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# Hypoxia-Inducible Factors Link Iron Homeostasis and Erythropoiesis

**Yatrik M. Shah**[1,2,*] and **Liwei Xie**[1]

[1]Department of Molecular & Integrative Physiology, Division of Gastroenterology, University of Michigan, Ann Arbor, Michigan

[2]Department of Internal Medicine, Division of Gastroenterology, University of Michigan, Ann Arbor, Michigan

## Abstract

Iron is required for efficient oxygen transport, and hypoxia signaling links erythropoiesis with iron homeostasis. Hypoxia induces a highly conserved signaling pathway in cells under conditions of low $O_2$. One component of this pathway, hypoxia-inducible factor (HIF), is a transcription factor that is highly active in hypoxic cells. The first HIF target gene characterized was *EPO*, which encodes erythropoietin—a glycoprotein hormone that controls erythropoiesis. The past decade has led to fundamental advances in our understanding of how hypoxia regulates iron levels to support erythropoiesis and maintain systemic iron homeostasis. We review the cell-type specific effects of hypoxia and HIFs in adaptive response to changes in oxygen and iron availability, as well as potential uses of HIF modulators for patients with iron-related disorders.

### Keywords

Hypoxia; HIF; Iron; Erythropoiesis

## Introduction

Iron, an essential nutrient, is required for oxygen delivery and is a cofactor in several enzymatic and redox reactions. In mammals, 70% of iron is found in red blood cells (RBCs), and 6% is a component of iron-containing proteins, which are required for respiration, energy metabolism, and endobiotic and xenobiotic metabolism. The remaining 24% is stored in ferritin. Iron levels are controlled by a multi-tissue homeostatic process in which dietary iron is absorbed through the proximal small intestine. Dietary iron ($Fe^{+3}$) is reduced by an apical ferric reductase duodenal cytochrome b (DCYTB) to ferrous iron ($Fe^{+2}$) and imported

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* To whom correspondence should be addressed. Tel: +1 734 6150567; Fax: +1 734 9368813; shahy@umich.edu.

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into the enterocyte via the apical iron transporter, divalent metal transporter-1 (DMT1, also known as NRAMP2 or DCT1 and encoded by *SLC11A2*) [1-3].

Tissues also have efficient mechanisms for use of heme iron—the major form of iron in meat. Although several transporters of heme have been identified, little is known about their in vivo functions [4-6]. Once heme is transported in enterocytes, it is broken down by heme oxygenases to release ferrous iron [5]. Iron can either be stored in ferritin or mobilized to circulation by the iron exporter ferroportin (encoded by *SLC40A1*) located on the basolateral surface of enterocytes [7-9]. Once in circulation, iron is loaded onto transferrin [10, 11], where a large proportion of iron is used in RBC synthesis. Splenic macrophages recycle iron from senescent RBCs, limiting the amount of iron required from dietary sources to as little as 1–2 mg per day [12].

The liver is central to iron homeostasis because hepatocytes produce hepcidin, a circulating factor that regulates iron homeostasis. Furthermore, the liver can store and mobilize iron, depending on systemic demands[13]. We review how the hypoxia response affects systemic iron homeostasis and iron-related disorders.

## Iron and Oxygen

Changes in atmospheric oxygen concentration have linked to key evolutionary events. A major hurdle in the evolution of multicellular organisms was the efficient delivery of $O_2$. $O_2$ has low solubility in water, resulting in selection for proteins that could efficiently transport oxygen. Hemoglobin, a highly conserved protein, can efficiently and reversibly bind oxygen and deliver it from the lungs to peripheral tissues. Hemoglobin is composed of 4 chains; each chain contains a heme group [14, 15] The charged iron ion in the heme group binds 1 molecule of $O_2$, so a single hemoglobin protein has the capacity to bind 4 $O_2$ molecules. The heme group provides a unique molecular characteristic—under a condition of high partial pressure of oxygen ($pO_2$), such as in the lung alveoli, $O_2$ binds with high affinity, whereas under a condition of low $pO_2$, such as in peripheral tissues, $O_2$ is released [16, 17]. Most iron is used for RBC synthesis; iron deficiency reduces numbers of RBCs and oxygen transport, leading to tissue hypoxia.

## Oxygen Sensing and Transcription

Tissues maintain specific levels of $O_2$ for respiration and homeostasis. Cells respond to conditions of low $O_2$ by altering gene expression patterns, which affects levels of several hundred proteins involved in cell survival. These changes are initiated by a heterodimeric nuclear transcription factor, hypoxia-inducible factor (HIF). HIF comprises an oxygen-dependent α-subunit (HIF1α [18, 19], HIF2α [20], or HIF3α [21]) and a constitutively expressed β-subunit, aryl hydrocarbon nuclear translocator (ARNT) [22]. In cells, adequate levels of oxygen cause rapid degradation of HIFα subunits by prolyl hydroxylase domain-containing enzymes (PHD). Three isoforms have been reported [23-25].

PHDs are 2-oxoglutarate-dependent dioxygenases; this activity requires iron binding at an active site and oxygen as a co-substrate, so they are an important link between levels of oxygen and iron [26]. At normal levels of oxygen, PHDs hydroxylate HIF α subunits at conserved prolines. Oxygen-dependent prolyl-hydroxylation is required for HIF to bind von Hippel-Lindau tumor suppressor protein (VHL), and then to the E3 ubiquitin ligase complex, leading to ubiquitination and degradation of HIF α subunits [27-29]. Inactivation of VHL in normoxic cells results in HIF activity demonstrating VHL is required for HIF degradation[30].

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A decrease in cellular oxygen or iron availability inhibits PHD-dependent proline hydroxylation of HIF α subunits. VHL can no longer bind to HIF α subunits, resulting in its stabilization. Reactive oxygen species (ROS) in the mitochondria are also required for HIF stabilization. Mitochondrial electron transport chain inhibitors or cells depleted of mitochondrial DNA do not stabilize HIF under hypoxic conditions [31-33]. Following stabilization, HIF α subunits interact with ARNT and other transcription factors, such as p300/CBP, to activate transcription of genes that regulate the response to hypoxia [34] (Figure 1). HIF1α and HIF2α regulate distinct yet overlapping target genes.

Specific stimuli can have different effects on HIF signaling. Low levels of iron, reductions in $O_2$, or increases in mitochondrial ROS lead to activation of different subsets of HIF target genes. For example, low levels of iron in the intestine lead to activation of HIF2α and its target genes, including *DMT1* and *CYBRD1* (which encodes DCYTB), but do not alter expression of vascular endothelial growth factor (*VEGF*), which is frequently induced under conditions of hypoxia [35]. The mechanisms of these differential gene expression patterns are not clear, might involve specific or relative inhibition of PHDs. In addition to regulating the transcriptional response to hypoxia, HIF2α binds to mRNAs and increases cap-mediated translation [36]. Disruption of *Hif1a*, *Hif2a*, *Arnt*, or *Vhl* causes embryonic lethality in mice, demonstrating the importance of an appropriate hypoxic response in vivo [37-40].

## Hypoxia and Regulation of Erythropoietin

Over a century ago, studies conducted during high-altitude expeditions associated levels of $O_2$ with numbers of RBCs. Further studies into the mechanisms of erythropoiesis led to the discovery and isolation of erythropoietin, encoded by *EPO* [41,42]. *EPO* expression is tightly regulated by developmental, physiological, and cellular mechanisms to maintain RBC homeostasis. The liver is the major source of erythropoietin during embryogenesis; after birth, production begins from the kidneys [43]. Hypoxia and HIF signaling are the primary regulators of *EPO* in the kidney, but in response to anemia or hypoxia, *EPO* can be reactivated in hepatocytes [44, 45]. In vitro promoter assays have shown that HIF1α and HIF2α bind and activate the *EPO* promoter [18] via canonical HIF response elements[18, 46-48]. However, subsequent studies in mice demonstrated that HIF2α specifically activates of *EPO* expression [49]. In mice with disruption of *Hif2a* specifically in kidney produce lower levels of erythropoietin [44, 50]. Moreover, in mice with hepatic disruption of *Hif2a,* extra-renal erythropoietin production is regulated by Hif2α (but not Hif1α)[49]. This demonstrated that HIF2α signaling is an important regulator of erythropoietin production.

Hepatocyte-specific disruption of *Vhl* in mice resulted in Hif1α and Hif2α activity and increased expression of *Epo*, leading to polycythemia [44, 49]. Hif2α is therefore required and sufficient to activate expression of *Epo*. HIF2α is required not only for renal and hepatic expression of *EPO*, but also for its expression in neurons, astrocytes, and osteoblasts—these cellular sources regulate erythropoiesis independently of renal function. [51, 52]

## Hypoxia and Hematopoiesis

The discovery of *EPO* as a direct target of HIF[18, 53, 54] provided the first evidence that HIF could regulate iron homeostasis by inducing erythropoietin and RBC production. Erythropoietin regulates RBC production by binding to the erythropoietin receptor on early and late erythroid progenitors, reducing apoptosis and increasing proliferation and differentiation, respectively [42]. Disruption of *Hif2a* leads to severe anemia, pancytopenia, and hematopoietic defects [44, 50, 55]. The alterations in the erythroid lineages result from decreased levels of erythropoietin. However, studies have demonstrated the role of hypoxia and HIF signaling in hematopoietic stem cell (HSC) maintenance. Quiescent HSCs are localized in hypoxic foci, and it has been proposed that $O_2$ levels regulate their

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activity [56, 57]. Deletion of ARNT, which prevents HIF1α and HIF2α function, reduces proliferation in hematopoietic progenitors [58]. Exogenous VEGF rescues the hematopoietic proliferative defects in *Arnt*$^{-/-}$ mice [58]. *VEGF* expression is also activated under conditions of hypoxia [59]. The *VEGF* promoter contains canonical HIF response elements. Deletion of the VEGF receptor (such as in *Flk1*$^{-/-}$ mice) reduces numbers of hematopoietic progenitors, as observed in the *Arnt*$^{-/-}$ mice [60].

HIF2α was also shown to regulate hematopoiesis through a separate mechanism. In mice, disruption of *Hif2a* causes embryonic lethality. However crossing 129S6/SVEvTac and C57BL/6J mice results in survival of 20% of *Hif2a*$^{-/-}$ mice until 1 month after birth, and pancytopenia was observed in these mice [55]. Interestingly, transplantation of bone marrow from *Hif2a*$^{-/-}$ mice into wild-type mice did not significantly alter differentiation or numbers of HSCs. A recent study of mice with hematopoietic cell-specific disruption of *Hif2a* demonstrated similar results [61]. However, *Hif2a*$^{-/-}$ mice that received bone marrow transplants from wild-type mice had defects in hematopoiesis. HIF2α is therefore critical in maintaining a functional microenvironment during hematopoiesis, rather than a bone marrow cell factor, required for proliferation and differentiation of HSCs [55].

## Hypoxia and Hepcidin

Hepcidin (encoded by *HAMP*) is a peptide hormone that is the master regulator of iron levels in humans and other mammals. It was initially discovered as an anti-microbial peptide that was mainly synthesized in the liver, could be detected in the urine, and was induced by inflammation [62-64]. Hepcidin has antifungal activity against *Candida albicans*, *Aspergillus fumigatus*, and *Aspergillus niger* and antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and group B *Streptococcus* [62, 63]. Shortly after its discovery, the link between hepcidin to iron homeostasis was uncovered when levels of *hepcidin* mRNA were found to change with levels of systemic iron. High systemic levels of iron, such as in patients with iron overload, increased hepcidin expression [64].

*Usf2*-knockout mice have multi-tissue iron overload (heart, pancreas, and liver)[65]. However, concentrations of iron in spleen were lower than in wild-type mice. Usf2 is a transcription factor, and analyses of gene expression patterns showed that hepcidin expression was virtually absent from livers of *Usf2*-knockout mice. However, Usf2 does not regulate hepcidin expression directly–the close proximity of *Usf2* to *Hamp* resulted in both genes being disrupted in the *Usf2*-knockout mice. Mice with disruption of *Hamp* but intact *Usf2* have demonstrated the role for hepcidin in progressive iron overload [66]. Moreover, overexpression of *Hamp* leads to hyposidermia [67].

In subsequent years, the mechanisms by which hepcidin regulates iron homeostasis were uncovered. A landmark study demonstrated that hepcidin binds to the only known mammalian iron exporter, ferroportin [68], resulting in rapid internalization and degradation of hepcidin[68, 69]. Therefore, in the presence of hepcidin, small amounts of iron are mobilized from stores, whereas in the absence of hepcidin, iron is rapidly mobilized, leading to iron overload (such as in patients who produce low levels of hepcidin). The function of hepcidin correlates with its expression, which is regulated by systemic levels of iron. Low systemic levels of iron reduce hepcidin expression and increase iron mobilization. On the other hand, high systemic levels of iron lead to expression of hepcidin and reduce iron mobilization (Figure 2).

**Hypoxic Repression of Hepcidin—**Systemic hypoxia increases levels of erythropoietin and erythropoiesis. To maintain RBC synthesis, it is essential to have a reciprocal mechanism that increases systemic levels of iron. Over the last decade, we have greatly

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increased our understanding of hepcidin regulation. Several pathways have been shown to be involved in hepcidin regulation. There are reviews of these pathways in health and disease [70, 71]. Here, we focus on hypoxic regulation of hepcidin.

Liver hypoxia is a strong repressor of hepcidin expression. Hypoxia can override the upregulation of hepcidin during liver inflammation and interleukin (IL)6 production [72]. Shortly after hepcidin was discovered, it was shown in hepatoma-derived cell lines and mice that hypoxia could repress transcription of *Hamp*[73]. Moreover, prolonged hypoxia activates iron absorption, which coordinates with decreased *Hamp* expression in liver [74]. In blood samples collected from healthy volunteers at sea level and then during acute and chronic exposure to high-altitude hypoxia, there was a rapid decrease in serum levels of hepcidin under the hypoxic conditions[75].

In addition to hypoxia, inhibition of PHDs also represses hepcidin expression in hepatoma-derived cells, indicating that HIFs are involved in suppression [76]. However, the precise mechanism by which hypoxia decreased hepcidin expression in the liver was not known. Activation of HIF in hepatocytes in mice decreased hepcidin expression and activated intestinal and macrophage expression of ferroportin [72]. This demonstrated that HIF activation in the absence of hypoxia could repress hepcidin expression in vivo. In mouse hepatoma-derived cell lines, HIF1α bound and repressed the *Hamp* promoter [72].

However, this may not be true for the human *HAMP* promoter, and other mechanisms of gene regulation are likely involved [77-80].

Subsequent studies have identified erythropoietin-dependent and -independent pathways that affect HIF-induced repression of *HAMP*. Increases in liver or systemic expression of HIF2α led to robust activation of *EPO* and concomitant decrease in *HAMP* expression. Inhibition of erythropoietin with neutralizing antibodies, or disruption of *Epo* in mice, significantly reversed HIF2α-mediated repression of *Hamp* [78, 79]. These findings are consistent with a recent study of indigenous populations who live at high altitudes [81]. Although exposure of volunteers to high-altitude hypoxia significantly decreased levels of hepcidin and increased in erythropoietin and RBC production, [75] indigenous highlanders with stable erythropoietic iron requirements did not have reduced levels of hepcidin compared to their lowland counterparts[81]. These findings support the concept that erythropoietin and/or erythropoiesis are involved in hypoxia-mediated suppression of *HAMP*. Erythropoietin can directly inhibit hepcidin expression, by binding to erythropoietin receptors on hepatocytes [82]. Moreover, several studies demonstrated that erythropoietin-induced erythropoiesis could inhibit hepcidin expression indirectly, through erythroid-derived factors (Figure 3) [83].

mRNA encoding growth differentiation factor 15 (GDF15) is highly induced during erythroblast differentiation [84]. Clinical studies found that levels of GDF15 were increased in patients with disorders of erythropoiesis and hematopoiesis [83, 84]. In cultured hepatocytes, high levels of GDF15 contribute to *HAMP* repression [84]. However, when cells from serum samples of patients with erythropoiesis disorders were depleted of GDF15, *HAMP* was still repressed, indicating the involvement of other erythroid factors.

Similar to GDF15, twisted gastrulation (TWSG1) was identified as an erythroid-derived factor that can inhibit hepcidin expression [85]. The mechanisms by which GDF15 and TWSG1 regulate hepcidin expression are not fully defined. However, TWSG1 can inhibit BMP-mediated activation of SMAD, an important transcriptional activator of *HAMP* [86].

Hypoxia can also inhibit hepcidin expression through an erythropoietin-independent pathway. Patients with Chuvash polycythemia have a specific mutation in *Vhl* that results in increased expression of HIF1α and HIF2α, increased numbers of RBCs (due to increased

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expression of erythropoietin) , and reduced expression of hepcidin [87]. However, in linear regression analysis, after correcting for serum levels of ferritin, erythropoietin, and RBC count, the decrease in hepcidin levels persisted [87]. This indicates that hypoxia-induced repression of hepcidin is independent of erythropoietin or RBC production. Consistent with these findings, mice that overexpress Hif1a only in hepatocytes do not have increased serum levels of erythropoietin or erythropoiesis. However, levels of hepcidin are reduced in these mice[80]. The erythropoietin-independent mechanism is initiated through a hypoxia-mediated degradation of C/EBPα, a critical transcription factor required for basal expression of hepcidin[80]. Hypoxic macrophages can also inhibit SMAD and C/EBPα in hepatocytes to reduce hepcidin expression [88] (Figure 3).

Because oxygen levels regulate iron levels and erythropoiesis, it is not surprising that hypoxia regulates hepcidin expression via several mechanisms. The goal is to understand under which physiologic and pathologic conditions these pathways are activated, and whether concomitant activation of these pathways has stronger effects on hepcidin expression.

## Intestinal Oxygen Sensing and Iron Absorption

High systemic demand for iron leads to reductions in hepcidin and increases in apical and basolateral proteins involved in iron transport. In the intestine, hepcidin directly regulates degradation of ferroportin [68]. However, 2 local pathways regulate ferroportin, DMT1, and DCYTB expression. The first pathway is mediated by the iron regulatory protein (IRP)1 and IRP2. The biochemical function and activity of IRPs, and their roles in vivo, were extensively reviewed [89].

IRP1 and IRP2 bind to specific elements of mRNAs called iron response elements (IRE). Binding of IRPs to IREs can block translation or increase mRNA stability, and affect levels of several proteins involved in iron homeostasis. Intracellular levels of iron regulate expression and activity of IRPs. Decreased cellular levels of iron activate IRPs, whereas high levels decrease IRP function (Figure 4). Mouse embryos that lack Irp1 and Irp2 die in utero, so these proteins are required for early development [90]. Studies in which the genes encoding Irp1 or Irp2 were disrupted in mice demonstrated their redundancy, as well as their specific roles in cell physiology [91-94]. Moreover, biochemical studies showed that the proteins have overlapping and specific mRNA targets [95].

IRP1 and IRP2 target the mRNA that encodes ferroportin (*SLC40A1* mRNA), binding to the IRE motif in its 5′-UTR to reduce its translation [8, 9]. Intestinal disruption of genes encoding Irp1 and Irp2 in mice increased levels of ferroportin protein[96]. Activation of IRPs by iron deficiency would be expected to reduce translation of intestinal ferroportin. However, intestinal cells can express an *SLC40A1* mRNA from an alternate promoter; this mRNA does not contain the IRE, allowing for its translation under conditions of iron deficiency [97].

Levels of DMT1 in the small intestine are also affected by intracellular iron levels. Four DMT1 isoforms have been identified, and are transcribed via alternate promoters[98]. These isoforms have similar transport activities, although they differ in the cells that express them, subcellular localization, and response to intracellular levels of iron [99]. mRNAs encoding 2 isoforms that are regulated by iron level contain a functional IRE in the 3'-UTR and are stabilized by IRPs. Mice with a disruptions in genes encoding Irp1 or Irp2 have no changes in duodenal levels of Dmt1, likely due to their overlapping functions. However, intestine-specific deletion of Irp1 and Irp2 reduced levels of the mRNA encoding Dmt1 (*Slc11a2* mRNA)[96]. The mechanism by which IRPs regulate levels of DMT1 is not clear, but is believed to involve a mechanism similar to IRP-mediated stabilization of mRNA encoding transferrin receptor-1 (*TFRC*) [100].

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The ability of IRPs to stabilize the transcript and increase protein levels of DMT1 could be coordinated with hepcidin-mediated repression of *SLC40A1*. Under conditions of adequate systemic levels of iron, hepcidin degradation of ferroportin limits mobilization of dietary iron into the circulation. This could increase intracellular levels of iron in enterocytes, decreasing IRP function and reducing levels of DMT1. This model is consistent with what is observed during hepcidin deficiency. The increase in ferroportin leads to increased basolateral export of iron. This results in reduced iron within enterocytes, activation of intestinal IRPs, and increased levels of DMT1[101].

**HIF2α Regulation of Iron Transporters—**Biochemical and knockout studies have characterized the roles of IRPs in local regulation of intestinal iron absorption. However, genes such as *CYBRD1* (encodes DCYTB) have no IREs, yet levels of protein and mRNA greatly increase at low iron concentrations [3]. In addition, transcription of *SLC40A1* (encodes ferroportin) and *SLC11A2* (encodes DMT1) are also induced following systemic increases in iron demand [102]. These findings indicate that other mechanisms must be involved in regulating local iron absorption.

Studies have demonstrated the role of intestinal Hif2α in regulating iron absorption [35, 103, 104]. Intestinal epithelial cells of mice that overexpress Hif2α have increased levels of ferroportin protein and mRNA [103]. In mice with an intestine-specific disruption of *Hif2a* or littermate controls on normal or low-iron diets, Hif2α was required for increases in *SLC40A1* mRNA in response to acute changes in iron levels. Surprisingly, low-iron induced expression of ferroportin was completely lost when *Hif2a* was disrupted intestines of mice[103]. Long-term deprivation of iron led to increases in *Slc40a1* mRNA, in a Hif2α-dependent manner. However, mice with intestine-specific disruption of *Hif2a* and wild-type mice placed on continuous low-iron diets increased intestinal levels of ferroportin [103]. This means that the acute, adaptive induction of ferroportin occurs via increased transcription of *Slc40a1*, and that long-term responses to systemic iron demand occur through a Hif2α-independent mechanism—most likely through a decrease in hepcidin-mediated degradation of ferroportin. This biphasic regulation provides an efficient system to maintain a rapid and sustained response to increase systemic iron requirements.

Overexpression of Hif2α greatly increases expression of Dmt1 and Dcytb. Upregulation of Dmt1 and Dcytb following acute or long-term iron deprivation is completely lost with disruption of intestinal Hif2α signaling [35, 104]. Moreover, the adaptive increase in Dmt1 and Dcytb following increased erythropoiesis is also lost following disruption of Hif2α signaling in the intestine [105]. Together, these findings demonstrate the role of HIF2α in the regulation of iron absorption following changes in systemic iron levels. Promoter regions of *SLC40A1, SLC11A2*, and *CYBRD1* contain a canonical HIF response element, and chromatin immunoprecipitation assays have shown that HIF2α binds these promoters, to directly regulate these genes during increased demand for systemic iron [35, 103, 104].

**Interactions Between IRP1 and HIF2α—**The 5'-UTR of *HIF2a* mRNA contains a phylogenetically conserved IRE that binds IRP1 and IRP2 (although IRP1 appears to the major regulator of the *HIF2a* IRE ) [106, 107] to control iron-dependent regulation of the transcript. Translation of *HIF2a* mRNA was inhibited in iron-deficient cells, due to a high activity of the IRPs[106, 107]. Selective inhibitors of *HIF2a* translation have been developed, which promote binding of IRP1 binding to the 5'-UTR of the mRNA [107].

However, not all IRE-containing transcripts appear to be regulated by IRPs in vivo, so ancillary sequences could be involved. An in vivo study reported that iron deficiency in liver significantly increased activity of IRP1 and IRP2, and thereby decreased translation of *HIF2a* mRNA[108]. Two recent studies demonstrated the importance of IRPs in *Hif2a*

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regulation using knockout mice. Irp1- (but not Irp2-) knockout mice developed transient polycythemia, due to increased levels of *Hif2a* mRNA and renal *Epo* mRNA. In addition, *Slc40a1*, *Dmt1*, and *Dcytb* were induced in the small intestine of the Irp1-knockout mice [109, 110]. These findings indicate that Irp1, through regulation of *Hif2a* mRNA, fine tunes the erythropoietic response to hypoxia.

Hypoxia responsive erythropoietin-producing cells in the liver and kidney must be able to selectively decrease the level of HIF2α, depending on availability of oxygen and iron. In hepatic and renal cells that are hypoxic, activation of HIF2α leads increases production of erythropoietin. Under conditions of sufficient systemic iron, RBC production occurs. However, the increase in RBC production under conditions of hypoxia, during systemic iron deficiency, would result in hypochromic and microcytic RBCs. Therefore, the interaction between IRP1 and HIF2α could be an important mechanism that limits erythropoiesis under conditions of hypoxia and low systemic levels of iron. In the intestine, Hif2α translation is not regulated by Irps; levels of Hif2α increase on low-iron diets and is essential for the adaptive increase in ferroportin, Dmt1, and DcytB [35, 103, 104]. This suggests an integrative mechanism where hypoxia and low level of iron might lead to a repression of HIF2α only in erythropoietin-producing cells. However, in the intestine, HIF2α increases iron absorption and systemic levels of iron. This results in release of the translational inhibition of hepatic and renal HIF2α, and an increase in erythropoietin and erythropoiesis (Figure 5). Studies are needed to determine how these pathways integrate into an optimal hypoxia and iron-regulated response.

## Hepcidin and Intestinal Hypoxia Signaling

Several in vivo studies have demonstrated that hepcidin deficiency (hepcidin-deficient mice) increases iron absorption, via increases in ferroportin, DMT1, and DCYTB proteins and mRNAs. Interestingly, these increases were lost when hepcidin-deficient mice were crossed to mice with intestinal disruption of *Hif2a*[101]. This was the first study to show that hepcidin and intestinal Hif2α signaling interact. However, it is unclear how hepcidin deficiency activates intestinal HIF2α signaling.

Researchers have identified 2 mechanisms important for activation of intestinal HIF2α following increased requirement for systemic iron [35, 103-105]. During iron deficiency, it is thought that the low level of intestinal epithelial iron reduces the activity of PHDs, leading to increased expression of HIF2α [35, 103, 104]. However, during increased erythropoiesis, intestinal levels of iron do not change, but intestinal levels of $O_2$ decrease, resulting in activation of intestinal HIF2α [105]. It is not clear if hepcidin affects either intestinal iron or $O_2$ availability. These mechanisms require testing so we can better understand how HIF2α is activated during hepcidin deficiency.

## HIFs and Iron-related Disorders

Heredity hemochromatosis is a genetic disorder that leads to iron overload. Mutations in genes such as *HFE*, *TFR2*, *HFE2, SLC40A1,* or *HAMP* reduce expression of hepcidin or, in the case of mutant forms of ferroportin, reduce hepcidin-mediated degradation of ferroportin [13]. Although some patients have hereditary forms of hemochromatosis, others have secondary hemochromatosis, which mostly comprises acquired disorders in erythropoiesis [13]. In these cases, iron overload can result from increased hemolysis, repeated blood transfusions, and hyper-absorption of iron. Similar to hereditary hemochromatosis, several types of secondary hemochromatosis are associated with reduced levels of hepcidin; correcting hepcidin levels reduces iron accumulation in tissues [111, 112]. Moreover, secondary hemochromatosis disorders such as β-thalassemia are similar to hepcidin-deficient mice in that intestinal Hif2α signaling is activated [113]. In patients with hereditary hemochromatosis

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or some forms of secondary hemochromatosis, iron overload is treated with several rounds of phlebotomy and/or administration with iron chelators [114, 115].

Intestinal HIF2α could be a therapeutic target for hereditary and secondary hemochromatosis; inhibiting its function could reduce iron absorption in the intestine. Since HIF2α regulates apical and basolateral iron transporters, blocking its action would shut down iron import and export, leading to a rapid response. Genetic deletion of *Hif2a* in the intestine improves iron overload in secondary hemochromatosis [113]. To the best of our knowledge, no study has assessed the ability of chemical inhibitors of HIF2α to reduce iron overload, although several selective HIF2α inhibitors have been identified [107]. In addition, HIF2α contains a pseudo ligand-binding pocket that can be used to allosterically inhibit HIF2α function [116]. New specific inhibitors have been designed to block HIF2α function by targeting the pseudo ligand-binding pocket [117]. Further studies are needed to determine the efficacy and potency of these compounds in vivo.

**Anemia—**Anemia, the most prevalent blood disorder worldwide, is defined as a decrease in RBCs and/or hemoglobin. Iron-deficient anemia is a common form of anemia caused by low dietary intake of iron, poor absorption, or loss of blood [118]. Chronic anemia, also called anemia of inflammation, is a general anemic phenomenon observed in many patients with chronic diseases [119]. The chronic inflammatory response can reduce serum levels of iron by increasing expression of hepcidin. This condition encompasses many distinct diseases, and mechanisms are specific for each disease. However, IL6 signaling via STAT3, which is induced by inflammation, increases hepcidin expression [120]. This increase in hepcidin expression following acute inflammation protects tissues from pathogens, due low iron availability (pathogens require iron for proliferation). However, chronic inflammation thereby leads to anemia, and in certain cases can exacerbate progression of the patient’s primary disease.

In addition to an increase in hepcidin expression from chronic disorders, loss-of-function mutations in the *TMPRSS6* gene increase expression of hepcidin, leading to iron-refractory iron-deficiency anemia (IRIDA) [121]—a form of iron deficiency anemia that is unresponsive to oral iron therapy. *Tmprss6*-knockout mice and mice with a premature stop codon in *Tmprss6* develop microcytic anemia, associated with high levels of hepcidin expression [122-124]. *Tmprss6* encodes a type II transmembrane serine protease that is highly expressed in liver and involved in membrane cleavage of hemojuvelin (HFE2)[125, 126]. Mutations in *Tmprss6* reduce protease activity of the product and increase membrane hemojuvelin, leading to an increase in hepcidin expression [126]. In most cases, anemia is efficiently treated with oral iron supplements. For cases such as chronic anemia of inflammation and IRIDA, more aggressive intravenous iron supplementation may be needed.

These disorders might be treated with reagents that activate HIF2α, which would decrease hepcidin in the liver and increase erythropoietin, leading to RBC production. Moreover, activation of intestinal HIF2α signaling could promote iron absorption. PHD inhibition activates HIF target genes; several PHD inhibitors exist and have been well characterized [127]. These inhibitors are well tolerated by mice, and genetic disruption of PHDs increases HIF expression and reactivates hepatic expression of erythropoietin in mice [128]. Several PHD inhibitors are being tested in clinical trials, and have shown some positive effects in patients with anemia [129]. One major concern about PHD inhibitors is that constitutive activation of HIF can lead to inflammation and cancer [130, 131]. Isoform-specific PHD inhibitors might be developed for selective modulation of HIF1α vs HIF2α activity to reduce these risks [132].

**Polycythemia—**Polycythemia is characterized by an increase in proportion of RBC volume, either from an increase in production of RBCs or a decrease of plasma volume [133]. This affects iron homeostasis, because RBC production is tightly linked to systemic level of iron.

Polycythemia associated with high altitude is a physiologic response to hypoxia. However, several heart and lung diseases, which cause chronic systemic hypoxia, can lead to polycythemia [134, 135]. Specific genetic alterations also activate HIF2α to cause polycythemia. Hemoglobin Chesapeake and Hemoglobin Kempsey are conditions in which a specific mutation in the α- or β-chain of hemoglobin greatly increases their affinity for oxygen, reducing oxygen delivery to the kidneys and causing polycythemia [136-138]. Polycythemic diseases also arise through mutations in factors that regulate HIF activity. Chuvash polycythemia caused by a specific mutation in the *VHL* gene [139]. In mouse models of this disorder, disruptions in Vhl increase Hif1α and Hif2α activity and production of erythropoietin. Mutations in PHD2 and gain-of-function mutation in HIF2α have also been found to cause polycythemia [140-142]. Similar to HIF2α-based therapeutics for iron overload, inhibitors of HIF2α might be developed to treat polycythemia.

### Future Directions

Hypoxia signaling is an important component of iron homeostasis and oxygen delivery. It regulates hepcidin, erythropoietin, and proteins that control intestinal iron absorption. However, many questions remain. Hypoxia, IRPs, and hepcidin mediate local and systemic iron responses. Disruption or overexpression of any these factors in cells or animals affects iron homeostasis. Studies are needed to determine how local and systemic hypoxic responses cooperate with hepcidin and IRP to control iron homeostasis.

Although HIF1α and HIF2α repress *HAMP* under hypoxic conditions, HIF2α specifically upregulates other genes that control iron absorption. However, we understand little about the precise mechanisms by which HIF1α and HIF2α control gene expression. HIF1α and HIF2α bind the same response element (a core sequence of A/G CGTG) but regulate different sets of genes. *SLC40A1*, *SLC11A2, DCTYB*, and *EPO* contain consensus HIF response elements in their proximal promoter regions that are specifically activated by HIF2α in vivo. It has been a challenge to identify the mechanisms that determine binding specificity. For example, in vitro reporter assays showed that the proximal promoters of *SLC40A1*, *SLC11A2*, and *DCTYB* specifically bind HIF2α, whereas the *EPO* proximal promoter is activated by HIF1α and HIF2α. However endogenous *EPO* expression is regulated by only HIF2α.

Increasing our understanding of the process of HIF gene regulation and its response to hypoxia could lead to development of reagents that control HIF activity with few side effects. It is also important to learn more about existing pharmacologic agents, as well as the new generation of HIF modulators, to determine if they might be effective therapeutics for iron-related disorders.

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## Abbreviations

| | |
|---|---|
| **ARNT** | aryl hydrocarbon nuclear translocator |
| **DMT1** | divalent metal transporter-1 |
| **DCYTB** | duodenal cytochrome B |
| **GDF15** | growth differentiation factor 15 |
| **HIF** | hypoxia inducible factor |
| **HIF1α** | hypoxia inducible factor1-α |
| **HIF2α** | hypoxia inducible factor-2α |
| **HJV** | hemojuvelin |
| **HSC** | hematopoietic stem cell |
| **IL6** | interleukin-6 |
| **IRE** | iron response element |
| **IRIDA** | iron-deficiency anemia |
| **IRP1/2** | iron-response protein 1/2 |
| **PHD** | prolyl hydroxylase |
| **RBC** | red blood cell |
| **ROS** | reactive oxygen species |
| **TMPRSS6** | Transmembrane protease, serine 6 |
| **Tfr1** | transferrin receptor-1 |
| **TWSG1** | twisted gastrulation 1 |
| **USF2** | upstream stimulating factor 2 |
| **UTR** | untranslated region |
| **VEGF** | vascular endothelial growth factor |
| **VHL** | von Hippel-Lindau |

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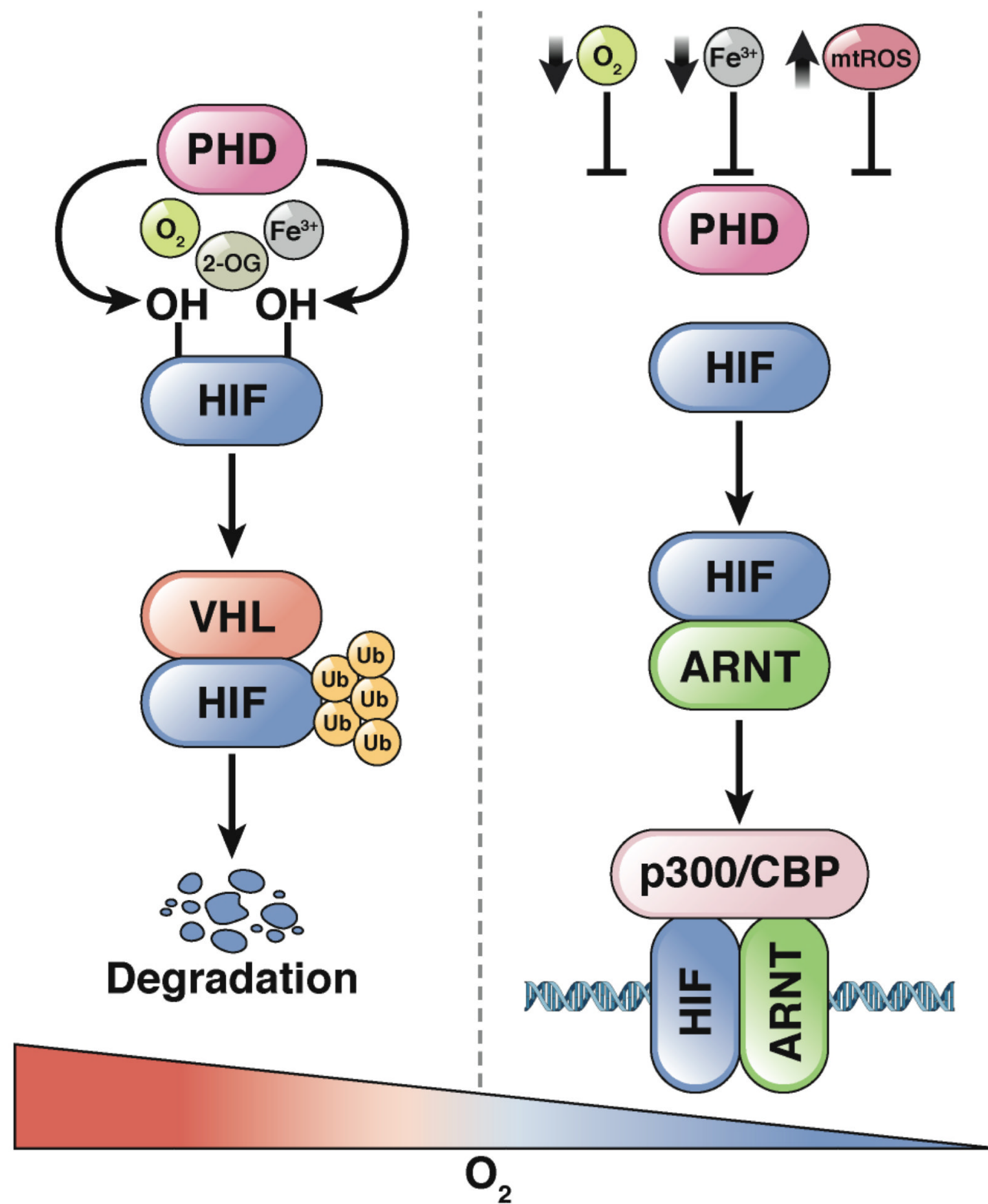


**Figure 1. Iron- and Oxygen-dependent Regulation of HIF Protein Stability**

HIF1α and α2 subunits are hydroxylated on 2 proline residues by PHD enzymes, which require oxygen ($O_2$), iron ($Fe^{3+}$) and 2-oxogluatrate (2-OG). Hydroxylation leads to VHL binding and rapid ubiquitination and degradation. Under low iron concentrations, hypoxic conditions, or increase in mitochondrial ROS (mtROS), the HIFα subunit is no longer hydroxylated, and is therefore stabilized.

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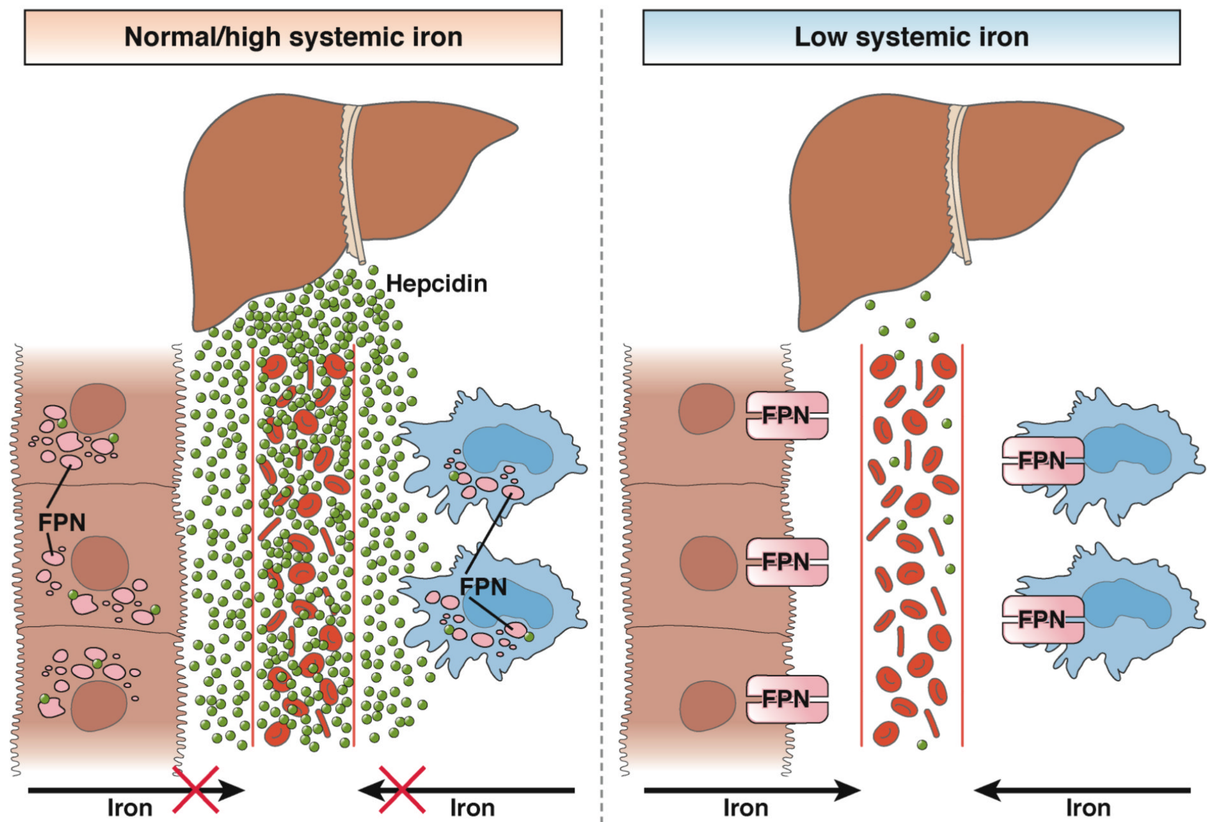


**Figure 2. Hepcidin-mediated Mobilization of Iron**

During normal–high systemic levels of iron, hepcidin is expressed, leading to degradation of ferroportin and reduced mobilization of iron from the intestine and macrophage into circulation. Under conditions of low systemic iron, hepcidin expression is inhibited, stabilizing ferroportin and increasing mobilization of iron from the intestine and macrophage.

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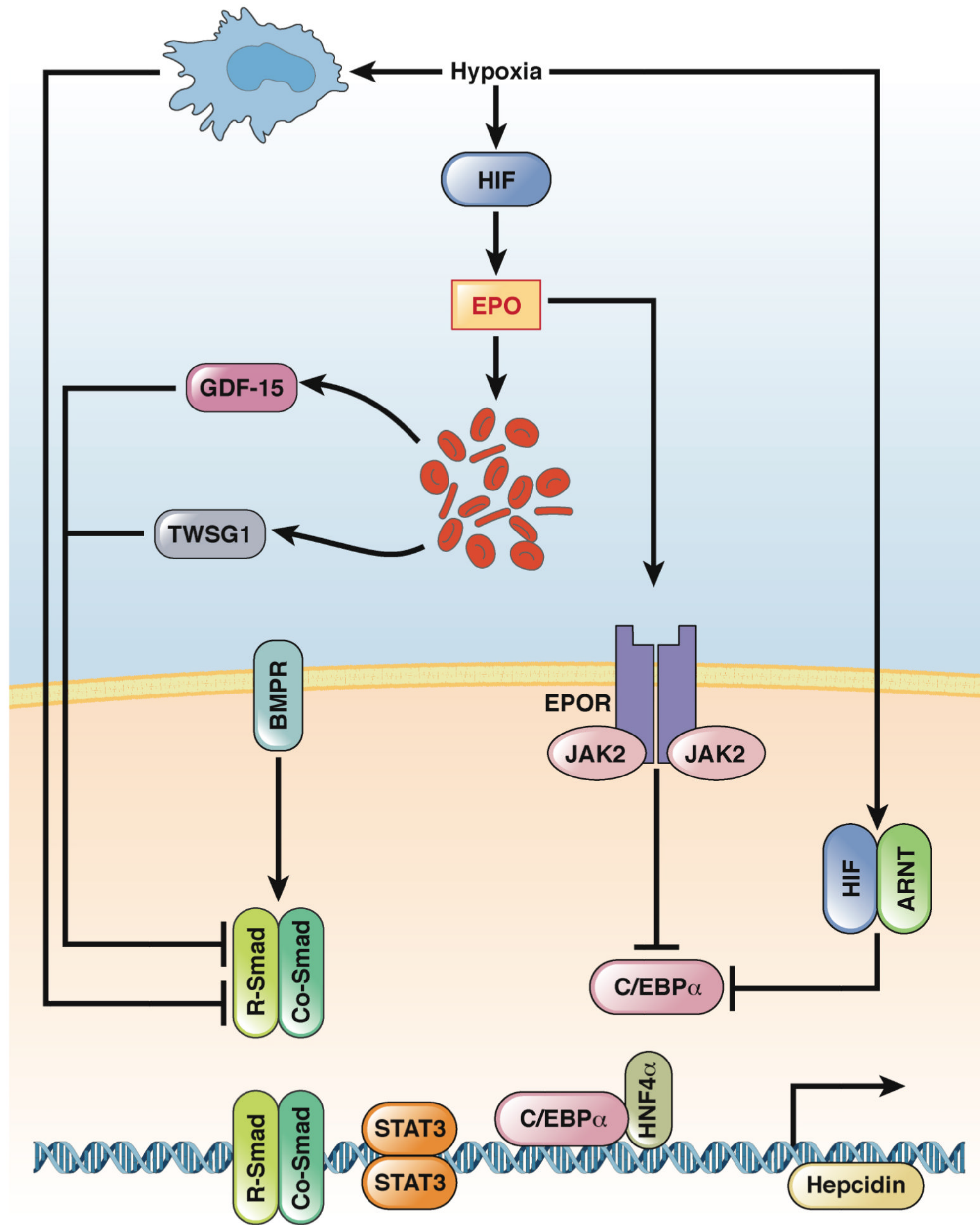


**Figure 3. Transcriptional Regulation of *HAMP* by HIFs**

The gene encoding hepcidin (*HAMP*) is controlled by bone morphogenetic protein (BMP) signaling via its receptor (BMPR) to SMAD (R-SMAD) and CO-SMAD. The transcription factors STAT3, hepatic nuclear factor 4α (HNF4α), and C/EBPα are all required for basal expression of hepcidin. Hypoxia represses transcription of *HAMP*, via erythropoietin-dependent and erythropoietin-independent mechanisms. Systemic hypoxia leads to hepatic or renal activation of HIF, which increases expression of erythropoietin. Erythropoietin binding to its receptor, EPOR, reduces levels of C/EBPα mRNA and protein in hepatocytes. In addition, erythropoietin, by increasing erythropoiesis and erythroid-derived factors GDF15 and TWSG1, can prevent BMP signaling and activation of SMAD. Two erythropoietin-independent mechanisms have been described. Systemic hypoxia inhibits expression of C/EBPα and regulatory SMAD (R-SMAD) in hepatocytes through a

macrophage-mediated mechanism; a local increase in HIF signaling in hepatocytes results C/EBPα degradation by proteasomes.

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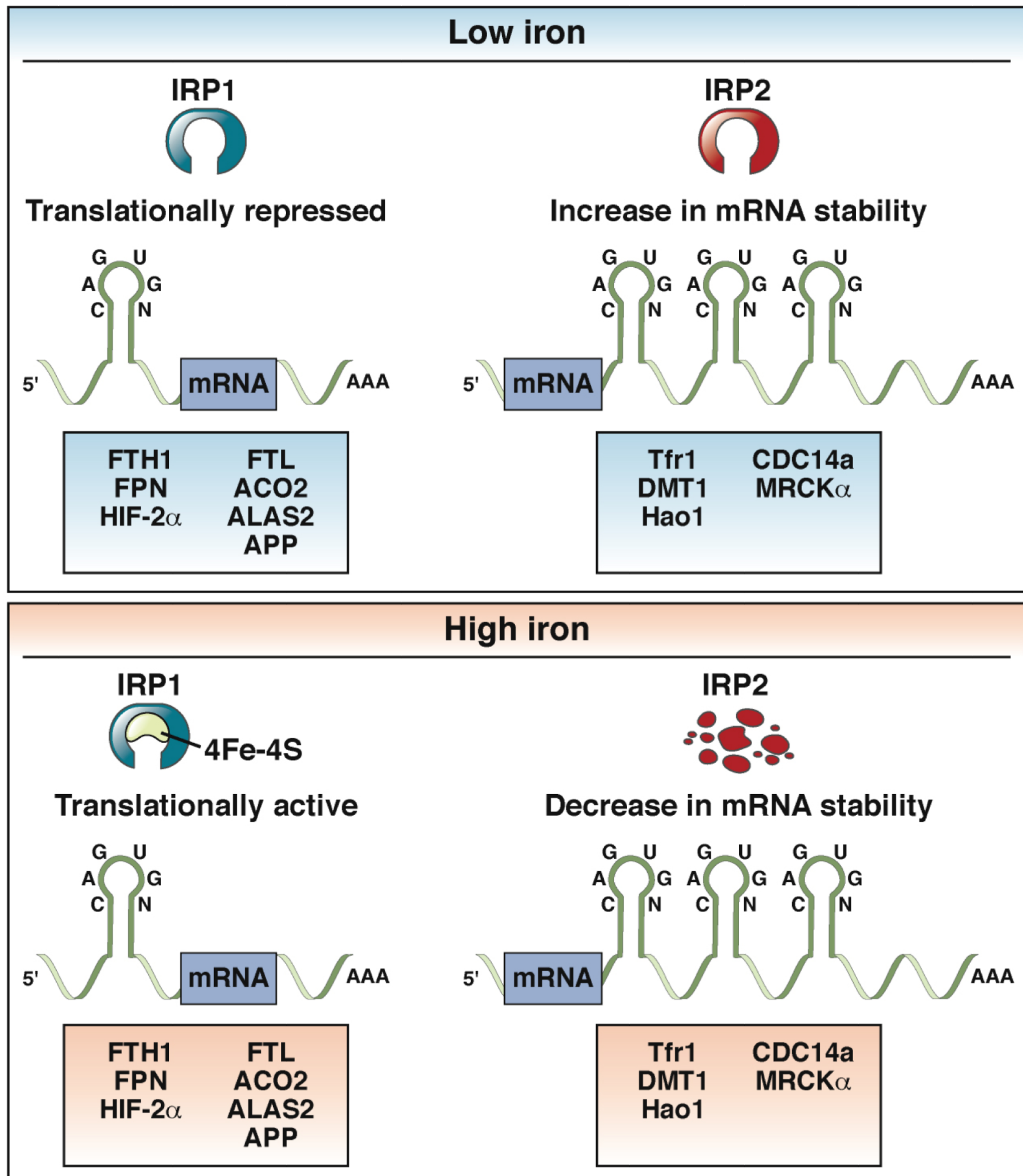


**Figure 4. Regulation of IRE-containing Genes by IRP**

Low concentrations of iron increase binding of IRP1 and IRP2 to IRE-containing mRNAs. Transcripts containing an IRE in the 5'-UTR, such as *FTH1*(encodes H-ferritin), *FTL* (encodes L-ferritin), *SLC40A1*, *ACO2* (encodes mitochondrial aconitase), *ALAS2* (encodes aminolevulinate synthase 2), or *APP* (encodes amyloid precursor protein) bind IRPs to repress translation. Alternatively, transcripts with an IRE in the 3'-UTR, such as *SLC11A2I* (encodes DMT1), *TFRC* (encodes the transferrin receptor), *HAO1* (encodes hydroxyacid oxidase 1), *CDC14A* (encodes cell division cycle 14A), or CDC42BPA (encodes myotonic dystrophy kinase-related Cdc42-binding kinase α) are stabilized by IRP binding. High cellular levels of iron lead to assembly of iron-sulfur clusters (4Fe-4S) in the IRE binding pocket of IRP1, which inhibit its interaction with IREs. IRP2 is degraded under high concentrations of cellular iron. Loss of IRP1 and IRP2 activity result in translation of

mRNAs that contain IREs in the 5’-UTR and decreased stability of mRNAs with IREs in the 3’-UTR.

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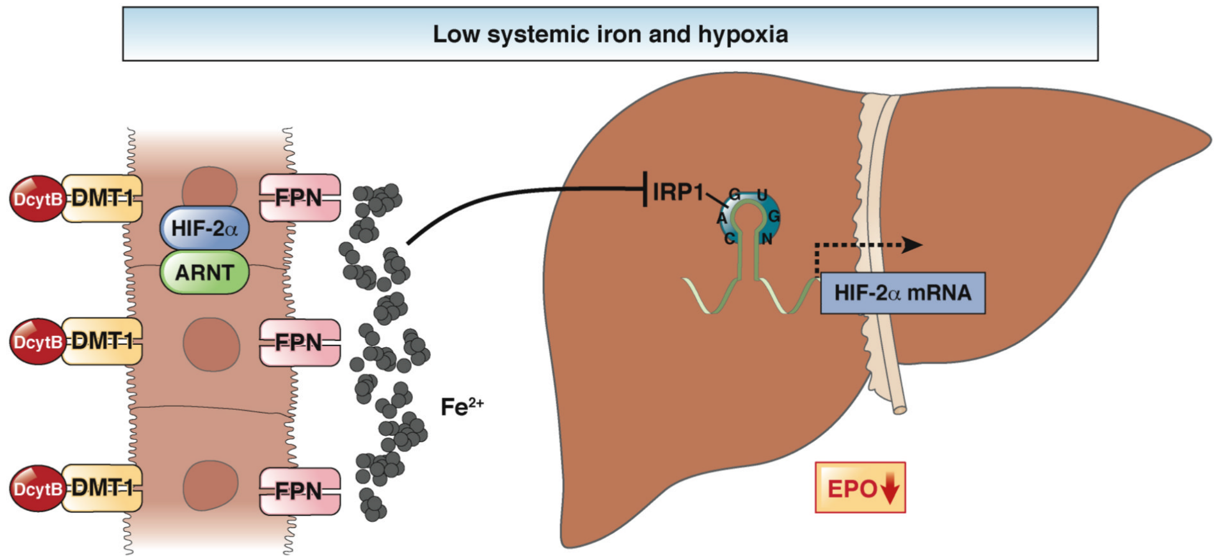


**Figure 5. Responses to Hypoxia and Low Systemic Levels of Iron in Liver and Intestine**

Hypoxia increases expression of erythropoietin and oxygen delivery. However, under conditions of low systemic iron, hypoxic activation of *EPO* would lead to microcytic RBCs. In the liver, low concentrations of iron reduce translation of HIF2α, to limit expression of *EPO* during times of hypoxia and low systemic iron. Coordinately, activation of intestinal HIF2α increases iron absorption. This increase in systemic level of iron relieves the translational block of HIF2α mRNA, increasing its protein levels in liver and/or kidney (not shown). This results in expression of *EPO* and erythropoiesis.