Severe Microcytic Anemia but Increased Erythropoiesis in Mice Lacking Hfe or Tfr2 and Tmprss6

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Abstract

Cell surface proteins Hfe, Tfr2, hemojuvelin and Tmprss6 play key roles in iron homeostasis. Hfe and Tfr2 induce transcription of hepcidin, a small peptide that promotes the degradation of the iron transporter ferroportin. Hemojuvelin, a co-receptor for bone morphogenic proteins, induces hepcidin transcription through a Smad signaling pathway. Tmprss6 (also known as matriptase-2), a membrane serine protease that has been found to bind and degrade hemojuvelin *in vitro*, is a potent suppressor of hepcidin expression. In order to examine if Hfe and Tfr2 are substrates for Tmprss6, we generated mice lacking functional Hfe or Tfr2 and Tmprss6. We found that double mutant mice lacking functional Hfe or Tfr2 and Tmprss6 exhibited a severe iron deficiency microcytic anemia phenotype mimicking the phenotype of single mutant mice lacking functional Tmprss6 (*Tmprss6* $^{msk/msk}$ mutant) demonstrating that Hfe and Tfr2 are not substrates for Tmprss6. Nevertheless, the phenotype of the mice lacking Hfe or Tfr2 and Tmprss6 differed from Tmprss6 deficient mice alone, in that the double mutant mice exhibited much greater erythropoiesis. Hfe and Tfr2 have been shown to play important roles in the erythron, independent of their role in regulating liver hepcidin transcription. We demonstrate that lack of functional Tfr2 and Hfe allow for increased erythropoiesis even in the presence of high hepcidin expression, but the high levels of hepcidin levels significantly limit the availability of iron to the erythron, resulting in ineffective erythropoiesis. Furthermore, repression of hepcidin expression was unaffected by loss of functional Hfe, Tfr2 and Tmprss6.

Keywords

Introduction

In the past few years, the understanding of the roles of hepcidin, ferroportin, Hfe, transferring receptor 2 (Tfr2), hemojuvelin, and Tmprss6 (matriptase-2) in iron regulation

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has been rapidly growing. Hepcidin is a 25 amino acid peptide that promotes internalization and degradation of ferroportin, an iron transporter molecule [18]. Hfe, Tfr2 and hemojuvelin are positive regulators for transcriptional expression of the hepcidin gene, *Hamp*. In the current model, transferring receptor 1 (Tfr1) sequesters Hfe under low levels of holo-transferrin [13;14;23]. At high levels of holo-transferrin, the Hfe Tfr1 complex is destabilized and Hfe interacts with Tfr2 and holo-transferrin. The Hfe Tfr2 holo-transferrin complex promotes hepcidin expression through a currently unknown pathway [13;14;23]. Hemojuvelin is a co-receptor for Bmps, particularly Bmp6, the Bmp induced by iron [1;2]. Hemojuvelin upregulates hepcidin expression through activation of the Smad1/5/8 and Smad4 signaling pathway [1;2]. Tmprss6, a membrane associated serine protease was shown to be a potent repressor of *Hamp* expression [7]. *In vitro*, Tmprss6 was found to bind and promote the cleavage of hemojuvelin [24]. Nevertheless, Tmprss6 protease activity has been demonstrated to be promiscuous *in vitro*, cleaving a variety of proteins including fibronectin, type I collagen, fibrinogen, pro-urokinase plasminogen activator and artificial peptides corresponding to cleavage sites predicted in filaggrin, Cub-Domain Containing Protein 1 (CDCP1) and $\alpha_E\beta_7$ integrin [3;27]. Furthermore, Krijt et al have demonstrated that mice lacking Tmprss6 do not express elevated levels of hemojuvelin protein, which would have been expected if hemojuvelin was indeed the major substrate for Tmprss6 [16]. This raised the question as to whether Tmprss6 might cleave Hfe or Tfr2, the other two positive regulators of *Hamp* expression. We previously reported that mice lacking functional hemojuvelin and Tmprss6 exhibited repression of the *Hamp* gene and severe iron overload mimicking mice lacking hemojuvelin alone [26]. The phenotype of these mice provided *in vivo* evidence that hemojuvelin or a protein upstream of hemojuvelin (possibly Hfe or Tfr2) was the major substrate for the serine protease Tmprss6.

It remains unclear how the Hfe/Tfr2 regulates *Hamp* gene expression. Hfe deficient mice exhibit inappropriately low levels of Smad1/5/8 phosphorylation despite high levels of endogenous Bmp6 suggesting that Hfe is required for Bmp6 signaling [15]. In that hemojuvelin is the putative endogenous receptor for Bmp6, this would suggest that Hfe and Tfr2 merge with the hemojuvelin/Bmp/Smad pathway in regulating the expression of *Hamp*. Hfe and Tfr2 were also found to regulate levels of furin through phosphorylation of erk1/2 [21]. Upregulation of furin would increase the maturation of hepcidin, and Bmps leading to increased hepcidin expression, as well as promote the generation of the soluble form of hemojuvelin to act as a feedback inhibitor of hepcidin expression.

Evidence is also emerging that Hfe and Tfr2 might have functional roles in the erythron independent of the hepatocyte. Ramos et al demonstrated that Hfe modulates transferrin receptor mediated iron uptake in erythroid precursor cells. They suggested that this contributes to the increased hemoglobin levels, mean corpuscular volume and mean cell hemoglobin levels observed in patients and mice lacking functional Hfe, since the magnitude of red cell changes were not observed in age matched *Hamp* deficient mice exhibiting higher liver iron content [22]. Forejtnikova et al identified Tfr2 by mass spectroscopy to be a component of the erythropoietin receptor complex [12]. Tfr2 was shown to be required for efficient cell surface expression of the erythropoietin receptor. Erythroid progenitor cells from mice lacking functional Tfr2 exhibited diminished responsiveness to erythropoietin and higher levels of serum erythropoietin. Silencing of Tfr2 in human erythroid progenitor cells resulted in a delay, but not inhibition of hemoglobinization resulting in a slight increase in total cell number [12]. In addition, genome wide association meta analysis identified Hfe, Tfr2 and Tmprss6 to play a role in MCV, red cell number, and mean cell hemoglobin, respectively [25].

In this report, we describe the phenotype of mice lacking Hfe or Tfr2 and Tmprss6 and their response to normobaric chronic hypoxia.

Methods

Mice

Tfr2 Y245X mutant mice (*Tfr2*$^{tm1slu/tm1slu}$) and the AKR control strain were a generous gift from Dr. Robert Fleming at Saint Louis University Medical School [10]. *Hfe*–/– mice in a C57BL/6 background (*Hfe*$^{tm1sly/tm1sly}$) were a generous gift from Dr. William Sly of Saint Louis University [11]. *Hfe*–/– mice were backcrossed for 10 generations into a 129SvJ background. *Tmprss6* Mask mutant mice (*Tmprss6*$^{msk/msk}$) were previously described [7]. All mice were used in accordance with the IACUC regulations and with Institutional Department of Animal Resource approval.

Male *Tmprss6*$^{msk/msk}$ mice were bred with female *Hfe*–/– mice or *Tfr2*$^{Y245X/Y245X}$ mice maintained on a standard mouse chow diet. Double heterozygous offspring were mated to each other and their progeny genotyped to identify *Hfe*–/–;*Tmprss6*$^{msk/msk}$ offspring and wildtype offspring 129SvJ × C57 BL/6J (*Hfe*$^{+/+}$;*Tmprss6*$^{+/+}$) or *Tfr2*$^{Y245X/Y245X}$; *Tmprss6*$^{msk/msk}$ offspring and wildtype AKR × C57BL/6 (*Tfr2*$^{+/+}$ *Tmprss6*$^{+/+}$). Double mutant or double wildtype mice were used for the current studies. Since iron deficient double mutant female mice do not breed well, the mice strains were maintained by breeding double knockout males with *Tfr2*$^{Y245X/Y245X}$;*Tmprss6*$^{+/msk}$ or *Hfe*–/–;*Tmprss6*$^{+/msk}$ females.

Serum iron and transferrin saturation determinations were made according to the method described by Fielding [8]. Hemoglobin, mean corpuscular volume (MCV), hematocrit, mean corpuscular hemoglobin (MCH), red blood cell distribution width (RDW), and red blood cell count (RBC) were determined using the Drew Scientific Hemavet® 950 Hematology Analyzer System (Oxford, CT). Tissue iron levels and *Hamp* mRNA expression in isolated livers from the various mouse strains was determined as previously described [26].

Chronic hypoxia was induced by placing mice in a hypoxia chamber at 8% oxygen for up to 8 consecutive days. The oxygen level was regulated by nitrogen gas infusion and monitored with a Biosperix (Lacona, NY) oxycycler controller (Model A84XOV). Control mice were exposed to similar environmental conditions within the same room except that they were kept at normoxia for the length of the experiment.

Results

We generated mice lacking functional Tfr2 and Tmprss6/matriptase-2 as well as mice lacking functional Hfe and Tmprss6 to determine if Tfr2 and/or Hfe might also be endogenous substrates for Tmprss6 proteolytic activity since Tmprss6 has been shown to be a promiscuous protease in vitro [3;27]. We found that mice lacking functional Hfe or Tfr2 and Tmprss6 exhibited severe iron deficiency microcytic anemia compared to controls (Table 1). Both double mutant mice had reduced hemoglobin (Hg) levels, lower mean corpuscular volume (MCV), lower serum iron levels, reduced transferrin saturation, and higher expression of *Hamp* mRNA. In some respects, the microcytic anemia (as indicated by hemoglobin levels, MCV, mean corpuscular hemoglobin (MCH)) of the double mutant mice, appeared to be more severe than in the single mutant mice lacking only functional Tmprss6 (*Tmprss6*$^{msk/msk}$ mutant). The iron deficient phenotypes of the mice lacking functional Hfe or Tfr2 and Tmprss6 is in stark contrast to the iron overload phenotype exhibited by mice lacking both functional hemojuvelin and Tmprss6 [26]. Although Bmp6 levels have been reported to be elevated in *Hfe* and *Tfr2* single mutant mice, we found that Bmp6 levels were not elevated in *Tfr2/Tmprss6* and *Hfe/Tmprss6* double mutant mice and therefore Bmp6 does not contribute to the elevated expression of hepcidin.

There were several notable differences between the double mutant and single mutant *Tmprss6* mice. *Tfr2/Tmprss6* double mutant mice exhibited high red blood cell count that essentially normalized the hematocrit (Table 1). *Hfe/Tmprss6* double mutant mice, like *Tmprss6* single mutant mice did not exhibit nearly as high red cell counts and had lower hematocrits. Increased erythropoiesis in the double mutant mice was indicated by the reticulocyte percent. *Tfr2/Tmprss6* and *Hfe/Tmprss6* double mutant mice (males +females) had a mean reticulocyte % of 7.25±0.5 and 6.25±1.2, respectively, as compared to wild type controls of 4.55±0.2 and 4.05±0.4 % respectively. In contrast, *Tmprss6* single mutant mice exhibited 4.86±0.2% reticulocytes compared to control mice with 5.19±0.1%. Only the difference between *Tfr2/Tmprss6* double mutant mice and their control wildtype strain reached statistical significance (P=0.0011). Spleen iron levels in the double mutant strains were the same as wildtype controls but the *Tmprss6* single mutant mice exhibited significantly higher spleen iron content than control mice (P=0.0006). Liver iron content was lower in *Hfe/Tmprrs6* double mutant and *Tmprss6* single mutant mice as compared to wildtype controls, but the liver iron content of *Tfr2/Tmprss6* double mutant mice was not significantly different from wildtype mice (P=0.2671) (Table 1). Erythropoietin levels were also significantly different between the *Tmprss6* double and single mutant mice. We found that *Tmprss6* single mutant mice exhibited significantly higher levels of erythropoietin than either of the double mutant strains (Figure 1).

We used normal barometric pressure hypoxia (8% oxygen) for 4 or 8 days in order to examine if hepcidin could be effectively repressed in these mutant strains. Hypoxia significantly reduced *Hamp* expression in single and both double mutant mice and controls demonstrating that the responsiveness of *Hamp* to hypoxia did not require functional Hfe, Tfr2, or Tmprss6 (Figure 2). Furthermore, hypoxia significantly increased the % reticulocytes, spleen weight, RDW and RBC count of *Tfr2/Tmprss6, Hfe/Tmprss6* double mutant mice, and *Tmprss6* single mutant mice indicating substantial erythropoiesis (Table 2, Figure 2). Hypoxia was effective in correcting the hematocrit and hemoglobin concentration in both double mutant and the single mutant Tmprss6 mice, however, the mice remained hypochromic and microcytic as indicated by low mean corpuscular volume (MCV) and mean cell hemoglobin (MCH) (Figure 2). Subjecting *Tfr2/Tmprss6* double mutant mice to three consecutive 8 day exposures of hypoxia separated by 2 week recovery times in normoxia was insufficient to correct the hypochromia and microcytosis (Table 3).

Discussion

Silvestri et al demonstrated *in vitro* that hemojuvelin is a substrate for Tmprss6/matriptase-2 [24]. These data suggested that *Tmprss6* mutant and knockout mice and humans should be associated with increased hemojuvelin expression, causing elevated hepcidin expression leading to microcytic anemia. Recently, Krijt et al demonstrated that this was not the case. Tmprss6 defective mice do not express elevated levels of hemojuvelin [16]. *In vivo* studies in our lab demonstrated that mice lacking functional hemojuvelin and Tmprss6 exhibit an iron overload phenotype highly similar to hemojuvelin deficient mice supporting that hemojuvelin or some substrate upstream of hemojuvelin was the substrate for Tmprss6 [26]. To determine if Tfr2 or Hfe might be substrates of Tmprss6, we bred double mutant mice defective in Tfr2 or Hfe and Tmprss6.

We reasoned that if either Hfe or Tfr2 were substrates for Tmprss6 proteolytic activity, we would expect the double mutant strain to be iron overloaded in the same manner as the double mutant mice lacking functional hemojuvelin and Tmprss6 [26]. The phenotype of the *Tfr2/Tmprss6^{mskmsk}* double mutant or *Hfe −/− / Tmprss6^{mskmsk}* strain would be more similar to the single *TfR2* mutant or *HFE −/−* strain alone. Alternatively, if Hfe and Tfr2 were independent of the Tmprss6 and hemojuvelin pathway, we would expect mice to have a near

normal phenotype since the suppression of *Hamp* by loss of Tfr2 or Hfe might be partially or totally rescued by upregulation of *Hamp* from increased hemojuvelin. In fact, we found a severe iron deficient phenotype in both *Tfr2/Tmprss6* and *Hfe/Tmprss6* double mutant mice, demonstrating that Hfe and Tfr2 are not substrates for Tmprss6 protease activity, and that Hfe and Tfr2 are upstream of hemojuvelin in the hemojuvelin/BMP/SMAD pathway regulating expression of hepcidin.

While these studies were in progress, Finberg et al reported that *Hfe/Tmprss6* double mutant mice were iron deficient and indistinguishable from the *Tmprss6* single mutant mice [9]. Our work described here is the first description of the *Tfr2/Tmprss6* double mutant mice. Our datademonstrate differences between the double mutant mice strains, particularly in the extent of ineffective erythropoiesis, and the *Tmprss6* single mutant strain.

Tfr2 has been demonstrated to play a role in erythropoiesis since it modulates cell surface localization of the erythropoietin receptor [12]. Loss of Tfr2 is associated with decreased responsiveness to EPO and a compensatory increase in plasma EPO levels. Genome wide association studies have identified Tfr2 to be significantly associated with red cell number [25]. We found that functional loss of both Tfr2 and Tmprss6 was associated with increased erythropoiesis (indicated by increased reticulocytosis, increased red cell number, and increased hematocrit). These data support that Tfr2 plays an important role in the erythron that is independent of its role in regulating hepcidin expression in the hepatocyte.

Mutations in Hfe have been shown to be associated with increased mean corpuscular volume and mean hemoglobin levels [4;25]. We found that loss of functional Hfe and Tmprss6 was not associated with a significant increase in MCV or hemoglobin levels as compared to loss of Tmprss6 alone. These data suggest that the affect of Hfe on MCV and hemoglobin levels is not able to manifest under iron deficiency conditions or high hepcidin levels caused by loss of Tmprss6. Nevertheless, loss of both Hfe and Tmprss6 resulted in an increase in reticulocytosis but not to the extent as loss of Tfr2 and Tmprss6 as indicated by a smaller basal level elevation of spleen weight and red cell number.

Nicolas et al was the first to demonstrate that long-term hypoxia *in vivo* was able to repress hepcidin expression [19]. *In vitro*, hypoxia has been inconsistent in demonstrating direct repression of hepcidin expression [5;6;17;20;28]. Our data clearly demonstrated that *in vivo* chronic hypoxia mediated repression of hepcidin expression did not require functional Tmprss6, Hfe or Tfr2. Since expression levels of EPO did not correlate with the degree of erythropoiesis, the repression of hepcidin expression was likely mediated through erythropoietin-independent hypoxia induced erythropoiesis.

Genome wide studies indicated an association of *TMPRSS6* polymorphisms and mean cell hemoglobin levels [25]. In this study, we find that loss of functional Tmprss6 was associated with a significant reduction in mean cell hemoglobin. Furthermore, while hypoxia was able to restore hemoglobin and hematocrit levels of both double and single *Tmprss6* mutant mice, mean cell hemoglobin levels and mean corpuscular volume improved but remained significantly lower than normal.

In conclusion, we found that upregulation of hepcidin expression by Tmprss6 deficiency supercedes the down regulation of hepcidin expression associated with lack of Hfe or Tfr2, demonstrating that Hfe and Tfr2 are not substrates for Tmprss6 and are likely upstream of hemojuvelin in regulating hepcidin expression. Mice lacking functional Hfe or Tfr2 and Tmprss6 exhibited increased ineffective erythropoiesis despite iron deficiency. Furthermore, we demonstrate that loss of Tmprss6, Hfe and Tfr2 does not affect the repression of hepcidin expression in response to hypoxia.

Acknowledgments

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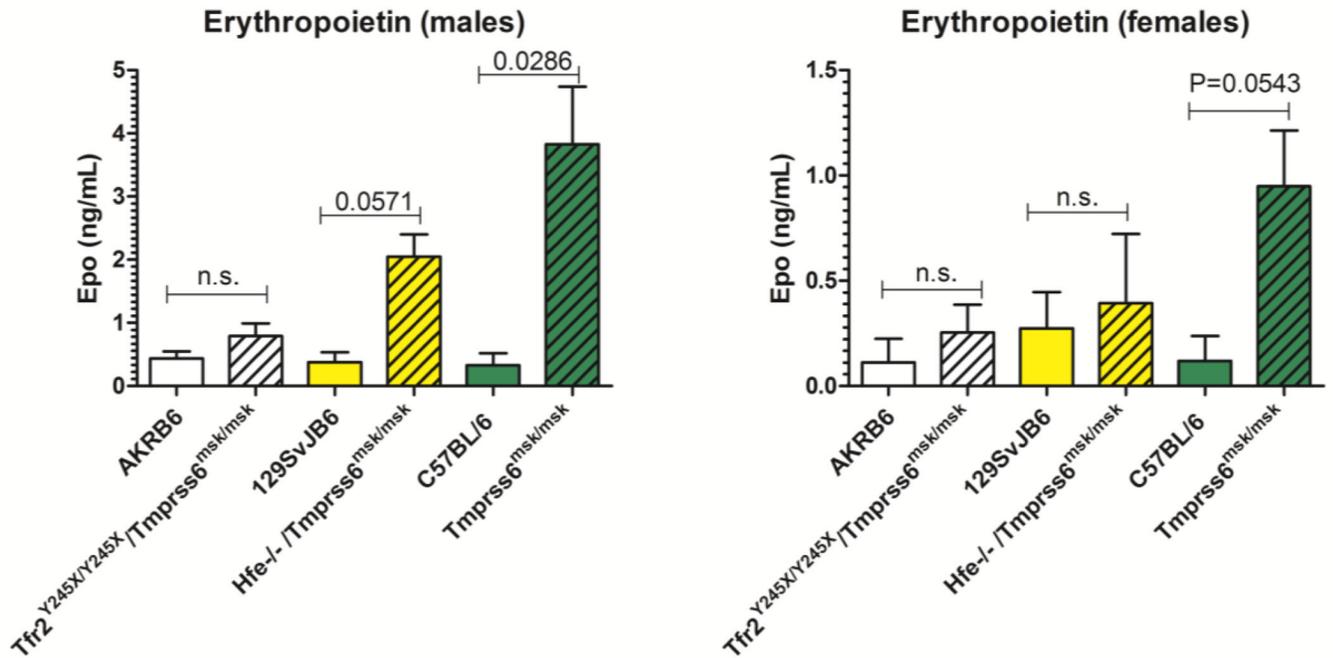
Figure 1.
Endogenous plasma erythropoietin levels in wildtype and mutant mice. Plasma erythropoietin levels were measured by ELISA as described in Methods. Each group represents n=4 mice. Means and standard errors are shown. Statistical significance was determined using Mann Whitney test (GRAPHPAD PRISM 5.0).

Figure 2.
Response to hypoxia by control and mutant mice. Homozygous $Tfr2^{Y245X}Tmprss6^{msk}$ double mutant, $Hfe^{-/-}Tmprss6^{msk}$ double mutant, $Tmprss6^{msk}$ single mutant mice and their respective control strains were exposed to 4 or 8 days of 8% oxygen under normal pressure. For each group, n 4, approximately equal numbers of males and females. The effect on hepcidin gene (*Hamp*) expression, spleen weight and hematological indices were determined as previously described [26]. Means and standard errors are shown. Statistical significance was determined using Mann Whitney test (GRAPHPAD P$_{RISM}$ 5.0). * P 0.0005; † P 0.005; ‡ P 0.05.
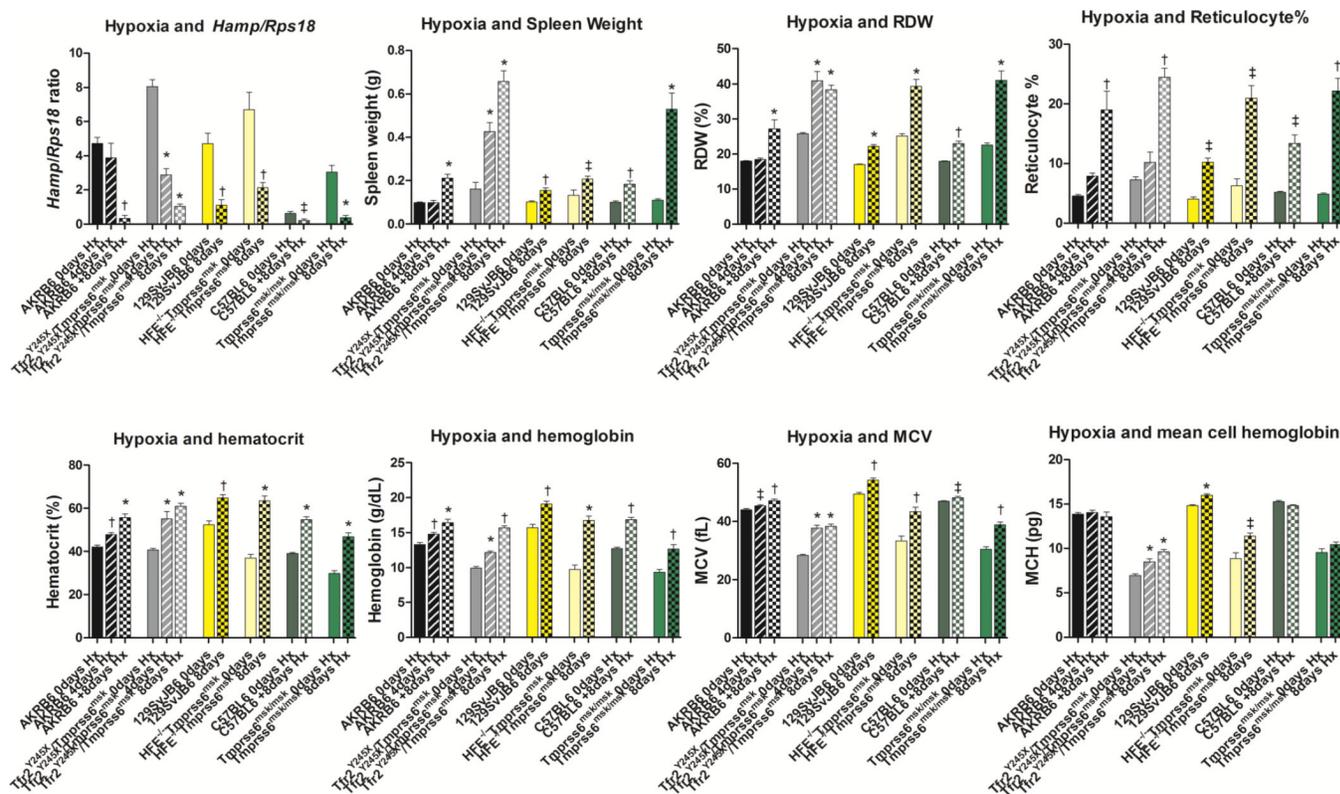
Table 1

Characteristics of $Tfr2^{Y245X/Y245X}/Tmprss6^{msk/msk}$ and $Hfe-/-/Tmprss6^{msk/msk}$ mice.

	strain	Mean ± SE (n)	P value
Hemoglobin (g/dL)	AKRB6	13.3 ± 0.3 (28)	
	$Tfr2^{Y245X/Y245X}/Tmprss6^{msk/msk}$	9.9 ± 0.3 (20)	<0.0001
	129SvJB6	15.8 ± 0.5 (21)	
	$Hfe-/-/Tmprss6^{msk/msk}$	9.8 ± 0.6 (13)	<0.0001
	C57BL/6	12.4 ± 0.2 (8)	
	$Tmprss6^{msk/msk}$	9.3 ± 0.3 (8)	<0.0001
MCV (fL)	AKRB6	44.0 ± 0.5 (28)	
	$Tfr2^{Y245X/Y245X}/Tmprss6^{msk/msk}$	28.4 ± 0.3 (20)	<0.0001
	129SvJB6	49.6 ± 0.7 (21)	
	$Hfe-/-/Tmprss6^{msk/msk}$	32.8 ± 1.6 (13)	<0.0001
	C57BL/6	47.2 ± 0.2 (8)	
	$Tmprss6^{msk/msk}$	31.7 ± 0.9 (8)	<0.0001
Hematocrit (%)	**AKRB6**	**42.2 ± 0.9 (28)**	
	$\boldsymbol{Tfr2^{Y245X/Y245X}/Tmprss6^{msk/msk}}$	**40.8 ± 0.8 (20)**	**0.439**
	129SvJB6	52.6 ± 1.9 (21)	
	$Hfe-/-/Tmprss6^{msk/msk}$	37.0 ± 1.7 (13)	<0.0001
	C57BL/6	39.6 ± 0.7 (8)	
	$Tmprss6^{msk/msk}$	32.7 ± 1.8 (8)	0.0086
MCH (pg)	AKRB6	13.8 ± 0.2 (28)	
	$Tfr2^{Y245X/Y245X}/Tmprss6^{msk/msk}$	7.0 ± 0.2 (20)	<0.0001
	129SvJB6	14.8 ± 0.1 (21)	
	$Hfe-/-/Tmprss6^{msk/msk}$	8.9 ± 0.6 (13)	<0.0001
	C57BL/6	15.3 ± 0.2 (8)	
	$Tmprss6^{msk/msk}$	9.6 ± 0.4 (8)	0.0009
RDW (%)	AKRB6	18.0 ± 0.1 (28)	
	$Tfr2^{Y245X/Y245X}/Tmprss6^{msk/msk}$	25.8 ± 0.4 (20)	<0.0001
	129SvJB6	17.0 ± 0.2 (21)	
	$Hfe-/-/Tmprss6^{msk/msk}$	25.2 ± 0.7 (13)	<0.0001
	C57BL/6	17.9 ± 0.1 (8)	
	$Tmprss6^{msk/msk}$	22.6 ± 0.6 (8)	0.0009
RBC (M/μl)	**AKRB6**	**9.7 ± 0.2 (28)**	

	strain	Mean ± SE (n)	P value
	Tfr2$^{Y245X/Y245X}$/*Tmprss6*$^{msk/msk}$	**14.4 ± 0.4 (20)**	**<0.0001**
	129SvJB6	10.6 ± 0.4 (21)	
	Hfe−/− /Tmprss6$^{msk/msk}$	11.5 ± 0.5 (13)	0.1099
	C57BL/6	8.3 ± 0.1 (8)	
	Tmprss6 $^{msk/msk}$	9.8 ± 0.4 (8)	0.0281
Plasma Iron (µ/dL)	AKRB6	88.9 ± 4.7 (29)	
	Tfr2$^{Y245X/Y245X}$/*Tmprss6*$^{msk/msk}$	24.7 ± 3.2 (19)	<0.0001
	129SvJB6	88.1 ± 8.8 (18)	
	Hfe−/− /Tmprss6$^{msk/msk}$	29.9 ± 5.7 (17)	<0.0001
	C57BL/6	94.6 ± 5.0 (6)	
	Tmprss6 $^{msk/msk}$	21.5 ± 4.9 (10)	0.0002
Tf Saturation (%)	AKRB6	39.6 ± 2.5 (28)	
	Tfr2$^{Y245X/Y245X}$/*Tmprss6*$^{msk/msk}$	8.2 ± 1.2 (16)	<0.0001
	129SvJB6	42.2 ± 3.6 (18)	
	Hfe−/− /Tmprss6$^{msk/msk}$	13.2 ± 3.3 (17)	<0.0001
	C57BL/6	31.9 ± 3.1 (6)	
	Tmprss6 $^{msk/msk}$	6.6 ± 2.2 (6)	0.0022
spleen iron (mg/g)	**AKRB6**	**1.0 ± 0.2 (7)**	
	Tfr2$^{Y245X/Y245X}$/*Tmprss6*$^{msk/msk}$	**1.2 ± 0.2 (6)**	**0.4452**
	129SvJB6	**2.1 ± 0.3 (18)**	
	Hfe−/− /Tmprss6$^{msk/msk}$	**2.1 ± 0.2 (16)**	**0.9039**
	C57BL/6	0.8 ± 0.2 (8)	
	Tmprss6 $^{msk/msk}$	2.3 ± 0.2 (8)	0.0006
liver iron (mg/g)	AKRB6	0.4 ± 0.0 (32)	
	Tfr2$^{Y245X/Y245X}$/*Tmprss6*$^{msk/msk}$	0.3 ± 0.0 (30)	0.0008
	129SvJB6	**0.7 ± 0.1 (29)**	
	Hfe−/− /Tmprss6$^{msk/msk}$	**0.6 ± 0.1 (28)**	**0.2671**
	C57BL/6	0.6 ± 0.1 (10)	
	Tmprss6 $^{msk/msk}$	0.2 ± 0.1 (9)	0.0057
***Hamp/Rps18* ratio**	AKRB6	4.7 ± 0.4 (8)	
	Tfr2$^{Y245X/Y245X}$/*Tmprss6*$^{msk/msk}$	8.2 ± 0.5 (10)	0.0002
	129SvJB6	6.8 ± 0.8 (10)	
	Hfe−/− /Tmprss6$^{msk/msk}$	11.8 ± 1.3 (10)	0.0011

	strain	Mean ± SE (n)	P value
	C57BL/6	5.9 ± 0.7 (10)	
	Tmprss6 $^{msk/msk}$	10.5 ± 1.5 (10)	0.0021
Bmp6/Rps18 ratio	AKRB6	0.004 ± 0.00 (16)	
	Tfr2 $^{Y245X/Y245X}$/*Tmprss6* $^{msk/msk}$	0.006 ± 0.00 (18)	0.0943
	129SvJB6	0.006 ± 0.00 (10)	
	Hfe−/− /*Tmprss6* ^{msk}	0.006 ± 0.00 (10)	0.7959
	C57BL/6	0.001 ± 0.00 (10)	
	Tmprss6 $^{msk/msk}$	0.001 ± 0.00 (10)	0.0524

Hematological values were measured using a Hemavet® 950 Hematology Analyzer System (Drew Scientific). Plasma iron, transferrin saturation, spleen and liver iron content were measured as previously described [26]. *Hamp*, *Bmp6* and *Rps18* mRNA expression were measured as described previously [26]. Data include approximately equal numbers of male and female mice between 8–14 weeks old. Mean and standard error are shown, along with the number of mice (n) used for each analysis. Statistical significance was determined using Mann Whitney test (GRAPHPAD PRISM 5.0).

Table 2

Reticulocytosis following hypoxia for 4 or 8 days

	% reticulocytes		
	normoxia	4 days hypoxia	8 days h ypoxia
AKRB6	4.32 (females)	7.62 (females [*])	14.20 (females [*])
	5.20 (males)	8.13 (males [*])	23.85 (males [*])
$Tfr2^{Y245X/Y245X}/Tmprss6^{msk/msk}$	7.18 (females)	8.34 (females [*])	26.95 (females [*])
	9.00 (males)	12.05 (males [*])	22.00 (males [*])
129SvJB6	4.65 (females)	nd	9.77 (females)
	3.45 (males)	nd	10.84 (males)
$Hfe-/-/Tmprss6^{msk/msk}$	6.52 (females)	nd	19.13 (females)
	5.98 (males)	nd	23.53 (males)
C57BL6	5.21 (females)	nd	10.21 (females)
	5.25 (males)	nd	16.5 (males)
$Tmprss6^{msk/msk}$	4.76 (females)	nd	26.2 (females)
	4.94 (males)	nd	18.2 (males)

For each value, n 4, unless indicated with *, where n=2. Statistics are shown with means and standard errors (males+females) in Figure 2 (panel 4).

Table 3

Response to repeated hypoxia by control and *Tfr2/Tmprss6* double mutant mice.

strain	Normoxia Mean ± SE (n)	3 × Hypoxia Mean ± SE (n)	P value no hpx (Cont vs mut)	P value 3x Hpx (Cont vs mut)	P value control (no vs 3x Hpx)	P value mutant (no vs 3x Hpx)
Hemoglobin (g/dL)						
AKRB6	12.1 ± 0.7 (8)	14.3 ± 0.6 (8)				
Tfr2Y245X/Y245X/Tmprss6msk/msk	10.4 ± 0.1 (8)	10.6 ± 0.5 (8)	0.0133	0.002	0.0356	0.4913
MCV (fL)						
AKRB6	45.6 ± 2.5 (8)	49.3 ± 1.3 (8)				
Tfr2Y245X/Y245X/Tmprss6msk/msk	28.8 ± 0.5 (8)	37.9 ± 0.6 (8)	0.0027	0.0009	0.1557	0.0009
Hematocrit (%)						
AKRB6	39.6 ± 2.1 (8)	47.4 ± 2.3 (8)				
Tfr2Y245X/Y245X/Tmprss6msk/msk	39.0 ± 0.9 (8)	41.1 ± 1.5 (8)	0.3823	0.0585	0.0458	0.1949
MCH (pg)						
AKRB6	13.9 ± 0.8 (8)	14.9 ± 0.4 (8)				
Tfr2Y245X/Y245X/Tmprss6msk/msk	7.7 ± 0.4 (8)	9.7 ± 0.3 (8)	0.0013	0.0009	0.0806	0.0073
RDW (%)						
AKRB6	17.9 ± 0.5 (8)	21.7 ± 1.3 (8)				
Tfr2Y245X/Y245X/Tmprss6msk/msk	24.5 ± 0.4 (8)	33.5 ± 0.7 (8)	0.0009	0.0009	0.0051	0.0002
RBC (M/µl)						
AKRB6	8.8 ± 0.3 (8)	9.7 ± 0.6 (8)				
Tfr2Y245X/Y245X/Tmprss6msk/msk	13.6 ± 0.5 (8)	10.9 ± 0.4 (8)	0.0002	0.1049	0.5737	0.003
Liver Iron (mg/g)						
AKRB6	0.39 ± 0.1 (8)	0.74 ± 0.1 (8)				
Tfr2Y245X/Y245X/Tmprss6msk/msk	0.29 ± 0.0 (8)	0.78 ± 0.1 (8)	0.3823	0.7209	0.0281	0.0006
Spleen Iron (mg/g)						
AKRB6	4.3 ± 0.8 (8)	7.2 ± 1.0 (8)				
Tfr2Y245X/Y245X/Tmprss6msk/msk	2.9 ± 0.3 (8)	3.6 ± 0.5 (8)	0.1275	0.0104	0.0379	0.1560

Mice were exposed to 8% oxygen under normal pressure three times for 8 consecutive days separated by 2 weeks of normoxia. Means and standard errors are shown. Eight mice were used in each group. Statistical significance was determined using Mann Whitney test (GRAPHPAD PRISM 5.0).