Because the dTip60 complex acetylated nucleosomal phospho-H2A in vitro, we tested dependence of H2A acetylation on dTip60 complex components in vivo. We probed chromatin extracts from γ-irradiated double-stranded RNA (dsRNA)-treated S2 cells as well as dMrg15 mutant embryos with antibodies against H2A(aeK5), which recognized H2Av(aceK5) (Fig. 3C). We detected transient acetylation of a protein band that exhibits the migratory properties of phospho-H2Av (Fig. 4D). This acetylation was most prominent 15 min after γ irradiation and was not detected in extracts of cells lacking dTip60 or dMrg15. Similar observations were made by immunolabeling dMrg15 mutant embryos (Fig. 4E and fig. S9). We conclude that the dTip60 complex acetylates nucleosomal phospho-H2Av at Lys6 in a DSB-dependent manner.

The Drosophila dTip60 complex is structurally homologous to its human counterpart (Fig. 1 and table S3) (17, 22, 23). Both complexes share factors that are linked to cancer, transcription, and DNA repair, including Pontin, Reptin, Mrg15, Tra1, E(Pc), Gas41, and Tip60. We also identified the histone variant H2A.X within the Drosophila dTip60 complex. The human Tip60 complex is essential for DSB repair and regulation of apoptosis, two processes that have been linked to histone H2Av in flies (13, 17). Also the yeast NuA4 complex appears to accumulate at DSBs (24).

We demonstrated that the Drosophila dTip60 complex acetylates nucleosomal phospho-H2Av and exchanges it with an unmodified H2Av (Figs. 2 and 3 and figs. S4 to S6). The histone-exchange reaction catalyzed by the ATPase Domino is enhanced by dTip60-mediated acetylation of nucleosomal phospho-H2Av. It appears likely that phospho-H2Av recruits the dTip60 complex to DSBs to facilitate chromatin remodeling during DNA repair. In yeast, the DNA damage–dependent H2A kinase Mec1 genetically interacts with subunits of the NuA4 complex (21, 25), and cells missing NuA4 subunits are sensitive to DSB-inducing agents (25, 26).

The physiological roles of the dTip60-mediated phospho-H2Av removal at sites of DSBs could not be clearly separated from a potential function of this complex in DSB repair because of the intimate temporal link between DSB repair and phospho-H2Av clearance (20). However, the overexpression of phospho-H2Av did not induce G2/M arrest or affect DSB-dependent G2/M arrest (fig. S10) (14, 21), suggesting that this signal is not sufficient for damage checkpoint control.

The loss of human Tip60 leads to the accumulation of DSBs and is linked to a growing number of cancer types (26, 27). The histone variant H2A.X is essential for genomic stability and a candidate tumor suppressor (13, 28, 29). Thus, our findings help to understand the functional link between DNA damage–dependent H2A.X phosphorylation and the role of Tip60-type complexes during DSB repair in chromatin.

**References and Notes**

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18. Materials and methods are available as supporting material on Science Online.

## References and Notes

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primary cultures of macrophages and lymphocytes from WT, IRP1−/−, and IRP2−/− animals. In both cell types, IRP1 appeared to be the predominant IRE-binding protein when cells were cultured in atmospheric oxygen (21% O2). Because mammalian tissue oxygen concentrations are closer to 3 to 6% than 21% (11–14), and because IRP activities can be affected by oxygen (15), these experiments were carried out at oxygen concentrations ranging from 3 to 21% to evaluate how IRP activities vary accordingly. In macrophages, the change in oxygen concentration from 3 to 21% led to a dramatic switch in the proportion of IRE-binding activity contributed by each IRP. IRP2 was the predominant source of IRE-binding activity at 3% O2, whereas IRP1 became the major source of IRE-binding activity at 21% O2. As IRP1 activity increased at 21% O2, IRP2 levels decreased. In addition, at 3% O2, IRP1 lost most of its ability to respond to changes in iron status (Fig. 2A and fig. S1). Thus, the relative importance of each IRP in the regulation of iron metabolism varied greatly with changes in oxygen concentration.

In spleen-derived lymphocytes, IRE binding activity of IRP1 also increased markedly as the oxygen concentration rose from 3 to 21% (Fig. 2C). IRP2 levels were highly regulated by iron at all oxygen concentrations examined, although IRP2 protein levels decreased gradually as the oxygen concentration increased (Fig. 2D). Thus, oxygen exposure is a critical variable that determines the potential regulatory activity of IRP1 and IRP2 and their relative importance in both macrophages and lymphocytes.

To evaluate the effect of oxygen-dependent changes of IRP activity on regulated targets, we compared ferritin and TIR expression levels in WT, IRP1−/−, and IRP2−/− primary cell cultures grown in 21 or 3 to 6% O2 concentrations. WT macrophages decreased ferritin and increased TIR expression in response to iron deficiency at both low and high oxygen concentrations (Fig. 3, A and B). In contrast, iron-deficient macrophages from IRP2−/− animals could not repress ferritin or
increase TIR expression when grown at 3% O₂. However, at 21% O₂, iron-deficient IRP2−/− macrophages almost fully regulated ferritin and TIR expression, suggesting that the regulatory activity of IRP1 at 21% O₂ allowed IRP1 to substitute for IRP2 in regulation of IRP targets.

Increased ferritin and decreased TIR levels were observed in IRP2−/− macrophages immunoblots grown in 3% O₂ (Fig. 3, A and B) and were attributable to changes in the biosynthetic rates of ferritin and TIR grown at low oxygen concentrations (Fig. 3C). The observation that ferritin and TIR regulation were normal in IRP1−/− macrophages maintained at 3% O₂ implied that IRP2 provided almost all of the regulatory activity in macrophages with minimal contribution from IRP1 at these oxygen concentrations. Levels of IRP2 did not change in IRP1−/− macrophages compared to WT at 3% O₂ (Fig. 3D).

Unlike macrophages, which relied almost exclusively on IRP2 for regulation at physiologically relevant oxygen concentrations, lymphocytes appeared to depend on both IRP1 and IRP2 for normal regulation. The ability to regulate ferritin was significantly reduced in IRP2−/− lymphocytes (Fig. 4A). In IRP1−/− lymphocytes grown at 21% O₂, ferritin levels were also significantly elevated compared to levels in WT controls, and in IRP1−/− lymphocytes grown at 6% O₂, ferritin was slightly elevated at the lowest iron concentration points. These results revealed that IRP1 contributes a substantial fraction of IRRE-binding activity in lymphocytes, even though it is minimally sensitive to changes in iron status at low oxygen concentrations (Fig. 2C), similar to its previously observed role in brown fat and kidney (9). In lymphocytes, IRP2 levels increased in IRP1−/− cells (Fig. 4B), perhaps because loss of IRP1 resulted in a slight increase in ferritin expression (Fig. 4A) that produced a state of subtle functional cytosolic iron deficiency and a concomitant decrease in the rate of iron-dependent IRP2 degradation (5, 6). The increase in IRP2 allowed cells and tissues to partially compensate for loss of IRP1, as was observed in lymphocytes (Fig. 4B) and in tissues of IRP1−/− mice (9). Thus, IRP1 contributes significantly to baseline regulation of iron metabolism in some cells, consistent with the increased severity of neurodegeneration in IRP1−/− IRP2−/− animals (16), and the embryonic lethality of IRP1−/− IRP2−/− mice (17).

IRP1 and IRP2 operate on a continuum in which the partial pressure of oxygen determines relative activity. IRP1 contains an oxidation-sensitive Fe-S cluster that is enzymatically assembled in cells that have sufficient iron and sulfur (18, 19). However, once the Fe-S cluster of IRP1 is assembled, it may remain stable unless it is exposed to oxidants, including oxygen, superoxide, hydrogen peroxide, nitric oxide, and peroxynitrite (4, 20–23). At the ambient oxygen concentrations in tissues of healthy animals (13–15), the Fe-S cluster of IRP1 appears to be stable, and IRP1 is therefore poorly suited to function as an iron sensor (9). In contrast, IRP2 has a different mechanism of iron sensing that depends on iron-dependent degradation (5, 6, 24–27). Iron-dependent turnover is intact at physiologically relevant oxygen concentrations, which enables IRP2 to dominate normal regulation of iron homeostasis in mammals.

IRP1 plays a minor role in the regulation of iron metabolism by contributing a fraction of
baseline IRE-binding activity in tissues. However, the observation that IRE-binding activity can be recruited by oxidative disassembly of its Fe-S cluster means that in some pathological situations, including those in which inflammation results in release of reactive oxygen species, the vast reservoir of IRP1 in the aconitase form (Fig. 1C) may be converted to the IRE-binding form (20, 21). This can lead to inappropriate repression of ferritin synthesis, increased TIR expression, and cellular iron toxicity. Increased iron content coupled with decreased ferritin has been observed in degenerating regions of the brain in Parkinson’s disease (28). Thus, pathologic activation of IRP1 by reactive oxygen species may play an important role in some disease processes.

Our findings provide genetic evidence that in mammals, the tissue oxygen concentrations are a critical variable in regulating genes that are important in iron metabolism. Results obtained from tissue culture cells grown in room air may lead to conclusions that are not relevant to normal physiology. Iron- and oxygen-based chemistries affect many cellular processes, including mitochondrial function, DNA replication, and the response to hypoxia (29). Our work demonstrates that the IRP-regulatory system has evolved to tightly regulate iron metabolism at oxygen concentrations that exist in normal mammalian tissues. Even though the two IRPs are highly homologous (1), their activities are only partially redundant, and they occupy different regulatory niches. In normal physiology, tissue oxygen tension determines the contribution of each IRP to the regulation of iron homeostasis.

References and Notes
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Hepcidin Regulates Cellular Iron Efflux by Binding to Ferroportin and Inducing Its Internalization

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Hepcidin is a peptide hormone secreted by the liver in response to iron loading and inflammation. Decreased hepcidin leads to tissue iron overload, whereas hepcidin overproduction leads to hypoferremia and the anemia of inflammation. Ferroportin is an iron exporter present on the surface of absorptive enterocytes, macrophages, hepatocytes, and placentas. Here we report that hepcidin bound to ferroportin in tissue culture cells. After binding, ferroportin was internalized and degraded, leading to decreased export of cellular iron. The posttranslational regulation of ferroportin by hepcidin may thus complete a homeostatic loop: Iron regulates the secretion of hepcidin, which in turn controls the concentration of ferroportin on the cell surface.

The liver-produced hormone hepcidin controls plasma iron levels by regulating the absorption of dietary iron from the intestine, the release of recycled hemoglobin iron by macrophages, and the movement of stored iron from hepatocytes [for a review, see (1,2)]. During pregnancy, fetal hepcidin controls the transfer of maternal iron across the placenta to the fetus. In turn, hepcidin levels are homeostatically regulated by hepatic iron and the need for erythropoiesis as sensed by liver oxygenation. Hepcidin is also induced during inflammation, in which hepcidin’s effect on iron transport causes the characteristic decrease in blood iron (hypoferremia of inflammation). The hypoferremia is thought to increase host resistance to microbial infection but also leads to the anemia of inflammation (often referred to as the anemia of chronic disease).

Ferroportin (Fpn) is an iron exporter on the surface of absorptive intestinal enterocytes, macrophages, hepatocytes, and placental cells, all of which release iron into plasma (3–5). To determine whether hepcidin interacts with Fpn, we generated a stable cell line (HEK293-Fpn) expressing mouse Fpn with a C-terminal green fluorescent protein (GFP) under the control of the cedysone-inducible promoter. In the absence of the inducer ponasterone, there was no detectable synthesis of Fpn-GFP. Within 24 hours of ponasterone addition, there was abundant GFP fluorescence outlining the surface of cells (Fig. S1). To determine whether Fpn-GFP was functional, we examined the effect of ferroportin induction on cellular iron levels, as measured by the accumulation of ferritin, the cytosolic iron storage protein. Incubation of cells with ferric ammonium citrate (FAC) alone resulted in a large increase in ferritin levels. FAC loading with simultaneous induction of Fpn-GFP prevented ferritin accumulation (Fig. 1A). Similar results were obtained when diterfer transferrin was used as an iron source and levels of IRP2, inversely regulated by cytosolic iron (6,7), were measured as an indicator of cellular iron levels (fig. S2). Induction of Fpn-GFP resulted in an increase of IRP2 levels when compared to uninduced cells, indicating that cytosolic iron levels decreased after the induction of Fpn-GFP. To show that reduced cytosolic iron levels re-

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