Tissue-Specific Regulation of Ferroportin in Wild-Type and Hjv-/- Mice Following Dietary Iron Manipulations

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Hepcidin is a liver-derived peptide hormone that limits iron egress from tissues to the bloodstream. It operates by binding to the iron exporter ferroportin, which blocks iron transport and tags ferroportin for degradation. Genetic hepcidin inactivation leads to hereditary hemochromatosis, a disease of iron overload. We used wild-type and Hjv-/- mice, a model of hemochromatosis, to examine the expression of ferroportin and other proteins of iron metabolism in hepcidin target tissues. The animals were previously subjected to dietary iron manipulations. In Hjv-/- mice, hepcidin messenger RNA correlated significantly with hepatic iron load ($r = 0.8211$, $P < 0.001$), but was substantially lower compared with wild-type controls. Duodenal ferroportin and divalent metal transporter 1 (DMT1), as well as splenic and hepatic ferroportin, were overexpressed in these animals. A high-iron diet (2% carbonyl iron) suppressed duodenal DMT1 levels in both wild-type and Hjv-/- mice; however, it did not affect duodenal ferroportin expression in Hjv-/- mice, and only reduced it in wild-type mice. In contrast, the high-iron diet decreased splenic ferroportin exclusively in Hjv-/- mice, whereas it induced hepatic ferroportin exclusively in wild-type mice. Conclusion: Our data show that dietary iron differentially affects ferroportin expression in mouse tissues and are consistent with hepcidin-dependent and hepcidin-independent mechanisms for ferroportin regulation. In the Hjv-/- mouse model of hemochromatosis, duodenal ferroportin remains unresponsive to iron but DMT1 is appropriately iron-regulated. (Hepatology Communications 2021;5:2139-2150).
respectively. Iron overload varies among the hemochromatosis genotypes and correlates with the degree of hepcidin inactivation.

Hepcidin is induced transcriptionally primarily in response to iron or inflammatory signals; conversely, it is suppressed by iron deficiency or increased erythropoietic demand. Iron-dependent activation of the hepcidin pathway is mediated by bone morphogenetic protein (BMP)/mothers against decapentaplegic (SMAD) signaling, which involves phosphorylation of SMAD1/5/8, recruitment of SMAD4, and translocation of the complex to the nucleus for activation of the HAMP (hepcidin) promoter. It appears that increased transferrin (Tf) saturation is sensed by TfR2. On the other hand, elevated tissue iron stores promote secretion of BMP6 from liver sinusoidal endothelial cells, which in turn binds to BMP receptors and induces a potent SMAD response. BMP2 is likewise secreted from liver sinusoidal endothelial cells but is less responsive to iron and appears essential for basal hepcidin expression.

HJV is a BMP co-receptor and enhances basal and iron-dependent signaling to hepcidin. Pathogenic HJV mutations cause juvenile hemochromatosis, a disease characterized by early onset iron overload due to severe hepcidin deficiency. Ubiquitous or liver-specific Hjv-/- mice recapitulate this phenotype. Hjv-/- mice accumulate high levels of ferroportin in duodenal enterocytes and in macrophages of the spleen and the liver, as a result of hepcidin suppression. Nevertheless, they retain a limited capacity for hepcidin up-regulation by dietary iron.

We used wild-type and Hjv-/- mice to address functional implications of intact and impaired hepcidin responses, respectively, to dietary iron manipulations. We analyzed expression of ferroportin and other iron proteins in tissues targeted by hepcidin. Our data suggest that ferroportin is regulated by hepcidin-dependent and hepcidin-independent mechanisms in a tissue-specific manner.

**Animals and Methods**

**ANIMALS**

Wild-type and Hjv-/- mice, both in C57BL/6 genetic background, were housed in macrolone cages (up to 5 mice/cage; 12-hour/12-hour light-dark cycle [7 AM to 7 PM]; 22° ± 1°C; 60% ± 5% humidity) according to standard institutional guidelines. Ten-week old male animals (n = 10 per each group) were placed on diets with variable iron content (Harlan Laboratories, Indianapolis, IN). The standard diet contained 225 ppm iron (2018 Teklad; Envigo, Indianapolis, IN). The low-iron diet contained 75-100 ppm iron (TD.05616), while the high-iron diet was the standard, enriched with 2% carbonyl iron (TD.09521). After 4 weeks, the mice were killed by cervical dislocation. Experimental procedures were approved by the Animal Care Committee of McGill University (protocol 4966).

**SERUM BIOCHEMISTRY**

Blood was collected with cardiac puncture. Serum was separated by centrifugation and used to determine iron concentration and Tf saturation by a Roche Hitachi 917 Chemistry Analyzer (Basel, Switzerland).

**TISSUE IRON QUANTIFICATION**

Tissue nonheme iron content was quantified by the ferrozine assay. Results are expressed as micrograms of iron per gram of dry tissue.
HISTOLOGICAL ANALYSIS

Tissue specimens from 3 animals per experimental group were fixed in 10% buffered formalin and embedded in paraffin. One section per animal was prepared for liver and spleen specimens, and three sections per animal for duodenal specimens. Samples were cut at 4 µm, placed on SuperFrost/Plus slides (Thermo Fisher Scientific, Waltham, MA), and dried overnight at 37°C. The slides were then loaded onto the Discovery XT Autostainer (Ventana Medical Systems, Oro Valley, AZ) and underwent deparaffinization and heat-induced epitope retrieval for automated immunohistochemistry. Immunostaining was performed by using a 1:500 diluted custom-made ferroportin antibody (16) and the Omnimap rabbit polyclonal horseradish peroxidase (#760-4311) detection kit. (17) Slides were counterstained with hematoxylin, blued with Bluing Reagent, washed in warm soapy water, dehydrated through graded alcohols, cleared in xylene, and mounted with Permount (Thermo Fisher Scientific). Immunostained tissue slides were scanned using the Aperio ScanScope System (Leica BioSystems [Aperio], Vista, CA) at ×20 or ×40 magnifications, to provide high-resolution digital images. Slides with hematoxylin-stained splenic sections were analyzed and quantified for red and white pulp content by using the Aperio ImageScope software (Leica Biosystems).

WESTERN BLOTTING

The livers and spleens were washed with ice-cold phosphate-buffered saline (PBS) and dissected into pieces. Tissue aliquots were snap-frozen in liquid nitrogen and stored in a −80°C freezer. Duodenal segments were washed with ice-cold PBS and cut longitudinally. The mucosal layers were obtained by scraping with mild pressure using the short edge of a glass slide; subsequently, they were snap-frozen in liquid nitrogen and stored in a −80°C freezer. For western blot analysis, frozen tissue aliquots or mucosal scrapings were suspended in a lysis buffer containing 50 mM trishydroxymethylaminomethane (Tris)–Cl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), an ethylene diamine tetraacetic acid–free protease inhibitor cocktail (Roche) and a Halt phosphatase inhibitor Cocktail (Thermo Fisher Scientific). The suspensions were immediately homogenized with a TissueRuptor handheld homogenizer (Qiagen, Hilden, Germany). Cell debris was cleared by centrifugation and the protein concentration was quantified with the DC-Protein Assay (Bio-Rad Laboratories, Hercules, CA). Lysates containing 40 µg protein were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) after boiling in a loading buffer containing 25 mM Tris-Cl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.0002% bromophenol blue. For detection of or liver ferroportin, we used 100 µg of protein lysates. Ferroportin and DMT1 are transmembrane proteins prone to aggregation in response to heat or reducing agents; thus, the samples were not boiled before loading on the gel, and β-mercaptoethanol was omitted from the loading buffer. After termination of SDS-PAGE, the proteins were transferred from the polyacrylamide gel onto nitrocellulose filters. The blots were saturated with 10% nonfat milk in Tris-buffered saline buffered saline (TBS) containing 0.1% (vol/vol) Tween-20 (TBS-T) and probed with the following primary antibodies (all 1:1,000 diluted and raised in rabbit, unless otherwise indicated); custom-made ferroportin, (16) custom-made DMT1, (18) mouse monoclonal TfR1 (Zymed 13-6800, lot #VH307351), 1:500 diluted ferritin (NB600-920, lot #15656; Novus Biologicals, Littleton, CO), β-actin (A2066, lot #019M4777V; Sigma-Aldrich, St. Louis, MO), and villin (2369S; Cell Signaling Technology, Danvers, MA). After three washes with TBS-T, the blots were incubated with 1:20,000 goat anti-rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or 1:5,000 diluted peroxidase-coupled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). The peroxidase signal was visualized by enhanced chemiluminescence with the Western Lightning ECL kit (PerkinElmer, Waltham, MA). All original western blots are provided as supplemental material. Immunoreactive bands were quantified by densitometry with the ImageJ software and normalized to expression of β-actin. Densitometric data are represented as fold changes relative to samples from mice on standard diet.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was isolated from frozen tissues using the RNeasy Mini kit (Qiagen). Purity was assessed by 260/280 nm absorbance ratios, and quality was monitored by agarose gel electrophoresis. Complementary
DNA was synthesized from 1 μg RNA by using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR was performed in a 7500 Fast Real Time PCR System (Applied Biosystems, Waltham, MA) with gene-specific primers provided in Supporting Table S1. Each primer pair was validated by dissociation curve analysis and demonstrated amplification efficiency between 90% and 110%. SYBR Green (Bioline AgroSciences, Essex, United Kingdom) and primers were used to amplify products under the following cycling conditions: initial denaturation at 95°C for 10 minutes, 40 cycles of 95°C for 5 seconds, 58°C for 30 seconds, 72°C for 10 seconds, and final cycle melt analysis between 58°C and 95°C. Relative messenger RNA (mRNA) expression was calculated by the comparative Ct method. Data normalized to β-actin or ribosomal protein L19 (Rpl19) are represented as fold changes to samples from wild-type mice on standard diet.

**STATISTICS**

Quantitative data were expressed as mean ± SEM. The GraphPad Prism software (v. 7.0e) was used for statistical analysis with analysis of variance (ANOVA) and for Spearman rank correlation analysis. A probability value $P < 0.05$ was considered to be statistically significant.

**Results**

As expected, in wild-type mice fed with diets of variable iron content, hepcidin mRNA levels correlated significantly with both Tf saturation ($r = 0.5599$, $P < 0.05$) and hepatic iron levels ($r = 0.7772$, $P < 0.001$) (Fig. 1A,B). In Hjv−/− mice, hepcidin mRNA levels were substantially lower and correlated significantly to hepatic iron load ($r = 0.8211$, $P < 0.001$), whereas Tf

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**FIG. 1.** Hepcidin mRNA levels correlate with hepatic iron load in Hjv−/− mice subjected to dietary iron manipulations. Ten-week-old male Hjv−/− and wild-type mice (n = 10 for each group) were fed for 4 weeks with diets of variable iron content (low: 75–100 ppm; standard: 225 ppm; high: 225 ppm plus 2% carbonyl iron). Hepcidin mRNA expression is plotted against Tf saturation (A,C) and hepatic iron content (B,D). Spearman’s rank correlation analysis reveals a highly significant correlation of hepcidin mRNA with hepatic iron load of Hjv−/− mice ($r = 0.8211$, $P < 0.001$). Squares represent low-iron diet; triangles represent standard-iron diet; and circles represent high-iron diet.
saturation reached a plateau under all dietary regimens (Fig. 1C,D). These findings confirm that Hjv−/− mice can sense alterations in dietary iron levels and mount limited iron-dependent induction of hepcidin. (13,14,20)

HIGH-IRON DIET SUPPRESSES SPLENIC FERROPORTIN IN Hjv−/− BUT NOT WILD TYPE MICE

Ferroportin appears to be expressed at higher levels in splenic macrophages of Hjv−/− versus wild-type mice (Fig. 2A-C), in agreement with published data, (10,17) and in spite of minor decreases in mRNA content (Fig. 2D). Western blot (Fig. 2A) and immunohistochemical data (Fig. 2C) show that high-iron diet did not significantly affect splenic ferroportin expression in wild-type mice, notwithstanding hepcidin induction (Fig. 1). Nevertheless, high-iron diet led to substantial and significant (2.8-fold, \( P < 0.05 \)) reduction in splenic ferroportin content in Hjv−/− mice (Fig. 2B), in line with their augmented iron-retaining capability in this

**FIG. 2.** Dietary iron loading down-regulates splenic ferroportin expression in Hjv−/− but not wild-type mice. Splenic protein extracts, tissue sections, and RNA were prepared from the Hjv−/− and wild-type mice described in Fig. 1 and used for western blotting, immunohistochemistry, and quantitative real-time PCR, respectively. (A, B) Analysis of ferroportin, ferritin, and control β-actin expression in representative samples from each group (\( n = 3 \)) of wild-type (A) and Hjv−/− (B) mice. Densitometric quantification of ferroportin/β-actin (corresponding to \( n = 6 \) mice for each group) is shown below the western panels. (C) Immunohistochemical detection of splenic ferroportin (original magnification: \( \times 20 \)). Representative ferroportin-expressing cells are indicated by arrows. (D) Analysis of splenic ferroportin mRNA (\( n = 10 \) for each group). All quantitative data are presented as the mean ± SEM. Statistical analysis was performed by two-way ANOVA. Statistically significant differences across genotypes in (D) are indicated by **\( P < 0.01 \). Abbreviation: wt, wild-type.
organ under similar experimental conditions. These responses were associated with significant increases in ferroportin mRNA (1.4-fold, \(P < 0.001\) in Hjv-/- mice; and 2-fold, \(P < 0.01\) in wild-type mice, respectively) (Fig. 2D). Interestingly, low-iron diet diminished splenic ferroportin content in wild-type mice (Fig. 2A) but elicited opposite effects in Hjv-/- animals (Fig. 2B). Ferritin levels (Fig. 2A, second panel) corroborated splenic iron load in wild-type mice but were close to detection limit in Hjv-/- mice (Fig. 2B, second panel). We did not observe any differences in cellularity of the spleen between wild-type and Hjv-/- mice under all dietary regimens (Supporting Fig. S1).

**HIGH-IRON DIET STIMULATES LIVER FERROPORTIN EXPRESSION IN WILD-TYPE BUT NOT Hjv-/- MICE**

Immunohistochemical analysis of liver sections showed robust expression of ferroportin in Kupffer cells, with apparently stronger signal intensity in Hjv-/- mice (Fig. 3A, arrows). The staining was predominant

![Fig. 3.](image)
in periportal areas (Fig. S2). Hepatocellular ferroportin expression was also evident in Hjv-/– livers, and especially in hepatocytes around portal triads (Supporting Fig. S2). However, the signal was weaker compared with that in Kupffer cells, while slight variations were noted among samples (not shown). Western blot analysis shows that the high-iron diet induced significantly \( P < 0.05 \) total liver ferroportin expression in wild-type (Fig. 3C) but not Hjv-/– mice (Fig. 3D). Nevertheless, a more intense ferroportin staining in Kupffer cells was evident in both genotypes after high dietary iron intake (Fig. 3A). The low-iron diet did not affect liver ferroportin expression in wild-type mice, but slightly reduced it in Hjv-/– animals. As expected, ferritin content was augmented in the liver of iron-loaded wild-type mice and remained elevated in the liver of all Hjv-/– mice independently of dietary regimen (Fig. 3C,3D). Liver ferroportin mRNA levels did not significantly differ among the genotypes and did not change in response to diets (Fig. 3B).

**BOTH WILD-TYPE AND Hjv-/– MICE SUPPRESS DUODENAL DMT1, BUT ONLY THE FORMER INHIBIT DUODENAL FERROPORTIN EXPRESSION IN RESPONSE TO HIGH-IRON DIET**

DMT1 is expressed on the apical membrane of enterocytes\(^{21}\) and mediates dietary iron absorption from the intestinal lumen.\(^{22}\) Duodenal extracts from Hjv-/– mice appeared to contain considerably higher DMT1 levels compared with wild-type controls (Fig. 4A,B, upper panel). Purity of the duodenal extract preparation is indicated by the presence of villin, a marker of brush border enterocytes.\(^{23}\) Importantly, duodenal DMT1 was strongly induced in Hjv-/– mice on low-iron diet and, conversely, dramatically inhibited in mice fed with high-iron diet (Fig. 4B). Induction of DMT1 was also observed in iron-poor wild-type controls, whereas dietary iron loading appeared to further reduce DMT1 content (Fig. 4A). These effects were associated with alterations in DMT1 mRNA. Thus, Hjv-/– mice exhibited 6-fold \( P < 0.01 \) higher expression of duodenal DMT1 mRNA compared with wild-type controls on standard diet (Fig. 5C). DMT1 mRNA levels were increased by the low-iron diet (2.4-fold, \( P < 0.05 \) in Hjv-/– mice; and 2-fold, \( P < 0.05 \) in wild type mice, respectively) and reduced by the iron-enriched diet in Hjv-/– mice (2.9-fold, \( P < 0.05 \)). These results highlight an important role of DMT1 in the development of iron overload in Hjv-/– mice. In addition, they suggest that the iron-dependent regulation of DMT1 largely operates at the transcript level.

Ferroportin was markedly induced in the duodenum of Hjv-/– versus wild-type mice (Figs. 4 and 5A), in agreement with previous findings.\(^{10,11}\) In animals on standard diet, this was associated with moderate (0.7-fold) but significant \( P < 0.05 \) up-regulation of ferroportin mRNA (Fig. 5B). In wild-type animals, duodenal ferroportin decreased by the high-iron diet and, conversely, increased by the low-iron diet (Fig. 4A); the latter was associated with a significant \( P < 0.05 \) 2-fold mRNA induction (Fig. 5B). In contrast, duodenal ferroportin did not respond to dietary iron alterations in Hjv-/– mice (Fig. 4B), whereas the induction of its mRNA by the lower iron diet was preserved (Fig. 5B). The immunohistochemical analysis in Fig. 5A validates the western blot data. Taken together, the findings in Figs. 2–5 highlight tissue-specific differences in the regulation of splenic, hepatic, and duodenal ferroportin, and demonstrate a failure of Hjv-/– mice to mount appropriate tissue ferroportin responses to high-iron diet.

**TfR1** is expressed on the basolateral membrane of intestinal crypt cells and mediates uptake of Tf-bound iron from the bloodstream.\(^{24}\) TfR1 and ferritin are coordinately and reciprocally regulated post-transcriptionally by the “iron-responsive element/iron regulatory protein” system.\(^{25}\) Western blot analysis shows the expected pattern of duodenal TfR1 and ferritin expression in both wild-type and Hjv-/– mice (Fig. 4A,B; quantification of the western blots is shown in Supporting Fig. S3). The apparently higher TfR1 and lower ferritin levels in the Hjv-/– duodenum may reflect the known iron deficiency of this tissue in hereditary hemochromatosis.\(^{26}\) Importantly, the high-iron diet triggered a noticeable induction of duodenal ferritin in Hjv-/– mice, which indicates iron retention. Indeed, quantification revealed a significant \( P < 0.01 \) 0.6-fold increase in duodenal iron content under these conditions (Fig. 5D). Thus, high-iron diet promotes at least partial duodenal iron retention in Hjv-/– mice, in spite of the maintenance of pathological ferroportin levels.
Discussion

We show that wild-type and Hjv−/− mice differentially regulate ferroportin expression in major iron-exporting tissues in response to dietary iron manipulations. Hjv−/− mice express higher ferroportin levels in the spleen, liver, and duodenum compared with age-matched and gender-matched isogenic wild types, as a result of severe genetic inactivation of hepcidin. We previously demonstrated that these animals retain a capacity for regulating residual hepcidin in response to iron\(^{14,20}\) but also to inflammatory signals\(^{17}\). The data in Fig. 1 corroborate the published data and, furthermore, raise the possibility that

![Graphs and Images]

**FIG. 4.** Differential expression and iron-dependent regulation of duodenal DMT1 and ferroportin in wild-type and Hjv−/− mice. Duodenal mucosal lysates were prepared from Hjv−/− and wild-type mice described in Fig. 1 and used for western blotting. (A, B) Expression of DMT1, ferroportin, TfR1, villin, ferritin, and β-actin in representative samples from each group (n = 3) of wild-type (A) and Hjv−/− (B) mice. Densitometric quantification of DMT1/β-actin and ferroportin/β-actin (corresponding to n = 6 mice for each group) is shown below the western panels; quantification of TfR1/β-actin and ferritin/β-actin is shown separately in Supporting Fig. S3. All quantitative data are presented as the mean ± SEM. Statistical analysis was performed by two-way ANOVA.
iron-dependent induction of residual hepcidin may account for the decreased ferroportin content in the spleens of Hjv-/- mice on high-iron diet (Fig. 2B). A contribution of splenic hepcidin cannot be formally excluded but is unlikely because its levels are minuscule. (14)

In spleens of wild-type mice, ferroportin was not significantly reduced following a high-iron diet, even though a trend was apparent (Fig. 2A). We speculate that hepcidin-dependent degradation of splenic ferroportin may be counterbalanced by translational de-repression of its mRNA, which contains a “translation-type” IRE and is highly expressed in iron-replete conditions. (27) The induction of ferroportin mRNA (Fig. 2D), possibly through transcriptional mechanisms, (28,29) would further augment this response. Conversely, suppression of ferroportin mRNA translation would explain the decrease in ferroportin steady-state levels observed in wild-type mice on low-iron diet. Presumably, splenic ferroportin mRNA is likewise poorly translated in Hjv-/- mice. The reason is that macrophages fail to retain iron in hemochromatosis, and, consequently, the spleen becomes iron-deficient. Nevertheless, the dramatic

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**FIG. 5.** Immunohistochemical detection of duodenal ferroportin and analysis of duodenal ferroportin and DMT1 mRNAs and iron content. Duodenal sections, RNA, and tissue segments were prepared from Hjv-/- and wild-type mice described in Fig. 1 and used for immunohistochemistry, quantitative real-time PCR, and iron quantification, respectively. (A) Immunolocalization of ferroportin (original magnification: ×40). Representative ferroportin-expressing cells are indicated by arrows. (B) Analysis of ferroportin mRNA (n = 10 for each group). (C) Analysis of DMT1 mRNA (n = 10 for each group). (D) High-iron diet promotes partial duodenal iron retention in Hjv-/- mice. Duodenal segments from the Hjv-/- mice described in Fig. 1 were isolated and used for quantification of nonheme iron by the ferrozine assay (n = 10 for each group). Data are presented as the mean ± SEM. Statistical analysis was performed by one-way ANOVA. Statistically significant differences across genotypes in (B) and (C) are indicated by *P < 0.05 or **P < 0.01.
suppression of hepcidin in Hjv-/− mice may allow unrestricted accumulation of even inefficiently synthesized protein.

Ferroportin is expressed in red pulp macrophages of the spleen; in fact, we have shown that the ferroportin signal co-localizes with the macrophage marker c-fms in immunohistochemical experiments. (17) On the other hand, liver ferroportin is expressed in macrophages (Kupffer cells) (17) as well as in parenchymal cells (hepatocytes). (30) Hepatocellular ferroportin is not always detectable by immunohistochemistry but very likely contributes to the apparent increase in biochemically detectable liver ferroportin content in Hjv-/− mice compared with controls (Fig. 3). Presumably, hepatocellular ferroportin also accounts for the increase in liver ferroportin content in wild-type mice on a high-iron diet. In light of the strong up-regulation of hepcidin under these conditions (Fig. 1), this response appears counterintuitive. Nonetheless, by analogy to a similar but less pronounced effect observed in the spleen (Fig. 2), the ferroportin-degrading action of hepcidin may be negated by increased ferroportin synthesis due to translational de-repression of its mRNA. It is also conceivable that hepcidin remains operational by occluding iron efflux from ferroportin under these conditions. This interpretation is supported by the induction of ferritin, which is indicative of cellular iron retention and overload.

Liver sections from Hjv-/− mice exhibit more intense ferroportin staining in Kupffer cells compared with wild-type controls (Fig. 3), which is a result of hepcidin deficiency. (10) The intensity of the staining tends to increase in response to high-iron diet, even though the overall ferroportin content remains unchanged. This may reflect increased ferroportin synthesis in Kupffer cells, assuming that they can retain some iron by analogy to splenic macrophages under similar experimental conditions. (14, 20) We speculate that the increased ferroportin expression in Kupffer cells does not lead to an overall increase in liver ferroportin content because of a “dilution effect” in more abundant and highly expressed hepatocellular ferroportin. Liver ferroportin mRNA levels remained unresponsive to dietary iron manipulations in both wild-type and Hjv-/− mice, highlighting that iron-dependent ferroportin expression in this organ is regulated by post-transcriptional mechanisms.

In the duodenum, ferroportin is expressed on the basolateral membrane of enterocytes. (31) In wild-type mice, duodenal ferroportin is appropriately suppressed by high-iron and induced by low-iron diets, respectively (Fig. 4A). These responses are consistent with hepcidin regulation. Moreover, the increased ferroportin mRNA levels in the duodenum of wild-type mice fed iron-poor diet is in line with transcriptional induction through hypoxia inducible factor 2α (HIF-2α). (32) Because duodenal ferroportin is encoded by an alternatively spliced transcript that lacks an iron-responsive element, (27) iron loading is not expected to stimulate its translation.

Although residual iron-dependent induction of hepcidin in Hjv-/− mice may have functional implications in the spleen, it clearly does not affect duodenal ferroportin levels (Fig. 4B). This may indicate tissue-specific differences in ferroportin regulation by hepcidin, as proposed in previous studies. (33, 34) However, it is also possible that duodenal ferroportin requires a higher threshold of hepcidin to undergo degradation. In this case, its high expression in Hjv-/− mice on high-iron diet would indicate hepcidin insufficiency. On the other hand, the increased duodenal iron retention in Hjv-/− on a high-iron diet (Fig. 5D) may suggest that up-regulated residual hepcidin in these animals operates by occluding iron efflux. Pharmacological experiments with synthetic hepcidin or analogues can shed light on the responsiveness of overexpressed duodenal ferroportin to hepcidin and the underlying mechanisms.

The data in Fig. 4 show that duodenal DMT1 is also overexpressed in Hjv-/− mice and, furthermore, it is profoundly suppressed by iron. Previous reports identified increased duodenal DMT1 mRNA expression in Hfe-/− mice (35) as well as in patients with HFE hemochromatosis, (36) suggesting an important contribution of this transporter in the development of genetic iron overload. Nevertheless, DMT1 mRNA and protein levels were physiological in another Hfe-/− mouse strain, and it was argued that the augmentation of DMT1 mRNA in patients may be a secondary effect to therapeutic phlebotomies. (37) Our results are in agreement with the increased DMT1 expression seen in Hamp-/− mice, (38) an additional model of juvenile hemochromatosis, and indicate that duodenal DMT1 levels correlate with the degree of systemic iron overload and intestinal iron deficiency.

Duodenal DMT1 is subjected to iron regulation by transcriptional and post-transcriptional mechanisms. (39) Thus, under iron deficiency, its mRNA is
transcriptionally activated by HIF-2α, whereas IRE-containing isoforms are post-transcriptionally stabilized by iron regulatory proteins. Our DMT1 mRNA analysis (Fig. 5C) is consistent with both scenarios. At the protein level, duodenal DMT1 has been proposed to be an additional molecular target of hepcidin, but it is unclear whether circulating hepcidin can reach DMT1, which is expressed on the apical membrane of duodenal enterocytes. We speculate that the suppression of duodenal DMT1 in Hjv−/− mice on high-iron diet is driven by the relatively increased duodenal iron content in these animals. The possibility that residual hepcidin contributes to this effect is intriguing but requires experimental validation.

In conclusion, our data suggest that ferroportin expression is iron-regulated by hepcidin-dependent and hepcidin-independent mechanisms in iron-exporting mouse tissues. Understanding these mechanisms is critical in the context of pharmacological targeting of hepcidin pathways for the treatment of iron overload disorders. Moreover, duodenal DMT1 may offer another legitimate pharmacological target against hemochromatosis.

Acknowledgment: The authors thank Dr. Naciba Benlimame and Lilian Canetti for assistance with the histology.

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