Published in final edited form as:
Gastroenterology. 2009 October ; 137(4): 1489–1497. doi:10.1053/j.gastro.2009.06.057.

Bone morphogenetic protein signaling is impaired in an *Hfe* knockout mouse model of hemochromatosis

Elena Corradini[1,2], **Cinzia Garuti**[2], **Giuliana Montosi**[2], **Paolo Ventura**[2], **Billy Andriopoulos Jr.**[1], **Herbert Y. Lin**[1], **Antonello Pietrangelo**[2,*], and **Jodie L. Babitt**[1,*]

[1]Program in Membrane Biology, Division of Nephrology, Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA

[2] Center for Hemochromatosis, University Hospital of Modena and Reggio Emilia, Modena Italy

Abstract

Background and Aims—Mutations in *HFE* are the most common cause of the iron-overload disorder hereditary hemochromatosis (HH). Levels of the main iron regulatory hormone, hepcidin, are inappropriately low in HH mouse models and patients with *HFE* mutations, indicating that HFE regulates hepcidin. The bone morphogenetic protein 6 (BMP6)-SMAD signaling pathway is an important endogenous regulator of hepcidin expression. We investigated whether HFE is involved in BMP6-SMAD regulation of hepcidin expression.

Methods—The BMP6-SMAD pathway was examined in *Hfe* knockout (KO) mice and in wild-type (WT) mice as controls. Mice were placed on diets of varying iron content. Hepcidin induction by BMP6 was examined in primary hepatocytes from *Hfe* KO mice; data were compared with those of WT mice.

Results—Liver levels of *Bmp6* mRNA were higher in *Hfe* KO mice; these were appropriate for the increased hepatic levels of iron in these mice, compared with WT mice. However, levels of hepatic phosphorylated Smad 1/5/8 protein (an intracellular mediator of Bmp6 signaling) and *Id1* mRNA (a target gene of Bmp6) were inappropriately low for the body iron burden and *Bmp6* mRNA levels in *Hfe* KO, compared with WT mice. BMP6 induction of hepcidin expression was reduced in *Hfe* KO hepatocytes compared with WT hepatocytes.

Conclusions—HFE is not involved in regulation of BMP6 by iron, but does regulate the downstream signals of BMP6 that are triggered by iron.

* These authors contributed equally. Address correspondence to: Jodie L. Babitt, Program in Membrane Biology, Division of Nephrology, Center for Systems Biology, Massachusetts General Hospital, 185 Cambridge St., CPZN-8218, Boston, MA 02114, babitt.jodie@mgh.harvard.edu, tel (617)-643-3181, fax (617)-643-3182; or Antonello Pietrangelo, MD, PhD, Department of Internal Medicine, Center for Hemochromatosis, University Hospital of Modena, Policlinico, Via del Pozzo 71, 41100 Modena, Italy. E-mail: antonello.pietrangelo@unimore.it, tel: 059-4224356, fax:059-4223041.

Disclosures: None

Author Contributions: E.C., C.G., G.M., A.P., and J.L.B. designed the experiments. E.C., C.G., G.M., and B.A.J. performed the experiments. E.C., C.G., G.M., H.Y.L., A.P., and J.L.B. analyzed and interpreted the data, and P.V. performed the statistical analysis. E.C. and J.L.B. wrote the manuscript. C.G., G.M., P.V., H.Y.L., and A.P. edited the manuscript. A.P. and J.L.B. conceived and oversaw the entire project.

Introduction

Hereditary hemochromatosis (HH) is a genetically heterogeneous hereditary disorder of iron metabolism caused by a failure to prevent unneeded iron from entering the circulatory pool and characterized by progressive iron overload with potential for multi-organ damage and disease[1]. A common polymorphism in *HFE* is associated with the most frequent and first characterized form of HH, affecting approximately five per one thousand individuals of northern European descent[2]. Mouse models with disruptions of the *Hfe* gene have a similar iron overload phenotype[3,4,5].

Although the discovery of the *HFE* gene[6] dates back more than 10 years, the pathophysiology of HFE-HH is still not fully understood. *HFE* encodes an atypical class I major histocompatibility complex molecule that requires β2 microglobulin for appropriate localization to the cell surface[6-8]. HFE binds to transferrin receptor 1 (TfR1) [9-11] at a site that overlaps with the transferrin binding site[12-15]. HFE also binds to the liver-specific homologue transferrin receptor 2 (TfR2)[16,17]. Selective disruption of the *Hfe* gene in the liver recapitulates the phenotype of *Hfe* KO mice, suggesting that the liver is the predominant organ for HFE action in the regulation of iron metabolism[18]. Notably, both mouse models and human patients with *HFE* mutations have inappropriately low expression of the key iron regulatory hormone hepcidin[19-23], which is produced in the liver[24-26]. Hepcidin downregulates the iron exporter ferroportin on the surface of duodenal enterocytes, macrophages, and hepatocytes, thereby inhibiting iron release into the bloodstream[27]. It is now widely accepted that impaired regulation of hepcidin expression by HFE plays a central role in the pathogenesis of HFE-HH. It has recently been postulated that TfR1 in the liver serves to sequester HFE, and that HFE is displaced from TfR1 by holotransferrin[28]. This displaced HFE is then able to generate a signal to upregulate hepcidin via an interaction with TfR2 and transferrin[16,28,29]. However the precise molecular mechanism by which HFE affects hepcidin expression is still unknown.

A severe juvenile form of HH is caused by mutations in the gene encoding hemojuvelin (*HFE2*) [30]. We have recently discovered that hemojuvelin is a co-receptor for the bone morphogenetic protein (BMP) signaling pathway[31] and that the BMP signaling function of hemojuvelin is important for its role in iron metabolism[31,32]. Members of Tranforming Growth Factor-β (TGF-β) family, BMPs exert their effects by binding 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 common mediator SMAD4, and the SMAD complex translocates to the nucleus to affect transcription of target genes such as *ID1*[33-35]. We and others have shown that *HAMP* (encoding hepcidin) is also a target gene that is upregulated by BMP signals at the transcriptional level *in vitro*[31-32, 36-39]. In mice, BMP ligands increase hepatic *Hamp1* mRNA expression and reduce serum iron, while BMP inhibitors reduce hepatic *Hamp1* mRNA expression, mobilize reticuloendothelial cell iron stores, and increase serum iron[32,40-41]. Impaired hepatic BMP signaling, through mutations in genes encoding the BMP co-receptor hemojuvelin[42-43], the common BMP/TGF-β intracellular mediator Smad4[36] or the ligand Bmp6[41,44], leads to low hepcidin levels and iron overload in mouse models. Recent evidence also suggests that BMP signaling in the liver is modulated by iron[40,45]. Parenteral iron increases hepatic Smad1/5/8 phosphorylation within 1 hour[40], and longterm changes in dietary iron modulate hepatic *Bmp6* mRNA expression in mice concordantly with *Id1* and *Hamp1* mRNA[45]. Collectively these data indicate that the BMP-SMAD signaling is a main regulatory pathway acting in iron metabolism.

Here, we dissect the BMP-SMAD signaling pathway in an *Hfe* KO mouse model to determine whether HFE regulation of hepcidin expression involves the BMP-SMAD signaling pathway. Our data show that hepatic *Bmp6* mRNA expression is appropriately regulated by dietary iron

content and tissue iron burden in *Hfe* KO mice. However, HFE appears to be involved in the downstream signals of BMP6 that are triggered by iron.

Materials and methods

Animals

WT 129S6/SvEvTac mice and *Hfe* KO mice bred on a 129S6/SvEvTac background[4], kindly provided by Dr. Nancy C. Andrews, (Duke University Medical Center, received humane care under approved experimental protocols according to University Hospital of Modena guidelines. Six *Hfe* KO (3 males, 3 females) and six WT (2 males, 4 females) mice were maintained on a standard rodent diet containing 330 ppm iron (2018, Harlan, Teklad) until 12 weeks of age (Control diet, CTR). Dietary iron loading was achieved by feeding six 8 week-old *Hfe* KO and six WT mice (3 males, 3 females each) the same standard diet supplemented with 2% carbonyl iron for 4 weeks (Iron-enriched diet, IR). Six 7 week-old *Hfe* KO and six WT mice (4 males, 2 females each) were fed with a diet with negligible iron content (customized diet, Harlan, Teklad) for 5 weeks (Iron-deficient diet, ID). All mice were sacrificed at 12 weeks of age and blood and livers were harvested for analysis.

Hematological and iron parameters

Blood was obtained by retroorbital puncture. Serum iron and total iron binding capacity (TIBC) were determined using a kit (Pointe Scientific Inc.) according to the manufacturer's instructions. Transferrin saturation (Tf Sat) was calculated as (serum iron ÷ TIBC) × 100%. Red blood cells, hemoglobin and hematocrit were determined using an automated cell counter at the clinical-chemical laboratory of the University Hospital of Modena.

Tissue Iron Content

Tissue specimens were analysed for non-heme iron content by the method of Torrance and Bothwell[46]. Liver iron concentration (LIC) for each animal was the mean of measurements performed on two different tissue specimens from each liver.

Quantitative real-time RT-PCR

Total RNA was isolated from mouse liver tissue using the RNeasy Mini Kit (QIAgen), with DNAse digestion with the RNase-Free DNase Set (QIAgen) or TRIzol reagent (Invitrogen) according to the manufacturers' instructions as previously described[41,47]. Real time quantification of *Bmp6*, *Bmp4*, *Bmp2*, *Id1*, *Hamp1*, *Rpl19*, *and Gapdh* mRNA transcripts was performed using two-step quantitative real-time RT-PCR as previously described[41,47]. Samples were analyzed in duplicate or triplicate and expression levels were normalized to the housekeeping genes *Rpl19 or Gapdh*.

Western blot

Livers were disrupted with a manual homogenizer in lysis buffer (1× TBS, 0.1% SDS, 10 μl/ml Triton x-100, 1g/100 ml Na Deoxycholate, 2 μl/ml EDTA) containing protease inhibitors (Complete Mini, Roche Diagnostic) and phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail, Thermo Scientific). Western blot of liver lysates for P-Smad1/5/8 relative to total Smad1 protein and chemiluminescence quantitation was performed as previously described[32].

Primary hepatocyte cultures and BMP6 treatment

Primary hepatocytes from 8-12 week-old male 129S6/SvEvTac WT and *Hfe* KO mice maintained on a standard rodent diet (300 ppm iron, Harlan Teklad) were isolated as previously described[47] with the following modifications. Hepatocytes were plated on plastic multiwell

plates at a density of 300,000-400,000 cells/ml in DMEM (GIBCO_BRL Life Technologies) supplemented with 100 IU/ml penicillin, 30 mg/ml streptomycin, and 10% fetal calf serum (FCS). Twenty-four hours after isolation, hepatocytes were serum starved for 6 hours in DMEM with 1% FCS, followed by stimulation with 0.1 to 2.0 ng/ml of BMP6 (R&D systems) in DMEM with 1% FCS for 16 hours.

Statistics

All data were controlled for normal distribution (Kolgomorov-Smirnov's and Shapiro-Wilk's tests). In cases of skewed distribution, log-transformed data were used. Two-tailed independent Student's t-test was used to compare normalized mean values of considered variables between strains under the same diet regimen. Two-way ANOVA (with Holm-Sidak post-hoc test for pair wise multiple comparisons) was used to compare, between and within considered groups, the mean levels of the considered variables as a function of diet, strain and the interaction between these two factors. One-way ANOVA (with LSD post-hoc test for pair wise multiple comparisons) was used to compare mean values for the BMP6 dose-response curves in primary hepatocyte cultures. The Pearson's r coefficient was used to assess the correlation between continuous variables, and z-statistic to compare r coefficients. For small sample sizes, Spearman's r coefficient was also calculated to confirm the results. In all statistical analyses, $P < .05$ was considered significant. All analyses were conducted using SPSS® v.17.0 (SPSS inc., Chicago,Illinois) and SigmaStat® v.3.5 (Systat Software Inc., Richmond, California) statistical software.

Results

Hepatic *Bmp6* mRNA is increased, while P-Smad1/5/8 protein and *Id1* mRNA are not increased, in *Hfe* KO mice compared with WT mice on a standard diet

To examine the role of HFE in the BMP-SMAD signaling pathway, we analyzed 12 week-old *Hfe* KO mice on a 129/SvEvTac background compared with matched WT mice maintained on a standard diet for blood and tissue iron parameters, hepatic *Bmp6* mRNA, P-Smad1/5/8 protein, *Id1* mRNA, and hepcidin (*Hamp1*) mRNA expression (Fig.1). *Hfe* KO animals manifested a well-known iron overload phenotype[3,4,5] with increased liver iron concentration (LIC), serum iron, and transferrin saturation (Tf Sat) compared with WT controls (Fig.1, bottom). Hepatic *Bmp6* relative to *Rpl19* mRNA levels were significantly higher in *Hfe* KO compared to WT mice (black bars). This is consistent with a recent paper showing that hepatic *Bmp6* mRNA expression correlates with dietary iron content and tissue iron burden in mice with C57BL/6 and DBA/2 backgrounds[45]. Similar to the previous study[45], we did not find changes in hepatic *Bmp2* and *Bmp4* mRNA levels in *Hfe* KO mice despite significant iron overload (data not shown). In contrast to the findings for *Bmp6* mRNA, hepatic P-Smad1/5/8 (the intracellular mediator of BMP6 signaling) and *Id1* mRNA (a target gene upregulated by BMP6) were not increased in *Hfe* KO compared with WT mice (Fig.1, gray and white bars). Consistent with previous studies [19-22], *Hamp1* mRNA levels were lower in *Hfe* KO compared with WT mice, and inappropriately low for LIC (Fig.1, striped bars).

Hfe KO mice have equivalent hepatic *Bmp6* mRNA expression, but lower P-Smad1/5/8 protein and *Id1* mRNA expression, compared with WT mice with equivalent iron burden

To further examine the modulation of the BMP6-SMAD pathway by iron and to compare animals with similar body iron burden, we fed *Hfe* KO mice and matched WT mice an iron-deficient (ID) diet, a control diet, (CTR) or an iron-enriched diet (IR) for 4 or 5 weeks ($N = 6$ per group). WT mice on an ID diet had lower mean cellular volume (54.6 +/- 0.63 versus 59.8 +/- 1.78, $P = .0004$) and a trend toward lower hematocrit (47.75 +/- 4.3 versus 52.43 +/- 2.4, $P = .05$) compared to WT mice on a CTR diet, but no other significant differences in erythroid parameters were found among animals receiving different diets (data not shown). Dietary iron

caused a dose-dependent increase in serum iron in WT mice, but was less effective at modulating serum iron parameters in *Hfe* KO mice, where iron burden was already high (Fig. 2, bottom). Dietary iron also significantly modulated LIC in both WT and *Hfe* KO mice (Fig. 2) Notably, WT and *Hfe* KO mice on an IR diet had equivalent LIC and serum iron parameters (Fig. 2, black bars). We therefore examined the BMP6-SMAD signaling pathway in WT and *Hfe* KO mice on an IR diet to compare mice with a similar body iron burden.

Hepatic *Bmp6* mRNA showed a trend toward higher levels in *Hfe* KO compared with WT mice maintained on IR diet (Fig.3, black bars). This is consistent with the non-significant trend toward increased LIC in the *Hfe* KO mice. In contrast, hepatic P-Smad1/5/8 protein and *Id1* mRNA levels (Fig.3, gray and white bars) were significantly lower in *Hfe* KO compared with WT mice on an IR diet. As expected, *Hamp1* mRNA expression was significantly lower in *Hfe* KO compared with WT mice on an IR diet (Fig.3, striped bars).

Hepatic *Bmp6* mRNA expression is appropriately upregulated by iron in *Hfe* KO mice

To further understand the regulation of the BMP6-SMAD pathway by iron in *Hfe* KO mice, we compared hepatic *Bmp6* mRNA, P-Smad1/5/8 protein, *Id1* mRNA, and *Hamp1* mRNA expression in *Hfe* KO mice maintained on ID, CTR, and IR diets. The same parameters were examined in WT mice as a control. In *Hfe* KO mice, hepatic *Bmp6* mRNA levels were significantly modulated by dietary iron, and were regulated concordantly with LIC in a manner similar to WT mice (compare Fig. 4A to Fig. 2). To examine whether the increase in *Bmp6* mRNA levels in *Hfe* KO mice was appropriate for iron burden, we compared the ratio of *Bmp6* mRNA levels to LIC. *Bmp6* mRNA levels relative to LIC in *Hfe* KO mice were not significantly different from those in WT mice across all diets (Fig.4B). The Pearson's chi-square test showed a significant correlation between *Bmp6* mRNA levels and LIC in the overall animal population ($r = 0.726$, $P = .0001$). This correlation was maintained after separate analysis in WT ($r = 0.726$, $P = .001$) and *Hfe* KO mice ($r = 0.730$, $P = .001$), and was not significantly different between the two genotypes ($z=-0.0233$, $P = .9814$). Due to the small sample size, nonparametric statistics were also used, with similar results. These data suggest that hepatic *Bmp6* mRNA correlates with LIC and is appropriately increased relative to body iron burden in *Hfe* KO mice.

Intracellular mediators and targets of BMP6 signaling in the liver are not appropriately increased relative to iron burden and *Bmp6* mRNA expression in *Hfe* KO mice

Dietary iron also significantly modulated hepatic P-Smad 1/5/8 protein (Fig. 5A-B), *Id1* mRNA (Fig. 6A), and *Hamp1* mRNA expression (Fig. 7A) in *Hfe* KO mice. The latter results are consistent with a prior study showing that hepcidin levels can be modulated by dietary iron in *Hfe* KO mice[48]. However, the levels of P-Smad 1/5/8 protein relative to body iron burden (LIC) were significantly reduced by ~30-40% in *Hfe* KO compared with WT mice across all diets (Fig. 5C), and there was a similar trend towards reduced P-Smad1/5/8 protein relative to *Bmp6* mRNA in Hfe KO versus WT mice that reached significance for the IR diet group (Fig. 5D). *Id1* mRNA expression relative to LIC or to *Bmp6* mRNA expression were even more significantly reduced in *Hfe* KO compared with WT mice by ~65% across all diets (Fig.6B and C). *Hamp1* mRNA expression relative to LIC and *Bmp6* mRNA levels reinforced the well-known inappropriately low hepcidin levels in *Hfe* KO mice (Fig.7B and C). These results suggest that although hepatic P-Smad1/5/8 protein, *Id1* mRNA, and *Hamp1* mRNA can be regulated by dietary iron, they are all inappropriately low relative to iron burden and hepatic *Bmp6* mRNA levels in *Hfe* KO mice.

BMP6 induction of hepcidin expression is impaired in *Hfe* KO mouse hepatocytes

To further confirm whether HFE plays a role in hepcidin induction by BMP6, we tested the ability of BMP6 to induce hepcidin expression in primary hepatocyte cultures from *Hfe* KO

versus WT mice. Basal *Hamp1* mRNA expression was ~40% lower in *Hfe* KO compared with WT hepatocytes, consistent with prior studies[37]. Low doses of BMP6 ranging from 0.5 to 2 ng/mL significantly increased *Hamp1* mRNA expression up to 2-fold in WT primary hepatocyte cultures (Fig.8, closed rhombus). However, *Hamp1* mRNA induction by BMP6 was significantly lower in *Hfe* KO hepatocytes (Fig.8, open squares) compared with WT hepatocytes treated with identical BMP6 concentrations.

Discussion

Mutations in the gene encoding *HFE* are the most common cause of HH[2]. Recent data suggest that one mechanism by which *HFE* mutations cause HH is by causing inappropriately low expression of the key iron regulatory hormone hepcidin[18-23]. However, the mechanisms by which HFE affects hepcidin expression are still unknown. We and others have recently shown that BMP-SMAD signaling is a main regulatory pathway acting in hepcidin regulation and iron metabolism[31-32, 36-41, 44-45]. We therefore investigated whether HFE was involved in the BMP-SMAD signaling pathway.

In the present work we show that *Hfe* KO mice have higher hepatic *Bmp6* mRNA expression than WT mice. Furthermore, we show that dietary iron modulates hepatic *Bmp6* mRNA levels in both WT and *Hfe* KO mice in an equivalent manner. Similar modulation of hepatic *Bmp6* mRNA by dietary iron content, and a similar correlation between hepatic *Bmp6* mRNA levels and liver iron content was seen previously in two other mouse strains[45]. These data suggest that the increased hepatic *Bmp6* mRNA in *Hfe* KO mice is appropriate for the increased iron burden, and that HFE is not necessary for regulation of *Bmp6* mRNA levels in response to iron.

We did not find any changes in hepatic *Bmp2* and *Bmp4* mRNA levels in *Hfe* KO compared with WT mice, despite significant iron overload. We also did not find any changes in hepatic *Bmp2* and *Bmp4* mRNA levels in *Hfe* KO or WT mice maintained on an iron enriched diet compared with their own control diet groups. These data are in agreement with a prior study showing that hepatic *Bmp2* mRNA was increased only under extreme iron overload conditions and *Bmp4* mRNA was not altered by dietary iron in two other mouse strains[45]. Together with the recent finding that BMP6 is a ligand for the BMP co-receptor hemojuvelin, and that *Bmp6* null mice have an iron overload phenotype that resembles juvenile hemochromatosis due to hemojuvelin mutations[41,44], these data reinforce the idea of the preeminent role of endogenous BMP6 in iron metabolism *in vivo*.

In contrast to the findings for *Bmp6* mRNA expression, hepatic P-Smad 1/5/8 protein and *Id1* mRNA levels are the same in *Hfe* KO animals and WT animals on a standard iron diet, and therefore inappropriately low relative to the higher body iron and *Bmp6* mRNA levels in the *Hfe* KO mice. Indeed, when we examined WT versus *Hfe* KO mice with comparable *Bmp6* mRNA expression, serum iron, transferrin saturation and LIC, that is WT mice on an IR diet versus *Hfe* KO on an IR diet, we found that *Hfe* KO mice had significantly lower hepatic P-Smad1/5/8 protein and *Id1* mRNA levels compared with WT mice. Furthermore, we found that across all dietary iron groups, *Hfe* KO mice had significantly lower hepatic P-Smad1/5/8 protein and *Id1* mRNA levels relative to LIC, significantly lower *Id1* relative to *Bmp6* mRNA, and a trend toward lower P-Smad1/5/8 protein relative to *Bmp6* mRNA that reached statistical significance for the IR diet, compared with WT mice. These data suggest that the downstream signals of BMP6 triggered by iron are impaired in *Hfe* KO mice. The impairment in *Hfe* KO mice was more pronounced for *Id1* mRNA compared with P-Smad1/5/8 expression, and even more pronounced for *Hamp1* mRNA expression. These findings can be explained in several ways. First, the kinetics of SMAD phosphorylation in response to BMP ligand is much faster and more transient compared with the regulation of downstream mRNAs. It may therefore be more difficult to capture the transient SMAD phosphorylation state when using a model looking

at the effects of chronic changes in dietary iron levels and iron burden. Second, there may be a "cascade effect" through the BMP-SMAD signaling pathway that is able to amplify the impairment step-by-step. In support of one or both of these hypotheses, the modulation of P-Smad1/5/8 by iron in WT mice was less pronounced than the modulation of *Id1* mRNA and *Hamp1* mRNA. Another possibility is that HFE may affect the BMP signaling pathway downstream of SMAD phosphorylation. Finally, it is possible that HFE also affects hepcidin expression through an additional non-BMP-SMAD pathway.

The involvement of HFE in BMP6-mediated induction of hepcidin expression is further supported by the relative resistance of *Hfe* KO primary hepatocyte cultures to *Hamp1* mRNA induction by BMP6 ligand. It is unlikely that the iron loaded state of these *Hfe* KO hepatocytes per se impairs BMP-SMAD signaling because WT mice with higher LIC due to dietary iron loading had increased hepatic P-Smad1/5/8 protein and *Id1* mRNA levels appropriate for their increased *Bmp6* mRNA levels. Interestingly, a prior study showed that *Hfe* KO primary mouse hepatocytes exhibited equivalent increases in *Hamp* mRNA expression in response to BMP2, BMP4, and BMP9 ligands compared with WT hepatocytes[37]. One explanation for these findings is that we used relatively low doses of BMP6 ligand, while Truska *et al*.[37] used relatively high doses of BMP ligands. We hypothesize that low doses of endogenous BMP6 ligand are expressed under normal physiologic conditions, and that HFE is important for the downstream signals to BMP6 under these physiologic conditions. Under nonphysiologic conditions of high levels of exogenous BMP ligand, the large excess of BMP ligand can bypass the role of HFE in optimizing the BMP6 response via BMP type I and type II receptors and the co-receptor hemojuvelin. Additionally, the role of HFE in regulating downstream BMP signals might specific to BMP6 ligand. Further experiments will be needed to determine the ligand specificity of HFE involvement in the BMP pathway.

In summary, we propose the following model for the role of HFE in iron metabolism. Our data, in conjunction with others'[28,45], suggest that iron sensing occurs at multiple levels. First, chronic iron loading increases hepatic *Bmp6* mRNA. The mechanism for this upregulation remains to be determined, however, it does not appear to require HFE. Second, recent work suggests that HFE may participate in the sensing of increased serum iron levels by becoming displaced from TfR1 by holotransferrin and by interacting with a TfR2/transferrin complex, which then acts to increase hepcidin expression[28-29]. We have previously shown that BMP6-mediated signaling by the co-receptor hemojuvelin in complex with BMP type I and type II receptors causes phosphorylation of SMAD proteins, which in turn increases transcription of hepcidin mRNA[31-32,39, 49]. Based on our data here, we propose that HFE increases hepcidin expression through an interaction with the BMP6/hemojuvelin/SMAD signaling pathway. Future studies will be needed to determine the precise molecular mechanisms by which HFE affects downstream BMP6 signals in hepatocytes and whether TfR2 plays a role in this process.

Acknowledgments

Grant support and acknowledgements: AP and EC were supported in part by EU (EUROIRON Ct.2006-037296) and the Italian Ministry of University and Research (MIUR-2006) grants. HYL was supported in part by NIH grants RO1 DK-069533 and RO1 DK-071837. JLB was supported in part by NIH grant K08 DK-075846 and by a Claflin Distinguished Scholar Award from the Massachusetts General Hospital.

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Abbreviations

HH
: hereditary hemochromatosis

BMP
: bone morphogenetic protein

KO
: knockout

WT
: wild-type

TfR1
: Transferrin receptor 1

TfR2
: Transferrin receptor 2

P-Smad1/5/8
: phosphorylated Smad1/5/8

CTR
: control

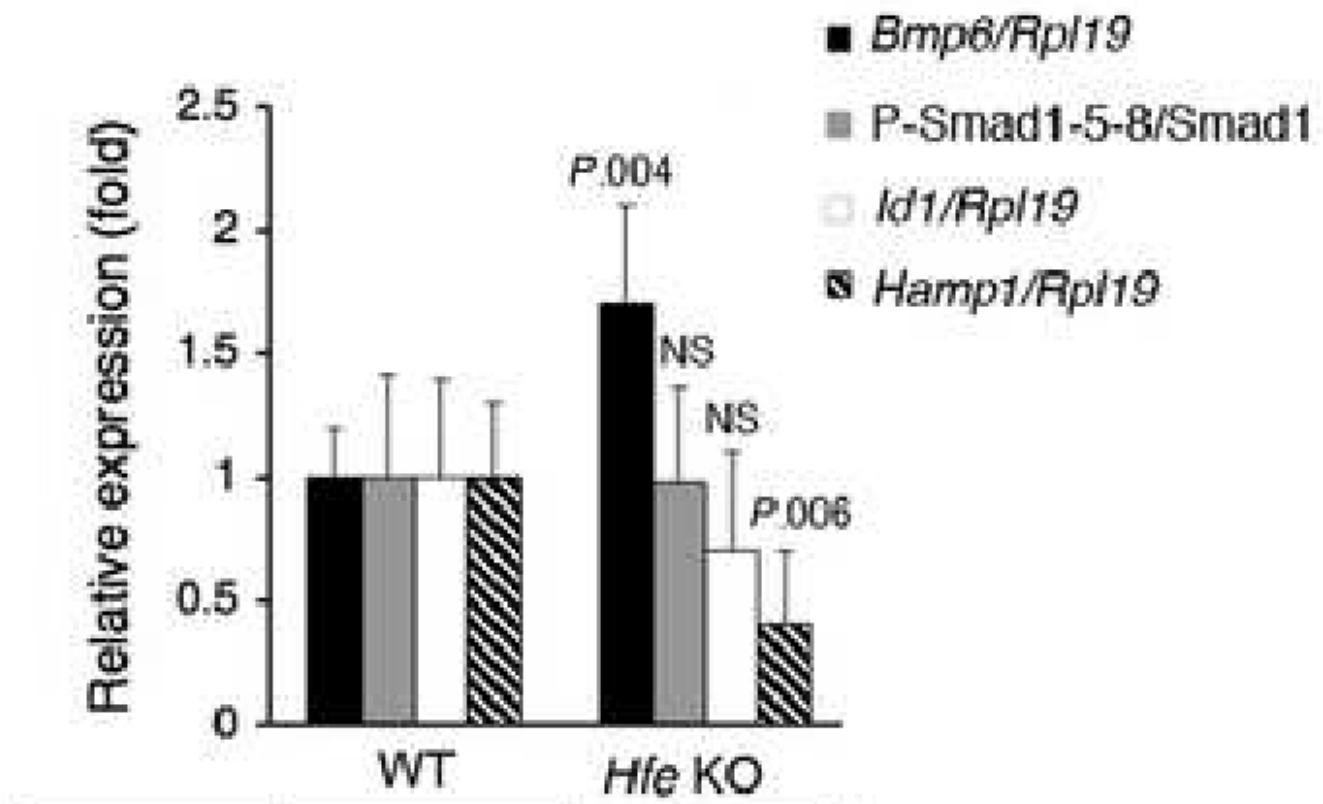
IR
: iron-enriched

ID
: iron-deficient

TIBC
: total iron binding capacity

Tf Sat
: transferrin saturation

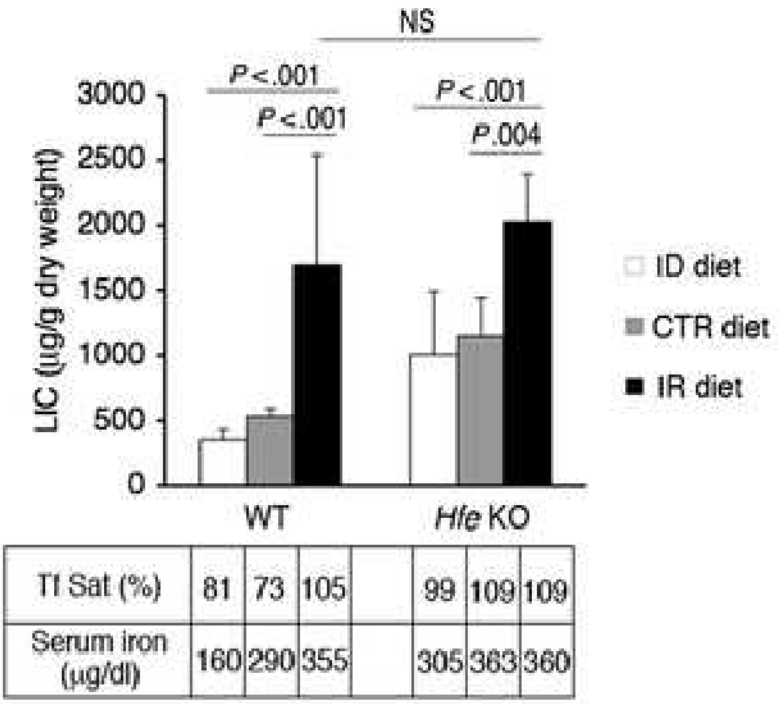
LIC
: liver iron concentration



Serum iron (μg/dL)	290	363
Tf Sat (%)	73	109
LIC (μg/g)	535	1147

Figure 1. Liver iron concentration and *Bmp6* mRNA levels are increased, phosphorylated Smad1/5/8 protein and *Id1* mRNA are unchanged, and *Hamp1* mRNA expression is decreased in *Hfe* KO versus WT mice on a standard diet

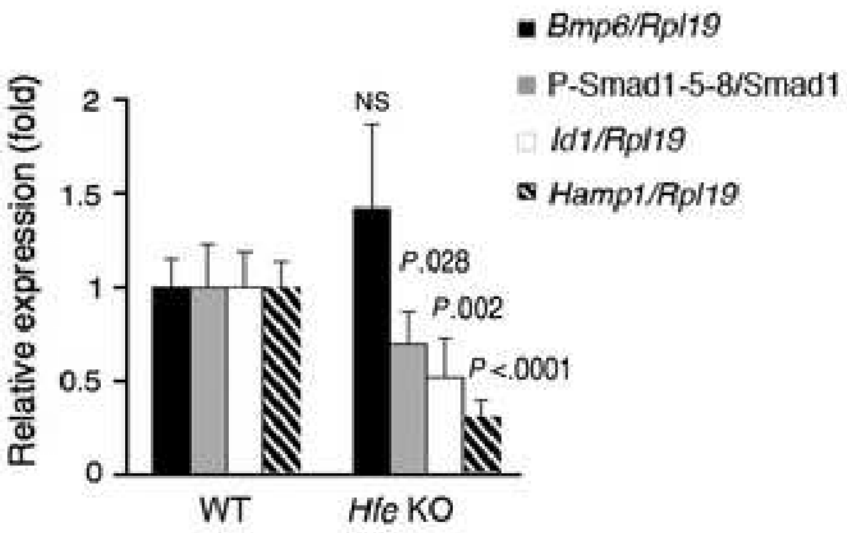
Twelve-week-old *Hfe* KO mice (*N* = 6) and WT control mice (*N* = *6*) were analyzed for hepatic *Bmp6, Id1* and *Hamp1* relative to *Rpl19* mRNA expression by quantitative real-time RT-PCR, and for P-Smad1/5/8 relative to total Smad1 protein expression by Western blot quantified using IPLab Spectrum software. Results are reported as the mean ± s.d for the fold change from WT mice. Exact *P* values determined by 2-tailed independent Student's t-test are shown for the comparisons between *Hfe* KO mice and WT mice. Serum iron, serum Tf Sat, and LIC were also determined. Results from pooled samples are shown for serum iron and Tf Sat. Mean values are shown for LIC.



Tf Sat (%)	81	73	105		99	109	109
Serum iron (µg/dl)	160	290	355		305	383	360

Fig.2. Dietary iron modulates the iron burden of WT and *Hfe* KO mice

Seven to eight week-old *Hfe* KO mice and WT control mice were maintained on an ID, CTR, or IR diet for 4 to 5 weeks ($N = 6$ per group). Mice were analyzed for LIC, serum Tf Sat, and serum iron. Results for serum Tf Sat and serum iron are from pooled samples. LIC is expressed as the mean ± s.d. Exact *P* values determined by 2-way ANOVA (or NS if not significant) are shown.



Serum iron (μg/dL)	355	360
Tf Sat (%)	105	109
LIC (μg/g)	1694	2029

Fig.3. *Hfe* KO mice have adequate hepatic *Bmp6* mRNA expression, but lower P-Smad1/5/8 protein, *Id1* mRNA, and *Hamp1* mRNA expression compared to WT mice with equivalent iron burden

Eight week-old *Hfe* KO mice and WT control mice were maintained on an IR diet for four weeks (*N* = 6 mice per group). Mice were analyzed for *Bmp6, Id1* and *Hamp1* relative to *Rpl19* mRNA expression, and for P-Smad1/5/8 relative to total Smad1 protein expression as in Figure 1. Results are reported as the mean ± s.d for the fold change from WT mice. Exact *P* values determined by 2-tailed independent Student's t-test are shown for the comparisons between *Hfe* KO mice and WT mice.

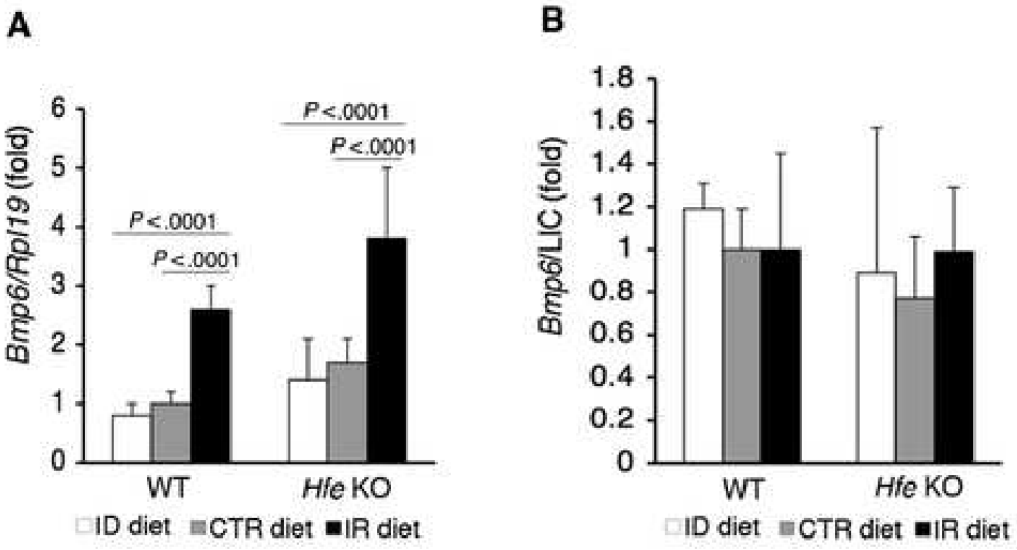


Fig.4. Dietary iron appropriately upregulates hepatic *Bmp6* mRNA expression in *Hfe* KO mice

A) WT and *Hfe* KO mice on an ID, CTR, or IR diet from Figure 2 ($N = 6$ per group) were analyzed for *Bmp6* relative to *Rpl19* mRNA expression as in Figure 1. B) Ratio of *Bmp6* mRNA from panel A relative to LIC. Results are expressed as the mean ± s.d for the fold change from WT mice on a CTR diet. Exact *P* values determined by 2-way ANOVA are shown. For panel B, *P* values were not significant for comparisons among all groups.

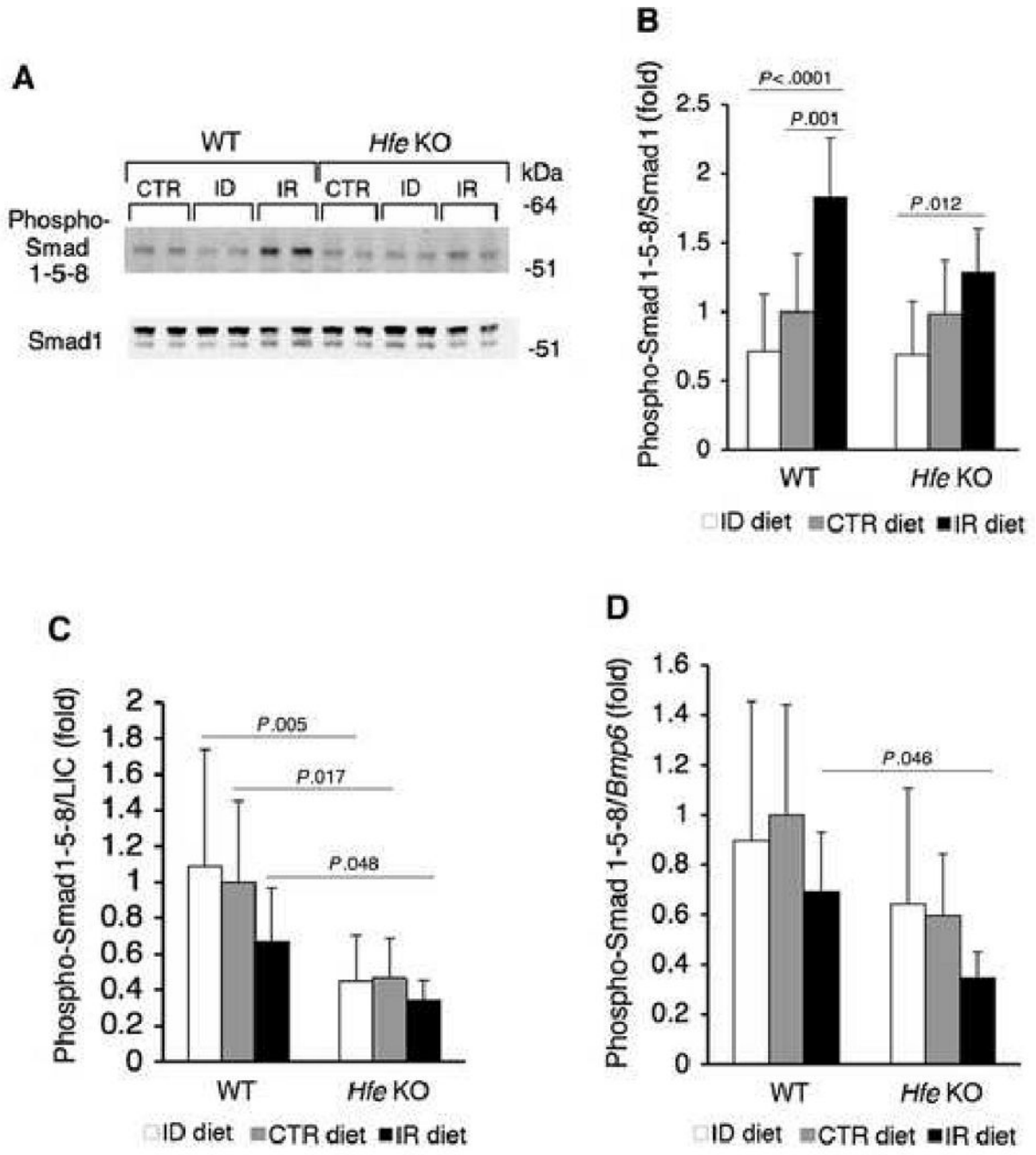


Fig.5. In *Hfe* KO mice, dietary iron modulates hepatic P-Smad1/5/8 protein expression, but P-Smad1/5/8 is not appropriately increased relative to iron burden compared with WT mice

A-B) WT and *Hfe* KO mice on an ID, CTR, or IR diet from Figure 2 ($N = 6$ per group) were analyzed for hepatic P-Smad1/5/8 relative to total Smad1 expression by Western blot (A, representative blot) quantitated by IPLab Spectrum software (B). C-D) Ratio of P-Smad1/5/8 from panel B relative to LIC (C) or hepatic *Bmp6* mRNA (D). Results are expressed as the mean ± s.d for the fold change from WT mice on a CTR diet. Exact *P* values determined by 2-way ANOVA are shown.

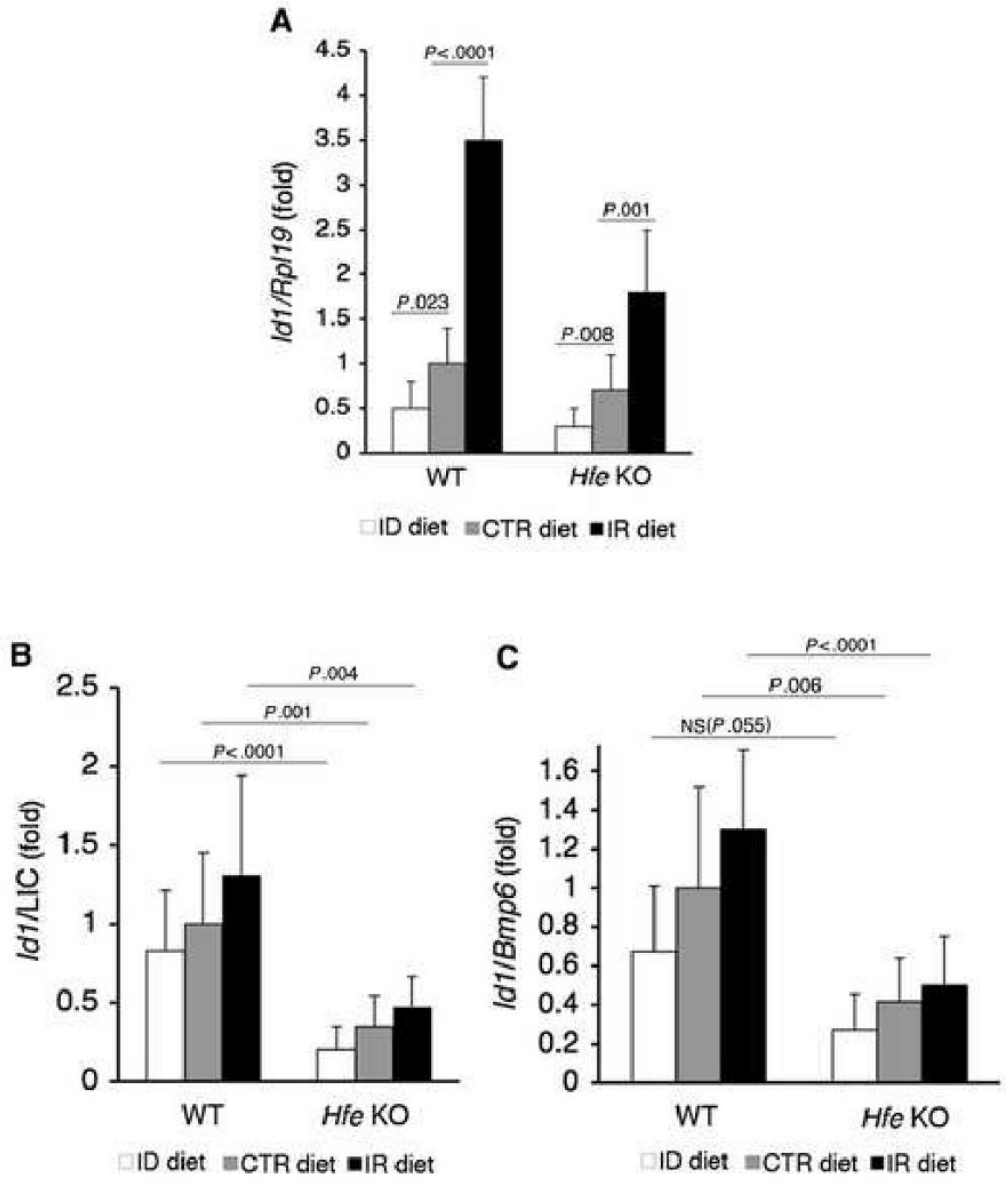


Fig.6. In *Hfe* KO mice, dietary iron modulates hepatic *Id1* mRNA expression, but *Id1* mRNA is not appropriately increased relative to iron burden and *Bmp6* mRNA expression compared with WT mice

A) WT and *Hfe* KO mice on an ID, CTR, or IR diet from Figure 2 ($N = 6$ per group) were analyzed for *Id1* relative to *Rpl19* mRNA as in Figure 1. B-C) Ratio of *Id1* mRNA from panel A relative to LIC (B) or hepatic *Bmp6* mRNA (C). Results are expressed as the mean ± s.d for the fold change from WT mice on a CTR diet. Exact *P* values determined by 2-way ANOVA are shown.

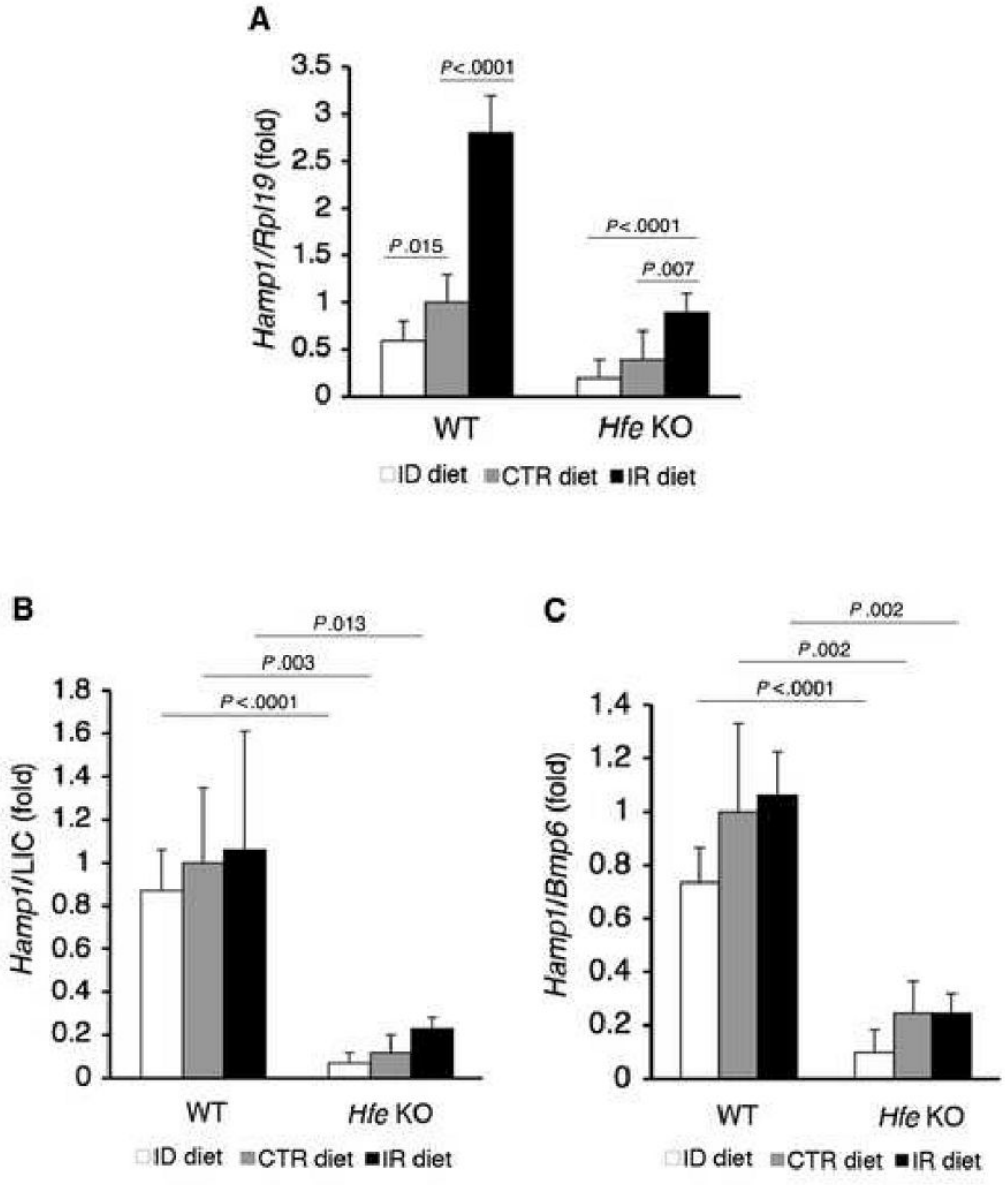


Fig.7. In *Hfe* KO mice, dietary iron modulates hepatic *Hamp1* mRNA expression, but *Hamp1* mRNA is not appropriately increased relative to iron burden and *Bmp6* mRNA expression compared with WT mice

A) WT and *Hfe* KO mice on an ID, CTR, or IR diet from Figure 2 ($N = 6$ per group) were analyzed for *Hamp1* relative to *Rpl19* mRNA as in Figure 1. B-C) Ratio of *Hamp1* mRNA from panel A relative to LIC (B) or hepatic *Bmp6* mRNA (C). Results are expressed as the mean ± s.d. for the fold change from WT mice on a CTR diet. Exact *P* values determined by 2-way ANOVA are shown.

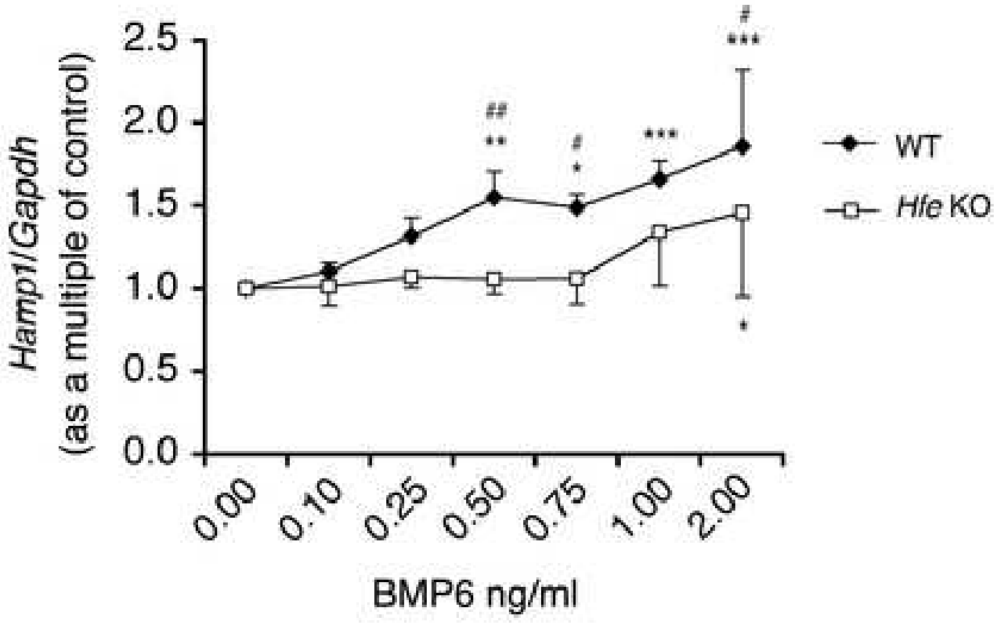


Figure 8. Hepcidin induction by BMP6 is impaired in *Hfe* KO hepatocytes

Primary hepatocyte cultures from *Hfe* KO versus WT control mice were treated with low doses of exogenous BMP6 ligand ranging from 0.1 to 2.0 ng/ml. *Hamp1* relative to *Gapdh* mRNA was analyzed by quantitative real-time RT-PCR. Results are expressed as the mean ± s.d. for the fold increase over untreated cells from five independent experiments each conducted in triplicate. * $P < .05$, ** $P < .01$, *** $P < .001$ compared with untreated cells for a given genotype; # $P < .05$, ## $P < .01$ for *Hfe* KO compared with WT cells treated with identical concentrations of BMP6 as determined by one-way ANOVA.