Genes that modify the hemochromatosis phenotype in mice

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Hereditary hemochromatosis (HH) is a prevalent human disease caused by a mutation in *HFE*, which encodes an atypical HLA class I protein involved in regulation of intestinal iron absorption. To gain insight into the pathogenesis of hemochromatosis, we have bred *Hfe* knockout mice to strains carrying other mutations that impair normal iron metabolism. Compound mutant mice lacking both Hfe and its interacting protein, beta-2 microglobulin (B2m), deposit more tissue iron than mice lacking Hfe only, suggesting that another B2m-interacting protein may be involved in iron regulation. Hfe knockout mice carrying mutations in the iron transporter DMT1 fail to load iron, indicating that hemochromatosis involves iron flux through DMT1. Similarly, compound mutants deficient in both Hfe and hephaestin (Heph) show less iron loading than do *Hfe* knockout mice, indicating that iron absorption in hemochromatosis involves the function of Heph as well. Finally, compound mutants lacking Hfe and the transferrin receptor accumulate more tissue iron than do mice lacking Hfe alone, consistent with the idea that interaction between these two proteins contributes to the control of normal iron absorption. In addition to providing insight into the pathogenesis of HH, our results suggest that each of these genes might be a candidate modifier of the human hemochromatosis phenotype.

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Introduction

Hereditary hemochromatosis (HH) is a prevalent genetic disorder, affecting up to one in 250 individuals of European descent (1). It is characterized by iron deposition in hepatocytes, cardiac myocytes, and other cells, as a consequence of a small, chronic increase in intestinal iron absorption. Over several decades, patients with HH accumulate up to tenfold more body iron than do normal individuals. Iron deposition leads to tissue damage and fibrosis and ultimately to organ failure if the disease is not recognized and treated. HH is inherited as a monoallelic, recessive disorder, in which clinical signs and symptoms are manifest in homozygotes. However, there is clear evidence that both genetic and environmental factors modify disease severity (2).

Insight into the molecular basis of HH first came almost 25 years ago, when the phenotype was shown to be linked to the HLA locus on human chromosome 6 (3). This observation led to the identification of the causative gene, *HFE*, in 1996 (4). *HFE* encodes an atypical MHC class I–like molecule that is widely expressed and forms a heterodimer with beta-2 microglobulin (B2m). Its role in regulation of intestinal iron absorption is not well understood. It appears to act through a high-affinity protein-protein interaction with the transferrin receptor (Trfr) (5–9). Most patients with HH are homozygous for a missense mutation in the *HFE* gene that results in a cysteine→tyrosine substi-

tution at amino acid 282 of the HFE protein (C282Y) (4). However, there is marked variability in the phenotype of C282Y homozygotes and heterozygotes that is only partially explained by environmental factors. Not all individuals homozygous for the C282Y mutation develop iron overload. At the other extreme, some individuals with marked iron overload are heterozygous for the C282Y mutation, with one apparently normal *HFE* allele. The biologic basis for this phenotypic variability is unknown.

Although many tissues are affected in hemochromatosis, the liver is the primary storage depot for iron in excess of immediate body needs. Liver non-heme iron increases in linear relationship to increasing body iron stores until late in the disease, when liver iron storage sites become saturated (10). For this reason, liver iron content is a useful measure of total body iron stores during the early, iron-loading phase of hemochromatosis (reviewed in ref. 11).

We previously generated two murine models of HH (12). The *Hfe* gene was disrupted by targeted removal of essential coding sequence and transmitted through the germline to produce mice homozygous for a null *Hfe* allele. In parallel, the disease-causing C282Y mutation was introduced into the murine *Hfe* allele. Both mutations result in loss of protein function and in an iron overload similar to that seen in the human disease. The null mutation produces a more severe

phenotype than does the missense mutation (12). Other investigators have independently shown that *Hfe* knockout mice develop iron overload (13, 14). We wanted to use our mutant mice to study the effects of mutations in other genes on the expression of the hemochromatosis phenotype.

To evaluate potential genetic modifiers of mutations at the *Hfe* locus, we crossed *Hfe* mutant mice with mice carrying other spontaneous and induced mutations affecting defined steps in iron metabolism. We have used two spontaneous mutant strains, *mk* and *sla*. Mice homozygous for the microcytosis (*mk*) trait have a severe loss-of-function mutation in the gene encoding DMT1 (formerly Nramp2/DCT1), the major transmembrane iron import molecule (15–17). This results in a defect in absorption of dietary iron from the intestinal lumen and in defective iron utilization by erythroid precursors (reviewed in ref. 18). Mice homozygous or hemizygous for the sex-linked anemia (*sla*) mutation carry a deletion in the *Hephaestin* gene (*Heph*), found on the X chromosome. These animals have a defect in export of iron from epithelial cells due to deficiency of the protein Hephaestin (Heph). Heph is homologous to ceruloplasmin and is postulated to act as a membrane-bound, copper-dependent ferroxidase (19). In *sla* mice, dietary iron appears to enter absorptive duodenal enterocytes normally but is not efficiently transferred across the basolateral membrane to reach the circulation.

We have also tested two strains of knockout mice carrying null mutations in the genes encoding B2m (20) and Trfr (21). B2m is a dimerization partner for typical and atypical major histocompatibility class I proteins, including Hfe. Disruption of the *B2m* gene results in immunological abnormalities, as well as iron loading (22). Knockout mice homozygous for a null mutation in the *Trfr* gene die from severe anemia during embryonic development (21). The formation of most tissues does not appear to be impaired, with the exception of the nervous system and the erythron. Surprisingly, heterozygous mice carrying only one functional *Trfr* allele develop mild tissue iron depletion (21). Decreased levels of tissue iron may result from a lower "set point" for iron absorption in the heterozygous animals.

Here, we report the iron status of animals carrying mutations in the *Hfe* locus in combination with mutations in other genes important in iron metabolism. The results of our studies help to define the relationship between genotype and phenotype in this murine model of hereditary hemochromatosis and give insight into the pathogenesis of iron overload. In addition, these observations identify candidate modifier genes that may influence the course of HH in human patients.

Methods

Animal care and genotyping. Mice were housed in the barrier facility at Children's Hospital and maintained on standard mouse diet. All mouse production and experimentation were in compliance with the guidelines of the Institutional Animal Care and Use Committee. $Hfe^{-/-}$ and $Trfr^{+/-}$ mice used in this study have been described previously (12, 21). C57BL/6J-$B2m^{-/-}$ and MK/ReJ-*mk*/+ mice were derived from breeding stocks purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). The C57BL/6J-*sla* mutant stock was obtained from a colony maintained by L. Peters at The Jackson Laboratory.

Animals were housed for different lengths of time prior to analysis. Some investigations were done at 6 weeks of age, and others were done at 10 to 11 weeks of age. Because iron accumulation is progressive, the size of the iron stores is dependent upon age. For this reason, experimental and control animals of the same age were used for each comparison.

DNA was prepared from mouse tail fragments using a DNeasy Tissue kit (QIAGEN Inc., Valencia, California, USA). *Trfr* mutant mice were genotyped as described previously (21). *Hfe* mutant mice were genotyped using a PCR assay. Radiolabeled primers were used to amplify a 202-bp product from the wild-type allele (5′-ATCAAATGGCATCTCTGGCA-3′ and 5′-GTGGCGAGT-CACTTTCACCA-3′), and a 190-bp product from the targeted allele (5′-CTAGCTTCGGCCGTGACG-3′ and 5′-AGTTGGGAGTGGTGTCCGA-3′). PCR products were electrophoresed through 6% acrylamide gels and detected by exposure of the gels to x-ray film. The *mk* mutant mice were genotyped using radiolabeled primers (5′-GAAATCGTCCCTGGGTTTTT-3′ and 5′-CCCCTGGCCT-TACCATATTT-3′) for PCR amplification of DMT1 DNA flanking the G185R missense mutation (15). The resulting 129-bp PCR products from the wild-type and mutant DMT1 alleles were distinguished by differential migration through 5% nondenaturing polyacrylamide gels (23). *B2m* mutant mice were genotyped using primers that amplify a 262-bp product from the wild-type *B2m* allele (5′-CTGAGCTCTGTTTTCGTCTG-3′ and 5′-TATCAGTCTCAGTGGGGGTG-3′) and a 768-bp product from the targeted allele (5′-GCTATTCGGCTATGACTGGG 3′ and 5′-TATCAGTCTCAGTGGGGGTG-3′). In this case, the PCR products were fractionated on 1% agarose gels. The mutant *sla* mice were maintained in our laboratory as homozygous *sla/sla* females and hemizygous *sla*/Y males. The breeding scheme used in crosses with *Hfe* mutant mice involved breeding homozygous *sla/sla* females to $Hfe^{-/-}$ males and then backcrossing the obligate *sla*/Y, $Hfe^{+/-}$ male progeny to their *sla/sla* mothers. The progeny from this cross were bred to obtain animals with various *Hfe* genotypes. Because all of these animals were homozygous or hemizygous for *sla*, molecular genotyping was not necessary. Animals of similar genetic background were generated as $Hfe^{-/-}$ controls with wild-type *sla* alleles.

Liver iron determinations and histology. Liver non-heme iron content was determined as described previously (12). Histological sections were prepared and stained with the Perl's stain for iron as described previously (21).

Statistical analysis. Statistical significance was evaluated using the unpaired Student's *t* test with the Welch correction for comparisons between two means. A
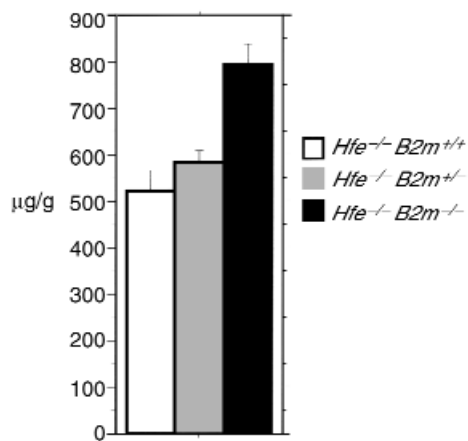
Figure 1

Liver iron in mice carrying *Hfe* and *B2m* mutations. In Figures 1, 2, 3, and 5, histograms show levels of liver iron expressed as micrograms of non-heme iron per gram wet weight of liver. Genotypes are specified by color coding. Absolute quantities are given in parentheses as mean + SE followed by the number of samples analyzed. *P* values were calculated by the unpaired Student's *t* test with the Welch correction, with *P* values < 0.05 considered significant. This figure shows liver iron levels in mice carrying mutant alleles of *Hfe* and *B2m* in various combinations: *Hfe*^{-/-}*B2m*^{+/+} (526.18[A] + 40.39; *n* = 16), *Hfe*^{-/-}*B2m*^{+/-} (581.71[B] + 26.08; *n* = 24), *Hfe*^{-/-}*B2m*^{-/-} (794.12[C] + 45.40; *n* = 15). *P* values are as follows: A vs. C, *P* = 0.0001; B vs. C, *P* = 0.0005; A vs. B, *P* = NS.

value of *P* < 0.05 was regarded as significant. Statview (SAS Institute, Inc., Cary, North Carolina, USA) and InStat (Graph Pad Software, San Diego, California, USA) software were used for statistical evaluation.

Results

Absence of B2m increases iron loading in Hfe knockout mice. B2m heterodimerizes with MHC class I proteins, including Hfe. It has been presumed that Hfe requires B2m for its regulatory effect on iron metabolism, particularly because mice lacking B2m (*B2m*^{-/-}) develop iron overload (24). We wanted to determine how iron loading in mice lacking both Hfe and B2m compared with iron loading in mice lacking Hfe alone. To study the effect of *B2m* mutations on the *Hfe*^{-/-} phenotype, we interbred *B2m*^{-/-} and *Hfe*^{-/-} mice. We studied compound mutant animals whose genetic background was mixed, averaging half C57BL/6J and half 129/SvEvTacfBR. Mice were analyzed at 10–13 weeks of age. Surprisingly, liver iron deposition was greater in mice lacking both Hfe and B2m than in mice lacking Hfe alone (Figure 1). The difference was highly significant.

Iron loading of Hfe knockout mice requires the apical iron transporter DMT1. The severe phenotype of homozygous *mk* mice, carrying a deleterious missense mutation in the gene encoding DMT1, indicates that DMT1 is an important component of the major pathway for intestinal iron absorption in normal animals. We wanted to determine whether the increased iron absorption seen in HH resulted from increased iron flux through DMT1 or from the activation of an alter-

native, accessory, iron absorption pathway. To answer this question, we interbred mice carrying the *mk* mutation in DMT1 with *Hfe*^{-/-} mice. The compound mutant offspring had a mixed genetic background that included contributions from MK/ReJ (25), C57BL/6J, and 129/SvEvTacfBR strains. Liver iron deposition was analyzed at 4.5–6.5 weeks of age (Figure 2). There is a marked difference in iron accumulation between *Hfe*^{-/-} mice that carry at least one wild-type allele of the DMT1 gene and *Hfe*^{-/-} mice that are homozygous for the *mk* allele of DMT1. In these animals, mutation of the *Hfe* gene did not improve the extremely low iron stores of homozygous *mk* mutant mice. This indicates that the iron accumulation seen in this model of hemochromatosis is prevented by mutations in DMT1, suggesting that iron accumulation occurs predominantly through an absorption pathway mediated by DMT1.

Interestingly, although liver non-heme iron levels were indistinguishable from those of age-matched *mk/mk* mice with normal Hfe protein, the animals that were homozygous for both the *mk* mutation in DMT1 and the *Hfe* knockout allele appeared healthier. Typically, fewer than 10% of *mk/mk* mutant mice survive to weaning (ref. 26, and M.D. Fleming, unpublished study). In contrast, on the *Hfe* knockout background, most *mk/mk* mice survived (data not shown). We have previously shown that DMT1 protein containing the *mk* amino acid substitution retains a low level of iron transport function (17). We speculate that, in the absence of *Hfe* protein, there is increased intestinal iron absorption in *mk/mk* mice. However, the increased absorption is
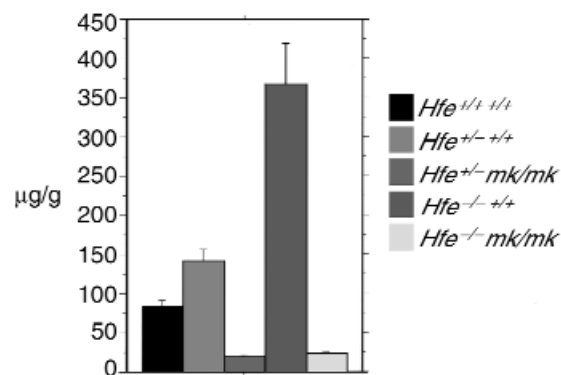

Figure 2

Liver iron in mice carrying *Hfe* and DMT1 mutations. This figure shows liver iron levels in mice carrying mutant alleles of *Hfe* and the gene encoding DMT1 in various combinations. The notation +/+ is used for animals that are wild type at both DMT1 alleles; *mk/mk* is used for animals that carry the *mk* mutation (G185R) in both of their DMT1 alleles. Values are given here as described for Figure 1. *Hfe*^{+/+} +/+ (84.02[A] + 7.72; *n* = 6), *Hfe*^{+/-}+/+ (142.35[B] + 14.31; *n* = 7), *Hfe*^{+/-}*mk/mk* (19.24[C] + 1.39; *n* = 9), *Hfe*^{-/-}+/+ (365.96[D] + 51.87; *n* = 5), *Hfe*^{-/-}*mk/mk* (22.32[E] + 2.46; *n* = 8). *P* values: C vs. E, *P* = NS; A vs. B, *P* = 0.0059; A vs. C, *P* = 0.0004; A vs. D, *P* = 0.0058; A vs. E, *P* = 0.0003; B vs. C, *P* = 0.0001; B vs. D, *P* = 0.0142; B vs. E, *P* = 0.0002; C vs. D, *P* = 0.0026; D vs. E, *P* = 0.0027.
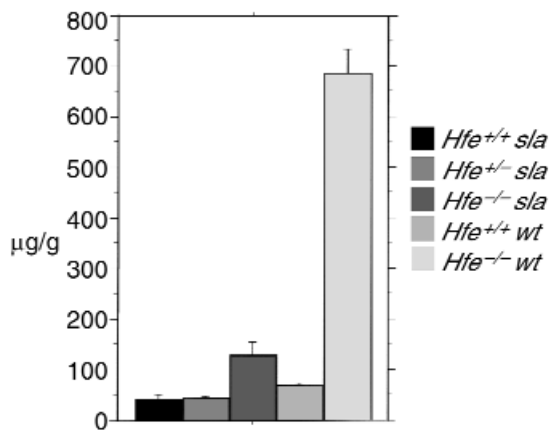
Figure 3

Liver iron in mice carrying *Hfe* and *Heph* mutations. This figure shows liver iron levels in mice carrying mutant alleles of *Hfe* and *Heph* in various combinations. *sla* mice indicates a genotype of either *Heph^sla/sla^* or *Heph^sla/Y^*. Wild-type (wt) indicates a *Heph* genotype that is either *Heph^+/+^* or *Heph^+/Y^*. Values are given here as described for Figure 1. *Hfe*^+/+^ *sla* (41.23[A] + 9.54; *n* = 4), *Hfe*^+/−^ *sla* (45.93[B] + 3.90; *n* = 21), *Hfe*^−/−^ *sla* (128.47[C] + 27.02; *n* = 22), *Hfe*^+/+^ *wt* (70.64[D] + 4.10; *n* = 9), *Hfe*^−/−^ *wt* (685.97[E] + 47.88; *n* = 8). *P* values: A vs. C, *P* = 0.0057; B vs. C, *P* = 0.0065; C vs. D, *P* = 0.0464; C vs. E, *P* < 0.0001; A vs. B, *P* = NS; A vs. D, *P* = 0.0472; A vs. E, *P* < 0.0001; B vs. D, *P* = 0.0003; B vs. E, *P* < 0.0001; D vs. E, *P* < 0.0001.

marginal and not detectable as an increase in liver iron stores because the iron is diverted to the erythron, where it is used for erythropoiesis.

Compound mutant mice lacking Heph and Hfe. Heph is thought to be a component of the basolateral iron transport apparatus, probably acting as a ferroxidase to facilitate transmembrane iron transport. Mice that are homozygous or hemizygous for the X-linked *sla* phenotype have a partial disruption of intestinal iron absorption. Although the *sla* deletion in *Heph* is presumed to be a null mutation (19), *sla* mice are less iron deficient than are *mk* mice lacking functional DMT1. If the *sla* mutation does result in total loss of protein function, then Heph must aid in intestinal iron absorption, but not be strictly required. To determine whether increased iron absorption in hemochromatosis involves the iron uptake pathway that is enhanced by Heph function, we bred *Hfe*^−/−^ mice to *sla* mice and studied 10- to 12-week-old mice. In this case, the genetic background was a mix of C57BL/6J and 129/SvEvTacfBR. Mice homozygous for mutations in both *Heph* and *Hfe* had less hepatic iron than did *Hfe* knockout mice at the same age (Figure 3). Mutations in *Hfe* modified the *sla* phenotype; loss of the *Hfe* gene ameliorated the iron deficiency of *sla* mice; iron stores in mice lacking both Heph and Hfe were higher than in mice lacking Heph alone and were higher than in wild-type mice (Figure 3). It appears likely that iron loading occurs through a pathway involving Heph, but, as previously inferred, Heph is not absolutely required for intestinal iron absorption.

Homozygous and hemizygous *sla* mice accumulate iron in the intestinal mucosa, as a result of diminished basolateral transfer (27). Iron is reported to be decreased in the villus enterocytes of patients with HH (28). We examined the distribution of iron in duodenal epithelial cells from mice with the *sla* mutation in *Heph*, the *Hfe* knockout mutation, and both mutations combined. As shown in Figure 4, all mice carrying the *sla* mutation accumulate mucosal iron, regardless of whether they do or do not have Hfe. This has implications for models of Hfe function, as discussed later here.

Transferrin receptor interacts functionally with Hfe to regulate iron homeostasis. In a previous study, we showed that animals lacking one *Trfr* allele (*Trfr*^+/−^ mice) had decreased hepatic iron stores, indicating altered iron homeostasis (21). Because Trfr has no role in apical intestinal iron absorption, this result cannot simply be explained by a direct effect on uptake of dietary iron from the intestinal lumen. One explanation is that decreased gene dosage for *Trfr* might change the stoichiometric ratio of Trfr/Hfe and that this somehow downregulates intestinal iron absorption. This possibility is difficult to investigate using biochemical assays because the in vivo function of the Hfe-Trfr complex is not fully understood. Alternatively, it might be that there is less Trfr expression on hepatocytes, and, therefore, less liver iron uptake. This seems unlikely, because hepatocytes do not normally have a large complement of cell surface Trfr, and posttranscriptional regulation of Trfr by iron regulatory proteins (IRPs) is thought to adjust the amount of Trfr mRNA in response to iron needs (reviewed in ref. 29).

To learn more about the role of Trfr in iron homeostasis and the functional consequences of the Hfe-Trfr interaction, we bred mice heterozygous for a null *Trfr* allele (*Trfr*^+/−^ mice) to mice carrying mutations in *Hfe*. In this case, all strains had a pure 129/SvEvTacfBR background. Mice were analyzed at 4–6 weeks of age. As shown in Figure 5, we found that absence of one *Trfr* allele did not inhibit iron loading in *Hfe*^−/−^ mice. Surprisingly, *Hfe*^−/−^ mice lacking one *Trfr* allele actually have significantly greater hepatic iron deposition than do *Hfe*^−/−^ mice with a normal complement of Trfr. This result is paralleled by the result of the cross between *Trfr* mutant mice and *Hfe*^C282Y/C282Y^ mice. The fact that heterozygosity for *Trfr* has opposite effects on mice that have or lack Hfe has implications for the function of the Hfe/Trfr protein complex, as discussed later here.

Discussion

Genetic variability in human patients with HFE-associated HH has led to a complex spectrum of phenotypic expression. Although there is no doubt that the C282Y mutation in *HFE* is a disease-causing mutation, there is a small subgroup of C282Y homozygotes who do not develop clinically significant iron overload (2). On the other hand, some C282Y homozygotes develop severe iron overload early in life, and some C282Y heterozygotes develop hemochromatosis that is indistin-

guishable from that of the homozygotes. The variability cannot be explained by environmental factors alone; there must be other modifier genes that influence the HH disease phenotype.

Mice carrying targeted mutations in the *Hfe* gene provide an excellent model system to study the pathogenesis of HH. They have the advantage that they are inbred, and *Hfe* mutations have been engineered in the context of a defined genetic background. Furthermore, there are a variety of mouse strains with spontaneous and induced mutations in other genes affecting iron metabolism, and interbreeding offers an opportunity to test selectively for modifying effects. In this study, we have taken advantage of mouse genetics to investigate the pathogenesis of HH and to examine the influence of possible modifying genes.

Hfe belongs to a large family of MHC class I–like proteins. At present, it remains unclear how a molecule from this group acts to regulate intestinal iron absorption. The tantalizing finding that it interacts with Trfr offers a clue to its activity, but has not yet elucidated its mode of action. The first question that we addressed was whether Hfe is unique among MHC class I–like proteins, or whether there are other, related proteins affecting iron homeostasis. We previously observed that *Hfe* knockout mice still have substantial regulation of intestinal iron absorption. As they age, their iron overload proceeds, until the amount of tissue iron reaches a plateau (data not shown). Although similar to human patients (30), this is in striking contrast to another mouse model of iron overload (31). Homozygous *hpx* mice carry a mutation in the transferrin (Trf) gene that severely abrogates

Trf expression. Age-matched *hpx* mice accumulate 15–20 times as much hepatic iron as do *Hfe* knockout mice (32). They have accelerated intestinal iron absorption that continues indefinitely as the animals age; tissue iron levels do not appear to plateau. These findings suggest that there are additional regulatory pathways governing intestinal iron absorption. Our finding that mice lacking both B2m and Hfe develop more iron overload than do mice lacking Hfe alone suggests that there may be another, still unidentified, molecule that interacts with B2m and regulates intestinal iron absorption. It is possible that it is an atypical MHC class I–like molecule, similar to Hfe. Alternatively, it is possible that the compromised immune system of B2m knockout mice exacerbates the iron loading phenotype of Hfe$^{-/-}$ mice by some indirect mechanism that is not yet understood.

Studies of human patients provide genetic evidence for an additional locus on chromosome 6p, distinct from HFE, that modifies the hemochromatosis phenotype (33). Furthermore, a second polymorphism in *HFE*, resulting in a histidine→aspartic acid substitution (H63D), is highly prevalent but only rarely associated with clinical hemochromatosis in the homozygous state (reviewed in ref. 34). It is possible that the H63D polymorphism does not, itself, significantly perturb HFE function. Rather, a unique H63D haplotype might be genetically linked to a mutation in a nearby gene, within the HLA complex, that encodes a molecule that functions in a manner similar to HFE. In this case, H63D would not be a disease-causing mutation, but rather a marker in linkage disequilibrium with a disease-causing mutation in another gene. On the basis of
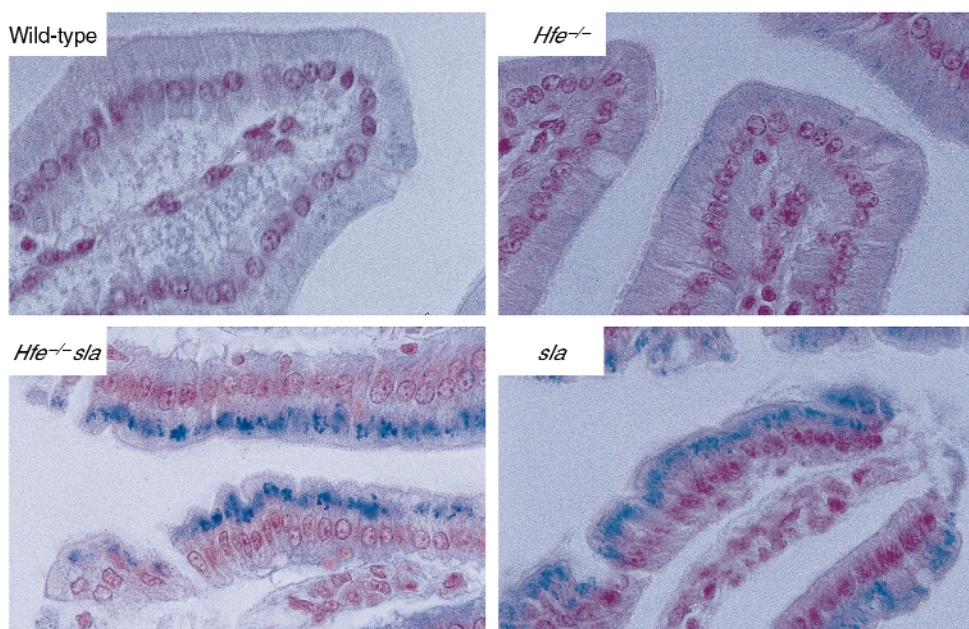

Figure 4

Iron stain of proximal duodenum in mice carrying *Hfe* and *Heph* mutations. Tissues were fixed in formalin and stained with the Perls' stain for iron. Non-heme iron stains blue. Photomicrographs were taken through an Olympus BX50 microscope at ×2,500. *sla* indicates a sample from a mouse that was *Heph$^{sla/sla}$* or *Heph$^{sla/Y}$*.
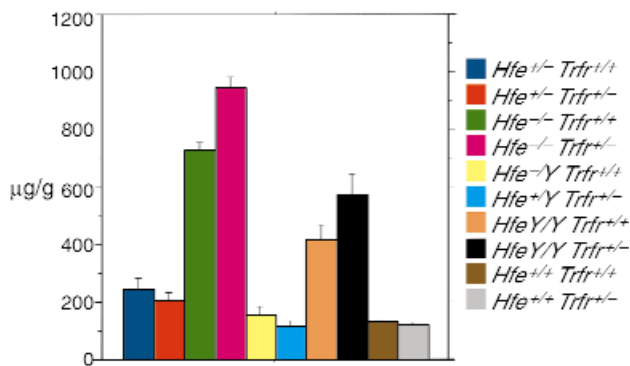
Figure 5

Liver iron in mice carrying *Hfe* and *Trfr* mutations. This figure shows liver iron levels in mice carrying mutant alleles of *Hfe* and *Trfr* in various combinations. *Hfe* mutant mice carrying the C282Y mutation are listed as *Hfe$^{Y/Y}$* or *Hfe$^{+/Y}$* . *Hfe* mutant mice carrying the null mutation are listed as *Hfe$^{-/-}$* or *Hfe$^{+/-}$*. Values are given here as described for Figure 1. Navy blue*: Hfe$^{+/-}$Trfr$^{+/+}$* (246.71 + 34.73; *n* = 6). Red: *Hfe$^{+/-}$Trfr$^{+/-}$* (208.17 + 26.08; *n* = 6). Green: *Hfe$^{-/-}$Trfr$^{+/+}$* (725.36[A] + 32.04; *n* = 25). Pink: *Hfe$^{-/-}$Trfr$^{+/-}$* (947.20[B] + 36.64; *n* = 29). Yellow: *Hfe$^{+/Y}$Trfr$^{+/+}$* (155.76 + 25.03; *n* = 3). Light blue: *Hfe$^{+/Y}$Trfr$^{+/-}$* (113.07 + 17.92; *n* = 5). Orange: *Hfe$^{Y/Y}$Trfr$^{+/+}$* (416.93 + 50.38; *n* = 5). Black: *Hfe$^{Y/Y}$Trfr$^{+/-}$* (574.73 + 67.19; *n* = 4). Brown: *Hfe$^{+/+}$Trfr$^{+/+}$* (127.75 + 6.99; *n* = 8). Gray: *Hfe$^{+/+}$Trfr$^{+/-}$* (115.02 + 12.63; *n* = 3). *P* value: A vs. B, *P* < 0.0001. The difference in iron loading between *Hfe$^{+/+}$Trfr$^{+/+}$* and *Hfe$^{+/+}$Trfr$^{+/-}$* animals was not statistically significant in this experiment, because only three *Hfe$^{+/+}$Trfr$^{+/-}$* animals were analyzed. However, the trend in the data is consistent with our previous work, in which we have shown that there is a statistically significant difference between these two genotypes (21).

the increased iron loading observed in *Hfe$^{-/-}$ B2m$^{-/-}$* compound mutant mice, we speculate that there may be another nearby MHC class I–like gene that similarly regulates iron absorption. Alternatively, another iron regulatory, B2m-interacting protein may be encoded by a gene located on a different chromosome. If so, it might be a candidate for the causative gene in non-HFE hemochromatosis (35, 36) or in juvenile hemochromatosis (37). Each of these diseases resembles HH clinically, but can be distinguished genetically.

Several different iron uptake pathways have been proposed to explain intestinal iron absorption (38). Studies of severely iron-deficient *mk* mice (39) and *b* rats (40), carrying a severe loss-of-function mutation in DMT1 (15, 41), indicate that DMT1 is the primary apical iron transporter involved in the uptake of dietary iron in normal mammals. We wanted to determine whether increased iron absorption associated with hemochromatosis involved DMT1 or induction of an alternative transport mechanism. To answer this question, we bred *Hfe$^{-/-}$* mice to *mk* mice. Our results unequivocally show that DMT1 mediates iron absorption in HH. It is not yet clear whether increased iron uptake in hemochromatosis is associated with increased levels of DMT1 protein. Although there are reports that levels of DMT1 mRNA are increased in patients with HH (42) and *Hfe* knockout mice (43), we have not found significant induction of

DMT1 levels in our *Hfe* mutant mice (M.D. Fleming, J.E. Levy, and N.C. Andrews, unpublished study). We favor a model supported by several clinical studies, in which basolateral iron transfer is increased in HH (28, 44). However, regardless of the mechanism, the fact that iron overload in HH depends on DMT1 has an important clinical implication. If a compound were to selectively block DMT1 activity at the brush border, it might be developed into an oral pharmaceutical agent to prevent iron absorption in patients with hemochromatosis.

To investigate basolateral iron transfer in *Hfe* knockout mice, we bred *Hfe$^{-/-}$* animals to *sla* animals, which have impaired basolateral iron transport as a result of a mutation in *Heph*. The decreased liver iron loading seen in compound mutant mice carrying loss-of-function mutations in both *Hfe* and *Heph* indicate that Heph is also a component of the enterocyte iron uptake apparatus functional in HH. However, loss of Heph function does not block iron transfer completely, either in *sla* mice, in which the mutation was initially described, or in *Hfe$^{-/-}$/Heph$^{sla/sla}$* compound mutants.

The absorptive enterocyte is reported to be relatively iron depleted in HH (28). This has fueled speculation that cellular iron deficiency might lead to activation of IRPs, which might in turn induce the expression of DMT1 (43). In contrast, the *sla* mutation in *Heph* results in iron accumulation within duodenal enterocytes. We examined these cells in animals with mutations in both *Hfe* and *Heph* and found that the compound mutants have abundant, stainable, non-heme iron, similar to that of *sla* animals. Although we have not measured the activity of IRPs in the duodenal mucosa, and it remains possible that the abundant intracellular iron is somehow sequestered from the IRP regulatory system, the fact that enterocyte non-heme iron is increased should be taken into account in making models for Hfe function. We observe that compound mutants deficient in both Hfe and Heph have larger liver iron stores than do wild-type animals, indicating that they absorb more iron in spite of their increased mucosal iron content.

The most intriguing results come from analysis of mice with the genotype *Hfe$^{-/-}$/Trfr$^{+/-}$*. These animals lack Hfe and have only one functional *Trfr* allele. Surprisingly, they accumulate more hepatic iron than do mice lacking Hfe alone. This result was unexpected because we previously found that mice with intact *Hfe* alleles but missing one *Trfr* allele have lower body iron stores than do normal mice (21).

This apparent paradox can be resolved in the following way. We hypothesize that an interaction between Hfe and Trfr establishes a dominant set point for iron homeostasis. In the absence of one functional *Trfr* allele, the set point would be decreased, as a result of the changed stoichiometry (Hfe relative to Trfr). In the absence of Hfe, this set-point mechanism is disrupted and iron absorption increases, unregulated by this level of control. When Hfe is absent and only one *Trfr* allele is functional, the Hfe/Trfr-related set point is already

perturbed and cannot be further affected because there is no Hfe to form the complex. In this setting, an alternative regulatory mechanism is revealed, and iron stores increase beyond the level seen in Hfe knockout mice. We cannot yet identify the putative, alternative regulatory mechanism, but we speculate that it may be related to the "erythroid regulator," which modulates intestinal iron absorption in response to the needs of the erythron (45, 46). We have previously shown that mice heterozygous for the null *Trfr* allele have small erythrocytes that contain less hemoglobin than normal red cells because erythroid precursors have reduced levels of Trfr and therefore cannot take up enough iron for normal hemoglobinization (21). *Hfe*$^{-/-}$/*Trfr*$^{+/-}$ mice also have iron deficient erythropoiesis, in spite of tissue iron overload (data not shown). It is well established that erythroid iron deficiency stimulates intestinal iron absorption through an unknown humoral signal. We suggest that this signal, in response to decreased iron uptake by erythroid precursors, acts independently of Hfe to further increase iron absorption in *Hfe*$^{-/-}$/*Trfr*$^{+/-}$ mice.

In summary, we have used mice with defined genetic backgrounds to explore the relationship between *Hfe* and other genes important for the regulation of iron metabolism. We have shown that B2m, DMT1, Heph, and Trfr can act as modifiers of the HH phenotype in mice. Our results give insight into the pathogenesis of hemochromatosis, by suggesting the existence of another B2m-associated protein involved in iron homeostasis. Furthermore, we have established that increased iron absorption in HH occurs through an iron transport pathway involving DMT1 and Heph. We have begun to characterize the molecular aspects of complex regulatory mechanisms controlling intestinal iron absorption. It seems likely that variability in the human orthologues of the genes we have studied could similarly modify HH in human patients. It will be important to search for polymorphisms and mutations in these genes in patients with exceptionally severe and exceptionally mild cases of HH. It is possible that loss-of-function mutations in B2M exacerbate the phenotype of C282Y homozygotes. In addition, mild loss-of-function mutations in the genes encoding DMT1 and HEPH may ameliorate HH. Furthermore, mutations in TRFR and other proteins important for erythroid iron utilization may have complex effects on patients who are heterozygous or homozygous for the C282Y mutation in HFE.

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