Iron Regulation of Hepcidin Despite Attenuated Smad1,5,8 Signaling in Mice Without Transferrin Receptor 2 or Hfe

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BACKGROUND & AIMS: HFE and transferrin receptor 2 (TFR2) are each necessary for the normal relationship between body iron status and liver hepcidin expression. In murine *Hfe* and *Tfr2* knockout models of hereditary hemochromatosis (HH), signal transduction to hepcidin via the bone morphogenetic protein 6 (Bmp6)/Smad1,5,8 pathway is attenuated. We examined the effect of dietary iron on regulation of hepcidin expression via the Bmp6/Smad1,5,8 pathway using mice with targeted disruption of *Tfr2*, *Hfe*, or both genes. **METHODS:** Hepatic iron concentrations and messenger RNA expression of Bmp6 and hepcidin were compared with wild-type mice in each of the HH models on standard or iron-loading diets. Liver phospho-Smad (P-Smad)1,5,8 and *Id1* messenger RNA levels were measured as markers of Bmp/Smad signaling. **RESULTS:** Whereas Bmp6 expression was increased, liver hepcidin and Id1 expression were decreased in each of the HH models compared with wild-type mice. Each of the HH models also showed attenuated P-Smad1,5,8 levels relative to liver iron status. Mice with combined *Hfe/Tfr2* disruption were most affected. Dietary iron loading increased hepcidin and Id1 expression in each of the HH models. Compared with wild-type mice, HH mice demonstrated attenuated (*Hfe* knockout) or no increases in P-Smad1,5,8 levels in response to dietary iron loading. **CONCLUSIONS: These observations show that Tfr2 and Hfe are each required for normal signaling of iron status to hepcidin via the Bmp6/Smad1,5,8 pathway. Mice with combined loss of Hfe and Tfr2 up-regulate hepcidin in response to dietary iron loading without increases in liver Bmp6 messenger RNA or steady-state P-Smad1,5,8 levels.**

Keywords: Bone Morphogenetic Protein 6; Id1.

Hereditary hemochromatosis (HH) is a genetically heterogeneous hereditary disorder caused by elevated iron absorption from the diet, with consequent iron overload and tissue injury.[1,2] The most common form of HH is caused by mutation in the *HFE* gene. A much more rare form of HH (type 3) results from mutations in the gene for transferrin receptor 2 (*TFR2*).[3] It is now widely accepted that impaired regulation of hepcidin expression plays a central role in the pathogenesis of HH. Hepcidin acts to down-regulate the iron exporter ferroportin on the surface of duodenal enterocytes and macrophages, thereby inhibiting iron release from these cells.[4] Human patients and mouse models of *TFR2*-related[5,6] and *HFE*-related[7–10] HH each show inappropriately low expression of hepcidin. The mechanisms by which TFR2 and HFE influence hepcidin expression remain unclear. Several observations suggest a model in which TFR1 may participate as well. In this model, as the transferrin saturation increases, diferric transferrin displaces HFE from TFR1, thereby making HFE available to bind to TFR2.[11–13] The complex of HFE and TFR2 is then postulated to influence hepcidin expression differently than TfR2 alone.[14] Mice with inactivating mutations in both *Hfe* and *Tfr2* show a severe HH phenotype and very low hepcidin expression, raising the possibility that each may serve to regulate hepcidin expression even in the absence of the other.[15]

A bone morphogenetic protein 6 (BMP6)-dependent signaling pathway has been shown to play a key role in regulation of hepcidin expression.[16,17] BMPs bind to type I and type II serine threonine kinase receptors, which phosphorylate specific intracellular SMAD proteins (SMAD1,5,8). Phosphorylated SMAD1,5,8 (P-SMAD1,5,8) binds to the common mediator SMAD4, and the SMAD complex translocates to the nucleus to affect transcription of target genes such as *ID1*.[18,19] *HAMP* (encoding hepcidin) is transcriptionally up-regulated by BMPs.[20–23] Impaired hepatic Bmp signaling, through mutations in genes encoding either the ligand Bmp6,[16,17] the Bmp coreceptor hemojuvelin (Hjv),[24,25] or Smad4[26] leads to low hepcidin levels and iron overload in mice. Conversely, dietary iron loading increases hepatic *Bmp6* messenger RNA (mRNA) expression in mice concordantly with *Hamp1* and *Id1* mRNAs.[27] Collectively, these data show that BMP-SMAD signaling is an important regulatory pathway for hepcidin expression and thus iron metabolism. In *Hfe* knockout mice[28,29] and in patients with *HFE*-associated HH,[30,31] the induction of *Bmp6* mRNA by iron is intact, but Smad1,5,8 signaling to hepcidin is impaired.

Abbreviations used in this paper: ANOVA, analysis of variance; BMP, bone morphogenetic protein; HH, hereditary hemochromatosis; Hjv, hemojuvelin; RT-PCR, reverse-transcription polymerase chain reaction; Tfr2, transferrin receptor 2.

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Impaired Bmp6 signaling to hepcidin has also been reported in murine models of *Tfr2*-associated HH.[15,32]

The goal of this study was to investigate the Bmp6-Smad-hepcidin signaling pathway in the *Tfr2* and *Hfe* mutant mouse models of HH under standard iron diets and with dietary iron loading. We observed the expected impaired signaling to hepcidin via the Bmp6/Smad pathway in *Tfr2* and *Hfe* HH mouse models. Signaling to hepcidin via the Bmp/Smad pathway was more impaired in *Hfe/Tfr2* mice than in mice with loss of either gene product individually. Dietary iron loading increased hepcidin expression in each of the murine HH model systems. In *Tfr2* mice and *Hfe/Tfr2* mice, hepcidin up-regulation occurred without an increase in liver P-Smad1,5,8 levels. Taken together, these results indicate that Hfe and Tfr2 are each necessary for normal signaling from Bmp6 to hepcidin, that each can influence hepcidin expression independent of the other, and that mechanisms regulating hepcidin expression in response to dietary iron exist that do not require Hfe or Tfr2.

Materials and Methods

Animal Care

Hfe knockout mice[33] and *Tfr2*^{Y245X} mice[34] were bred to uniformity on an FVB background for more than 7 generations. The *Tfr2*^{Y245X} mice have no detectable Tfr2 or truncated form of the protein in hepatocellular membrane preparations and are a functional knockout.[34] These 2 mouse lines were crossed with each other and bred to homozygosity for each mutant allele. Colonies were maintained as homozygotes for each allele individually (hereafter referred to as *Hfe* mice or *Tfr2* mice) and as compound mutant homozygotes (*Hfe/Tfr2* mice). Mice were fed standard chow (Purina 5001, containing 270 ppm iron; Purina Mills, Richmond, IN) ad libitum after weaning at 21 days. Dietary iron loading was achieved by weaning mice onto a diet containing an additional 25,000 ppm of carbonyl iron. At 5 weeks of age, the mice were killed by exposure to hypercarbia followed by exsanguination, and tissues were harvested. To minimize potential variability related to sex, samples from only male mice were used in subsequent studies. Sample sizes unless otherwise indicated in figure legends were as follows: 13 wild-type on a standard diet, 4 wild-type on high iron, 3 *Hfe* knockout, 3 *Hfe* knockout on high iron, 5 *Tfr2* knockout, 3 *Tfr2* knockout on high iron, 5 *Hfe/Tfr2*, and 3 *Hfe/Tfr2* on high iron. The murine studies were performed under protocols approved by the Institutional Animal Care and Use Committee of Saint Louis University and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Liver Iron Content

Liver specimens were homogenized, and a portion was desiccated overnight at room temperature and analyzed for nonheme iron content by the method of Torrance and Bothwell.[35] Data were expressed as micrograms of iron per gram dry weight of liver.

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from mouse liver tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA), with deoxyribonuclease digestion using the RNase-Free DNase Set (Qiagen). Quantitation of murine *Bmp6*, *Hamp1*, *Id1*, and *Rpl19* messenger RNA transcripts was performed using 2-step quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) as previously described.[28] Samples were analyzed in triplicate, and expression levels were normalized to the housekeeping gene *Rpl19*. Additional quantitation of *Bmp6*, *Hamp1*, and *β-actin* mRNA transcripts were performed using One-Step Quantitative Real-Time RT-PCR (TaqMan, Applied Biosystems, Carlsbad, CA ABI7700) and the following probes and primers: Hamp1 forward CCTATCTCCATCAACAGGTG, reverse AACAGATACCACACTGGGAA, and probe 6FAM-CCCTGCTTTCTTCCCCGTGCAAAGT-TAMRA; β-actin forward CCGTGAAAAGATGACCCAGATCATG, reverse TCTTCATGAGGTAGTCCGTCAGGTC, and probe 6FAM-TACGAGGGCTATGCTCTCCCTCACGCT-TAMRA. Hamp1 expression relative to β-actin expression was compared across groups using both the ΔCt method and the method described by Pfaffl et al[36] using REST software (Qiagen). Similar results were obtained using each analytical and real-time PCR method.

Western Blot Analyses

Liver specimens were homogenized in lysis buffer (1× Tris-buffered saline, 0.1% sodium dodecyl sulfate, 10 μL/mL Triton X-100, 1 g/dL sodium deoxycholate, 2 μL/mL EDTA) containing protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail; Thermo Fisher Scientific, Pittsburgh, PA). Western blots of liver lysates for P-Smad1,5,8 protein (relative to total Smad1 protein and to β-actin) and chemiluminescence quantitation were performed as previously described.[28] P-Stat3 was quantified by Western blot using the PhosphoPlus Stat3 Antibody Kit (Cell Signaling, Danvers, MA) per the manufacturer's instructions and normalized to β-actin.

Statistical Analyses

Statistical analyses across multiple groups were performed by analysis of variance (ANOVA) with Dunnett's test comparing each experimental group with each control (wild-type) group or (in separate experiments) by ANOVA with Newman–Keuls test when comparing across each group. For iron loading studies, comparison was made within each genotype between mice on a high-iron diet or standard diet by 2-tailed Student *t* test. $P < .05$ was considered statistically significant.

Results

Elevated Bmp6 *mRNA Expression is Associated With Hepatic Iron Loading in Mice With Loss of Hfe and/or Tfr2*

Functional loss of Hfe or Tfr2 is known to result in inappropriately low hepatic expression of hepcidin and consequent iron overload. Several lines of evidence suggest that up-regulation of Bmp6 contributes to iron-dependent regulation of hepcidin. We measured the hepatic expression of *Bmp6* mRNA in *Hfe*, *Tfr2*, and *Hfe/Tfr2* mice to assess if the decreased hepcidin expression could be attributed to decreased hepatic Bmp6 expression. To provide a comparison group for the degree of hepatic iron loading observed in the murine HH models, wild-type mice were placed on a high-iron (25,000 ppm) diet. As seen in Figure 1, *Hfe*, *Tfr2*, and *Hfe/Tfr2* mice on a standard iron diet had the expected elevated liver iron con-

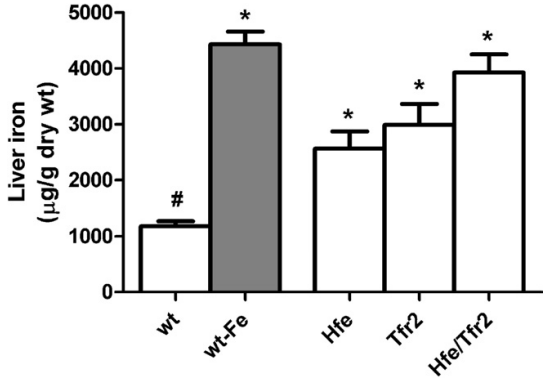

Figure 1. Hepatic iron concentrations. Hepatic nonheme iron concentrations were measured in wild-type (wt) mice and murine models of hemochromatosis (*Hfe*, *Tfr2*, and *Hfe/Tfr2*) on a standard iron diet (270 ppm iron). As an iron-loaded control, wt mice were fed a diet containing 25,000 ppm iron (wt-Fe). ANOVA $P < .0001$. $^{*}P < .05$ compared with wt. $^{\#}P < .05$ compared with wt-Fe.

centrations. Hepatic iron concentrations were highest in the *Hfe/Tfr2* mice although only statistically increased when compared with the *Hfe* mice and wild-type mice. The wild-type mice placed on a high-iron diet had hepatic iron concentrations comparable to the *Hfe/Tfr2* mice and not statistically different from the other mouse HH models.

We compared hepatic *Bmp6* expression in each of the previously described groups. As expected, dietary iron loading of the wild-type mice led to a significant increase in hepatic *Bmp6* mRNA (Figure 2). No increase in *Bmp6* expression in the small intestine was observed with dietary iron loading (Supplementary Figure 1). Hepatic expression of *Bmp6* mRNA in each of the murine HH models was similar to that observed in the iron-loaded wild-type mice and increased over wild-type mice on the standard diet. Although the difference in Bmp6 expression between *Hfe* knockout and wild-type mice on a standard iron diet was not statistically significant in this multi-group analysis, separate analysis of mice from these 2 groups showed a statistically significant increase in *Bmp6* mRNA in the *Hfe* knockout mice ($P < .05$, Student *t* test). This observation is in agreement with observations by other groups.[29,32] These data indicate that hepatic iron loading is associated with increased hepatic *Bmp6* mRNA expression despite the absence of Hfe and/or Tfr2.

Impaired Relationship Between Hepatic Bmp6 and Hepcidin Expression in Mice With Loss of Hfe and/or Tfr2

The observation that Bmp6 is up-regulated in the HH mouse models led us to examine in these mice the relationship between Bmp6 expression and hepcidin expression. *Hamp1* mRNA expression was measured by real-time reverse-transcription PCR (Figure 3*A*) and expressed relative to that in wild-type mice. We observed an absolute decrease in liver *Hamp1* mRNA expression with loss of Hfe or Tfr2 individually as expected. Mice with a combined loss of each protein had the lowest liver hepcidin mRNA content; however, in this multi-group analysis, the difference between the *Hfe/Tfr2* mice and the single *Hfe* or *Tfr2* mutant mice did not achieve statistical significance. We directly compared the *Hfe/Tfr2* mice with mice carrying each mutation individually in a separate set of experiments. We found that indeed the *Tfr2/Hfe* mice had statistically lower hepcidin expression than did mice with only one of these mutations (Supplementary Figure 2). These observations show that Tfr2 and Hfe can influence hepcidin expression independently of each other.

We next expressed *Hamp1* mRNA relative to *Bmp6* mRNA content as a ratio for each of the groups (Figure 3*B*). The relationship between *Bmp6* mRNA and *Hamp1* mRNA was unchanged in the wild-type mice placed on an iron-loading diet. However, each of the HH mouse models showed markedly decreased *Hamp1* mRNA expression relative to *Bmp6* mRNA expression. This effect was most pronounced in the *Hfe/Tfr2* mice. These data show that loss of Hfe and/or Tfr2 negatively affects the relationship between hepatic *Bmp6* mRNA and hepcidin mRNA expression.

To further characterize the requirement for both Hfe and Tfr2 in the signaling between *Bmp6* mRNA and hepcidin, independent analysis of 3 additional *Tfr2*, *Hfe*, and *Hfe/Tfr2* mice was performed. Comparison of *Hamp1*/*Bmp6* mRNA ratios showed a significant decrease ($P < .05$) in the *Hfe/Tfr2* mice compared with the *Hfe* mice alone. Although the *Hamp1*/*Bmp6* mRNA ratio was also decreased in the *Hfe/Tfr2* mice compared with the *Tfr2* mutant mice, this difference was not statistically significant. These observations indicate that Hfe and Tfr2 each contribute to the normal relationship between *Bmp6* mRNA and hepcidin mRNA expression.

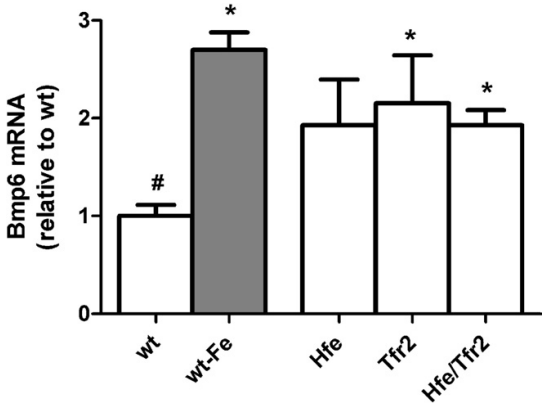

Figure 2. Liver *Bmp6* mRNA expression. *Bmp6* mRNA was quantified by real-time RT-PCR, normalized to *Rpl19* mRNA, and expressed relative to the mean value obtained in wild-type (wt) mice on a standard diet. As an iron-loaded control, wt mice were fed a diet containing 25,000 ppm iron (wt-Fe). ANOVA $P < .001$. $^{*}P < .05$ compared with wt. $^{\#}P < .05$ compared with wt-Fe.

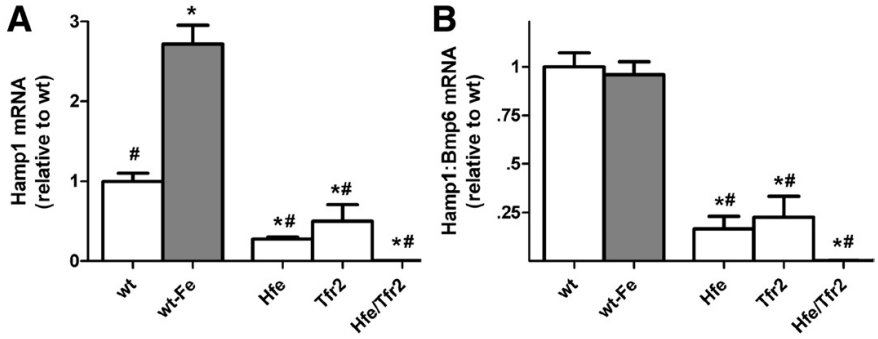

Figure 3. Liver hepcidin mRNA expression. (*A*) Liver *Hamp1* mRNA was quantified by real-time RT-PCR, normalized to *Rpl19* mRNA, and expressed relative to the mean value obtained in wild-type (wt) mice on a standard diet. (*B*) The ratio between *Hamp1* and *Bmp6* mRNA expression was determined individually for each sample and expressed relative to the mean ratio obtained from wt mice on a standard diet. Iron-loaded wt mice (wt-Fe) were fed a diet containing 25,000 ppm iron. ANOVA $P < .0001$. $^{*}P < .05$ compared with wt. $^{\#}P < .05$ compared with wt-Fe.

Attenuated Hepatic P-Smad1,5,8 Levels in Mice With Loss of Hfe and/or Tfr2

Bmp6 induces hepcidin expression via phosphorylation of Smad1,5,8. We compared steady-state levels of P-Smad1,5,8 in wild-type mice on standard and iron-loading diets and observed the anticipated increase in response to iron loading (Figure 4). We then compared levels of P-Smad1,5,8 in each of the murine HH models with the iron-loaded wild-type mice. Although P-Smad1,5,8 levels were not significantly lower in the HH mouse models compared with wild-type mice, they were significantly less that those observed in the iron-loaded wild-type mice. This was the case despite similar increases in *Bmp6* mRNA levels in the HH mouse models and iron-loaded wild-type mice. These data are consistent with an impairment in the relationship of hepatic iron status and *Bmp6* mRNA levels to P-Smad1,5,8 levels in the murine HH models.

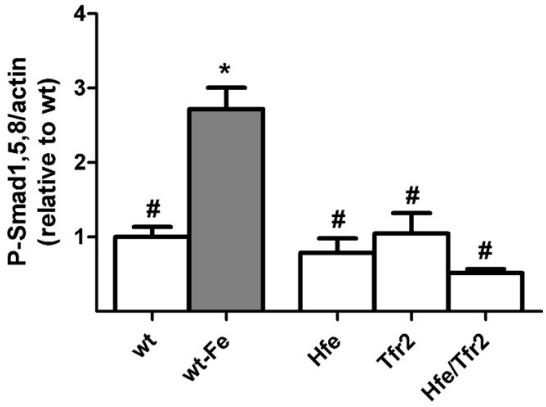

Figure 4. Liver P-Smad1,5,8 levels. P-Smad1,5,8 levels obtained by Western blot analysis were quantified, normalized to the signal obtained for β-actin, and expressed relative to the mean value obtained from wt mice on a standard diet. As an iron-loaded control, wt mice were fed a diet containing 25,000 ppm iron (wt-Fe). Sample sizes: wt = 4, wt-Fe = 4, Hfe = 3, Tfr2 = 3, Hfe/Tfr2 = 5. ANOVA $P < .0001$. $^{*}P < .05$ compared with wt. $^{\#}P < .05$ compared with wt-Fe.

Decreased Expression of Id1 *Relative to* Bmp6 *With Loss of Hfe and/or Tfr2*

We next examined the expression of *Id1*, a Bmp-Smad signaling pathway target transcript that has been shown to be modified by dietary iron loading. Dietary iron loading in wild-type mice was associated with the expected increase in *Id1* mRNA expression (Figure 5*A*). However, in each of the murine HH models, *Id1* mRNA levels were significantly lower than those observed in the iron-loaded wild-type mice. The relationship between *Bmp6* mRNA and *Id1* mRNA levels was examined by comparing their ratios in each of the groups (Figure 5*B*). As seen with hepcidin, the expression of *Id1* mRNA relative to *Bmp6* mRNA was markedly decreased in each of the HH mouse models.

Up-regulation of Hepcidin and Id1 in Mice With Dietary Iron Loading in Mice Despite Loss of Hfe and/or Tfr2

Previous studies have shown that dietary iron is capable of up-regulating hepcidin expression in mice despite loss of Hfe. We performed studies to determine if mice in which *Hfe* and/or *Tfr2* have been disrupted show a response to dietary iron loading with an increase in *Bmp6*, hepcidin, or *Id1* mRNA expression. We found that there was a modest but not statistically significant increase in *Bmp6* mRNA expression in *Hfe* and *Tfr2* mice with dietary iron loading. *Hfe/Tfr2* mice showed no increase in *Bmp6* mRNA (Figure 6*B*). Nonetheless, each of the mutant mouse models, including *Hfe/Tfr2* mice, showed an increase in both hepcidin (Figure 6*C*) and *Id1* (Figure 6*D*) mRNA expression in response to dietary iron loading. Although the increase in hepcidin expression in the *Tfr2* mice with dietary iron in this sampling was not statistically significant, we have observed statistically significant up-regulation of hepcidin in response to dietary iron in other samplings. Interestingly, the increase in

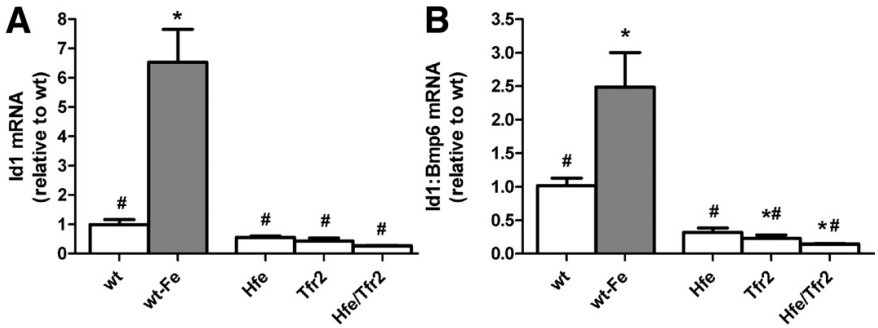

Figure 5. Liver *Id1* mRNA expression. (*A*) Liver *Id1* mRNA was quantified by real-time RT-PCR, normalized to *Rpl19* mRNA, and expressed relative to the mean value obtained in wild-type (wt) mice on a standard diet. (*B*) The ratio between *Id1* and *Bmp6* mRNA expression was determined individually for each sample and expressed relative to the mean ratio obtained from wt mice on a standard diet. Iron-loaded wt mice (wt-Fe) were fed a diet containing 25,000 ppm iron. ANOVA $P < .0001$. $^{*}P < .05$ compared with wt. $^{\#}P < .05$ compared with wt-Fe.

hepcidin and *Id1* mRNA expression in the iron-loaded *Tfr2* and *Hfe/Tfr2* mice was not associated with an increase in P-Smad1,5,8 levels. Indeed, when normalized to β-actin, the P-Smad1,5,8 levels in the iron-loaded *Hfe/Tfr2* mice were statistically lower than observed in these mice on a standard iron diet (Figure 7). Normalization of these data to total Smad1 rather than β-actin resulted in overall similar findings; however, the decrease in P-Smad1,5,8 in the iron-loaded *Hfe/Tfr2* mice was not statistically significant. These observations suggest that in *Hfe/Tfr2* mice, hepcidin up-regulation with dietary iron loading can occur independently of changes in steady-state P-Smad1,5,8 levels. The Jak/STAT pathway mediates an increase in hepcidin expression in response to interleukin-6–medi-

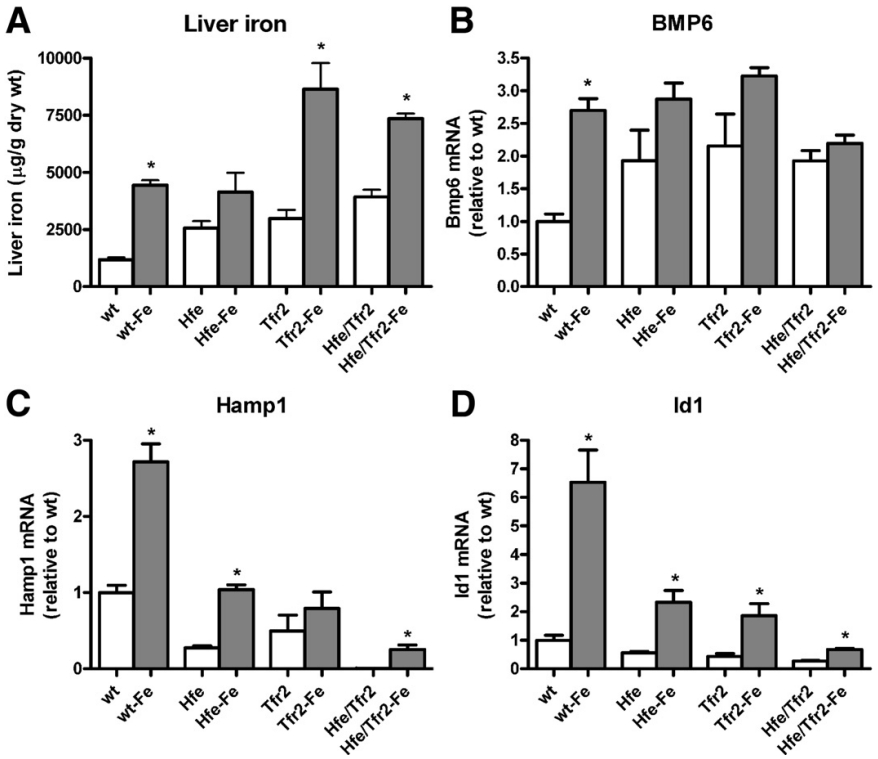

Figure 6. Effect of dietary iron loading in murine HH models. (*A*) Liver nonheme iron levels were measured after 2 weeks on a standard iron or high-iron (Fe) diet. (*B–D*) mRNA levels of the identified gene were quantified by real-time RT-PCR, normalized to *Rpl19* mRNA, and expressed relative to the mean value obtained in wild-type (wt) mice on a standard diet. $^{*}P < .05$ comparing mean values from mice on a standard diet with mice of like genotype on a high-iron diet.

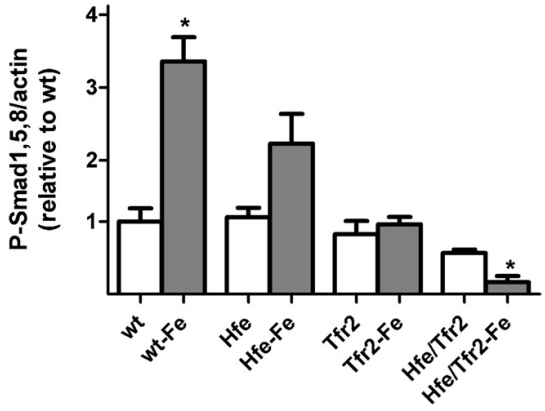

Figure 7. Effect of dietary iron loading on hepatic P-Smad1,5,8 levels in murine HH models. (*A*) Representative Western blot of P-Smad1,5,8 in total liver homogenates from 3 wild-type (wt) mice on a standard diet (*lanes 1–3*), 3 wt mice on a high-iron diet (*lanes 4–6*), 5 *Hfe/Tfr2* mice (*lanes 7–11*), and 3 *Hfe/Tfr2* mice on a high-iron diet (*lanes 12–14*). Blots were also reacted with antibodies to detect total Smad1 protein or β-actin. (*B*) P-Smad1,5,8 levels were quantified by Western blot analyses, normalized to expression of β-actin, and expressed relative to the mean value obtained from the wild-type (wt) mice on a standard iron diet. Sample sizes: wt = 3, Hfe = 3, Hfe-Fe = 3, Tfr2 = 3, Tfr2-Fe = 3, Hfe/Tfr2 = 5, Hfe/Tfr2-Fe = 3. ANOVA $P < .0001$. *$P < .05$ comparing mean values from mice on a standard diet with mice of like genotype on a high-iron diet.

ated inflammatory stimuli. To determine if this pathway was responsible for the increase in hepcidin mRNA in the iron-loaded mutant mice, we measured liver P-Stat3 levels in selected groups by Western blot analyses, with normalization to β-actin. There was no change in liver P-Stat3 levels in the iron-loaded wild-type mice compared with those on a standard iron diet (0.93-fold, $P = .71$). Moreover, there was no change in P-Stat3 expression in the *Hfe/Tfr2* mice on the high-iron diet compared with a standard diet (0.89-fold, $P = .78$).

Discussion

The normal relationship between body iron status and liver hepcidin expression requires the action of several identified genes, including *HFE*, *TFR2*, *HJV*, *BMP6*, and *SMAD4*.[26,37] Mutations in any one of these genes generate the classic HH phenotype, that is, excess dietary iron absorption, elevated serum iron concentrations, hepatocellular iron loading, and macrophage iron sparing.[1,2] TFR2 is the only one of these gene products known to directly interact with an iron-containing protein, that is, its ligand ferritransferrin.[38] As such, TFR2 has been proposed to be an essential component of a hepatocellular iron sensor. Recent observations support a model in which elevated body iron stores normally up-regulate BMP6 expression, leading to increased hepatocellular hepcidin expression via an intracellular pathway that involves SMAD signaling.[37] Although some evidence suggested the possibility that the intestine is the relevant source for iron-regulated Bmp6 expression,[39] our studies (Supplementary Figure 1) and others[40] implicate a hepatic rather than intestinal source.

In agreement with recent reports, our studies support a role for Tfr2 and for Hfe in the regulation of hepcidin downstream of the regulation of *Bmp6* mRNA. It has been proposed that HFE and TFR2 form a complex with the BMP receptor and HJV on the hepatocellular surface,[41] possibly affecting the interaction of BMP6 with its receptor. As another possibility, HFE or TFR2 might influence the function of genes responsible for the relative distribution of hepatocellular HJV. Whereas membrane-associated HJV serves as a coreceptor that augments BMP signaling, soluble HJV (released from the cell surface) serves instead to inhibit BMP signaling.[42] The release of HJV from the hepatocellular surface appears to be mediated at least in part by the serine protease matriptase 2.[43,44] Whether hepatic matriptase 2 activity is influenced by loss of HFE or TFR2 is unknown.

Several lines of evidence suggest that HFE and TFR2 function as a complex[11,14] and that the ability of TFR2 to regulate hepcidin via transferrin requires HFE.[13] Most recently, Gao et al used an adeno-associated viral delivery system to express Hfe or Tfr2 in vivo in the livers of mice in which the complementary gene was disrupted.[45] They found that Tfr2 overexpression had no effect on iron parameters in *Hfe* mice or wild-type mice. Moreover, they found that Hfe overexpression had no effect in *Tfr2* mice but did in wild-type mice. Such observations suggest that Tfr2 is functionally dependent on Hfe in the regulation of hepcidin. The studies we present here examine instead the effects of expression of Tfr2 and Hfe at endogenous levels when compared with mice in which neither is expressed. The *Hfe/Tfr2* mice had lower hepcidin mRNA expression than did mice with loss of either Hfe or Tfr2, consistent with the previously reported severe phenotypic abnormalities in *Hfe/Tfr2* mice[15] and in patients with mutations in both *HFE* and *TFR2* genes.[46] The observation that *Hfe/Tfr2* mice have lower hepatic hepcidin mRNA expression than mice with either gene individually knocked out is in agreement with our studies on serum hepcidin levels.[47] We thus find that Hfe and Tfr2 can each influence hepcidin expression in the absence of the other molecule. Our studies do not examine whether the functional properties of Tfr2 or Hfe might be enhanced by the presence of other molecules or whether they function as a complex when both are present. Indeed, observations by Gao et al in cell culture[13] and in vivo[45] suggest that the level of expression of Hfe might be a limiting factor in the regulation of hepcidin by Tfr2. Nonetheless, our observations indicate that Tfr2 retains functional activity in the regulation of hepcidin in the absence of Hfe. Moreover, Hfe influences liver hepcidin expression without a requirement for Tfr2.

We observe that dietary iron loading increases hepcidin and Id1 expression in each of the murine models tested, although the level of liver hepcidin expression in the *Hfe/Tfr2* mice on an iron-loading diet remains a small fraction of that observed in wild-type mice. The increase

in hepcidin and Id1 expression in the mice without Hfe or Tfr2 occurred without an increase in Bmp6 expression or P-Smad1,5,8 levels. The mechanism(s) by which hepcidin is increased in this mouse model has yet to be elucidated. However, multiple hepcidin signaling pathways have been identified, not each of which would be expected to require a change in P-Smad1,5,8 levels. These additional regulatory pathways include TWSG1,[48] TGF-β,[26] SMAD7,[49] members of the mitogen-activated protein kinase pathway,[15,50] the interleukin-6 receptor–Jak–STAT pathway,[51,52] and interleukin 1.[53] The transcription factor CREB-H mediates an increase in hepcidin expression in response to endoplasmic reticulum stress.[54] Signaling to hepcidin via one or more of these pathways might have been influenced by the very high iron concentrations in the iron-loaded *Hfe/Tfr2* mice. Substantial cross-communication may exist between these hepcidin regulatory signaling pathways and the iron-regulatory BMP/SMAD1,5,8 signaling pathway.

The effects of the HH gene mutations on expression of hepcidin were similar to those observed for Id1. However, they were not identical. For example, the Id1/Bmp6 mRNA ratio of the iron-loaded wild-type mice was higher than the ratio observed in the standard-diet wild-type mice. On the contrary, the hepcidin/Bmp6 mRNA ratios of iron-loaded wild-type mice and standard-diet wild-type mice were not different from each other. These observations suggest that changes in Bmp6 expression have greater influence on Id1 transcription compared with hepcidin, and/or that iron loading increases Id1 transcription by mechanisms in addition to the Bmp/Smad pathway. Supporting these concepts, loss of Hfe and/or Tfr2 had a greater effect on hepcidin expression than on Id1 expression.

In summary, our findings support a model in which Tfr2 and Hfe each modulate the signal between iron status and hepcidin expression via the Bmp/Smad pathway. The effects of these molecules on Bmp signaling occur downstream of the regulation of *Bmp6* mRNA by iron and influence the relative abundance of hepatocellular P-Smad1,5,8. Moreover, mice with combined loss of Hfe and Tfr2 show no increase in hepatic steady-state P-Smad1,5,8 levels with dietary iron loading yet show an increase in hepcidin expression. The mechanism by which iron regulates liver *Bmp6* mRNA expression remains to be elucidated but does not require Hfe or Tfr2.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.06.077.

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Conflicts of interest
The authors disclose no conflicts.

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