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Effect of Erythropoietin, Iron Deficiency and Iron Overload on Liver Matriptase-2 (TMPRSS6) Protein Content in Mice and Rats

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Abstract

Matriptase-2 (TMPRSS6) is an important negative regulator of hepcidin expression; however, the effects of iron overload or accelerated erythropoiesis on liver TMPRSS6 protein content *in vivo* are largely unknown. We determined TMPRSS6 protein content in plasma membrane-enriched fractions of liver homogenates by immunoblotting, using a commercial antibody raised against the catalytic domain of TMPRSS6. Plasma membrane-enriched fractions were obtained by centrifugation at 3000 g and washing. TMPRSS6 was detected in the 3000 g fraction as a 120 kDa full-length protein in both mice and rats. Feeding of iron-deficient diet as well as erythropoietin treatment increased TMPRSS6 protein content in rats and mice by a posttranscriptional mechanism; the increase in TMPRSS6 protein by erythropoietin was also observed in *Bmp6*-mutant mice. Administration of high doses of iron to mice (200, 350 and 700 mg/kg) decreased TMPRSS6 protein content. Hemojuvelin was detected in the plasma membrane-enriched fractions of control animals as a full length protein of approximately 52 kDa; in iron deficient animals, the full length protein was partially cleaved at the N-terminus, resulting in an additional weak band of approximately 47 kDa. In livers from hemojuvelin-mutant mice, TMPRSS6 protein content was strongly decreased, suggesting that intact hemojuvelin is necessary for stable TMPRSS6 expression in the membrane. Overall, the results demonstrate posttranscriptional regulation of liver TMPRSS6 protein by iron status and erythropoietin administration, and provide support for the interaction of TMPRSS6 and hemojuvelin proteins *in vivo*.

Introduction

Iron deficiency is the most common cause of anemia worldwide. Although most cases of iron deficiency anemia respond to oral iron supplementation, some patients suffer from iron-

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refractory iron deficiency anemia, which is refractory to dietary iron, and can be only partly corrected by parenteral iron administration [1]. Seven years ago it was discovered that this anemia can be caused by mutations in the *TMPRSS6* gene [2–6] which result in inappropriately high expression of hepcidin, the key iron-regulatory hormone [7]. Elevated hepcidin blocks iron export from enterocytes and macrophages, thus limiting iron availability for erythropoiesis.

The newly discovered role of *TMPRSS6* in iron metabolism has raised questions both about its mode of action, as well as about its physiological regulation. *TMPRSS6* encodes a serine protease, matriptase-2 (TMPRSS6), which has relatively low substrate specificity [8]. It has been convincingly demonstrated that, *in vitro*, TMPRSS6 cleaves hemojuvelin (HFE2), a GPI-bound hepatocyte membrane component of the hepcidin regulatory pathway [9]. HFE2 is a coreceptor for bone morphogenetic protein 6 (BMP6), which is transcriptionally induced by iron overload [10], and, through interaction with BMP receptors, activates phosphorylation of SMAD proteins and ultimately increases transcription of hepcidin [11]. Therefore, it is presumed that *TMPRSS6* mutations result in elevated HFE2 protein levels at the hepatocyte plasma membrane, leading to enhanced signaling through the BMP/HFE2/SMAD signal transduction pathway and, consequently, elevated expression of hepcidin [9,12].

The proposed scheme implies that TMPRSS6 could function as an important regulatory protein which determines hepcidin expression by controlled cleavage of hepatocyte HFE2. Since it is very well known that mouse liver hepcidin (*Hamp*) mRNA content sensitively reacts to iron deficiency, iron overload or the rate of erythropoiesis [7], it is essential to know how TMPRSS6 protein content changes in response to these stimuli. It has already been reported that, in rats, TMPRSS6 protein increases following short-term administration of iron-deficient diet [13], which supports the role of TMPRSS6 in hepcidin downregulation. In addition, *in vitro* studies demonstrated an increase in TMPRSS6 protein or *Tmprss6* mRNA following hypoxia [14,15], again in line with the proposed function of TMPRSS6 as a negative regulator of hepcidin expression. On the contrary, iron administration to mice has been reported to increase *Tmprss6* mRNA content [16], while administration of iron to rats, albeit at a relatively low dose, did not influence liver TMPRSS6 protein [13]. Overall, the *in vivo* regulation of TMPRSS6 has not yet been fully elucidated.

The aim of this study was to determine TMPRSS6 protein content in experimental animals subjected to iron deficiency, iron overload or erythropoietin (EPO) administration. In addition, TMPRSS6 protein content was determined in HFE2-deficient mice. The results demonstrate that all these stimuli influence TMPRSS6 protein content, generally supporting the important role of TMPRSS6 in hepcidin gene regulation. Data from HFE2-deficient mice suggest that HFE2 enhances TMPRSS6 protein stability, supporting the concept of an interaction between these two proteins.

Materials and Methods

Animals and Treatment

All animal experiments were approved by the Czech Ministry of Education, protocol MSMT-1461/2015-5. Efforts were made to limit animal suffering: Intraperitoneal injections were performed under ether anesthesia, animals were sacrificed by decapitation under ether anesthesia.

Female outbred Wistar rats (170–195 g, Anlab SRO, Prague, Czech Republic) and male C57BL/6 mice (25–30 g) received four daily doses of EPO (NeoRecormon, Roche, 500 IU/day and 50 IU/day respectively) and were sacrificed 24 hours after the last injection. For iron-deficiency experiments, young female Wistar rats (45–55 g) or weaned four week old male C57BL/6 mice (14–17 g) were placed on an iron deficient diet for three or four weeks respectively. For

iron overload experiments, iron was administered as iron dextran (Sigma Aldrich) to male adult C57BL/6 mice at 200, 350 and 750 mg/kg by single intraperitoneal injection; animals were sacrificed one week after treatment. For short-term iron overload experiments, animals were administered a single intraperitoneal injection of iron dextran at 1000 mg/kg and were sacrificed 24 h later [17].

Female mice with disruption of the *Hfe2* gene coding for hemojuvelin (*Hfe2*-/- mice), age 3–4 months, were originally a generous gift from Prof. Silvia Arber, Basel, Switzerland [18]. Female *Bmp6*-null mice were a generous gift from Prof. Roel Goldschmeding, University Medical Center Utrecht, the Netherlands [19]. The EPO administration scheme for *Hfe2*- and *Bmp6*-null mice was identical to C57BL/6 mice. Liver samples from *mask* mice [2], lacking the proteolytic domain of TMPRSS6, were a generous gift from Dr. Pauline Lee and Dr. Xin Du, Scripps Research Institute, La Jolla, CA, USA.

Sample Preparation for Immunoblotting

For all experiments, a plasma membrane-enriched fraction obtained by centrifugation at 3000 g was used. Samples of liver (approx. 250 mg) were homogenized with a 6 mm Ultra Turrax homogenizer (3 x 10 s at maximum speed) in 2 ml of 10 mM HEPES, pH 7.4, containing protease inhibitors and 2 mM EDTA. The homogenate was centrifuged for 10 min at 400 g, and the supernatant was centrifuged at 3000 g for 15 min. The pellet was resuspended (Ultra Turrax, 10 s) in 10 mM HEPES containing 2 M NaCl to deplete the sample of co-precipitated non-membrane proteins associated by ionic bonds [20] and recentrifuged at 3000 g for 15 min. Subsequently, the pellet was resuspended in 0.1 M sodium carbonate, agitated for 1 h at 4°C and recentrifuged. This step is postulated to remove soluble proteins trapped in vesicles formed during homogenization [20]. Next, the pellet was resuspended in 10 mM HEPES containing 4 M urea and 100 mM NaCl to wash out residual non-membrane proteins. Finally, the pellet was washed with 10 mM HEPES and resuspended by sonication in 125 μl of 2% SDS containing 25 mM of ammonium bicarbonate. Insoluble material was pelleted by centrifugation at 16 000 g for 10 min and the supernatant was aliquoted and stored at -80°C. Typical yield for the washed 3000 g fraction was about 1.5 mg of protein per 1 g of liver. Immunoblotting was performed under reducing conditions as previously described [21]. Primary antibodies were: Rabbit anti-TMPRSS6, Ab56182, Abcam, 1:750; goat anti-HFE2, AF 3634, R&D Systems, 1:1500; goat anti-neogenin, AF1079, R&D Systems, 1:1000, and rabbit anti- Na+/K+ATPase α, SC-28800 (ATP1A), Santa Cruz, 1:10 000. Secondary antibodies (anti-rabbit, 711-036-152, 1:40 000 and anti-goat, 705-036-147, 1:40 000) were from Jackson Immunoresearch.

Real-Time PCR, PNGase F Treatment, and Iron Determinations

Real-time PCR was performed on a Biorad IQ5 instrument using SYBR Green protocol, primers are listed in S1 Table. For the removal of N-linked oligosaccharides, PNGase F (New England Biolabs) was used according to manufacturers instructions. Iron was determined according to Torrance and Bothwell [22].

Statistical Analysis

For densitometric quantification, TMPRSS6 band densities obtained on a Biorad GS-800 densitometer were normalized to their respective loading control band densities; treated *vs*. control sample values were then compared using paired t-test. Graphed band density data, generally calculated from several blots, are expressed in % of control, control values were set as 100%. For small data sets (n = 3), values obtained from single 15-well gels were analyzed by unpaired t-test. PCR data were evaluated by the Mann-Whitney test. Data are expressed as means ± SD.

Results

HFE2 and TMPRSS6 are Present as Full Length Proteins in the Liver 3000 g Fraction

HFE2 has been clearly identified as a TMPRSS6 substrate in *in vitro* studies [9]. In order to examine the interaction between TMPRSS6 and HFE2 *in vivo*, it was necessary to select a cellular fraction which would allow reproducible quantification of both proteins. In our previous experiments, we found that HFE2 is easier to detect in microsomal fraction than in whole homogenate [23]. However, published studies on HFE2 processing indicate that HFE2 undergoes complex retrograde trafficking to the Golgi compartment [24]. As liver microsomes also contain parts of Golgi membranes, while the proposed cleavage of HFE2 occurs at the plasma membrane, liver microsomes do not appear to be ideally suited for the investigation of the TMPRSS6/HFE2 interaction. Therefore, a partially purified membrane fraction obtained by centrifugation at 3000 g was used for the current studies. The detection of TMPRSS6, and particularly the detection of HFE2, was significantly improved in this fraction, as was the detection of ATP1A, a marker of the plasma membrane (Fig 1). Whereas TMPRSS6 was detected as a 120 kDa band in all samples, the size of the HFE2 band was influenced by the method of

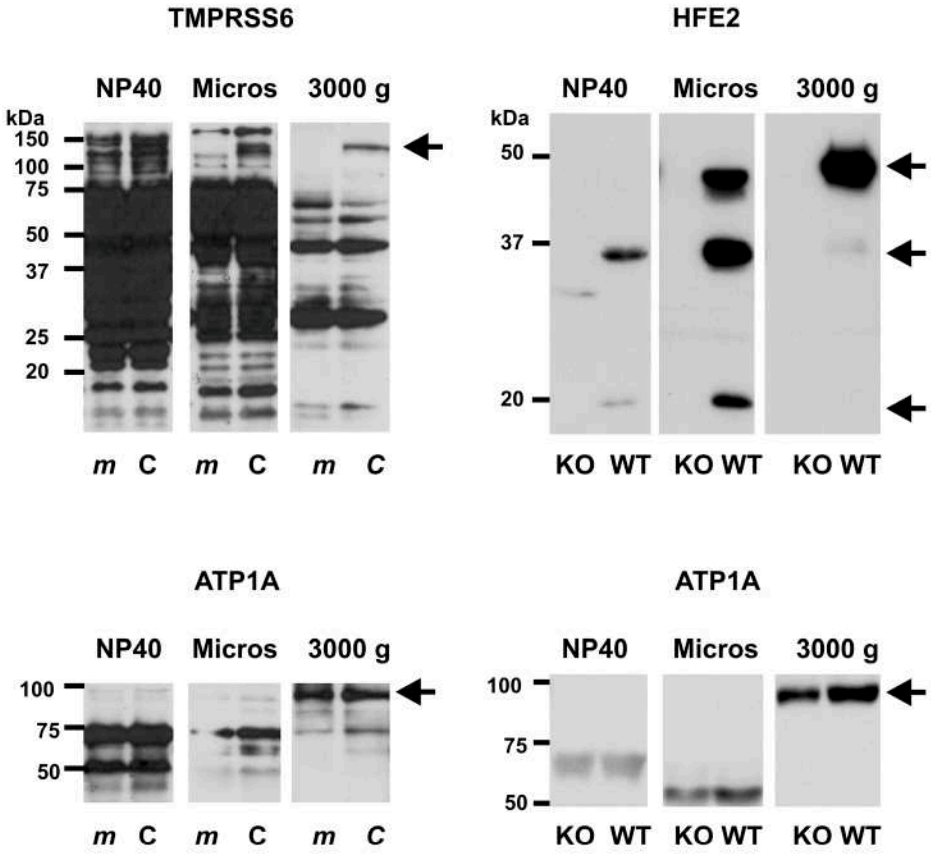

Fig 1. Immunoblot of TMPRSS6, HFE2 and ATP1A in the 3000 g fraction. Detection of TMPRSS6, HFE2 and ATP1A is enhanced in the 3000g fraction of mouse liver homogenates (3000g) in comparison with whole liver homogenates prepared in 1% NP40 (NP40) or microsomes (Micros). Samples were prepared from C57BL/6 mice (C), *mask* mice (*m*), *Hfe2*+/+ mice (WT) and *Hfe2*-/- mice (KO). Arrows denote the TMPRSS6-, HFE2- and ATP1A-specific bands. In contrast to the NP40 whole liver homogenate or liver microsomes, HFE2 is present in the 3000 g fraction solely as a full-length protein.

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sample preparation and the detergent used. In our previous studies using NP-40-containing liver homogenates, as well as in detergent-free microsomes, HFE2 was detected as a heterodimer composed of two bands of 35 and 18 kDa [21,23]; in the present study using the 3000 g fraction HFE2 was present as a strong band of approximately 52 kDa (Fig 1). This size corresponds to full-length, glycosylated HFE2 protein. These data suggest that whereas the major part of cellular HFE2 protein exists as a heterodimer, a small amount of the protein is present at the plasma membrane as the uncleaved full-length 52 kDa chain.

In vivo studies on TMPRSS6 protein have so far used mostly non-commercial antibodies [13,25]. In an attempt to identify a suitable commercially available antibody, we used liver samples from C57BL/6 and *mask* mice. The Ab56182 antibody, raised against the catalytic domain of TMPRSS6, enabled detection of a TMPRSS6-specific band of about 120 kDa which was not present in samples from *mask* mice (Fig 1). The observed size corresponds to reported TMPRSS6 size in rats and mice [13,25], and very probably represents the full-length mouse protein (811 amino acids, predicted molecular weight 91 kDa plus 7 glycosylations). On mouse liver blots, an additional lower band of approximately 110 kDa was occasionally seen; however, since this band was also weakly detectable in some *mask* mice samples, only the 120 kDa band was used for quantification.

TMPRSS6 is reported to undergo proteolytic activation which is proposed to produce a non-glycosylated catalytic domain fragment of about 26 kDa, connected via disulfide bridges to the membrane-bound part of the protein [26,27]. In agreement with published data [13,28], no TMPRSS6-specific band of this size was observed on immunoblots (Fig 1), suggesting that the amount of activated TMPRSS6 is small compared to the full-length protein.

TMPRSS6 Protein is Increased in Iron-deficient Rats and Mice

It has previously been reported that TMPRSS6 increases following short-term administration of iron-deficient diet to weaned rats [13]. For our experiments, we used young mice and rats kept on an iron-deficient diet for several weeks. The treatments resulted in iron-deficiency anemia and a significant decrease of liver iron concentration (Table 1).

The 120 kDa TMPRSS6-specific band was significantly increased in both mice and rats (Fig 2A and 2B). Liver *Tmprss6* mRNA content tended to increase in iron-deficient rats, but the changes did not reach statistical significance (Fig 2C). Rat liver samples probed with the Ab56182 antibody displayed, in addition to the 120 kDa TMPRSS6 band, a relatively strong

Table 1. Iron content in iron-deficient and EPO-treated experimental animals.

Group and Treatment	Hemoglobin g/litre	Hematocrit %	Liver Iron µg/g wet wt.
Mouse, control	141±12	40±3	46±13
Mouse, iron deficiency	110±8*	37±2	20±2*
Mouse, EPO	162±17*	48±4*	33±9*
Rat, control	140±1	42±1	157±12
Rat, iron deficiency	53±5*	17±3*	19±2*
Rat, EPO	161±5*	53±3*	111±26*
Tmprss6 +/+ mouse	140±8	44±4	66±5
Mask mouse	104±6*	37±3	21±3*

Data are expressed as mean ± SD, n = 5 for male mice, n = 4 for female rats and n = 3 for male *mask* mice. Asterisk denotes statistical significance compared to controls ($p<0.05$).

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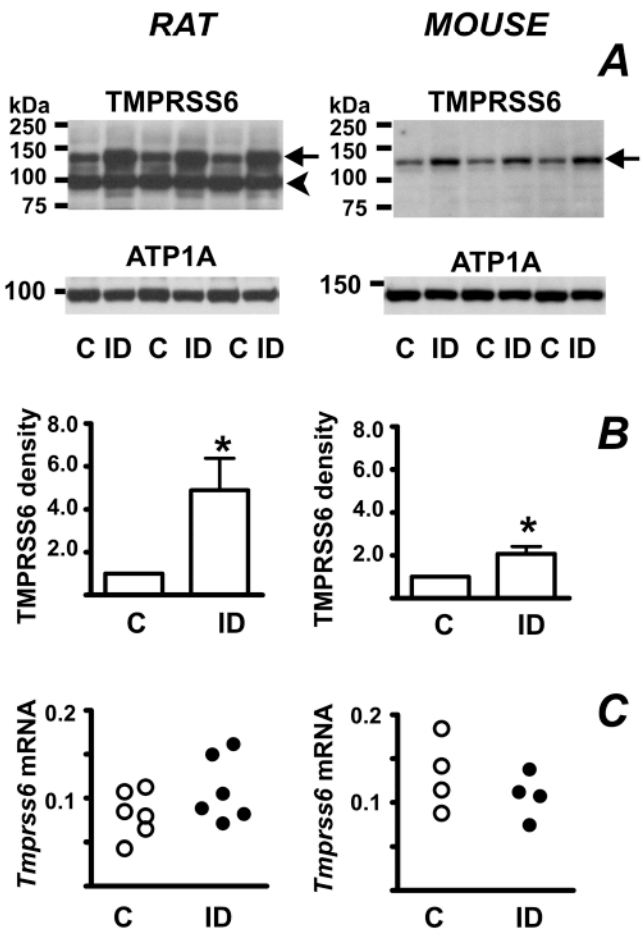

Fig 2. Iron deficiency increases TMPRSS6 protein content. (A) Increase of TMPRSS6 protein content in rats and mice kept on an iron-deficient diet. Control animals (C) were fed standard laboratory diet, iron-deficient animals (ID) were fed an iron-deficient diet since weaning. Arrows denote TMPRSS6-specific bands, arrowhead denotes a non-specific band present in rat liver samples. ATP1A is used as loading control. (B) TMPRSS6 band density quantifications. n = 4, asterisk denotes statistical significance ($p<0.05$). (C) Effect of iron deficiency anemia on liver *Tmprss6* mRNA content. *Tmprss6* mRNA content is determined relative to *Actb* mRNA.

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band at 100 kDa (Fig 2A). Since this band did not react to any treatment, and was not seen in mouse samples, we regard it as a nonspecific band.

TMPRSS6 Protein is Increased in Erythropoietin-treated Rats and Mice

Hepcidin expression is strongly affected by the rate of erythropoiesis [7]. Therefore, it was of interest to determine the effect of EPO administration on TMPRSS6 protein expression. As expected, administration of high doses of EPO for four days resulted in dramatic downregulation of *Hamp* mRNA (less than 0.1% of control values, S1 Fig). TMPRSS6 protein content increased in both rats and mice (Fig 3A and 3B); the effect in rats was more pronounced. The effect of EPO on TMPRSS6 protein was posttranscriptional, as *Tmprss6* mRNA content was not influenced by EPO treatment (Fig 3C).

Since the administration of EPO for four days resulted in a significant increase of blood hemoglobin and a decrease of liver iron (Table 1), it was not possible to determine whether the

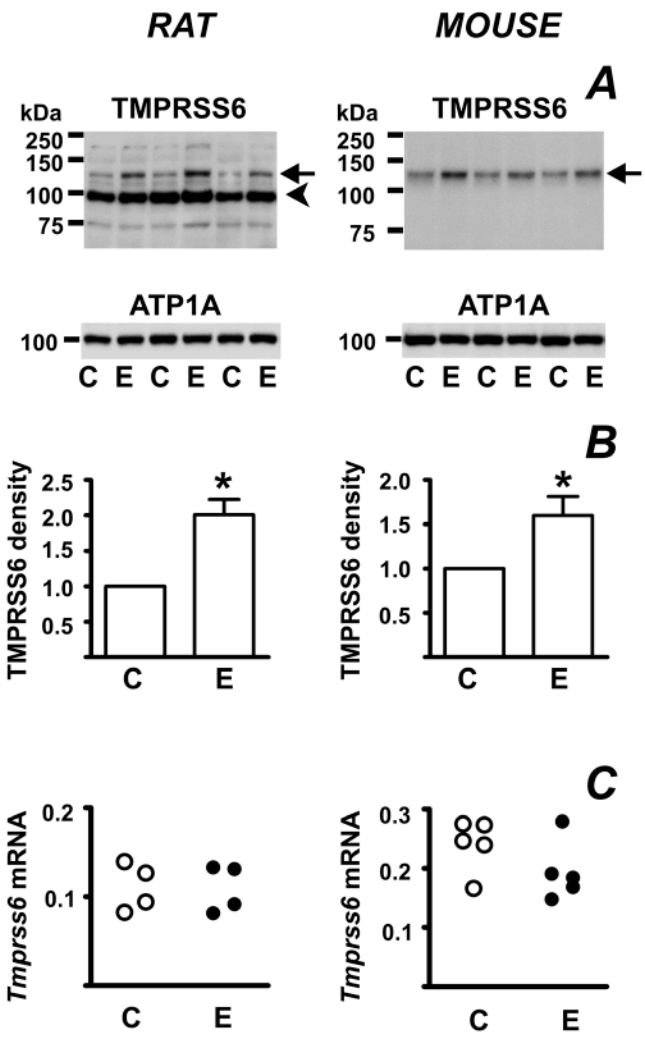

Fig 3. Erythropoietin treatment increases TMPRSS6 protein content. (A) Increase of TMPRSS6 protein content in rats and mice treated with saline (C) or EPO (E) at 500 or 50 IU/animal for four days. Arrows denote TMPRSS6-specific bands, arrowhead denotes a nonspecific band present in rat liver samples. ATP1A is used as loading control. (B) TMPRSS6 band density quantification. Asterisk denotes statistical significance ($p<0.05$), n = 5 for rats and n = 6 for mice. (C) Lack of effect of EPO treatment on *Tmprss6* mRNA. *Tmprss6* mRNA content is determined relative to *Actb* mRNA.

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effect of EPO on TMPRSS6 protein content was primarily related to accelerated erythropoiesis, or to decreased iron availability. Therefore, we performed an additional experiment, in which rats were sacrificed 24 hours after EPO administration. In this experimental setting, liver *Hamp* mRNA content decreased only to approximately 25% of controls and liver iron content was not affected (S2 Fig). Liver TMPRSS6 protein content was not significantly changed (S2 Fig), indicating that EPO influences TMPRSS6 protein content only after prolonged administration, which also substantially affects iron homeostasis.

Regulation of TMPRSS6 Protein by Erythropoietin is Intact in *Bmp6*-Mutant Mice

BMP6 protein is regarded as a key extracellular molecule which initiates iron-dependent signaling at the hepatocyte plasma membrane through the BMP6/HFE2/SMAD pathway,

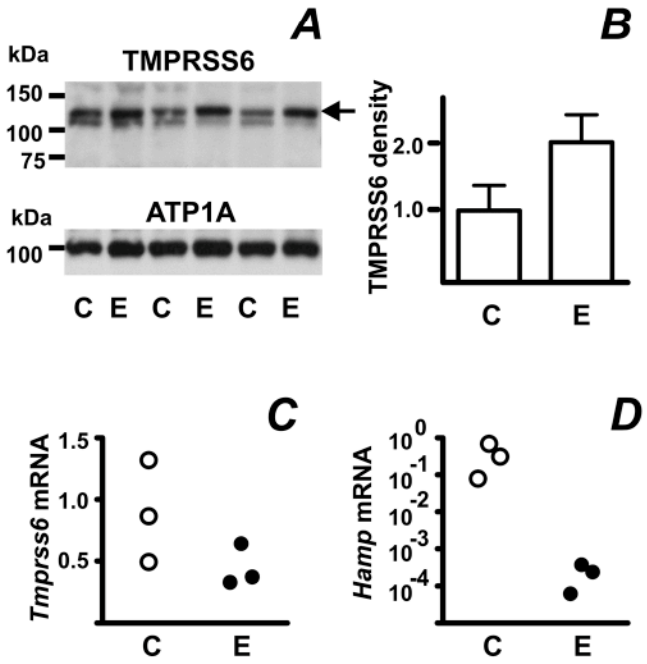

Fig 4. Erythropoietin treatment increases TMPRSS6 protein content in *Bmp6*-mutant mice. (A) Increase of TMPRSS6 protein content in *Bmp6*-mutant mice mice treated with saline (C) or EPO (E) at 50 I. U./mouse for four days. Arrow denotes TMPRSS6-specific band. ATP1A is used as loading control. (B) TMPRSS6 band density quantification. (C and D) Effect of EPO treatment on *Tmprss6* and *Hamp* mRNA content. *Tmprss6* and *Hamp* mRNA content is determined relative to *Actb* mRNA.

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ultimately leading to an increase in hepcidin transcription [29,30]. It was therefore of interest to determine whether the observed regulation of TMPRSS6 protein by EPO remains functional in *Bmp6*-null mice. In accordance with results obtained in C57BL/6 mice, EPO tended to increase TMPRSS6 protein content in *Bmp6*-null mice (Fig 4A and 4B) by a posttranscriptional mechanism (Fig 4C). Although the results did not reach statistical significance, due to limited number of available mouse pairs (n = 3), they nevertheless indicate that the EPO-mediated regulation of TMPRSS6 does not require functional BMP6 protein. Similarly to C57BL/6 mice, EPO treatment dramatically decreased *Hamp* mRNA in *Bmp6*-null mice (Fig 4D), demonstrating that BMP6 is dispensable for the regulation of *Hamp* expression by accelerated erythropoiesis.

Iron Decreases Liver TMPRSS6 Protein Content in Rats and Mice

Injection of iron dextran at 200, 350 and 700 mg/kg decreased TMPRSS6 protein (Fig 5A). However, lower doses of 50 and 100 mg/kg did not produce an effect (S3 Fig). As expected [7,10], iron treatment increased *Hamp* and *Bmp6* expression (S4 Fig). In contrast to the decrease of TMPRSS6 protein in mice treated with high doses of iron dextran (Fig 5A), *Tmprss6* mRNA content was slightly increased (S4 Fig). In livers from iron-treated mice, a strong additional dose-dependent band was apparent at 75 kDa (Fig 5A). Since this band did not react to PNGase F treatment, and was also seen in liver samples from *mask* mice kept on an iron-enriched diet (S5 Fig), we regard it as a non-specific band, probably related to the high intracellular liver iron content (S2 Table).

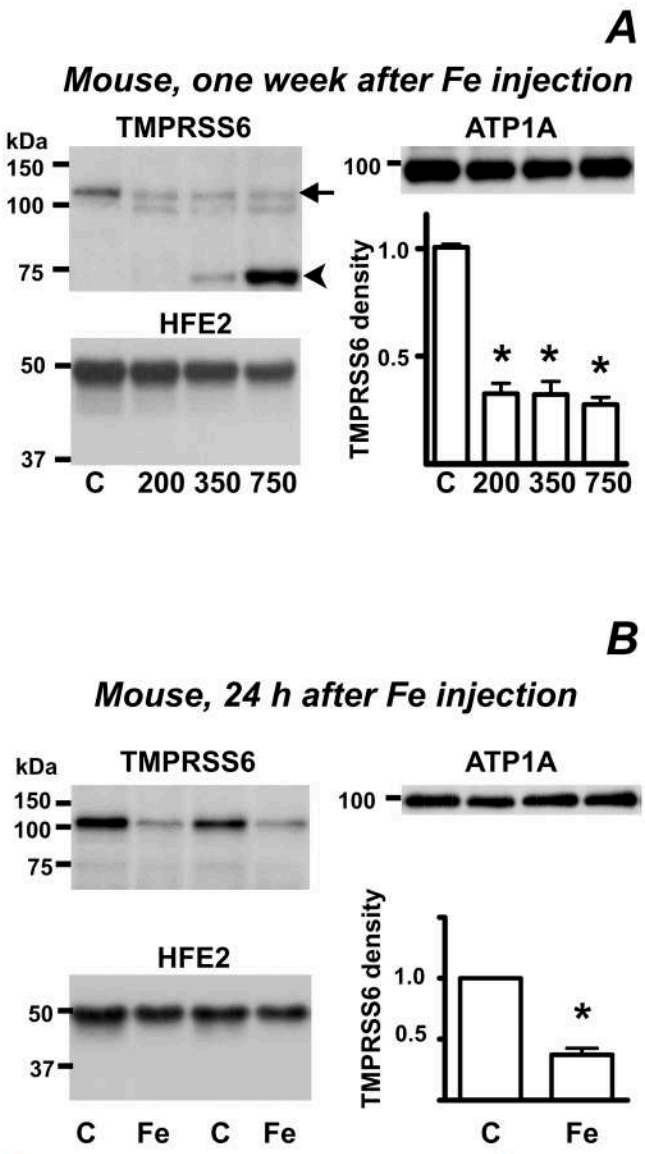

Fig 5. High dose of iron dextran decreases TMPRSS6 protein content. (A) Effect of iron on TMPRSS6 and HFE2 protein content. Mice were treated with 200, 350 and 750 mg/kg of iron dextran one week before sacrifice, control mice (C) received physiological saline. ATP1A is used as loading control. Arrow denotes the TMPRSS6-specific protein band, arrowhead denotes a nonspecific band. Graph represents TMPRSS6 band density quantification, asterisk denotes statistical significance ($p<0.05$, $n = 3$). (B) Effect of a 1000 mg/kg of iron dextran administered to mice 24 h prior to sacrifice. Graph represents TMPRSS6 band density quantification, asterisk denotes statistical significance ($p<0.05$, $n = 4$).

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It has been reported that the response of *Hamp* gene to iron overload differs with different time intervals, and different sites of liver iron accumulation [17,31–33]. It was therefore of interest to determine whether the observed iron-induced decrease in TMPRSS6 protein content also occurs early after iron administration. To this purpose, we used mice injected with 1000 mg/kg of iron dextran 24 h prior to sacrifice. In this experimental setting, iron is reportedly not upregulating liver *Bmp6* expression [17], and the response of *Hamp* mRNA to iron remains limited (S4 Fig). TMPRSS6 protein content was again decreased (Fig 5B), suggesting that iron influences TMPRSS6 protein content and *Bmp6* gene expression by different pathways.

Full Length HFE2 is Partially Cleaved in Iron-deficient Rats and Mice

In previous studies using liver microsomes, we found unchanged HFE2 protein content in samples from iron-deficient mice [34]. However, in the present study, the 3000 g samples from iron-deficient animals displayed, in addition to the main band of approximately 52 kDa, an additional weak truncated band of approximately 47 kDa (Fig 6A). Since TMPRSS6 protein was increased in the same samples from iron-deficient rats and mice (Fig 2A), it is theoretically possible that this shorter band represents a TMPRSS6-cleaved fragment of full-length HFE2. In contrast to the effect of iron deficiency, EPO treatment did not result in increased cleavage of HFE2, despite an increase in TMPRSS6 protein content (Fig 6B). These results possibly indicate that increased cleavage of HFE2 requires prolonged treatment and/or severe iron deficiency.

The modest shift of the cleaved band *vs*. the full-length protein (approximately 4 to 6 kDa) indicates a possible cleavage site at one of the several arginines at the N-terminus of HFE2. To determine whether the cleavage occurs in the glycosylated part of the protein, low-iron samples displaying distinct cleaved bands were treated with PNGase F, which removes N-linked oligosaccharides. This treatment reduced the apparent mass of the full length HFE2 protein by approximately 10 kDa (from 52 to 42 kDa); the size of the cleaved band present in iron-deficient samples was reduced by PNGase F treatment from 47 kDa to approximately 37 kDa (Fig 6C). These data demonstrate that the iron deficiency-induced cleavage of HFE2 does not affect the number of N-linked oligosaccharides present in the membrane-bound part of the protein, indicating that the cleavage site is located N-terminally to the first N-linked oligosaccharide (Fig 6D).

TMPRSS6 Protein is Decreased in *Hfe2*-/- Mice

HFE2 is the proposed target for the protease activity of TMPRSS6 [9]. However, in our previous studies with whole liver homogenates and liver microsomes [21], we found decreased, rather than increased, HFE2 protein content in *mask* mice. A similar decrease in full-length HFE2 protein in *mask* mice was confirmed in the present study utilizing the 3000 g membrane fraction (Fig 7B). *Vice versa*, the amount of TMPRSS6 protein was strongly decreased in liver samples from *Hfe2*-/- mice (Fig 7A). In addition, *Hfe2*-/- mice displayed strongly decreased content of neogenin (Fig 7C), another protein reportedly participating in *Hamp* gene regulation [25,35].

Although *Hfe2*-/- and *Bmp6*-/- mice are almost identical with respect to the iron overload phenotype [18,29,30], their liver TMPRSS6 protein content is markedly different, with *Hfe2*-/- mice displaying almost no detectable full length TMPRSS6 protein (Fig 7A) and no response of TMPRSS6 to EPO treatment (S6 Fig). In contrast, TMPRSS6 was readily detectable in *Bmp6*-/- mice (Fig 4A).

Similar to mice injected with iron, the anti-TMPRSS6 antibody detected a strong band at 75 kDa in *Hfe2*-/- mice (Fig 7A). As the position of this band did not change following PNGase F treatment (S5 Fig), it very probably represents a non-specific band.

Discussion

TMPRSS6 is a hepatocyte transmembrane serine protease whose mutations result in iron-refractory iron deficiency anemia [1]. The identification of TMPRSS6 as a potent negative regulator of hepcidin, the key iron-regulatory hormone, has raised the possibility that hepcidin expression could be regulated by modulation of TMPRSS6-dependent proteolytic activity. However, very little information is available on the *in vivo* regulation of TMPRSS6 protein levels.

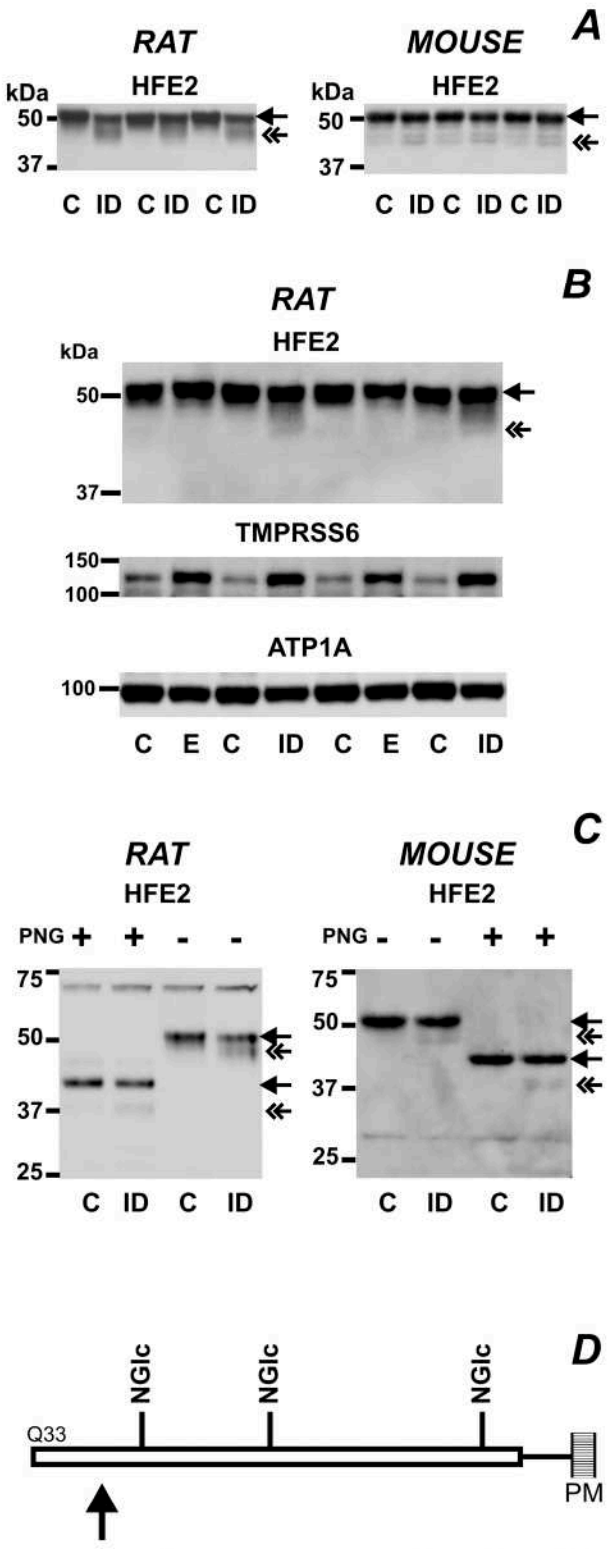

Fig 6. HFE2 is partially cleaved at the N-terminus in iron-deficient animals. (A) Partial cleavage of full length HFE2 (arrow) to an approximately 5 kDa shorter protein (double arrow) under iron deficiency conditions. Weaned rats and mice were kept on an iron deficient diet for three or four weeks respectively (ID), control animals (C) received standard laboratory diet. Loading controls are similar to Fig 2A. (B) Comparison of the effects of iron-deficient diet (ID) or EPO treatment (E) on TMPRSS6 protein content and HFE2

cleavage in rats. Both treatments increase TMPRSS6 protein content; cleaved HFE2 band (double arrow) is apparent only in iron-deficient animals. (C) Cleaved HFE2 protein has the same number of N-linked oligosaccharides as the full length protein. Samples from control (C) or iron-deficient (ID) rats and mice were incubated with PNGase F (PNG +) or water (-). PNGase F treatment reduced the size of full length HFE2 protein (arrow) by about 10 kDa; the same size reduction of approximately 10 kDa was observed for the cleaved HFE2 protein (double arrow) present in iron-deficient samples. (D) Schematic representation of full length mouse HFE2 protein. The 361 amino acid chain has Q33 as the N-terminal amino acid (UniProt entry Q7TQ32), and is bound with a GPI anchor to the plasma membrane (PM). Arrow denotes the approximate position of the observed cleavage site, which is located N-terminally of the three N-linked oligosaccharide (NGlc) chains.

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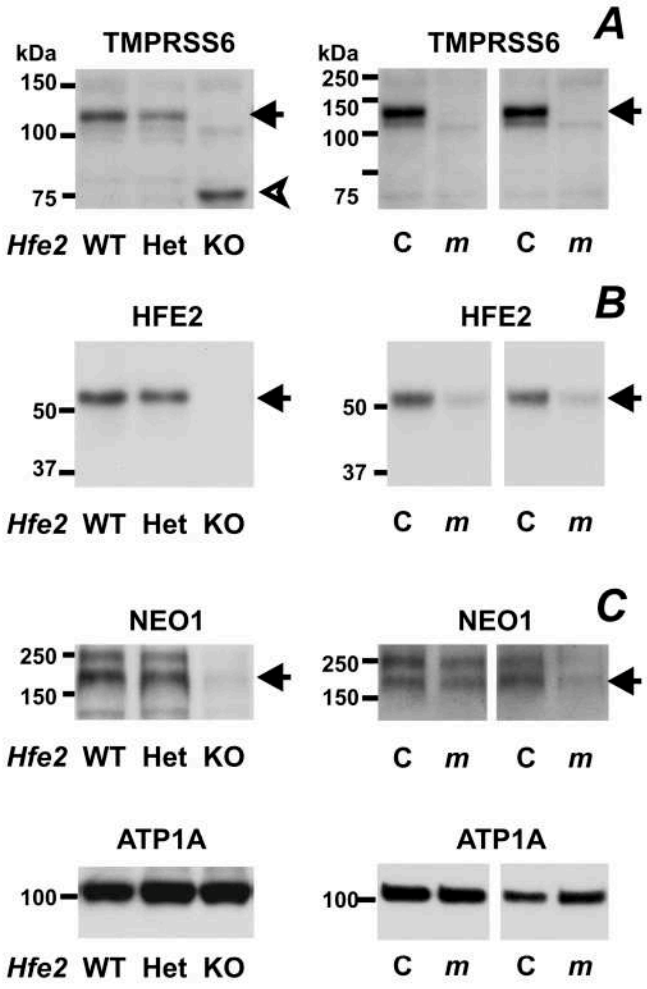

Fig 7. TMPRSS6 protein content and neogenin protein content is decreased in *Hfe2-/-* mice. (A) Immunoblot of TMPRSS6 protein in the liver 3000 g fraction of *Hfe2*+/+, (*Hfe2* WT), *Hfe2*-/+ (Het), *Hfe2*-/- (KO), C57BL/6 (WT) and *mask* (*m*) mice. Arrow denotes the TMPRSS6-specific band; arrowhead indicates a nonspecific band. *Mask* mice samples represent results from two experiments with two pairs of mice. (B) and (C) Immunoblots of HFE2, neogenin (NEO1) and ATP1A (loading control) in the same samples. Arrows denote HFE2- and NEO1-specific bands.

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Hepcidin expression is known to respond to iron overload and iron deficiency, as well as to the rate of erythropoiesis [7]. Therefore, the primary aim of this study was to determine TMPRSS6 protein content in plasma membrane-enriched fractions of liver samples from experimental animals subjected to manipulations of iron homeostasis or EPO administration. Since HFE2 is the proposed target of TMPRSS6, HFE2 protein levels were determined as well.

Analysis of samples from iron-deficient animals confirmed the previously reported [13] increase of TMPRSS6 protein by iron deficiency. *Tmprss6* mRNA did not significantly change in iron deficiency anemia, highlighting the importance of posttranscriptional regulation of TMPRSS6 [36]. Interestingly, immunoblotting of HFE2 protein indicated that in iron-deficient animals the full length HFE2 protein is partially cleaved. Since TMPRSS6 protein content was increased in the same samples, these results indirectly support the proposed concept that membrane HFE2 could be the physiological target of TMPRSS6 [9]. The modest band shift of about 3–6 kDa suggests that HFE2 is cleaved relatively close to the N-terminus, while treatment with PNGase F indicates that the resulting shortened membrane-bound fragment retains all three of its N-linked oligosaccharides (Fig 6D). In this respect, it is interesting to note that the ligand of HFE2, BMP6, was proposed to bind at the N-terminal part of HFE2 [37]. It is therefore possible that the partial cleavage of HFE2 at the N-terminus could diminish its ability to function as BMP6 coreceptor. In conditions of iron deficiency, increased TMPRSS6 protein content could thus result in decreased activity of the BMP6/HFE2 signaling pathway, contributing to the well-documented decrease of *Hamp* expression.

In sufficiently exposed blots, the cleaved band could also be seen in control samples, demonstrating that HFE2 cleavage occurs to some degree in control livers as well. However, even under conditions of severe iron deficiency, the intensity of the truncated band was much weaker than the intensity of the full-length protein band, suggesting that only a minor part of the full-lengh plasma membrane HFE2 is actually cleaved.

Injections of iron dextran to mice decreased liver TMPRSS6 protein content by a posttranscriptional mechanism. This response of TMPRSS6 to iron again confirms the inverse correlation between hepatic *Hamp* mRNA content and TMPRSS6 protein content, indicating that the modulation of TMPRSS6 activity could be an important factor in the regulation of hepcidin expression.

Clinical as well as experimental studies indicate that TMPRSS6 is particularly important for the appropriate downregulation of hepcidin expression by signals related to accelerated erythropoiesis. It has for a long time been known that administration of EPO to experimental animals downregulates *Hamp* transcription [38]. This EPO-mediated decrease of *Hamp* expression occurs not only in rats and wild-type mice, but also in HFE2-deficient mice [39,40], TFR2-deficient mice [40], and BMP6-deficient mice (Fig 4D). In striking contrast to these animals, TMPRSS6-deficient mice fail to decrease *Hamp* mRNA content following EPO treatment [41,42]. In addition, it has been reported that EPO administration does not improve anemia in patients with *TMPRSS6* mutations [43]. These reports strongly suggest that TMPRSS6 is a crucial component of the "erythroid regulator" which increases iron absorption in response to erythropoietic demand [44]. In agreement with this concept [45,46], we found increased TMPRSS6 protein content in the livers of EPO-treated mice and rats. Intriguingly, although liver TMPRSS6 protein increased both in response to EPO treatment or in response to iron deficiency, the partial cleavage of HFE2 protein was not observed in EPO-treated animals, possibly indicating that detectable cleavage occurs only in conditions of prolonged and severe iron depletion.

The exact pathway(s) which decrease *Hamp* expression following administration of EPO are at present under intensive investigation. Very probably, the dramatic downregulation of liver *Hamp* mRNA content observed in EPO-treated experimental animals results from a

combination of two main events: The EPO-induced decrease in liver and plasma iron content, which causes decreased signaling through the iron-sensing signaling pathways, and the synthesis, in the activated bone marrow and spleen, of specific factor(s) mediating *Hamp* downregulation by an yet unknown mechanism [40,47]. It is not clear whether the observed EPO-induced increase in TMPRSS6 protein is related solely to the EPO-induced efflux of liver and plasma iron into the erythron, as suggested by the observed lack of effect of short-term EPO administration, or whether EPO also increases TMPRSS6 protein content by some other mechanism. In any case, the increase of TMPRSS6 protein content observed in mice and rats treated with EPO for several days could, by downregulating BMP6/HFE2 signaling, contribute to the profound downregulation of *Hamp* expression in these animals.

Although HFE2 is the most probable target of TMPRSS6, we previously paradoxically found decreased, rather than increased, amount of the autocatalytically cleaved 35 kDa HFE2 fragments in microsomes from *TMPRSS6*-mutated mice [21,23]. In the present study, we confirm decreased HFE2 protein content in the liver 3000 g fraction from *mask* mice. To further study the interaction between TMPRSS6 and HFE2, we now reciprocally determined TMPRSS6 protein levels in *Hfe2*-/- mice (Fig 7A). Interestingly, disruption of the *Hfe2* gene resulted in a dramatic decrease of TMPRSS6 protein content. A possible explanation for this observation is that TMPRSS6 and HFE2, and possibly also neogenin, function as a protein complex in the hepatocyte plasma membrane, as previously reported by other groups [9,25]. Disruption of one component of this protein complex could then lead to accelerated degradation of the other component(s). This would explain the decreased liver TMPRSS6 and neogenin content in *Hfe2*-/- mice, as well as the decreased HFE2 content in *mask* mice.

Overall, the *in vivo* results reported in this study do support the reported [9] interaction between TMPRSS6 and HFE2 proteins. However, this does not exclude the possibility that, in addition to HFE2, TMPRSS6 could have other important physiological targets as well. While available literature data indicate that the presence of functional TMPRSS6 protein represents a *sine qua non* condition for the EPO-induced decrease of liver *Hamp* mRNA [41–43,45] it has also very recently been reported that EPO-mediated downregulation of liver *Hamp* expression does not occur in mice lacking the newly identified erythroblast-secreted factor erythroferrone [40]. This could indicate that both TMPRSS6 and erythroferrone are important components of one signaling pathway [46]. In this respect, it should be noted that despite the profound decrease in hepatic TMPRSS6 protein in *Hfe2*-/- mice (Fig 7A), these mice are still able to downregulate *Hamp* expression following EPO administration [39,40], possibly indicating that even a limited amount of Tmprss6 protein enables efficient erythroferrone signaling. Obviously, further studies will be necessary to elucidate the exact role of TMPRSS6, and to identify its possible additional substrates.

In conclusion, our study demonstrated, in both mice and rats, a posttranscriptional increase of liver TMPRSS6 protein by erythropoietin. In addition, the observed partial cleavage of HFE2 in liver samples with high TMPRSS6 protein content, as well as the substantial decrease of TMPRSS6 protein in *Hfe2*-/- mouse liver samples, strongly suggests that TMPRSS6 and HFE2 proteins interact at the hepatocyte plasma membrane.

Supporting Information

S1 Fig. Effect of iron deficiency and EPO administration on *Hamp* mRNA.
(DOC)

S2 Fig. Lack of effect of a single dose of EPO on TMPRSS6 protein content in rats.
(DOC)

S3 Fig. Lack of effect of low doses of iron-dextran on TMPRSS6 protein content in mice.
(DOC)

S4 Fig. Effect of iron on *Hamp*, *Bmp6* and *Tmprss6* RNA.
(DOC)

S5 Fig. The 75 kDa band observed in mouse liver samples with high iron content is not TMPRSS6-specific.
(DOC)

S6 Fig. Lack of effect of EPO on TMPRSS6 protein content in *Hfe2*-/- mice.
(DOC)

S1 Table. List of primers used for PCR analysis.
(DOC)

S2 Table. Liver iron content in iron-overloaded animals.
(DOC)

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Author Contributions

Conceived and designed the experiments: JK. Performed the experiments: JF PP LLF IG JK. Analyzed the data: JT MV JK. Contributed reagents/materials/analysis tools: JT XD. Wrote the paper: JT MV JK.

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Erythropoietin administration increases splenic erythroferrone protein content and liver TMPRSS6 protein content in rats

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ABSTRACT

Erythroferrone (ERFE) and TMPRSS6 are important proteins in the regulation of iron metabolism. The objective of the study was to examine splenic ERFE and liver TMPRSS6 synthesis in rats treated with a combination of iron and erythropoietin (EPO).

EPO was administered to female Wistar rats at 600 U/day for four days, iron-pretreated rats received 150 mg of iron before EPO treatment. Content of ERFE and TMPRSS6 proteins was determined by commercial antibodies.

Iron pretreatment prevented the EPO-induced decrease in hepcidin expression. Content of phosphorylated SMAD 1,5,8 proteins was decreased in the liver by both EPO and iron plus EPO treatment.

Fam132b expression in the spleen was increased both by EPO and iron plus EPO treatments; these treatments also significantly induced splenic *Fam132a* expression. ERFE protein content in the spleen was increased both by EPO and iron plus EPO to a similar extent.

EPO administration increased TMPRSS6 content in the plasma membrane-enriched fraction of liver homogenate; in iron-pretreated rats, this increase was abolished.

The results confirm that iron pretreatment prevents the EPO-induced decrease in liver *Hamp* expression. This effect probably occurs despite high circulating ERFE levels, since EPO-induced ERFE protein synthesis is not influenced by iron pretreatment.

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1. Introduction

Iron is indispensable for life. Although iron is required for the function of many proteins, the major part of iron in the body, about 75%, is contained in hemoglobin [1,2]. Therefore, regulation of iron metabolism must be closely related to the rate of erythropoiesis. Already in 1994, a classical paper [3] postulated the existence of an "erythroid regulator", which controls iron uptake according to erythropoietic needs.

At the turn of the century, the basal principles of iron metabolism regulation were elucidated by the discoveries of hepcidin, the iron regulatory hormone encoded by the *HAMP* gene [4,5], and its target protein ferroportin [6–9], which exports iron from enterocytes, macrophages and other cells. Shortly after the discovery of hepcidin, it was demonstrated [10] that *Hamp* mRNA content in mouse liver dramatically decreases following administration of erythropoietin (EPO). Since hepcidin blocks iron uptake from the duodenum, as well as the release of iron from macrophages, the EPO-induced downregulation of *Hamp* expression serves to increase iron absorption and recycling to provide adequate iron supply for erythropoiesis [1,2].

The pathway which downregulates *Hamp* expression in response to EPO administration remained elusive for a long time. Although it is known that EPO-mediated *Hamp* mRNA downregulation is mediated by EPO-induced erythropoietic activity, and not by EPO *per se* [11,12], the actual protein responsible for *Hamp* gene regulation by erythropoiesis has been discovered only very recently. Erythroferrone (ERFE), encoded by the *Fam132b* gene, had originally been named myonectin and had been regarded as a protein related mainly to glucose metabolism [13]; however, in 2014, it was convincingly demonstrated that ERFE, secreted by erythroblasts, efficiently decreases liver *Hamp* expression by an yet unidentified pathway [14]. Of particular significance is the reported observation that mice deficient in ERFE are unable to downregulate *Hamp* expression in response to administration of a high dose of EPO [14], which confirms the role of ERFE in iron regulation during stress erythropoiesis.

Interestingly, prior to the discovery of the role of ERFE, another protein participating in *Hamp* regulation by EPO had been identified: the serine protease matriptase-2 (TMPRSS6), encoded by the *TMPRSS6* gene [15–17]. In striking similarity with ERFE-deficient mice, it was shown that mice lacking TMPRSS6 proteolytic activity were also unable

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to downregulate *Hamp* expression following EPO administration [18, 19]. It has been hypothesized that TMPRSS6 could be a part of the ERFE signaling pathway [20]; however, the exact relationship between TMPRSS6 and ERFE is at present unclear and under investigation.

Since EPO and iron exert opposite effects on *Hamp* expression, several groups have examined the combined effect of these two stimuli in *Hamp* gene regulation [21–24]. The pathway regulating *Hamp* expression in response to iron is relatively well characterized, and includes the hepatocyte plasma membrane protein hemojuvelin (HJV) as its important component [25]. Signaling through the HJV pathway results in phosphorylation of the SMAD1, SMAD5 and SMAD8 proteins, ultimately leading to an increase in *Hamp* transcription. Originally, it had been demonstrated that the resulting effect of simultaneous administration of EPO and iron on the activity of the HJV/SMAD pathway depends on the doses used [21]. Subsequently, it was shown in mice that the dramatic downregulation of *Hamp* gene expression by EPO is prevented by iron pretreatment [22]. This experimental observation has been confirmed in a recent paper, which moreover showed that bone marrow *Fam132b* mRNA responds to EPO treatment, but not to iron administration [19].

The purpose of the present study was to further examine the effect of combined treatment with iron and EPO on *Hamp* gene expression and HJV/SMAD signaling, and to correlate the results with TMPRSS6 protein content in the liver and ERFE protein content in the spleen.

2. Material and methods

2.1. Animals and treatment

Animal experiments were approved by the Czech Ministry of Education, protocol MSMT-1461/2015-5; necessary efforts were made to limit animal suffering. Previous data have demonstrated that EPO-induced changes in TMPRSS6 protein content are easier to detect in rats than in mice [26]. Therefore, female outbred Wistar rats (200–225 g, Anlab SRO, Prague) were used for the experiments. Rats were pretreated with two doses of iron dextran (Sigma-Aldrich): two weeks before the start of EPO treatment, rats received an intraperitoneal injection of 100 mg Fe/rat, and one week before the start of EPO treatment, an additional injection of 50 mg Fe/rat was administered. EPO (NeoRecormon Roche) was administered at a daily dose of 600 IU/rat on days 1, 2, 3 and 4; rats were sacrificed by decapitation under halothane anesthesia on day 5.

2.2. Sample preparation and immunoblotting

Liver TMPRSS6 and HJV content was determined in a plasma-membrane enriched fraction of liver homogenate as previously described [26]. Briefly, a piece of liver (approx. 200 mg) was homogenized for 3 × 10 s in a 6 mm Ultra Turrax homogenizer in 2.5 ml of 10 mM Hepes, pH 7,4, containing 0.25 M sucrose, 5 mM EDTA and a protease inhibitor cocktail (Roche). After 30 min on ice, the homogenate was centrifuged at 400*g* for 10 min, the pellet was discarded and the supernatant centrifuged at 3000*g* for 15 min. The 3000 g pellet was homogenized in 1 ml of 10 mM Hepes containing 2 M NaCl, recentrifuged at 3000 g and homogenized in 1 ml of 0.1 M sodium carbonate. After 1 h agitation, the homogenate was centrifuged at 16,000*g*, and the pellet homogenized in 1 ml of 10 mM Hepes containing 4 M urea. After 30 min on ice, the homogenate was centrifuged at 16,000*g* and the final pellet washed in 10 mM Hepes, recentrifuged and resuspended in 125 μl of 2% SDS in 25 mM of ammonium bicarbonate. The protein content of this plasma membrane-enriched fraction was approximately 3–5 μg/μl.

For the determination of phosphorylated SMADs a 100 mg piece of liver was homogenized in 2 ml of 50 mM Tris, pH 8, containing 150 mM NaCl, 5 mM EDTA, 1% NP-40 detergent, a protease inhibitor cocktail (Roche) and 1% of phosphatase inhibitor cocktail 3 (Sigma Aldrich). After 1 h agitation at 4 °C, samples were centrifuged at 12,000*g* and the supernatant was used for analysis. For the determination of ERFE protein content in whole spleen, a similar procedure without the addition of phosphatase inhibitors was used. For the determination of ERFE in spleen microsomes, a piece of spleen (approx. 100 mg) was homogenized in 2.5 ml of 0.25 M sucrose, centrifuged at 8000*g* and the supernatant was centrifuged at 100,000*g* for 1 h. The resulting microsomal pellet was resuspended in 2% SDS in 25 mM of ammonium bicarbonate.

Immunoblots were performed as previously described [26]. For the determination of TMPRSS6, Abcam Ab56182 antibody was diluted 1:750; for the determination of HJV, R&D Systems AF 3634 antibody was diluted 1:1000; for the determination of phosphorylated SMADs, Cell Signaling Technology antibody 9511 was diluted 1:600. For the determination of ERFE, Santa Cruz antibody SC-246567, raised against mouse myonectin, was used at 1:200 dilution. Anti-Na+/K + ATPase α (ATP1A), Santa Cruz SC-28800, 1:10,000, and anti-GAPDH, Sigma G9545, 1:30,000, were used as loading controls. Secondary antibodies (anti-rabbit, 711-036-152, 1:40,000 and anti-goat, 705-036-147, 1:40,000) were from Jackson Immunoresearch. Densitometry was performed on a Bio-Rad GS 800 scanner.

For the removal of N-linked oligosaccharides, PNGase F (New England Biolabs) was used according to manufacturers' instructions.

2.3. Validation of the anti-ERFE antibody

Murine *Fam132b* was cloned from spleen cDNA of *mask* mice kept on an iron-deficient diet. The *Fam132b* gene was PCR amplified by using Q5 high fidelity polymerase and the gene specific primers CACCATGGCCTCGACCCGCCGCC and TCACAGGCCCAGGAGGATAGC ACTG, yielding a 1023 bp long PCR product. The PCR product was T/A cloned in to a mammalian expressing vector pTARGET (Promega), clones containing the *Fam132b* insert were identified by colony PCR and verified by restriction with EcoRI. Sequence was verified by Sanger sequencing.

Human hepatoma cell lines HepG2 and Hep3B were obtained from ATCC, cultivated in MEM/5% FBS, seeded on a 6 well plate at 150,000 per well and transfected *via* Lipofectamine LXT reagent according to manufacturer's protocol with DNA: transfection ratio 1:3. Cells were then cultivated for 2 days, lysed in RIPA buffer and immunoblot was performed with 50 μg of total protein. The membrane was then incubated with SC-246567 antibody (1:500), followed by 1:15,000 diluted secondary anti-goat HRP-conjugated antibody. The antigen was then visualized by Clarity ECL substrate and LAS4000 equipment. Validation of the anti-TMPRSS6 and anti-HJV antibodies has been reported previously [26].

2.4. Iron determinations and PCR

Determination of blood counts and liver iron content was performed as previously described [26]. Real-time PCR was performed on an Bio-Rad IQ5 instrument as previously described [26], results are expressed as a difference in PCR cycles (Δ CT) relative to *Actb* mRNA (Δ CT on graphs = *Actb* mRNA CT minus target mRNA CT; the higher the graphed Δ CT value, the higher the expression). Primer sequences are given in Supplementary Table 1.

2.5. Statistical analysis

When comparing PCR data (Δ CT values) or signal densities between the four groups, one way Anova followed by Newman-Keuls multiple comparison test was used. For immunoblot signal density comparison from various blots, the densities of control groups were set as 100%. Hematology parameters between two particular groups were compared using Students *t*-test.

3. Results

3.1. Iron pretreatment prevents the EPO-induced Hamp gene downregulation

Administration of EPO for four consecutive days resulted in an increase in spleen weight, hemoglobin content and hematocrit (Table 1). As expected, liver *Hamp* mRNA content was markedly decreased by EPO treatment; however, as previously reported for mice [19,22], pretreatment with iron almost completely prevented the EPO-induced *Hamp* gene downregulation (Fig. 1A). Although the difference between control and iron-treated group did not reach statistical significance, the amount of liver *Hamp* mRNA increased approximately two-fold after iron pretreatment. There was a statistically significant difference between the iron-treated and iron plus EPO-treated group, suggesting that the ERFE pathway is to some extent functional even in animals with marked iron overload.

3.2. Repeated EPO administration decreases SMAD-dependent signaling

EPO administration for four days decreased *Hamp* expression (Fig. 1A) and liver and plasma iron content (Table 1). It was therefore of interest to determine whether this decrease is mirrored by a decrease of *Id1* and *Smad7* mRNA, used as markers of SMAD signaling [27,28], as well as by a decrease in the content of phosphorylated SMAD1,5 and 8. Results presented in Fig. 2A and B show that the expression of both *Id1* and *Smad7* significantly decreased in the EPO-treated group; in addition, EPO-treatment significantly decreased the expression of both genes as compared to the iron-treated group.

In mice, the content of phosphorylated SMAD proteins is markedly increased by iron pretreatment [29]. However, in iron pretreated rats, the increase in phosphorylated SMADs was only modest and did not reach statistical significance (Fig. 2C). In addition, in contrast to values reported for mice [29], iron pretreatment had only a modest effect on rat liver *Bmp6* mRNA content, which increased to about 145% of control values (Fig. 2D); the increase did not reach statistical significance.

3.3. Repeated EPO administration increases Fam132b and Fam132a expression in the spleen

It has already been reported that, in mice, EPO administration increases bone marrow *Fam132b* expression regardless of pretreatment with iron dextran [19]. The same results were obtained in the present experiment in rat spleen, confirming that iron overload does not interfere with EPO-induced *Fam132b* expression (Fig. 3A).

It has been reported that, *in vitro*, ERFE forms heteromeric complexes with adipolin, a secreted protein encoded by the *Fam132a* gene [13]. Therefore, it was of interest to determine whether *Fam132a* is also expressed in the spleen. As can be seen in Fig. 3B, splenic *Fam132a* mRNA displayed a response to EPO administration; however, the observed increase in *Fam132a* mRNA was not as pronounced as the response of *Fam132b* mRNA. In a separate experiment in mice, the response of splenic *Fam132a* mRNA to EPO was confirmed in EPO-treated C57BL/6 mice (Supplementary Fig. 1).

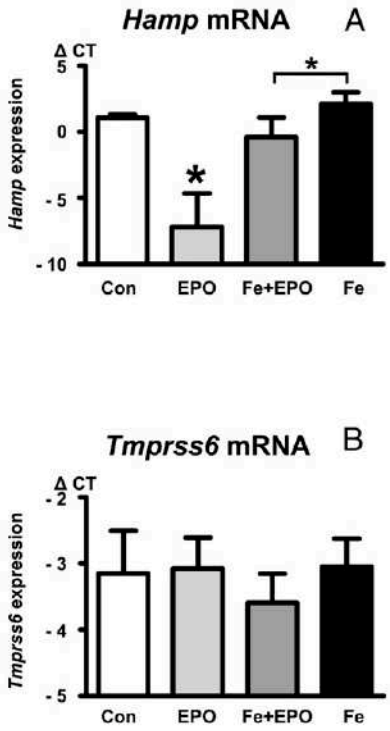

Fig. 1. Iron pretreatment prevents EPO-induced *Hamp* gene downregulation. A: Liver *Hamp* expression in control rats (Con), rats treated with EPO for four days (EPO), iron-pretreated rats treated with EPO for four days (Fe + EPO) and rats pretreated with iron (Fe). B: Liver *Tmprss6* expression in the same group. Values are expressed as means ± SD (n = 6) of Δ CT (CT *Actb* − CT target) obtained by PCR quantification (the higher the Δ CT value, the higher the expression). Asterisk denotes statistically significant difference ($p < 0.05$) from control group, asterisk plus bar a statistically significant difference between the Fe and Fe + EPO group.

3.4. Repeated EPO administration increases ERFE protein synthesis in the spleen

Although it has already been reported that iron does not interfere with EPO-induced *Fam132b* mRNA content [19], no data have so far been reported for ERFE protein content. As can be seen in Fig. 4A, the SC-246567 antibody enabled detection of ERFE protein in both EPO and iron plus EPO-treated spleen. The bands detected by the antibody *in vivo* are ERFE-specific, as similar bands were detected *in vitro* in ERFE-overexpressing cells (Fig. 4B). In control and iron-treated spleens, no bands corresponding to ERFE were detected. This pattern fully correlated with splenic *Fam132b* mRNA content, which displayed an increase

Table 1
Effect of erythropoietin and iron on hematologic parameters and iron concentrations in rats.

Treatment:	Control	EPO	EPO + Iron	Iron
Relative spleen weight (%)	0.22 ± 0.03	0.83 ± 0.07*	0.98 ± 0.12*	0.24 ± 0.05
RBC (10^6)	7.18 ± 0.29	7.72 ± 0.20*	7.54 ± 0.08	6.93 ± 0.13
Hb (g/l)	144.6 ± 7.0	164.6 ± 6.1*	167.4 ± 5.4*	143.8 ± 4.4
HCT (%)	32.6 ± 1.5	39.8 ± 0.9	40.4 ± 1.2	32.00 ± 0.71
MCV (fl)	45.2 ± 0.9	51.7 ± 2.5*	53.4 ± 1.7*	46.5 ± 0.9
Liver iron (μg/g)	244 ± 22	213 ± 75	4902 ± 1138*	6501 ± 174*
Plasma iron (mmol/l)	51.4 ± 10.0	7.8 ± 4.9*	15.7 ± 7.2*	69.9 ± 10.7

RBC, red blood cells; Hb, hemoglobin; HCT, hematocrit; MCV, mean cell volume.
Data are expressed as mean ± SD. Asterisks denote statistically significant difference from control group ($p < 0.05$, n = 4).

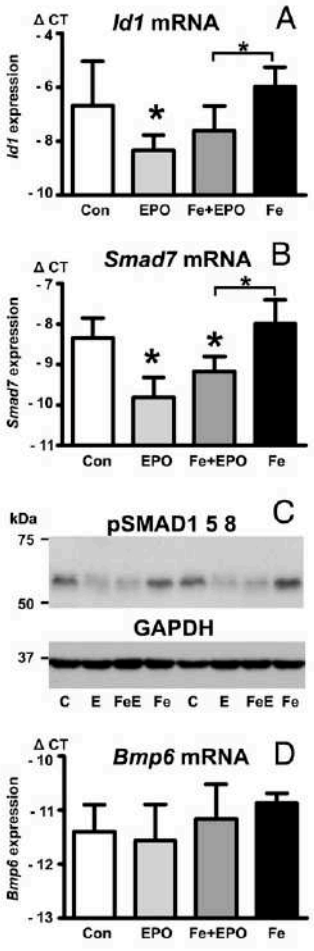

Fig. 2. Effect of erythropoietin and iron on SMAD-dependent signaling. A: Liver *Id1* expression in control rats (Con), rats treated with EPO for four days (EPO), iron-pretreated rats treated with EPO for four days (Fe + EPO) and rats pretreated with iron (Fe). B: Liver *Smad7* expression in the same groups. C: Liver phosphorylated SMAD 1, 5 and 8 protein content in control rats (C), EPO-treated rats (E), iron plus EPO-treated rats (FeE) and iron-treated rats (Fe). GAPDH is used as loading control. D: Liver *Bmp6* expression in the same groups as in panels A and B. Values in panels A, B and D are expressed as means ± SD (n = 6) of Δ CT (CT *Actb* − CT target) obtained by PCR quantification (the higher the Δ CT value, the higher the expression). Asterisk denotes statistically significant difference ($p < 0.05$) from control group, asterisk plus bar a statistically significant difference between the Fe and Fe + EPO group.

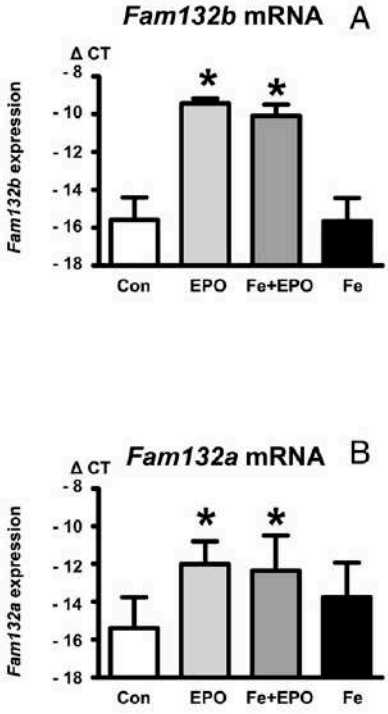

Fig. 3. EPO increases expression of *Fam132b* and *Fam132a* genes in the spleen regardless of iron pretreatment. A: Spleen *Fam132b* expression in control rats (Con), rats treated with EPO for four days (EPO), iron-pretreated rats treated with EPO for four days (Fe + EPO) and rats pretreated with iron (Fe). B: Spleen *Fam132a* expression in the same group. Values are expressed as means ± SD (n = 4) of Δ CT (CT *Actb* − CT target) of PCR quantification (the higher the Δ CT value, the higher the expression). Asterisk denotes statistically significant difference from control group ($p < 0.05$).

of more than an order of magnitude in EPO and iron plus EPO-treated spleens (Fig. 3A).

Since the ERFE bands seen in immunoblots of spleen lysates were relatively weak, an attempt was made to enhance ERFE detection by analyzing spleen microsomes. It was speculated that, as a secreted protein, ERFE will be present in endoplasmic reticulum, which is concentrated in the microsomal fraction. As can be seen in Fig. 4A, ERFE bands were clearly detected in microsomes from EPO-treated and iron plus EPO-treated rats; no bands were seen in control samples or samples from rats pretreated with iron only. The enhanced detection of ERFE in microsomes allowed studies on its glycosylation status. In spleen microsomes, ERFE was clearly present as two bands of approximately 40 and 42 kDa, suggesting different glycosylation of the two bands. Following treatment with PNGase F, which removes N-linked oligosaccharides, both ERFE bands were shifted to approximately 37 kDa (Fig. 4C), which is in rough agreement with the predicted FAM132B mass of 34 kDa. Similar results, *i.e.* an increase following EPO administration and a shift after PNGase F treatment, were observed in a supplementary experiment with EPO-treated mice (Supplementary Fig. 2).

3.5. Iron pretreatment prevents the EPO-induced increase in TMPRSS6 protein content

We have previously reported that EPO administration increases TMPRSS6 protein content in the plasma membrane-enriched fraction of rat liver homogenate [26]. Interestingly, in the present study, an increase in TMPRSS6 protein content was detected in EPO-treated rats, but not in iron plus EPO-treated rats (Fig. 5A and B). The effect was post-transcriptional, as there was no corresponding change in *Tmprss6* mRNA (Fig. 1B).

3.6. Increase in TMPRSS6 protein content has no apparent effect on HJV protein content

It has been proposed that HJV represents the physiological substrate of TMPRSS6-related proteolytic activity. It was therefore of interest to determine the effect of the EPO-induced TMPRSS6 content on HJV protein levels. As can be seen in Fig. 5C, the increase in TMPRSS6 protein content was not coupled with a decrease in HJV protein content.

4. Discussion

Although the marked effect of EPO on *Hamp* gene downregulation was described as early as 2002 [10], the exact pathways linking accelerated erythropoiesis in the spleen and bone marrow with *Hamp* expression in the liver are still largely unknown. The role of ERFE, a key protein in the *Hamp* response to EPO, has been discovered only very recently [14]. Mice with mutated *Fam132b* gene are unable to downregulate *Hamp* expression following EPO administration, which clearly establishes ERFE as an important molecule in the regulation of hepcidin synthesis. *Fam132b* mRNA increases in the spleen and bone marrow following EPO administration, and a recently

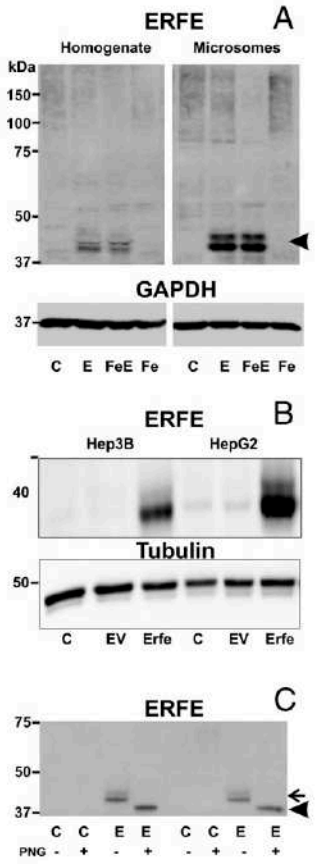

Fig. 4. EPO increases ERFE protein synthesis in the spleen regardless of iron pretreatment. A: Spleen ERFE protein content in control rats (C), rats treated with EPO for four days (E), iron-pretreated rats treated with EPO for four days (FeE) and iron-treated rats (Fe). ERFE was determined in spleen homogenate containing 1% NP40 detergent, or in spleen microsomes, by immunoblotting using Santa Cruz SC-246567 antibody. Arrowhead denotes the ERFE protein bands; GAPDH is used as loading control. B: Validation of the anti-ERFE antibody. Hep3B and HepG2 cells were transfected with an empty vector or with vector containing a 1023 bp *Fam132b* insert. Cells lysates from control cells (C), empty vector- (EV) or *Fam132b*-transfected cells (Erfe) were analyzed using the SC-246567 antibody. Tubulin is used as loading control. C: ERFE protein content in control rat (C) and erythropoietin-treated rat (E) spleen microsomes incubated with water (PNG−) or PNGase F (PNG+). Arrow denotes the glycosylated ERFE bands, arrowhead denotes deglycosylated ERFE protein band.

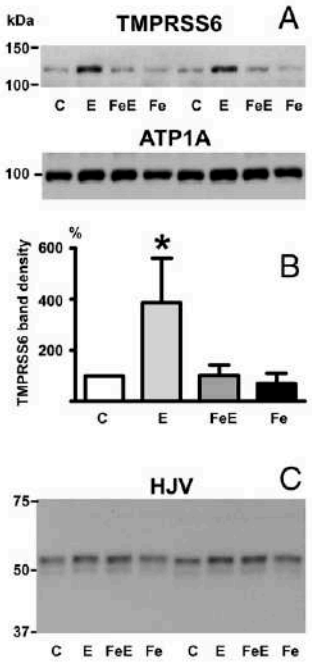

Fig. 5. TMPRSS6 protein content increases in the liver of EPO-treated rats. A: TMPRSS6 protein content in plasma membrane-enriched fractions from control rats (C), rats treated with EPO for four days (E), iron-pretreated rats treated with EPO for four days (FeE) and iron-treated rats (Fe). Na+/K+ ATPase α (ATP1A) is used as loading control. B: TMPRSS6 band density quantification relative to control group. Results are calculated from a total of five immunoblots. Control values are set as 100%. Asterisk denotes statistically significant difference from control group. C: Liver hemojuvelin (HJV) protein content. Stripped membrane from panel A. No significant changes in HJV expression were found between the groups.

developed ELISA assay demonstrated a dramatic increase of ERFE levels in blood following EPO treatment [30].

Another protein possibly participating in the response of *Hamp* to erythropoiesis is the transmembrane serine protease TMPRSS6. Patients with *TMPRSS6* mutations suffer from iron-refractory iron deficiency anemia, and *Tmprss6*-mutated mice are, in striking similarity to *Fam132b*-mutated mice, unable to downregulate liver *Hamp* mRNA following EPO administration [18,19]. However, whether TMPRSS6 participates in ERFE signaling is at present unknown.

The aim of the present study was to confirm, in a rat model, our previous observation that iron pretreatment prevents the dramatic downregulation of *Hamp* gene expression by EPO [22], and to examine the effect of the combined treatments on liver TMPRSS6 and spleen ERFE protein synthesis. Although EPO increases *Fam132b* expression in both the spleen and bone marrow [14,19], the study focused on the spleen since it provides sufficient amount of tissue for protein studies, and also because in rodents the spleen is the principal organ involved in stress erythropoiesis [31].

The results indicate that although iron pretreatment at some level interferes with ERFE signaling, as evidenced by the attenuation of EPO-induced *Hamp* downregulation by iron dextran pretreatment (Fig. 1A), iron overload does not affect splenic ERFE protein synthesis (Fig. 4A). Using the SC-246567 antibody, ERFE was detected both in spleen homogenate and spleen microsomes; although the microsomal fraction enabled more sensitive detection, the results were similar in both system, *i.e.* lack of effect of iron overload on EPO-induced ERFE protein synthesis. To our knowledge, the results represent first reported *in vivo* visualization of ERFE protein by immunoblot. Under reducing conditions, the protein is detected in EPO-treated animals as two bands, indicating different glycosylation. Splenic ERFE protein levels correlated with *Fam132b* mRNA, suggesting that splenic ERFE synthesis is not to a significant extent regulated at the posttranscriptional level. Splenic ERFE protein and splenic *Fam132b* mRNA were increased to the same extent in both the EPO-treated group and the iron plus EPO-treated group, demonstrating that the observed effect of iron on EPO-induced *Hamp* gene downregulation occurs independently of ERFE synthesis.

It has been reported that native ERFE could form higher molecular weight complexes, possibly also with other members of the CTRP family such as adipolin, encoded by the *Fam132a* gene [13]. Therefore, we have determined *Fam132a* expression in the spleens of EPO-treated rats. Interestingly, *Fam132a* mRNA content was increased in the spleen of EPO-treated rats and mice. Whether ERFE actually forms heterodimers with FAM132A protein, and whether it's possible binding to other members of the CTRP family could influence its function in the regulation of *Hamp* expression is at present unknown and requires further investigation.

It has not yet been unequivocally determined whether the ERFE signaling pathway intersects with the BMP/HJV/SMAD signaling pathway. The activity of the BMP/HJV/SMAD pathway can be monitored by determination of phosphorylated SMAD proteins, or by the determination of mRNA levels of its target genes, such as *Id1* and *Smad7*; both phosphorylated SMADs and *Id1* and *Smad7* expression are known to increase following iron treatment [29]. In experiments with short term EPO administration to mice, it has been reported that phosphorylated SMADs and *Id1* mRNA are increased to the same extent in animals

treated with iron, or a combination of iron plus EPO [19]. In contrast, the present results, employing repeated EPO injections to rats, demonstrated a decrease of phosphorylated SMADs and decreased content of *Id1* and *Smad7* mRNA in both EPO and iron plus EPO-treated groups. Thus, in rats, prolonged EPO administration is apparently able to downregulate SMAD signaling even in the presence of very high liver iron content.

Since the synthesis of ERFE, as well as the content of phosphorylated SMADs are approximately similar in both the EPO-treated and iron plus EPO-treated groups, these parameters probably cannot explain the marked difference in *Hamp* expression observed between the EPO-treated and iron plus EPO-treated groups. In this respect, an interesting result of the study is the EPO-induced increase in TMPRSS6 protein, which was observed only in the EPO-treated group, and not in the iron plus EPO-treated group. TMPRSS6 protein content thus displayed a negative correlation with *Hamp* mRNA content (Figs. 1A and 5B). Therefore, it is theoretically possible that the iron-mediated attenuation of *Hamp* gene downregulation by EPO is related to the failure of EPO to elicit sufficient increase in TMPRSS6 protein content in iron-pretreated animals.

During the last years, TMPRSS6 is emerging as an important negative regulator of *Hamp* expression. Liver TMPRSS6 increases posttranscriptionally both following iron deficiency [32] and EPO administration [26]. According to the current model, TMPRSS6 modulates BMP/SMAD signaling by cleaving the BMP coreceptor HJV [33]. Because only one member of the BMP family, BMP6, is known to increase in iron overload [29], TMPRSS6 can be regarded mainly as a modulator of BMP6-dependent signaling. However, very recently a new concept of BMP-dependent signaling has been proposed, which suggests that, rather than to function as a BMP6 coreceptor, HJV serves as a coreceptor for other BMP proteins [34]. This would imply that TMPRSS6 does not affect signaling through BMP6. In this respect, it is interesting to note that in our experimental setting the response of *Bmp6* mRNA to iron administration did not reach statistical significance, whereas TMPRSS6 protein increased following EPO administration approximately four fold. This could indicate that, in female rats, signaling through BMP proteins other than BMP6 could be the main factor determining *Hamp* gene expression, and that this signaling could be modulated by TMPRSS6 protein levels. In contrast, in mice, the response of *Hamp* expression to iron is probably influenced mainly by BMP6 signaling, as evidenced by the well-documented increase in *Bmp6* mRNA following iron treatment [29,35,36]. As to the mechanism of TMPRSS6 action, it is interesting to note that despite the marked increase of TMPRSS6 in EPO-treated rats, no significant change was seen in HJV protein content. This apparently indicates that if the TMPRSS6-mediated cleavage of HJV occurs *in vivo*, the actual amount of cleaved HJV is small. Although it is tempting to speculate that TMPRSS6 could modulate ERFE signaling through proteolytic processing of ERFE [20], in analogy with the reported physiological role of furin-mediated proteolytical processing of FAM132A protein [37], this mode of action of TMPRSS6 is not compatible with the report that *Hamp* expression is efficiently downregulated by EPO administration in mice lacking both HJV and TMPRSS6 [38], or with the report that blocking of SMAD-dependent signaling in *Tmprss6* −/− mice enables partial downregulation of *Hamp* mRNA content by EPO [19]. Obviously, more experiments are necessary to elucidate the possible interaction between TMPRSS6 and ERFE-mediated signaling.

Finally, it should be noted that although the dramatic downregulation of *Hamp* mRNA content by repeated EPO administration is almost completely prevented by iron pretreatment both in mice [22] and rats (Fig. 1A), *Hamp* expression in animals treated with the combination of iron plus EPO is significantly lower than *Hamp* expression in animals treated with iron alone. Despite the marked iron overload caused by injection of high iron doses, EPO is apparently still to some extent able to downregulate the iron-induced *Hamp* mRNA content. This is in agreement with the concept that the “erythroid regulator” is to some extent stronger than the “stores regulator” [23].

In conclusion, the study confirms, in a rat model, the observation that iron pretreatment prevents the dramatic downregulation of *Hamp* expression by EPO. This effect probably occurs despite high circulating ERFE levels, since EPO-induced ERFE protein synthesis is not influenced by iron pretreatment. In addition, the study demonstrates that iron overload decreases the EPO-induced liver TMPRSS6 protein content.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bcmd.2017.02.007.

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IG and JF participated equally in the study. IG, JF and JK performed experiments, ZR and JT provided and validated the ERFE antibody, MV, JT and JK analyzed data, MV and JK designed the experiments, JK wrote the manuscript. The technical assistance of Martin Molík and Napatsorn Thewaran, as well as the proofreading by Filip and Tom is gratefully acknowledged.

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