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Iron-related disorders are among the most prevalent diseases worldwide. Systemic iron homeostasis requires hepcidin, a hepatic-derived hormone that controls iron mobilization through its molecular target, ferroportin (FPN), the only known mammalian iron exporter. This pathway is perturbed in diseases that cause iron overload. Additionally, intestinal HIF-2α is essential for the local absorptive response to systemic iron deficiency and iron overload. Our data demonstrate a hetero-tissue crosstalk mechanism, where liver hepcidin regulated intestinal HIF-2α in iron deficiency, anemia, and iron overload. We show that FPN controlled cell autonomous iron efflux to regulate the activity of iron-dependent, intestinal prolyl hydroxylase domain enzymes to stabilize HIF-2α. Pharmacological blockade of HIF-2α using a clinically relevant and highly specific inhibitor successfully treated iron overload in a mouse model. These findings demonstrate a molecular link between liver hepcidin and intestinal HIF-2α that controls physiological iron uptake and drives iron hyperabsorption during iron overload.

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Hepatic Hepcidin/Intestinal HIF-2α Axis Maintains Iron Absorption During Iron Deficiency and Overload

Andrew J. Schwartz¹, Nupur K. Das¹, Sadeesh K. Ramakrishnan¹, Chesta Jain¹, Mladen T. Jurkovic¹, Jun Wu¹,², Elizabeta Nemeth³, Samira Lakhal-Littleton⁴, Justin A. Colacino⁵,⁶, and Yatrik M. Shah¹,⁷,*

¹Department of Molecular & Integrative Physiology, ⁵Environmental Health Sciences, ⁶Nutritional Sciences, ²Life Sciences Institute, ⁷Division of Gastroenterology, University of Michigan, Ann Arbor, MI, 48109.
³Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA, 90095.
⁴Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom

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* Correspondence author: Tel: +1 734 6150567; Fax: +1 734 9368813; Email: shahy@umich.edu
Abstract

Iron-related disorders are among the most prevalent diseases worldwide. Systemic iron homeostasis requires hepcidin, a hepatic-derived hormone that controls iron mobilization through its molecular target, ferroportin (FPN), the only known mammalian iron exporter. This pathway is perturbed in diseases that cause iron overload. Additionally, intestinal HIF-2α is essential for the local absorptive response to systemic iron deficiency and iron overload. Our data demonstrate a hetero-tissue crosstalk mechanism, where liver hepcidin regulated intestinal HIF-2α in iron deficiency, anemia, and iron overload. We show that FPN controlled cell autonomous iron efflux to regulate the activity of iron-dependent, intestinal prolyl hydroxylase domain enzymes to stabilize HIF-2α. Pharmacological blockade of HIF-2α using a clinically relevant and highly specific inhibitor successfully treated iron overload in a mouse model. These findings demonstrate a molecular link between liver hepcidin and intestinal HIF-2α that controls physiological iron uptake and drives iron hyperabsorption during iron overload.
Introduction

Over one billion people worldwide are affected by iron overload, iron deficiency, and states of malnutrition that perturb iron homeostasis (1). Diseases of iron overload are among the most common genetic disorders in humans (2). The morbidity and mortality in patients with genetic iron overload is a result of the pathological hyperabsorption of dietary iron, which leads to systemic iron accumulation and iron-induced oxidant damage (3). The master regulator of systemic iron metabolism in mammals is hepcidin, a highly conserved peptide hormone that is predominately synthesized and secreted by the liver. The function of hepcidin is to bind to the only mammalian iron exporter, ferroportin (FPN), resulting in FPN occlusion, internalization from the plasma membrane, and intracellular degradation (4). In states of normal systemic iron and oxygen, hepcidin is abundantly produced, FPN is degraded, and iron mobilization into plasma is limited. Conversely, during iron demand or systemic hypoxia, hepcidin production is repressed to enable FPN stabilization and iron mobilization into circulation (5). FPN is predominately expressed and regulated in tissues that maintain systemic iron homeostasis, namely intestine, liver, and macrophages of the reticuloendothelial system (6). The hepcidin-FPN interaction is the essential mechanism by which physiological iron homeostasis is maintained. Genetic mutations that disrupt the hepcidin-FPN axis give rise to all known forms of iron overload in mammals, referred to as hereditary hemochromatosis (2, 3, 5, 6). These data have fueled much research over the last decade on the molecular mechanisms that regulate liver hepcidin production in order to gain insight into how systemic iron homeostasis is maintained.

In addition to hepcidin, local intestinal regulation of iron handling plays an essential role in the maintenance of systemic iron homeostasis. Hypoxia inducible factor-2α (HIF-2α) is also sensitive to cellular iron and oxygen levels as the master intestinal transcriptional regulator of apical and basolateral iron transporters and is essential to maintain postnatal systemic iron levels (7, 8). HIF-2α is necessary and sufficient to mediate the adaptive increase in iron
absorption during both systemic iron deficiency and erythropoietic demand under systemic hypoxia through direct transcriptional activation of iron absorptive machinery (9-11). HIF-2α also controls the hyperabsorption of dietary iron that leads to systemic iron accumulation in diseases of iron overload, such as β-thalassemia and sickle cell disease (12-14). However, the precise molecular cues that initiate and maintain intestinal HIF-2α during normal physiology and in disease are poorly understood. Moreover, it is unclear if there is a concerted molecular integration of the systemic hepcidin pathway to local intestinal HIF-2α signaling in the regulation of iron homeostasis.

This paper establishes that intestinal HIF-2α signaling is regulated by liver hepcidin dynamics. Through temporal in vivo and in vitro models of hepcidin and FPN modulation, this work demonstrates that the liver hepcidin and intestinal HIF-2α crosstalk is essential during iron overload, systemic iron deficiency, and anemia. Through an unbiased whole genome RNA-seq analysis it was demonstrated that the canonical HIF-2α transcriptional response in the intestine is mediated by liver hepcidin. Mechanistically, the hepcidin-FPN axis controls HIF-2α in a cell autonomous fashion by limiting the activity of iron-dependent prolyl hydroxylase domain (PHD) enzymes. A pharmacological inhibitor of HIF-2α that is in clinical Phase 2 trials for clear cell renal cell carcinoma (NCT03108066) demonstrated reversal of iron overload in a mouse model.
Results

Inducible Deletion of Liver Hepcidin Leads to Activation of Intestinal HIF-2α and Rapid Iron Accumulation.

To understand the molecular connection between liver hepcidin and intestinal HIF-2α, mice that express a tamoxifen inducible CreERT2 fusion protein under the control of the serum albumin promoter (Alb) were crossed to hepcidin 1 (Hamp) floxed mice (AlbCreERT2;Hampfl/fl), giving rise upon tamoxifen administration to mice null for liver hepcidin (HampΔLiv) (Figure 1A). This inducible model allows for temporal, in vivo investigation of hepcidin action on HIF-2α without confounding effects that arise in later stages of hepcidin-deficiency iron overload, namely accumulation of reactive oxidant species (15). Moreover, hepatocyte-specific deletion of hepcidin leaves intact the sources of hepcidin that exist outside of the liver, such as in the heart where cell autonomous regulation of cardiac iron homeostasis has recently been shown to exist (16). In this model, the hepcidin transcript (Hamp) was significantly decreased in livers as early as two weeks following tamoxifen treatment (Figure 1B). Interestingly, liver expression of the serum iron uptake receptor, transferrin receptor (Tfrc), was significantly decreased at 4 weeks, while the FPN transcript (Fpn) was increased at 2 weeks (Supplemental Figure 1A). Prussian blue stain revealed progressive liver iron overload. Liver iron accumulation at 2 weeks was minimal, but a time dependent increase was detected at 4 and 12 weeks. Histological analysis revealed minimal morphological differences across all time points (Figure 1C). Iron assay revealed a significant increase in serum iron by as quickly as 2 weeks, with no further increase at 4 and 12 weeks, suggesting that serum iron is rapidly saturated following disruption to hepcidin (Figure 1D). A major complication for patients with hemochromatosis is cardiac dysfunction (17). Prussian blue staining did not detect heart iron accumulation at 2 and 4 weeks following hepcidin deletion, but significant iron accumulation and disruptions in cellularity and tissue architecture were observed by 12 weeks (Supplemental Figure 1B). Similar tissue iron...
loading was observed in the heart and the pancreas (Figure 1, E and F, and Supplemental Figure 1B). In order to assess the regulation of intestinal HIF-2α by liver hepcidin prior to confounding effects that are associated with iron overload, immunohistochemistry analysis was performed on duodenal sections two weeks following tamoxifen treatment. A robust increase in HIF-2α protein expression was observed (Figure 1G). Consistent with this data, activation of iron-absorptive HIF-2α-specific target genes and proteins were also observed (Duodenal cytochrome b (Dcytb; gene name Cybrd1), divalent metal transporter-1 (Dmt1; gene name Slc11a2), Fpn, and Ankrd37), as well as the expression of duodenal Tfrc, an indicator of low cellular iron status (Figure 1, H and I) (10). There was no change in the expression of HIF-1α-target genes (Supplemental Figure 1C) or HIF-2α inflammatory target genes (Supplemental Figure 1D) (18, 19). Furthermore, no change in HIF-2α-regulated transcripts was observed in the kidney and the spleen, while Tfrc expression was decreased in both organs, suggesting that the hepcidin-HIF-2α axis was specific to the intestine (Supplemental Figure 2, A and B). These data suggest changes to hepcidin regulate HIF-2α stability and activity in physiology and disease.

**FPN is Necessary for the Activation of Intestinal HIF-2α During Systemic Iron Deficiency.**

To address the molecular mechanism by which liver hepcidin regulated intestinal HIF-2α, we investigated the intestinal iron exporter and only target of hepcidin, FPN, in a context of systemic iron demand. Fpn-floxed mice were bred to mice that express a tamoxifen-inducible CreERT2 fusion protein under the control of the Villin (Vil) promoter (VilCreERT2;Fpnfl/fl), giving rise upon tamoxifen administration to mice null for FPN in the intestinal epithelium (FpnΔIE). Fpnfl/fl and VilCreERT2;Fpnfl/fl mice were placed on 350 PPM (iron-replete) or < 5 PPM (low-iron) iron diets for one week, injected with tamoxifen for three consecutive days, and sacrificed after an additional week on the respective diets (Figure 2A). This model mimics hepcidin excess at the
intestinal level and leads to iron retention in intestinal epithelial cells, despite a state of systemic iron demand (Figure 2B). Duodenal Fpn was significantly decreased following tamoxifen treatment (Figure 2C) and duodenal iron retention was detected by Western blot for the intracellular iron storage protein, ferritin (FTH1) (Figure 2D). Hepcidin transcript (Hamp) was significantly decreased in mice on the low-iron diet and in FpnΔIE mice (Figure 2E). This time point did not induce anemia as red blood cell numbers (RBC), hemoglobin counts (HB), hematocrit (HCT), mean corpuscular hemoglobin (MCH), and mean corpuscular volume (MCV) were all unchanged across cohorts (Figure 2F and Supplemental Figure 3A). As complete blood count parameters were unaffected, this model dissociated the enterocyte cellular response to iron deficiency from hypoxia. As expected, duodenal HIF-2α was stabilized and HIF-2α-specific iron absorptive genes were increased in Fpnfl/fl mice on a low-iron diet. These responses were abrogated in FpnΔIE mice (Figure 2, G and H). There were also no changes observed in the expression of HIF-1α target genes or HIF-2α inflammatory target genes (Supplemental Figure 3, B and C). These data demonstrate that the intestinal HIF-2α response to systemic iron demand is downstream of the hepcidin-FPN axis.

**FPN is Essential for the Intestinal HIF-2α Response to Erythropoietic Demand**

Intestinal HIF-2α is critical for the adaptive increase in iron absorption that enables efficient erythropoiesis (9, 10). This has been postulated to be regulated by changes in intestinal epithelial oxygen levels. A phenylhydrazine (Phz) hemolytic anemia model was utilized, which stimulates massive erythropoiesis. Fpnfl/fl and VilCreERT2;Fpnfl/fl mice were injected with tamoxifen and allowed to recover for 1 week. Two consecutive injections of either vehicle or Phz were administered and mice were sacrificed 48 hours later (Figure 3A). Erythropoietin transcript (Epo) was significantly elevated in kidneys of Phz treated mice, indicating a state of erythropoietic drive and systemic hypoxia (Figure 3B). Hepcidin transcript (Hamp) was significantly decreased
in Phz treated mice and in $Fpn^{ΔIE}$ mice treated with vehicle (Figure 3C). Duodenal ferritin abundance was decreased following Phz treatment in $Fpn^{fl/fl}$ mice, indicating mobilization of intestinal iron, while this response was blunted in $Fpn^{ΔIE}$ mice (Figure 3D). Interestingly and consistent with the low-iron response, activation of intestinal HIF-2α and HIF-2α-specific iron absorptive genes during stress erythropoiesis were completely dependent on intact intestinal FPN (Figure 3, E and F). There was also no observed change in the expression of HIF-1α target genes or HIF-2α inflammatory target genes (Supplemental Figure 4, A and B). These data demonstrate that the hepcidin-FPN axis is essential for the response of intestinal HIF-2α to low systemic oxygen levels.

**Intestinal Epithelial Iron Regulates the HIF-2α Response to Changes in Systemic Iron and Oxygen**

To clearly demonstrate that enterocyte iron flux was the major mechanism by which the hepcidin-FPN axis regulated HIF-2α, we sought to modulate luminal versus enterocyte iron levels. To modulate luminal enterocyte levels, $Dmt1$-floxed mice were bred to mice that express a tamoxifen-inducible Cre$^{ERT2}$ fusion protein under the control of the Villin (Vil) promoter ($Vil^{CreERT2};Dmt1^{fl/fl}$), giving rise upon tamoxifen administration to mice null for DMT1 in the intestinal epithelium ($Dmt1^{ΔIE}$). Enterocyte iron levels were modulated using $Fpn^{ΔIE}$ animals explained above. Long-term disruption of DMT1 or FPN gives rise to a state of systemic iron-deficiency anemia, with differences only in the compartment of iron trapping (i.e. luminal vs. enterocyte iron retention) (Figure 4A). $Vil^{CreERT2};Fpn^{fl/fl}$, $Vil^{CreERT2};DMT1^{fl/fl}$ and littermate controls were assessed 3 months following tamoxifen treatment. $Fpn$ and $Dmt1$ transcripts were significantly decreased in $Fpn^{ΔIE}$ and $Dmt1^{ΔIE}$ mice, respectively (Figure 4B). The $Fpn$ transcript was significantly elevated in the $Dmt1^{ΔIE}$ mouse, while there was no change in the $Dmt1$ transcript in $Fpn^{ΔIE}$ mice. As expected, the hepcidin transcript ($Hamp$) was potently repressed in the $Fpn^{ΔIE}$
and $DMT1^{\Delta IE}$ cohorts, as compared to $Fpn^{\text{fl/fl}}$ and $Dmt1^{\text{fl/fl}}$, respectively (Figure 4C). Furthermore, decreased RBC, HB, HCT, MCH, and MCV were observed in the $Fpn^{\Delta IE}$ and $Dmt1^{\Delta IE}$ mice compared to littermate controls, which indicated a state of systemic iron deficiency anemia (Figure 4D and Supplemental Figure 5A). Duodenal ferritin abundance was decreased in $Dmt1^{\Delta IE}$ mice, with the opposite response observed in $Fpn^{\Delta IE}$ mice (Supplemental Figure 5B). Immunohistochemistry revealed significant stabilization of the HIF-2$\alpha$ protein in $Dmt1^{\Delta IE}$ mice, with no change in $Fpn^{\Delta IE}$ mice (Figure 4E). Moreover, expression of the HIF-2$\alpha$-specific iron genes, $Dcytb$ and $Ankrd37$, were significantly elevated in the $Dmt1^{\Delta IE}$ mice, but not in $Fpn^{\Delta IE}$ mice (Figure 4F). The expression of $Tfrc$ was unchanged in $Fpn^{\Delta IE}$ and significantly increased in $Dmt1^{\Delta IE}$ mice compared to their littermate controls (Figure 4F). These data convincingly demonstrate that intestinal epithelial iron levels regulate HIF-2$\alpha$ during contexts of systemic iron and oxygen deficiency and during iron-deficiency anemia.

The Intestinal Transcriptome During Systemic Iron Demand Matches The Intestinal Response to Hepcidin Deficiency

The data thus far suggested the entire intestinal HIF-2$\alpha$ response to systemic iron and erythropoietic demand was controlled by liver hepcidin. However, the outputs for these experiments relied on the measurement of canonical HIF-2$\alpha$ target genes involved in intestinal iron handling. An unbiased, high-throughput RNA-sequencing (RNA-seq) approach was utilized to compare the duodenal transcriptome during systemic iron demand to that of hepcidin-deficiency iron overload (Supplemental Table 2). $Hamp^{\text{fl/fl}}$ and $Alb^{\text{CreERT2}};Hamp^{\text{fl/fl}}$ mice were placed on iron-replete and low-iron diets and were sacrificed two weeks following tamoxifen treatment (Figure 5A). The hepcidin transcript was significantly decreased in $Hamp^{\text{fl/fl}}$ mice on a low-iron diet and both $Hamp^{\Delta Liv}$ cohorts (Figure 5B). To assess the most significantly changed transcripts when comparing genotype/diet interactions, samples were clustered hierarchically in
an unsupervised manner based on expression of genes that were differentially expressed between conditions at a high-stringency false discovery rate (FDR) of < 0.01. Iron-replete \( \text{Hamp}^{\text{fl/fl}} \) samples clustered separately from the iron-replete \( \text{Hamp}^{\text{ALv}} \), low-iron \( \text{Hamp}^{\text{fl/fl}} \), and low-iron \( \text{Hamp}^{\text{ALv}} \) samples (Figure 5C). This demonstrated in an unbiased fashion that the intestinal transcriptomes during systemic iron demand and iron overload were statistically similar to one another because the nine treatment samples did not segregate into discrete, experimental clusters. A heatmap was then generated plotting scaled gene expression of the same differentially expressed genes to assess the identity of the genes used for unsupervised hierarchical clustering (Figure 5D). Importantly, the canonical HIF-2\( \alpha \) iron-regulated genes were identified (i.e. \( \text{Slc11a2} \), \( \text{Cybrd1} \), and \( \text{Ankrd37} \)). In order to identify novel transcripts in the RNA-seq dataset, a lower stringency differential expression analysis was performed (FDR < 0.1). Using this approach, genes that were exclusively regulated by iron deficiency (e.g. \( \text{Nos2} \), \( \text{Ccl20} \), and \( \text{Serpine1} \)), hepcidin deficiency (e.g. \( \text{Wdr72} \), \( \text{A4gn7} \), and \( \text{Gkn3} \)), and novel targets regulated in both contexts (e.g. \( \text{Mir7082} \), \( \text{Slc34a2} \), and \( \text{Itpr1} \)) were identified (Figure 5E). Collectively, these data demonstrate that the most robustly changed intestinal transcripts during systemic iron demand resemble those in primary hepcidin deficiency iron overload.

**FPN Activates HIF-2\( \alpha \) in a Cell Autonomous Manner that is Dependent on Intracellular Iron Efflux**

To interrogate the molecular mechanism of HIF-2\( \alpha \) stabilization downstream of the hepcidin-FPN axis, an in vitro system was utilized that models the cellular response to low levels of systemic hepcidin. Most cell lines express very low levels of the FPN protein and some cell lines appear to be resistant to hepcidin-mediated FPN degradation (10). Hepcidin-sensitive doxycycline-inducible human FPN\(^{\text{GFP}} \) HEK293 cells were assessed, as described previously (20). Upon doxycycline treatment, robust FPN\(^{\text{GFP}} \) expression was detected by Western blot
(Figure 6A). This mimicked a cellular environment of low systemic hepcidin, comparable to the intestine as the fold induction of FPN protein in the FPN\textsuperscript{GFP} HEK293 cells was similar to the fold induction observed in duodens of Hamp\textsuperscript{ALv} mice (Supplemental Figure 6, A and B). To determine if the regulation of HIF-2\(\alpha\) by hepcidin-FPN is cell autonomous, FPN\textsuperscript{GFP} cells were treated with doxycycline for 24 hours and cytosolic and nuclear fractions were generated. Cells were also treated with FG4592, a 2-oxoglutarate analog and chemical inhibitor of the prolyl hydroxylase domain-containing (PHD) enzymes that regulate HIF, serving as positive control. HIF-2\(\alpha\) was robustly stabilized in the nucleus of FPN\textsuperscript{GFP} cells (Figure 6B). Importantly, the HIF-2\(\alpha\) protein was stabilized to the same extent as FG4592 treatment, suggesting maximal activation. The HIF-2\(\alpha\) response was blunted by iron loading with ferric ammonium citrate (FAC) and recombinant hepcidin treatment, indicating that the activity of HIF-2\(\alpha\) in the FPN\textsuperscript{GFP} cells was dependent on intracellular iron concentration, downstream of hepcidin-mediated FPN degradation (Figure 6C). Together, this data demonstrates cell autonomous activation of HIF-2\(\alpha\) by iron efflux. This mechanism shows some differences as compared to HIF activation by intracellular iron chelation using compounds such as deferoxamine (DFO), which disrupts mitochondrial function and results in significant cell death (Supplemental Figure 6C) (21-23).

The protein stability of HIF-2\(\alpha\) is regulated by PHD enzymes. PHD enzymes were downstream of hepcidin-FPN in the regulation of HIF-2\(\alpha\) as FG4592 restored the HIF-2\(\alpha\) response in FPN\textsuperscript{GFP} cells following FAC or recombinant hepcidin treatment (Figure 6D). PHD enzymes require both iron and oxygen for their function. In order to address if PHD enzyme activity was decreased following FPN stabilization, an adenoviral-based reporter construct to measure PHD enzyme activity was generated by fusing luciferase to a canonical PHD hydroxylation domain (PHD Reporter) (Figure 6E). A significant increase in luciferase activity was detected in FPN\textsuperscript{GFP} cells following doxycycline treatment, to the same extent as chemical inhibition of PHD enzymes by FG4592 treatment (Figure 6F). This response was rescued by loading with FAC and by
treatment with recombinant hepcidin. These data demonstrated that stabilization of FPN in contexts of low liver hepcidin led to cellular iron efflux, decreased PHD enzyme activity, and ultimately cell autonomous stabilization of HIF-2α. PHD enzymes regulate both HIF-2α and HIF-1α. However, HIF-1α protein was stabilized submaximally following FPN overexpression compared to treatment with FG4592, suggesting selectivity of the hepcidin-FPN axis for HIF-2α over HIF-1α (Supplemental Figure 6D). HIF-2α contains a 5’ UTR iron-responsive element (IRE) that is responsible for translational inhibition during decreases in intracellular iron (24).

Using a HIF-2α IRE luciferase construct, we demonstrated HIF-2α inhibition following FPN overexpression via doxycycline, with DFO and FAC as controls, suggesting a negative feedback mechanism on FPN-mediated activation of HIF-2α (Supplemental Figure 6E). There are two major pools of intracellular iron: i) labile “free” iron, and 2) iron bound by the intracellular iron storage protein, ferritin. The mobilization of ferritin bound iron requires the lysosomal degradation of ferritin via the rate-limiting cargo protein, nuclear receptor coactivator 4 (NCOA4) (25, 26). To address which pool of iron is limited for PHD enzymes by FPN, two unique NCOA4 knockout cell lines were generated and sequence verified in FPNGFP cells. FPN overexpression led to ferritin degradation in an NCOA4-dependent manner (Figure 6G). However, NCOA4 deletion did not prevent the decreased PHD enzyme activity following FPN overexpression (Supplemental Figure 6F). While doxycycline-inducible FPNGFP HEK293 cells have been widely used to interrogate hepcidin-FPN dynamics (20, 27, 28), we sought to interrogate the hepcidin-FPN-HIF-2α axis in an intestinal epithelial cell line. IEC-6 cells are a normal rat small intestinal cell line and a doxycycline-inducible human FPNGFP IEC-6 cell line was generated. FPNGFP IEC-6 cells demonstrated FPN stabilization after doxycycline treatment to similar levels observed in duodenums of HampΔLiv mice (Supplemental Figure 6B). Moreover, FPNGFP IEC-6 cells were highly sensitive to hepcidin (Figure 6H). Several HIF-2α antibodies that were tested did not detect a specific HIF-2α band by Western blot analysis in rat lysates (data not shown). An
ELISA approach with a HIF-2α antibody that could detect native recombinant HIF-2α was assessed. Robust HIF-2α stabilization was observed following FPN overexpression via doxycycline treatment and co-treatment of doxycycline and hepcidin completely rescued this response, with DFO serving as positive control (Figure 6I). Similar to what was observed in vivo, there was no increase in HIF-1α following FPN overexpression in the IEC-6 FPNGFP cells, while DFO treatment significantly increased HIF-1α, further suggesting a difference in the mechanism of action of iron efflux through FPN and iron chelation by DFO (Supplemental Figure 6G). The mechanism of HIF-2α activation by FPN overexpression in IEC-6 cells was the same as in HEK293 cells, as PHD enzyme activity was decreased by FPN overexpression in an iron and hepcidin-dependent manner (Figure 6J). Collectively, these data demonstrate that in the absence of hepcidin, stabilization of membrane FPN regulates HIF-2α in a cell autonomous manner through depleting the cellular labile iron pool and limiting the activity of PHD enzymes.

**Inhibition of HIF-2α with PT2385 Decreases Systemic Iron Accumulation in Hepcidin-Deficient Iron Overload.**

Current therapeutic approaches for patients with iron overload rely on iron chelators and phlebotomy, which lead to significant off target effects and causes fatigue. We sought to determine if the liver hepcidin-intestinal HIF-2α axis can be therapeutically targeted to treat iron overload. A HIF-2α-specific inhibitor was recently developed, PT2385. PT2385 binds to HIF-2α and prevents its heterodimerization with aryl hydrocarbon receptor nuclear translocator (ARNT), thus preventing the transcriptional activity of HIF-2α (29). PT2385 is currently in a Phase 2 clinical trial for the treatment of clear cell renal cell carcinoma (NCT03108066). Hampfl/fl and AlbCreERT2;Hampfl/fl mice were injected with tamoxifen; two weeks later, HampΔLiv cohorts were orally gavaged with vehicle or PT2385 daily for two weeks (Figure 7A). No change in body weight was observed during the treatment protocol (Supplemental Figure 7A). The hepcidin
transcript was decreased in both HampΔLiv cohorts (Figure 7B). Decreased intestinal iron absorption in mice with a genetic disruption of intestinal HIF-2α led to anemia (10). Thus, we sought to determine if prolonged treatment with PT2385 would lead to systemic anemia. Kidney erythropoietin (Epo) transcript was decreased in vehicle treated HampΔLiv mice, while this decrease was abrogated in PT2385 treated HampΔLiv mice (Figure 7B). There was a significant expansion of red blood cells and increased hemoglobin and hematocrit in vehicle treated HampΔLiv mice that was rescued in the PT2385 cohort (Figure 7C). MCV and MCH were unchanged between all groups (Supplemental Figure 7B). The membrane stabilization of HIF-2α iron absorptive targets, FPN, DMT1, and DCYTB, were elevated in HampΔLiv mice but were completely absent in PT2385 treated HampΔLiv mice (Figure 7D). Prussian blue staining for iron in the liver was decreased in the PT2385 treated HampΔLiv mice compared to vehicle treatment (Figure 7E). Additionally, quantitative iron assays revealed significant decreases in serum, liver, and pancreas iron content, with a trend in decrease of heart iron in the PT2385 treated HampΔLiv mice (Figure 7F). These data demonstrate HIF-2α as a pharmacological target downstream of the hepcidin-FPN axis in patients with iron overload (Figure 7G).
Discussion

Systemic iron homeostasis requires multiple organs working in concert to maintain cellular iron concentrations for metabolism and red blood cell levels for systemic oxygen transport. The last decade of research demonstrated that this system was centrally regulated by the liver-derived hormone hepcidin and required intestinal iron absorption for the maintenance of postnatal systemic iron levels. However, the complete biological link between the liver and the intestine during iron deficiency and in diseases of iron overload has remained unclear. The present work demonstrates that the liver controls the intestine through a liver hepcidin-intestinal HIF-2α axis that regulates physiological iron uptake during systemic iron deficiency and drives pathological iron absorption during iron overload caused by hepcidin deficiency. Paradoxically, we show using unbiased, high throughput RNA-seq that the intestinal response to systemic iron deficiency and hepcidin deficiency-mediated iron overload are largely the same. The physiological repression of hepcidin during iron demand, and the perturbation of hepcidin during genetic iron overload, directly triggers iron efflux through intestinal FPN to limit the activity of iron-dependent PHD enzymes. This stabilizes intestinal HIF-2α to activate genes that are necessary and sufficient for intestinal iron absorption. Interestingly, HIF-2α activation downstream of hepcidin was intestinal specific, as HIF-2α-dependent transcripts were unchanged in the kidney and the spleen following liver hepcidin deletion. Potential explanations include the presence of intestinal specific co-activators and/or genetic suppressors and enhancers. In context with data that HIF-2α is necessary for the adaptive increase in intestinal FPN during iron deficiency (10), the present data suggests a feedforward loop whereby intestinal FPN stabilization following a decrease in hepcidin activates HIF-2α to maintain the FPN transcript during systemic iron demand and in iron overload. Although the hepcidin-FPN-HIF-2α axis is the major trigger for the intestinal transcriptional response following iron demand, the discovery of smaller subsets of genes that are either regulated by systemic iron deficiency
or hepcidin deficiency indicates differences in the intestinal response to a decrease in dietary iron compared to iron hyperabsorption during iron overload. A recent report indicated that the HIF response can be modulated by microbiota-derived short-chain fatty acids, which could explain in part the difference between luminal and systemic cues to the intestine (30). More work needs to be done to understand the regulation of this small subset of genes.

Previous work showed a critical role for HIF-2α in the hyperabsorption of iron in primary and secondary hemochromatosis (12-14). However, the field has relied largely on germline knockout strategies to study hepcidin disruption, which gives rise to iron loading that begins during embryonic development and can cause reactive oxygen species that are known inducers of HIF. Furthermore, full-body hepcidin deficiency disrupts sources of hepcidin outside of the liver, which have recently been shown to establish a cell-autonomous mechanism of local iron regulation, particularly in the heart, an organ critical for systemic oxygen transport (31). The use of our inducible model of liver hepcidin deletion has characterized for the first time the kinetics by which iron overload progresses. There is data to show that iron overload is toxic to red blood cell survival at later stages of hemoglobinopathies (12, 32), while our data demonstrates that there is a significant expansion of the red blood cell pool in early stages that occurs in a HIF-2α-dependent manner due to rescue with PT2385 treatment. Recent work has discovered erythroid-derived factors that regulate hepcidin to facilitate erythropoiesis (33-35). This present data conversely suggests that hepcidin restricts the red blood cell pool, potentially by limiting intestinal iron absorption or through the direct regulation of signaling downstream of FPN-mediated cellular iron efflux in other organs and cell types. Future work will need to determine the mechanism by which this red blood cell expansion occurs and whether it is a physiologically relevant process of iron storage during early iron overload.
The dioxygenase superfamily of PHD enzymes regulate the protein stability of both HIF-2α and HIF-1α. However, our lab, among others, has demonstrated that intestinal HIF-2α, but not HIF-1α, is stabilized, transcriptionally active, and necessary and sufficient for iron absorption during systemic iron demand (7, 8). Recent reports have shown that certain PHD isoforms exert selectivity for HIF-2α over HIF-1α, namely PHD3 (36). Furthermore, small intestine HIF-2α is more sensitive to pharmacological inhibition of all PHD isoforms, as compared to HIF-1α (37). This selectivity could explain the differential activation of small intestinal HIF-2α over HIF-1α downstream of hepcidin-FPN-PHDs. Future work will need to establish the Km of intestinal PHD enzymes for iron to determine if iron efflux through FPN limits the activity of a HIF-2α-specific PHD.

In addition to hepcidin and HIF-2α, another mammalian iron sensing axis exists via iron-regulatory protein (IRP)/iron-responsive element (IRE) machinery. This system modulates translation via the binding of IRPs with IREs that exist in the 5' or 3' untranslated region of target transcripts involved in cellular iron handling. Duodenal enterocytes produce a FPN transcript that evades IRP-mediated repression in contexts of low intracellular iron by lacking an IRE (38). This variant might function alongside HIF-2α-mediated transcriptional upregulation of Fpn to maintain the FPN protein following intestinal iron efflux. Interestingly, IRP1 is activated following decreases in intracellular iron to negatively regulate HIF-2α translation via action on an IRE in the 5'-UTR of the HIF-2α mRNA, which was shown in vitro (24) and in vivo (39-41). Recently, this pathway was shown to be pharmacologically targeted to treat HIF-2α-induced polycythemia (42). We also observed repression of the HIF-2α IRE in our in vitro model following FPN\textsuperscript{GFP} stabilization. Taken together, the hepcidin-FPN-PHD axis may control HIF-2α during systemic iron deficiency and IRP1-mediated repression of HIF-2α translation may limit the level of
activation. More work will need to be done to fully understand the interaction between the IRP/IRE system and the hepcidin-FPN-PHD-HIF-2α axis during systemic iron demand.

Iron chelators have been shown for decades to regulate HIF, although these molecules dramatically disrupt mitochondrial function and can strip iron from iron-containing proteins. These data are the first to show that cell autonomous, biologically relevant iron efflux regulates intestinal HIF-2α-mediated iron absorption in vivo, in contexts of both systemic iron deficiency and low systemic oxygen. The present work shows that the FPN-mediated efflux of iron is a cell autonomous trigger to stabilize HIF-2α. Moreover, this finding demonstrates in vivo that a liver-derived endocrine signal plays an essential role in the activity of intestinal enzymes that regulate HIF-2α. Numerous reports have recently begun to characterize the function of FPN in organs that do not play a role in maintaining systemic iron homeostasis (16, 43, 44). It will therefore be vital to determine if FPN-mediated iron efflux directly regulates iron dependent proteins and downstream signaling pathways in other cell types, either triggered by changes to liver hepcidin or other factors.

Patients with iron overload currently rely on iron chelators and/or phlebotomy to decrease systemic iron levels. However, these therapies exhibit suboptimal patient adherence because iron chelators have off-target effects and chronic phlebotomy can cause fatigue. A selective inhibitor of HIF-2α, PT2385, has recently been developed and is currently under Phase 2 clinical trials for patients with clear cell renal cell carcinoma (NCT03108066). Here, we sought to address whether oral administration of PT2385 could be utilized to blunt intestinal iron absorption for the treatment of iron overload. Our data demonstrated that as quickly as two weeks of treatment with PT2385 decreased systemic iron levels in mice with established iron overload. This finding provides an exciting impetus for the utilization of PT2385 in the treatment
of human diseases of iron overload, many of which are characterized by dysfunction of the hepcidin-FPN axis and intestinal iron hyperabsorption.

In conclusion, our work demonstrated that systemic iron deficiency and hepcidin-deficiency-mediated iron overload activated the same liver hepcidin-intestinal HIF-2α axis. Moreover, cellular iron efflux through the hepcidin target, FPN, regulated the activity of iron dependent enzymes and directly activated HIF-2α. Lastly, these data suggest that a therapeutic currently in development in humans should be repurposed for the treatment of patients with iron overload.
Methods

Animals and Treatments

For temporal, hepatocyte-specific disruption of hepcidin, mice floxed for Hamp1 (Hamp\(^{fl/fl}\)) on a C57BL/6J background were crossed with C57BL/6J mice harboring Cre\(^{ERT2}\) recombinase under the control of the serum albumin promoter (Alb\(^{CreERT2}\)) to generate Alb\(^{CreERT2}\);Hamp\(^{fl/fl}\) mice. Alv\(^{CreERT2}\);Fpn\(^{fl/fl}\) and Alv\(^{CreERT2}\);Dmt1\(^{fl/fl}\) mice are on a 129S4/SvJae background. Wild-type littermates were used as controls for all animal studies (Hamp\(^{fl/fl}\), Fpn\(^{fl/fl}\), and Dmt1\(^{fl/fl}\)) and analysis began on mice that were between 2 and 2.5 months of age for each of our respective experiments. Mice were injected with tamoxifen (Sigma-Aldrich, St. Louis, MO) at 100 mg/kg of body weight via i.p. for three consecutive days to ensure Cre-mediated recombination. Phenylhydrazine (Sigma-Aldrich, St. Louis, MO) was administered as described previously (9), at 60 mg/kg of body weight via i.p. injection. PT2385 (MedChemExpress, Monmouth Junction, NJ) was prepared and administered daily as described previously (45), at 20 mg/kg of body weight via oral gavage. All mice were fed ad libitum and kept in a 12 hour dark/light cycle. All mice were fed with standard chow (Research Diets, New Brunswick, NJ) unless indicated as being fed a purified AIN-93G iron-replete (350 PPM) or low-iron (< 5 PPM) diet (Dyets, Bethlehem, PA). All mice were housed at the Unit for Laboratory Animal Management (ULAM) at the University of Michigan.

Cell Culture

Stable doxycycline-inducible human FPN\(^{GFP}\) HEK293 cells were generated previously (20). To generate stable doxycycline-inducible human FPN\(^{GFP}\) IEC-6 cells, IEC-6 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). pLenti rtTA3 (Addgene, Cambridge, MA) and pLVX-Tight-Puro hFpnGFP plasmids (20) were prepared into lentivirus by the University of Michigan Vector Core, co-infected into IEC-6 cells, and selected with 10 µg/mL of blasticidin and 1 µg/mL of puromycin. Cells were maintained 37°C in 5% CO\(_2\) and 21% O\(_2\).
Cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic. NCOA4 knockout cells were generated with the lentiCRISPR v2 construct (Addgene, Cambridge, MA) using two unique sgRNAs against NCOA4 (NCOA4 sg1 and NCOA4 sg2, respectively, Supplemental Table 1). Briefly, oligos were cloned into the lentiCRISPRv2 backbone. Empty vector, NCOA4 sg1, and NCOA4 sg2 constructs were prepared into lentivirus by the University of Michigan Vector Core. FPN\textsuperscript{GFP} cells were infected at 10 MOI and selected with 1 µg/mL of puromycin. Knockout cells were verified by sequencing. The PHD enzyme activity luciferase reporter was previously described (46). Briefly, FPN\textsuperscript{GFP} cells were infected at 10 MOI overnight and treated the next day with doxycycline at 250 ng/mL, 100 µM FG4592 (Selleckchem, Houston, TX), 200 µM ferric ammonium citrate (FAC) (Sigma-Aldrich, St. Louis, MO) and/or 1 mg/mL human recombinant hepcidin (Bachem, Bubendorf, Switzerland) or 200 µM DFO (Sigma-Aldrich, St. Louis, MO). The HIF-2α IRE luciferase construct was generated previously (24).

**Hematological and Iron Analysis**

The Unit for Laboratory Animal Medicine Pathology Core at The University of Michigan performed complete blood count analysis. Non-heme iron was quantified as described previously (12).

**Quantitative Reverse-Transcription PCR**

The mRNA was measured by real-time RT-PCR (Life Technologies, Carlsbad, CA). Primers are listed in Supplemental Table 1. Quantification cycle (Cq) values were normalized to β-actin and expressed as fold change.
Whole Genome RNA-sequencing and Analysis

RNA sequencing libraries were prepared using the TruSeq RNA library prep kit v2 (Illumina, San Diego, CA) following the manufacturer's recommended protocol. The libraries were sequenced using single-end 50-cycle reads on a HiSeq 2500 sequencer (Illumina) at the University of Michigan DNA Sequencing Core Facility. RNA-sequencing analysis was performed as described previously (18). Briefly, quality control of raw fastq files was performed using FastQC v 0.11.5. Fastq files were mapped to the mouse genome (mm10) using STAR-2.5.3.a using the options “outFilterMultimapNmax 10” and “sjdbScore 2”. Gene expression levels were quantified using Subread v1.5.2 package FeatureCounts. Differential expression testing was conducted with the Bioconductor package edgeR v3.16.5 using glmLRT. To reduce the dispersion of the dataset due to lowly expressed genes, genes with a mean aligned read count less than five across all samples were excluded from analysis. Genes were considered differentially expressed as indicated, either at a false discovery rate (FDR) of < 0.01 or < 0.1 to yield high and low stringency approaches. The sequence data is publically available through ArrayExpress (accession number E-MTAB-7329).

Western Blot

Whole-cell, nuclear, and membrane lysates were prepared as described before (10, 47). In brief, lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed overnight at 4°C with antibodies for: FPN (ADI #MTP11-A), DMT1 (ADI #NRAMP21-A), DCYTB (ADI #DCYTB-11A) (ADI, San Antonio, TX), and TFR1 (Invitrogen #13-6800, Carlsbad, CA) for mouse tissue lysates; FPN (Novus # NBP1-21502, Littleton, CO) FTH1 (Cell Signaling #3998S, Danvers, MA), GAPDH (#sc-47724), GFP (#sc-996) (Santa Cruz, Dallas, TX), Lamin A/C (Active Motif #3A6-4C11, Carlsbad, CA), HIF-2α (Bethyl #BL-95-1A2, Montgomery, TX),
and HIF-1α (Abcam #179483, Cambridge, United Kingdom) for human lysates, and Actin (Proteintech #60008-1, Chicago, IL) for mouse tissue and rat cell lysates.

**Enzyme-linked Immunosorbent Assay (ELISA)**

Protein lysates were coated onto high binding polystyrene microtiter plates overnight. Plates were washed in 1X PBS with 0.1% Tween-20 (PBST) and blocked with 5% BSA, incubated with primary antibodies for HIF-2α (Novus #AF2997, Littleton, CO) HIF-1α (Abcam #179483, Cambridge, United Kingdom), washed in PBST, incubated with appropriate HRP-conjugated secondary antibodies, developed, and read at 450 nm in a plate reader.

**Histology, Tissue Iron Staining, and Immunohistochemistry**

Histologic analysis was performed on H&E-stained formalin-fixed paraffin-embedded sections. Tissue iron detection was performed in formalin-fixed paraffin-embedded sections stained with Prussian blue. For immunohistochemistry analysis, frozen sections were probed with polyclonal rabbit anti- HIF-2α antibody (Novus #100-122, Littleton, CO), as previously described (47).

**Luciferase Assay**

Cells were lysed in reporter lysis buffer (Promega, Madison, WI), and firefly luciferase activity was measured as described previously (48).

**Statistics**

Results are expressed as mean ± SEM. Significance between two groups was tested using 2-tailed unpaired t test. Significance among multiple groups was tested using one-way or two-way ANOVA followed by Tukey’s post hoc for multiple comparisons. A p value less than 0.05 was
considered significant. Prism 7.0 software (GraphPad Software, La Jolla, CA) was used to conduct analyses.

**Study Approval**

All animal procedures were approved by the University of Michigan Institutional Animal Care and Utilization Committee (IACUC).

**Author Contributions**


**Acknowledgements**

This work was supported by grants from the NIH (R01CA148828 and R01DK095201 to Y.M.S.; F31DK116555 to A.J.S.; K99DK110537 to S.K.R.; R01DK107309 to E.N; R01DK107583 to J.W.; and R01ES028802 to J.A.C) and the American Diabetes Association (1-18-IBS-281 to J.W.) and the University of Michigan GI SPORE Molecular Pathology and Biosample Core (P50CA130810). We would also like to thank Dr. Matthias Hentze (Heidelberg University, Heidelberg, Germany) for kindly providing the HIF-2α IRE luciferase construct (24).

**Disclosures**

E.N. is a consultant and shareholder of Intrinsic LifeSciences and Silarus Therapeutics, and consultant for La Jolla Pharmaceutical Company, Keryx Pharmaceuticals and Protagonist Therapeutics.
References


Figure 1.

A) Schematic diagram showing the genetic modification:

- Serum Albumin
- CreER²
- Hamp1
- Exons 2 + 3

B) Bar graph showing relative expression over time:

- 0 Wk.
- 2 Wk.
- 4 Wk.
- 12 Wk.

C) Images showing Prussian Blue and H & E staining:

- 0 Wk.
- 2 Wk.
- 4 Wk.
- 12 Wk.

D) Bar graph showing serum iron levels:

- 0 Wk.
- 2 Wk.
- 4 Wk.
- 12 Wk.

E) Bar graph showing heart iron levels:

- 0 Wk.
- 2 Wk.
- 4 Wk.
- 12 Wk.

F) Bar graph showing pancreas iron levels:

- 0 Wk.
- 2 Wk.
- 4 Wk.
- 12 Wk.

G) Immunofluorescence images showing DAPI/HIF-2α:

- Hamp⁺/⁻⁻
- HampΔLiv

H) Western blots showing protein expression:

- FPN
- DMT1
- DCTYTB
- TFR1
- Ponceau

I) Bar graph showing relative expression:

- Dcytb
- Dmt1
- Fpn
- Ankrd37
- Tfrc

[Legend: ****: p < 0.0001; ***: p < 0.001; **: p < 0.01; *: p < 0.05; ns: not significant]
**Figure 1. Temporal disruption of liver hepcidin activates intestinal HIF-2α and leads to rapid iron accumulation.** (A) Schematic representation of mice with temporal disruption of hepatocyte hepcidin. (B) qPCR analysis for liver hepcidin (*Hamp*) transcript (n = 3 to 8 per group). (C) Representative Prussian blue iron stain and H&E analysis of livers from *Hamp*ΔLiv mice. Images, 20x (n = 3 per group). (D-F) Serum (D), heart (E), and pancreas iron content (F) (n = 3 to 14 per group). (G) Representative HIF-2α staining in duodenal sections two-weeks after tamoxifen injection in *Hamp*fl/fl and *Hamp*ΔLiv mice. Images, 20x (n = 3 per group). (H) Western blot analysis for FPN, DMT1, DCYTB, and TFR1 in duodenal membrane fractions (n = 2 to 3 per group). (I) qPCR analysis for duodenal HIF-2α-specific and iron-handling transcripts two-weeks after tamoxifen injection in *Hamp*fl/fl and *Hamp*ΔLiv mice (n = 5 to 8 per group). Mean ± SEM are plotted. Male samples are designated as squares and female samples are designated as circles. Significance determined using either one-way ANOVA with Tukey’s post hoc (B, D-F) or 2-tailed unpaired t test (I). *p < 0.05; ***p < 0.001; ****p < 0.0001 compared to *Hamp*fl/fl group.
Figure 2.

A. Iron-replete vs. Low-iron

B. DCYTB vs. DMT1

C. Fpn

D. Fpnnull vs. FpnaE

E. Hamp

F. RBC, HB, HCT

G. Fpnnull vs. FpnaE

H. Dcytb, Dmt1, Ankrd37, Tfrc
Figure 2. Intestinal epithelial FPN is necessary for the activation of intestinal HIF-2α during systemic iron deficiency. (A and B) Schematic representation of the experimental design (A) and of intestinal epithelial iron retention following ferroportin deletion (B). (C) qPCR analysis for duodenal ferroportin (Fpn) transcript (n = 4 to 7 per group). (D) Western blot analysis for duodenal ferritin (FTH1) (n = 3 per group). (E) qPCR analysis for liver hepcidin (Hamp) transcript (n = 4 to 7 per group). (F) Analysis of red blood cells (RBC), hemoglobin (HB), and hematocrit (HCT) (n = 4 to 7 per group). (G) Representative HIF-2α staining in duodenal sections. Images, 20x (n = 3 per group). (H) qPCR analysis for HIF-2α-specific and iron-handling transcripts in duodenal samples (n = 4 to 6 per group). Male samples are designated as squares and female samples are designated as circles. Mean ± SEM are plotted. Significance determined using two-way ANOVA with Tukey’s post hoc. ***p < 0.001; ****p < 0.0001 compared to iron-replete Fpn^fl/fl. # #p < 0.01; # ## #p < 0.0001 compared to low-iron Fpn^fl/fl. ^p < 0.05 compared to iron-replete Fpn^ΔIE.
Figure 3.

A) 

\[ \text{Vehicle} \quad \text{Phz} \]

\[ Fpn^{fl/fl} \, \text{vs.} \, \text{Vil}^{CreERT2};Fpn^{fl/fl} \]

\[ \text{Tamoxifen} \quad \text{Veh} \quad \text{Phz} \quad \text{Sacrifice} \]

Day -3 0 8 9 11

B) 

\[ \begin{align*}
\text{Epo} & \quad \text{****} \\
\text{Relative Expression} & \quad \text{Fpn}^{fl/fl} \quad \text{Fpn}^{ΔE} \\
\end{align*} \]

C) 

\[ \text{Hamp} \]

\[ \text{Relative Expression} \]

\[ \text{Fpn}^{fl/fl} \quad \text{Fpn}^{ΔE} \]

D) 

\[ \text{Fth1} \quad \text{Actin} \]

\[ \begin{array}{c}
\text{Veh} \quad \text{Phz} \\
\text{Veh} \quad \text{Phz} \\
\end{array} \]

E) 

\[ \text{Fpn}^{fl/fl} \quad \text{Fpn}^{ΔE} \]

\[ \text{Veh} \quad \text{Phz} \quad \text{Veh} \quad \text{Phz} \]

DAP/HIF-2α

F) 

\[ \text{Dcytb} \quad \text{Dmt1} \quad \text{Ankrd37} \quad \text{Tfrc} \]

\[ \text{Relative Expression} \]

\[ \text{Fpn}^{fl/fl} \quad \text{Fpn}^{ΔE} \]

\[ \text{Fpn}^{fl/fl} \quad \text{Fpn}^{ΔE} \]

\[ \text{Fpn}^{fl/fl} \quad \text{Fpn}^{ΔE} \]

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\[ \text{Fpn}^{fl/fl} \quad \text{Fpn}^{ΔE} \]

\[ \text{Fpn}^{fl/fl} \quad \text{Fpn}^{ΔE} \]

\[ \text{Fpn}^{fl/fl} \quad \text{Fpn}^{ΔE} \]
Figure 3. Deletion of intestinal epithelial FPN blocks the intestinal HIF-2α response to erythropoietic demand. (A) Experimental design for phenylhydrazine (Phz)-induced hemolytic anemia model. (B and C) qPCR analysis for kidney erythropoietin (Epo) (B) and liver hepcidin (Hamp) transcript (C) (n = 5 to 9 per group). (D) Western blot analysis for duodenal ferritin (FTH1) (n = 3 per group). (E) Representative HIF-2α staining in duodenal sections. Images, 20x (n = 3 per group). (F) qPCR analysis for HIF-2α-specific and iron handling transcripts in duodenal samples (n = 5 to 7 per group). Male samples are designated as squares and female samples are designated as circles. Mean ± SEM are plotted. Significance determined using two-way ANOVA with Tukey’s post hoc. *p < 0.05; **p < 0.01; ****p < 0.0001 compared to vehicle Fpn<sup>fl/fl</sup>. #p < 0.05; # # # p < 0.0001 compared to vehicle Fpn<sup>fl/fl</sup>.
Figure 4. The intestinal HIF-2α response to changes in systemic iron and oxygen is driven by epithelial iron levels. (A) Schematic of three-month, inducible iron trapping in animals lacking intestinal epithelial ferroportin (FpnΔIE) or DMT1 (Dmt1ΔIE). (B) qPCR analysis of the ferroportin (Fpn) and DMT1 (Dmt1) transcripts (n = 4 per group). (C) qPCR analysis of liver hepcidin (Hamp) transcript (n = 4 per group). (D) Analysis of red blood cells (RBC), hemoglobin (HB), and hematocrit (HCT) (n = 3 to 5 per group). (E) Representative HIF-2α staining in duodenal sections. Images, 20x (n = 3 per group). (F) qPCR analysis for HIF-2α-specific and iron handling transcripts in duodenal samples (n = 4 per group). Male samples are designated as squares and female samples are designated as circles. Mean ± SEM are plotted. Significance determined using 2-tailed unpaired t test. *p < 0.05; **p < 0.01; ****p < 0.0001 compared between Fpnfl/fl and FpnΔIE cohorts. #p < 0.05; # #p < 0.01; # # # #p < 0.0001 compared between Dmt1fl/fl and Dmt1ΔIE cohorts.
Figure 5.

A. \( \text{Hamp}^{+/+} \) vs. \( \text{Alb}^{CreERT2};\text{Hamp}^{-/-} \)

- Tamoxifen
- Day -3, 0, 14
- Iron-replete vs. Low-iron

B. Relative Expression

C. Tree diagram showing iron-replete vs. low-iron conditions for \( \text{Hamp}^{+/+} \) and \( \text{Hamp}^{ALy} \)

D. Heatmap showing expression levels of genes such as Stc11a2, Cybrd1, Ankrd37, Tfr2, etc., under iron-replete and low-iron conditions for \( \text{Hamp}^{+/+} \) and \( \text{Hamp}^{ALy} \)

E. Scatter plot showing log FC of low-iron vs. \( \text{Hamp}^{ALy} \) on the x-axis and log FC of Hamp on the y-axis, with genes such as Nos2, Col20, Serpine1, Caf9, etc., highlighted.
Figure 5. The intestinal transcriptome during systemic iron deficiency resembles that of hepcidin-deficiency-mediated iron overload. (A) Experimental design for samples used in whole genome RNA-sequencing. (B) qPCR analysis of liver hepcidin (Hamp) transcript on iron-replete (IR) and low-iron (LI) diets (n = 8 to 9 per group). (C) Dendrogram comparing genotype/diet interactions following unsupervised hierarchical clustering of genes differentially expressed at a high stringency false discovery rate (FDR) of < 0.01 (n = 3 per group). (D) Heatmap of genes used for unsupervised, hierarchical clustering (n = 3 per group). (E) Lower stringency differential expression analysis at a false discovery rate (FDR) of < 0.1 to uncover transcripts in the RNA-seq dataset unique to iron deficiency (iron def.) and hepcidin-deficiency (hepcidin def.). Genes in red text are novel intestinal transcripts regulated by both low-iron and hepcidin deficiency (n = 3 per group). Male samples are designated as squares and female samples are designated as circles. Mean ± SEM are plotted. Significance determined using two-way ANOVA with Tukey’s post hoc. ****p < 0.0001 compared iron-replete Hamp^{fl/fl}. 
Figure 6.

A. Dox - +
GFP
GAPDH

B. Cytosol Nuclear
V D FG V D FG
HIF-2α GFP GAPDH Lamin A/C

C. Cytosol Nuclear
V D D+F D+H D+F D+H
HIF-2α GFP GAPDH Lamin A/C

D. Cytosol Nuclear
V D D+F D+H D+F D+H
FG4592
HIF-2α GFP GAPDH Lamin A/C

E. Fe^{2+} PHD
O_2 P P Degradation
Hydroxyl Domain Luciferase

F. PHD Reporter Activity

H. Veh. Dox Dox + Hepcidin
FPN Actin

I. HIF-2α

J. PHD Reporter Activity
Figure 6. FPN activates HIF-2α in a cell autonomous manner dependent on efflux of cellular labile iron pool. (A) Western blot analysis in FPN<sup>GFP</sup> HEK293 cells following 24-hour doxycycline treatment. (B) Western blot analysis in cytosolic and nuclear fractions of FPN<sup>GFP</sup> HEK293 cells treated with vehicle (V), 250 ng/mL doxycycline (D), or 100 µM FG4592 (FG) for 24-hours. (C and D) Western blot analysis in cytosolic and nuclear fractions of FPN<sup>GFP</sup> HEK293 cells treated with vehicle (V), doxycycline (D), 200 µM ferric ammonium citrate (FAC) and doxycycline (D+F), or doxycycline and 1 mg/mL hepcidin (D+H) for 24-hours (C); Separate D+F and D+H conditions were also co-treated with FG4592 for 24-hours, as indicated (D). (E) Schematic of luciferase-based prolyl hydroxylase domain-containing (PHD) enzyme activity reporter. (F) Fold change in luciferase activity in FPN<sup>GFP</sup> HEK293 cells infected with the PHD reporter and treated with vehicle (V), doxycycline (D), FG4592 (FG), FAC and doxycycline (D+F), or doxycycline and hepcidin (D+H) for 24-hours. (G) Western blot analysis in FPN<sup>GFP</sup> HEK293 cells stable for empty lentiCRISPRv2 (Empty) or unique NCOA4 short guide RNAs (NCOA4 sg1 and NCOA4 sg2). Cells were treated with FAC for 24-hours and then treated with doxycycline for 24-hours. (H) Western blot analysis in FPN<sup>GFP</sup> IEC-6 cells treated with vehicle, doxycycline, or doxycycline and hepcidin for 24-hours. (I) ELISA in lysates from FPN<sup>GFP</sup> IEC-6 cells treated with vehicle (V), doxycycline (D), doxycycline and hepcidin (D+H), or deferoxamine (DFO) for 24-hours. (J) Fold change in luciferase activity in FPN<sup>GFP</sup> IEC-6 cells infected with the PHD reporter and treated with vehicle (V), doxycycline (D), FAC and doxycycline (D+F), or doxycycline and hepcidin (D+H) for 24-hours. All cell culture experiments were repeated at least three times. Mean ± SEM are plotted. Significance determined using one-way ANOVA with Tukey’s post hoc. **p < 0.01; ****p < 0.0001 compared to vehicle. # p < 0.05; ##p < 0.01; ### #p < 0.0001 compared to doxycycline.
Figure 7.

A. Alb<sup>CreERT2;Hamp<sup>fl/fl</sup></sup> Tamoxifen Veh vs. PT2385 Sacrifice

Day -3 0 14 28

B. Relative Expression

Hamp Epo

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D. Immunoblot

FPN
DMT1
DCYTB
Ponceau

E. Prussian Blue

Vehicle Vehicle PT

F. Serum Iron

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H. Heart Iron

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I. Pancreas Iron

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G. DCYTB
Fe<sup>3+</sup> Fe<sup>2+</sup>
HIF-2α
Ferroportin

Low liver hepcidin
Figure 7. Inhibition of HIF-2α using PT2385 reverses iron accumulation in multiple tissues in hepcidin-deficient hemochromatosis. (A) Experimental design for oral gavage of vehicle or PT2385 in HampΔLiv mice. (B) qPCR analysis for liver hepcidin (Hamp) and kidney erythropoietin (Epo) transcripts (n = 5 to 7 per group). (C) Analysis of red blood cells (RBC), hemoglobin (HB), and hematocrit (HCT) (n = 5 to 7 per group). (D) Western blot analysis for FPN, DMT1, DCYTB in duodenal membrane fractions (n = 3 per group). (E) Representative Prussian blue stain for iron in livers. Images, 20x (n = 3 per group). (F) Serum, liver, heart, and pancreas iron content (n = 5 to 7 per group). (H) Schematic representation of liver hepcidin-intestinal HIF-2α axis. Male samples are designated as squares and female samples are designated as circles. Mean ± SEM are plotted. Significance determined using one-way ANOVA with Tukey’s post hoc. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 compared to vehicle Hampfl/fl. #p < 0.05; # #p < 0.01; # # #p < 0.001 compared to vehicle HampΔLiv.