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Hypoxia-Inducible Factor-2 α Mediates the Adaptive Increase of Intestinal Ferroportin During Iron Deficiency in Mice

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Abstract

Background & Aims—Iron deficiency and iron overload affect over a billion people, worldwide. Dietary iron absorption in the small intestine is required for systemic iron homeostasis. Ferroportin (FPN) is the only characterized, mammalian, basolateral iron exporter. Despite the importance of FPN in maintaining iron homeostasis, its *in vivo* mechanisms of regulation are unclear.

Methods—Systemic iron homeostasis was assessed in mice with intestine-specific disruption of genes encoding the von Hippel-Lindau tumor suppressor protein (Vhl), hypoxia-inducible factor (HIF)-1 α , HIF-2 α , and aryl hydrocarbon nuclear translocator (ARNT).

Results—We observed biphasic regulation of Fpn during iron deficiency. *Fpn* was rapidly induced under conditions of low iron, which required the transcription factor HIF-2 α . Targeted disruption of HIF-2 α in the intestine inhibited *Fpn* induction in mice with low iron, through loss of transcriptional activation. Analysis of the *Fpn* promoter and *in vivo* chromatin immunoprecipitation assays demonstrated that HIF-2 α directly binds to the *Fpn* promoter and induces its expression, indicating a mechanism of transcriptional regulation of *Fpn* following changes in systemic levels of iron. During chronic iron deficiency, FPN protein levels also increased, via increased stability through a HIF-2 α -independent pathway.

Conclusion—In mice, expression of the gene that encodes Fpn and its protein levels are regulated by distinct pathways to provide a rapid and sustained response to acute and chronic iron deficiency. Therapies that target FPN might be developed for patients with iron-related disorders..

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Keywords

ChIP assay; Hepcidin; diet; metabolism

Introduction

Dietary iron is absorbed in the small intestine and utilized mainly in the production of red blood cells (RBC). In an iron-deficient state, iron is mobilized by increasing its dietary absorption from the small intestine. This is accomplished by an adaptive increase in duodenal cytochrome b (DcytB), an apical ferric reductase that reduces dietary ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). Ferrous iron is transported into the enterocyte by the divalent metal transporter-1 (DMT-1, also known as Nramp2, SLC11a2 and DCT1)¹⁻⁵. Iron is exported from the enterocyte into circulation by the only known mammalian iron exporter ferroportin (FPN, also known as SLC40A1)⁶⁻⁹. DMT-1 and FPN are expressed in cells that are critical in maintaining iron homeostasis, mainly macrophages, hepatocytes and enterocytes. FPN is post-transcriptionally regulated through an iron-regulated hepatic peptide hormone, hepcidin¹⁰⁻¹⁴. Under normal physiological iron levels, hepcidin is highly expressed and secreted from the liver into the bloodstream. Hepcidin binds to FPN leading to an increase in degradation^{15,16}. When serum iron levels are low, hepcidin is potently repressed correlating with an increase in FPN expression^{12,13}. Hepcidin signaling is the best-characterized pathway shown to regulate FPN during changes in systemic iron requirements. Hepcidin promotes endocytosis and degradation of FPN in HEK293, HeLa and macrophages^{15,17,18}. However, intestinal-derived cell lines are not responsive to hepcidin-induced FPN downregulation^{19,20}. Furthermore, short-term hepcidin treatment did not have an effect on duodenal iron transport, whereas multiple treatments for 72 hours significantly decreased duodenal iron transport²¹. These data suggest that other mechanisms may be critical in the intestine to regulate FPN expression. Iron deprivation can induce intestinal FPN mRNA through an unknown mechanism⁹. Recently, other transition metals such as zinc have been shown to induce FPN mRNA through direct binding of the metal transcription factor-1 on the promoter of FPN²². However, this pathway is not involved in the iron-dependent increase in FPN mRNA. Moreover, the relative contribution that FPN mRNA and protein stability play in the total change in intestinal FPN protein expression following an increase in systemic iron requirements are not clear.

In contrast to FPN, regulation of DMT-1 and DcytB following changes in iron levels have been recently characterized. The transcription factor, hypoxia-inducible factor (HIF)-2 α regulates expression of DMT-1 and DcytB by binding to consensus HIF response elements (HREs) on their respective promoters. In addition, HIF-2 α was shown to be critical in regulating DMT-1 and DcytB expression during iron deficiency in vivo^{23,24}. In the present study, whole genome microarray analysis was performed in the small intestines of iron-deprived wild-type and HIF-2 α intestine-specific knockout mice in order to identify additional roles HIF-2 α may play in regulating systemic iron homeostasis. Surprisingly, over 95% of the most enriched gene expression changes following low-iron diet were mediated via a HIF-2 α -dependent pathway. HIF-2 α was shown to be the critical regulator of FPN mRNA following changes in systemic iron requirements. The data establishes a novel bimodal mechanism for acute and long-term regulation of FPN. The increase in FPN transcription via HIF-2 α is essential for acute regulation of iron export, whereas a chronic increase in systemic iron requirements is regulated through protein stability via a HIF2 α -independent mechanism.

Methods

Luciferase assay

The mouse FPN1B luciferase reporter plasmid and the HRE mutant promoters were constructed by cloning the upstream regions as previously described by Zhang et al.²⁵ into the pGL3-basic vector (Promega, Madison WI) using primers listed in Supplementary Table 1. The luciferase reporters were co-transfected with HIF-2 α expression vector or empty vector into Caco-2 and HCT116 cells with Eugene 6 transfection reagent (Roche, Indianapolis, IN). Standard dual luciferase assay was used and normalized to a co-transfected control reporter (Promega).

Animals and diets

Vhl^{F/F}, *Vhl* ^{Δ IE}, *Arnt*^{F/F}, *Arnt* ^{Δ IE} and *Vhl* ^{Δ IE}/*Hif-1 α* ^{Δ IE} mice were previously described^{24, 26–29}. For disruption of HIF-2 α , *Hif-2 α* ^{F/F} mice were crossed with mice harboring the Cre recombinase under control of the villin promoter and backcrossed to C57BL/6 for five generations. For the HIF-2 α and VHL double knockout, *Vhl*^{F/F}/*Hif-2 α* ^{F/F} mice hemizygous for villin-cre were mated to each other. The mice were used between the ages of 6–8 weeks old for all experiments. The mice were housed in temperature and light-controlled rooms, and were given water and pelleted LabDiet 5001chow ad libitum (PMI Nutrition, St. Louis, MO). In the low-iron studies, mice were given iron-replete AIN93G diet containing 350 ppm of iron or iron-deficient AIN93G diet (less than 5 ppm of iron) for 2-weeks or 8-weeks (Dyets, Bethlehem, PA). All animal studies were carried out in accordance with Institute of Laboratory Animal Resources guidelines and approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Histological analysis

Small intestines and livers were processed in paraffin. Macroscopic assessment was performed on routine hematoxylin and eosin or Perl's iron stained sections.

RNA analysis

RNA was extracted from mucosal scrapings of the duodenum or from whole livers and kidneys and analyzed by qPCR as previously described²⁴. Primer sequences are listed in Supplementary Table 1.

Western blot analysis

For detection of HIF-2 α , nuclear proteins were isolated using the NE-PER nuclear extraction kit (Thermo Scientific, Rockford, IL). For detection of FPN and DcytB, scraped duodenal mucosa were dounce homogenized and passed through a 27-gauge needle several times in lysis buffer (5mM Tris pH 8.0, 2mM EDTA, 250mM of sucrose and protease inhibitor). The nuclei were pelleted and the supernatant was centrifuged at 100,000 $\times$ g for 45 min. The resulting membrane pellet was resuspended in storage buffer (75mM Tris pH8.0, 12.5mM MgCl₂, 5mM EDTA and protease inhibitor). Membrane extracts were mixed with 2X sample buffer and incubated at 65 C° for 5 min, for HIF-2 α and RAN detection the nuclear extracts were boiled for 5 min. The proteins were separated and transferred to nitrocellulose membranes using standard Western blotting techniques. The membranes were incubated with antibodies against HIF-2 α (Novus Biologicals, Littleton, CO), FPN and DcytB (Alpha Diagnostic International, San Antonio, TX); the signals obtained were normalized to RAN (Santa Cruz Biotechnology Inc, Santa Cruz, CA) for nuclear extracts and to Coomassie staining for total membrane proteins. For assessment of hepcidin-induced FPN degradation, intestine-derived Caco-2 and HCT116 cells and the hepcidin-responsive HeLa and Hek293 cells were transfected with green fluorescent protein-tagged FPN (FPN-GFP). The plasmid

construct is previously described³⁰ and was a kind gift from Dr. William Griffiths. 48 hours post-transfection the cells were treated with 1 µg/mL of hepcidin (Bachem, Torrance, CA) for 8 hours. Cells were lysed using RIPA buffer and FPN-GFP was detected using an antibody for GFP and the blot was normalized to GAPDH (Santa Cruz).

cDNA microarray analysis

cDNA microarrays were performed as previously described²⁴. Briefly, RNA was extracted from duodenal mucosal scrapings from *Hif2a*^{F/F} and *Hif2a*^{ΔIE} mice on control or low-iron diet for 2-weeks. Labeled cDNA was hybridized to whole mouse genome 60-mer oligonucleotide microarray (Agilent Technologies, Santa Clara, CA).

Serum iron and hematological analysis

Routine erythrocyte and serum iron analysis was performed as previously described²⁴. Tissue iron was quantified by the Diagnostic Center for Population and Animal Health at Michigan State University.

Chromatin immunoprecipitation (ChIP) assay

Duodenal epithelium scrapings from *Vhl*^{F/F} and *Vhl*^{ΔIE} mice were crosslinked in 1% formaldehyde in 1X PBS for 20 min. Nuclei were isolated and lysed in an SDS lysis buffer (50mM Tris-HCl pH 8.1, 10 mM, EDTA, 1% SDS, and protease inhibitors). Sheared soluble chromatin was immunoprecipitated with primary antibody for HIF-2α (Novus Biologicals). Decrosslinked samples were incubated with RNase A and proteinase K and 1 µL of purified DNA was used for PCR or qPCR with primers listed in Supplementary Table 2.

Data analysis

Results are expressed as mean ± S.D. P values were calculated by Independent t-test and ANOVA, Dunnett's t-test. $p < 0.05$ was considered significant.

Results

HIF-2α is a major regulator of iron-responsive genes in the intestine

Intestinal HIF-2α overexpressing mice (*Vhl*^{ΔIE}) generated by an intestine-specific disruption of the E3 ubiquitin recognition protein of HIF-1α and HIF-2α, the von Hippel-Lindau tumor suppressor protein (*Vhl*)^{24,27,31,32}. *Vhl*^{ΔIE} mice demonstrated a systemic dysregulation of iron homeostasis²⁴. These data suggest that HIF-2α may regulate genes other than DMT-1 and DcytB to control iron homeostasis. To better characterize the systemic response mediated by intestinal HIF-2α, mice were generated that lacked HIF-2α specifically in the intestine (*Hif-2a*^{ΔIE}) (Supplemental Figure 1). Duodenal gene expression profiles of *Hif-2a*^{ΔIE} and wild-type mice on 2-week low-iron diet were compared. By analyzing the most enriched changes (genes induced or repressed over 3-fold), 187 out of 195 genes (95%) were regulated in a HIF-2α-dependent manner (Figure 1A). To demonstrate that low-iron diet was working properly in wild-type and *Hif-2a*^{ΔIE} mice, a well-characterized iron-responsive gene was assessed. Transferrin receptor (TFRC), a gene known to be induced by low-iron diet was increased to the same level in *Hif-2a*^{ΔIE} mice and wild-type littermates (Figure 1B). In addition, both *Hif-2a*^{ΔIE} mice and wild-type littermates had a robust repression of liver hepcidin mRNA expression (Supplemental Figure 2A). The iron-responsive HIF-2α target genes DcytB and DMT-1, were increased in the small intestine of wild-type mice following low-iron diet, but not elevated in *Hif-2a*^{ΔIE} mice (Figure 1C). Expression of several genes that were significantly altered as assessed by microarray analysis were confirmed by qPCR. Ankyrin repeat domain 37 (ANKRD37), an iron-responsive gene containing putative HIF response elements in the promoter^{33,34} was

significantly induced following low-iron diet in a HIF-2 α -dependent manner (Figure 1D). In addition, the data identified novel iron responsive genes; ankyrin repeat and SOCS box-containing 11 (ASB11), solute carrier family 38, member 1 (Slc38a1), tripartite motif-containing 27 (Trim27), and gamma glutamyl hydrolase (GGH) (Gene list containing expression changes of 2-fold or more are in Supplementary Table 2). Indeed, the microarray data correlated well with the qPCR quantification, demonstrating a central role for HIF-2 α in mediating the intestinal response following iron deprivation.

Iron-induced FPN transcription is dependent on HIF-2 α

Through microarray analysis, FPN mRNA was shown to be significantly induced in a HIF-2 α -dependent manner following 2-weeks of a low-iron diet treatment. Recently, a study revealed that FPN exists as two isoforms, FPN1A and FPN1B, and that both are induced following low-iron treatment²⁵. To confirm the microarray results and determine the specific FPN isoform regulated by HIF-2 α , qPCR was performed using primers to detect total FPN and FPN1A and FPN1B isoforms. Total FPN, FPN1A, and FPN1B mRNAs were significantly increased following 2-weeks of low-iron diet treatment in wild-type mice; no increase was found in the *Hif-2 α ^{ΔIE}* mice (Figure 2A). FPN is regulated by transcriptional as well as post-transcriptional mechanisms, we sought to delineate the relative contributions that mRNA expression and protein stability play in regulating total FPN expression in the intestine. *Hif-2 α ^{ΔIE}* mice completely lose their ability to induce FPN mRNA expression following a low-iron diet treatment (Figure 2A), and therefore are an ideal model to address this question. Membrane proteins were isolated from *Hif-2 α ^{ΔIE}* and wild-type mice following 2-week low-iron diet treatment and Western blot analysis was performed. An induction of FPN expression was observed in wild-type mice. In addition, a significant increase was observed in the iron responsive ferric reductase, DcytB. In the *Hif-2 α ^{ΔIE}* mice, as expected, the increase in DcytB following 2-week low-iron treatment was completely abolished (Figure 2B and C). Interestingly, 2-week low-iron diet treatment did not increase membrane expression of FPN in the *Hif-2 α ^{ΔIE}* male (Figure 2B) and female (Figure 2C) mice. These data demonstrate that following 2-week low-iron diet treatment, FPN induction requires intact HIF-2 α signaling. Furthermore, the data suggest that the increase in FPN is dependent on the iron induced transcriptional response of FPN rather than an increase in protein stability.

Disruption of VHL in the small intestine induces FPN expression

To investigate the role of intestinal HIF-2 α overexpression on FPN regulation, duodenal FPN mRNA and protein expression were assessed in the *Vhl^{ΔIE}* and littermate control mice. Our previous study did not demonstrate that FPN was significantly induced in the *Vhl^{ΔIE}* mice, although there was a trend toward increased expression²⁴. However, using more specific primers that detect total FPN, FPN1A, and FPN1B, a significant induction of intestinal FPN expression was observed in the *Vhl^{ΔIE}* mice compared to littermate control *Vhl^{F/F}* mice (Figure 3A). Next, to assess FPN protein expression, duodenal membrane extracts were prepared from *Vhl^{ΔIE}* and wild-type mice. A robust induction of FPN protein was observed in duodenal extracts from *Vhl^{ΔIE}* mice compared to *Vhl^{F/F}* mice (Figure 3B). To assess the influence of the HIF-dependent pathways on FPN expression in the small intestine, mice with a double disruption of VHL and HIF-1 α or VHL and HIF-2 α were generated. Disruption of VHL and HIF-1 α inhibits HIF-1 heterocomplexes, but not HIF-2 heterocomplexes, whereas disruption of VHL and HIF-2 α prevents the formation of functional HIF-2. Disruption of both VHL and HIF-2 α (*Vhl^{ΔIE}/Hif-2 α ^{ΔIE}*) completely abrogated the induction of FPN mRNA, whereas disruption of both VHL and HIF-1 α (*Vhl^{ΔIE}/Hif-1 α ^{ΔIE}*) had no effect on FPN mRNA as compared to *Vhl^{ΔIE}* mice (Figure 3C and data not shown). Consistent with the gene expression data, the increase in FPN protein expression observed in *Vhl^{ΔIE}* duodenal membrane extracts was abrogated in the *Vhl^{ΔIE}/*

Hif-2 α ^{Δ IE} mice, whereas FPN protein expression was significantly induced in the *Vhl* ^{Δ IE}/*Hif-1 α* ^{Δ IE} mice compared to littermate controls (Figure 3D and E). These data further demonstrate that HIF-2 α regulates FPN expression.

HIF-2 α regulates FPN1B promoter in the duodenum by directly binding to the regulatory regions in vivo

Duodenal epithelial cells use the FPN1B promoter²⁵ and the upstream sequence from the transcription initiation site of FPN1B contained two putative HIF response elements (HRE) (Figure 4A). The FPN1B promoter containing the putative HREs was analyzed by transient transfection using a luciferase reporter construct (Figure 4B). Co-transfection with a mammalian expression plasmid for HIF-2 α strongly increased the luciferase activity in intestine-derived Caco-2 and HCT116 cells compared to empty vector (Figure 4B). Mutating the putative HREs inhibited the HIF-2 α -mediated induction, thus confirming that these sites were functional HREs (Figure 4C). Next, to demonstrate that HIF-2 α could directly bind the FPN1B promoter, in vivo ChIP assays were performed in duodenal samples from *Vhl*^{F/F} or *Vhl* ^{Δ IE} mice. HIF-2 α was immunoprecipitated from crosslinked soluble chromatin isolated from *Vhl*^{F/F} or *Vhl* ^{Δ IE} mice. Using PCR primers in close proximity to both HREs, a significant increase in HIF-2 α promoter occupancy was observed at the FPN1B HREs in the *Vhl* ^{Δ IE} mice compared to the *Vhl*^{F/F} mice (Figure 4D). As a control, the previously characterized HREs of DMT-1 were assessed and an increase in HIF-2 α binding was observed in the *Vhl* ^{Δ IE} mice compared to the *Vhl*^{F/F} mice. The promoter occupancy was quantitated using qPCR ChIP analysis, and this data demonstrates that HIF-2 α is able to bind the FPN1B regulatory region in vivo similarly to what is observed on the DMT-1 promoter region (Figure 4E).

Increased expression of FPN following chronic treatment with low-iron is not HIF-2 α -dependent

Together, the data suggest that FPN regulation in the intestine is independent of hepcidin, and mainly controlled by HIF-2 α through transcriptional regulation. This conclusion is in agreement with studies assessing FPN expression in intestine-derived cell lines. In Caco-2 and HCT116 cells hepcidin had no inhibitory effect on FPN protein expression, whereas in Hek293 and HeLa cells hepcidin significantly inhibited FPN protein expression (Figure 5A and 19,20). However, there are a number of studies that demonstrate the importance of hepcidin-induced FPN degradation in regulating iron homeostasis^{35,36}. In mouse models, hepcidin deficiency is linked to hemochromatosis and increased expression of hepcidin is associated with anemia^{10,11,14}. Moreover, mice that have decreased hepcidin levels, demonstrate an increase in duodenal FPN protein expression³⁷. To understand these discrepancies, HIF-2 α -dependent regulation of FPN expression was examined following long-term iron deprivation. Consistent with the 2-week low-iron diet treatment, wild-type mice under an 8-week low-iron diet treatment, significantly induced FPN, FPN1A, and FPN1B mRNA expression; no increase was noted in 8-week low-iron diet treated *Hif-2 α* ^{Δ IE} mice (Figure 5B, and data not shown). In addition, increases in DMT-1 and DcytB following long-term low-iron treatment were HIF-2 α dependent (Supplemental Figure 3A). However, FPN protein expression was induced following 8-week low-iron diet treatment in both wild-type and *Hif-2 α* ^{Δ IE} mice (Figure 5C). These data correlated well with hematological and serum iron parameters. The *Hif-2 α* ^{Δ IE} mice were highly susceptible to iron-deficiency-induced anemia. The most significant drop in iron levels, RBCs, hematocrit, and hemoglobin in *Hif-2 α* ^{Δ IE} mice were observed following a 1-month low-iron diet treatment (Figure 5D–E). The RBC and serum iron levels, as well as hematocrit and hemoglobin measurements stabilized following 2- and 3-month low-iron diet treatment. To further confirm the role of HIF-2 α in the biphasic regulation of FPN, mice with an intestine-specific disruption of ARNT were assessed. ARNT is an obligate heterodimer partner for HIF-1 α

and HIF-2 α , and therefore inactivation of ARNT ablates both HIF-1 α and HIF-2 α function³⁸. The intestine-specific ARNT knockout mice (*Arnt* ^{Δ IE}) were fed low-iron diet for 2-weeks and FPN protein expression was measured. Similar to that observed in the *Hif-2 α* ^{Δ IE} mice, inactivation of ARNT inhibited FPN induction in response to 2-week iron starvation (Figure 6A). In addition, The *Arnt* ^{Δ IE} mice demonstrated a similar response to the *Hif-2 α* ^{Δ IE} mice on long-term low-iron diet. FPN protein expression was induced following 8-week low-iron diet treatment of both wild-type and *Arnt* ^{Δ IE} mice (Figure 6B). The *Arnt* ^{Δ IE} mice were susceptible to iron deficiency-induced anemia, where the most significant drop in iron levels, RBCs, hematocrit, and hemoglobin in *Arnt* ^{Δ IE} mice was observed following a 1-month low-iron treatment (Figure 6C–D). This response was not due to a dysregulation of the hepcidin-FPN axis since liver hepcidin expression was significantly inhibited in both *Hif-2 α* ^{Δ IE} and *Arnt* ^{Δ IE} mice following acute and chronic low-iron diet treatment (Supplemental Figure 2A and data not shown). Together, these studies demonstrate that distinct pathways are activated that regulate intestinal FPN expression during acute and chronic iron-deficient states.

Discussion

Iron supplementation is the most widely used therapy for iron deficiency. However, a significant number of anemic patients are refractory to iron³⁹. Moreover, the main treatment for hemochromatosis is phlebotomy. Since several rounds of treatment are required, this is a slow-acting treatment option⁴⁰. Therefore, alternative treatment strategies are needed in iron refractory anemia and hemochromatosis. FPN provides an optimal target in iron-related disorders. Hemochromatosis and several other iron-related disorders are due to dysregulation of FPN expression^{41–43} and conditional disruption of FPN in the intestine demonstrates its central role in regulating systemic iron homeostasis⁸. Despite the importance of FPN in maintaining iron homeostasis, the mechanism for its induction in the intestine following an increase in systemic iron requirements was hitherto unclear. The present data demonstrates a novel role for HIF-2 α in iron homeostasis by directly regulating FPN during acute increases in systemic iron requirements. This conclusion is based on the following data: (i) Intestinal ablation of HIF-2 α leads to loss of the adaptive induction of intestinal FPN gene expression following 2-week low-iron diet treatment. (ii) *Vhl* ^{Δ IE} mice demonstrate an increase in FPN mRNA and protein expression. (iii) HIF-2 α activates the FPN1B regulatory region in two different intestinal epithelial cell lines. (iv) ChIP assays using chromatin isolated from the duodenum of *Vhl* ^{Δ IE} mice demonstrate direct binding of HIF-2 α to the promoter in vivo. (v) Lastly, long-term iron deprivation leads to an increase in intestinal FPN protein expression in wild-type and *Hif-2 α* ^{Δ IE} mice.

The present study provides a novel model for FPN regulation. Acute decreases in dietary iron activate intestinal HIF-2 α signaling, which stimulates an adaptive increase in apical iron transport through DMT-1 and DcytB and mobilizes iron into circulation through an increase in basolateral iron export via FPN. Moreover, the data in the present work suggests that the acute adaptive induction of FPN is due to the transcriptional increase of FPN mRNA. However, during chronic states of low-iron, FPN is activated independent of HIF-2 α signaling. FPN protein is highly induced independent of mRNA induction. This biphasic model of FPN regulation allows for rapid as well as sustained response to increase systemic iron requirements. Currently, the mechanism by which FPN is regulated following chronic iron starvation is unknown. However, measuring kidney erythropoietin levels in 2-week and 8-week low-iron treated mice suggest that both erythropoiesis or whole body hypoxia are not activated (Supplemental Figure 3B). Transgenic mice that overexpress hepcidin or mice that are disrupted for hepcidin demonstrate dramatic changes in intestinal FPN expression^{37,44}. Therefore, it is possible that effects of hepcidin on intestinal FPN are delayed or hepcidin does not directly regulate intestinal FPN expression. During the

preparation of this manuscript it was demonstrated that in Caco-2 cells and in duodenal segments hepcidin did not decrease FPN expression, but directly decreased DMT-1 protein expression⁴⁵. Therefore, it is possible that chronic changes in hepcidin could modulate intestinal FPN indirectly through changes in DMT-1 expression.

Hepcidin expression in the liver is significantly lower in the *Hif-2 α ^{ΔIE}* mice compared to *Hif-2 α ^{F/F}* mice on the control matched AIN93G diet supplemented with 350 ppm of iron (Supplemental Figure 2A). This data is consistent with a previous publication using an independent intestine-specific HIF-2 α knockout mouse line²³. However, a significant change in liver hepcidin was not observed in *Hif-2 α ^{ΔIE}* mice on the standard grain-based chow (Supplemental Figure 2B). This may be due to the source of iron, as the controlled match AIN93G diet contains iron from a ferric (Fe³⁺) source, whereas 5001 diet the iron is from a ferrous (Fe²⁺) source. Therefore, iron in the grain-based diet is significantly easier to absorb since it does not need to be reduced. Lower basal levels of hepcidin on control AIN93G diet correlated with a slight increase in basal FPN protein expression observed in *Hif-2 α ^{ΔIE}* mice compared to the *Hif-2 α ^{F/F}* mice following 8-weeks on the control AIN93G diet. This increase was not observed in *Hif-2 α ^{ΔIE}* mice on 2-weeks of control AIN93G diet. These data further provide evidence that the effect of hepcidin on intestinal FPN levels may be delayed, consistent with the biphasic regulation of FPN proposed in this study. Interestingly, the significant decrease in liver hepcidin levels in the *Hif-2 α ^{ΔIE}* mice, did not correlate to an increase liver iron staining, further demonstrating the importance of HIF-2 α in intestinal iron absorption (Supplemental Figure 2C).

The present work established that the FPN1B isoform is directly regulated by HIF-2 α . The FPN1B transcript encodes FPN with an identical open reading frame as FPN1A. FPN1B lacks the iron response element and is not repressed under iron-deficient conditions, and therefore it likely contributes to FPN protein expression in the duodenum of iron-deficient mice²⁵. In assessing the regulatory region, two HREs were identified and mutating their sequences inhibited FPN1B promoter induction following HIF-2 α overexpression. However, the HIF-2 α activity on the FPN1B promoter was not completely lost following mutation of the HREs, suggesting that other ancillary HRE-like sequences are involved in FPN1B promoter regulation. Interestingly, FPN1A mRNA is also increased after low-iron treatment in a HIF-2 α dependent manner. However, no consensus HRE was found within this promoter area. The current focus is to understand the precise mechanism by which FPN1A and FPN1B are induced by HIF-2 α and assess the contributions of FPN1A and FPN1B to total FPN expression.

In conclusion, FPN is a pivotal transporter in regulating iron homeostasis. The current study provides a unique and novel mechanism to control FPN expression independent of hepcidin signaling. These findings may lead to novel HIF-2 α -based therapies in hemochromatosis and iron deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANKRD37	ankyrin repeat domain 37
ARNT	aryl hydrocarbon nuclear translocator
ASB11	ankyrin repeat and SOCS box-containing 11
ChIP	chromatin immunoprecipitation
DcytB	duodenal cytochrome b
DMT-1	divalent metal transporter-1
FPN	ferroportin
GFP	green fluorescent protein
GGH	gamma glutamyl hydrolase
HIF	hypoxia inducible factor
HRE	HIF response element
ppm	parts per million
qPCR	quantitative RT-PCR
RBC	red blood cell
Slc38a1	solute carrier family 38, member 1
TFRC	transferring receptor
Trim27	tripartite motif-containing 27
Vhl	von Hippel-Lindau tumor suppressor protein

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Figure 1. HIF-2 α is critical in the regulation of iron responsive genes

(A) Global gene expression profiling was assessed in duodenal RNAs isolated from *Hif-2 α ^{ΔIE}* or *Hif-2 α ^{F/F}* mice receiving a low-iron diet for 2-weeks. (B) qPCR analysis of TFRC, (C) DMT-1 and DcytB and (D) selected genes from microarray results in duodenal epithelial cells from *Hif-2 α ^{ΔIE}* or *Hif-2 α ^{F/F}* mice receiving a low-iron diet for 2-weeks. Expression was normalized to β -actin and each bar represents the mean value $\pm$ S.D. (*)= $P < .05$.

Figure 2. HIF-2 α signaling activates FPN expression during iron deficiency

(A) qPCR analysis of FPN, FPN1A, and FPN1B in duodenal epithelial cells from *Hif-2 α ^{ΔIE}* or *Hif-2 α ^{F/F}* mice receiving a low-iron diet for 2-weeks. Expression was normalized to β -actin and each bar represents the mean value $\pm$ S.D. (*)= P <.05. (B and C) Western blot analysis of FPN and DcytB in membrane extracts from duodenal epithelial cells isolated from (B) male and (C) female *Hif-2 α ^{ΔIE}* or *Hif-2 α ^{F/F}* mice receiving a low-iron diet for 2-weeks. Expression was normalized to Coomassie stained gels.

Figure 3. Intestine-specific disruption of VHL increases FPN expression through a HIF-2 α -dependent mechanism

(A) qPCR measuring FPN, FPN1A, and FPN1B expression in duodenal epithelium from *Vhl*^{ΔIE} or *Vhl*^{F/F} mice. Expression was normalized to β -actin and each bar represents the mean value $\pm$ S.D. (*)= P <.05. (B) Western blot analysis measuring FPN and DcytB expression in duodenal epithelium membrane extracts from *Vhl*^{ΔIE} or *Vhl*^{F/F} mice. Expression was normalized to Coomassie stained gels (C) qPCR measuring FPN, FPN1A, and FPN1B expression in duodenal epithelium from *Vhl*^{ΔIE}/*Hif-2α*^{ΔIE} double knockout mice or littermate controls. Expression was normalized to β -actin and each bar represents the mean value $\pm$ S.D. (*)= P <.05. (D and E) Western analysis measuring FPN expression in duodenal epithelium membrane extracts from (D) *Vhl*^{ΔIE}/*Hif-2α*^{ΔIE} double knockout mice or (E) *Vhl*^{ΔIE}/*Hif-1α*^{ΔIE} double knockout mice and littermate controls. Expression was normalized to Coomassie stained gels

Figure 4. HIF-2 α directly binds to the endogenous promoter of FPN

(A) Schematic diagram of FPN1B promoter illustrating the HREs in the regulatory region. The upstream regions are numbered in relation to the translation initiation site. The black arrowheads correspond to primers used for ChIP analysis. (B and C) Luciferase-reporter constructs under the control of the regulatory region of the mouse (B) FPN1B (C) or the FPN1B promoter with mutated HREs. Caco-2 and HCT116 cells were transiently transfected with the luciferase construct, and co-transfected with empty vector or HIF-2 α expression plasmids. Standard dual luciferase assays were performed on cell extracts as described in the Materials and Methods. Each bar represents the mean value $\pm$ S.D. (*)= $P < .05$. (D and E) In vivo ChIP assays on duodenal extracts from *Vhl^{F/F}* and *Vhl^{ΔIE}* mice using primers amplifying HREs of the FPN1B and DMT-1 promoter and detected by (D) ethidium stained agarose gel or (E) quantitated by qPCR and the data is expressed as fold enrichment over control IgG and normalized to input. Each bar represents the mean value $\pm$ S.D. (*)= $P < .05$.

Figure 5. Long-term low-iron diet treatment increases FPN expression independent of HIF-2 α
 (A) Intestine-derived Caco-2 and HCT116 and the hepcidin-responsive HeLa and HEK293 cells were transfected with a mammalian expression construct for human FPN tagged with green fluorescent protein (FPN-GFP). Following transfection the cells were treated with 1 $\mu\text{g/mL}$ of hepcidin for 8 hours and FPN-GFP was detected by Western blot analysis. Expression was normalized to GAPDH. (B) qPCR analysis measuring FPN mRNA expression in duodenal epithelial cells in *Hif-2 $\alpha^{\Delta IE}$* or *Hif-2 $\alpha^{F/F}$* mice receiving low-iron diet for 8 weeks. Expression was normalized to β -actin and each bar represents the mean value $\pm$ S.D. (*)= $P < .05$. (C) Western blot analysis measuring FPN expression from duodenal epithelial cells in *Hif-2 $\alpha^{\Delta IE}$* or *Hif-2 $\alpha^{F/F}$* mice receiving low-iron diet for 8 weeks. (D) Serum iron analysis and red blood cell (RBC) count from *Hif-2 $\alpha^{\Delta IE}$* or *Hif-2 $\alpha^{F/F}$* mice receiving low-iron diet for 1, 2 or 3 months. (E) hematocrit and hemoglobin analysis from

Hif-2 $\alpha^{\Delta E}$ or *Hif-2 $\alpha^{F/F}$* mice receiving low-iron diet for 1, 2 or 3 months. Each time point represents the mean value $\pm$ S.D. (*)= $P<.05$.

Figure 6. Low-iron diet treatment increases FPN expression by an Arnt-dependent and independent mechanism

Western blot analysis measuring FPN expression from duodenal epithelial cells in *Arnt*^{ΔIE} or *Arnt*^{F/F} mice receiving low-iron diet for (A) 2-weeks or (B) 8-weeks. (C) Serum iron analysis and red blood cell (RBC) count from *Arnt*^{ΔIE} or *Arnt*^{F/F} mice receiving low-iron diet for 1, 2, or 3 months. (E) hematocrit and hemoglobin analysis from *Arnt*^{ΔIE} or *Arnt*^{F/F} mice receiving low-iron diet for 1, 2, or 3 months. Each time point represents the mean value $\pm$ S.D. (*)= $P < .05$.