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A Red Carpet for Iron Metabolism

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200 billion red blood cells (RBCs) are produced every day, requiring more than 2 × 10^{15} iron atoms every second to maintain adequate erythropoiesis. These numbers translate into 20 mL of blood being produced each day, containing 6 g of hemoglobin and 20 mg of iron. These impressive numbers illustrate why the making and breaking of RBCs is at the heart of iron physiology, providing an ideal context to discuss recent progress in understanding the systemic and cellular mechanisms that underlie the regulation of iron homeostasis and its disorders.

Iron deficiency is the most common cause of anemia and represents a global health problem. Iron-deficiency anemia is defined by low numbers of small (microcytic) and hypoferremic erythrocytes. In addition to erythropoiesis, iron is essential for mitochondrial function, DNA synthesis and repair, and many enzymatic reactions required for cell survival. Iron deficiency may contribute to cognitive developmental defects in children, poor physical performance, and unfavorable pregnancy outcomes (Camaschella, 2015).

Iron overload is also common and equally detrimental, affecting parenchymal organs including the liver, heart, and pancreas. In Western populations iron overload is mostly genetic due to hereditary hemochromatosis (HH), caused by mutations in genes involved in the sensing of systemic iron levels (such as *HFE*, *HJV*, and *TFR2*), or to disorders that cause ineffective erythropoiesis and secondary iron loading (e.g., thalassemias). There is increasing awareness that acquired metabolic disorders can also cause iron overload, which may exacerbate pathogenesis (Pietrangelo, 2016).

Focusing on red cells, we will first discuss cellular mechanisms of iron management and regulation before turning to the systemic processes that ensure an appropriate balance between iron supply and demand.

Rolling out the Red Carpet: Iron Metabolism in Erythroid Cells

Red blood cells (RBCs) are formed from lineage-committed progenitors during erythropoiesis, a highly regulated process. During definitive erythropoiesis, hematopoietic stem cells give rise to burst-forming unit erythroid (BFU-E) progenitors that progress into colony-forming unit erythroid cells (CFU-E). CFU-E progenitors generate proerythroblasts that progress to basophilic, polychromatophilic, and orthochromatic forms around a central "nurse macrophage" within an extravascular niche, the erythroblastic island. Erythroblasts then enucleate to form reticulocytes, which move out into the bloodstream and become mature RBCs that circulate until senescent (Figure 1). Like other cell types, erythroid precursors express molecules dedicated to the transport, utilization, and storage of iron. They are, however, characterized by extreme iron requirements to sustain hemoglobin synthesis. These requirements are mostly satisfied by iron recycling of senescent red blood cells by macrophages. Dietary iron absorption compensates for iron losses (bleeding) or increased needs (pregnancy, childhood, hypoxia) and hepatic iron stores serve as a buffer.

Iron Acquisition

To acquire such high amounts of iron, erythroid cells depend on transferrin (Tf). Tf is a glycoprotein with two high-affinity sites for Fe(III) that renders the metal bioavailable in the circulation while preventing the formation of toxic radicals and limiting iron access to invading pathogens, which also require iron. In healthy individuals, Tf is about 30% saturated with iron. Although diferric Tf can be used as an iron source by most cells, its main destination is the erythroid marrow. Diferric Tf binds to the high-affinity Tf receptor 1 (TFR1, also known as TFRC, CD71) on the surface of developing RBCs, followed by endocytosis of the Tf-Fe_2/TFR1 complex. The acidic environment in the early endosome promotes iron release from Tf, which itself remains bound to its receptor. The freed metal is then reduced to the ferrous form by STEAP3 (six-transmembrane epithelial antigen of prostate 3) (Ohgami et al., 2005) and transported into the cytosol by DMT1 (divalent metal transporter 1, also known as NRAMP2, SLC11A2) (Fleming et al., 1997; Gunshin et al., 1997); other members of the STEAP family may act as ferrireductases in non-erythroid cells. The Apo-Tf/TFR1 complex returns to the cell surface, where apo-Tf dissociates. This recycling process is crucial for optimal hemoglobin production. The sorting of Tf/TFR1 complexes into recycling endosomes requires sortin nexin 3 (SNX3) (Chen et al., 2013), while trafficking of the Tf/TFR1 complex from recycling endosomes to the cell surface is

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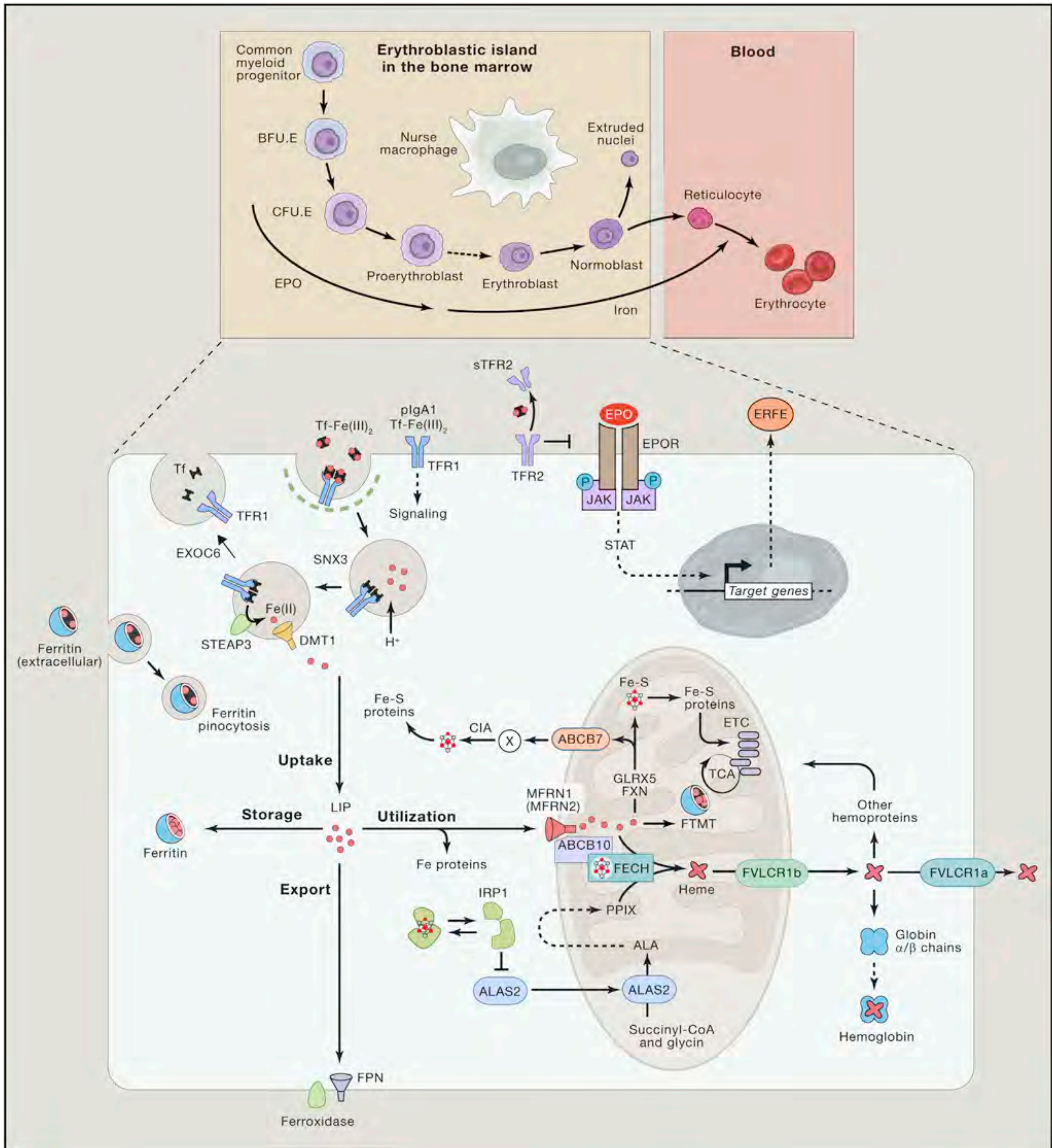

Figure 1. Iron Metabolism in Erythroid Cells

Top: In the bone marrow, macrophages are surrounded by erythroid cells at different stages of differentiation (erythroblastic islands). These macrophages support erythroid cell proliferation and maturation. The erythropoietic (EPO)- and iron-dependent phases of erythropoiesis are indicated. At steady state, most of the daily amount of iron needed for erythropoiesis is recycled by specialized macrophages in the spleen and liver.

Bottom: Iron acquisition in erythroid cells is dependent on endocytosis of diferric transferrin (Tf-Fe_2) via the transferrin receptor (TFR1). In acidified endosomes, iron is freed from Tf and exported into the cytoplasm by DMT1 after reduction of the metal by STEAP3 (six-transmembrane epithelial antigen of prostate 3). The recycling of Tf and TFR1 is crucial for optimal iron uptake and requires SNX3 (sortin nexin 3) and EXOC6 (exocyst complex component 6). Pinocytosis of extracellular ferritin may also contribute iron but cannot substitute for TFR1-mediated Tf-Fe_2 uptake. The binding of Tf-Fe_2 and/or pIgA1 (polymeric

(legend continued on next page)

dependent on the EXOC6 member of the exocyst protein complex (Figure 1; Lim et al., 2005).

Erythroid cells may also acquire iron by other means. Ferritin released from macrophages can enter erythroblasts by pinocytosis and support their differentiation in the absence of Tf in vitro (Leimberg et al., 2008). However, erythroid iron acquisition in vivo requires the Tf cycle, since profound iron-deficiency anemia ensues in patients or mice with atransferrinemia. Further illustrating the critical importance of Tf-Fe_2 uptake for erythropoiesis, partial or complete deficiency for other molecules of the Tf endocytic cycle also causes anemia (Bruno et al., 2015).

Intriguingly, TFR1 also has a role in intracellular signaling. In some cases mutations can show a selective role in B and T cells versus erythoid cells (Jabara et al., 2016); in another scenario, this function is not abrogated by mutations that impair TFR1 endocytosis (Coulon et al., 2011) or show an effect independently from its canonical role of binding Tf-Fe_2 (Chen et al., 2015). In erythroblasts, the binding of A1 isotype immunoglobulins to TFR1 potentiates the effect of erythropoietin (EPO) on erythroid development (Coulon et al., 2011). In *Drosophila* and in HeLa cells, TFR1 induces mitochondrial fragmentation via the JNK pathway even in the absence of iron in the milieu; this process is inhibited by modification of TFR1 by stearic acid and may link mitochondrial function to nutrient status (Senyilmaz et al., 2015). Physiologically, the signaling activities of TFR1 seem important for the maintenance of the intestinal epithelium (Chen et al., 2015). They may also help explain why TFR1-null mice that die in utero (Levy et al., 1999) display a more severe phenotype than atransferrinemic animals, which succumb shortly before weaning (Bernstein, 1987). The signaling functions of TFR1 are reminiscent of those of its homolog TFR2. TFR2 has a lower affinity for Tf and does not significantly contribute to iron import. Instead, it serves as a sensor of Tf saturation. It is highly expressed in erythroblasts, where it modulates EPO receptor signaling possibly to adjust RBC production to Tf-Fe_2 availability (Nai et al., 2015). In hepatocytes, TFR2 regulates hepcidin expression (see below).

Genetic ablation of TFR1 in mice demonstrates the importance of the Tf endocytic cycle for iron import into erythroid cells (Levy et al., 1999) as well as cardiomyocytes (Xu et al., 2015), muscle cells (Barrientos et al., 2015), and dopaminergic neurons (Matak et al., 2016); combined immunodeficiency in patients with a Y20H substitution in TFR1 also reveals a role of TFR1 in B and T cells (Jabara et al., 2016). However, most non-erythroid tissues can develop without TFR1 (Li et al., 2009; Ned et al., 2003), showing that Tf-independent routes of iron acquisition exist.

When the amount of iron exceeds the binding capacity of Tf, non-Tf-bound iron accumulates in the plasma and in parenchymal cells (see below). Cells also acquire heme iron. Heme-Responsive Gene 1 (HGR1; also known as SLC48A1) is the only known heme iron importer (Rajagopal et al., 2008). While it is expressed in erythroblasts (An et al., 2014), it is not clear whether it contributes to erythroid iron acquisition. Macrophages obtain heme indirectly from the phagocytosis of dying RBCs (see below).

Finally, other forms of protein-bound iron are taken up by receptor-mediated endocytosis. L-ferritin (FTL) and H-ferritin (FTH1), respectively, can enter the lysosomal compartment through the Scara5 (Scavenger receptor class A member 5) and TIM-2 (T Cell Immunoglobulin And Mucin Domain Containing 2) receptors (Chen et al., 2005; Li et al., 2009) in mouse cells; the TIM2 gene is absent from the human genome and human cells may instead take up FTH1 via TFR1 (Li et al., 2010b). Kidney cells have been reported to take up lipocalin 2 (also known as NGAL, siderocalin) bound to catechol-Fe(III) complexes (Bao et al., 2010) via the SLC22A17 (Solute Carrier Family 22 Member 17, also known as 24p3R) and megalin (also known as Low-Density Lipoprotein Receptor-Related Protein 2) membrane receptors (Devireddy et al., 2005; Mori et al., 2005). Free heme and hemoglobin arising from intravascular hemolysis are bound by the plasma molecules hemopexin and haptoglobin, respectively, and the complexes are cleared from the circulation by endocytosis via the CD91 and CD163 receptors. Different iron acquisition systems thus function in parallel with the ubiquitous Tf endocytic cycle. Some might contribute to normal (or pathological) erythroid iron acquisition but cannot substitute for the uptake of diferric Tf via TFR1.

Intracellular Iron Trafficking and Utilization

Iron taken up by cells enters a cytosolic pool termed the "labile iron pool" (Figure 1). The labile iron pool is destined for storage, export (see below), or metabolic utilization. In the cytoplasm, elemental iron is used by diverse Fe(II)-dependent proteins, apparently helped by poly(rC)-binding proteins (PCBPs) that facilitate iron loading onto the hypoxia-inducible factor (HIF) prolyl and asparginyl hydroxlases (Nandal et al., 2011), and the deoxyhypusine hydroxylase (Frey et al., 2014). However, most of the labile iron pool is routed to mitochondria, where it is incorporated into heme and Fe-S clusters. Of note, iron acquired from Tf-Fe_2 endocytosis may be transferred from endosomes to mitochondria by "kiss-and-run" via direct inter-organellar contacts (Hamdi et al., 2016), although the relative quantitative contribution of this pathway to mitochondrial iron acquisition remains to be clarified (Shvartsman and Ioav Cabantchik, 2012).

immunoglobulin A1) to TFR1 triggers signaling events independent of iron uptake. TFR2 is involved in sensing plasma Tf-Fe_2 concentration and in turn modulates erythropoietin receptor (EPOR) signaling.

The metabolically active labile iron pool is used directly for incorporation into iron proteins or transported into mitochondria via mitoferrin 1 (MFRN1, MFRN2 in non-erythroid cells), which forms a complex with ABCB10. In mitochondria, iron is inserted into protoporphyrin IX (PPIX) by ferrochelatase (FECH) to produce heme. Heme is transported outside of mitochondria via the 1b isoform of FLVCR (feline leukemia virus subgroup C cellular receptor) for incorporation into hemoproteins, mostly hemoglobin; the FLVCR1a isoform exports heme. Iron is also used for Fe-S cluster synthesis, which among other factors involves frataxin (FXN) and GLRX5 (Glutaredoxin-related protein 5). Fe-S clusters are incorporated into client proteins throughout the cell. Maturation of cytosolic Fe-S proteins by the CIA (cytosolic Fe-S cluster assembly) system depends on an unknown compound (designated X) generated during mitochondrial Fe-S cluster biosynthesis. Its export into the cytosol requires the ATP-binding cassette protein ABCB7. Fe-S proteins play key roles in the regulation of iron metabolism. For instance, under conditions of iron or GLRX5 deficiency, iron regulatory protein 1 (IRP1) is devoid of a Fe-S cluster and inhibits translation of ALAS2, the first and rate-limiting enzyme of heme biogenesis, thereby preventing the accumulation of toxic heme intermediates.

In the mitochondria, excess iron can be stored in the organelle-specific form of ferritin (FTMT). In the cytosol, excess iron is sequestered within heteropolymers of ferritin H and L chains. Cellular iron efflux is mediated by ferroportin (FPN) and requires iron oxidation on the extracellular side.

The molecular nature of the labile iron pool and its transport to mitochondria is one of the least well understood aspects of iron cell biology. Glutathione-Fe(II) conjugates have been proposed to be a major component of the labile pool (Hider and Kong, 2011) but may not play a major role in erythroid iron trafficking (Shvartsman and Ioav Cabantchik, 2012). Gentisic acid (GA) was identified as a potential mammalian siderophore critical for iron delivery to mitochondria. Inhibition of GA synthesis impairs heme and hemoglobin synthesis in zebrafish embryos (Devireddy et al., 2010), associated with delayed erythroid maturation (Davuluri et al., 2016). However, GA depletion results in relatively mild anemia in mice (Liu et al., 2014) and does not alter mitochondrial iron supply in erythroleukemia cells (Shvartsman and Ioav Cabantchik, 2012), indicating the existence of GA-independent iron trafficking mechanisms.

While iron trafficking to mitochondria is still undefined, molecules that facilitate iron influx into the organelle are known. Mitoferrin 1 (MFRN1, also known as SLC25A37) is the main mitochondrial iron importer in developing erythroblasts (Shaw et al., 2006). It is stabilized at the inner mitochondrial membrane by ABCB10 (ATP-Binding Cassette Sub-Family B Member 10) (Chen et al., 2009), and deficiency for either MFRN1 or ABCB10 causes severe anemia (Chung et al., 2014; Troadec et al., 2011; Yamamoto et al., 2014). The MFRN1 paralog mitoferrin 2 (MFRN2, SLC25A28) is ubiquitously expressed and mediates mitochondrial iron import into non-erythroid tissues (Shaw et al., 2006).

Within mitochondria, iron is used for conjugation with protoporphyrin IX (PPIX) to form heme. Heme is then exported to the cytoplasm by the mitochondrial isoform of the Feline Leukemia Virus Subgroup Receptor 1 (FLVCR1, also known as Mfsd7b) (Chiabrando et al., 2012) for incorporation into hemoglobin and other hemoproteins (reviewed by Hamza and Dailey, 2012); like iron, heme trafficking/chaperoning and delivery to target proteins is still poorly understood.

Iron is also delivered to specific scaffold proteins, probably with the help of frataxin (FXN), for de novo synthesis of Fe-S clusters that are then inserted into their client apoproteins. Nonetheless, FXN may have additional roles independent of iron metabolism (Busi and Gomez-Casati, 2012). The synthesis of Fe-S clusters and the maturation of Fe-S cluster proteins are complex and occur both in mitochondria and in the cytoplasm (see Lane et al., 2015, and Stehling et al., 2014, for comprehensive reviews); however, Fe-S cluster synthesis in the cytoplasm requires one or more compounds whose production in mitochondria and export into the cytosol are dependent on glutaredoxin 5 (GLRX5) and ABCB7 (ATP-Binding Cassette Subfamily B Member 7), respectively. Importantly, the heme and Fe-S cluster biosynthetic pathways intersect. Ferrochelatase (FECH), the terminal enzyme in heme synthesis responsible for the metalation of PPIX, is itself an Fe-S cluster protein (Wu et al., 2001). Furthermore, ALAS2 (∂-aminolevulinic acid synthase 2 that catalyzes the first and rate limiting step of heme synthesis in erythroid cells) mRNA translation is repressed by iron regulatory protein (IRP) 1 when deprived of its Fe-S cluster (see below). Impaired Fe-S cluster biogenesis in the GLRX5-deficient zebrafish mutant *Shiraz* and in patients causes anemia by hyperactivation of IRP1 and inhibition of ALAS2 translation (Wingert et al., 2005; Ye et al., 2010). The dependence of the proximal step of heme synthesis on Fe-S clusters formation may represent a stratagem to prevent the accumulation of toxic protoporphyrin intermediates when mitochondrial iron assimilation is insufficient (Chung et al., 2014). Non-erythroid cells express ALAS1. Contrary to ALAS2, ALAS1 is not subject to IRP regulation and is instead feedback regulated by heme itself.

Iron Export

Cells export elemental iron in its ferrous state via Ferroportin (FPN, also known as SLC40A1) (reviewed by Drakesmith et al., 2015). Biocomputational models predict a FPN topology with 12 transmembrane helices, with both the C and N termini of the protein facing the cytoplasm. The crystal structure of BbFPN, a putative bacterial homolog of FPN, suggests that the protein is divided into two halves forming two lobes separated by a cytosolic loop (Taniguchi et al., 2015). The two lobes form a central cavity with the iron-binding site. Depending on the spatial organization of the two lobes, the central cavity is either facing the intracellular space and is not accessible from the outside or is open to the extracellular space and cannot be accessed from the intracellular side. Transition between these inward- and outward-facing conformations could account for the iron export activity of FPN (Figure 2B).

Mammalian FPN is particularly abundant in cells that maintain plasma iron levels, namely duodenal enterocytes, macrophages, and hepatocytes (see below). Genetic disruption of FPN in these cell types indicates that FPN is their only path to export elemental iron (Donovan et al., 2005; Zhang et al., 2011). FPN also seems important for local iron management by cells that do not contribute to plasma iron, such as cardiomyocytes (Lakhal-Littleton et al., 2015). While FPN transports ferrous iron, efficient iron export requires its extracellular oxidation. Three multi-copper iron oxidases with distinct expression patterns are known: ceruloplasmin (CP), hephaestin (HEPH), and zyklopen (HEPHL1). Deficiency for CP and/or HEPH causes cellular iron retention and iron-restricted anemia in patients and/or mice. Zyklopen is expressed in placental cells and is thought to play a role in fetal iron supply (Chen et al., 2010); zyklopen (*Hephl1*) mouse models shall help address this possibility.

Although they require large amounts of iron for heme synthesis, erythroid cells paradoxically express FPN (Cianetti et al., 2005; Zhang et al., 2009). Whether iron export helps to purge excess iron during erythroid development and/or participates in maintaining plasma iron levels to prevent iron deficiency in other tissues is not known. Similarly, erythroid precursors express FLVCR, and the protein is required for optimal erythroid development (Doty et al., 2015; Mercurio et al., 2015). As for iron and FPN, heme export may serve in heme detoxification (and/or intercellular heme transport).

Iron Storage

Iron from the labile iron pool that is not utilized or exported is stored within a cytosolic heteropolymer made of 24 subunits of heavy (FTH1) and light (FTL) ferritin chains. FTH1 and FTL are ubiquitous, but their expression ratios vary between tissues and in response to physiological conditions. FTH1 displays ferroxidase activity required for iron mineralization into the ferritin nanocage, each of which can accommodate up to 4,500 iron atoms, while the enzymatically inactive light chain promotes

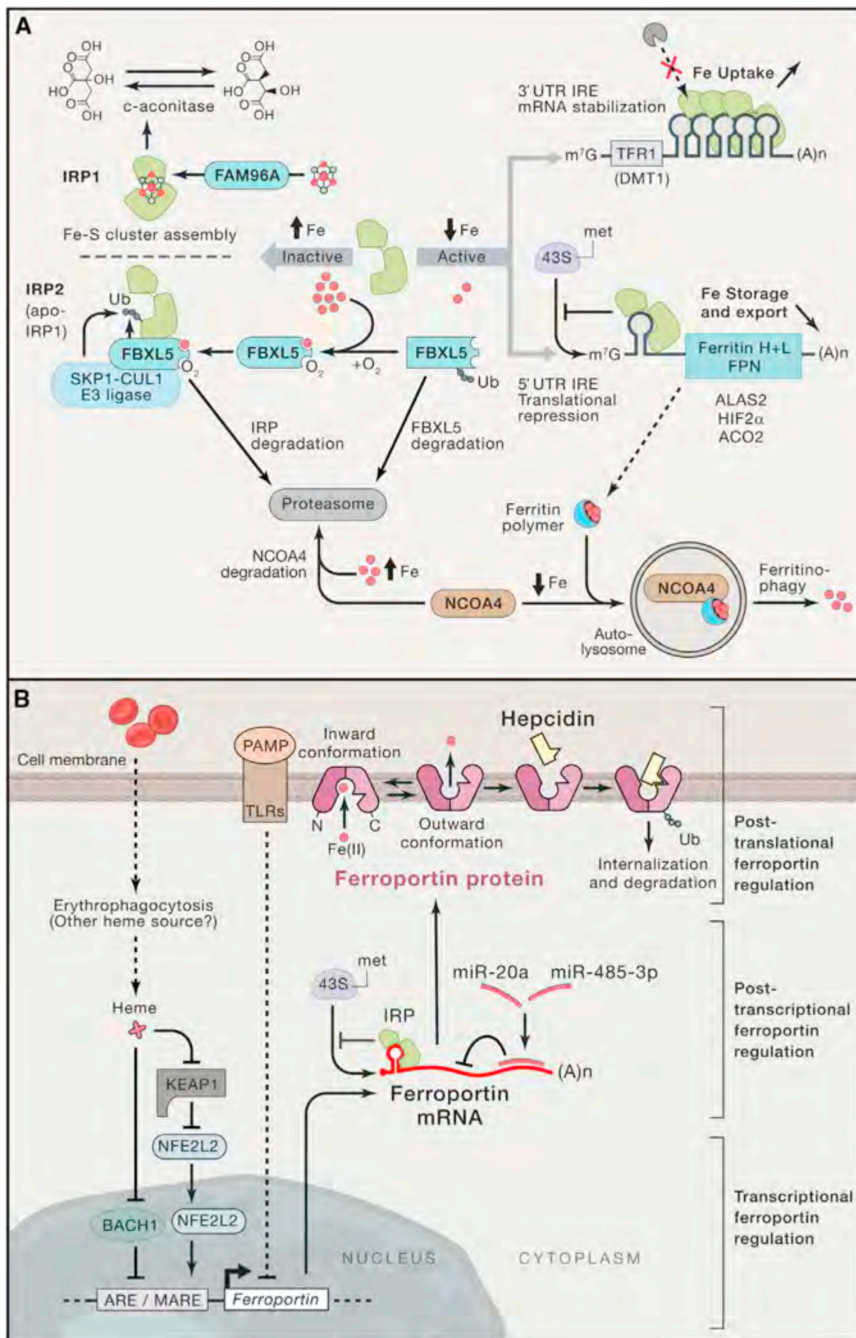

Figure 2. Regulation of Cellular Iron Metabolism

(A) In iron-deficient cells (right), IRP1 or IRP2 bind to *cis*-regulatory iron-responsive elements (IREs) present in the untranslated regions (UTRs) of target mRNAs. The binding of IRPs to single IREs in the 5′ UTRs of ferritin and FPN mRNAs inhibits their translation, whereas IRP interaction with multiple 3′ UTR IREs in the TFR1 mRNA inhibits its decay; ferritin protein is targeted to autolysomal degradation by NCOA4 (Nuclear receptor co-activator 4) to release iron. The ensuing increase in TFR1-mediated iron uptake and decrease in iron storage in ferritin and export via FPN augment the labile iron pool. In iron-loaded cells (left), IRP binding to IREs is suppressed. IRP1 assembles a cubane Fe-S cluster with the assistance of FAM96A and adopts a conformation that precludes IRE binding while conferring aconitase activity to interconvert citrate and isocitrate. IRP2 lacks a Fe-S cluster and is instead regulated by interaction with FBXL5, which bears an iron- and oxygen-sensing domain. FBXL5 accumulates in iron-replete cells and triggers the proteasomal degradation of IRP2 by recruiting a SKP1-CUL1 E3 ubiquitin ligase complex. In parallel, NCOA4 is destabilized and ferritin accumulates. IRPs also modulate the translation of the mRNAs that encode the erythroid-specific ALAS2 enzyme involved in heme biosynthesis, mitochondrial aconitase (ACO2), and of the transcription factor HIF2α. A single 3′ UTR IRE motif is present in the DMT1 mRNA, but its mechanism of action is not fully resolved.

(B) FPN is subject to multilayered regulation in macrophages. *FPN* transcription is suppressed by BACH1 (Btb and Cnc Homology 1) and stimulated by NFE2L2 (Nuclear Factor Erythroid 2-like). BACH1 and NFE2L2, respectively, bind MARE (Maf recognition elements) and ARE (Antioxidant Response Elements) cassettes in the *FPN* promoter. Heme derived from erythrophagocytosis stimulates *FPN* transcription by alleviating the inhibitory effect of BACH1 and by promoting the dissociation of NFE2L2 from KEAP1 (Kelch-like ECH-associated protein 1). The binding of specific pathogen-associated molecular patterns (PAMPs) to toll-like receptors (TLRs) leads to suppression of *FPN* transcription; the exact signaling events between TLRs and *FPN* are not known. Once transcribed, the FPN mRNA is regulated by the IRPs (see above) and the mIR-485-3p and miR-20a microRNAs; mIR-485-3p is induced by iron deficiency. At the plasma membrane FPN exports iron, possibly by switching between cytoplasmic-versus extracellular space-facing conformations. Hepcidin inhibits cellular iron efflux by binding to FPN, which leads to ubiquitination and degradation of the iron transporter.

the transfer of electrons across the protein shell of the polymer (Carmona et al., 2014). Iron entry into ferritin is facilitated by PCBPs in vitro as well as in engineered yeast models and mammalian cell lines (see above and Leidgens et al., 2013; Shi et al., 2008), but the role of PCBPs in iron management in vivo has not been addressed yet.

While the *Fth1* gene is essential in mice (Ferreira et al., 2001), a patient with a loss-of function mutation in the FTL gene suffering from idiopathic generalized seizures and atypical restless leg syndrome has been described (Cozzi et al., 2013). Frameshift mutations altering the FTL C terminus cause neurodegeneration with brain iron accumulation (recently reviewed by Levi and Rovida, 2015), and mutations in its 5′ mRNA leader (see below) result in the dominantly inherited hyperferritinemia-cataract syndrome.

A mitochondrial form of ferritin is expressed in some cell types. The protein accumulates in erythroblasts from patients with sideroblastic anemia, and may protect mitochondria against

iron-mediated toxicity (Levi et al., 2001); however, it is dispensable for baseline erythropoiesis in mice (Bartnikas et al., 2010).

Ferritin stores iron in a non-toxic form and contributes to intracellular iron bioavailability. Iron retention in ferritin can inhibit the passage of iron across absorptive enterocytes (Galy et al., 2013; Vanoaica et al., 2010) and activates the iron- and O_2-dependent HIF signaling pathway even under normoxic conditions (Siegert et al., 2015). Iron sequestered in ferritin constitutes a store that can be mobilized by ferritin degradation. In iron-deficient cells, NCOA4 (Nuclear Receptor Coactivator 4) interacts with FTH1 and targets the ferritin complex for degradation into autolysosomes, a process called "ferritinophagy" (Figure 2A); ferritinophagy is conversely suppressed in iron-loaded cells due to increased NCOA4 turnover (Dowdle et al., 2014; Mancias et al., 2014, 2015). NCOA4 is highly expressed during terminal erythroid differentiation (An et al., 2014) and is important for hemoglobin synthesis in cultured erythrocytes and zebrafish embryos (Mancias et al., 2015). Lack of ferritinophagy in NCOA4-null mice leads to ferritin accumulation and iron-deficiency anemia. This anemia is best explained by iron retention within ferritin, reducing iron availability in erythroblasts and/or a decreased iron release from other cells that help sustain systemic iron levels (Bellelli et al., 2016). Future work will indicate whether hereditary forms of anemia are linked to *NCOA4*.

Destruction of Senescent RBCs and Iron Recycling

Erythroid cells maintain an intimate relationship with macrophages from birth to death (de Back et al., 2014). Macrophages promote erythrocyte development within erythroblastic islands, especially under stress conditions (see below; Chow et al., 2013; Ramos et al., 2013). After ~120 and ~40 days in humans and mice, respectively, senescent RBCs are phagocytosed by specialized macrophages found mostly in the bone marrow, liver, and spleen (de Back et al., 2014). When the number of damaged red cells is increased (i.e., hemolytic anemia), macrophages in the liver contribute heavily to erythrocyte disposal and iron recycling, helping to prevent damage from uncontrolled iron, heme, and hemoglobin organ deposition (Theurl et al., 2016). RBC-containing phagosomes fuse with lysosomal vesicles to form erythrophagolysosomes, where RBCs are degraded. After the breakdown of hemoglobin, heme is exported into the cytosol by the heme transporter HRG1 (White et al., 2013). In the cytosol, iron is released from protoporhyrin by heme oxygenase 1 (HMOX1) and either stored in ferritin or exported for reuse by FPN. Of note, heme itself can trigger the differentiation of monocytes into iron recycling macrophages by activating the heme-binding transcriptional repressor BACH1 (Btb and Cnc Homology 1) and subsequent induction of a SPI-C-transcription factor-regulated cell differentiation program (Haldar et al., 2014). Because dietary iron absorption alone cannot sustain erythropoiesis (see below), the recycling of heme iron by HMOX1 (Kovtunovych et al., 2014) and its release by FPN (Zhang et al., 2011) in macrophages are critical for sustaining physiological erythropoiesis.

Regulation of the Cellular Iron Balance

Erythroid cells require mechanisms to coordinate the production of heme with iron availability. Central aspects of iron homeostasis in immature erythroid precursors and in most cells of the body are orchestrated posttranscriptionally by the RNA-binding proteins (RBPs) IRP1 (also known as ACO1) and IRP2 (also known as IREB2) (Figure 2A; reviewed by Wilkinson and Pantopoulos, 2014). These two orthologous RBPs control the fate of mRNAs encoding key iron metabolism proteins by interacting with *cis*-regulatory RNA hairpin structures, iron-responsive elements (IREs). Either of the two IRPs inhibits translation initiation when bound to the IRE located in the 5′ untranslated region (UTR) of the mRNAs encoding FTH1, FTL, FPN, ALAS2, or mitochondrial aconitase (ACO2); the mRNA encoding the transcription factor HIF2α (hypoxia inducible factor 2α, also known as EPAS1) seems to respond predominantly to IRP1 (see below). IRP binding to multiple IREs within the 3′ UTR prevents endonucleolytic cleavage of the TFR1 mRNA and its subsequent degradation. The IRPs also appear to stimulate DMT1 mRNA expression via a single 3′ UTR IRE; DMT1 regulation by the IRPs seems context dependent (Galy et al., 2013) and is mechanistically not understood. Intriguingly, some isoforms of the DMT1 and FPN mRNAs lack the IRE and thus escape IRP regulation (Cianetti et al., 2005; Gunshin et al., 1997; Zhang et al., 2009). Although FPN expression is partly repressed by the IRPs in the intestine (Galy et al., 2008, 2013), the expression of a FPN mRNA isoform lacking the IRE would in principle allow enterocytes to export iron under conditions of cellular iron deficiency and high IRP activity (Zhang et al., 2009).

A typical IRE consists of a hexanucleotide apical loop (5′-CAGWGH-3′) on a stem of five paired nucleotides, an unpaired cytosine on the 5′ strand of the stem, and an additional lower stem of variable length; the HIF2α and DMT1 IREs harbor an unpaired 3′ bulged nucleotide in the upper stem. An as yet unresolved key question is the scope of the IRP regulon. In vitro affinity chromatography experiments uncovered the existence of multiple IRP-binding mRNAs from different cell types and mouse tissues (Sanchez et al., 2011), but whether and how they are subject to IRP regulation in vivo remains to be defined.

IRP binding to IREs is regulated primarily by cellular iron availability, although the IRPs do not sense iron fluctuation directly. In iron-replete (and oxygenated) cells, IRP2 (and the apo form of IRP1, see below) interacts with FBLX5 (F-Box And Leucine-Rich Repeat Protein 5), which recruits an E3 ligase complex promoting IRP ubiquitination and proteasomal degradation (Salahudeen et al., 2009; Vashisht et al., 2009). FBXL5 is regulated in a reciprocal manner: it is stabilized in iron-loaded cells and degraded by the proteasome when iron is scarce. Iron sensing lies in the iron-binding N-terminal hemerythrin-like (Hr) domain of FBXL5. When not bound by iron, the Hr domain adopts a conformation that promotes FBXL5 polyubiquitination and degradation, leading to IRP2 accumulation (Thompson et al., 2012). As FBXL5-mediated degradation of IRP2 requires oxygen, IRP2 also accumulates in hypoxia. The control of IRP2 by FBXL5 is essential and aberrant gain of IRP2 function in $FBXL5^{-/-}$ mice is lethal (Moroishi et al., 2011).

IRP1 (but not IRP2) is most prominently regulated by an Fe-S cluster switch. In iron-loaded cells, Fe-S clusters produced by the cytoplasmic assembly machinery are delivered to apo-IRP1 by FAM96A (Family With Sequence Similarity 96 Member A) (Stehling et al., 2013). Ligation of the Fe-S cluster confers aconitase activity to the holo-protein and precludes IRE binding. Upon iron starvation, IRP1 loses its Fe-S cluster and enzymatic

activity and adopts its IRE-binding conformation (Walden et al., 2006). In contrast to IRP2, low-oxygen conditions stabilize holo-IRP1, decreasing IRP1 binding to IREs. As mentioned above, loss-of-function mutations in general components of Fe-S cluster biogenesis (FXN, GLRX5, ABCB7) hyperactivate IRP1. While this may inhibit heme synthesis as seen in GLRX5 deficiency (see above), IRP1 activation increases the lifespan of mice with hepatic FXN deficiency, suggesting that it can be beneficial in other contexts (Martelli et al., 2015).

Mouse embryos lacking both IRP1 and IRP2 die in utero, showing that the IRP/IRE system is essential for early development. Stage- and/or cell-specific co-ablation of both proteins further uncovered important roles of the IRPs in hepatocytes and enterocytes (reviewed by Wilkinson and Pantopoulos, 2014) as well as in macrophage-mediated immunity (Nairz et al., 2015). IRP mouse models also revealed key functions in erythropoiesis. In spite of normal serum iron levels, IRP2-null mice have small erythrocytes containing less hemoglobin (hypochromic microcytic anemia) and increased porphyrin levels compared to controls. Most likely, IRP2 secures TFR1-mediated iron acquisition and prevents unwanted production of heme intermediates by blockade of ALAS2 translation. IRP function may be particularly important during early erythroid development, but predictions of the IRP/IRE paradigm appear not to be met during terminal erythropoiesis (Schranzhofer et al., 2006). IRP1-null animals, on the other hand, present with increased red blood cell counts and extramedullary hematopoiesis during early life. This effect is secondary to derepression of HIF2α translation in kidney and liver and ensuing stimulation of EPO (Anderson et al., 2013a; Ghosh et al., 2013; Wilkinson and Pantopoulos, 2013). The control of HIF2α by IRP1 may allow adjustment of the rate of erythropoiesis to iron availability, and thereby prevent the production of faulty RBCs.

In spite of its relevance, the IRP/IRE system is not the sole conductor of cellular iron homeostasis, and iron metabolism is subject to multilayered regulation. An excellent example of this is *FPN*, which responds to iron signals at the transcriptional (via BACH1/NRF2 or HIF2), posttranscriptional (such as through miR-485-3p), translational (via the IRPs), and posttranslational (via hepcidin) levels (see below) (Figure 2B). Different iron regulatory mechanisms act in concert and intersect. For example, HIF2α and IRPs have common target genes (for example *Fpn* and *Dmt1*), and HIF2α itself is controlled by the IRPs. Also, the IRPs and NCOA4 may converge to modulate ferritin synthesis and turnover, respectively, for fine-tuned control of iron storage (Figure 2A). A key challenge for the future is to understand when and where these regulatory mechanisms are at work and how they intersect.

Controlling Supply and Demand: Iron Acquisition for Red Blood Cells

Erythrocytes satisfy their iron demands by receptor-mediated endocytosis of iron-bound transferrin, which needs to be maintained in a physiological range between 16% and 45%. The daily iron requirement is met by FPN-mediated export from intestinal enterocytes (1–2 mg), iron-recycling macrophages (20–25 mg), and iron storage tissues and is regulated by the hormone hepcidin (Figure 3). The supply of and demand for iron are affected by hypoxia, inflammation and infection, and genetic or nutritional iron deficiency and in response to abnormal erythropoiesis.

Dietary Iron Absorption by Duodenal Enterocytes

For lack of regulated hepatic or renal iron excretion, body iron levels are controlled solely by intestinal iron absorption. Following initial ferrokinetic studies, the responsible iron intestinal transporters were discovered during the last two decades. In severe iron deficiency, iron absorption can increase up to 15-fold (Pootrakul et al., 1988). Dietary non-heme iron, typically ferric (Fe(III)), is reduced by the ferrireductase Dcytb to ferrous iron (Fe(II)) for transport across the apical membrane of enterocytes mainly via DMT1 (Fleming et al., 1997). Control of enterocytic iron transfer involves ferritin, because mice with an intestinal ferritin H gene deletion show increased intestinal iron absorption and iron overload (Vanoaica et al., 2010). Once at the basolateral side, iron is exported into the bloodstream via FPN (Donovan et al., 2000; McKie et al., 2000). The trans-membrane ferroxidase hephaestin co-localizes with FPN and oxidizes Fe(II) to Fe(III), which then binds to serum transferrin (Fuqua et al., 2014). While the intestinal absorption of non-heme iron is now understood in some detail, mechanisms involved in dietary heme uptake require further investigation using animal models that, unlike mice, significantly absorb dietary heme.

Iron Release from Stores

Most iron required for erythropoiesis is recycled by macrophages from aged or damaged red blood cells. If recycling cannot satisfy the demand, iron stored in hepatocytes is released. By contrast, when serum iron is not used for erythropoiesis, it is stored in the liver for later use. Hepatic iron deposition can physiologically range between 300 mg and 1 g, but reaches up to 25–30 g in patients suffering from hereditary hemochromatosis (Pietrangelo, 2016). Hepatocytes can take up Tf-Fe_2 via TFR1 and store iron bound to ferritin. On demand, stored iron is mobilized from hepatocytes by release from ferritin through NCOA4-mediated ferritinophagy (see above).

When serum iron levels exceed the buffering capacity of transferrin at about 60% saturation, non-transferrin-bound iron appears. Non-transferrin-bound iron is imported into hepatocytes via SLC39A14 (Solute Carrier Family 39 Member 14, also termed ZIP14) (Liuzzi et al., 2006). *Slc39a14*-deficient mice display strongly reduced uptake of non-transferrin-bound iron by the liver and pancreas, directing it to extra-hepatic tissues such as the kidney (Jenkitkasemwong et al., 2015).

Hypoxia, Iron, and Control of Erythropoiesis

Exposure to high altitude, severe blood loss, or the production of too few or malfunctioning erythrocytes cause hypoxia, which the bone marrow aims to compensate by increased erythropoiesis. EPO release by the kidney triggers red blood cell proliferation and terminal differentiation. Under hypoxic conditions, EPO transcription is augmented in peritubular fibroblasts of the renal cortex by binding of HIF-2 heterodimers to hypoxia-responsive elements (HRE) of the *EPO* gene (Kuhrt and Wojchowski, 2015).

In the presence of O_2, Fe(II) and 2-oxoglutarate, HIF-1α and HIF-2α are hydroxylated by prolyl hydroxylases (PHDs). The hydroxylated HIFs associate with the von Hippel-Lindau tumor suppressor protein (pVHL), generating a complex polyubiquitinated by an E3 ligase for proteasomal degradation. Under hypoxic conditions, PHDs lack the essential O_2 and HIF-2α

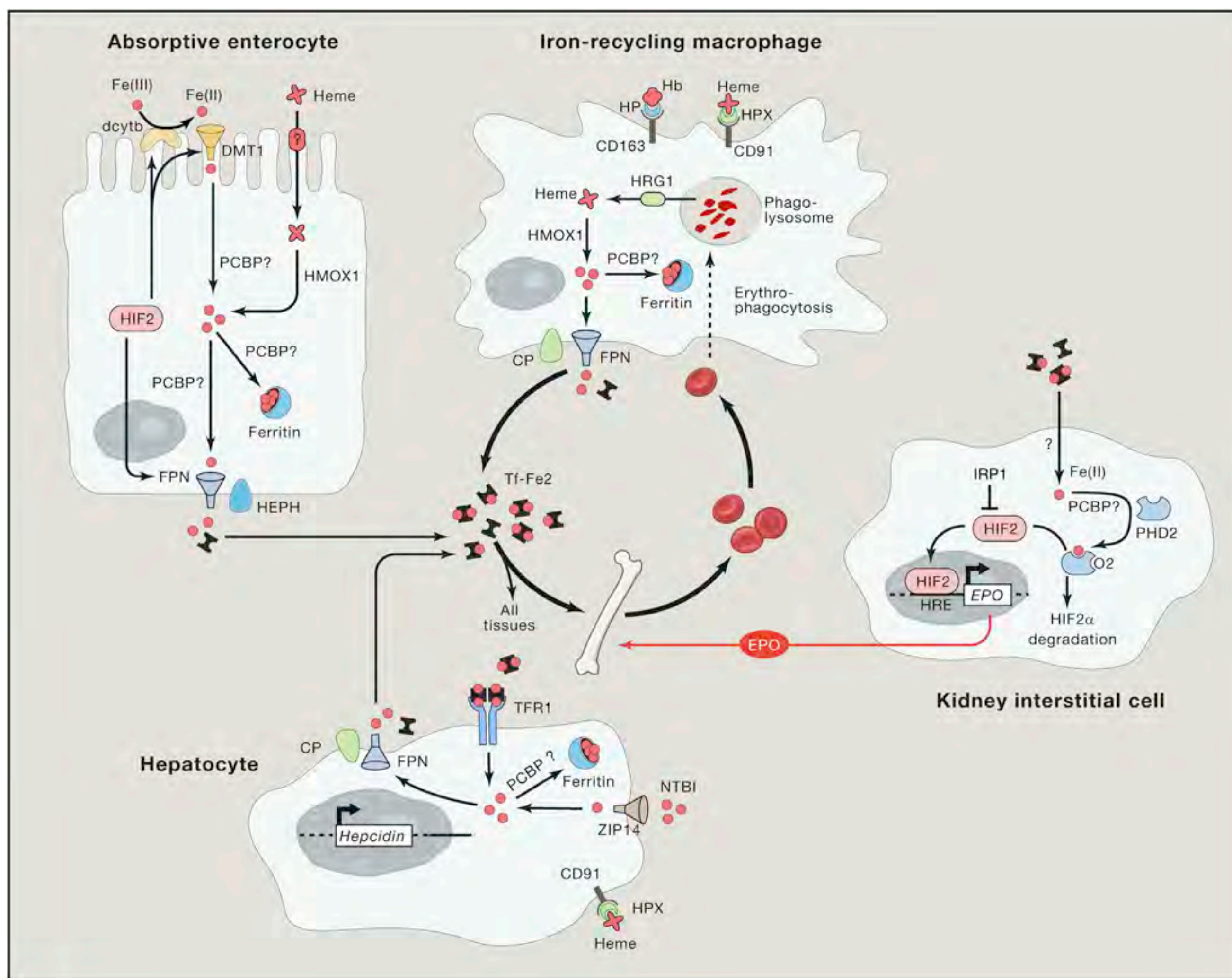

Figure 3. Systemic Iron Homeostasis

Enterocytes take up elemental iron from the intestinal lumen via DMT1 after reduction of the metal by e.g., Dcytb at the apical membrane. Dietary heme is taken up via an unknown transport system and degraded inside the cell by heme oxygenase 1 (HMOX1). The export of iron into the bloodstream via FPN and its loading onto Tf requires oxidation of the metal by hephaestin (HEPH) at the basolateral side. Iron passage across the enterocyte is transcriptionally stimulated by the hypoxia-inducible factor 2α (HIF2α) and inhibited through iron sequestration in ferritin. Whether PCBP is involved as an iron-binding chaperone during iron transport is unclear.

Diferric transferrin (Tf-Fe_2) is delivered to tissues via the bloodstream and is mostly used for the hemoglobinization of new red blood cells (RBCs). Specialized tissue macrophages engulf and degrade senescent RBCs to release heme into the phagolysosome. Heme is then exported into the cytoplasm by the heme transporter HRG-1 (SLC48A1) and processed by HMOX1 to release iron, which is either stored in ferritin or exported into the circulation via FPN with the assistance of the ceruloplasmin (CP) ferroxidase. Iron utilization by the erythroid marrow and its recycling by RES macrophages account for the major iron fluxes in the body. If not used, iron can be stored in hepatocytes. Hepatocytes can acquire Tf-Fe_2 and non-transferrin-bound iron, respectively, via TFR1 and ZIP14. Hepatocytes store iron in ferritin and they also express FPN and CP for iron export. Heme and hemoglobin (HB) present in the circulation interact with hemopexin (HPX) and haptoglobin (HP), respectively, and are scavenged via the CD163 and CD91 receptors.

Peritubular fibroblasts of the kidney sense iron and oxygen deficiency and release EPO to enhance erythropoiesis. When bound to iron and oxygen, prolyl hydroxylase 2 (PHD2) normally triggers the degradation of the HIF2α; iron supply to PHD2 may be facilitated by Poly(rC)-binding proteins (PCBP). Low iron and/or oxygen conditions inactivate PHD2, leading to HIF2α accumulation and stimulation of *EPO* transcription. In addition to PHD2, HIF2α is also translationally inhibited by IRP1.

accumulates in the nucleus recruiting HIF-1β, which itself is constitutively expressed and stable (Haase, 2010). Importantly, lack of iron also inhibits the PHD proteins, plausibly explaining how iron chelators promote EPO expression in cultured cells (Wang and Semenza, 1993) as well as in humans (Salvarani et al., 1996). Since excessive EPO synthesis in sustained iron deficiency would be detrimental, IRP1 can inhibit Hif-2α mRNA translation (see above).

When erythropoiesis intensifies, dietary iron absorption and iron release from stores must increase. For some years, it was postulated that a soluble factor released from the erythron would communicate the increase in iron demands to decrease hepcidin

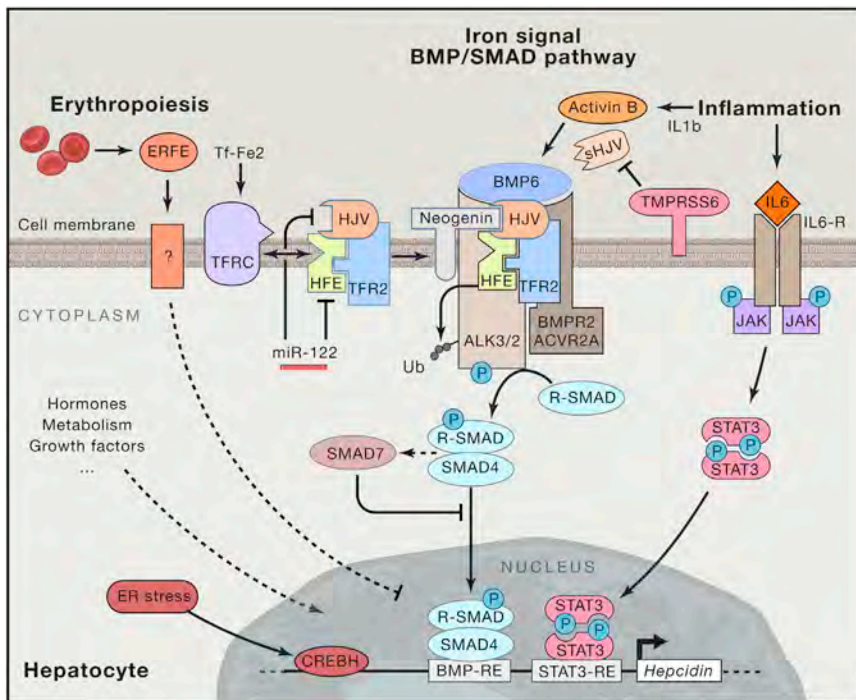

Figure 4. Pathways Involved in *Hepcidin* Regulation

Hepcidin expression is regulated by iron signals (middle), erythropoiesis (left), and inflammation (right).

High iron stores augment BMP6 (bone morphogenetic protein 6), which together with its co-receptor hemojuvelin (HJV) activates type 1 (Alk2/3) and type 2 (BMPR2, ACVR2A) BMP serine threonine kinase receptors, leading to phosphorylation of receptor-activated SMAD (R-SMAD) proteins and formation of active transcriptional complexes with SMAD4. The HJV-BMP interaction may be facilitated by neogenin. High concentrations of Tf-Fe_2 displace HFE from TFR1, which then forms a complex with TFR2 and HJV to promote BMP/SMAD signaling to *Hepcidin*. HFE additionally interacts with ALK3 and prevents its ubiquitination (Ub) and degradation. BMP/SMAD signaling to *Hepcidin* is suppressed by matriptase 2 (TMPRSS6), a serine protease that cleaves and generates a soluble form of HJV (sHJV). The pathway is also feedback inhibited by SMAD7 and dampened by the miR-122 microRNA, which lowers HJV and HFE expression.

In inflammation, the interaction of interleukin 6 (IL-6) with its receptor (IL-6R) activates JAK tyrosine kinases, triggering the formation of STAT3 (Signal transducer and transcription activator 3) complexes that bind to the *Hepcidin* promoter in the nucleus. *Hepcidin* stimulation by activin B is dependent on the BMP/SMAD signaling pathway.

High erythropoietic activity increases plasma levels of ERFE, which suppresses *Hepcidin* via as yet unknown signaling pathways.

Additional signals modulate *Hepcidin* expression. These include protein misfolding (ER stress), which activates *Hepcidin* transcription through CREBH (cyclic AMP-responsive element-binding protein 3-like protein 3, also known as CREB3L3), as well as hormones/growth factors and various metabolic cues.

The STAT3- and BMP-response elements (REs) in the *Hepcidin* promoter are depicted.

expression. Interestingly, hepcidin repression under these conditions depends on EPO and a functional bone marrow (Pak et al., 2006). Recently, an EPO-responsive "erythroid regulator" of hepcidin, named erythroferrone (ERFE, also known as FAM132B), was identified (Kautz et al., 2014). EPO increases ERFE secretion by erythroblasts via the JAK2/STAT5 pathway (Kautz et al., 2014). ERFE-deficient mice ($Erfe^{-/-}$) fail to suppress hepcidin in response to hemorrhage or EPO injections (Kautz et al., 2014). In humans, the role of ERFE still awaits clarification. ERFE may not be the only factor that controls hepcidin suppression under conditions of increased erythropoiesis (Kautz et al., 2015). GDF15 (Growth Differentiation Factor 15) has been implicated (Tanno et al., 2007), but its role has also been questioned (Casanovas et al., 2011). Human volunteers subjected to exercise under hypoxic conditions show elevated concentrations of the platelet-derived growth factor (PDGF)-BB, which inversely correlate with hepcidin levels. Mice injected with PDGF-BB suppress hepcidin and raise plasma iron (Sonnweber et al., 2014).

In addition, proteins associated with iron sensing in the liver are also expressed in erythroid cells. TFR2 regulates RBC production by direct interaction with the EPO receptor, modifying EPO sensitivity in an iron-dependent manner (Forejtnikovà et al., 2010; Nai et al., 2015). HFE also modulates erythroid iron homeostasis (Ramos et al., 2011). EPO may control IRP2 expression in erythroid cells, possibly coordinating cellular activity with erythroid iron intake (Kerenyi et al., 2008). In addition, hypoxia controls iron absorption directly in duodenal enterocytes, stabilizing Hif2, which in turn increases the transcription of genes involved in iron acquisition, including *Dcytb*, *Dmt1*, and *Fpn* (Anderson et al., 2013b; Mastrogiannaki et al., 2009; Taylor et al., 2011). Thus, links between the cellular and systemic iron control systems and oxygen sensing operate at multiple levels, ensuring that iron supplies are adequate for erythropoiesis (Gassmann and Muckenthaler, 2015). Future research will define the molecular details and hierarchy of these links.

Inflammation/Infection

Anemia of inflammation is a multifactorial, acquired disorder of iron homeostasis associated with infections, malignancies, and other causes of inflammation (Ganz and Nemeth, 2015). The causes for these mild to moderate anemias are multifactorial, including the diminished proliferative capacity of erythroid progenitor cells and the removal of iron-containing hemoproteins from the circulation. Iron retention within macrophages due to decreased iron export are hallmarks of this disease.

During inflammation, high plasma hepcidin levels are induced via the IL (interleukin)-6/STAT3 pathway (Verga Falzacappa et al., 2007) triggering FPN degradation; other cytokines such as IL-1β (Shanmugam et al., 2015) and activin-B (Besson-Fournier et al., 2012; Canali et al., 2016a) are also involved (Figure 4). This inflammatory pathway depends on the integrity of SMAD signaling that is induced by members of the transforming growth factor-β superfamily (TGFβ, see later). In a mouse model of inflammatory bowel disease, a complex interplay of cytokines, erythropoietic activity, and gut microbiota determines the degree of hepcidin expression and dysregulated iron homeostasis (Shanmugam et al., 2014).

The dogma that hypoferremia rapidly develops after infectious or inflammatory insults in response to hepcidin-controlled FPN degradation has recently been challenged. Transcriptional downregulation of *Fpn* in the liver and spleen of mice can suffice to induce hypoferremia (Guida et al., 2015). Decreased FPN mRNA expression is induced by binding of bacterial lipopeptide FSL1 to TLR (Toll Like Receptor) 2 and TLR6 (Guida et al., 2015) or lipopolysaccharides (LPS) ligation to TLR4 (Deschemin and Vaulont, 2013). The quantitative contribution of hepcidin-dependent and -independent mechanisms of *Fpn* control to inflammation-induced hypoferremia may depend on the nature of the pathogen and the time course of infection (Arezes et al., 2015).

Inflammation also induces proteins that sequester and relocate iron and thus limit iron supplies for erythropoiesis. These include extracellular proteins such as lactoferrin, lipocalin 2, haptoglobin, and hemopexin (Soares and Weiss, 2015). Hypoferremia limits iron as a critical growth factor for microbial proliferation. Additionally, lower levels of serum iron may free transferrin for binding non-transferrin-bound iron that is released during tissue injury and hemolysis and that could otherwise augment proliferation of pathogens and tissue damage (Ganz and Nemeth, 2015). Hypoferremia will develop into anemia if the inflammatory condition persists and hepatocytes become iron depleted with time, preventing (for example) superinfection by plasmodia during a second exposure to malaria-bearing mosquitos (Portugal et al., 2011).

Iron Refractory Iron-Deficiency Anemia

In addition to acquired conditions of iron deficiency, iron-restricted erythropoiesis may also be caused genetically by inactivating mutations in proteins suppressing hepcidin. This is the case in the rare genetic disorder iron refractory iron-deficiency anemia (IRIDA), in which mutations in the serine protease TMPRSS6 (Transmembrane Protease Serine 6, also known as matriptase-2) cause elevated hepcidin levels. High hepcidin levels render these patients refractory to both oral and parenteral iron substitution (Heeney and Finberg, 2014). In cultured cells, matriptase-2 cleaves the BMP (Bone Morphogenetic Protein) co-receptor hemojuvelin (HJV), physiologically attenuating the BMP-SMAD signaling pathway and reducing hepcidin expression (see below) (Figure 4; Silvestri et al., 2008). Genetic inactivation of TMPRSS6 preserved the iron overload of $Hjv^{-/-}$ mice, supporting the notion that HJV is a likely substrate for TMPRSS6 (Finberg et al., 2010). Genetic experiments further indicate that TMPRSS6 requires functional BMP6/SMAD signaling for its activity (Nai et al., 2016). EPO was tested as a therapeutic for IRIDA. The lack of success is consistent with findings in mice that an activated BMP/SMAD signaling pathway in the liver—as observed in response to TMPRSS6 inactivation—is incompatible with EPO/ERFE function (Lehmberg et al., 2013; Nai et al., 2016).

A role for TMPRSS6 in the control of erythropoiesis in healthy individuals was suggested by genome-wide association studies linking common *TMPRSS6* variants (rs855791) within the catalytic protease domain (A736V) with high hepcidin levels, reduced serum iron, and altered erythrocyte indices (mean corpuscular volume and mean corpuscular hemoglobin) (Heeney and Finberg, 2014; Nai et al., 2011). The exact physiological function of TMPRSS6 remains poorly understood. Its increased expression observed in hypoxia and iron deficiency suggests a contribution to decreasing hepcidin levels in these conditions. However, TMPRSS6 is also induced by BMP6 and iron (Meynard et al., 2011). Taken together, these data suggest that TMPRSS6 serves as a negative feedback inhibitor to avoid inappropriate hepcidin expression under conditions of both iron demand or increased iron accumulation (Meynard et al., 2011).

Dietary Iron Restriction

Dietary iron restriction diminishes transferrin saturation and hepatic iron stores. These parameters are sensed by the liver, involve proteins mutated in hereditary hemochromatosis (HFE, TFR2, and HJV) as well as the BMP6-SMAD signaling pathway, and regulate hepcidin levels (see below). Low hepcidin expression in response to iron deficiency thus stabilizes FPN in duodenal enterocytes, allowing for compensatory increases in iron acquisition.

Regulation of Systemic Iron Fluxes by the Hepcidin-Ferroportin Axis

Common diseases of systemic iron overload or deficiency are caused by misregulation of the hepcidin/FPN axis that coordinates iron export to the bloodstream from duodenal enterocytes, iron-recycling macrophages, and periportal hepatocytes (Figure 3). The hepcidin/FPN regulatory system further operates in placental syncytiotrophoblasts to control iron flux from the placenta to the fetal circulation (Donovan et al., 2005).

The N-terminal nine amino acids of hepcidin are sufficient to bind FPN. This insight was instrumental for the development of ‘‘minihepcidins,’’ therapeutic candidates being tested for indications hallmarked by hepcidin deficiency (Casu et al., 2016a; Ramos et al., 2012). Hepcidin binds to a predicted extracellular loop of FPN including cysteine 326 (Fernandes et al., 2009). Mutation of C326 confers severe hemochromatosis in patients (Sham et al., 2009) and mice (Altamura et al., 2014). Hepcidin binding to FPN triggers its endocytosis, which requires ubiquitination of multiple lysines within a cytoplasmic FPN loop (Qiao et al., 2012; Ross et al., 2012). The important role of hepcidin for controlling FPN expression is best reflected in hepcidin knock-out (Viatte et al., 2005) and hepcidin-resistant C326S *Fpn* knock-in mice (Altamura et al., 2014), in which FPN is stably expressed on the cell surface, causing increased dietary iron uptake and macrophage iron release. Conversely, hepcidin overexpression (Roy et al., 2007; Weinstein et al., 2002) or injection (Rivera et al., 2005) cause hypoferremia in wild-type mice.

Systemic disruption of the *Fpn* gene in mice causes embryonic lethality, likely because of lacking iron transfer to the embryo (Donovan et al., 2005). Conditional *Fpn* deletion from intestinal enterocytes causes severe anemia, indicating the critical role of FPN for iron uptake. By contrast, the selective deletion of FPN from macrophages or hepatocytes is not lethal, but impairs iron mobilization, causing anemia when dietary iron is limiting. These findings imply a compensatory role for intestinal iron absorption when sufficient dietary iron is supplied (Zhang et al., 2011, 2012). Interestingly, FPN deletion from cardiomyocytes impairs their function and causes premature death (Lakhal-Littleton et al., 2015), suggesting that iron export is essential, possibly as a protection from iron toxicity. Taken together, FPN-mediated iron export is critical for systemic and/or local organ iron management, which may explain the

complexity of its regulation in response to multiple (patho-) physiological signals.

Sensing/Signaling Machinery Regulating Hepcidin Expression

The concentration of circulating hepcidin is determined by the liver (Figure 4), and critical aspects of the underlying mechanisms were revealed by molecular analyses of HH. The most common form of HH is hallmarked by mutations in the *HFE* gene, an atypical major histocompatibility complex (MHC) class I-like molecule that heterodimerizes with β2-microglobulin. Rarer forms of HH result from mutations in TFR2, hemojuvelin (HJV), or hepcidin. Common to these subtypes of HH is an inappropriately low expression of hepcidin, suggesting that HFE, TFR2, and HJV are upstream regulators of hepcidin. Protein-protein interactions between HFE, TFR1, and TFR2 play roles in iron sensing, as high concentrations of iron-bound transferrin dissociate HFE from TFR1, which then binds to TFR2 (Core et al., 2014). However, Hfe and TFR2 may regulate hepcidin independently of each other in mice (Schmidt and Fleming, 2012; Wallace et al., 2009).

BMP6, a member of the TGFβ superfamily, is also important for activating hepcidin expression. Genetic impairment of BMP6 causes severe iron overload in mice (Andriopoulos et al., 2009; Meynard et al., 2009) and in a patient (Daher et al., 2016). Elevated iron stores augment BMP6 expression predominantly in non-parenchymal cells of the liver, which sense iron levels/fluxes and release BMP6 to control hepcidin expression in a paracrine manner (Rausa et al., 2015). Consistently, endothelial BMP6 and BMP2 expression is required to maintain iron homeostasis in mice (Koch et al., 2016; Canali et al., 2016b). BMP6 binds to type 1 (Alk2 and Alk3) (Steinbicker et al., 2011) and type 2 (BMPR2 and ActR2a) (Mayeur et al., 2014) serine threonine kinase receptors to activate Smad1/5/8 phosphorylation, binding to SMAD4, and nuclear translocation to induce hepcidin transcription (Wang et al., 2005). BMP6 directly binds its co-receptor HJV (Babitt et al., 2006) but may also act independently of HJV (Latour et al., 2015). HFE and TFR2 may further cross-talk with the BMP6/HJV pathway, as evidenced by a multi-protein complex between HFE, TFR2, and HJV on the surface of hepatocytes (D'Alessio et al., 2012). HFE further interacts with ALK3 to prevent its ubiquitination and proteasomal degradation, effecting ALK3 stabilization (Wu et al., 2014), which activates BMP/SMAD signaling and hepcidin transcription. Hepcidin induction by the HJV-BMP receptor complex may be facilitated by neogenin that may act as a scaffold by binding to both HJV and ALK3 (Lee et al., 2010; Zhao et al., 2016) and suppressed by SMAD7 (Mleczko-Sanecka et al., 2010) and miR122 (Castoldi et al., 2011).

While the molecules involved in iron sensing, iron-dependent protein interactions between them, and links to BMP/SMAD signaling have been identified, the exact mechanism of how the liver senses and responds to changes in iron remains an important question to be addressed. Further progress may require the development of cell-based assays that reflect the activation of hepcidin by transferrin-bound iron. This could be aided by the finding that more than one liver cell type appears to be involved in this process. Future work will also have to address the importance and underlying mechanisms of the emerging regulation of hepcidin by intermediary metabolism, growth factor, and hormone signaling (Mleczko-Sanecka et al., 2014; Goodnough et al., 2012; Guo et al., 2013; Latour et al., 2014; Li et al., 2016).

Hepcidin-Independent Regulation of Fpn

FPN expression is also controlled in cell-autonomous ways (Figure 2B). In macrophages, *Fpn* is transcriptionally activated by heme released from digested erythrocytes, via BACH1/NRF2 to promote iron recycling (Marro et al., 2010). *Fpn* transcription also responds to hypoxia and iron deficiency via HIF-2 in duodenal enterocytes (Anderson et al., 2013a; Mastrogiannaki et al., 2009, 2012; Taylor et al., 2011) but not in macrophages (Mathieu et al., 2014). By contrast, *Fpn* transcription is repressed by inflammatory signaling via TLRs4/2/6 (see above). Interestingly, pharmacological activation of NRF2 reverses *Fpn* transcriptional attenuation by inflammatory signals (Harada et al., 2011), suggesting that iron signals dominate over inflammatory cues.

FPN translation is repressed in iron deficiency by IRPs (Galy et al., 2013; Lymboussaki et al., 2003; Nairz et al., 2015), while iron accumulation in erythrophagocytic macrophages stimulates FPN translation and iron release. *Fpn* expression in macrophages is further affected by signals that they receive within their niches. These signals effect macrophage differentiation into phenotypically and functionally diverse subtypes and affect the expression of genes involved in iron management (Haldar et al., 2014; Kohyama et al., 2009; Vinchi et al., 2016). Finally, two miRNAs modulate FPN mRNA expression via its 3′ UTR: miR-485-3p, which is induced in iron deficiency (Sangokoya et al., 2013), and miR-20a, which plays a role in FPN-mediated iron export in lung cancer cells (Babu and Muckenthaler, 2016). The control of *Fpn* expression by multiple signals and mechanisms reflects its importance in determining plasma iron levels, which in turn are critical for erythropoiesis, cell growth, and innate immunity.

Iron Metabolism under Conditions of Abnormal Erythropoiesis

Disorders associated with aberrant erythropoiesis have proven highly instructive regarding the relationship between erythropoiesis and iron metabolism. β-thalassemia, one of the most frequent hemoglobinopathies due to β-globin mutations, causes insufficient RBC synthesis. The ensuing hypoxia triggers EPO release and erythroid precursor proliferation in an unsuccessful attempt to compensate for the anemia, a condition defined as ineffective erythropoiesis (Rivella, 2015). By contrast, polycythemia vera is characterized by excess RBC production (Crielaard and Rivella, 2014). Both disorders exhibit stress erythropoiesis with an increase in the absolute number of erythroid precursors, extramedullary hematopoiesis, and splenomegaly (Crielaard and Rivella, 2014). Diminished hepcidin expression has now been recognized as one of the key mechanisms responsible for the increased iron absorption observed in these disorders (Gardenghi et al., 2007; Weizer-Stern et al., 2006a, 2006b).

The ineffective erythropoiesis in β-thalassemic mice is associated with ERFE-mediated hepcidin suppression (Kautz et al., 2014) and augmented activities of DMT1, DCTYB, and FPN in the duodenum mediated by HIF-2 (Das et al., 2015). The absorbed iron is only partially utilized for erythropoiesis, and excess iron accumulates in parenchymal organs.

The problematic role of iron in pathological stress erythropoiesis has been demonstrated by crossing thalassemic animals

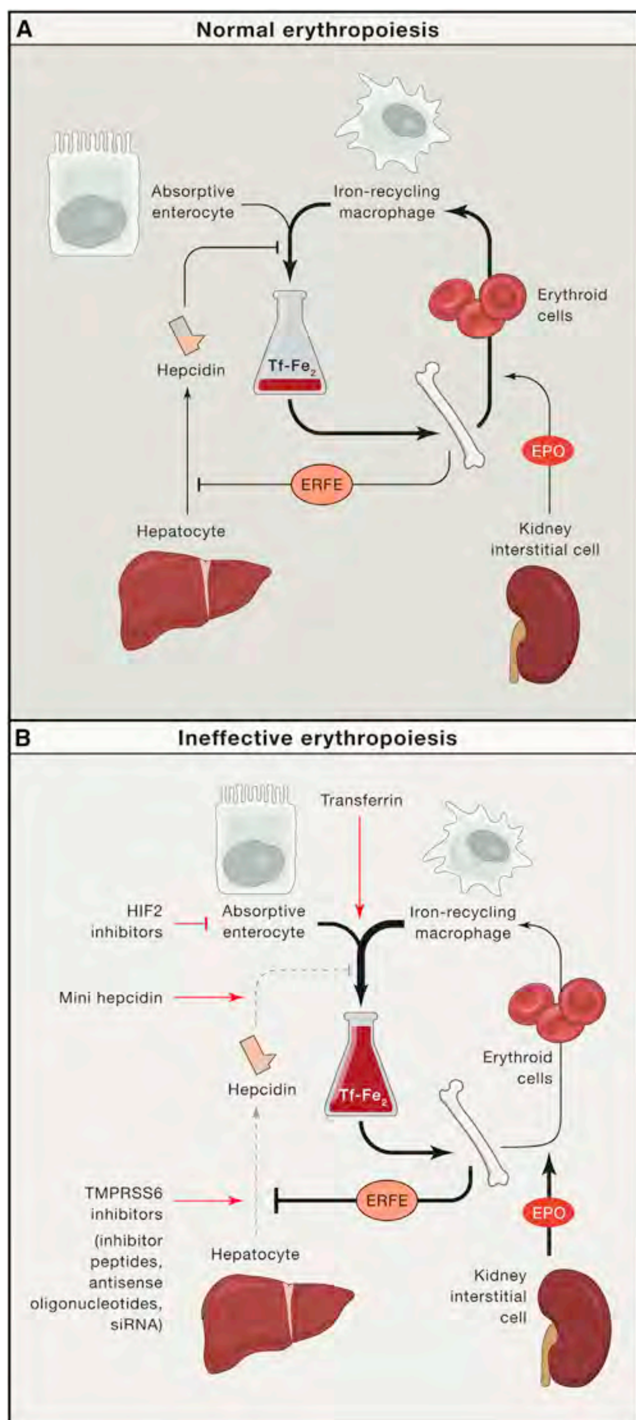

Figure 5. Therapeutic Strategies in Abnormal Erythropoiesis

(A) Under baseline conditions, iron flows mostly through the erythron and iron-recycling macrophages. EPO controls the rate of erythropoiesis, which in turn inhibits Hepcidin expression through erythroferrone (ERFE) to adjust iron efflux from macrophages and enterocytes.

(B) A situation of ineffective erythropoiesis is depicted. Hypoxia stimulates erythropoiesis through EPO in an attempt to compensate for decreased oxygen transport. The ensuing increase in ERFE production suppresses *Hepcidin* and results in iron overload due to enhanced dietary iron absorption and iron release from macrophages. Therapeutic strategies to prevent iron overload while improving erythropoiesis can be envisaged. Those include engineered minihepcidins, which limit dietary iron absorption by inhibiting FPN iron export. Similarly, siRNAs, antisense oligonucleotides (ASO), and inhibitors of TMPRSS6 aim at enhancing endogenous hepcidin activity. Targeting ERFE also seems a logical choice to alleviate *Hepcidin* suppression, but ERFE deficiency is insufficient to correct the anemia of thalassemic mice. Thus, this approach may require combination with other therapies. If selectively delivered to intestinal enterocytes, HIF2 inhibitors may also have the potential to limit dietary iron assimilation. Injection of apo-transferrin was shown to prevent hemichrome formation and ameliorate RBC survival in mice.

with mice engineered to overexpress hepcidin (Gardenghi et al., 2010; Nai et al., 2012), which ameliorated iron overload, erythropoiesis, and splenomegaly. The tissue-specific deletion of HIF-2α in the duodenum of thalassemic animals also decreased iron absorption (Anderson et al., 2013a).

Further studies in mouse models of β-thalassemia, polycythemia vera, and stress erythropoiesis induced by phlebotomy underscore the role of macrophage-erythroblast adhesion interactions (indicated as stress erythropoiesis macrophage-supporting activity [SEMA]) within the erythroblast island. SEMA is essential for the numeric expansion of erythroid progenitors and RBC development in physiological and disease conditions (Chow et al., 2013; Ramos et al., 2013). While macrophage depletion in phlebotomized mice impairs their recovery from the induced anemia, this depletion improves the phenotype of β-thalassemia and polycythemia vera mouse models. Macrophage depletion reduces the number of erythroblasts in polycythemia vera, normalizing the hematocrit and splenomegaly by the combined effect of diminished iron recycling and macrophage-erythroblast interactions (Chow et al., 2013; Ramos et al., 2013). The decreased transferrin saturation resulting from reduced iron recycling also shows a beneficial effect in β-thalassemia, decreasing hemichrome formation and ameliorating RBC lifespan, anemia, and splenomegaly (Chow et al., 2013; Li et al., 2010a; Ramos et al., 2013).

Sickle cell anemia (SCA) represents another common genetic hemoglobinopathy resulting from a missense mutation of the β-globin gene. Sickling RBCs elicit severe vaso-occlusive episodes, hemolysis, and anemia (Rees et al., 2010). Exposure of macrophages to high levels of heme from extracellular RBC lysis triggers their polarization toward a pro-inflammatory phenotype. Once scavengers such as hemopexin (binding heme) and haptoglobin (binding hemoglobin) are saturated, heme ligation by TLR4 and oxidative stress-induced mechanisms (Vinchi et al., 2016) may contribute to the hypercoagulable state observed in SCA (Eldor and Rachmilewitz, 2002), exacerbating the disease's condition. Duodenal deletion of Hif2α also ameliorates the phenotype of mice with SCA (Das et al., 2015). Decreased iron absorption may reduce the sickling component of the RBCs associated with mean corpuscular hemoglobin concentration (MCHC), and in turn, the component of hypercoagulable state mediated by heme, iron toxicity, and inflammation.

Development of Therapeutics

Hepcidin decreases duodenal iron absorption and, ultimately, transferrin saturation with potential clinical benefits in a variety of disorders. Consequently, new therapeutics are being developed around this concept (Figure 5).

Duodenal HIF2α and ERFE represent potential therapeutic targets. In the case of HIF2α, the development of drugs that

specifically target HIF2α in the duodenum poses a major challenge. ERFE deficiency is insufficient to ameliorate the anemia in thalassemic animals and to prevent iron overload (Kautz et al., 2015). However, if humans also utilize ERFE to suppress hepcidin under conditions of stress or ineffective erythropoiesis, it is possible that complete normalization of iron absorption may be achieved by targeting additional molecules or mechanisms, such as PDGF-BB or duodenal iron absorption. The administration of apo-transferrin significantly improves the phenotype of thalassemic animals by decreasing hemichrome formation and normalization of RBC survival (Li et al., 2010a).

Minihepcidins (MHs) are short peptide mimetics (9 aa long) that target FPN iron export, ameliorating the iron overload in animals affected by Hfe- or Hepcidin-related hemochromatosis (Preza et al., 2011; Ramos et al., 2012). Administration of these compounds is also beneficial in mice affected by β-thalassemia and polycythemia vera where they, respectively, can improve the anemia and normalize the hematocrit (Casu et al., 2016a). Similarly, both use of antisense oligonucleotides (Tmprss6-ASO) and RNA interference (Tmprss6-siRNA) can increase endogenous hepcidin activity by degrading Tmprss6 mRNA (Guo et al., 2013). These compounds also ameliorate the phenotype of Hfe-mediated hemochromatosis and β-thalassemia mouse models (Guo et al., 2013). They can be administered together with iron chelators, showing that the effective removal of excess iron by chelation can be combined with their ability to improve erythropoiesis (Casu et al., 2016b).

Increased hepcidin expression represents a hallmark of some anemias (e.g., AI and IRIDA). Recent preclinical studies have described multiple approaches to diminish hepcidin overexpression, including neutralizing antibodies and antisense/siRNA strategies and BMP pathway inhibitors (Rivella, 2015). However, the apparent involvement of additional, hepcidin-independent mechanisms in AI suggests that more comprehensive therapeutic strategies may be needed (Gardenghi et al., 2014).

Perspectives

While the area of iron therapeutics is developing, recent progress in iron biology has been immense, regarding both the molecular physiology and the understanding of disease mechanisms. As the major consumer and the largest physiological reservoir of iron in the body, red cells are particularly suited to illustrate the key aspects of cellular and systemic iron homeostasis. Nonetheless, most of the “general principles” discussed against the background of the erythron apply to the other cell and organ systems. Specifically, the brain, heart, kidneys, and the vasculature are subject to intense investigation. Moreover, multi-faceted pathologies like cell and tissue degeneration, inflammatory disorders, and cancer involve abnormal iron management. With the cornerstones of systemic and cellular iron regulation now largely having been defined, attention to the specifics of different cell types and the involvement of iron in complex pathologies promises a rich harvest for the future.

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