The role of hypoxia-inducible factors in the regulation of systemic iron homeostasis

by

Erik Ryan Anderson

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Doctoral Committee:

Assistant Professor Yatrik M. Shah, Chair
Professor Deborah L. Gumucio
Professor Bishr Omary
Assistant Professor Lei Yin
I dedicate this thesis to my family, lab, and mentor.
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alcoholic liver disease (ALD), acute myeloid leukemia (AML), aryl hydrocarbon receptor nuclear translocator (ARNT), bone morphogenetic protein (BMP), bone marrow transplantation (BMT), cyclin-dependent kinase (Cdk), CCAAT/enhancer binding protein alpha (C/EBPα), duodenal ferric reductase (DcytB), Dulbecco’s modified Eagle’s medium (DMEM), divalent metal transporter 1 (Dmt1), enzyme-linked immunosorbent assay (ELISA), erythropoietin (EPO), fetal bovine serum (FBS), feline leukemia virus subgroup C cellular receptor 1 (FLVCR1), ferroportin (Fpn1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), growth-differentiation factor 15 (GDF15), green fluorescent protein (GFP), hepcidin antimicrobial peptide (HAMP), hemoglobin (Hb), hematocrit (HCT), hereditary hemochromatosis (HH), hypoxia-inducible transcription factors (HIFs), hemoujuvelin (Hjv), heme oxygenase 1 (HO-1), interleukin-6 (IL-6), iron-responsive element (IRE), iron-refractory iron deficiency anemia (IRIDA), iron-regulatory protein (IRP), juvenile hemochromatosis (JH), Moloney murine leukemia virus (MMLV), multiplicity of infection (MOI), nitrotetrazolium blue (NBT), oxygen-dependent-degradation domain (ODD), prolyl hydroxylase domain enzymes (PHD), phenylhydrazine (PhZ), quantitative real-time reverse transcription-PCR (qPCR), red blood cells (RBCs), radio immune precipitation assay (RIPA), reverse transcriptase (RT), sodium-dodecyl sulfate polyacrylamide
gel electrophoresis (SDS-PAGE), solute carrier family 46 member 1 (Slc46a1), SMAD family member 4 (SMAD4), signal transducer and activator of transcription 3 (STAT3), six-transmembrane epithelial antigen of prostate (Steap), transferrin receptor 1 (Tfr1), transferrin receptor 2 (Tfr2), transforming growth factor beta 1 (TGF-β), transmembrane protease serine 6 (TMPRSS6), twisted gastrulation (TWSG1), upstream transcription factor 2 (USF2), untranslated region (UTR), von Hippel-Lindau tumor suppressor (VHL), zymosan-induced generalized inflammation (ZIGI), Zrt-and Irt-like protein 14 (Zip14)
Abstract

Disorders of iron homeostasis affect over a billion people worldwide. These disorders can be grouped according to iron deficiency, the major cause of anemia, and iron overload, the major cause of death in patients with hemochromatosis. The past decade has been termed, “The Golden Age in Iron Biology”, due to the significant increase in the understanding of the molecular underpinnings of systemic iron homeostasis. However, therapeutic options remain static. Anemia is managed with blood transfusions or iron supplementation, and hemochromatosis is treated with chelation and phlebotomy. These treatments are effective in many cases, but are often poorly tolerated and can take years to improve the quality of life for patients.

Hepcidin is an antimicrobial peptide produced in the liver and secreted into the bloodstream. Several seminal studies from the past decade have suggested that hepcidin is the master regulator of iron homeostasis. Hepcidin functions by binding to ferroportin-1 (Fpn1), the sole mammalian iron exporter, which induces its internalization and degradation. Fpn1 is highly expressed on the basolateral side of duodenal enterocytes, and also on macrophages. Thus, hepcidin acts to restrict iron availability by blocking intestinal iron absorption and release of iron from macrophages, which recycle iron from senescent erythrocytes. However, data from our lab demonstrate that oxygen signaling is a local effector that controls iron homeostasis both upstream and downstream of hepcidin.
The present work describes a novel signaling pathway by which intestinal iron absorption can be controlled by oxygen signaling. The hypoxia-inducible factor (HIF)-2α is a critical regulator of iron absorption during iron deficiency. In the intestine, HIF-2α directly activates divalent metal transporter 1 (Dmt1), duodenal ferric reductase (DcytB), and Fpn1, which are iron transporters critical for adaptive changes in iron absorption. The present work builds on this to show that during increased iron demands from erythropoiesis, iron absorption is activated in a HIF-2α-dependent manner, and loss of intestinal HIF-2α inhibits hematopoietic recovery. In addition, β-thalassemia is a genetic disorder characterized by ineffective erythropoiesis causing severe anemia and iron overload. Using a mouse model of the disease, this study shows that loss of intestinal HIF-2α prevents iron overload. Together, these data demonstrate that intestinal HIF-2α activity is essential for the adaptive changes in iron absorption and could be targeted therapeutically to treat anemia and iron overload.

In addition to the critical role of intestinal HIF-2α in iron homeostasis, liver HIF signaling is also a critical regulator of iron homeostasis. Hepcidin is regulated by multiple signaling pathways, and one of the least understood regulators of hepcidin expression is hypoxia. The present work demonstrates that hypoxic repression of hepcidin requires HIFs and is mediated by a decrease in CCAAT-enhancer binding protein alpha (C/EBPα) protein in the liver. Subsequently, this work shows that ethanol-mediated hepcidin repression, which exacerbates liver injury, requires HIF activity. Together, these models demonstrate that HIFs are
the central regulators of iron homeostasis and can be targeted therapeutically to manage diseases of both anemia and iron overload.
Chapter 1

Introduction

Iron is an essential micronutrient found in nearly all forms of life and functions as a cofactor for enzymes in a multitude of pathways. In humans, most iron is found as a component of hemoglobin, the oxygen-carrying complex present in red blood cells (RBCs). Thus, iron deficiency leads to anemia, which is highly prevalent worldwide. While iron is essential for the ability of RBCs to carry oxygen, it can be harmful when it is present in excess amounts. To attest to this, there are multiple human disorders of iron overload, which can progress nearly asymptotically until organ injury has occurred (1). In this chapter, I will first cover general iron homeostasis and focus on mechanisms critical for intestinal iron absorption. Following this, I will review the role of hepcidin, which is a major regulator of systemic iron homeostasis. Then, I will discuss iron-related diseases associated with insufficient or excess hepcidin levels. Lastly, I will cover the novel role of hypoxia signaling in regulating both intestinal iron absorption and hepcidin expression.

1.1 Iron homeostasis and intestinal iron absorption

Nearly all known living organisms require iron in order to survive. This is due to its role in electron transfer, oxygen binding, and catalysis for enzymatic
reactions (2). However, this inherent versatility is associated with toxicity when iron is present in excess amounts, primarily due to its ability to produce hydroxyl radicals through the Haber-Weiss-Fenton sequence (3).

\[
\begin{align*}
\text{Fe}^{2+} + \text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{O}_2^- \\
2\text{O}_2^- + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{OH}^- + \text{OH}^- + \text{Fe}^{3+}
\end{align*}
\]

The hydroxyl radical will react with nucleic acids, carbohydrates, and proteins, which leads to chain propagating lipid peroxidation (4). Thus, cellular iron levels must be tightly controlled in order to limit iron-mediated damage. Cellular iron is stored in a large, multi-subunit complex called ferritin. The importance of ferritin in iron storage is evidenced by embryonic lethality in ferritin knockout mice (5).

While iron is toxic in excess amounts, it is also necessary for cell survival and there is a regulated mechanism for cellular iron uptake. Iron in circulation is normally bound to transferrin. Transferrin is an 80 kDa protein primarily produced by the liver (6). Transferrin binds with high affinity to ferric, but not ferrous iron (7). Circulating iron-loaded transferrin binds to the transferrin receptor (Tfr1), which leads to endocytosis of the ligand-receptor complex (8). Iron in the endosome is then reduced by the six-transmembrane epithelial antigen of prostate 3 (Steap3) metalloreductase (9) before being transported into the cytosol by divalent metal transporter 1 (Dmt1) or Zrt- and Irt-like protein 14 (Zip14) (10, 11).

Most iron in the human body is present in hemoglobin contained in RBCs and their progenitors (12). Iron is also present in myoglobin of muscle,
macrophages of the reticuloendothelial system, and excess iron is stored in the liver (13). In humans, 20 mg of iron each day is necessary for production of new RBCs, and most of this iron is made available by the recycling of senescent RBCs by macrophages (14). 1-2 mg of iron is lost each day due to shedding of skin and intestinal epithelial cells, which is replaced through intestinal iron absorption (15).

Iron absorption in the small intestine is a tightly regulated process. Dietary iron, which is primarily in the ferric (Fe$^{3+}$) form, must first be reduced to ferrous iron (Fe$^{2+}$) by the apical duodenal ferric reductase (DcytB) (16-18). However, DcytB has been shown to be unnecessary for systemic iron homeostasis, suggesting that other proteins may compensate for its activity (19, 20). Dmt1 then imports ferrous iron into the enterocyte (17, 21, 22). The central role of Dmt1 in iron homeostasis is underscored in mice lacking Dmt1 in the intestine, which leads to progressive anemia and decreased survival (23). Cytosolic iron is loaded into the protein ferritin, which serves to compartmentalize iron (24). In order for iron to exit the enterocyte and enter the bloodstream, Fe$^{2+}$ must first be oxidized to Fe$^{3+}$ by the multicopper ferroxidase hephaestin (25) before exiting through the only known mammalian basolateral iron exporter, ferroportin (Fpn1) (26-29) (Fig. 1.1A). The central role of Fpn1 in intestinal iron absorption is evidenced in mice lacking intestinal Fpn1, which rapidly succumb to severe anemia (30). One of the major mechanisms controlling expression of these transporters is the iron-responsive element/iron-regulatory protein (IRE/IRP) system.
Regulation by the IRE/IRP system

The IRE/IRP system is a complex regulatory network by which intracellular iron levels control transcript stability and translation of several proteins involved in maintenance of iron homeostasis (31, 32). See Muckenthaler et al. for a comprehensive review of this pathway (33). Briefly, iron regulatory proteins 1 and 2 (IRP1 and IRP2) become active under conditions of intracellular iron deficiency, which allows IRPs to bind to a hairpin iron-responsive element (IRE) present in either the 5’ or 3’ untranslated region (UTR) of target mRNAs (Fig. 1.2). Binding of IRP1 or 2 leads to an increase in transcript stability or a block in translation. The major Dmt1 isoform expressed in the duodenum has an IRE in its 3’ UTR (34). Mice lacking both IRP1 and IRP2 expression in the intestine have a robust decrease in Dmt1, suggesting that the activated IRE/IRP system serves to stabilize Dmt1 (34). Since the IRP proteins are activated by intracellular iron deficiency, stabilization of Dmt1 under these conditions serves to bring more iron into the enterocyte to correct the deficiency.

Iron that enters the cell becomes rapidly trapped in the iron storage complex ferritin (24). Ferritin is composed of heavy and light chains, and both subunits are regulated by the IRP system. When IRP activity is inhibited, the ferritin subunits are upregulated (35). The IRP system serves to decrease ferritin translation under conditions of intracellular iron deficiency. Since ferritin serves to sequester and store iron, ferritin downregulation leads to a more readily available intracellular iron pool. Iron exits the enterocyte and enters the circulation through the basolateral iron exporter, Fpn1. The Fpn1 transcript
contains a functional IRE in its 5’ UTR, and intracellular iron deficiency leads to an IRP-mediated block in Fpn1 translation (36, 37). This mechanism serves to keep intracellular iron levels high, but low intestinal Fpn1 levels during dietary iron deficiency causes anemia (37). However, in the duodenum the major Fpn1 transcript lacks an IRE, leading to efficient translation under iron-deficient conditions where IRPs are active (38). Therefore, dietary iron deficiency through the IRE/IRP system can increase Dmt1 and decrease ferritin to transport in more iron, and Fpn1 can evade repression through alternative transcription to export iron into the circulation.

1.2 Regulation of hepcidin

Hepcidin is a 25 amino acid antimicrobial peptide that is produced in the liver and secreted into the bloodstream (39). Hepcidin is translated as an 84 amino acid pro-protein, and cleaved by furin to produce the active peptide (40). Hepcidin is regulated by a complex network of signaling pathways which will be further described in this section (Fig. 1.3). The role of hepcidin in iron homeostasis was first demonstrated in 2001, when upstream transcription factor 2 (USF2) knockout mice were shown to have decreased hepcidin levels and severe iron overload (41). This is due to the close genomic proximity of the USF2 and hepcidin genes, such that both genes were deleted in the USF2 knockout mice. In a subsequent paper, mice with a specific disruption of hepcidin, but having normal USF2 expression, developed severe

* This section is adapted from a published manuscript: Anderson ER and Shah YM (2013). “Iron homeostasis in the liver.” Comprehensive Physiology
hemochromatosis (42). Conversely, animals that overexpress hepcidin in the liver demonstrate severe iron-deficiency anemia (43).

A clue to the function of hepcidin was discovered after subjecting rats to an iron-deficient diet. Following iron deficiency, rats rapidly downregulate liver hepcidin and upregulate the intestinal iron transporters Dmt1, DcytB, and Fpn1 (44). Hepcidin was believed to regulate iron homeostasis due to an interaction with iron transporters. A breakthrough occurred when the hepcidin receptor was discovered. Through careful in vitro analysis, it was determined that hepcidin binds to Fpn1 which leads to its internalization and proteasomal degradation (45). With the connection between hepcidin and Fpn1 solidified, its role in iron homeostasis became clear. Hepcidin is increased in iron loading, which leads to limitation of duodenal iron absorption to normalize iron levels. In the case of iron deficiency anemia, hepcidin is repressed and Fpn1 is stabilized, which increases iron export from the enterocyte. In addition, Fpn1 is increased in macrophages following erythrophagocytosis, and hepcidin is able to lead to Fpn1 degradation in these cells (46). Thus, hepcidin was determined to be the master regulator of both duodenal iron absorption and RBC iron recycling.

One major discovery in the regulation of hepcidin was the involvement of SMAD family member 4 (SMAD4). Liver-specific knockout of SMAD4 leads to near abolition of hepcidin expression (47). These mice die of severe iron overload in multiple tissues. Liver SMAD4 knockout mice are unable to increase hepcidin expression in response to iron loading, suggesting that SMAD4 mediates the response of hepcidin to systemic iron requirements. It was
subsequently shown that bone morphogenetic protein (BMP), but not transforming growth factor beta 1 (TGF-β) signaling, both of which function through SMADs, induced hepcidin expression in cultured liver cell lines and in vivo (48). It is known that signaling through the BMP receptor leads to SMAD1/5/8 phosphorylation, which is required for SMAD4 transcriptional activity (49). In times of increased systemic requirements for iron, phosphorylated SMAD1/5/8 drops dramatically, and it is substantially increased in conditions of iron overload. However, the precise molecular mechanisms for how hepcidin is regulated by iron remain unclear. Studying mutations identified in iron-related disorders provided further insight into how BMP/SMAD signaling modulates hepcidin expression. The significance of these mutations and the proteins they encode will be discussed in more detail below.

In addition to iron, increased production of RBCs also modulates hepcidin expression. In a phenylhydrazine (PhZ)-induced hemolytic anemia model, which induces erythropoiesis, duodenal iron absorption is increased following a decrease in hepcidin expression (50). In addition, phlebotomy, or repeated bleeding also causes hepcidin repression (51). The mechanism was further delineated demonstrating that treatments such as PhZ and phlebotomy could only decrease hepcidin expression in situations where erythropoiesis can proceed (52). Several mechanisms have been attributed to erythropoietic repression of hepcidin (52-54), however this is still incompletely understood.
1.3 Iron related disorders

Anemia

Anemia is defined as a decrease in RBCs or abnormally low hemoglobin levels. One common cause of anemia is iron deficiency, which is especially prevalent in children and pregnant women (55). Maternal iron supplementation is effective in reducing anemia and incidence of low birth weights (55). However, iron deficiency anemia is also common in infants that are breastfed, particularly in developing countries (56, 57). In addition, iron is important in early psychomotor and cognitive development, increasing the negative impact of iron deficiency in young children (58). While iron supplementation is highly effective in combating anemia, distribution of iron supplements is poor in developing countries (59). Moreover, in iron-refractory iron deficiency anemia (IRIDA) iron supplements are not effective (60). Studying this disease has shed light on novel mediators of hepcidin expression. Patients with IRIDA have high hepcidin expression, which is the primary cause leading to anemia and non-responsiveness to iron supplementation.

Recently, mutations in transmembrane protease serine 6 (TMPRSS6), which encodes a type II transmembrane serine protease (matriptase-2), were identified in patients with IRIDA. This was confirmed in the mouse TMPRSS6 knockout, which had hair loss and microcytic anemia associated with high levels of hepcidin expression (61, 62). As discussed above, BMP signaling is essential for hepcidin expression (Section 1.2), and the connection between TMPRSS6

* This section is adapted from a published manuscript: Anderson ER and Shah YM (2013). “Iron homeostasis in the liver.” Comprehensive Physiology
and BMP signaling was made with the discovery that matriptase-2 cleaves membrane hemojuvelin (Hjv). Hjv is a BMP6 co-receptor. Functional matriptase-2 restricts hepcidin expression by cleaving Hjv. However, the mutated form found in IRIDA cannot cleave Hjv, which leads to increased expression of hepcidin and iron deficiency (63).

One highly prevalent disorder of anemia is the anemia of chronic disease, which encompasses several diseases including kidney disease, inflammatory disease, cancer, and aging (64). In healthy human volunteers and mice, studies demonstrate that inflammatory agents cause a robust and rapid decrease in serum iron levels (65-68). Hepcidin is induced in response to inflammatory stimuli (69-71). The mechanism for the induction of hepcidin by inflammation is mediated by interleukin-6 (IL-6)-induced signal transducer and activator of transcription 3 (STAT3) activity, which increases hepcidin expression in cultured cells, mice, and humans (67, 72-74) (Figure 1.3). The increase in hepcidin is thought to be a protective mechanism that restricts iron to infectious pathogens. However, in chronic disorders this leads to anemia, which can have detrimental effects in the primary disease pathogenesis. Similarly, in most cancers there is a decrease in serum iron levels and increased anemia (75). The best treatment for anemia of chronic disease is resolving the primary chronic disease. In severe cases blood transfusion, erythropoietin (EPO), or intravenous administration of iron is used (76).

Iron overload
Iron overload diseases are characterized by pathological iron accumulation and can be caused by a wide variety of genetic factors. One very common cause of iron overload is hereditary hemochromatosis (HH), which is a genetic disorder that affects 1 in 200 people (77). The most common mutation observed in HH is in HFE. The Hfe gene encodes an atypical major histocompatibility complex protein, and the C282Y mutation is the most common cause of HH in humans (78, 79). Mice with either a deletion of Hfe or a knock-in C282Y allele demonstrate iron overload which is associated with decreased hepcidin expression (80). HFE is expressed in many tissues important for iron homeostasis, including the liver and intestine. Conditional disruption of HFE in the liver, but not intestine, recapitulates the iron overload phenotype, demonstrating that the primary role of HFE is to regulate hepcidin expression in the liver (81, 82). It is thought that HFE controls hepcidin expression in iron-loaded conditions, and lack of HFE leads to a blunted increase in hepcidin expression during iron overload (83).

Juvenile hemochromatosis (JH) is a rare autosomal recessive disorder of iron overload and symptoms become apparent before the age of 30. JH is caused by mutations in the gene for Hfe2, which encodes for Hjv (84), the BMP6 coreceptor, as discussed above in more detail. The Hjv knockout mouse model demonstrates severe iron overload associated with a dramatic decrease in hepcidin expression, similar to that observed in patients with Hjv mutations (84, 85). Similar to Hjv mutations, mutations in the hepcidin antimicrobial peptide (Hamp) gene, which encodes for hepcidin, are a very rare cause of JH. Currently
12 known mutations occur on the *Hamp* gene leading to a decrease in the normal production of hepcidin (86). Since hepcidin function or expression is dramatically diminished, the iron overload symptoms are observed before the age of 30.

Transferrin receptor 2 (Tfr2) mutations lead to an autosomal recessive iron overload disease similar to the HFE-related HH phenotype. Tfr2 is capable of binding to HFE and this interaction is critical in maintaining hepcidin expression (87). The knockout mouse model and the liver-specific disruption of Tfr2 confirm its importance in regulating hepcidin levels (88). Hepcidin levels are decreased significantly in these mouse models compared to littermate controls, and tissue iron is increased.

If left untreated, hemochromatosis causes liver cirrhosis, cardiomyopathy, diabetes, and arthritis (79). The most common form of treatment is therapeutic phlebotomy, which is effective in decreasing iron but is complicated by poor compliance with treatment and is contraindicated in patients with anemia and other diseases (89). Another treatment option is oral iron chelation using deferasirox, which also reduces iron levels but is associated with the side effects of diarrhea, headache, and nausea (90).

**Thalassemia**

In addition to hereditary hemochromatosis, there exist diseases of secondary hemochromatosis such as β-thalassemia in which iron overload is a complication of the primary disorder. β-thalassemia is a genetic disorder in which
the β-globin gene is partially or completely deleted, which leads to β-thalassemia intermedia and Cooley’s anemia, respectively (91). Patients with β-thalassemia demonstrate anemia that is controlled with blood transfusions when necessary. Blood transfusions, however, constitute a major source of iron which contributes to the pathological consequence of iron overload in thalassemic patients (92). Interestingly, iron overload and its pathological consequences are prevalent in patients who do not receive regular transfusions, suggesting that increased iron absorption could contribute to iron overload in β-thalassemia (93). Indeed, mouse models of the disease demonstrate increased iron absorption that is accompanied by a decrease in hepcidin expression (94).

Since hepcidin is a major regulator of iron homeostasis, much work has gone into investigating the role of hepcidin in the pathogenesis of β-thalassemia (95). Growth-differentiation factor 15 (GDF15) and twisted gastrulation (TWSG1) are two factors produced by erythroid cells that are correlated to decreases in hepcidin expression in thalassemia (53, 54). However, recent data demonstrate that neither GDF15 nor TWSG1 are likely to be involved in the suppression of hepcidin expression during erythropoiesis (96). More recently we have shown that suppression of BMP/SMAD signaling is important for hepcidin repression in β-thalassemia (97). The mechanism of hepcidin repression in β-thalassemia remains incompletely understood. However, the potential of hepcidin as a treatment tool for iron overload disorders is clear. Deletion of TMPRSS6, which leads to increased hepcidin expression, substantially ameliorates iron overload and anemia in β-thalassemia (98). In addition, transgenic overexpression of
hepcidin in the liver also improves the disease (99). However, alternative therapeutic strategies are sought due to the ability of hepcidin to induce anemia (43).

1.4 Regulation of iron homeostasis by hypoxia signaling

In addition to regulation of iron absorption by hepcidin and the IRE/IRP system, there are also mechanisms that induce intestinal iron transporters at the transcriptional level. In multiple mouse strains, the duodenal iron transporters Dmt1, DcytB, and Fpn1 are induced during dietary iron deficiency (100). Subsequent work shows that oxygen signaling is essential for intestinal iron transporter gene expression. Oxygen signaling is primarily controlled by a family of hypoxia-inducible transcription factors (HIFs). The HIF complex consists of a constitutively present HIF-β subunit, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), and an oxygen-regulated HIF-α subunit (101). Under normal oxygen and iron levels, the HIF-α subunit is hydroxylated by a family of prolyl hydroxylases, and this allows the von Hippel-Lindau tumor suppressor (VHL) to bind HIF-α and promote its ubiquitination and proteasomal degradation (102) (Fig. 1.4).

HIF-2α specifically is essential for the induction of the iron absorption genes Dmt1, DcytB, and Fpn1 in response to an iron-deficient diet (103, 104) (Fig. 1.1B). Through promoter luciferase and chromatin immunoprecipitation assays, all three genes have been demonstrated to be direct HIF-2α targets (103-105). Expression of these iron transporters in the intestine is induced
during erythropoietic stress, although the mechanism remained unclear until our recent work (106). In addition to dietary iron deficiency and erythropoiesis, HIF-2α is necessary for iron absorption in hepcidin knockout mice, a model of iron overload (107).

At the beginning of my work, hepcidin was widely accepted to be the primary regulator of iron homeostasis under all pathological and physiological conditions. My thesis builds upon and expands the observation that intestinal HIF-2α is an important local regulator of iron absorption during iron deficiency (103). Moreover, HIF-2α is essential in iron absorption to meet systemic iron needs during erythropoietic stress (106). Chapter 2 of this thesis examines the role of intestinal HIF-2α during erythropoietic iron absorption.

Increased iron absorption in β-thalassemia is attributed to a decrease in hepcidin expression (94), but the role of intestinal HIF-2α in this disease has not been examined. Chapter 3 of this thesis demonstrates that intestinal HIF-2α is essential for iron overload in β-thalassemia, calling into question the dominant role hepcidin is purported to play in the disease. Furthermore, loss of intestinal HIF-2α allows rapid mobilization of iron stores in response to erythropoietic stress, suggesting that intestinal HIF-2α could be a therapeutic target in disorders of iron overload.

In addition to hypoxic regulation of local iron absorption in the intestine, hypoxia is important in systemic iron regulation as well. Hypoxia strongly represses hepcidin expression both in cultured cells and in mice (108). Moreover, a human mutation that leads to HIF stabilization, Chuvash
polycythemia, causes decreased hepcidin levels without a significant association with erythropoiesis (109). Many mechanisms have been proposed to play a role in hypoxic hepcidin repression (110-117) (Figure 1.3). However, the mechanism for hypoxic regulation of hepcidin is still unclear. In Chapter 4 we demonstrate that HIFs regulate hepcidin expression by decreasing the expression of CCAAT/enhancer binding protein alpha (C/EBPα), which is required for basal hepcidin expression. Moreover, patients with alcoholic liver disease (ALD) have liver iron accumulation (118). Chapter 4 of this thesis also shows that hypoxia is the major regulator of hepcidin expression during ethanol consumption (119).

The work presented in this thesis strongly challenges the dominant role of hepcidin in controlling systemic iron homeostasis, and proposes that HIF signaling plays an essential role in the maintenance of iron homeostasis. Intestinal HIF-2α, not hepcidin, is responsible for increased iron absorption during erythropoiesis. Moreover, iron overload in β-thalassemia is abolished with loss of intestinal HIF-2α, despite an intact hepcidin response. Lastly, HIF signaling plays a critical role in modulating hepcidin expression during ethanol consumption, with implications for iron overload in ALD. Thus, activation or inhibition of HIF signaling in the liver or intestine could be used therapeutically to control systemic iron homeostasis and control diseases of anemia and iron overload.
Normal Iron diet  Dietary iron deficiency

Figure 1.1. Regulation of intestinal iron transport. A) Luminal iron, primarily in ferric form (Fe$^{3+}$) is reduced by the duodenal ferric reductase DcytB. Ferrous (Fe$^{2+}$) iron is transported into the enterocyte the divalent metal transporter-1 (DMT1). Intracellular iron is stored in the ferritin complex or exported through the only basolateral iron exporter, ferroportin (FPN). B) Under conditions of dietary iron deficiency, intestinal HIF-2α is stabilized, leading to increased transcription of its target genes Dmt1, DcytB, and Fpn. This leads to increased apical iron absorption and basolateral iron transfer. [Reproduced with permission from (Shah et al, 2009 Cell Metabolism)].
Figure 1.2. Control of iron homeostasis by iron regulatory proteins (IRPs).
Under conditions of iron repletion, the IRP proteins are inactivated, leading to decreased stability of the iron importer Dmt1, and increased stability of the iron exporter Fpn1 and the iron storage protein ferritin. Under conditions of low iron, the IRP proteins become active and bind to a hairpin in the 5’ or 3’ UTR of target mRNAs. This increases stability of Dmt1 mRNA and decreases translation of Fpn1 and ferritin mRNA, resulting in an increased level of cellular iron to normalize iron levels.
Figure 1.3. Regulation of hepcidin expression. Transferrin/transferrin receptor interactions and Bmp signaling lead to increased SMAD1/5/8 phosphorylation, which is required for SMAD4 activation of the hepcidin promoter. The inflammatory cytokine IL-6 binds to its receptor, leading to STAT3 phosphorylation and promoter activation. Hypoxia represses hepcidin through stimulation of erythropoiesis, which is mediated by an erythroid factor, removal of C/EBPα from the hepcidin promoter, and direct HIF binding to the hepcidin promoter. [Reproduced with permission from (Anderson and Shah, 2013 Comprehensive Physiology)].
Figure 1.4. Regulation of HIF activity. Under conditions of normal oxygen and iron levels, prolyl hydroxylase domain enzymes (PHD) hydroxylate specific proline residues on the HIFα protein allowing VHL to bind, which leads to polyubiquitination and proteasomal degradation of HIFα. Under conditions of iron or oxygen deficiency, PHD activity is inhibited and HIFα is not hydroxylated. This prevents VHL binding, which allows HIFα to become stabilized, translocate to the nucleus, and bind ARNT to activate target gene transcription.
References


Chapter 2

Intestinal Hypoxia-inducible Factor-2α is Critical for Efficient Erythropoiesis

Abstract:

Erythropoiesis is a coordinated process by which RBCs are produced. Erythropoietin, a kidney-derived hormone, and iron are critical for the production of oxygen-carrying mature RBCs. To meet the high demands of iron during erythropoiesis, small intestinal iron absorption is increased through an undefined mechanism. In this study, erythropoietic induction of iron absorption was further investigated. Hypoxia-inducible factor-2α (HIF-2α) signaling was activated in the small intestine during erythropoiesis. Genetic disruption of HIF-2α in the intestine abolished the increase in iron absorption genes as assessed by quantitative real-time reverse transcription-PCR and Western blot analyses. Moreover, the increase in serum iron following induction of erythropoiesis was entirely dependent on intestinal HIF-2α expression. Complete blood count analysis demonstrated that disruption of intestinal HIF-2α inhibited efficient erythropoiesis; mice disrupted for HIF-2α demonstrated lower hematocrit, RBCs, and Hb compared with wild-type mice. These data further cement the essential role of HIF-2α in regulating iron absorption and also demonstrate that hypoxia sensing

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in the intestine, as well as in the kidney, is essential for regulation of erythropoiesis by HIF-2α.

Introduction

Erythropoiesis is a tightly regulated process involved in maintaining and modulating RBC production under both normal and pathological conditions. Erythropoiesis is controlled by erythropoietin (EPO), which is produced by the kidney and secreted into the bloodstream (1). EPO acts on red blood cell progenitors in the bone marrow to stimulate their differentiation (2). When large numbers of erythrocytes are lost due to disease or injury, EPO production increases dramatically, leading to increased erythrocyte production to restore the RBC population (3). Increased erythrocyte production requires an increase in available iron to produce Hb (4). Erythropoiesis stimulates expression of iron absorption genes in the small intestine, although the mechanism by which this occurs is unknown (5-7). Erythropoiesis-stimulating agents are commonly used to increase serum iron levels to combat iron deficiency anemia, particularly in cancer patients and patients with chronic kidney disease (8).

In addition, β-thalassemia is a common genetic disorder caused by mutations in the β-globin gene, which result in ineffective erythropoiesis leading to iron overload (9). In β-thalassemia patients, intestinal iron absorption is three to four times greater than in normal control patients, and the increase in circulating and tissue iron is the major cause of death in β-thalassemia patients (9, 10).
Intestinal iron absorption is tightly regulated by the small intestine. Dietary iron is primarily in ferric (Fe$^{3+}$) form and is reduced to ferrous (Fe$^{2+}$) iron by the duodenal ferric reductase (DcytB) (11-13). Ferrous iron is then transported into the enterocyte by Dmt1 (divalent metal transporter 1) (12, 14, 15). To enter circulation, ferrous iron passes through the only characterized basolateral iron exporter, Fpn1 (ferroportin-1) (16-19). Hepcidin, a liver-derived peptide that regulates iron homeostasis through Fpn1 degradation (20-22), has been implicated in the increase in iron absorption following erythropoiesis and in thalassemia patients (10, 23-25). However, other mechanisms that regulate intestinal iron absorption are thought to be involved because hepcidin expression does not completely ameliorate the iron overload in β-thalassemic mouse models (23), and the effect of hepcidin on intestinal iron absorption does not appear to be direct and rapid (26-30).

In addition to hepcidin, the transcription factor hypoxia-inducible factor-2α (HIF-2α) is necessary for intestinal iron absorption following iron deficiency (31, 32). HIF-2α is a heterodimeric transcription factor consisting of an iron- and oxygen-regulated α-subunit and a constitutively expressed β-subunit, the aryl hydrocarbon receptor nuclear translocator (also known as HIF-β) (33). Under normal cellular oxygen and iron levels, HIF-2α is hydroxylated at specific proline residues, which are required for Von Hippel-Lindau (VHL) tumor suppressor binding. VHL is the substrate recognition portion of an E3 ubiquitin ligase complex and is necessary for ubiquitination and subsequent proteasomal degradation of HIF-2α. The prolyl hydroxylases responsible for hydroxylation of
HIF-2α require oxygen and iron as cofactors (34). During iron deficiency, prolyl hydroxylase activity is inhibited, and HIF-2α accumulates, dimerizes with the aryl hydrocarbon receptor nuclear translocator, and activates transcription of its target genes. HIF-2α binds to consensus hypoxia response elements in the promoters of genes involved in maintaining iron homeostasis, such as Dcytb, Dmt1, and Fpn1 (27, 31, 32). Because HIF-2α is critical in maintaining iron homeostasis following iron deficiency, in this study, we assessed whether similar mechanisms are required for the erythropoietic induction of iron absorption genes in the small intestine. Wild-type and intestinal HIF-2α knock-out mice were administered phenylhydrazine (PhZ), which causes hemolysis and stimulates erythrophoiesis (5-7). It is shown that intestinal HIF-2α is critical not only for the erythropoietic induction of iron absorption genes in the small intestine but also for the increase in serum iron, which in turn is necessary for efficient erythrophoiesis.

**Experimental Procedures**

*Animals and Treatments*

Vhl<sup>F/F</sup> (35), Vhl<sup>ΔIE</sup> (36), Hif-2α<sup>F/F</sup>, and Hif-2α<sup>ΔIE</sup> (27) mice were described previously. The mice were housed in a light- and temperature-controlled room and were given water and chow *ad libitum*. PhZ (Sigma) was dissolved in neutralized saline. Mice were injected at 60 mg/kg of body weight on 2 consecutive days and were killed 2, 3, and 7 days following the second injection. Tissues were harvested and used fresh or flash-frozen in liquid nitrogen and stored at −80 °C for future use. 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.

**Western Blot Analysis**

Tissues were homogenized and lysed in radio immune precipitation assay buffer for whole cell extracts, and nuclear proteins were isolated using the NE-PER nuclear extraction kit (Pierce). Membrane proteins were isolated as described previously (27). Membrane extracts were incubated for 5 min at 65 °C for detection of DcytB, whereas all other extracts were heated at 95 °C for 5 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using standard methods. Membranes were incubated with antibodies against HIF-1α and HIF-2α (Novus Biologicals, Littleton, CO), Dmt1 and DcytB (Alpha Diagnostic International Inc., San Antonio, TX), and RAN (Santa Cruz Biotechnology, Santa Cruz, CA). For in vivo hypoxia detection, nitroimidazole was dissolved in normal saline with 2.4% ethanol and injected at 200 mg/kg of body weight 90 min before harvesting tissues. The signal was measured by using an antibody to nitroimidazole-protein adducts (HPI Inc., Burlington, MA) in whole cell extracts.

**Quantitative Real-time Reverse Transcription-PCR (qPCR)**

RNA was isolated from fresh or frozen tissue using Isol-RNA lysis reagent (5 PRIME, Gaithersburg, MD) and reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Fisher). cDNA was quantified using SYBR Green dye and run on a 7900HT fast real-time PCR system (primers are listed in
Table 2.1. $C_t$ values were normalized to β-actin and expressed as -fold difference from controls.

Iron and Hematological Analysis

Serum iron was analyzed using the QuantiChrom iron assay kit (Bioassay Systems, Hayward, CA) following the manufacturer's protocol. Tissue iron in small intestinal epithelium was measured as described previously (37). Briefly, tissues were digested in 3 M HCl and 10% trichloroacetic acid at 65 ºC for 20 h and then compared against an iron standard by a colorimetric assay. Complete blood count analysis was performed by the Unit for Laboratory Animal Medicine Pathology Core for Animal Research.

Immunohistochemistry

Duodenum was Swiss-rolled, frozen in cryo-embedding medium, and sectioned at 7 µm. The sections were fixed in 4% paraformaldehyde in PBS and incubated overnight at 4 ºC with rabbit anti-mouse Fpn1 antibody (1:100; Alpha Diagnostic International Inc.) diluted in PBS with 2% BSA. Slides were washed twice with PBS and then incubated for 1 h at room temperature with Alexa Fluor® 488-labeled goat anti-rabbit IgG (1:500; Molecular Probes, Inc., Eugene, OR) diluted in PBS with 2% BSA. After incubation with the secondary antibody, all sections were washed three times for 5 min with PBS and mounted with ProLong® gold antifade reagent with DAPI (Molecular Probes, Inc.). Immunofluorescence was visualized using a Nikon Eclipse TE200 microscope with a ×20 objective. Images were acquired using an Olympus DP71 microscope.
digital camera and processed using an Olympus DP Controller Version 3.2.1.276 software package (Olympus America Inc., Center Valley, PA).

Cell Culture

Caco-2 cells were maintained at 37 °C in 5% CO₂ and 21% O₂. Cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic. For EPO experiments, cells were switched to serum-free medium 24 h before EPO incubation. Recombinant human erythropoietin (Prospec, East Brunswick, NJ) was used at a concentration of 10 or 100 IU/ml, and cells were incubated for 24 h. Cells were lysed in radioimmune precipitation assay buffer, and Western blot analysis was performed. For hypoxia experiments, cells were incubated in 1% O₂ and 5% CO₂ with balance N₂ at 37 °C for 24 h.

Results

PhZ Treatment Induces Expression of Epo in the Kidney and Iron Absorption Genes in the Small Intestine

PhZ is commonly used to study hemolytic anemia-induced erythropoiesis (5-7, 26). A dramatic decrease in RBCs following PhZ treatment leads to hypoxia in the kidney (1). This stabilizes HIF-2, activating EPO expression (1), and stimulates RBC differentiation and maturation into erythrocytes (2). Wild-type mice were treated with PhZ for 2 consecutive days and killed 48 h following final treatment. Epo mRNA expression was highly induced in the kidney, but not in the liver. Also, liver hepcidin mRNA expression was strongly suppressed (Fig. 2.1A), consistent with a previous report (25). Expression of Dmt1a and Dcytb
was significantly increased in the duodenums of treated mice compared with
untreated controls as assessed by qPCR. *Fpn1* expression was not affected,
however (Fig. 2.1B). Because erythropoiesis increases the demand for iron,
these integrative changes provide an efficient system to increase the available
iron stores. Moreover, PhZ treatment provides an ideal model to assess the
mechanism by which iron absorption is induced following erythropoiesis.

*Erythropoietic Induction of Iron Absorption Genes in the Small Intestine is HIF-
2α-dependent*

Chronic low iron treatment induces the expression of iron absorption
genes in the small intestine through a HIF-2α-dependent mechanism (27, 31,
32). However, the precise molecular mechanisms by which iron absorption is
increased to satisfy systemic iron requirements during erythropoiesis are
unknown. To investigate whether HIF-2α is also critical in erythropoietic induction
of iron absorption, mice with an intestinal disruption of HIF-2α (*Hif-2αΔIE*) and
wild-type littermate controls (*Hif-2α F/F*) were assessed following PhZ treatment.

*Hif-2αΔIE* and *Hif-2α F/F* mice were treated with PhZ for 2 days and then killed 2 or
3 days post-treatment. In *Hif-2α F/F* mice, PhZ increased iron absorption genes in
the duodenum as assessed by qPCR. However, PhZ induction of intestinal
*Dmt1a* and *Dcytb* was completely abolished in the *Hif-2αΔIE* mice (Fig. 2.2A).

Kidney *Epo* expression was highly induced to the same extent in *Hif-2αΔIE* mice
and wild-type littermates, demonstrating that these changes are not due to
dysregulation of kidney *Epo* expression (Fig. 2.2B). Western blotting was
performed on protein extracts from the duodenum. In *Hif-2α F/F* mice, DcytB and
Dmt1 expression was strongly induced in membrane duodenal extracts 2 and 3 days post-PhZ treatment. Consistent with the mRNA data, in Hif-2αIE mice, there was no increase in either Dmt1 or DcytB protein expression following PhZ treatment (Fig. 2.2C). Because Fpn1 expression was not detected by Western blotting consistently after PhZ treatment (data not shown), a highly sensitive immunohistochemistry assay was performed using intestines from PhZ-treated wild-type and Hif-2αIE mice. Fpn1 expression was similar following PhZ administration in Hif-2αIE mice compared with Hif-2αF/F mice, consistent with qPCR data (Fig. 2.2D). These data demonstrate that the induction of the apical iron absorption genes in the small intestine is dependent on HIF-2α expression.

**Erythropoiesis Stabilizes HIF-2α, but Not HIF-1α**

The gene expression data strongly suggest that HIF-2α is necessary for PhZ-induced expression of intestinal iron absorption genes. Consistent with these data, Western blotting of nuclear extracts prepared from the duodenums of PhZ-treated and untreated wild-type mice demonstrated a robust induction of HIF-2α, whereas expression of HIF-1α was not detected in duodenal nuclear extracts from PhZ-treated mice (Fig. 2.3A). Intestinal HIF-2α is rapidly stabilized by iron deficiency (27, 31, 32); therefore, the possibility that a decrease in duodenal iron stabilizes HIF-2α was assessed. Non-heme iron was extracted from PhZ-treated and untreated duodenal epithelia and quantified (Fig. 2.3B). No significant difference was detected, demonstrating that a decrease in tissue iron is not responsible for HIF-2α stabilization following PhZ treatment.

**Intestinal Hypoxia Stabilizes HIF-2α following PhZ-induced Erythropoiesis**
*Hif-2α* has recently been identified as a direct STAT5 target gene in hematopoietic stem cells (38). EPO is a known activator of the JAK2/STAT5 signaling cascade (39), and therefore, *Hif-2α* mRNA was assessed. No significant increase was detected in duodenal epithelial cells from PhZ-treated and untreated wild-type mice (Fig. 2.4A). Although *Hif-2α* mRNA was unchanged, EPO stabilization of HIF-2α protein was assessed. Caco-2 cells, a human colon cancer cell line, were incubated for 24 h in the presence or absence of recombinant human EPO. No change in HIF-2α expression was observed following EPO incubation (Fig. 2.4B). In addition, we assessed whether EPO and hypoxia could synergistically stabilize HIF-2α. Hypoxia strongly stabilized HIF-2α in Caco-2 cells. However, EPO incubation did not potentiate the increase in HIF-2α protein expression (Fig. 2.4B). Moreover, acute treatment of EPO (2, 4, and 8 h) in Caco-2 cells was performed, and EPO did not alter HIF-2α expression at any time points that were assessed (data not shown). These data clearly establish that circulating EPO is not involved in intestinal HIF-2α expression. An initiating event in erythropoiesis is kidney hypoxia stimulating *Epo* expression in a HIF-2α-dependent manner (40, 41). Therefore, the possibility that oxygen sensing in the small intestine is responsible for HIF-2α stabilization was evaluated. The hypoxia-detecting reagent nitroimidazole was used to investigate tissue hypoxia. Nitroimidazole forms protein adducts only in tissues with low oxygen tension but will not bind to proteins under normoxic conditions (42, 43). It was shown recently that Western blotting performed on extracts from cells treated with nitroimidazole is a sensitive and quantitative method for
measuring hypoxic adduct formation (44). Wild-type mice were treated with PhZ for 2 days and injected with nitroimidazole 90 min prior to tissue collection. Nitroimidazole-protein adducts were detected by Western blotting in PhZ-treated and control duodenums, whereas no signal was detected in the oxygen-replete lung. In the duodenum, nitroimidazole staining was significantly stronger in the PhZ-treated mice compared with the control mice (Fig. 2.4C). These data suggest that following PhZ treatment, HIF-2, but not HIF-1, is stabilized in the intestine by hypoxia, a distinct mechanism from HIF-2α stabilization during iron deficiency.

*Intestinal HIF-2α Is Necessary for the Erythropoietic Increase in Serum Iron*

To determine whether HIF-2α-dependent regulation of iron absorption genes is required for the increase in systemic iron levels during erythropoiesis, serum iron was assessed in control and PhZ-treated wild-type and *Hif*-2αΔIE mice. No significant difference in basal serum iron levels was observed in *Hif*-2αΔIE mice compared with wild-type mice. At 2 days post-PhZ treatment, serum iron levels were significantly increased in wild-type mice, and the adaptive increase in serum iron following PhZ treatment was completely abolished in *Hif*-2αΔIE mice. At 7 days post-PhZ treatment, serum iron levels were back to normal in wild-type mice (Fig. 2.5). These data demonstrate that erythropoietic induction of iron absorption is dependent on HIF-2α.

*Intestinal HIF-2α Expression Is Required for Efficient Erythropoiesis*

The erythropoietic induction of iron absorption is completely abolished in *Hif*-2αΔIE mice; therefore, these mice provide an ideal model to assess the
significance of intestinal HIF-2α and iron absorption in erythropoiesis. To observe how inhibition of iron absorption in Hif-2αΔIE mice could affect erythropoiesis, complete blood count analysis was performed on Hif-2αF/F and Hif-2αΔIE mice treated with PhZ. Whole blood was taken 2 and 7 days following PhZ treatment. Control Hif-2αF/F and Hif-2αΔIE mice had normal RBC, hematocrit (HCT), and Hb levels (Fig. 2.6, A-C). Two days following PhZ treatment, RBC and HCT levels decreased significantly in both Hif-2αF/F and Hif-2αΔIE mice, and Hb levels were increased. At 7 days post-PhZ treatment, HCT and Hb levels were completely recovered in wild-type mice, and RBCs were increased compared with 2 days post-treatment. However, Hif-2αΔIE mice demonstrated significantly reduced RBC, HCT, and Hb levels compared with wild-type littermates. The changes observed in RBCs, HCT, and Hb in the Hif-2αΔIE mice reflect a critical role of intestinal HIF-2α in erythropoiesis.

**Discussion**

The induction of iron absorption genes in the intestine by erythropoiesis has been described previously, but a mechanism had not been provided prior to this study (5-7). Similar to iron absorption induced by iron deficiency, HIF-2α is a critical regulator of iron absorption during erythropoiesis (31, 32). Intestinal HIF-2α signaling is essential for the increase in serum iron following induction of erythropoiesis by PhZ treatment. Furthermore, the increase in serum iron mediated by HIF-2α is essential for erythropoiesis, as it is severely inhibited in Hif-2αΔIE mice compared with Hif-2αF/F mice. The results of this study and of
previous work demonstrate a central role of HIF-2α in erythropoiesis. Together, these data provide a working model for the induction of erythropoiesis (Fig. 2.7). A decrease in RBCs results in hypoxic kidneys due to decreased delivery of oxygen, which activates HIF-2α expression and subsequently Epo mRNA (40). The decrease in RBCs stabilizes intestinal HIF-2α, which activates iron absorption genes, leading to an increase in serum iron. Activation of HIF-2α signaling in the kidney and intestine is required for efficient RBC synthesis. Moreover, EPO acts on the bone marrow to stimulate erythrocyte differentiation and maturation, and the renewal of bone marrow-derived hematopoietic stem cells is also activated in a HIF-2α-dependent pathway (38).

One finding of note is that basal erythropoiesis appears to be largely unaffected in Hif-2αΔIE mice compared with Hif-2αF/F mice under control conditions. Following erythropoietic stress, hematologic parameters are significantly different between Hif-2αΔIE and Hif-2αF/F mice, albeit the changes are moderate. Given the essential role of HIF-2α in intestinal iron absorption, one might expect a dramatic difference in RBCs, Hb, and HCT in basal as well as stress-induced erythropoiesis. One explanation is that a grain-based laboratory diet contains 350 ppm of ferrous iron, which is 10-fold higher than the normal dietary intake of mice. Moreover, ferrous iron does not require ferric reductase activity to be absorbed in the duodenum. It is possible that a diet consisting of ferric iron, which makes up the majority of normal dietary iron intake, would lead to more striking changes in hematologic parameters in Hif-2αΔIE mice.
Fpn1 is up-regulated in a HIF-2α-dependent manner following low iron treatment (27). However, HIF-2α activation following PhZ treatment did not increase Fpn1 expression. Also, an increase in Fpn1 does not appear to be required for erythropoiesis in a PhZ-induced erythropoiesis mouse model. It is possible that the acute HIF-2α activation following PhZ treatment is not sufficient to increase Fpn1 expression in comparison with the chronic activation seen with a low iron diet (27). In addition, basal Fpn1 expression appears to be sufficient to transport iron, despite increased iron uptake due to increased DcytB and Dmt1 expression. Together, these data suggest that regulation of Fpn1 expression is more important in low iron treatment than erythropoiesis, despite the increase in iron absorption in both cases.

Interestingly, in PhZ-treated mice, liver hepcidin expression was strongly repressed despite the mice having high serum iron levels. A similar phenomenon is observed in mouse models and patients with β-thalassemia. Hypoxia and EPO have been shown to repress hepcidin levels, and the increase in hypoxia and or EPO due to ineffective erythropoiesis may override the increase in hepcidin due to high serum iron levels (45, 46). Further experiments are needed to elucidate the role of hepcidin in erythropoiesis. Although the role of hepcidin in erythropoiesis is unclear, this study has clearly demonstrated an essential role of intestinal hypoxia sensing and HIF-2α activity in the regulation of erythropoiesis. HIF-2α-dependent increases in DcytB and Dmt1 at the mRNA and protein levels are required for the increased systemic iron demand during erythropoiesis. These findings present new therapeutic opportunities for treatment of injuries and
diseases involving erythropoiesis. Thalassemia major leads to severe iron overload and is treated with chelation therapy (9, 10). It would be beneficial to explore targeted abrogation of intestinal HIF-2α activity, which would reduce iron absorption and could alleviate iron overload. Conversely, intestinal HIF-2α agonists could be used to increase intestinal iron absorption, which could help control iron deficiency anemia without requiring oral iron supplementation. HIF-2α is a central regulator of intestinal iron absorption, and more studies are needed to determine how modulation of intestinal HIF-2α activity can be used to treat diseases of iron overload and iron deficiency anemia.

**Acknowledgments**

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**Abbreviations**

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>EPO</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>hypoxia-inducible factor-2α</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel-Lindau</td>
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<td>PhZ</td>
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<td>qPCR</td>
<td>quantitative real-time reverse transcription-PCR</td>
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Table 2.1 Primers used for qPCR analysis
Figure 2.1. PhZ-induced hemolysis activates expression of EPO in the kidney and iron absorption genes in the small intestine. Wild-type mice were injected twice 24 h apart with 60 mg of PhZ/kg of body weight or with normal saline (Control) and killed 2 days later. qPCR was performed in the kidney or liver for Epo and hepcidin (Hepc) (A) or in the duodenum for Dmt1, Dcytb, and Fpn1 (B). Expression was normalized to β-actin. Values are expressed as -fold change compared with untreated controls. Four to five animals for each treatment group were assessed. Statistical analyses were performed using Student's t test. Each bar represents the mean ± S.D. **, p < 0.01; ***, p < 0.005; n.s., not significant.
Figure 2.2. Erythropoietic induction of iron absorption genes in the small intestine is HIF-2α-dependent. Wild-type (Hif-2α^{F/F}) and intestinal HIF-2 knock-out (Hif-2α^{ΔIE}) mice were treated with PhZ or saline (control) and killed 48 h post-treatment. qPCR was performed in the duodenum for Dmt1, Dcytb, and Fpn1 (A) or in the kidney for Epo (B). Expression was normalized to β-actin. Values are expressed as fold change compared with untreated controls. C, Hif-2α^{F/F} and Hif-2α^{ΔIE} mice were treated with PhZ or saline (control (Con)) and killed 48 and 72 h post-treatment. Western blot analysis measuring Dmt1a and DcytB expression in membrane extracts and Coomassie Blue staining of total membrane proteins were assessed for loading controls. D, immunofluorescence staining of Fpn1 in the duodenums of Hif-2α^{F/F} and Hif-2α^{ΔIE} mice following PhZ treatment. Four to five animals for each treatment group or time point were assessed. Statistical analyses were performed using Student’s t test. Each bar represents the mean ± S.D. *, p < 0.05; **, p < 0.01; n.s., not significant. For Western blot analysis and immunofluorescence, a representative image from an individual mouse for each treatment group or time point is shown.
Figure 2.3. PhZ treatment induces HIF-2α (but not HIF-1α) expression in the small intestine. A, Western blot analysis of duodenal nuclear extracts for HIF-1α and HIF-2α following PhZ or saline (Control) treatment. Hypoxia-treated Caco-2 cells were used as a positive control (+C) for HIF-1α. Expression was normalized to RAN protein expression. B, tissue non-heme iron quantification of the duodenums of PhZ- and saline-treated mice. Four to five animals for each treatment group were assessed. For Western blot analysis, representative images from two individual mice for each treatment group are shown. n.s., not significant.
Figure 2.4. HIF-2α is stabilized by hypoxia in the small intestine following PhZ treatment. A, qPCR analysis of duodenal Hif-2α mRNA expression following PhZ or saline (Control) treatment in wild-type mice. B, Western blot analysis of Caco-2 cells treated with recombinant human EPO under normoxia and hypoxia for 24 h. Expression was normalized to GAPDH protein expression. C, Western blot analysis of nitroimidazole-protein adducts in extracts from the duodenums and lungs of PhZ-treated and control (Con) wild-type mice. Expression was normalized to GAPDH. Four to five animals for each treatment group were assessed. For Western blot analysis, a representative image from an individual mouse for each treatment group is shown. n.s., not significant.
Figure 2.5. Intestinal HIF-2 expression is critical for the erythropoietic increase in serum iron. Wild-type (Hif-2α^{F/F}) and intestinal HIF-2 knock-out (Hif-2α^{ΔIE}) mice were treated with PhZ or saline (Control), and sera were collected 2 (2d) and 7 (7d) days after the last injection. Serum was assayed for iron content. Four to five animals for each treatment group were assessed. Statistical analyses were performed using Student's $t$ test. Error bars indicate S.D. *, $p < 0.05$; n.s., not significant.
Figure 2.6. Intestinal HIF-2 expression is essential for erythropoiesis. Wild-type (Hif-2α<sup>F/F</sup>) and intestinal HIF-2 knock-out (Hif-2α<sup>ΔIE</sup>) mice were treated with PhZ or saline (Control), and blood was collected 2 (2d) and 7 (7d) days after the last injection. Complete blood count analysis was performed measuring RBCs (A), HCT (B), and Hb (C). Four to five animals for each treatment group were assessed. Statistical analyses were performed using Student's t test. Error bars indicate S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.005; n.s., not significant.
Figure 2.7. HIF-2 is a central regulator of erythropoiesis. Following hemolysis, the kidney and intestine experience hypoxia due to poor oxygen delivery. In the kidney, HIF-2α is stabilized, allowing it to bind to and activate the Epo promoter. In the small intestine, HIF-2α activates the Dcytb and Dmt1 genes, increasing iron absorption. Increased levels of circulating EPO and iron stimulate erythropoiesis and supply new erythrocytes with iron for hemoglobin production. ARNT, aryl hydrocarbon receptor nuclear translocator.
References


Chapter 3
Intestinal HIF-2α Promotes Iron Overload and Inhibits Iron Mobilization in β-thalassemia*

Abstract

β-thalassemia is a highly prevalent genetic disorder characterized by anemia caused by inefficient erythropoiesis, which leads to pathological iron overload. The increase in tissue iron is the major cause of morbidity and mortality in patients with β-thalassemia, however the mechanism is unclear. In this study we demonstrate that expression of the intestinal apical iron absorption genes ferric reductase (DcytB) and divalent metal transporter-1 (DMT1) were increased in β-thalassemic mice, correlating to the robust iron overload that is observed. Systemic hypoxia and intestinal hypoxia-inducible factor 2α (HIF-2α) activation was evident early in mouse models of β-thalassemia. Intestinal HIF-2α is a transcription factor that is essential for increased iron absorption during iron deficiency and erythropoiesis. To assess the role of the intestine and HIF-2α in thalassemic iron overload, mice with an intestinal HIF-2α disruption were assessed in two models of β-thalassemia. The increase in intestinal iron

absorptive genes was completely abolished in β-thalassemic mice containing a genetic disruption of intestinal HIF-2α. Moreover, these mice did not display tissue iron overload. Further mechanistic studies demonstrate that loss of intestinal HIF-2α signaling led to decreased iron absorption and increased iron mobilization from the liver without worsening the anemia. These data demonstrate that dysregulation of intestinal hypoxia and HIF-2α signaling is critical for iron overload during the progression of β-thalassemia and may be a novel therapeutic target.

Introduction

β-Thalassemia is a congenital blood disorder characterized by partial or complete loss of the β-globin gene product. Patients that exhibit suboptimal expression of the β-globin gene do not require blood transfusion for survival, but exhibit increased iron absorption and iron overload. This condition has been defined as β-thalassemia intermedia or non-transfusion dependent thalassemia (1). Increased iron loading in the disease, particularly in the liver, is associated with increased morbidity (2). Since the main cause of death in β-thalassemia is iron overload, a major focus has been to investigate the mechanisms involved in iron accumulation. A mouse model of β-thalassemia intermedia (Hbb<sup>th3/+</sup>) was developed that recapitulates the human phenotype (3). These mice have a heterozygous deletion in the β-minor and β-major chain of hemoglobin. Hbb<sup>th3/+</sup> mice develop severe anemia. Moreover, this model develops progressive iron overload in the absence of blood transfusions, demonstrating that increased iron
absorption plays a critical role in the disease pathogenesis. The mechanism of increased iron absorption is unclear and has not been targeted therapeutically in humans (4).

The liver is a central sensor and regulator of iron homeostasis, and examination of gene expression in thalassemic livers revealed a strong decrease in hepcidin expression (5, 6). Hepcidin is a small peptide produced in the liver and secreted into the bloodstream that controls systemic iron homeostasis (7). Hepcidin acts by binding to ferroportin (Fpn1), the only known mammalian iron exporter, which leads to its internalization and degradation (8). Fpn1 is primarily expressed on enterocytes and macrophages of the reticuloendothelial system, thus hepcidin acts to limit both duodenal iron absorption and release of iron from stores (9, 10). The prevailing thought is that the decrease in liver hepcidin during the progression of β-thalassemia is a critical factor in the hyperabsorption of iron. Increasing hepcidin levels inhibited iron overload in mouse models of β-thalassemia (11), however serum and tissue iron are not completely corrected. Moreover, the decrease in hepcidin expression is age dependent. A significant decrease in hepcidin expression is observed at 2-months of age, but normalized similar to wild-type mice at 5-months of age (6). Hepcidin expression does not correlate to intestinal Fpn1 expression in mouse models of β-thalassemia. An increase in intestinal Fpn1 expression is observed in older (12-months) but not young (2-months or 5-months) β-thalassemic mice, whereas tissue iron overload is evident in young mice. (6). Lastly, it has been shown that hepcidin exerts a limited effect on intestinal Fpn1 expression (12). Together, these data suggest
that other pathways in addition to hepcidin may mediate the increased iron absorption in β-thalassemia.

Intestinal oxygen sensing is essential for adaptive increases in iron absorption. Intestinal hypoxia-inducible factor 2 alpha (HIF-2α) is a critical regulator of iron absorption in the settings of iron deficiency, erythropoiesis, and hepcidin deficiency (13-16). HIF-2α is a member of the family of hypoxia-inducible transcription factors which control the cellular and systemic response to oxygen deficiency (17). Recently, we have shown that the apical ferric reductase and iron importer, DcytB and Dmt1 respectively (18, 19), and the basolateral iron exporter Fpn1 (20) are direct HIF-2α target genes (13, 14). Despite the critical role of these apical transporters in iron absorption, no studies have examined the expression of these genes in β-thalassemia in mechanistic detail. Since the β-thalassemic mice develop iron overload without blood transfusions, it is an ideal model for studying the role of iron absorption in the progression of this disease. In this study, an intestinal hypoxia-HIF-2α signaling axis was shown to be critical in the iron overload associated with β-thalassemia. Blocking intestinal HIF-2α signaling led to decreased iron absorption and increased iron mobilization from the liver, providing a novel therapeutic target for patients with β-thalassemia.

Results and Discussion

β-Thalassemic mice exhibit systemic and duodenal hypoxia

Consistent with previous reports, 2-month old Hbb<sup>th3/+</sup> mice had lower liver hepcidin expression compared to littermate controls Hbb<sup>+/+</sup> (Fig. 3.1A) (6).
However, no change in duodenal Fpn1 protein expression was observed, suggesting that mechanisms in addition to hepcidin are critical for the iron overload in β-thalassemic patients (Fig. 3.1B). β-thalassemia is characterized by severe anemia and iron overload due to ineffective erythropoiesis. A mouse model of anemia demonstrated increased iron absorption in an intestinal HIF-2α-dependent manner (15). In order to determine if mice with β-thalassemia are similarly hypoxic, the hypoxia reporter mouse, ODD-luc (21), was crossed with the Hbbth3/+ mice. In vivo luminescence imaging of mice from this cross demonstrated that 3-week old thalassemic mice are systemically hypoxic (Fig. 3.1C). In order to obtain better resolution, the duodenum was excised and imaged ex vivo. The duodenum was more hypoxic in the β-thalassemic model compared to wild-type littermates (Hbb+/+), suggesting that the increased iron absorption in β-thalassemia could be due to intestinal hypoxia (Fig. 3.1D). Since intestinal HIF-2α is a critical regulator of iron absorption (13, 14, 22), Western blot for duodenal HIF-2α was performed on Hbb+/+ and Hbbth3/+ mice. HIF-2α protein was stabilized in the duodenum of Hbbth3/+ mice, demonstrating that increased iron absorption could be mediated by HIF-2α (Fig. 3.1E).

**β-Thalassemic mice hyperabsorb iron in a HIF-2α-dependent manner**

Bone marrow transplantation (BMT) of Hbbth3/+ bone marrow into irradiated wild-type syngeneic mice leads to stable ineffective erythropoiesis and iron overload, hallmarks of β-thalassemia. This provides a powerful and rapid means to assess genes critical in the disease progression of β-thalassemia (3). In order to
investigate the role of intestinal HIF-2α in thalassemic iron absorption, intestinal HIF-2α knockout mice (Hif-2αΔIE) or littermate controls (Hif-2αF/F) were lethally irradiated and transplanted with Hbbth3/+ bone marrow. One-month following BMT, expression of duodenal iron transporter genes was analyzed using Quantitative Real-Time PCR (qPCR). The mRNA level of Fpn1 was not significantly changed, however the expression of the apical iron transporters Dmt1 and DcytB were significantly increased in Hif-2αF/F mice that were subjected to BMT from Hbbth3/+ donors (Table 3.1). These data demonstrate that the apical iron transporters are increased early in the pathogenesis of iron overload in β-thalassemia. To further understand the mechanistic basis for the increase in Dmt1 and DcytB, bone marrow from Hbbth3/+ mice was transplanted into Hif-2αΔIE mice. The increase in Dmt1 and DcytB was completely abolished in the Hif-2αΔIE mice (Table 3.1). Consistent with these changes, the increase in liver iron was entirely ameliorated in the Hif-2αΔIE mice. Interestingly, inhibition of iron absorption in Hif-2αΔIE mice did not adversely impact hematologic parameters in comparison to Hbbth3/+ transplanted bone marrow in wild-type littermates (Table 3.1).

*Increased iron absorption and tissue iron overload in a genetic model of β-thalassemia is dependent on HIF-2α.*

Radiation that is required for the BMT model of β-thalassemia can cause small intestinal atrophy and apoptosis (23). To confirm the role of the intestinal HIF-2α response in thalassemic iron overload without the confounding effects of
radiation, the \( \text{Hif-2a}^{\text{ΔIE}} \) and \( \text{Hif-2a}^{\text{F/F}} \) mice were crossed to the \( \text{Hbb}^{\text{th3/+}} \) mice. The increase in Dmt1 and DcytB mRNA that was observed in the BMT model of \( \beta \)-thalassemia was recapitulated in the \( \text{Hbb}^{\text{th3/+}}/\text{Hif-2a}^{\text{F/F}} \) mice and this increase was abolished in \( \text{Hbb}^{\text{th3/+}}/\text{Hif-2a}^{\text{ΔIE}} \) mice (Fig. 3A-B). Protein levels of the iron transporters Dmt1 and DcytB were decreased in 2-month old \( \text{Hbb}^{\text{th3/+}}/\text{Hif-2a}^{\text{ΔIE}} \) mice compared to \( \text{Hbb}^{\text{th3/+}}/\text{Hif-2a}^{\text{F/F}} \) mice (Fig. 3C). When assessed for iron content by enhanced Prussian blue staining, \( \text{Hbb}^{\text{th3/+}}/\text{Hif-2a}^{\text{F/F}} \) mice exhibited significantly higher accumulation of liver iron and spleen iron than wild-type littermates at 2-months of age (Fig. 3D). Disruption of intestinal HIF-2\( \alpha \) in the \( \beta \)-thalassemia model resulted in normalization of liver iron similar to the levels observed in wild-type mice (Fig. 3D). Furthermore, \( \text{Hbb}^{\text{th3/+}}/\text{Hif-2a}^{\text{ΔIE}} \) mice demonstrate a slight, but not statistically significant improvement in hematological parameters compared to \( \text{Hbb}^{\text{th3/+}}/\text{Hif-2a}^{\text{F/F}} \) mice (Fig. 3E-G).

Together, the data demonstrate that HIF-2\( \alpha \) is critical in the pathogenesis of iron accumulation in \( \beta \)-thalassemia. In addition, disruption of HIF-2\( \alpha \) did not worsen anemia, suggesting that HIF-2\( \alpha \) may be an efficacious therapeutic target in the treatment of \( \beta \)-thalassemia.

**Loss of intestinal HIF-2\( \alpha \) promotes tissue iron mobilization**

Our data demonstrate that activation of HIF-2\( \alpha \) signaling is critical for tissue iron accumulation in mouse models of \( \beta \)-thalassemia. Blocking intestinal HIF-2\( \alpha \) restricts iron absorption leading to a decrease in tissue iron accumulation.

However, it is not clear if existing iron that is accumulated in the liver can be
cleared or mobilized more efficiently through inhibition of intestinal HIF-2α and blocking iron absorption. To answer this question two models were used that increased liver iron independent of intestinal absorption. Phenylhydrazine (PhZ)–induced hemolysis leads to an increase in liver iron that is poorly mobilized over time (24). Hif-2α^{F/F} mice were treated with PhZ and liver iron was measured at two- and seven-days after treatment. Hif-2α^{F/F} mice had a significant increase in liver iron two-days after PhZ treatment, and levels remained high one-week following treatment (Fig. 3.3A). Similar to wild-type mice, PhZ treatment of Hif-2α^{ΔIE} mice causes substantial liver iron loading two-days following treatment (Fig. 3.3A). However, seven days after PhZ treatment liver iron content dropped to normal levels in the Hif-2α^{ΔIE} mice (Fig. 3.3A). In the second model, iron stores were boosted by a 100 µg injection of iron dextran four-days prior to bleeding for three consecutive days. Mice were then analyzed five-days after the last phlebotomy. Hif-2α^{F/F} mice effectively mobilized spleen iron, but were unable to mobilize liver iron (Fig. 3.3B). Hif-2α^{ΔIE} mice were able to effectively mobilize iron from the liver and spleen (Fig. 3.3B) Together these data demonstrate that inhibition of iron absorption by intestinal HIF-2α disruption allows rapid mobilization of iron to meet the demand of erythropoiesis. The data presented here demonstrate that inhibition of intestinal HIF-2α, in conjunction with existing therapies, would drastically accelerate mobilization of iron stores in the treatment of multiple diseases complicated by iron overload.

Methods
**Animals and Treatments**

\( Hif-2\alpha^{\Delta IE}, Hbb^{th3/+}, \) and ODD-luc animals were described previously (3, 14, 21). All mice were analyzed at 2-months of age. PhZ (Sigma, St. Louis MO) treatment was performed as previously described (15). For BMT, mice received 12 Gy of total body irradiation split into two doses of 6 Gy in an interval of 4-hours, and BMT was performed the day after irradiation. In vivo and ex vivo luminescence imaging was performed as previously described in 3-week old mice (21). Iron-dextran (Sigma) was diluted in sterile normal saline and administered by intrascapular injection. For phlebotomy experiments, 200-300 µL of blood was removed by submandibular bleeding for 3 consecutive days. Complete blood count (CBC) analysis was performed by the Unit for Laboratory Animal Medicine Pathology Core for Animal Research. Tissues were flash frozen in liquid nitrogen and stored at -80°C until analysis. 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.

**Western Blot**

Tissues were homogenized and lysed in radioimmunoprecipitation assay buffer (RIPA) for detection of HIF-2α and GAPDH. Membrane protein isolation was performed for the detection of Dmt1, DcytB, and Fpn1 as described previously (14). Antibodies against HIF-2α (Novus Biologicals, Littleton CO),
GAPDH (Santa Cruz Biotech, Santa Cruz CA), Dmt1, DcytB, and Fpn1 (Alpha Diagnostic International Inc., San Antonio TX) were used.

Iron Staining

Tissues were fixed in 10% Formalin in PBS overnight. Tissues were processed, embedded in paraffin, and sectioned at 7 µm thickness. Sections were deparaffinized and hydrated before incubation in a solution of 1% HCl and 1% potassium ferrocyanide for 30 minutes and then washed in deionized water before treatment with 0.025% 3,3-diaminobenzidine and .005% H$_2$O$_2$ for 30 minutes. Slides were counterstained with Nuclear Fast Red. Reagents were purchased from Sigma.

Real-time quantitative PCR (qPCR)

RNA isolation, reverse transcription, qPCR, and primers used for qPCR were described previously (15).

Non-heme iron quantitation

Tissues were weighed and homogenized in ddH$_2$O to a concentration of 100 mg/mL. The homogenate was mixed 1:1 with a solution of 1M HCl and 10% trichloroacetic acid (TCA) before incubation at 95º C for 1 hour. Debris was pelleted and iron in the supernatant was quantified using ferrozine in comparison to known standards.
**Statistics**

Results are expressed as mean ± S.D. P values were calculated by independent t-test, 1-way ANOVA, and two-way ANOVA. p<0.05 was considered significant.

**Acknowledgments**

This work was supported by the Rackham predoctoral fellowship to E.R.A., NIH grants (CA148828 and DK095201) and The University of Michigan Gastrointestinal Peptide Center to Y.M.S.
Figure 3.1. β-thalassemia leads to an increase in systemic and duodenal hypoxia. Control and thalassemic mice were analyzed 1-month after bone marrow transplant. A) mRNA was extracted from liver and hepcidin was quantified by qPCR. Expression was normalized to β-actin. B) Membrane protein was extracted from duodenum and Western Blot was performed for Fpn1. Loading was confirmed by coomassie stain. C) ODD-luc mice were crossed with Hbb^{th3/+} mice and examined at 3-weeks of age. In vivo analysis of hypoxia in Hbb^{th3/+} mice compared with littermate controls. D) Ex vivo imaging of the isolated duodenum from Hbb^{th3/+} mouse and a littermate control. E) Western blot for duodenal HIF-2α in wild-type and Hbb^{th3/+} mice at two-months of age. GAPDH was used as a loading control. Each bar represents the mean value ± S.D. *p<0.05 compared to Hbb^{th3/+} mice.
Table 3.1 Iron-related parameters in wild-type and thalassemic mice.

<table>
<thead>
<tr>
<th>Gene Expression</th>
<th>$Hif-2\alpha^{F/F}$</th>
<th>$Hbb^{th3/+}/Hif-2\alpha^{F/F}$</th>
<th>$Hbb^{th3/+}/Hif-2\alpha^{AIE}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duo Fpn1</td>
<td>1.00 ± 0.53</td>
<td>1.26 ± 0.61</td>
<td>1.37 ± 0.66</td>
</tr>
<tr>
<td>Duo Dmt1</td>
<td>1.00 ± 0.40</td>
<td>6.95 ± 3.03*</td>
<td>2.78 ± 1.62T</td>
</tr>
<tr>
<td>Duo DcytB</td>
<td>1.00 ± 0.04</td>
<td>4.62 ± 1.88*</td>
<td>1.26 ± 0.73T</td>
</tr>
<tr>
<td>CBC and Iron</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver Iron (µg/</td>
<td>0.09 ± 0.03</td>
<td>0.28 ± 0.04*</td>
<td>0.08 ± 0.09</td>
</tr>
<tr>
<td>mg tissue)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCT (%)</td>
<td>42.76 ± 1.58</td>
<td>31.50 ± 4.17*</td>
<td>29.80 ± 1.94*</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>14.34 ± 0.55</td>
<td>11.03 ± 1.13*</td>
<td>10.68 ± 0.46*</td>
</tr>
<tr>
<td>RBC (M/µL)</td>
<td>10.00 ± 0.58</td>
<td>7.53 ± 0.48*</td>
<td>7.36 ± 0.47*</td>
</tr>
</tbody>
</table>

* $< 0.05$ vs. $Hif-2\alpha^{F/F}$
$^T$ $< 0.05$ vs. $Hbb^{th3/+}/Hif-2\alpha^{F/F}$
Figure 3.2. Intestinal HIF-2α is essential for tissue iron overload in β-thalassemia. Hif-2α<sup>F/F</sup> and Hif-2α<sup>ΔIE</sup> mice crossed with the Hbb<sup>th3/+</sup> mice and analyzed at 2-months of age. qPCR analysis for duodenal A) DcytB and B) Dmt1. Expression was normalized to β-actin. C) Western blot analysis for Dmt1 and DcytB in Hbb<sup>th3/+</sup>/Hif-2α<sup>F/F</sup> and Hbb<sup>th3/+</sup>/Hif-2α<sup>ΔIE</sup> mice. Loading was determined by Coomassie stain. D) Hbb<sup>th3/+</sup>/Hif-2α<sup>F/F</sup> and Hbb<sup>th3/+</sup>/Hif-2α<sup>ΔIE</sup> mice were analyzed for spleen and liver iron content by DAB-enhanced prussian blue staining. Liver non-heme iron quantitation is in parenthesis below stained sections. E) Red blood cell (RBCs) count, F) hemoglobin (Hb), G) and hematocrit were analyzed from Hbb<sup>th3/+</sup>/Hif-2α<sup>F/F</sup> and Hbb<sup>th3/+</sup>/Hif-2α<sup>ΔIE</sup> mice. Each bar represents the mean value ± S.D. not significant (n.s), *p<0.05 compared to Hif-2α<sup>F/F</sup> mice, and †p<0.05 compared to Hbb<sup>th3/+</sup>/Hif-2α<sup>F/F</sup> mice.
Figure 3.3. Intestinal disruption of HIF-2α leads to rapid mobilization of liver iron. A) *Hif-2α<sup>F/F</sup>* and *Hif-2α<sup>ΔIE</sup>* mice were treated with phenylhydrazine (PhZ) and liver non-heme iron was quantitated 2 and 7 days following the last PhZ injection. B) *Hif-2α<sup>F/F</sup>* and *Hif-2α<sup>ΔIE</sup>* mice were loaded with 100 µg iron dextran, bled 3 consecutive days, and allowed to recover for 5 days. Liver and spleen non-heme iron content was quantitated from phlebotomized *Hif-2α<sup>F/F</sup>* and *Hif-2α<sup>ΔIE</sup>* mice. Each bar represents the mean value ± S.D. *p<0.05 compared to untreated or un-bled *Hif-2α<sup>F/F</sup>* and *Hif-2α<sup>ΔIE</sup>* mice.
References


Chapter 4

The Hypoxia-Inducible Factor-C/EBPα Axis Controls Ethanol-Mediated Hepcidin Repression

Abstract

Hepcidin is a liver-derived peptide hormone and the master regulator of systemic iron homeostasis. Decreased hepcidin expression is a common feature in alcoholic liver disease (ALD) and in mouse models of ethanol loading. Dysregulation of hepcidin signaling in ALD leads to liver iron deposition, which is a major contributing factor to liver injury. The mechanism by which hepcidin is regulated following ethanol treatment is unclear. An increase in liver hypoxia was observed in an acute ethanol-induced liver injury model. The hypoxic response is controlled by a family of hypoxia-inducible transcription factors (HIFs), which are composed of an oxygen-regulated alpha subunit (HIFα) and a constitutively present beta subunit, aryl hydrocarbon receptor nuclear translocator (HIFβ/Arnt). Disruption of liver HIF function reversed the repression of hepcidin following ethanol loading. Mouse models of liver HIF overexpression demonstrated that both HIF-1α and HIF-2α contribute to hepcidin repression in vivo. Ethanol treatment led to a decrease in CCAAT-enhancer-binding protein alpha (C/EBPα)

* This chapter represents a published manuscript: Anderson ER, Taylor M, Xue X, Martin A, Moons DS, Omary MB, Shah YM (2012). “The hypoxia-inducible factor-C/EBPα axis controls ethanol-mediated hepcidin repression.” Molecular and Cellular Biology
protein expression in a HIF-dependent manner. Importantly, adenoviral rescue of C/EBPα in vivo ablated the hepcidin repression in response to ethanol treatment or HIF overexpression. These data provide novel insight into the regulation of hepcidin by hypoxia and indicate that targeting HIFs in the liver could be therapeutic in ALD.

**Introduction**

Patients with alcoholic liver disease (ALD) accumulate iron in the liver (1). Free iron enhances reactive oxygen species (ROS) production in the liver, leading to oxidative stress, which is a major contributing factor to alcohol-induced liver injury (2). The development of liver fibrosis positively correlates with liver iron staining in ALD, and the presence of iron deposits in the liver of patients with alcoholic cirrhosis is predictive of death (3). There is compelling evidence that iron-mediated oxidative stress may be an important pathological mechanism for the increased incidence of hepatocellular carcinoma in individuals with hepatic iron overload who consume alcohol (4). Lastly, hepatic iron overload increases the risk of insulin resistance and diabetes due to hepatic inflammation (5).

Hepcidin is a small antimicrobial peptide produced in the liver and secreted into the bloodstream which regulates systemic iron homeostasis (6, 7). Hepcidin functions by binding to the only known mammalian iron exporter, ferroportin (FPN), which leads to its internalization and degradation (8). FPN is primarily expressed on macrophages of the reticuloendothelial system and absorptive enterocytes in the small intestine (9). Thus, hepcidin acts to restrict
intestinal iron absorption and prevent release of iron from stores. Conversely, hepcidin deficiency leads to increased iron absorption and mobilization of iron stores, which can cause iron overload (9, 10). Previous publications have shown that in rodents and humans, hepcidin is downregulated in response to ethanol treatment (11-18). Moreover, it has been shown that the increase in liver iron following alcohol consumption in ALD patients is directly due to low hepcidin expression (11-18). However, the mechanism(s) by which hepcidin is dysregulated following ethanol exposure remains unclear.

The present study demonstrated that alcohol treatment in mice led to a robust hypoxic response compared to that seen with vehicle-treated mice and that this hypoxia was associated with a strong inhibition of hepcidin expression. Changes in gene expression during hypoxia are primarily controlled by a family of hypoxia-inducible transcription factors (HIFs) (19). HIFs consist of an oxygen-regulated alpha subunit (HIFα) and a constitutively expressed beta subunit, aryl hydrocarbon receptor nuclear translocator (HIFβ/Arnt). Under conditions of normal oxygen tension, the HIFα subunit is hydroxylated by prolyl hydroxylase enzymes (PHDs) at specific proline residues. This modification is recognized by the protein coded by the Von Hippel-Lindau tumor suppressor gene (Vhl), which is part of an E3 ubiquitin ligase complex. VHL facilitates ubiquitination of the HIFα subunit, which leads to its subsequent degradation by the proteasome. Genetic disruption of Vhl results in the presence of constitutively active HIF in vivo and is a well-characterized model to study HIF function (20, 21). Under conditions of low oxygen, PHD activity is decreased and the HIFα subunit is stabilized, which
allows it to accumulate in the cytoplasm and then translocate into the nucleus
and heterodimerize with Arnt. The HIF complex binds to promoters of many
genomes involved in the hypoxic response and activates their transcription (22).

Hypoxia is known to repress hepcidin, and several mechanisms for the
hypoxic repression have been proposed (23-26). However, the precise
mechanism is still unclear. Moreover, the role of hypoxia in the repression of
hepcidin in ALD has not been examined. The present report clearly demonstrates
that alcohol-induced hepcidin repression can be blocked by a conditional
disruption of Arnt in the hepatocytes, which leads to total loss of HIF
transcriptional activity. Furthermore, we identified a novel signaling cascade that
involved HIF-mediated degradation of CCAAT-enhancer-binding protein alpha
(C/EBPα). C/EBPα overexpression rescued the repression of hepcidin observed
with ethanol loading and partially reversed the repression caused by HIF
overexpression. These data demonstrate a novel connection between hypoxia
and hepcidin expression in vivo and could lead to development of therapies
targeting HIFs or C/EBPα in the liver of ALD patients.

Materials and Methods

Animal experiments

VhlF/F, VhlF/F Hif1αF/F, VhlF/F Hif2αF/F, and VhlF/F ArntF/F mice were
previously described (27) and are in the same genetic background
(129S6/SvEv). Temporal liver-specific knockouts constructed using serum
albumin enhancer-driven tamoxifen-inducible estrogen receptor-conjugated Cre
(SA-Cre-ERT2) were achieved as previously described (28). In these animals, tamoxifen treatment causes the estrogen receptor Cre fusion protein to translocate to the nucleus, which leads to Cre-mediated excision of floxed genes. The hypoxia reporter mouse (oxygen-dependent-degradation domain–luc [ODD-luc]) animal model was obtained from Jackson Laboratories (Bar Harbor, ME) and has been described previously (29). For viral expression, animals were administered 1 × 10^9 PFU of purified virus in 200 µl of normal saline solution by tail vein injection. Blood was collected by submandibular bleeding. For ex vivo imaging experiments, animals were administered d-luciferin (Promega Corp., Madison, WI) (50 mg/kg of body weight) by intraperitoneal injection, and tissues were imaged after 15 min using an IVIS 200 imaging system (Caliper Life Sciences, Hopkinton, MA). For tamoxifen experiments, tamoxifen (Sigma-Aldrich, St. Louis, MO) (2 mg in corn oil) was injected intraperitoneally once daily for two consecutive days and mice were sacrificed 5 days or 2 weeks later. For ethanol experiments, mice were gavaged every 12 h with increasing doses of 25% ethanol starting at 5 mg/kg and increasing by 0.5-mg/kg increments each treatment. Animals were euthanized 12 h after the last gavage of 7.5 mg/kg. The mice were housed in a light- and temperature-controlled room and were given water and chow ad libitum. Tissues were harvested and used fresh or were flash-frozen in liquid nitrogen and stored at −80°C for future use. 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.
**Cell culture**

Huh7 and HEK293A cells were maintained at 37°C in 5% CO$_2$ and 21% O$_2$. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics-antimycotics. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, and Western blot analysis was performed. For transfection experiments, HEK293A cells were transfected using FuGENE 6 transfection reagent (Promega Corporation, Madison, WI) 24 h before hypoxia treatment. For adenoviral infection experiments, cells were treated at a multiplicity of infection (MOI) of virus of 100 24 h before sample collection. Viruses were purified by the University of Michigan Vector Core. MG132 (Cayman Chemical, Ann Arbor, MI) was used at a concentration of 10 µM for 24 h. For hypoxia experiments, cells were incubated in 1% O$_2$ and 5% CO$_2$ with balance N$_2$ at 37°C for 24 h.

**Western blotting**

Tissues were homogenized and lysed in RIPA buffer for whole-cell extracts, and nuclear proteins were isolated using an NE-PER nuclear extraction kit (Pierce, Rockford, IL). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes using standard methods. Membranes were incubated with antibodies against C/EBPα (sc-61), histone H1 (sc-8030), HIF-1α (sc-10790), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sc-25778; Santa Cruz Biotech, Santa Cruz, CA). Histone H3 (catalog no. 4499) and pSmad1, -5, and -8 (pSmad1/5/8; catalog no. 9511) antibodies were from Cell Signaling
Technology (Danvers, MA). HIF-2α (NB100-122) antibody was from Novus (Novus Biologicals, Littleton, CO). All antibodies were used at a 1:1,000 dilution.

**Real-time quantitative PCR**

RNA from fresh or frozen tissue and cells was isolated using Isol-RNA lysis reagent (Prime, Gaithersburg, MD) and subjected to reverse transcription using Moloney murine leukemia virus (MMLV) reverse transcriptase (RT; Fisher Scientific, Waltham, MA). cDNA was quantified using SYBR green dye and run on a 7900HT Fast real-time RT-PCR system (primers are listed in Table 4S1 in the supplemental material). Threshold cycle (C<sub>T</sub>) values were normalized to β-actin and expressed as fold differences from control values.

**Site-directed mutagenesis**

Site-directed mutagenesis was performed using a QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) (primers are listed in Table 4S1 in the supplemental material).

**Measurement of serum EPO**

Serum erythropoietin (EPO) was measured using a mouse enzyme-linked immunosorbent assay (ELISA) kit per the manufacturer's recommendation (Abcam, Cambridge, MA).

**Results**

*Repression of hepcidin during ethanol loading is independent of the Bmp/Smad signaling pathway*
Mice were treated with increasing concentrations of ethanol by oral gavage every 12 h for a total of 5 doses. This model parallels the binge drinking observed in a significant proportion of ALD patients. Livers collected from control and ethanol-loaded mice were examined for gene expression. Hepcidin expression was strongly repressed in response to ethanol treatment (Fig. 4.1A), whereas EPO expression in the kidney was not statistically significant ($P = 0.23$) (Fig. 4.1A). In order to determine the mechanism by which hepcidin is repressed, a number of known hepcidin regulatory genes were examined by real-time quantitative PCR (qPCR). No changes in bone morphogenetic protein (Bmp) ligands or their respective receptors were observed following ethanol treatment (Fig. 4.1B). There was an increase in transferrin receptor 1 (Tfr1) expression (Fig. 4.1C). Moreover, ethanol treatment significantly decreased expression of Tmprss6, which is a critical regulator of hepcidin expression (Fig. 4.1C) (30). However, these changes could not account for the repression of hepcidin expression seen following ethanol treatment, as Tmprss6 is a negative regulator of hepcidin expression and an increase in Tfr1 expression should increase liver iron uptake and thus increase hepcidin expression. There was a significant repression of mothers against decapentaplegic homolog 9 (Smad8) (Fig. 4.1D). The Smad family of transcription factors is well characterized in their regulation of hepcidin expression (31). However, when phosphorylated proteins Smad1, -5, and -8 were examined by Western blot analysis, no significant changes were found (Fig. 4.1E), demonstrating that an alternate mechanism is responsible for the repression of hepcidin.
**Hypoxic signaling is critical in ethanol-mediated hepcidin repression**

In order to test the possibility that hypoxia is involved in the repression of hepcidin during ethanol loading, the hypoxia reporter mouse (ODD-luc) was utilized. This mouse model expresses a fusion protein consisting of the oxygen-dependent-degradation domain (ODD) from HIF-1α fused to luciferase. This protein is constitutively expressed in all tissues but is stable only under conditions where endogenous HIF would also be stabilized. Thus, this model allows detection of hypoxia in live mice or specific detection of hypoxia in a tissue ex vivo (29). ODD-luc mice treated with ethanol showed a strong induction of hypoxia in the liver, as seen by ex vivo imaging (Fig. 4.2A). In addition, Western blot analysis was performed on liver nuclear extracts from control and ethanol-treated mice. HIF-2α expression was strongly increased in response to ethanol treatment (Fig. 4.2B). HIF-1α was not detected, although it is possible that HIF-1α is stabilized acutely following ethanol treatment and is degraded more rapidly than HIF-2α. To assess if liver hypoxia is critical for hepcidin repression following ethanol treatment, intravenous administration of adenoviral Cre, which traffics specifically to the liver (32), was used to induce recombination in mice containing loxP recombination sites flanking exon 6 of Arnt, referred to as Arnt^F/F mice (33). Arnt is the obligate heterodimer partner for HIF1α, HIF2α, and HIF3α; thus, disruption results in complete inactivation of HIF signaling. This model of Arnt knockdown is preferable to an albumin-Cre-mediated recombination, as it eliminates the possibility of developmental effects on hepcidin expression (34). Arnt knockdown was validated by qPCR (Fig. 4.2C). Control mice with intact
Arnt expression in the liver had decreased hepcidin expression following ethanol treatment. Disruption of Arnt in the liver completely blocked repression of hepcidin in response to ethanol loading (Fig. 4.2C). These data suggest that HIF signaling is essential in hepcidin repression by ethanol treatment.

Repression of hepcidin expression by HIFs does not require erythropoiesis

Hypoxia is a well-known but poorly understood repressor of hepcidin expression. An initial connection between hypoxia and hepcidin expression was made in which direct HIF binding to the hepcidin promoter led to repression (25). However, this finding was not uniformly observed (26), thereby leaving the relationship between HIF and hepcidin unclear. A recent study demonstrated that overexpression of HIFs in the liver caused a decrease in hepcidin expression that required erythropoiesis (35). However, reports of studies that examined erythropoietic hepcidin repression make no mention of the repression of hepcidin in alcohol loading (36-38). In addition, a study of VhIR200W homozygote humans, who have activated HIF signaling, demonstrated reduced hepcidin levels that did not correlate with an increase in serum EPO, suggesting that HIFs could repress hepcidin independently of erythropoiesis (39).

To understand the role of HIFs in hepcidin repression and to avoid the confounding developmental defects of Vhl deletion, a model of inducible liver gene knockout was used (28). Tissues that lack Vhl are unable to degrade the HIFα subunit under normal oxygen conditions, leading to constitutive HIF stability and activity. VhfF/F mice were crossed with SA-Cre-ERT2 transgenic mice to generate a temporal and conditional disruption of Vhl (VhlLivKO) using the
tamoxifen-inducible cre fusion protein (40). In these mice, Cre-mediated recombination specifically in the liver is induced by tamoxifen treatment, allowing temporal control over gene expression. Moreover, to assess the influence of HIF-dependent pathways on iron-regulatory gene expression in the liver, mice with a double disruption of Vhl and Hif-1α or Hif-2α (Vhl/Hif-1αLivKO and Vhl/Hif-2αLivKO) were also assessed. These compound knockouts remove the possibility that the effects of Vhl ablation on hepcidin expression are the result of a HIF-independent mechanism. By the use of compound conditional liver knockouts, mice with loss of Vhl alone or in conjunction with HIF-1α demonstrated a robust decrease in hepcidin levels, high EPO expression, and a large increase in circulating EPO 2 weeks after induction of Cre by tamoxifen treatment (Table 4.1). Double disruption of Arnt and Vhl (Vhl/ArntLivKO) completely inhibits all HIFα function, since it is an obligate heterodimer partner for both HIF-1α and HIF-2α. The livers of the Vhl/ArntLivKO mice showed a loss of hepcidin repression, demonstrating a HIF-dependent mechanism. Surprisingly, compound deletion of Vhl and HIF-2α still led to strong hepcidin repression despite the absence of any increase in circulating EPO levels (Table 4.1). These data suggest that there is a HIF-dependent mechanism of hepcidin repression that does not require enhanced erythropoiesis.

Examination of gene expression in the liver Vhl knockout mice after 5 days demonstrated no change in Bmp ligand or receptor expression (Fig. 4.3A). Consistent with ethanol treatment, Tfr1 expression was increased and Smad8 mRNA expression was significantly decreased following liver Vhl knockout (Fig. 84
However, there was no detectable decrease in the levels of phosphorylated Smad proteins (Fig. 4.3D). These data establish that there is a HIF-dependent but Bmp/Smad- and erythropoietin-independent pathway for hepcidin repression.

A decrease in liver C/EBPα protein expression is responsible for hepcidin repression by HIFs

C/EBPα is a transcription factor that is also critical in maintaining basal hepcidin expression (41). In order to determine whether C/EBPα is involved in the repression of hepcidin expression by HIFs, conditional deletion of Vhl was performed for different amounts of time. Five days following induction of Cre recombination by tamoxifen treatment, hepcidin expression was strongly repressed without a decrease in C/EBPα mRNA levels (Fig. 4.4A). Two weeks after recombination, hepcidin mRNA and C/EBPα mRNA levels were both decreased (Fig. 4.4A). Examination of C/EBPα protein expression by Western blot analysis showed that liver Vhl knockout alone or in conjunction with HIF-1α led to a significant decrease in the protein expression (Fig. 4.4B). EPO has been shown to decrease C/EBPα expression (42). To completely rule out the role of EPO, C/EBPα protein was examined in the mice with the compound Vhl and HIF-2α knockout. These mice had no increase in EPO or erythropoiesis. The Vhl and HIF-2α double-knockout mice demonstrated lower C/EBPα protein expression (Fig. 4.4B), whereas the Vhl and Arnt double-knockout mice showed no change in C/EBPα protein expression (Fig. 4.4B). C/EBPα protein expression was decreased as early as 1 day after induction of Cre by tamoxifen, demonstrating
that the decrease in C/EBPα expression is an early event (Fig. 4.4C). To test whether C/EBPα could rescue hepcidin expression in liver Vhl knockout mice, animals were infected with an adeno-viral construct expressing C/EBPα. Successful adenoviral overexpression was confirmed by Western blot analysis (Fig. 4.4D). Infection of Vhl knockout animals with C/EBPα led to a substantial derepression of hepcidin expression (Fig. 4.4E). To confirm that the green fluorescent protein (GFP)-expressing adenovirus infection did not affect hepcidin levels, mice infected with adenovirus expressing GFP demonstrated levels of expression of hepcidin similar to those seen with uninfected mice (Fig. 4S1). These data demonstrate that the repression of hepcidin expression by HIFs is mediated by a decrease in C/EBPα protein expression.

_Hypoxia promotes proteasomal degradation of C/EBPα_

Since the decrease in C/EBPα protein expression was observed independently of a change in mRNA, the possibility that hypoxia affects C/EBPα protein stability was tested. Huh7 cells, representing a human hepatoma cell line, have been used in multiple studies to examine regulatory mechanisms of hepcidin expression (9, 43-45). Treatment of Huh7 cells with hypoxia caused no change in endogenous hepcidin expression (Fig. 4.5A). However, adenoviral overexpression of C/EBPα increased hepcidin expression, and this increase was abolished by hypoxia treatment (Fig. 4.5A). In addition, hypoxia treatment did not decrease endogenous or exogenous C/EBPα mRNA expression (Fig. 4.5B). Western blot analysis of Huh7 protein extracts showed that hypoxia led to a decrease in exogenous levels of C/EBPα (Fig. 4.5C). There was no substantial
decrease in the presence of endogenous C/EBPα, which is expressed at very low levels in this cell line. This could explain why hypoxia did not decrease endogenous hepcidin expression. Studies have shown that C/EBPα is degraded through the ubiquitin-proteasome pathway (46). To determine if this process is responsible for hypoxia-dependent C/EBPα destabilization, cultured Huh7 cells were treated with the proteasome inhibitor MG132 under hypoxia conditions. Proteasome inhibition drastically increased C/EBPα protein stability under hypoxia conditions (Fig. 4.5D). A previous publication showed that phosphorylation at serine 193 was critical for proteasomal degradation of C/EBPα (46). To determine if phosphorylation at this site was important for HIF-mediated C/EBPα degradation, we transfected HEK293A cells with either a wild-type or an S193A-mutated C/EBPα expression construct and subjected the cells to normoxia and hypoxia. HEK293A cells were used, since these cells are easily transfected with plasmid DNA. Similar to Huh7 cells, wild-type C/EBPα is degraded under hypoxia conditions in HEK293A cells (Fig. 4.5E). However, the S193A mutation abolished the protein degradation, indicating that phosphorylation at this site is essential for HIF-mediated C/EBPα degradation. Cyclin-dependent kinase 4 has been shown to phosphorylate C/EBPα at S193 and is regulated by cyclin D. To determine whether HIFs could cause an increase in CDK activity, Western blot analysis was performed on liver whole-cell extracts from liver Vhl knockout mice. These animals have an increase in liver cyclin D1 protein (Fig. 4.5F). Moreover, an increase in cyclin D1 protein was also detected in the livers of ethanol-treated mice (Fig. 4.5G). In conjunction with the in vivo
data, these experiments demonstrate that HIF-dependent hepcidin repression is mediated by destabilization of C/EBPα protein.

*A HIF-dependent decrease in C/EBPα protein stability is responsible for hepcidin repression in ethanol loading*

To determine whether the HIF-mediated C/EBPα protein decrease was relevant to hepcidin repression following ethanol treatment, Western blot analysis for C/EBPα was performed on livers from control and ethanol-treated mice. Ethanol treatment caused a decrease in C/EBPα protein expression in the liver (Fig. 4.6A). However, mice deficient for Arnt expression in the liver showed no decrease in C/EBPα protein expression following ethanol treatment (Fig. 4.6A). To test if C/EBPα overexpression could rescue the ethanol-induced repression of hepcidin, mice were infected with an adenoviral vector expressing C/EBPα and then treated with ethanol. C/EBPα overexpression prevented the decrease in hepcidin expression following ethanol treatment (Fig. 4.6B).

**Discussion**

Iron overload in ALD contributes to oxidative stress and tissue damage (1). Investigating the mechanisms behind ethanol-mediated hepcidin repression could lead to new therapies to combat iron overload in ALD. This report provides novel insight not only into the mechanisms of hepcidin repression following ethanol loading but also into the role of HIFs in controlling hypoxic hepcidin repression. Mechanisms of hepcidin repression that involve hypoxia, such as high-altitude exposure, hemolytic anemia, and phlebotomy, are all complicated
by increased erythropoiesis (36, 47, 48). This study took advantage of a
temporal liver-specific double disruption of Vhl and HIF-2α, which strongly
represses hepcidin expression without an increase in the presence of circulating
erthropoietin. This model allowed delineation of hypoxic and erythropoietic
pathways for hepcidin expression. Also, the ethanol-loading model described in
this report represents the first demonstration of the physiological consequence of
liver hypoxia in the absence of increased erythropoiesis.

The present report demonstrates that ethanol-mediated repression of
hepcidin is accomplished through a HIF-dependent decrease in C/EBPα protein
stabilization. The proposed mechanism is that ethanol treatment stabilizes HIF in
hepatocytes, leading to C/EBPα degradation by the proteasome and thus
preventing it from activating hepcidin (Fig. 4.6C). The HIF-dependent changes
that lead to this destabilization remain unclear. Previous studies have shown that
C/EBPα can be phosphorylated by cyclin-dependent kinases (Cdk) at serine 193,
subsequently leading to its ubiquitination and degradation through the ubiquitin-
proteasome pathway (46). Mutation of serine 193 to alanine blocks hypoxia-
induced degradation, suggesting that the HIF and Cdk pathways may interact to
control C/EBPα stability. Notably, cyclin D1, an important cofactor for Cdk
activity, is upregulated in a HIF-dependent manner (49, 50).

Prior studies have shown that ethanol treatment can lead to a decrease in
C/EBPα mRNA and DNA-binding activity (11, 15, 51). Moreover, EPO has been
shown to decrease levels of C/EBPα mRNA. In the present study, 5 days after
induction of HIF overexpression, in experiments utilizing the conditionally and
temporally disrupted Vhl mice, hepcidin expression was potently repressed and
C/EBPα mRNA expression was unchanged (Fig. 4.4A). Two weeks after HIF
overexpression, both C/EBPα expression and hepcidin mRNA expression were
strongly repressed (Fig. 4.4A). At that later time point, the decrease in C/EBPα
mRNA expression was likely mediated by EPO signaling through its receptor, as
a previous publication demonstrated that an EPO receptor antibody blocked
erthropoietic C/EBPα mRNA repression (42). The rapid destabilization of
C/EBPα protein by HIFs represents a novel mechanism for HIF-C/EBPα
interactions. The data presented herein demonstrate that HIF-mediated C/EBPα
destabilization plays an important role in the liver hypoxic response in vivo. This
raises the question of whether the mechanism for C/EBPα degradation during
hypoxia is intact in other tissues as well. For example, mutations in C/EBPα have
been identified in multiple cases of acute myeloid leukemia (AML) (52) and
C/EBPα is dysregulated in more than half of patients with the disease (53). In
addition, it has been shown that HIF inhibition can selectively kill human AML
cancer stem cells (54). Further exploration of this pathway in additional cell
types could lead to a better understanding of the pathogenesis of C/EBPα-related
diseases, which could improve therapy.

HIF repression of hepcidin is a well-documented observation, and several
putative mechanisms have been proposed (23-26). Through an in vivo
mechanistic assessment, the data in the present paper identify a novel
erthropoietin-independent mechanism by which HIF represses hepcidin
expression. It is possible that low levels of hepcidin allow increased activity of
iron-dependent enzymes and processes, which could be beneficial, but the physiological role of this repression is completely unclear. No publications to date have shown the importance of HIF repression of hepcidin in maintaining systemic iron homeostasis. Although its normal physiological role is not apparent, the present report definitively demonstrates that alcohol induces pathological hypoxia and that HIF activity is essential in alcohol-mediated hepcidin repression. This finding may be critical in identifying therapies to limit iron-induced injury in ALD patients.

**Acknowledgments**

This work was supported by a Rackham predoctoral fellowship (E.R.A.), NIH grants CA148828 and DK095201 and the University of Michigan Gastrointestinal Peptide Center (Y.M.S.), NIH grant DK47918 (M.B.O.), and institutional NIH P30 grant DK34933. We declare no conflicts of interest related to this work.
Fig. 4.1. Repression of hepcidin during ethanol treatment is independent of Bmp/Smad and EPO signaling. Following ethanol treatment, gene expression was determined by qPCR analysis of (A) kidney (Kid) EPO and liver (Liv) EPO and hepcidin (Hepc) expression, (B) liver expression of Bmp ligands and receptors, (C) hepcidin regulatory genes, and (D) Smads. mRNA expression was normalized to β-actin. (E) Western blot analysis of pSmad1/5/8 protein from whole-cell extracts of liver following ethanol treatment. Loading was normalized to GAPDH. n.d., not detected. n = 6 to 8 animals per group. Each bar represents the mean ± standard error (SE). *, P < 0.05 as determined by Student's t test; **, P < 0.01.
Fig. 4.2. HIF signaling in the liver is required for repression of hepcidin expression by ethanol treatment. ODD-luc mice were treated with ethanol and imaged using an IVIS 200 imaging system. (A) Representative livers from two control and two ethanol-treated ODD-luc mice. (B and C) Western blot analysis of HIF-1α, HIF-2α, and histone 3 in liver nuclear extracts from control (Con) and ethanol-treated (EtOH) mice. Liver-specific Arnt disruption was induced by tail vein injection of adenovirus expressing Cre recombinase (Ad-Cre) 3 days before ethanol treatment, and (B) hepcidin and (C) Arnt gene expression was analyzed in control mice (Arnt^F/F) and Arnt knockout mice (Arnt^F/F+Ad-Cre). Gene expression was normalized to β-actin. n = 6 to 8 animals per group. Each bar represents the mean ± SE. Asterisks denote statistical significance versus control results at P < 0.05 as determined by Student’s t test.
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**Liver EPO\(^T\)**

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**Circulating EPO\(^L\)**

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\(^T\)mRNA normalized to \(\beta\)-actin

\(^L\)units of ng/mL

*denotes significance at \(p < .05\) versus control by Student’s T-test

** denotes significance at \(p < .01\) versus control by Student’s T-test

Table 4.1 HIF represses hepcidin through EPO-dependent and – independent mechanisms
Fig. 4.3. Hepcidin repression by HIFs is independent of Bmp/Smad signaling. (A, B, and C) Vhl^{F/F} and Vhl^{F/F;AlbERcre} mice were administered tamoxifen (TM), and then mRNAs from livers of the mice were examined 5 days later by qPCR analysis of (A) Bmp ligand and receptor expression, (B) hepcidin regulatory genes, and (C) Smads. mRNA expression was normalized to β-actin. (D) Western blot analysis was performed on whole-cell extracts of livers from Vhl^{F/F} and Vhl^{F/F;AlbERcre} mice treated with tamoxifen (TM) for 5 days and examined for pSmad1/5/8 expression. Loading was normalized to GAPDH. n = 6 to 8 animals per group. Each bar represents the mean ± SE. Asterisks denote statistical significance versus control results at P < 0.05 as determined by Student's t test.
Fig. 4.4. Hepcidin repression by HIF requires a decrease in C/EBPα protein expression. (A) Vhl<sup>F/F</sup> and Vhl<sup>F/F;AlbERcre</sup> mice were treated with tamoxifen (TM) and sacrificed either 5 days or 2 weeks following Cre recombination, and liver expression of C/EBPα and hepcidin was determined by qPCR and normalized to β-actin. (B) Western blot analysis was performed on nuclear extracts to detect C/EBPα in Vhl<sup>F/F</sup> and Vhl<sup>F/F;AlbERcre</sup> mice or in the Vhl compound-disrupted mice following 2 weeks of tamoxifen (TM) treatment. (C) C/EBPα protein expression in the livers of Vhl<sup>F/F</sup> and Vhl<sup>F/F;AlbERcre</sup> mice was examined by Western blot analysis 1 day following tamoxifen (TM) treatment. (D) Mice were infected with recombinant C/EBPα adenovirus (Ad-C/EBPα) and 3 days later examined for C/EBPα protein expression by Western blot analysis. Loading was normalized to histone H1 or histone H3. (E) Vhl<sup>F/F</sup> and Vhl<sup>F/F;AlbERcre</sup> mice were infected with adenovirus for 3 days and then treated with tamoxifen (TM) for an additional 5 days, and hepcidin expression was assessed by qPCR normalized to β-actin. n = 6 to 8 animals per group. Each bar represents the mean ± SE. Single asterisks denote statistical significance versus control results at P < 0.05 as determined by Student's t test. **, P < 0.01.
Proteasomal degradation of C/EBPα is necessary for HIF-dependent hepcidin repression. (A, B, and C) Huh7 cells were infected with Ad-C/EBPα at an MOI of 100 1 day before exposure to hypoxia (1% O₂) for 24 h. Gene expression was examined by qPCR for (A) hepcidin and (B) C/EBPα, and (C) Western blot analysis was performed on whole-cell extracts to detect C/EBPα. (D) Western blot analysis for C/EBPα in Huh7 cells infected with Ad-C/EBPα at an MOI of 100 1 day before exposure to hypoxia (Hyp) (1% O₂) and 10 µM MG132 for 24 h. (E) HEK293A cells were transfected with wild-type or S193A mutant C/EBPα expression constructs and exposed to normoxia (Norm) or hypoxia (Hyp) for 24 h, and C/EBPα expression was examined by Western blot analysis. (F) Liver Vhl knockout mice were analyzed for liver cyclin D1 expression by Western blot analysis in whole-cell extracts 2 weeks after Cre recombination. (G) Control and ethanol-treated animals were examined for liver cyclin D1 expression by Western blot analysis in whole-cell extracts. Loading was normalized to GAPDH. n = 3 samples per group; the experiments were repeated at least three times. Each bar represents the mean ± SE. Single asterisks denote statistical significance versus control results at $P < 0.05$ as determined by Student's $t$ test. **, $P < 0.01$. 
Fig. 4.6. C/EBPα overexpression in vivo blocks repression of hepcidin by ethanol treatment. (A) Liver-specific Arnt disruption was induced by tail vein injection of adenovirus expressing Cre recombinase (Ad-Cre) 3 days before ethanol treatment, and Western blot analysis was performed to detect C/EBPα in control and ethanol-treated livers. Protein loading was normalized to histone H3. (B) Mice were infected with adenovirus expressing C/EBPα (Ad-C/EBPα) 3 days before ethanol treatment, and hepcidin expression was analyzed by qPCR. Expression was normalized to β-actin. (C) A schematic diagram illustrating the mechanism by which alcohol-induced activation of HIF controls hepcidin repression through Cdk-dependent C/EBPα degradation. n = 6 to 8 animals per group. Each bar represents the mean ± SE. **, P < 0.01 (Student's t test).
Fig. 4S1. Adenoviral infection does not induce hepcidin expression. Mice were infected with GFP-expressing adenovirus and analyzed 3 days after infection. Gene expression was examined by qPCR for hepcidin and normalized to β-actin. n = 3 animals per group.
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<td>Human β-actin R</td>
<td>GCACAGACCTCGCCTT</td>
</tr>
<tr>
<td>Mouse β-actin F</td>
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<tr>
<td>Human hepc F</td>
<td>CTCCTTCGCTCTGGAACAT</td>
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<tr>
<td>Human hepc R</td>
<td>AGTGGCTCTGTCTTTTCACCACA</td>
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<tr>
<td>Mouse EPO R</td>
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<td>Mouse Arnt F</td>
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<tr>
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<tr>
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<tr>
<td>C/EBPα S193A BOT</td>
<td>GTGCGCGGGAGCCCGCTGCGGGT</td>
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**Table 4S1. Primers for qPCR analysis and cloning**
References


Chapter 5
Conclusions and Future Directions

The work presented in this thesis provides convincing data that local iron sensing through the hypoxic pathway is a critical systemic regulator of iron homeostasis. Hypoxia-inducible factor 2 alpha (HIF-2α) is an essential regulator of iron absorption in the setting of iron deficiency (1). In addition to iron deficiency, iron absorption is increased during erythropoiesis, and this was thought to be controlled by erythropoietic hepcidin repression (2). However, the work in this thesis extends the role of intestinal hypoxic sensing and HIF-2α as the major physiological regulator of iron homeostasis during erythropoiesis. Intestinal HIF-2α disruption inhibits the increase in intestinal iron transporters during erythropoiesis and blunts the production of red blood cells (RBCs) compared to wild-type littermates, likely due to decreased iron availability (3). Intestinal hypoxia appears to be the major signal that leads to activation of HIF-2α during high levels of erythropoiesis. This is a unique mechanism compared to dietary iron deficiency, in which a decrease in intestinal iron levels likely causes HIF-2α stabilization in this condition (Erik Anderson, unpublished data).

In diseases of ineffective erythropoiesis such as β-thalassemia, increased iron absorption that leads to iron overload is commonly observed, and again the major driving mechanism leading to hyper-absorption of iron was thought to be
mediated by a decrease in hepcidin expression (4). To test whether intestinal HIF-2α is important in pathological iron absorption in β-thalassemia, thalassemic mice were crossed to mice with HIF-2α disrupted in the intestinal epithelium. The loss of intestinal HIF-2α completely inhibited the increase in iron absorption genes and prevented iron overload in mouse models of β-thalassemia. In addition, loss of intestinal HIF-2α promoted mobilization of liver iron in models of erythropoietic stress. This suggests that inhibition of intestinal HIF-2α could be combined with existing treatments to rapidly reduce liver iron levels (Anderson et al, 2012 submitted).

In addition to local hypoxic effects in the intestine, hypoxia robustly represses liver hepcidin expression. Under physiological conditions that regulate hepcidin, such as iron deficiency and erythropoiesis, HIFs are entirely dispensable (1) (Matthew Taylor, unpublished data). While HIF signaling is not necessary for physiological hepcidin expression, a pathological condition in which hepcidin deficiency contributes to iron absorption and iron overload is alcoholic liver disease (ALD) (5). Several mechanisms have been proposed to explain hepcidin repression in this disease (6-9). However, the mechanism remained unclear. In this thesis we demonstrate that CCAAT/enhancer binding protein alpha (C/EBPα), a transcription factor that is essential for hepcidin expression (10), is decreased in ethanol loading in a HIF-dependent manner (11). This clearly places hypoxia signaling upstream of hepcidin signaling in ALD, revealing new therapeutic targets and reinforcing the central role of hypoxia in the regulation of iron homeostasis.
The work presented in this thesis clearly demonstrates that hypoxia signaling plays an essential role in maintaining systemic iron homeostasis under physiological and pathological conditions. The laboratory is currently working to identify novel compounds that inhibit intestinal HIF-2α in vivo, which could be added to existing therapies for iron overload to rapidly reduce tissue iron levels. In addition, the laboratory is investigating whether targeting intestinal divalent metal transporter 1 (Dmt1), a major HIF-2α target involved in iron absorption, could be therapeutic in conditions of iron overload.

This thesis work demonstrates that the intestine, through HIF-2α signaling, is both a sensor and regulator of systemic iron homeostasis, and has led to further questions that will need to be addressed. While the following sections are outside the scope of this thesis work, they will serve as jumping-off points for future endeavors to discover additional roles for hypoxia signaling and iron homeostasis in the regulation of human physiology and disease.

**HIF-2α-hepcidin crosstalk in iron absorption**

Erythropoiesis and iron deficiency activate intestinal iron absorption, and it was previously thought that a decrease in hepcidin was entirely responsible for meeting this increased iron demand (12). However, this thesis work clearly demonstrates that intestinal HIF-2α is essential for increased iron absorption (3). In addition, the function of hepcidin with respect to iron homeostasis requires intact HIF signaling (13). While short term decreases in hepcidin expression may not be responsible for increased iron absorption, animal work has demonstrated
that hepcidin deficiency over a period of time leads to enhanced iron absorption and severe iron overload (14). Together the data suggest that HIF-2α must be stabilized in the intestine following a chronic decrease hepcidin expression.

One condition which is well known to stabilize HIF-2α is enterocyte iron deficiency (1). This could be achieved through upregulation of duodenal Fpn1 expression, but multiple publications have shown that hepcidin does not directly influence stability of Fpn1 in the intestine (13, 18). This conflicts with the observation that hepcidin treatment leads to inhibition of enterocyte iron export (19). One possibility is that hepcidin is able to exert an effect on the activity of Fpn1 without directly influencing its stability. Hepcidin could bind to Fpn1 and block iron export, and the absence of hepcidin could allow Fpn1 to export iron more efficiently. This possibility was suggested to occur using the human intestinal Caco-2 cell line, in which hepcidin treatment blocked iron export without influencing Fpn1 protein expression (20).

If this model is true, then HIF-2α activation by hepcidin deficiency would occur through intracellular iron depletion (Fig. 5.1). Under conditions of normal hepcidin expression, hepcidin would interact with Fpn1 and limit its ability to export iron. Blocking iron export will maintain enterocyte iron at high levels, allowing prolyl hydroxylase enzymes (PHD) to hydroxylate HIF-2α and lead to its proteasomal degradation. Under hepcidin deficiency, Fpn1 would export iron more efficiently, leading to intracellular iron deficiency. Low intracellular iron inactivates the PHD enzymes, stabilizing HIF-2α and subsequent increase in
target genes Dmt1, duodenal ferric reductase (DcytB), and Fpn1 (1, 21). This would lead to a robust increase in iron absorption and iron overload that is observed with hepcidin deficiency (17).

The other known stimulus that can increase HIF-2α activity in the intestine is hypoxia (3). Hepcidin exerts an effect on the erythropoietic compartment, given that erythroblasts express the hepcidin receptor, Fpn1 (22). Hepcidin regulates expression of Fpn1 on erythroid progenitors (23). Thus, hepcidin deficiency could lead to increased Fpn1 expression on developing RBCs, depleting them of iron and compromising their ability to carry oxygen. While prior publications have shown that Fpn1 expression in erythroid precursors decreases as they differentiate, we have been able to detect high levels of Fpn1 protein on the membranes of mature RBCs and are able to observe a response to hepcidin (Fig. 5.2A). In addition, a physiological stimulus that reduces hepcidin levels, dietary iron deficiency, leads to a substantial upregulation of Fpn1 expression on mature RBCs (Fig. 5.2B).

To investigate if hepcidin deficiency stabilizes HIF-2α through its effect on RBCs, mice were generated which overexpress a green fluorescent protein (GFP)-tagged Fpn1 protein (24) which is mutated to be resistant to hepcidin degradation (25), specifically in RBCs (26). This mouse model should mimic one effect of hepcidin deficiency, increased expression of Fpn1 on mature RBCs and erythroid progenitors. If this leads to RBC iron deficiency and subsequent hypoxia, then intestinal HIF-2α should be stabilized. Founder lines are currently being analyzed. While iron deficiency and hypoxia are the most well
characterized inducers of intestinal HIF-2α, it is possible that another unknown mechanism could mediate stabilization of HIF-2α by hepcidin deficiency.

**HIF and anemia of inflammation**

In humans, acute infection leads to a drop in serum iron and anemia (27). It is thought that this is primarily controlled by hepcidin and its ability to degrade the iron exporter, Fpn1 (28). While iron sequestration provides a short term benefit during inflammation by depriving pathogens of this critical nutrient (29), anemia becomes a critical factor in the setting of chronic disease (30). Intriguingly, the model of intestinal HIF-2α overexpression leads to substantial iron overload (1) despite having appropriate increases in hepcidin expression in response to iron loading (Yatrik Shah, unpublished data). This raises the possibility that intestinal HIF-2α overexpression could overcome the effect of hepcidin on iron sequestration.

In a model of zymosan-induced generalized inflammation (ZIGI) (31) serum iron drops precipitously following zymosan treatment (Fig. 5.3A). This correlates with decreases in both Fpn1 mRNA and protein in the duodenum (Fig. 5.3B-C). Since mice overexpressing HIF-2α in the intestine greatly increase Fpn1 expression (21), it is possible that these mice would be protected from the drop in serum iron observed after zymosan treatment.

Future plans entail assessing newly weaned wild-type or intestinal HIF-2α overexpressing mice following zymosan treatment. Serum iron levels, intestinal iron transporter gene expression, and tissue iron levels will be assessed. If the
drop in serum iron is attenuated by intestinal HIF-2α overexpression, mice could
be allowed to survive past 24 hours in order to determine if the subsequent
anemia observed in this model is also affected (31). If stimulating iron absorption
through increasing intestinal HIF-2α counteracts the anemia, intestinal HIF-2α
agonists could be used therapeutically in critically ill patients.

**Intestinal HIF-2α in the mothers of nursing neonates is essential for
production of milk with adequate iron concentrations**

Iron deficiency is common in infants that are breastfed, particularly in
developing countries, which can lead to anemia (32, 33). Iron absorption is very
efficient in young mammals, likely due to the importance of iron in early
psychomotor and cognitive development (34). Thus, iron deficiency in suckling
infants must be due to low levels of iron in breast milk. Maternal iron absorption
is increased during pregnancy, but maternal iron absorption postpartum has not
been investigated. Supplementing infant diets with iron is effective in reducing
the prevalence of anemia in infants, but widespread infant dietary
supplementation could be difficult to achieve in developing countries where risk
of iron deficiency is greatest (35). Previous studies have demonstrated that a
maternal diet deficient in macro and micronutrients translates to deficiencies in
milk nutrient content and lower weight gain in breastfed infants (36). Thus,
maternal dietary intervention, either through distribution of knowledge or
supplements, could substantially improve infant health, growth, and development
in developing countries.
A healthy person will absorb 1-2 mg/day of iron from the diet, mostly in the duodenum of the small intestine (37). At 1 day postpartum, breast milk iron concentrations are approximately 90 µg/dL, which declines to approximately 30 µg/dL at 6 months postpartum (38). Infant breast milk consumption is on average about 800 mL/day, which will deplete a lactating mother of about 700 µg/day of iron early after birth, and about 250 µg/day as infants approach weaning age (39). Despite this high demand of iron from the mother, 6 months postpartum maternal iron levels are increased compared to 1 day postpartum (38). Together, these data suggest that lactating mothers may increase iron absorption in order to meet this high demand for iron. Investigating this possibility will lead to new knowledge that can be used to combat anemia and growth retardation in infants.

It is well-established that intestinal HIF-2α deletion (Hif-2αΔIE) in mice blocks adaptive increases in iron absorption (1, 3, 21). Over the course of breeding these mice, it was observed that mice born to mothers lacking intestinal HIF-2α expression have a defect in hair growth and weigh significantly less than weanlings from wild-type mothers, which are related to iron deficiency (40) (Fig. 5.4A and C). Fostering mice born from a Hif-2αΔIE mother to a wild-type mother leads to complete recovery of the phenotype, demonstrating that the phenotype is not due to fetal development (Fig. 5.4B). One explanation for this recovery could be due to some nutritional deficiency in breast milk from Hif-2αΔIE mothers. Since it is known that these mice have a deficiency in iron absorption, breast milk was collected and pooled from wild-type and Hif-2αΔIE mothers with pups that
were 7 days old and analyzed for iron content. Milk from \textit{Hif-2α\textsuperscript{ΔIE}} mothers is severely deficient for iron (Fig. 5.4C). While these data are preliminary, they strongly suggest that studying iron absorption in mothers with nursing offspring could improve knowledge and prevent anemia in infants worldwide. Moreover, there are many foods that inhibit iron absorption, including eggs, cocoa, coffee, tea, and nuts (41-43). On the other hand, ascorbic acid can enhance dietary iron absorption (44). Investigating the mechanisms of maternal iron absorption postpartum could lead to dietary interventions which could prevent anemia and developmental delays in breastfed infants.

\textbf{Steap4 in the intestine}

DcytB is the only known mammalian ferric reductase in the intestine which responds to increases in systemic iron requirements. However, deletion of DcytB does not affect iron homeostasis, suggesting that other ferric reductases must compensate. Since intestinal deletion of HIF-2α completely abrogates ferric reductase activity in the intestine following an increase in systemic iron requirements, mice overexpressing HIF-2α are an ideal model to identify additional ferric reductases in the intestine. Microarray analysis from duodenal mRNA from wild-type mice compared to mice overexpressing HIF-2α identified six-transmembrane epithelial antigen of prostate 4 (Steap4) as a gene highly induced by HIF-2α (Fig. 5.5A)(1). Steap4 contains metalloreductase activity (45).

Similar to other proteins involved in iron homeostasis, Steap4 expression in the intestine requires HIF-2α (Fig. 5.5B). However, analysis of protein
expression demonstrates that Steap4 is only induced in the colon, despite loading substantially more duodenal extract (Fig. 5.5C). This suggests that Steap4 is unlikely to play a role in iron homeostasis, but could be involved in homeostasis of the colon. Consistent with these data Steap4 is highly induced in intestinal samples from ulcerative colitis patients compared to normal controls. Moreover, Steap4 is induced in mouse models of colitis (Fig. 5.5D). These data suggest that Steap4 may be an important protein linking nutritional cues to inflammation. To directly investigate the role of Steap4 in the intestine in vivo, transgenic mice were generated which overexpress a Steap4-GFP fusion protein (46) under control of the villin promoter (47), which drives expression throughout the entire intestine. Mouse lines with both high and moderate expression of the transgene were identified and confirmed by quantitative polymerase chain reaction (qPCR) for transgene mRNA, Western blot for the fusion protein, and by the functional reductase assay using nitrotetrazolium blue (NBT) (1) (data not shown). Currently these mice are being crossed to the C57BL/6 background, and we plan to assess the role of Steap4 in mouse models of colitis and intestinal tumors (48).

**Regulation of heme iron absorption**

The vast majority of literature on iron absorption focuses on dietary non-heme iron, despite heme iron making up the majority of iron absorbed from a Western diet (49). While mechanisms of non-heme iron absorption are very well characterized (50) little is known about how heme iron absorption is regulated.
One potential mechanism for heme to enter the enterocyte is through the heme transporter, solute carrier family 46 member 1 (Slc46a1) (51). However, it was subsequently determined that the primary role of Slc46a1 is to serve as a folate transporter (52). It was also thought that heme taken up in this manner could be exported through feline leukemia virus subgroup C cellular receptor 1 (FLVCR1), a heme exporter (53). However, an experiment in which dogs were administered an intragastric dose of radiolabeled hemoglobin demonstrated that most of the radiolabeled iron in circulation was present in non-heme form, suggesting that heme is broken down in the enterocyte prior to export to the circulation (54).

Electron microscopic data indicate that heme uptake in the intestine occurs through endocytosis, and heme is broken down within endocytic vesicles (55). While no receptor for heme has been identified in the intestine, the breakdown of heme is known to be catalyzed by heme oxygenase 1 (HO-1) (56).

Following release of iron from heme, it must be exported from the vesicle into the cytosol and into circulation by Fpn1 (57). Interestingly, iron deficiency increases dietary heme absorption, although mechanistically it is not known how this is achieved (58). One possibility is that HIF-2α, which mediates adaptive increases non-heme iron absorption following iron deficiency, could also be involved in increasing heme absorption.

HIF-2α may promote heme iron absorption through a number of mechanisms. Dmt1 is a HIF-2α target (1), and could be involved in the release of iron from endocytic vesicles after heme breakdown. Also, Fpn1 is a HIF-2α target (21) that is necessary for export of iron from the enterocyte to the serum.
In addition, HIF-2α could regulate the as yet undiscovered heme receptor, since many genes are regulated by HIF-2α that have been identified by microarray but not yet characterized (21). To test this, mice that overexpress HIF-2α in the intestine will be maintained on a diet deficient for non-heme iron, but enriched with heme iron. After maintaining mice on the diet for two weeks, they will be analyzed for serum, intestinal, and liver iron content. If iron stores are higher in the HIF-overexpressing mice, then it would suggest that HIF-2α is an important mediator of increased heme absorption.

It will also be important to determine if loss of HIF-2α affects heme iron absorption as well. To test this, we will first determine a diet that is deficient in non-heme iron, but is supplemented with a small amount of heme iron, such that the intestine activates genes involved in the response to iron deficiency. Wild-type mice should be able to increase heme absorption and maintain normal iron levels. Mice lacking intestinal HIF-2α on the diet should become progressively anemic if HIF-2α is important for adaptive increases in heme iron absorption. Regardless of the data, the diets used for these experiments offer a novel model for investigating the mechanisms of heme iron absorption, as no laboratory has previously used diets deficient for non-heme iron that are supplemented with heme iron.

**Conclusions**

The data in this thesis challenge the central role of hepcidin in regulating iron homeostasis. Hepcidin expression certainly correlates very well with
systemic iron levels and requirements, and animal models of hepcidin knockout or overexpression lead to iron overload and anemia, respectively. The present work demonstrates that HIFs are local effectors controlling the response to systemic iron levels, and that HIFs control both hepcidin expression itself as well as the response to fluctuations in hepcidin expression. Hepcidin could be used therapeutically to limit iron levels, but as a consequence hepcidin traps iron in tissues such as macrophages, liver, and bone marrow, which could lead to iron-mediated oxidative stress and tissue damage. Targeting HIF-2α in the intestine, however, only affects the iron entering the body, which is then regulated by hepcidin and other factors to ensure proper distribution in a way that limits organ damage. Further studies into pharmacologic HIF-2α inhibitors or agonists could lead to novel therapies to combat diseases of iron overload and anemia.
Figure 5.1. Putative model by which hepcidin regulates HIF-2α expression. Under conditions of normal hepcidin expression, intracellular iron levels are kept high by hepcidin's ability to block export of iron through Fpn1. This allows prolyl hydroxylase enzymes (PHD), which require iron as a cofactor, to hydroxylate HIF-2α, leading to its degradation. Under conditions of hepcidin deficiency, the inhibitory effect of hepcidin on Fpn1 function is relieved, leading to a depletion of intracellular iron. This inactivates the PHD enzyme, leading to HIF stabilization and activity. HIF then increases expression of Dmt1, DcytB, and Fpn1, leading to greatly increased iron absorption and contributing to iron overload observed in hepcidin-deficient animals.
Figure 5.2. Hepcidin acts on mature RBCs to degrade Fpn1. RBCs were collected from an animal in K$_2$EDTA tubes and aliquoted. RBCs were then incubated with the indicated hepcidin concentrations for 24 hours before harvesting cells and performing a membrane protein extraction. A) Fpn1 protein was detected by western blot on membrane and cytosolic fractions. Blood was collected from mice maintained on a normal diet, or an iron-deficient diet (3.5 ppm Fe$^{3+}$) for 2 weeks. B) Fpn1 protein was detected by western blot from membrane extracts. (dark) indicates longer exposure time. Even protein loading was determined by Coomassie stain.
Figure 5.3. Duodenal Fpn1 expression decreases during the hypoferremia of inflammation. Mice were injected with 0.64 mg/g zymosan and analyzed 24 hours later. A) Serum iron levels in control and zymosan-treated (ZIGI) mice. mRNA was extracted from the duodenum and reverse transcribed. B) qPCR analysis of Fpn1 expression in the duodenum. Expression was normalized to β-actin. Membrane protein was extracted from the duodenum and analyzed. C) Fpn1 expression in the duodenum of control and ZIGI mice. Even loading was determined by Coomassie stain. *p-value < 0.05 vs. control as determined by Student’s t-test.
Figure 5.4. Maternal iron absorption determines offspring growth and development. Mice from Hif-2α^{F/F} and HIF-2α^{ΔIE} mothers were compared after weaning. A) Mice weaned from Hif-2α^{ΔIE} mothers demonstrate a defect in hair growth. B) Fostering mice from Hif-2α^{ΔIE} mothers to Hif-2α^{F/F} mothers at 1 week of age rescues the hair growth defect. C) Milk non-heme iron content pooled from Hif-2α^{F/F} mothers and Hif-2α^{ΔIE} was analyzed. Pups from Hif-2α^{F/F} and Hif-2α^{ΔIE} mice were weighed after weaning. p-value was determined by Student’s t-test.
Figure 5.5. Steap4 is induced in the intestine of HIF-overexpressing mice (VhlΔIE). Mice with targeted disruption of Vhl in the intestine (VhlΔIE) were analyzed for Steap4 expression by qPCR. A) Steap4 mRNA is upregulated in both the small intestine and colon of VhlΔIE mice. B) Loss of HIF-2α in addition to Vhl (VhlΔIE Hif-2αΔIE) completely abrogates the induction of Steap4 mRNA. C) Western Blot analysis of Steap4 demonstrates colonic, but not duodenal induction of Steap4 protein. D) Steap4 expression in the colon of mice treated with C. rodentium (C. rod). Coomassie stain was used as a loading control. mRNA expression was normalized to beta-actin.* p-value < 0.05 as determined by Student’s t-test.
References


