Mechanistic and Regulatory Aspects of Intestinal Iron Absorption

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Abstract

Iron is an essential trace mineral that plays a number of important physiologic roles in humans, including oxygen transport, energy metabolism and neurotransmitter synthesis. Iron absorption by the proximal small bowel is a critical check point in the maintenance of whole-body iron levels as, unlike most other essential nutrients, no regulated excretory systems exist for iron in humans. Maintaining proper iron levels is critical to avoid the adverse physiological consequences of either low or high tissue iron concentrations, as commonly occurs in iron-deficiency anemia and hereditary hemochromatosis, respectively. Exquisite regulatory mechanisms have thus evolved to modulate how much iron is acquired from the diet. Systemic sensing of iron levels is accomplished by a network of molecules which regulate transcription of the HAMP gene in hepatocytes, thus modulating levels of the serum-borne, iron-regulatory hormone hepcidin. Hepcidin decreases intestinal iron absorption by binding to the iron exporter ferroportin 1 on the basolateral surface of duodenal enterocytes, causing its internalization and degradation. Mucosal regulation of iron transport also occurs during low-iron states, via transcriptional (by HIF2α) and post-transcriptional (by the iron-sensing IRP/IRE system) mechanisms. Recent studies demonstrated that these regulatory loops function in tandem to control expression or activity of key modulators of iron homeostasis. In health, body iron levels are maintained at appropriate levels; however, in several inherited disorders and in other pathophysiological states, iron sensing is perturbed and intestinal iron absorption is dysregulated. The iron-related phenotypes of these diseases exemplify the necessity of precisely regulating iron absorption to meet body demands.

Key Words: duodenum, divalent metal-ion transporter 1, ferroportin 1, hephaestin, hepcidin
Iron is abundant in the Earth’s crust, and most forms of life, including mammals, have evolved to utilize iron in many biological processes. For example, iron is required to support oxygen delivery to tissues, for the control of cellular growth and differentiation, and for energy metabolism. The role of iron in these physiological processes revolves around the ability of the metal to exist in two stable oxidation states (ferric [Fe$^{3+}$] and ferrous [Fe$^{2+}$]). This chemical property of iron underlies its ability to participate in reduction-oxidation (electron transfer) reactions, and also leads to its potential toxicity if not properly managed by cells and tissues. Free iron can participate in Fenton chemistry, whereby oxygen free-radicals are produced, and these in turn can damage numerous biological molecules (e.g. membrane lipids, proteins, DNA). Mammals have thus developed complex regulatory mechanisms that manage iron absorption, transport and recycling.

Unlike most essential nutrients, no active excretory mechanisms exist for iron in humans, although small amounts are lost via exfoliation of skin and gastrointestinal cells, and in bile and urine. Body iron levels are thus principally controlled by modulation of iron absorption in the duodenum and proximal jejunum, which allows absorption to be precisely matched to unregulated losses. The mechanisms which regulate iron absorption also allow for appropriate increases or decreases according to physiologic demand. Large variations in iron status and stores among individuals could, however, be misconstrued as indicative of imprecise regulation of intestinal iron absorption. This might be true if one assumed that iron status/stores were directly related to the capacity of the small intestine to absorb iron. This is not the case though since the amount of bioavailable iron is often limiting, which can partially explain variations in iron stores. Moreover, various pathologies can influence the relationship between the rate of iron absorption and body iron levels. Absorption of dietary
Iron by the proximal intestine is thus accurately regulated by cellular and systemic factors to ensure that overall body iron levels are maintained at adequate levels.

Iron balance is controlled in part by the liver-derived, serum-borne, peptide hormone hepcidin (HEPC), which functions to block intestinal iron absorption and inhibit iron release from stores. Molecules that regulate HEPC expression, such as transferrin receptor 2 (TFR2), HFE, hemojuvelin (HJV) and matriptase-2, “sense” body iron levels allowing appropriate modulation of iron absorption. HEPC exerts its influence on iron homeostasis by binding to the iron export protein ferroportin 1 (FPN1) and causing its internalization and degradation (80). FPN1 is expressed in cells that absorb (enterocytes) and store (hepatocytes and reticuloendothelial [RE] macrophages) iron. HEPC expression is induced by high body iron stores, and by infection and inflammation. During iron deficiency and tissue hypoxia, when HEPC production is very low, additional regulatory mechanisms are invoked to upregulate intestinal iron absorption.

Perturbations in iron absorption can have significant physiologic consequences. Many iron-related disorders in humans occur when dietary iron absorption is inappropriately regulated, resulting in excessive iron accumulation in various tissues and subsequent oxidative damage or, in some cases, serum iron depletion. The most common of these pathologies is a group of genetic, iron-overload disorders, collectively referred to as hereditary hemochromatosis (HH). In most forms of HH, HEPC expression is inappropriately low leading to increased iron absorption. Diseases associated with reduced iron absorption are much less common, attesting to the essentiality of the metal. Anemias that don’t respond to oral iron supplementation have also been described, with one form, iron-refractory iron-deficiency anemia (IRIDA), being recently linked to alterations in HEPC expression (27).
Forms of Iron in the Diet and Bioavailability

Iron in foods exists principally as heme and non-heme (or inorganic) iron. Heme iron is derived predominantly from hemoglobin and myoglobin in meats. Heme iron absorption is efficient and largely uninfluenced by other dietary constituents. A candidate intestinal heme transporter, heme carrier protein 1 (HCP1) was proposed (109), but recent evidence suggests that it is most likely a folate transporter (and not a physiologically relevant heme transporter) (94). This issue is still, however, not fully resolved (64). Conversely, non-heme (and largely ferric) iron, found in both meat and plant foods, is highly insoluble and its bioavailability is influenced by many dietary components. Gastric acid and ascorbic acid promote reduction and solubilization of dietary ferric iron and thus improve absorption. Furthermore, dietary factors commonly found in plants, such as phytate, oxalate, polyphenols and tannins, decrease the absorption of non-heme iron. The chronic use of proton pump inhibitors for gastric acid reflux, Helicobacter pylori infection and inflammatory conditions (e.g. celiac disease) also decrease non-heme iron absorption (16).

Non-Heme Iron Transport by Duodenal Enterocytes

Although multiple dietary sources of iron exist, the transport of non-heme iron has been most intensively studied and will be the focus of this review. Many of the proteins which mediate non-heme iron absorption have been identified (Table 1, Figure 1).

Ferric Iron Reduction

Dietary non-heme iron exists predominantly in the oxidized or ferric (Fe$^{3+}$) form, yet ferrous iron (Fe$^{2+}$) is likely the form that is transported into enterocytes. Ferric iron, therefore, must be reduced prior to transport. Recent investigations have identified a brush-border membrane (BBM) ferrireductase, duodenal cytochrome b [DCYTB] (72), which may mediate
this process. Interestingly, DCYTB facilitates the reduction of ferric iron via electron transfer
from intracellular ascorbate (66, 118), providing one potential mechanism by which vitamin
C enhances iron absorption. DCYTB is strongly upregulated in duodenal enterocytes during
iron deficiency (17, 62) and acute hypoxia (63). Moreover, DCYTB can account for the
inducible ferrireductase activity in the mouse intestine during iron deficiency, pregnancy and
hypoxia (14). These data are suggestive of an important physiological role for DCYTB in
intestinal iron transport. However, recent investigations in knockout mice showed that
DCYTB is not required for efficient iron absorption (50). It is thus likely that, under basal
conditions, other reductases (e.g. a STEAP protein) (84) are important, or that dietary
components or gastrointestinal secretions provide the reducing power necessary to promote
iron absorption. Enzymatic reduction may not, therefore, be rate-limiting. It is also important
to consider that these observations were made in laboratory rodents. It is a logical prediction
that the physiology of non-heme iron transport could be different among mammalian species,
especially since rats and mice can synthesize ascorbic acid and humans cannot.

Iron Uptake Across the BBM

Subsequent to reduction of dietary ferric iron, ferrous iron (Fe\(^{2+}\)) is transported across
the BBM of enterocytes via divalent metal-ion transporter 1 (DMT1; encoded by the
\textit{SLC11A2} gene) (67). DMT1 is a multi-pass, transmembrane protein that mediates proton-
coupled, ferrous iron uptake. Protons, which provide the driving force for iron transport, are
likely provided by the action of a BBM sodium-hydrogen exchanger (probably NHE3) which
acidifies the unstirred water layer. DMT1 is so named because it can transport other divalent
cations (49), including manganese and cobalt (108). Some studies suggest DMT1 also
transports copper (4, 116), but others refute this contention (68). Irrespective of its substrate
profile in different model systems, physiological data implicate DMT1 as being an essential
intestinal iron importer, as exemplified by the severe iron-deficiency anemia that results from deletion (48) or mutation (34, 35) of the gene in rodents. Humans with SLC11A2 mutations also suffer from severe systemic iron deficiency (6, 75), exemplifying the essentiality of DMT1 for efficient acquisition of dietary non-heme iron.

Iron Trafficking and Storage Within Enterocytes

After ferrous iron is transported across the BBM into enterocytes, it is likely chelated by small molecular weight organic acids (e.g. citrate), amino acids, or intracellular proteins. The poly-r(C)-binding proteins are iron-trafficking proteins (chaperones) that have been identified recently (65, 110), but whether they are expressed in mammalian enterocytes is unknown. How iron trafficks to different intracellular compartments (e.g. mitochondria) within enterocytes is thus unclear. Irrespective of specific trafficking mechanisms, intracellular iron is rapidly transferred across the basolateral membrane (BLM) by FPN1 when body iron demands are high. When demand is low, iron can be stored in ferritin, an intracellular iron storage protein complex consisting of heavy (H) and light (L) chain subunits that form a hollow sphere accepting up to 4,500 iron atoms. Most iron stored in ferritin is likely lost via subsequent exfoliation of intestinal epithelial cells. Interestingly, a recent study described a protective role for ferritin H in regulation of intestinal iron absorption during conditions of iron overload (117). These authors showed that intestine-specific ferritin H deletion led to a 2-fold increase in iron absorption in iron-loaded mice, suggesting that ferritin H works in conjunction with systemic signals (e.g. HEPC) to limit iron flux.

Iron Efflux Across the BLM

Ferroportin 1 (FPN1; encoded by the SLC40A1 gene) is the only ferrous iron export protein identified to date in mammals (73). FPN1 is highly expressed in enterocytes,
reticuloendothelial (RE) macrophages and hepatocytes, consistent with its established roles in iron absorption and recycling. The essentiality of FPN1 is exemplified by knockout of the gene in mice, which causes severe iron-deficiency anemia (25). SLC40A1 gene mutations have been described in humans, and, although rare, they collectively represent an important subset of iron-loading disorders (8, 24, 71). Affected individuals have varying phenotypes depending upon how the mutations alter FPN1 protein function. Importantly, these observations clearly exemplify the critical, non-redundant role of FPN1 in intestinal iron absorption.

Iron Oxidation and Transferrin Binding

Ferrous iron exits enterocytes via FPN1-mediated transport, but ferric iron is required for binding to transferrin in the interstitial fluids. Transferrin-bound iron is then distributed via the circulation throughout the body. Iron efflux must thus be coupled to oxidation. Earlier studies postulated that the oxidation step required an enzyme catalyst, as chemical oxidation is likely inadequate to supply the large amount of iron required by the bone marrow to maintain erythropoeisis (85, 86). In the intestine, iron oxidation is, in part, mediated by hephaestin (HEPH), which is a membrane-anchored, multi-copper ferroxidase (FOX) (11). Mice harboring a mutation in the Heph gene (sex-linked anemia [sla] mice) exhibit moderate iron deficiency, particularly during the rapid growth period in early life (2). HEPH has homology in the FOX domain (~50% amino acid identity) to the liver-derived, circulating, multi-copper FOX ceruloplasmin (CP) (119). In vitro (51, 126) and in vivo (125) approaches have demonstrated FPN1 and HEPH colocalization on or near the BLM of duodenal enterocytes. Furthermore, recent investigations reported immunoreactive HEPH and FOX activity in the cytosolic (soluble) fraction of isolated rodent enterocytes (98). This protein-mediated FOX activity was only partially contributed by HEPH, as robust activity remained...
in enterocytes isolated from *Heph* knockout (KO) mice (97). Although the nature of this
cytosolic FOX is currently unknown, this redundant FOX activity likely complements HEPH,
thus ensuring adequate iron absorption during times of increased demand.

CP may also influence intestinal iron transport, as it is undoubted present in the
interstitial fluids within the lamina propria of intestinal villi. *Cp* KO mice, however, do not
show a noticeable disruption in iron absorption (52). Moreover, CP added to the basal side of
differentiated Caco-2 cells grown in bicameral cell culture inserts did not influence iron
transport (127). Conversely, CP was necessary for increased iron absorption accompanying
stimulated erythropoiesis (13), as iron absorption did not increase to the same extent in *Cp*
KO mice following bleeding as it did in wild-type mice. Furthermore, copper contained
within CP and HEPH is required for enzymatic activity (i.e. electron transfer), perhaps
explaining why copper-deficient animals absorb less iron (20). Additional experimentation is
required to determine the relative contributions of enterocyte (membrane-bound HEPH and
cytosolic FOXs) and serum-derived (CP) FOXs to intestinal iron transport.

**Regulation of Intestinal Iron Absorption**

Iron absorption is precisely regulated by a range of systemic and cellular mediators
(Figure 2). Liver-derived, circulating HEPC regulates duodenal iron absorption and also
modulates iron release from stores in hepatocytes and RE macrophages (which recycle iron
from senescent red blood cells). Iron demand to support erythrocyte production in the bone
marrow is the strongest stimulator of iron absorption, but overall physiologic requirements
for iron also influence this process. Iron absorption thus increases when body iron stores are
low or when the erythropoietic rate is high, and decreases in the reverse situations.
Absorption is also increased during chronic hypoxia, pregnancy and in the suckling period (as
discussed below).
Early studies established that intestinal iron absorption is stimulated when body iron stores decrease, and the term *stores regulator* was coined to describe this phenomenon (33). Iron absorption is also enhanced when erythropoiesis is stimulated (e.g. by blood loss or acute hemolysis), as hemoglobin production in developing erythrocytes requires large amounts of iron (31). The term *erythroid regulator* has been used to described this physiological adaptation (33). Iron absorption also increases in response to tissue hypoxia. While in part this may relate to changes in the erythropoietic rate, a component of the response relates specifically to oxygen levels (96). For example, iron absorption increases during hypoxia prior to an increase in red blood cell production (53), demonstrating that hypoxia exerts a direct effect on the gut. Consistent with this observation, iron regulatory molecules in the liver and genes encoding iron transporters in the duodenum respond directly to hypoxia (90).

Iron absorption also increases during pregnancy (5). During gestation, iron requirements are high due to expansion of the maternal erythroid mass and the iron needed by the developing fetus. The underlying trigger probably relates to both a reduction in maternal iron stores and relative tissue hypoxia. Moreover, during the perinatal and neonatal periods, iron requirements of humans are high and iron absorption from breast milk is very efficient. The high iron absorption of neonates appears to be predominantly due to active transport mechanisms, as occur in adults, but the relative ‘leakiness’ of the neonatal epithelium, which allows passive absorption of solutes, likely makes some contribution. A recent study also suggested an anatomical adaptation at this developmental stage, whereby more iron is absorbed in the distal portions of the GI tract in neonatal rats than in adult animals (39). Furthermore, recent work has also demonstrated that iron absorption in neonates is refractory to HEPC (21), despite the fact that HEPC signaling is intact at this developmental stage.
Although the mechanisms are not fully understood, this leads to a high capacity to absorb iron from an iron-poor diet at a time of great physiologic need.

**Intestinal Iron Homeostasis During Iron-Overload and Infection/Inflammation**

The liver-derived peptide hormone HEPC has emerged as an important regulator of systemic iron homeostasis (81). HEPC decreases circulating iron levels by blocking intestinal iron absorption and inhibiting iron release from stores. Thus, mice overexpressing Hepe develop severe iron-deficiency anemia (82). HEPC expression is induced when body iron stores are elevated, and during infection and inflammation, with the net result being lower serum iron levels. An inverse relationship between hepatic HEPC secretion and expression and activity of duodenal iron transporters (e.g. DMT1, FPN1) has been established (40). Furthermore, the importance of HEPC in maintaining iron balance is exemplified by the phenotype of humans with mutations in the HAMP gene (104), who develop an early onset, severe iron-loading disorder (termed juvenile hemochromatosis) due to unregulated (and inappropriately increased) intestinal iron absorption.

The physiologic effects of HEPC are mediated by interaction of the circulating peptide with FPN1 on the plasma membrane of target cells, resulting in internalization and eventual degradation of FPN1 (22), thus limiting iron efflux (80). Duodenal enterocytes represent an important HEPC target, probably explaining the effect of this peptide on intestinal iron transport (103). Interestingly, recent studies have also supported novel roles for HEPC in the intestine. For instance, using *in vitro* and *ex vivo* approaches, it was shown that HEPC promotes the proteosomal degradation of DMT1 expressed on the BBM of duodenal enterocytes (7). Conceivably, additional FPN1-independent aspects of how HEPC modulates intestinal iron flux may be discovered in the future.
In accordance with its role in regulating iron homeostasis, HEPC expression is altered according to body iron needs. As might be anticipated, HEPC production decreases during iron deficiency (83), hypoxia and pregnancy (74), and when erythropoiesis is stimulated, allowing adequate iron absorption and efficient iron release from stores. Conversely, HEPC expression increases when body iron levels are adequate or elevated, effectively reducing intestinal absorption and allowing excess iron to be stored in RE macrophages and hepatocytes. Inflammation also induces HEPC expression, likely explaining the hypoferremia that accompanies chronic inflammatory conditions (83).

Much knowledge of how HEPC regulates systemic iron homeostasis has been attained from investigation of several inherited iron-loading disorders, collectively referred to as hereditary hemochromatosis (HH). In healthy individuals, iron absorption can be appropriately downregulated when iron stores are replete, but in patients with HH, iron absorption is chronically increased, allowing accumulation of the metal in liver, pancreas, heart and other tissues. In addition to HAMP (104), mutations in the hemjuvelin (HJV; also called HFE2) gene may also cause an early-onset (juvenile) hemochromatosis (88). The most common form of HH, however, results from mutations in the HFE gene, which causes a less severe, adult-onset iron loading (32). HFE encodes a membrane-bound MHC class I-like protein. In northern Europeans, ~1 in 200 individuals are homozygous for the most common HFE mutation that is linked to iron loading. Many individuals that carry HFE mutations, however, do not develop iron-related tissue damage, suggesting that genetic modifiers exist or that environmental or dietary factors are also important for disease pathogenesis. Less commonly, adult-onset HH may result from mutations in the transferrin receptor 2 (TFR2) gene (9). The phenotype of these patients is essentially indistinguishable from that of individuals with HFE-associated iron loading.
Although the severity of symptoms varies in patients harbouring these genetic mutations, the resulting phenotypes have several shared features: 1) iron absorption is inappropriately high; 2) iron accumulates with a periportal distribution in the liver (predominantly in hepatocytes); 3) macrophages have low iron content, particularly in early stages of the disease; and 4) the mutant genes are all highly expressed in hepatocytes. This latter observation suggests that the proteins encoded by these genes may form parts of a common regulatory network. Consistent with this prediction, it was subsequently shown that patients with these various subtypes of HH all display inappropriately low expression or absence of HEPC, even though body iron levels are elevated (36). Thus, HFE, TFR2 and HJV are all integral parts of the signaling pathway that regulates expression of the \textit{HAMP} gene in hepatocytes. These studies, and others not mentioned here, have paved the way for a detailed analysis of how HEPC expression is modulated in response to changes in body iron status, changes in erythropoietic demand for iron, and during infection and inflammation. Accordingly, specific mechanisms of HEPC regulation have recently emerged, whereby various signaling pathways (e.g. the BMP/SMAD pathway) influence transcription of the \textit{HAMP} gene in hepatocytes. This topic has been reviewed recently (43, 60).

\textbf{Regulation of Intestinal Iron Absorption During Iron Depletion and Hypoxia}

Additional control of iron absorption occurs at the level of the enterocyte, which ultimately determines how much iron is acquired from the diet. Regulation of iron homeostasis in enterocytes goes beyond the FPN1-HEPC axis, involving specific physiologic adaptations that have evolved to maximize iron extraction from the diet when the demand for iron increases. These adaptations involve direct effects on enterocyte gene transcription, post-transcriptional control of mRNA stability, and morphological remodeling of the epithelium, probably all in response to alterations in intracellular iron levels in enterocytes. Moreover, a
role for copper in the control of duodenal iron absorption, with direct effects upon
enterocytes, has recently emerged.

The Iron-Regulatory Protein/Iron-Response Element (IRP/IRE) System

Intestinal DMT1 expression is strongly upregulated by iron deprivation and
consequent hypoxia (17, 38, 100), probably via transcriptional and post-transcriptional
mechanisms. The DMT1 transcript contains an iron-responsive element (IRE) (a stem-loop
structure) in its 3’ untranslated region, which interacts with cytosolic iron-sensing proteins
(iron-regulatory proteins [IRP1 and/or IRP2]). When intracellular iron is low, IRPs bind to
the DMT1 3’ IRE and stabilize the transcript, ultimately leading to increased protein
production (1, 42, 59). Conversely, when iron is abundant, the IRPs do not bind to the IRE
and the transcript becomes less stable, resulting in lower DMT1 protein levels. Interestingly,
two DMT1 3’ splice variants exist, with and without the IRE (55). The variant with the IRE
(+IRE) is the form predominantly expressed in the duodenum (115). Recent data, however,
suggest that the DMT1 3’IRE may be functional (i.e. bound by IRPs during iron deprivation)
only in suckling mice and not in adults (41). Whether this is representative of other
mammalian species as well is unknown.

DMT1 expression is also transcriptionally regulated by hypoxia-inducible factor 2α
(HIF2α) (69, 107) (as detailed below). Interestingly, the HIF2α transcript contains a 5’ IRE
(105). IRP binding in this case would be expected to repress translation. A recent study
provides evidence that iron-regulatory protein 1 (IRP1) specifically regulates HIF2α
translation (3) in mouse duodenum and in part, thus regulates iron flux. This and other recent
studies provide emerging evidence that the IRP/IRE system and HIF-mediated regulation of
gene transcription function in tandem in the duodenum to provide precise control of iron
absorption (3, 41).
FPN1 regulation in the duodenum, like DMT1, occurs via transcriptional and post-transcriptional mechanisms. First, FPN1 protein levels are modulated via interaction with serum-borne HEPC, which triggers internalization and eventual degradation of FPN1. This effectively blocks iron efflux from duodenal enterocytes. Second, as one FPN1 transcript variant contains a 5’IRE (128), FPN1 protein expression may be attenuated by a translational block via the IRPs. Although the FPN1 variant with the IRE and the one lacking the IRE are both expressed in the duodenum, recent evidence in mice suggests that the +IRE variant predominates, even under conditions of iron restriction (41). This counterintuitive observation is suggested by these authors to imply that IRP regulation of FPN1 protein translation is of less functional significance than HEPC-mediated control of FPN1 protein turnover, at least in the duodenum (of mice). However, the IRP/IRE system contributes at least in part to the regulation of FPN1 expression in the duodenum, as mice lacking both IRPs specifically in the intestine fail to fully repress FPN1 under conditions of iron excess, when HEPC expression and secretion is enhanced (41). Lastly, FPN1 is also regulated at the level of gene transcription, like DMT1, via transactivation by HIF2α (114). Importantly, FPN1 thus represents a unique molecular link connecting cell-specific control of iron homeostasis via the IRP/IRE system, and HIF2α-mediated transcriptional regulation with systemic regulation via HEPC.

Hypoxia and Iron Absorption

The intestinal epithelium exists in a natural state of hypoxia, with enterocytes on the upper part of the villus that are furthest from the capillary bed in the lamina propria being most significantly affected (15). Epithelial hypoxia increases between meals when blood flow to the gut decreases. More significant intestinal hypoxia results from iron deprivation which impairs hemoglobin synthesis (and thus oxygen transport). Early studies showed that iron
transport increased in rats (87) and mice (95) that were deprived of oxygen. As the identity of intestinal iron transporters was unknown at this time, these investigations did not provide mechanistic insight into how hypoxia increased iron absorption. Not surprisingly then, hypoxia is an important driver of intestinal iron absorption.

The molecular response to hypoxia is mediated by Hypoxia-Inducible transcription Factors (or HIFs). HIFs form heterodimers containing a hypoxia-responsive α subunit (HIF1, 2 or 3α) and a constitutively expressed β subunit (called HIF1β [or ANRT]). When oxygen levels are adequate (~21% O2), the cytosolic α subunits are hydroxylated, ubiquinated and rapidly degraded in lysosomes. During hypoxic conditions, however, the HIFα subunits are stabilized (i.e. not hydroxylated), allowing them to translocate to the nucleus and interact the β subunit. The net result is DNA binding by the complex and modulation of gene transcription. Interestingly, the enzymes that hydroxylate the HIFα subunits (causing their subsequent degradation), the prolyl hydroxylases (PHDs), are iron-dependent enzymes (30). So when iron is low, prolyl hydroxylase activity goes down and the HIFα subunits are more stable. Activity of the HIFs is thus regulated by oxygen and iron levels.

Recent studies showed that HIF2α was upregulated in the intestinal epithelium of mice during iron deprivation, while HIF1α levels were unaltered (54, 87). Furthermore, DCYTB, DMT1 and FPN1 were shown to be direct HIF2α targets (69, 107, 114); their induction presumably mediates the increase in iron absorption noted during low-iron/low-oxygen conditions. HIF2α was also preferentially stabilized in iron-deprived Caco-2 cells (54). Moreover, a genome-wide gene expression screen, combined with bioinformatics analysis of the promoters, suggested that many transactivated genes in iron-deprived Caco-2 cells were direct HIF2α targets. These studies collectively emphasize the important role that HIF2α signaling plays in the intestinal epithelium during alterations in iron or oxygen levels. Additionally, it is important to consider whether this regulatory mechanism also influences
iron absorption during other pathological situations with concurrent hypoxia (e.g. various inflammatory conditions or cancer). One recent study indeed showed that HIF2α transactivated DMT1 expression in colonic tumors (which are hypoxic), supporting the concept that iron plays an important role in the pathogenesis of cancer (124).

Morphological Adaptations of the Intestinal Mucosa to Iron Deprivation

In addition to HIF-mediated changes in gene transcription, morphological adaptations occur in the intestinal epithelium as part of the compensatory response to iron deprivation. Under normal conditions, enterocytes in the upper half of the villus make the largest contribution to iron absorption; however, during iron deficiency, enterocytes from the lower half of the villus participate in iron absorption (111). In this study, increased villus width and length was also documented. Another investigation performed using iron-deficient rats demonstrated that villus height, mucosal thickness and epithelial surface area increased in the jejunum (121). An additional, more recent investigation reconfirmed these previous observations and extended them to show increased cell proliferation in the crypts of iron-deficient rats, as indicated by an increase in the number of noted mitotic cell divisions (19). The latter study hypothesized that induction of a lipoxygenase (Alox15) altered eicosanoid biosynthesis in the gut during iron deficiency, perhaps providing a mechanistic explanation for these morphological adaptations. Whether similar compensatory, morphological adaptations occur in iron-deficient humans is unknown.

The Influence of Copper on Intestinal Iron Absorption

Recognition of physiologically-relevant interactions between iron and copper stem from observations made during the industrial revolution in England. It was noted that young women working in copper factories did not suffer from a common affliction at that time,
called chlorosis (i.e. probably iron-deficiency anemia) (37, 44). More recent investigations have shown that copper is redistributed to tissues vital for control of iron homeostasis during iron deprivation, including the intestinal mucosa (29), the liver (100) and blood (28, 112). Serum ferrooxidase activity is also enhanced in iron-deprived rodents, which corresponded with higher hepatic CP protein expression (99, 100). Moreover, the expression/activity of HEPH in duodenal enterocytes is also affected by copper levels (10, 12, 101). These data suggest that copper, either directly or indirectly, modulates intestinal iron homeostasis.

Several recent lines of experimental pursuit provide evidence that copper influences iron absorption directly at the level of duodenal enterocytes. First, copper homeostasis-related genes (such as those encoding copper-transporting ATPase 1 [ATP7A] and an intracellular copper binding protein [metallothionein]) are upregulated in parallel with iron transport-related genes (e.g. DMT1, DCYTB, FPN1) in the proximal small intestine of iron-deprived rats (17, 18). ATP7A was also induced in mice fed a low-iron diet (46) and in iron-deprived rat IEC-6 cells (123). Furthermore, the Atp7a gene is transactivated by HIF2α during low iron/hypoxia (122, 123), demonstrating coordinate regulation with DCYTB, DMT1 and FPN1 (69, 107, 114). Another copper transporter (CTR1) expressed in the intestine may also be regulated by HIF2α (92). These data have led to the postulate that ATP7A is a molecular link between iron and copper in the intestinal mucosa.

Altered ATP7A expression likely influences intracellular copper levels (or distribution), as it has dual functions in enterocytes: pumping copper into the trans-Golgi network (TGN) (to support cuproenzyme synthesis) and pumping copper across the BLM (to mediate copper efflux) (89). Since HEPH is a copper-containing protein that may be synthesized in the TGN, ATP7A may be required for HEPH expression/activity. This was directly tested by silencing ATP7A in rat IEC-6 cells (47). ATP7A knock down caused a significant reduction in membrane FOX activity (presumably mediated by HEPH), but
surprisingly, transepithelial iron flux increased. So, although ATP7A may not be absolutely required to deliver copper for the biosynthesis of HEPH, at least in this model system, diminished HEPH activity did not negatively influence iron transport. Transcriptional induction of FPN1 expression was suggested to be the mechanism by which iron flux was enhanced in cells lacking ATP7A (47). Other studies also suggested that FPN1 expression was influenced by copper levels (58, 70). Collectively, these studies reveal molecular details of iron-copper interactions in enterocytes, and provide rationale for further investigation.

Perturbations in Intestinal Iron Transport Associated with Various Pathological States

Intestinal iron absorption is altered in several important clinical conditions. These pathologies may result from mutations in genes encoding iron transporters or regulatory molecules, or alternatively, iron absorption may be secondarily perturbed by physiological changes associated with different diseases. Relevant human disorders which display altered intestinal iron absorption are summarized in Table 2.

Genetic Defects in Intestinal Iron Transporters

SLC11A2 and SLC40A1 mutations have been infrequently identified in humans. As DMT1 and FPN1 are expressed in many cell types, these mutations have wide-ranging pathophysiologic effects. Mutations in the SLC40A1 gene result in so-called Ferroportin Disease (or Type 4 hemochromatosis), which is an autosomal dominant form of iron loading (91). The mutant FPN1 protein may have a reduced capacity to export iron due to transport or trafficking defects, or its interactions with HEPC may be perturbed (23, 26). In the first case, lack of FPN1 activity traps iron in enterocytes, causing an initial systemic iron-deficiency in affected individuals. Ultimately, however, lack of FPN1-mediated iron export leads to iron loading in cells (and tissues) which store iron, including hepatocytes and RE macrophages.
Eventually, the bone marrow is deprived of iron required for red blood cell production and anemia ensues. Moreover, a compensatory increase in FPN1 expression from the remaining functional allele in the intestine occurs, enhancing iron absorption, and exacerbating tissue iron accumulation (120). The second class of SLC40A1 mutations perturbs the interaction of the FPN1 protein with HEPC so endocytosis from the BLM is impaired; iron absorption thus remains inappropriately high, leading to body iron accumulation (26).

SLC11A2 mutations in humans are exceedingly rare (56), which attests to the non-redundant role of DMT1 in iron metabolism. Development of severe microcytic, hypochromic anemia typifies these patients, but surprisingly, some of them load iron in the liver (75). This unexpected phenotype would be unlikely if DMT1 activity was abolished. In these patients, relative tissue hypoxia and downregulation of HEPC expression likely trigger increases in intestinal iron absorption, which must secondarily lead to hepatic iron accumulation. In this scenario, mutant DMT1 must retain residual activity (or an alternative iron transport pathway must be activated, which seems unlikely). The phenotype of another patient with an SLC11A2 mutation supports this possibility, as this individual suffers from severe iron-deficiency anemia but does not load iron in the liver. This SLC11A2 mutation leads to an amino acid substitution in a highly conserved residue in the first transmembrane domain (G75R), which is predicted to abolish iron transport activity (57).

Pathological Conditions Which Alter the Regulation of Iron Absorption

As detailed above, most inherited disorders of iron homeostasis involve mutations in the HAMP gene, or in genes encoding proteins which regulate HAMP transcription. HAMP or HJV mutations lead to extremely low or absent HEPC expression, secondarily resulting in enhanced iron absorption and subsequent severe tissue iron loading (88, 104). In patients with HFE or TFR2 mutations, HEPC reduction is less profound, and, as a result, the development
of the iron-overload phenotype is more gradual (36, 79). These autosomal-recessive disorders, with the exception of HFE-related hemochromatosis, are all quite rare.

HEPC deficiency leads to iron loading, so HEPC overexpression would be postulated to cause iron deficiency. This is indeed the case, as is exemplified by mutations in the \textit{TMPRSS6} gene, which cause many clinical cases of iron-refractory, iron-deficiency anemia (IRIDA) in humans (45). The \textit{TMPRSS6} gene encodes matriptase-2, an enzyme which functions via the BMP/SMAD signalling pathway to negatively regulate \textit{HAMP} transcription (77). Interestingly, single nucleotide polymorphisms in the \textit{TMPRSS6} gene have been linked to variations in iron homeostasis among different human populations (113), exemplifying the important role that matriptase-2 plays in determining body iron levels. Furthermore, a recent investigation suggests that TPMRSS6 could be a therapeutic target to treat iron-overload diseases (106).

\textbf{Altered Iron Absorption Secondary to Primary Pathological States}

Iron absorption may be secondarily perturbed in a number of pathological conditions. One common cause of inefficient iron absorption (and consequent anemia) relates to a reduction in the absorptive surface area of the gut, as commonly occurs in Celiac disease, inflammatory bowel diseases and short bowel syndrome. Blood loss associated with various GI disorders and diarrhea may also result in anemia. In other situations, morphological changes in the gut epithelium are not apparent, but additional physiological disturbances may alter HEPC expression and activity, as discussed below.

\textit{Iron-Loading Anemias}. Since the bone marrow is the predominant site of iron usage to support erythropoiesis, perturbations in red blood cell production can result in changes in HEPC expression and consequential alterations in iron absorption. Moreover, it has been known
for decades that ineffective erythropoiesis increases iron absorption (102). In disorders such as β-thalassemia, sideroblastic anemia or acute hemolysis, the enhanced erythroid drive reduces HEPC expression, leading to excessive intestinal iron absorption.

Chronic Liver Disease. Iron levels are altered in many chronic liver diseases, but the underlying physiological mechanism(s) are not fully understood. It is likely, however, that altered HEPC expression and subsequent changes in intestinal iron absorption are involved. For example, diminished HEPC synthesis and hepatic iron loading have been linked with excessive alcohol consumption (61) and hepatitis C virus infection (93). Furthermore, HEPC levels are increased in obesity, which is associated with reduced body iron levels (76). This likely reflects the inflammatory underpinnings of the metabolic syndrome.

Anemia of Chronic Disease. It is widely accepted that acute or chronic inflammation, associated with infection, cancer, rheumatoid arthritis and other inflammatory conditions, causes hypoferremia. Reduced plasma iron results from cytokine-mediated induction of HEPC expression (e.g. by IL-6), resulting in decreased iron absorption and iron release from RE macrophages (78). The development of HEPC antagonistic drugs would be potentially very useful in treating this common form of anemia.

Conclusions

Among essential dietary nutrients, the homeostasis of iron is unique given that free iron is highly reactive and no active excretory mechanisms have evolved in humans. The lack of a process for ridding the body of iron demonstrates the critical requirement for this nutrient and probably reflects the fact that early humans did not have consistent, readily available sources of highly bioavailable iron. As a result, humans and other mammals have developed
complex regulatory mechanisms to control body iron content at the level of absorption in the proximal small intestine. In health, iron absorption is precisely matched to iron losses, but in numerous clinical conditions this balance is disrupted, resulting in the pathological consequences of iron overload and iron deficiency.

Iron absorption is regulated systemically by HEPC. Many of the complexities of HEPC regulation have been revealed, and this has shown that HEPC is an integral part of each of the signaling pathways formerly known as the “stores”, “erythropoietic” and “inflammatory” regulators of iron homestasis. HEPC is induced when body iron stores are high, and during infection and inflammation, and is reduced when erythropoietic demand increases and during iron deficiency. Furthermore, discovery of HIF2α-mediated regulation of genes encoding iron transporters has provided a mechanistic explanation as to why hypoxia increases assimilation of dietary iron. HIF2α also alters copper homeostasis in duodenal enterocytes, supporting the possibility that copper is important in some respect for control of intestinal iron transport.

Despite these advances, many aspects of intestinal iron homeostasis remain to be elucidated and numerous pertinent questions remain to be answered. A few of those will be delineated here.

1) Given the physiologic importance of iron and the absorption process, do redundant backup systems exist? This is likely the case since intestine-specific inactivation of the SLC11A2 and SLC40A1 genes does not result in lethality (25, 48). Yet, mice without these intestinal transporters suffer from significant iron-deficiency anemia, demonstrating the near essentiality of these two iron transport systems.
2) Is DCYTB required for basal iron transport into enterocytes, or is it only required when demand increases? Its coregulation by HIF2α-mediated transcriptional activation (along with DMT1 and FPN1) and its robust induction during iron deficiency hint at an important role for this BBM ferrireductase. Additionally, is enzymatic iron reduction rate-limiting for iron absorption, or do dietary factors and/or GI secretions supply the necessary reducing power?

3) How does iron traffic within enterocytes after it is absorbed, and how it is delivered to FPN1? Given its propensity to mediate production of reactive oxygen species, free iron is likely very low in cells. Do specific iron-binding proteins or chaperones thus exist in enterocytes? Are the poly-r(C)-binding proteins expressed in enterocytes?

4) FPN1 and HEPH have been detected within the cytosol of enterocytes. Where within the cells are they located and what is their function there? Do they physically and/or functionally interact? How is export coupled to iron oxidation by HEPH?

5) What are the relative contributions of HEPH, CP and cytosolic FOXs to intestinal iron transport? Is enzymatic oxidation of ferrous iron rate-limiting for iron absorption? Can ceruloplasmin compensate for the loss of HEPH activity? What is the nature of the cytosolic FOXs in enterocytes? Are they required to complement HEPH activity when iron absorption increases? How do intracellular copper concentrations or distribution influence iron transport? Is ATP7A required for the biosynthesis of HEPH?

6) Does HEPC have additional biologically relevant effects on enterocytes, independent of its action on FPN1? How might HEPC regulate DMT1 protein trafficking on the BBM of duodenal enterocytes?
These unresolved issues and important questions will undoubtedly occupy the efforts of many investigators in this area of scientific pursuit over the next several years.

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Figure 1: Mechanisms of iron absorption in the mammalian duodenum. A single enterocyte is depicted with the transport machinery responsible for assimilation of dietary iron. Iron may be derived from heme or ferritin or it may occur as free non-heme iron. Heme iron transport is probably mediated by endocytosis of heme followed by iron liberation from heme within endosomes by heme oxygenase (HO). How heme traverses the brush border or endosomal membrane has yet to be elucidated. Non-heme ferric iron must be reduced, possibly by DCYTB or other cell surface ferrireductases, and subsequently transported into cells via DMT1. The proton gradient which fuels DMT1 activity is maintained by the combined actions of an apical sodium-hydrogen exchanger (NHE) and the basolateral Na⁺/K⁺ ATPase. Iron from ferritin is absorbed into enterocytes via an unknown mechanism and is likely then freed within lysosomes. Iron derived from all three dietary sources likely forms a single intracellular iron pool. Whether iron chaperones exist in enterocytes is unknown and thus how iron trafficks within cells after absorbance is not clear. Iron destined for export traverses the basolateral membrane (BLM) via FPN1. The exit of ferrous iron is functionally coupled with iron oxidation via HEPH and possibly other ferroxidases. Ultimately, ferric iron then binds to transferrin in the interstitial fluids or in the vasculature and is distributed throughout the body.

Figure 2: Systemic and mucosal regulation of iron absorption. During iron deficiency, morphological adaptations occur within the intestinal mucosa (A). These include increased mucosal thickness, increased villus length and width, enhanced mitosis of stem cells in the crypts, and absorption of iron via enterocytes over a greater length of the villus. The net result is to maximize the capacity of the small intestine to extract iron from the diet. Additional adaptations occur within duodenal enterocytes in response to changes in intracellular metal-ion concentrations or to alterations in body iron status (B). When body iron stores are replete
or during iron overload, HEPC binds to FPN1 on the BLM of enterocytes and causes its internalization and degradation, effectively blocking iron export (I). Iron absorption is also regulated locally by changes in intracellular iron levels, which alter interactions between stem-loop structures in mRNA transcripts (IREs) and cytosolic iron-sensing proteins (IRPs) (II). The result is that the translation of certain transcripts is blocked (e.g. ferritin) and other transcripts are stabilized (e.g. DMT1). Moreover, during iron deficiency and/or low oxygen conditions, a hypoxia-inducible trans-acting factor, HIF2α, is stabilized in enterocytes, promoting its dimerization with a HIF1β subunit and induction of gene transcription (III). This results in increased iron transport via apical and BLM transport processes. Alterations in intracellular copper homeostasis, which occur during iron deficiency, have also been shown to affect iron transport in enterocytes (IV). Knockdown of a copper transporter (ATP7A), which presumably alters copper distribution and/or cuproenzyme synthesis, increases FPN1 gene transcription and enhances iron efflux.
References


46. Gulec S and Collins JF. Investigation of iron metabolism in mice expressing a mutant Menke’s copper transporting ATPase (Atp7a) protein with diminished activity (Brindled; Mo (Br) (y)). *PLoS One* 8: e66010, 2013.


### Table 1: Proteins Involved in the Absorption of Non-Heme Iron

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenal cytochrome B</td>
<td>DCYTB</td>
<td>Ferric iron reduction for absorption via DMT1</td>
</tr>
<tr>
<td>Solute carrier family 11 (proton-coupled</td>
<td>DMT1</td>
<td>BBM ferrous iron/proton cotransporter; also transports a range of other</td>
</tr>
<tr>
<td>divergent metal ion transporter), member 2</td>
<td></td>
<td>divalent metal ions, including Mn$^{2+}$ and Cd$^{2+}$; may transport</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cu during iron deprivation</td>
</tr>
<tr>
<td>Solute carrier family 40 (iron-regulated</td>
<td>FPN1</td>
<td>BLM ferrous iron exporter; HEPC target</td>
</tr>
<tr>
<td>transporter), member 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferritin, light polypeptide/ Ferritin, heavy</td>
<td>FTL/FTH1</td>
<td>Intracellular iron storage</td>
</tr>
<tr>
<td>polypeptide 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hephaestin</td>
<td>HEPH</td>
<td>BLM ferroxidase; a soluble form may also exist as well as additional,</td>
</tr>
<tr>
<td>HEPC antimicrobial peptide</td>
<td>HEPC</td>
<td>unidentified FOXs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver-derived, circulating peptide hormone; binds to FPN1 and mediates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>internalization and degradation</td>
</tr>
</tbody>
</table>
## Table 2. Selected Pathological States Associated with Perturbed Iron Absorption

<table>
<thead>
<tr>
<th>Hereditary Hemochromatosis</th>
<th>Classification</th>
<th>Gene*</th>
<th>Iron Absorption</th>
<th>HEPC Level*</th>
<th>Resulting Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>Primary iron overload</td>
<td><em>HFE</em></td>
<td>Increased</td>
<td>L</td>
<td>Parenchymal iron overload, liver disease, arthropathy</td>
</tr>
<tr>
<td>Type 2A</td>
<td>Primary iron overload</td>
<td><em>HFE2</em> <em>HJV</em></td>
<td>Increased</td>
<td>VL</td>
<td>Severe parenchymal iron overload, cardiac disease, liver cirrhosis, endocrine failure, diabetes, arthropathy</td>
</tr>
<tr>
<td>Type 2B</td>
<td>Primary iron overload</td>
<td><em>HAMP</em></td>
<td>Increased</td>
<td>VL- Ab</td>
<td>Same as type 2A</td>
</tr>
<tr>
<td>Type 3</td>
<td>Primary iron overload</td>
<td><em>TFR2</em></td>
<td>Increased</td>
<td>L</td>
<td>Parenchymal iron overload, liver disease</td>
</tr>
<tr>
<td>Type 4; “transport defective”</td>
<td>Primary iron overload</td>
<td><em>SLC40A1</em></td>
<td>Reduced</td>
<td>N-H</td>
<td>Parenchymal and reticuloendothelial iron overload in the liver</td>
</tr>
<tr>
<td>Type 4; “HEPC resistance”</td>
<td>Primary iron overload</td>
<td><em>SLC40A1</em></td>
<td>Increased</td>
<td>N-H</td>
<td>Parenchymal iron overload</td>
</tr>
</tbody>
</table>

### Iron-Related Anemias

| Refractory iron-deficiency anemia | Iron-deficiency anemia | *TMPRSS6* | Reduced | H | Hypochromic, microcytic anemia |
| Refractory iron-deficiency anemia | Iron-deficiency anemia | *SLC11A2* | Reduced | L-N | Hypochromic, microcytic anemia |
| Hereditary atransferrinemia       | Iron-loading anemia    | *TF*      | Increased | L | Hemosiderosis of the heart and liver |
| β-thalassaemia                   | Iron-loading anemia    | *HBB*     | Increased | L-N | Parenchymal and reticuloendothelial iron overload, anaemia, reticulocytosis |
| Sickle cell anemia               | Iron-loading anemia    | *HBB*     | Increased | L-N | Same as β-thalassaemia |
| X-linked sideroblastic anemia    | Iron-loading anemia    | *ALAS2*   | Increased | L** | Same as β-thalassaemia |

*Mutated gene causing disorder; *L, low; VL, very low; Ab, absent; N, normal; H, high. **Predicted levels based on current knowledge of HEPC regulation.