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*EDITORIAL*

# Function of the hemochromatosis protein HFE: Lessons from animal models

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## Abstract

Hereditary hemochromatosis (HH) is caused by chronic hyperabsorption of dietary iron. Progressive accumulation of excess iron within tissue parenchymal cells may lead to severe organ damage. The most prevalent type of HH is linked to mutations in the *HFE* gene, encoding an atypical major histocompatibility complex class I molecule. Shortly after its discovery in 1996, the hemochromatosis protein HFE was shown to physically interact with transferrin receptor 1 (TfR1) and impair the uptake of transferrin-bound iron in cells. However, these findings provided no clue why *HFE* mutations associate with systemic iron overload. It was later established that all forms of HH result from misregulation of hepcidin expression. This liver-derived circulating peptide hormone controls iron efflux from duodenal enterocytes and reticuloendothelial macrophages by promoting the degradation of the iron exporter ferroportin. Recent studies with animal models of HH uncover a crucial role of HFE as a hepatocyte iron sensor and upstream regulator of hepcidin. Thus, hepatocyte HFE is indispensable for signaling to hepcidin, presumably as a constituent of a larger iron-sensing complex. A working model postulates that the signaling activity of HFE is silenced when the protein is bound to TfR1. An increase in the iron saturation of plasma transferrin leads to displacement of TfR1 from HFE and assembly of the putative iron-sensing complex. In this way, iron uptake by the hepatocyte is translated into upregulation of hepcidin, reinforcing the concept that the liver is the major regulatory site for systemic iron homeostasis, and not merely an iron storage depot.



**Key words:** Hepcidin; Iron metabolism; Transferrin; Hemojuvelin; Bone morphogenetic proteins

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## PHYSIOLOGY AND PATHOPHYSIOLOGY OF IRON HOMEOSTASIS

Iron is essential for various physiological and metabolic pathways. However, unshielded iron is toxic, as a catalyst of free radical generation[1,2]. The adult human body contains a pool of 3-5 g of iron (about 55 mg and 44 mg per kilogram body weight in males and females, respectively), the majority of which (> 70%) is utilized by erythroid cells for heme synthesis and integration into hemoglobin[3]. A daily requirement of about 20-30 mg iron for erythropoiesis is mainly covered by recycling of the metal from senescent erythrocytes *via* reticuloendothelial macrophages. These cells metabolize heme and release iron into the circulation, where it is scavenged by plasma transferrin and delivered to tissues. A considerable amount of iron (about 1 g) is stored in the liver. Dietary iron absorption by duodenal enterocytes compensates for losses through bleeding or desquamation; a physiological rate of 1-2 mg/d suffices to maintain the body iron pool. This is subjected to feedback regulation and may adjust to fluctuations in iron demands.

In hereditary hemochromatosis (HH), disruption of this homeostatic loop leads to unrestricted dietary iron absorption at a rate that may reach 8-10 mg/d[4,5]. This

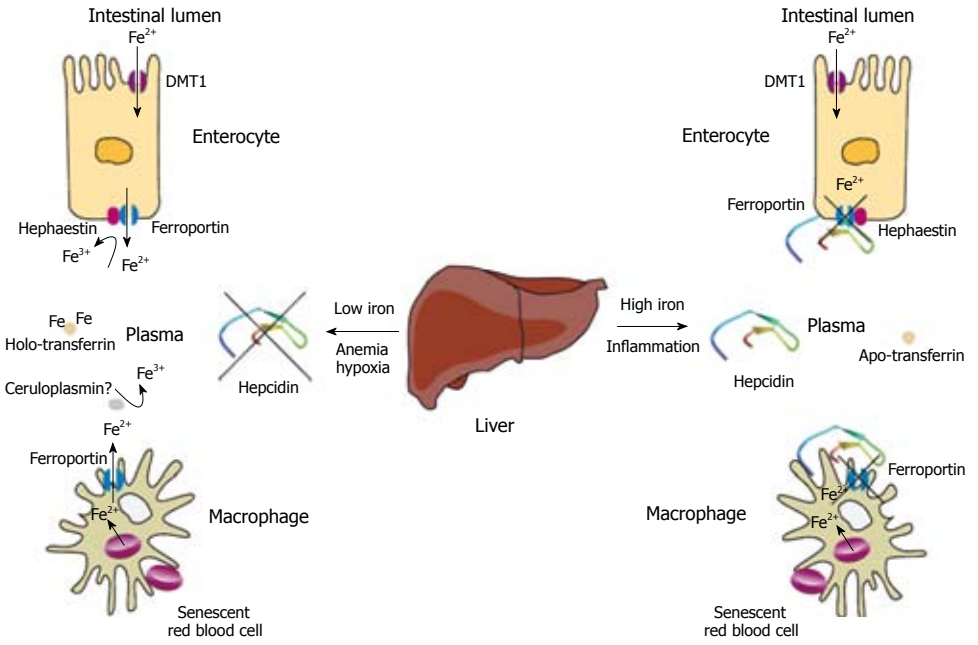


**Figure 1 Regulation of iron efflux from enterocytes and macrophages by hepcidin.** Duodenal enterocytes absorb dietary iron *via* DMT1 and reticuloendothelial macrophages phagocytose iron-loaded senescent red blood cells. Both cell types release ferrous iron ($Fe^{2+}$) into plasma via ferroportin, which is incorporated into transferrin following oxidation into the ferric form ($Fe^{3+}$) *via* hephaestin or ceruloplasmin. The secretion of the iron-regulatory hormone hepcidin from the liver in response to high body iron stores or inflammatory signals results in internalization and degradation of ferroportin, and retention of iron within enterocytes and macrophages. A decrease in body iron stores, a requirement of iron for erythropoiesis, or hypoxia, inhibit hepcidin expression, permitting dietary iron absorption by enterocytes and iron release from macrophages.

is accompanied by a gradual increase in the saturation of transferrin with iron (from physiological 30% up to 100%), a buildup of non-transferrin-bound iron and excessive accumulation of the metal in parenchymal cells of the liver, pancreas, pituitary, heart, joints and skin. Notably, macrophages and absorptive duodenal enterocytes are spared from iron loading and exhibit increased rates of iron release. Excessive iron deposition in the liver constitutes a risk factor for fibrosis, cirrhosis and hepatocellular cancer[6-8], and may exacerbate other types of liver disease[9,10]. Iron overload may also lead to cardiomyopathy, diabetes mellitus, hypogonadism, arthritis and skin pigmentation[3]. HH is efficiently treated by phlebotomy.

## HORMONAL REGULATION OF IRON TRAFFIC BY HEPCIDIN

The discoveries of the divalent metal transporter (DMT1), the iron exporter ferroportin, and the iron regulatory hormone hepcidin provided a framework to understand the molecular mechanisms for systemic iron traffic and homeostasis[11,12]. DMT1 accounts for the absorption of ferrous ions across the apical membrane of duodenal enterocytes, but also for intracellular transport of transferrin-derived iron across the endosomal membrane in many cell types. Ferroportin mediates efflux of ferrous iron from enterocytes and macrophages to plasma transferrin. The transport of iron by DMT1 requires its reduction by ferric reductases (such as Dcytb or the Steap proteins), while its export by ferroportin is coupled by re-oxidation *via* ferroxidases (such as ceruloplasmin or hephaestin).

The ferroportin-mediated efflux of iron from enterocytes and macrophages defines a key regulatory checkpoint for iron homeostasis. This process is negatively controlled by hepcidin, a cysteine-rich peptide hormone that binds to ferroportin and promotes its internalization and lysosomal degradation[13]. Hepcidin is synthesized in hepatocytes as a pro-peptide, which undergoes proteolytic processing to form a bioactive molecule of 25 amino acids[14]. The mature peptide is secreted into plasma and orchestrates homeostatic responses to iron, erythropoiesis, hypoxia and inflammation. An increase in hepcidin levels, commonly encountered following dietary iron intake or in inflammation[15,16], impairs iron absorption by duodenal enterocytes and promotes retention of the metal within macrophages (Figure 1), limiting its availability for erythropoiesis. Excessive hepcidin expression, in response to prolonged inflammation, contributes to the anemia of chronic disease[17]. On the other hand, low hepcidin levels triggered by iron deficiency, hypoxia or phlebotomy[18] facilitate duodenal iron absorption and iron release from macrophages (Figure 1). Importantly, HH patients fail to mount an appropriate upregulation of hepcidin expression, despite high transferrin saturation and elevated body iron stores[19,20]. Thus, HH is largely based on the loss of feedback control in dietary iron absorption due to defects in the hepcidin pathway.

Juvenile hemochromatosis, a rare but severe form of hereditary iron overload results from genetic inactivation of the hepcidin gene[21] or mutations in hemojuvelin (HJV) associated with profound hepcidin deficiency[22]. The most prevalent form of HH is linked to mutations in HFE[23], while another less common but phenotypically indistinguishable HH subtype is caused by mutations in transferrin receptor 2 (TfR2)[24]. Iron overload patients with either HFE or TfR2 mutations exhibit inappropriately decreased hepcidin levels or blunted hepcidin responses[19,20,25,26]. Similar results were obtained with mouse models of iron overload, bearing targeted disruptions of the *HFE*[27-30], *HJV*[31,32] or *TfR2*[33] genes. These findings suggest that HFE, HJV and TfR2 are upstream regulators of hepcidin expression.

## REGULATION OF HEPCIDIN EXPRESSION

Hepcidin is transcriptionally activated by distinct iron-

and cytokine-dependent pathways. The latter is mediated by IL-6 (and IL-1) *via* STAT3[34-36]. The iron-dependent pathway is less well characterized and involves proximal and distal promoter elements[37,38]. The lack of hepcidin expression, accompanied by iron overload, in mice carrying a hepatocyte-specific disruption of SMAD4[39] has linked iron-sensing with bone morphogenetic protein (BMP) signaling. In fact, BMP-2, -4 and -9 are potent inducers of hepcidin transcription, while hemojuvelin stimulates this pathway as a BMP co-receptor[40-42]. The CCAAT/enhancer-binding protein α (C/EBPα) appears necessary for basal hepcidin transcription[43].

Hepcidin expression is suppressed in anemia by a mechanism that requires erythropoietic activity[44,45]. At least in thalassemia patients, the silencing of hepcidin is mediated by overexpression of growth differentiation factor 15 (GDF15), a member of the transforming growth factor β (TGFβ) superfamily[46]. Erythropoietin (EPO) directly reduces the binding of C/EBPα to the hepcidin promoter *via* EPO receptor signaling[47]. Hepcidin is also negatively regulated by hypoxia[18]. Experiments in mice with hepatic disruption of HIF-1α provided evidence for the involvement of this transcription factor in the underlying pathway[48]. However, other reports suggested that the hypoxic downregulation of hepcidin is HIF-independent[49,50] and involves oxidative stress-mediated repression of C/EBPα and STAT3[49], or inhibition of 2-oxoglutarate dependent oxygenases[50]. Recent work revealed that the transmembrane serine protease TMPRSS6 negatively regulates signaling to hepcidin[51-55], by a yet unknown mechanism.

What is the role of HFE in hepcidin regulation?

## DISCOVERY OF HFE AS THE HEMOCHROMATOSIS GENE

The *HFE* gene was elucidated by linkage disequilibrium and haplotype analysis from a large group of HH patients[23], culminating lengthy efforts to map the hemochromatosis locus. It encodes an atypical major histocompatibility complex (MHC) class I protein, which is processed *via* the Golgi network to the cell surface, following interaction with β2-microglobulin. Structural analysis revealed that in contrast to typical MHC class I homologues, HFE formed a smaller groove between the α1 and α2 subunits, which was predicted to preclude peptide antigen presentation[56]. The majority of HH patients carry an *HFE* C282Y substitution. This abrogates a disulphide bridge and prevents the association of HFE with β2-microglobulin, a necessary step for its processing and transport to the plasma membrane[57,58]. Unprocessed *HFE* C282Y undergoes proteasomal degradation following retention in the endoplasmic reticulum (ER), which promotes ER stress[59]. An *HFE* H63D mutation may also lead to HH, especially in the compound heterozygous state with C282Y. Homozygosity for the *HFE C282Y* genotype is highly prevalent (1:200) in populations of Northern European ancestry; however, the clinical penetrance is lower and remains a matter of debate[4-6,60,61]. It appears that HH is a multifactorial disease and the development of iron overload in individuals bearing disease-associated *HFE* mutations requires the contribution of additional, yet incompletely understood environmental, genetic and/or epigenetic factors[62]. Nevertheless, mice with either targeted disruption of the *HFE*[63,64] or β2-microglobulin[65,66] genes, or expressing orthologues of the *HFE* C282Y[67] or H63D[68] mutants, develop progressive iron overload, the degree of which depends on the genetic background of the animals[69-71]. Collectively, these findings underlie the significance of HFE in the control of body iron homeostasis.

## EARLY MODELS FOR THE FUNCTION OF HFE

Biochemical[72,73] and crystallographic[74] studies revealed that HFE interacts with TfR1 (Kd about 60 nmol/L) and competes for the binding of transferrin to its receptor, which has a Kd of about 1 nmol/L[75]. However, considering that the physiological concentration of plasma diferric holotransferrin is about 5 μmol/L[76], HFE is unlikely to affect the rate of TfR1 endocytosis *in vivo*. In transfected cell lines, overexpressed HFE reduced the efficiency of the transferrin cycle[77] and promoted an iron-deficient phenotype[78-81], without or with co-expression of β2-microglobulin[82]. Notably, a similar phenotype was observed with an *HFE* W81A mutant that is unable to bind to TfR1, suggesting that the HFE-mediated decrease of intracellular iron levels is independent of the HFE/TfR1 interaction[83].

The above data did not shed much light on how HFE controls systemic iron homeostasis and rather created some confusion. The immunohistochemical detection of HFE in precursor enterocytes of the intestinal crypts[84] and its association with TfR1 in these cells[85] laid the foundation for the "crypt-programming model"[86]. This postulates that iron absorption is regulated by signals that are sensed by precursor enterocytes, which undergo maturation and migrate along the crypt-villus axis. An iron deficient status in the crypt cells would program mature enterocytes to absorb more dietary iron from the lumen. According to the crypt-programming model, HFE would serve to promote iron retention within crypt cells, possibly by increasing the uptake of plasma transferrin[87] and/or inhibiting iron efflux[88]. The model is supported by the iron deficient status manifested in duodenal biopsies from HH patients[89,90]. Experimental evidence has been provided that HFE may also facilitate iron accumulation[91] or retention[92] within macrophages, which are likewise iron-deficient in HH patients[93]. In the pre-hepcidin era, these findings have highlighted the enterocytes and macrophages as possible primary sites of the HFE regulatory function. Nonetheless, HFE is expressed in multiple cell types, including hepatocytes[94], the major producers of hepcidin.

## LESSONS FROM ANIMAL MODELS I: THE SITE OF HFE REGULATORY FUNCTION

Definite clues as to the site of HFE regulatory function

in the context of systemic iron homeostasis were recently provided by experiments with genetically engineered mice, bearing a targeted, tissue-specific disruption of the *HFE* gene. The technology is based on the generation of animals carrying a "floxed" *HFE* allele, surrounded by loxP sites, which are specific targets of the Cre recombinase. Crossing of "floxed" *HFE* mice with a transgenic line expressing the Cre recombinase under the control of the villin promoter resulted in intestinal-specific disruption of HFE in the progeny[95]. Importantly, mice lacking HFE expression in the intestine did not show any signs of abnormal iron metabolism, at least with regard to liver iron content, serum iron parameters and serum ferritin levels. Moreover, they exhibited physiological expression of the mRNAs encoding liver hepcidin and the intestinal iron transporters DMT1 and ferroportin[95]. By showing that intestinal HFE expression is dispensable for the regulation of body iron homeostasis, these data challenge the validity of the "crypt-programming model" and raise the possibility for a critical role of HFE in the liver.

In a follow-up study, "floxed" *HFE* mice were crossed with transgenic animals expressing the Cre recombinase under the control of either the hepatocyte-specific albumin promoter, or the macrophage-specific lysozyme M promoter[96]. While HFE ablation in macrophages did not affect body iron metabolism, the disruption of HFE in hepatocytes recapitulated the hemochromatosis phenotype of null *HFE*-/-mice. Thus, mice lacking HFE expression in hepatocytes exhibited hyperabsorption of dietary iron, increased serum iron, transferrin saturation and iron deposition in the liver[96]. Taken together, the tissue-specific knock-out experiments demonstrate that hepatocyte HFE is necessary to promote appropriate hepcidin responses and thereby prevent iron overload.

These data also corroborate clinical findings, showing that the iron status of recipients of a liver transplant was largely dependent on the *HFE* genotype of the donors[97,98]. Nevertheless, a contribution of macrophage HFE to hepcidin regulation cannot be completely ruled out. While macrophages are dispensable for hepcidin expression in response to iron or inflammatory signals[99,100], bone marrow transplantation from wild type mice into irradiated *HFE*-/-counterparts corrected iron parameters and significantly increased hepcidin levels in the recipients[101]. Conceivably, this could be the result of intercellular communication and signaling to hepatocytes and/or HFE-mediated autocrine production of hepcidin in macrophages[102].

## LESSONS FROM ANIMAL MODELS II: THE ROLE OF TFR1 IN THE CONTROL OF HFE ACTIVITY

How does HFE modulate signaling to hepcidin? Biochemical work showed that HFE not only interacts with TfR1, but also with TfR2[103]. Moreover, the HFE/TfR2 interaction leads to an increase in TfR2 levels[104]. TfR2 is primarily expressed in hepatocytes[105] and stabilized by diferric holo-transferrin[106,107]. While TfR1 mediates cellular iron uptake from circulating transferrin, TfR2 is thought to function as an upstream regulator of hepcidin, and possibly an iron sensor[14]. A testable prediction arising from the capacity of HFE to interact with both TfR1 and TfR2 would be that the choice of its binding partner is regulated by transferrin and, furthermore, this event is crucial for signaling to hepcidin.

This hypothesis was explored in a recent study, based on the idea to induce or abolish HFE/TfR1 interactions *in vivo*[108]. To this end, transgenic mice were engineered for expression of TfR1 mutants that prevent the binding of either transferrin (R654A) or HFE (L622A). In light of the early embryonic lethality of *TfR1*-/-mice[109], indicating an utmost importance for the interaction of TfR1 with transferrin, a TfR1 R654A cDNA was integrated by homologous recombination into the heterologous ROSA26 locus, maintaining endogenous wild type *TfR1* expression (thus, the transgenic product did not disrupt the transferrin cycle, excluding abnormalities of erythropoiesis). In contrast, the L622A mutation was introduced by homologous recombination within the TfR1 locus ("knock-in").

TfR1 R654A, that is unable to bind to transferrin, would be expected to constitutively associate with HFE. Transgenic mice expressing *TfR1* R654A developed iron overload, associated with decreased hepcidin mRNA levels, closely resembling the *HFE*-/-phenotype. On the other hand, TfR1 L622A, that is unable to bind to HFE, would be expected to be highly efficient in the uptake of transferrin-bound iron. Interestingly, transgenic mice expressing TfR1 L622A developed a mild hypochromic microcytic anemia, and exhibited decreased serum iron and elevated hepcidin mRNA levels. These results suggest that HFE stimulates hepcidin expression when it is free of TfR1. In support of this notion, the hepatocyte-specific transgenic overexpression of an HFE cDNA in *HFE*-/-mice substantially induced hepcidin mRNA expression to the extent that it not only corrected hepatic iron overload, but also promoted hypochromic microcytic anemia.

## A MODEL FOR THE IRON REGULATORY FUNCTION OF HFE

A model accommodating the above findings postulates that under low serum iron conditions, hepatocyte HFE is predominantly bound to TfR1 (Figure 2A). An increase in transferrin saturation triggers the release of HFE from TfR1 and concomitantly stabilizes TfR2[106,107]. In that way, TfR1 becomes accessible for the binding and endocytosis of holo-transferrin, resulting in cellular iron uptake. At the same time, HFE associates with stabilized TfR2 and possibly other proteins, such as hemojuvelin and BMPs and their receptor (BMPR), to form a putative iron signaling complex that induces hepcidin transcription *via* Smad proteins (Figure 2B). Thus, an increase in the iron content of the hepatocyte is

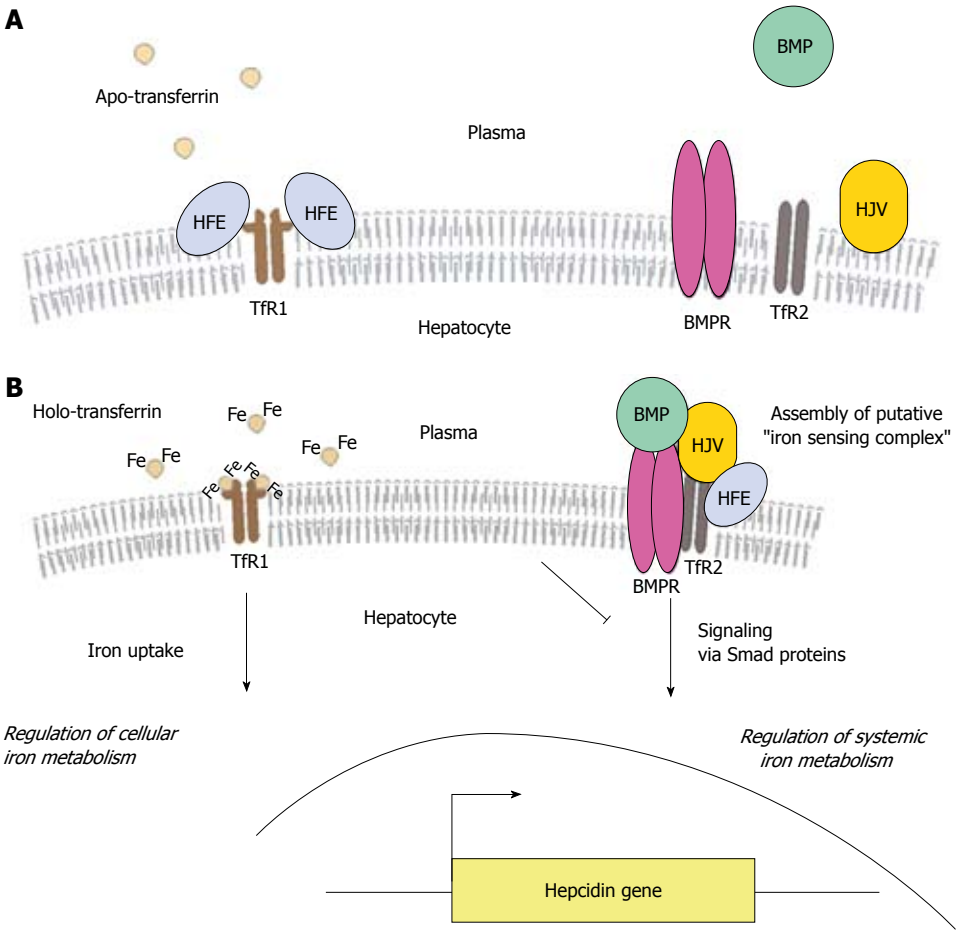


**Figure 2 A model for HFE-mediated signaling to hepcidin in hepatocytes.** A: At low plasma iron concentration, HFE is bound to TfR1 and other proteins involved in signaling to hepcidin remain silent; B: An increase in plasma iron levels results in displacement of HFE from TfR1, followed by iron uptake. This triggers the assembly of a putative "iron-sensing" complex, comprising of HFE, TfR2, BMPs (such as BMP-2, BMP-4 and BMP-9) and their receptor BMPR, and Hjv, which mediates signaling to activate hepcidin transcription via Smad proteins. Thus, the hepatocyte integrates signals for regulation of iron metabolism at the cellular and systemic level.

indirectly translated into a systemic regulatory response *via* hepcidin. Iron-dependent degradation of TfR1 mRNA by iron regulatory proteins[110] would terminate this process in a feedback loop. According to this model, HFE serves to sense alterations in transferrin saturation.

Considering that a number of HH patients with HFE C282Y mutations[25] and some *HFE*-/-mice[29] express normal (or close to normal) basal hepcidin mRNA levels but exhibit blunted hepcidin responses to dietary iron, it is conceivable that the role of HFE is somehow restricted to the fine-tuning of iron-dependent signaling to hepcidin. Along these lines, BMP-2, -4 and -9 can induce hepcidin mRNA transcription in *HFE*-/-and *TfR2*-/-hepatocytes[41]. Several reports have also shown that HFE is dispensable for signaling to hepcidin *via* the inflammatory pathway[29,41,111,112], even though opposing views exist[113].

Recent animal studies[95,96,108] have not entirely solved the mystery of HFE function, but have significantly advanced our understanding on how this protein regulates systemic iron homeostasis. First, they uncovered HFE as a hepatocyte iron sensor, necessary to prevent iron overload and sufficient to control hepcidin expression (at least at the mRNA level). And second, they demonstrated that HFE-dependent signaling to hepcidin is regulated by the interaction of HFE with TfR1.

## OUTLOOK AND PERSPECTIVES

Several outstanding issues remain to be addressed. For example, the proposed function of HFE as a sensor of transferrin saturation requires experimental validation. The functional significance of the interaction between HFE and TfR2, as well as the role and composition of the putative iron-sensing complex await further investigation. It will be interesting to explore a potential functional redundancy between HFE and classical MHC class I molecules with regard to iron regulation, considering that mice lacking such molecules develop iron overload[114]. Conversely, the proposed capacity of HFE to engage into immune responses, following recognition by cytotoxic T lymphocytes[115], deserves additional attention, especially in light of immunological abnormalities of HH patients[116]. A possible connection between the unfolded protein response, caused by defective processing of HFE C282Y, and the hepcidin pathway would not be totally unexpected[117]. Finally, it will be important to examine whether HFE may also affect the maturation of hepcidin; this would necessitate analytical methods for direct measurement of the peptide in plasma[118,119] and in cell culture.

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