The erythroid function of transferrin receptor 2 revealed by *Tmprss6* inactivation in different models of transferrin receptor 2 knockout mice

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ABSTRACT

Transferrin receptor 2 (TFR2) is a transmembrane glycoprotein expressed in the liver and in the erythroid compartment, mutated in a form of hereditary hemochromatosis. Hepatic TFR2, together with HFE, activates the transcription of the iron-regulator hepcidin, while erythroid TFR2 is a member of the erythropoietin receptor complex. The *TMPRSS6* gene, encoding the liver-expressed serine protease matriptase-2, is the main inhibitor of hepcidin and inactivation of *TMPRSS6* leads to iron deficiency with high hepcidin levels. Here we evaluate the phenotype resulting from the genetic loss of *Tmprss6* in *Tfr2* total (*Tfr2*$^{-/-}$) and liver-specific (*Tfr2*^{LCKO}) knockout mice. *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ and *Tmprss6*$^{-/-}$*Tfr2*^{LCKO} mice have increased hepcidin levels and show iron-deficiency anemia like *Tmprss6*$^{-/-}$ mice. However, while *Tmprss6*$^{-/-}$*Tfr2*^{LCKO} are phenotypically identical to *Tmprss6*$^{-/-}$ mice, *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ mice have increased red blood cell count and more severe microcytosis than *Tmprss6*$^{-/-}$ mice. In addition hepcidin expression in *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ mice is higher than in the wild-type animals, but lower than in *Tmprss6*$^{-/-}$ mice, suggesting partial inhibition of the hepcidin activating pathway. Our results prove that hepatic TFR2 acts upstream of TMPRSS6. In addition *Tfr2* deletion causes a relative erythrocytosis in iron-deficient mice, which likely attenuates the effect of over-expression of hepcidin in *Tmprss6*$^{-/-}$ mice. Since liver-specific deletion of *Tfr2* in *Tmprss6*$^{-/-}$ mice does not modify the erythrocyte count, we speculate that loss of *Tfr2* in the erythroid compartment accounts for the hematologic phenotype of *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ mice. We propose that TFR2 is a limiting factor for erythropoiesis, particularly in conditions of iron restriction.

Introduction

Transferrin receptor 2 (TFR2) is a transmembrane protein homologous to transferrin receptor 1 (TFR1) which is mutated in hereditary hemochromatosis type 3.[1,2] TFR2 is expressed in the liver and, to a lower extent, in erythroid cells.[3,4] TFR2 protein is stabilized on cell surface by binding to its ligand, diferric transferrin (holo-TF),[5] and, in a complex with the hemochromatosis protein HFE, is considered a sensor of circulating iron. In the current model in conditions of iron deficiency HFE associates with TFR1; inversely, when transferrin saturation increases, competitive binding of holo-TF displaces HFE from TFR1 and the HFE-TFR2 complex activates *HAMP* transcription.[6,7] However, the phenotype of the HFE and TFR2-related disease is different[8] and the association between the two proteins has recently been questioned.[9]

Hepcidin blocks dietary iron absorption and iron recycling from senescent erythrocytes by inducing the degradation of the iron exporter ferroportin on enterocytes and macrophages, respectively.[10] The mechanism of *HAMP* activation by TFR2 and HFE is still unclear. Both proteins probably contribute to *HAMP* upregulation by bone morphogenetic proteins (BMP) in response to increased tissue iron.[11,12] BMP6, using hemojuvelin as a co-receptor, signals through sons-of-mothers-against-decapenthaplegic 1/5/8 (SMAD1/5/8) proteins. In agreement, HFE and TFR2 *in vitro* may form a multi-protein complex with hemojuvelin.[13] The role of hepatic TFR2 as a regulator of *HAMP* transcription is confirmed by the phenotype of the *Tfr2* total (*Tfr2*$^{-/-}$) and liver-specific (*Tfr2*^{LCKO}) knockout mouse models. Both mice are characterized by iron overload and low *Hamp* levels relative to their high iron stores, with *Tfr2*^{LCKO} having more severe liver iron accumulation than *Tfr2*$^{-/-}$ animals.[14,15]

Recently TFR2 has been identified as a component of the erythropoietin receptor (EPOR) complex. *TFR2* and the *EPOR* are co-expressed during erythroid differentiation, TFR2 associates with EPOR in the endoplasmic reticulum and is required for the efficient transport of the EPOR to the cell surface. Moreover *TFR2* knockdown *in vitro* delays the terminal differentiation of erythroid precursors[16] indicating that TFR2 is required for efficient erythropoiesis.

The BMP6-hemojuvelin-HAMP pathway is inhibited by matriptase-2, a type II transmembrane serine protease encoded by the *TMPRSS6* gene. By cleaving hemojuvelin,[17] TMPRSS6 strongly impairs BMP-mediated *HAMP* activation in the liver. *TMPRSS6* mutations both in humans[18] and in mice[19,20] cause excessive *HAMP* production and iron-refractory, iron-deficiency anemia (IRIDA).[21] The important role of TMPRSS6 in erythropoiesis is also highlighted by genome-wide association studies: indeed, common *TMPRSS6* genetic

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variants associate with iron and erythrocyte traits in different populations.[22-27] By studying *Tmprss6* haploinsufficient mice[28] and hepcidin levels of normal individuals and the *TMPRSS6* common single nucleotide polymorphism (rs855791)[29] we demonstrated that even a partial inability to modulate hepcidin influences iron parameters and, indirectly, erythropoiesis.

The regulation of TMPRSS6 and its activity is incompletely understood: besides hypoxia,[30] iron and BMP6, through the BMP-SMAD pathway, induce *TMPRSS6* expression, likely as a negative feedback loop to limit excessive increases of HAMP.[31] However, the regulation of *TMPRSS6 in vivo* according to iron needs remains to be clarified. A possible role of Tmprss6 in iron overload was demonstrated by Finberg *et al.*[32] who showed that *Hfe*$^{-/-}$ mice with complete loss of *Tmprss6* revert from a phenotype of iron overload to one of iron-deficiency anemia with high *Hamp* levels. These findings suggest that HFE acts genetically upstream of TMPRSS6 in the modulation of the BMP-SMAD pathway and of *HAMP* expression. In analogy with these results and given the role of TFR2 in erythropoiesis[16] we wondered whether TFR2 is involved in the regulation of TMPRSS6. To answer this question, we back-crossed *Tmprss6*$^{-/-}$ mice with animals with a complete deletion of *Tfr2 (Tfr2*$^{-/-}$*)* and analyzed the hematologic phenotype and the *Bmp-Smad-Hamp* pathway of the double mutant mice. Moreover, in order to discriminate between the hepatic and extra-hepatic functions of TFR2, we performed the same analysis in *Tmprss6*$^{-/-}$ mice lacking *Tfr2* specifically in the liver (*Tfr2*^{LCKO}).[15]

Methods

Mouse models

Mice were maintained in the animal facility of the Department of Clinical and Biological Sciences, University of Turin (Italy) in accordance with European Union guidelines. Each study was approved by the Institutional Animal Care and Use Committee (IACUC) of the same institution.

A *Tmprss6*$^{-/-}$ mouse model on a mixed C57BL/6-Sv129 background was kindly provided by Prof. C. Lopez-Otin (University of Oviedo, Spain) and maintained by brother-sister mating for more than ten generations. *Tfr2*$^{-/-}$ and *Tfr2*^{LCKO} mice on a pure 129S2 background were generated as previously described.[15] For the experimental work described we bred *Tfr2*$^{-/-}$ or *Tfr2*^{LCKO} mice with *Tmprss6*$^{+/-}$ mice and then intercrossed the F1 progeny to generate various genotype combinations (F2: wild-type, *Tmprss6*$^{+/-}$, *Tmprss6*$^{-/-}$, *Tfr2*$^{-/-}$, *Tmprss6*$^{+/-}$*Tfr2*$^{-/-}$, *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$, *Tfr2*^{LCKO}, *Tmprss6*$^{+/-}$*Tfr2*^{LCKO}, *Tmprss6*$^{-/-}$*Tfr2*^{LCKO}). Mice were given a standard diet (480 mg iron/Kg) and only male mice were analyzed when 10 weeks old. Blood was collected for hematologic analyses, transferrin saturation and erythropoietin levels. After sacrifice livers and spleens were dissected, weighed, and snap-frozen immediately for RNA analysis or dried for tissue iron quantification.

Hematologic analyses

Blood was obtained by retro-orbital puncture from anesthetized mice. Red blood cell and white blood cell counts, hemoglobin concentration, hematocrit and erythrocyte indices (mean corpuscular volume, mean corpuscular hemoglobin) were measured using an ADVIA®120 Hematology System (Siemens Diagnostics).

Transferrin saturation was calculated as the ratio of serum iron and total iron binding capacity levels, using the Total Iron Binding Capacity kit (Randox Laboratories Ltd.), according to the manufacturer's instructions. Serum erythropoietin levels were measured using a mouse Erythropoietin Quantikine set (R&D System), 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 3M HCl, 0.6M trichloroacetic acid for 20 h 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 min 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, Darmstadt, Germany).

Quantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted from the 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 (PCR) 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 (20x) and TaqMan Master Mix (2x) from Applied Biosystems were used, and the reactions were run on a 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 the quantitative reverse transcriptase PCR are listed in *Online Supplementary Table S1*.

Statistical analysis

Data are presented as mean ± standard deviation. Unpaired two-tailed Student t-tests were performed using GraphPad PRISM 5.0 and a *P* value less than 0.05 was considered statistically significant.

Results

Tmprss6$^{-/-}$Tfr2$^{-/-}$ mice are anemic and have increased red cell numbers

Ten-week old *Tfr2*$^{-/-}$ mice had higher hemoglobin levels than controls, while *Tfr2*^{LCKO} mice had levels comparable to those in wild-type animals, as previously reported.[15] Conversely *Tmprss6*$^{-/-}$ mice had the hematologic phenotype of microcytic anemia with increased red blood cell and reticulocyte counts accompanied by low levels of hemoglobin, hematocrit, mean corpuscular volume and mean corpuscular hemoglobin. The heterozygous loss of *Tmprss6* in *Tfr2*$^{-/-}$ mice slightly reduced hemoglobin levels although the difference from the levels in *Tfr2*$^{-/-}$ mice was not statistically significant. On the contrary, *Tmprss6*$^{+/-}$*Tfr2*^{LCKO} mice had hemoglobin levels comparable to those of *Tfr2*^{LCKO} animals. Both *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ and *Tmprss6*$^{-/-}$*Tfr2*^{LCKO} mice were anemic and had hemoglobin levels similar to those of *Tmprss6*$^{-/-}$ mice. However, the *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ mice had higher numbers of red blood cells than did *Tmprss6*$^{-/-}$ animals, resulting in more severe microcytosis, while this was not the case for *Tmprss6*$^{-/-}$ mice with specific liver deletion

Table 1. Hematologic data of all the genotype combinations analyzed.

	WBC (x10⁶ cells/μL blood)	RBC (x10⁶ cells/μL blood)	Reticulocytes (x10⁹ cells/L blood)	Hb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)
Wild-type	7.2±2.6	8.6±0.7	302.8±149.9	13.1±0.4	43.3±2.7	50.8±1.5	15.1±1.4
Tmprss6$^{+/-}$	5.6±1.4	9.3±0.8	243.7±28.8	13.2±0.8	45.3±3.3	48.5±0.8^{b}	14.1±0.5
Tmprss6$^{-/-}$	5.9±4.1	11.0±1.4^{b}	771.1±265.5^{a}	8.0±0.9^{b}	32.3±2.2^{b}	29.5±2.5^{b}	7.3±0.7^{b}
TfR2$^{-/-}$	10.4±2.9	9.5±0.8	324.8±116.7	15.1±1.4^{a}	48.9±4.8^{a}	51.7±0.9	16.0±0.6
Tmprss6$^{+/-}$TfR2$^{-/-}$	10.2±3.6	8.8±0.6	293.8±119.4	14.2±0.9^{a}	46.7±3.0	52.4±1.8	16.0±0.9
Tmprss6$^{-/-}$TfR2$^{-/-}$	8.1±5.8	13.9±2.0^{b,d,e}	2032.7±1063.7^{a,c,e}	8.3±0.9^{b,d}	35.6±4.6^{a,c}	25.7±2.5^{b,d,e}	6.0±0.3^{b,d,e}
TfR2^{LCKO}	6.8±3.2	9.0±0.3	229.9±48.5	13.2±0.6	45.4±1.8	50.3±1.4	14.6±0.3
Tmprss6$^{+/-}$TfR2^{LCKO}	8.1±2.8	9.5±0.6	264.0±68.0	13.9±0.9	47.7±1.9	50.1±1.3	14.7±0.4
Tmprss6$^{-/-}$Tfr2^{LCKO}	10.4±3.5	10.7±1.5^{a,g,i}	1047.0±456.8^{a,h}	8.5±1.2^{b,h}	30.0±1.9^{b,h,i}	28.5±2.4^{b,h,i}	8.2±1.5^{b,h,i}

White blood cells (WBC), red blood cells (RBC), reticulocyte counts, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) are shown as means ± standard deviations of the results in four to seven male mice. [a]P<0.05, [b]P<0.005 relative to wild-type (Tmprss6$^{+/+}$Tfr2$^{+/+}$) *controls; [c]P<0.05, [d]P<0.005 relative to* Tfr2$^{-/-}$; *[e]P<0.05, [f]P<0.005 relative to* Tmprss6$^{-/-}$; *[g]P<0.05, [h]P<0.005 relative to* TfR2^{LCKO}; *[i]P<0.05 relative to* Tmprss6$^{-/-}$Tfr2$^{-/-}$. *For the complete statistical analysis see* Online Supplementary Table S2.

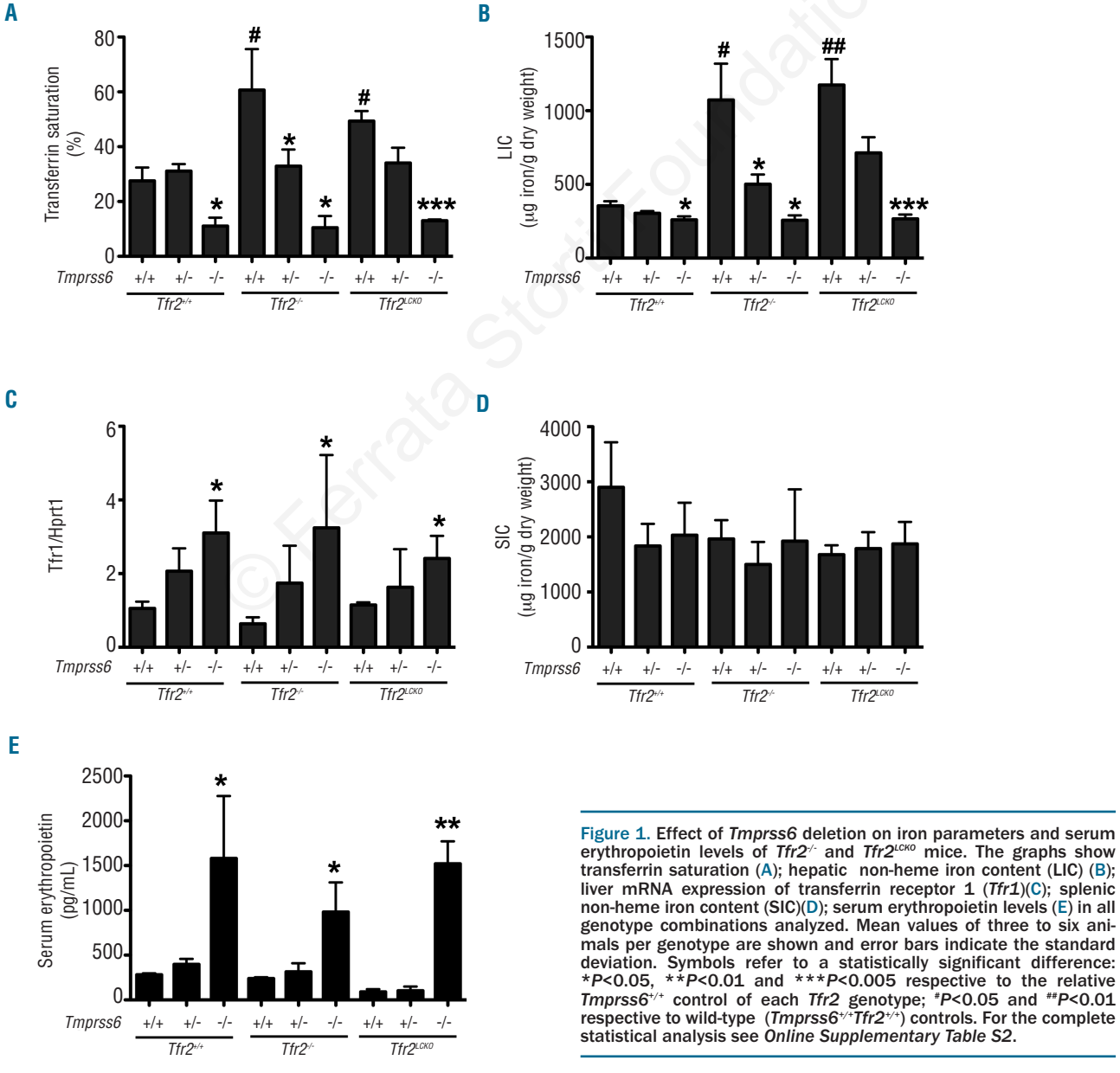

Figure 1. Effect of *Tmprss6* deletion on iron parameters and serum erythropoietin levels of *Tfr2*$^{-/-}$ and *Tfr2*^{LCKO} mice. The graphs show transferrin saturation (A); hepatic non-heme iron content (LIC) (B); liver mRNA expression of transferrin receptor 1 (*Tfr1*)(C); splenic non-heme iron content (SIC)(D); serum erythropoietin levels (E) in all genotype combinations analyzed. Mean values of three to six animals per genotype are shown and error bars indicate the standard deviation. Symbols refer to a statistically significant difference: *P<0.05, **P<0.01 and ***P<0.005 respective to the relative *Tmprss6*$^{+/+}$ control of each *Tfr2* genotype; #P<0.05 and ##P<0.01 respective to wild-type (*Tmprss6*$^{+/+}$*Tfr2*$^{+/+}$) controls. For the complete statistical analysis see *Online Supplementary Table S2*.

of *Tfr2* (Table 1). In the absence of *Tmprss6*, reticulocytes were increased only in *Tfr2*$^{-/-}$ animals.

Homozygous loss of Tmprss6 *reduces systemic and tissue iron levels of* Tfr2$^{-/-}$ *and* Tfr2^{LCKO} *mice*

Transferrin saturation (Figure 1A) and liver iron content (LIC) (Figure 1B) were significantly lower in the iron-deficient *Tmprss6*$^{-/-}$ mice than in wild-type mice (defined as *Tmprss6*$^{+/+}$*Tfr2*$^{+/+}$ in Figures 1 and 2), while *Tfr2*$^{-/-}$ and *Tfr2*^{LCKO} animals showed an important iron overload.[15] Deletion of the *Tmprss6* gene in both *Tfr2*$^{-/-}$ and *Tfr2*^{LCKO} mice had a dose-dependent effect. The loss of a single allele slightly reduced transferrin saturation and LIC in both models, although the differences were statistically significant only for the *Tfr2*$^{-/-}$ animals. The homozygous inactivation of *Tmprss6* lowered LIC of both *Tfr2*$^{-/-}$ and *Tfr2*^{LCKO} animals to the levels of *Tmprss6*$^{-/-}$ mice. The difference in LIC of the various genotypes was confirmed by analysis of the *Tfr1* mRNA levels, which are known to be inversely related to the cell iron content. *Tfr1* mRNA levels were high in *Tmprss6*$^{-/-}$, *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ and *Tmprss6*$^{-/-}$*Tfr2*^{LCKO} animals and reduced according to gene-dosage of *Tmprss6* (Figure 1C). We observed no differences in the spleen iron content among all the genotypes analyzed (Figure 1D). In addition spleen and liver sizes were similar between *Tmprss6*$^{-/-}$, *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ and *Tmprss6*$^{-/-}$*Tfr2*^{LCKO} animals (*data not shown*).

Tfr2$^{-/-}$ and *Tfr2*^{LCKO} mice had serum erythropoietin levels comparable to those of wild-type mice. As expected, anemic *Tmprss6*$^{-/-}$ mice had erythropoietin levels higher than those of wild-type mice and comparable to those of *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ and *Tmprss6*$^{-/-}$*Tfr2*^{LCKO} animals (Figure 1E).

Hamp *levels are less inappropriately high in* Tmprss6$^{-/-}$ Tfr2$^{-/-}$ *mice than in* Tmprss6$^{-/-}$ *mice*

The expression of *Bmp6* reflected LIC in all the genotypes analyzed, being high in *Tfr2*$^{-/-}$ and *Tfr2*^{LCKO} animals and low in *Tmprss6*$^{-/-}$ as compared to wild-type controls, although in the latter case the difference was not statistically significant. *Bmp6* in *Tmprss6* haploinsufficient *Tfr2*^{LCKO} was indistinguishable from that in *Tfr2*^{LCKO} animals (Figure 2A). The *Bmp6*/LIC ratio was comparable among all the genotypes analyzed proving that *Bmp6* expression is adequate to the hepatic iron content (*Online Supplementary Figure S1*).

As expected, *Hamp* (Figure 2B) was over-expressed in *Tmprss6*$^{-/-}$ mice while comparable to wild-type levels in both *Tfr2*$^{-/-}$ and *Tfr2*^{LCKO} mice. As a consequence the iron-

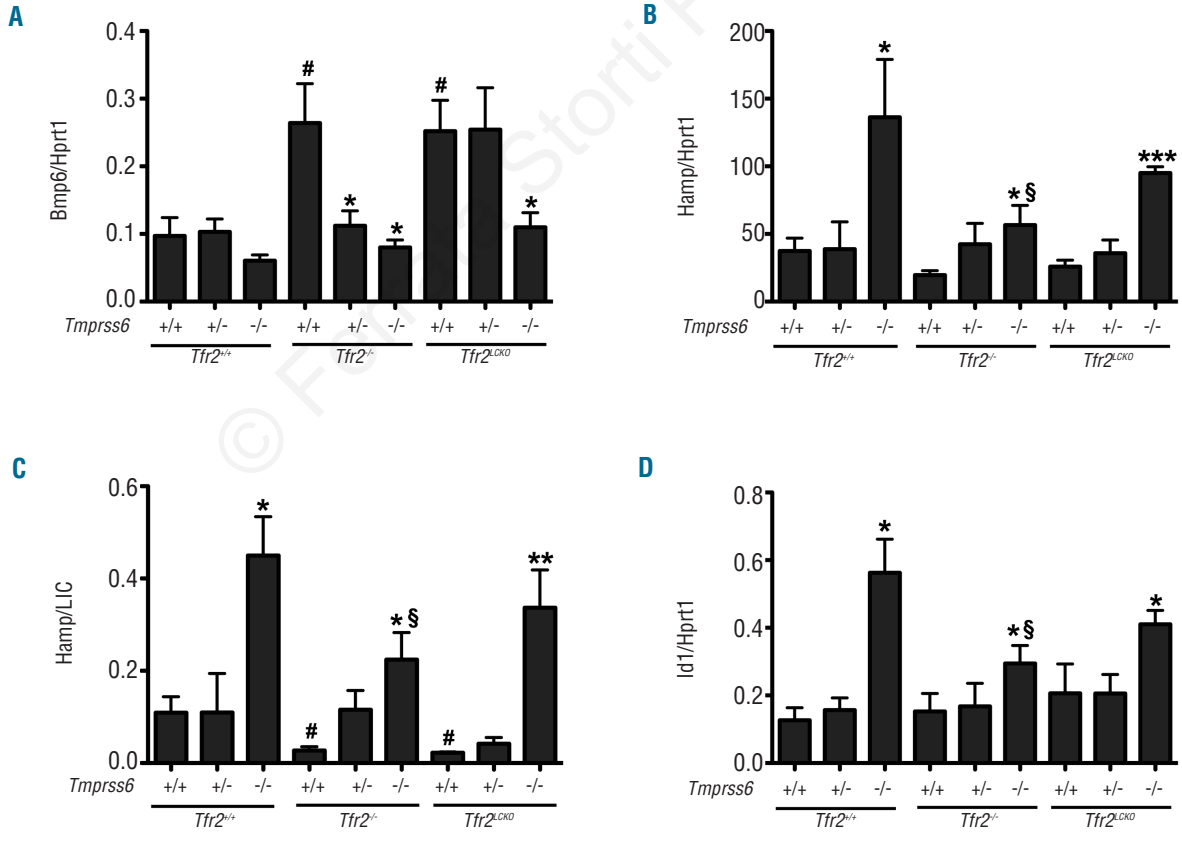

Figure 2. Effect of *Tmprss6* deletion on the Bmp-Smad pathway of *Tfr2*$^{-/-}$ and *Tfr2*^{LCKO} mice. The graphs show liver mRNA expression of: bone morphogenetic protein 6 (*Bmp6*; A); hepcidin (*Hamp*; B); *Hamp* normalized on LIC (*Hamp*/LIC; C) and inhibitor of DNA binding 1 (*Id1*; D) in all genotype combinations analyzed. Mean values of three to six animals per genotype are shown and error bars indicate the standard deviation. Symbols refer to a statistically significant difference: **P*<0.05, ***P*<0.01 and ****P*<0.005 respective to the relative *Tmprss6*$^{+/+}$ control of each *Tfr2* genotype; $^{\#}$*P*<0.05 respective to wild-type (*Tmprss6*$^{+/+}$*Tfr2*$^{+/+}$) animals; §*P*<0.05 respective to *Tmprss6*$^{-/-}$ mice. For the complete statistical analysis see *Online Supplementary Table S2*.

deficient *Tmprss6*$^{-/-}$ mice had a *Hamp*/LIC ratio higher than that of wild-type animals, while both the iron-loaded *Tfr2* knockout mice had a *Hamp*/LIC ratio lower than that of controls (Figure 2C).

In *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ mice *Hamp* expression was higher than in wild-type mice, but lower than in *Tmprss6*$^{-/-}$ animals, while levels in *Tmprss6*$^{-/-}$*Tfr2*^{LCKO} were comparable to those of *Tmprss6*$^{-/-}$ animals. This resulted in a *Hamp*/LIC ratio that was higher in *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ mice than in controls, but lower than in *Tmprss6*$^{-/-}$ animals, while the *Hamp*/LIC ratio of *Tmprss6*$^{-/-}$*Tfr2*^{LCKO} was comparable to that of *Tmprss6*$^{-/-}$ animals (Figure 2C). The mRNA levels of inhibitor of differentiation 1 (*Id1*), another target of the Bmp-Smad pathway, followed the same pattern as that of *Hamp* expression (Figure 2D), proving that in the double *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ mice the Bmp-Smad pathway is more active than in wild-type mice, but less active than in *Tmprss6*$^{-/-}$ mice.

Discussion

The analysis of animal models of *Tfr2*-hemochromatosis suggests that low hepcidin is due to an attenuated Bmp-Smad pathway. In theory TFR2 might promote BMP-SMAD signaling for hepcidin production by inhibiting the activity of TMPRSS6, as was hypothesized for HFE,[32] by up-regulating *BMP6* or through other unknown mechanisms. For this reason we compared the effect of *Tmprss6* inactivation in mice with a total deletion of *Tfr2* (*Tfr2*$^{-/-}$) with that in mice with specific ablation of *Tfr2* in the liver (*Tfr2*^{LCKO}). Since the latter animals maintain Tfr2 function in other organs, the comparison of the phenotypes of the double knockout mice may provide clues to the extra-hepatic functions of TFR2.

We found that in adult *Tfr2*$^{-/-}$ mice the heterozygous loss of *Tmprss6* slightly reduces the severity of hepatic iron overload and partially reverts the hematologic phenotype, reducing hemoglobin levels. In contrast, *Tmprss6* haploinsufficiency does not correct the iron-overload phenotype of *Tfr2*^{LCKO} mice. This might be compatible with a more severe iron burden reported for the liver-specific knockout,[14,15] although in the present study, in which only males were examined, LIC was similar in *Tfr2*$^{-/-}$ and *Tfr2*^{LCKO}. Homozygous loss of *Tmprss6* led to systemic iron deficiency and severe anemia in both genotypes with low levels of hemoglobin, transferrin saturation and LIC and enhanced hepatic *Tfr1* expression, in analogy to what has been observed in *Hfe* knockout mice with deletion of *Tmprss6*.[32] Similar results were also previously published for *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ mice,[33] although differences of genetic backgrounds made the genotype comparison problematic.

The phenotype modification of *Tfr2*$^{-/-}$ and *Tfr2*^{LCKO} from iron overload to iron deficiency in the absence of *Tmprss6* demonstrates that TFR2 in the liver acts upstream of the serine protease and might control its activity, thus raising the possibility that pharmacological inhibition of TMPRSS6 is effective in limiting dietary iron absorption and redistributing iron to macrophages in *TFR2*-hemochromatosis, as shown for *HFE*-hemochromatosis.[34,35]

Loss of the protease activity of Tmprss6 leads to increased expression of hepcidin in *Tfr2* iron-loaded animals which explains reduced iron absorption and iron deficiency. However, in *Tmprss6*$^{-/-}$ mice with complete loss of *Tfr2* the hepatic mRNA levels of *Hamp* and *Id1*, although increased, do not reach the high levels observed in *Tmprss6*$^{-/-}$ mice. In contrast, the expression levels of *Hamp* and *Id1* in *Tmprss6*$^{-/-}$*Tfr2*^{LCKO} are comparable to those in *Tmprss6*$^{-/-}$ mice. These differences are not mediated by an altered expression of *Bmp6* since *Bmp6* levels reflect the hepatic iron burden in all the genotypes analyzed. This appropriate regulation of *Bmp6* in *Tfr2*$^{-/-}$ animals indicates that Tfr2 is not required for adequate *Bmp6* response to increased tissue iron, a finding discordant from that in a recent report of *Bmp6* being inappropriately low in *Tfr2*$^{-/-}$ mice.[36] Based on our results we speculate that in *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ mice an inhibitory signal partially affects the efficiency of the Bmp-Smad pathway downstream of Bmp6 leading to a lower than expected hepcidin activation.

Since inhibition of hepcidin is largely dependent on erythropoietic signals we analyzed the hematologic phenotype of our models. The functional loss of both Tmprss6 and Tfr2 in the whole organism is associated with the same degree of iron deficiency as *Tmprss6*$^{-/-}$. However, as compared to mice lacking *Tmprss6* alone, *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ mice showed a consistent increase of red cell number and hematocrit, which was not observed when *Tfr2* was specifically deleted in the liver. This observation supports the hypothesis that the hematologic phenotype of *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ is dependent on the lack of a still unknown extra-hepatic function of Tfr2.

We speculate that the loss of *Tfr2* in the erythroid compartment accounts for the increased number of red cells observed in *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ mice and that the expansion of erythropoiesis is responsible for the partial inhibition of the Bmp-Smad pathway exclusively observed in these double mutant mice.

Iron-loaded *Tfr2*$^{-/-}$ mice are not characterized by increased red blood cell counts, but do have increased hemoglobin, as shown here and by others,[15] as compared with *Tfr2*^{LCKO} mice, indicating deregulated erythropoiesis. Indeed, the normal hemoglobin levels in the iron-loaded *Tfr2*^{LCKO} mice indicate that the high hemoglobin levels observed in *Tfr2*$^{-/-}$ animals are not only due to their elevated iron burden, but to some other factors likely related to the absence of *Tfr2* in the erythroid compartment.

In the attempt to verify whether the high red blood cell counts of *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ mice are due to increased erythropoietin levels, we measured serum erythropoietin in all the models. Since *Tmprss6*$^{-/-}$, *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ and *Tmprss6*$^{-/-}$*Tfr2*^{LCKO} mice, which have the same degree of anemia, have comparable serum erythropoietin levels we conclude that erythroid precursors lacking *Tfr2* might have enhanced sensitivity to erythropoietin stimulation. Our data seem discrepant with those reported by Foretnikova *et al.*,[16] who found higher serum erythropoietin levels in *Tfr2*$^{-/-}$ mice than in *Tfr2*^{LCKO} ones. However, the latter results were obtained in young animals (4-weeks old), while our data refer to adult, 10-weeks old mice. It is of interest that the same authors observed that *TFR2*-knockdown in human erythroid precursors led to a slight increase of total cell numbers after 12 days of cell culture.

In conclusion we propose that TFR2 is a modulator of erythropoiesis in keeping with its function as an EPOR partner. It is possible that TFR2, as an iron sensor, modulates the erythropoietin sensitivity of the erythroid precursors. The increased red cell numbers might be the result of this function in iron-deficient *Tmprss6*$^{-/-}$*Tfr2*$^{-/-}$ animals.

More specifically, since iron-loaded *Tfr2*$^{-/-}$ mice are not characterized by increased red blood cell counts, we propose that TFR2 is a limiting factor for erythropoiesis, which controls red cell numbers to avoid excessive production in conditions of iron-restriction. Further studies in mice with specific erythroid deletion of *Tfr2* will clarify this possibility.

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