Brief Report

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Deletion of BMP6 worsens the phenotype of HJV-deficient mice and attenuates hepcidin levels reached after LPS challenge

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Key Points

- Loss of Bmp6 further represses hepcidin expression in the liver of *Hjv* knockout mice and markedly worsens the iron phenotype of females.
- Induction of hepcidin by LPS is not prevented by lack of Bmp6 and/or Hjv but its level poststimulation is blunted compared with controls.

Lack of either bone morphogenetic protein 6 (BMP6) or the BMP coreceptor hemojuvelin (HJV) in mice leads to a similar phenotype with hepcidin insufficiency, hepatic iron loading, and extrahepatic iron accumulation in males. This is consistent with the current views that HJV is a coreceptor for BMP6 in hepatocytes. To determine whether BMP6 and HJV may also signal to hepcidin independently of each other, we intercrossed *Hjv*$^{-/-}$ and *Bmp6*$^{-/-}$ mice and compared the phenotype of animals of the F2 progeny. Loss of Bmp6 further repressed Smad signaling and hepcidin expression in the liver of *Hjv*$^{-/-}$ mice of both sexes, and led to iron accumulation in the pancreas and the heart of females. These data suggest that, in *Hjv*$^{-/-}$ females, Bmp6 can provide a signal adequate to maintain hepcidin to a level sufficient to avoid extrahepatic iron loading. We also examined the impact of Bmp6 and/or Hjv deletion on the regulation of hepcidin by inflammation. Our data show that lack of 1 or both molecules does not prevent induction of hepcidin by lipopolysaccharide (LPS). However, BMP/Smad signaling in unchallenged animals is determinant for the level of hepcidin reached after stimulation, which is consistent with a synergy between interleukin 6/STAT3 and BMP/SMAD signaling in regulating hepcidin during inflammation. (*Blood*. 2017;130(21):2339-2343)

Introduction

Liver sinusoidal endothelial cells produce bone morphogenetic protein 6 (BMP6), which acts on the hepatocyte coreceptor hemojuvelin (HJV) to regulate hepcidin production in a paracrine fashion.[1,2] Both are required to ensure optimal maintenance of iron homeostasis. In humans, mutations in the HJV gene are responsible for juvenile hemochromatosis, an early onset form of hereditary hemochromatosis caused by profound hepcidin insufficiency.[3] Whereas loss-of-function mutations in BMP6 have not been described so far, heterozygous mutations in the propeptide have been identified in patients with iron overload and lead to inappropriate hepcidin synthesis.[4,5] In the mouse, deletion of either *Bmp6* or *Hjv* leads to a similar phenotype characterized by hepcidin insufficiency, severe iron loading, and extrahepatic iron accumulation in males,[6-11] which is consistent with the current views that HJV is a coreceptor for BMP6.

Whether BMP6 and HJV may also signal to hepcidin independently of each other is still a topic of discussion.[11] To provide direct evidence that BMP6 and HJV can separately stimulate hepcidin, we intercrossed *Hjv* and *Bmp6* knockout mice and looked at whether deletion of both *Bmp6* and *Hjv* in mice of the F2 progeny was aggravating the phenotype of single knockout animals. Whether BMP6 and HJV are both required for the upregulation of hepcidin by inflammatory stimuli is another unresolved issue. We took the opportunity of having genetically comparable single and double knockout animals to challenge them with lipopolysaccharide (LPS) and examine the impact of Bmp6 and/or Hjv deletion on Smad signaling and hepcidin expression after stimulation.

Study design

Mouse crosses

Hjv$^{-/-}$ mice on a 129S6/SvEvTac background[9] were bred to *Bmp6*^{tm1Rob} mice (*Bmp6*$^{-/-}$) on a CD1 background.[12] Experiments were done on 12-week-old wild-type, *Bmp6*$^{-/-}$, *Hjv*$^{-/-}$, and *Bmp6*$^{-/-}$*Hjv*$^{-/-}$ littermates of the F2 progeny. Experimental protocols were approved by the Midi-Pyrénées Animal Ethics Committee. Animals were given free access to tap water and standard laboratory mouse chow diet (250 mg of iron per kilogram; SAFE, Augy, France). Littermates carrying the different genotypes were also challenged with an intraperitoneal injection of LPS (1 μg/g body weight) and livers were harvested 4 hours later.

Quantitation of liver messenger RNA levels

Quantitative polymerase chain reactions (PCRs) were run on a LightCycler 480 System (Roche Diagnostics), using primers previously referenced.[13] Δ Cycle threshold (ΔCt) values were obtained by subtracting the *Hprt* reference gene Ct to the target gene Ct.

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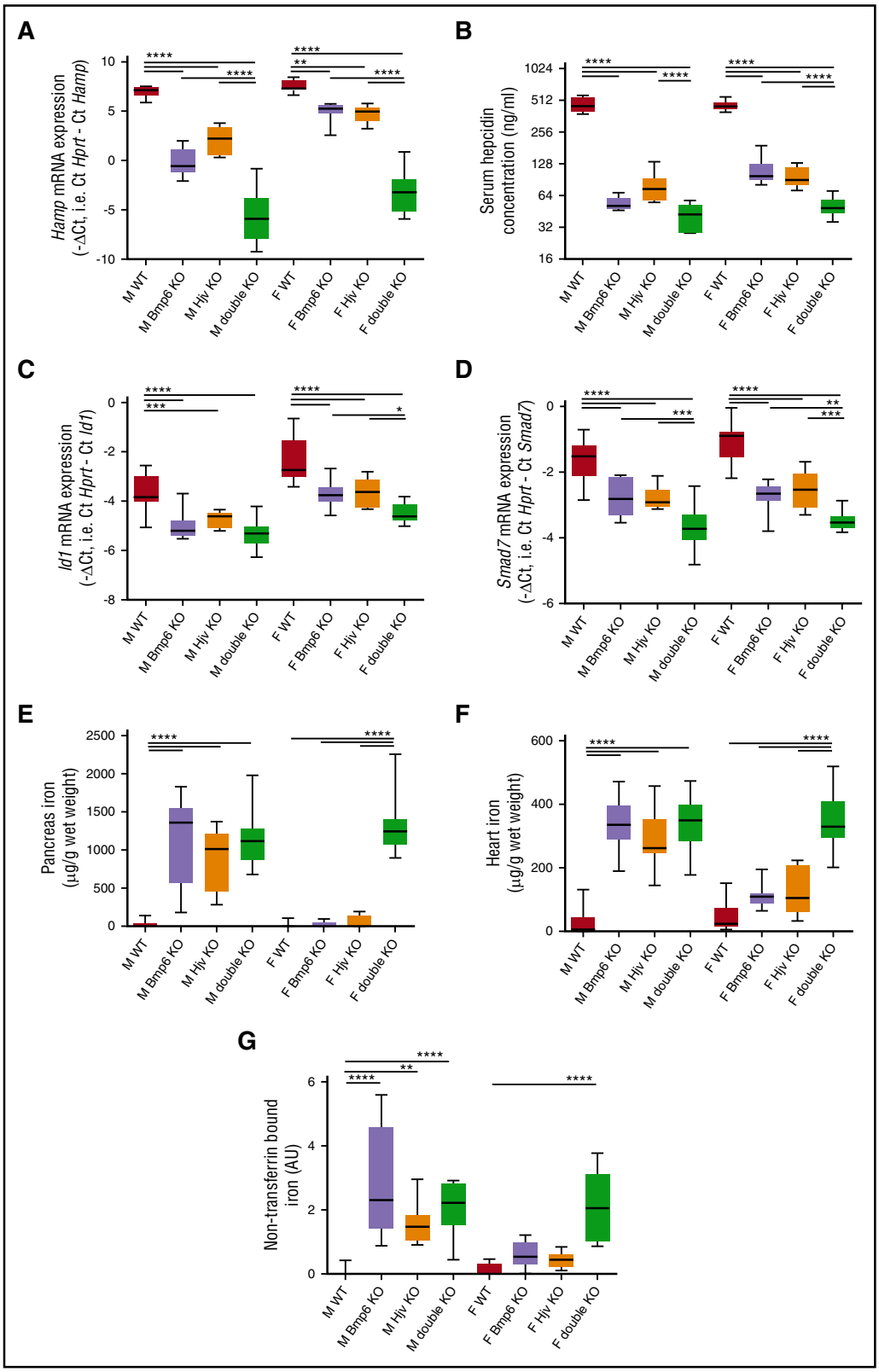

Figure 1. Deletion of Bmp6 further represses hepcidin expression in *Hjv* knockout mice and worsens the iron overload phenotype of females. (A) Hepcidin (*Hamp*), (C) *Id1*, and (D) *Smad7* mRNA expression were measured by quantitative PCR in the liver of male (M) and female (F) littermates that had the wild-type (WT), *Bmp6*$^{-/-}$, *Hjv*$^{-/-}$, or double knockout (KO) genotype. (B) Serum hepcidin levels were measured by competitive ELISA on the same mice. Quantitative measurement of nonheme iron in the pancreas (E), and the heart (F) was performed according to the method recommended by Torrance and Bothwell.[14] NTBI (G) was measured using the FeROS eLPI kit (Aferrix LTD). Box-and-whisker plots are shown on the graphs. Data were obtained on 10 WT, 10 *Bmp6*$^{-/-}$, 10 *Hjv*$^{-/-}$, and 18 double knockout males, and on 11 WT, 10 *Bmp6*$^{-/-}$, 8 *Hjv*$^{-/-}$, and 11 double knockout females. Means of quantitative PCR ΔCt values, log-transformed serum hepcidin, tissue iron content, and NTBI arbitrary units (AUs) in mice of the different genotypes were compared with 2-way ANOVA followed by Sidak multiple comparison tests of planned contrasts between pairs of means. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$. Hprt, hypoxanthine-guanine phosphoribosyltransferase.

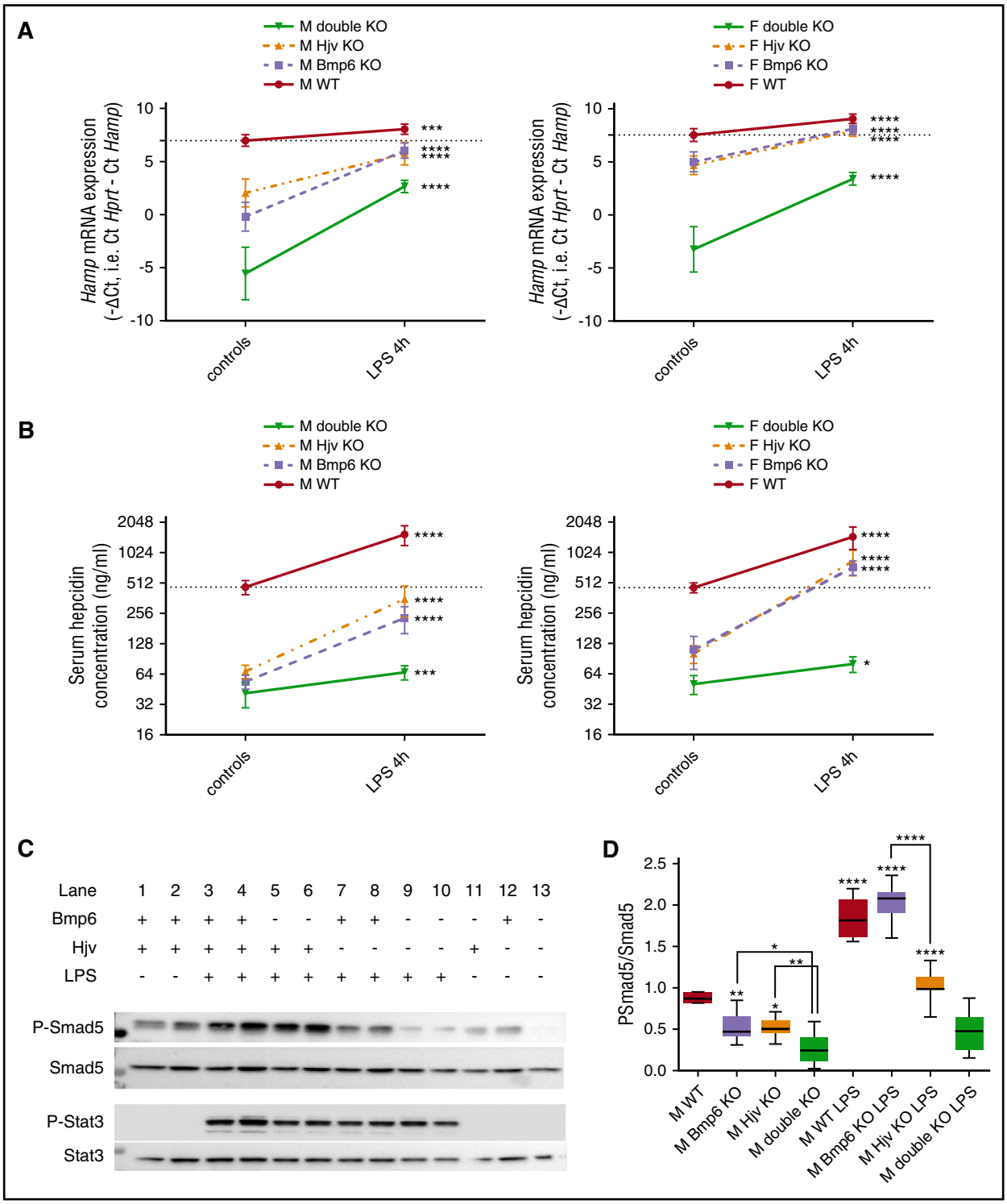

Figure 2. The lack of Bmp6 and/or Hjv does not prevent the induction of hepcidin by LPS but the level of hepcidin reached after stimulation is linked to baseline BMP/Smad signaling in unchallenged animals. (A) Hepcidin (*Hamp*) mRNA expression was measured by quantitative PCR before and after LPS stimulation in the liver of male (M) and female (F) littermates that had the WT, *Bmp6*$^{-/-}$, *Hjv*$^{-/-}$, or double knockout (KO) genotype. (B) Serum hepcidin levels were measured by competitive ELISA on the same mice. Means ± standard deviation (SD) are shown on the graphs. Data on LPS-challenged mice were obtained on 8 WT, 4 *Bmp6*$^{-/-}$, 9 *Hjv*$^{-/-}$, and 5 double knockout males, and on 6 WT, 4 *Bmp6*$^{-/-}$, 7 *Hjv*$^{-/-}$, and 4 double knockout females. Means of quantitative PCR ΔCt values and log-transformed serum hepcidin before and after LPS were compared with Student *t* tests. (C) Representative western blot of 7 independent experiments for phospho-Smad5, total Smad5, phospho-Stat3, and total Stat3 expression in the liver of WT (Bmp6^{+}/Hjv^{+}), *Bmp6*$^{-/-}$(Bmp6^{-}/Hjv^{+}), *Hjv*$^{-/-}$ (Bmp6^{+}/Hjv^{-}), and double knockout (Bmp6^{-}/Hjv^{-}) male littermates before (LPS^{-}) and after (LPS^{+}) LPS stimulation. Western blots were analyzed on a Chemidoc MP Imaging System (Bio-Rad). (D) Quantification was obtained with Image Laboratory Software. Means of PSmad5-to-Smad5 ratios were compared with 2-way ANOVA. Results of comparisons of unchallenged knockout with unchallenged WT mice as well as of LPS-challenged with unchallenged mice of the same genotype are shown above the bars. Results of comparisons between knockout mice are shown by connecting lines. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

Serum hepcidin

Serum hepcidin levels were quantified using the Intrinsic LifeSciences (La Jolla, CA) Hepcidin-Murine Compete competitive enzyme-linked immunosorbent assay (ELISA).

Quantitative iron measurement and tissue iron staining

Transferrin saturation was obtained through the determination of serum iron and latent iron-binding capacity. Quantitative measurement of nonheme iron in the liver, heart, and pancreas was performed according to the method of Torrance and Bothwell.[14] Nontransferrin-bound iron (NTBI) was measured using the FeROS eLPI kit (Aferrix).

Protein extraction and western blot analysis

Protein extraction and western blot analysis were performed as previously described.[13]

Statistical analyses

Means of quantitative variables (log-transformed for serum hepcidin) were compared with 2-way analysis of variance (ANOVA) followed by Sidak multiple comparison tests of planned contrasts between pairs of means.

Results and discussion

We first measured by quantitative PCR the amount of hepcidin messenger RNA (mRNA) in the liver of the genetically comparable F2 littermates (Figure 1A). As previously reported in mice of other genetic backgrounds,[11] hepcidin expression in the liver of *Bmp6*$^{-/-}$ and *Hjv*$^{-/-}$ mice is much lower than in wild-type mice, particularly in males. However, deletion of Bmp6 more dramatically repressed the already reduced hepcidin expression of *Hjv*$^{-/-}$ mice, regardless of sex. These observations were confirmed at the protein level (Figure 1B). Gene expression of *Id1* (Figure 1C) and *Smad7* (Figure 1D), 2 targets of Bmp/Smad signaling, was also further repressed in double knockout mice, compared with single knockouts, providing indirect evidence that deletion of Bmp6 further alters Smad1/5/8 signaling in the liver of *Hjv* knockout mice. This was confirmed by western blot (Figure 2C lanes 11-13).

In line with the repression of hepcidin, targeted disruption of the *Bmp6* and/or the *Hjv* gene leads to a strong increase in transferrin saturation (supplemental Figure 1A, available on the *Blood* Web site) and liver iron accumulation (supplemental Figure 1B) in mice of both sexes. However, whereas *Bmp6*$^{-/-}$ or *Hjv*$^{-/-}$ males have iron deposits in acinar cells of the exocrine pancreas (Figure 1E; supplemental Figure 2A) and in the heart (Figure 1F; supplemental Figure 2B), females do not accumulate iron in these extrahepatic tissues, which reflects the fact that, in the absence of testosterone, their hepcidin is less strongly repressed[10] and their NTBI is lower (Figure 1G) than in males. Nevertheless, the concomitant loss of Bmp6 suppresses this hepcidin advantage over males. As a consequence, and in contrast to single *Bmp6*$^{-/-}$ or *Hjv*$^{-/-}$ females, double knockout females have substantial NTBI amounts and massive iron loading in all extrahepatic tissues examined (supplemental Figure 2A-B).

Interestingly, although LPS significantly induces hepcidin mRNA (Figure 2A) and protein (Figure 2B) not only in wild-type males and females but also in single and double knockout animals, the level reached after stimulation depends on basal expression of hepcidin. It is highest in wild-type mice, intermediate in *Bmp6*$^{-/-}$ and in *Hjv*$^{-/-}$ mice, and lowest in double knockout animals. This is compatible with the proposed synergy between interleukin 6/STAT3 and BMP/SMAD signaling in regulating hepcidin during inflammation.[15] Smad5 signaling was previously shown to be activated by activin B as a consequence of LPS stimulation[16] but to have no impact on hepcidin induction.[13] Here, although activation of Smad5 by LPS was similar in wild-type and in *Bmp6*$^{-/-}$ mice (Figure 2C-D lanes 3-6), the level of hepcidin reached after stimulation was much lower in *Bmp6*$^{-/-}$ mice. In contrast, *Hjv*$^{-/-}$ mice present with reduced Smad5 activation compared with *Bmp6*$^{-/-}$ mice (Figure 2C-D lanes 5-8) but have similar induction of hepcidin. These data confirm recent in vitro observations showing that HJV augments Smad5 signaling by activin B.[17] They also definitely indicate the lack of relationship between activation of Smad1/5/8 signaling by inflammatory stimuli, which is facilitated by HJV, and elevation of hepcidin expression.

In conclusion, analysis of this *Bmp6*$^{-/-}$ × *Hjv*$^{-/-}$ intercross clearly shows that deletion of both *Bmp6* and *Hjv* further represses hepcidin and aggravates the phenotype of single knockout animals. This indicates that, when 1 actor of the major hepcidin signaling pathway is lacking, alternative pathways, although less efficient to activate Smad1/5/8, succeed in maintaining hepcidin to a level avoiding extrahepatic iron accumulation in females. These can be initiated by interaction of HJV with liver sinusoidal endothelial cell–produced BMP2 whose deletion in mice also leads to iron overload,[18,19] or by direct binding of BMP6 to preformed BMP type I/type II receptor complexes that exist at the membrane in the absence of ligands and coreceptors.[20-22] The suppression of these alternative pathways, as here in *Bmp6/Hjv* double knockout animals, leads to greater repression of hepcidin in mice of both sexes and substantial exacerbation of the extrahepatic iron overload phenotype in females. Our data also show that induction of hepcidin by LPS in vivo is linked to BMP/Smad signaling before but not after stimulation. Notably, the less severely affected *Bmp6*$^{-/-}$ or *Hjv*$^{-/-}$ females produce, when challenged with LPS, more hepcidin than unchallenged wild-type mice. Thus, in females, treatments targeting BMP type I receptors, previously shown to attenuate induction of hepcidin gene expression by various inflammatory stimuli,[23-25] will be more effective against anemia of inflammation than treatments that would target only HJV, as the latter would not prevent BMP6 to signal to hepcidin independently of HJV.

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Authorship

Contribution: C.L. genotyped the mice; C.L. and O.G. killed the mice and sampled the different tissues; C.L. performed reverse transcription–PCR experiments and hepcidin ELISAs; C.B.-F. did quantitative iron measurements and western blots; O.G. performed

Perls Prussian blue staining of deparaffinized tissue sections; D.M. helped with data analysis and interpretation; and M.-P.R. and H.C. led and supervised the project through all stages, helped in data analyses, and wrote the manuscript with suggestions and comments from all authors.

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