-spectrometric proteomic profiling of amniotic fluid to identify biomarkers of intra-amniotic infection/inflammation (IAI) in patients with spontaneous preterm labor and intact membranes who delivered at term and patients who delivered preterm with IAI. We conducted the proteomic profiling through several simultaneous surface-enhanced laser desorption/ionization (SELDI) mass-spectrometry conditions, which we combined into a single proteomic fingerprint. We used a novel computational approach to reduce the complexity of the data and identify individual features as well as patterns related to the clinical phenotype. At an overall accuracy of 90 percent (28/31), 39 unique mass-spectrometric peaks discriminated between patients with preterm labor/delivery with IAI and patients with preterm labor and term delivery. We have thus demonstrated that proteomic analysis of amniotic fluid allowed the identification of mass-spectrometry features that can distinguish patients with preterm labor with IAI from patients with preterm labor without inflammation or infection who subsequently delivered at term.

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Detection of a microbial biofilm in intra-amniotic infection

Romero, Gotsch; in collaboration with Kusanovic, Erez, Kim CJ, Espinoza, Gonçalves, Vaisbuch, Mazaki-Tovi, Hassan, Costerton

We previously described the sonographic finding of dense aggregates of particulate matter in the amniotic fluid close to the uterine cervix of (1) women with an episode of preterm labor and (2) asymptomatic patients at risk for spontaneous preterm delivery. We proposed the term amniotic fluid "sludge" to refer to this sonographic finding and provided evidence that sludge is an independent risk factor for impending and early preterm delivery, preterm prelabor rupture of membranes (PROM), histologic chorioamnionitis, and microbial invasion of the amniotic cavity. Until now, intra-amniotic infection has been attributed to planktonic (free-floating) bacteria. The biofilm theory, however, states that most bacteria growing in exopolymeric matrix–enclosed biofilms differ from their planktonic counterparts. Microbial biofilms, which are communities of sessile microorganisms formed by cells irreversibly attached to a substratum or interface or to each other and embedded in a hydrated matrix of extracellular polymeric substances, have been implicated in over 80 percent of human infections, such as periodontitis, urethritis, endocarditis, and device-associated infections. To determine the nature of amniotic fluid sludge, we have been collecting the material by transvaginal needle amniotomy under ultrasound guidance in patients with imminent preterm delivery. Amniotic fluid sludge is macroscopically similar to pus and is associated with a markedly elevated white blood cell count in amniotic fluid as well as with positive Gram stain and a culture positive for microorganisms. In a case of spontaneous preterm labor and clinical chorioamnionitis at 28 weeks' gestation, we retrieved amniotic fluid sludge and identified bacteria with scanning electron microscopy and fluorescence in situ hybridization for conserved regions of the microbial genome; we identified the exopolymeric matrix histochemically with the wheat germ agglutinin lectin method. We imaged amniotic fluid sludge with confocal laser scanning microscopy and scanning electron microscopy, which allowed the identification of bacteria embedded in an amorphous material and inflammatory cells. Our work represents the first report of a microbial biofilm in the amniotic cavity. The recognition that bacteria can form biofilms within the amniotic cavity is important because the diagnosis of microbial invasion in the presence of biofilms is extremely difficult, and current cultivation techniques to detect such infections are limited. Thus, the frequency of intra-amniotic infection may be underestimated, and molecular microbiologic techniques will be necessary to improve diagnosis. Moreover, bacteria within biofilms exhibit increased resistance to antimicrobial agents. Thus, the difficulties in the treatment of intra-amniotic infection may be attributable to the refractoriness of biofilms to conventional antibiotic treatment.

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Microbial prevalence, diversity, and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation

Romero, Gotsch, Edwin; in collaboration with DiGiulio, Kusanovic, Kim CJ, Erez, Relman

Intra-amniotic infection is a common and important mechanism of disease in obstetrics and has been identified in the amniotic fluid of asymptomatic women in the mid-trimester of pregnancy as well as in patients with preterm labor with intact membranes, preterm PROM, cervical insufficiency, idiopathic vaginal bleeding, premature rupture of membranes at term, spontaneous labor at term with intact membranes, and clinical chorioamnionitis, in which unrecognized intra-amniotic infections caused by cultivation-resistant microbes may play a role. Molecular methods can detect, characterize, and quantify microbes independently of traditional culture techniques. Our group, in collaboration with investigators at Stanford University, used broad-range end-point and real-time PCR assays to amplify, identify, and quantify ribosomal DNA (rDNA) of bacteria, fungi, and archaea from the amniotic fluid of 166 women in preterm labor with intact membranes. We sought correlations among microbial prevalence, diversity, and abundance on the one hand and host inflammation and gestational and neonatal outcomes on the other. The combined use of molecular and culture methods revealed a greater prevalence (15 percent of subjects) and diversity (18 taxa) of microbes in amniotic fluid than did culture alone (10 percent of subjects; 11 taxa). A positive PCR was associated with histologic chorioamnionitis and funisitis, and the positive predictive value of PCR for preterm delivery was 100 percent. A temporal association between a positive PCR and delivery was supported by a shortened amniocentesis-to-delivery interval. We found a dose-response association between bacterial rDNA abundance and gestational age at delivery. The amniotic cavity of women in preterm labor harbors DNA from a greater diversity of microbes than previously suspected, including as yet uncultivated, previously uncharacterized taxa. The strength of association (relative risk), temporality, and biological gradient (dose-response relationship) with which these microbial sequences are associated with preterm delivery support a causal relationship.

DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, Kim CJ, Erez O, Edwin S, Relman DA. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PLoS ONE* 2008;3:e3056.

Soluble receptor for advanced glycation end products and endogenous secretory RAGE in amniotic fluid: modulation by infection and inflammation

Romero, Gotsch, Edwin; in collaboration with Espinoza, Hassan, Kusanovic, Erez

Advanced glycation end products (AGE) have been implicated in the pathogenesis of diabetes, renal failure, and aging. The receptor for advanced glycation end products (RAGE) has been proposed to participate in innate and adaptive immune responses. RAGE can induce the production of pro-inflammatory cytokines and chemokines as well as neutrophil chemotaxis in a manner that may be suppressed or stimulated by the soluble form of RAGE (sRAGE) and endogenous secretory RAGE (esRAGE). RAGE is expressed in the amnion epithelium, extravillous trophoblast, and decidual cells in patients without chorioamnionitis as well as in the neutrophils in the choriodecidua in cases of histologic chorioamnionitis. Our group conducted a study to determine if intra-amniotic infection/inflammation is associated with changes in the amniotic fluid concentration of sRAGE and esRAGE in the mid-trimester of pregnancy, in term and preterm labor, and in patients with preterm PROM. The amniotic fluid concentrations of sRAGE and esRAGE increased as a function of gestational age and decreased in the presence of labor at term; they were significantly elevated in the presence of intra-amniotic infection/inflammation in patients with preterm labor and intact membranes and patients with preterm PROM, suggesting that changes in the amniotic fluid concentration of sRAGE and esRAGE may represent part of the immune response to intra-amniotic infection/inflammation.

Romero R, Espinoza J, Hassan S, Gotsch F, Kusanovic JP, Avila C, Erez O, Edwin S, Schmidt AM. Soluble receptor for advanced glycation end products (sRAGE) and endogenous secretory RAGE (esRAGE) in amniotic fluid: modulation by infection and inflammation. *J Perinat Med* 2008;36:388-398.

The anti-inflammatory limb of the immune response in preterm labor, intra-amniotic infection/inflammation, and spontaneous parturition at term: a role for interleukin-10 (IL-10)

Romero, Gotsch, Edwin; in collaboration with Kusanovic, Erez, Espinoza, Kim CJ, Vaisbuch, Than, Mazaki-Tovi, Gomez, Mittal, Hassan

Spontaneous parturition at both term and preterm labor are characterized by inflammation in the cervix, membranes, and myometrium. The anti-inflammatory limb of the immune response is crucial for dampening inflammation. In particular, the anti-inflammatory cytokine IL-10 participates in negative feedback to reduce inflammation. We designed a cross-sectional study of 301 pregnant women to determine whether there is a relationship between amniotic fluid concentrations of IL-10 and gestational age, parturition (term and preterm), and intra-amniotic infection/inflammation. We detected IL-10 in amniotic fluid and noted that its concentration did not change with gestational age from mid-trimester to term. Labor at term, however, was associated with a significantly elevated amniotic fluid IL-10 concentration. Moreover, the presence of intra-amniotic infection/inflammation was associated with a significantly higher amniotic fluid concentration of IL-10 in patients at term and patients with preterm labor with intact membranes as compared with patients without intra-amniotic infection/inflammation. The results suggest that IL-10 plays an important role in the control of labor and regulation of the immune response in vivo by initiating actions that dampen inflammation.

Gotsch F, Romero R, Kusanovic JP, Erez O, Espinoza J, Kim CJ, Vaisbuch E, Than NG, Mazaki-Tovi S, Chaiworapongsa T, Mazor M, Yoon BH, Edwin S, Gomez R, Mittal P, Hassan SS, Sharma S. The anti-inflammatory limb of the immune response in preterm labor, intra-amniotic infection/inflammation, and spontaneous parturition at term: a role for interleukin-10. *J Matern Fetal Neonatal Med* 2008;21:529-547.

Visfatin/Pre-B cell colony-enhancing factor in amniotic fluid in normal pregnancy, spontaneous labor at term, preterm labor, and prelabor rupture of membranes: an association with subclinical intrauterine infection in preterm parturition

Romero, Gotsch, Edwin; in collaboration with Mazaki-Tovi, Kusanovic, Erez, Mittal, Than, Vaisbuch, Chaiworapongsa, Nien, Gomez, Espinoza

Compelling evidence supports the view that cytokines play a central role in the mechanism of infection-induced preterm labor. Visfatin is a novel adipokine produced by visceral adipose tissue. The expression of its gene is elevated in several inflammatory conditions, including sepsis, metabolic syndrome, and rheumatoid arthritis. Visfatin is expressed by amniotic epithelium, cytotrophoblast, and decidua and overexpressed in fetal membranes exposed to mechanical stress and/or pro-inflammatory stimuli; its expression by fetal membranes is dramatically upregulated after normal spontaneous labor. We conducted a study to determine whether visfatin is detectable in amniotic fluid and whether its concentration changes with gestational age, spontaneous labor, and preterm PROM and in the presence of microbial invasion of the amniotic cavity. We found that visfatin is a physiologic constituent of amniotic fluid and that its concentrations in amniotic fluid increase with advancing gestational age and in patients with microbial invasion of the amniotic cavity, regardless of membrane status. This is the first in vivo study describing the presence of visfatin in amniotic fluid and its association with acute infection in humans. The findings suggest that visfatin plays a role in normal gestation as well as in the inflammatory response associated with infection. Increased visfatin secretion from fetal membranes, and possibly from intra-amniotic leukocytes in the presence of infection, may represent an attempt to protect the epithelial cells from apoptosis.

Mazaki-Tovi S, Romero R, Kusanovic JP, Erez O, Gotsch F, Mittal P, Than NG, Nhan-Chang CL, Hamill N, Vaisbuch E, Chaiworapongsa T, Edwin SS, Nien JK, Gomez R, Espinoza J, Kendal-Wright C, Hassan SS, Bryant-Greenwood G. Visfatin/Pre-B cell colony-enhancing factor in amniotic fluid in normal pregnancy,

spontaneous labor at term, preterm labor and prelabor rupture of membranes: an association with subclinical intrauterine infection in preterm parturition. *J Perinat Med* 2008;36:485-496.

Region-specific gene expression profiling: novel evidence for biological heterogeneity of the human amnion

Romero, Gotsch; in collaboration with Han, Kim JS, Tarca, Kusanovic, Mittal, Hassan, Kim CJ

The amnion plays an important role during pregnancy and parturition. Though referred to as a single structure, this fetal tissue is regionally divided into placental amnion, reflected amnion, and umbilical amnion. Our empirical observations regarding the regional histological differences in meconium-exposed placentas, especially between placental amnion and reflected amnion, led us to hypothesize that the human amnion is biologically heterogeneous depending on its anatomical region in the placenta. We compared the gene expression profiles of placental amnion and reflected amnion in patients at term with and without spontaneous labor. Real-time quantitative reverse transcriptase (RT) PCR revealed a higher expression of IL-1beta mRNA in reflected amnion than in placental amnion in patients at term not in labor but not in patients in labor. Extended screening with microarray showed differential expression of 17 genes in labor. Interestingly, 839 genes were differentially expressed between placental and reflected amnion. Pathway analysis identified 19 signaling pathways, such as mitogen-activated protein kinase and transforming growth factor–beta pathways associated with region. Lipopolysaccharide (LPS) treatment of the amnion explants showed more robust activation of mitogen-activated protein kinase 3/1 (extracellular signal-regulated kinase 1/2) in placental amnion of patients at term not in labor but not in patients in labor. Placental amnion from term–no labor and term–in labor cases showed a significant difference in the amplitude of IL-1beta mRNA induction by LPS. We report that the anatomical region of the amnion has a substantial impact on its transcriptional program and biological properties and that a particular feature of placental amnion is labor-associated switching to a pro-inflammatory signature. Our study's novel observations strongly suggest that the apparently homogeneous amnion is biologically heterogeneous and compartmentalized, with implications for the physiology of pregnancy and parturition.

Han YM, Romero R, Kim JS, Tarca AL, Kim SK, Draghici S, Kusanovic JP, Gotsch F, Mittal P, Hassan S, Kim CJ. Region-specific gene expression profiling: novel evidence for biological heterogeneity of the human amnion. *Biol Reprod* 2008;79:954-961.

Gene expression profiling demonstrates a novel role for fetal fibrocytes and the umbilical vessels in human feto-placental development

Romero, Gotsch; in collaboration with Kim JS, Tarca, Han, Mittal, Kusanovic, Hassan, Kim CJ

The fetal inflammatory response syndrome (FIRS) is characterized by elevated IL-6 concentrations in the fetal plasma and multi-organ involvement. It is associated with an increased risk of preterm delivery and both short- and long-term perinatal morbidities, such as chronic lung disease and cerebral palsy. Acute funisitis, a component of acute chorioamnionitis, is the histologic hallmark of FIRS. A key feature of funisitis is acute umbilical vasculitis. Interestingly, umbilical phlebitis almost invariably precedes umbilical arteritis. The mechanisms responsible for the higher inflammatory response in the umbilical vein (UV) versus the umbilical arteries (UA) remain to be elucidated. Accordingly, we hypothesized that there is an intrinsic difference in the pro-inflammatory response between UA and UV. We collected and examined segments of the UA and UV from women with normal pregnancies at term not in labor, women with normal pregnancies at term in labor, women with preterm labor/delivery, women with intrauterine growth restriction (IUGR) at term, and women with preterm IUGR. Real-time quantitative RT-PCR and microarray analysis revealed higher expression of IL-1β and IL-8 mRNA in the UV than in the UA and differential expression of 567 genes between the UA and UV associated with distinct biological processes, including the immune response. Unexpectedly, HLA-DR(+) cells migrated via the umbilical vessels into Wharton's jelly more frequently in the UV than in the UA. A significant proportion of these cells co-expressed CD45 and type I pro-collagen and acquired CD163 or α-smooth muscle actin immunoreactivity in

Wharton's jelly. We also found migrating cells in the chorionic and stem villous vessels. Furthermore, the extent of migration increased with advancing gestation but diminished in cases of IUGR. Our observations strongly suggest that circulating fetal fibrocytes, routing via umbilical and placental vessels, are a reservoir for key cellular subsets in the placenta. For the first time, we report fibrocytes in the human umbilical cord and placenta as well as a novel role for both circulating fetal cells and the umbilical vessels in placental development, which is disturbed in IUGR.

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Galectin-1 expression in preterm PROM, histologic chorioamnionitis, severe preeclampsia, and small-for-gestational age neonates

Romero, Gotsch, Edwin; in collaboration with Than, Han, Tarca, Erez, Kusanovic, Montenegro, Mazaki-Tovi, Espinoza, Hassan

Galectin-1 is an anti-inflammatory protein with pleiotropic intra- and extracellular functions; it is expressed by chorioamniotic membranes and the decidua and overexpressed in activated immune and endothelial cells as well as in sites of inflammation, such as fibroblasts in chronic pancreatitis. Galectin-1 can also facilitate immune tolerance and tumor immune escape and has been successfully used for the suppression of experimental autoimmune diseases as well as for graft-versus-host disease in murine models. Given that an abnormal immune response in some pregnancy complications might be associated with changes in placental and membrane expression of galectin-1, we designed a series of studies to determine galectin-1 mRNA and protein expression in (1) the chorioamniotic membranes in women with a normal pregnancy and women with preterm PROM with and without histologic chorioamnionitis and (2) the placenta of women with normal pregnancies, women with severe preeclampsia, and women who delivered a small-for-gestational age (SGA) neonate. In normal term placentas, we detected galectin-1 mRNA or immunofluorescence signals in the trophoblasts, villous stromal cells, Hofbauer cells, endothelial cells of the villous blood vessels, and the villous stroma. In patients with preterm PROM, we detected galectin-1 mRNA and protein in the amniotic epithelium, chorioamniotic fibroblasts/myofibroblasts and macrophages, chorionic trophoblasts, and decidual stromal cells. Patients with preterm PROM and histologic chorioamnionitis exhibited 2-fold–higher galectin-1 mRNA expression in the fetal membranes than patients with preterm PROM without histologic chorioamnionitis. Moreover, histologic chorioamnionitis was associated with strong galectin-1 immunostaining in amniotic epithelium, chorioamniotic mesodermal cells, and apoptotic bodies. These findings suggest that galectin-1 may be involved in regulating inflammatory responses to chorioamniotic infection. Furthermore, placental galectin-1 mRNA expression was significantly higher in severe preeclampsia (with or without SGA) than in controls and SGA; trophoblasts in placentas of patients with severe preeclampsia exhibited the most intense galectin-1 immunostaining. Placental galectin-1 expression is higher in severe preeeclampsia than in normal pregnancy regardless of the presence of SGA but is not altered in SGA without preeclampsia. This is the first report of placental expression and localization of galectin-1 mRNA. We propose that the increased placental expression of galectin-1 in patients with severe preeclampsia may represent a fetal response to an exaggerated systemic maternal inflammation. Thus, galectin-1 may be implicated in maternal-fetal immune tolerance.

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First-trimester maternal serum PP13 in the risk assessment for preeclampsia

Romero, Gotsch, Edwin; in collaboration with Kusanovic, Than, Erez, Espinoza, Gomez, Nien, Hassan

Placental Protein 13 (PP13) is a member of the galectin family and is predominantly expressed by the placenta, specifically by the syncytiotrophoblast, where it is localized on the brush-border membrane at the maternal-fetal interface. Recently, we found that maternal serum PP13 concentrations were significantly reduced during the first trimester among women who subsequently developed preeclampsia. We designed a nested case-control study to determine whether first-trimester maternal serum PP13 concentrations may be used to assess risk for preeclampsia. Our study included 50 patients with preeclampsia and 250 patients with normal pregnancies from whom we collected maternal blood samples between 8 and 13 weeks of gestation. The results showed that serum PP13 concentration in the first trimester was significantly lower in patients who developed preterm and early-onset preeclampsia than in those with normal pregnancies and that, when specificity was fixed at 80 percent, a cutoff of 0.39 MoM[1] had a sensitivity of 100 and 85 percent, respectively, for prediction of early-onset preeclampsia and preterm preeclampsia. We concluded that maternal serum concentration of PP13 in the first trimester appears to be a reasonable marker for risk assessment for preterm preeclampsia.

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A longitudinal study of angiogenic and anti-angiogenic factors in normal pregnancy and patients destined to develop preeclampsia and deliver an SGA neonate

Romero, Gotsch, Edwin; in collaboration with Erez, Espinoza, Kusanovic, Nien, Chaiworapongsa, Mittal, Mazaki-Tovi, Than, Gomez, Hassan

An imbalance between pro-angiogenic factors, i.e., vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), and anti-angiogenic factors, i.e., soluble VEGF receptor-1 (sVEGFR-1, also referred to as sFlt1) and soluble endoglin (s-Eng), is involved in the pathophysiology of preeclampsia. We designed a longitudinal nested case-control study to determine whether changes in maternal plasma concentration of these angiogenic and anti-angiogenic factors differ before the development of disease in patients with normal pregnancies and patients destined either to develop preeclampsia (preterm and term) or deliver an SGA neonate. The study included 144 singleton pregnancies from which we collected longitudinal blood samples at each prenatal visit, scheduled at 4-week intervals from the first or early second trimester until delivery. Patients destined to deliver an SGA neonate had higher plasma concentrations of s-Eng throughout gestation than those with normal pregnancies. Similarly, patients destined to develop preterm preeclampsia and term preeclampsia exhibited significantly higher concentrations of s-Eng than those with normal pregnancies at 23 and 30 weeks, respectively, and a significantly higher plasma concentration of sVEGFR-1 at 26 and 29 weeks of gestation, respectively. Moreover, patients destined to develop preeclampsia (term or preterm) and those who delivered an SGA neonate had lower plasma concentrations of PlGF than those with a normal pregnancy throughout gestation, and the maternal plasma concentration of this analyte was detectable later among patients with pregnancy complications than in normal pregnant women. Interestingly, we observed no significant differences in the plasma concentrations of sVEGFR-1 between patients destined to deliver an SGA neonate and those with normal pregnancies. Our longitudinal study demonstrated that changes in the maternal plasma concentration of s-Eng, sVEGFR-1, and PlGF precede the clinical presentation of preeclampsia but that only changes in s-Eng and PlGF precede the delivery of an SGA neonate. The differences in the profile of angiogenic and anti-angiogenic response to intrauterine insults may determine whether a patient will deliver an SGA neonate, develop preeclampsia, or both.

Given that changes in the maternal plasma concentration of angiogenic and anti-angiogenic factors precede the clinical presentation of preeclampsia and the delivery of an SGA neonate, we aimed

to determine whether changes in the profile of angiogenic and anti-angiogenic factors in maternal plasma between the first and second trimesters are associated with a high risk for the subsequent development of preeclampsia and/or delivery of an SGA neonate. We conducted a case-control study that included 402 singleton pregnancies in the following groups: patients with normal pregnancies, patients who delivered an SGA neonate, and patients who developed preeclampsia. The study included two samples per patient, with the first sample obtained between 6 and 15 weeks of gestation ("first trimester" sample) and the second sample obtained between 20 and 25 weeks of gestation ("second trimester" sample). We compared changes in the maternal plasma concentrations of the angiogenic and anti-angiogenic factors among normal patients and those destined to develop preeclampsia or deliver an SGA neonate while adjusting for maternal age, nulliparity, and body mass index. We used general linear models and polytomous logistic regression models to relate the analyte concentrations, ratios, and products to the subsequent development of preeclampsia and SGA. Our study showed that an increase in the maternal plasma concentration of s-Eng between the first and second trimesters conferred risk for the development of preterm preeclampsia and SGA. Similarly, an increase in the maternal plasma concentration of sVEGFR-1 between the first and second trimester conferred risk for the development of preterm preeclampsia, whereas a subnormal increase in maternal plasma PlGF concentration between the first and second trimester was a risk factor for the subsequent development of preterm and term preeclampsia. In addition, the combination of the three analytes into a pro-angiogenic versus anti-angiogenic ratio [PlGF/(s-Eng x VEGFR-1)] conferred an elevated risk for subsequent development of preterm preeclampsia. In conclusion, changes in the maternal plasma concentrations of s-Eng, sVEGFR-1, PlGF, or their ratios between the first and second trimester confer an increased risk to deliver an SGA neonate and/or develop preeclampsia.

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Maternal-plasma sVEGFR-1 concentration is elevated in SGA, and the magnitude of the increase relates to Doppler abnormalities in the maternal and fetal circulation

Romero, Gotsch, Edwin; in collaboration with Chaiworapongsa, Espinoza, Gonçalves, Kusanovic, Erez, Than, Hassan

Preeclampsia and pregnancy complicated by SGA fetuses share some pathophysiologic derangements, including failure of physiologic transformation of the spiral arteries, endothelial cell dysfunction, and leukocyte activation. An antagonist to vascular endothelial growth factor and placental growth factor, sVEGFR-1, has been implicated in the pathophysiology of preeclampsia (see above). We designed a study with two purposes. The first was to determine whether plasma concentrations of sVEGFR-1 in mothers with SGA fetuses without preeclampsia at the time of diagnosis differ from plasma concentrations of sVEGFR-1 in patients with preeclampsia or in patients with a normal pregnancy. The second purpose was to examine the relationship between plasma concentrations of sVEGFR-1 and Doppler velocimetry of uterine and umbilical arteries in patients with preeclampsia and patients with SGA. Mothers with SGA fetuses had a higher mean plasma concentration of sVEGFR-1 than normal pregnant women but a lower concentration than patients with preeclampsia. Among mothers with preeclampsia and those with SGA fetuses in whom Doppler velocimetry was performed, those with abnormalities in both uterine and umbilical artery velocimetry had the

highest mean deviation of sVEGFR-1 plasma concentrations from the mean for gestational age. These observations provide support for the participation of the soluble receptor of VEGF in the pathophysiology of SGA with abnormal uterine artery Doppler velocimetry and preeclampsia.

Chaiworapongsa T, Espinoza J, Gotsch F, Kim YM, Kim GJ, Gonçalves LF, Edwin S, Kusanovic JP, Erez O, Than NG, Hassan SS, Romero R. The maternal plasma soluble vascular endothelial growth factor receptor-1 concentration is elevated in SGA and the magnitude of the increase relates to Doppler abnormalities in the maternal and fetal circulation. *J Matern Fetal Neonatal Med* 2008;21:25-40.

Distinct genomic signatures of adaptation in pre- and postnatal environments during human evolution

Romero; in collaboration with Erez, Goodman, Grossman

The human genome evolution project seeks to reveal the genetic underpinnings of key phenotypic features that are distinctive of humans, such as a greatly enlarged cerebral cortex, slow development, and long life spans. We hypothesized that adaptive genotypic changes during earlier periods of evolutionary history also helped shape the distinctive human phenotype. Focusing predominantly on genotypic changes during the 6-million-year descent from the last common ancestor of humans and chimpanzees, we set out to identify the positively selected genetic changes that may have helped shape key, distinctive (and, in some cases, unique) phenotypic features of humans. Using comparative genome sequence data from 10 vertebrate species, we found a signature of human ancestry–specific adaptive evolution in 1,240 genes during their descent from the last common ancestor with rodents. We also found that the signature of adaptive evolution differs significantly for highly expressed genes in human fetal and adult-stage tissues. Functional annotation clustering showed that, on the ape stem lineage, an especially evident, adaptively evolved biological pathway contains genes that function in mitochondria, that are crucially involved in aerobic energy production, and that are highly expressed in two energy-demanding tissues—heart and brain. In addition, the ape stem lineage evidenced adaptive evolution among genes associated with human autoimmune and aging-related diseases. Interestingly, during more recent human descent, the adaptively evolving, highly expressed genes in the fetal brain are involved in mediating neuronal connectivity. Our comparison of adaptively evolving genes from pre- and postnatal-stage tissues suggests that different selective pressures act on the development as opposed to the maintenance of the human phenotype.

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The human progesterone receptor shows evidence of adaptive evolution associated with its ability to act as a transcription factor

Romero; in collaboration with Erez, Goodman, Grossman

The steroid hormone progesterone is a vital regulator of reproduction in mammals. In primates, progesterone participates in the regulation of normal menstrual cycles, ovulation, and embryo implantation. Many of progesterone's biological actions are mediated through the progesterone receptor. The gene encoding the receptor (*PGR*) arose early in vertebrate evolution via a series of duplications of an ancestral estrogen receptor; it acts as a transcription factor and participates in the regulation of reproductive processes, including menstruation, implantation, pregnancy maintenance, parturition, mammary development, and lactation. Unlike other mammals, primates do not exhibit progesterone withdrawal at the time of parturition. Given that progesterone-mediated reproductive features vary among mammals, *PGR* is an attractive candidate gene for studies of adaptive evolution. We sequenced the progesterone receptor–coding regions in a diverse range of species, including apes, Old World monkeys, New World monkeys, prosimian primates, and other mammals. We investigated three aspects of *PGR* evolution: (1) the strength of positive selection in human and chimpanzee lineages, (2) the prevalence of positive selection within primates, and (3) the specific isoforms and domains at which amino acids were replaced. Adaptive evolution occurred in the

human and chimpanzee lineages, as evidenced by statistically significant higher non-synonymous substitution rates than synonymous substitution rates. We rarely observed the positive selection seen in other lineages. In humans, amino acid replacements occurred mostly in a region of the gene shown to inhibit the ability of the progesterone receptor to act as a transcription factor. Moreover, many of the non-synonymous substitutions in primates occurred in the N-terminus, suggesting that co-factor interaction surfaces might have been altered as reflected in altered progesterone-regulated gene transcriptional effects. It is possible that changes in the inhibitory function region uniquely modulate expression and interaction among the different progesterone isoforms in functional progesterone withdrawal during human and chimpanzee parturition. Positive selection provides the basis for the hypothesis that changes in structure and function of the progesterone receptor during evolution contribute to the diversity of primate reproductive biology, especially in parturition.

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Twin-to-twin transfusion syndrome: an anti-angiogenic state?

Romero, Gotsch, Edwin; in collaboration with Kusanovic, Espinoza, Nien, Kim CJ, Mittal, Erez, Mazaki-Tovi, Than, Gomez, Hassan

Twin-to-twin transfusion syndrome (TTTS) affects approximately 10 to 15 percent of monochorionic-diamniotic twin pregnancies; its diagnosis is based on strict sonographic criteria. An imbalanced chronic blood flow between the donor and recipient twin through placental vascular anastomoses is the accepted pathophysiology of TTTS. Recently, others demonstrated that VEGFR-1 mRNA is overexpressed in some cases of TTTS only in the donor twin's syncytiotrophoblast. We conducted a case control study to determine maternal plasma concentrations of the angiogenic factor PlGF and of the anti-angiogenic factors sVEGFR-1 and s-Eng in monochorionic-diamniotic pregnancies between 16 and 26 weeks' gestation with and without TTTS. As compared with patients without TTTS, patients with TTTS had significantly higher median plasma concentrations of s-Eng and sVEGFR-1 and lower median plasma concentrations of PlGF. This novel finding suggests that an anti-angiogenic state may be present in some cases of TTTS.

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Individualized growth assessment of fetal thigh circumference using three-dimensional ultrasonography

Romero; in collaboration with Lee, Espinoza, Gonçalves

Estimated fetal weight and growth are usually determined by using formulas that incorporate fetal biometric parameters measured by two-dimensional ultrasound. Using three-dimensional ultrasonography, we conducted a prospective, longitudinal sonographic study of 30 fetuses beginning at 18 weeks' menstrual age to develop individualized growth assessment (IGA) standards for upper and middle fetal thigh circumferences. We measured second-trimester sonographic parameters from three-dimensional volume data to establish IGA standards, subsequently predicted third-trimester growth trajectories and birth measurements for upper and middle fetal thigh circumferences, and determined the 95 percent ranges for Growth Potential Realization Index (GPRI) values for both types of thigh circumference. The 30 newborns showed no postnatal evidence of abnormal growth. Our measurements demonstrated that fetal thigh circumference may be evaluated and measured reliably with standard IGA methods. Both upper and middle thigh circumferences yielded similar results in the third trimester. However, the use of middle thigh circumferences improved neonatal thigh circumference predictions. Corresponding GPRI values for fetal middle thigh circumference are closer to the ideal value of 100 percent and may be used in calculations for assessing neonatal growth outcome.

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A systematic approach to the use of the multiplanar display in evaluation of abnormal vascular connections to the fetal heart using four-dimensional ultrasonography

Romero, Gotsch; in collaboration with Espinoza, Hassan, Kusanovic, Lee, Erez, Gonçalves

Congenital heart disease is the leading cause of infant death among newborns with congenital anomalies. Conotruncal anomalies represent one-fifth of all congenital heart defects diagnosed prenatally. Simultaneous display of standard views used in fetal echocardiography and their orthogonal planes may provide novel ultrasonographic views for examining the fetal heart and its vascular connections. We designed a study to determine the clinical utility of the multiplanar display in the examination of abnormal vascular connections to the fetal heart. We reviewed four-dimensional volume data sets, acquired with the spatiotemporal image correlation technique, from four patients with abnormal vascular connections to the fetal heart. We used multiplanar views of the fetal heart to display simultaneously the standard planes used in fetal echocardiography and their corresponding orthogonal planes. This method facilitated the identification of the abnormal vessels and their spatial relationships with other vascular structures. Thus, we concluded that multiplanar imaging may be used to assess abnormal vascular connections to the fetal heart and may provide novel ultrasonographic planes for fetal echocardiography with three- and four-dimensional ultrasonography.

Espinoza J, Hassan SS, Gotsch F, Kusanovic JP, Lee W, Erez O, Gonçalves LF, Schoen ML, Romero R. A systematic approach to the use of the multiplanar display in evaluation of abnormal vascular connections to the fetal heart using 4-dimensional ultrasonography. *J Ultrasound Med* 2007;26:1461-1467.

The use of inversion mode and three-dimensional manual segmentation in volume measurement of fetal fluid-filled structures: comparison with Virtual Organ Computer-aided AnaLysis (VOCAL)

Romero; in collaboration with Kusanovic, Nien, Gonçalves, Espinoza, Lee, Erez

Volume measurements obtained by three-dimensional ultrasonography are considered more accurate than those obtained by two-dimensional ultrasonography that uses the ellipsoid formula, among other formulas. We conducted a study to compare agreement among three techniques (VOCAL, inversion mode, and manual segmentation) as well as inter- and intra-observer agreements for volume measurements of fetal fluid-filled structures. We obtained 50 three-dimensional volume data sets of the fetal stomach and bladder; two observers independently performed volume measurements. The three techniques yielded a high degree of reliability and good agreement. We obtained manual segmentation and inversion mode measurements significantly faster with the VOCAL technique than with the other techniques. We concluded that the inversion mode is a reliable method for volume calculations of fluid-filled organs, whereas manual segmentation may be used when volume measurements by VOCAL or the inversion mode are technically difficult to obtain.

Kusanovic JP, Nien JK, Gonçalves LF, Espinoza J, Lee W, Balasubramaniam M, Soto E, Erez O, Romero R. The use of inversion mode and 3D manual segmentation in volume measurement of fetal fluid-filled structures: comparison with Virtual Organ Computer-aided AnaLysis (VOCAL). *Ultrasound Obstet Gynecol* 2008;31:177-186.

[1]Multiples of the median

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TISSUE BIOPHYSICS AND BIOMIMETICS

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We try to understand fundamental relationships between function and structure in soft tissues, "engineered" tissue constructs, and tissue analogues—how microstructure, hierarchical organization, composition, and material properties of tissue affect biological function and dysfunction. To investigate biological and physical model systems at different time and length scales, we make physical measurements in tandem with analytical and computational models. Primarily, we use water to probe both equilibrium and dynamic interactions among tissue constituents over a wide range of time and length scales. To determine the equilibrium osmo-mechanical properties of well-defined model systems, we vary water content or ionic composition. To probe tissue structure and dynamics, we employ small-angle X-ray scattering (SAXS), small-angle neutron scattering (SANS), static light scattering (SLS), dynamic light scattering (DLS), and nuclear magnetic resonance (NMR) relaxometry. We use mathematical models to understand how changes in tissue microstructure and physical properties affect essential transport processes (e.g., mass, charge, and momentum). The most direct non-invasive method for characterizing these transport processes in vivo is magnetic resonance imaging (MRI), which can characterize normal tissue microstructure and follow its changes in development, degeneration, and aging. Another goal is to translate our new quantitative methodologies from bench to bedside.

Virtual in vivo tissue biopsy

Basser, Pierpaoli, Komlosh, Nevo, Özarslan; in collaboration with Assaf, Cohen, Freidlin, Jarisch, Pajevic, Pikalov

We continue to develop novel magnetic resonance (MR) methods that allow us to probe tissue microstructure and diagnose neurological and developmental disorders in vivo. Diffusion Tensor MRI (DT-MRI or DTI) is the most mature such technology that we have developed. With it, we measure a diffusion tensor of water, $\boldsymbol{D}$, on a pixel-by-pixel basis within tissue. Information derived from $\boldsymbol{D}$ includes the local fiber-tract orientation, the mean-squared distance that water molecules diffuse in any given direction, the orientationally averaged mean diffusivity, and other intrinsic scalar (invariant) quantities that are independent of the laboratory coordinate system. The scalar parameters behave like quantitative histological "stains" but are "developed" without exogenous contrast agents or dyes. The bulk or orientationally averaged diffusivity is the most successful imaging parameter proposed to date for identifying ischemic tissue regions in the brain during

acute stroke. Furthermore, measures of diffusion anisotropy are useful in identifying white matter degeneration (e.g., Wallerian degeneration) associated with chronic stroke. DTI also provides new information about early developmental changes in cortical gray and white matter that cannot be detected with other imaging methods.

We have also pioneered the use of color maps to encode nerve fiber orientation, allowing us to identify the main association, projection, and commissural white matter pathways in the brain and even to differentiate white matter pathways with similar structure and composition but distinct spatial orientations. To assess anatomical connectivity between different functional brain regions, we developed DTI fiber tractography to track nerve fiber trajectories by continuously following the direction along which the apparent diffusivity is at a maximum. While the reliability of DTI tractography is high in coherently organized primary white matter pathways, artifactual tracts may be generated when following white matter pathways with a more complex underlying fiber topology (e.g., pathways that "kiss," cross, merge, or branch). To assess the reliability of individual computed tracts, we have used Point-wise Assessment of Streamline Tractography Attributes (PASTA). We are using other MR data and more detailed models of water diffusion in tissue to compensate for artifacts.

We have proposed several advanced in vivo MR methods to measure fine microstructural features of nerve fascicles that previously could be measured only by using laborious histological methods. One approach is CHARMED (Composite Hindered And Restricted Model of Diffusion), a combined experimental and modeling framework that allows us to describe water diffusion in white matter. More specifically, CHARMED permits us to distinguish between water diffusing within the intra-axonal spaces and extra-axonal compartments, thereby improving the angular resolution in tract tracing and resolution of fibers that cross. Recently, we extended CHARMED (and dubbed it AxCaliber) in order to measure the axon diameter distribution within large fascicles in vivo.

While gray matter appears featureless in DTI, its microarchitecture is rich and varied. We have been working toward developing a non-invasive, in vivo method to perform Brodmann parcellation of the cerebral cortex. To this end, we are developing advanced MR sequences to probe correlations between displacements of water molecules within brain tissue and various gray matter compartments at microscopic resolution as well as sophisticated mathematical models describing water displacements in those compartments; we use the latter to infer key microstructural and morphological features.

To permit analysis of novel multidimensional data sets, clinical and biological applications of DTI (and of other novel displacement MRI methods) generally require new mathematical, statistical, and image-sciences concepts and tools. We have developed algorithms for continuous, smooth approximation to the discrete, noisy, measured DT field data; the algorithms reduce noise and enable us to follow fibers more reliably. We recently derived a new Gaussian distribution for tensor-valued random variables that we used in designing optimal DTI experiments. In addition, we have developed non-parametric empirical (e.g., Bootstrap) methods for determining the statistical distribution of DT–derived quantities in order to study, for example, the inherent variability and reliability of white matter fiber tract trajectories. These parametric and non-parametric statistical methods enable us to apply powerful statistical hypothesis tests to a wide range of important biological and clinical questions that previously could be examined only with ad hoc methods. More recently, we have been developing a variety of economical empirical methods to describe the effect of noise on DTI data, permitting the assessment of data variability with a clinically feasible MRI acquisition.

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MRI study of normal brain development

Pierpaoli, Basser, Chang, Sarlls, Walker, Koay, Özarslan; in collaboration with The Brain Development Cooperative Group

Four NIH Institutes (NICHD, NIMH, NINDS, and NIDA) have jointly sponsored a multicenter study to advance our understanding of brain development in typical, healthy children and adolescents. The study has enrolled approximately 500 children, ranging from infancy to young adulthood, who have been seen at different times over a six-year period (2001–2007) at several clinical centers around the United States. The group has acquired MR images of the brain and will relate the imaging findings to the results of standardized neuropsychological tests.

Our role in this project is to serve as a diffusion tensor data processing center (DPC). We process and analyze all DTI data that the various clinical centers acquire during the course of the study. During the past several years, we have been developing and implementing a novel DTI data processing pipeline. Specifically, we have developed procedures for sorting, displaying, and co-registering the raw diffusion-weighted images from which the DTI data are computed. The image registration procedure removes the effects of subject motion and eddy current distortion and aligns the images to a given template with only one interpolation step, ensuring minimal loss of data quality. We also proposed a new strategy for robust estimation of the diffusion tensor and the quantities derived from it. We undertook the task of registering DTI data to other structural MRI data in the database (which is public) by using rigid body and linear (affine) transformations. More recently, we addressed the issue of correcting residual image distortion originating from the Echo-Planar Image (EPI) acquisition used for DTI.

In 2005, the project received Neuroscience Blueprint funds to expand the DTI portion of the study. The original DTI scan was limited to a 10-minute protocol performed on those subjects able to tolerate additional time in the scanner following the collection of the core structural MRI data. The study's expansion is increasing the quality and quantity of data collected through longer or additional scanning sessions for older subjects and through the recruitment of new subjects and scanning sessions for the younger cohort. Our group has designed the expanded DTI protocol and coordinated its implementation at each acquisition site. We completed data collection in 2007 and are now receiving raw diffusion data and structural MRI targets for processing through our pipeline. We plan to produce processed diffusion tensor data to be made available to the scientific community approximately one year after receiving all the raw data.

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Biopolymer physics: water-ion-biopolymer interactions

Horkay, Lin, Basser; in collaboration with Geissler, Hecht, Tasaki

To help us understand the nature of physical/chemical interactions within biomolecules and among biomolecular assemblies, we developed an experimental approach to study assembly structure (morphology) and thermodynamic properties as a function of length scale (i.e., spatial resolution). The methodology combines macroscopic (coarse length–scale) osmotic swelling pressure measurements and various high-resolution scattering experiments. Macroscopic swelling pressure measurements provide information about the overall thermodynamic response, whereas SANS, SLS, and DLS collectively provide information about the sizes of structural elements and their respective contribution to macroscopic osmotic properties. Combining these measurements allows us to (1) separate the scattering intensity caused by thermodynamic concentration fluctuations from large static superstructures (e.g., aggregates) and (2) determine the length scales of fluctuations that give rise to macroscopic thermodynamic properties.

Moreover, in solutions of large charged polymers, counter-ions form a cloud surrounding the polymer chain. With increasing salt concentration, a fraction of these ions adsorb onto the macro-ion. For monovalent ions (such as sodium), their distribution can be approximated by the Poisson-Boltzmann theory; however, the theory fails to describe the counter-ion atmosphere in the presence of multivalent counter-ions (such as calcium), whose effect on chain conformation remains poorly understood. Knowledge of the counter-ion distribution is therefore essential to understanding the behavior of biomolecules in the physiological milieu. Recently, we developed a method based on measurement of the X-ray scattering signal in the vicinity of the absorption edge of the counterions that allows us to determine the distribution of ions around charged biopolymer molecules.

Specifically, we have applied the above multiscale approach to the interactions of multivalent cations, particularly calcium, on the structure and morphology of various biomolecules. Divalent cations are ubiquitous in the biological milieu, yet existing theories do not adequately explain their effect on and interactions with charged polymers or biomolecules. Moreover, experiments to study the interactions are difficult to perform, particularly in solution, because multivalent cations above a low concentration threshold generally cause phase separation or precipitation of charged molecules. Given that macroscopic phase separation does not occur in cross-linked gels, we overcame this limitation by cross-linking our biopolymers, thereby extending the range of ion concentrations over which the system remains stable and may be studied. We have applied the new method in pilot studies of DNA and of cross-linked gels of the model synthetic polymer polyacrylic acid. Concentrated DNA solutions and novel DNA gels have never before been investigated with SANS in conjunction with osmotic measurements. Our method also provides a unique framework for analyzing the osmotic and scattering behavior of other biomolecular systems.

We also investigated cross-linked gels of the biopolymer hyaluronic acid (HA) to determine the size of the structural elements that contribute to osmotic concentration fluctuations. We combined SAXS and SANS to estimate the osmotic modulus of HA solutions in the presence of monovalent and divalent counterions. We also investigated the diffusion processes in biopolymer solutions, using DLS to determine the osmotic modulus independently from the dynamic response.

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Functional properties of extracellular matrix

Horkay, Lin, Silva, Basser; in collaboration with Dimitriadis

The swelling behavior of cartilage is exquisitely sensitive to biochemical and microstructural changes during development, disease, degeneration, and aging. Given that primarily the osmotic properties of its constituents allow cartilage to resist applied loads and express or imbibe fluid, understanding the mechanisms affecting cartilage hydration is also essential to predicting cartilage's load-bearing and lubricating ability,

Previously, we showed that controlled hydration or swelling of cartilage is a means of determining functional properties of cartilage's extracellular matrix (ECM), thus permitting an independent measurement of important physical/chemical properties of the collagen network and proteoglycans (PG) within the ECM. This approach involves (1) modeling the ECM as a composite material consisting of two distinct phases—a collagen network and a proteoglycan solution trapped within it; (2) applying various known levels of equilibrium osmotic stress; and (3) using physical-chemical principles and additional experiments to determine independently a pressure-volume relationship for both the PG and collagen phases. In pilot studies, we used this approach to determine pressure-volume curves for the collagen network and PG phases in native and trypsin-treated normal human cartilage specimens as well as in cartilage specimens from osteoarthritic (OA) joints. In both normal and trypsin-treated specimens, collagen network stiffness appeared unchanged, whereas collagen network stiffness was reduced in OA specimens. Our findings highlighted the role of the collagen network in limiting normal cartilage hydration and ensuring a high PG concentration in the matrix, which are both essential for effective load bearing in cartilage and lubrication but are lost in OA. The data also suggest that the loss of collagen network stiffness, rather than the loss or modification of PGs, may be the incipient event leading to the subsequent disintegration of cartilage observed in OA.

One shortcoming of this approach, however, was that it took many days to analyze a single cartilage specimen, making the technique unsuitable for routine pathological analysis or tissue engineering applications. Furthermore, a significant amount of tissue was required to perform these osmotic titration experiments. Therefore, we developed a new tissue micro-osmometer to perform such experiments practically and rapidly. The instrument can measure minute amounts of water absorbed by small tissue samples (less than 1 μg) as a function of the equilibrium activity (pressure) of the surrounding water vapor. A quartz crystal detects the water uptake of a specimen attached to the crystal's surface. The high sensitivity of the crystal's resonance frequency to small changes in the amount of adsorbed water allows us to measure precisely the mass of water taken up by the tissue specimen. Varying the equilibrium vapor pressure surrounding the specimen induces controlled changes in the tissue layer's osmotic pressure.

To illustrate the applicability of the new apparatus, we measured the swelling pressure of a tissue-engineered cartilage specimen. We also used atomic force microscopy (AFM) in tandem to determine and map the tissue's local-mechanical properties and biochemical analysis to determine the concentration of the main biopolymer components. In addition, we study the contribution from individual components to the tissue's osmotic and mechanical properties. Our current research focuses on aggrecan, which is the most abundant cartilage proteoglycan and is most responsible for the tissue's load-bearing ability. We study the static properties of aggrecan and aggrecan/hyaluronic acid aggregates by SLS, SANS, and SAXS and by osmotic pressure measurements and we probe the dynamics by DLS and neutron spin-echo measurements.

The micro-osmometer will eventually permit us to obtain a simultaneous profile of the osmotic compressibility or stiffness of several cartilage specimens as a function of depth from the articular surface to the cartilage/bone interface, to quantify the contributions of individual components of ECM (e.g., aggrecan, hyaluronic acid, and collagen) to total osmotic pressure, and to assess the osmotic compatibility and mechanical integrity of developing tissues and tissue-engineered cartilage (or ECM) for improved integration following implantation in reconstructive or restorative surgical procedures.

We recently developed an AFM technique for mapping the local elastic properties of tissues and successfully addressed many of the issues that previously hindered use of AFM as a high-throughput probe of inhomogeneous samples, particularly of biological tissues. The technique uses the precise scanning capabilities of a commercial AFM to generate large volumes of compliance data and automatically extracts the relevant elastic properties. Used in conjunction with results from micro-osmometry and biochemical analysis, AFM will allow us to map spatial variations in the load-bearing capacity of cartilage specimens.

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BIOPHYSICS OF LARGE MEMBRANE CHANNELS

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To address fundamental questions of membrane transport, we apply physical theory to experiments on membrane channels reconstituted in planar lipid bilayers, focusing on channel-facilitated transport of metabolites and other large solutes across cell and organelle membranes. The corresponding large ion channels are not only the gateways of metabolite exchange between different cellular compartments and cells but are also recognized as multifunctional membrane receptors and components of many toxins. We explore channel-forming proteins such as anthrax protective antigen (from *Bacillus anthracis)*, VDAC (voltage-dependent anionic channel from the outer membrane of mitochondria), OmpF (general bacterial porin from *Escherichia coli*), LamB (sugar-specific bacterial porin from *Escherichia coli*), alpha-hemolysin (toxin from *Staphylococcus aureus*), OprF (porin from *Pseudomonas aeruginosa*), alamethicin (amphiphilic peptide toxin from *Trichoderma viride*), and syringomycin E (lipopeptide toxin from *Pseudomonas syringae*). To study these channels under precisely controlled conditions, we purify and then reconstitute the channel-forming proteins in planar lipid bilayer membranes. Our main goal is to elucidate the physical principles and molecular mechanisms responsible for metabolite flux regulation under normal and pathological conditions. Our research will provide the basis for the development of new approaches to treat various diseases for which regulation of transport through ion channels plays the key role.

Role of cytosolic proteins in the regulation of mitochondrial respiration
Rostovtseva, Sheldon, Bezrukov; in collaboration with Sackett, Saks

Positioned at the interface between mitochondria and the cytosol, the VDAC is at the control point of mitochondrial life and death. This large channel functions as a "switch" that defines whether mitochondria will engage in suppression of mitochondrial metabolism, which leads to apoptosis and cell death, or in normal respiration. VDAC, the most abundant protein in the mitochondrial outer membrane (MOM), is responsible for ATP/ADP exchange and for the fluxes of other metabolites across MOM. VDAC controls exchange and fluxes by switching between "closed" states, which are virtually impermeable to ATP and ADP, and open states. This control has dual importance in maintaining normal mitochondrial respiration and in triggering apoptotic signals that lead to release of cytochrome c and other apoptogenic factors from the intermembrane space into the cytosol. Emerging evidence indicates that VDAC closure promotes apoptotic signals, although VDAC itself is not directly involved in the permeability transition pore or hypothetical Bax-containing, cytochrome *c*–permeable pores. We analyzed closure of VDAC induced by dissimilar cytosolic proteins such as pro-apoptotic tBid and dimeric tubulin to show that the involved

mechanisms are distinct. While tBid largely modulates VDAC voltage gating, tubulin blocks the channel with the efficiency of voltage-controlled blockage. With high-affinity binding of tubulin to isolated mitochondria reported long ago, we are now able to demonstrate the mechanism of VDAC regulation by tubulin in vitro—by reconstituting the channel in planar lipid membranes in the presence of dimeric tubulin—and to relate the regulation to the action of this protein in vivo. We found that blockage of VDAC by tubulin is highly voltage-dependent and may be described by a first-order reaction. Interpolated to zero applied voltage, the reaction is characterized by an equilibrium binding constant as high as inverse nanomoles. We confirmed our findings in experiments with isolated mitochondria from mouse heart and brain. Accordingly, we believe that we have identified a natural cytoplasmic regulator of mitochondrial respiration in permeabilized cells—the potent but evasive "Factor-X." With such control, tubulin may selectively regulate metabolic fluxes between mitochondria and the cytoplasm. Thus, our results not only reveal a novel mechanism of mitochondrial respiration regulation but also uncover a new functional role for the cytoskeleton protein dimeric tubulin.

We also investigated the long-standing question of how VDAC voltage gating observed in planar lipid bilayers at relatively high potentials (greater than 30 mV) could be relevant to the situation in vivo and in isolated mitochondria wherein the presence of the VDAC should make the potential difference across MOM very low. This potential is most likely just the Donan potential stemming from the high concentration of charged polyions (such as cytochrome *c*) in the intermembrane space, which was estimated at close to 10 mV. As now follows from our findings, cytosolic concentrations of tubulin could markedly increase VDAC sensitivity to voltage and induce significant VDAC closure at transmembrane potentials even smaller than 10 mV. To conclude, we note that tubulin, as a newly discovered and potentially active player, adds another level of complexity to the VDAC's regulation of mitochondrial signals, suggesting possible competition between tubulin and hexokinase for VDAC binding. Interestingly, others found that paclitaxel, a well-known antitumor drug that inhibits the dynamics of microtubules and subsequently induces apoptosis, induced cytochrome *c* release from mitochondria in intact human neuroblastoma cells and isolated mitochondria. It is likely, however, that paclitaxel and other microtubule-active antitumor drugs might modify interactions of microtubules and/or tubulin with VDAC and thus deliver a signal for mitochondria permeabilization and apoptosis induction.

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Peptide-lipid interactions revealed by kinetics of a model channel

Rostovtseva, Bezrukov; in collaboration with Petrache, Weinrich

Critical to biological processes such as secretion and transport, protein-lipid interactions within the membrane and at the membrane-water interface still raise many questions. Residing within the inner oily part of the membrane, transmembrane proteins are significantly affected by non-specific, hydrophobic interactions. To quantify tractable energetic contributions of these interactions within an ingenuous physical model, others introduced the concept of hydrophobic mismatching. In essence, in this model, any mismatch in hydrophobic dimensions between the protein and the lipid incurs an energetic penalty that causes lipid and protein deformations or structural adaptations. Many studies, including ours, support this model. We used the benchmark antibiotic gramicidin A to show that the polar part (the "other part") of the lipid bilayer claims its own role in lipid-channel interactions. We compared the dissociation rate of single gramicidin channels in "solvent-free" planar bilayers made of dioleoyl-phosphatidylcholine (DOPC), dioleoyl-phosphatidylethanolamine

(DOPE), and diether-DOPC (DEPC) lipids (or mixtures); we also evaluated the effect of monovalent salt concentration. While headgroup demethylation from DOPC to DOPE decreases the life time of gramicidin channels by an order of magnitude in accordance with the currently accepted hydrophobic mismatch mechanism, our results for diether-DOPC suggest the importance of the headgroup-peptide interactions. According to our X-ray diffraction measurements, this lipid has the same hydrophobic thickness as DOPC but increases gramicidin channel life time by a factor of 2. Thus, using gramicidin channels as a probe, we showed that peptide-headgroup interactions may dominate over the effect of hydrophobic mismatch in regulating protein function. To conclude, our findings demonstrate that, even in this simple case, the channel regulation involves both non-specific (hydrophobic mismatch) and specific (headgroup-peptide) interactions, thus highlighting the importance of the latter in the function of membrane proteins.

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Physics of channel-facilitated transport

Bezrukov; in collaboration with Berezhkovskii, Pustovoit, Szabo, Zitserman

Our effort in physical theory of channel-facilitated membrane transport concentrated on further development of the continuum diffusion model of solute dynamics in a membrane channel. The first important advance of the past year was the development of an analytical approach that permits calculation of the optimal intrachannel potential of mean force that maximizes the channel-facilitated flux driven by the solute concentration gradient. Paradoxically, it would be preferable for a solute to "bind" more strongly near the exit rather than near the entrance of the channel. Another interesting observation is that the optimum value of the interaction potential depends on the concentrations of the solute outside the channel, suggesting that, in a given organism and depending on solute concentration, channel proteins designed to transport the same molecule might have different amino acid sequences. One gene might encode a channel protein that functions at high solute concentrations while another might encode a channel protein that works at low concentrations.

Second, we were able to formulate a fluctuation theorem for membrane transport. By counting translocations of single solutes through an arbitrarily biased channel, we established the statistical relation between particle transport and particle dynamics in the channel and reservoirs. We showed that the same fluctuation theorem is true for both single- and multichannel transport of non-interacting particles and particles that strongly repel each other. The theory provides the fine statistics of solute translocation through the channel and, in the limit of increasing time, leads to the expression for the steady-state flux derived earlier. The results of our work are helpful in interpretation of single-molecule translocation experiments.

Third, we analyzed relaxation of the particle number in a membrane channel at arbitrary particle-channel interactions and derived general expressions for the relaxation time and low-frequency limit of the power spectral density. The expressions simplify significantly when the channel is symmetric. For a square-well potential of mean force that occupies the entire channel, we verified the accuracy of the analytical predictions by Brownian dynamics simulations. Interestingly, we showed that, for such a channel, as the depth of the well increases, the familiar scaling of the relaxation time with the channel length squared is transformed into a linear dependence on the length.

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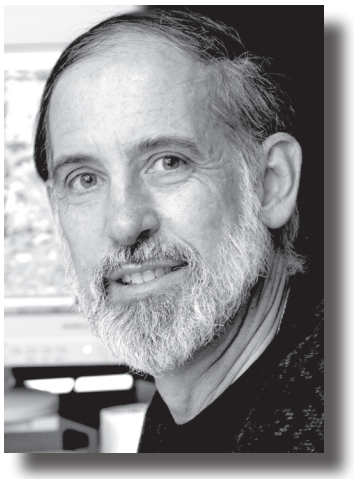
MEDICAL BIOPHYSICS



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Currently, we are developing optical technologies to characterize early stages of disease and to monitor responses to therapy in cancer and age-related macular degeneration (AMD). By integrating multispectral, non-invasive, clinical retinal autofluorescence imaging with automated image analysis, we seek to map the distribution of several intrinsic photochemicals implicated in health preservation and early disease. In pilot clinical studies, we are applying our new technology to understanding how changes in retinal spectral irradiance affect the balance between retinal photochemical pathways that, we hypothesize, drive early age-related maculopathy. Our broader goals are to quantify earlier stages of local molecular imbalance throughout the retina and to develop reliable means for classifying and quantifying early disease in order to evaluate the effectiveness of strategies to prevent disease progression. We are further developing our inventions of expression microdissection and laser capture microdissection for better integration with multiplex molecular analyses of specific cells and organelles extracted from complex tissue.

Laser microdissection and molecular diagnostics technology development
Majumdar, Ory, Bonner; in collaboration with Emmert-Buck, Pohida

Integrative molecular biology requires an understanding of interactions of large numbers of pathways. Similarly, molecular medicine increasingly relies on complex macromolecular diagnostics to guide therapeutic choices. A fundamental argument for laser capture microdissection (LCM) of tissues is that, without separation of specific cell populations from complex tissues, we will miss critical control functions of thousands of regulated transcription factors, cell regulators, and receptors that are expressed at low copy number. By detecting changes in these critical effectors, we expect to improve our integrative understanding of tissue function and pathology. In complex tissues—particularly among pathological variations—it is exceptionally difficult to measure the majority of molecules that are at low copy numbers per cell without first isolating specific cell populations. With our newest inventions, we are increasing the microdissection resolution to the organelle level.

The LCM techniques that we started developing 12 years ago are now widely used in molecular analysis of genetics and gene expression changes within target cells within complex tissues. However, in global proteomic and lipid studies without molecular amplification methods, the quantity of isolated cells sufficient to perform accurate characterization of less abundant species is problematic because the microscopic visualization, targeting, and isolation in laser microdissection has a maximal rate of 1–20 cells per second, depending on the cells' microscopic distribution within the tissues.

Recently, in collaboration with NCI and CIT, we invented and are now refining an automatic "target-directed microtransfer" technique based on macromolecule-specific staining of cells. The technique (patent pending) is built on our understanding of the physics of thermoplastic microtransfer and uses a much simpler device and transfer films than do commercial laser microdissection microscopes. Given that the new microbonding process does not require microscope-based visualization and targeting, it is capable of much higher throughput rates. Our current prototype is capable of isolating all specifically immunolabeled cells within a 1 cm^2 region of an immunostained tissue section in about 5 seconds, which corresponds to specific separation from approximately 100,000 cells per second. This technique allows cell separation rates exceeding fluorescence-activated cell sorting (FACS) of labeled cell suspensions while preserving our ability to harvest specifically stained targets directly from sections of complex tissues. This rapid, automated microtransfer method has a spatial resolution determined by stain localization (less than 1 μ), not by optical diffraction. Consequently, it is uniquely suited to isolating highly dispersed, specific cell populations (e.g., stem cells or only those neurons in the supra-optic nucleus that express vasopressin) or specific organelles (e.g., neuronal nuclei in the brain). The spatial relationships (morphology) among the specific cells in the tissue are preserved on the transfer film. As the technology becomes more robust, we will integrate the automated microtransfer with molecular profiling of specific cells within tissues in our NIH LCM Core Facility.

United States Patents

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Gene expression during normal development and pathology progression

Majumdar, Ehler, Ory, Bonner; in collaboration with Emmert-Buck, Brooks, McQueen

If microdissection and molecular analysis can be made clinically practical, the expression levels of sets of approximately 20–100 critical, stage-specific disease markers within a selected cell population might provide reliable diagnosis and intermediate endpoints of response to molecular therapies in individual patients. Our analysis of large gene expression and protein databases suggests that a significant fraction of all genes is expressed in any specific cell type and that the levels of gene products universally exhibit a highly skewed power-law distribution similar to those characterizing many other complex systems (Kuznetsov VA, *Signal Processsing* 2003;83:889). We have developed mathematical models for the evolution of such distributions—models that predict the observed distributions of genes, protein domains, and gene expression in species of increasing biological complexity (Kuznetsov et al., *Genetics* 2002;161:1321). In collaboration with Jacob Brown and Brian Brooks, we devised a method for using LCM to integrate small regions from the developing eye around the time of topological closure in the mouse embryo for comprehensive gene expression analysis in order to identify candidate genes associated with coloboma, a common human development defect. We are applying dimensionality reduction algorithms to identify temporal classes within Affymetrix® gene expression data from LCM-dissected target tissue for eight distinct time points around the time of normal retina closure. We will then apply such analysis to databases of gene pathway, transcription factor–related expression, and so forth in order to develop a more integrative understanding of the normal developmental program and extend the list of candidate genes that may be associated with the occurrence of coloboma.

To allow more routine and simpler multiplex molecular diagnostics, we are attempting to develop new approaches for better integration of our thermoplastic microtransfer methods of microdissection

with downstream macromolecular analysis. Using a variety of confocal microscopy techniques, we are systematically measuring the complex physical interactions among scanned laser dosimetry, tissue stain density, thermoplastic polymer film, and underlying tissue sections. Using finite-element modeling of thermal and fluid flow to fit the observed microbonding, we are refining our understanding of expression microdissection physics in order to automatically predict and optimize the process for any given tissue sample. Coupling the robust and simple automatic microdissection with rapid purification and detection of species might provide unique abilities for following macromolecular changes in normal tissue development and in pathologies such as cancer progression within prostate, colon, breast, lung, and ovary tissues. In continuing collaborations with NCI, we have developed standard procedures for isolating normal and pathological cells from clinical specimens. We have used our models of the statistics of expression levels in cell populations to identify genes differentially expressed in temporal patterns such as cancer progression. To date, our analysis points to a critical role for many less abundantly expressed genes at a critical stage of ovarian cancer progression, suggesting that, for most cancers, critical diagnostic marker sets should include such low-abundance transcripts. This notion is guiding our research in statistics of less abundant gene products and suitable detection methods.

We foresee an evolution of molecular diagnosis from one based on qualitative or quantitative analysis of a few key macromolecules to one in which dimensionality reduction and classification algorithms analyze complex multivariate databases. Such analyses should allow a more complete identification of highly correlated clinical cases and the characterization of their response to molecular therapies specifically designed to prevent progression.

Prevention of progression of age-related macular degeneration through photoprotection
Majumdar, Ory, Meyers, Kisseleva, Ostrovsky, Bonner; in collaboration with de Monasterio, Cunningham, Chew, Wong, Avetisov

Our clinical studies are designed to test our biophysical model and clinical hypothesis that spectral imbalances of light reaching the retina lead to increased levels of toxic photochemicals within the retinal pigment epithelium, thereby driving early stages of AMD. If our hypothesis is correct, we believe that, during the earliest stages of retinal pathology, this process may be reversible. Therefore, progression to more advanced AMD might be prevented with the use of spectral sunglasses in bright daylight or by otherwise altering the spectrum of ambient light reaching the retina. Using our specifically designed bicolored sunglasses, we have begun non-invasive clinical studies in subjects who have undergone bilateral cataract surgery. Our aim is to image expected changes in the distribution of toxic photochemicals within the human retina. We are improving the autofluorescence imaging protocols and multispectral image analysis in order to provide high-resolution, molecularly specific images of early microscopic changes, thereby allowing us to follow the lesions' changes over time in relation to photochemicals present in various retinal cell layers. In collaboration with the University of Maryland's experts in the mathematical analysis of hyperspectral images and clinicians in NEI's Clinical Branch, we are developing and testing new quantitative tools for following non-invasively the earliest changes in macular pathology in AMD and other retinal diseases. Our goal is to provide better early diagnosis and methods to evaluate early intervention and disease prevention strategies.

In our clinical collaborations at the NIH and with the Institute of Eye Diseases in Moscow, we are seeking to apply non-invasive spectral imaging methods to study early photochemical injury and the means of limiting it. Lipofuscin (including undigestible fluorescent photoproducts of the A2E pathway) accumulates with age in the RPE and co-localizes with the acute injury caused by photosensitization of reactive oxygen intermediates (ROI) in the primate retina when exposed to unnaturally high doses of blue light. This observation along with a variety of epidemiological studies has led to a widely held hypothesis that cumulative blue-light photochemistry is harmful

and plays a role in AMD progression. We have developed an alternative biophysical model of (1) the normal accumulation during aging of potentially damaging photoproducts in the RPE and (2) changes in this "natural" aging associated with external spectral filtering (sunglasses) or cataract surgery with intra-ocular lens implantation. The precursors (principally A2PEH2) of A2E and its oxidation products that segregate within lipofuscin granules in the RPE originate from reactions of all-*trans*-retinal within the photoreceptor outer segments (ROS) when in bright daylight. Although RPE lysosomal processing enzymatically digests over 99 percent of shed ROS contents, A2E and related fluorophores are not broken down but rather concentrate into lipofuscin granules. By age 60, the average steady-state concentrations within the RPE reach approximately 200 μM in normal eyes. However, A2E is toxic to cellular membranes at much lower concentrations.

Our novel hypothesis (Meyers et al., *Trans Am Ophthalmol Soc* 2004;102:83) posits that blue light photosensitizes singlet oxygen generation within lipofuscin granules. The singlet oxygen efficiently oxidizes A2E within the lipofuscin granule in which it was generated and is the principal means of controlling steady-state levels of A2E in the RPE. As short-wavelength macular irradiance decreases with age, the rate of A2E photo-oxidation falls by approximately up to 20-fold, causing the syeady-state concentration ($[A2E]_{ss}$) in the normal phakic eye to increase even as rod bleaching and A2E production decrease. Our theoretical model of macular aging reproduces the normal age dependence of lipofuscin and A2E. It provides a primary cytotoxic mechanism in which, once A2E reaches a threshold concentration in the RPE cell, A2E redistribution into critical membranes causes damage with or without additional photo-activation. The model also predicts that, in normal RPE, nearly constant levels of A2E are maintained at a given age and lens color, irrespective of total ambient light exposure. It is primarily the yellowing of the lens with age that distorts the original spectral balance between the rate of production and rate of photo-oxidation found in youth, allowing $[A2E]_{ss}$ to rise with age. If our model is correct, then restoring or optimizing the spectral balance with external spectrally selective sunglasses could significantly lower A2E levels and may prevent associated macular degeneration. Our clinical studies using non-invasive, multispectral autofluorescence imaging to map the levels of A2E and other retinal photochemicals are designed to test the predictions of our model and the benefits of specific spectral photoprotective filters.

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MEMBRANE FUSION MEDIATED BY VIRAL AND DEVELOPMENTAL PROTEIN FUSOGENS

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While our earlier work on mechanisms of protein-mediated membrane fusion focused on membrane rearrangements yielding a fusion pore (FP), we have recently studied the mechanisms of FP expansion. Transition from nanometer-sized nascent FPs to an open lumen of cell-size diameter is an important and unexplored stage of cell-to-cell fusion in both development and pathophysiology. Using confocal microscopy, we studied expansion of the micron-scale FPs in syncytium formation initiated by baculovirus gp64. In contrast to the opening of a pore, FP growth requires cell metabolism. While the actin cortex has been proposed to drive FP expansion, our model demonstrates that actin structures slow expansion and disassemble under the pore. We plan to test the applicability of the mechanisms of syncytium formation to biologically relevant cell fusion processes. We also explored intracellular delivery of the chemical conjugates of RNase-resistant phosphorodiamidate morpholino oligomers (PMO), which are promising reagents for redirecting the splicing machinery. We varied the charge and hydrophobicity of these conjugates and monitored their cellular uptake and splicing correction efficiency. Our work identifies the stage of endosomal escape of PMO as a limiting stage in PMO delivery.

Fusion pore expansion during syncytium formation is restricted by an actin network
Chen, Leikina, Melikov, Chernomordik; in collaboration with Kozlov, Podbilewicz

Cell-cell fusion in animal development involves expansion of nascent fusion pores formed by protein fusogens to yield an open lumen of cell-size diameter. Earlier work on fusion mechanisms focused on the initial stages of the fusion pathways, which yield fusion pores of a few nanometers in diameter. In syncytium formation, these pores expand to ones readily detectable by fluorescence microscopy (diameter greater than about 0.2 μm) and finally yield an open lumen with an approximate cell-size diameter of 10–15 μm. Little is known about the properties of these larger pores and the mechanisms that underlie the enlargement of cytoplasmic bridges from early fusion pores to syncytia. For instance, we still do not know whether the enlargement is driven by the fusogens, proceeds spontaneously, or is driven by the cytoskeleton, membrane tension, or other as yet unidentified cell machinery.

In our most recent work, we explored the enlargement of micron-scale pores in syncytium formation initiated by baculovirus gp64, a well-characterized fusogen. Low pH–triggered conformational change in gp64 results in a fast opening of fusion pores and inactivation of the fusogen. Thus, these properties of gp64-mediated cell fusion allowed effective uncoupling of fusogen-specific early fusion

stages from the pore expansion stages. To visualize the three-dimensional morphological changes in the contact zone during the opening and expansion of the fusion pore(s), we labeled $Sf9^{Op1D}$ cells expressing gp64 with fluorescent lipids and imaged the cells with three-dimensional time-lapse confocal microscopy as they underwent fusion. In both live-cell experiments and after fixing cells, we analyzed the development of fusion pores large enough to be detected by light.

The pores were roughly circular and expanded radially until they came close to one another, whereupon their shapes begin to distort. Fusion pore expansion within the zone of tight contact was accompanied by an increase in the cell contact area, suggesting that fusion pores grow by displacement of membrane material toward the contact zone periphery. Pore growth is driven neither by membrane tension nor by microtubule cytoskeleton but rather depends on cell metabolism and is accompanied by a local disassembly of the actin cortex under the pores. Polymerization and depolymerization of actin filaments inhibited and promoted pore expansion and syncytium formation, respectively, indicating that the actin cytoskeleton restricts rather than drives the expansion of fusion pores. We propose that the growth of the strongly bent fusion pore rim is restricted by dynamic resistance of the actin network and driven by membrane-bending proteins involved in the generation of highly curved intracellular membrane compartments.

Chen A, Leikina E, Melikov K, Podbilewicz B, Kozlov MM, Chernomordik LV. Fusion pore expansion during syncytium formation is restricted by an actin network. *J Cell Sci* 2008;121:3619-3628.

Delivery of steric block morpholino oligomers by $(R\text{-}X\text{-}R)_4$ peptides: structure activity studies

Yang, Chernomordik; in collaboration with Abes R, Moulton, Clair, Abes S, Kamran, Melikov, Prevot, Youngblood, Iversen, Lebleu

Several arginine-rich peptides have been shown to traverse the cell membrane and reach the cytosol and nucleus. These peptides, referred to as cell-penetrating peptides (CPP), have been shown to facilitate intracellular delivery of conjugated (or fused) macromolecules while retaining their biological activity. In our earlier work, we established that cationic CPP enter the cell by endocytosis, although the mechanisms by which CPP cross the endosomal membrane to be delivered into the cytosol and nucleus remain unknown. Recent studies have indicated that cationic peptides such as oligoarginines and Tat 48-60 are relatively inefficient in transporting uncharged oligonucleotide (ON) analogues such as peptide nucleic acids (PNA) or phosphorodiamidate morpholino oligomers (PMO) largely because CPP-conjugated material remains trapped in endocytic vesicles. Given that redirecting the splicing machinery through the hybridization of high-affinity, RNase-incompetent PNA and PMO might lead to important clinical applications, our collaborators recently explored the efficiency of splicing correction by a number of PMO conjugates. Among these conjugates, $(R\text{-}Ahx\text{-}R)_4$ (Ahx stands for 6-aminohexanoic acid) was the most efficient in the tested group. Importantly, $(R\text{-}Ahx\text{-}R)_4$-PMO conjugates are effective in mouse models of various viral infections and Duchenne's muscular dystrophy.

To identify structural features facilitating efficient nuclear delivery and to uncover limiting steps in the internalization pathway of conjugated PMO, we carried out structure-activity characterization. We observed a significant correlation among splicing correction efficiency, affinity for heparin, and the ability to destabilize model synthetic vesicles but found no correlation with efficiency of cellular uptake. To evaluate the ability of CPP-PMO conjugates to escape from endosomes, we employed a liposome leakage assay. It is known that late endosomes are characterized by an unusual lipid composition—they are enriched in bis(monooleoylglycero) phosphate—and that their lumen is acidic (pH 5.5). We therefore prepared liposomes from the lipid mixture mimicking the lipid composition of late endosomes and explored the effect of low pH on the CPP-PMO–induced dye escape. In correlation with the data on splicing activity, the $(R\text{-}Ahx\text{-}R)_4$-PMO conjugate induced significantly more low pH-dependent leakage than other tested conjugates. Interestingly, the more

hydrophobic (R-AbuL-R)$_4$-PMO (Abu stands for 4-aminobutyric acid) induced considerably less leakage than (R-Ahx-R)$_4$-PMO. Our findings indicate that the efficiency of delivery of the splice-redirecting ON analogues correlates with their ability to destabilize membranes with a lipid composition mimicking that of the late endosomal membranes.

Our work substantiates the hypothesis that entry of CPP proceeds through binding to cell-surface heparin sulfates, endocytosis, and escape from endosomal compartments, with the escape stage dramatically limiting the efficiency of entry. We expect future work on the mechanisms of low pH– and lipid composition–dependent endosomal escape to bring about better cell-penetrating reagents required for drug delivery and to provide new insights into intracellular trafficking of internalized material.

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QUANTITATIVE BIOPHOTONICS FOR TISSUE CHARACTERIZATION AND FUNCTION

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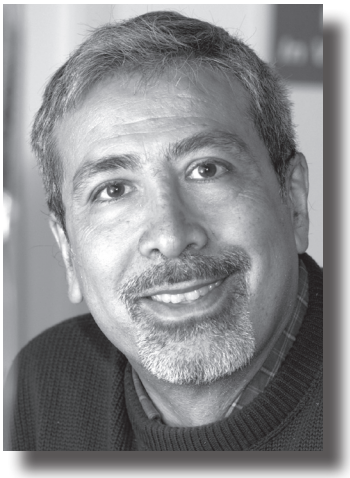

Our objectives are to devise quantitative biophotonics methodologies and associated instrumentations in order to study (1) biological phenomena at different length scales—from nanoscopy to microscopy—and (2) diffuse biophotonics. We take advantage of our expertise in stochastic modeling to study complex biological phenomena whose properties are characterized by elements of randomness in both time and space, such as light-tissue interactions. We explore various properties of light-matter interactions as sources of optical contrasts, such as polarization properties, endogenous or exogenous fluorescent labels, absorption (e.g., hemoglobin or chromophore concentration), and/or scattering. We have used these contrast mechanisms for tomographic and spectroscopic methods to develop benchtop instrumentation for preclinical and clinical uses. We are identifying physiological sites where optical techniques might be clinically practical and offer new diagnostic knowledge and/or less morbidity than existing diagnostic methods.

Quantitative characterization of tissue

Sviridov, Ulissi, Chernomordik, Gandjbakhche; in collaboration with Hebden, Weiss, Smith

Many components of biological tissues—such as collagen, muscle fibers, keratin, retina, and glucose—demonstrate polarization properties attributable to their anisotropy. As a result, depolarization of initially polarized light is observable and strongly dependent on the tissue's bulk optical properties. Mapping the degree of polarization may yield valuable diagnostic information about the superficial and subsurface structures of the skin and other tissues. A statistical analysis of data can exclude distortions in the polarization degree maps and enhance the observability of hidden structures, permitting quantification of the structures' characteristic scales and directionality.

We use the pattern of the surface distribution of the degree of polarization to detect and analyze structures of initial skin fibrosis at the early stages of abnormality when it is barely visible. We devised a new data-processing algorithm that involves Pearson correlation analysis (Sviridov et al., *J Biomed Opt* 2005;10:051706). The technique reduces blurring of features by unpolarized backscattered light, permitting visualization of regions of high statistical similarities within the noisy tissue images. We are developing a hand-held camera for polarization imaging of biological tissue structures. Phantom experiments have demonstrated the ability of the designed system to visualize the collagen network at the scale of 10 to 20 μ. One potential application of the methodology is for

characterizing the state of the uterus to predict preterm birth. In collaboration with physicians, we plan to analyze the results of in vivo tests to optimize the potential diagnostic content of the data.

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Preclinical studies on fluorescence imaging of HER2-positive breast cancer

Hassan, Riley, Chernomordik, Gandjbakhche; in collaboration with Capala, Lee, Gannot, Smith

Advances in the molecular biology of disease processes, new immunohistopathological techniques, and the development of fluorescently labeled cell surface markers have led to a revolution in (1) specific molecular diagnosis of disease by histopathology and (2) research into the molecular origins of disease processes. As a result, an exceptional level of specificity is now possible in the design of exogenous markers, permitting molecules to be engineered to bind only to specific receptor sites in the body. Such receptor sites may be antibodies or other biologically interesting molecules. Fluorophores bound to the engineered molecules preferentially concentrate at the sites of interest after injection. Such concentration parallels the delivery of some novel drugs, e.g., Trastuzumab, to the diseased site and opens a new era of the "treat and image" paradigm.

To optimize the course of the treatment of human epidermal growth factor receptor 2 (HER2)–positive breast cancer (and exclude from HER2-based chemotherapy those patients with other forms of breast carcinoma), there is a need for molecular probes capable of in vivo monitoring of a receptor downregulated as an immediate response to such therapy. In collaboration with Jacek Capala, we developed fluorescent markers, based on HER2-specific affibody molecules, for in vivo monitoring of the level of overexpression of *HER2*. These small molecules possess high affinity for HER2 and do not compete with trastuzumab because they target a different epitope of the receptor, making them an appropriate choice as a contrast agent. The potential application is based on fluorescence intensity distributions originating from specific markers in the tumor area. The measured time course of marker variation after injection of the contrast agent can provide information on the status of *HER2* expression in the tumor cells—information that may guide adjustments to the treatment regimen. For preclinical studies, we used a mouse model with xenografts of various carcinomas implanted into the mouse flank and tested two types of affibodies: albumin-binding domain (ABD) and monomer. Results showed that ABD performed better based on both marker accumulation in the tumor and reasonable clearance times. The developed methodology allows us to investigate the pharmacokinetics of the contrast agent and potentially assess the status of HER2 receptors. We have initiated a comparative study in the mouse model for several types of breast carcinoma (BT474, MDA-MB361, U251, and MCF7) that are characterized by different levels of amplification and overexpression of *HER2*.

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Fluorescence life-time imaging

Hassan, Riley, Chernomordik, Gandjbakhche; in collaboration with Capala, Gannot, Smith

With specially designed fluorophores, it is possible to probe environmental conditions (e.g., pH) at a given site, based on local variations in fluorophore life time (the time required for an electron to return from an excited state to its initial state). To use fluorophores to target abnormalities that are likely to be located deep inside biological tissues, we developed reconstruction algorithms that take

into account the highly scattering nature of the medium. Using our well-established small animal time-resolved imaging system, along with a quantitative time-resolved fluorescence diffuse inverse method, we showed that, for tissue-like phantoms, we could reconstruct intrinsic fluorescence life times from targets deeply embedded in a turbid medium, e.g., tissue. Three-dimensional localization of the fluorophores with the life-time mapping may be used to characterize tumor status; the technique provides useful clinical information, e.g., pH maps obtained with pH-sensitive dyes, and can help localize cancer cells and monitor treatment.

To investigate three-dimensional reconstruction algorithms, we used generalized analytical formulas for excitation/emission photon migration as a forward model. Experimental data obtained with our time-resolved instrumentation confirmed limitations of data analysis because of noise. For example, a low signal-to-noise ratio for photon times of flight greater than 5 to 6 ns precludes use of this time range to quantify fluorescence characteristics. The accuracy of the three-dimensional reconstruction critically depends on the noise sensitivity of input parameters. We have suggested that a new local set of data types is likely to provide more stability to noise than classical statistical moments (global data types). The equivalent forms of the moments in the "local" data types, based on the geometry (shape) of the temporal point spread function (TPSF), are the peak value, peak point, full width at half maximum (FWHM), and slope of the curve. To compare various data types, we created a numeric model of time-resolved fluorescence from fluorophores inside a turbid medium and showed that the local data types were much more resilient to noise than their standard global counterparts. After demonstrating this as the appropriate noise type versus the expected Poisson noise, we performed the analysis by using Gaussian noise.

To test further the relative merits of the two sets of data types (global and local), we developed a generic reconstruction algorithm that allowed substitution of the data types. Though initial results for mathematical models and phantoms are encouraging, we need to investigate distributed fluorescent dyes. When life times were comparable to photon migration times, we have found scaling relationships for time-of-flight distributions of emission photons at different depths. Our phantom data substantiate the findings. Taking into account that background optical parameters of the medium may be estimated from multiple-source detector data sets, we suggested a multistep methodology of intrinsic life-time quantification based on the scaling relationships.

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Using multimodality imaging techniques to monitor tissue vasculature in Kaposi's sarcoma lesions

Hassan, Vogel, Riley, Chernomordik, Kainerstorfer, Gandjbakhche; in collaboration with Demos, Yarchoan, Binzoni

An intense effort is currently under way to develop novel targeted therapies for use in a variety of diseases, with a strong focus on adequate methods to monitor responses that correlate with or even predict the outcome of illnesses of interest. We have developed and are using three non-invasive imaging techniques: near-infrared multispectral imaging, thermography, and laser Doppler imaging (LDI). Combining multispectral imaging and LDI allows us to determine changes in blood volume, oxygenation state, and blood velocity of the microvasculature and location of an abnormality.

The near-infrared multispectral imaging system used in our laboratory was designed and built in collaboration with Lawrence Livermore National Laboratory. To exclude specular reflection from the skin, we detect photons in cross-polarized mode by using a pair of polarizers with orthogonal

orientations in the illumination and detection arms. A spectral filter placed in front of a CCD[5] camera permits the consecutive acquisition of six wavelength images (700, 750, 800, 850, 900, and 1,000 nm). With our two-layer skin model, we then use the images in a reconstruction of the distribution of analytes such as oxy- and deoxy-hemoglobin and melanin in the tissue. To exclude non-physiological contributions to the image, we developed a curvature-correction, rigid-registration algorithm that uses a Canny edge detector to extract the edges of the imaged body part and to make alignment more accurate. Our reconstruction algorithm allows us to extract physiological information from spectral images and has undergone substantiation by Monte Carlo simulations of photon migration. We have shown that expected differences in photon trajectories attributable to variations in tissue optical parameters at considered wavelengths result in minor errors in the quantification of physiological parameters such as blood volume/oxygenation. We have demonstrated that combining the three non-invasive imaging systems (multispectral imaging, thermography, and LDI) provides clinically useful information to evaluate follow-up treatment efficiency in Kaposi's sarcoma patients.

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Cellular dynamics of angiogenesis

Amyot, Small, Ulissi, Gandjbakhche; in collaboration with Sackett, Boukari, Plant, Camphausen, Neagu

Much debate remains on which form of vascular endothelial growth factor (VEGF) guides chemotactic migration of cells to form a capillary network and on the roles played by the extracellular matrix and matrix metalloproteases (MMP). Given that growth factor gradients guide chemotactic migration, we devised a system of reaction-diffusion equations to model VEGF's diffusion, binding, and cleavage in vivo. Our simulations showed that rapid binding to the matrix led to short-range gradients of matrix-bound VEGF, whereas slower binding led to longer-range gradients. Our findings are consistent with in vivo observations that the vasculature is disorganized around tumors that produce the fast-binding isoforms of VEGF. Only the slowly binding forms of VEGF produce long-range gradients that can guide cell migration to form an efficient and organized network. We also found that the cleaved form of VEGF, removed from the matrix by the action of MMPs, is distributed with a gradient that points away from the tumor, calling into question whether the cleaved form of VEGF plays a chemotactic role in tumor-induced angiogenesis. Our observation is consistent with the findings that cleaved VEGF molecules have a weaker chemotactic effect on receptors. Our simulations showed that, without MMPs, a gradient of matrix-bound VEGF cannot be sustained, consistent with findings that the production of MMPs by cells near the parent capillary is necessary to initiate the formation of new vasculature.

Growth factor distribution, MMP release, and cell migration, differentiation, and aggregation are major components of tumor-induced angiogenesis. We have developed a model that includes these phenomena and the interaction of endothelial cells (EC) with the extracellular matrix (ECM). ECs switch among growth, differentiation, motility, and apoptotic behavior in response to the ECM's local topology and composition. Based on the assumption that the ECM medium is a statistically inhomogeneous medium (not all areas support sprout growth), we showed that the ECM can be a natural barrier to angiogenesis. We studied vascular network formation for several ECM distributions

and topologies and found an analogy with percolation theory in network formation. A threshold exists under which sprouts cannot reach the tumor. During the growth of the vascular network, attraction exerted by the tumor competes with the preferred path created by the ECM. We also examined the influence of branching on tumor vascularization; branching is a natural phenomenon that helps the tumor become vascularized. By increasing the number of sprouts, the vascular network increases the probability of reaching the tumor, as it can explore more pathways. Our simulations showed that, after two branching events, the vascular network is highly likely to reach the tumor.

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Virtual quantification of confocal microscopy systems

Riley, Gandjbakhche; in collaboration with Combs, Smirnov, Knutson, Balaban, Nossal, Boukari

In collaboration with Robert Balaban and colleagues, we have been working on quantification of photonic behavior in two-photon microscopy. That work, in turn, has led to a collaboration with Hacène Boukari to quantify photon behavior in fluorescence correlation spectroscopy.

Recent developments in two-photon imaging have aimed at pushing back the depth limit of this remarkable imaging technique. In particular, we have been involved in the development and quantification of a new approach to enhance two-photon microscopy. The basic tenet of the approach is to obtain total emission detection (TED) as opposed to the usual epi/trans detection of two-photon imaging. Others have developed an instrument called "MoreLight" that uses optics to capture light from all directions; it represents an advance over the small solid angle usually used in two-photon imaging. To quantify the instrument, our group developed a simple emission-only fluorescence Monte Carlo model. Given that we are dealing with scattered light (light emitted through tissue), we use a photonic model rather than a wave model. We can safely ignore the diffraction blurring because we are at the boundary of photonic/wave behavior of light and are considering only the gain of the instrument. The initial results from our algorithm matched well with data from a phantom study (given a scaling factor based on known instrumental losses due to efficiency of optical components). Use of the virtual modeling tool also permitted further quantification of the effective gain in different tissue types.

We are now collaborating with Ralph Nossal and Hacène Boukari in the Program in Physical Biology to quantify the effects of scattering in fluorescence correlation spectroscopy (FCS). It is common to use microbeads in FCS to examine a molecule's diffusion rates; however, the microbeads' effect on imaging needs further consideration. It is generally noted that some blurring occurs, but no quantification is currently available. We have thus created a second Monte Carlo simulation that looks at the source field for a confocal instrument and noted that the effects of scatter on an FCS illumination field are more complex than just blurring. The scattering (and relative anisotropy) has significant effects on the shape and location of the illuminated region, thus biasing the monitored diffusion rate. We have shown how scattering can blur the confocal spot entirely and how relatively high anisotropy factors can reduce this effect. It is thus important to understand the size and refractive index of the microbeads and how they affect the illumination field in FCS.

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HIV PATHOGENS IN HUMAN LYMPHOID TISSUE

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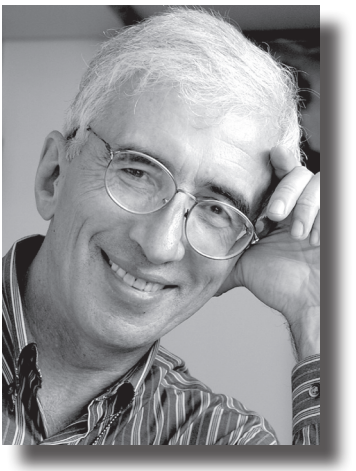

Host and viral factors largely determine the course of HIV infection. Among host factors, tissue immuno-activation plays a critical role. Among viral factors, viral phenotype is important, as illustrated by accelerated HIV disease progression associated with the evolution of CXCR4-using HIV-1 at the late stages of disease and by the exclusive transmission of CCR5-using HIV-1 clade B. Non–HIV pathogens are important host factors that affect HIV infection. To investigate the role of immuno-activation in HIV infection, to decipher the mechanisms of HIV interactions with other microbes, and to use the interactions to contain HIV infection in complex tissue microenvironments, we studied viral pathogenesis in various human tissues infected ex vivo with HIV-1 alone or in combination with other viruses. The first study focused on the role of immuno-activation in HIV-1 pathogenesis; the second study investigated the mechanisms of interactions of non–HIV microbes with HIV-1 in co-infected tissues; and the third study exploited the ability of herpesviruses to transform an antiherpetic drug into an HIV suppressor. The results of these studies provide an understanding of basic mechanisms of HIV transmission, evolution, and interactions with other viruses in human tissues and lay the foundation for designing new strategies to prevent and contain HIV infection.

Critical role of tissue activation in HIV infection

Biancotto, Grivel, Lisco, Vanpouille, Margolis; in collaboration with Lederman

Paradoxically, infection with HIV, the virus that eventually leads to immunodeficiency, depends on tissue immuno-activation, and HIV itself immuno-activates tissues. We propose that immune activation in secondary lymphoid tissues is central to the pathogenesis of immune deficiency in chronic HIV-1 infection. In vivo, the level of activation may predict the pace of CD4 depletion and the onset of clinical immune deficiency. Earlier, we found significant perturbation of cytokine secretion in HIV-infected lymph nodes. We also observed a dramatic alteration in the normal pattern of constitutive cytokine expression, with some cytokines such as interleukin-1β, interleukin-2, interferon-γ, and interleukin-15 characteristically found in increased concentrations and some others such as MIP-1α and SDF-1β substantially diminished.

In addition, we found a profound upregulation of the activation marker CD38 in naive, central-memory, and effector $CD4^+$ and $CD8^+$ T cells. The distorted activation patterns of lymphocytes in lymph nodes and tonsils from HIV-1–infected individuals emphasize the important role of tissue activation in HIV-1 disease progression. To study this phenomenon in a laboratory setting,

we used a system of blocks of human lymphoid tissue developed earlier and a newly developed system of cervico-vaginal tissue ex vivo. (In vivo, both tissues are critical in HIV transmission and pathogenesis.) Both types of tissue revealed a similar response to HIV infection: HIV-1 infection was associated with the activation of both HIV-1-infected and uninfected (bystander) T cells in infected tissues. Given that productive HIV infection is limited to activated cells, HIV-1 infection facilitates its own production by creating new cellular targets for viral infection. Moreover, with a selective increase in apoptosis in productively infected T cells expressing the activation markers CD25/HLA-DR, the vicious circle of HIV infection-activation-infection contributes to T-cell depletion, the hallmark of HIV infection. Such a mechanism, especially in cervico-vaginal tissue, the main site for HIV transmission, may play a critical role in susceptibility of the vagina to HIV transmission. The targeting of individual elements of this cycle may constitute a new basis for preventive and therapeutic strategies against HIV infection.

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Effect of non–HIV pathogens on HIV infection in human lymphoid tissue

Biancotto, Sassi,[1] Grivel, Vanpouille, Brichacek, Margolis; in collaboration with Sher, Golding

We continue to study the effect of non–HIV pathogens on HIV infection in human lymphoid tissue. Earlier, on the basis of our studies on HIV interactions with human herpesviruses (HHV) 6 and HHV7 and vaccinia and measles viruses, we found that these microbes affect HIV-1 by inducing the release of various cytokines (chemokines) in tissue as well as by altering the expression of cell-surface molecules relevant to HIV infection. In this study, we investigated HIV interactions with two HIV-1 co-pathogens, human cytomegalovirus (HCMV) or HHV-5 and the cellular parasite *Toxoplasma gondii.* In vivo, the interactions occur in the context of co-infected tissue. To investigate these phenomena under controlled laboratory conditions, we co-infected human lymphoid tissue ex vivo with HIV-1 of different co-receptor usages and with one of the non–HIV pathogens.

HCMV is typically latent in healthy individuals; however, reactivation frequently occurs in immuno-compromised individuals and can lead to severe organ dysfunction. Therefore, HCMV can become an important HIV co-pathogen. HCMV induces disease only in humans; thus, there is currently no adequate model to study the replication of HCMV in tissues. To support HCMV replication, we optimized the system of human tonsillar tissue ex vivo that was developed earlier to study HIV-1 pathogenesis in human tissue. To monitor HCMV infection, we used a recombinant strain of HCMV that expresses the green fluorescent protein CMVPT30-gfp. In addition, we used detection of HCMV DNA to characterize chronic infection in controls and analyzed infection of individual cells by flow cytometry. HCMV readily replicated in tissue blocks, as revealed by the release of HCMV viral DNA and a rise in the number of viral-positive cells. Immuno-phenotyping of HCMV-infected cells showed the preferential infection of activated lymphocytes ($CD3^+$, $HLA\text{-}DR^+$, and $CD3^+$ $CD25^+$) and of plasmocytoid and myeloid dendritic cells ($CD3^-$ $CD123^+$ $CD11c^+$). The number of these cells significantly increased in HIV-1 co-infected tissues. Accordingly, HCMV replication was enhanced two- to three-fold; such upregulation occurred in tissues infected with either CXCR4- or CCR5-using HIV-1. Thus, HIV-1 creates new targets for HCMV, perhaps explaining the strong association of HCMV with HIV-1 infection in vivo. Human lymphoid tissue infected ex vivo constitutes a model

for the study of mechanisms of HCMV tissue pathogenesis and its interactions with HIV-1 and may provide new targets for anti–HIV-1 therapy.

Human lymphoid tissue ex vivo also provides a model to study HIV interaction with non-viral HIV pathogens. The apicomplexan parasite *Toxoplasma gondii* is a common HIV co-pathogen associated with AIDS development. We examined the interaction of *T. gondii* and HIV in co-infected human lymphoid tissue ex vivo. Both pathogens readily replicate in ex vivo–infected blocks of human tonsillar tissue. Surprisingly, we found that live *T. gondii* preferentially inhibits replication of CCR5-using HIV-1 in co-infected tissues. This effect is reproduced in treatment of the tissue blocks with recombinant C-18, a *T. gondii*–encoded cyclophilin that binds to CCR5. These ex vivo findings raise the possibility that, in addition to functioning as a co-factor in HIV disease, *T. gondii* may influence the outcome of viral infection by preferentially suppressing CCR5-using HIV-1 variants.

Our studies of HIV interactions with non–HIV pathogens in human tissues, together with earlier published results, revealed diverse mechanisms of local HIV interactions with non–HIV viruses in lymphoid tissues. The mechanisms involve activation of T lymphocytes, thereby providing for new targets for HIV, competition for T cells between HIV and other microbes, downregulation of HIV receptors and co-receptors as well as upregulation of HIV co-receptor ligands (both tissue- and pathogen-encoded), and depletion of target cells. HIV co-pathogens may differentially generate positive and negative signals for HIV replication. We attempted to mimic one of the negative signals for HIV-1 of different phenotypes, thus affecting selection of particular HIV variants and eventually changing the course of HIV disease.

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Up- and downregulation of HIV-1 replication in cervico-vaginal tissue

Lisco, Vanpouille, Grivel, Biancotto, Margolis; in collaboration with Anton, Balzarini, McGuigan, Schinazi, Shattock, Münch, Kirchhoff

Sexual intercourse is the major route of HIV transmission. Therefore, it is important to identify the endogenous factors that affect the efficiency of sexual viral transmission and to develop strategies to prevent and contain infection at the critical sites of HIV transmission and pathogenesis. We participated in the identification of a semen factor that facilitates HIV replication and showed that the factor upregulates HIV replication in infected T cells, macrophages, ex vivo human tonsillar tissues, and cervico-vaginal tissues. The factor consists of naturally occurring fragments of the abundant semen marker prostatic acidic phosphatase, which form amyloid fibrils. Thus, the fibrils may play an important role in sexual transmission of HIV and could represent new targets for its prevention.

Earlier, we found that, in HIV-infected tissues, it is also possible to generate factors that downregulate HIV-1 infection. These factors may be generated by other pathogens (see above). Here, we suggest a new strategy for generating such factors: we exploit the unique ability of herpesvirus kinases to phosphorylate acyclovir (ACV), the first step in transforming this otherwise inert compound into an inhibitor of herpesvirus DNA polymerases. Surprisingly, we found that in a cell-free system, phosphorylated ACV directly suppresses HIV reverse transcriptase (RT). We showed that HIV-1 RT incorporates ACV-TP into the nascent HIV-1 DNA chain with a level of efficiency similar to that of its natural equivalent dGTP. Incorporation of ACV-TP results in the formation of a dead-

end complex and traps RT at the site of termination, leading to the complete termination of reverse transcription. Accordingly, we found that, in tissues co-infected with HHVs that are capable of phosphorylating ACV, HIV-1 was inhibited. We demonstrated such inhibition in cervico-vaginal tissue as well as in tonsils, lymph nodes, and colorectal tissue, where the critical events of HIV-1 pathogenesis and transmission occur in vivo. Various HHVs, including the ubiquitous HHV-6 (detected in all but one tissue ex vivo), may mediate HIV-1 suppression by ACV. We could detect no anti–HIV ACV activity in either the HHV-free tissue or HHV-free cell lines. However, when we added HHV-6–infected cells to HIV-1–infected cultures, ACV became a potent suppressor of HIV-1 replication. In summary, the following mechanism seems to be responsible for ACV suppression of HIV-1 in human tissues ex vivo, the majority of which carry one or several HHVs, including HHV-6: ACV is monophosphorylated by herpesviral enzymes in HHV-infected cells, and then cellular kinases convert it into ACV-TP, which suppresses HIV in co-infected tissues by inhibiting HIV-1 RT. Our results suggest that ACV may be therapeutically beneficial for various HIV-1–infected patients, as the majority of humans are already infected with various HHVs that transform ACV, at least during reactivation. However, clinical trials are needed to test this concept in vivo. In general, the combination of ACV with an endogenous HHV infection to suppress HIV may constitute a new principle of anti–HIV therapy: a "binary weapon" in which one inert component is converted by another, endogenous component into an active therapeutic compound

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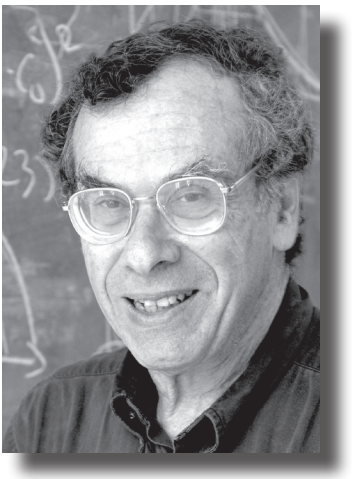
CELL BIOPHYSICS



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We study elements of cell processes involved in signal transduction, protein trafficking, and cell motility. We are particularly interested in the way cellular activities are coordinated in space and time. We develop and apply novel methodologies based on mathematical and physical principles in order to foster a better understanding of such phenomena. We focus on the origination and transformations of supramolecular cellular assemblages such as protein-coated endocytic vesicles, metabolic signaling complexes, and cytoskeletal structures. We thus have constructed specialized fluorescence-based optical instrumentation to study dynamic supramolecular processes and use advanced electromagnetic scattering techniques to examine structures on nanoscopic length scales. We have also developed mathematical models of specific aspects of cellular behavior.

Biophysical methods and models

Boukari, Nossal, Sackett; in collaboration with Michelman-Ribeiro, Horkay, Silva, Brichacek, Margolis, Gandjbakhche, Riley

We continue to expand the use of physics-based methodologies to study aspects of complex biological phenomena. For example, we develop new analytical methods based on fluorescence correlation spectroscopy (FCS), Fourier transform analysis, and quantitative microscopy to study biological structure and behavior. In particular, we devised new techniques that permit us to examine the movement of particles—varying in size from small metabolites to viruses—through concentrated polymer solutions and dense, interconnected polymer matrices. Initially, our studies used models of non-biological origin to establish the physical basis of the methods. Measurements indicated that the diffusion of materials through a polymer gel depends not only on the polymer concentration but also on the cross-link density of the matrix. Recently, to develop a fuller comprehension of underlying physical mechanisms, we focused on discerning how the swelling or shrinking of a cross-linked hydrogel affects the translation of embedded nanoparticles. The knowledge gained should ultimately lead to, for example, a deeper understanding of the behavior of extracellular matrix material in edematous tissue. We are now employing related techniques to examine the movement of antibodies and viruses through vaginal secretions. In the case of viruses, our goal is to understand how HIV and other viruses involved in sexually transmitted diseases penetrate cervical mucus and other protective barriers to reach the cells they infect. Particle-tracking analysis of individual virions indicates that most slow down over 100-fold when compared with their movement in water and that typical diffusion does not drive the virions' translocation. Rather, a major factor is relaxation of the matrix following mechanical perturbations such as stretching and twisting of a sample. Such

relaxation gives rise to measured, ensemble-averaged displacements of virions, demonstrating a combination of anomalous diffusion and occasional jumps influenced by microenvironment properties sensed by individual virus particles.

The above phenomena occur in relatively dense media, where significant light scattering from the surrounding matrix might cause problems. In FCS measurements, for example, analysis requires reliable determination of the illuminated volume, but the incident beam might be distorted by multiple scattering. Hence, we carried out Monte Carlo simulations to examine the effects of scattering induced by a crowded solution of spherical nanoparticles and found that, as the concentration or size of the particles increases, the beam-spot broadens and its focal point is shifted toward the direction of the source. We also found a concomitant reduction of the local laser beam flux. Such effects may need to be accounted for when using FCS to investigate optically dense tissue. One goal of these studies is to provide metrics that enable us to make quantitative corrections where appropriate.

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Clathrin lattice and other supramolecular assembly

Galanis, Boukari, Sackett, Nossal; in collaboration with Ferguson, Lafer, Prasad, Krueger, Harries, Schuck

Many biological functions involve the assembly of large supramolecular structures that, at first approximation, are large oligomers of specialized proteins. Receptor-mediated endocytosis, however, is a special case that involves the formation of vesicles surrounded by closed polyhedral, cage-like structures assembled from a three-legged heteropolymer, which is a triskelion composed of three clathrin heavy/light chain complexes joined at a common hub. In developing physical theories to understand how such vesicles (clathrin-coated vesicles, or CCVs) arise, we have quantitatively analyzed previously published data and have acquired our own data. Most recently, we used novel computer-based structural modeling, combined with dynamic light scattering (DLS), static light scattering (SLS), and small angle neutron scattering (SANS), to examine conformations of clathrin triskelia in solution. We were interested in determining if and how clathrin triskelia change their shape when they leave solution to assemble into coat-associated cages. We showed that triskelia are puckered when free in solution, although they exhibit a somewhat different conformation than when incorporated into a reconstituted clathrin basket, suggesting that the mechanical properties of triskelia indeed must be taken into account. Therefore, we developed a novel method based on SANS to assess the flexibility of the triskelia, permitting us to determine quantities that previously could be deduced only by somewhat indirect methodology. Results substantiate our earlier hypotheses and inferences, establishing that triskelia are semiflexible polymeric structures capable of bending when integrated into polyhedral coats of various sizes and shapes. In addition, the clathrin lattice by itself is unlikely to be sufficiently strong to cause vesicles to form with the high curvature noted in cell-biological observations. We are now applying the knowledge gained from these and related studies to investigate clathrin pit formation at the plasma membrane, with a focus on discovering how the binding of clathrin to accessory proteins such as AP-2 and AP-180 influences clathrin coat nucleation.

In another, somewhat more abstract project, we set out to study the effects of steric interference between microtubules apart from other intermolecular interactions. We aim to obtain a basic understanding of how physical boundaries influence spatial patterns that arise in concentrated ensembles of rod-shaped objects such as microtubules, amyloid plaques, and similarly shaped biological assemblies. We constructed a biomimetic analogue composed of small hard rods confined within enclosures of various sizes and shapes and subjected them to mechanical shaking to mimic thermal excitation. We found that, when the rods are confined to containers whose dimensions are

of the same order of magnitude as the lengths of the rods, conditions permit the rods to self-organize and experience a density-dependent isotropic-nematic structure transition. Recently, to investigate the role of factors that act as molecular crowders in processes such as the formation of protein aggregates on membrane surfaces, we added small spheres to such rod assemblies. Given that macroscopic models can provide information about the assembly of complex molecular structures not easily obtained by other means, we found that, for sufficiently low rod densities, "vibrofluidized" rods self-assemble in a "solution" of sphere crowders to form linear polymer-like structures. We also found that Monte Carlo simulations show similar patterns in thermally equilibrated binary mixtures. Unlike the known equilibrium phases for rod-sphere mixtures in three dimensions, our two-dimensional simulations and experiments show finite-sized aggregates, indicating that lower dimensionality prevents true phase separation and that thermodynamic depletion interactions create an effective attractive force between the rods.

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Tubulin polymers and cytoskeletal organization

Sackett, Boukari, Begaye; in collaboration with Bezrukov, Rostovtseva, Yergey, Bates, Fojo, Park, Li, Werbovetz, Fecik, Schriemer

We study both fundamental and applied aspects of tubulin biology. Tubulin polymers are central to various critical cell functions, including mitosis, intracellular transport, maintenance of cell morphology, cell motility, and perhaps mitochondrial function. We have devoted considerable effort to determining the ability of small molecules to alter the formation of microtubules (MT), which are the most rigid of the cytoskeletal polymers and thus are central to establishing non-spherical cell morphology. In addition, MTs have intrinsic polarity, and their cytoplasmic array provides the substrate for directional intracellular movement. Thus, small molecules that alter MT integrity and/or dynamics can alter the physical properties of the cytoplasm. We have been studying anti-mitotic peptides, often of marine origin, because they are among the most potent anti–MT agents known; moreover, several such peptides are available for study. In particular, certain peptides induce MT subunits to assume unusual ring-shaped supramolecular structures, which we have been studying by analytical ultracentrifugation, cryo-electronmicroscopy, fluorescence correlation spectroscopy, and protease mapping. We are also examining the effects on microtubule polymerization of synthetic analogues of thalidomide and combrestatin A and have demonstrated both the role of post-translational modifications by deacetylases and the effects of proteosome inhibitors on microtubule stability.

Recently, we identified several new modified peptides derived from the natural peptide tubulysin; these peptides show a range of anti-microtubule activities in assays with purified proteins as well as in cells. In addition, focusing on the natural compound peloruside, we examined a new type of microtubule stabilizer and demonstrated that an old chemotherapy agent, a nitrosourea, may indirectly affect microtubule stability by altering the activity of the protein stathmin. In additionally, in an effort to look for molecules that would target *Leishmania*, the infectious agent of an important group of human diseases, we are seeking to identify small molecules that do not bind well to mammalian tubulin but do bind to parasite tubulin. We have thus far identified several small molecules that bind to *Leishmania* tubulin preferentially over mammalian tubulin, preventing parasite multiplication inside human macrophage cells.

Finally, we have been studying the interactions between tubulin and supramolecular entities that affect metabolic activities of mitochondria—organelles that move along microtubules in mammalian cells. We examined the effect of tubulin binding on the function of the major mitochondrial outer membrane (MOM) channel protein VDAC[1] and found that nanomolar concentrations of tubulin

cause closure of the channel. This closure, which requires the C-terminal tail peptides found on alpha and beta tubulin, is expected to restrict exchange of ADP and ATP across the MOM. Using isolated intact mitochondria, we confirmed the effect. Added tubulin reduces the oxygen uptake associated with added ADP as reflected in a nearly 100-fold increase in K_M for ADP. Our results show that tubulin can be involved in regulating respiration in mammalian cells by interacting with VDAC on the outer membrane of mitochondria

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Complex systems biophysics

Krsko, Sackett, Nossal; in collaboration with Skupsky, Losert, Kolenbrander, Palmer

We continue to use advanced physical and mathematical methods to understand the biophysics of complex cellular processes. The role of phosphoinositide metabolism in eukaryotic cell processes such as gradient sensing in chemotactic cells and the biogenesis of coated vesicles involved in endocytosis and other intracellular transport processes is one such phenomenon. We also study the structural organization of multicellular biofilms arising from the attachment of prokaryotes to surfaces, with a focus on the extracellular polymeric substance (EPS) that fills the space between bacterial colonies. Our studies are of basic interest but are also relevant to disease processes and normal and abnormal tissue development. Each study requires the integration of several complicated processes and relies on information obtained through reductionist studies while focusing on behaviors emerging from both synergistic and competitive interactions.

We previously devised a mathematical model—based on non-linear reaction-diffusion—that captures the major kinetic behaviors of the gradient sensing observed in the chemotactic response of amoeboid cells such as *Dictyostelium* and neutrophils. Among these behaviors are the establishment of cell polarity in shallow gradients of stimulus, adaptation to changes in uniform background levels of signaling ligand, and the ability of a cell to rapidly follow movements of an excitatory chemical source. A recent extension of our work has provided an explanation for the distinct zig-zag motions occasionally observed when a neutrophil travels toward a source of chemotaxins. In addition to their involvement in gradient sensing, 3′ phosphoinositides are implicated in the biogenesis of clathrin-coated and other endocytic vesicles. We are constructing a complex, multi-element model of receptor-mediated endocytosis that encompasses cargo recognition, phosphoinositide metabolism, and clathrin coat formation and dissolution. Our analysis, which demonstrates how interrelated kinetic elements of these processes determine whether an endocytic vesicle will form, will permit us to explain how vesicle biogenesis is triggered by, for example, the binding of ligands to receptors at specific sites and subsequent recruitment of clathrin-associated proteins. We are also interested in rationalizing the observed probabilistic quality of cell response in the presence of a stimulus.

We are currently investigating various characteristics of bacterial biofilms. These films are surface-attached communities of microorganisms that express a polymer coating (EPS) that mediates material transport to the attached bacterial colonies and protects them from antimicrobial agents. Biofilms are ubiquitous in nature, yet little is known about their formation and viability. We do know that,

in human disease, many bacterial pathogens form biofilms that resist destruction, causing serious difficulties for infected patients. We have focused on measuring the mechanical and transport properties of EPS as a function of environmental parameters such as pH and externally induced shear forces and have linked the parameter to morphological features of the films. To characterize the mechanical properties of EPS, we have developed methods involving atomic force microscopy that allow us to take into account the spatial distribution of bacterial colonies within the film. We found that the soft, hydrated EPS gel, which consists mainly of polysaccharides and other macromolecules carrying labile charges, softens and stiffens according to the proton concentration in the surrounding environment, concomitantly changing the mechanical properties of the colonies. Using scanning electron microscopy, we have developed an improved technique for morphological analysis of the biofilms that preserves the structure of these fragile and highly hydrated materials upon drying, hence revealing finer details of their in situ architecture and cellular adhesion. Finally, we fabricated a multichannel culture chamber from cast PDMS[2] that allows examination of biofilms with optical instruments as well as with direct contact instruments such as an atomic force microscope.

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[1]voltage-dependent anion channel
[2]polydimethylsiloxane

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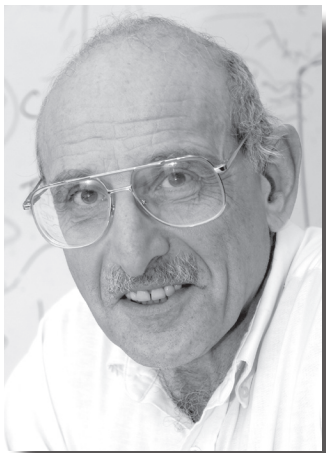
PHYSICAL FORCES ORGANIZING BIOMOLECULES



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With a long-term goal of building a practical physics of biological material, we measure, characterize, and codify the interactions that govern the organization and self-assembly of different types of biological molecules. Connected in part with the recent NIH-wide interest in nanotechnology, we are building on our experience with van der Waals fluctuation forces to formulate interactions involving carbon nanotubes not only in their assembly but also, and more important, as substrates for biopolymers such as DNA. Our undertaking is strengthened by its strong connection with physical theory. Through a series of measurements and analyses of the different types of interactions as revealed in vivo, in vitro, and in computation, we are working with DNA assemblies such as those observed in viral capsids and in vitro; polypeptides and polysaccharides in suspension; and lipid/water liquid crystals. In all these systems, we simultaneously observe the structure of packing and measure intermolecular interaction energies. We have also worked with researchers who create repulsive as well as attractive charge fluctuation forces that have implications for nanotechnology.

van der Waals forces

Parsegian, Podgornik; in collaboration with French, Mkrtchian, Rajter, Ching, Capasso, Munday

Even though van der Waals forces are (1) the dominant interaction that coheres membranes and proteins, (2) the source of the powerful surface tension at membrane interfaces, and (3) the attraction that creates membrane multilayers or allows membranes to adhere to artificial surfaces, the forces are only now undergoing systematic study in a collaboration between quantum physicists and biophysicists. This year, we used the quantum mechanical density functional theory (DFT) solved for several carbon nanotubes in order to compute the forces that cause membranes to cohere and serve as a substrate for many materials. It is remarkable that quantum chemistry combined with our expertise in macromolecular interactions is allowing us to see properties that include torque and force between carbon nanotubes.

By beginning with the elements of physical theory that relate the polarizability of materials to the fluctuations of charges within them, we have been able to design experiments that show how macromolecular organization responds to deliberate changes in solution properties. Progress thus depends on a tight coupling of modern quantum theory of structured materials with experiments and measurements that reveal electromagnetic properties.

We have teamed with groups that measure absorption spectra in order to formulate and compute van der Waals forces involving lipids, water, and ions as well as synthetic structures such as carbon

nanotubes. We have shown how charge fluctuation forces conferred by ions in solution can modify forces between lipid membranes. We have measured those forces and computed van der Waals charge fluctuation forces in those same membrane systems.

To compute forces between bodies with extended interfaces, we also extended the Lifshitz theory of van der Waals interactions in stratified media such as lipid multilamellar systems. These systems can range from the practical, such as the composite media of electric insulators, to the biological, such as the action of extended polymer layers on biological membranes. In addition, we have developed ways to measure repulsive van der Waals forces in systems in which the properties of the medium are intermediate between those of the interacting bodies. The subsequent ability to create switchable systems offers promising applications in nanotechnology.

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Molecular assembly in vitro and in viruses

Bezrukov, Harries,[1] Parsegian, Petrache,[2] Podgornik; in collaboration with Stanley, Todd, Rau, Gelbart, Knobler, Zemb

Beginning with direct measurements of forces between large molecules, proceeding with observations of molecules under confinement, and then building on the statistical physics of molecular organization under the action of organizing forces, we have developed new theories and methods of macromolecular organization. One application was to observe DNA under the osmotic stress of large polymers or confined within the hard walls of a virus capsid.

In our most recent work, we observed the ejection of DNA from capsids subjected to various salt conditions. Expansive pressures may vary up to many tens of atmospheres and thus cause initial ejection of DNA. These forces are the product of variable ionic conditions. An unrecognized feature of many viruses is that ionic conditions can penetrate the virus and even modify the expansive force within. At one extreme, DNA in simple salts is under great pressure to expand and be ejected from the capsid; however, under other conditions that permit the DNA-condensing ions to enter the capsid, DNA may be under no expansive pressure. We have begun to measure the motion of DNA within capsids subject to differing ionic conditions and are observing how ejection might be controlled by ionic surroundings. Whether these manipulations ultimately affect viral infectivity is a worthy of pursuit.

To see the connections between DNA pressure and ejection, we have been growing viruses of various lengths for insertion into the same-size capsid. Using X-ray diffraction, we are able to follow the packing and pressure of DNA within the capsid. We have also succeeded in following the condensation of triple-helical DNA, which is implicated in the behavior of telomers. The response to various divalent ions in various tsDNA constructs reveals differences in packing and liquid crystalline tendencies.

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INTERMOLECULAR FORCES, RECOGNITION, AND DYNAMICS

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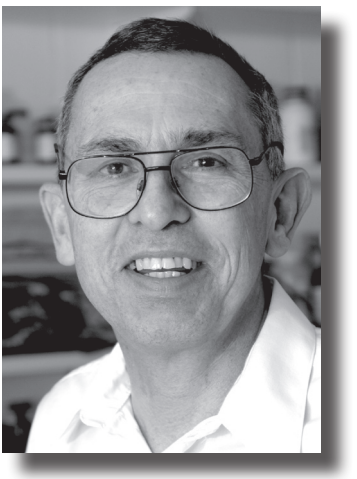

Our laboratory focuses on the coupling of forces, structure, and dynamics of biologically important assemblies. The next challenge in structural biology is to understand the physics of interactions between molecules. The ability to take advantage of an increasing number of available protein and nucleic acid structures will depend critically on establishing the link between structure and energy. A fundamental and quantitative knowledge of intermolecular forces is essential for (1) understanding the interactions among biologically important macromolecules that control cellular function and (2) rationally designing agents that can effectively compete with the interactions associated with disease. We have shown that measured forces differ from those predicted by current theories, and we have interpreted the observed forces as indicating the dominant contribution of water-structuring energetics. The observation that interacting macromolecules tenaciously retain their hydration waters unless the surfaces are complementary has profound implications for recognition reactions. To investigate the role of water in binding, we measure and correlate changes in binding energies and hydration that accompany recognition reactions of biologically important macromolecules, particularly sequence-specific DNA-protein complexes. We observe a strong correlation between retained water and binding energy; stronger binding means less water retained at the DNA-protein interface.

Direct Force Measurements

The ability to measure forces directly between biopolymers in macroscopic condensed arrays has greatly changed our understanding of how molecules interact at close spacings, that is, at the last 1 to 1.5 nm of separation. The universality of the force characteristics observed for a wide variety of macromolecules, charged or uncharged, including DNA, proteins, lipid bilayers, and carbohydrates, and for the interaction of small solutes and salts with macromolecules has led us to conclude that intermolecular forces are dominated by the energy associated with changes in structuring water.

DNA-DNA attractions

Rau, Todd

DNA packaging by multivalent ions is a critical testing ground for understanding forces between charged molecules. If a sufficient concentration of multivalent ions is present, DNA will spontaneously assemble into an ordered array. The helices do not collapse to touching but rather are separated by 0.5 to 1.5 nm of solvent depending on the nature of the condensing ion. Attractive and repulsive

forces balance at the equilibrium spacing. By combining osmotic stress/pushing experiments with single-molecule/magnetic tweezers/pulling experiments, we were able to separate the attractive and repulsive free energies at the equilibrium spacing for the commonly used condensing agents cobalt hexa-ammine, the biogenic alkylamines spermidine and spermine, and a synthetic +6-charged alkyl hexa-amine (essentially two spermidines joined by a butyl linker). The results confirmed our previous hypotheses for hydration forces. The 0.2 nm decay–length exponential repulsive force is the hydration equivalent of the image-charge repulsion in electrostatics. The hydration "atmosphere" extending from a solvated surface stabilizes water structuring at the surface. Disruption of the atmosphere simply by replacing water with another surface lowers hydration energies regardless of the water structuring on the other surface. The repulsion amplitude should depend predominately on the water structuring of DNA-surface groups and perhaps on the mode of binding, but not on the correlations of these groups with apposing helices. The attractive force is a 0.4 nm decay–length exponential force resulting from the direct interaction of surface hydration structures. Perturbations in water structure around one surface resulting from the close presence of another surface can either weaken or strengthen hydration energies depending on the mutual structuring water. We postulated that the attractive force had the same exponential 0.4 nm decay length as previously observed for repulsive hydration forces but that the force was now attractive because of correlations in complementary water structuring on apposing helices.

To confirm and expand on the alkylamine results, we are examining two other sets of homologous compounds, arginine and lysine peptides. As with the alkylamine series, we observed the same limiting 0.2 nm decay–length exponential repulsion for mono-arginine to hexa-arginine and poly-arginine regardless of charge. The force amplitude, however, differs for the alkylamine and arginine series; repulsion depends on the hydration properties of the bound counterion. Given that equilibrium spacing decreases with larger charge while repulsion remains constant, attraction increases with charge. Attraction is presumably caused by complementary patches on apposing helices; thus, the greater the attraction the closer the correlation between hydrated amine charges on one helix with phosphate groups on another. We observe that calculated attractive energies for the alkylamine and arginine series vary linearly with the inverse of the cation charge. This finding is consistent with a constant loss in entropy from correlating a single molecule regardless of charge but a gain in interaction energy that increases with the number of charges.

We also began measuring the packaging forces of salmon protamine–assembled DNA. Protamines are small, arginine-rich peptides used to package DNA in sperm heads. Protamine-DNA forces resemble polyarginine-DNA interactions. The attraction with protamine, however, corresponds to tetra- or penta-arginine, not to the 21 charges actually present. The sensitivity of equilibrium spacings of protamine-DNA complexes to the anion of the added salt indicates that salt does not operate through a screening or overcharging mechanism to weaken attraction but rather through anion binding to protamine to lower the net protein charge. This finding is consistent with our previous work on the resolubilization of DNA assemblies at high concentrations of spermidine and spermine (Yang and Rau, *Biophys J* 2005;89:1932). By measuring the change in interhelical spacing as a function of salt concentration at constant osmotic pressure, we can quantitate anion binding.

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Hydration Changes Linked to Sequence-Specific DNA-Protein Recognition Reactions

Our ultimate goal is to apply the lessons from direct force measurements to the recognition reactions that control cellular processes. We focused on differences in water sequestered by complexes of sequence-specific DNA binding proteins bound to different DNA sequences, with particular emphasis on the correlation between binding energy and incorporated water and on the energy necessary to remove hydrating water from complexes. We determine differences in sequestered water between complexes by examining how changing osmotic pressure affects binding constants or dissociation rates.

EcoRV binding to specific and non-specific DNA sequences

Sidorova, Muradymov, Rau

We have applied our unique perspective and experimental tools to the controversial type II restriction enzyme EcoRV. Restriction endonucleases typically show a noticeably high stringency in DNA sequence recognition. We have obtained puzzling results for the relative specific–non-specific binding constant of EcoRV. Estimates of $K_{sp\text{-}nsp}$ for EcoRV range from 1 to 100, with most estimates less than 10. The possible causes for the inability of EcoRV to distinguish between specific and non-specific sequences remain unclear. X-ray crystal structures are available for both cognate and non-cognate EcoRV complexes. The interface of the specific complex is essentially anhydrous, with many direct DNA-protein interactions, and differs dramatically from the non-cognate complex, which has a large water-filled gap at the protein-DNA interface. The substantial difference in structure would suggest a large difference in binding energy.

We used our self-cleavage assay to quantitate EcoRV-DNA binding. The association kinetics of EcoRV shows at least two components: one is rapid as is typical for specific binding while the other is unusually slow with a half-life of about 20 minutes—an observation that differs markedly from our previous results with EcoRI. We plan to use other techniques to investigate further the consequence of the unusual association kinetics for the estimation of binding constants.

Our measurements of relative EcoRV specific–non-specific binding constants indicate significant osmotic pressure dependence. Our estimate of the difference in sequestered water between cognate and non-specific complexes is about 160 water molecules, a value that correlates well with the difference in structures of the complexes determined by crystallography. We estimate a relative binding constant in the absence of osmolyte of about 400. EcoRV can effectively distinguish between specific and non-specific sequences. We plan further measurements of relative binding and dissociation rate constants in the presence of other solutes.

EcoRI sliding rates

Sidorova, Rau

Many sequence-specific DNA-binding proteins locate their target sequence by first binding to DNA non-specifically and then diffusing linearly along DNA until the protein either dissociates from the DNA or finds the recognition sequence. We completed our measurements of the sliding rate of EcoRI along DNA by calculating sliding rates from the ratio of dissociation rates of EcoRI from DNA fragments containing one and two specific binding sites. We have several two-site fragments with varied distances between sites and can vary the non-specific dissociation rate widely by altering the salt concentration. We calculate that the one-dimensional diffusion coefficient of EcoRI is about 1,000-fold slower than the diffusion of free protein in water. We hypothesize that the extra drag is attributable to transient breaking of charge-charge contacts between DNA and protein. To test such a hypothesis, we plan to measure sliding rates for the closely related nuclease BamHI, which is characterized by many fewer charge-charge pairs.

Stabilizing labile DNA-protein complexes in polyacrylamide gel using osmolytes
Sidorova, Hung, Rau

Polyacrylamide gels stabilize DNA-protein or protein-protein complexes by a crowding or caging mechanism. Many non-specific DNA-protein complexes, however, are weak enough that they dissociate during electrophoresis, yielding smeared bands that are difficult to quantitate precisely. We find that adding osmolytes directly to the gel can further stabilize weak complexes. Given that complex dissociation is accompanied by a change in solvent-accessible surface area, different osmolytes have differing efficacies depending on their extent of exclusion from the newly exposed surfaces. Experiments with the weak, non-specific complexes of EcoRI and BamHI show that triethylene glycol is particularly effective at inhibiting dissociation; it does not interfere with normal gel polymerization and does not significantly slow normal migration. Extension of this approach to other techniques for separating complex and free components, such as gel chromatography and capillary electrophoresis, is straightforward.

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MEMBRANE REMODELING DURING VIRAL INFECTION, PARASITE INVASION, AND APOPTOSIS; COMPONENTS AND KINETICS IN EXOCYTOSIS

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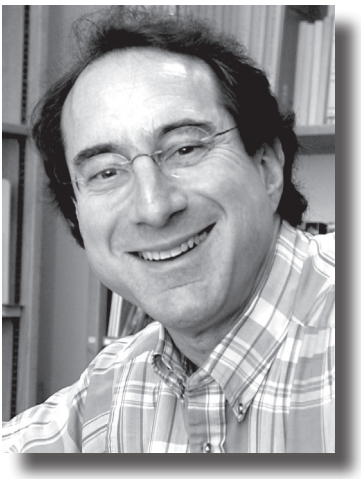

We study membrane mechanics, intracellular molecules, membranes, viruses, organelles, and cells in order to understand viral and parasite infection, exocytosis, and apoptosis. This year, we report on four projects. For malaria, we study the mechanisms by which proteases take part in the release of parasitic cells from erythrocytes, leading to our suggestion for a protease inhibitor therapy for malaria. Second, in enveloped viral disease, we discovered how lipids forming the envelope of the influenza virus gel at cooler temperatures, suggesting that the gel protects the virus during airborne transmission and explaining the winter-time occurrence of flu epidemics. Third, using electrophysiology and fluorescence microscopy, we found that cholesterol plays a 2-fold role in viral fusion mediated by the hemagglutinin of influenza: as a lipid curvature agent helping hemifusion and as a specific widener of the fusion pore. Fourth, we investigate the theoretical effect of protein wetting to determine its role in protein domain formation in cell membranes. We find that wetting can provide important features seen in vivo.

Irreversible effect of cysteine protease inhibitors on the release of malaria parasites from infected erythrocytes

Glushakova, Hama,[1] Blank, Humphrey, Kapnik,[2] Mazar, Zimmerberg; in collaboration with Hohmann-Marriott

Given the absence of an effective vaccine for malaria and *Plasmodium falciparum*'s resistance to many drugs, there is interest in developing inhibitors that target cysteine and serine proteases. Such drug inhibitors are known to interfere with the parasitic asexual life cycle by trapping maturing parasites in clusters and diminishing de novo infection of erythrocytes. A deeper understanding of the role of proteases in the pathophysiology of malaria is critically needed; despite decades of research, controversy still surrounds the proteases' function in the malarial erythrocyte cycle, particularly in parasite release. Two recent reports identified two parasite proteases that may mediate the cascade of final cycle events—the cysteine protease dipeptidyl peptidase 3 (DPAP3) and the subtilisin-family serine protease PfSUB1—although the precise time and place of the proteases' action in the release process remains enigmatic.

The release of parasites appears to be a two-step process. Parasites are released first from the erythrocyte's parasitophorous vacuole within the erythrocyte and then from the erythrocyte. After breaching the parasitophorous vacuolar membrane and the erythrocyte membrane, the parasites are free to invade fresh erythrocytes. While the release of parasites is crucial for the parasite's life cycle, the mechanism of release remains unknown, and only limited technologies exist for its analysis. Imaging of the release process in vitro shows that the process occurs rapidly and is highly sensitive to a variety of conditions, thus limiting possible experimentation in living cells at the end of the erythrocyte cycle. Wide-spectrum cysteine- and cysteine/serine-protease inhibitors such as E-64, bADA, and leupeptin (the last alone or in combination with chymostatin and antipain) block the cycle. Even though such treatment does not interfere with parasite maturation and thus permits analysis of the accumulated parasite clusters, it blocks rupture of one of the two membranes surrounding the parasites. Given disagreement as to the origin of the limiting membrane that preserves the remaining cluster, the order of membrane rupture during parasite release remains subject to debate.

We developed a new approach for the differential labeling of the membrane of live infected erythrocytes as well as a quantitative parasite release assay, coupled with morphological analysis of live infected cells undergoing a cycle transition. Using confocal microscopy of living cells and electron microscopy of high-pressure–frozen and freeze-substituted cells to study inactivation of malaria parasite culture by cysteine protease inhibition, we confirmed that cysteine proteases likely regulate the opening of the erythrocyte membrane, causing release of malaria parasites. Inhibition of cysteine proteases within the last few minutes of cycle does not affect rupture of the parasitophorous vacuole but irreversibly blocks the subsequent rupture of the host cell membrane, thereby locking in resident parasites, which die within a few hours of captivity. In contrast to the findings of Salmon and colleagues, both reversible and irreversible cysteine protease inhibitors irreversibly inactivate clustered parasites. Such irreversible inactivation of mature parasites inside host cells makes plasmodial cysteine proteases attractive candidates for antimalarials, as parasite-specific cysteine protease inhibitors may significantly augment multitarget drug cocktails.

It is likely that cysteine proteases suddenly enter the erythrocyte cytoplasm upon disruption of the parasitophorous vacuole membrane. Once active in the compartment facing the plasma membrane of the erythrocyte, the proteases disrupt the plasma membrane. In the presence of protease inhibitor, parasites degrade and die within clusters. It is unlikely that E-64 has a direct toxic effect on parasites because clustered parasites locked inside erythrocytes in E-64–containing medium have approximately the same life span as parasites in normal medium in vitro. We believe that E-64 is deleterious to parasites by fatally prolonging the otherwise transient cytoplasmic "cluster stage" of parasites that have broken through the vacuolar membrane. Given the short life span of mature parasites, prolongation of the cluster stage leads to irreversible blockage of the plasmodial erythrocyte cycle. The withdrawal of irreversible and reversible cysteine protease inhibitors does not restart parasite release from clusters as previously suggested but rather permits schizonts to release parasites in the future. Thus, a dosing schedule, as informed by our studies, may be important for testing protease inhibitors as antimalarial drugs. Hopes for antimalarial protease inhibitor drugs are augmented by the finding that relatively low concentrations of calpeptin, a reversible inhibitor, had a potent inhibitory effect on parasite release.

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Progressive ordering with decreasing temperature of the phospholipids of influenza virus

Farrington, Fera, Blank, Frolov, Bezrukov, Polozov, Zimmerberg; in collaboration with Gawrisch, Hess, Reese

Membranes of most enveloped viruses form by budding out a highly select subset of plasma membrane components from the plasma membranes of the viruses' host cells. Compared with

the plasma membrane, the envelope of influenza contains higher amounts of both cholesterol and glycosphingolipids, lipids known to partition into the liquid-ordered phase (l_o) and characterized by extended hydrocarbon chains with a lower *gauche-trans* isomerization than the liquid disordered (l_d) phase, but with similar rotational and translational mobility.

Using NMR,[3] we found that phospholipids in viral membranes form ordered lipid phases over a wide range of physiologically relevant temperatures. We have evidence that both l_o and gel (s_o) phases co-exist with the l_d phase. At 37°C, lipids with broadened spectra (the sum of l_o and s_o phases) represent a minute fraction of the membrane, whereas, at 4°C, almost the entire membrane is in the ordered state. These data rule out the hypothesis that the influenza envelope is created entirely from an ordered lipid domain. We observed co-existence of ordered and disordered lipid domains in both the intact viral envelope and liposomes prepared from viral lipids. A reduction in cholesterol reversibly increases gel-phase lipids. It is remarkable that, despite the wide temperature dependence of the fraction of ordered domains, the extracted lipids exhibit the same fraction of ordered domains as the intact virus at any given temperature, suggesting that the properties of membrane lipids primarily determine the fraction of ordered domains. Thus, proteins may play only a limited role in the phase behavior of lipids in the intact viral envelope, although they may affect line tension at boundaries between co-existing phases.

Three independent lines of evidence support the notion of solid ordered domains at ambient temperatures. First, in liposomes formed from viral lipids, gel lipids were confined to areas smaller than the size of the liposomes and exhibited the characteristics of solid-ordered domains; this finding is similar to our earlier observations for gel phase–fluid phase co-existence in SOPC-POPE[4] lipid mixtures. Second, the ragged edges seen by fluorescence in both supported viral lipid bilayers and giant unilamellar vesicles suggest that crystalline packing shapes domains. Third, we assigned methylene resonances wider than 1.5 kHz (3 ppm) to the solid-ordered lipid phase. The presence of such broad components is visible in the first-order spinning side band of the virus spectrum even at temperatures higher than ambient. While we may not entirely exclude the possibility that protein resonances contribute to those spectra, the rapid increase in their intensity with decreasing temperature confirms that they are dominated by contributions from lipids. Taken together, the above evidence strongly supports the presence of s_o. We speculate that s_o phase formation is related to the high phosphatidylethanolamine (PE) content of viral envelopes; PE is a lipid with low affinity for cholesterol and high gel-fluid phase transition temperatures.

At physiological temperature and higher, MAS[5] NMR provides little evidence for ordered lipid, and, even though we continue to assay for fusion to cell membranes, we have not been able to detect ordered lipid in the host or target membrane. Thus, phase properties of complex, biologically relevant lipid mixtures need to be studied at the appropriate temperatures. An issue of particular interest is why the viral envelope lipid composition is set to be near an apparent lipid phase boundary with respect to temperature. It may be instructive to consider that the lipid composition of the influenza virus differs dramatically from that of vesicular stomatitis virus (VSV), another virus that buds from the same cells. Both enveloped viruses bud from the plasma membrane, albeit from different locations. The difference in location relates to the viruses' mode of host-to-host transmission—apical budding for aerial transmission of influenza and serosal budding for transmission through animal bite for VSV (and for rabies, the other virus of the *Rhabdoviridea* pathogenic genus). A virus would be at room temperature during aerial transmission but not during transmission by animal bite; the ordered phases we documented here may be important for stability. Indeed, a recent report shows that airborne transmission of influenza virus by guinea pigs increases at lower temperatures, a result predicted by our proposal that the progressive ordering of lipids with decreasing temperature is important during the low-temperature stages of the influenza life cycle.

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Cholesterol promotes hemifusion and pore widening in membrane fusion induced by influenza hemagglutinin

Biswas,[6] Shnyrova, Frolov, Yin, Pekkurnaz, Zimmerberg

Successful infection by influenza virus requires the envelope spike protein hemagglutinin (HA) both to catalyze fusion between the viral envelope and the target cell's intracellular endosomal membrane and to create a pore large enough to release the viral genome. Experiments on and theory of lipid composition in relationship to membrane monolayer curvature stress strongly suggest that membrane lipids play a role in this critical event. Recently, attention has focused on the role of membrane phase behavior and membrane microdomains in the lateral distribution, sorting, and interactions of lipids with membrane proteins in general and with viral envelope glycoproteins in particular. Cholesterol is a major and vital constituent of eukaryotic cell membranes. Its unique structure—a small hydrophilic head group and rigid, hydrophobic fused rings—favors preferential association with saturated acyl-chain lipids and sphingolipids to form liquid-ordered microdomains (termed lipid rafts) in phospholipid bilayer membranes of the appropriate composition. Lipid rafts are hypothesized to exist in the cell plasma membrane at specialized sites where proteins concentrate, creating favorable associations with the ordered, cholesterol-rich environment.

Cholesterol regulates specific intermediates of membrane fusion catalyzed by the influenza virus protein HA. The extent of early lipid transfer in Sf9 cells expressing HA (HAS cells) is similar to that previously observed in mammalian cell systems, but the initial fusion pore is small and pore expansion is stunted. A 3-fold increase in cellular cholesterol leads to (1) faster lipid dye transfer kinetics, (2) increased aqueous dye transfer kinetics and extent, and (3) an increase in the rate of pore conductance. The cholesterol-dependent increase in fusion efficiency requires an intact HA transmembrane domain (TMD) and optimal pH. Overall, our results support the hypothesis that host-cell cholesterol acts at two stages in membrane fusion: at an early, lipidic stage before fusion pore opening and at a later stage during fusion pore expansion.

We are interested in how the physical properties of cholesterol influence fusion. We have shown that cholesterol promotes both lipid transfer (hemifusion) and fusion pore expansion in the cell-cell membrane fusion mediated by influenza HA. We hypothesize that cholesterol promotes fusion pore expansion by virtue of its negative intrinsic curvature and the same specific cholesterol/lipid/HA interactions that mediate (1) the 1 to 10 nm scale clustering of HA in the plane of the membrane, (2) the mobility of HA in fibroblasts, and (3) the phase behavior of the influenza envelope. These structural forces act at the fusion stage of viral invasion to facilitate fusion pore widening.

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Domain formation in membranes caused by lipid wetting of protein

Frolov, Zimmerberg; in collaboration with Akimov, Chizmadzhev, Cohen

The classic fluid mosaic model views the lipid environment of a plasma membrane as essentially homogeneous. Developments over the past decade, however, have increasingly shown that non-homogeneities in membranes are central to biological functions that depend on protein-protein interactions within membranes. Given that lipid and protein interactions mediated by hydrophobic, van der Waals, electrostatic, and chemical forces cause some lipids and proteins to cluster into domains and others to repel, it is obvious that membrane non-uniformities must exist. Over 30 years ago, researchers demonstrated by electron spin resonance the existence of small spatial inhomogeneities. They found that "boundary lipids" surrounding a protein exhibit about a 10-fold lower hop time than lipids not associated with proteins. In our project, formation of rafts and other domains in cell membranes is considered as wetting of proteins by lipids, with the membrane modeled as a continuous elastic medium. Our approach yields the conditions necessary for a macroscopic wetting film to form and permits us to determine the film's thickness.

Using a mean-field theory of liquid crystals as adapted to biomembranes, we calculated thermodynamic functions of wetting lipid films. We showed that either molecular or macroscopic films can form, depending on the values of parameters such as membrane thickness, hydrophobic height mismatches, spontaneous curvature of lipids, and protein radius. We demonstrated that a single protein of approximately 1 nm radius is not large enough to induce a local phase transition to form a protein-lipid raft but that a macroscopic wetting film can form around a lipid/protein aggregate of more than tens of nanometers in diameter. We have assumed that the lipids in such aggregates are in a liquid-ordered state, analogous to wetting of a solid surface. Calculations and simulations in the context of the capillary wave model of Mouritsen support the validity of our assumption. Wetting films that coat an aggregate could be particularly important because they facilitate the merger of domains. Moreover, a wetting film prevents a protein from leaving an aggregate, thereby promoting protein accumulation and clustering.

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REGULATION OF GENE EXPRESSION, CELLULAR PROLIFERATION, AND DIFFERENTIATION IN MAMMALIAN DEVELOPMENT

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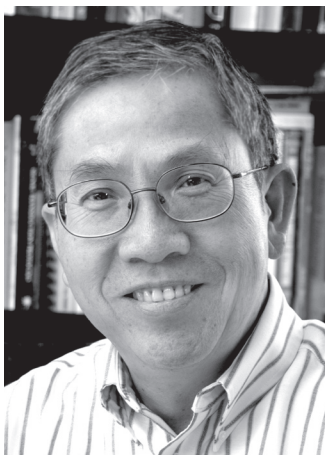

Using functional genomic and systems-biological approaches to gene expression and epigenomic changes during development, we seek to understand genomic regulation of development in health and disease. At different stages of spermatogenesis, male germ cells have distinct morphological features or genetic markers that permit their easy identification and preparation as well as characterization of stage-specific gene expression and biological pathways. Such knowledge can help us (1) delineate the network of genes that regulates renewal and differentiation of germ cells and (2) develop novel methods of fertility control and aids for the infertile. Vitamin A deficiency is known to cause spermatogenic arrest at the spermatogonia stage. By studying the transcriptome of spermatogonia in the vitamin A–deficient animal, we hope to identify the elements that propel differentiation of these cells. Abnormalities of gene regulation during spermatogenesis, such as abnormal genomic methylation, lead to the development of testicular tumors. Studying changes in the epigenome of testicular tumors can help us further understand normal genetic regulation of germ cell development. To investigate selected clinical conditions, particularly those with abnormal cell growth, we applied genomic approaches to the role of mutated luteinizing hormone/choriogonadotropin receptor in testicular tumor development. Another study examines the role of mitochondria in tumorigenesis.

Regulation of Gene expression, Cellular Proliferation, and Differentiation in Male Germ Cell Development

Global expression mapping of developing male germ cells

Lee, Cheung, Davis, Tarn, Baxendale, Chan W-Y; in collaboration with Rennert, Culty, Claus, Sastry

Understanding the functional significance of protein and regulatory networks requires a complete description of the cellular transcriptome. After sequencing the genes from several mammalian species, the identification of functional genes within a network is based on in silico predictions substantiated by evidence of transcription in vivo. Conservative estimates point to about 20,000 protein-encoding genes in the mammalian genome. However, in-depth analyses of the transcriptional outputs from a range of experimental approaches suggest that the information content of the genome

is much more complex than previously thought. We use developing male germ cells as a model system to study important developmental processes that lead to the transition from one cell type to another. To explore and identify transcriptome complexity in male germ cells, we previously applied serial analysis of gene expression (SAGE) to profile the expression signature of the major stages of spermatogenesis, including type A spermatogonia (Spga), pachytene spermatocytes (Spcy), and round spermatids (Sptd). We identified a large number of novel transcripts, alternative splicing transcript variants, and antisense transcripts.

To provide an unbiased and higher-resolution profile of the transcriptome, we expanded our SAGE findings by incorporating whole-genome 25 bp–resolution tiling expression arrays (Affymetrix®). Using 45 million oligonucleotide probes and 35-bp probe spacing, we generated a high-definition transcriptome map of developing male germ cells with an unbiased and germ cell–specific whole-genome expression map. Preliminary data proved that combining the SAGE data set with the tiling platform is a powerful approach that provides new insights into germ cell stages. In summary, we found that over 45 percent of transcripts were not annotated; current annotation accounts for only about 30 percent of our data set, with the remainder of the data set containing mostly expressed sequence tags (EST). We identified thousands of transcript cluster units located within introns and internal exons of protein-coding genes, indicating that promoter sites are common and that transcriptional organization is complex. The transcriptional architecture implies that most genomic regions serve many functions. Although a large proportion of human transcription occurs outside the boundaries of known genes, the functional significance of such transcription remains unknown.

To facilitate further data analysis of the high-throughput genomic assay generated in our laboratory, we developed custom bioinformatics tools. GermSAGE, which was generated by SAGE, is a comprehensive web-based database of the major stages of mouse male germ cell development, with a sequence tag coverage of 150,000 in each SAGE library. It includes a total of 452,095 tags derived from Spga, Spcy, and Sptd and provides web-based tools for browsing, comparing, and searching male germ cell transcriptome data at various stages with customizable search parameters. The data may be visualized in tabulated format or further analyzed by alignment with various annotations available in the UCSC[1] Genome Browser. As a flexible platform, GermSAGE will be useful for developing a better understanding of the genetic networks that regulate spermatogonial cell renewal and differentiation and will allow novel gene discovery. It is available to the public at http://germsage.nichd.nih.gov/.

We are also developing an online tool called TransfragMap to map tiling array data to various gene annotations and to mark particular gene features. The tool, which will accelerate qualitative analysis of tiling microarray data, is platform-independent and compatible with tiling microarrays from major tiling array platforms, including Affymetrix® and NimbleGen™. It will support the human and mouse genome in its initial version and will be expanded to other species.

Lee TL, Cheung HH, Claus J, Sastry C, Singh S, Vu L, Rennert OM, Chan WY. GermSAGE: a comprehensive SAGE database for transcript discovery on male germ cell development. *Nucleic Acids Res* 2008 [E-pub ahead of print].

Characterization of spermatogonial stem cell signaling

Lee, Chan W-Y; in collaboration with Dym, Kokkinaki

Previous work has shown that GDNF-receptor-α-1 (GFRA1) is specifically expressed in spermatogonial stem cells (SSC) and required for their stem-cell properties. To characterize the molecular phenotype of SSCs, we isolated GFRA1(+) and GFRA1(−) spermatogonia from 6-day-old mice by using magnetic-activated cell sorting with an antibody to GFRA1. In comparing microarray-based expression profiles, we found the expression of several genes to be upregulated in GFRA1(+) spermatogonia. The most overexpressed gene was *Csf1r*, which encodes the receptor for granulocyte-macrophage colony–stimulating factor (GM-CSF) and plays a long-established role

in hematopoietic stem cell function. We also found that several chemokine ligands were highly overexpressed in SSCs. Analysis revealed the potential role of chemokine signaling in SSCs and suggested a common pathway for GFRα-1 and Csf1r, which may lead to the cells' self-renewal.

Kokkinaki M, Lee TL, He Z, Jang J, Golestaneh N, Hofmann MC, Chan WY, Dym M. The molecular signature of spermatogonial stem cells in the 6-day-old mouse testis. *Biol Reprod* 2008 [E-pub ahead of print].

Characterization of mammalian RNA-dependent RNA polymerase activity
Wu

Analyses of antisense transcripts suggested the existence of RNA-dependent RNA polymerase (RdRP) activity in mouse germ cells. We identified antisense transcripts complementary to several coding exons for *Tcte3*, *Ldh3*, and *Calm2*. The *Calm2* antisense transcript was present in mouse testis and three mouse cell lines, namely, CRL-2576 (mouse spermatogonia cell line), CRL-1715 (mouse Sertoli cell line), and CRL-6436 (mouse kidney cell line). In a knockdown experiment, we confirmed the antisense transcript as a product of the sense transcript. Specifically, we knocked down the sense transcript of *Calm2* by using siRNA and demonstrated reduced levels of both sense and antisense transcripts, indicating that the synthesis of the *Calm2* antisense transcript was dependent on the sense transcript. We observed that *Calm2* antisense was not synthesized starting from the 3′ end of the sense mRNA, defined the sequence representing the potential start site of the action of RdRP, generated a hybrid RNA containing this sequence ligated to *EGFP* on its 5′ end, and introduced the hybrid into CRL-6436 cells. Orientation-specific RT-PCR[2] showed production of an antisense RNA derived from hybrid RNA. The level of *Calm2* antisense transcript appeared to be independent of the stage of growth of cultured cells and was ubiquitous in all mouse tissues examined (testis, ovary, liver, lung, kidney, spleen, thymus, heart, brain, and embryo). Our results provide further proof of the existence of RdRP activity in mammalian cells. Experiments to isolate and purify RdRP activity are under way.

Chan WY, Wu S, Ruszczyk LM, Lee T, Rennert OM. Antisense transcription in developing male germ cells. In: Lau YFC, Chan WY, eds. *Y Chromosome and Male Germ Cell Biology in Health and Diseases*. World Scientific Publishers, 2007;201-220.

Identification and functional characterization of novel spermatogenesis stage–specific genes
Tang, Lee, Tarn, Cheung, Chan W-Y; in collaboration with Rennert

Through algorithmic analysis of mouse tiling array signals of the three main cell stages of spermatogenesis with reference sequences, we identified a pool of stage-specific genes. Two of the novel genes were transmembrane and coiled-coil domain 5A (*Tmco5A*) and 4930563P21Rik (*Tmco5B*), which are expressed exclusively in mouse testis during the post-meiotic stage of male germ cell development. Both genes have a single CAGE[3] tag in the testis. Comparative genomics showed conservation of the two genes in different species, including human. Evidence from protein structure prediction, gene mapping, sequence alignment, and expression profiles suggested that *Tmco5A* and *Tmco5B* likely belong to a single gene family with a specific role in male post-meiotic development or sperm function. Our studies aim to characterize the two genes in mouse testis.

Epigenomic changes in testicular cell transformation
Cheung, Lee, Davis, Taft, Young, Chan W-Y; in collaboration with Rennert

Unlike most cancers, which peak in old age, testicular germ cell tumors (TGCT) are common in young males. While genomic mutations may be one of the causes of familial TGCT, accumulating information suggests that aberrant epigenetic changes may contribute to tumorigenesis of various cancers, including TGCT. DNA methylation is one of the epigenetic hallmarks affecting chromatin structure, genomic stability, or altered transcriptional activity. Using methylated DNA immunoprecipitation and tiling array hybridization (MeDIP-chip), we compared global DNA methylation alterations between normal and testicular tumor cells. We obtained a high-resolution

cytosine methylation map of the human germ cell cancer and identified more than 6,000 differential methylated regions (DMR) between normal and TGCT cells. More than 70 percent of DMRs resided in intergenic regions, with promoter methylation accounting for 9 percent. About one-third (27 percent) of these genes demonstrated the perceived relationship between promoter methylation and gene expression, i.e., hypermethylation associated with suppression of gene expression.

A focal analysis of DMRs located in the regulatory regions of annotated genes yielded 207 differentially methylated genes. We selected three candidate genes for further characterization in primary tumor tissues: *APOLD1*, *RGAG1*, and *PCDH10*. The open reading frame of *APOLD1* encodes an apolipoprotein L domain–containing protein whose function is unknown. Remarkably, *APOLD1* is located in 12p13.1, a TGCT-susceptibility locus identified previously by genetic linkage analysis. *RGAG1* is an X-linked retrotransposon-derived neogene with unknown function. Our studies revealed that ESTs of *RGAG1* were located predominantly in testis, suggesting that this retrogene might be important in male germ cell development. *PCDH10* encodes a protocadherin10 membrane protein for cell adhesion, and had been implicated as a tumor suppressor gene in nasopharyngeal, esophageal, breast, colorectal, cervical, lung, and hepatocellular carcinomas. We used bisulfite sequencing and methylation-sensitive PCR to confirm differential methylation of these three genes observed in cultured tumor cells in primary testicular tumor tissues. RT-PCR confirmed differential expression of the genes in tumor and control tissues. Thus, cultured cells could be used as a model for studying the mechanism of altered methylation in tumors, and the genes may serve as novel noninvasive epigenetic markers for molecular diagnosis of TGCT.

The role of intergenic DMRs is not clear—they could simply be a consequence of inappropriate epigenetic establishment during primordial germ cell differentiation, or they may play a hidden regulatory role in the maintenance of genomic stability or chromatin condensation. Another possible function of non-genic DMRs is the regulation of non-coding RNAs. We mapped the non-genic DMRs to a current non-coding RNA database and found that three miRNAs (miR-199a-2, miR-124a-2, and miR-184) were hypermethylated in TGCT. Notably, miR-124a was first identified in embryonic stem cells, suggesting that it may be important in differentiation. Coincidentally, it was found that miR-124a-2 was epigenetically silenced in colon cancer, resulting in activation of the oncogene *CDK6*. Furthermore, we found that three snoRNAs (hb11-240, aca22, and aca8) were hypomethylated. Epigenetic changes of non-coding RNAs may lead to deregulation of genetic networks in a wider spectrum, as a single miRNA is capable of regulating several target genes.

Lee TL, Cheung AHH, Davis AJ, Rennert OM, Chan WY. The paradox of DNA methylation in transcriptional control. *PLoS Biol* 2008;6.

Li YM, Tabatabai ZL, Lee TL, Hatakeyama S, Ohyama C, Chan WY, Looijenga LHJ, Lau YFC. The Y-encoded TSPY protein: a significant marker potentially plays a role in the pathogenesis of testicular germ cell tumors. *Human Pathol* 2007;38:1470-1481.

Effect of vitamin A deficiency on male germ cells

Boucheron, Baxendale, Chan W-Y; in collaboration with Rennert

Vitamin A deficiency (VAD) is known to cause spermatogenesis arrest, but the molecular mechanism of VAD's effect is not known. We plan to employ expression profiling to delineate the genetic basis of VAD-induced arrest of spermatogonial stem cell differentiation and the potential regeneration of spermatogenesis following restoration of vitamin A in the diet. We initiated a timed study of the VAD diet in which we measure the vitamin A status of the animal, the RBP^4 level in the serum, and mRNA levels of the retinoic acid receptors RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ in the liver and testis. Given the close relationship between thyroid hormones and vitamin A metabolism, we will also study the paternally imprinted enzyme deionidase iodothyronine type III (Dio3) during VAD. Dio3 is highly expressed in developmental tissues, but its expression and function in the testis have yet to undergo study. We will begin expression profiling and epigenomic studies of the germ cells once we have established the chronology of VAD.

Biological Studies of the Luteinizing Hormone/Choriogonadotropin Receptor

The luteinizing hormone/choriogonadotropin receptor (LH/CG-R) plays a central role in human male sexual development. Mutation of LH/CG-R results in abnormal production of testosterone and disorders of sexual development. Though the role of LH/CG-R in transducing the signal of luteinizing LH/CG binding is well established, the mechanism of its action is still not fully understood. We have studied the molecular genetics of LH/CG-R in a large number of patients with activating and inactivating mutations of the receptor. Studies of these naturally occurring mutations continue to help us understand the varied disease phenotype as well as the molecular mechanism of receptor signal transduction.

Function of hCG/LH and its receptor in mammalian nervous system
Meng, Chan W-Y; in collaboration with Rennert

Individuals with activating mutations in LH/CG-R develop familial male-limited precocious puberty and often exhibit behavioral problems that may be related to dysfunction of brain cells caused by the mutated receptor. Recent studies demonstrated that human choriogonadotropin (hCG) and its receptor (LH/CG-R) may have non-gonadal functions that could be physiologically important. In the nervous system, LH/CG-R is expressed in the mammalian brain in a temporal and spatial pattern. Administration of hCG promoted nerve regeneration and neurite outgrowth in vivo and survival of primary neurons in vitro. To date, the function of hCG and its receptor in the nervous system remains unclear.

To understand the role of hCG and its receptor in the development of the mammalian nervous system, we studied the effect of the transgenes on the differentiation of the bipotent cell line PC12, which is derived from rat adrenal pheochromocytoma. Our studies showed that the differentiation effect of hCG was dependent on ligand dose and time. Both the extracellular signal–regulated kinases (ERK) and p38 mitogen–activated protein kinase (MAPK) were indispensable for the differentiation process. In addition, the phospholipase C (PLC) pathway was partially involved in hCG-induced PC12 differentiation. These findings suggest a role for hCG/LH and LH/CG-R in the neurogenesis of the mammalian nervous system. They also suggest that hCG promotes myelination by inducing myelin protein zero (P0) expression and that hCG/LH and its receptor participate in the development and maintenance of the mammalian nervous system. Thus, hCG may be a potential regeneration drug for treatment of acute neural injuries or neurodegenerative disorders.

Meng XL, Rennert OM, Chan WY. Human chorionic gonadotropin induced neuronal differentiation of PC12 cells through activation of stably expressed lutropin/choriogonadotropin receptor. *Endocrinology* 2007;148:5865-5873.

Molecular basis of tumorigenic activity of constitutively activated LH/CG-R mutants
Chan W-Y, Lee; in collaboration with Rennert, Leung, Su

Discovery of the presence of LH/CG-R with germline and somatic, activating mutations in patients with testicular tumor raised the question of the potential tumorigenic effect of mutated LH/CG-R. Two patients with the activating Asp578Gly mutation developed Leydig cell neoplasia; we identified a somatic, activating Asp578His mutation in several testicular tumor patients. Animal studies have so far failed to establish lines of male or female transgenic founder mice carrying LH/CG-R with the Asp578His mutation, which would reveal inherent differences between the germline and the somatic mutations. To explore the difference in the biological effects between the two mutations, we established an MA-10 Leydig cell model by stable transfection with the two mutant human receptors LH/CG-R-Asp578Gly and LH/CG-R-Asp578His. By examining gene expression profiles with cDNA microarray and using a systems-biological approach, hierarchical clustering, and multidimensional scaling analysis, we could distinguish the wild type and the two mutants. The presence of the LH/CG-R Asp578Gly mutation altered the expression of 54 genes while 49 genes were changed in the presence of the LH/CG-R Asp578His mutation. Comparing both mutants to the wild type,

we found that 132 genes were differentially expressed. We also found novel regulatory pathways unique to each mutation—9 networks in LH/CG-R-Asp578Gly–expressing cells and 12 in LH/CG-R-Asp578His–expressing cells. Further analyses showed that *c-Myc* and *c-Src* were the key regulators associated with Asp578Gly and Asp578His mutants, respectively. We confirmed the involvement of these two factors by molecular and functional assays. The results open a new dimension and provide a novel explanation for the role of LH/CG-R mutation in testicular tumorigenesis.

Pang ALY, Martin MM, Martin A, Chan WY. Molecular basis of diseases of the endocrine system. In: Coleman WB, Tsongalis GJ, eds. *Molecular Pathology: The Molecular Basis of Human Disease*. Elsevier-Academic Press, 2009, in press.

Other Factors Affecting Gene Expression

Effects of mitochrondria on nuclear gene expression

Chan W-Y, Lee; in collaboration with Wong, Ma

Mitochondrial dysfunction is one of the most notable features of cancer cells. There are fewer mitochondria in rapidly growing tumor cells than in normal cells and they are smaller and exhibit a variety of altered morphologies. Somatic mutations in mitochondrial DNA are found in 30 to 100 percent of all tumors studied. The role of the altered mitochondrial genome in tumor growth is unclear. Recent studies with transmitochondrial cybrids revealed that cancer cell–derived mitochondria could alter the behavior of the host cell. To understand the genetic basis of this phenomenon, we examined the host-cell gene profile in transmitochondrial cybrids containing mitochondria derived from breast cancer cells. Preliminary results indicated distinct differences in nuclear gene expression between cybrids with normal mitochondria and cybrids with cancer cell–derived mitochrondria. We also observed marked differences in nuclear gene expression patterns between cybrids harboring various cancer cell–derived mitochrondria that behave differently with respect to tumorigenicity and various key regulators in energy metabolism, with gene signatures mimicking the breast cancer profile. To reveal the potential transcriptional regulators of the nuclear gene set (from the cybrids harboring cancer-cell derived mitochondria), we performed promoter analysis. Of the 12 known genes validated by real-time PCR, five genes demonstrated the presence of TP53 binding site while NF-kappaB binding sites were present in six genes. Four genes contain sites for both factors. Using a systems-biological analysis, we reconstructed gene networks to also confirm the potential regulation by p53 or NF-KappaB. The results suggest that mitochondria derived from breast cancer cell lines can communicate with and affect transcription activities of the nucleus. Thus, genetically altered mitochondria may actively participate in breast cancer development.

Genomic effects of naked ceria nanoparticles

Chan W-Y Lee; in collaboration with Chan S-W, Tuan

Despite the myriad applications of nanoparticles and possibly high human exposure, the impact of manufactured nanoparticles on the environment and human health is not clear. Most studies on the biological effects of nanoparticles have focused on the activities of functional components, such as drugs, proteins, antibodies, and so forth, conjugated to the nanoparticles. More recently, it has been reported that some naked nanoparticles are cytotoxic and affect cellular activities. Envirox, a trade name for naked cerium oxide (CeO_2; ceria) nanoparticles used as a diesel fuel additive, reduces fuel consumption and the emission of carbon dioxide and particulates from vehicles. However, the health impact of CeO_2 nanoparticles is poorly understood. To generate a detailed, unbiased assessment of the molecular responses to CeO_2 nanoparticles (CeO-6, 6 nm), we conducted a genomic study to identify gene expression signatures associated with nanoparticle exposure in a murine neuronal cell (HT22) model. We controlled for the effect of particle size by using 1,000 nm diameter cerium oxide (CeO-M) and for the effect of chemical property by using 300 nm diameter aluminum oxide particles (AlO). We demonstrated that cerium oxide nanoparticles induced changes in the transcriptome of murine neuronal cells—changes that depended on the chemistry and size

of the nanoparticles. The genes whose expression is affected by the presence of CeO-6 are related to neurological disease, cell cycle control, and growth. Similar studies with human mesenchymal stem cells revealed the induction of osteogenic factors by CeO-6. Our observations indicate that nanoparticles are capable of inducing size- and chemistry-specific changes in cells, underscoring the need for in-depth assessment of the potential health risks of nanoparticle exposure.

Publication Related to Other Work

Ohta S, Lai EW, Morris JC, Pang ALY, Watanabe M, Yazawa H, Zhang R, Green JE, Chan WY, Sirajuddin P, Taniguchi S, Powers JF, Tischler AS, Pacak K. Metastasis-associated gene expression profile of liver and subcutaneous lesions derived from mouse pheochromocytoma cells. *Mol Carcinog* 2008;47:245-251.

[1]University of California Santa Cruz
[2]Reverse transcriptase–polymerase chain reaction
[3]cap analysis gene expression
[4]retinol binding protein

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REPRODUCTIVE ENDOCRINOLOGY AND SCIENCE

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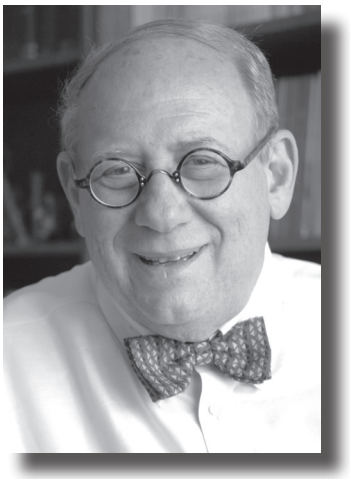

Deviations from the norm can prevent the establishment of pregnancy or contribute to obstetrical disorders associated with aberrant placentation. Through basic and translational research, our mission is to identify critical cellular and molecular events required for successful implantation and to understand their relationship to the pathologies of early pregnancy. Our objective is therefore to understand the biology of the developing blastocyst, which requires the differentiation of the trophectoderm—its outer epithelium—into invasive trophoblast cells and the maintenance of a cohort of pluripotent embryonic stem cells. We focus on the developing blastocyst, trophectoderm, and pluripotential embryonic stem cells and investigate the interactions between the uterine endometrium and implanting blastocyst at the inception of pregnancy. We are evaluating agents for cellular and molecular evidence of successful and pathologic implantation.

Regulation of blastocyst development
Armant, DeCherney; in collaboration with Brenner

Using in vitro approaches with both mouse and primate animal models, we investigated the role of the maternal environment on development of pre- and peri-implantation embryos. Previous work with mouse blastocysts revealed signaling pathways regulated by oxygen, growth factors, and the extracellular matrix; these pathways advance the trophoblast's intrinsic developmental program. In the coming year, we plan to extend our studies of trophoblast differentiation and examine how the microenvironment sustains the pluripotent embryonic stem cell population within the blastocyst. Our collaborator Carol Brenner has established the rhesus monkey pre-implantation embryo model and works with several rhesus embryonic stem cell lines. Our upcoming studies will provide needed information on the comparative physiology of mouse and primate embryonic development and implantation.

Nuclear and cytoplasmic damage of pre-implantation embryos
Armant, DeCherney; in collaboration with Brenner

Fluorescent in situ hybridization analysis of blastomeres from pre-implantation embryos from young rhesus macaque females revealed a high chromosomal abnormality frequency that was surprisingly similar to rates demonstrated by in vitro–produced (IVP) human embryos. In the coming year, we plan to study monkey IVP embryos from older female animals and animals subjected to various ovulation-induction regimes. We are assessing mitochondrial replication and function to determine

their impact on cytoplasmic cellular components, as the mitochondrion is central to energy and toxicity management. The proportion of mtDNA deletions in stimulated oocytes and embryos from rhesus macaques is significantly higher than that of mtDNA deletions in immature, unstimulated oocytes derived from ovaries of age-matched monkeys. These findings validate non-human primates as a model for investigating reproductive mechanisms relevant to human infertility. Our studies will have an impact on human in vitro fertilization where aneuploidy in embryos is common.

The evaluation of trophoblast differentiation and survival during human implantation is critical to the successful establishment of a pregnancy. We are studying multifunctional growth factors involved in normal trophoblast development and associated pathologies.

DeUgarte CM, Li M, Surrey M, Danzer H, Hill D, DeCherney AH. Accuracy of FISH analysis in predicting chromosomal status in patients undergoing preimplantation genetic diagnosis. *Fertil Steril* 2008;90:1049-1054.

Metabolomics of frozen/thawed immature human oocytes

Tong, DeCherney

Given that ATP is a principal donor of free energy and phosphate in many intracellular metabolic reactions and signal transduction pathways, we measured the ATP level in oocytes after a freezing/thawing process and compared the level to that in fresh oocytes (human oocytes from a stimulated IVF cycle that are discarded owing to their nuclear immaturity). ATP levels from surviving frozen/thawed oocytes are significantly lower than those in fresh oocytes. The low level results from cryopreservation, but, once the mitochondria begin to function after thawing, the ATP level rises to close to that of fresh oocyte. We are continuing our study to measure the ATP level in a larger number of oocytes and will extend our study by incubating post-thawed oocytes for either 2 or 3 hours to demonstrate the effect of the incubating time after thawing.

Manipalviratn S, DeCherney A. Clinical application of human oocyte cryopreservation. *Rev Recent Clin Trials* 2008;3:104-110.

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TARGET TISSUE GLUCOCORTICOID SIGNALING: BASIC AND CLINICAL IMPLICATIONS

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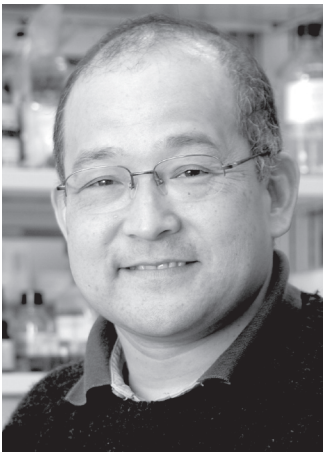

The glucocorticoids' life-sustaining functions are many and they play an important role in the therapy of several inflammatory, autoimmune, allergic, and lymphoproliferative disorders. Changes in tissue sensitivity to glucocorticoids may develop into pathologic states and influence disease course. We thus continue to investigate the glucocorticoid signaling system and the clinical implications of changes in tissue sensitivity to glucocorticoids. In addition, we serve as the world's referral center for analyzing pathologic mutations of the glucocorticoid receptor (GR). We also conduct research into the molecular mechanisms underlying the AIDS-related lipodystrophy and insulin resistance syndrome and strive to increase our understanding of the physiologic/pathophysiologic interactions among the endocrine, immune, and central nervous systems.

Pathologic glucocorticoid receptor mutations causing familial/sporadic glucocorticoid resistance syndrome

Kino, Nader; in collaboration with Charmandari, Chrousos

This past year, we described a glucocorticoid-resistant patient from Colombia in whom we identified a novel heterozygous point mutation in the GR gene that results in the replacement of phenylalanine (F) by leucine (L) at amino acid position 737 (GRF737L). We also indentified in another Colombian patient a heterozygotic mutation that replaced histidine (H) by aspartic acid (D) at amino acid position 401 (GRD401H). Using panels of assay methods, we characterized details of molecular defects of GRD401H and found that this mutant receptor demonstrated two to three times stronger transcriptional activity than the wild-type receptor while the patient showed a mixed phenotype characteristic of both glucocorticoid resistance and hypersensitivity. Thus, this mutant receptor causes tissue-specific alterations of glucocorticoid sensitivity. In another glucocorticoid kindred from Greece, we further identified a pathologic mutation leading to substitution of valine (V) by alanine (A) at amino acid position 423 (GRV423A) at the base of the first zinc finger of the DNA-binding domain (DBD).

Charmandari E, Ichijo T, Jubiz W, Zachman K, Chrousos GP, Kino T. A novel point mutation in the amino terminal domain of the human glucocorticoid receptor (hGR) gene enhancing hGR-mediated gene expression. *J Clin Endocrinol Metab* 2008;93:4963-4968.

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Identification of the intracellular molecules that alter tissue glucocorticoid action and their pathophysiologic implications for common diseases

Kino, Nader, Pavlatou; in collaboration with Almeida, Chrousos, Edelman, Pant, Rother, Skarulis

We discovered that the brain-specific serine/threonine kinase cyclin–dependent kinase 5 (CDK5) phosphorylates the GR at several serine residues located in the immunogenic domain and then modulates the transcriptional activity of this receptor in a target gene–specific fashion. CDK5 is essential for brain development and has implications for the pathogenesis of neurodegenerative disorders such as Alzheimer's disease and amyotrophic lateral sclerosis. Given that glucocorticoids are pivotal for brain function and that excess amounts of these hormones cause neuronal death and emotional and psychiatric problems, our findings suggest that aberrant activation of CDK5 may exert its pathologic actions also by modulating glucocorticoid activity. To examine the clinical implications of our findings, we have begun to evaluate the expression/activity of CDK5 along with tissue glucocorticoid action in brain tissues from patients with major depression.

We also found that SET/TAF-Ibeta, which others identified as both a part of the SET-CAN oncogene product and a component of the inhibitor of the histone acetyltransferases (INHAT) complex, acted as a negative regulator of GR transcriptional activity. In addition, we discovered that ligand-activated GR stimulated transcription by displacing the INHAT complex from histones via physical interaction through the receptor's DBD. In contrast to Set/TAF-Ibeta, the Set-Can fusion protein bound to glucose response elements (GRE) regardless of ligand availability and strongly suppressed GR-induced transcriptional activity/histone acetylation, possibly participating in the development of glucocorticoid insensitivity in acute undifferentiated leukemia with *Set-Can* translocation.

We have almost completed our examination of the non–protein-coding RNA growth arrest–specific 5 (Gas5), which accumulates in growth-arrested cells but whose physiologic roles remain unknown. We found that Gas5 has a GRE-like sequence in the 3′ portion of its intramolecular double helical structure, through which this ncRNA interacts competitively with the GR DBD by mimicking DNA GREs and suppressing GR's transcriptional activity. Thus, Gas5 is a growth arrest–related co-repressor of the GR harboring an RNA mock "GRE," restricting the expression of steroid-responsive genes. This novel concept suggests competition between ncRNA and genomic DNA for the DBD of steroid receptors. Our work also suggests that Gas5 contributes to the cellular adaptive reaction to starvation, preventing apoptosis and saving energy resources. In addition to these molecules (cyclin-dependent kinase 5, Set/TAF-Ibeta, and Gas5), we have examined the influence of the energy sensor AMK-activated protein kinase (AMPK) and the circadian rhythm transcription factors CLOCK/BMAL1 on GR-mediated transcriptional activity. We found that they modulate tissue glucocorticoid action in vitro and in vivo by acetylating and phosphorylating GR, respectively. We further found that the nuclear liver X receptor, which transduces biologic signals of cholesterol and fatty acid metabolism, suppresses GR transcriptional activity. These results indicate that glucocorticoid action in local tissues is adjusted, in a circadian fashion, according to cellular energy levels and the supply of energy resources.

To integrate our novel findings on regulators of tissue glucocorticoid action into clinically meaningful information, we have begun collaborative work that is examining expression of the genes known to regulate the transcriptional activity of the GR in tissues obtained from anorexic, lean, and obese individuals and diabetic patients. Our clinically oriented research will reveal factor(s) and mechanism(s) responsible for alterations of target tissue glucocorticoid action in these common diseases.

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Viral effects on host endocrine systems

Kino, Ng; in collaboration with Chrousos, Kopp, Ozato

This year, we examined the effect of the HIV-1 accessory protein Vpr on the activity of the nuclear receptor PPARgamma; PPARgamma is a critical regulator of lipid and glucose metabolism. We discovered that Vpr, whether administered extracellularly or expressed in cells, suppressed PPARgamma agonist-induced adipocyte differentiation by suppressing the receptor's transcriptional activity. The results suggest that circulating Vpr, or, alternatively, Vpr produced as a consequence of direct infection of adipocytes, alters insulin sensitivity by suppressing PPARgamma activity, possibly contributing to the development of the lipodystrophy and insulin resistance observed in HIV-1–infected patients.

In collaboration with Keiko Ozato, we have begun to examine the expression of all nuclear receptors, including the GR and numerous transcriptional co-regulators, in dendritic cells in response to viral infection. These cells play a central role in the initiation of both innate and acquired immunity by capturing/presenting viral antigens to regulatory T cells and B cells. In addition, they secrete a variety of cytokines that further activate downstream defense mechanisms. Our preliminary studies suggest that the endocrine nuclear receptor system immediately responds to viral infection and changes its profile in response to specific viruses. Our research will provide information relevant to the hypothesis that some viruses alter host pathogenesis to their advantage by changing the activities of the endocrine/nuclear receptor system in immune cells. We believe that our work will provide a new understanding of chronic infection, inflammation, allergy, autoimmunity, senescence, and cancer and will allow us to explore rational therapeutic approaches to treating virus-related pathologies through nuclear hormone receptor ligands.

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Using spontaneous 46,XX primary ovarian insufficiency as a model condition, we investigate genetic, immunological, and molecular aspects of disorders of the menstrual cycle and human reproduction. Once considered an irreversible condition similar to normal menopause and previously referred to as "premature menopause," "premature ovarian failure," or "POF," primary ovarian insufficiency (POI) causes young women to develop amenorrhea and infertility before age 40. In 90 percent of cases, the mechanism of the ovarian insufficiency remains a mystery even after thorough investigation. We investigate POI with the use of mouse models of the condition and by recruiting patients to research protocols that are designed to gain insight into the mechanisms of ovarian follicle dysfunction and to guide the development of appropriate treatments for such patients.

Genetic mechanisms of spontaneous 46,XX POI

Tong, Vanderhoof, Corrigan, Nelson; in collaboration with Wittenberger, Reijo Pera, Castrillon

Genetic studies have identified several loci at Xq22, Xq26–28, and Xp11.2–p22.1 whose disruption has been associated with the development of spontaneous POI. Fragile X syndrome, an X-linked disorder, is the most common hereditary cause of mental retardation and developmental delay. In nearly all cases, the disorder is caused by an expansion of CGG trinucleotide repeats in the 5′ untranslated region of *FMR1* (Fragile Site Mental Retardation 1 gene). Interestingly, pre-mutations in the *FMR1* gene, located at Xq27.3, have been associated with the development of spontaneous 46,XX POI. We previously reported a case of a young woman with established spontaneous POI who conceived subsequent to the diagnosis and had a child who manifests mental retardation due to fragile X syndrome. The case illustrates the need to inform patients with POI about their increased risk of carrying a pre-mutation in *FMR1*, their options for testing, and the potential implications for family members with regard to diagnosis of menstrual irregularity, developmental delay, and neurological symptoms.

The forkhead transcription factor Foxo3 is a master regulator and potent suppressor of primordial follicle activation. Loss of Foxo3 function in the mouse leads to primary ovarian insufficiency due to global follicle activation. The mouse *Foxo3* locus is haploinsufficient, and *Foxo3*$^{-/+}$ females undergo early reproductive senescence consistent with an increased rate of primordial follicle utilization. As part of an international collaboration, we sought to determine if heterozygous or homozygous polymorphisms or mutations of the human orthologue *FOXO3* contribute to primary ovarian insufficiency. We sequenced the exons and flanking splice sequences of the gene in a large number (302) of women with idiopathic primary ovarian insufficiency. We identified a total of eight

single-nucleotide polymorphisms (SNPs), revealing a substantial amount of genetic variation at the *FOXO3* locus. Allelic frequencies in control samples excluded several of the variants as causal. For the remaining variants, we performed site-directed mutagenesis to assess their functional impact but observed that these rare sequence variants were not associated with significant decreases in FOXO3 activity. Taken together, our findings suggested that, despite the potential for *FOXO3* haploinsufficiency to cause POI, *FOXO3* mutations are not a common cause of primary ovarian insufficiency.

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Testosterone deficiency and sexual function in young women with spontaneous 46,XX POI
Kalantaridou, Vanderhoof, Corrigan, Nelson; in collaboration with Calis, Troendle

The normal premenopausal ovary is an important source of androgen as well as of estrogen production. In premenopausal women, daily testosterone production is approximately 300 μg, of which approximately half is derived from the ovaries and half from the adrenal glands. Thus, young women with spontaneous POI would be expected to have lower serum testosterone levels than normal women. However, there is considerable controversy as to whether female androgen deficiency syndrome in fact exists and whether such a syndrome has clinical consequences for women, with effects on bone, quality of life, or libido. We sampled regularly menstruating control women during the mid-follicular phase. While off estrogen therapy, patients with POI had median serum free testosterone concentrations that were significantly lower than those in controls. While on physiologic transdermal estradiol therapy, the patients' median serum free testosterone dropped significantly lower even though their sex hormone binding globulin levels did not change. While on estradiol replacement, 13 percent of the women had serum free testosterone levels below the lower limit of normal.

No controlled studies to date have specifically evaluated sexual function in women with spontaneous 46,XX POI. We sought to determine whether women with this condition who have been given estradiol replacement experience sexual dysfunction as compared with young women of similar age with normal ovarian function. Sexual dysfunction can have harmful effects on relationships, self-esteem, and quality of life. Normal sexual function involves the interaction of emotional, cultural, personal, interpersonal, contextual, and medical factors. Young women find the diagnosis of 46,XX spontaneous POI particularly traumatic. Disorders of mental health, especially depression, frequently underlie the presentation of sexual dysfunction. The traditional separatist notion that sexual dysfunction has either psychological or organic origins has given way to an understanding that the two are closely linked and must be taken into consideration together.

We assessed sexual function in 143 women with spontaneous 46,XX POI after at least three months of a standardized hormone replacement regimen (100 μg/day estradiol patch and oral medroxyprogesterone acetate 10 mg for 12 days each month) and compared the findings with those of 70 control women with normal ovarian function and regular menstrual periods. We employed the Derogatis Interview for Sexual Function Self Report (DISF-SR), a validated self-administered questionnaire. Women with POI had significantly lower DISF-SR composite scores than control women. Their serum total testosterone levels were significantly correlated with their DISF-SR

composite scores, but accounted for only 4 percent of the variance in the score. Patients with testosterone levels below normal tended to have lower DISF-SR composite scores. As assessed by the DISF-SR, sexual function is in the normal range for most young women with 46,XX spontaneous POI who are receiving physiologic estradiol replacement. However, as a group, these women score significantly lower on this sexual function scale than control women. We are currently investigating the relative contributions of psychosocial factors and testosterone deficiency to the sexual function of these patients.

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Needs of young women with spontaneous 46, XX primary ovarian insufficiency

Popat, Ventura, Fitzgerald, Covington, Vanderhoof, Nelson; in collaboration with Orshan, Calis, Koziol, Troendle

We investigate women's psychological response to the diagnosis of spontaneous POI. In general, given that the inability to reproduce creates a profound loss for most women and affects their self-esteem and relationships, the literature has thoroughly documented the psychological distress of women with infertility. Most commonly, women discover that they are infertile in a gradual manner after many failed attempts at conception. However, in cases such as POI, medical conditions that preclude normal fertility may be uncovered during the course of investigation of other presenting complaints. Thus, the clinician is confronted with communicating information about a sudden, unexpected diagnosis that is life-altering but not life-threatening. The manner in which bad news is communicated can have a profound effect on patient satisfaction, treatment compliance, quality of life, and other health outcomes. We previously demonstrated that over two-thirds of women with POI were unsatisfied with the manner in which they were informed of the diagnosis. Nearly 90 percent reported that they experienced moderate to severe emotional distress at the time, the degree of which was positively correlated with the degree of dissatisfaction with the manner in which they had been informed of the diagnosis. They perceived that thorough and accurate medical information on POI, support of others, and spirituality were all helpful in coping. Patients perceived a need for clinicians to spend more time with them and provide more information about premature ovarian failure.

In structured interviews, 90 percent of women with spontaneous POI reported to us that spirituality plays an important role in how they cope with the emotional sequelae of the diagnosis. In a follow-up study, we demonstrated a statistically significant positive correlation between functional well-being and spiritual well-being by employing an instrument specifically designed and validated to measure spirituality apart from religiosity. Our findings suggest a need for a controlled interventional clinical trial to test the hypothesis that strategies to assist women in finding meaning and purpose in the diagnosis of spontaneous POI would improve their functional well-being and quality of life. A group of women with the disorder who receive standard management would serve as controls.

We tested the hypothesis that women with spontaneous POI differ from control women with regard to perceived social support. Using validated self-reporting instruments (Personal Resource Questionnaire-85 [PRQ85], Rosenberg Self-Esteem Scale), we investigated the relationship between perceived social support and self-esteem by comparing 154 women diagnosed with spontaneous POI at a mean age of 27 years with 63 healthy control women. We found that women with POI had significantly lower scores than controls on the perceived social support scale and self-esteem scale. The findings remained significant after modeling with multivariate regression for differences in age, marital status, and having children. The modeling showed a significant positive correlation between self-esteem scores and perceived social support and no significant differences in perceived social support or self-esteem related to marital status, number of children, or time since diagnosis. The evidence supports the need for prospective controlled studies. Strategies to improve social

support and self-esteem might provide a therapeutic approach for reducing the emotional suffering that accompanies the life-altering diagnosis of spontaneous POI.

We also investigated methods that might improve fertility in women with POI. Normally, ovarian follicles grow in response to FSH stimulation; then, the mid-cycle LH surge induces follicle rupture, ovulation, terminal differentiation of granulosa cells into luteal cells, and formation of the corpus luteum. In up to 78 percent of women with POI, the normal process of ovulation usually fails despite the presence of antral follicles. We demonstrated previously that many of these follicles fail to function normally; instead, they become prematurely luteinized because of associated chronically elevated serum LH levels. The process is akin to "luteinized unruptured follicle syndrome." Estrogen replacement therapy might improve ovulation rates in women with spontaneous POI by lowering serum LH levels to normal. We found that a regimen of 100 μg per day of transdermal estradiol replacement achieves normal serum LH levels in approximately half of women with spontaneous POI. Theoretically, by avoiding inappropriate luteinization, physiologic estradiol replacement therapy might improve follicle function in women with POI. Accordingly, we are undertaking controlled studies to assess the effect of estrogen replacement on follicle function in such women.

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INVESTIGATION OF ADRENAL GLAND DISORDERS AND DISORDERS OF FEMALE REPRODUCTION

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Over the past decade, we have made major contributions to the differential diagnosis of hypercortisolism. We established the corticotropin releasing hormone (CRH) test and inferior petrosal sinus sampling (IPSS) as major diagnostic tools in the identification of pituitary adenomas causing Cushing's syndrome. However, the detection of Cushing's syndrome remains difficult, as does the localization of ectopic ACTH-producing tumors. We also evaluate the pathophysiology and potential new treatments for fibroids in women. This reproductive disorder is common, poorly understood, and lacks optimal medical treatments.

Adrenal Gland Disorders

Localization of ectopic ACTH-secreting tumors

Nieman, Gundabolu; in collaboration with Pacak, Chen, Carrasquillo, Gharib, Sinaii

Imaging studies are the cornerstone for tumor localization in patients with Cushing's syndrome caused by ectopic adrenocorticotropin hormone (ACTH) secretion (EAS). Despite routine use of computed tomography (CT) and magnetic resonance imaging (MRI), tumors remain occult in up to 50 percent of patients with EAS. Up to half of the EAS patients do not respond to medical therapy of hypercortisolism and must undergo bilateral adrenalectomy with life-long replacement therapy. Thus, there is a need for improved imaging techniques to identify ACTH-secreting tumors.

Nuclear medicine techniques permit in vivo imaging of pathophysiological processes. Among these techniques, positron emission tomography (PET) studies are finding increasing use in oncology. We previously evaluated the utility of [^{18}F]-fluorodeoxyglucose (FDG) positron emission tomography (PET) or [^{111}In-DTPA-D-Phe]-pentetreotide (OCT) at higher-than-standard doses of radionuclide (18 mCi; H-OCT) in all consecutive patients with EAS (thus, including those in whom we found a tumor later, as well as those who were occult). We found that FDG-PET did not detect tumors that were occult on CT/MRI; H-OCT rarely identified a lesion. Thus, conventional modalities of CT and MRI should be used in these patients. FDG-PET does not provide additional information. H-OCT may be useful and needs more investigation. We are now evaluating the utility of [^{18}F]- L-3,4-dihydroxyphenylalanine (^{18}F-DOPA) PET to identify tumors in any patient presenting with EAS. This compound is a precursor for serotonin production in neuroendocrine tumors and is therefore a good candidate for PET examination given that most occult ACTH-secreting tumors are neuroendocrine.

Evaluation of guidelines for the diagnosis and treatment of Cushing's syndrome

Nieman

The literature provides little guidance regarding the diagnosis and treatment of Cushing's syndrome. In collaboration with international experts, we developed guidelines for diagnosis that call for four first-line tests (urine cortisol, salivary cortisol, and dexamethasone 1 and 2 mg tests), along with suggestions about their use and caveats. Similarly, we developed guidelines that advocated transsphenoidal surgery and adjunctive therapy for treatment.

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Disorders of Female Reproduction

CDB-2914 as a potential therapeutic agent for fibroids

Nieman, Levens, Wei, Blocker; in collaboration with Blithe, Nansel, Armstrong, Premkumar, Chow, Wesley, Gladwin

We have investigated the effect of single doses of the progestin receptor modulator CDB-2914 on the menstrual cycle in women and showed that doses of 100 or 200 mg retard folliculogenesis and precipitate menses in the follicular and luteal phases, respectively. In a small study of 18 women, we have shown that CDB-2914, at a 10 or 20 mg daily dose, can decrease fibroid size. Ongoing studies evaluate longer-term use. We are also using gene arrays to evaluate the pathophysiology of leiomyomata.

We have explored whether the endometrium of women with endometriosis is abnormal and whether such abnormality might account for decreased fertility rates in this condition. We recently demonstrated lower level of progesterone-dependent markers of implantation in the luteal phase endometrium of women with endometriosis than in with healthy volunteers. In addition, a placebo-controlled randomized trial of raloxifene in women with endometriosis showed earlier return of pain with the agent despite anti-endometrial effects in healthy control women.

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DIAGNOSIS, LOCALIZATION, PATHOPHYSIOLOGY, AND MOLECULAR BIOLOGY OF PHEOCHROMOCYTOMA

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We conduct patient-oriented research into the etiology, pathophysiology, genetics, diagnosis, and treatment of pheochromocytoma (PHEO) and paraganglioma (PGL). Projects include not only translational research—applying basic science knowledge to clinical diagnosis, pathophysiology, and treatment—but also "reverse translation research" by which clinical findings lead to new concepts for pursuit by basic researchers in the laboratory. Our goals are to (1) establish new and improved methods and strategies for diagnosis and localization of PHEO and PGL; (2) explain the molecular basis for varying clinical presentations of PHEO and PGL and establish the pathways of tumorigenesis; (3) search for new molecular and genetic markers for diagnosis and treatment of malignant PHEO and PGL; and (4) facilitate new and improved collaborations and interdisciplinary studies. To achieve these goals, we base our strategy on multidisciplinary collaborations with investigators from several NIH Institutes and outside medical centers. We link a patient-oriented component with two bench-level components (Figure 10.1). The patient-oriented component (medical neuroendocrinology) is the driving force for our hypotheses and discoveries. The two bench-level components (tumor pathogenesis and chemistry; biomarkers) emphasize, first, technologies of basic research tailored for pathway and target discovery and, second, the development of discoveries into clinical applications.

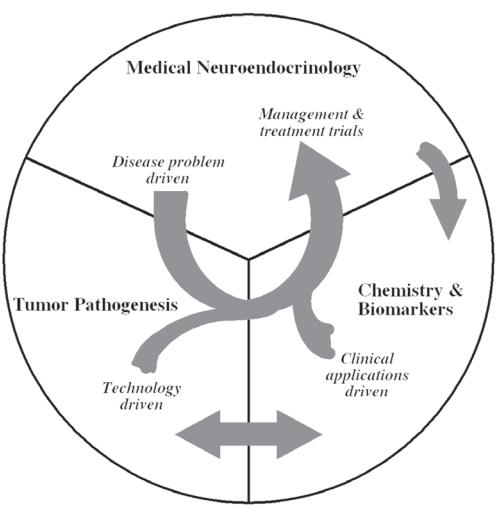

FIGURE 10.1

Hereditary PHEOs and PGLs

Pacak, He, Adams, Lai; in collaboration with Timmers, Huynh, King, Chen, Reynolds, Ling, Fojo, Linehan, Libutti, Merino, Raygada, Morris, Munson, Lubensky, Wesley, Lenders, Widimsky, Eisenhofer, Tischler

Advances in genetics and the recognition of the high prevalence of PHEO/PGL in certain familial syndromes dictate mandatory tumor screening in patients with those syndromes, irrespective of the

presence of classical clinical signs and symptoms. Accumulating data also indicate that many more PHEOs/PGLs result from germline mutations than previously recognized, raising the importance of considering an underlying hereditary condition even in those patients without an obvious family history. To date, mutations in five main genes have been identified as responsible for familial PHEOs/PGLs (Table 10.1): (1) the von Hippel-Lindau (*VHL*) gene in VHL syndrome; (2) the *RET* gene in multiple endocrine neoplasia type 2 (MEN 2); (3) the neurofibromatosis type 1 (*NF-1*) gene associated with von Recklinghausen's disease; and (4) mutations in genes encoding mainly the B and D subunits of mitochondrial succinate dehydrogenase (*SDHB* and *SDHD*) associated with familial PHEOs/PGLs.

Table 10.1: Hereditary PHEO/PGL: Facts and figures

Gene	***VHL***	***RET***	***NF1***	***SDHD***	***SDHB***
Chromosome	3p25-26	10q11.2	17q11.2	11q23	1p36.13
Exons	3	21	59	8	4
Frequency in "sporadic" tumors (%)	2-11	1-5	unknown	3-10	4-7
Predisposition to malignancy (%)	~3	<3	11	<2	66-83
Tumor catecholamine phenotype	NE	EPI	EPI	unknown	NE/DA
Adrenal disease	++	++	++	+	+
Extra-adrenal disease	+	rare	+	++	++

Low (+) to high (++) frequency; NE: norepinephrine; EPI: epinephrine; DA: dopamine

Patients with mutations of *SDHB* and *SDHD* genes are more likely to develop extra-adrenal rather than adrenal tumors. Furthermore, *SDHB* mutations appear to be associated with more aggressive tumor behavior and a higher rate of malignancy than are *SDHD* mutations. In an initial study, we examined the frequency of *SDHB* mutations in patients with malignant PHEOs/PGLs and found pathogenic *SDHB* mutations in 30 percent of such patients. In patients presenting initially with primary abdominal PGLs, mutations of the *SDHB* gene were associated with about one-half of all malignancies. The high frequency of *SDHB* germline mutations among patients with malignant disease, particularly when originating from PGL, justifies a high priority for *SDHB* germline mutation testing in these patients. Recently, however, malignant PGLs were also reported among a small minority of Dutch carriers of the *SDHD* founder mutation D92Y. Therefore, we investigated which *SDHD* mutations are associated with malignant PGL and found that germline S*DHD* mutations underlying metastatic PGL include G148D, Y114X, L85X, W43X, D92Y, and IVS2+5 G→A.

We also performed a retrospective analysis of 71 subjects with metastatic PHEO/PGL (30 subjects with *SDHB* mutation and 41 subjects without *SDHB* mutation), including three subjects with mutation of the *RET* gene, three subjects with mutation of the *VHL* gene, and one subject with mutation of the *SDHD* gene. Sixty-nine percent presented with bone metastases (77 percent of those with *SDHB* mutation and 63 percent of those without *SDHB* mutation), 39 percent with liver metastases, and 32 percent with lung metastases. The most common sites of bone involvement were thoracic spine (80 percent), lumbar spine (78 percent), and pelvic and sacral bones (78 percent). Subjects with *SDHB* mutation showed significantly higher involvement of long bones and pelvic and sacral bones than those without the mutation.

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Imaging of various PHEOs and PGLs

Pacak, He, Havekes, Adams; in collaboration with Timmers, Chen, Reynolds, Ling, Fojo, Linehan, Morris, Wesley, Carrasquillo, Lenders, Ilias, Widimsky, Eisenhofer

We compared different imaging methods in various PHEOs/PGLs. Of 11 non-metastatic lesions detected by any technique, we detected eight lesions by ^{18}F-DOPA PET, seven by ^{18}F-FDA PET/CT, 8 by ^{18}F-FDG PET/CT, and nine by ^{123}I-MIBG scintigraphy. Computed tomography (CT) and/or magnetic resonance imaging (MRI) identified 108 lesions in 19 patients with metastatic PGL. Functional imaging identified 80 additional lesions. Discrepant readings between the two nuclear-medicine physicians were solved by consensus for seven lesions on ^{18}F-DOPA PET and seven lesions on ^{18}F-FDA PET. Of 188 metastatic lesions, we detected 116 by ^{18}F-DOPA PET, 110 by ^{18}F-FDA PET/CT, 107 by ^{18}F-FDG PET/CT, and 67 by ^{123}I-MIBG scintigraphy. In reference to lesions detected by CT and/or MRI, sensitivities were 52 percent for ^{18}F-DOPA PET, 64 percent for ^{18}F-FDA PET/CT, 72 percent for ^{18}F-FDG PET/CT, and 54 percent for ^{123}I-MIBG scintigraphy. Sensitivities in reference to the total number of lesions detected by any technique were 62 percent for ^{18}F-DOPA PET, 59 percent for ^{18}F-FDA PET/CT, 57 percent for ^{18}F-FDG PET/CT, and 36 percent for ^{123}I-MIBG scintigraphy.

The best overall sensitivity in detecting bone metastases was ^{18}F-FDA PET (90 percent), followed by bone scintigraphy (82 percent), CT/MRI (78 percent), and $^{123/131}$I-MIBG scintigraphy (71 percent). In subjects with *SDHB* mutation, imaging modalities with the best sensitivities for detecting bone metastases were CT/MRI (96 percent), bone scintigraphy (95 percent), and ^{18}F-FDG PET (92 percent). In subjects without *SDHB* mutation, the modality with the best sensitivity for detecting bone metastases was ^{18}F-FDA PET (100 percent). We concluded that bone scintigraphy should be used in the staging of patients with malignant PHEO/PGL, particularly in those with *SDHB* mutation. We highly recommend ^{18}F-FDG PET as a PET imaging modality in *SDHB* mutation patients but ^{18}F-FDA PET in patients without the mutation.

In another study, we investigated whether carbidopa also improves ^{18}F-DOPA PET imaging of adrenal PHEOs. Two patients with non-metastatic adrenal PHEO and nine patients with extra-adrenal abdominal PGL (one non-metastatic, eight metastatic) underwent whole-body CT, MRI, baseline ^{18}F-DOPA PET, and ^{18}F-DOPA PET with oral pre-administration of carbidopa. We recorded the dynamics of tracer uptake by the lesions and the physiological distribution of ^{18}F-DOPA in normal tissues. Seventy-eight lesions were detected by CT and/or MRI, 54 by baseline ^{18}F-DOPA PET, and 57 by ^{18}F-DOPA PET plus carbidopa. In reference to findings on CT and MRI, the sensitivities of baseline ^{18}F-DOPA PET were 47.4 percent for lesions and 55.6 percent for positive body regions versus 50.0 percent (lesions) and 66.7 percent (regions) for ^{18}F-DOPA PET plus carbidopa (both non-specific versus baseline). Compared with baseline, carbidopa resulted in the detection of additional lesions in 3 of 11 patients. Carbidopa increased the mean ±SD peak standard uptake value

in index tumor lesions from 6.4±3.9 to 9.1±5.6. Carbidopa blocked the pancreas's physiological uptake of ^{18}F-DOPA, perhaps masking adrenal PHEO. We concluded that carbidopa enhances the sensitivity of ^{18}F-DOPA PET for adrenal PHEOs and extra-adrenal abdominal PGLs by increasing the tumor-to-background ratio of tracer uptake. The sensitivity of ^{18}F-DOPA PET for metastases of PGL appears to be limited.

We also evaluated seven VHL patients with adrenal PHEO confirmed by histopathology. We localized the adrenal PHEOs by using CT, MRI, [$^{123/131}$I]-MIBG scintigraphy, and [^{18}F]-DA PET. [^{18}F]-DA PET and CT localized the PHEOs in all seven patients. In contrast, three out of the seven patients had negative results with [$^{123/131}$I]-MIBG scintigraphy, and one out of six patients had negative MRI results. We found that [^{18}F]-DA PET gives more promising results than [$^{123/131}$I]-MIBG scintigraphy in the diagnostic localization of VHL-related adrenal PHEO, with a 100 percent rate of localization. Thus, [^{18}F]-DA PET in conjunction with CT/MRI should be considered an effective method for the proper localization of VHL-related adrenal PHEO.

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Other findings

Pacak, He, Adams; in collaboration with Fojo, Chen, Reynolds, Carrasquillo, Wesley, Puri, Lubensky, Zhuang, Morris, Merino, Eisenhofer, Lenders, Tischler, Widimsky

Patients who undergo successful surgery for apparently benign PHEO/PGL have a lower life expectancy than the general population because patients may still develop metastatic disease during post-surgical follow-up. Hypertension persisted in two-thirds of recurrence-free patients. Life-long follow-up is therefore warranted in benign PHEO/PGL surgery patients.

In a non-randomized, single-arm trial, we conducted a long-term follow-up of 18 patients with a diagnosis of PHEO/PGL who were treated with a combination of cyclophosphamide, vincristine, and dacarbazine (CVD chemotherapy). We found that combination chemotherapy with CVD produced objective tumor responses in patients with advanced malignant PHEO/PGL, but we observed no difference in overall survival between patients whose tumors objectively shrank and those with stable or progressive disease. However, patients reported improvement in symptoms, exhibited objective improvements in blood pressure, and evidenced tumor shrinkage that made surgical resection possible. We concluded that CVD therapy was not indicated in every patient with metastatic PHEO/PGL; nevertheless, such therapy should be considered in the management of patients with symptoms and when tumor shrinkage might be beneficial.

In collaboration with other medical centers, we found that catecholamine excess in PHEO was accompanied by an increase in carotid-femoral pulse-wave velocity, which was reversed by successful tumor removal. Age, mean blood pressure, and norepinephrine levels were independent predictors of pulse-wave velocity in patients with PHEO.

Through our initial microarray profiling data, we detected a high expression of IL13Rα2 mRNA in various PHEOs, indicating that IL13Rα2 could be a potential target for treatment of metastatic PHEO. Therefore, we initiated a collaboration with Raj Puri to study the expression of IL13Rα2 by various mouse pheochromocytoma (MPC) cells and PHEOs. If we detected such expression, we would investigate the utility of IL13Rα2-*Pseudomonas* exotoxin fusion protein (IL13-PE38) in mice. IL13-PE38 binds to IL13Rα2 and kills cells that carry it. Our initial results, based on RT-PCR, confirmed the presence of IL13Rα2 on human PHEO cells. Thus, we plan to begin preclinical studies on our mouse model to evaluate the therapeutic potential of IL13-PE38. If successful, the therapy may offer a treatment option for IL13Rα2-positive metastatic PHEO patients.

We hypothesized that the differences in catecholamine release between VHL- and MEN 2–related PHEOs may reflect the presence of different pathways of catecholamine secretion, namely, regulated versus constitutive pathways. Our initial microarray and proteomic databases indicate underexpression of numerous components of the regulated secretory pathway in VHL compared with MEN 2 tumors (e.g., SNAP25, syntaxin, rabphilin 3A, calcium-dependent secretion activator, syntaphilin, chromogranin A and B, secretogranin II, and neuropeptide Y). Many of these components are downregulated by the transcriptional repressor REST (RE1-silencing transcription factor) and/or have genes with REST binding sites in regulatory regions. Using QRT-PCR, we confirmed our microarray evidence of higher expression of REST in VHL than in MEN 2 tumors. We found that overexpression of REST in PC12 cells reduces catecholamine stores, the number of secretory vesicles, and the expression of chromogranins and increases baseline catecholamine secretion while inhibiting calcium-dependent secretion. These findings are congruent with our data—already published or in press—comparing VHL with MEN 2 tumors. The constitutive secretory pathway (shown in Figure 10.2) is spontaneous, rapid, and not responsive to secretagogues. Secretion by the regulated pathways is calcium-dependent. The pathways consist of vesicles that sort peptides and pack proteins—both processes being dependent on a V-ATPase-mediated proton gradient and under the control of REST.

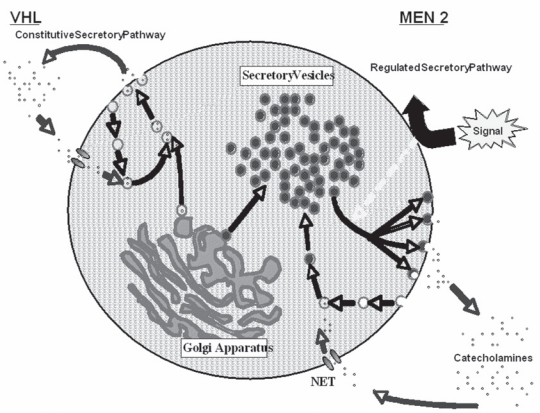

FIGURE 10.2

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An animal model of PHEO and cell culture studies

Pacak, Martiniova, Lai, Tischler, Puri, Morris, Merino, Green, Chan, Albanase, Fliedner, Porter, Elkahloun

The use of suitable laboratory animal models enhances the speed with which new treatments for PHEO/PGL may be evaluated, particularly in the case of uncommon malignancies that are typically not the subject of clinical trials. Previously, we reported a new mouse model of metastatic PHEO based on a tail vein injection of a MPC line that reproducibly generated several liver tumors in mice. Recently, we found that microCT and MRI were approximately equal in their ability to detect hepatic metastases at a size threshold of 350 mm. In the lungs, MRI was more sensitive than microCT, detecting lesions 0.6 mm in diameter versus 1 mm for microCT. In addition, MRI was more sensitive than microCT to lesions in the kidneys, bone, ovaries, and adrenal glands. MRI demonstrated higher contrast-to-noise ratio than microCT. We concluded that, in addition to the advantage of not exposing an animal to ionizing radiation, MRI rather than microCT provided a more complete assessment of the extent of metastases in our mouse model.

In another study, we enhanced the animal model by modifying the number of injected cells, the length of trypsin treatment, and the temperature for cell storage before injection; we enhanced tumor growth by removing metastatic lesions, subjecting them to serial passage, and re-selecting metastases. We evaluated the effect of these modifications on tumor growth by using in vivo MRI. The results showed that the number of injected cells, the temperature for cell storage before injection, and the length of trypsin treatment are important factors in producing faster-growing, more aggressive tumors that yield secondary metastatic lesions at other sites. Serial culture and selection of metastatic liver lesions produced even more aggressive PHEO cells that retained their biochemical phenotype. Microarray comparison of the more aggressive cells with the parental cell line identified genes important for the cells' rapid metastatic process.

ErbB-2/Her2/Neu is a growth factor receptor tyrosine kinase frequently overexpressed in tumors; clinical evidence suggests that enhanced ErbB-2 growth factor receptor signaling may play a role in PHEO. An ongoing study showed that ectopic expression of an activated ErbB-2 transgene resulted in bilateral adrenal PHEO. Analyses of tumor samples and normal adrenal tissue revealed a great reduction in the levels of the Pten tumor suppressor protein in PHEOs while levels of the cell cycle–regulatory protein cyclin D1 usually increased. In addition, levels of phospo-AKT were higher in PHEOs than in normal adrenal tissue. Biochemical analyses established that PHEOs were functionally active, producing abundant levels of the catecholamines epinephrine and norepinephrine. These data establish that increased ErbB-2 growth factor receptor signaling in the adrenal medulla can lead to PHEO through combined influences on Pten, AKT, and cyclin D1.

Recombinant adenoviruses (rAd) have found wide application as gene transfer vectors in both the laboratory and human clinical trials. We are investigating the effects of adenoviral-mediated gene transfer in primary bovine adrenal chromaffin cells (BACC) and an MPC line. We infected cells with one of three non-replicating E1/E3-deleted ($E1^{-}/E3^{-}$) rAd vectors: Ad.GFP, which expresses a green fluorescent protein; Ad.null, which expresses no transgene; or Ad.C2.TK, which expresses the *herpes simplex* virus-1 thymidine kinase gene (*TK*). Forty-eight hours after exposure to Ad.GFP, the percentage of GFP-expressing BACCs ranged from 23.5 to 97 percent in a dose-dependent manner and similarly from 1.06 to 84.4 percent in the MPC, indicating that adrenomedullary cells are a

potentially valuable target for adenoviral-mediated gene transfer. Ultrastructural analysis, however, revealed profound changes in the nucleus and mitochondria of cells infected with rAd. Furthermore, infection of BACCs with Ad.null was accompanied by a time- and dose-dependent decline in cell survival due solely to the vector. Specific whole-cell norepinephrine uptake also rose in a time- and dose-dependent manner in BACCs. Infection of MPC cells with the Ad.C2.TK vector sensitized them to the cytotoxic effect of the antiviral drug ganciclovir in direct proportion to the fraction of cells infected with the virus. We conclude that rAd may alter the structural and functional integrity of adrenomedullary cells, potentially interfering with the normal stress response. At the same time, given the vector's ability to deliver and express genes effectively in PHEO cells, it may be useful in the gene therapy of adrenomedullary tumors.

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GENETIC AND GENOMIC STUDIES IN NORMAL DEVELOPMENT AND DISEASES

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We focus on translating genetic and genomic technologies, acquired through basic studies, into research on clinical problems. One of our missions is to train physicians in the application of genomic and genetic approaches to studies of human diseases. Using the cutting-edge methylation tiling array technology, we are studying the transcription regulation of *mArd2*, a novel testis-specific gene that was first cloned in our laboratory. We also study *Lin 28*, a gene that has been shown to regulate developmental timing. Proteomic approaches helped the identification of RNAs bound to the protein encoded by this gene. Gene knockdown experiments are under way to study the function of *Lin 28* in determining cell fate. Relevant to our missions is our attempt to define global approaches for identifying and screening for the risk factors for complex disorders. To this end, we have designed two-hybridization–based high-throughput screening methods to detect susceptibility risk factors for thrombophilia and age-related macular degeneration. Besides basic and translational research, we are involved in clinical protocols to study patients with genetic and metabolic disorders, allowing us to deliver clinical genetics training to our fellows in what is a critical component of our work.

Transcription Regulation and Functional Studies of Testis-Specific Genes

Genetic, genomic, and proteomic studies of a testis-predominant isoform of a mouse acetyltransferase

Pang, Fang, Rennert; in collaboration with Chan, Taft

Based on expression analysis of mouse type A spermatogonia, pachytene spermatocytes, and round spermatids, we cloned a novel *mArd1* (*Arrest Defective 1*) homologue, which we named *mArd2* (also known as *Ard1b*), that demonstrated testis specificity and elevated expression in pachytene spermatocytes. The mArd1 protein is known to interact with an auxiliary protein subunit mNAT1 to constitute a functional *N*-acetyltransferase. We showed that the protein encoded by *mArd2* is functionally similar to its homologue mArd1 in vitro. However, the two homologous genes displayed grossly different expression patterns in various cell lines and mouse tissues. Specifically, the *mArd2* gene is expressed in a developmentally regulated manner during spermatogenesis. To elucidate the molecular mechanism of the gene's tissue specificity, we performed an *mArd2* promoter assay in two *mArd2* non-expressing cell lines, namely, NIH/3T3 and GC-2spd(ts), which represent cells of somatic and early germline origin, respectively. The detection of *mArd2* promoter activities in both cell types indicates that the transcriptional machinery required for *mArd2* expression is intact;

the absence of *mArd2* expression would thus result from other regulatory mechanisms. We hypothesize that DNA methylation could play a role in the regulation of *mArd2* expression.

Using 5-aza-deoxycytidine treatment in non-expressing cells, in vitro methylation, and bisulfite sequencing analysis, we showed that *mArd2* transcription is regulated epigenetically. Comparison of the methylation status of the two CpG islands spanning the proximal promoter and half of the coding region of the *mArd2* gene showed that its proximal promoter was hypermethylated in mouse tissues that do not express *mArd2* but hypomethylated in male germ cells, which express the gene. On the other hand, we found that the distal CpG island was hypomethylated in germ cells at stages that display higher *mArd2* expression but hypermethylated in non-expressing tissues as well as in mitotic germ cells, in which *mArd2* expression is minimal. Thus, our findings confirmed the hypothesis that testis-specific expression of *mArd2* is epigenetically regulated. The *Ard1-Ard2* expression system could be a paradigm for studying epigenetic regulation of mammalian spermatogenic gene expression.

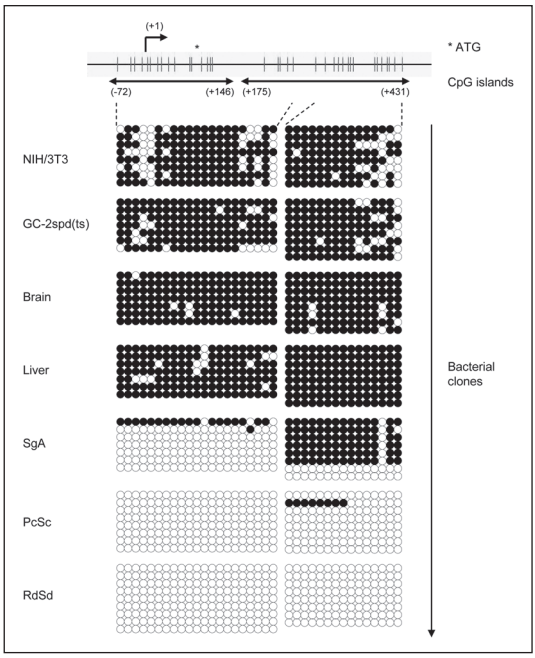

FIGURE 10.3

Methylation status of the two CpG islands spanning the proximal promoter and half of the coding region of the *mArd2* gene in different tissues determined by bisulfite sequencing. Filled circles represent methylated cytosine and empty circles represent unmethylated cytosine. GC-2spd(ts) = spermatogonial cell line; SgA = spermatongonia; PcSc = pachytene spermatocyte; RdSd = round spermatid.

Despite the above findings, the involvement of other protein factors in the activation of *Ard2* transcription in testis remains unknown. We are therefore attempting to identify the minimal *Ard2* promoter. Our data indicate that such a promoter is proximal to the transcriptional start site of the gene and overlaps with one of the gene's CpG islands. We are now isolating the factors that are bound to this sequence in order to understand how activation and suppression of *Ard2* transcription is regulated.

We also demonstrated that the translation of *Ard2* in vivo is delayed during spermatogenesis; the transcript level peaks in meiotic germ cells, but the protein is not expressed until after the appearance of round spermatids. We hypothesized that the 3′ untranslated region (UTR) of *Ard2* confers a repressive effect on the gene's translation. To test the UTR's ability to repress reporter gene expression, we established a cell culture model and identified several regions on the 3′ UTR that are involved in the suppression of reporter translation. We are isolating the testicular protein factors that would be bound to these sequences and will study the involvement of the factors in regulating the translation of other spermatogenic transcripts.

Using gene knockdown experiments in mammalian cells, we are also studying the global effect of a deficiency of protein N-alpha terminal acetylation on cell functions.

Examination of the biological role of Lin28 in mitotic male germ cells

Pang, Rennert; in collaboration with Chan, Taft

Lin28 is a heterochronic gene involved in the temporal control of cell fate determination in *C. elegans*. In mammals, *Lin28* is detected mostly in cells that possess proliferative capacity, e.g., embryonic

stem cells, embryonal carcinoma cells, and mouse type A spermatogonia. Lin28 is known to be localized predominantly in the cytoplasm. However, a recent study showed that Lin28 is involved in a microRNA maturation process in the nucleus, suggesting that the protein may play a dual role in the cell by interacting preferentially with nuclear and cytoplasmic proteins. However, the gene's biological function, especially its role in mammalian development, remains largely unknown.

We hypothesized that Lin28 acts as an RNA chaperone to control the availability of transcripts that encode functions important to cell fate determination. In a cell culture model, we have identified a set of RNA transcripts that bind to Lin28; using gene knockdown, we are now studying the corresponding change in protein levels of the transcripts. We are also isolating the proteins that interact with Lin28 in the nuclear and cytoplasmic fractions of expressing cells in order to examine the protein's biochemical role.

The selectivity of gene expression in certain cell types suggests that transcription of *Lin28* is strictly regulated. Our preliminary data showed that DNA methylation does not regulate *Lin28* transcription. Instead, a specific genomic region about 1 kilobp upstream of the transcriptional start site showed promoter activity only in *Lin28*-expressing cells. We are screening a panel of *Lin28*-expressing and –non-expressing cells to identify the genetic factors involved in activating or suppressing *Lin28* transcription.

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Genomic and Genetic Studies of Heritable Disorders

In pursuit of our goal to develop global approaches for identifying and screening for risk factors of complex disorders, we designed two-hybridization–based high-throughput methods that can screen for susceptible risk factors of thrombophilia and age-related macular degeneration. Besides basic and translational research, we have instituted clinical protocols to study patients with genetic and metabolic disorders.

Application of high-throughput approaches in the study of complex disorders

Dogulu, Rennert; in collaboration with Chan, Su

Venous thrombosis (VT) is one of the leading causes of mortality and morbidity, resulting in approximately 300,000 hospitalizations and 50,000 fatalities per year in the United States, with an incidence of 141 per 100,000 African Americans, 104 per 100,000 Caucasians, 55 per 100,000 Hispanics, and 21 per 100,000 Asian/Pacific Islanders. Our calculations demonstrated that concurrent use of a panel of 11 genetic tests increases by at least 30-fold the positive predictive value of testing for VT. We have patented a Method Evolved for Recognition of Thrombophilia [MERT] that is currently under evaluation for proof of principle for potential prediction and accurate assessment of hereditary thrombophilia in several ethnic populations. The methodology involves rapid, concurrent screening of an array of all known 145 venous thrombosis–associated recurrent mutations and polymorphisms in nine genes [antithrombin III (AT III), protein C, protein S, fibrinogen, factor V (FV), prothrombin (factor II), methylenetetrahydrofolate reductase (MTHFR), angiotensin 1–converting enzyme (ACE), and plasminogen activator inhibitor-1 (PAI-1)]. We have designed 291 oligonucleotide 25-mer probes to be spotted onto the microarray and, using Multiplex PCR assay, amplified 40 amplicons covering the variation sequences from nine genes in a single amplification reaction. We are now verifying our method's analytic validity.

We have patented a similar approach for the design of a microarray screening for susceptibility to age-related macular degeneration (Method Evolved for Recognition and Testing of Age-Related Macular Degeneration [MERT-ARMD]). Among people 65 years of age and older, age-related macular degeneration (ARMD) is the most common cause of severe vision loss in the United States and developed countries. It is a multifactorial disorder. We have designed a MERT-ARMD that, using hybridization-based, high-density oligonucleotide array technology, will screen concurrently for 105 known age-related macular degeneration–associated mutations and polymorphisms in 16 genes (CFH, LOC387715, BF, C2, ABCR, Fibulin 5, VMD2, TLR4, CX3CR1, CST3, MnSOD, MEHE, paraoxonase, APOE, ELOVL4, and hemicentin-1).

Clinical protocol on studies of pediatric patients with genetic and metabolic disorders
Raygada, Dogulu, Rennert; in collaboration with Kaler, Stratakis

In support of the Institute's research mission, we are expanding the spectrum of diseases seen in our clinics and inpatient units, thereby broadening training opportunities for our clinical fellows. We are conducting two active protocols. Protocol # 02-CH-0023 provides diagnostic and consultative services to patients with a variety of rare genetic disorders; in addition, it supplements and offers additional training in clinical genetics, dysmorphology, and human biochemical genetics in NICHD and other Institutes. Under the protocol, we have evaluated more than 450 patients with a broad spectrum of metabolic and genetic conditions. For five years, we have accommodated pediatric residents from Georgetown University Hospital in our protocol. Starting in July 2003, the residents have rotated in our clinic every month. In addition, during the past year, we began the rotation of all Intramural Training awardees in NIH (three per week). We perform evaluations of local and out-of-state patients with suspected or diagnosed genetic/metabolic disorders; we have established a well-known resource for patients and physicians who need counseling and/or evaluation of these disorders. Clinical services include genetic counseling, risk assessment, diagnostic testing, and discussion of preventive interventions. We often perform standard, medically indicated laboratory or radiological studies to confirm a diagnosis or to aid in the management of patients. In some cases, patients receive medical or surgical treatment for their disorder, according to current clinical practice. Patients and/or family members with genetic disorders may offer their DNA for storage and/or testing.

Protocol #06-CH-0119 aims to unravel the contributions of insulin and insulin-related actions (e.g., insulin resistance, abdominal fat) to breast cancer risk, with the ultimate goal of early screening of at-risk patients and development of new strategies for risk reduction. Our study plan focuses on (1) determining whether factors associated with or governing insulin function may be involved in the modification of breast cancer risk in specific patient populations; (2) determining the role of abdominal fat and other anthropometric measures as related to, first, breast cancer risk in patients with and without a positive family history (classified by menopausal status) and, second, to interaction with insulin and other hormones; (3) providing sufficient data for future studies that investigate mediators of the actions of insulin on breast cancer (IRS-1, IRA isoform) and gene polymorphisms in this patient population; and (4) developing rational, cost-efficient guidelines for risk-reducing and screening strategies for a subset of patients responsive to the growth-promoting actions of insulin. As of this writing, we have evaluated more than 20 patients. The reduction in funds to cover patient costs has hindered patient recruitment. We are analyzing our pilot data and will subsequently determine any modifications that might be required for continuation of the protocol.

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REPRODUCTIVE ENDOCRINOLOGY AND INFERTILITY

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The laboratory supports basic, translational, and clinical studies of clinically relevant reproductive disorders. We focus on uterine leiomyoma (commonly known as fibroids), endometriosis, and reproductive disorders leading to infertility in women. The laboratory also supports graduate medical education for visiting students and residents as well as the NICHD-sponsored Clinical Training Program in Reproductive Endocrinology and Infertility. Our objective is to conduct reproductive research while training young clinical investigators who will continue to pursue a career in reproductive research.

Uterine leiomyomas

Armstrong, Browne, Feinberg, Segars; in collaboration with Christman, Tuan, Chen, Payson, Catherino, Leppert

Uterine leiomyomas disproportionately affect African American women and are associated with adverse pregnancy outcomes such as pre-term delivery, miscarriage, and cesarean section. The condition adversely affects the health of millions of women, leading to excessive menstrual bleeding and pelvic pain. Despite their prevalence, leiomyoma growth and development remain poorly understood, with few non-surgical, long-term treatment options.

To gain insight into strategies that may be developed for medical treatment of fibroids, we have used a gene-profiling approach to uncover genes associated with leiomyoma development. The approach led to the realization that the expression of genes encoding the connective tissue between cells, the extracellular matrix (ECM), is altered in uterine leiomyomas. Further studies showed that the structure of the ECM is altered in leiomyomas. Together, our findings suggest that the altered ECM might contribute to the growth of fibroids through mechanical signaling; the ECM is a load-bearing structure, and increases in loading could affect the growth rate of cells. To test our hypothesis, we collaborated with Rocky Tuan and Faye Chen to measure tissue stiffness. We found that cells within fibroid tumors were exposed to increased mechanical stress; in turn, the increased mechanical stress was accompanied by cellular changes, such as alteration in the actin cytoskeleton and biochemical evidence of activation of solid-state signaling. In brief, our studies support the conclusion that cells within a fibroid exhibit fundamental alterations in mechanical signaling and that altered mechanical signaling may contribute to the growth of uterine leiomyomas. In the coming year, we plan to characterize in greater detail both the altered mechanical signaling in fibroids and the factors that might be responsible for altered cell growth.

We also continued our clinical studies related to leiomyomas. Information about the possible impact of myomectomy on ovarian function is important in the clinical management of patients with fibroids who desire fertility, yet no systematic investigation of the impact of myomectomy has been performed; in particular, there are no estimates of subsequent fertility rates in fertile women (or at least in women not proven to be infertile). Therefore, we examined the impact of leiomyomas on fertility and ovarian reserve. We plan to expand the investigation to evaluate anti–Müllerian hormone pre- and post-myomectomy.

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Role of *BRX* (also known as *AKAP13*) in cardiac development

Segars, Mayers; in collaboration with Kino, Westphal, Wu, Driggers

Our interest in the molecular mechanism of estrogen action in reproductive tissues led to the cloning of a large modular protein (from a breast cancer expression library), which we called "Brx" for Breast Receptor Auxiliary Factor (the gene is also known as *AKAP13*). In addition to discovering the enhancing ligand-dependent activation of estrogen receptors alpha and beta, we found that Brx augmented in vivo activation of the glucocorticoid receptor (GR) and that mice haplo-insufficient for *Brx* had an impaired response to glucocorticoids.

In collaboration with Heiner Westphal, we have continued studies of *AKAP13*-homozygmous null mice (knockout). We found that mice died in utero due to a failure of heart development. It was known that the general family of AKAPs was involved in heart function, but a specific requirement for AKAP13 (Brx) for proper heart formation had not been reported. We thus focused on the requirement for Brx in cardiac development and found that cell differentiation of ventricular cardiomyocytes in *Brx*-null mice was greatly impaired. In addition, mice haplo-insufficient for *Brx* displayed a structurally altered actin cytoskeleton, a subtle impairment in other responses to nuclear factors that regulate cytoskeletal structure, and actin polymerization in response to stress.

In the coming year, we plan to continue examining the mechanism(s) that may be responsible for the failure of differentiation of developing ventricular cardiomyocytes in *Brx*-null mice in the hope of finding clues to the role of the gene in reproductive tissues, such as the ovary, where *Brx* is highly expressed. In addition, using the cre-lox approach, we plan to develop a conditional targeted loss of function for the gene in mice.

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Endometriosis and chronic pelvic pain

Stratton, Stegmann, Sinaii,[1] Segars; in collaboration with Nieman

Chronic pelvic pain significantly affects the health of up to 10 percent of women with endometriosis. The diagnosis of endometriosis is established at a surgical procedure. Given the variable appearance

of lesions, one persistent issue in surgical diagnosis is whether histologic confirmation of the disease should be obtained. In the past year, we have correlated biopsy results with lesion appearance in two ways. First, given varying lesion characteristics, we reported on histologic confirmation, illustrating that no single color was associated with endometriosis and that surgeons should biopsy any suspicious lesion. Subtle lesions were as likely to contain endometriosis as classic lesions (64 percent), and 40 percent of women with only small, subtle lesions had biopsy-proven endometriosis. Mixed-color lesions and endometriomas were the only two lesion types that were more commonly biopsy-proven (78 percent). Second, we created a logistic model to predict endometriosis. The model identified characteristics indicating a high and low probability of biopsy-proven endometriosis. It was useful as a guide in choosing appropriate lesions for biopsy but is not a substitute for histologic confirmation.

In collaboration with Rebecca Greene and Ninet Sinaii, a group led by Pamela Stratton described the diagnostic experience of women with chronic pelvic pain and endometriosis who responded to the Endometriosis Association Survey. The group examined the relationship between disease severity and patient characteristics by analyzing questionnaires from 1,000 women in the Oxford Endometriosis Gene (OXYGENE) study. In the coming year, the group will continue examining aspects of the health of women with endometriosis by analyzing the Endometriosis Association Survey, defining the pain outcomes in endometriosis clinical trials, and analyzing the hypothalamic pituitary-adrenal-axis in women with chronic pelvic pain and endometriosis.

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Assessment and preservation of ovarian function in women and girls undergoing cancer treatment

Armstrong, Browne, Segars; in collaboration with Pavletic, Nelson

As a result of the rising success of cancer treatments, many young girls and women are now cancer survivors but find that their reproductive function is irreparably damaged by their treatment. Alicia Armstrong led an initiative to devise strategies to enable women who are cancer survivors to maximize and preserve reproductive function, with an emphasis on treatment regimens designed to minimize the effect of cancer treatments on the later reproductive function of women. The group helped initiate a large multicenter, randomized trial of gonadotropin-releasing hormone antagonists in young women before cancer treatments. In addition, basic science studies will help elucidate the mechanism of the possible protective effect of such treatment.

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Infertility and reproductive health disparities

Armstrong, Segars, Stegmann, Feinberg; in collaboration with Catherino, DeCherney

In an effort to devise strategies to optimize reproductive outcomes across racial and ethnic groups, we have been examining the causes and consequences of reproductive health disparities. In the past year, we continued our study of reproductive health disparities in the context of multicenter investigations. Specifically, we examined racial disparities across over 130,000 IVF cycles. The results confirmed the findings of disparate outcomes among African American women. We also evaluated ART[2] utilization and outcome among Hispanic couples. The evaluation suggested that, contrary to some claims that economic barriers account for lower rates of ART utilization, other factors may be responsible for reproductive health disparities. In the coming year, we plan to continue investigating racial disparities in reproductive health in multicenter trials of women in assisted reproduction and infertility care.

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GENETIC DISORDERS OF BONE AND EXTRACELLULAR MATRIX

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In an integrated program of laboratory and clinical investigation, we study the molecular biology of the heritable connective tissue disorders osteogenesis imperfecta (OI) and Ehlers-Danlos syndrome (EDS). Our objective is to elucidate the mechanisms by which the primary gene defect causes skeletal fragility and other connective tissue symptoms and then apply this knowledge to the treatment of children with these conditions. Last year, we identified the long-sought cause of recessive OI. Discoveries of defects in collagen modification generated a new paradigm for collagen-related disorders of matrix. We have established that structural defects in collagen cause dominant OI while defects in the components of a complex in the endoplasmic reticulum that modifies collagen cause recessive OI. Our challenge now is to understand the cellular and biochemical mechanisms of recessive OI. We have also generated a knockin murine model for OI with a classical collagen mutation and are using the model to study disease pathogenesis and the skeletal matrix of OI, the effects of pharmacological therapies, and approaches to gene therapy. Our clinical studies involve children with types III and IV OI, who form a longitudinal study group enrolled in age-appropriate clinical protocols for treatment of their condition.

Identification of the genetic cause of type VII OI

Barnes, Chang, Cabral, Marini; in collaboration with Leikin

Structural defects of the heterotrimeric type I collagen molecule are known to cause the dominant bone disorder osteogenesis imperfecta. A severe recessive form of OI was first postulated in 1979. More recently, investigators noted that they were unable to detect defects in the type I collagen sequences in some patients with clinical OI. Such patients may be divided into those with normal or abnormal collagen biochemistry. We hypothesized that the cause of recessive OI with abnormal collagen biochemistry and normal collagen gene sequence would involve a gene(s) whose products interacted with type I collagen. We demonstrated that deficiency of either of two components of the collagen prolyl 3-hydroxylation complex, which interacts with collagen in the endoplasmic reticulum and modifies a single prolyl residue in the α1(I) chain at Pro986, was the cause of recessive OI.

Among the patient cells in the Bone and Extracellular Matrix Branch (BEMB) cell repository that did not contain a mutation in either of the type I collagen genes, we identified three patients with null mutations for *CRTAP*. Their clinical findings overlapped with those of lethal type II OI but with distinctive features, including white sclerae. All three *CRTAP*-null patients had a severe recessive form of OI, which was lethal in the first year of life. As a result of abnormal modeling, their long

bones were extremely osteoporotic and deformed, with prenatal fractures and a tubular shape. In addition, the infants had a narrow thorax with several prenatal rib fractures. We found all three patients to be homozygous or compound heterozygotes for mutations that resulted in altered splicing and introduction of premature termination codons in transcripts, leading to nonsense-mediated mRNA decay of the *CRTAP* transcripts. Parents of *CRTAP* probands were heterozygous carriers. Probands exhibited absence of protein on Western blot and absence of Pro986 hydroxylation on mass spectrometry analysis.

We are now focusing on understanding the structure of the prolyl 3-hydroxylation complex and the stabilizing effect of CRTAP, the complex's "helper protein," on prolyl 3-hydroxylase 1 (P3H1). We are investigating the stress response of cells in the absence of CRTAP.

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Identification of the genetic cause of type VIII OI

Cabral, Chang, Barnes, Marini; in collaboration with Leikin

Together with with cyclophilin B and P3H1/leprecan (encoded by *LEPRE1*), CRTAP forms a complex in the endoplasmic reticulum that interacts with and 3-hydroxylates collagen. We identified seven cases of null *LEPRE1* mutations in the BEMB cell repository. The probands from whom the cells derived had bone dysplasia overlapping lethal type II OI and severe type III OI but exhibited distinctive features, including white sclerae, as found in CRTAP defects. We screened patients for null mutations with real-time RT-PCR by using primary fibroblast mRNA. The patients were homozygous or compound heterozygotes for mutations resulting in altered splicing and introduction of premature termination codons in transcripts. The seven null *LEPRE1* patients had severe to lethal recessive bone dysplasia, characterized by shortened long bones, generalized bone disorganization, and extremely low bone mineral density (BMD; L1-L4 DEXA z-score -7). Parents of *LEPRE1* probands were heterozygous carriers. Furthermore, P3H1 protein was absent from these patients' fibroblasts on Western blots. Patient type I collagen exhibited severely reduced or completely abolished 3-hydroxylation of the α1(I) Pro986 residue as demonstrated by mass spectrometry of collagen tryptic peptides. In contrast, the extent of overhydroxylation of proband collagens by lysyl hydroxylase was comparable to collagens with a structural defect in the carboxyl terminus of the helical region, consistent with delayed folding of the molecule. The results of differential scanning calorimetry of patient collagens were consistent with an increased post-translational modification in the absence of a primary structural defect, with patient collagens showing a 1°C higher thermal stability than normal control collagen. Proband collagen secretion was moderately delayed, but total collagen secretion increased. These recessive null mutations of *CRTAP* and *LEPRE1* result in a novel metabolic disorder of bone and demonstrate that the 3-hydroxylation complex is crucial for normal bone development.

For type VIII OI, we are investigating a special *LEPRE1* mutation, IVS5^{+}1G→T, that results in five alternatively spliced forms of the transcript, each leading to a premature termination codon. Homozygotes for the mutation die within the first few months of life while compound heterozygotes survive with extremely severe bone disease. The mutation occurs only in African Americans and West Africans. We are interested in determining the incidence of carriers for the mutation and in understanding its molecular anthropology. We have used a restriction enzyme digestion assay and a custom SNP (single nucleotide polymorphism) assay to identify carriers among contemporary African Americans from Pennsylvania, Maryland, and Washington, DC, and among contemporary West Africans. The carrier rate among the American populations is 1 in 200 to 300 individuals, predicting an estimated incidence of type VIII OI from the *LEPRE1* mutation of 1 in 165,000 to 320,000. Among contemporary West Africans, we were surprised to find a carrier frequency of 1.37

percent. This particularly high incidence predicts that the rate of recessive OI in West Africa will equal the incidence of dominant OI. We are currently using haplotype analysis to determine the age of the mutation, which presumably arrived in the Americas via the Atlantic slave trade.

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Insights from the Brtl mouse model for OI

Uveges,[1] Forlino, Cabral, Marini; in collaboration with Leikin, Osdoby

We continue to investigate the Brtl mouse model for OI in order to understand the pathological and cellular mechanisms of OI. We have already investigated the cellular contribution to Brtl bone properties. Brtl cortical and trabecular bone decreases before and after puberty, with BV/TV (bone volume/total volume) reduced by 40 to 45 percent. Brtl osteoblast surface is comparable to wild type, and Brt marrow and wild-type marrow generate equivalent numbers of osteoblast precursors before and after puberty. Interestingly, both before and after puberty, the osteoclast surface in Brtl mice is augmented compared with wild type (36 to 45 percent), as is the number of TRAP-positive cells (57 to 47 percent). After puberty, Brtl osteoblast matrix production falls to half of wild-type values, yet osteoclasts remain significantly higher than in wild type. When the Brtl mice reach 6 months of age, the combination of osteoblast and osteoclast changes results in a decline in BFR (bone formation rate) from normal to half of wild-type values. We investigated whether the RANKL/OPG signaling pathway is responsible for the uncoupled cell numbers. Both immunohistochemistry and real-time RT-PCR[2] showed an increase in RANK, RANKL, and OPG levels in Brtl but a normal RANKL/OPG ratio. Surprisingly, we found that $TRAP^+$ precursors are markedly elevated in Brtl marrow cultures and form more osteoclasts, suggesting that osteoclast increases result from elevated numbers of precursors. The data suggest that OI pathology is attributable to a combination of deficient bone formation by osteoblasts and a rise in the numbers of osteoclasts triggered independently by abnormal bone matrix. We are currently investigating the pathways that stimulate the increase in osteoclasts.

Given that the Brtl mouse provides a model for the phenotypic variability seen in human α1(I) mutations, we also studied the basis for phenotypic variability in Brtl mice. Brtl has two discrete phenotypes; about 30 percent of pups die within hours of birth while the survivors exhibit a moderately severe skeletal defect. We used microarrary and two-dimensional proteomics for complementary studies of calvarial bone from lethal and surviving Brtl pups and found that Gadd153 expression was 2- to 3-fold higher in lethal Brtl mice than in surviving and wild-type littermates. We detected the increase in Gadd153 expression in bone tissue only, not in skin or lung. Furthermore, Western blots showed that increased Gadd153 was present in calvarial protein extracts. Gadd153 is a member of the C/EBP family that can pair with CEBPβ or CEBPα to inhibit osteoblast differentiation or promote cellular apoptosis, respectively. Conversely, we found 1.6-fold higher expression of the αB-crystallin chain and a doubling of protein in the non-lethal Brtl pups. The role of αB crystallin is opposite to that of Gadd153; it is a small heat-shock protein that is known to confer resistance to apoptosis. Our data suggest that apoptosis is the primary factor in the phenotypic variability of Brtl mice. We are currently exploring the dimerization partners of Gadd153 in Brtl osteoblasts in culture.

We also completed a collaborative study showing that the Brtl mouse develops an early and rapidly progressive osteoarthritis (OA). We detected knee joint OA by micro-MRI and micro-CT and confirmed it with histology and immunohistochemistry. Brtl mice show thinning of subchondral bone by 2 months of age; by 12 months, they have severe OA with joint-space narrowing.

Safranin O staining showed lower cartilage proteoglycans than in wild-type mice of the same age. We demonstrated immunohistochemically that cartilage degeneration was not a consequence of abnormal spatial expression of collagen. Our studies are an important contribution to our understanding of the common condition OA as well as of the rare dysplasia OI and demonstrate that abnormalities in subchondral bone can cause OA in mice with initially normal cartilage.

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The Consortium for Collagen Mutations in OI

Forlino, Barnes, Cabral, Marini; in collaboration with Leikin, San Antonio

We led an international consortium of connective tissue laboratories to assemble and analyze a database of structural mutations in type I collagen causing OI. The consortium assembled over 830 mutations, including 682 glycine substitutions and 150 splice site defects. Genotype-phenotype modeling revealed distinct functional relationships for each chain of type I collagen. Importantly, we observed that two exclusively lethal regions in the α1 chain coincide with the Major Ligand Binding Regions (MLBR) of several ligands on the collagen monomer, including integrins and fibronectin. On average, glycine substitutions in the α2 chain versus the α1 chain have a more moderate outcome, with fewer than 20 percent of occurrences resulting in lethality. As for α1, occurrences in the amino third of the chain are non-lethal. Thereafter, the lethal mutations occur in eight clusters regularly spaced along the chain. The distribution of lethal mutations in the α2 chain continues to follow the pattern we described in the Regional Model for that chain and correctly predicts the phenotype of 86 percent of cases in α2. The lethal regions coincide with the binding regions for matrix proteoglycans on the collagen fibril. The consortium's modeling provides testable hypotheses for the mechanisms of OI.

We have participated in several collaborations that have extended our understanding of genotype-phenotype relationships. Our long and continuing collaboration with Sergey Leikin's laboratory has yielded insight into structural heterogeneity in the type I collagen triple helix and its role in osteogenesis imperfecta. Using differential scanning calorimetry and circular dichroism, we measured changes in collagen melting temperature for 41 glycine substitutions and observed no correlation between melting temperature (Tm) and substituting residue. Instead, the variations in Tm defined regions along the triple helix that we further confirmed with an activation energy map. We deduced the presence of two large, flexible regions important for collagen fibril assembly and ligand binding, one of which aligned with a lethal region for glycine substitutions in the α1(I) chain.

We also extended the mapping of collagen functional domains in terms of cell and matrix interaction domains on the collagen fibril. The effort took advantage of a database of hundreds of type I collagen ligand-binding sites and mutations on a two-dimensional model of the fibril. The fibril-based model supports important roles for the interaction with integrins in a cell-interaction domain and in a matrix-interaction domain important for fibril connections with proteoglycans. Using OI mutations to validate the importance of a major and secondary binding site, we undertook a collaboration, complementary to the larger mapping enterprise, with investigators mapping the osteonectin binding sites on type I collagen.

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Bisphosphonate treatment of children with types III and IV OI

Abukhaled, Obafemi, Marini; in collaboration with Paul

We undertook the first randomized controlled trial of bisphosphonate in children with types III and IV OI. We set out to test both primary skeletal gains (increased bone density and decreased fractures) and secondary gains (improved functional level and muscle strength and decreased pain) reported in observational trials. Children in the treatment group received pamidronate for 18 to 23 months and experienced improvement in vertebral parameters, including BMD z-scores, central vertebral height, and vertebral area. However, the increment in vertebral BMD in the treatment group tapered off after one to two years of treatment. Furthermore, the treatment group did not experience a decrease in long-bone fractures. We observed no functional effect from bisphosphonate treatment and no significant change in ambulation level, lower-extremity strength, or pain in children with OI treated with pamidronate. Some patients reported increased endurance or decreased back pain, but most reported no perceptible changes. Hence, the changes previously reported in these parameters appear to have been a placebo effect in the uncontrolled trials. The data from our controlled trial were in accord with three other controlled trials conducted in the same time frame. We now recommend limiting pamidronate treatment of children with types III and IV OI to one to two (or at most three) years, with subsequent follow-up of bone status. Furthermore, we are currently engaged in a dose-comparison trial, using both the dose from our first trial and a lower dose. The trial has now entered the data analysis phase; our hypothesis posits that the children will gain a comparable benefit from the lower dose with decreased detrimental effects.

We also conducted a clinical radiographic investigation that parallels our studies of recessive OI. We demonstrated that popcorn calcifications occur in recessive OI; we are now delineating the calcifications' incidence and progression in dominant OI. We retrospectively examined serial lower-limb radiographs of 45 children with known dominant mutations in type I collagen. The mean age of onset of popcorn calcifications was 7 years, with a range of 4 to 14 years. We always found such calcifications in the distal femora, with the greatest number in the proximal tibiae. We discovered that unilateral popcorn calcifications contribute to leg length discrepancy but not to OI's severe linear growth deficiency. The type I collagen mutations associated with popcorn calcifications occur equally in both COL1A1 and COL1A2 and demonstrate no preferential location along the chains.

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NERVOUS SYSTEM DEVELOPMENT AND PLASTICITY

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Our research concerns molecular and cellular mechanisms by which functional activity in the brain regulates development of the nervous system during late stages of fetal development and early postnatal life. Areas of study include interactions between neurons and glia; the mechanisms of learning and memory; effects of neural impulses on cell proliferation, differentiation, neurite outgrowth, synaptogenesis, synapse plasticity, and myelination; and regulation of gene expression in neurons and glia by specific patterns of neural impulses.

Activity-dependent neuron-glia interactions: myelin regulation by neural impulse activity
Lee, Fields; in collaboration with Ishibashi[2]

The importance of neural impulse activity in regulating the development of neurons is widely appreciated, but such is not the case for the development of non-neuronal cells in the brain (glia). With little attention historically focused on activity-dependent neuron-glia interactions outside the synapse, our laboratory is working to identify the mechanisms and functional significance of activity-dependent communication between axons and glia.

Myelin is the spiral wrapping of membrane around axons that provides electrical insulation essential for rapid impulse conduction. Our research suggests that myelin participates in cognition, psychiatric disorders, and learning by affecting the speed and synchrony of neural impulse transmission between cortical regions. Our findings show that myelinating glia can detect electrical activity in axons and that such detection influences glial development and myelination. We have identified several molecular mechanisms that enable myelinating glia to sense impulse activity in axons and to respond with changes in cell proliferation, differentiation, and myelination. These findings are relevant to early childhood development and offer new approaches to treating a demyelinating disease such as multiple sclerosis. At the same time, our work expands current concepts of activity-dependent plasticity and learning in the brain to include nonsynaptic and non-neuronal communication.

We have shown that ATP (adenosine triphosphate) is released from axons firing action potentials and that the release activates purinergic receptors on myelinating glia, causing an increase in intracellular calcium, activation of transcription factors, regulation of gene expression, and control of glial development, cell proliferation, and myelination. In the central nervous system (CNS), we find that impulse activity promotes myelination. Adenosine, derived from the breakdown of ATP released from electrically active axons, stimulates differentiation of oligodendrocyte progenitor cells (OPC) to a promyelinating stage and promotes myelination by acting on P1 receptors. After OPCs

mature, electrical activity can increase myelination in a different manner, which is dependent on ATP stimulation of another type of glial cell—astrocytes—to release the cytokine LIF (leukemia inhibitory factor) that promotes myelination by mature oligodendrocytes. Our research shows that, in the peripheral nervous system (PNS), ATP released from axons firing action potentials inhibits Schwann cell proliferation, arresting cellular differentiation at an immature stage and inhibiting myelination. Our cellular and electron microscopic studies on LIF knockout mice show impairment of astrocyte proliferation and differentiation and reduced myelination.

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Neuron-glia signaling in astrocyte development and plasticity

Cohen, Lee, Bukalo, Ni, Fields

In the early postnatal hippocampus, glial progenitor cells proliferate and differentiate into astrocytes. This developmental process is correlated with the onset of spontaneous neural activity and may be relevant to how early experience can affect development of the brain. Our work shows that bidirectional signaling between neurons and glial cells contributes to the activity-dependent regulation of astrocyte development and in turn affects neuronal development, synaptogenesis, and synaptic function. Purinergic signaling (via extracellular ATP) has broad effects on both proliferation and differentiation of glial progenitor cells. We have found that ATP can be released from neurons following action potential firing and from astrocytes in response to neuronal activity; however, the mechanisms of ATP release from neurons and astrocytes are unclear. We are investigating the underlying mechanism of ATP release from axons and the response of astrocytes, oligodendrocytes, Schwann cells, and microglia and the subsequent effects on neuronal development and function. Recent findings indicate that interactions between purinergic and cytokine signaling regulate hippocampal glial and neuronal development in an activity-dependent manner, with effects on long-term potentiation of synaptic strength in the hippocampus. Similar processes also regulate synaptic development and function in the PNS at synapses with muscle (neuromuscular junction).

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Hippocampal synaptic plasticity

Bukalo, Cohen, Lee, Fields

It is widely appreciated that there are two types of memory: short-term and long-term. It has been known for decades that gene expression is necessary for converting short-term into long-term memory, but it is not known how signals reach the nucleus to initiate this process or which genes make memories permanent. Long-term potentiation (LTP) and long-term depression (LTD) are two widely studied forms of synaptic plasticity that can be recorded electrophysiologically in the hippocampus, and these phenomena are believed to represent a cellular basis for memory. We use cDNA microarrays to investigate the signaling pathways, genes, and proteins involved in LTP and

LTD. We work to understand how regulatory networks are controlled by the appropriate patterns of impulses leading to different forms of synaptic plasticity and to identify new molecular mechanisms regulating synaptic strength.

We have identified sets of transcription factors, structural genes, and signaling pathways that are regulated by activity patterns leading selectively to different types of synaptic plasticity. One finding of particular interest is that expression of the gene *BDNF*, which encodes a growth factor, depends on whether or not firing of postsynaptic CA1 neurons is coincident with excitatory synaptic firing from presynaptic neurons.

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Regulation of gene expression by action potential firing patterns

Lee, Cohen, Fields

All information in the nervous system is encoded in the pattern of neural impulse activity. Given that experience regulates nervous system structure and function, gene activity in neurons must be regulated by the pattern of neural impulse activity. We have tested this hypothesis by using custom cDNA arrays for gene expression profiling. We stimulated nerve cells to fire impulses in different patterns by delivering electrical stimulation through platinum electrodes in specially designed cell culture dishes. After stimulation, we measured mRNA and protein expression by gene arrays, quantitative RT-PCR,[3] Western blot, and immunocytochemistry. The results confirm our hypothesis that precise patterns of impulse activity can turn specific genes on or off. The experiments reveal signaling pathways and gene-regulatory networks that respond selectively to appropriate temporal patterns of action potential firing in neurons. Temporal aspects of intracellular calcium signaling are particularly important in regulating gene expression according to neural impulse firing patterns in normal and pathological conditions. We are also analyzing the role of post-transcriptional gene regulation mediated by mRNA stability and transport in hippocampal and DRG[4] neurons. Our findings provide a deeper understanding of how nervous system development and plasticity may be regulated by information coded in the temporal pattern of impulse firing in the brain.

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Nerve sheath tumor

Lee, Cohen, Fields

Neurofibromatosis type 1 (NF-1) is an autosomal dominant condition leading to nerve sheath tumors that can develop into malignancies. We are using gene expression profiling in combination with quantitative RT-PCR[3] and biochemical methods to investigate the processes involved in the transition from a benign to a malignant peripheral nerve sheath tumor. Our studies identify hundreds of genes that are dysregulated in malignant Schwann cells, including those associated with all aspects of cellular function involved in malignancy, and including, for example, those regulating cell proliferation, motility, growth factor, and immune system responses. We have observed widespread suppression of many genes involved in immune responses in nerve sheath tumor cells, which may help them escape detection by the immune system.

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Ampullae of Lorenzini: electroreception in sharks
Fields

Ampullae of Lorenzini are sense organs of sharks, rays, and chimeras that detect weak electric fields generated by many physiological and biological processes in the aquatic environment. These sense organs can detect voltage gradients as weak as 5 billionths of a volt/cm by a mechanism of sensory transduction that is only partly understood. The organs are only distantly related to the sense cells in the human inner ear, but the anatomical features of these electrosensory organs provide an excellent model for studying the anatomical and physiological properties of sensory transduction and synaptic plasticity. Our research concerns the mechanisms of sensory transduction in these sense cells.

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[1]No longer in the laboratory.
[2]Tomoko Ishibashi, *former Postdoctoral Fellow*
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PHYSICAL PRINCIPLES OF BIOMOLECULAR RECOGNITION, SELF-ASSEMBLY, AND REGULATION

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In living organisms, recognition and self-assembly reactions are some of the most fundamental molecular processes responsible for, among other reactions, folding, interactions, and aggregation of proteins and nucleic acids. Our ultimate goal is to arrive at a better understanding of the common physical principles governing such reactions. We laid the foundation for our work by studying molecular mechanisms relating the structural organization of molecules to the stability of collagen fibers, sequence homology recognition and condensation of DNA, and several other reactions. We continue to build our understanding of DNA self-assembly. However, in recent years, we have shifted the major focus of our research to extracellular matrix proteins and proteoglycans. We investigate their role in osteogenesis imperfecta (OI), Ehlers-Danlos syndrome (EDS), chondrodysplasias, osteoporosis, and other connective tissue pathologies and in common diseases such as cancer. Together with NICHD and extramural clinicians and scientists, we strive to gain better knowledge of molecular mechanisms contributing to these diseases. We hope to use the resultant knowledge for diagnostics, characterization, and treatment, bringing our expertise in physical biochemistry and theory to clinical research and practice.

Structure and stability of the triple helix in the function and pathology of collagen
Makareeva, Mertz, Vera, Leikin; in collaboration with McBride, Barnes, Cabral, Marini

Type I collagen is a triple-helical protein that forms the stable matrix of bone, skin, and other tissues. We discovered, however, that the equilibrium state of collagen as well as its procollagen precursor at body temperature is a random coil rather than the triple helix. Even in a crowded environment mimicking the endoplasmic reticulum (ER), procollagen triple helix folding occurs only below 35°C. Apparently, cells must use specialized chaperones to fold procollagen within the ER. Once procollagen is secreted from cells, it begins to unfold. Local weakening of its least stable regions and concurrent cleavage of N- and C-terminal propeptides appear to trigger self-assembly of fibers. In fibers, collagen helices are protected from complete unfolding, but local unfolding/refolding of the triple helix still occurs and plays an important physiological role. Over 80 percent of moderately severe to lethal collagen mutations are substitutions of a single glycine in the $(Gly\text{-}X\text{-}Y)_n$ sequence of the triple helix. By disrupting the stability and, consequently, the folding of the triple helix, the mutations result in abnormal collagen production, secretion, and fibrillogenesis. Our studies

revealed that the effect of a glycine substitution on collagen folding and stability is determined by the structural region within which the substitution is located rather than by the type of substituting residue. To understand the latter observations, we proposed a model for extracting the activation energy of local helix unfolding from reported measurements of triple-helical peptide stability. We tested the model and determined its parameters by measuring the H-D[1] exchange rate for glycine NH residues involved in interchain hydrogen bonds in collagen. From comparison of the peptide-based and mutation-based maps of the stability of type I collagen, we found evidence for several distinct structural regions within the triple helix.

Over the last year, we expanded our study to over four dozen mutations at different sites along the helix. To date, we have observed three "flex" regions with low triple-helical stability and two "anchor" regions with high stability. Mutations in the first 90 amino acids (aa) at the N-terminal end of type I collagen triple helix disrupt the most stable "N-anchor" region, preventing normal cleavage of the adjacent N-propeptide. The resulting uncleaved pN-collagen incorporates into fibrils and leads to a distinctive OI/EDS phenotype in patients. The "N-flex" region with low triple-helical stability, which is located about 150 aa away from the N-terminal end, appears to be responsible for binding of several ligands. The "mid-flex" region, about 400 aa from the N-terminal end, may be crucial for triple helix bending in collagen fibers. The "C-flex" region, which surrounds the collagenase cleavage site, binds to many collagen ligands and aligns with the largest known region of lethal Gly substitutions in the α(I) chain. The "C-anchor" region close to the C-terminal end of the triple helix, which is likely to be important for proper initiation of procollagen folding, aligns with the other region of predominantly lethal α1(I) Gly substitutions.

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Interactions of collagen fibrils with other matrix proteins

Makareeva, Leikin; in collaboration with Forlino, Visse, Nagase, Marini, Tenni

Interactions of collagen with ligands, particularly other extracellular matrix proteins and proteoglycans, are believed to be important in connective tissue diseases. In tissue, ligands interact primarily with collagen fibrils rather than with triple-helical monomers. We recently developed a new confocal microscopy assay with differential fluorescent labeling that permits one to visualize and measure the binding of various matrix proteins to individual collagen fibrils under physiological conditions. We found that MMP[2]-1 attacks damaged and poorly assembled rat tail–tendon collagen fibers via preferential binding to microfibrils, which become more exposed at fiber defects. Given that MMP1 is responsible for remodeling type I collagen fibers, such preferential binding may be physiologically important. In another set of experiments, we demonstrated high reproducibility of the new assay for measuring recombinant decorin binding to human collagen fibrils. During the past year, we found that decorin and biglycan bind to surfaces of collagen fibrils of different size with the same affinity, suggesting that the proteins regulate fibril size by favoring a larger surface area rather than a specific fibril diameter. The measurements also demonstrated similar binding of decorin and biglycan to fibrils reconstituted from collagen with truncated terminal peptides (telopeptides), suggesting that the telopeptides do not contain or block important binding sites on fibril surfaces. We are now focusing on measuring decorin and biglycan interactions with fibers containing mutant collagen molecules that cause OI and other connective tissue disorders.

Type I collagen homotrimers and their role in tissue remodeling

Han, Makareeva, Leikin; in collaboration with McBride, Phillips, Pace, Byers

While normal type I collagen is a heterotrimer of two α1(I) chains and one α2(I) chain, insufficient or abnormal synthesis of the α2(I) chain results in the chain's replacement with α1(I) homotrimers in a variety of pathological conditions. For instance, synthesis of α1(I) homotrimers in individuals with a polymorphism in the SP1 binding region of COLIA1 may contribute to osteoporosis. Homotrimer synthesis is also observed in, for example, cancer, glomerular sclerosis, and several forms of OI and EDS. To understand how α1(I) homotrimers contribute to pathology, we investigated murine and human homotrimers and their mixtures with the corresponding heterotrimers. The most significant findings during the last several years showed (1) the segregation of the homo- and heterotrimers at a subfibrillar level and (2) significantly reduced susceptibility of the homotrimers to cleavage by tissue collagenases. A more detailed study with rhMMP^3-1 revealed that absence of the α2(I) chain does not alter the enzyme binding but hinders opening of the triple helix necessary for presenting the unwound chains to the catalytic site. In mixtures, rhMMP-1 degraded the normal type I heterotrimers before noticeable cleavage of the homotrimers. We hypothesize that subfibrillar segregation of the homotrimers and selective proteolytic degradation of the heterotrimers may alter tissue remodeling, resulting in accumulation of MMP-resistant fibers.

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Translational studies of patients with novel/unusual OI mutations

Makareeva, Vera, Leikin; in collaboration with Cabral, Barnes, Marini

To gain further insight into molecular mechanisms of bone pathology associated with abnormal folding, function, and interactions of collagens, we continued our collaboration with clinical researchers in the Bone and Extracellular Matrix Branch on characterization of pathology in patients with unusual OI mutations and/or phenotypes. The goal of the collaboration is to find and characterize molecular abnormalities of collagen from these patients. Previously, such studies revealed, among other findings, the molecular mechanism of OI/EDS caused by mutations within the N-anchor domain of the triple helix. More recently, we suggested a possible molecular origin of pathology in patients with a familial R888C substitution in the α1(I) chain and a unusual combination of OI and EDS symptoms. Over the past year, we continued a study of severe/lethal skeletal dysplasia reminiscent of OI in patients with mutations in the cartilage-associated protein (CRTAP) and prolyl-3-hydrohylase (P3H1), which form a tight three-protein complex with cyclophilin B in the ER. Disruptions of this complex significantly delay type I procollagen folding, resulting in overhydroxylation and overglycosylation of Lys residues, which are also observed in many OI cases. Surprisingly, further measurements revealed an abnormally high rate of synthesis of the collagen chains and delayed secretion of the mature protein by dermal fibroblasts from patients lacking P3H1. The combination of faster chain synthesis, slower triple helix folding, and delayed secretion caused accumulation of up to five times the normal amount of collagen within the cells. In dermal fibroblasts from patients lacking CRTAP, we also observed faster chain synthesis and delayed procollagen folding, causing accumulation of two to three times the normal amount of collagen (which was more efficiently secreted than in P3H1-null mutants). The excessive collagen accumulation in the ER likely leads to ER stress, contributing to the severe/lethal outcome in the patients.

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Insights into OI mechanisms from murine models

Mertz, Han, Leikin; in collaboration with Forlino, Uveges, McBride, Phillips, Marini

Murine OI models offer unique opportunities for systematic studies of the molecular mechanism of pathology in animals with the same mutation but different genetic background, age, sex, and so forth. We currently work with all three existing murine models, including the oim mouse with nonfunctional α2(I) chains, the Brtl IV mouse with a knockin G349C substitution in the α1(I) chain, and a mouse model with a knockin G610C substitution in the α2(I) chain. Our previous studies revealed several changes in the physical and chemical properties of collagen from oim and G610C animals, including altered thermal stability (increased in oim and decreased in G610C) and abnormal collagen-collagen interactions. In Brtl IV animals, we found no significant abnormalities in the stability, folding, or interactions of type I collagen, but we discovered selective retention and intracellular degradation of molecules with a single mutant chain. The retention of the mutant collagen within the ER produced visible swelling of ER cisterns, potentially resulting in ER stress and malfunction of collagen-producing cells, including osteoblasts. To understand more fully how these molecular defects may cause bone failure, we continued to characterize animal bones by high-definition, hyperspectral, infrared microimaging as well as by polarization, reflection, and Raman microscopy. We found that bones from oim and G610C mice had higher mineral and lower collagen content in the extracellular matrix than age- and sex-matched normal controls. Interestingly, reduced synthesis of the mutant α2(I) chain in G610C animals, with a large intron insertion used to knock in the mutation (neo cassette), resulted in synthesis of a fraction of α1(I) homotrimers, milder OI phenotype, and closer-to-normal matrix mineralization. In Brtl IV animals, the mineral and collagen content of the extracellular matrix appeared to fall within the normal range, but the spatial organization of collagen and mineral was more irregular than in the other mice, potentially explaining their more severe bone phenotype. So far, our findings indicate that the severity of the bone phenotype in the three animal models may be related primarily to osteoblast malfunction rather than to abnormal interactions of mutant collagen molecules in the extracellular matrix. However, more studies are needed to verify our hypothesis.

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High-definition infrared micro-spectroscopy of cartilage in mice with normal and impaired diastrophic dysplasia sulfate transporter

Mertz; in collaboration with Forlino, Lupi, Rossi

We recently developed high-definition, hyperspectral, infrared microimaging technology for label-free imaging of thin, solvated tissue sections, permitting significantly increased chemical resolution and spectral reproducibility. This technology, which consist of an infrared spectrometer with a two-dimensional detector array attached to a microscope, simultaneously measures infrared absorption spectra at 16,000 μ–size spots in a tissue section. Chemical composition, orientation, and interactions of chemical groups within each spot are determined from unique spectral fingerprints and plotted as two-dimensional images. In extracellular matrix, the technology distinguishes collagen from other proteins, resolves different glycosaminoglycans (GAG), and even quantifies the extent of GAG sulfation. Last year, we further improved spectrochemical resolution of different types of sulfated GAG chains by combing infrared and HPLC[4] characterization of model chondroitins and hyaluronate. We also developed a new approach to quantitative mapping of collagen orientation across cartilage

sections by polarized infrared hyperspectral imaging. Specifically, we undertook studies of a knockin mouse model of diastrophic dysplasia (DTD) caused by mutations in the SLC26A2 sulfate/chloride antiporter. The mutations result in deficient sulfate uptake by chondrocytes, leading to undersulfation of proteoglycan GAG chains crucial for cartilage development and integrity. As with other inborn chondrodysplasias, DTD involves delayed skeletal development but exhibits an unusual progression. Even though age compensates for the undersulfation, the articular cartilage degrades with age. To understand the mechanism of cartilage degradation, we collected 5 μ–resolution quantitative images of distributions of major extracellular matrix components across femur head cartilage and growth plate in newborn DTD and wild-type (WT) mice. In DTD mice, GAG sulfation was lower than in WT in the articular and proliferative zones but almost normal in the resting zone. The undersulfation in DTD appeared to be associated with a faster growth rate in the articular and proliferative zones, consistent with the sulfation normalization that occurs when cartilage growth slows with age. Polarized infrared hyperspectral imaging revealed a dense layer of tangentially oriented collagen fibrils at the articular surface. The layer was present in WT and disrupted in DTD mice. The severity of the disruption correlated with the extent of GAG undersulfation but not with densities of collagen, noncollagenous proteins, and GAG chains, suggesting that GAG sulfation might be crucial for synthesis of the oriented matrix by cells. The tangential collagen layer likely reduces friction and protects the cartilage from synovial enzymes. The layer's disruption may cause cartilage degradation with age despite normalization of GAG sulfation. Based on these observations, we proposed a kinetic model for the regulation of GAG sulfation and new potential targets for DTD treatment.

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Sequence-dependent interactions between duplex DNA

Leikin; in collaboration with Baldwin, Brooks, Lee, Wynveen, Seddon, Kornyshev

For years, recognition that nucleotides are buried inside DNA's double helix and shielded by the highly charged sugar-phosphate backbone fostered the presumption that interactions between double-stranded (duplex) DNA were independent of the DNA's structure and base-pair sequence. This presumption was based on intuition rather than on experiments or rigorous theory. In reality, experimental evidence, e.g., overwinding from 10.5 base pairs (bp) per helical turn in solution to 10.0 bp per turn in aggregates, suggested otherwise. To account for possible effects of the structure of the sugar-phosphate backbone on DNA-DNA interactions, we have been developing a theory of electrostatic interactions between macromolecules with helical patterns of surface charges. Our theory suggests explanations for the observed counter-ion specificity of DNA condensation, DNA overwinding in aggregates, nontrivial cholesteric pitch behavior upon compression of DNA aggregates, subsequent transition from the cholesteric to hexagonal (hexatic) phase, and several quasi-crystalline phases of even more densely packed DNA aggregates. Most important, the theory predicts that the dependence of the backbone structure on the nucleotide sequence would be sufficiently strong to affect DNA-DNA interactions and even result in direct recognition of sequence homology between sequences of duplex DNA that are at least 100 bp long.

Last year, we tested the predicted sequence dependence of interaction between duplex DNA. In one study, we performed statistical analysis and comparison of known structures of DNA oligonucleotides in crystals (determined by X-ray diffraction) and in solution (determined by nuclear magnetic resonance). We evaluated the helical coherence length—a cumulative parameter quantifying sequence-dependent deviations from the ideal double-helical geometry. We found, among other observations, that the solution structure of synthetic oligomers is characterized by a 10 to 20 nm coherence length, which is similar to the 15 nm coherence length of natural salmon-sperm DNA, a

length extracted from fiber X-ray diffraction patterns. Packing of oligomers in crystals dramatically alters the oligomers' helical coherence, increasing coherence length to between 80 and 120 nm, consistent with theoretically predicted role of helical coherence in interactions between DNA at close separations.

In another study, we continued experiments that probed sequence-dependent DNA-DNA interactions in vitro. We imaged various mixtures of two fluorescently tagged, double-helical DNA molecules with identical nucleotide composition and length but different sequences. In electrolytic solution at minor osmotic stress, the DNAs formed discrete liquid-crystalline aggregates (spherulites). We observed spontaneous segregation of the two types of 294 bp–long DNA within each spherulite, revealing nucleotide sequence recognition between double helices separated by water in the absence of proteins. We also observed the segregation for several binary mixtures, suggesting that segregation was not specific to a particular pair. Consistent with our theoretical predictions, the segregation was much weaker for 150 bp DNAs. While the experiments unequivocally demonstrated the possibility of sequence homology recognition without unzipping the double helix, much work remains to be done to test whether the mechanism of recognition is indeed as predicted by the theory and whether such recognition plays any role in DNA pairing in vivo. Further experiments are currently in progress.

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[1]hydrogen-deuterium
[2]matrix metalloproteinase
[3]recombinant human MMP
[4]high-performance liquid chromatography

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SCIENTIFIC SOFTWARE SUPPORT AND BIOINFORMATICS CORE FACILITY

Jonathan Epstein, MS, *Head, Unit on Biologic Computation*

We provide end-user scientific software support for laboratories within NICHD's Division of Intramural Research. Operating as a core facility, we perform most of our work in conjunction with or on behalf of these laboratories.

Mass spectrometry
Epstein; in collaboration with Yergey

We continue to collaborate with the Mass Spectrometry Core Facility. Our previous development of software for the de novo identification of peptides included a large data set (LIPCUT) that exhaustively enumerates all possible amino acid combinations falling into a given mass range. We recently developed software to enumerate the elemental composition of peptides in an analogous fashion and are able to leverage favorable combinatorics to perform such enumeration for higher-mass peptides (4,500 daltons versus 1,750 daltons for LIPCUT). We seek to combine these resources with a newly developed isotopic clustering algorithm to improve existing peptide fragmentation database searches by submitting refined versions of the original spectrum to the search engine.

As a byproduct of these efforts, we developed Perl software for automatically submitting peptide fragmentation spectra for analysis by the NIH Mascot server. We also developed visualization software for isotopic clustering.

Short-read genome assembly and analysis
Epstein; in collaboration with Strunnikov, Maraia

We aligned short Solexa DNA reads to the *X. tropicalis* genome on behalf of the Program in Cellular Regulation and Metabolism's Unit on Chromosome Structure and Function. We formatted the resulting alignments so that they could be viewed within the University of Santa Cruz's genome browser.

We are actively involved in a joint project with the Program in Genomics of Differentiation's Section on Molecular and Cell Biology. Thus far, we have aligned Solexa reads for *S. pombe* to its reference genome and have remediated a set of *S. pombe* third-party genome corrections. We are working with three strains of *S. pombe*, one of which is a parent of the other two strains. We hope to identify differences between the parent strain and the canonical reference strain and, more importantly, to identify differences between the parent strain and its two mutants. Recently acquired computer hardware should aid in the analysis.

Protein sequence analysis
Epstein; in collaboration with Sackett

We performed a phylogenetic study of protein tails from a collection of selected bacterial and mammalian species. The study is a continuation of a past collaboration under which we performed extensive proteome-wide analysis of protein tails.

Radiation hybrid mapping

Epstein; in collaboration with Dawid

Surprisingly, even in 2008, we see demand for radiation hybrid mapping in zebrafish despite the availability of a draft zebrafish genome. We recently dismantled the LN54 radiation hybrid panel mapping web site (for technical reasons) and now receive requests to perform radiation hybrid mapping on a semimanual basis.

Microarrays

Epstein; in collaboration with Toyama, Dawid, Buonanno

We continue to function as a core facility for microarray analysis for several NICHD laboratories. On behalf of the Program in Genomics of Differentiation's Laboratory of Mammalian Genes and Development, we have performed considerable analysis of Affymetrix microarrays, providing information derived from the *Gene Ontology* database for selected upregulated and downregulated genes as well as considerable statistical analysis and development of web-based software tools. We provided microarray statistical consultation and quality validation for the Program in Developmental Neuroscience's Section on Molecular Neurobiology.

Three-dimensional protein structure

Epstein

The SASH method is a multi-dimensional data structure that has been proposed as a heuristic for *k*–nearest-neighbor problems, especially for problem domains where a true metric is unavailable. In contrast to more traditional methods that require $\mathbf{O}(n^2)$ construction time, SASH requires $\mathbf{O}(n \log n)$ construction time, at the price of some loss of sensitivity in identifying true neighbors. We applied SASH to a comparison of three-dimensional protein structures, using two substantially different methods (*DaliLite* and *SSEF*) for pairwise comparison of two protein structures. Potential benefits include reduced runtime and the ability to operate effectively in a problem domain where the triangle inequality does not apply.

General bioinformatics

Epstein; in collaboration with Howard

We provided ongoing consultation on DNA and protein sequence analysis and on general bioinformatics issues to the Program in Genomics of Differentiation and have consulted with regard to evolving high-throughput DNA sequencing technology.

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MICROSCOPY AND IMAGING CORE FACILITY

James T. Russell, DVM, *Director*
Louis (Chip) Dye, BS, *Staff Scientist*
Vincent Schram, PhD, *Staff Scientist*

In February 2004, the Institute created the NICHD Microscopy and Imaging Core (MIC) to provide high-end light and electron microscopy services to all NICHD scientists. In just a few years, the MIC has grown into a full-service facility offering a diversified portfolio of state-of-the art instruments and imaging methodologies. The MIC is a multi-user facility where investigators can, with minimum time and effort, prepare, image, and analyze their samples. Located on the fifth and sixth floors of Building 49 on the NIH campus, the facility is staffed by two full-time microscopists working under the supervision of James Russell. Vincent Schram oversees the light microscopy operations and information technology infrastructure, and Chip Dye manages the electron microscopy branch.

Mode of operation
The facility's equipment and staff are available to everyone within NICHD free of charge, provided that existing resources are distributed equally among investigators requesting MIC services. The MIC's mission is to ensure that only reliable, high-quality data are recorded on its instruments. For every new project, MIC staff meet with the principal investigator (PI) and postdoctoral scientists involved in the study to discuss details of the experimental design and the project's background and imaging goals in order to determine the most appropriate techniques and instrumentation. Users must sign a document outlining the policies to be followed when using MIC equipment (http://mic.nichd.nih.gov/pdf/mic_rules_of_use.pdf). Long-term user feedback indicates that the high level of interaction between MIC staff and researchers has greatly improved the efficiency of each imaging project.

Light microscopy
After consultation on specimen preparation and staining, each user undergoes hands-on training on the light microscope required by the project at hand. Once the MIC staff are confident that the user can collect high-quality data, the user may proceed unsupervised but with continuous support and guidance. The facility is accessible 24 hours a day/7 days a week, and users may reserve time on each instrument by using an online calendar (https://dircal.nichd.nih.gov/calendar/login.html).

The MIC operates several confocal laser scanning microscopes that address different needs:

1. *Zeiss LSM 510* inverted with UV and spectral detection capabilities, optimal for high-resolution imaging
2. *Zeiss Live DuoScan* inverted, for high-speed, high-sensitivity imaging, photobleaching, and photoactivation of live cells
3. *Zeiss LSM 510* upright with two-photon excitation and an integrated electrophysiology platform, for combined physiology with deep-tissue confocal imaging on live tissue sections and whole animals

4. *Perkin-Elmer* spinning disk for low-light imaging of photosensitive specimens
5. High-performance dual-channel *Olympus Total Internal Reflection Fluorescence* (TIRF) microscope

Live imaging is fully supported, with temperature control, heated perfusion, and solution change available on every microscope. Most instruments have an environmental chamber with temperature, CO_2, and humidity control.

To ensure that it addresses users' needs optimally, the MIC continuously upgrades its equipment portfolio with new capabilities. An important ongoing project is the subresolution imaging device (F-PALM) developed under the trans–NIH Imaging Initiative. Pending availability of funds for completion, this single-molecule imaging device should be available for general use by late spring 2009. Another recently completed instrument upgrade is the replacement of the older wide-field Leica fluorescence microscope with a modern, fully automated Olympus wide-field microscope. The data analysis software has also been significantly upgraded during the past 12 months with the addition of Bitplane's Imaris software, which offers advanced three-dimensional rendering, quantitation, and filament tracing.

Electron microscopy

Given that sample processing for Electron Microscopy (EM) is more sophisticated than for light microscopy, the EM division of the MIC operates differently from the light microscopy division. Typically, Chip Dye carries out all EM processing (fixation, embedding, sectioning, and staining) in-house in a full-fledged EM laboratory with an LKB Pyramitome, a Leica CM3050-S Cryostat, and a Reichert Ultracut-E Ultramicrotome. Imaging on the facility's JEOL 1010 transmission electron microscope is the responsibility of either the microscopist or, for users with the inclination and proper training, the end user. Given the labor involved with EM, the number of projects undertaken in the EM division is necessarily small. A recently acquired microwave-assisted programmable incubator (PELCO BioWave Pro, Ted Pella, Inc.) has been instrumental in reducing incubation times and allowing Mr. Dye to support more investigators. Consistent and controlled incubation parameters have also significantly improved the quality of both ultrastructure and immunolabeling studies. Mr. Dye has recently set up techniques for EM-level immunohistochemistry, and the use of the new microwave technology permits excellent preservation of both morphology and immunoreactivity.

Ancillary support

Given that the NICHD's Division of Intramural Research laboratories are scattered across the NIH campus, the MIC provides all necessary techniques and facilities within one building: tissue culture hood, 5 and 10 percent CO_2 incubators, animal holding and preparative space, and vibratomes for live and fixed tissues. The MIC also operates a data analysis center with three high-end workstations and image analysis software (Metamorph, Volocity, Imaris, and Zeiss AIM), an enterprise-strength file server, and a data backup server. The MIC offers training and support on all imaging software and, as necessary, develops custom macros and high-throughput analysis programs.

Community outreach

The MIC is committed to promoting light and electron microscopy to NICHD's Division of Intramural Research. We educate investigators on the benefits and pitfalls of advanced imaging techniques by (1) coaching users on the principles of confocal microscopy during training and publishing comprehensive operating protocols for each microscope; (2) conducting on-campus demonstrations of new instruments and software developed by vendors such as Zeiss, Photometrics, Nikon, and Perkin-Elmer; and (3) providing on-site assistance to investigators operating their own imaging equipment in order to optimize the quality of the data recorded. Moreover, the MIC website (http://mic.nichd.nih.gov/) is an important resource for tutorials and protocols for both fixed and live microscopy. In parallel with these efforts, the staff have developed collaborations with other Institutes

to promote the exchange of information and bring new imaging technologies to NICHD. Ongoing collaborations include those with Afonso Silva (NINDS) and Richard Saunders (NIMH).

Facility usage

The MIC currently serves 113 registered users associated with 40 NICHD PIs and 4 PIs of sister Institutes on the campus. During any given week, approximately 20 users spend half a day or more on an MIC microscope. As of August 2008, users had logged more than 22,000 hours on MIC equipment, a level of usage resulting in more than 44 publications, 6 of which were co-authored by MIC personnel (see http://mic.nichd.nih.gov/publications.htm for a complete list).

Looking ahead

Several technology developments, such as multiple immunolabeling for EM and subresolution light imaging, are currently in progress. The MIC is also investigating a replacement of its 14-year-old JEOL 1010 electron microscope with a modern instrument that will allow for tomography and cryo–EM imaging, two technologies requested by several investigators. The required capital investment, although significant, would be commensurate with the level of use and service provided by the EM division to the Institute.

An important consideration for the MIC's future is resource and budget allocation. The facility is currently operating at capacity in terms of both instruments and personnel. Expanding and diversifying the MIC mission, such as by increasing technology development or providing more support for sample preparation, could be achieved with modest increases in funding and personnel. Although expansion could be viewed as a challenge in the current fiscal environment, a centralized imaging facility provides significant cost savings to the Institute by maximizing the return on investment for both equipment and personnel. With ongoing support from the Institute, the MIC will continue to provide the NIH with the competitive edge that is so important in today's research environment.

Publications with Assistance from the MIC

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MASS SPECTROMETRY FACILITY

Alfred L. Yergey, PhD, and **Peter S. Backlund, PhD**, *Co-Directors*
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The NICHD Mass Spectrometry Facility was created under the auspices of the Office of the Scientific Director to provide high-end mass spectrometric services to all NICHD scientists, particularly in the area of protein characterization. The facility is located in the 9D Corridor of Building 10 on the NIH campus.

Mode of operation

The facility's staff are available for consultation to everyone within the Institute free of charge, provided that existing resources are distributed equally among investigators requesting our services. Except for the ABI Voyager MALDI TOF[1] instrument, none of the equipment is available for general use. The philosophy of the Mass Spectrometry Facility is to ensure that its instruments record only reliable, high-quality data and that its clients receive only statistically meaningful analyses. For every new project, staff members meet with the Principal Investigator (PI) and the postdoctoral scientists involved in the study to discuss details of the experimental design. The discussion focuses on the project's background and analytical goals in order to determine the most appropriate techniques for sample preparation before the delivery of samples to the laboratory. We digest the proteins, typically gel-separated by the investigator, in situ and isolate peptides for mass spectrometric analysis. We subject separated peptides to analysis by fragmenting them in a second stage of mass spectrometer operation and then use the fragmentation spectra as input to database search engines to determine the protein(s) present in the original sample. Upon completion of an analysis, we provide the investigators with the results and review the findings. Long-term user feedback indicates that our high level of interaction with researchers has greatly improved the efficiency of each project.

Instrumentation

The facility currently has four mass spectrometers used for specific areas of analysis.

1. *ABI 4800 MALDI TOF/TOF*. This state-of-the-art high-performance instrument can be operated in both positive and negative ion modes. We typically use it for peptide mixtures without chromatographic separation. Additional uses include accurate determination of peptide masses in complex mixtures and the determination of sequences of unusual peptides through de novo sequencing techniques.
2. *Thermo Finnigan LCQ Deca*
3. *LCQ DecaXP*. Both the Thermo Finnigan LCQ Deca and the LCQ DecaXP employ liquid chromatography and electrospray ionization to separate and ionize peptides that are then fragmented.

4. *ABI Voyager MALDI TOF*. This instrument is used for analysis of higher–molecular-weight species—equal to or greater than 125 kDa—and to verify the molecular weight of intact proteins. The instrument is also available for general use after a potential user has undergone appropriate training.

Community outreach

The Mass Spectrometry Facility is committed to promoting mass spectrometric aspects of proteomics in NICHD's Division of Intramural Research. We make serious efforts to educate investigators on the benefits and pitfalls of the techniques used in the facility. In particular, we provide coaching on the principles of appropriate methods for the isolation and staining of gels and support an NIH-wide seminar series featuring nationally known experts in proteomics. In parallel, the staff have developed collaborations with other Institutes to promote the exchange of information and bring new mass spectrometric techniques to NICHD.

Facility usage

The Mass Spectrometry Facility currently serves 15 Sections within the Institute, representing about 40 projects, as well as four PIs of sister Institutes on the campus.

Major projects during Fiscal Year 2008

NUCLEOLAR PROTEIN B23/NUCLEOPHOSMIN. Novel protein-protein interactions can be detected by co-immunoprecipitation of specific proteins with a target protein of interest. In collaboration with Mary Dasso's group (see page 26), we analyzed proteins from *Xenopus* egg extracts that specifically bind to the Ulp1p-like SUMO proteases xSENP3 and xSENP 5. Analysis of gel-separated proteins by tryptic digestion followed by LC/MS/MS^2 permitted the identification of several novel binding partners. We identified a protein that strongly bound to xSENP3 as the *Xenopus* B23/nucleophosmin homologue. Further work showed that such binding (carried out in LGRD) was essential for stable accumulation of SENP3 and SENP5 in mammalian tissue culture cells.

Yun C, Wang Y, Mukhopadhyay D, Backlund P, Kolli N, Yergey A, Wilkinson K, Dasso M. Nucleolar protein B23/Nucleophosmin regulates the proteases. *J Cell Biol* 2008;183:589-595.

LIPID QUANTIFICATION IN SERUM. In association with the Program in Developmental and Molecular Immunity, we have continued to develop a mass-spectrometric methodology for quantifying cardiolipins in human serum. The effort supports a clinical study in the Institute to evaluate the effects of antibiotic treatment of pregnant women colonized with the Group B streptococcal (GBS) organism. The study's hypothesis posits that typical perinatal penicillin treatment gives rise to a large increase of circulating cardiolipins in the infant and then leads to respiratory distress. Other studies in newborn sheep have demonstrated that the GBS organisms secrete a specific cell-wall membrane cardiolipin upon penicillin treatment and that the substance causes respiratory distress at levels of about 100 pmole per mL serum. It is not known whether the respiratory distress observed in a fraction of infants born to GBS-colonized mothers results from a similar effect or perhaps from a release of endogenous cardiolipins stimulated by bacterial death. The analytical approach involves the addition of an internal standard to a 200 µl serum sample and is a combination of liquid-liquid and solid-phase extractions, followed by an LC-MS analysis that incorporates an extraction/recovery standard to monitor system quality control. We have shown that cardiolipin may be extracted from serum with greater than 75 percent efficiency and that cardiolipin serum levels of less than 100 pmole per ml may be determined. We continue to refine mass-spectrometric methods for the determination of these materials in order to increase analytical robustness and the sample through-put rate.

TUBULIN POST-TRANSLATIONAL MODIFICATIONS. Continuing work on the characterization of post-translational modifications of tubulins recently yielded interesting results with respect to phosphorylation sites. A characteristic addition of 80Da to a chicken erythrocyte tubulin C-terminal

CNBr digest fragment showed the presence of phosphorylation, although we have yet to identify the modified residue. In another study employing a kinase known to phosphorylate tubulin in the ratio of a 2 mole phosphate to 1 mole tubulin, we identified phosphorylation sites of HeLa tubulins. We showed that two beta-tubulins found in HeLa cells phosphorylate not in the C-terminal region but rather in the main sequence of the proteins in a region that, shown by X-ray crystallography, evidences a loop external to the protofilaments; the loop plays an active role in microtubule assembly. To date, however, we have not identified analogous sites on alpha-tubulins. Our observation might have substantial therapeutic implications, particularly in light of the presence or absence of phosphorylation in the analogous region of alpha tubulins—a loop known to be the site in which tamoxifen inhibits microtubule assembly. This work was carried out in collaboration with Dan Sackett (see page 340).

IMPROVEMENT IN SPECTRAL RELIABILITY. We have continued to make advances in our efforts to develop methods for obtaining more robust mass spectra of samples by generating consensus spectra from several replicates. When applied to peptide fragmentation spectra as part of an effort to infer the presence of proteins in gel samples, our approach—particularly when applied to peptide sequencing de novo—shows substantial improvement as reflected in our preliminary results. Our novel algorithm for deducing peptide sequences de novo often yields useful results for a particular peptide precursor undergoing fragmentation and seems to yield meaningful results with each application, generating a consensus spectrum from several replicate spectra of the same peptide precursor. Accordingly, we plan to extend the approach to routine protein inference from unknown samples in both MALDI TOF-TOF[1] and LC/MS-MS[2] methodologies.

Olson MT, Blank PS, Sackett DL, Yergey AL. Evaluating reproducibility and similarity of mass and intensity data in complex spectra—applications to tubulin. *J Am Soc Mass Spect* 2008;19:367-374.

[1]MALDI: matrix-assisted laser desorption/ionization; TOF: time-of-flight
[2]LC: Liquid chromatography; MS: mass spectrometry

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