Bmp6 Expression Can Be Regulated Independently of Liver Iron in Mice

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Abstract
The liver is the primary organ for storing iron and plays a central role in the regulation of body iron levels by secretion of the hormone Hamp1. Although many factors modulate Hamp1 expression, their regulatory mechanisms are poorly understood. Here, we used conditional knockout mice for the iron exporter ferroportin1 (Fpn1) to modulate tissue iron in specific tissues in combination with iron-deficient or iron-rich diets and transferrin (Tf) supplementation to investigate the mechanisms underlying Hamp1 expression. Despite liver iron overload, expression of bone morphogenetic protein 6 (Bmp6), a potent-stimulator of Hamp1 expression that is expressed under iron-loaded conditions, was decreased. We hypothesized that factors other than liver iron must play a role in controlling Bmp6 expression. Our results show that erythropoietin and Tf-bound iron do not underlie the down-regulation of Bmp6 in our mice models. Moreover, Bmp6 was down-regulated under conditions of high iron demand, irrespective of the presence of anemia. We therefore inferred that the signals were driven by high iron demand. Furthermore, we also confirmed previous suggestions that Tf-bound iron regulates Hamp1 expression via Smad1/5/8 phosphorylation without affecting Bmp6 expression, and the effect of Tf-bound iron on Hamp1 regulation appeared before a significant change in Bmp6 expression. Together, these results are consistent with novel mechanisms for regulating Bmp6 and Hamp1 expression.

Introduction
Iron metabolism is a complex yet highly coordinated process. Hamp1, a short peptide secreted primarily by the liver [1], is vitally important for maintaining iron homeostasis throughout the body. Hamp1 binds to the non-heme iron exporter ferroportin 1 (Fpn1) and induces its internalization and degradation; Fpn1 is the only known non-heme iron exporter [2–3]. Many factors regulate Hamp1 expression, including iron overload [4] and iron deficiency, which induce and inhibit, respectively, the expression of this protein. Hamp1 expression is also regulated by inflammatory cytokines and erythropoietic factors [5]. However, the mechanisms that regulate Hamp1 expression remain poorly understood.

Bone morphogenetic proteins (BMPs) play an essential role in Hamp1 regulation [6–7]. The binding of BMPs to BMP receptors and the co-receptor Hemojuvelin (Hjv) triggers the phosphorylation of the transcription factors Smad1/5/8. Phosphorylated Smad1/5/8 (p-Smad1/5/8) then forms a complex with Smad4 and translocates to the nucleus, where it binds the Hamp1 promoter and modulates Hamp1 expression [8]. Bmp6 has been shown to be a key regulator of Hamp1 expression in vivo [9], and Bmp6 expression is usually correlated with liver iron content [10–11]. Mice with Bmp6 deficiency [12], Hjv deficiency [13–14], or hepatocyte-specific Hjv [15] or Smad4 [16] deletion all have severely reduced Hamp1 expression and a phenotype that closely resembles Hamp1-deficient mice [1]; therefore, the Bmp6/Hjv/p-Smad1/5/8 pathway clearly plays a key role in Hamp1 regulation [17].

Another critical regulator of Hamp1 expression is transferrin (Tf)-bound iron, the primary source of iron for erythroid cells [18]. The levels of Tf-bound iron generally reflect the body’s balance between iron supply and iron demand [19]. Both patients and mice with mutations in the Tf gene develop a condition called hypotransferrinemia, in which the body produces little or no Tf-bound iron and develops severe anemia [20–21]. Under these conditions, Hamp1 levels are reduced considerably. Moreover, Tf supplementation restores Hamp1 expression, demonstrating the essential role that Tf-bound iron plays in Hamp1 regulation [22]. In addition, patients and mice with hypotransferrinemia also exhibit massive erythropoietic drive and hypoxia, both of which

Citation: Zhang Z, Guo X, Herrera C, Tao Y, Wu Q, et al. (2014) Bmp6 Expression Can Be Regulated Independently of Liver Iron in Mice. PLoS ONE 9(1): e84906. doi:10.1371/journal.pone.0084906

Editor: Tracey Rouault, National Institute of Child Health and Human Development, United States of America

Received: June 28, 2013; Accepted: November 27, 2013; Published: January 13, 2014

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Funding: This work was supported by research grants from the Ministry of Science and Technology of China (973 Program) (grant numbers 2011CB946200, 2012BAAD33835); the National Natural Science Foundation of China (grant numbers 31200892 to ZZ, 31225013, 31300356, and 31300039 to FW); National Institutes of Health (NIH) grant to TBB (grant number R00DK084122); The Postdoctoral Research Program of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (grant number 2012XKPS12) and SA-SIBS Scholarship Program. This work was also supported by the Distinguished Professorship Program from Zhejiang University (to FW); FW is Principal Investigator at the Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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are factors that inhibit Hamp1 regulation [5]. Studies have shown that Ti-bound iron regulates Hamp1 expression through the phosphorylation of Smad1/5/8 (p-Smad1/5/8) [23]. This regulation is mediated by an interaction between Ti-bound iron and transferrin receptor 1 (TfR1) or 2 (TfR2), with the membrane-bound protein Hfe acting as an intermediate factor [24]. Mutations in Hfe [23] or TfR2 [26] lead to Hamp1 down-regulation, and the combined deletion of both Hfe and TfR2 results in extremely severe Hamp1 down-regulation, with decreased phosphorylation of Smad1/5/8, Erk1 and Erk2 [27]. Together, these results suggest that Ti-bound iron can modulate Hamp1 expression through the Hfe/TfR complex, with p-Smad1/5/8 and p-Erk1/2 serving as intermediate signaling molecules.

In this study, we dissected the factors involved in Hamp1 regulation and found that factors other than liver iron levels can regulate the expression of Bmp6. Our data suggest that the signal for Bmp6 down-regulation may arise from the driving force of high iron demand. Our study also confirms previous reports that Ti-bound iron regulates Hamp1 expression via p-Smad1/5/8 without affecting Bmp6 expression.

Materials and Methods

Ethics statement

All mice were handled in accordance with our institution’s humane animal care policies. The experimental protocols were approved by the Institutional Animal Care and Use Committee of The Institute for Nutritional Sciences, Shanghai Institutes for Biologic Sciences, and Chinese Academy of Sciences.

Animals and animal treatment

The generation of Fpn1Alb Alb and Fpn1Alb Alb LysM Alb LysM mice was described previously [29]. Tek-Cre mice [29], which express Cre recombinase under the Tek receptor tyrosine kinase promoter/enhancer, were purchased from the Jackson Laboratory and maintained on a 129/SvEvTac background, then crossed with Fpn1 Flexflor mice to generate Fpn1 Tek Tek mice. Fpn1 Flexflor mice were also maintained on a 129/SvEvTac background. Fpn1 was systemically deleted in embryonic Fpn1 Tek Tek mice due to maternal Cre recombinase expression [29–30]. The mice were fed a normal rodent laboratory diet (containing 232 mg iron/kg) obtained from SLRC Laboratory Animal Co., Ltd. (Shanghai, China) unless otherwise specified. Age-matched male mice were used in separate experiments. To induce various serum levels of Ti-bound iron, 2-month-old Fpn1 Flexflor and Fpn1 Alb Alb Alb Alb Alb LysM Alb LysM mice were fed an iron-deficient diet for 0, 2, 4 or 8 days. The mice then received an intraperitoneal injection of 10 mg human holo-Tf (or an equal volume of PBS), then given ad libitum access to normal rodent diet overnight to ensure high saturation of the supplemented Tf. For experiments requiring iron loading followed by the mobilization of iron from internal stores, 3-week-old Fpn1 Flexflor and Fpn1 Alb Alb mice were fed an iron-rich diet for one week, and then placed on an iron-deficient diet for one month. Standard (50 mg iron/kg), iron-deficient (0.9 mg iron/kg), and iron-rich (8.3 g of carboxyl iron/kg) diets were egg white-based AIN-76A diets (Research Diets, Inc., New Brunswick, NJ).

Measurement of serum iron, hematological parameters, tissue non-heme iron, and tissue iron staining

Assays to measure serum iron levels, hematological parameters, quantitative measurements of tissue non-heme iron, and Perl’s Prussian Blue and DBA iron staining were performed as previously described [31].

Western blot analysis and qRT-PCR

Western blot analyses and qRT-PCR were performed as previously described [31]. All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Western blots were analyzed with densitometry using Bio-Rad Quantity One software. The qRT-PCR data were normalized to the internal control (β-actin) and are presented as the relative expression level (calculated using the 2ΔΔCt method). The primers used for the qRT-PCR experiments are listed in Table S1 in File S1.

Statistical analysis

Summary data are presented as the mean ± standard deviation (SD). The Student’s t-test was used to compare the groups, and differences with P<0.05 are considered to be statistically significant.

Results

Bmp6 and Hamp1 expression are decreased in iron-loaded Fpn1 Tek Tek mice with severe iron-deficiency anemia

The mechanisms that regulate Hamp1 expression are not fully understood. Therefore, we used mouse models with a conditional knockout in the non-heme iron exporter Fpn1 to establish stable iron levels in specific tissues. We first established a baseline phenotype of Fpn1 deficiency to which we could compare the results of subsequent experiments. Because global Fpn1 deletion is embryonic lethal, we used Fpn1 Tek Tek mice [29–30], in which the expression of Cre recombinase is driven by the receptor tyrosine kinase Tek promoter/enhancer, resulting in the deletion of Fpn1 in the maternal germline and in endothelial cells, without causing embryonic lethality. Thirteen-to-fifteen-day-old Fpn1 Tek Tek mice had decreased Fpn1 mRNA levels in the liver, spleen, and duodenum; moreover, the expression of other genes implicated in iron metabolism was unchanged (Figure 1A, 1B). Total iron levels were increased in the liver and spleen of the Fpn1 Tek Tek mice, and iron accumulation was detected primarily in macrophages and duodenal enterocytes. The iron levels in the hepatocytes were also increased, but this increase was not robust, as we could only detect the change using the highly sensitive DAB iron staining method (Figure 1C). Despite liver and spleen iron loading, the Fpn1 Tek Tek mice were anemic with decreased serum iron levels and Tf saturation (Figure 1D, 1E, Figure 2A, 2B, Table S2 in File S1), suggesting that Fpn1 deficiency impairs iron absorption and the mobilization of iron stores for erythropoiesis. Next, we measured gene expression in the livers of these mice (Figure 2C–E). Surprisingly, Hamp1 mRNA was barely detectable in the Fpn1 Tek Tek mice (Figure 2G), despite liver iron loading.

Even more surprisingly, the mRNA levels of Bmp6, a potent stimulator of Hamp1 expression typically expressed in abundance during iron overload, were significantly lower in the Fpn1 Tek Tek mice (Figure 2D), despite liver iron overload. This result suggests that factors other than liver iron level regulate Bmp6 expression in these mice. As expected given their anemia, the Fpn1 Tek Tek mice had significantly higher erythropoietin (Epo) mRNA levels in the liver and kidneys (Figure 2E and data not shown). Given that p-Smad1/5/8 [8, 16], p-Erk1/2 [27] and p-Stat3 [32] are transcription factors that regulate Hamp1 expression, we used western blot analysis to measure these proteins in liver lysates. The levels of p-Smad1/5/8 and p-Erk1/2—but not p-Stat3, a transcription factor implicated in inflammation-mediated stimulation of Hamp1 expression—were reduced in the Fpn1 Tek Tek mice (Figure 2F, 2G), consistent with decreased Hamp1 levels. Taken together, these
data suggest that liver iron levels are not the sole factor involved in regulating Bmp6 and Hamp1 expression.

Factors other than liver iron play a role in Bmp6 down-regulation

The growth of Fpn1Tek/Tek mice is retarded, and these mice develop severe anemia (Figure 1D, 1E). Because the majority of these mice do not live beyond three weeks, we used Fpn1flox/flox mice—which have the combined deletion of Fpn1 in hepatocytes and macrophages—to examine Hamp1 regulation. These mice had high levels of iron in the liver and spleen (Figure 3A). At 2–3 weeks of age, the Fpn1flox/flox mice were more susceptible to iron deficiency than the Fpn1flox/flox mice. When a heterozygous mother was fed an iron-abundant diet, the pups developed virtually no anemia phenotype (data not shown). In contrast, pups born from a mother with the Fpn1Tek/Tek genotype developed anemia (Table S3 in File S1), although the anemia was less severe than in the Fpn1Tek/Tek mice (Table S2 and S3 in File S1). After weaning, the anemia resolved, possibly reflecting sufficient absorption of iron from the iron-sufficient diet to meet iron demand [28]. We then characterized the phenotype of these anemic, 3-week-old Fpn1flox/flox mice. Similar to Fpn1Tek/Tek mice, the Fpn1flox/flox mice had increased liver and spleen iron levels, decreased levels of serum Tf-bound iron, decreased Tf saturation and Hamp1 expression, and increased Epo mRNA levels (Figure 3). Despite having liver iron overload (Figure 3A), liver Bmp6 expression was decreased in the Fpn1flox/flox mice (Figure 3D), consistent with the Fpn1Tek/Tek mice (Figure 2D). Moreover, the levels of liver p-Smad1/5/8 and p-Erk1/2—but not p-Stat3—were also reduced in the Fpn1flox/flox mice (Figure 3F, 3G). Taken together, the phenotype of the Fpn1flox/flox mice provides further evidence that Bmp6 and Hamp1 are down-regulated under conditions of increased liver iron levels. This finding was not the result of aberrant behavior of the Fpn1flox allele, as feeding Fpn1flox/flox mice an iron-rich diet increased their levels of liver iron and serum iron, increased Hamp1 and Bmp6 expression, and increased the levels of the downstream transcription factor p-Smad1/5/8 (data not shown).

Signals derived from the driving force of high iron demand may underlie Bmp6 down-regulation

The aforementioned experiments using Fpn1Tek/Tek and Fpn1flox/flox mice suggest that factors other than liver iron levels regulate the expression of Bmp6. Because Tf-bound iron [23], anemia, Epo, and erythropoietic activity [5,33–34] are all factors that influence Hamp1 expression, we hypothesized that some or all of these factors might be involved in regulating Bmp6 expression in our mouse models. To dissociate the effect of anemia, we established a liver iron-loaded mouse model that did not develop anemia. We first fed 3-week-old Fpn1flox/flox and Fpn1flox/flox mice an iron-rich diet for one month (see Methods). Our prediction was that after changing to an iron-deficient diet, iron would be mobilized from both the Fpn1-intact hepatocytes and macrophages in the Fpn1flox/flox mice, but would accumulate in the Fpn1-deficient hepatocytes in the Fpn1flox/flox mice. As we expected, compared to the Fpn1flox/flox mice, the Fpn1flox/flox mice had a 5-fold higher level of liver iron and lower spleen iron levels (Figure 4A). The Fpn1flox/flox mice showed no overt signs of anemia (Table S4 in File S1), suggesting that the pre-loaded iron could be mobilized and used. The Fpn1flox/flox mice also had decreased levels of serum Tf-bound iron, decreased Tf saturation (Figure 4B), and decreased Hamp1 and Bmp6 expression (Figure 4C, 4D). Consistent with their lack of anemia, Epo expression was not decreased in the Fpn1flox/flox mice (Figure 4E). Consistent with decreased liver Bmp6 expression...
(Figure 4D), p-Smad1/5/8 levels were decreased in the *Fpn1Alb/Alb* mice (Figure 4F, 4G); in contrast, the levels of p-Erk1/2 and p-Stat3 were not significantly different between the *Fpn1Alb/Alb* and *Fpn1flox/flox* mice (Figure 4F, 4G). Thus, we surmised that the decreased *Hamp1* expression in *Fpn1Alb/Alb* mice was not due to the effects of anemia or increased *Epo* expression [35], but rather to decreased *Bmp6* expression and/or decreased serum levels of Tf-bound iron.

*Bmp6* expression is generally correlated with liver iron content [10–11]. Our observation that *Fpn1Alb/Alb* mice had 5-fold higher levels of liver iron but lower *Bmp6* expression levels compared to *Fpn1flox/flox* mice (Figure 4A, 4D) provides further evidence that liver iron levels are not the sole factor regulating *Bmp6* expression. Although the *Fpn1Alb/Alb* mice were not overtly anemic (Table S4 in File S1) and did not differ significantly from the *Fpn1flox/flox* mice (Figure 4F, 4G), we surmised that the decreased *Hamp1* expression in *Fpn1Alb/Alb* mice was not due to the effects of anemia or increased *Epo* expression [35], but rather to decreased *Bmp6* expression and/or decreased serum levels of Tf-bound iron.

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such as sinusoidal endothelial cells) in regulating Bmp6 expression [36–37].

Serum Tf-bound iron regulates Hamp1 expression via p-Smad1/5/8 without affecting Bmp6 expression

Because the levels of Tf-bound iron were correlated with Bmp6 expression in the Fpn1Tek/– (Figure 2B, 2D), Fpn1Alb/Alb;LysM/LysM (Figure 3B, 3D), and Fpn1Alb/Alb (Figure 4B, 4D) mice, we hypothesized that Tf-bound iron may regulate Bmp6 expression in our animal models. To dissect the effect of Tf-bound iron on Bmp6 expression, we first placed adult Fpn1flox/flox mice on a short-term iron-deficient diet. These mice developed decreased levels of liver iron, serum Tf-bound iron, and Tf saturation, decreased Bmp6 and Hamp1 mRNA, and decreased p-Smad1/5/8 levels (Figure S2). We next placed Fpn1Alb/Alb;LysM/LysM mice on a short-term iron-deficient diet. Unlike the Fpn1flox/flox mice, the Fpn1Alb/Alb;LysM/LysM mice (which have hepatocyte- and macrophage-specific Fpn1 deletion) had relatively stable liver and spleen iron levels (Figure 5A); however, circulating Tf-bound iron levels and Tf saturation decreased immediately upon starting the iron-deficient diet (Figure 5B). Nevertheless, these mice did not develop obvious signs of anemia (Table S5 in File S1). Liver Hamp1 expression was dramatically down-regulated in these mice (Figure 5C), whereas both Bmp6 and Epo expression had not reached significance on the iron-deficient diet (Figure 5D, 5E). Finally, the levels of p-Smad1/5/8 decreased in parallel with the Tf-bound iron levels and liver Hamp1 expression, whereas p-Erk1/2 and p-Stat3 levels were unchanged (Figure 5F, 5G). These results suggest that serum Tf-bound iron regulates Hamp1 expression via p-Smad1/5/8 with no detectable effect on Bmp6 expression.

To further investigate the role of Tf-bound iron in Bmp6 and Hamp1 regulation, we performed two additional experiments. First, we used a previously characterized mouse model [38]. Tfpx/hpx Hjv2/2 mice lack both Tf and Hjv expression. We previously reported that treating Tfpx/hpx Hjv2/2 and Tfpx/hpx Hjv2/2 mice with Tf normalized the hemoglobin levels of both mouse models; in contrast, Hamp1 expression increased robustly in the Tfpx/hpx Hjv2/2 mice only (but not in the Tfpx/hpx Hjv2/2 mice). Bmp6 expression was not affected by Tf treatment in either genotype [38]. To determine whether any signaling pathways in these mice were affected by Tf treatment, we measured the protein levels of several transcription factors in these mice and found increased p-Smad1/5/8 levels in the Tfpx/hpx Hjv2/2 mice only (but not in the Tfpx/hpx Hjv2/2 mice). Bmp6 expression was not affected by Tf treatment in either genotype [38]. To determine whether any signaling pathways in these mice were affected by Tf treatment, we measured the protein levels of several transcription factors in these mice and found increased p-Smad1/5/8 levels in the Tfpx/hpx Hjv2/2 mice only (but not in the Tfpx/hpx Hjv2/2 mice). Bmp6 expression was not affected by Tf treatment in either genotype [38]. To determine whether any signaling pathways in these mice were affected by Tf treatment, we measured the protein levels of several transcription factors in these mice and found increased p-Smad1/5/8 levels in the Tfpx/hpx Hjv2/2 mice only (but not in the Tfpx/hpx Hjv2/2 mice). Bmp6 expression was not affected by Tf treatment in either genotype [38]. To determine whether any signaling pathways in these mice were affected by Tf treatment, we measured the protein levels of several transcription factors in these mice and found increased p-Smad1/5/8 levels in the Tfpx/hpx Hjv2/2 mice only (but not in the Tfpx/hpx Hjv2/2 mice). Bmp6 expression was not affected by Tf treatment in either genotype [38]. To determine whether any signaling pathways in these mice were affected by Tf treatment, we measured the protein levels of several transcription factors in these mice and found increased p-Smad1/5/8 levels in the Tfpx/hpx Hjv2/2 mice only (but not in the Tfpx/hpx Hjv2/2 mice). Bmp6 expression was not affected by Tf treatment in either genotype [38]. To determine whether any signaling pathways in these mice were affected by Tf treatment, we measured the protein levels of several transcription factors in these mice and found increased p-Smad1/5/8 levels in the Tfpx/hpx Hjv2/2 mice only (but not in the Tfpx/hpx Hjv2/2 mice). Bmp6 expression was not affected by Tf treatment in either genotype [38]. To determine whether any signaling pathways in these mice were affected by Tf treatment, we measured the protein levels of several transcription factors in these mice and found increased p-Smad1/5/8 levels in the Tfpx/hpx Hjv2/2 mice only (but not in the Tfpx/hpx Hjv2/2 mice). Bmp6 expression was not affected by Tf treatment in either genotype [38]. To determine whether any signaling pathways in these mice were affected by Tf treatment, we measured the protein levels of several transcription factors in these mice and found increased p-Smad1/5/8 levels in the Tfpx/hpx Hjv2/2 mice only (but not in the Tfpx/hpx Hjv2/2 mice). Bmp6 expression was not affected by Tf treatment in either genotype [38]. To determine whether any signaling pathways in these mice were affected by Tf treatment, we measured the protein levels of several transcription factors in these mice and found increased p-Smad1/5/8 levels in the Tfpx/hpx Hjv2/2 mice only (but not in the Tfpx/hpx Hjv2/2 mice). Bmp6 expression was not affected by Tf treatment in either genotype [38]. To determine whether any signaling pathways in these mice were affected by Tf treatment, we measured the protein levels of several transcription factors in these mice and found increased p-Smad1/5/8 levels in the Tfpx/hpx Hjv2/2 mice only (but not in the Tfpx/hpx Hjv2/2 mice). Bmp6 expression was not affected by Tf treatment in either genotype [38]. To determine whether any signaling pathways in these mice were affected by Tf treatment, we measured the protein levels of several transcription factors in these mice and found increased p-Smad1/5/8 levels in the Tfpx/hpx Hjv2/2 mice only (but not in the Tfpx/hpx Hjv2/2 mice). Bmp6 expression was not affected by Tf treatment in either genotype [38]. To determine whether any signaling pathways in these mice were affected by Tf treatment, we measured the protein levels of several transcription factors in these mice and found increased p-Smad1/5/8 levels in the Tfpx/hpx Hjv2/2 mice only (but not in the Tfpx/hpx Hjv2/2 mice). Bmp6 expression was not affected by Tf treatment in either genotype [38].
intraperitoneally with 10 mg holo-Tf, and then analyzed the following day. Consistent with increased Tf-bound iron levels, Hamp1 expression and p-Smad1/5/8 levels increased approximately 2-fold in the holo-Tf-injected mice (Figure S4), despite having no increase in Bmp6 expression [38]. These results suggest that Tf-bound iron did not play a role in Bmp6 regulation in these mice. Thus, we conclude that Tf-bound iron regulates Hamp1 expression via p-Smad1/5/8 without affecting Bmp6 expression, which is consistent with previous reports [23].

In conclusion, factors other than liver iron levels regulated Bmp6 expression in our experiments. We postulate that the Bmp6-regulating signals arose from the driving force of high iron demand. However, we cannot exclude the possibility that the iron status of other hepatic cell types might also influence Bmp6 regulation. Our results also support previous findings that Tf-bound iron regulates Hamp1 expression via p-Smad1/5/8 without affecting Bmp6 expression.

**Discussion**

The liver is the major organ for storing iron. Liver iron levels reflect the whole-body iron status and correlate nicely with Hamp1 expression [10]. Under physiological conditions, iron overload increases Hamp1 expression, restricting intestinal iron absorption and iron release from iron storage sites; on the other hand, iron deficiency has the opposite effect. However, because Hamp1 deficiency is a known feature in mouse models of b-thalassemia [39] and hypotransferrinemia [22] (both of which are conditions associated with iron overload), other factors must be involved in Hamp1 regulation. Tf-bound iron [23,40] and Epo [34] are known regulators of Hamp1 expression. However, because these—and other—regulatory factors are often present together, we used conditional Fpn1-knockout mouse models that have relatively stable tissue iron levels, and we subjected these animals to various stressors to dissect the roles and interactions of various factors in regulating Hamp1 expression.

Both Fpn1Tek/Lek and actively growing Fpn1Alb/LysM/LysM mice developed an intriguing phenotype, namely decreased Hamp1 expression (Figure 2C, Figure 3C) in the context of liver iron loading (Figure 2A, Figure 3A). This phenotype has been observed previously in mouse models of hypotransferrinemia and other diseases [22,39]. However, a clear difference between hypotransferrinemic mice and our Fpn1 mouse models is that Bmp6 expression was decreased—not increased—in our mice.
To our knowledge, this is the first report of such a phenotype, and it suggests that factors other than liver iron must play a role in controlling \textit{Bmp6} expression in these mice. However, it should be noted that decreased \textit{Bmp6} expression relative to liver iron levels has been reported in \textit{b}-thalassemic mice [41].

We first hypothesized that the decreased \textit{Bmp6} expression in our \textit{Fpn1} mouse models might be due to anemia and/or Epo. However, this seemed unlikely, given that \textit{Fpn1Alb/Alb} mice (which were first pre-loaded with iron, and then transferred to an iron-poor diet to drive tissue iron mobilization) had liver iron loading and decreased \textit{Bmp6} and \textit{Hamp1} expression, yet developed no significant signs of anemia (Table S4 in File S1) or changes in Epo expression compared to control \textit{Fpn1flox/flox} mice (Figure 4E). We then theorized that decreased \textit{Bmp6} expression could be attributed directly to the lack of \textit{Fpn1} expression. However, we found no significant difference in \textit{Bmp6} expression between adult \textit{Fpn1flox/flox} and \textit{Fpn1Alb/Alb} mice that were fed an iron-rich diet (data not shown). Thus, we could exclude a direct role for \textit{Fpn1} in regulating \textit{Bmp6} expression. When \textit{Fpn1flox/flox} and \textit{Fpn1Alb/Alb} mice were pre-loaded with iron and then placed on an iron-deficient diet, we reasoned that iron demand would be high in the \textit{Fpn1Alb/Alb} mice, given that iron mobilization from their hepatocytes was impaired. Therefore, we inferred that the signal for \textit{Bmp6} down-regulation in \textit{Fpn1Alb/Alb} mice might arise from the driving force of high iron demand. This notion was validated in adult \textit{Fpn1Alb/Alb;LysM/LysM} mice that were fed an iron-replete or iron-deficient diet (Figure S1). Additionally, if the signal for iron demand indeed affects \textit{Bmp6} expression, the phenotype of hypotransferrinemic (\textit{Tfhpx/hpx}) mice must be rectified, given that these mice are profoundly anemic yet still have increased \textit{Bmp6} expression [22]. However, the increased \textit{Bmp6} expression in these mice could be explained by their extremely high liver iron levels, which could overwhelm the effect of high iron demand on \textit{Bmp6} regulation.

Moreover, mice with phenylhydrazine-induced hemolysis have high iron demand, yet still have high \textit{Bmp6} expression, which can also be explained by an increase in liver iron levels following phenylhydrazine treatment [42]. Similarly, \textit{b}-thalassemic mice have high iron demand and increased \textit{Bmp6} expression, although the magnitude of the increase in \textit{Bmp6} expression was not as large as expected [41–42]. Thus, we hypothesize that the factors that signal iron overload and erythroid iron demand have opposing and competing effects on \textit{Bmp6} expression in these contexts. Of course, we cannot exclude the possibility that other factors might play a role in altering \textit{Bmp6} expression in these model systems. For
example, hepatic sinusoidal endothelial cells are a more robust source of Bmp6 expression than hepatic stellate cells, Kupffer cells, and hepatocytes [36–37]. Finally, the iron status of hepatic non-parenchymal cells might affect Bmp6 expression in our mouse models, and this possibility merits further study.

Because Tf-bound iron levels can reflect the balance between iron supply and erythropoietic demand, we also investigated the role of serum Tf-bound iron in regulating Bmp6 and/or Hamp1 expression. Placing adult Fpn1Alb/Alb;LysM/LysM mice on an iron-deficient diet had no short-term effect on liver or spleen iron concentrations, yet Tf-bound iron levels decreased significantly, as were both Hamp1 expression and p-Smad1/5/8 (Figure 5). However, no significant change was found with respect to Hamp1 expression (Figure 5D), although a slight trend towards decreased expression was noted, which may reflect the higher iron demand status. Because the change in Bmp6 was negligible compared to the significant changes in both p-Smad1/5/8 and Hamp1 mRNA, these results suggest that Tf-bound iron down-regulated Hamp1 expression primarily via Smad1/5/8 phosphorylation.

To further investigate the role of Tf-bound iron in Bmp6 regulation, we also treated Fpn1Alb/Alb;LysM/LysM mice with Tf. This treatment increased the levels of p-Smad1/5/8 (Figure S3) and Hamp1 mRNA, but had no effect on Bmp6 expression [38]. This finding was confirmed in Fpn1Alb/Alb mice that were injected with holo-Tf (Figure S4). Together, these data do not necessarily support a role for Tf-bound iron in Bmp6 regulation, but they do reinforce the previous report that Tf-bound iron regulates Hamp1 via p-Smad1/5/8 [23].

While the Smad pathway plays a central role in regulating Hamp1 expression, p-Erk1/2 has also been implicated in mediating Hamp1 expression [27,40]. However, with the exception of 13–15-day-old anemic Fpn1Tek/Tek mice and 3-week-old Fpn1Tek/Tek mice (Figure 2F and 3F), no significant changes in p-Erk1/2 levels were observed in our mouse models, regardless of whether they were subjected to changes in dietary iron or injected with holo-Tf (Figure 4F, Figure S2F, S3F, S4F). Overall, our findings do not support a role for p-Erk1/2 in Hamp1 regulation in this context. However, at this time we cannot explain the significant decrease in p-Erk1/2 levels in the anemic Fpn1Tek/Tek and Fpn1Tek/Tek mice. It is possible that p-Erk1/2 plays a more important role in Hamp1 expression in early development or during overt anemia. Furthermore, in some cases, the variability in the p-Erk1/2 signal was too large to allow conclusive results to be drawn (Figure 5F, Figure S3F, S4F). Studying Erk1/2-deficient mice may be a more definitive approach for determining whether Erk1/2 plays a role in regulating Hamp1 expression.

In conclusion, our results suggest that factors other than liver iron can regulate Bmp6 expression. The Bmp6 down-regulation observed in our Fpn1Alb-deficient mice was not due to the effect of Tf-bound iron, anemia, or Epo expression. We speculate that the signals that underlie the decreased Bmp6 expression in our mouse models arose from the driving force of high iron demand. In addition, our results support previous findings that serum Tf-bound iron regulates Hamp1 expression via p-Smad1/5/8 without affecting Bmp6 expression. Given that other factors such as Epo expression, inflammatory factors, and reactive oxygen species have been reported to play important roles in regulating Hamp1 expression, the interplay between these factors under various physiological conditions warrant further study.

Supporting Information

Figure S1 Bmp6 expression is down-regulated in mice with high-iron demand. Liver mRNA levels of Bmp6 were measured in 2-month-old male Fpn1flex/flex and Fpn1 Tek/ Tek/LysM/LysM mice without anemia (A) or in 2-month-old Fpn1flex/flex and Fpn1 Tek/ Tek/LysM/LysM mice that were fed an iron-deficient diet for two months (B). n = 5 per group. Data are presented as mean $\pm$ SD. *P<0.05. (TIF)

Figure S2 Liver iron, serum Tf-bound iron and Hamp1 and Bmp6 expression levels are decreased in adult Fpn1flex/flex mice placed on a short-term iron-deficient diet. (A) Liver and spleen non-heme iron concentrations. (B) SI and TS% levels. (C) Liver mRNA levels of Hamp1, D) Bmp6 and (E) Epo. (F) Liver p-Smad1/5/8, Smad1, p-Erk1/2, Erk1/2, p-Stat3, Stat3, and $\beta$-actin protein levels were measured in 2-month-old male Fpn1flex/flex mice fed an AIN-76A (iron-deficient) diet for 0, 2, 4, or 8 days (n = 5 per group). Summary data are presented as mean $\pm$ SD. **P<0.01. (TIF)

Figure S3 Tf stimulates Hamp1 expression via p-Smad1/5/8. Mouse deficient in Tf (Tfp–/–, Hje–/–) or Tf and Hje (Tfp–/–, Hje–/–) were treated with 10 mg Tf or an equivalent volume of PBS every other day for two weeks; the organs were then harvested for analysis. Protein levels of (A) p-Smad1/5/8 and Smad1, (B) p-Erk1/2 and Erk1/2, (C) p-Stat3 and Stat3 were measured by western blot analysis of lysates prepared from the harvested livers; $\beta$-actin was measured as a loading control. The blots were analyzed by densitometry, and the ratios of phosphorylated protein to total protein are expressed graphically in the right panels. Summary data are presented as mean $\pm$ SD. *P<0.05. (TIF)

Figure S4 Holo-Tf supplementation regulates Hamp1 through p-smad1/5/8 without influencing Bmp6 expression. (A) Liver and spleen non-heme iron concentrations. (B) SI and TS% levels. (C) Liver mRNA levels of Hamp1, D) Bmp6 and (E) Epo. (F) Liver p-Smad1/5/8, Smad1, p-Erk1/2, Erk1/2, p-Stat3, Stat3, and $\beta$-actin protein levels were measured in 2-month-old male Fpn1flex/flex mice that were injected with 10 mg holo-Tf in PBS (or an equal volume of PBS) and then fed ad libitum overnight to facilitate saturation of Tf with iron (n = 5 per group). Summary data are presented as mean $\pm$ SD. *P<0.05; **P<0.01. (TIF)

File S1 Tables S1–S5. Table S1. Sequences of primers. Table S2. Hematologic parameters of Fpn1flex/flex and Fpn1 Tek/Tek mice. Hematologic parameters were measured in 13–15-day-old male Fpn1flex/flex and Fpn1 Tek/Tek mice. RBCs, Red Blood Cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration. Data are presented as mean $\pm$ SD. *P<0.05; **P<0.01. Table S3. Hematologic parameters of three-week-old Fpn1flex/flex and Fpn1 Tek/Tek mice. Hematologic parameters were measured in 3-week-old Fpn1flex/flex and Fpn1 Tek/Tek/LysM/LysM mice. Data are presented as mean $\pm$ SD. P<0.05; **P<0.01. Table S4. Hematologic parameters of Fpn1flex/flex and Fpn1 Tek/Tek/LysM/LysM mice preloaded with iron then maintained on an iron-deficient diet for one month. Three-week-old male Fpn1flex/flex and Fpn1 Tek/Tek/LysM/LysM mice were fed an iron-rich diet (3.5 g of carboxyl iron/kg) for one week, and then transferred to an iron-deficient diet (0.9 mg iron/kg) for one month. Blood was harvested for hematologic parameters analysis. Results are presented as
mean ± SD. *P<0.05; **P<0.01. Table S5. Hematologic parameters of Fpn1^Alb/Alb;LysM/LysM mice maintained short-term on an iron-deficient diet. Two-month-old male Fpn1^Alb/Alb;LysM/LysM mice were fed an AIN-76A (iron-deficient) diet (0.9 mg iron/kg) for 0, 2, 4, or 8 days (n = 5 per group). Blood was then harvested for hematologic parameter analysis. Blood parameters of Fpn1^Alb/Alb;LysM/LysM mice at day 0 were measured as a control. Results are presented as mean ± SD. *P<0.05; **P<0.01.

Acknowledgments
The authors are grateful to Dr. Nancy C. Andrews for providing the Fpn1 floxed mice, and other members of the Wang laboratory for helpful discussions.

Author Contributions
Conceived and designed the experiments: ZZ, FW. Performed the experiments: ZZ, XG, YT, QW, AW, HW, TB. Analyzed the data: ZZ, FW. Contributed reagents/materials/analysis tools: FW. Wrote the paper: ZZ, FW.

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