# Iron-Sensing Proteins that Regulate Hepcidin and Enteric Iron Absorption

Mitchell D. Knutson

Food Science and Human Nutrition Department, University of Florida, Gainesville, Florida 32611-2710; email: mknutson@ufl.edu

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hemochromatosis, bone morphogenetic protein, transferrin receptor, matriptase-2, hypoxia-inducible factor

## Abstract

The human body cannot actively excrete excess iron. As a consequence, iron absorption must be strictly regulated to ensure adequate iron uptake and prevent toxic iron accumulation. Iron absorption is controlled chiefly by hepcidin, the iron-regulatory hormone. Produced by the liver and secreted into the circulation, hepcidin regulates iron metabolism by inhibiting iron release from cells, including duodenal enterocytes, which mediate the absorption of dietary iron. Hepcidin production increases in response to iron loading and decreases in iron deficiency. Such regulation of hepcidin expression serves to modulate iron absorption to meet body iron demand. This review discusses the proteins that orchestrate hepatic hepcidin production and iron absorption by the intestine. Emphasis is placed on the proteins that directly sense iron and how they coordinate and fine-tune the molecular, cellular, and physiologic responses to iron deficiency and overload.

## Contents



## INTRODUCTION

No discovery has energized the study of iron metabolism more than that of hepcidin, the iron-regulatory hormone. Since its discovery ten years ago, remarkable progress has been made in our understanding of its function, regulation, and relation to diseases of iron overload. This review examines the proteins that regulate hepcidin expression in response to iron. Because hepcidin, in turn, regulates iron absorption, the proteins involved in the assimilation of dietary iron are also discussed. Emphasis is placed on the regulatory proteins that directly sense iron and how they coordinate the complex molecular and physiologic responses to iron deficiency and repletion. Although the regulation of hepcidin expression by inflammation (33) and erythropoiesis (76) also merits discussion, it is beyond the scope of the present review.

Hepcidin is a 25 amino acid peptide that is produced primarily by hepatocytes and secreted into the circulation (54, 87). Hepcidin controls systemic iron metabolism by binding to its receptor, ferroportin, causing its internalization and degradation (79). As ferroportin is the sole known iron-export protein (20), its removal from the cell surface reduces iron release. Such interactions between ferroportin and hepcidin regulate the amount of iron released into the plasma from macrophages of the reticuloendothelial system (53), hepatocytes (95), and duodenal enterocytes (97).

**BMPs:** bone morphogenetic proteins

**TFR:** transferrin receptor

## PROTEINS THAT MEDIATE HEPCIDIN EXPRESSION

Intensive research has been directed toward understanding the proteins that orchestrate hepcidin production. At present, two main iron-related signaling pathways for hepcidin expression have been identified: One involves bone morphogenetic proteins (BMPs), and the other is dependent upon transferrin receptor (TFR) 2.

### The BMP Signaling Pathway and Hepcidin Expression

BMPs comprise a group of at least 20 soluble secreted molecules that belong to the transforming growth factor-β superfamily. Initially identified as proteins that induce bone and cartilage formation, BMPs are now recognized as multifunctional autocrine and paracrine factors that regulate a constellation of processes during development as well as maintain specialized functions later in adult life (17). BMPs bind to cell-surface BMP receptor (BMPR) complexes, each composed of two type I (BMPRI) and type II (BMPRII) serine/threonine kinase receptors.

Upon ligand binding, BMPRII phosphorylates BMPRI, which then propagates the signal by catalyzing the phosphorylation of a subset of cytosolic transcription factors [homolog of the Sma protein in *C. elegans* and the MAD protein in *Drosophila* (SMAD)]1/5/8. Phosphorylation of SMAD1/5/8 promotes its association with SMAD4 and translocation into the nucleus, where the heteromeric complex binds to specific enhancers in target genes, activating transcription (**Figure 1**).

The link between the BMP/SMAD4 signaling pathway and hepcidin expression was discovered in 2005 in studies designed to assess the role of SMAD4 in liver development and maintenance. Wang et al. (125) observed that mice genetically engineered to lack *Smad4* in hepatocytes had no obvious defects in liver histology, indicating that SMAD4 does not play an essential role in liver development. Unexpectedly, the knockout mice were found to develop severe hepatic iron overload. The mutant animals also had 100-fold lower levels of hepatic hepcidin mRNA, suggesting that SMAD4 positively regulated hepcidin expression. Studies of primary hepatocytes further revealed that *Smad4*-null hepatocytes did not produce hepcidin in response to BMP4, a potent inducer of hepcidin expression in wild-type hepatocytes (130).

Following this work connecting hepcidin and BMP4, a number of in vitro and in vivo studies demonstrated increased hepcidin expression in response to a variety of other BMP ligands, including BMP2, BMP5, BMP6, BMP7, and BMP9 (5, 6, 113, 125). However, the key endogenous BMP regulator of hepcidin expression was found to be BMP6, as discovered by two independent groups of researchers (3, 72). Both groups found that targeted disruption of *Bmp6* in mice resulted in massive iron overload that was associated with nearly undetectable levels of hepcidin mRNA in the liver. Hepatic levels of phosphorylated Smad1/5/8 were also significantly reduced, indicative of diminished BMP signaling. BMP6 appears to be a direct mediator of hepcidin expression because injection of exogenous BMP6 into mice increased hepcidin mRNA abundance, whereas injection of a neutralizing antibody to BMP6 decreased it (3). Taken together, these studies indicate that BMP6 is essential for hepcidin expression and is functionally nonredundant with other members of the BMP family.

Full activation of hepcidin expression through the BMP signaling pathway requires hemojuvelin (HJV), a glycosylphosphatidylinositol (GPI)-linked cell-surface protein also known as repulsive guidance molecule C (RGMC). Mutations in *HJV* are responsible for ~95% of cases of juvenile hemochromatosis (85), a severe form of iron overload that affects individuals before the age of 30. Juvenile hemochromatosis patients have very low levels of hepcidin (86). Babitt et al. (5) showed that HJV increases hepcidin expression by enhancing BMP signaling. They proposed that, like the other two RGM family members, RGMA and RGMB, HJV (i.e., RGMC) functions as a BMP coreceptor, binding BMPs at the cell surface and facilitating their interaction with their canonical receptor, BMPR. The liver expresses at least five different BMPs, but only BMP6, BMP4, and BMP2 are endogenous ligands for HJV in hepatic cells (130). Interestingly, lack of HJV does not affect the responsiveness of hepcidin expression to BMPs; instead, it markedly reduces the overall basal level of hepcidin (110). The amount of HJV can be regulated by matriptase-2 [also called transmembrane protease, serine 6 (TMPRSS6)], a cell-surface serine protease expressed primarily in the liver (117). When activated, matriptase-2 cleaves HJV into small fragments (107), abrogating its function as a BMP coreceptor.

In addition to the GPI-anchored form, HJV exists as a secreted soluble form lacking the GPI tail (62). The two different forms of HJV exert opposite effects: plasma membrane HJV increases hepcidin expression, whereas s-HJV decreases it. The reciprocal regulation arises because s-HJV binds BMPs, preventing their interaction with HJV at the cell surface. Accordingly, injection of mice with s-HJV decreases BMP signaling and suppresses hepcidin expression (6). The subcellular origin of s-HJV is controversial. Although some data imply

**SMAD:** homolog of the Sma protein in *C. elegans* and the MAD protein in *Drosophila*

**Hemojuvelin (HJV):** mutations in the *HJV* gene cause a severe form of iron overload in individuals under 30 years of age

**TMPRSS6:** transmembrane protease, serine 6. Also called matriptase-2

**Holotransferrin:** transferrin that contains bound iron (up to two Fe atoms). Holotransferrin in blood plasma delivers iron to tissues. The percent saturation of transferrin with iron is a commonly used clinical indicator of iron status

***HFE*:** the high Fe gene. The C282Y point mutation in *HFE* causes hereditary hemochromatosis, an iron overload disorder resulting from excessive iron absorption and toxic iron accumulation in the liver, pancreas, and heart. Clinical symptoms usually become manifest after 50 years of age



that s-HJV originates from cleavage of the protein at the cell surface (56), it is more likely that s-HJV arises from cleavage of HJV at an intracellular site (106) in a process involving endocytosis of and retrograde trafficking (66). Cleavage of the GPI anchor in HJV is mediated by the protease furin (106).

The release of s-HJV requires the presence of neogenin (133), a membrane protein that is the classical receptor for RGMA, a close family member of HJV. As with RGMA, HJV also binds to neogenin (131). Neogenin's role in iron metabolism is suggested by the observation that the most common mutation in HJV causing juvenile hemochromatosis prevents the interaction between HJV and neogenin (134). In HepG2 cells, suppression of endogenous neogenin reduced the release of s-HJV but did not affect the expression of HJV at the cell surface (135). Neogenin knockdown also reduced BMP4-stimulated hepcidin expression as well as phospho-SMAD1/5/8 levels in HepG2 cells expressing HJV, suggesting that an interaction between neogenin and HJV is required for BMP-dependent signaling of hepcidin expression (136).

## The TFR2-Dependent Signaling Pathway and Hepcidin Expression

TFR2, a homolog of transferrin receptor 1 (TFR1), is expressed predominantly in the liver (49). Like TFR1, TFR2 can facilitate the cellular uptake of iron from holotransferrin, but it cannot compensate for TFR1 function, as indicated by the fact that mice lacking *TfR1* die in utero (60). In human liver, TFR2 protein levels are approximately five times higher than TFR1 (14). The first clue that TFR2 played a key role in iron metabolism came from the identification of six hemochromatosis patients who shared a single nonsense mutation in the *TFR2* gene (10). The orthologous mutation in murine *TfR2* recapitulated the iron-loaded phenotype, confirming the participation of TFR2 in iron homeostasis (28). Both humans and mice with disabling *TFR2* mutations were subsequently shown to express low levels of hepcidin (48, 78), indicating that TFR2 is essential for hepcidin production. Hepatocyte-specific ablation of *Tfr2* in mice confirmed that hepatic TfR2 is required for hepcidin expression (122). Studies in hepatic cell lines and primary mouse hepatocytes further revealed that TFR2-mediated hepcidin production additionally required holotransferrin (35, 96) and serum (96).

Similar to a lack of functional TFR2, genetic disruption of the high-Fe gene (*HFE*) results in diminished hepcidin production (74, 115). It is likely that HFE modulates hepcidin expression through its association with TFR2 and perhaps indirectly by its interaction with TFR1 (**Figure 1**). HFE binds to TFR2 through its extracellular α-3 domain (12) and to TFR1 through its extracellular α-1 and α-2 domains (7). The binding sites for holotransferrin and HFE overlap on TFR1 but not on TFR2 (12, 128). Mice engineered to carry a mutation that prevented the interaction of Hfe and TfR1 displayed high levels of hepcidin, whereas those with mutations that favored constitutive binding of Hfe to TfR1 exhibited low levels of hepcidin (101). These observations support a model proposed by Schmidt et al. (101) in which TFR1 serves to sequester HFE, preventing its interaction with TFR2, thereby decreasing signaling for hepcidin expression. In high iron conditions, binding of holotransferrin to TFR1 displaces HFE and allows it to interact with TFR2. In human liver, HFE is present in substoichiometric amounts relative to TFR1, indicating that all available HFE could be bound by TFR1 (14). In HepG2 cells, stimulation of hepcidin expression by holotransferrin was associated with an interaction between HFE and TFR2 (35). The interaction additionally involved the binding of holotransferrin, suggesting that holotransferrin, HFE, and TFR2 form a signaling complex that increases hepcidin expression. Consistent with this model, holotransferrin failed to induce hepcidin expression in primary mouse hepatocytes from mice lacking either *Hfe* or *TfR2* (35). Other studies, however, report that holotransferrin can induce hepcidin expression in *Hfe*-null primary mouse hepatocytes (96).

In contrast to our ample understanding of the intracellular signaling pathway elicited by BMP6, our knowledge is only starting to emerge regarding the signal transduction pathways that link TFR2 and HFE to hepcidin expression. Unlike TFR1, TFR2 at the cell surface is present in lipid rafts (9), membrane microdomains that are known to participate in cellular signal transduction. In the K562 erythroleukemia cell line, binding of holotransferrin to TFR2, but not TFR1, resulted in the activation of the mitogen-activated protein kinase (MAPK) signaling pathway, as indicated by increased phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/ERK2) and p38 (9). An increase in ERK1/2 phosphorylation in response to holotransferrin was also observed in freshly isolated mouse hepatocytes (96). Because holotransferrin induces hepcidin expression, it was proposed that ERK signaling may be involved in hepcidin production (9). Indeed, the holotransferrin-stimulated expression of hepcidin by hepatocytes could be blocked by using an ERK-specific inhibitor, directly implicating ERK1/2 in hepcidin expression (96). Additional evidence linking HFE, TFR2, and MAPK signaling to hepcidin is provided by a study of mice lacking *Hfe* or *TfR2* or both (121). Iron-loaded *Hfe*- or *TfR2*-null mice had diminished levels of hepatic phospo-ERK1/2, which were even lower in livers of mice lacking both *Hfe* and *TfR2*.

## Crosstalk Between BMP and TFR2 Signaling Pathways

In studies of isolated mouse hepatocytes, the addition of holotransferrin not only increased levels of phospho-ERK1/2, but it also increased the abundance of phospho-Smad1/5/8, the intracellular mediators of BMP signaling (96). Moreover, ERK inhibition prevented the increase in phospho-Smad1/5/8 (at least in the short term), suggesting that ERK signaling is essential for holotransferrin-related Smad phosphorylation. Consistent with this possibility is the demonstration that mice lacking both *Hfe* and *TfR2* had significantly reduced levels of Smad1/5/8 (121). Interestingly, the induction of hepatocyte hepcidin by holotransferrin was completely blocked by BMP2/4 antibodies or by noggin, a soluble protein that binds to BMP ligands and prevents their interaction with BMP receptors (96). Thus, it appears that BMP signaling is required for holotransferrin activation of hepcidin expression. There are at least two possible explanations for the apparent crosstalk between the BMP and TFR2-dependent signaling. First, the two pathways may induce hepcidin expression by ultimately using the same intracellular signaling molecules—i.e., Smad 1/5/8 and Smad4. Second, it is possible that the cell-surface proteins involved in the two pathways associate in a large signaling complex consisting of holotransferrin, TFR2, HFE, HJV, BMP6, and BMPR (76).

## Iron Status and Hepcidin Expression

In the first paper that linked hepcidin expression to iron, Pigeon et al. (91) showed that hepatic hepcidin mRNA levels increased as liver iron concentrations increased and decreased after a reduction in liver iron content. Hepcidin expression has also been shown to increase after iron dextran injection (91) and even after ingestion of a single 65 mg dose of supplemental ferrous sulfate by human volunteers (64).

To identify which iron-regulated molecules may contribute to the modulation of hepcidin expression, Kautz et al. (47) performed a genome-wide expression array study of livers from mice fed iron-deficient, iron-sufficient, and iron-loaded (8% carbonyl iron) diets. Surprisingly, only four transcripts were identified to be regulated like hepcidin: BMP6, Smad7, Id1, and Atoh8. Smad7 and Id1 are known to be direct target genes of the BMP/SMAD signaling pathway; Atoh8 is a basic helix-loop-helix transcription factor involved in cell differentiation. The upregulation of BMP6, Smad7, and Id1 underscores the preponderant role of BMP6 and BMP signaling in response to iron overload. BMP6 was also found to be upregulated in liver-specific *Smad4*-null

mice (47), which develop severe hepatic iron overload while consuming a normal-iron diet, indicating that upregulation of hepatic BMP6 is not solely due to the consumption of an iron-loaded diet. In contrast, Smad7, Id1, and Atoh8 were not upregulated, but downregulated, in liver-specific *Smad4*-null mice, suggesting that the expression of these genes is related more to SMAD signaling than to iron. SMAD4 and HJV are essential components of the iron-sensing pathway because iron dextran injection failed to induce hepcidin expression in mice lacking either of these proteins (81, 125).

Efficient BMP/SMAD signaling of hepcidin expression in response to iron requires HFE. Although mice lacking HFE had higher BMP6 expression, consistent with their elevated hepatic iron concentrations, levels of phospho-SMAD 1/5/8 and Id1 mRNA were not elevated (16, 46). Thus, the well-known blunted hepcidin response in humans and mice lacking HFE (8, 74, 92, 119) appears to result, at least in part, from impaired BMP/SMAD signaling. It should be noted, however, that iron regulation of hepcidin expression can occur in the absence of HFE, albeit with reduced efficiency (36, 121).

The concentration of iron transferrin (holotransferrin) in plasma has been proposed to be a regulator of hepcidin expression (44, 98). Support for such a role is provided by the observation that increases in urinary hepcidin levels after an oral iron supplement were proportional to increases in serum transferrin saturations (64). In addition, a direct effect of holotransferrin on hepcidin expression was demonstrated in primary mouse hepatocytes (64). Exposure of the cells to increasing transferrin saturations resulted in dose-dependent increases in hepcidin mRNA levels. Neither apotransferrin nor nontransferrin-bound iron affected hepcidin levels, demonstrating that hepcidin induction from holotransferrin does not respond to elemental iron or elevated cellular iron concentrations. In mice, serum transferrin saturations correlated with changes in hepatic hepcidin expression (31). Notably, these changes occurred before changes in liver iron concentrations became evident. It is probable that holotransferrin regulates hepcidin expression through TFR2, as holotransferrin has been shown to increase TFR2 levels in a dose-dependent manner (44, 98). In *TfR2*-null mice, intraperitoneal injection of iron failed to induce hepcidin expression (48), indicating that TfR2 is required for hepcidin production in response to iron. Holotransferrin also influences hepcidin expression by affecting BMP signaling, as holotransferrin inhibits the release of s-HJV (63, 133), likely by downregulating the expression of furin (106).

Although numerous proteins are known to participate in mediating hepcidin expression in response to iron loading, the mechanisms of hepcidin downregulation in response to iron deprivation are less well understood. It is clear, however, that matriptase-2 plays a role by cleaving cell-surface HJV, reducing BMP signaling. Mice and humans with mutations in matriptase-2 that impair its protease activity are unable to downregulate hepcidin and therefore become anemic (21, 24). A similar phenotype is observed in mice engineered to lack *matriptase-2* (29). Mice deficient in both *matriptase-2* and *Hjv* have an iron overload phenotype similar to mice lacking only *Hjv*, indicating that Hjv is likely the major substrate for matriptase-2 proteolytic activity in vivo (110). S-HJV additionally contributes to the downregulation of hepcidin expression during iron deficiency. Acting as a decoy receptor, s-HJV binds BMPs and prevents their interaction with cell-surface HJV, thereby decreasing BMP activation of hepcidin expression. In cultured cells, treatment with the iron chelator, desferrioxamine, increased the production and secretion of s-HJV into the culture medium (106). Levels of cell-surface HJV did not change, consistent with the model that s-HJV originates from an intracellular pool of HJV. Enhanced secretion of s-HJV during iron chelation results from an increase in the transmembrane protease furin, the enzyme responsible for the proteolytic event that causes s-HJV release (106). In iron deficiency, the increase in s-HJV release requires the presence of neogenin, whose levels do not change with iron status (133).

Insight into the relative contributions of the multiple proteins that regulate hepcidin expression is gained by comparing phenotypes of iron-loading disorders. Mice lacking *Bmp6*, *Smad4*, or *Hjv* are all characterized by very low hepcidin expression and a rapid and massive iron overload early in life (3, 81, 125), similar to juvenile hemochromatosis patients who harbor mutations in *HJV* (85). In contrast, mice or humans lacking functional HFE or TFR2 develop iron overload less rapidly and severely (61, 121). Mice without *TfR2* develop more severe iron overload than those without *Hfe* (121). Thus, it appears that the BMP signaling pathway involving BMP6, HJV, and SMAD4 represents a mandatory signaling pathway for hepcidin expression, whereas TFR2, and less importantly HFE, serves to modulate this response. That the combined disruption of TFR2 and HFE results in an iron overload similar to juvenile hemochromatosis (121) reinforces the possibility that BMP/SMAD/HJV signaling and holotransferrin/TFR2/HFE signaling interact or converge into a common pathway to ultimately orchestrate hepcidin expression in response to iron.

## Iron-Sensing Proteins that Regulate Hepcidin Expression

Among the numerous proteins that orchestrate the expression of hepcidin in response to iron, which ones directly sense iron? Certainly transferrin can be considered an iron-sensing protein by virtue of its ability to bind directly to atomic iron. The degree of saturation of circulating transferrin with iron has been proposed as the signal that relays information about body iron status to the liver, the site of hepcidin production (31). The concentration of holotransferrin is then "sensed" by hepatic TFR2, which binds holotransferrin at the hepatocyte cell surface (44, 98). HFE, which associates with TFR2 at the plasma membrane, appears to enhance the sensing and intracellular signaling for hepcidin expression, but it is not absolutely required (36, 121).

The interaction of HFE with TFR2 is promoted by the downregulation of TFR1—a process regulated in part by cytosolic iron-regulatory protein (IRP) 1 and 2. IRPs regulate the expression of TFR1 by interacting with iron-response elements (IREs) in the 3′ untranslated region (UTR) of TFR1 mRNA. Under iron-replete conditions, IRP1 assembles an iron-sulfur cluster and loses its ability to bind IRE, whereas IRP2 is degraded by the proteasome. Unbinding of IRPs destabilizes TFR1 mRNA and decreases levels of TFR1, allowing HFE to interact with TFR2. IRPs can therefore be considered as intracellular iron-sensing proteins that modulate hepcidin expression, albeit indirectly (75). Although IRP1 and IRP2 are largely functionally redundant, studies of mice engineered to selectively lack either *Irp1* or *Irp2* revealed that only IRP2 is essential for normal iron metabolism (59, 73). Mice lacking *Irp2* either globally or selectively in hepatocytes exhibit reduced hemoglobin levels and modestly elevated liver nonheme iron concentrations (80% higher than controls) (23). Surprisingly, in both animal models, hepatic hepcidin expression was unaffected, indicating that the increase in liver iron concentration was not sufficient to activate hepcidin production or that IRP2 is required for the hepcidin response.

**IRP:** iron-regulatory protein

Iron sensing by the BMP pathway appears to be mediated through BMP6, as its levels were found to increase with dietary iron loading and decrease with deficiency (47). BMP2, which also increased in iron loading, may contribute to activating BMP signaling, but the increase was not as great as that of BMP6. Exactly what triggers BMP6 and BMP2 in response to iron remains to be identified. The BMP response-enhancer region of the murine hepcidin promoter has been mapped to a region 1.6 and 1.8 kb upstream from the start of translation (114)—interestingly, the same region that is required for the in vivo responsiveness of iron (112). Recent finer mapping of this region has identified a 27-bp stretch that is critical for BMP responsiveness (111). Whether this same 27-bp region is critical for iron responsiveness remains to be determined.

**HIF:** hypoxia-inducible factor

The sensing of iron deficiency requires matriptase-2/TMPRSS6, which cleaves HJV and thereby decreases BMP signaling and hepcidin expression (107). Elucidation of how matriptase-2 "senses" the iron-deficient state will markedly enhance our understanding of hepcidin regulation. Indeed, a key role for matriptase-2 in body iron regulation seems highly probable in view of four recent genome-wide association studies that independently reported associations between single nucleotide polymorphisms (SNPs) in the human *TMPRSS6* gene and indicators of iron status [see review by Andrews (2)].

In iron deficiency, BMP signaling is additionally attenuated by s-HJV that arises from the furin-mediated cleavage of HJV (106). Furin levels increase in iron deficiency due to transcriptional activation by hypoxia-inducible factor (HIF)-1α (69), a member of the HIF family of transcription factors that mediate the cellular response to hypoxia. At the cellular level, iron deficiency mimics hypoxia, leading to HIF activation (123). Not surprisingly, a number of iron-related genes, including transferrin and TFR1, are positively regulated by HIF-1α. This recognition has led to a proposed model of iron metabolism in which HIF serves as an iron sensor and iron regulator (89). The observation that HIF-1α levels increase in iron-deficient mouse liver and that the murine hepcidin gene has two consensus hypoxia response elements (HREs) in its promoter prompted investigations into the role of HIFs in regulating hepcidin expression. Mice engineered to lack *Hif-1α* in hepatocytes had normal levels of hepatic hepcidin, but they were unable to downregulate hepcidin efficiently in response to iron deficiency (90). It was further demonstrated that HIF-1α was able to bind to and negatively transactivate the murine hepcidin promoter, suggesting that HIF-1α serves as a repressor of hepcidin expression in vivo. More recent studies, however, have failed to detect changes in hepcidin regulation in mice lacking hepatocyte *Hif-1α* (103) and did not find direct transcriptional suppression of hepcidin by HIF-1α (118). The discordant findings between these reports highlight the need for additional studies of the role of HIF-1α in iron sensing and hepcidin regulation.

## PROTEINS THAT MEDIATE IRON ABSORPTION

The process of dietary iron absorption can be divided into intestinal uptake (i.e., transport across the apical membrane of enterocytes) and transfer (i.e., translocation through the cytoplasm and across the basolateral membrane into the portal circulation). Dietary iron is found either as heme or nonheme iron. Heme iron is found in meat, whereas nonheme iron is generally found in foods of plant origin. However, roughly half of the total iron in meat is in the nonheme form as part of ferritin, iron-sulfur enzymes, and other iron-containing proteins. Accordingly, most dietary iron is nonheme iron.

### Apical Iron Uptake from the Lumen

Dietary nonheme iron in the lumen of the upper small intestine is taken up via the transmembrane protein divalent metal transporter 1 (DMT1) located at the apical membrane of enterocytes (26, 38) (**Figure 2**). DMT1 appears to be the only protein that serves this essential function, as mice lacking *Dmt1* specifically in the intestinal epithelium fail to absorb sufficient dietary iron and become progressively and severely anemic (37). Impaired iron absorption and anemia are also characteristic of the Belgrade (*b*) rat and the *mk* mouse, which both harbor a glycine-to-arginine substitution at position 185 in DMT1 (26, 27). This mutation in DMT1 has been shown to diminish iron transport activity and alter subcellular localization of the protein in the intestine (11, 108). In the duodenum, four different DMT1 transcripts have been identified that presumably code for distinct proteins referred to as DMT1A, DMT1A-IRE, DMT1B, and DMT1B-IRE (42). Alternative transcription start sites in distinct promoters give rise to the A and B variants, which

are alternatively spliced to yield variants with 3′IREs (DMT1A-IRE and DMT1B-IRE).

Transport studies in *Xenopus* oocytes show that DMT1 actively transports iron only as the divalent ferrous ($Fe^{2+}$) ion (38). Dietary nonheme iron, however, generally exists as the oxidized ferric ($Fe^{3+}$) species. Therefore, a reduction step is required before DMT1 can take up iron into the enterocyte. Reduction of $Fe^{3+}$ is probably mediated by the ferric reductase enzyme DCYTB (duodenal cytochrome B), which is abundantly expressed on the apical membrane of enterocytes, particularly during iron deficiency (67). Surprisingly, mice with targeted disruption of *Dcytb* were found to have normal iron levels, implying that DCYTB is not required for iron absorption (39). An important caveat to this interpretation is that the mice were fed a rodent chow diet containing a relatively high amount of iron (380 ppm), supplied as ferrous sulfate, as well as antioxidants that would help to maintain the iron in the reduced $Fe^{2+}$ state. To test the hypothesis that DCYTB serves as an essential ferric reductase in iron absorption, additional studies in *Dcytb*-null mice are needed in which purified diets are used that provide iron exclusively in the ferric form and in amounts more consistent with standard AIN formulations of rodent diets (i.e., ~45 ppm Fe). Recently, six-transmembrane epithelial antigen of the prostate (Steap) proteins have been identified as a new family of ferrireductases (84), with Steap3 being essential in ferric iron uptake by erythroid cells (83). Among the Steap proteins, only Steap2 is abundantly expressed in the mouse intestine (84). Its localization to epithelial cells of the gastroduodenal junction raises the possibility that Steap2 provides enzymatic ferric reductase activity in the intestine.

Intestinal uptake of iron as heme (predominantly from proteolytic degradation of hemoglobin and myoglobin) is less well understood. Studies in Caco-2 cells, a human intestinal cell line, provide evidence that heme is bound by specific cell-surface heme-binding proteins and then internalized by the cell (129). A candidate low-affinity heme transporter, heme carrier protein 1 (HCP1), was identified in the duodenum (104) but was later shown to serve in vivo as a folate transporter. Qiu et al. (93) independently identified HCP1 as a high-affinity proton-coupled folate transporter (PCFT) and further demonstrated that individuals with hereditary folate malabsorption harbored a loss-of-function mutation in this gene. Appropriately, the protein's name was amended to PCFT/HCP1. Studies in mice show that the duodenal uptake of folate was lower in the presence of a high concentration of heme, suggesting that folate and heme share a common intestinal transporter (58). Folate, however, did not inhibit intestinal uptake of heme. The contribution of PCFT/HCP1 to heme uptake in vivo will require additional study. A *Pcft/Hcp1* knockout mouse model would be particularly useful.

**PCBP1:** poly (rC)-binding protein 1

## Basolateral Iron Transfer into the Portal Circulation

After uptake, but before transfer into the circulation, nonheme iron must traverse the cytosol of the enterocyte. How this occurs is unknown, but given the reactive nature of iron, it is likely that iron-binding/carrier proteins are involved. It is well known that copper—also a highly reactive transition metal—is escorted throughout the cytoplasm bound to chaperone proteins (52). The existence of cytosolic iron chaperones has been postulated for many years, but only recently has one been identified. The protein, poly (rC)-binding protein 1 (PCBP1), has been shown to bind up to three iron atoms with high affinity and deliver them to ferritin (105). In human cells, suppression of PCBP1 expression reduced the incorporation of iron into ferritin and increased cytosolic iron concentrations. As PCBP1 is ubiquitously expressed, it may represent part of the translocation pathway of iron from the apical to basolateral membrane in the enterocyte.

At the basolateral membrane, iron is transported out of the enterocyte and into the portal blood circulation by the iron-export protein ferroportin (1, 19, 68). Mice with intestine-specific ablation of ferroportin present with

severe anemia and iron accumulation in enterocytes, emphasizing ferroportin's critical role in iron absorption (20). Efficient basolateral transfer of iron appears to involve the multi-copper ferroxidase protein, hephaestin, as supported by genetic studies of the sex-linked anemia (*sla*) mouse (120). The *sla* mouse is able to take up iron from the intestinal lumen, but its subsequent transfer into portal blood is diminished, resulting in moderate to severe anemia. A mutation in hephaestin in the *sla* mouse results in the production of a truncated protein that is detected only in a supranuclear compartment, unlike the wild-type protein, which is present both in a supranuclear compartment and the basolateral membrane in close proximity to ferroportin (41, 57). Hephaestin shares significant sequence homology to ceruloplasmin, a circulating copper-containing ferroxidase that enhances iron release from cells. By analogy, hephaestin is thought to aid in basolateral iron release by oxidizing $Fe^{2+}$ (transported by ferroportin) to $Fe^{3+}$, which is rapidly sequestered by apotransferrin in the portal circulation. In contrast to DMT1 and ferroportin, hephaestin is not absolutely required for iron absorption, as *sla* mice, though anemic at birth, manage to grow and reproduce, with the anemia resolving over time. Moreover, hephaestin has not yet been linked to any human diseases. Studies in ceruloplasmin-null animals suggest that ceruloplasmin, which exists as a soluble circulating ferroxidase as well as a membrane-bound GPI-anchored form (at least in some cell types; 18, 88), plays a role in intestinal iron absorption (13). It is thus possible that ceruloplasmin may be able to partially compensate for a lack of hephaestin. Due to the extreme insolubility of $Fe^{3+}$ at physiological pH, it has been proposed that the ferroxidases hephaestin and ceruloplasmin within or near the basolateral membrane may interact directly with transferrin to transfer the iron ligand. However, intensive studies using a variety of analytical techniques were unable to demonstrate a direct interaction between these proteins (43).

The export of heme across the basolateral membrane of the enterocyte seems possible in light of the identification of the transmembrane heme export proteins, feline leukemia virus subgroup C cellular receptor (FLVCR) (94) and ATP-binding cassette, subfamily G, member 2 (ABCG2) (55). FLVCR seems promising as an important heme exporter as it is abundantly expressed in the duodenum and has been shown to play a role in systemic iron homeostasis (50). ABCG2 also merits further investigation, as it was found to be strongly induced in iron-deficient rat duodenum (15). It is more likely, however, that very little heme is absorbed intact. In classic studies in dogs, dietary radiolabeled heme iron heme was shown to be taken up intact by epithelial cells of the small intestine, but it appeared in portal circulation as nonheme iron, indicating that iron was released from heme before basolateral export from the enterocyte (127). Apparently, intestinal heme oxygenase degrades heme to release iron, which then enters the cellular nonheme iron pool and is handled as such.

## Iron Status and Iron Absorption

Iron absorption increases during iron deficiency and decreases in iron overload. This apparently simple, reciprocal relationship between iron stores and iron absorption belies a complex network of proteins that responds to systemic signals reflecting body iron requirements and local signals reflecting cellular iron status of the absorptive enterocyte. The integration of these signals provides tight homeostatic regulation of dietary iron assimilation to ensure sufficient iron uptake and avoid toxic iron accumulation.

## Systemic Regulation of Iron Absorption by Iron Stores

A typical adult male with essentially normal body iron needs to absorb approximately 1 mg of iron per day to remain in iron balance. This amount of iron is required primarily to replace physiologic losses arising from occult blood loss and from the normal sloughing of cells that line the gastrointestinal tract. Females of reproductive age need an additional daily 0.5 mg on

average to account for the iron they lose in menstrual blood. A fraction of the iron absorbed is directed to storage sites, likely serving as an iron reserve in case of emergencies. In the United States, the typical adult male has approximately 1000 mg of iron stores, whereas a typical woman has about 300 mg. Iron absorption varies inversely with iron stores, largely represented by nonheme iron levels in the liver. Iron absorption is high when stores are depleted and is suppressed as iron stores enlarge. The correlation between iron stores and iron absorption is so strong that the concentration of serum ferritin, a commonly used indicator of iron stores, is a good predictor of iron absorption. In his classic review of regulators of iron balance, Finch (25) described in physiologic terms a "store regulator" that linked iron stores to iron absorption. This regulator not only modulated iron absorption to replace endogenous losses and to build an iron reserve, but it also served to prevent iron overload. A large and consistent body of evidence supports the hypothesis that hepcidin is the store regulator (77). That hepcidin could represent the long-sought store regulator was first proposed by Nicolas et al. (80), who discovered that mice lacking the hepcidin gene developed severe iron overload. This finding, coupled with the observation that hepatic hepcidin mRNA levels positively correlated with liver nonheme iron concentrations in mice (91), strongly implicated hepcidin as the store regulator.

To examine the role of hepcidin as the store regulator, Frazer et al. (31) investigated time-dependent changes in iron absorption and duodenal iron transporters in rats that were switched from an iron-replete diet to an iron-deficient diet. Six days after switching to the iron-deficient diet, hepatic hepcidin expression had decreased nearly tenfold and iron absorption had increased by twofold. These changes correlated precisely with an upregulation of duodenal Dcytb, DMT1, and ferroportin. Although these observations supported a causal link between hepcidin and iron absorption, the changes in iron absorption and duodenal gene expression preceded any detectable changes in liver nonheme iron concentrations. What correlated most closely with hepcidin was serum transferrin saturation, leading Frazer et al. (31) to propose that the effects of the store regulator are initially mediated through changes in transferrin saturation. In humans, serum hepcidin levels have been shown to positively correlate with the iron-store indicator serum ferritin (34) and inversely correlate with iron absorption (132, 139). However, serum hepcidin was found to be only a modest predictor of iron absorption—even less predictive than serum ferritin (139)—raising the possibility that other physiologic factors may contribute to the regulation of iron absorption.

## Local Regulation of Iron Absorption by the Enterocyte

It was first reported in the 1940s that the administration of a large oral dose of iron could inhibit the absorption of a second dose of iron given several hours later (40). This phenomenon is commonly referred to as the mucosal block. Due to the short time interval between doses, it seemed unlikely that the impairment in iron absorption resulted from a change in iron stores. It is more probable that the mucosal block resulted from a local downregulation or diminished activity of iron transport proteins in enterocytes in response to their increased cellular iron content. In rats, the decrease in iron absorption following the administration of a large oral iron dose was associated with a rapid reduction in mRNA levels of DMT1 and Dcytb, but not ferroportin or hephaestin, in isolated enterocytes, suggesting local control of apical iron uptake by changes in cellular iron levels (30). It is also possible that an acute increase in systemic hepcidin levels, causing a downregulation of basolateral ferroportin, contributes to the mucosal block. In humans, ingestion of 65 mg of iron caused an increase in serum hepcidin levels after 4–8 hours (34, 64).

The possibility that the cellular IRP/IRE network participates in the regulation of intestinal iron absorption was first explored by Schumann et al. (102), who examined

IRP-binding activity in duodenal enterocytes along the crypt-villus axis after the administration of an oral dose of iron (to increase local iron concentrations) or intravenous iron injection (to increase systemic iron load). They found that orally administered iron readily inactivated IRPs in enterocytes in the villus tip as well as the crypt-villus junctions. By contrast, systemic iron loading inactivated IRPs only in crypt-villus enterocytes. Taken together, these observations indicated that luminal iron, not systemic iron, can affect the IRP/IRE network in enterocytes at the duodenal villus tip, the site of the highest uptake of iron from the lumen.

Mice with ablations in *Irp1/Irp2* in intestinal epithelial cells have been used to define the roles of the individual IRPs in the control of proteins related to iron absorption. Enterocytes lacking IRPs were found to have markedly higher protein levels of ferroportin and ferritin H and L (32), which all contain 5′ IREs in their mRNAs. The substantially higher protein levels were not associated with elevated levels of their respective mRNAs, indicating that the increases probably resulted from translational derepression due to the lack of IRPs. Interestingly, intestinal ferroportin levels in IRP-deficient mice were elevated despite an increase in the expression of the negative effector hepcidin, suggesting that local control of ferroportin expression could override systemic regulation of iron absorption. IRP-deficient enterocytes also had fourfold lower DMT1 protein levels associated with reduced abundances of the IRE-containing DMT1 isoforms. Thus, lack of IRPs likely resulted in a destabilization of DMT1-IRE mRNAs analogous to TFR1, which contains multiple IRE motifs within its 3′UTR. These studies of the intestine-specific IRP-null mice conclusively demonstrate that IRPs coordinate the physiologic expression of iron-transport and iron-storage proteins in the enterocyte. IRP2 appears to be the indispensible IRP for ferritin expression in the intestine, as its selective ablation resulted in elevated levels of ferritin and nonheme iron in the duodenum (23). The role of IRP2 in modulating ferroportin translation/DMT1-IRE mRNA stability remains to be determined.

Although the work in IRP-deficient mice confirmed cell-autonomous translational control of enterocyte ferroportin through its 5′ IRE, it did not help to explain the response of intestinal ferroportin to iron status. In iron deficiency, for example, ferroportin levels increase in an effort to accelerate the absorption of dietary iron. Yet according to the IRP/IRE model, ferroportin levels should decrease in iron deficiency because IRPs are in their active IRE-binding state, repressing ferroportin translation. It now seems that the paradoxical intestinal ferroportin response can be largely explained by the presence of a previously unknown alternative ferroportin transcript that is highly expressed in the duodenum. Importantly, the newly identified transcript, named FPN1B, lacks an IRE and is therefore able to evade translational repression (137). Moreover, FPN1B expression increased in the duodenum of iron-deficient mice. As FPN1B encodes for a protein identical to canonical ferroportin, an increase in FPN1B expression could therefore account for the well-known increase in ferroportin levels in iron-deficient duodenum.

A consistent feature of iron-deficient duodenum is the dramatic upregulation of mRNA levels of DMT1 (38) and Dcytb (15, 22). Although DMT1-IRE mRNA may be stabilized post-transcriptionally through IRP binding during iron deficiency (32), the striking upregulation of DMT1-IRE mRNA levels implies increased transcription, a possibility supported by nuclear runoff experiments in Caco-2 intestinal cells (140). Unlike DMT1, DCYTB does not contain an identifiable IRE in its mRNA; its regulation therefore likely occurs at the transcriptional level.

On the basis of the observation that the mouse duodenum is mildly hypoxic under basal conditions, Mastrogiannaki et al. (65) hypothesized that HIF transcription factors may serve as key local regulators of intestinal iron absorption. The HIF family of transcription factors includes HIF-1$\alpha$, HIF-2$\alpha$, and HIF-3$\alpha$ (126). In normoxic situations, HIF is continually

produced but degraded (**Figure 3**). Degradation is initiated by hydroxylation of two proline residues on HIF by the prolyl hydroxylase domain (PHD) family of proteins (PHD1, PHD2, and PHD3). Hydroxylation of HIF promotes its interaction with the von Hippel-Lindau (VHL) tumor suppressor protein, which ubiquitinates HIF, marking it for degradation through the ubiquitin-proteasome pathway. During hypoxia, HIFs are not hydroxylated and thus accumulate and translocate into the nucleus, where they bind to aryl hydrocarbon receptor nuclear translocator, a constitutively expressed protein also known as HIF-1β. The active heterodimeric transcriptional complex then binds to hypoxia response elements (HREs) in target genes to modulate their transcription. Surprisingly, targeted deletion of *Hif-1α* in the intestine had no effect on iron status in mice; nor did it affect the expression of genes involved in iron absorption in the duodenum (65). In contrast, loss of *Hif-2α* was associated with dramatic reductions in the basal expressions of DMT1-IRE and Dcytb along with 50% lower levels of ferroportin mRNA. HIF-2α was also directly implicated in iron absorption by the observation that HIF-2α, but not HIF-1α, protein levels were markedly higher in duodenum of iron-deficient mice and in mice lacking *Vhl* (103). In vitro studies further demonstrated that HIF-2α directly binds to HREs in the promoters of DMT1 and Dcytb, activating their transcription (65, 103). Together, these studies identify HIF-2α as a local regulator of iron absorption, particularly by modulating the expression of enterocyte proteins involved in the apical uptake of iron.

## Iron-Sensing Proteins that Regulate Iron Absorption

As detailed above, the absorption of dietary iron by the intestine is regulated in response to systemic iron status (largely represented by iron stores and holotransferrin) and by the local iron status of the absorptive duodenal enterocyte. Long before the discovery of hepcidin, information about systemic iron status was believed to be conveyed to the intestine by circulating iron-transferrin (holotransferrin) in accordance with the "crypt programming" model. According to the model, the amount of iron taken up by progenitor cells in the intestinal crypt programmed the cell's iron status to reflect the body's iron status. As the crypt cell then matured and migrated to the absorptive region starting at the crypt-villus junction, it would be programmed with an appropriate number of iron transporters to meet body iron needs. Although the crypt program hypothesis provided for many years a testable model of the regulation of iron absorption, it failed to explain why hemochromatosis patients, who had high transferrin saturations and presumably high uptake of iron by the crypt cells, continued to display increased iron absorption. It is now generally accepted that the predominant regulator of iron absorption is hepcidin via its ability to promote the internalization and degradation of ferroportin, thus blocking basolateral transfer of iron (77). Indeed, the dominant role of hepcidin in controlling iron absorption now seems almost self-evident, as all the major forms of iron overload are characterized by inappropriately low levels of hepcidin (78, 85, 86, 115). Nonetheless, the ability of the enterocyte to autoregulate its expression of iron transport machinery may play more of a role in iron absorption than we realize.

Studies of mice lacking IRPs in enterocytes clearly demonstrate a role for these cellular iron-sensing proteins in iron absorption (23, 32). In iron-replete conditions, IRP1 directly senses iron by forming an iron-sulfur cluster and losing IRE-binding activity, whereas IRP2 is targeted for proteasomal degradation. Some data suggest that iron binds to a cluster of cysteines in IRP2, initiating a site-specific oxidation that targets the protein for proteasomal degradation (45), but other studies do not support this model (124). It is more likely that the "sensing" of iron by IRP2 is mediated by F-box and leucine-rich repeat protein 5 (FBXL5), an iron-dependent protein that directly binds to IRP2 (99, 116). Similar to VHL, FBXL5 is part of a protein complex that possesses ubiquitin

**PHD:** prolyl hydroxylase domain protein

**FBXL5:** F-box and leucine-rich repeat protein 5

ligase E3 activity, targeting proteins for proteasomal degradation. Under iron-replete conditions, iron binds to the hemerythrin-like domain of FBXL5, which stabilizes the protein, allowing it to associate with IRP2 and promote its degradation (**Figure 3**). In addition to iron-sulfur assembly of IRP1 and iron-dependent degradation of IRP2, free ferrous iron may directly affect the IRP/IRE regulatory network by binding to IRE-RNA structures, decreasing their association with IRPs (51).

In the iron-deficient enterocyte, transcription provides a second important point of local control in the regulation of iron absorption, mainly by HIF-2α-mediated transactivation of DCYTB, DMT1, and FPN1B (65, 103). HIF-2α levels, in turn, are regulated chiefly by PHD enzymes, which require ferrous iron for activity (82), thus suggesting that PHDs, rather than HIF-2α, serve as iron-sensing proteins (**Figure 3**). Enzymatic activity of PHDs additionally requires ascorbate, likely serving to keep iron in the ferrous state (82). PHD2 has been shown to have high affinity for ferrous iron, binding the metal cofactor via conserved histidine, aspartic acid, and glutamic acid residues (70). Targeted disruption of PHD2 in mice results in embryonic lethality, whereas mice engineered to lack PHD1 or PHD3 are viable, indicating that PHD2 is indispensible in vivo (109). It remains to be determined how the abundance or activity of PHD proteins varies with fluctuations in enterocyte iron (or ascorbate) concentrations. Although PHDs control the stability of HIF-2α, it should be noted that HIF-2α mRNA contains a 5′IRE (**Figure 3**), subjecting it to additional post-transcriptional regulation via IRPs (100). The contribution of IRP to HIF-2α expression is not readily apparent, however, given that translational repression of HIF-2α during iron deficiency would appear to antagonize its transcriptional activation of iron uptake genes. Translational control of HIF-2α by IRPs may be confounded by independent, and perhaps dominant, effects exerted by hypoxia, which has been shown to affect IRP binding to HIF-2α mRNA in renal cells (138).

The iron-binding protein PCB1 may also influence local control of iron absorption. It will be important to determine if this iron chaperone functions to deliver iron to ferritin in the enterocyte and if it plays a role in vectorial transport of iron throughout the absorptive cell. PCBP1 is of further interest in that it also localizes to the nucleus, where it is known to regulate and coordinate gene expression at multiple levels, including transcriptional activator, regulator of RNA splicing, and translational repressor (71).

Iron-sensing proteins in the enterocyte likely initiate the changes in proteins that partly account for the mucosal block phenomenon. For example, ingestion of a large dose of iron would load the enterocyte, leading to inactivation of IRP1 and FBXL5-mediated degradation of IRP2. Derepression of IRP binding would permit ferritin translation while decreasing the stability of DMT1 transcripts. Furthermore, the high-iron conditions would favor the PHD-initiated degradation of HIF-2α, leading to reduced levels of DMT1, DCYTB, and ferroportin. Collectively, these changes within the enterocyte would reduce iron uptake and favor iron sequestration in ferritin.

## Crosstalk Between the Liver and Small Intestine in Regulating Iron Absorption

Iron absorption is regulated and finely tuned by crosstalk between the liver and small intestine. In iron-replete conditions, hepcidin produced by hepatocytes is the dominant negative regulator of iron absorption by duodenal enterocytes. Hepcidin synthesis is positively regulated by iron stores and transferrin saturation, the latter reflecting a steady state between iron influx into plasma and iron uptake and utilization by the bone marrow and other tissues. In iron deficiency, hepatic hepcidin production virtually ceases, allowing enterocyte ferroportin levels to increase and facilitate basolateral export of iron. Also during iron deficiency, the liver produces and secretes into the circulation more apotransferrin and ceruloplasmin, both of which

favor the movement of iron into the portal blood.

Although it is clear that the liver communicates with the duodenum to regulate iron absorption, one recent study raises the intriguing possibility that the intestine may signal to the liver to influence iron absorption. Arndt et al. (4) found that in mice fed an iron-supplemented diet, hepatic BMP6 mRNA levels doubled compared to controls, whereas their levels in the small intestine were sixfold higher. Moreover, ex vivo incubation of tissue from liver and small intestine with ferrous sulfate or holotransferrin resulted in an upregulation of BMP6 in small intestine but not liver. Cocultures of small intestine from *Bmp6*$^{+/+}$ mice and liver from *Bmp6*$^{-/-}$ mice incubated with ferrous sulfate further demonstrated a ninefold upregulation in hepatic hepcidin mRNA levels compared to controls. Importantly, no induction in hepatic hepcidin mRNA abundance was observed in similar cocultures of small intestine from *Bmp6*$^{-/-}$ mice and liver from *Bmp6*$^{+/+}$ mice. From these observations, Arndt et al. concluded that the small intestine, not the liver, is the main in vivo source of BMP6 and that the small intestine is a key regulator of iron absorption. In their proposed model, iron in the small intestine induces the expression of BMP6, which then travels via the portal vein to the liver, where it activates BMP signaling, resulting in increased hepcidin expression. Indeed, the restricted expression of the BMP6 coreceptor, HJV, to periportal hepatocytes (81) would seem to optimally position it to receive signals coming directly from the duodenum. Ultimately, the generation of mice lacking *Bmp6* specifically in the intestinal epithelium or in hepatocytes will be needed to test this model.



## SUMMARY POINTS

1. BMP6, the main endogenous regulator of hepcidin expression, is positively regulated by iron. The binding of BMP6 to its receptor at the hepatocyte cell surface initiates a signaling cascade involving intracellular SMAD proteins, which translocate to the nucleus to activate hepcidin transcription. Targeted disruption of *Bmp6* in mice results in greatly diminished hepcidin expression and severe iron overload.
2. Hepcidin expression requires hepatic TFR2, a TFR1 homolog that is abundantly expressed in the liver. Hepatocyte TFR2 may serve as a body iron sensor, relaying information between circulating holotransferrin (an indicator of body iron status) and hepcidin production. TFR2, like TFR1, associates with HFE, which is also essential for hepcidin expression.
3. The sensing of iron deficiency, with the subsequent downregulation of hepcidin expression, requires matriptase-2, a cell-surface protease expressed primarily in liver. Matriptase-2 decreases hepcidin expression by cleaving HJV, abrogating its function as a BMP6 coreceptor.
4. Hepcidin serves as the iron stores regulator, modulating iron absorption to meet body iron demand while preventing iron overload.
5. Although liver-derived hepcidin is the dominant regulator of enteric iron absorption, the enterocyte itself partially controls iron absorption through local regulation of iron transport and storage proteins.
6. In iron deficiency, HIF-2$\alpha$ in the intestinal epithelium facilitates iron absorption by activating the transcription of DMT1 and DCYTB, proteins involved in apical iron uptake, and perhaps ferroportin, which mediates the basolateral transfer of enterocyte iron into portal blood.

7. By regulating HIF-2α, the PHD proteins, which require iron for activity, may serve as iron-sensing proteins in the enterocyte, contributing to local control of iron absorption.
8. The cellular iron-sensing proteins IRP1, and particularly IRP2, provide additional regulation of iron absorption in the enterocyte through the post-transcriptional control of DMT1, ferritin, and HIF-2α. By controlling IRP2 degradation in response to iron, the iron-dependent protein FBXL5 represents a key iron-sensing protein.

## FUTURE ISSUES

1. How does iron regulate the expression of BMP6? The identification of the iron-sensing protein(s) involved in mediating BMP6 expression is of paramount importance, for it will tell us how iron regulates its own metabolism by regulating hepcidin expression.
2. Is BMP6 the main regulator of hepcidin expression in humans? As of yet, no mutations in *BMP6* have been linked to iron overload in humans. One would predict a phenotype resembling juvenile hemochromatosis due to mutations in *HJV*, the coreceptor for BMP6.
3. What is the tissue/cellular origin of endogenous BMP6? Cell type–specific *Bmp6* knockout animals will help to elucidate this important question.
4. The intracellular signaling pathways that link TFR2 and hepcidin expression are poorly understood. Does TFR2-dependent signaling converge into a common intracellular pathway shared by BMP6-dependent signaling? Or do the two pathways converge at the plasma membrane through the association of TFR2 and cell-surface components of BMP signaling?
5. How does iron status affect the activity of matriptase-2? It is likely that matriptase-2 plays an important role in maintaining iron balance, as highlighted by the multiple independent associations between SNPs in the human *Matriptase-2/TMPRSS6* gene and iron status.
6. How does the abundance or activity of PHD proteins vary with fluctuations in enterocyte iron concentrations?
7. What is the in vivo role of the iron chaperone PCBP1? Does it participate in the apical-to-basolateral translocation of iron in the enterocyte?
8. Iron regulates the transcription of many key proteins in iron metabolism, including hepcidin, DMT1, DCYTB, and ferroportin. Future studies need to identify the set of transcription factors in the hepatocyte and enterocyte that mediates the response to iron.

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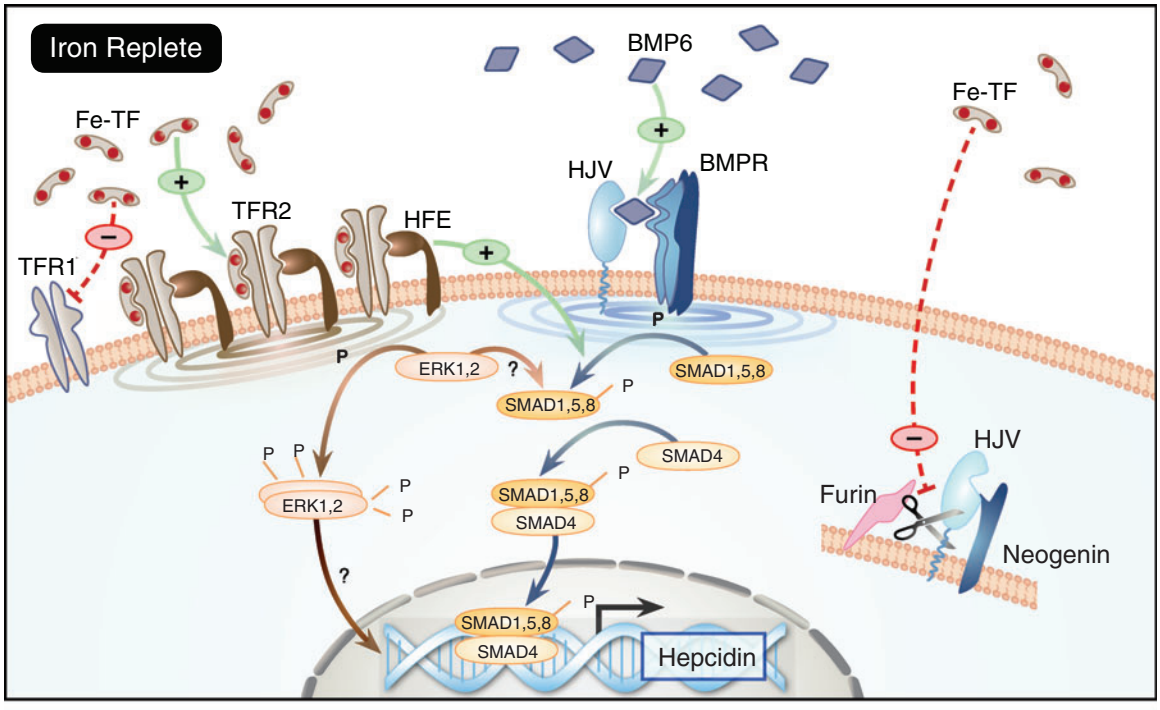


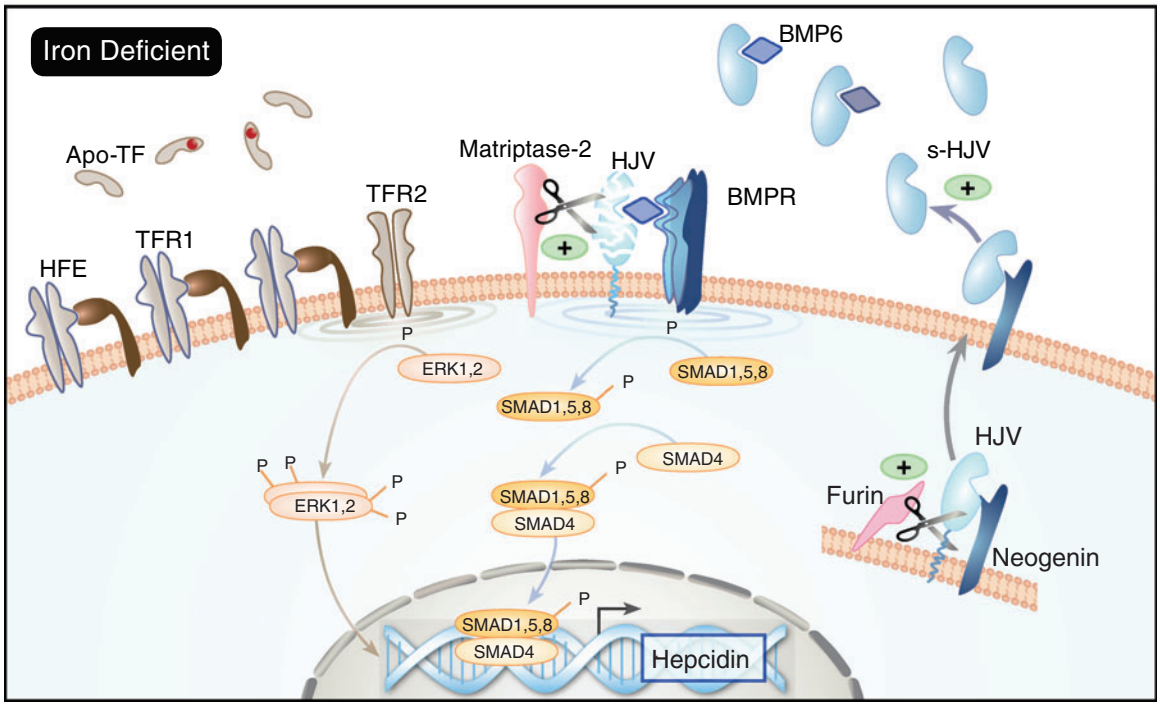
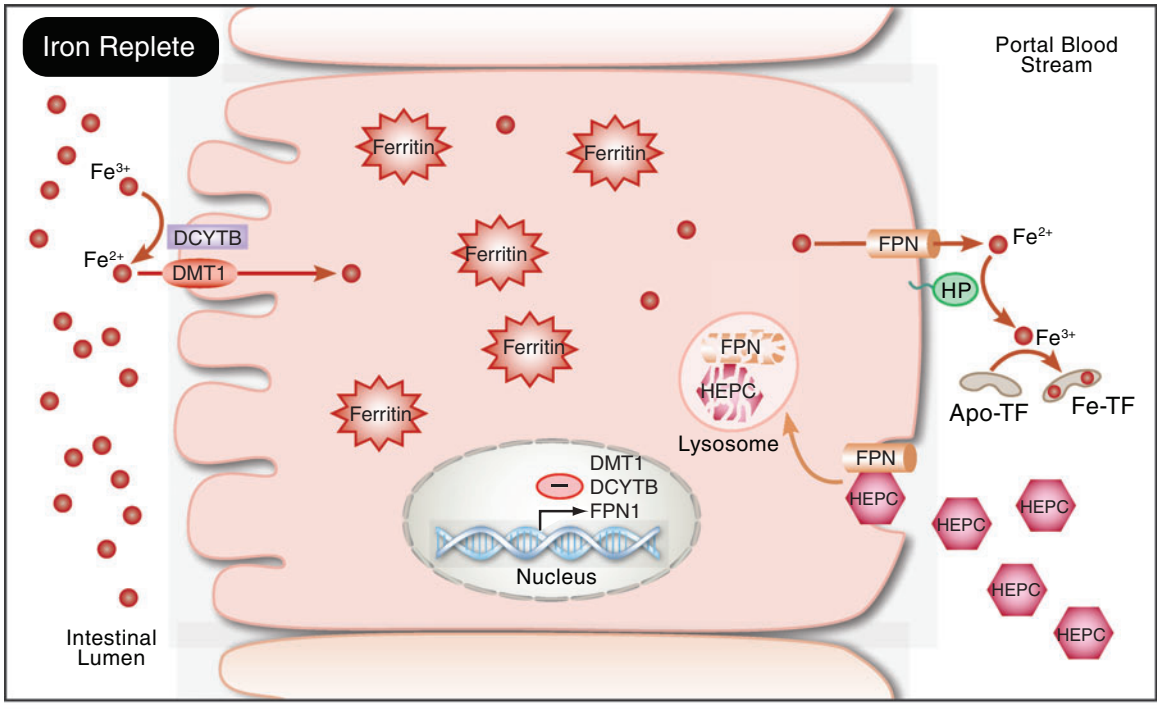



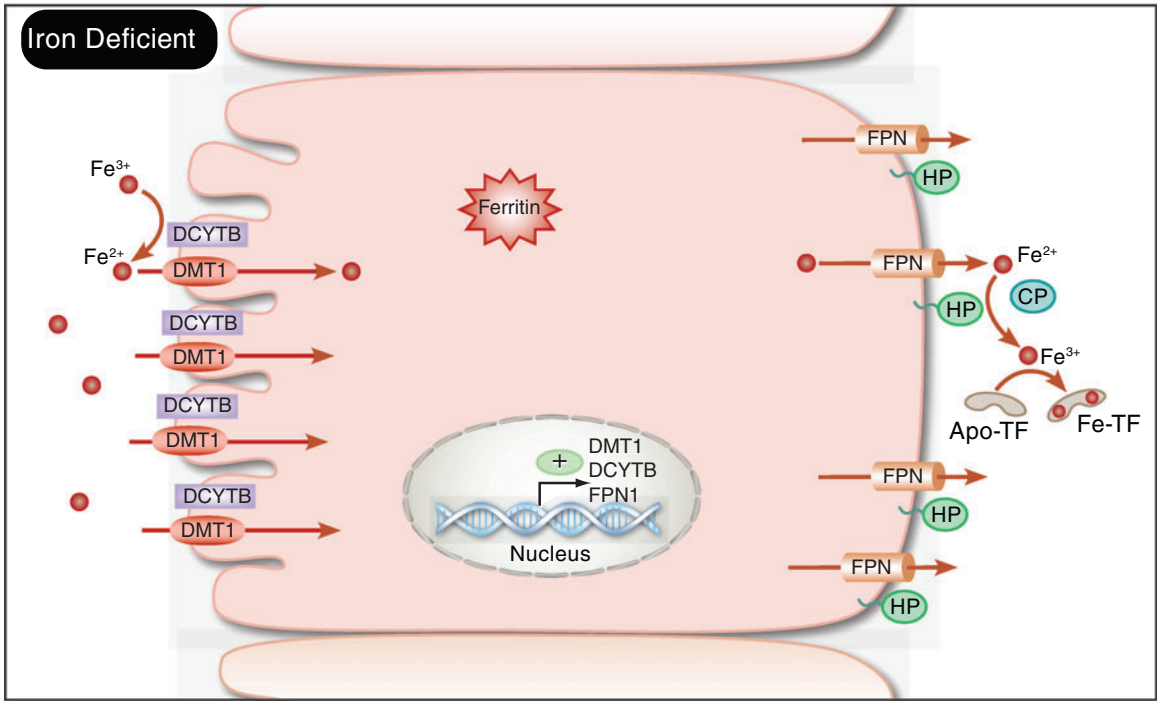
**Figure 1**

Proteins involved in mediating hepatocyte hepcidin production during iron-replete and iron-deficient conditions. BMP6, a key iron-responsive regulator of hepcidin, binds to BMPR, initiating an intracellular SMAD signaling cascade that activates hepcidin transcription. Transcriptional activation of hepcidin is also promoted by the binding of Fe-TF to TFR2 in a complex with HFE. The intracellular signaling pathways involved in this process are poorly understood but appear to involve crosstalk with BMP/SMAD signaling. Fe-TF further enhances hepcidin production by stabilizing TFR2 at the cell surface and by inhibiting the release of s-HJV. In iron deficiency, BMP signaling is impaired by matriptase-2-mediated cleavage of the BMP coreceptor, HJV, and by sequestration of BMP6 by s-HJV. The release of s-HJV is catalyzed by the protease furin, the levels of which increase in iron deficiency. TFR2-dependent signaling is decreased during iron deficiency because of a downregulation of TFR2 and an upregulation of TFR1, which sequesters HFE. Abbreviations: Apo-TF, apo-transferrin; BMP6, bone morphogenetic protein 6; BMPR; bone morphogenetic protein receptor; ERK, extracellular signal-regulated kinases; Fe-TF, iron transferrin; HFE, high Fe (hemochromatosis protein); HJV, hemojuvelin; P, phosphate; s-HJV, soluble hemojuvelin; SMAD, homolog of the Sma protein in *C. elegans* and the MAD protein in *Drosophila*; TFR, transferrin receptor.

**Figure 2**

Proteins involved in mediating enterocyte iron absorption during iron-replete and iron-deficient conditions. Liver-derived hepcidin controls iron entry into portal blood by binding to FPN, initiating its degradation. During iron deficiency, apical iron uptake increases due to upregulation of DMT1 and DCYTB, mainly resulting from enhanced transcription of these genes. Transcription of FPN also increases, and in the absence of hepcidin, basolateral FPN is not degraded. Iron release into portal blood is enhanced by the ferroxidase HP and by liver-derived, circulating CP and TF. Enterocyte iron that is not absorbed is sequestered in ferritin until the cell is sloughed into the intestinal lumen (normally within several days). Abbreviations: Apo-TF, apo-transferrin; CP, ceruloplasmin; DCYTB, duodenal cytochrome B; DMT1, divalent metal transporter 1; FPN, ferroportin; HEPC, hepcidin; HP, hephaestin.



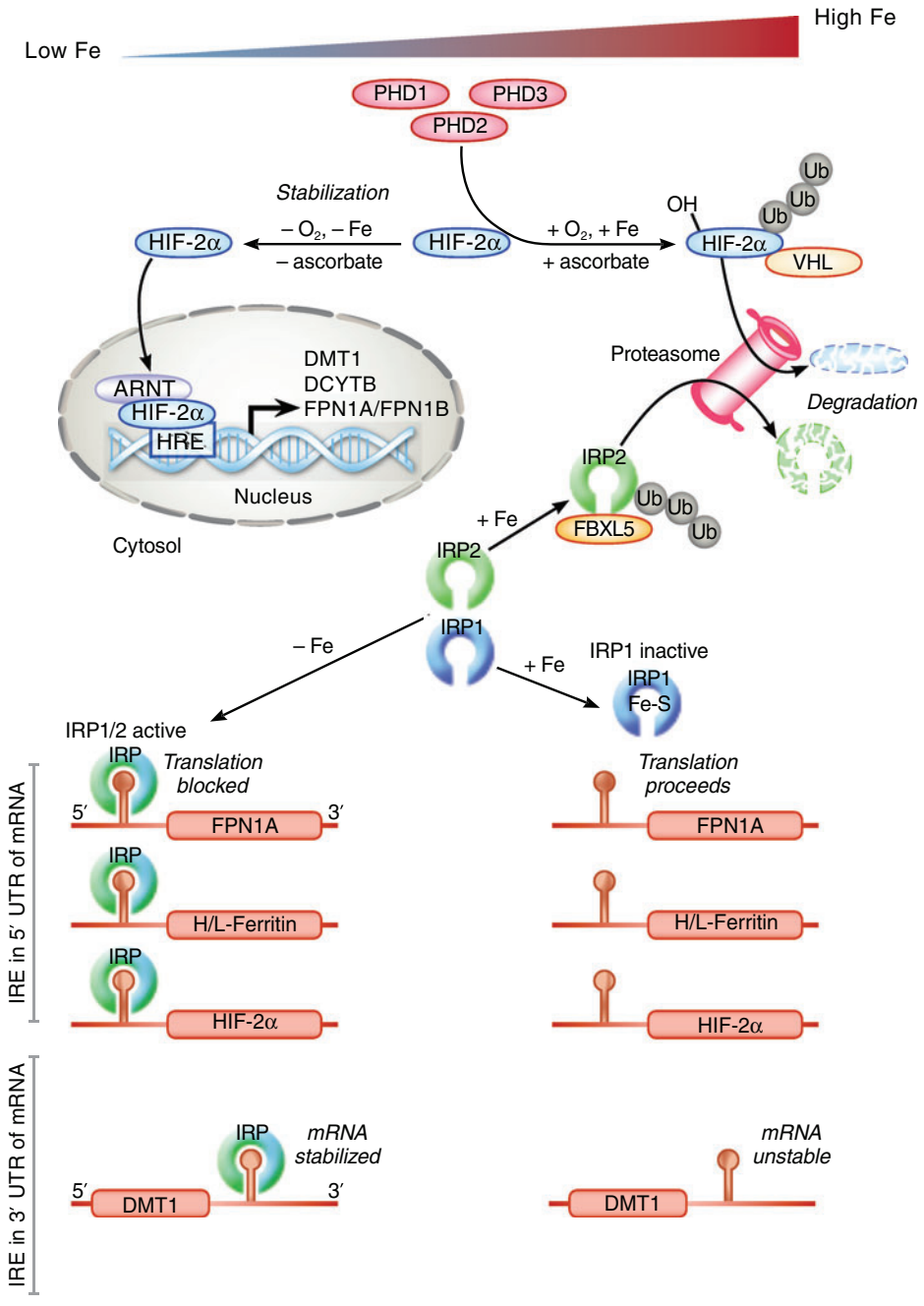


**Figure 3**

Cellular iron-sensing proteins PHDs, IRPs, and FBXL5 in the enterocyte coordinate and fine-tune the transcriptional and post-transcriptional response to iron. In low-iron conditions, HIF-2$\alpha$ activates the transcription of DMT1, DCYTB, and FPN. Unlike the FPN1A transcript, the FPN1B transcript (not shown) evades translational repression by IRPs, resulting in increased FPN levels. At the same time, DMT1 transcripts are stabilized by the binding of IRPs to 3′ IREs in their mRNAs, allowing for increased translation. In iron-replete conditions, PHDs, which require iron for activity, hydroxylate HIF-2$\alpha$, marking it for association with VHL, ubiquitination, and proteasomal degradation. Post-transcriptional control of iron-related, IRE-containing mRNAs is mediated by IRP2 (the dominant IRP) and IRP1. High-iron conditions are sensed by the iron-dependent protein, FBXL5, which targets IRP2 for proteasomal degradation. IRP1, on the other hand, forms an Fe-S cluster, which disrupts its iron-binding activity. Unbinding of IRPs to the 5′IREs in ferritin mRNAs permits translation of the iron-storage protein ferritin. Abbreviations: ARNT, aryl hydrocarbon receptor nuclear translocator; DMT1, divalent metal transporter 1; FBXL5, F-box and leucine-rich repeat protein 5; FPN, ferroportin; HIF-2$\alpha$, hypoxia-inducible factor 2$\alpha$; HRE, hypoxia-response element; IRE, iron-response element; IRP, iron-regulatory protein; PHD, prolyl hydroxylase domain protein; Ub, ubiquitin; VHL, von Hippel-Lindau tumor suppressor protein.

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**Errata**

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