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Mechanisms of Mammalian Iron Homeostasis

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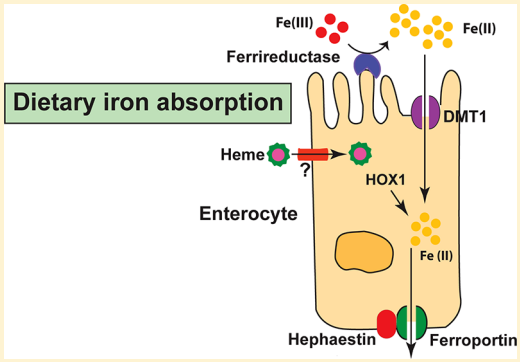
ABSTRACT: Iron is vital for almost all organisms because of its ability to donate and accept electrons with relative ease. It serves as a cofactor for many proteins and enzymes necessary for oxygen and energy metabolism, as well as for several other essential processes. Mammalian cells utilize multiple mechanisms to acquire iron. Disruption of iron homeostasis is associated with various human diseases: iron deficiency resulting from defects in the acquisition or distribution of the metal causes anemia, whereas iron surfeit resulting from excessive iron absorption or defective utilization causes abnormal tissue iron deposition, leading to oxidative damage. Mammals utilize distinct mechanisms to regulate iron homeostasis at the systemic and cellular levels. These involve the hormone hepcidin and iron regulatory proteins, which collectively ensure iron balance. This review outlines recent advances in iron regulatory pathways as well as in mechanisms underlying intracellular iron trafficking, an important but less studied area of mammalian iron homeostasis.



IMPORTANCE OF IRON IN MAMMALIAN PHYSIOLOGY

Iron is a *d*-block transition metal, and like many such metals, it can assume several oxidation states. The most common species are the divalent ferrous (Fe^{2+}) and the trivalent ferric (Fe^{3+}) iron. The redox potential of iron can be greatly modulated by the nature of attached ligands. This has significant physiological ramifications because other oxidation states, such as ferryl (Fe^{4+}), can be transiently generated as key intermediates during metal-mediated oxidative transformations. Other transition elements such as copper and manganese are likewise capable of participating in biological redox reactions.[1] Nevertheless, during evolution, organisms likely selected iron for the following reasons: (1) Iron is the second most abundant metal on earth's crust, falling closely behind aluminum. (2) Iron can exist in multiple oxidation states, which is essential for electron transfer, and for binding to biological ligands. (3) Iron's redox potential ranges from 1000 to −550 mV, depending on the ligand environment, whereas the range for other transition elements is narrower. (4) By exploiting the oxidation states, electron spin state, and redox potential, biological systems can adjust the chemical reactivity of iron to suit physiological needs.[1,2]

The indispensability of iron for living organisms is exemplified by the fact that it serves as a cofactor for several hemoproteins and non-heme iron-containing proteins, including many enzymes. Hemoproteins are involved in numerous biological functions such as oxygen binding and transport (hemoglobins), oxygen metabolism (catalases and peroxidases), and cellular respiration and electron transport (cytochromes). Proteins containing non-heme iron are important for fundamental cellular processes such as DNA synthesis, cell proliferation and differentiation (ribonucleotide reductase), gene regulation, drug metabolism, and steroid synthesis. Early in evolution, before oxygen emerged as an abundant constituent of the atmosphere, anaerobic cells acquired soluble ferrous iron with relative ease.[3] Later, the accumulation of oxygen oxidized ferrous to ferric iron, which is virtually insoluble at physiological pH. Moreover, the redox cycling of ferrous and ferric iron in the presence of H_2O_2 and $O_2^{\bullet-}$, which are physiologically produced during aerobic respiration and enzymatic reactions, yields hydroxyl radicals (Fenton chemistry). These in turn readily attack and damage cellular macromolecules. Thus, despite its abundance, the acquisition and transport of iron pose a challenge for cells and organisms because of its low solubility and high toxicity. To overcome these problems, unicellular organisms such as bacteria synthesize "siderophores", which are low-molecular weight, iron-specific chelating agents that capture extracellular iron and transfer it into the cell.[4,5] In contrast, mammalian cells acquire iron from extracellular carrier proteins.[6,7]

SYSTEMIC IRON METABOLISM

Distribution of Iron in the Body. The human body contains ∼3−5 g of iron.[6] Most of it is present as heme in hemoglobin of erythroid cells (>2 g) or myoglobin of muscles (∼300 mg). Macrophages in the spleen, liver, and bone marrow maintain a transient fraction of iron (∼600 mg), while excess iron is stored in the liver parenchyma within ferritin (∼1000 mg). All other cellular iron-containing proteins and

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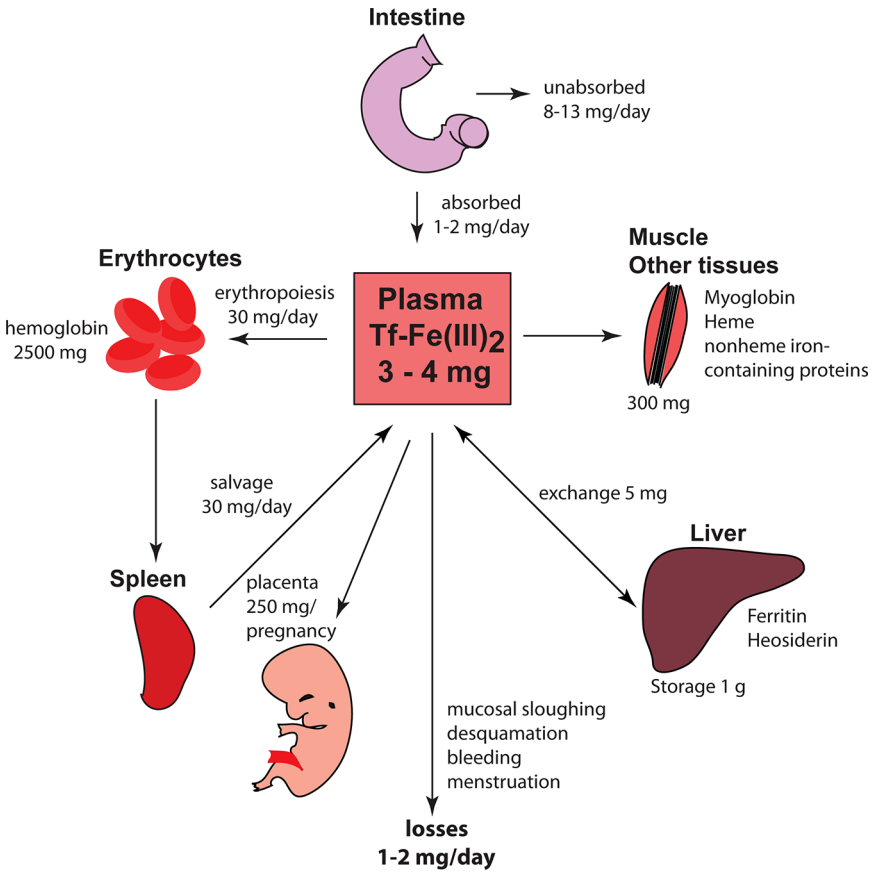

Figure 1. Iron absorption, distribution, and recycling in the body and quantitative exchange of iron between body iron sources. Body iron levels are maintained by daily absorption of ~1−2 mg of dietary iron to account for obligatory losses of a similar amount of iron through sloughing of mucosal and skin cells, hemorrhage, and other losses. Approximately 4 mg of iron is found in circulation bound to Tf, which accounts for 0.1% of the total body iron. A majority of the body iron is found in the erythroid compartment of bone marrow and in mature erythrocytes contained within the heme moiety of hemoglobin. Splenic reticuloendothelial macrophages, which recycle iron from senescent red blood cells, provide iron for the synthesis of new red blood cells. Tf delivers iron to developing erythroid precursors, as well as to other sites of iron utilization. Liver hepatocytes store iron in ferritin shells. During pregnancy, 250 mg of iron is transported across the placenta to the fetus. The distribution of iron in the body is altered in iron deficiency and iron overload (see the text).

enzymes are estimated to bind a total of ~8 mg of iron (ref 7 and Figure 1).

Iron is delivered to erythroblasts and to most tissues via circulating transferrin (Tf), which carries ~3 mg of the metal at steady state. Considering that Tf-bound iron turns over >10 times a day, it represents the most dynamic body iron pool. Plasma iron is predominantly replenished by reticuloendothelial macrophages, and to a small extent (~1−2 mg/day) by absorption from the diet, mediated by duodenal enterocytes. The macrophages acquire iron primarily via erythrophagocytosis and the enterocytes by internalization of heme or inorganic iron from the intestinal lumen.[8]

The absorption of inorganic iron involves reduction of Fe^{3+} to Fe^{2+} by ascorbate and/or membrane-associated ferrireductases such as duodenal cytochrome *b* (DcytB[9]), coupled to transport of Fe^{2+} across the apical membrane by divalent metal transporter 1 (DMT1/SLC11A2), solute carrier family 11 member 2, also known as natural resistance-associated macrophage protein 2 (NRAMP2), or divalent cation transporter (DCT1).[6,7] The mechanism of heme internalization remains poorly defined and may involve either direct transport of heme or receptor-mediated endocytosis.[10] Macrophages and enterocytes catabolize heme in a reaction catalyzed by heme oxygenase-1 and -2 (HO-1 and HO-2, respectively), which liberate inorganic iron.[11]

Both cell types export Fe^{2+} to plasma via the transmembrane transporter, ferroportin/SLC40A1,[6,7] in a process coupled to the reoxidation of Fe^{2+} to Fe^{3+}. This is mediated by the circulating ferroxidase ceruloplasmin or its homologue, hephaestin, which is expressed on the basolateral membrane of duodenal enterocytes and physically interacts with ferroportin.[6,7]

Exported iron is scavenged by plasma Tf, which maintains it in a redox inert state and delivers it to tissues. The loading of apo-Tf with iron may be facilitated by gastrins. These peptide hormones stimulate the secretion of gastric acid and have also been proposed to act as transient Fe^{3+} chaperones.[12] Tf contains two ferric binding sites and is only partially (30%) saturated with iron under physiological conditions. The concentration of differic Tf in plasma is ~5 μmol/L, corresponding to approximately one-tenth of the total circulating Tf.[13] The high abundance of unsaturated apo-Tf allows an efficient buffering of increased plasma iron levels and prevents the buildup of non-transferrin-bound iron (NTBI), which is taken up by tissue parenchymal cells and promotes oxidative injury.[14] NTBI is generated in iron overload states, such as in hereditary hemochromatosis, where transferrin gradually becomes fully saturated with iron and loses its buffering capacity.

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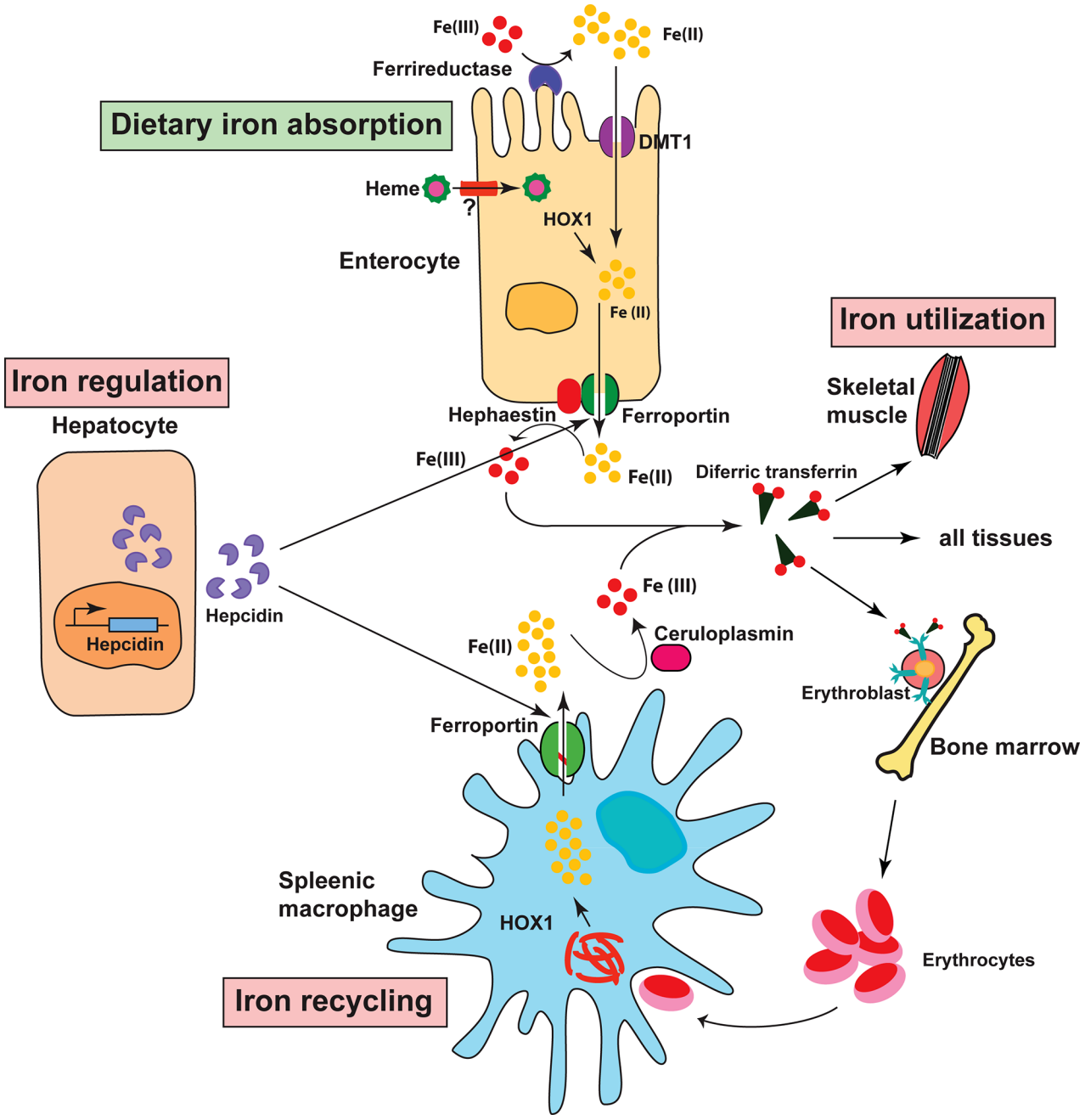

Figure 2. Regulation of systemic iron metabolism. Organs and cell types involved in systemic iron balance are shown. Duodenal enterocytes absorb dietary iron via DMT1 located on the apical surface upon reduction of Fe^{3+} to Fe^{2+} by DcytB. Spleenic reticuloendothelial macrophages recycle iron from senescent red blood cells. Both cell types release iron via ferroportin with the aid of hephaestin, which oxidizes Fe^{2+} to Fe^{3+}. Iron is also oxidized by ceruloplasmin in the circulation. Plasma Tf captures and circulates iron in the body. Hepatic hormone hepcidin regulates the efflux of iron from these cells by regulating the stability of ferroportin. The synthesis and secretion of hepcidin by hepatocytes are influenced by iron levels in the body as well as conditions that affect iron metabolism indirectly such as inflammation, ER stress, erythropoiesis, and hypoxia (see the text for additional details).

Regulation of Body Iron Metabolism. *Hormonal Regulation by Hepcidin.* The trafficking of iron into plasma is largely controlled by the iron regulatory hormone hepcidin, which was first purified from plasma[15] and urine[16] on the basis of its antimicrobial activity. This liver-derived peptide binds to ferroportin on the plasma membrane of enterocytes, macrophages, hepatocytes, and other cells, promoting its Jak-dependent phosphorylation and internalization that leads to its lysosomal degradation.[17,18] Ferroportin is the only exporter of inorganic iron in mammalian cells. Therefore, inactivation of ferroportin causes intracellular iron retention.[19,20] Hepcidin has also been shown to promote proteasomal degradation of DMT1.[19] Pathological overexpression of hepcidin under prolonged inflammatory conditions is associated with the anemia of chronic disease, which is characterized by diversion of iron to storage sites, hypoferremia and reduced iron availability for erythropoiesis (ref 20 and Figure 2). Even more severe iron redistribution occurs in patients with hereditary iron-refractory iron deficiency anemia (IRIDA), a disease caused by genetic disruption of the *TMPRSS6* gene encoding the

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transmembrane serine protease matriptase-2 that negatively regulates hepcidin expression.[21]

Conversely, hepcidin deficiency, or in rare cases ferroportin resistance to hepcidin, is a hallmark of hemochromatosis, which is associated with uncontrolled dietary iron absorption and progressive tissue iron overload.[22,23] Patients with uncommon nonsense mutations in the *HAMP*[24] or *high iron gene 2* (*HFE2*)[25] genes, encoding hepcidin or its upstream activator *hemojuvelin* (*HJV*), respectively, develop early onset juvenile hemochromatosis (JH). The most frequent type of hemochromatosis is associated with mutations in the *HFE* gene,[26] which leads to milder hepcidin insufficiency.[27] Other types of hemochromatosis are caused by inactivation of the *TFR2* gene encoding transferrin receptor 2 (TfR2)[28] or by specific mutations in the *SLC40A1* gene encoding ferroportin that prevent the binding of hepcidin.[29,30] The former is associated with inappropriately low levels of hepcidin expression and the latter with hepcidin resistance.[29,30]

Hepcidin is generated in hepatocytes and other cell types, as a precursor propeptide of 84 amino acids.[31] Following cleavage by the prohormone convertase furin,[32] a biologically active mature 25-mer is secreted to plasma where it is thought to interact with α2-macroglobulin.[33] The expression of hepcidin is regulated at the transcriptional level. C/EBPα (CCAAT enhancer-binding protein α) binds to a CCAAT motif within the *HAMP* promoter and maintains basal transcriptional activity.[34] Alk3, a type I bone morphogenetic protein (BMP) receptor, also appears to be critical for basal hepcidin expression.[35] Hepcidin transcription is induced by iron, inflammation, or ER stress and is inhibited by iron deficiency, increased erythropoietic drive, or hypoxia (reviewed in ref 22).

The responsiveness of the expression of hepcidin to iron was established during its initial discovery as an iron-induced peptide.[22] Subsequently, the oral administration of iron was found to increase the level of hepcidin mRNA in humans and mice.[22] Nevertheless, hemochromatosis patients exhibit impaired hepcidin responses to iron ingestion,[36,37] associated with low levels of hepcidin expression, which are disproportional to their iron load.[27,38] Likewise, mouse models of hemochromatosis demonstrate hepcidin insufficiency.[39−41] There is evidence that hepatic iron and circulating iron induce hepcidin expression by distinct pathways.[42,43]

Regulation of Hepcidin by Hepatic Iron. An increase in hepatic iron levels activates hepcidin transcription via the BMP signaling pathway.[44] The binding of BMP6 and other BMP ligands to type I (Alk2 and Alk3) and type II BMP receptors promotes the phosphorylation of SMAD1, SMAD5, and SMAD8 proteins.[35] The phosphorylated SMAD1−SMAD5−SMAD8 complex recruits SMAD4 and translocates to the nucleus, where it stimulates hepcidin transcription upon binding to BMP responsive elements (BMP-RE1 and BMP-RE2) at proximal and distal sites of the *HAMP* promoter.[45] The liver-specific disruption of Alk2, Alk3,[35] or SMAD4[46] in mice inhibits iron-induced hepcidin expression and leads to iron overload.[22] Induction of inhibitory SMAD7, another target of BMP signaling, antagonizes the interaction of the phosphorylated SMAD1−SMAD5−SMAD8 with SMAD4 and reduces the level of transcription of hepcidin in a feedback regulatory loop.[47]

The expression of *BMP6* is induced by hepatic iron, and its mRNA levels reflect the hepatic iron load.[42] Liver-specific *Bmp6*$^{-/-}$ mice develop iron overload and fail to upregulate hepcidin.[48,49] Other endogenous BMPs do not appear to be capable of rescuing this phenotype, despite the capacity of BMP2, -5, -7, and -9 to activate hepcidin expression in cell culture experiments.[44,46,50] Mice treated with recombinant BMP6 manifest increased levels of hepcidin expression and hypoferremia.[49] Conversely, a neutralizing BMP6 antibody[49] or the BMP inhibitor dorsomorphin[51] can reduce the level of hepcidin expression and trigger an increase in serum iron levels. These data highlight a function of BMP6 as the principal upstream regulator of hepcidin.

Hjv is a BMP coreceptor that plays an essential role in iron-induced activation of hepcidin by forming complexes with type I and II BMP receptors, which enhances downstream signaling.[44] It is primarily expressed in hepatocytes and skeletal muscle cells and associates with the plasma membrane via a C-terminal glycosylphosphatidylinositol (GPI) anchor. Soluble forms of Hjv have been detected in plasma,[52] while recombinant soluble Hjv competes with BMP signaling to hepcidin in vitro and in vivo.[50,52] Nevertheless, plasma Hjv seems unlikely to be of physiological importance for hepcidin regulation. Thus, mice with liver-specific disruption of *Hjv* develop defects in hepcidin expression and iron overload quantitatively similar to those of full *Hjv*$^{-/-}$ counterparts, while muscle-specific *Hjv*$^{-/-}$ animals exhibit normal iron homeostasis[53,54] (also see Table 1). These findings underline the role of hepatic Hjv as a major regulator of hepcidin.

Biochemical data suggest that matriptase-2 negatively regulates hepcidin by proteolytic inactivation of Hjv.[55] Nevertheless, *Tmprss6*$^{-/-}$ mice, a model of IRIDA with high levels of hepcidin expression[56,57] (Table 1), were reported to have lower hepatic Hjv content than wild-type animals.[58] Matriptase-2 expression is induced by iron and BMP6,[59] consistent with this protein functioning as a negative feedback regulator of hepcidin.

Regulation of Hepcidin by Plasma Iron. An increase in plasma iron levels activates the expression of hepcidin by an incompletely characterized pathway that likely involves hepatic HFE and TfR2. Evidence that these proteins are crucial for systemic iron homeostasis is provided by the iron overload that develops as a result of hepcidin suppression in mice with liver-specific disruption of either *Hfe*[60] or *Tfr2*.[61] The role of iron-loaded Tf in this pathway is illustrated by experiments in which injection of holo-Tf into mice caused a specific increase in the level of *hepcidin* mRNA within hours.[42] Moreover, acute administration of iron to mice enhanced Tf saturation and activated hepcidin expression without altering hepatic BMP6 levels.[43]

Hemochromatosis patients carrying *HFE* or *TFR2* mutations exhibit relatively higher levels of basal hepcidin expression than healthy subjects, yet this increase is only modest by comparison to their iron burden and does not prevent iron overload.[37,62] This suggests an at least partial preservation of hepcidin regulation by hepatic iron. Oral iron administration triggers a parallel increase in the levels of Tf saturation and hepcidin in healthy subjects, but a blunted hepcidin response in hemochromatosis patients, uncoupled from the increase in the level of Tf saturation.[37,63] Likewise, *Hfe*$^{-/-}$ and *Tfr2*$^{Y245X/Y245X}$ mice mount an impaired hepcidin response to acute dietary iron challenge.[42]

TfR1, which interacts with HFE,[64] is also involved in the regulation of hepcidin by holo-Tf. This became evident in studies of mice bearing mutations, which either favor or prevent HFE−TfR1 interactions. Animals in which HFE−TfR1 interactions were constitutive developed iron overload and

Table 1. Regulators of Systemic Iron Metabolism in Animal Models and Human Disorders

metabolic process	protein	gene	function	mouse model	phenotype	human disease	refs
iron absorption and recycling	hepcidin (HAMP)	*Hamp*	regulates intestinal iron absorption by controlling ferroportin	$Hamp^{-/-}$	iron overload, no hepcidin	juvenile hemochromatosis (HH type 2B)	24
	transferrin receptor 2 (TfR2)	*Tfr2*	sensor for diferric Tf; regulator of hepcidin	$Tfr2^{-/-}$	increased level of iron absorption; iron overload, decreased level of hepcidin	hemochromatosis (HH type 3)	61
	SMAD4	*Smad4*	regulates hepcidin expression	$Smad4^{-/-}$	iron overload; decreased level eof hepcidin	colorectal and pancreatic cancer predisposition	44, 50
	bone morphogenic protein 6 (BMP6)	*Bmp6*	regulates hepcidin expression	$Bmp6^{-/-}$	iron overload; decreased level of hepcidin	rare cases of hereditary hemochromatosis	44, 50
	mariptase-2 (TMPRSS6)	*Tmprs6*	regulates hemojuvelin	$Tmprss6^{msk/msk}$ $Tmprss6^{-/-}$	alopecia, microcytic anemia, infertility	microcytic anemia; low serum levels of iron; increased levels of hepcidin	21, 56, 57
	ferroportin (SLC40A1; MTP1)	*Slc40a1*	regulates efflux of iron from enterocytes	$Slc40a1^{-/-}$	embryonic lethality; conditional deficiency with iron deficiency	hemochromatosis, macrophage iron overload (HH type 4)	214
	hephaestin (HEPH)	*Heph*	ferroxidase, functions in concert with Fp	$Sla\ Heph^{-/-}$	microcytic anemia, iron deficiency	unknown	213
	ceruloplasmin (CP)	*Cp*	plasma ferroxidase; closely related to Heph	$Cp^{-/-}$	iron overload, anemia, impaired motor coordination	aceruloplasminemia	213
	high iron gene (HFE)	*Hfe*	regulator of HAMP; interacts with TfR1 and TfR2	$HFE^{-/-}$	iron overload, decreased level of hepcidin	HLA-linked hemochromatosis (HH type 1)	7, 26, 61
	hemojuvelin (HJV)	*Hfe2*	bone morphogenic protein coreceptor	$Hjv^{-/-}$	iron overload, decreased level of hepcidin	juvenile hemochromatosis (HH type 2A)	25, 53, 54
	divalent metal transporter 1 (*DMT1 Nramp1*)	*Slc11a2*	transmembrane iron transporter	$mk/mk\ DMT^{-/-}$	hypochromic, microcytic anemia; impaired iron absorption	iron overload in liver; anemia	105, 108, 109

failed to appropriately increase the level of hepcidin expression, similar to $Hfe^{-/-}$ mice.[65] By contrast, animals in which HFE−TfR1 interactions were abolished developed iron deficiency because of hepcidin overexpression, similar to hepatocyte-specific *Hfe* transgenic mice.[65] These findings suggest that the binding of TfR1 compromises HFE signaling to hepcidin, while the dissociation of TfR1 induces it. Earlier biochemical studies showed that TfR1 utilizes the same binding site for its interaction with both HFE and holo-Tf, while TfR2 interacts with HFE and holo-Tf in a noncompetitive manner.[66−68] Moreover, TfR1 and TfR2 compete for binding to HFE.[69] It should also be noted that holo-Tf stabilizes TfR2.[70,71]

It is therefore thought that HFE and TfR2 form a signaling complex that leads to hepcidin activation. When plasma iron levels are low, signaling to hepcidin is blocked by TfR1, which sequesters HFE and prevents its interaction with TfR2. Conversely, when plasma iron levels (and Tf saturation) increase, holo-Tf displaces HFE from TfR1, allowing formation of the HFE−TfR2 signaling complex. A prediction of this model is that inactivation of either HFE or TfR2 would yield a quantitatively similar iron overload phenotype. Nevertheless, the lack of HFE usually causes a milder form of hemochromatosis than the absence of TfR2 in human patients[72] and in mice.[73] Furthermore, compound pathogenic mutations in both HFE and TfR2 have been associated with early onset juvenile hemochromatosis in humans,[98] while mice lacking both *Hfe* and *Tfr2* accumulate more iron than mice lacking individual *Hfe* or *Tfr2* genes.[43,73] Taken together, these data suggest that HFE and TfR2 also exhibit independent functions.

The downstream events that occur following activation of the putative HFE−TfR2 signaling complex are far from being understood. Biochemical experiments have detected a link between HFE−TfR2 signaling and the ERK/MAP kinase pathway. Thus, the treatment of primary murine hepatocytes with holo-Tf activates this pathway and hepcidin expression, which could be blocked by the ERK-specific inhibitor U0-126.[74] Along similar lines, the simultaneous silencing of HFE and TfR2 was associated with reduced levels of ERK1/2 phosphorylation,[75] while low hepatic phospho-ERK1/2 levels were also observed in $Hfe^{-/-}$, $Tfr2^{-/-}$, and double $Hfe^{-/-}$/$Tfr2^{-/-}$ mice and in HFE hemochromatosis patients.[73]

There is also evidence of the involvement of the SMAD signaling pathway in the regulation of hepcidin by the HFE−TfR2 complex. Thus, holo-Tf triggered enhanced phosphorylation of the SMAD1−SMAD5−SMAD8 complex in primary murine hepatocytes.[74] In addition, the level of phosphorylation of the SMAD1−SMAD5−SMAD8 complex was significantly reduced in mice lacking HFE, TfR2, or both, and in HFE-related hemochromatosis patients.[43,73,76] In experiments with wild-type mice, only the SMAD but not the ERK/MAPK pathway was activated following both acute (plasma) and chronic (hepatic) iron loading.[43] Further work is required to clarify the exact role of these pathways in iron-dependent signaling to hepcidin.

Regulation of Hepcidin Expression during Inflammation and ER Stress. *Hepcidin* transcription is potently induced by IL-6 via a mechanism involving STAT3 phosphorylation, translocation to the nucleus, and binding to a STAT-specific site within the *HAMP* promoter.[77−79] This pathway leads to the accumulation of iron in macrophages and hypoferremia, which are considered as innate immune responses during systemic inflammation. However, persistent upregulation of hepcidin by IL-6 greatly contributes to the development of anemia of

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chronic disease.[20] Under inflammatory conditions or during infection, myeloid cells (macrophages and neutrophils) are also stimulated to generate hepcidin via IL-1β, TLR2, or TLR4 signaling.[6,31] ER stress is another cue that leads to transcriptional activation of hepcidin via the CREBH and CHOP pathways, possibly a part of a further protective innate immune strategy.[6,31]

Regulation of Hepcidin Expression during Anemia and Hypoxia. Increased erythropoietic drive during anemia and ineffective erythropoiesis is known to suppress the expression of hepcidin.[6] While erythropoietin (EPO) can reduce the level of expression of hepcidin by attenuating the binding of C/EBPα to the *HAMP* promoter, experiments with mice provided evidence that the inhibitory effects of EPO on hepcidin are indirect and require erythropoietic activity.[80,81] GDF15 (growth differentiation factor 15) and TWSG1 (twisted gastrulation-1) are potential mediators of bone marrow signaling, as they suppress hepcidin in thalassemias and other iron-loading anemias.[82,83] However, a general function of these proteins as "erythroid regulators" of hepcidin has been excluded.[84] Thalassemic patients express low hepcidin levels despite their high iron load, indicating a dominance of the erythroid signal over iron.[85,86] In line with these data, experiments in mice confirmed that erythropoietic drive could inhibit hepcidin induction in response to iron or inflammation.[6]

Hypoxia is also known to suppress the expression of hepcidin.[6] Genetic studies in mice with liver-specific disruption of the *Vhl* gene (von Hippel-Lindau), a negative regulator of HIFα (hypoxia inducible factor α) subunit expression, suggested a key role for HIFs in hepcidin regulation.[87] However, a direct transcriptional activity of HIFs on the HAMP promoter is not supported by biochemical data.[88] It is conceivable that the inhibitory effects of hypoxia on hepcidin expression are primarily triggered by increased erythropoietic drive. Consistent with this view, recent experiments suggest that hepatic HIF2α inhibits hepcidin expression via an EPO-mediated increase in the level of erythropoiesis.[89] Additionally, biochemical studies demonstrated that hypoxia downregulates hepcidin expression by inhibiting SMAD4 signaling.[90]

Hepcidin-Independent Regulation of Body Iron Metabolism. Hepcidin is the master regulator of both dietary iron absorption and systemic iron trafficking and homeostasis. However, these processes are fine-tuned by additional control mechanisms. Thus, the expression of DMT1 and DcytB in duodenal enterocytes is transcriptionally activated during iron deficiency via HIF2α.[91,92] Intestinal H-ferritin is also essential for proper dietary iron absorption, as the targeted ablation of this protein in the mouse intestine is associated with iron overload, despite functional preservation of the hepcidin–ferroportin axis.[93] In addition, *Ferroportin* and *DMT1* are subject to transcriptional[94,95] and post-transcriptional regulation by the IRE–IRP system.[96,97]

CELLULAR IRON METABOLISM

Cells have evolved metabolic strategies for safely importing and utilizing iron. Regulation of iron uptake, storage, intracellular trafficking, and utilization is critical for the maintenance of cellular iron homeostasis (Figure 3).

Cellular Uptake of Iron. Cell membranes and tight junctions between cells impede the free passage of iron. Cells therefore have transporters, chaperones, and chelators, which allow passage of iron across membranes and facilitate intracellular trafficking. With few exceptions, the majority of these transporters belong to the solute carrier (SLC) family of proteins. Most mammalian cells acquire iron from circulating Tf, upon binding to transferrin receptor 1 (TfR1).[6,7] Tf, an 80 kDa glycoprotein, is synthesized and secreted mainly by the liver. Varying amounts are also produced in lymph nodes, thymus, spleen, salivary glands, bone marrow, and testis. Tf binds iron in plasma and extracellular fluids such as lymph and cerebrospinal fluid. It is equally distributed between plasma and extracellular fluids and has a half-life of 8 days. Tf is a homodimeric β-globulin, which binds two molecules of ferric iron with high affinity ($K_d = 10^{-23}$ M). Binding and release are accompanied by a conformational change. Furthermore, the interaction between iron and Tf is pH-dependent: at physiological plasma pH, Tf binds iron very strongly, whereas the interaction is essentially abolished at acidic pH (<5). Under physiological conditions, only a fraction of Tf (~30%) is saturated with iron. Catabolism of Tf takes place mainly in the liver by lysosomal degradation or glomerular filtration followed by reabsorption and degradation in renal tubules.[99] Mice and humans bearing mutations in the *Tf* gene develop severe anemia.[7] Interestingly, nonhematopoietic cells display massive iron overload.[100] These studies highlight the importance of Tf-mediated iron delivery for erythropoiesis and imply the existence of alternative mechanisms for the uptake of cellular iron by nonhematopoietic cells.

TfR1 provides the physiological route for entry of Tf-bound iron into cells. It is expressed by most nucleated mammalian cells, and its levels correlate with cellular iron requirements. TfR1 is a disulfide-linked transmembrane glycoprotein that forms a homodimer with a molecular mass of 180 kDa, where each subunit binds one Tf molecule.[101] The efficiency of the interaction does not depend on temperature or energy but rather on the iron content of Tf, with diferric Tf having the greatest affinity, monoferric Tf intermediate, and apo Tf the lowest. In contrast, endocytosis of Tf–TfR1 complexes is an energy-dependent process that involves endocytosis via clathrin-coated pits. Iron is released from Tf within endocytic vesicles due to acidification through the action of a v-ATPase proton pump. In a cooperative manner, Tf and TfR1 undergo conformational changes in the acidified endosome leading to iron release.[102,103] The released Fe^{3+} is then reduced to Fe^{2+} by STEAP 3 (six-transmembrane epithelial antigen of prostate-3), a member of the metalloreductase family.[104] Fe^{2+} is transported across the endosomal membrane into the cytosol by DMT1/SLC11A2 in most cells or by its homologue Nramp 1 (natural resistance-associated macrophage protein-1) in macrophages.[105−107] In this regard, DMT1 is a dual-function protein, which regulates both systemic and cellular iron homeostasis. *Dmt1* inactivation in mice and humans leads to iron deficiency anemia.[105,108,109] Similarly, spontaneous mutations in *Steap3* or its genetic ablation in mice causes iron deficiency in the erythroblasts but not systemically, suggesting the presence of alternative routes for iron uptake.[110−112]

Newly assimilated cytosolic iron is transported to intracellular sites either for local use or for storage in ferritin (see below). The apoTf–TfR1 complex then returns to the cell surface where Tf dissociates from TfR1.[101] Targeted deletion of the mouse *Tfrc* (encoding TfR1) gene causes embryonic lethality at day E11.5 because of severe anemia, indicating that Tf-mediated iron delivery is critical for hematopoietic cells.[113] Nonhematopoietic tissues develop normally up to day E11.5, again highlighting the existence of alternative mechanisms for delivery of iron to cells in fetuses.[113] Mutations in the *TFRC* gene in humans have not been described. Nonetheless, an

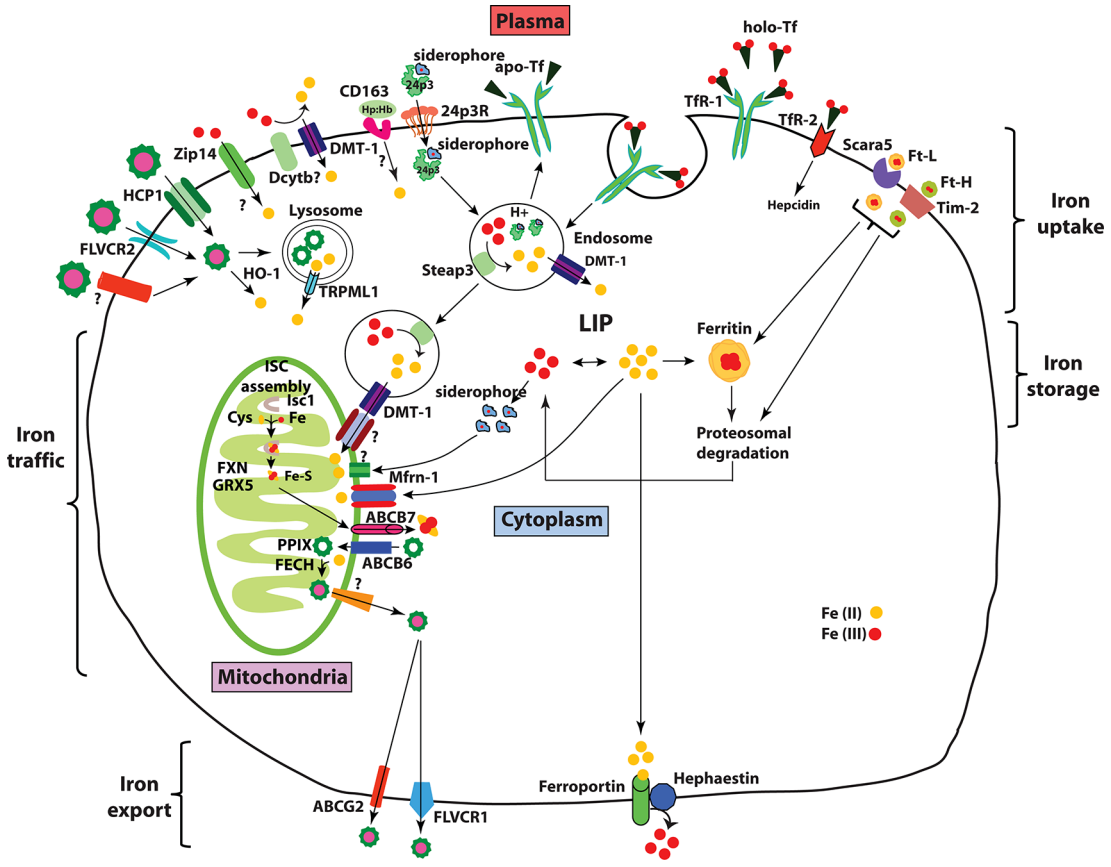

Figure 3. Cellular iron metabolism. Most cells in the body obtain iron from circulating differic Tf. Iron-loaded holo-Tf binds to TfR1 on the cell surface, and the complex undergoes endocytosis via clathrin-coated pits. A proton pump acidifies the endosome, resulting in the release of Fe^{3+}, which is subsequently reduced to Fe^{2+} by Steap3 and transported across the endosomal membrane to the cytosol by DMT1. DMT1 also facilitates dietary iron absorption. Apo-Tf is recycled back to the cell surface and released from TfR1 to plasma to repeat another cycle. Newly acquired iron enters into the cytosolic "labile iron pool" (LIP), which is redox-active. LIP is chelated by the intracellular siderophore that facilitates intracellular trafficking of iron to mitochondria via an unknown receptor for metabolic utilization (such as synthesis of heme and iron–sulfur clusters), and cellular iron that is not utilized is either stored in ferritin or exported via ferroportin. Cells also export iron contained in ferritin and heme.

autoimmune-like disease with antibodies to TfR1 can lead to severe anemia.[114]

Hepatocytes may take up Tf-bound iron via both TfR1 and TfR2. The amino acid sequence of the latter is 45% similar with that of TfR1, yet TfR2 differs significantly in terms of tissue distribution, affinity for Tf, and iron regulation.[115] TfR2 is also a type II transmembrane glycoprotein with cytoplasmic domains and ectodomains, and the Tf–TfR2 complex is likewise internalized via clathrin-mediated endocytosis. However, while TfR1 is almost ubiquitously expressed, the expression of TfR2 is restricted to liver hepatocytes and to differentiated erythroblasts. In addition, TfR2 binds Tf with an ~30-fold lower affinity than TfR1,[116] suggesting that it accounts for only a small fraction of iron uptake. Moreover, TfR2 is less stable than TfR1, mainly regulated post-translationally by protein degradation, and stabilized by differic Tf.[117] The major function of TfR2 appears to be regulatory. TfR2 is involved in sensing iron-laden Tf and the control of hepcidin expression.[118,119] Thus, mutations in the *TFR2* gene lead to non-HFE hemochromatosis due to hepcidin insufficiency in humans,[29] a phenotype that is recapitulated in *TfR2* mutant mice.[120]

NTBI comprises all forms of plasma iron that are bound to ligands other than Tf.[14,121] It is undetectable under physiological conditions but emerges in a variety of iron overload syndromes, when the iron binding capacity of transferrin is saturated, or in atransferrinemia. Although NTBI is a key player in hemochromatotic tissue iron overload, the underlying mechanism remains unknown. Chelators that display a lower affinity for iron than transferrin, such as lipocalin 24p3, are proposed to be NTBI ligands. This carrier protein sequesters iron-laden small hydrophobic molecules or siderophores (discussed in detail below). It has been proposed that 24p3-bound iron is important for epithelial morphogenesis, especially nephrogenesis.[122,123] Nonetheless, *24p3* null mice display no developmental abnormalities in their kidneys and apparently exhibit normal systemic and cellular iron metabolism.[124−127] Experiments with double HFE and 24p3 knockout mice excluded a role for 24p3 in NTBI uptake during hepatic iron overload.[127] Several biochemical and genetic studies identified other potential players in NTBI uptake, such as DMT1. Iron deficiency significantly enhances the expression of DMT1, and a gain-of-function mutation in DMT1 enhances NTBI uptake, suggesting the importance of DMT1 in the acquisition of elemental iron.[128,129] Additionally, in the microcytic (*mk*) mouse, mutant DMT1 behaves like a calcium channel to facilitate NTBI uptake,[130] a notion that may have broader implications in understanding the functional relationship between iron transporters and other ion channels (discussed in detail below). On the basis of these studies, there was speculation that DMT1 is a major facilitator of NTBI uptake. However, the excessive buildup of iron in tissues of *Dmt1* null mice points to the

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involvement of additional players.[108] Another candidate is the zinc transporter protein, Zip14/SLC39A14 (Zrt- and Irt-like protein 14), which facilitates NTBI-mediated iron delivery in cultured cells.[131] Interestingly, HFE regulates Zip14-dependent NTBI uptake by controlling the half-life of Zip14.[132] *Zip14/Slc39a14* null mice do not exhibit apparent abnormalities in iron metabolism,[133] which may indicate redundancy of Zip14 in NTBI uptake. Nevertheless, it would be interesting to cross these animals with $HFE^{-/-}$ mice and examine the effects of Zip14 ablation in iron loading. Consistent with studies of *mk* mice suggesting that similar transmembrane pores conduct Fe^{2+} and Ca^{2+} across membranes, the involvement in NTBI uptake has been documented for the widely expressed L-type voltage-gated calcium channels (LVGCCs), namely $Ca_v1.1-1.4$.[134,135] Furthermore, calcium channel blockers have been reported to mitigate iron overload by enhancing the activity of DMT1.[136] However, these data have been disputed.[137] Despite the evidence from in vitro studies supporting a role for LVGCCs in iron trafficking, mice lacking *$Ca_v1.2$* and *-1.3* do not exhibit alterations in iron metabolism.[138] Combined, these studies suggest a significant redundancy in mechanisms for NTBI uptake and the lack of a single exclusive pathway.

Cells can also acquire iron that is in complexes with proteins or small molecules. For instance, internalization of ferritin via ferritin-specific receptors such as T-cell immunoglobulin and mucin domain-containing protein-2 (TIM-2), or Scavenge receptor family class A, member 5 (Scara5), facilitates iron import.[139,140] However, *Tim-2* null mice do not display any overt abnormalities in iron homeostasis.[141] Silencing the H-ferritin receptor *SCARA5*, rather, contributes to cancer development.[142] Ingested heme also serves as an important source of iron. The importance of this route of iron uptake is exemplified by the enhanced heme uptake observed in iron deficiency. Cells are thought to internalize heme via a receptor-dependent process (directly) or indirectly with the aid of a heme carrier protein-1 (HCP-1).[143] HCP-1, also known as proton-coupled folate transporter (PCFT), is a member of the solute carrier family (SLC46A1), which imports heme or folate into cultured cells or isolated frog oocytes.[143,144] Imported heme is catabolized by heme oxygenases 1 and 2 in the endoplasmic reticulum (ER), and iron is released into the cytosol via DMT1 or transient receptor potential mucolipin 1 (TRPML1).[145,146] Mice deficient in *Hcp-1* do not exhibit iron deficiency but rather disturbances in folate metabolism.[144] This phenotype is quite perplexing, given the fact that iron deficiency augments the expression of HCP-1,[143] and may argue against a physiological role for this protein in dietary heme absorption. Heme can be transported from lysosomes to the cytosol by heme response gene-1 (HRG-1) (SLC48A1).[147] The in vivo functional relevance of HRG-1 in mammalian heme assimilation awaits the derivation of mice lacking this gene.

In summary, mammalian cells obtain iron via multiple routes tailored to meet their specific biochemical requirements.

Cellular Iron Utilization and Intracellular Iron Trafficking Pathways. One of the least understood aspects of cellular iron metabolism is the trafficking of iron within the cell. It is generally believed that iron taken up by the cell is routed to mitochondria via the cytoplasm. Facilitators of trafficking of cytosolic iron to mitochondria were unknown until recently.

Iron taken up by Tf-dependent or -independent routes presumably enters into a labile intermediary pool or labile iron pool (LIP). The LIP is also termed in the literature as the "exchangeable", "regulatory", or "chelatable" iron pool because its presence has been documented by using metal chelators.[46] It is defined as a low-molecular weight pool of weakly chelated iron,[148] including both Fe^{2+} and Fe^{3+}, and represents a minor fraction of the total amount of cellular iron (~3−5%). The LIP links cellular iron uptake with iron utilization, storage, or export.[148] The other source of iron for this pool is the degradation of non-heme and heme iron-containing proteins. Iron within the LIP is thought to be in steady state equilibrium and is proposed to bind diverse low-molecular weight chelates, such as organic anions (phosphates, citrates, and carboxylates) and polyfunctional ligands [polypeptides and siderophores (discussed in detail below)]. Organelles also contain the LIP, which is estimated to have a concentration from ~6 to 16 μM.[149] Mitochondria are the principal consumers of cellular iron. Even though mitochondrial iron is mostly bound to proteins or heme, chelatable iron has been detected within the organelle in cultured hepatocytes and cardiomyocytes.[150] An increase in the size of the mitochondrial LIP is suspected under certain pathological conditions where heme export is impaired. A subpopulation of endosomes and lysosomes also contains a large amount of LIP, with an estimated concentration of 16 μM. It may be derived from the degradation of iron carrier proteins.[149]

The LIP is readily available for iron utilization and may also contribute to adverse side effects as a source of redox-active iron for the Fenton reaction.[148,151] In addition, the LIP operates as a mediator of apoptosis. Thus, iron scarcity in the cell leads to apoptosis, while similar effects are observed by targeting the LIP with chemical chelators such as deferoxamine (DFO) or biological chelators such as lipocalin 24p3 (see below).[152,153] Moreover, apoptosis induced by these agents can be suppressed by exogenous supplementation with iron.[152]

Mitochondria are central for the regulation of cellular iron metabolism, and the majority of iron imported into the cell is utilized within this organelle for the synthesis of heme and iron−sulfur clusters (ISCs).[154] Very little is known about intracellular iron transport pathways that facilitate mitochondrial iron import. This is an important subject because iron in its free form must be shielded and escorted to the sites of utilization. Until recently, the nature and identity of molecules that chaperone iron and facilitate its intracellular trafficking were completely obscure. Nonetheless, early studies indicated the existence of low-molecular weight iron-binding compounds or siderophore-like molecules that were capable of binding iron in mammalian cells.[155,156] Interestingly, iron bound to siderophore-like molecules is a target for carrier proteins like 24p3.

Lipocalin 24p3, also known as lipocalin 2 (Lcn2) or siderocalin, is a member of the lipocalin family of proteins. This family includes more than 20 small secreted proteins that have a highly conserved squat β-barrel enclosing a cavity, which consists of a continuous eight-stranded, antiparallel β-sheet.[157] The cavity within the β-barrel binds, transports, and delivers small molecule ligands. By delivering this cargo via cell surface receptors, they are known to influence many cellular responses.[123,153] Lipocalin 24p3 is a unique iron binding protein, in that it lacks the ability to bind iron directly. Instead, iron binding is mediated by a cofactor. Bacterially derived 24p3 forms a complex with an enterobactin−iron species with a 1:1:1 stoichiometry.[158] *Escherichia coli* enterobactin is the prototype of all bacterial siderophores (from the Greek, meaning "iron carriers" or "iron bearers"), low-molecular weight, Fe^{3+}-specific chelating agents that are hyperexcreted at low iron concentrations. These compounds scavenge iron from the environment and transport it into bacteria by specific receptor proteins. Enterobactin

consists of three 2,3-DHBA (2,3-dihydroxybenzoic acid) and three L-serine molecules, cyclically linked to form a triserine−trilactone structure. Interestingly, 24p3 binds both 2,3-DHBA and enterobactin.[158] On the basis of these observations, it was proposed that 24p3 functions as a bacteriostat and operates in innate immunity.[158] In line with this prediction, 24p3 null mice are hypersensitive to bacterial septicemia.[124,125] This mode of host defense is limited by the ability of 24p3 to sequester bacterial siderophores. In addition, certain bacteria biochemically modify their siderophores to evade capture by 24p3.[159]

Several studies have demonstrated the association of mammalian 24p3 with siderophore-like molecules. It has been proposed that iron delivery or sequestration by 24p3 is crucial for nephrogenesis[122] and mediates apoptosis.[152] However, *24p3* deficient mice show normal kidney development and display no overt imbalances in iron metabolism but display multiple abnormalities in hematopoietic cells.[126] A recent study identified 2,5-DHBA as a mammalian cofactor that facilitates loading of iron onto 24p3. This molecule is remarkably chemically similar to 2,3-DHBA, which is generated by *E. coli* as a component of enterobactin.[160] The biosynthesis of enterobactin is mediated by a two-step process: First, a series of reactions convert chorismate into 2,3-DHBA. Second, enterobactin peptide bonds are formed from three molecules each of 2,3-DHBA and L-serine, by nonribosomal peptide synthetases.[161] Six enzymes participate in the biosynthesis of enterobactin. Among them, EntA, a NAD^+-dependent 2,3-dihydrobenzoate dehydrogenase, catalyzes the rate-limiting step. Elimination of EntA inhibits the synthesis of not only 2,3-DHBA but also enterobactin.[161,162] Interestingly, the mammalian siderophore (2,5-DHBA) is biosynthesized by an evolutionarily conserved pathway, and BDH2 (3-hydroxy butyrate dehydrogenase), a homologue of bacterial EntA, catalyzes the rate-limiting step. Elimination of *bdh2* inhibits 2,5-DHBA biosynthesis.[160] In contrast to 24p3, the majority of tissues synthesize 2,5-DHBA, suggesting that it has functions besides the loading of iron onto 24p3.[160] Siderophore-depleted mammalian cells, zebrafish, and yeast fail to synthesize heme, suggesting that 2,5-DHBA facilitates mitochondrial iron uptake.[160] Mitochondria are evolutionary relics of aerobic bacteria, which supply host cell energy in the form of ATP generated in an oxygen-dependent manner. Iron is integral to all the protein subunits involved in the respiratory chain. Thus, the presence of a phylogenetically conserved siderophore-like molecule whose structure and biogenesis are evolutionarily linked to bacterial siderophores makes good sense. Although a siderophore-like molecule in mammals has been identified, the transporters or receptors that mediate siderophore-dependent mitochondrial iron uptake remain unknown. Additional cytosolic chaperones that traffic iron in the cell have been reported. For instance, evolutionarily conserved glutaredoxins Grx3 and Grx4 play an important role in iron sensing and intracellular iron trafficking, and their absence impairs mitochondrial iron import.[163]

The mechanism of entry of iron into mitochondria has been explored recently. Iron must traverse both outer and inner mitochondrial membranes to the site of heme synthesis, the matrix. Mitochondrial outer membrane facilitators of iron import were identified by computational screening, which yielded several members of the SLC family, namely, SLC25a39 and SLC22A4, as well as a non-SLC transporter, TMEM14C.[164] All these genes are consistently and specifically coexpressed with genes encoding enzymes of the heme synthesis pathway.[164] Their targeted knockdown in the developing zebrafish embryo results in heme deficiency, thus validating their role in the import of iron into mitochondria.[164] Interestingly, members of the SLC family also facilitate the import of iron across the mitochondrial inner membrane. Most notable are SLC25A37 or Mitoferrin-1 (Mfrn1) and SLC25A38 or Mitoferrin-2 (Mfrn2), which are important for the import of mitochondrial iron into erythroid and nonerythroid cells, respectively.[165] Mitoferrin-1 levels in the developing erythron are stabilized by a member of the ATP-binding cassette transporter, ABCB10.[166] Although Mitoferrin-1 and Mitoferrin-2 are highly homologous, they exhibit no functional redundancy. Thus, overexpression of Mitoferrin-2 cannot rescue mitochondrial iron deficiency due to depletion of Mitoferrin-1.[167] Genetic ablation of *Mfrn1* confers embryonic lethality due to defective hemoglobinization.[165] However, conditional deletion of *Mfrn1* in the hematopoietic system results in defective erythroblast formation, leading to anemia. In contrast, targeted deletion of *Mfrn1* in liver hepatocytes results in no discernible phenotypic or biochemical abnormalities under normal conditions.[168] Despite these advances, how iron is transported across the outer mitochondrial membrane remains elusive. Siderophore-dependent iron transport supports mitochondrial heme biogenesis. The relationship between the SLC family of mitochondrial transporters and the mammalian siderophore is currently unknown.

An alternative to chaperone- or siderophore-mediated delivery of iron to the mitochondria has been proposed for the developing erythron. According to the "kiss-and-run" model, which is based on kinetic and imaging data, Tf-bound iron is delivered directly into the mitochondria upon contact of Tf-containing endosomes and mitochondria.[169] The mediators that could facilitate this process are unclear.

Finally, the iron regulatory proteins [IRPs (discussed in detail below)] whose primary function is to regulate cellular iron homeostasis are also critical for ensuring adequate iron supplies for mitochondria, at least in liver hepatocytes.[170] ISC limitations or even heme insufficiency is sensed as mitochondrial iron deficiency, and these conditions activate IRPs in the cytosol. Activated IRPs increase the level of cellular iron uptake and concomitantly reduce the extent of storage and export of iron.[7,170]

Mitochondrial Handling of Iron. As noted above, iron influences many aspects of mitochondrial metabolism. Imported iron is utilized for the biosynthesis of heme and ISCs, and excess iron is stored in the mitochondrion-specific iron storage protein mitochondrial ferritin or mitoferritin (Ftmt) (see below).

Heme synthesis begins and ends in mitochondria, but intermediate steps occur in the cytoplasm (reviewed in ref 171). The first step is the condensation of succinyl coenzyme A and glycine in the mitochondrial matrix to form 5-amino-levulinic acid (ALA), which is catalyzed by erythroid-specific ALA synthase 2 (ALAS2) or housekeeping ALAS1 in nonerythroid cells. Mutations in *ALAS2* result in anemia, heme deficiency, and mitochondrial iron overload, underscoring the importance of this enzyme.[172] ALA is then exported across mitochondrial membranes into the cytosol. The transporters or receptors that mediate ALA mitochondrial cross traffic are not known. Subsequently, ALA is converted to coproporphyrinogen in a series of reactions in the cytoplasm. The heme intermediate is then transported across the mitochondrial outer membrane for iron insertion by ABCB6, a member of the ATP-binding cassette (ABC) family.[173] The final step, insertion of iron into protoporphyrin, is catalyzed by mitochondrial ferrochelatase (Fech). Mutations that render *FECH* inactive cause erythropoietic

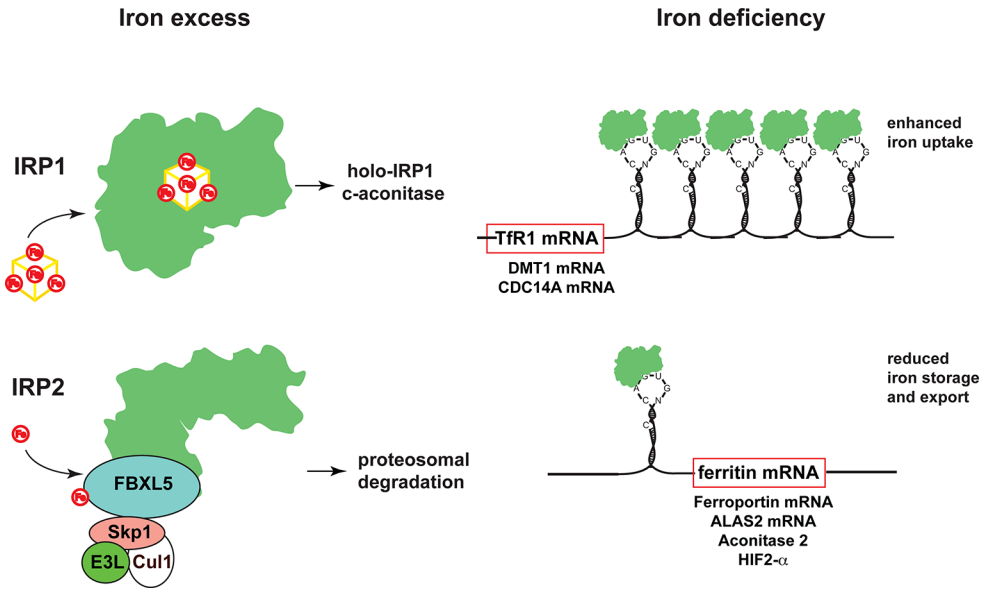

Figure 4. Cellular iron balance. A typical IRE motif consists of a hexanucleotide loop with the sequence 5′-CAGUGH-3′ (H could be A, C, or U) and a stem, interrupted by a bulge with an unpaired C residue. IREs post-transcriptionally control expression of regulators of cellular iron metabolism in concert with IRPs. Translational-type IRE−IRP interactions in the 5′ UTR modulate the expression of the mRNAs encoding H- and L-ferritin, ALAS2, m-aconitase, ferroportin, and HIF-2α, which in turn control iron storage, erythroid iron utilization, energy homeostasis, iron efflux, and hypoxic responses, respectively. Conversely, IRE−IRP interactions in the 3′ UTR stabilize the mRNAs encoding TfR1, DMT1, and Cdc14A, which are involved in iron uptake, iron transport, and the cell cycle, respectively. Under physiological conditions, IRP1 is regulated by a reversible ISC switch. Iron deficiency promotes ISC disassembly and a conformational rearrangement, resulting in conversion of IRP1 from c-aconitase to an IRE-binding protein. The ISC is regenerated in iron-replete cells. Hypoxia favors maintenance of the ISC, while H_2O_2 promotes its disassembly. When the ISC biogenesis pathway is not operational, iron leads to ubiquitination of apo-IRP1 by the FBXL5 E3 ligase complex (including Skp1, Cul1, and Rbx1), resulting in proteasomal degradation. IRP2 is stable in iron deficient and/or hypoxic cells; under these conditions, FBXL5 undergoes ubiquitination and proteasomal degradation. An increase in iron and oxygen levels stabilizes FBXL5 by formation of an Fe−O−Fe center in its hemerythrin domain, triggering the assembly of an E3 ubiquitin ligase complex together with Skp1, Cul1, and Rbx1. This complex ubiquitinates IRP2, leading to its recognition by the proteasome and its degradation.

protoporphyria and liver damage.[174] As discussed above, heme biosynthesis has been extensively studied, but the process by which heme is ferried across mitochondrial membranes into the cytosol for association with globins and apo cytochromes remains unknown. Likewise, no specific transporters or receptors that facilitate mitochondrial export of heme have been reported.

Mitochondrial iron is also utilized for the synthesis of ISCs. These cofactors consist of iron cations and sulfide anions. ISCs are assembled on scaffold proteins and are then targeted to specific proteins via sulfur of cysteine residues. In mammals, the scaffold protein is "ISC assembly protein U" (IscU).[175] Loading of iron onto IscU is very likely mediated by mitochondrial frataxin,[176] which thereby plays an important role in mitochondrial iron metabolism. Thus, mutations in the *FRDA* gene encoding frataxin result in Friedreich ataxia, a neurodegenerative disease characterized by profound mitochondrial abnormalities and iron toxicity.[177] In addition, targeted disruption of this gene in mice causes embryonic lethality, while its conditional deletion in brain or muscle results in neuronal or cardiac symptoms.[177] Although the majority of ISC biogenesis takes place inside mitochondria, ISCs may also be synthesized in the cytoplasm.[178] The biological relevance of the mediators of cytosolic ISC biogenesis is not well understood. Nonetheless, the mitochondrial inner membrane protein, ABCB7, is important for the maturation of cytosolic ISCs because targeted deletion of *Abcb7* results in altered cytosolic ISC levels and retards embryonic development.[179,180] ABCB7 may traffic ISCs (or ISC precursors) across mitochondrial membranes to the cytoplasm.[179] Multiple mechanisms ensure an adequate supply of iron to mitochondria. Perturbations of these processes affect iron homeostasis in both mitochondria and the cytoplasm.[181] Human frataxin deficiency severely disrupts mitochondrial ISC biogenesis, promotes mitochondrial iron overload, and causes increased sensitivity to oxidant stress.[177] Mutations in human *ABCB7* result in defective cytosolic ISC maturation and mitochondrial iron overload leading to X-linked sideroblastic anemia, which is also associated with developmental abnormalities in the brain.[182] The specific mechanism by which ABCB7 deficiency contributes to anemia is unclear. Nonetheless, ABCB7 interacts with Fech, and ABCB7 plays a role in supplying iron to Fech for incorporation into protoporphyrin.

Cellular Iron Balance. While systemic iron metabolism is predominantly regulated at the transcriptional level, cellular iron balance is largely controlled by post-transcriptional mechanisms. The cytoplasmic iron regulatory proteins (IRPs) determine the fate of several mRNAs upon binding to their iron-responsive elements (IREs). These structural motifs are within their untranslated regions (UTRs) (Figure 4). The mRNAs encoding TfR1 and ferritin define prototypic examples of coordinate post-transcriptional regulation by IRE−IRP interactions.[6,7]

IREs. IREs are evolutionarily conserved stem−loop structures of 25−30 nucleotides.[183] The stem is divided into an upper and a lower segment, which are separated by an unpaired C residue or an asymmetric UGC/C bulge/loop. The loop consists of a conserved 5′-CAGUGH-3′ sequence (H denotes A, C, or U), where the underlined C and G residues interact. TfR1 mRNA

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contains multiple IREs within its long 3′ UTR, while the mRNAs encoding H- and L-ferritin contain a single IRE in their 5′ UTRs.

In iron deficiency, IRPs bind with high affinity ($K_d \approx 10^{-12}$ M) to target IREs. This results in stabilization of TfR1 mRNA and steric inhibition of ferritin mRNA translation. Under these conditions, accumulation of TfR1 promotes the uptake of cellular iron from plasma Tf, while inhibition of ferritin biosynthesis prevents storage of iron, allowing its metabolic utilization. Conversely, in response to excess cellular iron, IRPs are inactivated, which leads to TfR1 mRNA degradation and ferritin mRNA translation. This minimizes further internalization of iron via TfR1 and promotes the storage of excessive intracellular iron into ferritin. Interestingly, mutations in the L-ferritin IRE that prevent IRP binding are causatively linked to hereditary hyperferritinemia-cataract syndrome (HHCS), an autosomal dominant disorder characterized by overexpression of serum ferritin (without iron overload) and early onset cataract.[184] Mutations, in the IRE of H-ferritin have been associated with a rare form of autosomal dominant iron overload in a Japanese pedigree, but the underlying pathogenic mechanism remains unclear.[185]

Functional IRE motifs have also been identified in mRNAs encoding proteins of iron transport (DMT1 and ferroportin) and erythroid heme synthesis (ALAS2), as well as others that appear to be less related to iron homeostasis [mitochondrial aconitase, *Drosophila* succinate dehydrogenase, HIF-2α, MRCKα, Cdc14A, β-amyloid precursor protein, and α-synuclein (reviewed in ref 6)]. It is notable that mRNAs encoding TfR2, Ftmt, and housekeeping ALAS1 as well as alternatively spliced transcripts of DMT1 and ferroportin do not possess an IRE. The expression of IRE or non-IRE isoforms of DMT1 and ferroportin mRNAs confers versatility and tissue-specific regulation. For instance, the prevalence of the non-IRE isoform of ferroportin mRNA in duodenal enterocytes and erythroid precursor cells[186] allows its expression during iron deficiency[187] via evasion of the translational IRP block. It should also be noted that in erythroid cells, which require very large amounts of iron for hemoglobinization, the expression of TfR1 mRNA is induced by transcriptional mechanisms, and its stability is uncoupled from iron supply and IRP regulation.[188,189] Transcriptional regulation of TfR1 as well as ferritin mRNA expression has also been established in other settings (reviewed in refs 6 and 7).

IRPs. IRP1 and IRP2 are homologous to mitochondrial aconitase and other members of the ISC isomerase family,[6,7] which possess a cubane ISC in their active site. In iron-replete cells, IRP1 assembles an ISC and functions as cytosolic aconitase (catalyzing the conversion of citrate to iso-citrate via a *cis*-aconitate intermediate). The ISC keeps IRP1 in a closed conformation, which precludes access of IREs. Iron deficiency promotes the loss of the ISC and a resulting structural rearrangement of the protein that acquires IRE binding activity. Thus, IRP1 is a bifunctional protein that is regulated by an ISC switch.

Despite its extensive homology with IRP1, IRP2 does not assemble an ISC and is regulated by a distinct mechanism. It remains stable in iron-starved or hypoxic cells and undergoes ubiquitination and proteasomal degradation in response to iron and oxygen. The mechanism involves FBXL5, an E3 ubiquitin ligase that contains an N-terminal hemerythrin domain with a Fe−O−Fe bridge.[190,191] Iron deficiency and/or hypoxia leads to disassembly of the Fe−O−Fe bridge, which destabilizes FBXL5 and allows accumulation of IRP2. Interestingly, FBXL5 may also target apo-IRP1 for iron-dependent degradation; however, this occurs only as a backup mechanism under conditions where ISC assembly is defective.[192,193]

The ablation of both IRPs leads to early embryonic lethality at the blastocyst stage.[194] Intestine-specific disruption of both IRPs has been associated with growth defects, intestinal malabsorption, dehydration, and weight loss.[195] Likewise, disruption of IRPs in hepatocytes causes premature death due to liver damage, mitochondrial abnormalities, and defects in heme and ISC biosynthetic pathways.[170]

A single disruption of either IRP1 or IRP2 is not lethal, indicating a functional redundancy of these proteins. *IRP1*$^{-/-}$ mice do not develop any apparent pathology but misregulate TfR1 and ferritin expression in the kidney and brown fat.[196] *IRP2*$^{-/-}$ mice exhibit microcytosis and hypochromic anemia, associated with iron overload in the duodenum and the liver, and relative iron deficiency in the spleen.[197,198] The deregulation of tissue iron homeostasis in these animals can be explained by cell-autonomous functions of IRP2.[199] Some aged IRP2$^{-/-}$ mice develop brain iron overload and deposition in overexpressed ferritin.[200] This leads to a progressive neurodegenerative disorder, possibly as a result of the decreased availability of iron (functional iron deficiency).

Mice lacking FBXL5 die early in embryogenesis, apparently from deleterious effects of iron accumulation and subsequent iron-induced damage to tissues. Their pathology resembles clinical features of neonatal hemochromatosis, which is fatal due to liver failure resulting from excessive deposition of iron in this organ.[201] Interestingly, ablation of IRP2 rescues the FBXL5 null phenotype, consistent with FBXL5 being a repressor of IRP2. Mice bearing liver-specific disruption of FBXL5 develop severe steatohepatitis, associated with hepatic iron overload and oxidative stress. Collectively, the data described above highlight the significance of the IRE−IRP system in the control of mammalian iron homeostasis.

Cellular Iron Storage. Cellular iron in excess of immediate needs is stored as an iron oxide within the nanocavity of ferritin. Ferritin is an evolutionarily conserved, ubiquitous protein that can accommodate up to 4500 iron atoms. In mammals, ferritin is a heteropolymer of 24 subunits of two types, heavy (H) and light (L), which assemble to make a hollow spherical shell (reviewed in ref 202). L-Ferritin is predominant in iron storing tissues, whereas H-ferritin is preferentially expressed in cells that take up and release iron rapidly. Different proportions of ferritin subunits give rise to the heterogeneity of the holoprotein in various tissue types. A glycosylated L-ferritin subunit circulates in serum and exhibits a low level of saturation with iron. Its origin is debated. The levels of serum ferritin increase in response to systemic iron load or infection.[6] Ferritin secretion may provide a mechanism for limiting iron storage after a shift from high to low iron concentrations, and prior to activation of IRPs.[202]

Channels in the ferritin shell may facilitate the entry and exit of iron. The ferroxidase activity of H-ferritin converts Fe^{2+} to Fe^{3+}, which is necessary for the deposition of iron into the nanocage. L-Ferritin induces iron nucleation and increases the rate of turnover of the ferroxidase activity. The mechanism by which iron is delivered to ferritin remains elusive. Experimental evidence suggests the involvement of the cytosolic iron chaperone poly(rC)-binding protein 1 (PCBP1).[203] The release of iron from ferritin is mediated by multiple mechanisms (reviewed in ref 202). Physiologically, degradation of ferritin is coupled to the supply of available metabolic iron under iron-limiting conditions.[204] Treatment with iron

Table 2. Regulators of Cellular Iron Metabolism in Animal Models and Human Disorders

metabolic process	protein	gene	function	mouse model	phenotype	human disease	refs
cellular iron and/or heme uptake	transferrin	*Tf*	plasma, lymph, and CSF ferric iron carrier	*hpx/hpx*	hypochromic, microcytic anemia	atransferrinemia	7, 100
	transferrin receptor 1	*Tfrc*	facilitates Tf-dependent iron uptake	$TfR1^{-/-}$	embryonic lethality	autoimmune-like with Abs to TfR1; anemia	113
	transferrin receptor 2	*TfR2*	regulates hepcidin; mediates Tf-bound and non-transferrin-bound iron uptake	$TfR2^{-/-}$	iron overload	hemochromatosis	61
	six-transmembrane epithelial antigen of the prostate	*Steap3*	erythroid endosomal ferrireductase	*nm:1054* $Steap3^{-/-}$	microcytic, hypochromic anemia; iron deficiency	unknown	110−112
	divalent metal transporter 1	*DMT1* *Nramp1* *Slc11a2*	endosomal ferrous iron transporter; transmembrane non-transferrin iron transporter	*mk/mk* $DMT^{-/-}$	hypochromic, microcytic anemia; impaired iron absorption	iron overload in liver; anemia	105, 108, 109
	duodenal cytochrome *b*	*DcytB*	intestinal ferrireductase	$Dcytb^{-/-}$	normal	unknown	9
	Zrt- and Irt-like protein 14	*Zip14*	non-transferrin iron transporter in liver	$Slc39A14^{-/-}$	Impaired gluconeogenesis; no abnormalities in iron metabolism	unknown	133
	L-type Ca^{2+} channels	$Ca_v1.1-1.4$	calcium/iron transporters	$Ca_v1.2^{-/-}$ $Ca_v1.3^{-/-}$	no abnormalities in iron metabolism	unknown	138
	T-cell Ig domain and mucin domain protein-2 (TIM-2)	*Tim-2*	H-ferritin receptor	$Tim2^{-/-}$	no overt abnormalities related to iron metabolism	unknown	141
	scavenger receptor class A member 5	*Scara5*	L-ferritin receptor	not available	unknown	predisposition to liver cancer	142
	feline leukemia virus subgroup C receptor 2	*FLVCR2*	heme importer	not available	unknown	Fowler syndrome	219
	heme carrier protein 1	*HCP1*	intestinal heme transporter	$HCP^{-/-}$	no alterations in iron homeostasis	folate malabsorption	144
	transient receptor potential mucolipidosis-associated protein	*TRPML1*	iron transporter from endosomes and lysosomes	not available	unknown	mucolipidosis	146
	heme responsive gene 1	*HRG1*	exporter of heme into cytoplasm from lysosomes	not available	unknown	unknown	7
	heme oxygenase-1	*HO-1*	heme iron reutilization	$HO\text{-}1^{-/-}$	iron overload	iron overload, hemolytic anemia	11
	heme oxygenase-2	*HO-2*	heme iron reutilization	$HO\text{-}2^{-/-}$	no disturbances in iron homeostasis	unknown	11
	24p3	*24p3*	facilitator of non-transferrin iron import	$24p3^{-/-}$	no observable alterations in iron homeostasis	unknown	127
cellular iron storage	H-ferritin (FT-H)	*Fth1*	iron storage protein subunit; ferroxidase	$Fth^{-/-}$	embryonic lethality	iron overload	185, 202
	L-ferritin (FT-L)	*Ftl*	iron storage protein subunit	not available	unknown	IRE mutation, hyperferritenemia-cataract syndrome; non-IRE mutation, neuroferritinopathy	185, 202
intracellular iron trafficking	ferrochelatase (FECH)	*Fech*	insertion of iron into porphyrin	$Fech^{m1\ Pas}$ $Fech^{-/-}$	microcytic, hypochromic anemia; embryonic lethality	erythropoietic protoporphyria	172, 174
	5-amino-levulinic acid synthase (ALAS2)	*Alas2*	heme biogenesis	$Alas2^{-/-}$	embryonic lethality; iron overload	X-linked sideroblastic anemia	171
	ATP-binding cassette family member B 10 (ABCB10)	*Abc-me or Abcb10*	interacts with Fech and Mitoferrin-1; transport ligand unknown	$Abcb10^{-/-}$	embryonic lethality; arrested erythroid differentiation	unknown	166
	ATP-binding cassette family member B 7 (ABCB7)	*Abcb7*	interacts with Fech; cytosolic ISC maturation	$Abcb7^{-/-}$	midgestational death; defective hematopoiesis; hepatic iron overload, ataxia	hereditary X-linked sideroblastic anemia, ataxia	180, 182
	ATP-binding cassette family member B 6 (ABCB6)	*Abcb6*	import of porphyrin into mitochondria?	unpublished	porphyria?	unknown	173
	Mitoferrin-1 (MFRN1)	*Mfrn1*	mitochondrial iron import	$Mfrn1^{-/-}$	embryonic lethality; conditional deletion confers anemia and defective hematopoiesis	erythropoietic protoporphyria	165, 168
	glutaredoxin 5 (GRX5)	*Grx5*	facilitator of ISC biogenesis	not available	unknown	anemia; iron overload	7

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Table 2. continued

metabolic process	protein	gene	function	mouse model	phenotype	human disease	refs
	frataxin (FXN)	*Fxn*	mitochondrial iron chaperone?	*Fxn*$^{-/-}$	embryonic lethality; conditional deletion confers brain and heart deformities	Friedreich ataxia	177
	3-hydroxy butyrate dehydrogenase-2 (BDH2)	*Bdh2 or Dhrs6*	siderophore biosynthesis	*Bdh2*$^{-/-}$	anemia, iron overload, splenomegaly	diabetes	unpublished
cellular iron and/or heme export	feline leukemia virus subgroup C receptor 1 (FLVCR1)	*FLVCR1*	heme exporter	*FLVCR1*$^{-/-}$	embryonic lethal; systemic iron overload and erythroid abnormalities in conditional deletion	Diamond Blackfan anemia? Neurological abnormalities	216, 218
	ATP-binding cassette family member C, G2 (ABCG2)	*Abcg2*	partially exports heme; PPIX exporter	*Abcg2*$^{-/-}$	elevated levels of PPIX; no iron overload	gout	220, 221
	ferroportin (SLC40A1; MTP1)	*Slc40a1*	regulates efflux of iron from cells	*Slc40a1*$^{-/-}$*pcm*	embryonic lethality; conditional deletion, iron deficiency	hemochromatosis, macrophage iron overload (HH type 4)	19, 215
cellular iron balance	iron regulatory protein 1 (IRP1)	*Aco1*	post-transcriptional regulation of target mRNAs via IREs; cytosolic aconitase	*IRP1*$^{-/-}$		unknown	194−200
	iron regulatory protein 2 (IRP2)	*Ireb2*	post-transcriptional regulation of target mRNAs via IREs	*IRP2*$^{-/-}$		unknown	194−200

chelators or expression of ferroportin accelerates ferritin degradation.[205,206]

Changes in iron availability regulate *ferritin* gene expression mainly at the post-transcriptional level via the IRE−IRP system (discussed above). Additionally, ferritin expression is regulated transcriptionally, which determines the tissue distribution of H and L chains. Iron homeostasis is profoundly altered during inflammation and infection. Although multiple mechanisms collectively influence iron balance (such as induction of hepcidin by IL-6), alteration of ferritin expression by proinflammatory cytokines such as TNFα or IL-1α substantially contributes to reprogramming of iron homeostasis.[6] Ferritin is transcriptionally activated under oxidative stress via an upstream antioxidant response element (ARE) in the promoter region of the *ferritin* genes.[207] In contrast, extracellular H_2O_2 inhibits ferritin mRNA translation by activating the IRE−IRP regulatory system.[6,7]

By sequestering redox-active iron, ferritin plays an important antioxidant role and promotes cell survival. Thus, overexpression of ferritin decreases the size of the LIP, impedes the generation of ROS, and confers resistance to oxidative damage.[208] Moreover, H-ferritin depletion triggers opposite effects.[207] Genetic deletion of *Fth1* (encoding H-ferritin) results in embryonic lethality, suggesting that its ferroxidase activity is critical.[185] Conditional ablation of *Fth1* in liver hepatocytes results in iron-induced oxidative damage because these cells cannot sequester and detoxify iron.[185] Mutations in L-ferritin are associated with an autosomal dominant neurological disorder (neuroferritinopathy), characterized by brain iron overload and entrapment of iron in mutant ferritin shells, which causes functional iron deficiency.[185]

Mitochondrial ferritin (Ftmt) is encoded by a distinct nuclear gene[209] and possesses ferroxidase activity, by analogy to H-ferritin. In contrast to that of cytosolic ferritin, the expression of Ftmt is restricted to few tissues and is not iron-regulated.[210] Ftmt serves as a molecular sink to prevent accumulation of unshielded iron in mitochondria, which protects the organelle against iron's toxicity. Ftmt levels are increased in sideroblastic anemia.[185] Forced overexpression or depletion of Ftmt results in profound alterations of mitochondrial as well as cytosolic iron levels.[211]

Cellular Iron Export. Cellular iron export routinely occurs in specialized cells such as enterocytes and macrophages that are involved in iron absorption and recycling, respectively. The main purpose of iron export is to maintain adequate plasma iron levels and to meet systemic needs. Iron export involves coordination between many enzymes and proteins. Ferroportin, a member of the solute carrier family, facilitates the export of iron from enterocytes, macrophages, hepatocytes, and the extraembryonic visceral endoderm (ExVE). As discussed above, iron is reduced in endosomes prior to being released into the cytoplasm. Thus, ferroportin exports Fe^{2+}, which has to be oxidized to Fe^{3+} upon its release into plasma for binding to Tf (reviewed in ref 212). This is mediated by the blue copper ferroxidases ceruloplasmin (soluble in serum or plasma membrane-associated in some cell types) and hephaestin (expressed on the plasma membrane of enterocytes and other cell types). These enzymes thus work in concert with ferroportin to coordinate iron export and oxidation. As copper is essential for ferroxidase activity, adequate levels of this metal are required for proper iron balance (reviewed in ref 213).

Complete disruption of ferroportin in mice is associated with early embryonic lethality, while conditional ablation leads to the

accumulation of iron in target cells, consistent with a function of this protein as the sole iron exporter.[214] In humans, ferroportin autosomal dominant mutations lead to "ferroportin disease". Mutations inhibiting iron efflux promote macrophage iron loading, low serum iron levels, and transferrin saturation. On the other hand, mutations that impair the binding of hepcidin cause parenchymal iron overload, relatively high serum iron levels, and transferrin saturation, as in classical hereditary hemochromatosis.[23,215]

Cells may also export iron bound to ferritin, especially in necroinflammation, or heme iron. Feline leukemia virus subgroup C receptor 1 (FLVCR1) facilitates the efflux of heme from hematopoietic cells. Suppression or forced expression of FLVCR1 enhances or depletes the cytoplasmic heme content, respectively.[216,217] Thus, it has been proposed that FLVCR1 mediates the efflux of heme from erythroblasts to ensure their survival and from macrophages to regulate hepatic and systemic iron homeostasis. *Flvcr1*$^{-/-}$ mice exhibit embryonic lethality and lack definitive erythropoiesis (Table 2). In contrast, neonatal deletions of *Flvcr1* cause severe macrocytic anemia with proerythroblast maturation arrest.[216] Point mutations in human *FLVCR1* are surprisingly not associated with erythroid disorders. Instead, affected patients exhibit neurological and vision abnormalities.[218] Interestingly, the related protein FLVCR2 appears to promote heme import.[219]

In addition to the list of heme exporters, an ATP binding cassette family member, ABCG2, has also been implicated in cellular heme export. Thus, genetic ablation of *Abcg2* results in the accumulation of the heme synthesis intermediate, protoporphyrin IX (PIX), suggesting a function in heme or porphyrin export. Protoporphyrin abnormally accumulates in mice deficient for *Abcg2*, suggesting that Abcg2 aids in porphyrin export.[220] Nevertheless, mutations in human *ABCG2* are not associated with heme abnormalities, but rather with elevated levels of uric acid.[221]

■ CONCLUSIONS

Iron is vital yet poorly bioavailable and potentially toxic. Cells and multicellular organisms have evolved sophisticated mechanisms for coping with the challenges of iron acquisition and handling. Remarkable progress has been made in understanding the regulation of mammalian iron metabolism. Mammals primarily control dietary iron absorption and systemic iron traffic via hepcidin, a hormonal peptide that responds to physiological stimuli. Cellular iron uptake, storage, and utilization are coordinately regulated by the IRE−IRP system. Fine-tuning is achieved by further regulatory pathways, which operate at the systemic and cellular levels. While basic mechanisms for mammalian iron homeostasis are now fairly well understood, several issues that remain open include the molecular characterization of iron sensing by hepcidin and the IRE−IRP pathways, the elucidation of iron trafficking inside cells, and the clarification of the physiological significance of the mammalian siderophore−24p3 network.

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