

Regulation of iron metabolism in *Hamp*^{-/-} mice in response to iron-deficient diet

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Abstract

Background Hepcidin, the liver-secreted iron regulatory peptide, maintains systemic iron homeostasis in response to several stimuli including dietary iron levels and body iron status. In addition, iron metabolism is controlled by several local regulatory mechanisms including IRP and Hif-2 α activities independently of hepcidin. However, the roles of these mechanisms and their interaction particularly in hepcidin-deficient individuals are not yet fully understood. We, therefore, aimed to explore whether *Hamp* disruption affects iron homeostatic responses to dietary iron deficiency. **Methods** *Hepcidin1* knockout (*Hamp*^{-/-}) mice and heterozygous littermates were fed with control or iron-deficient diet for 2 weeks. The expression of iron-related genes and proteins were determined by quantitative PCR and Western blot, respectively.

Results Two-week iron-deficient diet feeding in *Hamp*^{-/-} mice did not alter serum iron but significantly reduced liver non-heme iron levels. This was also associated with increased ferroportin protein expression in the duodenum and spleen, whereas decreased expression was

found in the liver. In addition, significant inductive effects of iron-deficient diet on Dcytb and DMT1 mRNA expression in the duodenum were noted with more pronounced effects in *Hamp*^{-/-} mice compared with controls. **Conclusions** *Hamp*^{-/-} mice exhibited a more dramatic increase in the expression of iron transport machinery, which may be responsible for the unaltered serum iron levels upon iron-deficient diet feeding in these mice. Despite the lack of hepcidin, *Hamp*^{-/-} mice can maintain a degree of iron homeostasis in response to altered dietary iron through several hepcidin-independent mechanisms.

Keywords Iron · Hepcidin · Iron deficiency · *Hamp*^{-/-} mice · Ferroportin

Introduction

Iron is one of the most important trace elements in biology since it not only is a major component of several biomolecules including hemoglobin but also plays many roles in fundamental biochemical reactions. As a result, the lack of iron can affect cell function and lead to iron deficiency anemia. However, excessive iron can result in oxidative stress. Therefore, iron homeostasis has to be tightly controlled through several mechanisms. Most of the circulatory iron is acquired from reticuloendothelial cells in the spleen, bone marrow, and liver through the reutilization of iron from senescent erythrocytes. In addition, the body also obtains iron from dietary iron absorption.

The absorption of non-heme iron involves four key proteins. Firstly, dietary iron is reduced by duodenal cytochrome b (Dcytb) [1] and transported into enterocytes by divalent metal transporter 1 (DMT1) [2]. Some of the absorbed iron may join the iron pool within the enterocytes,

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while the rest is exported through the basolateral iron transporter, ferroportin [3–5] with the aid of the basolateral oxidase, hephaestin [6]. Several factors have been shown to control intestinal iron absorption including body iron status, erythropoietic activity, hypoxia, and inflammation. The roles of body iron status, stores regulator, in the regulation of systemic iron homeostasis have been demonstrated in animal models with dietary iron deficiency, which demonstrated increased expression of *Dcytb*, *DMT1*, and ferroportin leading to increased intestinal iron absorption [7].

Hepcidin, a liver-secreted antimicrobial peptide, has been shown to play the central role in the control of systemic iron homeostasis in response to iron status, erythropoietic activity, inflammation, and hypoxia [8, 9]. The mechanism of hepcidin action is to induce ferroportin internalization and degradation thus inhibiting iron absorption and reducing reticuloendothelial iron recycling [10]. However, recent studies have revealed that iron transporter expression can be locally controlled by enterocyte iron status independently of hepcidin. Hypoxia-inducible factor-2 α (*Hif-2 α*) has been shown to alter iron absorption in response to iron deficiency by directly enhancing *Dcytb* and *DMT1* transcription [11, 12]. In addition, mice with intestinal-specific *IRP1* and *IRP2* ablation expressed higher ferroportin protein levels in the duodenum despite increased hepcidin expression indicating that the effects of hepcidin on duodenal ferroportin expression can be overridden by *IRP* loss [13].

The present study attempted to delineate local and systemic controls of iron homeostasis. *Hamp*^{-/-} mice [14] provide a good model to study the local iron regulatory mechanisms in the absence of hepcidin. In this study, *Hamp*^{-/-} mice and heterozygous littermates were fed with iron-deficient diet. *Dcytb*, *DMT1*, and ferroportin expression as well as iron parameters were then compared with mice fed with control diet.

Materials and methods

Animal care and dietary intervention

Male *Hamp*^{-/-} mice (acquired through collaboration with Dr. Sophie Vaulont, Institut Cochin, France) and heterozygous littermates (mixed C57BL/6 \times 129 background strain backcrossed for at least 5 generations on C57BL/6) aged 10 weeks were utilized. The mice were fed a low iron-purified diet (TD.80396, 4 ppm iron; Harlan Teklad; Madison, WI, USA) or control diet containing 48 ppm iron (TD.80394; Harlan Teklad) ad libitum for 2 weeks after which the mice were killed, and serum was collected along with duodenal mucosal scrapings, liver, and spleen tissues. All animal experiments were performed under the authority of the United Kingdom Home Office license.

Measurement of hemoglobin, serum iron, and tissue non-heme iron contents

Hemoglobin was determined spectrophotometrically using the methods described by Beutler [15]. Serum iron was measured with a liquid ferrozine-based iron reagent (Bio-Assay Systems; Hayward, CA, USA). Tissue non-heme iron levels were determined by a modification of the method of Foy et al. [16] as described by Simpson and Peters [17].

Real-time polymerase chain reaction (qPCR)

RNA was extracted from the duodenum, liver, and spleen using TRIzol reagent (Invitrogen, Paisley, UK), and complementary DNA was synthesized using a Transcriptor High Fidelity cDNA Kit (Roche Diagnostics, Mannheim, Germany). qPCR was performed using the ABI Prism 7000 (Applied Biosystems, Carlsbad, CA, USA) and Universal ProbeLibrary System (Roche Diagnostics). Messenger RNA expression was normalized to β -actin (*Actb*) mRNA. The sequence of the utilized primers is listed as follows:

<i>Actb</i>	forward CTAAGGCCAACCGTGAAAAG reverse ACCAGAGGCATACAGGGACA
<i>Cybrd1</i> (<i>Dcytb</i>)	forward GTGACCGGCTTCGTCCTC reverse TGGATGGATTTCATCAAGAGC
<i>Epas1</i> (<i>Hif-2α</i>)	forward GGTAAAGGAACCCAGGTGCT reverse GGGATTTCTCCTTCCTCAGC
<i>Hamp</i> (hepcidin)	forward AGAAAGCAGGGCAGACATTG reverse CACTGGGAATTGTTACAGCATT
<i>Slc11a2</i> (<i>DMT1</i>)	forward CACCGTCAGTATCCCAAGGT reverse CCAATGATTGCCAACTCCA
<i>Slc40a1</i> (ferroportin)	forward TTGTTGTTGTGGCAGGAGAA reverse AGCTGGTCAATCCTTCTAATGG
<i>Tfrc</i> (<i>TfR1</i>)	forward TCCTTTCCTTGTCATATTCTGG reverse CCAAATAAGGATAGTCTGCATCC

Western blot analysis

Protein was extracted by homogenizing tissues in lysis buffer (0.25 M Sucrose, 0.03 M L-histidine, 0.5 mM PMSF) containing protease inhibitor cocktail (Sigma Aldrich; Poole, United Kingdom). After incubating on ice for 20 min, the homogenate was centrifuged at 2,700g for 10 min at 4 °C. The resulting supernatant was decanted to new tubes and centrifuged at 28,500g for 1 h at 4 °C. The supernatant was used as cytoplasmic fraction, and the membrane pellet was resuspended in Sucrose-Histidine lysis buffer. Western blot analysis was performed using

anti-mouse MTP1 antibody (Alpha Diagnostic; San Antonio, TX, USA), anti-human IRP2 antibody (Novus Biologicals), and anti-actin antibody (Sigma Aldrich; Poole, UK) in order to detect ferroportin, IRP2, and actin, respectively. Blot densitometry was obtained using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data are presented as mean ± standard errors of the means (SEM). The comparison of multiple groups for significant effects of two variables (diet and genotype) was determined by two-way analysis of variance (two-way ANOVA) with Bonferroni’s post hoc test. A *P* value less than 0.05 was considered to be significant. All statistical analyses were performed using Graphpad Prism 4 software (GraphPad Software Inc; La Jolla, CA, USA).

Results

Effects of iron-deficient diet on iron parameters

The influence of iron-deficient diet on hemoglobin, serum iron, and tissue non-heme iron levels in *Hamp*^{-/-} mice and heterozygotes were determined. Two-week iron-deficient diet feeding resulted in a 30% decrease in serum iron in heterozygous mice; however, the change did not reach a statistically significant level (Table 1). *Hamp*^{-/-} mice had higher serum iron compared with heterozygous littermates, and the levels were not affected by dietary treatment. Liver non-heme iron levels were higher in *Hamp*^{-/-} mice while spleen non-heme iron levels were lower than in the heterozygotes. Iron-deficient diet caused significant reductions in liver non-heme iron and spleen non-heme iron levels in *Hamp*^{-/-} mice and heterozygotes, respectively (Table 1).

In addition to tissue non-heme iron levels, the effects of iron-deficient diet on iron metabolism were also confirmed through a significant suppression of hepcidin expression in the liver of heterozygous mice (Fig. 1).

Iron-deficient diet caused tissue-specific regulation of ferroportin in *Hamp*^{-/-} mice

As ferroportin is the sole iron exporter and potential hepcidin receptor, ferroportin protein expression was

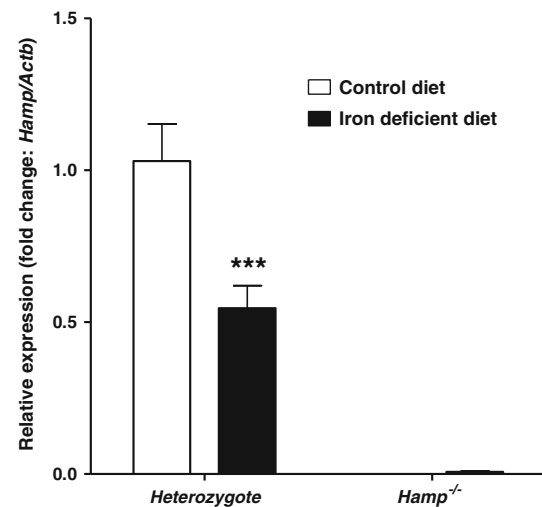


Fig. 1 Effects of iron-deficient diet on hepcidin mRNA expression in *Hamp*^{-/-} and heterozygous mice. Real-time PCR of hepcidin mRNA (*Hamp*) from the liver of *Hamp*^{-/-} and heterozygous mice fed with control/iron-deficient diet for 2 weeks. Relative mRNA expression was acquired by normalizing hepcidin mRNA to β-actin (*Actb*) mRNA. Values are means and SEM for the fold change as compared with heterozygote fed with control diet (*n* = 7 for *Hamp*^{-/-} fed with control diet and *n* = 6 for the rest). The samples were measured in triplicate. Statistical analysis was performed by two-way ANOVA with Bonferroni’s post hoc test. ****P* < 0.001 as compared to control diet-fed heterozygous mice. *P* < 0.0001 for the effect of genotype, *P* = 0.0022 and 0.0018 for diet and genotype × diet, respectively

Table 1 Iron parameters of 12-week-old male *Hamp*^{-/-} mice and heterozygous littermates fed with control or iron-deficient diet for 2 weeks

	Heterozygote control (<i>n</i> = 6)	Heterozygote Fe deficiency (<i>n</i> = 6)	<i>Hamp</i> ^{-/-} control (<i>n</i> = 7)	<i>Hamp</i> ^{-/-} Fe deficiency (<i>n</i> = 6)	<i>P</i> values (two-way ANOVA)		
					Genotype	Diet	Genotype × diet
Hemoglobin (g/dL)	16.67 ± 0.51	17.06 ± 0.71	17.10 ± 0.52	18.06 ± 0.92	0.3303	0.3627	0.6964
Serum iron (μM)	56.88 ± 3.77	40.08 ± 2.45	84.04 ± 14.23	79.97 ± 6.83	0.0012	0.2577	0.4859
Liver non-heme iron (nmol/mg wet weight)	1.63 ± 0.22	1.26 ± 0.22	20.59 ± 2.27	15.77 ± 1.29 ⁺	<0.0001	0.0848	0.1359
Spleen non-heme iron (nmol/mg wet weight)	5.05 ± 0.43	3.74 ± 0.37*	1.53 ± 0.09	1.61 ± 0.18	<0.0001	0.0475	0.0259

Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA with Bonferroni’s post hoc test

* *P* < 0.05 compared with heterozygote control; ⁺ *P* < 0.05 compared with *Hamp*^{-/-} control

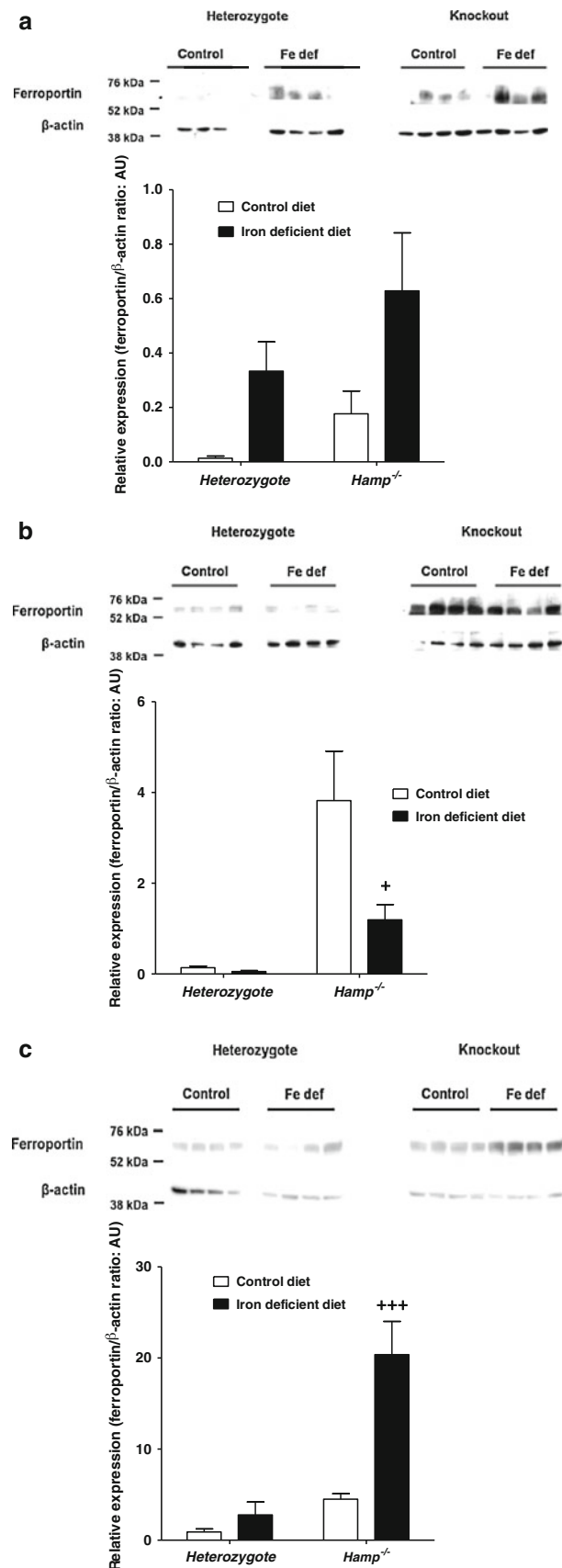
Fig. 2 Effects of iron-deficient diet on ferroportin protein expression in *Hamp*^{-/-} and heterozygous mice. Western blot analysis of ferroportin (100 μg crude membrane preparation) from duodenum (a), liver, (b) and spleen (c) of *Hamp*^{-/-} mice and heterozygotes fed with control or iron-deficient diet for 2 weeks. Ferroportin expression was normalized to the expression of β-actin and presented in arbitrary unit (AU). Densitometry is displayed below the blot. Values are means and SEM (*n* = 4). Statistical analysis was performed by two-way ANOVA with a Bonferroni's post hoc test. +*P* < 0.05, +++*P* < 0.001 as compared to control diet-fed *Hamp*^{-/-} mice. *P* values for the effects of genotype, diet, and genotype × diet; Duodenum: *P* = 0.0953, 0.0101 and 0.6118; Liver: *P* = 0.0012, 0.0349 and 0.0458; and Spleen: *P* = 0.0002, 0.0008 and 0.0043

determined in order to delineate differential changes in iron parameters between *Hamp*^{-/-} mice and heterozygotes. *Hamp* disruption results in increased ferroportin protein in liver and spleen as indicated by significant effects of genotype (*P* = 0.0012 and 0.0002 for liver and spleen ferroportin, respectively) (Fig. 2). Iron-deficient diet feeding increased ferroportin protein expression in the duodenum and the spleen (*P* = 0.0101 and 0.0008 for the effects of diet on duodenum and spleen ferroportin, respectively). In contrast, liver ferroportin was lower in mice fed with iron-deficient diet (*P* = 0.0349). Notably, the ferroportin response to iron-deficient diet was more pronounced in *Hamp*^{-/-} mice than in heterozygous littermates as shown by a significant interaction between genotype and diet (*P* = 0.0458 and 0.0043 for liver and spleen, respectively).

In order to investigate the mechanisms of ferroportin regulation, ferroportin expression was examined at the mRNA level by qPCR. Splenic ferroportin mRNA expression was not influenced by the genotype or diet (Fig. 3a). Since ferroportin mRNA contains a 5'-IRE, IRP2 protein levels were determined by Western blot analysis to explore the regulation at post-transcriptional level. Similar to ferroportin mRNA levels, IRP2 protein levels in the spleen were unaffected by the genotype or diet (Fig. 3b).

In contrast to the spleen, *Hamp* ablation resulted in ferroportin mRNA upregulation in the liver (Fig. 4a). Interestingly, ferroportin mRNA expression was unaffected by the diet despite the down-regulation at the protein level. IRP2 protein expression was therefore measured to delineate the mechanism underlying this discrepancy. As shown in Fig. 4b, IRP2 protein was decreased in *Hamp*^{-/-} liver (*P* = 0.0481 for the effect of genotype) while its expression was increased by iron-deficient diet feeding (*P* = 0.0054). Hence, the decrease in liver ferroportin protein expression could be explained through IRP regulation.

In the duodenum, iron-deficient diet feeding and *Hamp* disruption were associated with trends toward increased ferroportin mRNA expression. Notably, the ferroportin response in *Hamp*^{-/-} appeared to be more pronounced than in heterozygous littermates (Fig. 5). These did not, however, achieve statistical significance.



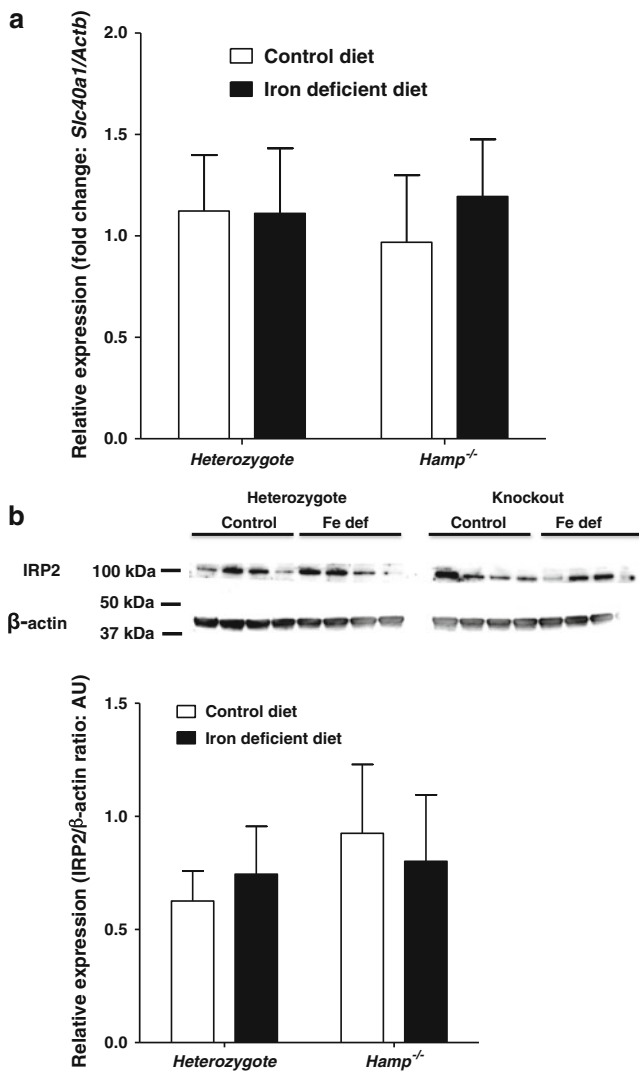


Fig. 3 **a** Effects of iron-deficient diet on splenic ferroportin mRNA expression in *Hamp*^{-/-} and heterozygous mice. Real-time PCR of ferroportin (*Slc40a1*) from the spleen of *Hamp*^{-/-} and heterozygous mice fed with control/iron-deficient diet for 2 weeks. Relative mRNA expression was acquired by normalizing to β -actin (*Actb*) mRNA. Values are means and SEM for the fold change as compared with heterozygote fed with control diet ($n = 7$ for *Hamp*^{-/-} fed with control diet and $n = 6$ for the rest). The samples were measured in triplicate. Statistical analysis was performed by two-way ANOVA with Bonferroni's post hoc test. $P = 0.9082, 0.7274,$ and 0.6978 for the effect of genotype, diet, and genotype \times diet, respectively. **b** IRP2 protein expression in the spleen of control and iron-deficient diet-fed *Hamp*^{-/-} and heterozygous mice. Western blot analysis of IRP2 (100 μ g cytosolic protein extract) from the spleen of *Hamp*^{-/-} mice and heterozygotes fed with control or iron-deficient diet for 2 weeks. IRP2 expression was normalized to the expression of β -actin and presented in arbitrary unit (AU). Densitometry is displayed below the blot. Values are means and SEM ($n = 4$). Statistical analysis was performed by two-way ANOVA with a Bonferroni's post hoc test. $P = 0.4768, 0.9912,$ and 0.6260 for the effect of genotype, diet, and genotype \times diet, respectively

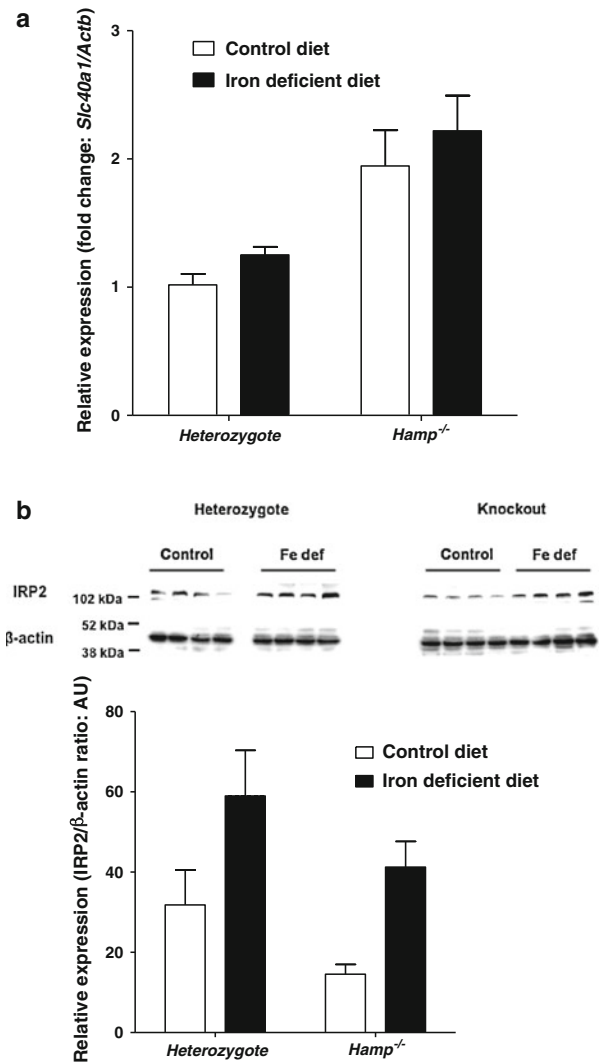
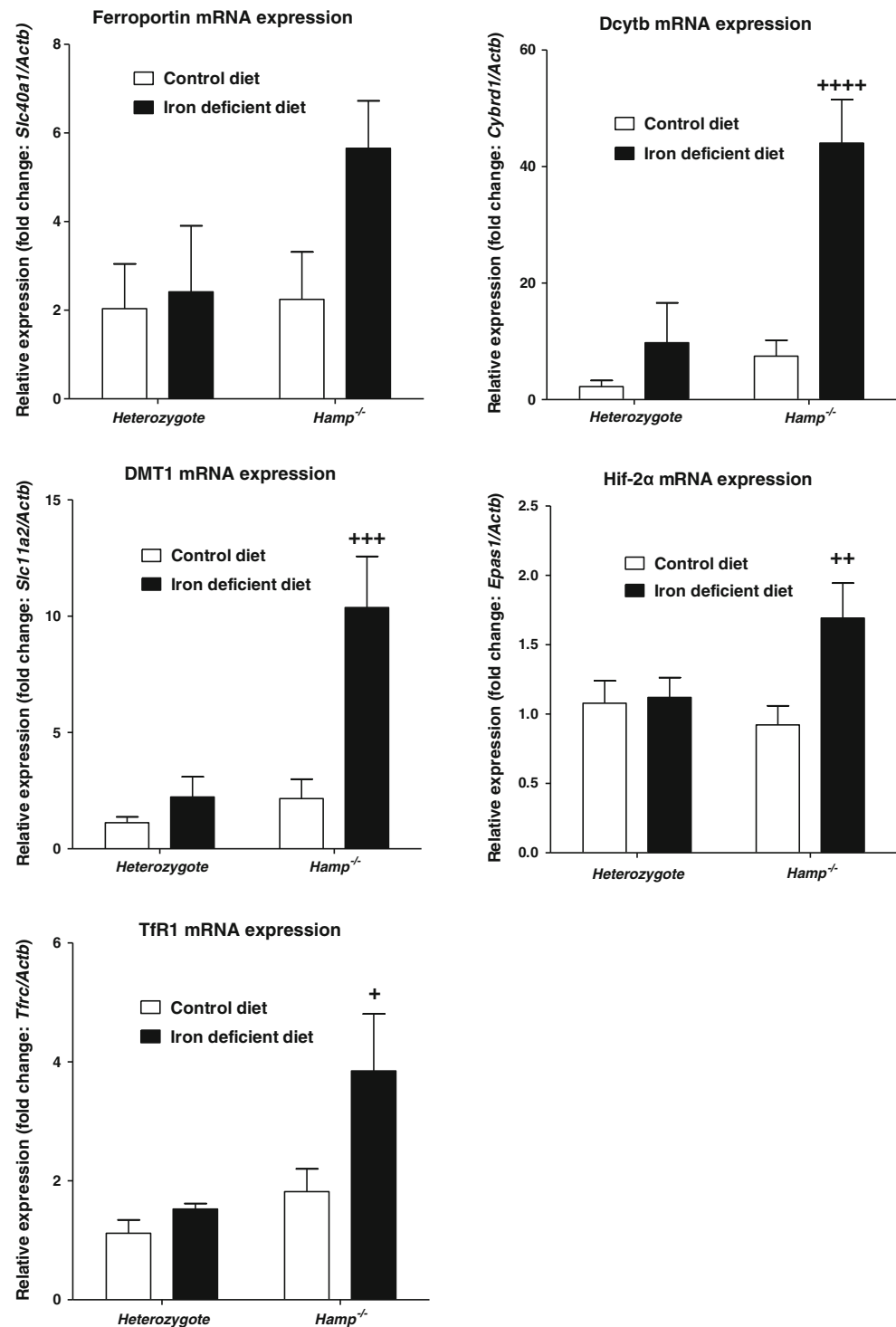


Fig. 4 **a** Effects of iron-deficient diet on liver ferroportin mRNA expression in *Hamp*^{-/-} and heterozygous mice. Real-time PCR of ferroportin mRNA (*Slc40a1*) from the liver of *Hamp*^{-/-} and heterozygous mice fed with control or iron-deficient diet for 2 weeks. Relative mRNA expression was acquired by normalizing ferroportin mRNA to β -actin (*Actb*) mRNA. Values are means and SEM for the fold change as compared with heterozygote fed with control diet ($n = 7$ for *Hamp*^{-/-} fed with control diet and $n = 6$ for the rest). The samples were measured in triplicate. Statistical analysis was performed by two-way ANOVA with Bonferroni's post hoc test. $P = 0.0002, 0.2436,$ and 0.9249 for the effect of genotype, diet, and genotype \times diet, respectively. **b** IRP2 protein expression in the liver of control and iron-deficient diet-fed *Hamp*^{-/-} and heterozygous mice. Western blot analysis of IRP2 (100 μ g cytosolic protein extract) from the liver of *Hamp*^{-/-} mice and heterozygotes fed with control or iron-deficient diet for 2 weeks. IRP2 expression was normalized to the expression of β -actin and presented in arbitrary unit (AU). Densitometry is displayed below the blot. Values are means and SEM ($n = 4$). Statistical analysis was performed by two-way ANOVA with a Bonferroni's post hoc test. $P = 0.0481, 0.0054,$ and 0.9761 for the effect of genotype, diet, and genotype \times diet, respectively

Fig. 5 Effects of iron-deficient diet on duodenal iron transport machinery, Hif-2 α and TfR1 mRNA expression in *Hamp*^{-/-} and heterozygous mice. Real-time PCR of ferroportin (*Slc40a1*), Dcytb (*Cybrd1*), DMT1 (*Slc11a2*), Hif-2 α (*Epas1*), and TfR1 (*Tfrc*) mRNA from the duodenum of *Hamp*^{-/-} and heterozygous mice fed with control/iron-deficient diet for 2 weeks. Relative mRNA expression was acquired by normalizing mRNA levels to β -actin (*Actb*) mRNA. Values are means and SEM for the fold change as compared with heterozygote fed with control diet ($n = 7$ for *Hamp*^{-/-}-fed with control diet and $n = 6$ for the rest). The samples were measured in triplicate. Statistical analysis was performed by two-way ANOVA with Bonferroni's post hoc test. $+P < 0.05$, $++P < 0.01$, $+++P < 0.001$, and $++++P < 0.0001$ as compared to control diet-fed *Hamp*^{-/-} mice. P values for the effects of genotype, diet, and genotype \times diet; ferroportin: $P = 0.1560, 0.1211$, and 0.2103 ; Dcytb: $P = 0.0063, 0.0011$, and 0.0211 ; DMT1: $P = 0.0061, 0.0012$, and 0.0117 ; Hif-2 α : $P = 0.2537, 0.0320$, and 0.0520 ; TfR1: $P = 0.0804, 0.0365$, and 0.0473



Effects of iron-deficient diet on transcripts of iron absorption machinery in duodenum

In order to study the effect of iron-deficient diet on duodenal expression of iron absorption machinery, qPCR was performed on the duodenal samples from *Hamp*^{-/-} mice or heterozygotes fed with control or iron-deficient diet for 2 weeks.

As shown in Fig. 5, Dcytb and DMT1 mRNA expression was higher in *Hamp*^{-/-} duodenum. Iron-deficient diet feeding was associated with increased mRNA expression of both Dcytb and DMT1 as indicated by significant effects of the diet on the expression of both transcripts. It is also noteworthy that these responses were more pronounced in *Hamp*^{-/-} mice as indicated by significant interactions between diet and genotype.

Duodenal Hif-2 α and IRP2 expressions were studied to examine the mechanism of the differential response of iron transport machinery mRNA expression. qPCR results indicated a significant effect of diet on Hif-2 α mRNA expression that was significantly upregulated in *Hamp*^{-/-} mice upon iron-deficient diet treatment (Fig. 5). Western blot analysis demonstrated a significant effect of genotype on duodenal IRP2 protein levels, whereas no significant effect of the diet was observed (Fig. 6). However, a trend toward increased IRP2 protein was found in *Hamp*^{-/-} mice fed with iron-deficient diet compared with control counterparts.

Additionally, transferrin receptor 1 (*Tfrc*) mRNA expression was measured to determine duodenal iron status. *Tfrc* mRNA expression was significantly increased by iron-deficient diet. Notably, the response of *Tfrc* mRNA expression to iron-deficient diet was also more pronounced in *Hamp*^{-/-} duodenum (Fig. 5).

Discussion

The regulation of mammalian iron homeostasis consists of a complex network of regulatory mechanisms at both cellular and systemic levels. While hepcidin is regarded as the major systemic iron regulator, the local control of iron

metabolism can be achieved through the IRP-IRE mechanism. In addition, Hif-2 α has recently been shown to regulate iron homeostasis in duodenal enterocytes. The interaction between these mechanisms is not clearly understood. We recently proposed that the effects of exogenous hepcidin might be influenced by dietary iron or systemic iron status [18]. The current study was conducted in order to explore whether *Hamp* disruption affects the responses to dietary iron deficiency. Heterozygous mice were used as control group in order to compare the results from the current study with our previous report. In addition, previous literature [14] as well as our unpublished findings found no significant difference in iron parameters or mRNA levels of iron transport machinery between wild type and heterozygous mice.

Our study demonstrates that *Hamp*^{-/-} mice and heterozygotes responded differently to iron-deficient diet. Two-week feeding of iron-deficient diet caused a trend toward reduced serum iron in heterozygous mice, whereas no change in serum iron was found in the knockout. It is noteworthy that similar response was also found when these mice were injected with synthetic hepcidin [18]. The expression of ferroportin, the sole cellular iron efflux molecule, was therefore studied. Remarkably, *Hamp*^{-/-} mice exhibited more dramatic changes in ferroportin protein expression in response to iron-deficient diet compared with heterozygotes. In addition, ferroportin protein response to the diet in *Hamp*^{-/-} mice was tissue specific with increased expression in the duodenum and spleen, but decreased expression in the liver. In order to delineate tissue-specific ferroportin responses, ferroportin mRNA expression was measured in the three tissues. In the spleen, ferroportin mRNA levels were unaffected by the genotype or the diet, suggesting that splenic ferroportin was upregulated at the post-transcriptional and/or post-translational levels. Reduced spleen non-heme iron levels (as found in iron-deficient diet-fed heterozygous mice) would be expected to increase IRP activity and result in suppressed ferroportin translation, which is opposite to the finding in this study. Hence, it is less likely that IRP-IRE interaction was responsible for the ferroportin response to iron-deficient diet in these mice. IRP2 is synthesized de novo under iron-deficient conditions, thus making IRP2 Western blot analysis a practical approach for IRP2 activity determination. Furthermore, results from IRP-disrupted mouse models suggested that IRP2 seems to be more important in vivo in iron metabolism in mice [19–21]. IRP2 protein levels were therefore measured by Western blot analysis in order to determine IRP2 activity. We found that spleen IRP2 protein levels remained unaffected by the diet or genotype. Thus, we conclude that increased splenic ferroportin protein expression in heterozygotes may be caused by hepcidin suppression in response to iron-deficient diet

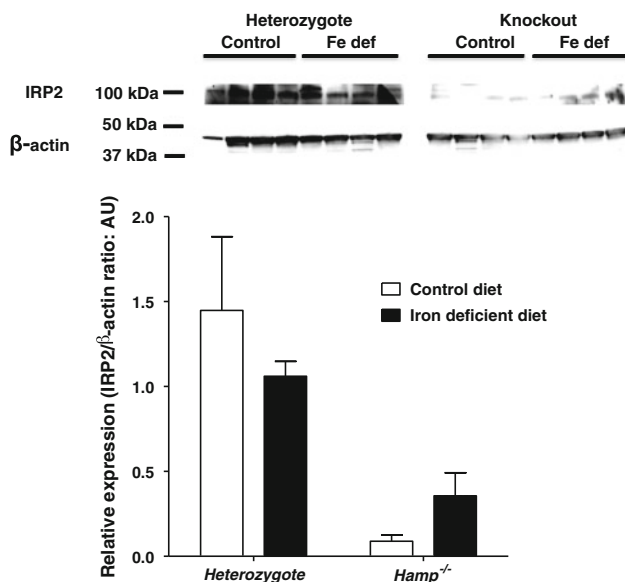


Fig. 6 IRP2 protein expression in the duodenum of control and iron-deficient diet-fed *Hamp*^{-/-} and heterozygous mice. Western blot analysis of IRP2 (100 μ g cytosolic protein extract) from the duodenum of *Hamp*^{-/-} mice and heterozygotes fed with control or iron-deficient diet for 2 weeks. IRP2 expression was normalized to the expression of β -actin and presented in arbitrary unit (AU). Densitometry is displayed below the blot. Values are means and SEM ($n = 4$). Statistical analysis was performed by two-way ANOVA with a Bonferroni's post hoc test. $P = 0.0008$, 0.8026 , and 0.1843 for the effect of genotype, diet, and genotype \times diet, respectively

feeding. In contrast, the change in *Hamp*^{-/-} mice was a result of post-transcriptional and/or post-translational mechanisms and was independent of hepcidin or IRP.

In the liver, ferroportin mRNA and protein expressions were increased in *Hamp*^{-/-} mice. Notably, a significant and suppressive effect of *Hamp* disruption on IRP2 protein levels was also found. Together with the lack of hepcidin, these findings suggest that increased ferroportin protein in *Hamp*^{-/-} liver was cumulatively caused by a combination of transcriptional, post-transcriptional and post-translational control. Interestingly, iron-deficient diet suppressed liver ferroportin protein expression in *Hamp*^{-/-} mice. This was associated with the significant and inductive effect of iron-deficient diet on IRP2 protein levels, thus suggesting that the post-transcriptional control of ferroportin could override the effect of the transcriptional control even in the absence of hepcidin.

In the duodenum, ferroportin protein levels were significantly increased by iron-deficient diet, while the effect of the genotype was not significant possibly due to a high individual variation. Similarly, only marginal effects of genotype and diet on ferroportin transcription were observed, possibly for the same reason. These findings suggest that the response of ferroportin in the duodenum was post-transcriptionally regulated; however, a transcriptional regulation cannot be excluded. Moreover, iron-deficient diet and *Hamp* disruption had significant effects on *Dcytb* and *DMT1* transcription, and the effects were more dramatic in *Hamp*^{-/-} mice. As *Dcytb* and *DMT1* shared a similar pattern of transcriptional response, it is possible that these iron transport machineries were regulated by a common mechanism.

Duodenal *Hif-2 α* has recently been demonstrated to transcriptionally regulate *Dcytb*, *DMT1*, and, possibly, ferroportin in response to iron deficiency [11, 12, 22]. In the present study, iron-deficient diet was demonstrated to significantly affect duodenal *Hif-2 α* mRNA levels in *Hamp*^{-/-} mice even in the absence of anemia. *Hif-2 α* induction was demonstrated following acute iron deficiency in the duodenum [22]. In agreement, duodenum of iron-deficient diet-fed *Hamp*^{-/-} mice appeared to be iron deficient as suggested by *Tfrc* mRNA levels. In addition, enterocytes have been shown to be hypoxic even under basal condition [11]. It is therefore possible that duodenal *Hif-2 α* can be induced in response to iron-deficient diet feeding regardless of the development of anemia. Interestingly, *Hif-2 α* mRNA expression was significantly increased in *Hamp*^{-/-} mice upon iron-deficient diet feeding which corresponded to changes in the expression of *Dcytb*, *DMT1* and ferroportin mRNA. This is, therefore, suggestive that *Hif-2 α* may be responsible for the transcriptional response of the three iron transport proteins in these mice. However, this remains to be confirmed by *Hif-*

2 α protein levels. It is also noteworthy that the control diet utilized in the present study contained only marginal amount of iron in contrast to iron-replete diet used in previous studies (48 ppm vs. 250–350 ppm) [12, 22]. The relatively low iron levels in control diet might result in the lack of *Hif-2 α* response in heterozygous mice upon iron-deficient diet feeding in the present study.

In addition to *Hif-2 α* , IRP2 protein expression was determined in the duodenum. It is noteworthy that the protein was acquired from duodenal scrapping that may contain other cell types apart from mature enterocytes. This may explain the negative effect of genotype on IRP2 protein levels as the scrapping from *Hamp*^{-/-} duodenum could contain other iron-loaded cells. Interestingly, iron-deficient diet feeding was associated with a trend for increased IRP2 protein levels in *Hamp*^{-/-} duodenum. In agreement, duodenal *Tfrc* mRNA expression, which is an indirect indicator of IRP activity, also appeared to correspond to the IRP2 response and mRNA expression of iron transport machineries. Upon iron-deficient diet feeding, *Tfrc* mRNA expression was significantly upregulated in *Hamp*^{-/-} duodenum (thus reflecting increased IRP activity). It is therefore possible that iron-deficient diet increase *DMT1* mRNA expression in the duodenum of *Hamp*^{-/-} mice through the IRP regulatory mechanism.

Collectively, the results suggest that iron-deficient diet or *Hamp* disruption transcriptionally increased *Dcytb*, *DMT1*, and, to a less extent, ferroportin in normal mice. In addition, ferroportin expression could also be post-translationally enhanced by reduced hepcidin levels. In contrast, iron-deficient diet increased the expression of iron transport machinery in *Hamp*^{-/-} duodenum through hepcidin-independent mechanisms. This might be partially caused by increased IRP activity particularly in the case of *DMT1* transcription. However, other mechanisms, including *Hif-2 α* , could also be involved since *Dcytb* mRNA does not contain an IRE.

In conclusion, our study demonstrates that the expression of ferroportin as well as *Dcytb* and *DMT1* in *Hamp*^{-/-} mice was regulated by iron-deficient diet through several hepcidin-independent mechanisms, and ferroportin response in these mice displayed a tissue-specific regulatory pattern. Moreover, *Hamp*^{-/-} mice exhibited a more pronounced increase in iron transport machinery expression that may be responsible for the unaltered serum iron levels upon iron-deficient diet feeding. Our findings indicate that despite the lack of hepcidin, the body is still capable of maintaining a degree of iron homeostasis in response to altered dietary iron through several hepcidin-independent mechanisms.

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Conflict of interest P.M., N.P., G.O.L., S.V., R.J.S. and A.T.M. have no conflicts of interest.

References

- McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E, Mudaly M, Richardson C, Barlow D, Bomford A, Peters TJ, Raja KB, Shirali S, Hediger MA, Farzaneh F, Simpson RJ (2001) An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science* 291(5509):1755–1759. doi:10.1126/science.1057206
- Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 388(6641):482–488. doi:10.1038/41343
- McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D, Miret S, Bomford A, Peters TJ, Farzaneh F, Hediger MA, Hentze MW, Simpson RJ (2000) A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. *Mol Cell* 5(2):299–309
- Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt SJ, Moynihan J, Paw BH, Drejer A, Barut B, Zapata A, Law TC, Brugnara C, Lux SE, Pinkus GS, Pinkus JL, Kingsley PD, Palis J, Fleming MD, Andrews NC, Zon LI (2000) Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* 403(6771):776–781. doi:10.1038/35001596
- Abboud S, Haile DJ (2000) A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *J Biol Chem* 275(26):19906–19912. doi:10.1074/jbc.M000713200
- Vulpe CD, Kuo YM, Murphy TL, Cowley L, Askwith C, Libina N, Gitschier J, Anderson GJ (1999) Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the *sla* mouse. *Nat Genet* 21(2):195–199. doi:10.1038/5979
- Frazer DM, Wilkins SJ, Becker EM, Vulpe CD, McKie AT, Trinder D, Anderson GJ (2002) Hepcidin expression inversely correlates with the expression of duodenal iron transporters and iron absorption in rats. *Gastroenterology* 123(3):835–844
- Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, Loreal O (2001) A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem* 276(11):7811–7819. doi:10.1074/jbc.M008923200
- Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A, Vaulont S (2002) The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J Clin Invest* 110(7):1037–1044. doi:10.1172/jci15686
- Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J (2004) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 306(5704):2090–2093. doi:10.1126/science.1104742
- Mastrogiannaki M, Matak P, Keith B, Simon MC, Vaulont S, Peyssonnaud C (2009) HIF-2alpha, but not HIF-1alpha, promotes iron absorption in mice. *J Clin Invest* 119(5):1159–1166. doi:10.1172/jci38499
- Shah YM, Matsubara T, Ito S, Yim SH, Gonzalez FJ (2009) Intestinal hypoxia-inducible transcription factors are essential for iron absorption following iron deficiency. *Cell Metab* 9(2):152–164. doi:10.1016/j.cmet.2008.12.012
- Galy B, Ferring-Appel D, Kaden S, Grone HJ, Hentze MW (2008) Iron regulatory proteins are essential for intestinal function and control key iron absorption molecules in the duodenum. *Cell Metab* 7(1):79–85. doi:10.1016/j.cmet.2007.10.006
- Lesbordes-Brion JC, Viatte L, Bennoun M, Lou DQ, Ramey G, Houbron C, Hamard G, Kahn A, Vaulont S (2006) Targeted disruption of the hepcidin 1 gene results in severe hemochromatosis. *Blood* 108(4):1402–1405. doi:10.1182/blood-2006-02-003376
- Beutler E (1971) Red cell metabolism: a manual of biochemical methods. Grune & Stratton, New York
- Foy AL, Williams HL, Cortell S, Conrad ME (1967) A modified procedure for the determination of nonheme iron in tissue. *Anal Biochem* 18:559–563
- Simpson RJ, Peters TJ (1990) Forms of soluble iron in mouse stomach and duodenal lumen: significance for mucosal uptake. *Br J Nutr* 63(1):79–89
- Masaratana P, Laftah AH, Latunde-Dada GO, Vaulont S, Simpson RJ, McKie AT (2011) Iron absorption in hepcidin1 knockout mice. *Br J Nutr* 105(11):1583–1591. doi:10.1017/s0007114510005507
- Galy B, Ferring D, Minana B, Bell O, Janser HG, Muckenthaler M, Schumann K, Hentze MW (2005) Altered body iron distribution and microcytosis in mice deficient in iron regulatory protein 2 (IRP2). *Blood* 106(7):2580–2589. doi:10.1182/blood-2005-04-1365
- Ferring-Appel D, Hentze MW, Galy B (2009) Cell-autonomous and systemic context-dependent functions of iron regulatory protein 2 in mammalian iron metabolism. *Blood* 113(3):679–687. doi:10.1182/blood-2008-05-155093
- Starzynski RR, Lipinski P, Drapier JC, Diet A, Smuda E, Bartlomiejczyk T, Gralak MA, Kruszewski M (2005) Down-regulation of iron regulatory protein 1 activities and expression in superoxide dismutase 1 knock-out mice is not associated with alterations in iron metabolism. *J Biol Chem* 280(6):4207–4212. doi:10.1074/jbc.M411055200
- Taylor M, Qu A, Anderson ER, Matsubara T, Martin A, Gonzalez FJ, Shah YM (2011) Hypoxia-inducible factor-2alpha mediates the adaptive increase of intestinal ferroportin during iron deficiency in mice. *Gastroenterology* 140(7):2044–2055. doi:10.1053/j.gastro.2011.03.007