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Deletion of *TMPRSS6* attenuates the phenotype in a mouse model of β-thalassemia

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Inappropriately low expression of the key iron regulator hepcidin (HAMP) causes iron overload in untransfused patients affected by β-thalassemia intermedia and Hamp modulation provides improvement of the thalassemic phenotype of the *Hbb*$^{th3/+}$ mouse. HAMP expression is activated by iron through the bone morphogenetic protein (BMP)–son of mothers against decapentaplegic signaling pathway and inhibited by ineffective erythropoiesis through an unknown "erythroid regulator." The BMP pathway is inactivated by the serine protease TMPRSS6 that cleaves the BMP coreceptor hemojuvelin. Here, we show that homozygous loss of *Tmprss6* in *Hbb*$^{th3/+}$ mice improves anemia and reduces ineffective erythropoiesis, splenomegaly, and iron loading. All these effects are mediated by Hamp up-regulation, which inhibits iron absorption and recycling. Because *Hbb*$^{th3/+}$ mice lacking Tmprss6 show residual ineffective erythropoiesis, our results indicate that Tmprss6 is essential for Hamp inhibition by the erythroid regulator. We also obtained partial correction of the phenotype in *Tmprss6* haploinsufficient *Hbb*$^{th3/+}$ male but not female mice and showed that the observed sex difference reflects an unequal balance between iron and erythropoiesis-mediated Hamp regulation. Our study indicates that preventing iron overload improves β-thalassemia and strengthens the essential role of Tmprss6 for *Hamp* suppression, providing a proof of concept that Tmprss6 manipulation can offer a novel therapeutic option in this condition. (*Blood*. 2012;119(21):5021-5029)

Introduction

The liver antimicrobial peptide hepcidin (HAMP) is the central regulator of systemic iron homeostasis. HAMP controls the surface expression of the iron exporter ferroportin on duodenal enterocytes and macrophages, modulating iron absorption and recycling. HAMP is activated by the bone morphogenetic proteins (BMP)–son of mothers against decapentaplegic (SMAD) signaling pathway, in response to increased body iron and by the IL-6–signal transducer and activator of transcription (STAT)3 pathway in inflammation.[1] The glycosylphosphatidylinositol (GPI)–anchored protein hemojuvelin (HJV) is a BMP coreceptor and homozygous mutations of *HAMP* or *HJV* cause juvenile hemochromatosis in humans[2,3] and severe iron overload in mice.[4,5]

The BMP pathway is inhibited by matriptase-2 (MT-2), a type II transmembrane serine protease encoded by the transmembrane proteaSe serine 6 (*TMPRSS6*) gene and mainly expressed in the liver.[6,7] MT-2, by cleaving HJV from the hepatocyte surface, attenuates the BMP-SMAD signaling and down-regulates *HAMP* expression.[8] Mice deficient for both *Tmprss6* and *Hjv* show markedly decreased *Hamp* mRNA levels and systemic iron overload, as do *Hjv* deficient mice,[9] in agreement with Hjv being the serine protease substrate.

TMPRSS6 plays an essential role for erythropoiesis: homozygous inactivation of the *Tmprss6* gene leads to excessive Hamp production, impaired dietary iron absorption and microcytic anemia in mice,[10,11] and iron-refractory iron deficiency anemia (IRIDA) in humans.[12-17]

The important role of TMPRSS6 in erythropoiesis is highlighted also by genome-wide association studies. Common *TMPRSS6* genetic variants, such as rs855791, associate with serum iron and transferrin saturation, hemoglobin (Hb), and erythrocyte (MCV and MCH) traits in different populations.[18-24] We and others have shown that *Tmprss6* haploinsufficient mice have an increased susceptibility to iron deficiency.[9,25] Altogether these results suggest that *TMPRSS6* gene dosage may modify erythropoiesis and influence *HAMP* expression.

β-thalassemias are severe recessive disorders because of mutations of β-globin genes that lead to defective globin chain synthesis, transfusion-dependent microcytic anemia, ineffective erythropoiesis, shortened red cell survival, and secondary iron overload. β-thalassemia intermedia results from globin gene mutations with less profound effects.[26] The degree of anemia is compatible with survival even without transfusions, but iron overload develops in the liver, heart, pancreas, and other organs. Excess iron leads to oxidative damage as a result of the generation of reactive oxygen species and cardiac iron toxicity is the primary cause of death in patients with β-thalassemia syndromes.[27]

The *Hbb*$^{th3/+}$ mouse exhibits features similar to β-thalassemia intermedia in humans, including Hb levels between 7 and 9 g/dL, aberrant erythrocyte morphology, increased reticulocyte count, ineffective and extramedullary erythropoiesis, hepato-splenomegaly, and liver and spleen iron overload,[28] a complex phenotype which worsens with aging.[29,30]

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HAMP is deficient in humans with thalassemia intermedia.[31,32] Liver *Hamp* mRNA is decreased in young *Hbb*$^{th3/+}$ mice,[29,33,34] but increases with age, eventually reaching the same level of WT mice.[30] However, the up-regulation of *Hamp* is not proportional to the increased iron accumulation observed in the *Hbb*$^{th3/+}$ animals,[30] suggesting that the inappropriately low Hamp plays a role in iron overload.

Iron manipulation may improve anemia in thalassemic mice.[35] Infusions of transferrin were first used to ameliorate anemia in a model (th1/th1) of mild thalassemia intermedia.[36] Limited dietary iron restriction might be beneficial in the short-term in *Hbb*$^{th3/+}$ mice, reducing iron overload and improving anemia and splenomegaly.[37] In *Hamp* transgenic animals transplanted with a thalassemic bone marrow, a partial correction of the phenotype was observed, but the positive effect was strictly dependent on the *Hamp* gene copy number.[37] Whether the effect is dependent solely on iron reduction, solely on an increase in Hamp, or a combination of both remains unclear.

These studies suggest that therapeutic strategies aimed at increasing HAMP levels or the use of HAMP agonists might decrease abnormal iron absorption and improve the anemia in humans with β-thalassemia.[35]

Here we asked whether inactivation of the *Hamp* inhibitor *Tmprss6* would up-regulate *Hamp* and rescue the phenotype of the *Hbb*$^{th3/+}$ mouse model.

Methods

Mouse models

C57BL6/Hbb^{th3} (th3/+) mice (The Jackson Laboratory) were maintained in heterozygosity by breeding with C57BL/6N mice (Charles River) and genetic screening.

Tmprss6$^{-/-}$ mouse on a mixed C57BL/6-Sv129 background was kindly provided by Prof C. Lopez-Otin (University of Oviedo, Spain). The animals were maintained in the animal facility of San Raffaele Scientific Institute (Milan, Italy) in accordance with the European Union guidelines. The study was approved by the Institutional Animal Care and Use Committee of the San Raffaele Scientific Institute.

We bred *Hbb*$^{th3/+}$ to *Tmprss6*$^{+/-}$ mice and then intercrossed the *Tmprss6*$^{+/-}$ *Hbb*$^{th3/+}$ and the *Tmprss6*$^{+/-}$ progeny to generate various genotype combinations. Mice were given a standard diet and males and females were analyzed separately. For hematologic analyses, blood was collected by tail vein puncture into tubes containing 40 mg/mL EDTA (ethylenediaminetetraacetic acid) in 1-, 2-, 4-, and 6-month-old animals. Only for 6-month-old animals, blood was collected for erythropoietin (Epo) quantification before sacrifice. After sacrifice, livers and spleens were weighed, dissected, and snap-frozen immediately for RNA analysis or dried for tissue iron quantification or processed for fluorescence-activated cell sorter (FACS) analysis.

Hematologic analysis

Hemoglobin (Hb) concentration, red blood cell (RBC) counts, and erythrocyte indexes (MCV, MCH) were measured on the Sysmex KX-21 automated blood cell analyser (Sysmex America). Blood smears were stained with May-Grunwald-Giemsa and photomicrographs were obtained using a Nikon Eclipse E6000 Microscope with a Nikon DXM1200 Digital Camera and analyzed with the Nikon Act-1 Version 2.20 software (Nikon).

Serum Epo was measured using mouse Epo quantikine set (R&D Systems), according to the manufacturer's instructions.

Tissue iron content

To measure iron concentration, tissue samples were dried at 110°C overnight, weighed, and digested in 1 mL of acid solution (3M HCl, 0.6M trichloroacetic acid) for 20 hours at 65°C. The clear acid extract was added to 1 mL of working chromogen reagent (1 volume of 0.1% bathophenanthroline sulfate and 1% thioglycolic acid solution, 5 volumes of water, and 5 volumes of saturated sodium acetate). The solutions were then incubated for 30 minutes at room temperature until color development and the absorbance measured at 535 nm. A standard curve was plotted using an acid solution containing increasing amounts of iron diluted from a stock solution of Titrisol iron standard (Merck).

Quantitative RT-PCR

Total RNA was extracted from murine liver and spleen using the guanidinium thiocyanate-phenol-chloroform method (Trizol Reagent), following the manufacturer's (Invitrogen) recommendations. RNA (2 μg) was used for quantitative polymerase chain reaction (qPCR) analysis for first-strand synthesis of cDNA with the High Capacity cDNA reverse transcription kit (Applied Biosystems), according to the manufacturer's instructions. For real-time PCR analysis, specific murine assays-on-demand products (20×) and TaqMan master mix (2×) from Applied Biosystems were used, according to the manufacturer's instructions, and the reactions were run on 7900HT Fast real-time PCR System (Applied Biosystems) in a final volume of 20 μL. Each cDNA sample was amplified in triplicate and the RNA level was normalized to the corresponding level of *Hprt1* mRNA. Primers used for qRT-PCR are shown in supplemental Table 1 (available on the *Blood* Web site, see the Supplemental Materials link at the top of the online article).

Flow cytometry

Splenic cells were incubated with purified rat anti–mouse CD16/CD32 antibody (Mouse BD FcBlock, 2.4G2; BD Pharmingen) and 1% FBS to block unspecific binding, stained with a phycoerythrin (PE)–conjugated rat anti–mouse CD71 antibody (C2; PharMingen) and a fluorescein isothiocyanate (FITC)–conjugated rat anti–mouse TER-119 antibody (TER-119; BD PharMingen). Analyses by FACS were performed using FACSCanto flow cytometer (Becton Dickinson).

Percentages of reticulocytes were determined by flow cytometry after staining with thiazole orange dye (BD Biosciences).

Statistics

Data are presented as mean ± SD. Unpaired 2-tailed Student t test was performed using Prism 4.0 (GraphPad). $P < .05$ was considered statistically significant.

Results

Homozygous loss of *Tmprss6* ameliorates anemia and improves ineffective erythropoiesis of *Hbb*$^{th3/+}$ mice

We generated double mutant animals by breeding and screening *Tmprss6* and *Hbb*$^{th3/+}$ knockout mice. First, we analyzed the effect of homozygous loss of *Tmprss6* on the phenotype of *Hbb*$^{th3/+}$ mice. We analyzed the hematologic parameters at 1, 2, 4, and 6 months of age, to explore possible age-dependent variation. In addition, because in wild-type (WT) males Hb levels are higher than in females at 1 ($P = .039$), 2 ($P = .044$), and 4 months ($P = .017$), we performed sex specific analysis. Consistent with previous studies, both male and female *Hbb*$^{th3/+}$ mice harboring 2 WT *Tmprss6* alleles have lower RBC count, Hb levels (Figure 1A), mean corpuscular volume (MCV), and mean corpuscular Hb (MCH, not shown) than WT littermates and all these parameters decrease at 6 months of age.

Six-month-old *Tmprss6*$^{-/-}$ mice have lower Hb levels, but RBCs comparable with those of WT littermates (Figure 1A), because of severe microcytosis. In thalassemic mice the homozygous loss of *Tmprss6* significantly increases RBC count of approximately 30% and Hb levels of approximately 15% at all ages analyzed (Figure 1A).

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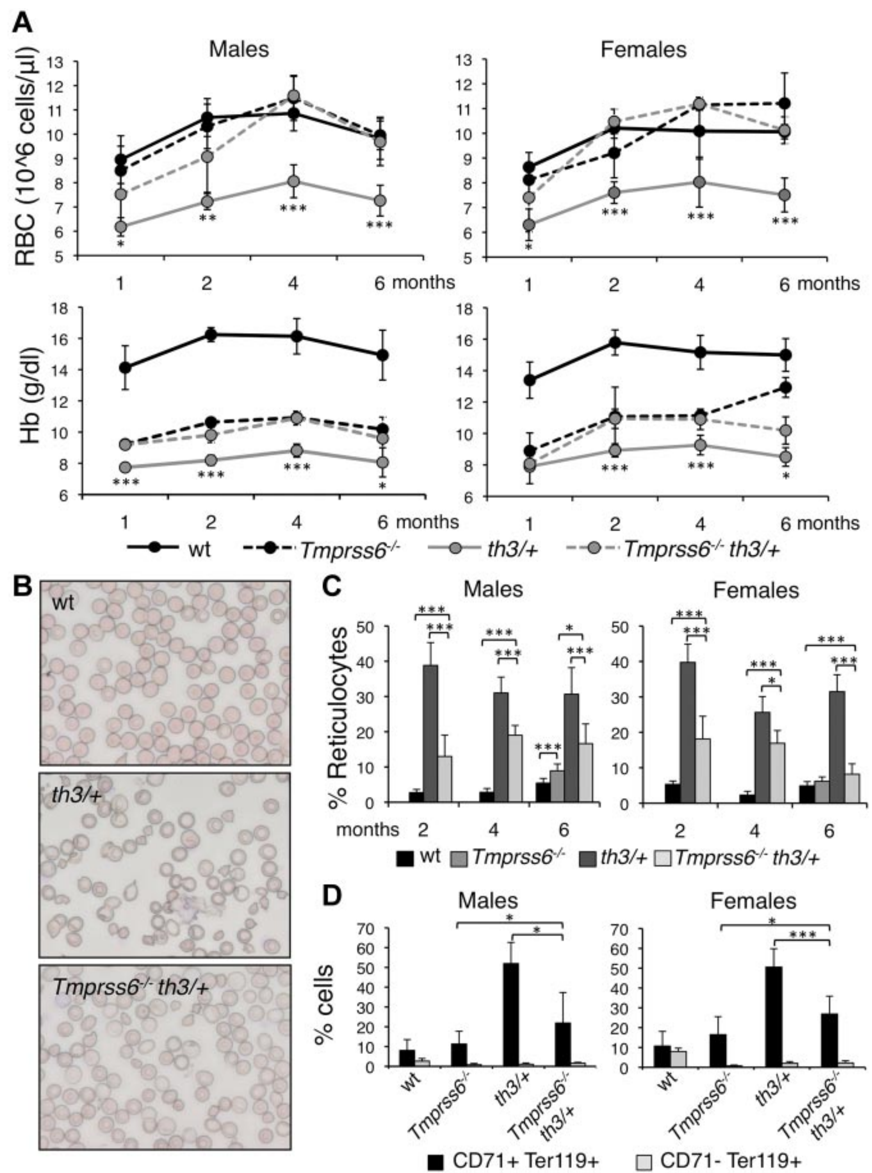

Figure 1. Effect of *Tmprss6* inactivation on hematologic parameters and erythroid maturation of thalassemic mice. (A) Time course (1, 2, 4, and 6 months of age) analysis of RBC count and Hb levels of male and female WT, *Tmprss6*$^{-/-}$, *Hbb*$^{th3/+}$ (*th3/+*), and *Tmprss6*$^{-/-}$ *Hbb*$^{th3/+}$ (*Tmprss6*$^{-/-}$ *th3/+*) mice. Asterisks refer to a statistically significant difference between *Hbb*$^{th3/+}$ and *Tmprss6*$^{-/-}$ *Hbb*$^{th3/+}$ mice. (B) Blood smears stained with May-Grunwald-Giemsa showing the morphology of RBC of representative WT, *Hbb*$^{th3/+}$ (*th3/+*), and *Tmprss6*$^{-/-}$*Hbb*$^{th3/+}$ (*Tmprss6*$^{-/-}$ *th3/+*) mice (Original magnification 40×). (C) Percentages of reticulocytes in peripheral blood of 2-, 4-, and 6-month-old male and female WT, *Tmprss6*$^{-/-}$ (only 6 months old), *Hbb*$^{th3/+}$ (*th3/+*), and *Tmprss6*$^{-/-}$*Hbb*$^{th3/+}$ (*Tmprss6*$^{-/-}$ *th3/+*) mice. (D) FACS analysis performed on splenic erythroid cells of 6-month-old male and female WT, *Tmprss6*$^{-/-}$, *Hbb*$^{th3/+}$ (*th3/+*), and *Tmprss6*$^{-/-}$*Hbb*$^{th3/+}$ (*Tmprss6*$^{-/-}$ *th3/+*) mice using CD71 (transferrin receptor 1) and Ter119 (erythroid specific) costaining. The graphs indicate the percentages of early erythroid precursors (CD71$^+$Ter119$^+$), which correspond to basophilic erythroblasts and late basophilic and chromatophilic erythroblasts, and those of mature erythroid cells (CD71$^-$ Ter119$^+$), which correspond to orthochromatophilic erythroblasts. Anucleated cells were excluded from analysis. Mean values of 4 to 8 animals for sex and genotype are graphed and error bars indicate SD. Asterisks refer to a statistically significant difference (*$P < .05$; **$P < .01$; ***$P < .005$). For complete statistical analysis see supplemental Table 2.

As expected, the erythropoiesis of 6-month-old *Hbb*$^{th3/+}$ mice was highly compromised, with all the hallmarks of ineffective erythropoiesis: abnormal RBC morphology (Figure 1B), increased percentage of reticulocytes (Figure 1C), high proportion of immature (CD71$^+$Ter119$^+$) erythroid progenitor cells in the spleen (Figure 1D), splenomegaly (Figure 2A) and remarkably high serum Epo levels (Figure 2B). Notably *Hbb*$^{th3/+}$ male mice have higher (1977.15 ± 780.40 pg/mL versus 1122.44 ± 253.36 pg/mL, $P = .022$) serum Epo levels than females in the presence of similar Hb levels (compatible with a greater difference in Hb levels between *Hbb*$^{th3/+}$ and WT mice in males than in females). Serum Epo levels are elevated as expected in the anemic *Tmprss6*$^{-/-}$ mice (Figure 2B). Genetic loss of *Tmprss6* significantly improves erythropoiesis in *Hbb*$^{th3/+}$ mice, ameliorating the erythrocytes morphology (Figure 1B), reducing the percentage of reticulocytes (Figure 1C), of immature cells of approximately 50% (Figure 1D) and spleen size (Figure 2A) without significantly changing serum Epo concentration (Figure 2B). In *Tmprss6*$^{-/-}$*Hbb*$^{th3/+}$ Epo levels persist higher in males than in females ($P = .014$).

Homozygous loss of Tmprss6 reduces tissue iron and increases *Hamp* mRNA levels of *Hbb*$^{th3/+}$ mice

In *Hbb*$^{th3/+}$ mice the erythropoietic abnormalities are accompanied by splenic (SIC; Figure 2C) and hepatic (LIC; Figure 2D) iron overload, which is more severe in females (LIC $P = .016$ vs males). Both parameters are reduced to levels similar to those in WT mice by loss of *Tmprss6* (Figure 2C-D).

At 6 months of age, *Hbb*$^{th3/+}$ and WT mice have similar liver *Hamp* mRNA levels (Figure 2E), as reported.[37] However, these levels are inappropriately low relative to LIC in *Hbb*$^{th3/+}$ mice (Figure 2F). The inactivation of *Tmprss6* increases *Hamp* expression in thalassemic mice, reaching levels comparable with those in *Tmprss6*$^{-/-}$ mutants (Figure 2E). In both sexes *Hamp* mRNA remains inappropriately high relative to LIC (Figure 2F). It should be noted that *Hamp* levels vary according to sex, with higher levels in females (Figure 2E), in response to their elevated iron stores (in control mice: Hamp $P = 4.31 \times 10^{-5}$; LIC $P = 3.21 \times 10^{-6}$; SIC $P = 3.54 \times 10^{-4}$; Figure 2C-D). This sex-dependent variation of total body iron was previously reported.[38,39]

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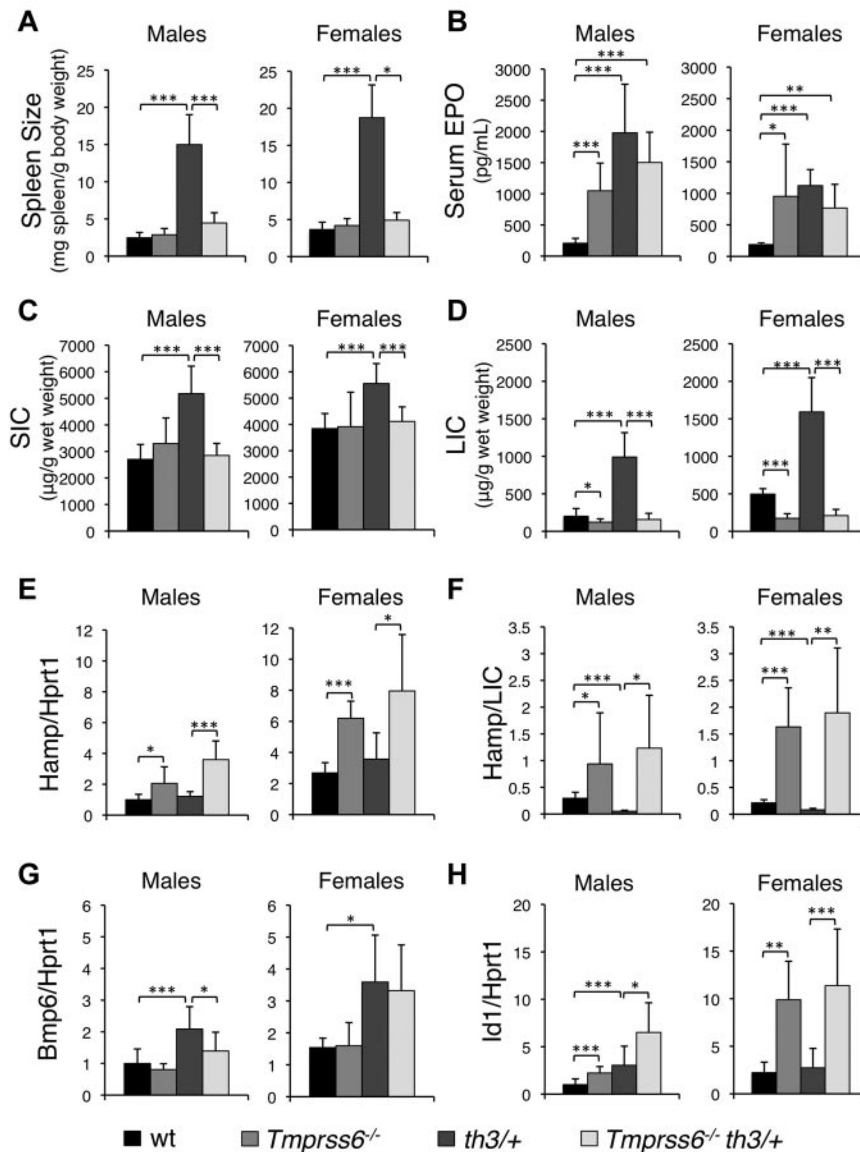

Figure 2. Effect of *Tmprss6* deletion on spleen size, serum EPO levels, and iron parameters of thalassemic mice. In the figure are graphed: spleen weights normalized to body weight (A); serum EPO levels (B); splenic (C); and hepatic (D) non-heme iron content (SIC and LIC); liver mRNA expression of hepcidin (*Hamp*; E), *Hamp* normalized on LIC (*Hamp*/LIC; F), *Bmp6* (G), and inhibitor of DNA binding 1 (*Id1*; H). mRNA expression ratio was normalized to a male WT mean value of 1. Mean values of 4 to 8 animals for sex and genotype (WT, $Tmprss6^{-/-}$, $Hbb^{th3/+}$ [*th3*/+], and $Tmprss6^{-/-}Hbb^{th3/+}$ [$Tmprss6^{-/-}th3/+$]) are graphed and error bars indicate SD. Asterisks refer to a statistically significant difference. (*$P < .05$; **$P < .01$; ***$P < .005$). For complete statistical analysis see supplemental Table 2.

Finally, we analyzed *Bmp6*, which is transcriptionally regulated by iron and controls both the expression of *Hamp* and other targets, such as *Id1*, *Smad7*, and *Atoh8*.[40] We noticed that *Bmp6* mRNA level in $Tmprss6^{-/-}$ overlaps that of WT mice (Figure 2G), in spite of significantly lower values of LIC (Figure 2D). As expected from the abnormal iron stores, and as previously observed[41] *Bmp6* is increased in thalassemic mice, especially in females ($P = .05$ vs males; Figure 2G). However, *Bmp6* levels are inappropriately low relative to LIC, a finding more evident in males (not shown). The expression of *Id1* (Figure 2H), *Smad7* (supplemental Figure 1A) and *Atoh8* (supplemental Figure 1B) is up-regulated in male $Hbb^{th3/+}$ compared with controls. Surprisingly, this is not the case among females. Compared with $Hbb^{th3/+}$, $Tmprss6^{-/-}Hbb^{th3/+}$ males have reduced *Bmp6* mRNA levels (Figure 2G), in agreement with the decreased LIC. They have elevated *Id1* (Figure 2H), *Smad7* (supplemental Figure 1A) and *Atoh8* (supplemental Figure 1B) as expected because lack of cleavage of Hjv from the plasma membrane of hepatocytes in the absence of *Tmprss6* induces a constitutive activation of the Bmp-Smad pathway. The same pathway is even more active in $Tmprss6^{-/-}Hbb^{th3/+}$ females that maintain high *Bmp6* levels despite a strong LIC reduction.

Tmprss6 haploinsufficiency ameliorates the phenotype of $Hbb^{th3/+}$ male mice

Because *Tmprss6* haploinsufficient mice show increased susceptibility to iron deficiency,[9,25] we analyzed the effect of the genetic loss of a single *Tmprss6* allele in $Hbb^{th3/+}$ mice. Among thalassemic males heterozygous loss of *Tmprss6* increases RBC count and reduces MCV at 6 months, with an even earlier positive effect on Hb (Figure 3 left panels). The increased number of RBC with a decreased size indicates that iron availability for erythropoiesis is restricted.[36] In females we observed the same trend documented in males for all parameters, but the difference between $Hbb^{th3/+}$ *and* $Tmprss6^{+/-}Hbb^{th3/+}$ mice was statistically significant only for RB count at 6 months of age (Figure 3 right panels).

In male mice, the improvement of the hematologic parameters is accompanied by a statistically significant reduction of spleen size (Figure 4A), serum Epo (Figure 4B), and LIC (Figure 4D). SIC, measured as micrograms iron/grams tissue, is not affected (Figure 4C). However, total splenic iron is reduced in $Tmprss6^{+/-}Hbb^{th3/+}$ mice compared with the $Hbb^{th3/+}$ after spleen volume reduction (not shown). In females the difference in spleen size, Epo levels,

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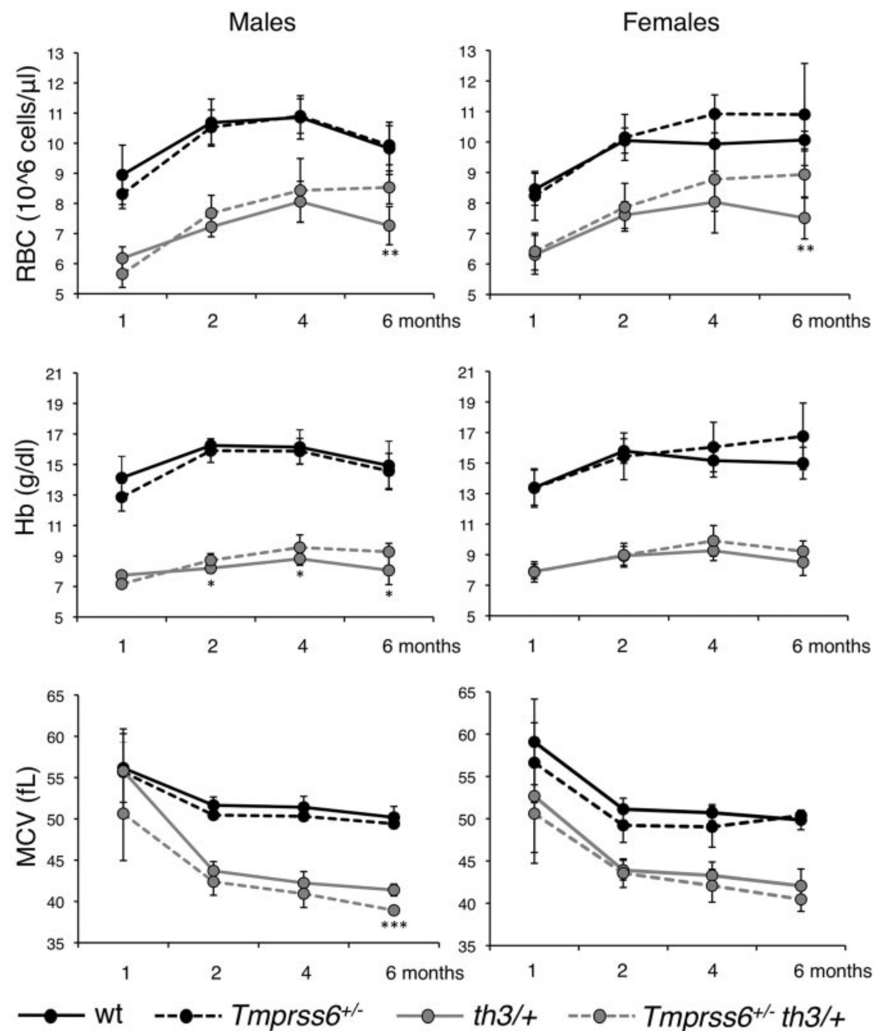

Figure 3. Effect of *Tmprss6* haploinsufficiency on hematologic parameters of thalassemic mice. Time course analysis of RBC count, Hb levels and MCV of male and female WT, *Tmprss6*$^{+/-}$, *Hbb*$^{th3/+}$ (*th3/+*) and *Tmprss6*$^{+/-}$*Hbb*$^{th3/+}$ (*Tmprss6*$^{+/-}$*th3/+*) mice. Mean values of 4 to 8 animals for sex and genotype are graphed and error bars indicate SD. Asterisks refer to a statistically significant difference between *Hbb*$^{th3/+}$ and *Tmprss6*$^{+/-}$*Hbb*$^{th3/+}$ mice (*$P < .05$; **$P < .01$; ***$P < .005$). For complete statistical analysis see supplemental Table 3.

SIC and LIC between *Tmprss6*$^{+/-}$*Hbb*$^{th3/+}$ and *Hbb*$^{th3/+}$ mice is not significant, although the trend is toward an improved phenotype associated with the heterozygous loss of *Tmprss6* allele (Figure 4A-D). The different effect of the *Tmprss6* haploinsufficiency in thalassemic male and female mice is probably because of the remarkably higher LIC in females (Figure 4D).

The loss of one *Tmprss6* allele does not affect liver *Hamp* mRNA (supplemental Figure 2A). However, the *Hamp*/LIC ratio is significantly higher in *Tmprss6*$^{+/-}$*Hbb*$^{th3/+}$ male mice than in *Hbb*$^{th3/+}$, whereas among females the difference is not statistically significant (Figure 4E).

Although the *Bmp6* expression was unchanged (Figure 4F), the expression of target genes *Id1* (Figure 4G), *Smad7* (supplemental Figure 2B), and *Atoh8* (not shown) was partially reduced in *Tmprss6*$^{+/-}$*Hbb*$^{th3/+}$ compared with *Hbb*$^{th3/+}$ males, mirroring LIC. However, the difference reached statistical significance only for *Id1*. Among females no difference was observed between *Tmprss6*$^{+/-}$*Hbb*$^{th3/+}$ and *Hbb*$^{th3/+}$ in the expression of genes of the *Bmp-Smad* pathway.

In attempt to understand why the heterozygous loss of *Tmprss6* has a different effect on the phenotype according to sex, we measured *Tmprss6* expression in thalassemic mice. Compared with WT, *Tmprss6* is up-regulated in *Hbb*$^{th3/+}$ males, but not in females, probably because *Tmprss6* mRNA is constitutively higher in females than in males both in WT mice ($P = 5.95 \times 10^{-5}$) and in Hbb$^{th3/+}$ mice ($P = 9.06 \times 10^{-6}$) because of higher LIC and *Bmp6* levels (Figure 4H).

Discussion

Iron overload is the primary cause of death in patients with β-thalassemia syndromes, because of heart toxicity.[27] Iron overload occurs not only in transfused patients but also in thalassemia intermedia patients who, because of milder genetic defects, survive without the need of blood transfusions.[42] In these patients and in the corresponding animal models, increased intestinal iron absorption and recycling occurs despite replete iron stores, driven by a signal from the expanded erythropoiesis that causes low/inappropriate hepcidin production (the so called erythroid regulator).[43] Indeed, moderate *Hamp* increase in β-thalassemia mice limits dietary iron absorption, improving not only iron overload but also ineffective erythropoiesis, and anemia.[37]

Here we show that the genetic loss of *Tmprss6,* the most important *Hamp* inhibitor, results in an impressive improvement of anemia, ineffective erythropoiesis, and splenomegaly in *Hbb*$^{th3/+}$ mice. This benefit is present early in life and persists up to 6 months in both sexes. In the *Tmprss6*$^{-/-}$*Hbb*$^{th3/+}$ mice RBC count and Hb levels are increased compared with *Hbb*$^{th3/+}$ mice, with a reduction in the number of immature erythroid cells and reticulocytes. The

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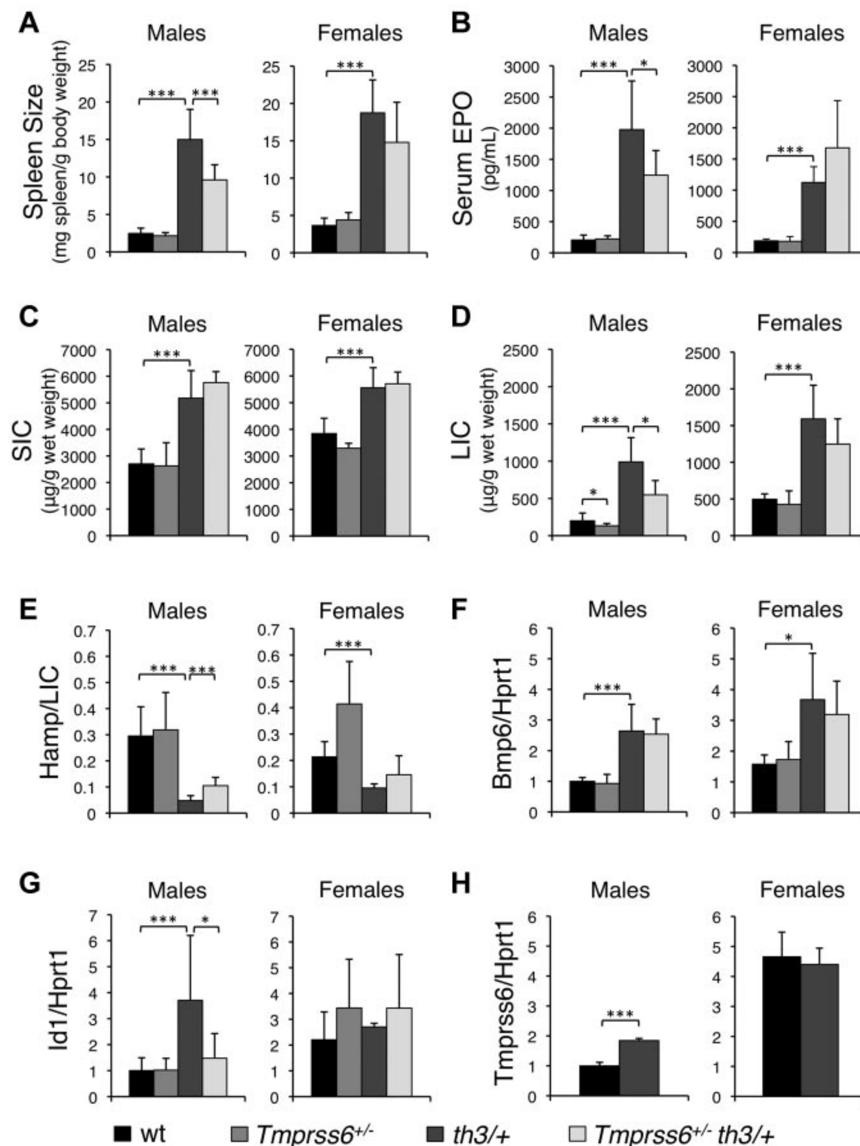

Figure 4. Effect of *Tmprss6* haploinsufficiency on spleen size, serum EPO levels, and iron-related parameters of thalassemic mice. In the figure are graphed: spleen weights normalized to body weight (A), serum EPO levels (B), splenic (C) and hepatic (D) non-heme iron content (SIC and LIC), liver mRNA expression of Hepcidin normalized on LIC (*Hamp*/LIC; E), *Bmp6* (F), inhibitor of DNA binding 1 (*Id1*; G), and *Tmprss6* (H). mRNA expression ratio was normalized to a male WT mean value of 1. Mean values of 4 to 8 animals for sex and genotype (WT, $Tmprss6^{+/-}$, $Hbb^{th3/+}$ [*th3/+*], and $Tmprss6^{+/-}Hbb^{th3/+}$ [$Tmprss6^{+/-}$ *th3/+*]) are graphed and error bars indicate SD. Asterisks refer to a statistically significant difference (*$P < .05$; **$P < .01$; ***$P < .005$). For complete statistical analysis see supplemental Table 3.

improved erythropoiesis was accompanied by profound changes in systemic iron homeostasis, with marked reduction of iron stores to levels comparable or even lower than WT mice. *Hamp* expression in the double-mutant mice is elevated, as in $Tmprss6^{-/-}$ mice, and was inappropriately high considering the LIC values.

Our results strengthen and extend previous findings on the beneficial effect of limiting iron supply in β-thalassemia. Compared with administration of transferrin[36] or dietary iron restriction,[37] that partially correct the established thalassemic phenotype of adult mice, a constitutionally high Hamp production prevents the full expression of the phenotype of the $Hbb^{th3/+}$ mouse from the first months of life. The effect is similar to that obtained in the *Hamp* transgenic mouse transplanted with a thalassemic bone marrow,[37] although our model is a more physiologic one. As expected, chronic increase of *Hamp* prevents iron overload in the $Hbb^{th3/+}$ mouse. However, the amelioration of anemia is not due to hepcidin increase but to the reduced body iron. The erythrocyte indexes (MCH and MCV) are further reduced in the double mutants compared with those in thalassemic mice, probably as the result of the heme synthesis reduction in the erythroblast. It was demonstrated that heme controls protein translation in erythroid cells through the modulation of the activity of the Heme-regulated eukaryotic initiation factor 2α (eIF2α) kinase (HRI). In conditions of heme deficiency HRI is activated and phosphorylates eIF2α, blocking the translation of proteins, in particular of globin chains.[44] For this reason it has been proposed that the reduced heme synthesis in iron deficiency activates HRI, partially correcting the globin chain imbalance that characterizes thalassemic syndromes.[37] Ours and previous results[36,37] indicate that reducing iron availability benefits thalassemic erythropoiesis reducing erythroblast premature death and improving RBC survival.

Our data clearly indicate that the modulation of iron absorption mediated by Tmprss6 is indispensable for the phenotype "iron overload/low hepcidin" observed in β-thalassemia. In addition, our results are relevant to define the erythroid regulator pathway. Anemia and some degrees of ineffective erythropoiesis, as shown by the proportion of immature red cell precursors compared with *Tmprss6* null mice, persist in the double-mutant mice and the Epo level is maintained high in accordance with similar findings in $Hbb^{th3/+}$ mice overexpressing *Hamp*.[37] $Tmprss6^{-/-}Hbb^{th3/+}$ animals seem resistant to the inhibitory effect of the erythroid regulator. The nature of this regulator is still under debate and how

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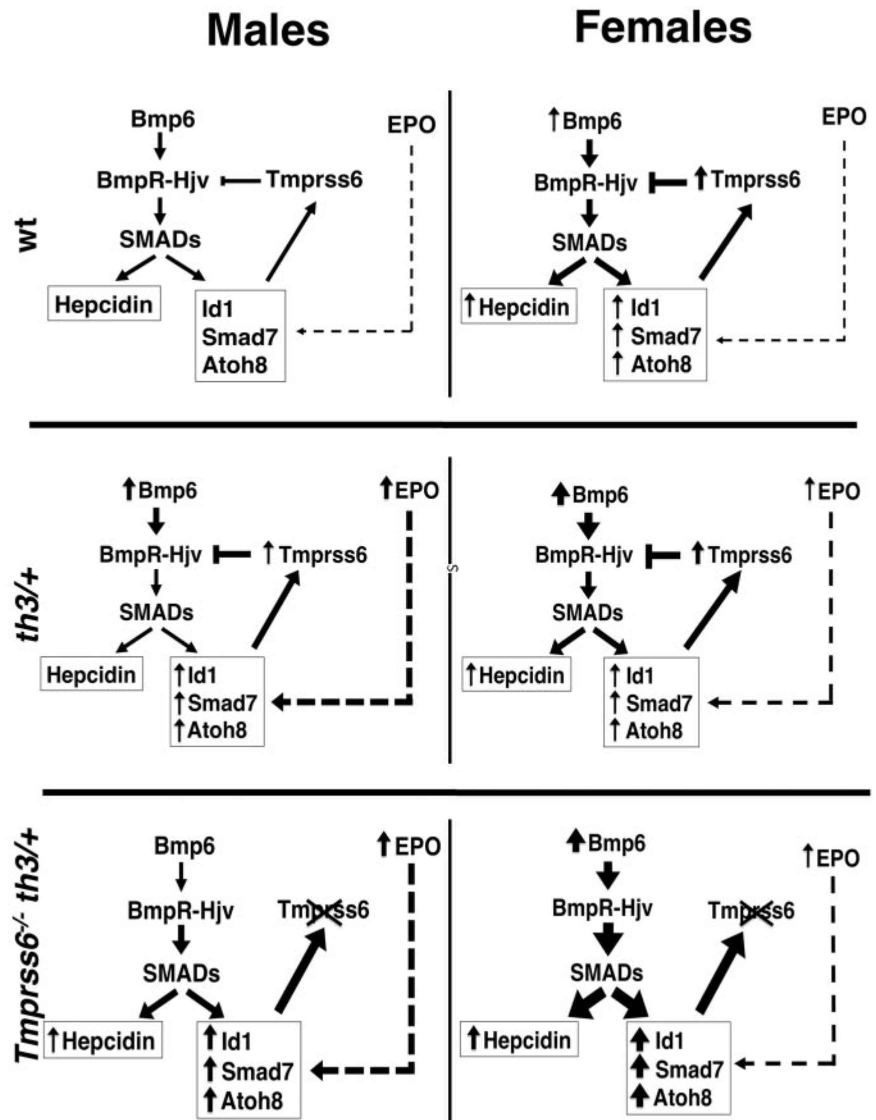

Figure 5. Schematic model of the proposed mechanism of hepcidin regulation. Representation of the proposed pathways of *Id1* control: the first Bmp6-dependent and the second EPO-dependent (see Discussion for details). The thickness of the arrows is proportional to the intensity of the signal. WT mice: low activation of the Bmp-Smad pathway causes basal transcription of *Hamp* (higher in females, because of their more abundant iron stores) and of the other targets (*Id1*, *Smad7*, and *Atoh8*). The latters are modulated also by the erythroid regulator through a still unknown pathway. Id1 induces transcription of *Tmprss6*, which by cleaving Hjv, inhibits *Hamp* transcription, activating a negative feedback loop.[47] $Hbb^{th3/+}$ (*th3/+*) mice: in males there is a significant iron overload which increases *Bmp6* production and a concurrent strong ineffective erythropoiesis which up-regulates *Id1* and consequently *Tmprss6* transcription. The balance between the positive (Bmp6) and the negative (Tmprss6) regulators of the Bmp-Smad pathway, leaves *Hamp* mRNA levels unchanged compared with WT mice. In the less anemic females the erythropoietic effect is lower and probably irrelevant on the *Id1* transcription, overcome by the strong up-regulation of *Bmp6* and resulting in Hamp levels similar to WT mice. $Tmprss6^{-/-}Hbb^{th3/+}$ mice: the loss of *Tmprss6* increases the activation of the Bmp-Smad pathway. The effect is more pronounced in females, because of their higher *Bmp6* levels. In the absence of *Tmprss6*, the erythroid factor is ineffective and *Hamp* transcription remains up-regulated.

it acts to suppress *Hamp* remains obscure. The prevalent view is that a role is played by cytokines, such as GDF15[45] or TWGS1,[46] released by an expanded erythroid marrow. However, liver and spleen *Gdf15* mRNA and spleen *Twsg1* mRNA of $Hbb^{th3/+}$ mice were not different from WT mice (data not shown). Independently from the nature of the regulator, we favor the hypothesis that Tmprss6 is a component of the erythroid regulator pathway.

How Tmprss6 might be related to the erythroid regulator activity? Recently it was demonstrated that increased *Id1* expression by increased Bmp6 may up-regulate *Tmprss6* transcription (and activity) in a negative feedback loop.[47] We noticed a striking sex difference in the activation of this pathway that could be informative to the Tmprss6/erythroid regulator relationship. In our model the *Bmp-Smad* pathway is properly regulated in the $Hbb^{th3/+}$ male mice with the only exception being *Hamp* expression. As expected from the high concentration of tissue iron, *Bmp6* and its target genes (*Id1*, *Smad7*, and *Atoh8*) are all up-regulated. On the contrary, in $Hbb^{th3/+}$ female mice *Bmp6* is correctly up-regulated in response to iron-loading, whereas *Hamp* and the other targets are all expressed at levels similar to those of WT mice. In an effort to understand the cause of the observed sex difference, we noticed that $Hbb^{th3/+}$ female mice are "less anemic" than males. Although male and female $Hbb^{th3/+}$ mice have similar Hb levels, WT females have lower levels than WT males. Thus, the difference between female $Hbb^{th3/+}$ mice and their WT counterparts is significantly smaller than the difference between $Hbb^{th3/+}$ and WT male mice. Accordingly, serum Epo levels, that roughly correspond to the degree of anemia and may be used as a surrogated signal of the expanded erythropoiesis, are significantly lower in female than in male $Hbb^{th3/+}$ mice. Because Epo is inversely related to *Hamp* levels, this is consistent with the higher levels of *Hamp* expression in females that have both increased *Bmp6* (high iron stores) and reduced activity of the erythroid regulator. According to the negative feedback loop, *Tmprss6* mRNA is higher in females than in males, secondary to high LIC and *Bmp6* levels. However, in our mixed background mice, *Tmprss6* is up-regulated in $Hbb^{th3/+}$ males (Figure 4H) compared with WT, but not in females, which have higher basal expression. These results are in accordance with the data of *Id1* mRNA expression and with previous reports[47]. With the limitation of the mixed genetic background of our $Hbb^{th3/+}$ mice and of expression level data obtained by RT-PCR, we propose a model for the interpretation of our findings, summarized in Figure 5. We hypothesize that 2 pathways control *Id1*: one is Bmp6-dependent within the negative feedback of *Hamp* regulation[47]; we

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speculate that the second is erythroid (Epo)–dependent and increases *Id1* and *Tmprss6* independently of *Bmp6* expression. In our model the former is prevalent in females with higher tissue iron, the second in males with lower *Bmp6*. Further studies will explore whether increased *Tmprss6* transcription corresponds to enhanced Tmprss6 activity, as proposed.[47]

In the double-mutant mice, the *Id1* pathway was greatly induced especially in females, which have higher *Bmp6* expression secondary to higher LIC, but it is inefficient because of the lack of the *Tmprss6* target.

Our interpretation is further supported by the sex differences in the phenotype of $Tmprss6^{+/-}Hbb^{th3/+}$ mice. We and others[9,25] previously demonstrated an increased susceptibility to iron deficiency in *Tmprss6* haploinsufficient mice. For this reason, we explored the effect of the genetic loss of a single *Tmprss6* allele in $Hbb^{th3/+}$ mice. Again we observed a striking sex difference in the response to *Tmprss6* haploinsufficiency. In male mice the loss of one *Tmprss6* allele partially rescued the thalassemic phenotype, improved anemia and erythropoiesis, reduced iron-loading, and increased *Hamp*/LIC ratio. In female mice we observed a trend toward the same behavior, but the differences between $Hbb^{th3/+}$ and $Tmprss6^{+/-}Hbb^{th3/+}$ mice did not reach statistical significance for all parameters examined (with the exception of RBC count at 6 months of age). We hypothesize that the different response observed was related to a more active Bmp6 pathway because of higher tissue iron loading in females compared with males. The genetic loss of a single *Tmprss6* allele has positive effects in males with higher activation of the Epo-dependent signaling, whereas in females with a more active *Bmp6* pathway the effect is less evident.

Bmp6 remains remarkably elevated in the double-mutant, notwithstanding the strong LIC reduction. A similar phenomenon was observed in our *Tmprss6* null mice of both sexes but not in another report.[9] Whether Tmprss6 loss leads to a different threshold for *Bmp6* activation by iron or may variably increase the stored iron requires further studies.

In conclusion, our results strengthen the relevance of the balance between *BMP6* and *TMPRSS6* activities in hepcidin regulation and indicate that manipulating the Hamp pathway is of benefit in β-thalassemia in terms of improvement of anemia and prevention of iron overload. In addition, the elevated *Hamp* levels in the double-mutant suggest that the erythroid regulator requires *Tmprss6* activity to inhibit *Hamp* in conditions characterized by ineffective erythropoiesis.

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Authorship

Contribution: A.N. designed the experimental work, performed research, and cowrote the paper; A.P., G.M., M.R.L., and L.S. performed research and analyzed data; G.F. contributed to the experimental design and to write the paper; and C.C. designed research and wrote the paper.

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