Genetic loss of *Tmprss6* alters terminal erythroid differentiation in a mouse model of β-thalassemia intermedia

In β-thalassemia intermedia (TI), circulating levels of the hepcidin hormone are inappropriately low in relation to hepatic iron stores, promoting excessive absorption of dietary iron. Additionally, precipitation of unpaired α-globin chains causes oxidative damage that increases erythroid precursor (EP) apoptosis and shortens erythrocyte lifespan.[1] TMPRSS6 is a hepatic protease that inhibits bone morphogenetic protein (BMP) signaling for hepcidin production.[2] In the $Hbb^{th3/+}$ TI mouse model, abolishing or reducing expression of TMPRSS6 has been shown to attenuate iron loading and improve anemia.[3-5] Here, we define the consequences of genetic loss of *Tmprss6* on erythroid maturation in $Hbb^{th3/+}$ mice on a C57BL/6 genetic background. We show that *Tmprss6* loss in $Hbb^{th3/+}$ mice decreases mean corpuscular hemoglobin (MCH), decreases membrane-bound globins in erythrocytes, attenuates erythroid expansion in bone marrow (BM) and spleen, and produces a terminal erythroid maturation profile indistinguishable from $Tmprss6^{-/-}$ mice. Additionally, *Tmprss6* loss in $Hbb^{th3/+}$ mice decreases apoptosis and reactive oxygen species (ROS) in late EP and erythrocytes, raises peripheral red blood cell (RBC) count, decreases reticulocytes, and decreases renal and hepatic *Epo* mRNA, indicating greater efficacy of erythropoiesis. These findings demonstrate that genetic loss of *Tmprss6* determines terminal erythroid differentiation in $Hbb^{th3/+}$ mice.

By breeding $Tmprss6^{+/-}$ and $Hbb^{th3/+}$ mice of C57BL/6 genetic background, we found that homozygous *Tmprss6* loss in $Hbb^{th3/+}$ mice produced organ iron deficiency, limited splenomegaly, raised hepatic expression of hepcidin (*Hamp*) and other BMP target genes (*Id1, Atoh8*), and lowered serum iron and transferrin saturation (*Online Supplementary Table S1* and *Online Supplementary Figures S1-S4*). Organ non-heme iron, serum iron, and transferrin saturation levels were similar in $Hbb^{th3/+}Tmprss6^{-/-}$ and $Hbb^{+/+}Tmprss6^{-/-}$ mice, and hepatic *Hamp, Id1*, and *Atoh8* in $Hbb^{th3/+}Tmprss6^{-/-}$ mice matched or exceeded levels in $Hbb^{+/+}Tmprss6^{-/-}$ mice. Heterozygous *Tmprss6* loss only modestly altered iron-related parameters of $Hbb^{th3/+}$mice.

Hemoglobin and hematocrit levels were not improved by homozygous *Tmprss6* loss in $Hbb^{th3/+}$ mice (Figure 1A and B and *Online Supplementary Tables S1-S3*). However, homozygous *Tmprss6* loss in $Hbb^{th3/+}$ mice altered erythrocyte morphology (*Online Supplementary Figure S5A*), raised RBC, and lowered mean corpuscular volume (MCV), MCH, red cell distribution width, and reticulocytes (which likely contributes to the lower MCV) (Figure 1C-F and *Online Supplementary Tables S1-S3*). While $Hbb^{th3/+}Tmprss6^{+/+}$ spleens showed disrupted follicular architecture with red pulp EP expansion, follicular architecture was retained in $Hbb^{th3/+}Tmprss6^{-/-}$ spleens (*Online Supplementary Figure S5B*). Red cell indices, erythrocyte morphology, and splenic histology of $Hbb^{th3/+}Tmprss6^{-/-}$ and $Hbb^{+/+}Tmprss6^{-/-}$ mice were similar. Erythrocyte α-globin precipitates were moderately and severely reduced by heterozygous and homozygous *Tmprss6* loss in $Hbb^{th3/+}$ mice, respectively (Figure 1G), despite persistent globin chain mRNA imbalance in BM and spleen (*Online Supplementary Figure S6A and B*).

By an established method that characterizes stages of erythroid maturation,[6] the percentage of total cells that correspond to EP (i.e. cells in regions I-IV) was higher in $Hbb^{th3/+}Tmprss6^{+/+}$ mice than wild-type in both BM (55% *vs.* 31%; $P<0.0005$) (Figure 2A) and spleen (47% *vs.* 3%; $P<0.00005$) (*Online Supplementary Figure S7A*). *Tmprss6* loss in $Hbb^{th3/+}$ mice lowered the percentage of total cells that correspond to EP in BM (43%; $P<0.005$) and spleen (6%; $P<5\times10^{-7}$). The fact that $Hbb^{th3/+}Tmprss6^{+/+}$ and $Hbb^{th3/+}Tmprss6^{-/-}$ females maintained similar hemoglobin levels, even though $Hbb^{th3/+}Tmprss6^{-/-}$ females showed attenuated splenomegaly and fewer EP in BM and spleen, indicates that *Tmprss6* loss improves effectiveness of erythropoiesis in $Hbb^{th3/+}$ mice.

In $Hbb^{th3/+}Tmprss6^{-/-}$ and $Hbb^{+/+}Tmprss6^{-/-}$ mice, the percentage of total cells that correspond to EP was remarkably similar in BM ($P=0.59$) and in spleen ($P=0.95$). In each early EP region (I-III) in BM, transferrin receptor (CD71) expression was significantly higher in $Hbb^{th3/+}Tmprss6^{-/-}$ mice than $Hbb^{th3/+}Tmprss6^{+/+}$ mice, paralleling the level of hypoferremia, while CD71 expression in $Hbb^{th3/+}Tmprss6^{-/-}$ and $Hbb^{+/+}Tmprss6^{-/-}$ mice was similarly elevated (Figure 2B). In splenic early EP, CD71 expression by genotype was more variable (*Online Supplementary Figure S7B*).

The distribution of EP among regions I-IV in both BM and spleen was affected by genotype ($P<0.0001$ and $P<0.0001$, respectively) (Figure 2A and *Online Supplementary Figure S7A*). In $Hbb^{+/+}Tmprss6^{+/+}$ BM, the relative size of each EP population (as a percentage of total BM cells) increased progressively from regions I to IV. By contrast, in $Hbb^{th3/+}Tmprss6^{+/+}$ mice, the percentage of BM EP was lower in region IV (20%) than region III (23%), compatible with the increased α-globin-mediated apoptosis described in $Hbb^{th3/+}$ region IV EP.[7] Indeed, *Tmprss6* loss reduced the percentage of apoptotic erythroid cells in regions IV and V in BM (Figure 2C) and spleen (*Online Supplementary Figure S7C*) of $Hbb^{th3/+}$ mice; *Tmprss6* loss also reduced ROS levels in these populations (Figure 2D and E and *Online Supplementary Figure S7D and E*). Nevertheless, in $Hbb^{th3/+}Tmprss6^{-/-}$ BM, the percentage of EP in region IV (15%) remained lower than in region III (19%), suggesting that factors other than apoptosis regulate the size of the region IV EP population in iron deficiency. The distribution of EP among regions I-IV in $Hbb^{th3/+}Tmprss6^{-/-}$ mice was remarkably similar to that of $Hbb^{+/+}Tmprss6^{-/-}$ mice in BM and spleen ($P=0.58$ and $P=0.95$, respectively), demonstrating that erythroid maturation was dictated by *Tmprss6* genotype, rather than effects of impaired β-globin production.

Reactive oxygen species promote cell membrane damage and impair erythrocyte deformability, decreasing oxygen-delivering capacity.[8] Consistent with improved tissue oxygenation due to a reduction in erythrocyte ROS, *Tmprss6* loss in $Hbb^{th3/+}$ mice caused a significant reduction in erythropoietin (*Epo*) mRNA in kidney (Figure 3A) and liver (*Online Supplementary Figure S8*). However, the high serum erythropoietin levels of $Hbb^{th3/+}$ mice were not lowered by *Tmprss6* loss (Figure 3B), perhaps because *Tmprss6* loss reduces the number of EP available to consume erythropoietin.

In $Hbb^{th3/+}Tmprss6^{-/-}$ and $Hbb^{+/+}Tmprss6^{-/-}$ mice, BM levels of specific mRNA expressed during terminal erythroid maturation (*Erfe, Gdf15, Ahsp*) were higher than corresponding levels in $Hbb^{th3/+}Tmprss6^{+/+}$ mice when normalized to α-globin to adjust for genotype-specific differences in BM erythroid content (Figure 3C-E). The respective BM expression of *Erfe, Gdf15*, and *Ahsp* was similar in $Hbb^{th3/+}Tmprss6^{-/-}$ and $Hbb^{+/+}Tmprss6^{-/-}$ mice. In spleen, *Erfe* levels in $Hbb^{th3/+}Tmprss6^{-/-}$ and $Hbb^{+/+}Tmprss6^{-/-}$ mice were higher than $Hbb^{th3/+}Tmprss6^{+/+}$ mice when adjusted for erythroid content (Figure 3F).

Transgenic hepcidin overexpression,[7] low-iron diet,[7]

and transferrin injection[9] improve erythropoiesis in TI mouse models; the underlying mechanism(s), while still uncertain, appear to be related to altered iron availability to EP. Because *Tmprss6* is expressed in liver but not hematopoietic cells, erythropoietic consequences of *Tmprss6* loss likely occur secondary to the primary physiological consequence of *Tmprss6* loss: hepcidin elevation leading to hypoferremia. Indeed, early EP of both $Hbb^{th3/+}Tmprss6^{-/-}$ and $Hbb^{+/+}Tmprss6^{-/-}$ mice showed markedly elevated CD71 expression, suggesting similar sensing of hypoferremia.

Given that treatment of $Hbb^{th3/+}$ mice with *Tmprss6*-siRNA improved erythrocyte survival,[4] the ability of *Tmprss6* genetic loss to raise RBC in $Hbb^{th3/+}$ mice likely reflects, at least in part, an improvement in erythrocyte lifespan. Because RBC and reticulocyte count in both $Hbb^{th3/+}Tmprss6^{-/-}$ and $Hbb^{+/+}Tmprss6^{-/-}$ mice were higher than wild type, we hypothesize that *Tmprss6* loss increases the rate of reticulocyte release into the circulation. However, because decreased erythrocyte lifespan has been reported in patients with iron deficiency anemia,[10] it is possible that the erythrocyte lifespan of mice with *Tmprss6* loss remains shorter than wild type.

Notably, on a C57BL/6 background, *Tmprss6* loss in $Hbb^{th3/+}$ mice not only raised RBC but indeed simultaneously lowered MCH; therefore, hemoglobin did not

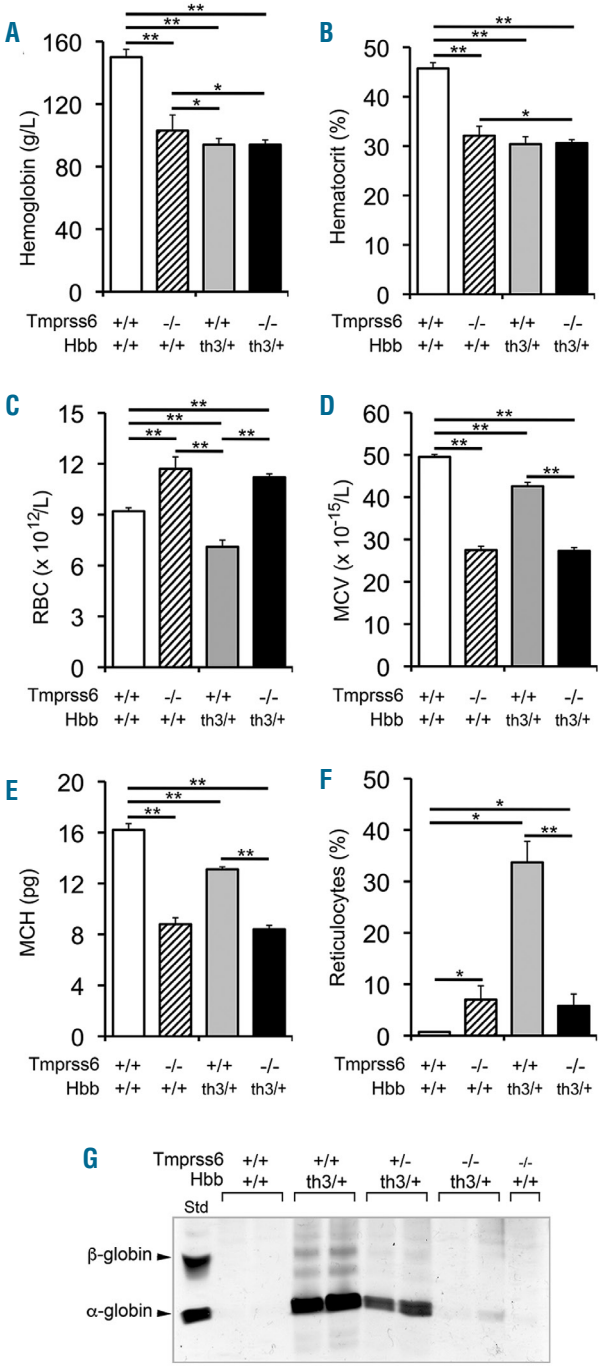

Figure 1. Genetic loss of *Tmprss6* in $Hbb^{th3/+}$ mice produces red blood cell indices consistent with iron-restricted erythropoiesis and reduces membrane-bound globins in erythrocytes. Shown for 8-week-old female mice of selected *Hbb-Tmprss6* genotypes are mean values obtained from analyses of hemoglobin level (A), hematocrit (B), RBC (C), MCV (D), MCH (E), and reticulocyte count as a percentage of total red blood cells (F). Error bars represent SD. **P*<0.05; ***P*<0.005. (G) Shown is analysis of membrane-bound globins prepared from peripheral red blood cells of mice of selected *Hbb-Tmprss6* genotypes (males, 8-9 weeks old). Globins were fractionated by triton-acetic acid-urea gel electrophoresis and visualized by Coomassie stain. Sample loading was adjusted by RBC (from complete blood count), so that 1.5 × 10^8 erythrocytes are represented per lane. Std, globin standard. For panels A-E, 7 $Hbb^{+/+}Tmprss6^{+/+}$, 9 $Hbb^{+/+}Tmprss6^{-/-}$, 7 $Hbb^{th3/+}Tmprss6^{+/+}$, and 6 $Hbb^{th3/+}Tmprss6^{-/-}$ mice were analyzed. Reticulocytes were counted in 3-5 female mice per genotype. Mice were fed a 270 p.p.m. iron diet in panels A-F and a 200 p.p.m. iron diet in panel G.

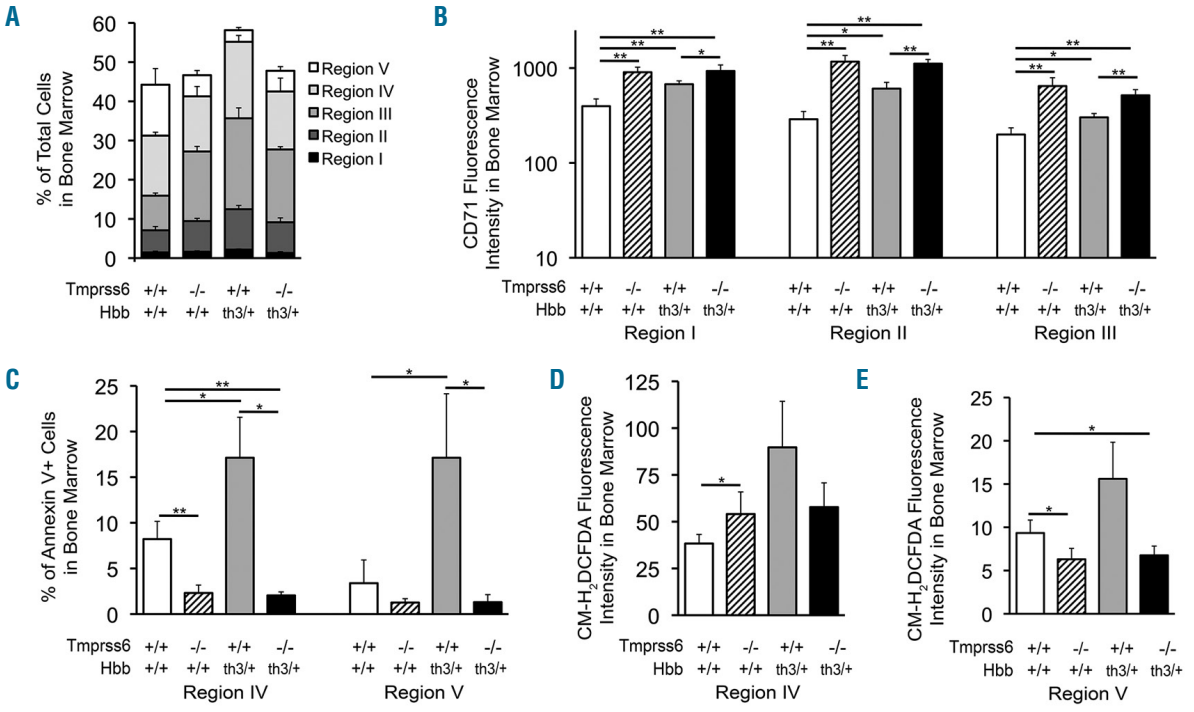

Figure 2. Genetic loss of *Tmprss6* alters the erythropoietic composition of BM of *Hbb*$^{th3/+}$ mice. Erythroid (TER-119^{+}) cells from BM of mice of selected *Hbb-Tmprss6* genotypes were grouped by stage of erythroid maturation based on forward scatter and CD44 expression, as illustrated in *Online Supplementary Figure 9*. This approach enables gating of populations that correspond morphologically to proerythroblasts (region I), basophilic erythroblasts (region II), polychromatic erythroblasts (region III), orthochromatic erythroblasts and immature reticulocytes (region IV), and mature erythrocytes (region V).(6) (A) Graphed is the mean percentage of total BM cells composed of TER-119^{+} cells distributed in regions I, II, III, IV, and V, respectively. (B) Graphed on a log scale is the mean fluorescence intensity of CD71 expression in BM erythroid cells distributed in regions I-III. (C) Graphed is the mean percentage of apoptotic (annexin V+) erythroid cells distributed in regions IV and V. Graphed is the mean fluorescence intensity of BM erythroid cells distributed in region IV (D) and region V (E) generated following incubation with the ROS indicator 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA). Error bars represent SD. For all panels, tissues from female mice (8-11 weeks old) were analyzed. *P<0.05; **P<0.005. For panel A, 5 *Hbb*$^{+/+}$*Tmprss6*$^{+/+}$, 6 *Hbb*$^{+/+}$*Tmprss6*$^{-/-}$, 4 *Hbb*$^{th3/+}$*Tmprss6*$^{+/+}$, and 6 *Hbb*$^{th3/+}$*Tmprss6*$^{-/-}$ mice were analyzed. For panel B, 4 *Hbb*$^{+/+}$*Tmprss6*$^{+/+}$, 5 *Hbb*$^{+/+}$*Tmprss6*$^{-/-}$, 3 *Hbb*$^{th3/+}$*Tmprss6*$^{+/+}$, and 5 *Hbb*$^{th3/+}$*Tmprss6*$^{-/-}$ mice were analyzed. For panel C, 5 *Hbb*$^{+/+}$*Tmprss6*$^{+/+}$, 6 *Hbb*$^{+/+}$*Tmprss6*$^{-/-}$, 4 *Hbb*$^{th3/+}$*Tmprss6*$^{+/+}$, and 5 *Hbb*$^{th3/+}$*Tmprss6*$^{-/-}$ mice were analyzed. For panels D and E, 4 *Hbb*$^{+/+}$*Tmprss6*$^{+/+}$, 5 *Hbb*$^{+/+}$*Tmprss6*$^{-/-}$, 3 *Hbb*$^{th3/+}$*Tmprss6*$^{+/+}$, and 4 *Hbb*$^{th3/+}$*Tmprss6*$^{-/-}$ mice were analyzed. Mice were fed a 200 p.p.m. iron diet.

improve. By contrast, on a C57BL/6-Sv129 mixed background, Nai *et al.* found that *Tmprss6* loss increased hemoglobin in *Hbb*$^{th3/+}$ mice.[3] Genetic background influences iron parameters, including hepcidin,[11] and *Tmprss6* loss produced a greater rise in hepcidin in our study. *Hbb*$^{th3/+}$*Tmprss6*$^{+/+}$ hemoglobin levels in our study, which were similar to 2-month old C57BL/6J *Hbb*$^{th3/+}$ mice that were also retro-orbitally-sampled,[12] were slightly higher than tail vein levels sampled by Nai *et al.*[3] Hemoglobin levels in *Hbb*$^{th3/+}$ mice decline with age,[12] which should be considered when comparing different studies employing this strain.[4,5]

Molecular chaperones, ubiquitin-mediated proteolysis, and autophagic pathways have been implicated in the degradation of excess α-globin in erythroid cells. In β-thalassemia, these protective mechanisms may become overwhelmed during erythroid maturation.[13] Because the MCH of *Hbb*$^{th3/+}$*Tmprss6*$^{-/-}$ mice is lower than *Hbb*$^{th3/+}$*Tmprss6*$^{+/+}$ mice, the absolute amount of excess α-globin per cell is also likely to be lower, enabling more effective cellular handling. Iron deficiency may also induce protective mechanisms;[14] indeed, the chaperone *α-hemoglobin stabilizing protein* (*Ahsp*) was up-regulated in total BM RNA from mice with *Tmprss6* loss.

Hepcidin suppression in TI has been attributed to effects of circulating factors, such as erythroferrone, that are released by erythroblasts.[1] The ability of *Tmprss6* loss to raise hepcidin in *Hbb*$^{th3/+}$ mice could indicate that: (i) the hepcidin-suppressing factors require TMPRSS6 for activity; or (ii) without functional TMPRSS6, production or efficacy of the hepcidin-suppressing factors is reduced. A recent study found that serum erythroferrone was markedly higher in *Hbb*$^{th3/+}$ mice than *Tmprss6*$^{-/-}$ mice, yet higher in *Tmprss6*$^{-/-}$ mice than wild-type controls.[15] All genotypes in our study, however, showed low or undetectable serum erythroferrone, possibly due to differences in blood collection methods or duration of sample storage (*E Nemeth, 2019, personal communication*). Notably, erythroferrone does not appear to modulate the hepcidin elevation caused by *Tmprss6* loss, as genetic loss of *Erfe* did not alter hepatic hepcidin mRNA or hematologic parameters in *Tmprss6*$^{-/-}$ mice.[15]

In summary, homozygous *Tmprss6* loss in *Hbb*$^{th3/+}$ mice results in terminal erythroid differentiation consistent with iron-restricted erythropoiesis. Our findings have relevance for application of *Tmprss6*-targeting therapies in TI, suggesting that TMPRSS6 inhibition will require careful titration to avoid exacerbation of anemia by iron restriction.

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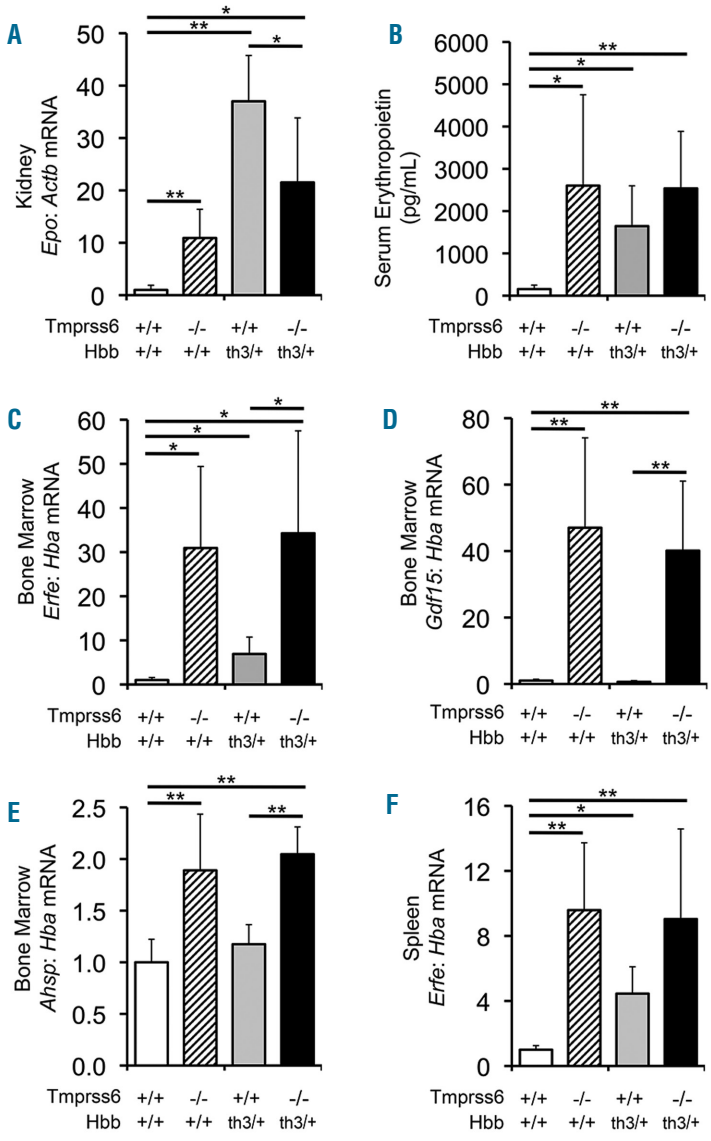

Figure 3. Genetic loss of *Tmprss6* lowers renal *Epo* expression and alters erythroid gene expression in *Hbb*$^{th3/+}$ mice. Graphed for selected *Hbb-Tmprss6* genotypes are mean values obtained from analyses of *Epo* mRNA relative to β-actin (*Actb*) in total kidney RNA (A), serum erythropoietin (B), *Erfe* mRNA relative to *Hba* in total BM RNA (C), *Gdf15* mRNA relative to *Hba* in total BM RNA (D), *Ahsp* mRNA relative to *Hba* in total BM RNA (E), and *Erfe* mRNA relative to *Hba* in total spleen RNA (F). Error bars represent SD. Eight-week-old female mice were analyzed in panels A-B, and 8-to-9-week-old male mice were analyzed in panels C-F. For panels A, C, D, E, and F, ratios of mRNA expression are normalized to an *Hbb*$^{+/+}$*Tmprss6*$^{+/+}$ mean value of 1. **P*<0.05; ***P*<0.005. For panel A, kidneys from 6 *Hbb*$^{+/+}$*Tmprss6*$^{+/+}$, 9 *Hbb*$^{+/+}$*Tmprss6*$^{-/-}$, 5 *Hbb*$^{th3/+}$*Tmprss6*$^{+/+}$, and 6 *Hbb*$^{th3/+}$*Tmprss6*$^{-/-}$ mice were analyzed. For panel B, serum from 6 *Hbb*$^{+/+}$*Tmprss6*$^{+/+}$ mice and 7 mice of all other genotypes was analyzed. For panels C-F, the number of mice analyzed was as follows: 7 *Hbb*$^{+/+}$*Tmprss6*$^{+/+}$, 7 *Hbb*$^{+/+}$*Tmprss6*$^{-/-}$, 5 *Hbb*$^{th3/+}$*Tmprss6*$^{+/+}$, and 7 *Hbb*$^{th3/+}$*Tmprss6*$^{-/-}$ (8 in panel F). Mice were fed a 200 p.p.m. iron diet.

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