Systemic Iron Homeostasis and the Iron-Responsive Element/Iron-Regulatory Protein (IRE/IRP) Regulatory Network

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Abstract

The regulation and maintenance of systemic iron homeostasis is critical to human health. Iron overload and deficiency diseases belong to the most common nutrition-related pathologies across the globe. It is now well appreciated that the hormonal hepcidin/ferroportin system plays an important regulatory role for systemic iron metabolism. We review recent data that uncover the importance of the cellular iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network in systemic iron homeostasis. We also discuss how the IRE/IRP regulatory system communicates with the hepcidin/ferroportin system to connect the control networks for systemic and cellular iron balance.

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SYSTEMIC IRON HOMEOSTASIS IN MAMMALS

Iron is essential for oxygen transport, cellular respiration, and DNA synthesis. Iron deficiency can cause cellular growth arrest and death. Conversely, iron excess and "free" reactive iron is toxic: Ferrous iron reacts with hydrogen peroxides or lipid peroxides to generate hydroxyl or lipid radicals, respectively. These radicals damage lipid membranes, proteins, and nucleic acids. Since both iron deficiency and iron overload are detrimental to the cell, anomalies in iron metabolism are frequent causes of clinical disorders. For example, iron overload in hereditary hemochromatosis (HH) and the thalassemias leads to potentially fatal liver or heart failure. Iron deficiency represents the most common cause of anemia worldwide and can cause developmental retardation in children. Thus, iron homeostasis must be tightly controlled on both the systemic and the cellular levels to provide just the right amounts of iron at all times.

The saturation of the plasma protein transferrin with iron is a major indicator and determinant of systemic iron homeostasis. Diferric transferrin circulates in the blood and provides iron to most cells of the body. Iron saturation of serum transferrin is predominantly determined by the amount of iron (*a*) absorbed from the intestine, (*b*) recycled from senescent red blood cells and released by macrophages, and (*c*) utilized for erythropoiesis, the main iron consumer (32). In addition, diferric transferrin affects the expression of hepcidin (Hamp1/Leap1) (48), a small hepatic peptide hormone that regulates intestinal iron absorption and iron release from macrophages and hepatocytes by virtue of controlling the expression of the iron exporter ferroportin (FPN1) (24). Thus, both iron-bound transferrin and hepcidin are important effectors of systemic iron homeostasis (**Figure 1**).

Cellular iron levels are predominantly balanced by the iron-responsive element/iron regulatory protein (IRE/IRP) regulatory system (see below). Interestingly, recent studies have begun to uncover important regulatory roles of the IRE/IRP network of systemic iron parameters, which is the focus of this review.

THE IRE/IRP REGULATORY SYSTEM

Cellular iron homeostasis is achieved by the coordinated and balanced expression of proteins involved in iron uptake, export, storage, and utilization. Although genetic control

is exerted at multiple steps, the posttranscriptional control mediated by the IRE/IRP system has emerged as central and essential (32). Cellular iron metabolism is coordinately controlled by the binding of IRP1 or IRP2 to *cis*-regulatory mRNA motifs termed IREs; IRE/IRP interactions regulate the expression of the mRNAs encoding proteins for iron acquisition [transferrin receptor 1 (TFRC-TFR1), divalent metal transporter 1 (SLC11A2-DMT1-DCT1-NRAMP2)], storage [ferritin H (FTH1) and ferritin L (FTL)], utilization [erythroid 5′-aminolevulinic acid synthase, mitochondrial aconitase (ACO2), *Drosophila* succinate dehydrogenase (SDH), hypoxia-inducible factor 2 (HIF2, Epas1)], and export (SLC40A1-FPN1-IREG1-MTP1) (74, 92) (**Figure 2**). Past and recent evidence suggests that additional mRNAs are regulated by the IRPs (10, 39, 73).

IRE/IRP complexes formed within the 5′UTR of an mRNA (e.g., FTH1, FTL, ALAS2, ACO2, FPN1) inhibit translation, whereas IRP binding to IREs in the 3′UTR of TFR1 mRNA prevents its degradation. The combined IRE-binding activity of both IRPs is high in iron-deficient cells and low in iron-replete cells. Distinct mechanisms control IRP1 and IRP2 activity in response to the cellular labile iron pool. Under iron-replete conditions, a cubane [4Fe-4S] cluster assembles in IRP1, preventing IRE binding. Interestingly, this assembly converts IRP1 to a cytosolic aconitase that interconverts citrate and isocitrate. The physiological importance of this cytosolic aconitase remains to be defined, and IRP1-deficient mice display no overt phenotype (21, 59). In iron deficiency, IRP1 binds to IREs as an apoprotein. Extensive conformational changes that occur in IRP1 during the conversion between the IRE-binding form and the cytosolic aconitase can be explained with the recently solved structure of the IRE/IRP complex (91). Components of the iron sulfur cluster assembly machinery, located mostly in mitochondria, are essential for the incorporation of the 4Fe-4S cluster. For example, lack of glutaredoxin 5 activity causes increased IRE-binding activity of zebrafish IRP1 with concomitant anemia (97) (see below). Likewise, hepatocyte-specific ablation of the mitochondrial ABC half-transporter Abcb7 in the mouse, which is mutated in X-linked sideroblastic anemia with ataxia in humans, impairs iron-sulfur cluster assembly in IRP1 (68). In addition, the iron-sulfur cluster of IRP1 can be destabilized by other stimuli, such as oxidative stress and nitric oxide (92).

In contrast, IRP2 does not contain an Fe-S cluster and is regulated by iron via proteasomal degradation. The precise details of the degradation mechanism are still debated (92). In summary, the regulation of the IRE-binding activities of IRP1 and 2 assures the appropriate expression of IRP target genes and cellular iron balance.

Although the role of the IRE/IRP regulatory system in cellular iron homeostasis has been extensively investigated in cultured cells during the past two decades, its in vivo functions have only recently become a focus of attention. Mouse embryos that lack both copies of the IRP1 and the IRP2 genes die early, indicating that the IRE/IRP regulatory network is essential, at least for early development (23, 82). By contrast, analysis of mice lacking either IRP1 or IRP2 reveals that the two IRPs can largely replace each other (see below) (11, 22, 59). In combination with biochemical data, these findings suggest that IRP1 and IRP2 can both effectively regulate their target mRNAs.

THE FUNCTION OF THE IRE/IRP REGULATORY NETWORK IN ERYTHROPOIESIS

In healthy individuals, some two million erythrocytes are produced every second in the bone marrow, and erythropoiesis requires large amounts of iron (25 mg daily) for heme synthesis. The iron required for erythropoiesis is almost exclusively provided by the plasma protein transferrin (**Figure 1**). The number and hemoglobin content of erythrocytes is a major determinant of tissue oxygenation, and a feedback loop involving erythropoietin (EPO) regulates erythropoiesis. EPO is produced in the

kidney and the liver in response to low oxygen levels and increases erythropoiesis. Enhanced erythropoietic activity in turn reduces hepcidin production by the liver, which then allows sufficient iron to be absorbed from the intestine (see **Figure 1**). IRPs control the expression of several critical components of this regulatory circuit.

Role of the IRE/IRP System in the Control of EPO Levels

Mammals adapt to changes in oxygen availability via a response pathway mediated by a family of transcription factors termed HIF (hypoxia-inducible factor). These transcription factors form heterodimers composed of the oxygen-regulated HIF1alpha or HIF2alpha subunit and a constitutively expressed HIF1ß subunit. Posttranslational control of HIF1/2alpha expression by iron- and oxygen-dependent hydroxylases couples oxygen sensing with the regulation of hypoxia-sensitive genes. Hypoxia abrogates HIF1/2alpha hydroxylation that would lead to proteasomal degradation, resulting in increased protein stability and activity (33). HIF transcription factors regulate more than 40 known target genes, including some involved in vascularization and oxygenation (77).

Additional mRNAs that are regulated by the IRE/IRP system were recently identified by an experimental strategy that integrates biochemical, biocomputational, and microarray approaches (73). Among those, a phylogenetically conserved IRE was discovered in the 5′UTR of the HIF2alpha mRNA, which mediates the iron-dependent translational control of HIF2alpha expression (74). Because the *EPO* gene is a major target of the HIF2alpha transcription factor, the IRE within the HIF2alpha mRNA may serve to modulate EPO levels indirectly, potentially allowing adjustment of the rate of red blood cell production to iron availability. Without such an adjustment, stimulation of erythropoiesis in iron-deficient individuals may lead to the accumulation of hypochromic and microcytic red blood cells, as iron is critical for heme synthesis and hemoglobin production. To avoid such a situation, the IRE in the 5′UTR of the HIF2alpha mRNA may serve to limit HIF2alpha expression and subsequent EPO production when iron is scarce (74) (**Figure 3**).

The IRE/IRP Regulatory System in Erythroid Cells

Erythroid cells satisfy their immense iron needs by taking up transferrin-bound iron. Diferric transferrin (Tf) binds to Tf receptor 1 (TfR1) that is highly expressed on the surface of erythroblasts. Tf-TfR1 complexes localize to clathrin-coated pits and internalize into endosomes. Early Tf-containing endosomes are acidified, which induces a conformational change in both Tf and TfR1 to release the iron. The recently identified ferrireductase Steap3a then reduces Fe^{3+} to Fe^{2+} to import the iron into the cytoplasm via DMT1 (15, 64) and into mitochondria for heme biosynthesis. Excess iron may be stored within ferritin (**Figure 3**). Apo-Tf and TfR1 return to the cell surface for further cycles of iron binding and uptake (32).

Mouse models reveal the importance of transferrin-mediated iron uptake for erythropoiesis: (*a*) Tf-deficient mice develop severe microcytic hypochromic anemia with tissue iron deposition (85); (*b*) TfR1-deficient mice show normal embryonic organ development but then succumb to severe anemia with microcytic hypochromic erythrocytes and decreased iron stores at mid gestation (46); (*c*) mice lacking functional DMT1 present with impaired iron uptake into erythroid precursors (15). Importantly, both the TfR1 and the DMT1 mRNAs contain IREs within their 3′ untranslated regions, suggesting a functional role of the IRE/IRP regulatory system in controlling iron uptake into erythroid precursor cells. This view is supported by the finding that mice lacking IRP2 show reduced TFR1 mRNA levels in the bone marrow together with a mild microcytosis (11, 22). The decreased TfR1 mRNA expression likely results from the failure to protect

TfR1 mRNA sufficiently against degradation in the absence of IRP2. Low TfR1 expression then hampers sufficient iron uptake and hemoglobinization leading to microcytosis. Apparently, the lack of IRP2 cannot be fully compensated for by IRP1 in erythroid cells, highlighting the importance of IRP2 for normal erythropoiesis. In addition, the enzyme 5-aminolevulinic acid synthase 2 (Alas2) is overexpressed in erythroid cells of IRP2-deficent mice (11). Alas2 mRNA contains a 5′UTR IRE that is subject to loss of IRP-dependent translational repression in *Ireb2*$^{-/-}$ mice, confirming previous studies in cultured cells (57). Alas2 is the first and rate-limiting enzyme of erythroid heme biosynthesis, and IRP-dependent translational control of this enzyme is thought to limit heme biosynthesis in erythroid precursors with insufficient iron supplies. Consistent with the elevated Alas2 expression in *Ireb2*$^{-/-}$ mice, protoporphyrin IX levels are increased, which suggests that IRP2 deficiency can potentially account for some cases of protoporphyrinemia (11).

Iron that enters erythroid precursor cells can also be stored within ferritin (84). Ferritin is a multimeric protein that sequesters iron from the intracellular labile iron pool into a chemically less-reactive ferrihydrite form for detoxification or further use. Ferritin consists of light (L)- and heavy (H)-chain subunits building an apoprotein shell that can hold up to 4500 iron atoms (31). Both the ferritin L- and H-chain mRNAs contain IREs in their 5′UTRs and are thus subject to IRP-mediated translational control. Ferritin expression is increased in erythroid precursors of *Ireb2*$^{-/-}$ mice, and increased iron sequestration into ferritin may contribute to the anemia observed in this mouse line (11, 22).

The analysis of IRP2-deficient mice reflects the importance of the IRE/IRP network in erythropoiesis. Recent analyses of the zebrafish mutant shiraz that succumbs to severe hypochromic anemia further support this view. In shiraz zebrafish, the hypochromic anemia is caused by a deficiency of glutaredoxin 5 (grx5) (97). Grx5 is located in the mitochondria of erythroid cells and is important for the biogenesis of Fe-S clusters. Loss of Fe-S cluster assembly in shiraz zebrafish activates IRP1 and blocks heme biosynthesis by inhibition of ALAS2 mRNA translation, whose expression is controlled by an IRE in its 5′UTR (see above). Overexpression of an ALAS2 mRNA variant without its 5′IRE sequence rescues the shiraz phenotype. Likewise, antisense-mediated knockdown of IRP1 restores hemoglobin synthesis in this animal model. These data point toward a link between heme biosynthesis and Fe-S cluster assembly and highlight the regulatory role of the IRE in eALAS mRNA for heme biosynthesis (97). Interestingly, a patient with a sideroblastic-like anemia and iron overload was recently identified to carry a mutation in the human grx5 gene. Consistent with the phenotype observed in shiraz zebrafish, patient cells show diminished cytosolic aconitase and H-ferritin expression whereas TfR1 levels are increased, which suggests that IRP1 is also pathologically activated in this patient (4).

Although there is a clear requirement for IRPs in orchestrating the expression of IRE-containing mRNAs during erythropoiesis (**Figure 3**), a recent study (75) proposes that the IRE/IRP system can be bypassed during the terminal differentiation of primary mouse erythroid progenitors when hemoglobin is synthesized and the demand for iron is unusually high. Using cultured primary murine erythroid progenitors from fetal liver, the authors demonstrate that the expression of IRE-containing mRNAs escapes the control by the IRPs in this experimental setting: The IRP-coordinated regulation of ferritin and eALAS is uncoupled, and high TfR1 mRNA levels persist even in the presence of high levels of iron-bound transferrin. In addition, IRP activity strongly declines and appears no longer to be regulated by the cellular iron content. Mechanistically, an imbalance between IRPs and IRE target sites could contribute to uncoupling ferritin and eALAS mRNA translation. Alternatively, massive mitochondrial iron import for heme biosynthesis may involve a mechanism(s) whereby iron is not sensed within the cytosol (79).

THE IRE/IRP REGULATORY NETWORK IN IRON RECYCLING AND ABSORPTION

In healthy individuals, more than two-thirds of the total body iron content is accounted for by hemoglobin in mature erythrocytes and in their precursors. About 20 to 25 mg of iron are needed every day to sustain the hemoglobinization of new erythrocytes. The vast majority of this comes from the recycling of iron from senescent or damaged erythrocytes by macrophages, while only a small fraction (about 1 to 2 mg of iron) is provided by the intestinal absorption of dietary iron (1). Nevertheless, in the absence of physiologically regulated iron excretion, intestinal iron absorption critically influences systemic iron availability and loading. By inhibiting iron efflux from enterocytes and macrophages via FPN1, hepcidin plays a central role in controlling systemic iron levels (**Figure 1**). Conversely, critical iron management proteins expressed in enterocytes and macrophages are encoded by IRE-containing mRNAs (**Figure 2**), raising the question of the role of the IRE/IRP regulatory network in iron absorption and recycling.

Iron Absorption

Absorption of dietary iron begins with its transport from the lumen of the gut across the apical membrane of absorptive enterocytes followed by its translocation through the cell and its basolateral release into the bloodstream, where it is loaded onto apo-transferrin. In adults, iron absorption mainly takes place in the duodenum. In neonatal rats, iron is also absorbed more distally, possibly explaining their highly efficient dietary iron assimilation (19). Two main forms of iron are taken up by the intestinal mucosa: heme and inorganic iron. In diets rich in meat, heme accounts for approximately two-thirds of the dietary iron supply of the body. Heme is translocated intact across the brush-border membrane of duodenal enterocytes and is subsequently degraded intracellularly by heme oxygenase to release the iron moiety and to feed the low-molecular-weight iron pool of the cell. A duodenal cDNA encoding a putative apical heme carrier with homology to bacterial metal-tetracyclin transporters was recently isolated (78). Solute carrier family 46 member A1 (SlC46A1, also named heme carrier protein 1, or HCP1) is able to carry heme into cells, and its expression or localization in the intestine responds to stimuli that affect iron absorption (e.g., hypoxia, dietary or systemic iron loading). However, the main function of SlC46A1 appears to be proton-coupled folate transport, and its importance for intestinal iron absorption has been challenged (70). Targeted mutagenesis of the murine *Slc46a1* gene could shed light on this issue. Before transport across the apical membrane of absorptive enterocytes, ferric iron must be reduced. Duodenal cytochrome b (DCYTB, also known as cytochrome b reductase 1, or CYBRD1), whose expression is increased by iron deficiency and hypoxia, was thought to fulfill this function (55), but DCYTB appears to be dispensable for iron absorption, at least in the mouse (29). Unlike DCYTB, DMT1 is essential for dietary iron acquisition, as demonstrated by the severe iron-deficiency anemia of mice with a selective ablation of DMT1 in the intestinal mucosa (28). By alternative transcription start site usage and posttranscriptional processing, at least four different DMT1 mRNA isoforms containing or lacking an IRE in the 3′UTR can be expressed (34). In the duodenum, the DMT1 isoforms encoded by IRE-containing mRNAs prevail (23) and are particularly enriched at brush-border membranes (54).

How iron is transferred intracellularly from the apical site to the basolateral iron export machinery is still largely unknown, and different models have been suggested (53). Iron is exported into the bloodstream via the basolaterally expressed efflux channel FPN1 in conjunction with the ferroxidase hephaestin. FPN1 is essential for iron export from enterocytes, as illustrated by the mucosal iron retention and the ensuing hypochromic anemia in mice lacking intestinal FPN1 expression (14). Hephaestin is also important for this process

(90) but can apparently be substituted for by the other ferroxidase, ceruloplasmin, at least under some stress conditions, such as acute bleeding (8).

Intestinal iron absorption can be modulated by the size of the body iron stores, by erythropoietic activity, and by recent dietary iron intake. The regulation of iron absorption involves complex transcriptional, posttranscriptional, and posttranslational mechanisms. Although SlC46A1, DCYTB, and hephaestin are not directly regulated by IRPs, other central iron management proteins in the intestine, including the ferritin H- and L-chains, TFR1, and the two essential iron transporters DMT1 and FPN1, are encoded by IRE-containing mRNAs. Both IRP1 and IRP2 are expressed in villi and in the crypts of Lieberkühn (76), and the IRE/IRP system is essential to maintain the structural and functional integrity of the intestine (23). In villus enterocytes able to take up dietary iron, IRP activity is responsive to changes in luminal iron levels; by contrast, precursor cells in the crypts cannot take up dietary iron but acquire it from the blood via TFR1 (76). TFR1-mediated iron uptake has been proposed to "inform" intestinal precursor cells about the body iron status and, via regulation of IRP activity, to program their iron absorption capacity while maturing to absorptive villus cells (76). This "crypt programming" hypothesis could in principle account for the negative correlation between dietary iron absorption and transferrin saturation. Moreover, impaired duodenal uptake of transferrin-bound iron in hemochromatosis (HFE)-deficient mice was initially seen as a possible molecular mechanism underlying HH, explained by the inability of nascent enterocytes to sense body iron overload and be appropriately programmed to reduce iron absorption (86). However, intestinal HFE has subsequently been shown to be dispensable for normal iron homeostasis (89), and the lag observed between a stimulus to alter iron absorption and the actual change in absorption more likely reflects the time required for adjusting hepcidin expression (18). Hence, IRP-dependent programming of crypt cells, if true, does not appear to play a primary role in the control of dietary iron absorption.

The regulation of DMT1 and FPN1 expression by, e.g., iron deprivation, increased erythropoiesis, or pregnancy (17) appears to involve distinct mechanisms that operate in mature enterocytes. Basolaterally expressed FPN1 responds directly to the humoral regulator hepcidin that triggers FPN1 internalization and degradation (61), although this mechanism has not yet been formally proven in vivo. Although it has been proposed that hepcidin could target apical rather than basolateral iron transport (43, 58, 98), DMT1 appears to be regulated locally by the amount of iron within enterocytes. Indeed, Sla mice with iron loading in enterocytes fail to increase their intestinal DMT1 expression in spite of systemic iron deficiency and severe anemia (6). Although no change in duodenal DMT1 expression has been reported in mice lacking either IRP1 (59) or IRP2 (22), likely due to the ability of the two IRPs to substitute for each other, simultaneous ablation of both IRPs in the intestinal mucosa elicits a strong and selective downregulation of the IRE-containing isoforms of DMT1 mRNA (23). This result suggests that the IRPs positively regulate DMT1 expression, possibly involving a mechanism similar to the stabilization of TfR1 mRNA. The precise molecular mechanisms by which the IRPs control DMT1 synthesis, however, remain to be determined. In cultured cells, iron regulation of DMT1 expression was attributed to transcriptional control (101), and direct positive evidence for the modulation of mRNA turnover has not been obtained (27, 83). It is possible that the mechanisms by which the IRPs regulate the fate of mRNAs bearing single (DMT1, human cell division cycle 14A) versus multiple (TfR1) IRE motifs in their 3′UTRs differ from each other (73).

The positive effect of the IRPs on DMT1 expression has been proposed to serve the coordination of apical iron uptake with the humoral (hepcidin-mediated) regulation of basolateral iron export (17). According to this model, DMT1 regulation is responsive to the

hepcidin-mediated control of iron efflux from enterocytes: The negative control of FPN1 by hepcidin would increase intracellular iron, reduce IRP activity, and decrease DMT1 expression. If correct, this model could explain why intestinal DMT1 is inappropriately high in HFE-associated HH (16, 100) that is characterized by low hepatic hepcidin expression (24). Consistent with this model, absorptive enterocytes are relatively iron-deficient in HH (56), and intestinal IRP activity has been reported to be elevated in HH patients (67, 71) and HFE-knockout mice (50). Because DMT1 is involved in iron loading of HFE-knockout mice (47), it would be interesting to test whether the positive regulation of DMT1 expression by the IRPs contributes to the HH phenotype, although no conclusive association between HH and polymorphisms in the *Aco1* and *Ireb2* genes have been identified so far (45).

Hepcidin-mediated control of FPN1 levels is viewed to represent the major regulatory pathway that controls cellular iron efflux and that is defective in all forms of HH (66). Notably, the FPN1 mRNA bears a functional IRE motif in its 5′UTR, which mediates IRP-dependent translational control in cultured cells (52). However, intestinal FPN1 expression appears not to be modulated in response to luminal iron loading, in spite of reduced IRP activity and the expected ferritin upregulation (20). Does this mean that intestinal FPN1 mRNA escapes the translational regulation by the IRPs in vivo? The simultaneous ablation of both IRPs in the mouse intestine causes a marked increase in FPN1 expression, although the hepatic expression of hepcidin mRNA is even increased (23). This result shows that the IRPs are as critical as hepcidin to secure physiological FPN1 expression in the intestine. Knockin mouse models expressing FPN1 mutants that lack a functional IRE or that escape regulation by hepcidin may help to determine the contribution of the two regulatory pathways to the control of iron efflux from enterocytes.

Both ferritin H- and L-chains are expressed in the gut, and iron sequestration into mucosal ferritin could in principle affect intestinal iron absorption. Both ferritin subunits are posttranscriptionally upregulated in IRP deficiency (22, 23, 44) or following IRP inhibition by intraluminal iron loading (20). However, the ability of intestinal ferritin to alter iron absorption remains controversial (22, 76); transgenic overexpression of ferritin H-subunits in the intestinal mucosa could help to define the role of ferritin in this tissue.

Iron Recycling

The major source of serum iron does not come from absorption but rather from internal recycling: the recovery of iron from damaged or senescent erythrocytes. Recycling takes place in the reticulo-endothelial system (RES), consisting of specialized macrophages present mainly in the spleen, the liver (Kupffer cells), and the bone marrow. Macrophages of the RES phagocytose and lyse effete erythrocytes. Heme is then catabolized by heme oxygenase 1 (69) to liberate inorganic iron. Although quantitatively the most prominent, erythrophagocytosis is not the only way by which macrophages acquire iron. CD163 is present on the surface of monocytes and macrophages and scavenges hemoglobin, which is derived from intravascular hemolysis, by endocytosis of the haptoglobin-hemoglobin complexes (42). Macrophages also express LDL receptor–related protein/CD91, the receptor for the heme-hemopexin complex (35), and they can acquire iron from transferrin via TfR1. The physiological importance of these different iron acquisition routes remains to be fully defined. The intracellular trafficking of erythrophagocytosis-derived iron involves Nramp1 and DMT1, but the mechanisms remain controversial. Iron that is not used for metabolic purposes can be stored in ferritin. The RES represents a major iron storage compartment, and the ferritin H- and L-chain mRNAs are among the most abundant mRNAs in monocyte-derived macrophages (37). Although the extent of erythrophagocytosis and the sequestration of iron within ferritin can

influence iron recycling by the RES, iron release appears to be a limiting step that positively correlates with the bone marrow requirements (37). Cells of the RES can also release iron in the form of hemoglobin, heme, or ferritin (37). However, targeted mutagenesis of the murine *Slc40a1* locus shows that FPN1 is the major conduit of iron release (14); it requires the ferroxidase ceruloplasmin (30).

Iron loading or chelation regulate the IRPs in cultured primary macrophages and cell lines. Both ferritin and FPN1 are upregulated in J774 cells (38) and in bone marrow–derived macrophages (BMDM) (13) following erythrophagocytosis. Although such a response would be compatible with IRP-dependent translational control, the FPN1 upregulation appears to occur at the transcriptional level in these experimental settings (13, 38). Although no overt macrophage abnormality has been reported in IRP1-deficient mice (59), *Ireb2*$^{-/-}$ animals display an unexpected iron deficiency in spleen and bone marrow macrophages, associated with reduced expression of FPN1 and of the ferritin H- and L-chains (22). The underlying mechanism remains to be identified, but these changes are unlikely to represent a direct consequence of IRP deficiency in RES cells: Mice with selective ablation of IRP2 in macrophages in which the *LysosymeM* promoter is active do not display these features, indicating that the phenotype is unlikely to be cell autonomous (D. Ferring, M.W. Hentze, & B. Galy, in preparation); in addition, BMDM lacking both IRP1 and IRP2 upregulate ferritin and FPN1 as predicted by an orthodox translational inhibition mechanism (B. Galy, D. Ferring, & M.W. Hentze, unpublished observations).

The consequences of abnormalities of iron recycling are best illustrated by two pathological conditions with opposite clinical outcomes. Patients with chronic inflammatory or autoimmune disorders frequently present with hypoferremia and develop the so-called anemia of chronic disease (ACD). ACD can be caused by iron retention, notably in the RES. Ferritin induction by inflammatory stimuli was long thought to impair iron recycling by diverting it into storage (40), which could be explained, at least in part, by reduced IRP activity in response to inflammatory signals (36, 72). In contrast to ACD, HH is characterized by relatively low macrophage iron levels until late in the disease. This condition is accompanied by high macrophage IRP activity (3, 71, 72). It has been suggested to be due to impaired TfR1-mediated iron uptake (60).

It now appears that the primary iron-recycling defect in ACD and HH affects the export of iron from the RES. In ACD, hepatic hepcidin production is increased, resulting in inhibition of FPN1-mediated iron efflux. Conversely, the inappropriately low hepcidin levels in HH patients and HFE-deficient mice fail to limit FPN1-mediated iron export in spite of systemic iron loading (24). This defect can be corrected by transgenic overexpression of hepcidin (62, 88). This result does not exclude, however, that IRP feedback on FPN1 synthesis may contribute to the control of iron efflux, although synthetic hepcidin fully overrides the FPN1 upregulation produced by iron loading of BMDM (12). As discussed above, genetic models that separate the IRP- from the hepcidin-dependent control of FPN1 expression may help to determine the actual contributions of the two regulatory pathways to controlling iron efflux from the RES.

THE IRE/IRP REGULATORY NETWORK IN IRON STORAGE AND RELEASE

In addition to iron recycling and absorption, the storage and potential release of (excess) iron are critical determinants of circulating iron levels. Under physiological conditions, approximately 20% (0.5 to 1 g) of the body's total iron content is stocked in the storage compartment, and only 1 to 2 mg of iron are lost each day. The iron stores become depleted when iron absorption does not meet the body's needs or in cases of excessive iron loss (e.g., bleeding, pregnancy); conversely, tissue iron overload occurs when intestinal iron absorption surpasses iron utilization and loss.

Iron Storage

Iron is mainly stored in macrophages of the RES and in hepatocytes. The liver (mostly hepatocytes) accounts for approximately 8% of plasma iron turnover. It has a high iron-storage capacity, and liver damage ensues normally only when hepatic iron levels are grossly (about tenfold) elevated (24). Iron accumulates mostly in the periportal regions with a decreasing gradient toward the centrilobular areas.

The liver is equipped with a complex array of molecules that affect iron metabolism. It expresses both IRP1 and IRP2 and most of the known IRP target genes (**Figure 2**). Both IRPs respond to dietary iron manipulation (7), although IRP2 seems to respond more strongly than IRP1, at least in the mouse (59). The liver is also the site of transferrin and ceruloplasmin synthesis and release; importantly, it expresses the iron hormone hepcidin and molecules that sense environmental cues (HFE, TfR2, hemojuvelin, etc.) to adjust its synthesis. The role of the IRE/IRP system in the regulation of hepcidin synthesis is discussed below.

Hepatocytes acquire iron by different means (**Figure 4**). Two receptors, TfR1 and TfR2, can bind diferric transferrin. TfR1 is encoded by an mRNA bearing five IRE motifs in its 3′UTR and is positively regulated by the IRPs. Although essential in erythroid cells, TfR1 is dispensable for fetal liver iron accumulation, as shown by the hepatic iron overload of *TfRC*$^{-/-}$ mice (46). Unlike TfR1, the TfR2 mRNA is not a direct target of the IRPs. Its affinity for diferric transferrin is 25–30 times lower than that of TfR1, and its direct contribution to liver iron uptake of transferrin-bound iron is likely to be minor. TfR2 appears to function mostly as a sensor of the saturation of transferrin with iron to adjust hepcidin expression (see below). Although TfR1-mediated iron uptake is important under normal conditions, hepatic iron overload in patients or in mice with atransferrinemia shows that liver cells can acquire iron independently of the transferrin cycle (1). Non-transferrin-bound iron (NTBI) builds up when the serum iron levels exceed the iron-binding capacity of transferrin (e.g., in atransferrinemia, patients with HH or transfusional iron overload). The liver is the major site of NTBI clearance, but the underlying mechanisms are still incompletely understood. DMT1 has been proposed to transport NTBI into hepatocytes (80). However, the iron-transport activity of DMT1 is weak at neutral pH, and targeted mutagenesis of the *Slc11a2* locus in the mouse revealed that DMT1 is dispensable for liver iron accumulation, at least during fetal life (28). Tissue-specific ablation of DMT1 in hepatocytes could help to define the precise role of DMT1 in adult liver iron uptake. ZIP14/SLC39A14, originally described as a zinc transporter, is also an interesting candidate for hepatocellular NTBI uptake (49), but its role in liver iron loading has not yet been ascertained in vivo. The liver can also take up other forms of iron, such as hemoglobin/haptoglobin and heme/hemopexin complexes, ferritin, and lactoferrin (26). Eighty percent of the iron that enters the liver is stored in ferritin and can eventually be mobilized; in conditions of iron overload, hepatocellular iron is also stored in hemosiderin.

In addition to IRP-mediated translational control, hepatic ferritin expression can be regulated at the transcriptional (95) and post-translational (87) levels. Hepatocytes can export iron via FPN1 (14) in conjunction with the ferroxidase ceruloplasmin (30). The IRE/IRP system can potentially influence hepatocellular iron fluxes by modulating the expression of at least TfR1, ferritin, and FPN1 (**Figure 2**). ABCB7-deficient mice, a model of human X-linked sideroblastic anemia, display liver iron overload with inappropriately high TfR1 expression (68). ABCB7 is a mitochondrial protein required for Fe-S cluster biogenesis. In the liver, IRP1 usually prevails in its aconitase form, but the impaired Fe-S cluster causes massive IRP1 activation in ABCB7-deficient mice. The high TfR1 levels may well result from the stabilization of TfR1 mRNA by activated IRP1 (68). Nonetheless, the importance

of IRP1 and TfR1-mediated iron uptake for liver iron loading in ABCB7-deficient mice has not yet been assessed directly.

IRP2-deficient mice display hepatic TfR1 downregulation (81) and ferritin upregulation associated with iron loading (22); no FPN1 misregulation was reported. Hepatic iron accumulation in *Ireb2*$^{-/-}$ mice could be plausibly explained by partial diversion of hepatocellular iron fluxes toward ferritin, whose quantity is abnormally augmented by the absence of the IRP2 translational repressor (22). As in other organs and cells, the hepatic phenotype of *Ireb2*$^{-/-}$ mice is relatively mild, likely due to functional substitution by IRP1. *Aco1*$^{+/-}$, *Ireb2*$^{-/-}$ animals display a slightly stronger phenotype than *Aco1*$^{+/+}$, *Ireb2*$^{-/-}$ mice (81). The combined ablation of both IRPs in the liver using (conditional) Cre/Lox technology offers an approach to better define the role of the IRE/IRP regulatory network in the control of hepatocellular iron fluxes.

Iron Loss and Excretion

Physiologically, iron is released from the body by sloughing of mucosal cells, desquamation of skin cells, blood loss, and urinary excretion. In principle, the IRPs could influence the quantity of iron that exits the body, e.g., by modulating the amount of metal retained in sloughing mucosal cells.

An interesting question that has hardly been explored so far is the potential role of the IRPs in urinary iron excretion (**Figure 4**). The kidney has been suggested to play a significant role in iron metabolism by filtration and reabsorption of iron (94). In the rat, iron reabsorption occurs in the thick ascending loop of Henle and in cortical collecting ducts (94). An increase of transferrin in the urine of patients suffering from deficient tubular protein reabsorption (63) indicates that Tf is present in the glomerular filtrate. Kidney cells are able to take up transferrin-bound iron via TfR1 and by megalin-dependent, cubilin-mediated endocytosis (41). Unlike TfR1, megalin and cubilin are not IRP targets. The kidney also expresses the IRE-containing variants of DMT1 mRNA (5). Renal DMT1 expression is concentrated in the kidney cortex, which could possibly reflect a role in the reabsorption of luminal iron. Renal DMT1 expression is responsive to dietary iron intake: It is increased in animals receiving a low-iron diet and conversely reduced by an iron-rich regimen (5, 93). In the rat, this is associated with changes in urinary iron excretion and an inverse relationship between renal DMT1 levels and urine iron levels (93). These data are consistent with a role of DMT1 in iron reabsorption. However, treatment of mice suffering from primary or secondary iron overload with nifedipine, an L-type calcium channel blocker that augments DMT1-mediated iron transport, was recently shown to enhance urinary iron excretion and to diminish the iron overload (51). This apparent discrepancy remains to be resolved, and a better understanding of DMT1 function in the kidney is needed to assess whether nifedipine treatment could benefit patients with iron overload.

The ferritin H- and L-chains are also expressed in the kidney. IRP1- or IRP2-deficient mice show increased renal ferritin expression (59, 99), but the importance of IRP-mediated control in renal and systemic iron metabolism is unknown and possibly masked by functional redundancies between the two IRPs. Transgenic overexpression of the ferritin-H chain in the mouse kidney locally alters renal iron metabolism but has no detectable effect on systemic iron availability (96). The FPN1 mRNA is also expressed in the kidney (9), but the distribution of the protein and its importance for renal iron metabolism have not been determined yet. To date, the renal IRE/IRP system remains poorly characterized. Given the potential of pharmacological modulation of DMT1 function for iron excretion (51), it will be of interest to better define how the IRPs control the expression of their target genes in the kidney and how this could affect renal iron excretion.

CROSS TALK BETWEEN THE IRE/IRP REGULATORY NETWORK AND THE HEPCIDIN/FERROPORTIN SYSTEM

Understanding the functional interconnections between hepcidin and the IRE/IRP system represents an important challenge to fully comprehend systemic iron homeostasis.

Role of the IRPs in the Regulation of Hepatic Hepcidin Expression

Hepcidin expression is predominantly regulated at the transcriptional level (48). The "hemochromatosis proteins" HFE, TfR2, and hemojuvelin stimulate hepcidin expression (24). These HH proteins and their connected signaling pathways appear not to be regulated directly by the IRPs. However, HFE, the protein most frequently affected in HH, competes with transferrin for binding to TfR1 (2). According to a current model, HFE dissociates from TfR1 when the transferrin saturation is high. It then binds to TfR2 and activates a signaling pathway to the nucleus that augments hepcidin transcription (25). The expression of TfR1 and TfR2 on the surface of hepatocytes may thus affect the equilibrium of HFE binding to the endocytic (TfR1) versus the signaling (TfR2) receptor. Because TfR1 expression is regulated by the IRPs, it is conceivable that they may indirectly adjust hepcidin expression; however, hepcidin mRNA expression seems to be unaffected in mice with IRP1 or IRP2 deficiency. It will be informative to study the effect of transgenic IRP overexpression in the liver on hepcidin expression.

Additionally, IRPs may fine-tune hepcidin gene transcription in response to iron deficiency and/or hypoxia. As described above, HIF2alpha mRNA translation is controlled by the IRPs (74). Recent work suggests that HIF2alpha may regulate hepcidin transcription during iron deficiency and hypoxia (65). The physiological consequences of such a regulatory circuit remain to be explored.

IRPs and the Response to Hepcidin

The hepcidin regulatory system and the IRE/IRP network converge on the regulation of FPN1. As demonstrated recently, intestinal IRP activity is required to limit duodenal FPN1 expression, directly demonstrating that the IRPs control FPN1 expression in intestinal enterocytes (23). Intestinal FPN1 levels are also thought to be controlled posttranslationally by hepcidin (61). Thus, FPN1 expression at the basolateral surface of enterocytes appears to be determined by at least two regulatory inputs, one reflecting systemic iron requirements (communicated by hepcidin) and the other responding to the amount of iron available within the intestinal enterocyte (mediated by the IRPs).

In principle, similar mechanisms may apply to other cell types in which FPN1 is expressed, like macrophages and hepatocytes. However, the role of the IRPs in regulating FPN1 expression in these cell types requires further investigation by analysis of mouse lines with combined selective IRP1 and IRP2 deficiency.

PERSPECTIVE

During the past 20 years, the IRE/IRP regulatory network emerged as the central system for the control of cellular iron homeostasis. Since the beginning of this century, the understanding of systemic iron homeostasis and its disorders has rapidly progressed, and regulation by the hepcidin/ferroportin system can now explain key features of systemic iron balance. Most recently, the influence of the IRE/IRP network on systemic iron parameters has become apparent. By contrast to the action of hepcidin that acts as a hormone, the IRE/IRP network appears to exert (many of) its effects on "systemic" iron parameters such as organ iron loading or erythropoiesis by "local," cell- or organ-autonomous interventions. The regulation of the iron exporter FPN1 by both IRE/IRP and hepcidin defines a first point of connectivity between the two regulatory systems.

From the viewpoint of the IRE/IRP network, the systems biology of iron metabolism now faces three challenges. First, the complete set of regulatory targets must be identified. Whereas it is conceivable that FPN1 may represent the only physiologically relevant target of hepcidin (an assumption that should presently not be considered an established fact), recent findings show that more than the "traditional" set of IRE-containing mRNAs is regulated by the IRPs. New regulatory targets may unveil additional functions of the IRE/IRP network and further our understanding of systemic iron regulation. Second, IRP-mediated control is largely exerted "locally" in cells and tissues. Very little is known about the role of local iron mismanagement in numerous inflammatory or degenerative disorders. The availability of conditional IRP knockout mouse strains should help to explore this territory. Third, the interconnectivities between IRE/IRP- and hepcidin-mediated controls must be defined beyond FPN1. In this regard, liver iron metabolism in particular may still hide interesting secrets.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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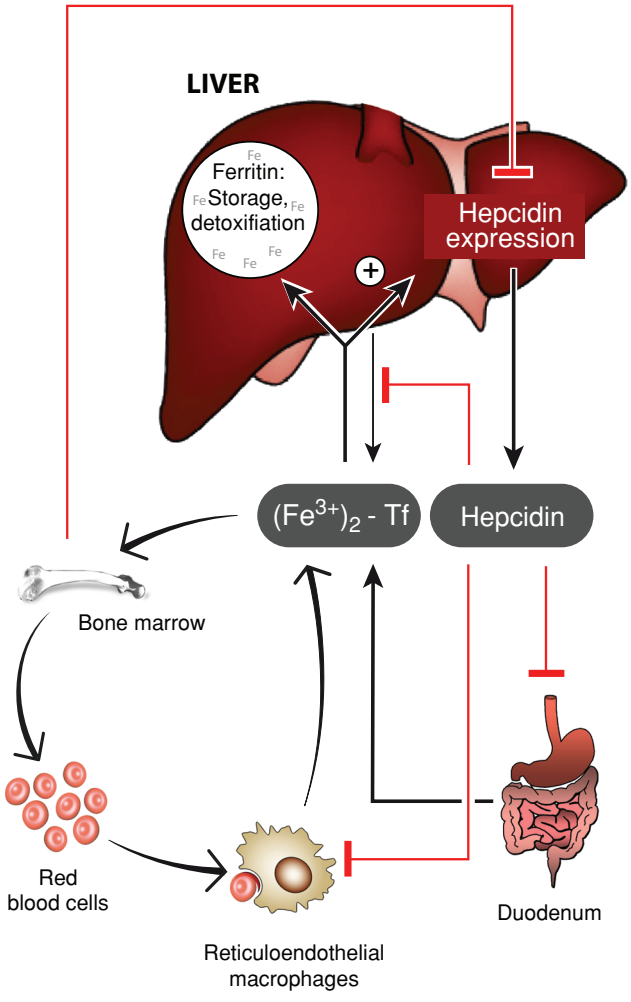

Figure 1

Systemic iron homeostasis. Major body iron fluxes and important regulatory effects are represented. Dietary iron is absorbed by duodenal enterocytes. It circulates bound to transferrin in the plasma and is mainly used for the hemoglobinization of newly synthesized red blood cells. Most body iron is recycled by macrophages that phagocytose effete erythrocytes and degrade their heme moiety to reload iron onto circulating apo-transferrin. Excess iron is stored within ferritin in the liver. Hepcidin controls the transferrin iron saturation by inhibiting iron efflux mainly from duodenal enterocytes and macrophages but also from hepatocytes. Its synthesis is positively regulated by the iron stores and negatively regulated by erythropoietic activity.

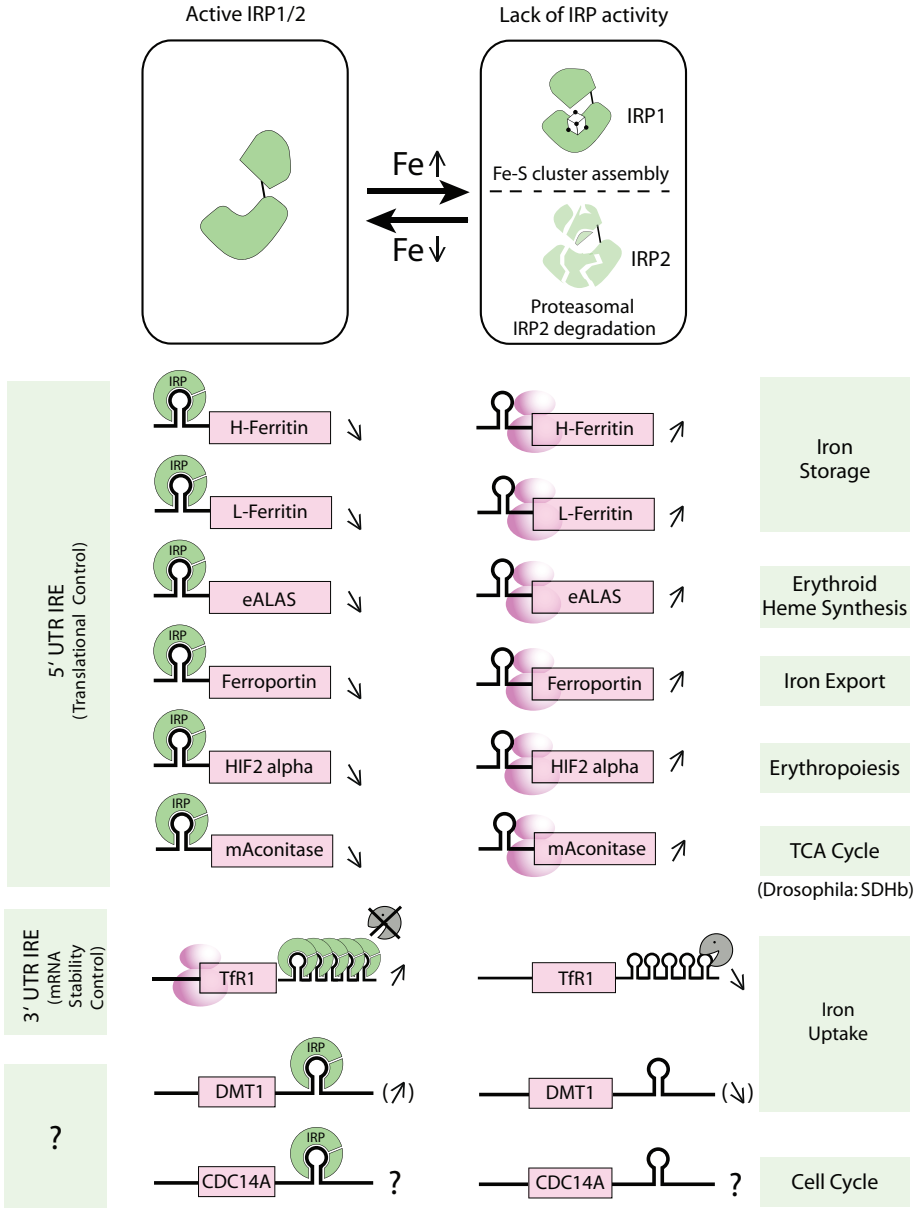

Figure 2

The iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network. IRPs-1 and -2 interact with IREs to coordinate the expression of proteins involved in iron uptake, export, and storage, as well as in erythroid proliferation and hemoglobinization, the tricarboxylic acid (TCA) cycle, and cell cycle control. IRP binding to single IREs located in 5′untranslated regions (UTRs) inhibits translation, whereas IRP binding to the multiple 3′UTR IREs of the transferrin receptor 1 (TfR1) mRNA increases its stability. Cellular iron loading switches IRP1 from its IRE-binding form to an Fe-S cluster containing cytoplasmic aconitase and triggers proteasomal degradation of IRP2. Low iron levels promote accumulation of active IRP1 in its apo form and stabilize IRP2. eALAS, erythroid 5-aminolevulinate synthase; CDC14a, human cell division cycle 14A; DMT1, divalent metal transporter 1; HIF2 alpha, hypoxia inducible-factor 2 alpha.

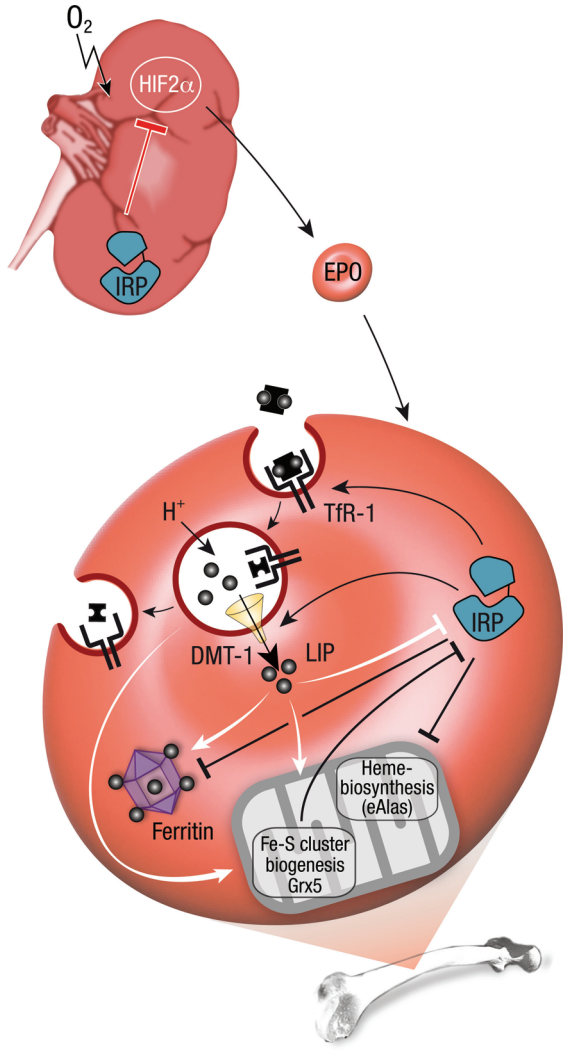

Figure 3

The iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network in erythropoiesis. Erythroid precursors in the bone marrow acquire iron via transferrin receptor 1 (TfR1)-dependent endocytosis of diferric transferrin. Iron is exported out of endosomes via DMT-1 to contribute to the cytoplasmic labile iron pool (LIP). Alternatively, iron may also be delivered to the mitochondria by direct contact with the endosomes. Iron that is not stored in ferritin within the cytoplasm is available for Fe-S cluster biogenesis and heme synthesis in the mitochondria. In addition to TfR1, divalent metal transporter 1 (DMT1), and ferritin, IRPs control the expression of erythroid 5-aminolevulinate synthase (eALAS), the first and rate-limiting enzyme in the heme biosynthetic pathway. IRP activity is modulated by the LIP (IRP1 + IRP2) and by Fe-S cluster biogenesis (IRP1). In the kidney, IRPs control the synthesis of hypoxia-inducible factor 2 alpha (HIF2α), the transcription factor that regulates erythropoietin (EPO) production. EPO then stimulates erythropoiesis. IRPs are thus implicated in the coordination of erythropoiesis and iron availability at two levels: by HIF2α-dependent modulation of EPO levels in the kidney and by controlling iron acquisition and heme synthesis in erythroid cells. Grx5, glutaredoxin 5.

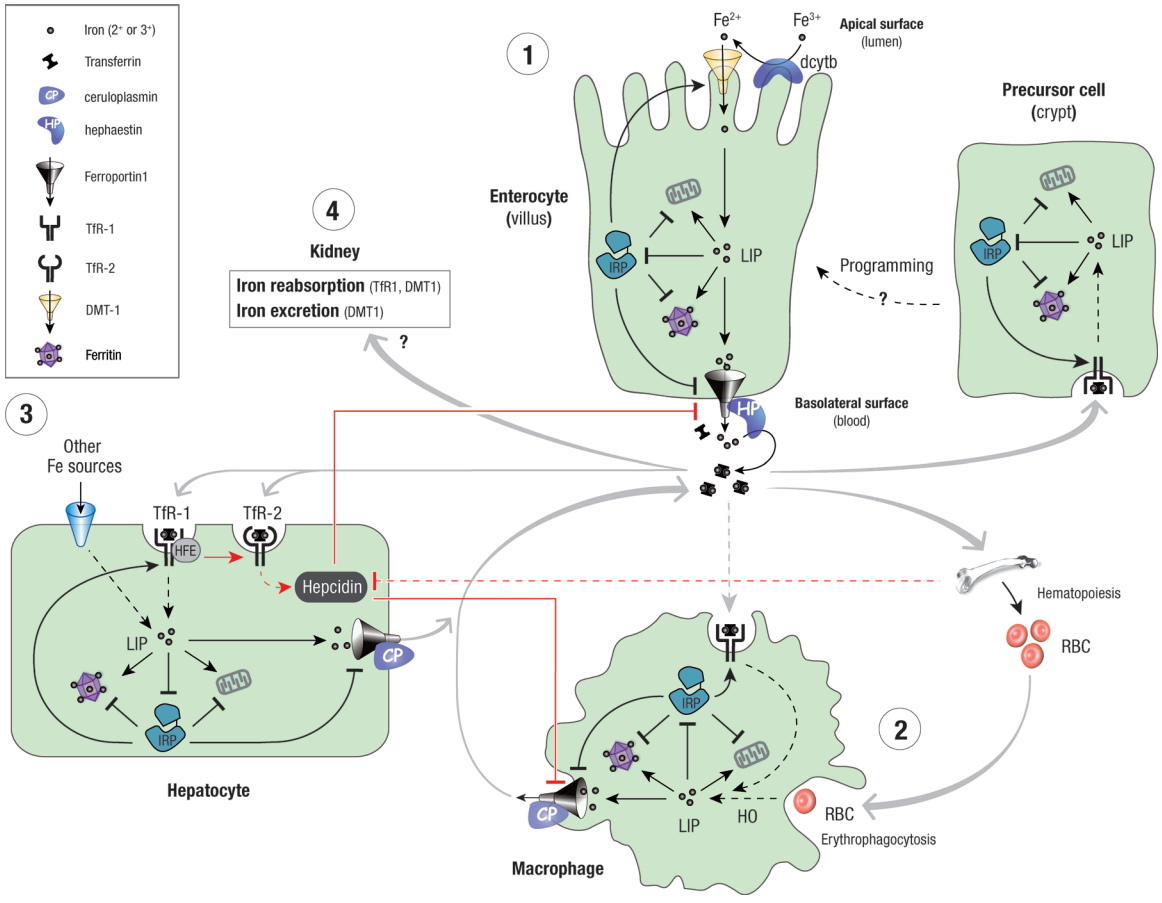

Figure 4

Control of body iron fluxes by the iron-responsive element/iron-regulatory protein (IRE/IRP) system. IRPs influence the transferrin iron saturation in the plasma by controlling the expression of specific iron management proteins that are critical for the absorption of dietary iron by duodenal enterocytes (*1*), iron recycling by tissue macrophages (*2*), iron storage in hepatocytes (*3*), and potentially iron excretion by the kidney (*4*). Within each tissue, IRP activity is modulated by the size of the labile iron pool (LIP) that is determined by the extent of iron uptake, utilization, storage in ferritin, and export. In enterocyte precursors that take up circulating transferrin-bound iron via transferrin receptor (TfR) 1, the size of the LIP may reflect the body iron status and modulate IRP activity accordingly; this has been proposed to "program" the dietary iron absorption machinery of daughter cells in an IRP-dependent manner. *Red arrows* refer to the hepcidin regulatory system. Hepcidin acts systemically by triggering ferroportin degradation in target tissues, while IRPs act locally by inhibiting ferroportin translation. Both regulatory systems are important to secure physiological ferroportin levels. Cellular iron utilization pathways are symbolized by a mitochondrium. CP, carrier protein; DMT, divalent metal transporter; dcytb, duodenal cytochrome b; HFE, hemochromatosis; HO, heme oxygenase; RBC, red blood cell.

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Errata

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