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Decreased hemojuvelin protein levels in *mask* mice lacking matriptase-2-dependent proteolytic activity

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Running title: Hemojuvelin protein levels in *mask* mice

Summary:

Matriptase-2, a membrane protein encoded by the *Tmprss6* gene, is a negative regulator of hepcidin expression. Although matriptase-2 has been proposed to cleave membrane hemojuvelin, we have recently found decreased hemojuvelin protein levels in *Tmprss6* *-/-* mice. The purpose of this study was to confirm this observation by determining hemojuvelin protein levels in another strain of mice with disrupted *Tmprss6* gene, and to determine the effect of matriptase-2 deficiency on the expression of other membrane proteins participating in the bone morphogenetic protein signal transduction.

Mask mice, which lack the proteolytic domain of matriptase-2, displayed decreased liver hemojuvelin protein content, while *Id1* mRNA level, an indicator of hemojuvelin-dependent signal transduction, was increased. Protein levels of bone morphogenetic protein receptors *Alk3* and *Acvr2a* were unchanged, and transferrin receptor 2 and neogenin protein levels were slightly decreased.

The results confirm that the loss of matriptase-2 increases bone morphogenetic protein-dependent signalling, while paradoxically decreasing liver hemojuvelin protein content. The regulation of transferrin receptor 2 protein levels by transferrin saturation was not affected in *mask* mice. How the loss of matriptase-2 proteolytic activity leads to decreased hemojuvelin protein levels is at present unclear.

Key words: Hepcidin, *Id1*, *Tmprss6*, *Hfe2*, Transferrin receptor 2, Neogenin

Introduction:

Iron-deficiency anaemia is the most common anaemia worldwide. Although the overwhelming majority of iron-deficiency anaemia cases are caused by chronic bleeding or inadequate dietary iron intake, a minor subgroup of iron-deficiency anaemias is inherited in an autosomal recessive manner. Recently, it has been demonstrated that rare cases of iron-refractory iron deficiency anaemia (IRIDA) are caused by mutations of the *TMPRSS6* gene (Finberg *et al.* 2008).

The exact function of the *TMPRSS6* gene is at present unknown. The gene encodes a serine protease, matriptase-2, which is expressed mainly at the hepatocyte plasma membrane. *In vitro*, matriptase-2 has been convincingly demonstrated to cleave hemojuvelin (Silvestri *et al.* 2008), another hepatocyte plasma membrane protein, which plays an essential role in the regulation of expression of hepcidin, the key iron-regulatory hormone. Hemojuvelin (Hjv) is a central component of the bone morphogenetic protein /hemojuvelin (Bmp/Hjv) signalling pathway, which upregulates hepcidin expression in response to increased iron stores (Babitt *et al.* 2006). In this pathway, hemojuvelin facilitates the binding of the Bmp6 protein (Meynard *et al.* 2009, Andriopoulos *et al.* 2009) to a set of bone morphogenetic protein receptors at the extracellular side of the hepatocyte plasma membrane, leading to intracellular receptor phosphorylation, phosphorylation of the Smad1/5/8 proteins and, ultimately, to transcriptional activation of hepcidin expression. By cleaving plasma membrane hemojuvelin, matriptase-2 is thus proposed to remove an important component of the Bmp/hemojuvelin pathway from the membrane, resulting in diminished hepcidin expression. In accordance with this concept, lack of matriptase-2, as in IRIDA patients, leads to inappropriate high expression of hepcidin (Finberg *et al.* 2008), which causes a decrease in iron absorption from the intestine.

At present, three well-characterised mouse models of matriptase-2 deficiency exist: *mask* mice on C57BL/6 background, which harbor an ethylnitrosourea-induced mutation a splice acceptor site upstream of exon 15 of the *Tmprss6* gene, resulting in the loss of the proteolytic domain of matriptase-2 (Du *et al.* 2008); *Tmprss6*^{-/-} mice on a mixed background, in which the expression of matriptase-2 was compromised by duplication of exons 3 to 6 (Folgueras *et al.* 2008), and finally *Tmprss6* mutant mice on a C57BL/6 background, obtained by deletion of a part of exon 17, again resulting in the loss of a part of the protease domain of matriptase-2 (Finberg *et al.* 2010). The phenotype of these strains is very similar, with truncal alopecia and iron-deficiency anemia as most prominent findings. All three strains display elevated expression of hepcidin, which is in accordance with enhanced signalling through the Bmp/Hjv pathway. Despite these similarities, there are also slight differences between the phenotypes of the mutant strains, particularly with respect to the survival of offspring (Folgueras *et al.*, 2008, Finberg *et al.* 2010) or expression of the *Bmp6* gene (Finberg *et al.* 2008, Nai *et al.* 2012).

When recently investigating Hjv protein levels in *Tmprss6*^{-/-} (Folgueras *et al.* 2008) mice, we surprisingly found decreased, rather than increased, levels of hemojuvelin protein (Krijt *et al.* 2011). This observation suggests that, *in vivo*, the actual function of matriptase-2 in iron metabolism regulation could be more complex than simple cleavage of plasma membrane hemojuvelin. The purpose of the present investigation was to confirm this observation by examining hemojuvelin protein levels in *mask* mice, and to determine the effect of matriptase-2 deficiency on the expression of other proteins participating in the bone morphogenetic protein signal transduction.

Methods

Animal experiments were approved by the Ethics Committee of the First Faculty of Medicine in Prague.

Liver samples from male *mask* mice, aged three to four months (Du *et al.* 2008), were processed to obtain either whole tissue homogenate or crude membrane fraction. For whole tissue homogenates, liver samples were homogenised in 150 mM sodium chloride, pH 7.8, containing protease inhibitors (Roche) and 1 % of Triton X-100. Homogenate was centrifuged for 15 min at 12 000 g, and 60 µg of the supernatant protein was used for electrophoresis. Crude membrane fraction was obtained by homogenisation of liver samples in 250 mM sucrose, pH 7.5, containing protease inhibitors. After low-speed centrifugation at 6000 g for 15 min, the crude membrane fraction was isolated by ultracentrifugation of the homogenate at 80 000 g for 50 min. The pellet was resuspended in Tris-buffered saline, pH 7.5, and a 60 µg protein aliquot was used for electrophoresis on 12% polyacrylamide gels. For neogenin determinations, 6% polyacrylamide gel was utilized. A total of four pairs of C57BL/6 and *mask* mouse livers, originating from age-matched mice kept on a standard laboratory diet, were used for the experiments.

Immunoblotting was performed on an Invitrogen semi-dry blotter, PVDF membrane was blocked for one hour in 5% milk in tris-buffered saline containing 0.1 % Tween 20, and incubated overnight with the primary antibody in 5% milk. The primary antibodies were: Goat anti-Hjv, AF 3634, R&D Systems, 1:500; rabbit anti-transferrin receptor 2 (Tfr2), Alpha Diagnostics International, 1:1000; rabbit anti-Acvr1 (Alk2), #4398, Cell Signaling Technology, 1:250; rabbit anti-Bmpr1a (Alk3), ab59947, Abcam, 1:300; rabbit anti-Actr-IIa (Acvr2a), sc-130679, Santa Cruz Biotechnology, 1:200, and rabbit anti neogenin (Neo1), sc-15337, Santa Cruz Biotechnology, 1:500. Rabbit anti pan-cadherin, #4068, Cell Signaling

Technology, 1:24 000, and rabbit anti-Gapdh, G9545, Sigma Aldrich, 1:250 000, were used as loading controls. Secondary antibodies were from Jackson ImmunoResearch.

RNA was isolated from samples stored at -80°C using Qiagen RNeasy Plus Mini Kit, and reverse transcribed by RevertAid First Strand cDNA synthesis kit (Thermo Scientific). Real-time PCR was performed on a Roche Light Cycler instrument as previously described (Krijt *et al.* 2011), target mRNA content is expressed relative to β -actin (*Actb*) mRNA. Primer sequences were (forward and reverse): *Actb* GAC ATG GAG AAG ATC TGG CA and GGT CTT TAC GGA TGT CAA CG, *Acvr1* AGG TTT ATG AGC AGG GGA AGA and CTG AGA GCA ACT CCA AGG ATG, *Bmpr1* TCG AGA CCT GAA GAG CAA AAA and GAT GTA GGG CTG GAA ATG GTT, *Hfe2* CAA TCC TGC GTC TTT GAT GTT and GAA GCA AAG CCA CAG AAC AAA, *Id1* CGA GGT GGT ACT TGG TCT GTC and CTG CAG GTC CCT GAT GTA GTC, *Neo1* CTT GAT GCC AGC AAC TGT GTA and ATT TCC CCA TTG CCA GAT AAC.

Results:

Hjv protein is decreased in *mask* mice

As previously reported (Krijt *et al.* 2011), the R&D AF3634 antibody detects, under reducing conditions, two Hjv-specific bands *in vivo* - a major band at approximately 35 kDa, and a minor band at approximately 18 kDa. Comparison of whole liver homogenates from *mask* mice and their wild-type littermates showed significant downregulation of the major 35 kDa band (Fig. 1A); the minor band was barely visible. When the same samples were run under non-reducing conditions, only one band at approximately 50 kDa, probably representing a disulfide bridge-bound Hjv heterodimer (Zhang *et al.* 2005), was apparent. The intensity of this band was decreased in *mask* mice (Fig. 1B).

To enhance the visualisation of the 35 kDa band, crude liver membrane fraction was prepared by ultracentrifugation. Hjv signal from the crude membrane fraction was stronger as compared to the whole homogenate; the intensity of both the 35 and 18 kDa bands (Fig. 1C, reducing conditions), as well as the single 50 kDa band (Fig. 1D, non-reducing conditions), reproducibly decreased in *mask* mice. These results confirm that the disruption of *Tmprss6* gene significantly decreases hemojuvelin protein content.

Matriptase-2 does not decrease membrane Tfr2, Alk3, Acvr2a and neogenin protein content

Since matriptase-2 is a hepatocyte membrane protein, it was of interest to determine whether disruption of the *Tmprss6* gene will affect the content of other membrane proteins participating in iron homeostasis. Of these, transferrin receptor 2 (Tfr2) is of particular interest, since it is also selectively expressed in the hepatocyte (Fleming *et al.* 2000), and

therefore represents a potential target for matriptase-2 activity. As can be seen in Fig 2A, the levels of transferrin receptor 2 slightly decreased in *mask* mice, as compared to wild-type littermates. To confirm that the antibody indeed allows reliable detection of Tfr2 protein, we determined Tfr2 protein levels in two female C57BL/6 mice fed an iron-deficient diet (Altromin C 1038, iron content 10 mg/kg) for three weeks since weaning, as well as in two three months old female C57BL/6 mice treated with a single intraperitoneal dose of iron dextran (200 mg iron/kg). In addition, we also determined Tfr2 protein in two male *Hjv*^{-/-} mice. In all cases, Tfr2 protein levels reacted to the changes in body iron levels (Supplementary Figure 1), which is in accordance with published data (Johnson and Enns, 2004).

The signal from bone morphogenetic proteins is transduced by bone morphogenetic protein receptors. Bmp6 has been reported to interact with Alk2 and Alk3, both type 1 Bmp receptors (Steinbicker *et al.* 2011), which are presumed to interact with type 2 Bmp receptors (Xia *et al.* 2008). Of the type 2 receptors, Acvr2a has been reported as the most abundant in human liver (Xia *et al.* 2008). Using commercially available antibodies, we detected protein bands corresponding to Alk3 and Acvr2a. The expression of these proteins was not significantly changed in *mask* mice (Fig. 2A). The Alk2 antibody produced only very weak bands at the expected band size of 60 kDa, although, as stated by the manufacturer, it showed strong bands of unknown origin at 95 kDa (Fig. 2A).

Several groups have reported that iron metabolism is influenced by neogenin (Zhang *et al.* 2005, Lee *et al.* 2010). In liver crude membrane preparations from *mask* mice, we detected slightly decreased membrane neogenin protein levels (Fig. 2A). Overall, these results suggest that Alk3, Acvr2a and neogenin are not physiological substrates of matriptase-2.

The decrease in *Hjv* protein occurs posttranscriptionally

To verify that the observed changes in HJV protein occur posttranscriptionally, levels of *Hfe2* mRNA, coding the HJV protein, were determined by real-time PCR. *Hfe2* mRNA content was unchanged in *mask* mice (Fig. 2B). In addition, no changes were observed in *Acvr1*, *Bmpr1a* and *Neol* mRNA, encoding the Alk2, Alk3 and neogenin proteins (results not shown).

Id1 mRNA levels in *mask* mice confirm upregulation of Bmp-dependent signal transduction, despite decreased HJV protein levels

Liver *Id1* mRNA levels are often used to monitor Bmp6-dependent signalling. In iron-deficient C57BL mice, liver *Id1* mRNA levels are decreased in comparison with control mice (Kautz *et al.* 2008). Despite low liver iron levels (Du *et al.* 2008) and low liver HJV protein (Fig. 1), liver *Id1* mRNA levels were increased in *mask* mice (Fig. 2B). These results are in accordance with data previously reported for other mice with disrupted *Tmprss6* gene (Finberg *et al.* 2010, Nai *et al.* 2012), confirming the positive effect of *Tmprss6* gene disruption on the activity of the Bmp6/HJV pathway.

Discussion

During the past decade, the iron regulatory hormone hepcidin has emerged as the key factor which controls iron metabolism in mammals (Andrews 2008, Ganz 2011). Consequently, the signalling pathways which regulate hepcidin expression in the hepatocyte represent a subject of intensive research. Hepcidin expression is influenced by several proteins present at the hepatocyte plasma membrane: Hemojuvelin, Tfr2, the Hfe protein and neogenin (Lee *et al.* 2010). Disruption of any of these proteins leads to decreased hepcidin expression and iron overload. Recently, another hepatocyte membrane protein, the serine protease matriptase-2, has been identified as the only known negative regulator of hepcidin expression. Mutations in the *Tmprss6* gene cause inappropriately high expression of hepcidin, resulting in IRIDA (Finberg *et al.* 2008). The currently accepted model of matriptase-2 function proposes that matriptase-2 cleaves hemojuvelin (Silvestri *et al.* 2008), a GPI-bound protein which serves as a coreceptor for the Bmp6 molecule. However, *in vivo* evidence for this mode of action of matriptase-2 is still lacking. Moreover, the possible discrepancy between the *in vivo* and *in vitro* effects of *Tmprss6*^{-/-} mutations has also recently been highlighted by the observation that, *in vitro*, hepcidin expression is repressed even by those *Tmprss6*^{-/-} mutant constructs which lack protease activity (Guillem *et al.* 2012). Therefore, it is still of importance to determine the effect of *in vivo* loss of matriptase-2 proteolytic activity on proteins participating in the regulation of hepcidin expression.

The Bmp6/hemojuvelin signalling pathway, which controls hepcidin expression in response to iron levels, has to a significant extent been elucidated. Iron overload transcriptionally increases the synthesis of Bmp6, which then binds at the extracellular side of the hepatocyte plasma membrane to a set of bone morphogenetic protein receptors. The bone morphogenetic

proteins responsible for Bmp6 binding have recently been identified as Alk2 and Alk3 (Steinbicker *et al.* 2011), both type 1 Bmp receptors. Type 1 Bmp receptors dimerize with type 2 receptors, of which Acvr2a is abundantly expressed in human liver (Xia *et al.* 2008). Theoretically, all these proteins - HJV, Alk2, Alk3, the type 2 Bmp receptors, as well as matriptase-2 - should interact at the hepatocyte plasma membrane in order to regulate hepcidin expression. Intriguingly, HJV protein level was significantly decreased in *mask* mice. In this respect, the presented results confirm our data previously obtained in another mouse model with *Tmprss6* gene disruption (Krijt *et al.* 2011). Although the results from both studies do not directly support hemojuvelin cleavage by matriptase-2, they nevertheless apparently confirm a specific interaction between matriptase-2 and hemojuvelin.

Immunoblotting of liver crude membrane fraction from *mask* mice has demonstrated a slight decrease in Tfr2 and neogenin protein levels. Tfr2 is known to positively modulate hepcidin expression (Nemeth *et al.* 2005), but the exact mode of its signal transduction, as well as its possible participation in the Bmp6/hemojuvelin pathway, has not yet been fully elucidated (Chen and Enns 2012, D'Alessio *et al.* 2012). Tfr2 protein levels are known to respond to plasma iron concentration (Johnson and Enns 2004). Since *mask* mice are known to display low liver iron levels and plasma iron levels (Du *et al.* 2008), it is plausible that the observed decrease in Tfr2 protein is caused by iron deficiency, rather than by an interaction between matriptase-2 and Tfr2. This conclusion is in accordance with the recently published finding that Tfr2 is not a substrate for matriptase-2 (Lee *et al.* 2012).

Neogenin is an ubiquitously expressed protein, which has been demonstrated to interact with hemojuvelin (Zhang *et al.* 2005). Disruption of the *Neo1* gene in mice has been reported to diminish Bmp-dependent signalling and to decrease hepcidin expression, resulting in massive iron overload (Lee *et al.* 2010). Therefore, matriptase-2 could theoretically exert its effect on iron metabolism by cleaving membrane neogenin. However, as can be seen in Fig. 2, liver

membrane neogenin protein content was actually slightly decreased in *mask* mice, confirming the recently published observation that neogenin is not cleaved by matriptase-2 (Enns *et al.* 2012).

Levels of *Id1* mRNA were increased in *mask* mice. *Id1* is a sensitive indicator of Bmp6-dependent signalling (Kautz *et al.* 2008), and the observed increase in *Id1* mRNA thus confirms the concept that matriptase-2 blocks Bmp6/hemojuvelin-dependent signalling (Silvestri *et al.* 2008). How this interference with the Bmp6/Hjv pathway would decrease total Hjv protein levels is at present unclear. Lack of matriptase-2 proteolytic activity could significantly change the ratio of hemojuvelin to other components of the Bmp6/hemojuvelin pathway, and this imbalance could then affect hemojuvelin protein levels as a compensatory mechanism. It is also possible that matriptase-2 selectively influences the ratio of the various hemojuvelin forms (Maxson *et al.* 2009) present at the membrane. As matriptase-2 represents the most recently identified component participating in the complex regulation of hepcidin gene expression, it is obvious that more research will be necessary to define its exact function *in vivo*.

In conclusion, the presented study confirmed decreased expression of hemojuvelin in mice with a mutation in matriptase-2 gene, while the overall activity of the Bmp6/Hjv pathway, as determined by the *Id1* gene expression, was increased. The regulation of Tfr2 protein levels by iron was apparently unaffected. The obtained results confirm the inhibitory effect of matriptase-2 on the Bmp6/hemojuvelin dependent signalling pathway, and suggest a complex relationship between matriptase-2 expression and hemojuvelin protein levels.

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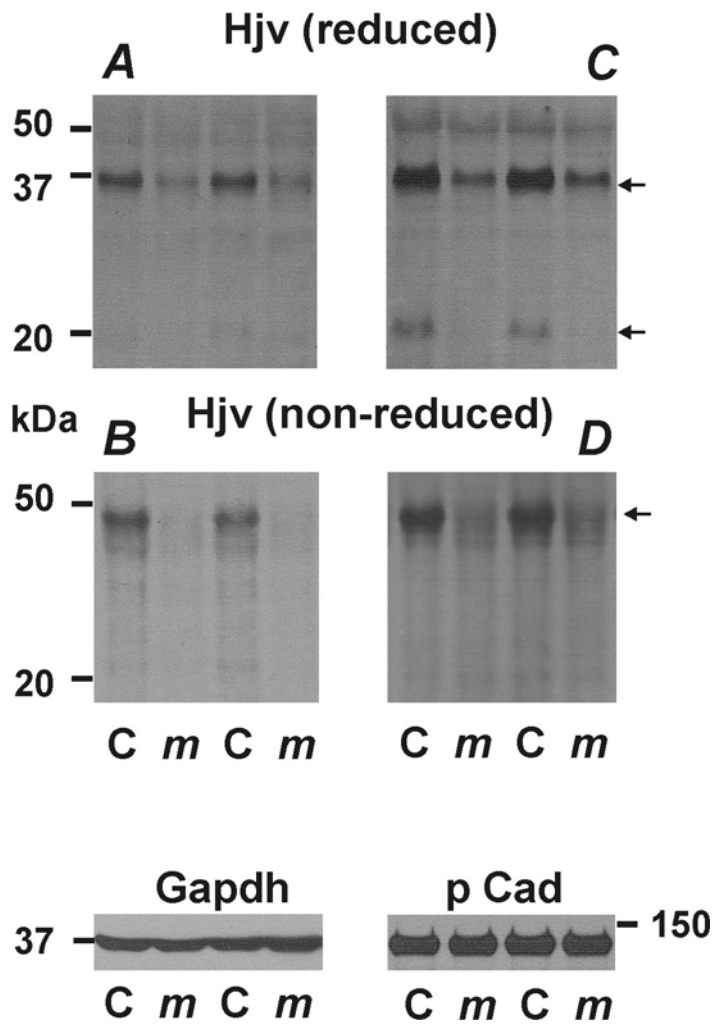
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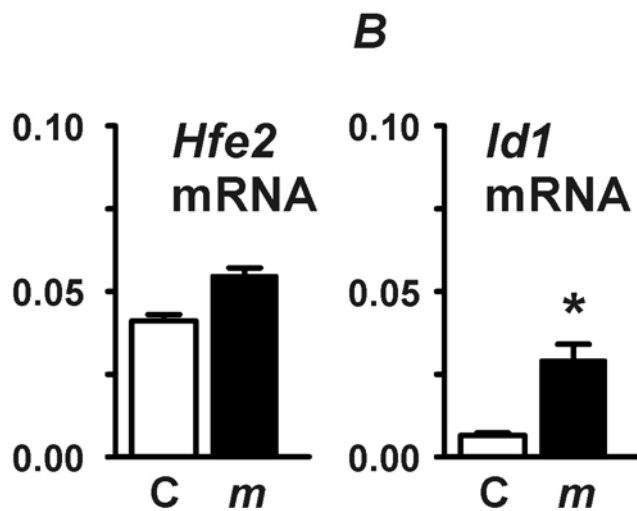
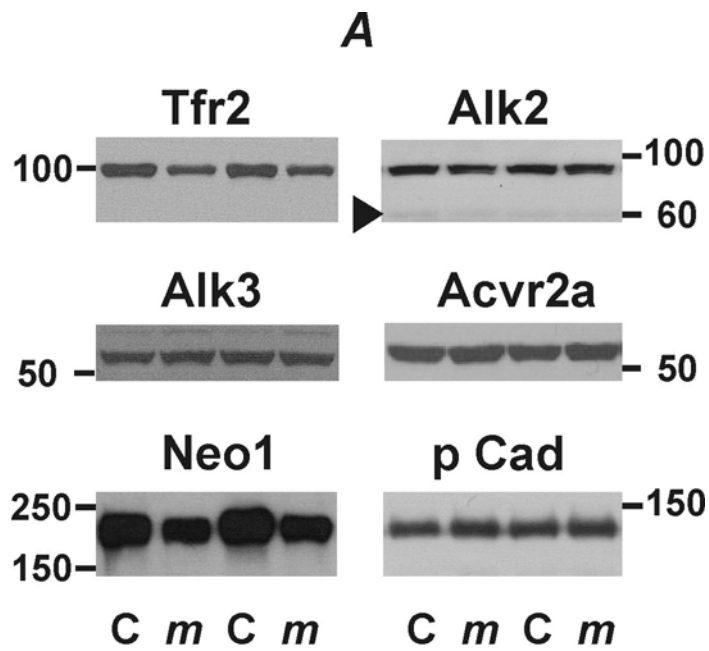
Fig. 1: Liver hemojuvelin protein levels in *mask* mice.

Panel A and B: Liver Hjv protein levels determined in whole tissue homogenate, under reducing and non-reducing conditions respectively.

Panel C and D: Liver Hjv protein levels determined in crude membrane fraction, under reducing and non-reducing conditions respectively.

C: male C57BL/6 mice, *m*: age-matched *mask* mice. Gapdh and pan-cadherin were used as loading controls. 60 μ g of protein was loaded per lane.

Fig. 2: Hemojuvelin-related proteins in the liver of *mask* mice.

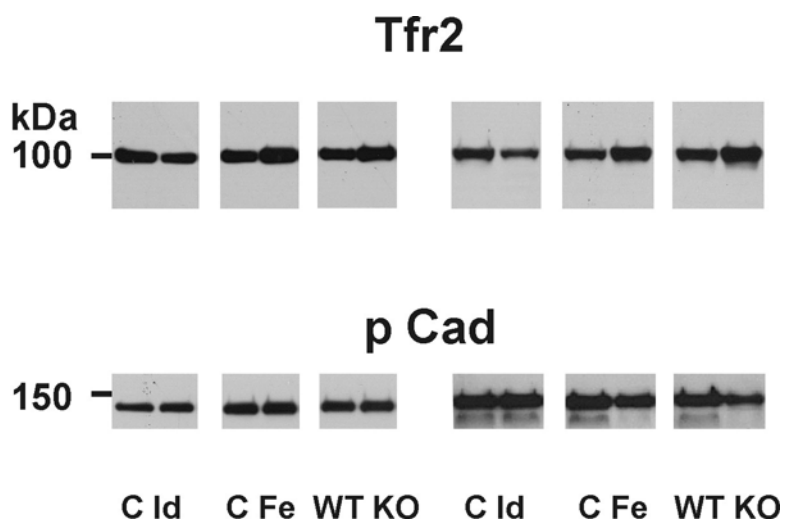


Panel A: Tfr2, Alk2, Alk3, Acvr2a and neogenin (Neo1) protein levels determined in crude membrane fraction under reducing conditions. C: C57BL/6 mice, *m*: *mask* mice. 60 μ g of protein was loaded per lane. Pan-cadherin is used as loading control. Arrowhead indicates putative Alk2-specific bands.

Panel B: Real-time PCR analysis of *Hfe2* and *Id1* gene expression in the livers of *mask* mice.

Target mRNA levels were determined relative to *Actb* mRNA. Asterisk denotes statistical significance ($P < 0.05$, $n = 3$).

Supplementary Figure 1: Response of liver Tfr2 protein to iron deficiency and iron overload.



Female C57BL/6 mice were kept on iron-deficient diet since weaning for three weeks (Id), or were injected with 200 mg/kg iron and sacrificed after three days (Fe). Male *Hjv*^{+/+} (WT) and *Hjv*^{-/-} (KO) mice were sacrificed at 5 months. Liver iron content in the respective groups was: Control (C), 80 $\mu\text{g/g}$; Id, 20 $\mu\text{g/g}$; Fe 1730 $\mu\text{g/g}$; WT, 40 $\mu\text{g/g}$ and KO, 1390 $\mu\text{g/g}$ (wet weight). 60 μg of crude membrane protein was loaded per lane, pan-cadherin was used as loading control.