COORDINATION OF IRON AND OXYGEN

SIGNALING THROUGH POST-

TRANSCRIPTIONAL REGULATION OF HYPOXIA

INDUCIBLE FACTOR-2ALPHA

By

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CHAPTER I

INTRODUCTION

Iron is necessary for DNA synthesis, key metabolic reactions, and respiration, and thus is an essential micronutrient for mammals. Despite the relative abundance of iron in the earth's crust, iron deficiency remains the world's most common nutrient deficiency, affecting an estimated 2 billion people worldwide [1]. Iron deficiency progresses in stages and can occur with and without anemia. Iron deficiency anemia occurs in the final stage of iron deficiency when iron depletion is severe. Symptoms of iron deficiency anemia include weakness, fatigue, reduced capacity for work, and reduced capacity to transport oxygen throughout the body. In contrast, there is considerable potential for iron toxicity as excess iron can lead to tissue and organ damage through the production of damaging free radicals [2].

Because iron is an essential nutrient, yet potentially toxic, iron homeostasis is controlled both systemically and at the cellular level. Systemic iron homeostasis is primarily regulated through alterations in absorption or utilization and can be thought of as a closed system since there is no regulated mechanism for iron excretion. Intestinal absorption of dietary iron is decreased in both iron overload and inflammatory conditions, and increased in response to deficiency, enhanced erythropoiesis, and inadequate levels of oxygen (or hypoxia) [3, 4]. Iron is recycled by specialized

macrophages within the reticuloendothelial system that phagocytose senescent or damaged red blood cells thereby removing them from circulation [5]. This conservation and recycling of body iron contributes to the regulation of iron status since it provides the majority of iron entering the plasma to support erythropoiesis, and for incorporation into iron-dependent enzymes [6]. Excess iron can also be redistributed to tissues and cells for storage in the iron storage protein ferritin [6].

Iron Regulatory Proteins (IRPs) are global regulators of cellular iron metabolism. This family of cytosolic RNA binding proteins regulates iron metabolism by binding to stem-loop structures termed Iron Responsive Elements (IREs), that are located in either the 5' Untranslated Region (UTR) or 3' UTR of mRNAs encoding proteins involved in iron metabolism [7]. These stem-loop IREs are present in the mRNAs encoding proteins of iron acquisition (transferrin receptor 1, TfR1; and divalent metal transporter 1, DMT1), iron storage (H- and L-ferritin), iron utilization (mitochondrial aconitase, m-Acon), and iron export (ferroportin, Fpn1) [8].

When iron is limiting, IRPs bind IREs with high affinity inhibiting the translation of mRNAs containing 5'IREs, such as ferritin, and stabilizing mRNAs containing 3'IREs, like TfR1 [7]. In contrast, when iron is adequate, IRPs fail to bind IREs, resulting in the activation of ferritin synthesis and degradation of TfR1 mRNA [7, 9]. IRP1 is a bifunctional protein that exists either as a high-affinity RNA binding protein or the *cytosolic* isoform of the tricarboxylic acid (TCA) cycle Fe-S cluster enzyme mitochondrial aconitase (m-acon). Under iron replete conditions, the formation of an [4Fe-4S] cluster promotes the enzymatic function of the protein cytosolic aconitase, c-acon). Iron deficiency however, results in loss of the Fe-S cluster promoting the high-

affinity RNA binding activity form of the protein. Independently of cellular iron status, IRP1/c-acon activity can also be regulated by oxidative stress and post-translational modification by phosphorylation [10].

Unlike IRP1, IRP2 lacks an [4Fe-4S] cluster, and therefore exhibits no aconitase activity. Instead, IRP2 high affinity RNA binding activity is regulated primarily through iron- and oxygen-dependent modulation of protein stability and degradation [7, 10] Under iron-replete conditions, IRP2 is targeted for proteasomal degradation through iron and/or heme-dependent oxidation mechanisms involving an iron- and oxygen-dependent prolyl hydroxylase and subsequent recognition by an E3-ubiquitin ligase required for proteasomal degradation [11, 12]. Under iron-deficient or hypoxic conditions, IRP2 is stabilized by the inhibition of prolyl hydroxylase activity. Redox-dependent regulation of IRP2 deserves consideration because of the potential relationship between the control of iron utilization and the hypoxic response.

Cells respond to low oxygen, or hypoxic conditions, by altering the expression of specific genes aimed at limiting the effects of decreased oxygen availability. This response is primarily mediated by a family of oxygen-regulated transcription factors called the hypoxia inducible factors (HIFs). There are at least three regulatory subunits of HIF (1α, 2α, and 3α) that heterodimerize with the constitutively expressed oxygen-insensitive HIF-1β (or ARNT) subunit [13]. HIFs function by binding to hypoxia-response elements (HREs) present in the promoters of target genes thereby regulating transcription [13]. HIF-1α and HIF-2α modulate the transcription of an overlapping, yet distinct set of target genes involved in cellular glucose metabolism, cell growth, apoptosis, as well as in restoration of the oxygen supply by promoting angiogenesis [13,

14]. HIF-2α is of particular interest in terms of systemic iron homeostasis as one of its direct target genes is erythropoietin (EPO), which is required for stimulating the proliferation of red blood cells [6, 8, 15].

Interestingly, HIFs share similar iron- and oxygen-dependent mechanisms of regulation as that exhibited by IRP2. Under normoxic conditions, HIFs are targeted for proteasomal degradation by prolyl hydroxylation and interaction with the von-Hippel Lindau protein [13, 14, 16]. Under either low oxygen or hypoxic conditions, HIFs are stabilized due to the inactivation of prolyl hydroxylation resulting in the regulation of target gene expression [8]. These regulatory mechanisms may also coordinate the physiological adaptation to iron deficiency, including the increased expression of EPO to stimulate erythropoiesis [8]. In the absence of adequate iron stores, however, stimulation of erythropoiesis could exacerbate the production of hypochromic microcytic red blood cells as a result of impaired heme synthesis and hemoglobin production [6, 8]. Therefore, under either iron-deficient or hypoxic conditions, the EPO-dependent stimulation of erythropoiesis may be regulated to prevent the formation of immature red blood cells.

The recent discovery of an IRE in the 5'UTR of the HIF-2α mRNA has led to the hypothesis that the IRP-dependent (and thus iron-dependent) regulation of HIF-2α could serve to modulate EPO levels, thereby adjusting the rate of red blood cell production (and iron utilization) to iron availability [8]. Thus far, these suggestions have been limited to results from *in vitro* studies [8, 17]. The focus of this work is to examine the *in vivo* IRP-dependent regulation of HIF-2α using an animal model of dietary iron deficiency. The primary hypothesis is that dietary iron deficiency will activate hepatic IRP RNA binding activity resulting in the repression HIF2-α mRNA translation. This research will provide

us with a better understanding of the regulation of iron metabolism and the utilization of

iron stores under hypoxic conditions.

CHAPTER II

REVIEW OF LITERATURE

Essentiality and Toxicity of Iron

The essentiality of iron for mammals is characterized by its vital role in many life preserving functions including oxygen transport (hemoglobin), cellular respiration (cytochromes), and DNA synthesis (ribonucleotide reductase). Iron is also necessary for cellular proliferation, energy metabolism, and has recently been implicated in the coordination of metabolic and circadian pathways [21, 22]. However, while many functions of iron are attributed to its properties as a transition metal and its ability to undergo oxidation and reduction, this redox property of iron also contributes to its ability to be potentially toxic through its participation in the generation of damaging free radicals by the Haber-Weiss reaction and Fenton chemistry [2, 23].

The body produces approximately 200 billion new red blood cells (RBCs) daily, with each RBC containing millions of hemoglobin molecules [4]. Hemoglobin, which is synthesized in reticulocytes, plays a major role in respiration because it carries about 95% of the total oxygen found in the body [6, 24]. The daily production of these hemoglobin containing RBCs accounts for the majority of iron utilization in the body and represents nearly 80% of the iron demand in humans [4].

A large part of the essentiality of iron is due to its presence in heme. It is the iron atom at center of heme which makes the transport of oxygen by hemoglobin possible. In heme-containing cytochromes, iron also enables the transport of electrons. In the electron transport chain, cytochromes, such as cytochromes b and c, pass along single electrons; the transfer of which is made possible by the change in the oxidation state of iron from the ferrous (Fe^{2+}) to ferric (Fe^{3+}) state [6]. The oxidation of nutrients through the electron transport chain is necessary to release their energy to the body, either in the form of heat or ATP. Other heme containing cytochromes (i.e., cytochrome P450) are involved in lipid drug metabolism and steroid hormone synthesis [6].

Iron plays a crucial role in DNA synthesis as a part of the iron-dependent enzyme ribonucleotide reductase, which converts adenosine phosphate into deoxyadenosine phosphate [6]. Iron has also been shown to be absolutely necessary for cellular proliferation because iron containing proteins are critical for catalyzing the reactions involved in oxygen sensing [25, 26]. Indeed, expression of many of the molecules involved in cell cycle progression, proliferation, and oxygen sensing (e.g., p53, n-myc, c-myc, and HIF-1α) are regulated by intracellular iron levels [25-27]. Additionally, iron also plays a catalytic role in other key metabolic reactions such as glycolysis (glycerol-3-phosphate dehydrogenase), gluconeogenesis (phosphoenolpyruvate carboxykinase or PEPCK), and the TCA cycle (mitochondrial aconitase or m-acon) [6].

More recently, the physiological role of iron has been extended to the coordination of mammalian circadian rhythm through a heme-dependent mechanism. Lazar and colleagues found that heme is a co-factor for the orphan nuclear receptor Rev-erbα, a negative regulator of the circadian clock [22]. When the ligand heme is bound to

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Rev-erbα, it enhances transcriptional repression by promoting the interaction between Rev-erbα and the nuclear co-repressor complex [22]. Thus, through the control of Rev-erbα, cellular iron status may alter the expression of two key gluconeogenic genes – glucose-6-phosphatase (G6Pase) and PEPCK [22]. The implication of these exciting findings is that iron may function as a central regulator of circadian rhythm thereby coordinating metabolic homoeostasis.

While iron is absolutely necessary, it is important to note that there is also considerable potential for iron toxicity. Because there are no regulated mechanisms to control iron excretion, excess iron can accumulate in body tissues and organs. As mentioned above, part of the essentiality of iron is based on its ability to accept or donate electrons, however, in excess this redox activity can lead to the generation of damaging free radicals via Fenton chemistry, and the subsequent production of reactive oxygen species [2].

Fenton chemistry describes the reaction between hydrogen peroxide (H_2O_2) and ferrous iron (Fe^{2+}) that can produce hydroxyl radicals ($OH^{\bullet}$) and other oxidizing species capable of creating biological injury [2]. In fact, elevated iron accumulation in the brain has been linked to oxidative damage and neurodegeneration associated with multiple sclerosis, Friedrich's ataxia, Parkinson's disease, and Alzheimer's disease [16].

Additionally, oxidative stress and damage has been implicated as a causative factor for several chronic diseases including cardiovascular disease and diabetes [28, 29]. The role of iron in the pathogenesis of these two diseases has been supported by epidemiological evidence indicating that elevated iron storage levels are associated with increased risk of coronary heart disease and insulin resistance [29, 30]. Although a

precise mechanism has not yet been elucidated, the potentially toxic characteristics of iron are suspected to play a critical role.

Iron overload and toxicity is most commonly observed in individuals with hemochromatosis, a condition characterized by inappropriate parenchymal iron deposition. Hereditary hemochromatosis (Classical or Type I) is the most common genetic iron overload disorder affecting approximately 1/200 individuals of northern European descent [31]. It is associated with mutations in the HFE gene and characterized by dysregulation of intestinal iron absorption resulting in excessive iron storage in organs such as the liver, heart and pancreas [32, 33]. Early symptoms of excess deposition of iron include fatigue, joint pain, depression, impotence, and increased skin pigmentation [33]. Left untreated, patients with hemochromatosis accumulate iron in tissues like the liver, heart, and pancreas resulting in the development of cirrhosis, cardiomyopathy, and diabetes, respectively [33].

Several other iron overload or hemochromatosis disorders exist that are not associated with mutations in the HFE gene. For example, "juvenile" or Type II hemochromatosis, is associated with the same phenotypic characteristics as type I hemochromatosis but is due to mutations in the gene encoding hemojuvelin (HJV) also known as HFE2 [4, 34]. Individuals with juvenile hemochromatosis accumulate iron at a much faster rate and tend to experience cardiomyopathy and other endocrinopoathies rather than severe liver disease [35]. In the absence of treatment, these patients typically suffer from heart failure before age thirty [33, 35]. Other types of hemochromatosis can result from mutations in genes encoding the iron hormone hepcidin (HAMP1), the iron uptake protein transferrin receptor 2 (TfR2), and the iron export protein ferroportin (Fpn

or SLC40A1) [4]. The importance of all of the proteins to the regulation of mammalian iron homeostasis will be discussed below.

<u>Iron Absorption, Transport, Utilization, and Uptake</u>

The control of iron homeostasis is primarily influenced by an individual's iron needs to support erythropoiesis and the status of body iron stores. Because there is no regulated means for iron excretion, homeostasis is primarily mediated through controlling dietary absorption. Intestinal absorption is largely influenced by organismal iron status, and may range from 10% (for an individual with a normal iron status) up to 35% (for an individual who is iron deficient) [6]. Iron absorption can occur throughout the entire length of the small intestine, but is most efficient in the duodenum [6]. Dietary iron exists as either heme iron or nonheme iron. Relatively little is known about heme iron absorption, but it has been suggested that it is internalized through the recently identified receptor heme carrier protein-1 and then disassembled by heme oxygenase [36, 37]. Non-heme ferric iron is reduced by an iron-regulated duodenal cytochrome reductase (dCytb) [38]. Ferrous iron is then transported across the luminal membrane of enterocytes by divalent metal transporter-1 (DMT1) [4, 39, 40].

Once in the enterocyte, iron has three fates: (1) storage/excretion, (2) utilization, or (3) transport to other tissues. Iron that is not transported out of the enterocyte can be incorporated into the iron storage protein ferritin for short-term storage [6]. If needed, iron can later be released from ferritin for transport and utilization. If iron is not needed, it can be "excreted" within the short-lived mucosal cells that are sloughed off every 2-3 days [6]. Iron can also be used in the intestinal cells as a component of other iron- or heme-containing enzymes. Iron that is neither stored nor utilized in the intestinal cells is

transported across the basolateral membrane by the iron export protein ferroportin (Fpn1) [39]. Intestinal iron absorption and export is markedly increased in individuals with hereditary hemochromatosis as mutations in HFE result in an increase in the expression of DMT1 and Fpn1 in the duodenum [41]. A diagram of intestinal iron absorption and cellular uptake is shown in **Figure 1**.

Following export across the basolateral membrane, ferrous iron (Fe^{2+})is oxidized to ferric iron (Fe^{3+}) by the multi-copper oxidase hephasestin (Heph) prior to being loaded onto the iron transport protein transferrin [6, 39]. Transferrin (Tf), a glycoprotein, binds a maximum of two iron atoms and serves as the primary means for interorgan transport [9]. Tf plays a critical role in iron transport as it has the capacity to reversibly bind iron. This is important because at physiological pH (pH ~7.4) iron is insoluble in its free state and is capable of generating free radicals. However, at physiological pH transferrin binds to iron with high affinity, making it safely available for use by the body tissues, such as the liver, muscles, and bone marrow [42].

Iron uptake in these tissue cells occurs through receptor-mediated endocytosis of transferrin via interaction with transferrin receptors (TfRs) 1 and 2 [9, 39]. TfR1 is expressed in nearly all tissues and has a higher affinity for diferric Tf than monoferric or apo-Tf [42]. TfR1 also has a 20-fold higher affinity for Tf than TfR2 [43]. Several other notable differences exist between TfR1 and TfR2. First, TfR2 expression is limited to hepatocytes and erythroid cells whereas TfR1 is ubiquitously expressed. Next, while TfR1 expression is regulated by iron status, TfR2 expression is instead modulated by cell growth rate [42]. Finally, TfR1 forms a complex with HFE while TfR2 is incapable of
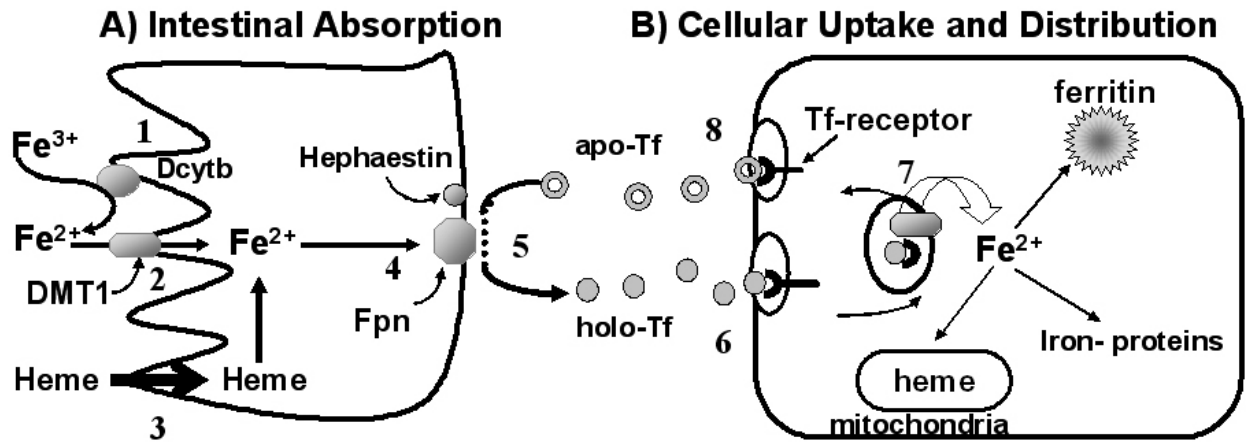
Figure 1 Intestinal absorption and cellular uptake of iron. (**A**) Dietary iron absorption. Non-heme (Fe^{3+}) is reduced by duodenal cytochrome B (Dcytb) and transported into the cytosol by divalent metal transporter-1 (DMT1). Dietary heme iron is thought to be internalized through assistance of heme carrier protein-1, where the Fe^{2+} is liberated by heme oxygenase. In the enterocyte, iron is stored (L- or H- Ferritin), utilized (m-Acon), or exported out of the cell by ferroportin-1 (Fpn1). Following export across the basolateral membrane, Fe^{3+} is oxidized by hephaestin before it is bound to transferrin for transport to various tissues. (**B**) Cellular iron uptake. The transferrin-bound iron binds to the transferrin receptor (TfR) on the surface of the cell. The Tf/TfR complex is then internalized through receptor-mediated endocytosis. The acidic pH of the endosome results in the release of iron from Tf so that it can be pumped into the cytoplasm, most likely through DMT1. Tf and TfR are recycled back to the cell surface where they dissociate upon encountering a neutral pH.

interacting with HFE [42]. Once bound, the Tf/TfR complex is endocytosed, the acidic pH of the endosome results in the release of iron, and Tf and TfR are recycled back to the cell surface where they disassociate upon encountering the nearly neutral pH [42, 44]. The released iron is then pumped into the cytoplasm, most likely by DMT1 [9]. Once in the cytoplasm, iron is either used by the cell (i.e., in the production of iron-containing proteins), exported out of the cell through ferroportin, or stored in ferritin, the primary storage form of iron in cells.

A striking feature of mammalian iron metabolism is the extent to which iron is conserved by the body following its absorption. Ferritin is a cytosolic protein involved in iron storage and detoxification in across many animal kingdoms including microbial, plant, and animal species [9]. In mammals, ferritin molecules are present as heteropolymers with 24 subunits of two types, H-subunits (heavy or heart) and L-subunits (light or liver). H-subunits exhibit ferroxidase activity that promotes the loading of iron into storage, whereas L-subunits are more efficient at promoting mineralization of protein nuclei [45, 46]. Different organs and tissues have different levels of expression of the two subunits [45, 47]. Ferritin can store up to 4500 iron atoms, though under most conditions only 20% of the storage capacity is utilized [48]. Reduction of Fe^{3+} by reductants such as riboflavin, niacin, and vitamin C is required for the release of iron from ferritin [6]. However, for transport out of the cell and subsequent transport by transferrin, Fe^{2+} must first be reoxidized by ceruloplasmin [6].

There is no regulated mechanism for iron excretion, but iron losses of around 1 mg per day occur primarily through obligatory losses (i.e., bleeding and sloughing off of mucosal and skin cells). About 1-2 mg of dietary iron is absorbed each day, but daily

erythrocyte production (200 billion RBCs) requires 20-24 mg of iron for hemoglobin synthesis [4]. Thus, while absorption of dietary iron is important to satisfy daily iron losses, it is the body's mechanism of conserving and recycling iron that ensures proper iron stores. Although the liver accounts for approximately 60% of the ferritin in the body, the remaining 40% is found in cells of the reticulendothelial system (RES) [49].

Iron is recycled by specialized macrophages within the RES that phagocytose senescent or damaged red blood cells and remove them from circulation [5]. Within the macrophages, red blood cells are lysed and hemoglobin is degraded by heme oxygenase which catalyzes the liberation of iron from heme [4, 23]. Macrophages can then either store the iron derived from hemoglobin in ferritin or release iron through the iron export protein Fpn1 with the aid of soluble multi-copper oxidase cerruloplasmin (Cp) [50]. The majority of iron entering the plasma for distribution or redistribution by transferrin is derived from the cells of the RES, sites of hemoglobin destruction and/or ferritin and hemoglobin degradation [6]. Thus, cells of the RES play a critical role in maintaining whole-body iron homeostasis.

The adult human body contains iron in two major pools: 1) functional iron in hemoglobin, myoglobin and enzymes and 2) storage iron in ferritin and transferrin [24]. The majority of iron is found in the functional pool, with only about 20% remaining as storage iron found primarily in hepatocytes and the macrophages of the reticuloendothelial system [34]. In order to maintain iron homeostasis and meet the iron requirements of the body, the movement of iron between these two pools must be tightly regulated.

<u>Control of Iron Homeostasis</u>

Because of iron's essential yet potentially toxic nature, iron homeostasis must be maintained at both systemic and cellular levels. As there is no regulated mechanism controlling iron excretion, systemic iron balance is tightly maintained through the regulation of absorption from the intestine. As previously mentioned, the rate and degree of absorption is directly related to existing body iron stores and the amount of iron needed to support erythropoiesis. Two regulators, a so-called "store regulator" and "erythroid regulator," are thought to regulate the degree of iron absorption [3, 51]. The store regulator inversely affects the rate of iron absorption based on body iron stores, such that when levels of this regulator are elevated, there is an overall decrease in the amount of dietary iron that is absorbed in the small intestine. The erythroid regulator, on the other hand, enhances iron absorption when an imbalance between iron supply and the rate of erythropoiesis in the marrow occurs [3].

Four situations lead to measurable changes in intestinal iron absorption: 1) abnormal iron availability (overload or deficiency), 2) accelerated erythropoiesis, 3) hypoxia, and 4) inflammation [50]. Therefore, iron absorption and plasma availability is decreased in response to iron overload and inflammation, and is increased in response to an inadequate iron status, enhanced erythropoiesis, and hypoxia [3, 4, 50].

Much enthusiasm was generated when the small peptide hormone hepcidin, initially thought to function as an antimicrobial agent, was shown to be a major regulator of both intestinal iron absorption and iron recycling within the RES [52-54]. The "stores regulator," hepcidin, is the key iron regulatory hormone secreted by the liver and is primarily responsible for coordinating iron absorption with existing iron stores to meet

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systemic iron needs. Hepcidin regulates systemic iron metabolism by promoting the degradation of the iron export protein Fpn1 in both enterocytes and macrophages in the RES [53, 55]. Thus, when iron stores are elevated, hepatic hepcidin expression and secretion are increased and iron absorption and release from stores is diminished. Conversely, when iron stores are low, hepcidin expression is decreased and intestinal iron absorption and iron release from the RES is enhanced. Mutations in HAMP1, the gene encoding hepcidin, are associated with the development of a type of juvenile hemochromatosis and illustrate the central role this peptide hormone plays in the regulation of systemic iron homeostatis [40, 52, 53].

Hepcidin regulation occurs at the transcriptional level. One means of regulating Hepcidin is dependent upon signaling through the bone morphogenetic protein/Smad (BMP/Smad) pathway. BMPs are cytokines which can bind to the cell surface Type I and Type II BMP receptors, thereby activating a signaling cascade that generates phoshporylated RSmads which dimerize with Smad4 [55]. The RSmad/Smad4 heterodimer can then translocate to the nucleus and activate transcription of the HAMP1 gene [55, 56]. Inflammatory cytokines such as IL-6 can also induce HAMP1 transcription in hepatocytes through activation of STAT3 (which also requires the presence of Smad4) and the subsequent binding of STAT3 to a regulatory element in the HAMP1 promoter [56, 57].

When iron levels are high, molecules such as the hemochromatosis gene product (HFE), hemojuvelin (HJV), and transferrin receptor 2 (TfR2) are associated with increased hepatic hepcidin expression. Mutations in these genes results in the absence of hepcidin and thus iron overload [55]. In response to situations such as anemia and

inflammation, hepcidin expression is increased, however, expression is decreased in response to hypoxia suggesting that hepcidin is a key regulator in iron homeostasis under various pathophysiological conditions [3, 55]. This evidence has been further supported by work demonstrating that complete lack of hepcidin in mice results in iron overload, while animals overexpressing hepcidin experience decreased body iron levels and severe anemia [58, 59]. Thus, an effective means of hepcidin administration in response to iron overload, or a means to counteract overexpression of hepcidin seen in inflammatory diseases, could have momentous pharmacological value, especially in view of the fact that iron deficiency, as seen in anemia of chronic disease, as well as nutritional iron deficiency, is a major public health issue.

Iron Deficiency

Dietary iron deficiency occurs when iron absorption from the diet fails to meet physiological requirements, and can result from several factors. Rapid growth coupled with a low iron diet can result in iron deficiency in children. Premenopausal women may become iron deficient due to frequent heavy menstrual blood loss. Other conditions associated with increased blood loss include infections, tumors, and inflammation. In under developed countries blood loss as a result of parasitic infection can also exceed dietary iron intake, resulting in iron deficiency [60]. In general and regardless of etiology, iron deficiency progresses in 3 stages: 1) iron stores are depleted, 2) erythropoiesis is diminished as the iron supply to the marrow is reduced, and 3) hemoglobin production falls leading to anemia [61]. Symptoms include weakness, fatigue, and reduced capacity

for work which are largely due to the decreased capacity to transport oxygen as result of low hemoglobin levels.

Iron deficiency is the world's most common nutrient deficiency, affecting an estimated 2 billion people worldwide, and posing as a major public health issue [1]. According to the Global Burden of Disease 2000 Project, iron deficiency potentially contributes to the death and disability of more than 800,000 individuals annually through increased risk of child mortality, maternal mortality, perinatal mortality, cognitive impairment, and decreased fitness and productivity [62]. The vast majority of this wide spread iron depletion is attributed to parasitic infection due to limited access to clean food and water in many developing countries. Billions of people worldwide are afflicted with intestinal worm infections which often results in anemia due to decreased nutrient loss, impaired nutrient absorption, and/or an overall decrease in food intake [63].

Iron deficiency disproportionally affects premenopausal and pregnant women, infants, and rapidly growing children. Over 50% of pregnant women in developing countries are anemic, and while prevalence in the U.S. has significantly decreased over the last thirty years, infants born to poor, minority, and/or immigrant mothers still remain at risk for becoming iron deficient [64]. This is significant because iron deficiency adversely affects development of the central nervous system through its involvement in important developmental processes such as myelination, dendritogenesis, synaptogenesis, and neurotransmission [64]. Indeed, infants born to iron deficient women have been found to have cognitive, motor, social, and emotional dysfunction, which may be only partially reversible with iron repletion [1, 64]. Studies in school age children have

shown iron deficiency affects cognition, and that anemic children struggle with social inattention and decreased motor activity and school performance [65, 66].

In adults, iron deficiency even in the absence of anemia can lead to fatigue and reduced work performance [1]. This decrease in work capacity is attributed to low hemoglobin levels observed in iron deficiency, leading to a decreased efficiency in moving oxygen from the lungs to the brain and muscles [67]. Support for this is provided by evidence showing that with severe anemia, oxygen transport is restricted to tissue oxidative function at anything other than a resting condition [68]. In developing countries the impact of iron deficiency comes with a heavy price as the resulting reduced work capacity is attributed to the loss of millions of dollars each year [69]. Consequently, organizations such as the World Health Organization and the International Labor Organization are working to establish an effective, cost efficient way of treating iron deficiency by means of dietary iron supplementation and treatment for causes such as parasitic infection.

The body utilizes various physiological mechanisms in an attempt to adapt to iron deficiency. First, intestinal expression of both the luminal iron transporter DMT1 and basolateral iron exporter Fpn1 is increased [4, 70]. Further, hepcidin expression is repressed leading to an enhanced release of iron stores through the de-repression of Fpn1-mediated iron export from the RES [40, 52]. Next, plasma levels of the iron transport protein transferrin are increased in an effort to maximize transport capacity [33, 71]. Finally, at the cellular level, iron deficiency enhances the expression of the iron uptake protein TfR [72]. The responses of various tissues to iron deficiency may differ significantly to most efficiently meet the needs of the organism and these tissue-specific

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responses are thought to be coordinated by iron regulatory proteins. In summary, these physiological adaptive mechanisms function to increase the acquisition and conservation of organismal iron under conditions when environmental iron is limiting.

Global Regulators of Iron Metabolism

As alluded to above, a number of proteins intimately involved in cellular iron homeostasis are post-transcriptionally regulated by the action of Iron Regulatory Proteins (IRPs). The coordination of iron uptake, storage, and utilization is critical in maintaining optimal levels of iron and the appropriate distribution of the intracellular iron pool. Through the actions of Iron Regulatory Protein 1 (IRP1) and Iron Regulatory Protein 2 (IRP2), which both act to "sense" the intracellular iron status, total body iron homeostasis is tightly controlled. IRPs regulate iron metabolism through high-affinity binding to Iron Responsive Elements (IREs), stem-loop structures in messenger RNA (mRNA) composed of a highly conserved CAGUGX hexanucleotide loop sequence [7, 73]. These conserved stem-loop structures are located in either the 5' Untranslated Regions (UTRs) or 3'UTRs of mRNAs encoding proteins of iron metabolism and alter protein translation or mRNA stability, respectively (**Table 1**) [7, 10, 73].

Both IRP1 and IRP2 function as high-affinity cytosolic RNA binding proteins that are regulated in an iron-dependent manner. IRP1 is a bifunctional protein exhibiting either high affinity RNA binding protein activity (IRP1) or enzymatic activity by functioning as the cytosolic isoform of the TCA cycle enzyme aconitase (c-acon) [7, 74]. The activity (or function) of the protein is largely dependent on the presence or absence

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Table 1 Established and newly identified targets of IRP action

mRNA	Function of Protein	Location of IRE
Transferrin Receptor	Iron transport	3'UTR
Divalent Metal Transporter 1	Iron uptake	3'UTR
H- and L-Ferritin	Iron storage	5'UTR
Ferroportin	Iron export	5'UTR
Mitochondrial-aconitase	Tricarboxylic Acid Cycle	5'UTR
Succinate Dehydrogenase[*]	Tricarboxylic Acid Cycle	5'UTR
Erythroid Aminolevulinic Acid Synthase	Heme biosynthesis	5'UTR
Hypoxia Inducible Factor-2α	Oxygen Sensing	5'UTR

IREs located in either the 5'UTR or 3'UTR of mRNAs alter protein translation or stability, respectively. [*]IRE present only in *Drosophila melanogaster*.

of the Fe-S cluster [7, 9]. Under iron replete conditions, the presence of a [4Fe-4S] cluster confers enzymatic (aconitase) activity and inhibits high-affinity RNA binding activity [75]. Conversely, under iron deficient conditions, the Fe-S cluster is "disassembled" resulting in the generation of high-affinity RNA binding activity (IRP1) [76]. This iron dependent regulation of IRP1 is shown in **Figure 2**. In contrast to IRP1, IRP2 does not contain a [4Fe-4S] cluster and lacks aconitase activity, functioning only as an RNA binding protein. Rather than being regulated through the assembly or disassembly of an Fe-S cluster, IRP2 is regulated primarily through iron- and oxygen-dependent modulation of protein stability (not protein function like IRP1) and degradation [77] (**Figure 3**).

Aside from the lack of an Fe-S cluster as in IRP1, IRP 2 also contains an additional 73 amino acid sequence that is necessary for its iron-dependent regulation and degradation [7]. Through an iron and/or heme-dependent oxidation mechanism involving prolyl hydroxylation and recognition by an E3-ubiquitin ligase, IRP2 is uibiqitylated and targeted for proteasomal degradation [11, 12]. Under iron-deficient or hypoxic conditions, prolyl hydroxylase activity is relatively low and IRP2 is not recognized by the E3-ubiquitin ligase. Thus, IRP2 is not ubiquitylated nor degraded leading to increased protein abundance as a function of enhanced stability [11]. This iron- and oxygen-dependent regulation of both IRPs deserves consideration because of the potential for coordinating both iron and oxygen sensing.

IRPs are cytosolic RNA binding proteins that bind to IREs and mediate the translation (when present in the 5'UTR) or stability (when the IRE is present in the

Figure 2 Iron dependent regulation of IRP1. When cells are iron deficient, IRP1 is converted to its active RNA binding form and binds to IREs with high affinity. Under iron replete conditions, IRP1 confers enzymatic activity through the assembly of a [4Fe-4S] cluster and is the cytosolic isoform of aconitase (c-Acon).
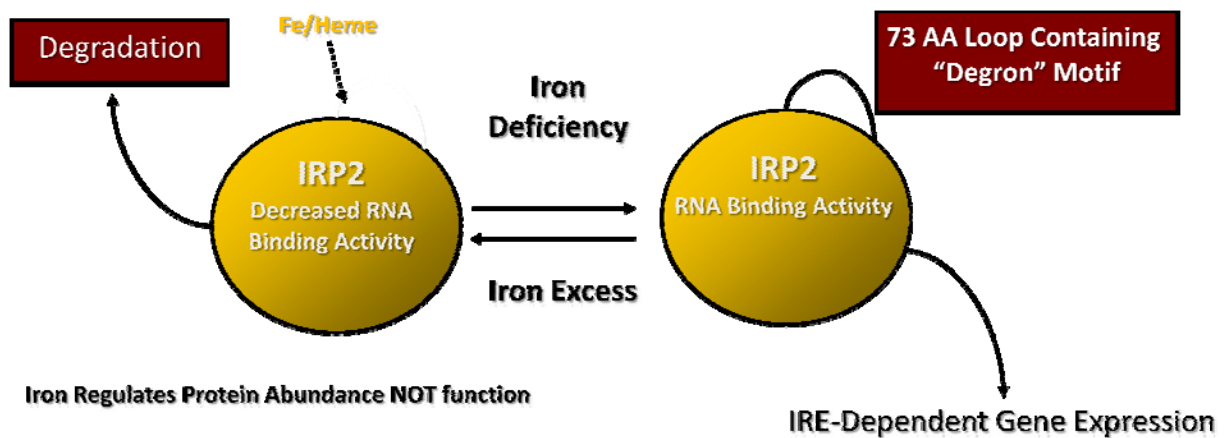
Figure 3 Iron dependent regulation of IRP2. Iron regulates IRP2 by enhancing protein stability or degradation through a mechanism involving prolyl hydroxylation and recognition by an E3-ubiquitin ligase. When iron is limiting prolyl hydroxylase activity is low and IRP2 is not recognized by the E3-ubiquitin ligase leading to an increase in protein abundance and thereby an increase in RNA binding activity. Excess iron results in recognition of IRP2 by the E3-ubiquitin ligase. IRP2 is then ubiquitylated and targeted for proteasomal degradation leading to a decrease in protein abundance and subsequent decrease in RNA binding activity.

3'UTR) of mRNAs encoding proteins involved in iron metabolism. In order to understand the mechanisms through which IRPs regulate translation, a brief review of protein translation is required. **Figure 4** illustrates how IRPs mediate translation under iron deficient and iron adequate conditions.

Translation initiation on mRNA occurs in three steps: 1) the 40S ribosomal subunit binds to the mRNA close to the m^7GpppG cap forming the 43S pre-initiation complex, 2) the 43S pre-initiation complex scans through the 5'UTR to the initiation codon, and 3) the 60S ribosomal subunit is recruited to form the 80S ribosome so that translation can begin [78, 79]. The formation of an IRP/IRE complex within the 5' UTR of mRNAs relatively close to the m^7GpppG cap can repress translation initiation through stearic hindrance thereby blocking the binding of the 40S ribosomal subunits to the mRNA [79-81]. Complexes that are farther away from the cap cannot affect binding of 40S subunit, but rather inhibit initiation by impeding ribosomal scanning [79]. There is, however, a positional requirement that exists in order for IRPs to be able to repress translation. If the IRE is positioned more than ~70nt distal to the m^7GpppG cap, IRPs are incapable of efficiently blocking translation [82]. This position effect may allow for tissue-specific or perhaps developmental regulation of various IRP targets. Further, differences in IRE location and/or thermodynamic stability may contribute to differential regulation of translation of mRNAs containing a single IRE in the 5'UTR. For instance, the 5'IRE in ferritin is very strictly regulated with changes in iron status whereas the 5'IRE in m-acon is less-sensitive to IRP-mediated repression [10, 73, 83, 84]. The differences observed in IRE functionality are likely attributed to structural differences in
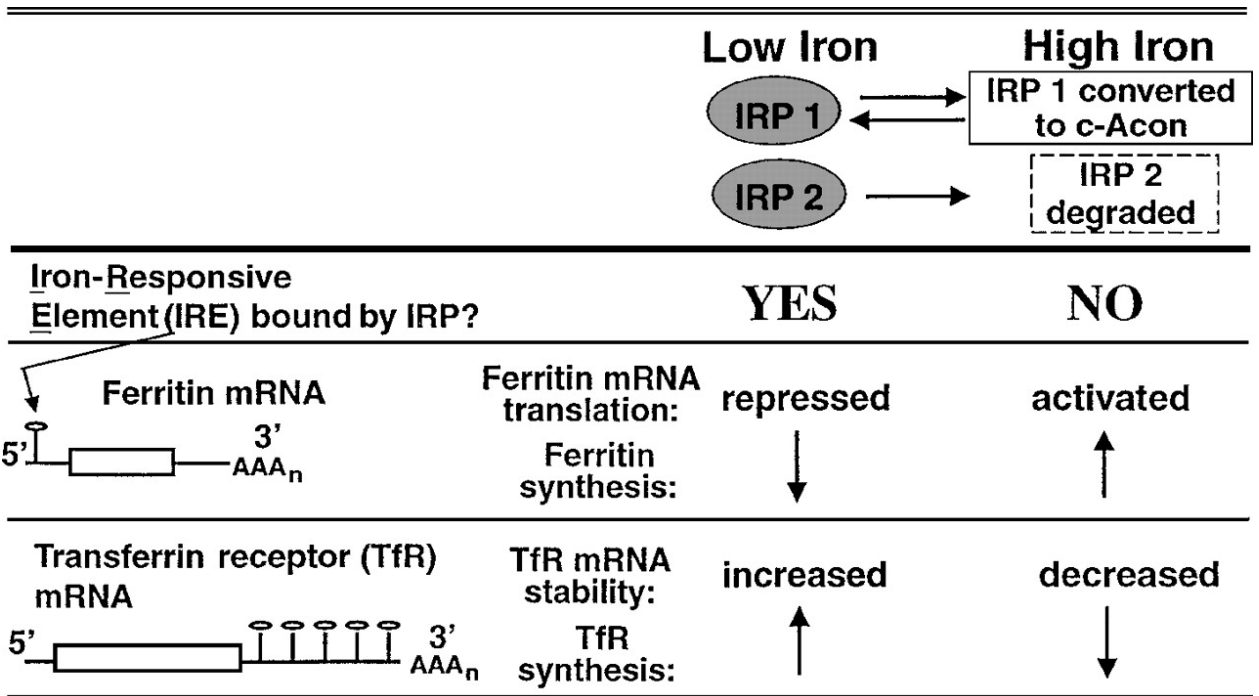
Figure 4 Iron regulatory protein (IRP) regulation of ferritin and transferrin receptor (TfR). Under low iron conditions, IRP1 is converted from its cytoscolic aconitase (c-Acon) form to its active RNA binding form and IRP2 protein is stabilized. IRP binding to the 5'IRE in ferritin and the 3'IRE in TfR results in repressed ferritin mRNA translation and enhanced TfR mRNA stability. Under iron adequate or high iron conditions, IRP is found mainly in its c-acon form and IRP2 protein is degraded. Consequently, the IRPs fail to exhibit high affinity RNA binding activity leading to the activation of ferritin synthesis and a decrease in TfR mRNA stability.

the IRE and is based on the observation that even slight variations in the IRE structure produces a variety of mRNA-specific responses to alterations in iron availability [73, 83]. Finally, unlike IREs present in the 5'UTR, the presence of IREs in the 3'UTR of mRNAs encoding TfR and perhaps DMT1, lead to an increase in mRNA stability [85, 86]. Upon IRP binding to the IRE in TfR mRNA, the mRNA is stabilized as a result of the interference with nuclease access to an endonucleolytic cleavage site in the 3'UTR [87].

IRPs coordinate the cellular response to depleted iron status by decreasing iron storage or increasing iron uptake through the regulation of ferritin and transferrin receptor mRNA, respectively [7, 10]. When cells are iron deficient, IRPs function as high-affinity RNA binding proteins and repress the translation of mRNAs containing 5'UTR IREs (i.e., ferritin) and increase the stability of mRNAs containing 3'UTR IREs (i.e., TfR) [10, 17, 88]. Under iron replete conditions, IRPs lose their high affinity RNA binding capacity and fail to bind IREs resulting in the de-repression of ferritin synthesis and a decrease in TfR mRNA stability [7].

In addition to regulating the expression of proteins involved in iron storage and acquisition, IRPs also regulate mRNAs encoding proteins involved in iron utilization. For example, erythroid 5'-aminolevulenic acid synthase (eALAS), the rate limiting enzyme in heme formation, contains an IRE in its 5'UTR. In iron deficiency eALAS translation is repressed decreasing the overall rate of heme biosynthesis [73]. Interestingly, IREs have also been identified in the 5'UTRs of mRNAs encoding mithochondrial aconitase (m-acon) and the iron-protein subunit of succinate dehydrogenase, SDH (in *Drosophila sp.* only), two TCA cycle enzymes [7, 10]. The regulation of m-acon and SDH via the IRE/IRP system provides a direct link between

iron and energy metabolism and is thought to play an important role in fuel utilization during iron deficiency [19, 89].

An IRE is also found in the 3'UTR of the mRNA encoding the iron uptake protein (DMT1), though it remains unclear how this IRE functions since there appear to be DMT1 splice-variants expressed that do not contain an IRE [90, 91]. In addition to regulating iron uptake (TfR and DMT1), IRPs also appear to regulate the translation of the iron export protein Fpn1 through an IRE in its 5'UTR (S.L. Clarke, K.L. Ross, R.S. Eisenstein, unpublished observations) [18]. Thus, under iron deficient conditions, Fpn1 translation is repressed, resulting in decreased iron release from hepatocytes, enterocytes, and cells of the RES. It remains unclear what role translational repression plays since the primary means of regulating Fpn1 activity is through hepcidin-dependent degradation. It is clear however, that both IRP1 and IRP2 are central regulators of iron metabolism, as they regulate proteins involved in the absorption, uptake, transfer, and storage of iron.

Although the interconversion of IRP1/c-acon via assembly and dissembly of the Fe-S cluster is thought to be the primary mechanism through which the protein's activity is regulated, IRP1 activity can also be regulated independent of iron by other means including oxidative stress and post-translational modification [92-94]. IRP1 is also be regulated by protein kinase C (PKC)-dependent phosphorylation at two PKC phosphorylation sites, S711 and S138 [95, 96]. The evidence of phosporylation-dependent regulation indicates that IRP1 is regulated by means dependent and independent of Fe-S cluster assembly/disassembly [92, 96]. Phosphorylation of S711 of IRP1 results decreased in aconitase activity and suggests that phosphorylation at this site may alter cytosolic citrate/isocitrate metabolism independent of iron status [20]. On the

other hand, phosphorylation at S138 of IRP1 results in decreased Fe-S cluster stability and through an unknown mechanism enhances protein turnover [92]. Whereas phosphorylation of IRP1 provides a means to alter protein function in response to extracellular stimuli, modulation of IRP activity in response to oxidative stress appears to be a more generalized response due to modification of the [4Fe-4S] cluster. Hydrogen peroxide, nitric oxide, and peroxynitrite are the most well-characterized cluster perturbants. They promote the loss or disassembly of the [4Fe-4S] cluster generating the RNA binding form of the protein [7, 10, 97]. IRP2 can also be regulated in an iron-dependent manner by phosphorylation [7]. Phosphorylation of IRP2 was shown to increase RNA binding activity through the activation of a latent pool of IRP2 rather than an increase in protein synthesis [98]. Thus, IRP2 appears to be able to switch from a high-affinity phoshphorylated RNA binding protein to a low-affinity dephosphorylated form through the regulation of phosphatases and protein kinases [7, 98]. Thus, various extracellular stimuli are capable of influencing IRP1/c-acon and IRP2 independent of cellular iron status.

Because both IRP1 and IRP2 exhibit similar genetic regulatory functions (as RNA binding proteins) and because the function of c-acon remains elusive, the question as to whether or not these two proteins are functionally redundant is often asked. To further investigate the specific roles, if any, IRP1 and IRP2, knock-out models have been developed (IRP1$^{-/-}$ and IRP2$^{-/}$, respectively). In work from the Rouault lab, IRP1$^{-/-}$ mice exhibited dysregulated iron metabolism in the kidney and brown fat [74]. Mice with a complete genetic ablation of IRP2 exhibited dysregulation of target gene expression in all tissues examined [74]. Additionally, while there was no obvious phenotype in IRP1$^{-/-}$

mice, IRP2$^{-/-}$ mice developed microcytic anemia, erythropoietic protoporhyria, and neurodegenerative disease [74, 99]. Other laboratories have obtained similar results, though the phenotype of IRP2$^{-/-}$ animals is considerably less severe [100]. The loss of both IRP1 and IRP2 genes is embryonic lethal [7, 74]. That IRP2 can support biological function in the absence of IRP1, but that IRP1 is not able to fully complement the loss of IRP2 activity in IRP1$^{-/-}$ animals suggests that these two proteins are not functionally redundant.

Hypoxia and HIF Regulation

One of the results of prolonged iron deficiency is the development of iron deficiency anemia. As previously discussed, in the absence of appropriate levels of iron, erythropoiesis and subsequent hemoglobin synthesis is repressed. Lack of hemoglobin impairs potential oxygen delivery to metabolically active tissues. Thus, it is important to investigate mechanisms coordinating the physiological response to low levels of oxygen (hypoxia). Hypoxia is a vital component in the pathophysiology of many common causes of disease and mortality such as myocardial and cerebral ischemia [101, 102]. Cellular hypoxia occurs when there is insufficient oxygen to generate enough ATP to sustain normal physiological functions like DNA, RNA, and protein synthesis, and key metabolic reactions [101]. Hypoxia occurs in a variety of disease states, such as vascular disease, chronic inflammation and cancer [101]. In disease states, target organs either fail to adequately adapt to hypoxia, or exhibit hypoxia-induced alterations in gene expression as a result of disruption of oxygen homeostasis that can further contribute to disease pathogenesis [102]. One example of the role hypoxia plays in contributing to the

pathogenesis of disease is the mechanism by which hypoxia increases angiogenic factors such as VEGF [103, 104]. In cancer, the hypoxic nature of tumors increases expression of VEGF promoting angiogenesis, which can lead to vascularization of the tumor further exacerbating the condition.

Hypoxia has a significant effect on gene transcription. In any given cell, several hundred genes are positively (VEGF) or negatively (HAMP1) regulated by hypoxia [105]. The body responds to hypoxia by transcriptionally up-regulating genes that enhance glycolytic ATP production and oxygen delivery to tissues [13, 101]. A family of oxygen-regulated transcription factors called the Hypoxia Inducible Factors (HIFs) is primarily responsible for regulating this transcriptional response [13, 101, 106].

To date, three HIF regulatory subunits have been identified, HIF-1α, 2α, and 3α along with the constitutively expressed oxygen-insensitive HIF-1β, also known as aryl hydrocarbon receptor nuclear translator or ARNT [12]. In the presence of oxygen, HIFs are targeted for proteasomal degradation by prolyl hydroxylation and interaction with the von-Hippel Lindau protein (pVHL), a protein with E3-ubiquitin ligase activity [14, 16, 107, 108]. The primary function of pVHL is to target ubiquitylated proteins for proteasomal degradation [109].

Briefly, three 2-oxoglutarate-dependent oxygenases known as prolyl hydroxylase domain protein (PHD) 1, PHD2, and PHD3 are responsible for catalyzing the prolyl hydroxylation of HIF [105]. pVHL recognizes and binds to hydroxylated proline residues, but fails to recognize unhydroxylated HIFs [106, 110]. Thus, pVHL is critical for regulating the stability of HIF regulatory subunits by orchestrating ubiquitylation and proteasomal degradation [14, 106, 107].

In contrast to prolyl hydroxylation, asparaginyl hydroxylation inhibits the transcriptional activation of HIF regulatory subunits independent of protein stability [105]. HIF subunits escaping the prolyl-hydroxylation and proteasomal degradation pathway can be post-translationally modified through hydroxylation of conserved aparagine resuides. Asparaginyl hydroxylation blocks the interaction of HIF with the CREB-binding protein/p300 co-activator (CBP)/p300 complex, preventing the transcriptional activation of HIF [79, 106]. Asparaginyl hydroxylation is catalyzed by the asparaginyl hydroxylase known as Factor Inhibiting HIF (FIH), which is also a member of the 2-oxoglutarate-dependent oxygenase superfamily [28, 29]. Thus, there is a direct link between iron and oxygen sensing, because in addition to iron, both PHDs and FIH require oxygen as a co-substrate.

Recently, mitochondria have also been implicated as having an important role in the regulation of HIF activation [111, 112]. Under normal oxygen conditions the mitochondria consume approximately 90% of available oxygen, leaving the remaining ~10% for other cellular processes (e.g., as a co-substrate for PHDs) [108]. However, under hypoxic condition, the mitochondria consume most of the available oxygen because cytochrome c oxidase has a very high affinity for molecular oxygen [101]. This oxygen utilization by mitochondria leads to insufficient oxygen for prolyl or asparaginyl hydroxylation, thus, HIF regulatory subunits accumulate and subsequently translocate to the nucleus [101]. Once in the nucleus, the HIF regulatory subunits heterodimerize with HIF-1β, and function by binding to hypoxia responsive elements (HREs) present in the promoters of target genes thereby activating transcription [8, 29].

Table 2 HIF target genes

HIF Target Genes	Cellular Process
Insulin-like growth-factor-binding protein-1 (IGFBP1)	Growth and apoptosis
BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3)	
Endoglin (ENG)	
Calcitonin-receptor-like receptor	
Telomerase reverse transcriptase (TERT)	
Membrane type 1 metalloprotease (MT1-MMP)*	
Erythropoietin (EPO)*	Oxygen supply
Vascular endothelial growth factor (VEGF)	
Inducible nitric-oxide synthase iNOS	
Endothelial nitric-oxide synthase (eNOS)	
Endothelin-1 (END1)	
Heme oxygenase (HOX)	
Vascular endothelial growth factor (VEGF)	Angiogenesis
Vascular-endothelial-growth-factor receptor (FLT1)	
Plasminogen-activator inhibitor-1 (PAI-1)*	
Cbp/p300-interacting transactivator (CITED2)*	Transcription
Inhibitor of differentiation 2 (ID2)	
Erythroblastosis virus E26 oncogene homoglog 1 (ETS1)	
Glucose transporter-1 (GLUT1)	Metabolism
Hexokinase-2 (HK2)	
Lactate dehydrogenase (LDH)	
Carbonic anhydrase-9 (CAIX)	
Phosphoglycerate kinase 1 (PGK1)	
6-Phospohfructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3)	
Transferrin (Tf)	Transport
Transferrin receptor (TfR)	
Ceruloplasmin (Cp)	
Multidrug-resistance P-glycoprotein	

Target genes transcriptionally activated by HIFs and their functional roles. *Denotes genes that are preferentially targeted by HIF-2α.

HIF-1α and HIF-2α regulate the transcription of an overlapping, but separate set of target

genes involved in cellular metabolism (**Table 2**) (glucose transporter 1 (GLUT1),

hexokinase 2 (HK2), and glyceraldehydes 3-phoshpate dehydrogenase (GAPDH)), cell

growth and apoptosis (insulin like growth factor 1 (IGF1), transforming growth factor β3

(TGFB3), and endoglin (ENG)), as well as in the restoration of the oxygen supply by

promoting angiogenesis (vascular endothelial growth factor (VEGF), plasminogen-

activator inhibitor-1 (PAI-1), and EPO) [12, 13, 28]. While HIF1α and HIF2α share an

overlapping set of target genes, it also appears that these two transcription factors exhibit

some degree of preference for similar HREs [17, 113]. HIF-1α primarily targets genes

encoding proteins involved in glucose metabolism (glycolytic enzymes and glucose

transport), whereas HIF-2α-selective target genes tend to be involved in cell growth and

oxygen restoration including membrane type 1 metalloprotease (MT1-MMP), PAI-1,

CITED2 and EPO [17, 114]. In terms of an interaction between iron and oxygen sensing,

HIF-2α is of particular interest because of its role in regulating EPO expression and the

stimulation of erythropoiesis, a process that accounts for the majority of iron use in the

body.

Interestingly, the use of iron chelators such as desferrioxamine can elicit the same

hypoxia transcriptional cascade observed when cellular oxygen is dropped from

normoxic conditions (21%) to hypoxic conditions (< ~3%) [8, 105, 115, 116]. Under low

iron conditions, HIFs are stabilized resulting in transactivation of target gene expression

[8, 105]. In regards to iron metabolism and utilization, the upregulation EPO and

subsequent increase in erythropoiesis is of particular interest. With adequate iron, the

hypoxic stabilization of HIF-2α and thereby stimulation of EPO synthesis and red blood

cell production serves to meet physiological needs by increasing oxygen transport [117, 118]. However, in the absence of adequate iron stores, stimulation of erythropoiesis can lead to the production of hypochromic microcytic red blood cells due to impaired heme synthesis and hemoglobin production potentially further depleting already low iron stores [6, 8]. Therefore, under either iron-deficient or hypoxic conditions, EPO stimulation of erythropoiesis is regulated to prevent the formation of immature red blood cells.

Mechanisms through which both iron and oxygen sensing are coordinated remain poorly characterized. However, the discovery of an IRE in the 5'UTR of HIF-2α mRNA by Hentze and colleagues has provided new insight into the iron-dependent regulation of HIF-2α [8]. This proposed oxygen- and iron-dependent regulatory mechanism is of biological significance because of the role of HIF-2α in the regulating EPO. The results from this work suggest the rate of red blood cell production (and iron utilization) is adjusted to iron availability though the iron-dependent regulation of HIF-2α translation [8].

To date, these suggestions have been limited to results from *in vitro* studies [8, 17]. We hypothesized that HIF-2α translation occurs through direct action of IRP RNA binding activity, and through this mechanism IRPs coordinate iron and oxygen signaling. In order to study the effects of dietary iron deficiency in the context of the whole organism we used a weanling rat model of diet-induced iron deficiency to investigate the extent to which hepatic HIF-2α mRNA translation is regulated in response to dietary iron deficiency. Furthermore, we examined the relationship between the iron-dependent translational regulation of HIF-2α mRNA and IRP RNA binding activity. Our results demonstrate that HIF-2α translation is repressed in the livers of iron deficient animals.

These findings are consistent with the activation of IRP RNA binding activity, suggesting that, *in vivo*, IRPs regulate the expression of HIF-2α providing a direct link between mechanisms controlling cellular iron and oxygen sensing.

CHAPTER III

METHODOLOGY

Animal care

Twenty-one day old male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 45-50 g were placed on either an iron adequate control (C, 50 mg Fe/kg) or iron deficient (ID, <5 mg Fe/kg) diet for 21 days. A third group was pair-fed (PF) the control diet to the level of food consumed by the ID animals. The powdered diets were purchased from Harland Teklad (Madison, WI; Control - TD.89300 and Iron Deficient - TD.80396). The composition of the iron deficient diet is summarized in **Table 3.** The iron adequate control diet had an identical composition, but was supplemented with 0.235 g/kg ferric citrate.

After 3 days of acclimation on the control diet, rats were randomly assigned to one of three treatment groups (n = 8 animals/group). Control and ID animals were allowed *ad libitum* access to diet and deionized water throughout the treatment period. Pair-fed animals were allowed *ad libitum* access to deionized water, but amount of diet provided was restricted to the amount consumed by the ID group. Food intake and individual body weight for each rat was measured and recorded daily.

Animals were housed individually in wire-bottomed cages at the OSU Laboratory Animal Research facility and maintained on a 12 hr light/dark cycle. Upon completion

Table 3 Composition of iron deficient (ID) diet.

Ingredient	Composition (g/kg)
Casein, low Cu & Fe	200.0
DL-Methionine	3.0
Sucrose	549.99
Corn Starch	150.0
Corn Oil	50.0
Mineral Mix, Fe Deficient	35.0
Vitamin Mix, AIN-76A	10.0
Choline Bitartrate	2.0
Ethoxyquin, antioxidant	0.01

The ID diet contained <5 mg Fe/kg diet. The control diet contained all of the same components of the ID diet, but was supplemented with ferric citrate to provide 50 mg Fe/kg diet. Diets were purchased from Harlan Teklad, Madison, WI.

of the treatment period, animals were anesthetized with a mixture of ketamine/xylazine (75 mg ketamine and 7.5 mg xylazine/kg body weight).   Following cardiac exsanguination via the abdominal aorta, tissues were removed, processed for polysome analysis, or snap frozen in liquid nitrogen. All animal studies were approved by the Institutional Animal Care and Use Committee of Oklahoma State University.

Assessment of iron status

Blood from each rat was collected in EDTA-coated tubes and assayed for hemoglobin, hematocrit, and percent reticulocytes (Stillwater Medical Center, Stillwater, OK).

Polysome profile analysis

Livers were excised, washed in ice-cold polysome buffer (PB: 40 mM HEPES pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 2 mM citrate, and 1 mM dithiothreitol), minced, and placed in a Potter-Elvehjem homogenizer. The liver was homogenized in 3 volumes PB using a fitted Teflon pestle. Approximately, 15 ml of the homogenate was transferred to a polypropylene tube and centrifuged (Beckman RC5C Centrifuge) in a SS-34 rotor at 5000 x g at 4˚C for 20 min. The upper 2/3 of the supernatant was collected and one volume of detergent (10% deoxycholate and 10% Triton X-100) was added to 9 volumes of the supernatant. After adding detergent, the samples were gently mixed by inverting the tubes 4-5 times. Lastly, 500 µL of the sample was layered onto an ice-cold 11 ml linear 10% to 43% sucrose gradient in PB. The samples were centrifuged (Thermo/Sorvall WX Ultra) at 180,000 x g in a Sorvall TH-641 swinging bucket rotor for

2 h at 4˚C with slow braking. Immediately following centrifugation, gradients were

fractionated using an ISCO gradient fractionator (Teldyne Isco, Inc. Lincoln, NE). The

absorbance at 254 nm was continuously monitored and 11 x 1 min (~1 ml) fractions were

collected and stored at -80˚C.

RNA isolation from sucrose gradients

Total RNA was isolated from 500 µL of each gradient fraction using STAT-60

(Teltest, Inc., Friendswood, TX). RNA collected from each fraction was resuspended in

20 µl diethyl pyrocarbonate (DEPC) treated water. The concentration of RNA was

determined by spectrophotometry (NanoDrop ND-1000, Thermo Fisher Scientific,

Wilmington, DE) and integrity of 18S and 28S rRNA in the gradient fractions was

analyzed by agarose gel electrophoresis. The agarose gel solution was pre-stained with

GelStar Stain (Lonza, Rockland, ME) and the RNA was visualized using a VersaDoc

imaging system (Bio-Rad, Hercules, CA) using a 520 Long Pass filter and UV

transillumination. It was determined from these images that fractions 1-3 represented the

free protein pool, some of the ribonucleoprotein (RNP) pool, and a portion of the

ribosomal subunits, fractions 4-5 primarily contained the ribosomal subunits (40S, 60S,

and 80S), while fractions 6-10 contained the light and heavy polysomes.

Real-Time PCR from gradient fractions

Two microliters, or one tenth of the total RNA collected from each fraction was

treated with DNase I (Roche, Indianapolis, IN – Catalog # 776 785) at 37˚C for 30 min,

75˚C for 10 min followed by a 4˚C soak cycle. RNA was then reverse transcribed using

Superscript II (Invitrogen, Carlsbad, CA – Catalog # 18064-022) to synthesize cDNA [8].

Each real-time reaction was performed in duplicate using SYBR green chemistry

(Applied Biosystems, Foster City, CA – Catalogue # 4367659). Primers were designed

using the Genebank database or published species-specific sequences and are listed in

Table 4. The standard used for primer design and validation was that the amplicon had

to span the intron, that template titration must form a single dissociation curve, and have

an efficiency slope of -3.3.

The relative abundance of cyclophilin, L-Ferritin, TfR, HIF-2α, Fpn1, and m-acon

mRNA and 18S mRNA was determined in each gradient fraction by real-time PCR using

an ABI Fast 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA).

Cycles included one 2 min hold (50˚C); one 10 min denaturation (95˚C); 40 cycles of

denaturation (95˚C for 15 sec) and annealing (60˚C for 1 min); and dissociation (95˚C for

15 sec followed by 60˚C for 15 sec). Additionally, 2 μg of total RNA isolated from the

post-mitochondrial supernatant was also DNase treated, reverse transcribed and subjected

to real-time PCR to examine gene expression. Real-time PCR reactions were performed

in duplicate using 25 ng cDNA at a final concentration of 2.5 ng/μl. All real-time PCR

results were analyzed using the comparative cycle number at threshold (C_T) method.

Total 18S rRNA and cyclophilin B were used as the controls for determining relative

mRNA abundance in gradient fractions and post-mitochondrial supernatant, respectively.

Table 4 Primer pairs used for real-time PCR

Primer Name	Sense Primer	Antisense Primer	Accession No
Cyclo	5'-GGTCTTTGGGAAGGTGAAAGAA-3'	5'-GCCATTCCTGGACCCAAAA-3'	NM_017101
L-Ft	5'-TTCGCGGTTAGCTCCATACTC-3'	5'-TCACGGCAGCTTCCACTTC-3'	NM_022500
TfR1	5'-TCGGCTACCTGGGCTATTGT-3'	5'-CCGCCTCTTCCGCTTCA-3'	XM_340999
HIF-2α	5'-GCGACAATGACAGCTGACAA-3'	5'-CGGCATCTCGGGATTTCT-3'	NM_023090

Primer pairs used to quantify polysomal distribution of mRNA in response to dietary iron deficiency. Cyclo, Cyclophilin; L-Ft, L-Ferritin; TfR1, Transferrin Receptor 1; HIF-2α, Hypoxia Inducible Factor-2α;

Electrophoretic Mobility Shift Assay

IRP RNA binding activity in the liver was determined by electrophoretic mobility shift assay. First, a plasmid encoding the rat L-Ferritin IRE (generously provided by Dr. Richard Eisenstein, University of Wisconsin-Madison, WI) was digested with SmaI to for synthesis of radiolabeled RNA. The vector p16Bgl, which contains the entire rat L-Ferritin cDNA was used for IRE synthesis as described by Eisenstein *et. al* [119]. A 73 nucleotide [^{32}P]-labeled RNA containing the IRE was produced by T7 RNA polymerase. The RNA was gel purified though a 10% acrylamide 8M urea gel and specific radioactivity of the RNA probe was determined (~4000 dpm/fmol). Secondly, cytosolic fractions were obtained by adding Triton X-100 (1% final) to the post-mitochondrial supernatant described above. After adding detergent, samples were centrifuged at 16,000 x *g* at 4°C for 25 minutes. The supernatant was collected and the concentration was determined using the bicinchoninic acid (BCA) protein assay [120].

Spontaneous IRP1 and IRP2 RNA binding activity was assessed by incubating 5 μg cytosolic extract for 10 minutes on ice with saturating levels of [^{32}P]-labeled RNA (1 nmol/L) in a final concentration of 5% glycerol, 1 mmol/L magnesium acetate, 20 mmol/L HEPES, 75 mmol/L potassium chloride and 20 mg/L of nuclease free bovine serum albumin in a final volume of 30 μL. Next, 3 μL of heparin (5 g/L in nuclease-free water) was added to each reaction and allowed to incubate an additional 10 minutes. Finally, 25 μL of each 33 μl reaction was loaded onto a 0.5X TBE 4% polyacrylamide (60:1 acrylamide/bisacrylamide) gel. During sample preparation, the polyacrylamide gel was pre-ran at 150 V for approximately 20 minutes. Samples were loaded onto gel

running at 90 V. After all samples were loaded, they were electrophoresed at 150 V for 90 minutes.

Total RNA binding activity was measured by adding 1 µg cytosolic extract in the presence of 2% β-mercaptoethanol, to saturating levels of $[^{32}P]$-labeled RNA as described above. Samples were incubated at room temperature (25˚C) for 30 minutes. Next, 3 µL of heparin was added and the samples were electrophoresed as described above. Gels were then vacuum-dried for two hours using a Model 583 Gel Dryer connected to a Hydrotech Vacuum Pump (Bio-Rad, Hercules, CA) and visualized using a Bio-Rad Phosphor K imaging screen and Personal Molecular Imager FX imaging system (Bio-Rad, Hercules, CA).

RNA binding activity was quantified using OptiQuant Acquisition & Analysis software (Packard Bioscience, Meridien, CT). Briefly, a standard curve of the $[^{32}P]$-RNA was included on each phosphoimaged gel. The digital light units (DLUs) for the standard curve were used to convert DLUs established by phosphoimaging to mol $[^{32}P]$-RNA by determining the counts per minute (cpm) of the standard curve using scintillation counting (Packard Tri-Carb 2900 Liquid Scintillation Analyzer, GMI, Inc., Ramsey, MN). Spontaneous and total RNA binding activities are expressed as fmol RNA bound/mg protein and pmol RNA bound/mg protein respectively.

Statistical analyses using ANOVA and Students T-test techniques using SPSS software (SPSS, Inc., Chicago, IL) were performed to determine the significance of treatment effects. All tests were done at the 95% confidence level ($\alpha = 0.05$). Descriptive statistics were calculated on all variables to include mean and standard deviation.

CHAPTER IV

FINDINGS

***Dietary iron deficiency alters total body weight and hematological parameters
in weanling male rats.*** To examine the extent to which an ID altered food intake and/or
weight gain during the study, daily food intake and weight were closely monitored.
Previous studies have established that a diet providing less than 5 mg Fe/kg diet results in
decreased food intake and weight gain, thus to control for this we included a pair-fed
group in the current study [73, 84, 121]. To determine the degree to which dietary
treatments were associated with the development of anemia, we examined hemoglobin,
hematocrit, and percent reticulocytes. The effect of dietary iron deficiency on weight
gain and indices of iron status among the different treatment groups of rats is shown in
Figure 5. The ID and PF groups weighed significantly less than the C group at the end
of the 21 day period (192.1 ± 2.0 g for the C group compared to 175.2 ± 5.6 g and 170.7
± 1.1 for the ID and PF groups, respectively, p < 0.001). The decline in weight gain was
statistically significant on day 14 and continued through the end of the study (p = 0.029).

Biochemical indicators of systemic iron status indicated that only animals
receiving the iron deficient diet were anemic (**Figure 6**). Blood hemoglobin and
hematocrit levels were significantly lower in ID animals. Hemoglobin was 66.0 ± 2.0
g/L for the ID animals compared to 135.0 ± 3.0 and 133.0 ± 2.0 g/L for the C and PF
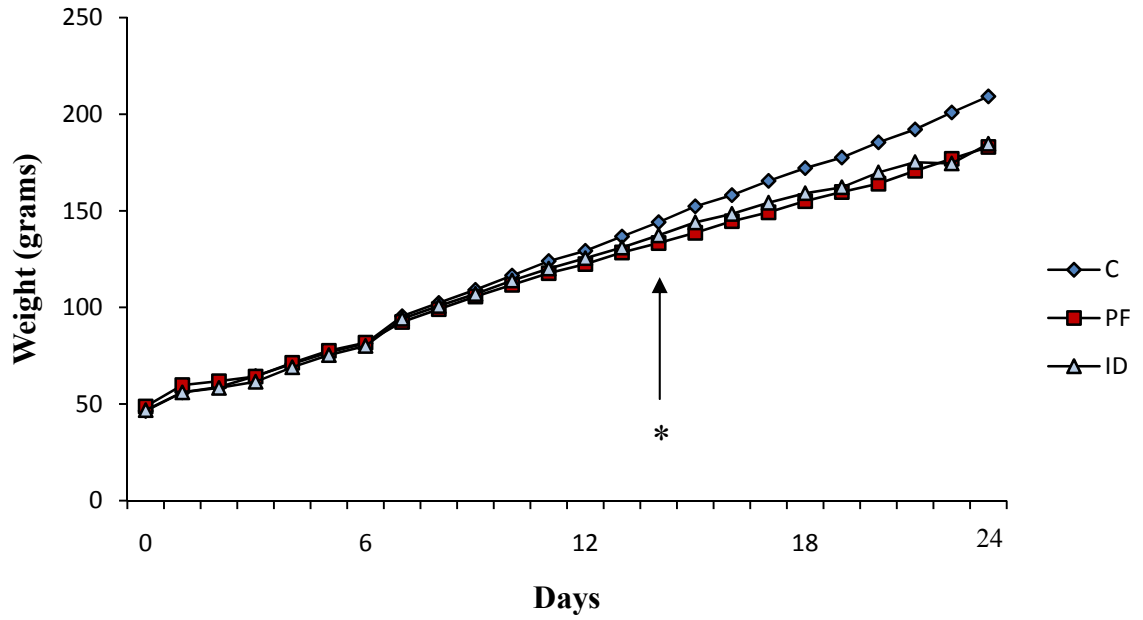
Figure 5 Effect of dietary iron deficiency on weight gain. Beginning on day 14 the PF and ID groups weighed significantly less than the C group ($p = 0.029$). This decline in growth rate continued and became more dramatic through the termination of the study. Asterisk denotes first statistical significance relative to control.
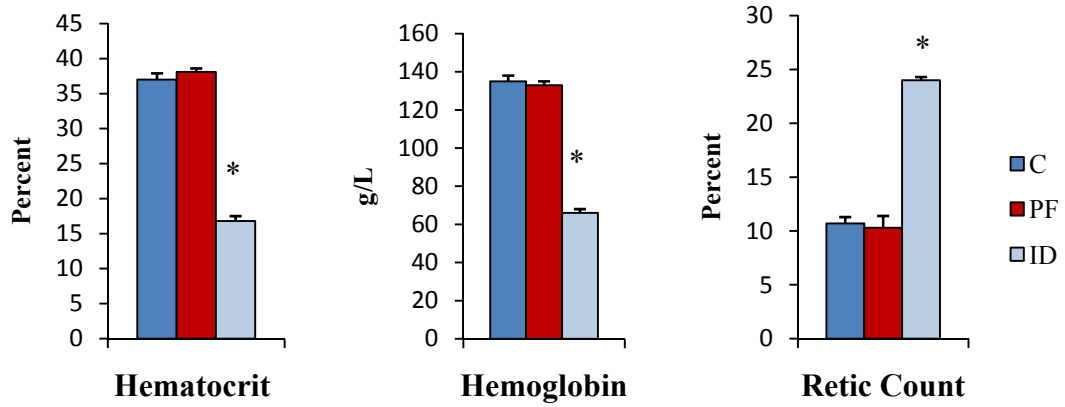
Figure 6 Hematological indices in response to dietary iron deficiency. Biochemical indicators of systemic iron status indicate the animals consuming an iron-deficient diet are anemic. The ID animals had significantly lower levels of hemoglobin and hematocrit than the PF and C animals. The formation of reticulocytes, or immature red blood cells, is another means of confirming the development of anemia. The ID animals had a significantly higher reticulocyte count than the PF and C animals. [*]Statistical significance (p < 0.001).

groups, respectively (p < 0.001) and hematocrit was 16.8 ± 0.7% for the ID animals and

37.0 ± 0.9 and 38.1 ± 0.7 for the C and PF groups, respectively (p < 0.001). In iron

deficiency anemia, the relative abundance of reticulocytes can be used to further confirm

the development of anemia. An increase in the number or percent of reticulocytes

reflects impaired erythropoiesis in response to iron deficiency. In animals receiving the

ID diet, the percent reticulocytes in blood was significantly elevated compared to C and

PF animals (24 ± 0.3% for the ID animals compared to 10.7 ± 0.6% and 10.3 ± 1.1% for

the C and PF animals, respectively). Further, all of the animals in this study were

experiencing rapid growth, and this is reflected by the higher-than-normal (for adult rats)

percent reticulocytes in all treatment groups.

IRP1 and IRP2 binding activity is increased in livers of iron deficient animals.

To determine the extent to which dietary iron deficiency affects hepatic IRP1 and IRP2

RNA binding activity, we quantitatively assayed spontaneous and total IRP binding

activity in the ID and PF animals. We compared RNA binding activity of the ID animals

versus the PF animals because we felt the PF animals served as a more appropriate

control to confirm that changes in binding activity were a result of iron deficiency and

not decreased nutrient intake. Spontaneous IRP1 and IRP2 RNA binding activity

increased from 455 ± 53 to 793 ± 60 fmol/mg protein for IRP1 and from 93 ± 21 to 324 ±

121 fmol/mg protein for IPR2 in the liver of the ID animals (**Figure 7A**). Thus, the

spontaneous RNA binding activity of both IRP1 and IRP2 is significantly elevated in

animals receiving the ID diet compared to the PF animals (p < 0.001).

Factors such as changes in iron status can influence the distribution between

active and inactive forms of IRPs. Under normal physiological conditions, less than

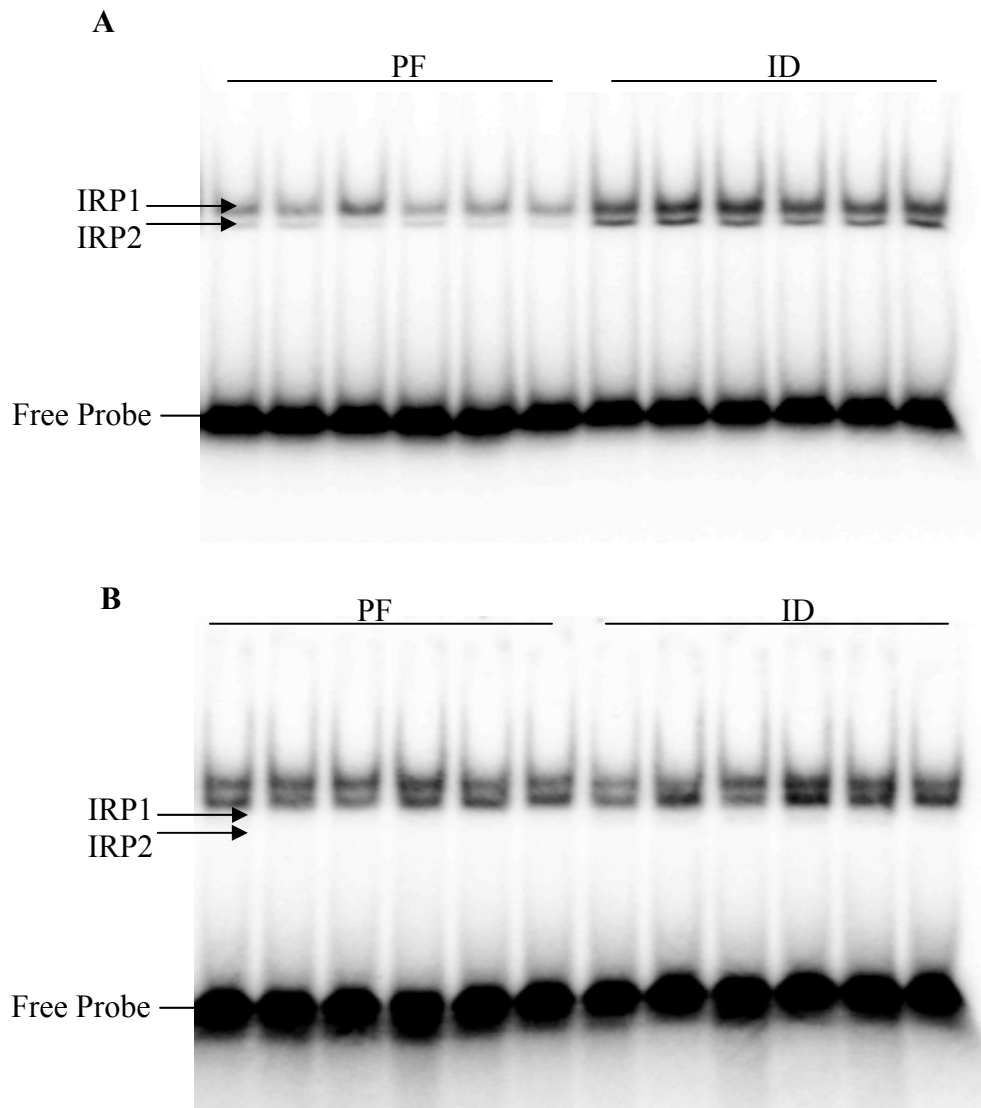**Figure 7** Effect of dietary iron deficiency on spontaneous and total binding activity of IRP1 and IRP2 in rat liver cytosol. (**A**) Spontaneous IRP1 and IRP2 RNA Binding Activity are increased 0.6 and 3.5-fold, respectively in ID animals (p < 0.001). (**B**) Total RNA binding activity (spontaneous + latent), as measured by the addition of β-mercaptoethanol, remains unchanged in response to dietary iron deficiency.

about 5% of IRPs in the rat liver are in their active RNA binding form [9]. Addition of the reductant β-Mercaptoethanol (β-ME) allows for the measure of total IRP protein present by recruiting inactive IRPs to their active RNA binding forms [84, 98, 122]. As IRP2 is regulated by degradation of the protein itself, the addition of β-ME does not significantly affect IRP2. Consequently, this assay offers a means of measuring the amount of IRP1 present in its inactive (c-aconitase) form [84]. To determine the influence of dietary treatment on total IRP binding capacity, we examined the inducible binding activity effect of β-ME on IRPs in hepatic cytosolic extracts of the PF and ID rats. Total RNA binding activity was unchanged in response to dietary iron deficiency **(Figure 7B)**, indicating that changes in spontaneous IRP binding activity were not a function of alterations in the total pool size of IRPs.

HIF-2α is translationally repressed in livers of iron deficient animals. Because HIF-2α mRNA possesses a functional IRE in its 5'UTR, we were interested in examining the extent to which hepatic HIF-2α mRNA was translationally regulated in response to dietary iron deficiency [8]. Given the increased IRP RNA binding activity exhibited by ID animals, we hypothesized that established and newly identified targets of IRPs (i.e., L-ferritin and HIF-2α) would be translationally repressed. We examined the translational control by assessing polysomal distribution of target mRNAs in livers of PF and ID animals. Using sucrose density centrifugation, we separated mRNA into inactively (ribonucleoprotein, RNP) and actively (polysome) translating fractions. A representative OD_{254} profile from a sucrose gradient and fractions corresponding to RNPs (1-3), ribosomal subunits (4-5), and polysomes (6-10) is shown in **Figure 8A-B**. After isolating total RNA from gradient fractions, RNA was reverse transcribed and the resulting cDNA

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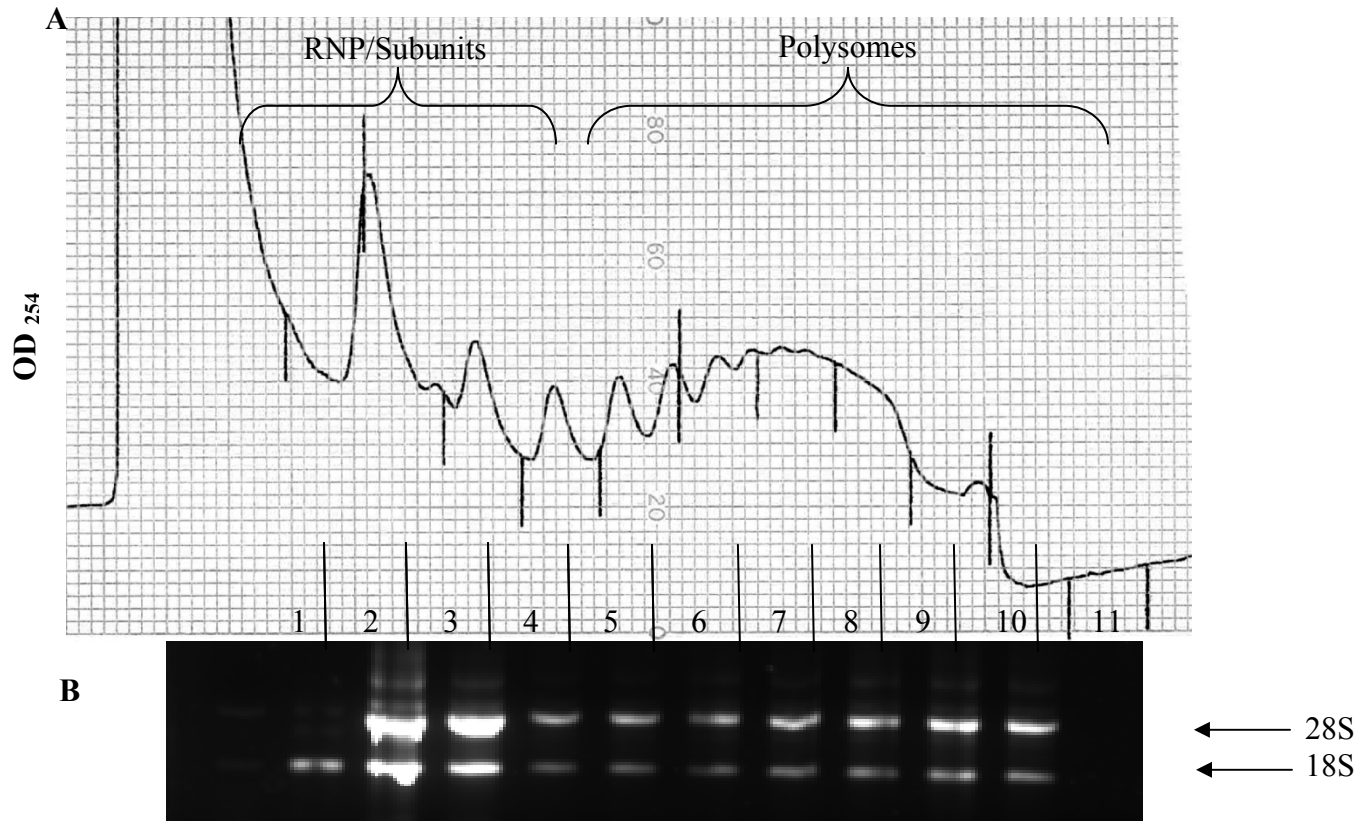
Figure 8 Representative polysome profile and agarose gel assesseing integrity and location of 18S/28S rRNA from sucrose gradient fractions. (**A**) The OD_{254} of each sucrose gradient was monitored continuously and 11 x 1 minute fractions were collected (~1ml). Fractions 1-5 correspond to RNPs and the ribosomal subunits, and fractions 6-11 correspond to the actively translating polysomes. (**B**) RNA was then isolated from each fraction and electrophosed through a 0.7% agarose gel to assess integrity and location of the 18S/28S rRNA.

was used as a template for real-time PCR to examine mRNA distribution across the sucrose gradient. Cyclophilin B (Cyclo B) and L-Ferritin (L-Ft) mRNA were used as negative and positive controls, respectively, for iron-dependent (i.e., IRP) translational regulation.

Real-time PCR analyses show that there is a significant change in the distribution of HIF-2α mRNA (**Figure 9A**) with a greater percentage found in the repressed pool and less on the polysomes in response to dietary iron deficiency (47.2% and 38.2% in the repressed pool and polysomal pool, respectively in the ID animals compared to 32.3% and 57.2% in the repressed and polysomal pools of the PF animals, $p < 0.03$, **Table 5**). Similarly, translation of L-Ft mRNA, a canonical IRP target, is repressed in the livers of ID rats with nearly 90% of mRNA present in the repressed mRNP fraction (85.8% in the RNP pool in ID animals compared to 74.8% in the RNP pool in PF animals, $p < 0.05$, **Figure 9B**, **Figure 10**, and **Table 5**). To determine if the translational repression of identified IRP targets is due to a global repression in translation, we examined the translational distribution of cyclophilin mRNA. Nearly 80% of cyclophilin mRNA is present in the polysomal fractions and importantly, the polysomal distribution of cyclophilin mRNA is unaffected by iron status (**Figure 9C** and **10**).

Although polysomal analysis can provide useful information regarding translational control, it does not accurately reflect changes in the total pool of a given mRNA. Thus, to determine that repression of HIF-2α mRNA was not simply due to HIF-2α transcription, we examined HIF-2α mRNA abundance from RNA isolated from an equal amount of post-mitochondrial supernatant layered onto the gradients. The relative abundance of HIF-2α mRNA was unaffected by diet ($p = 0.76$, **Figure 11**).
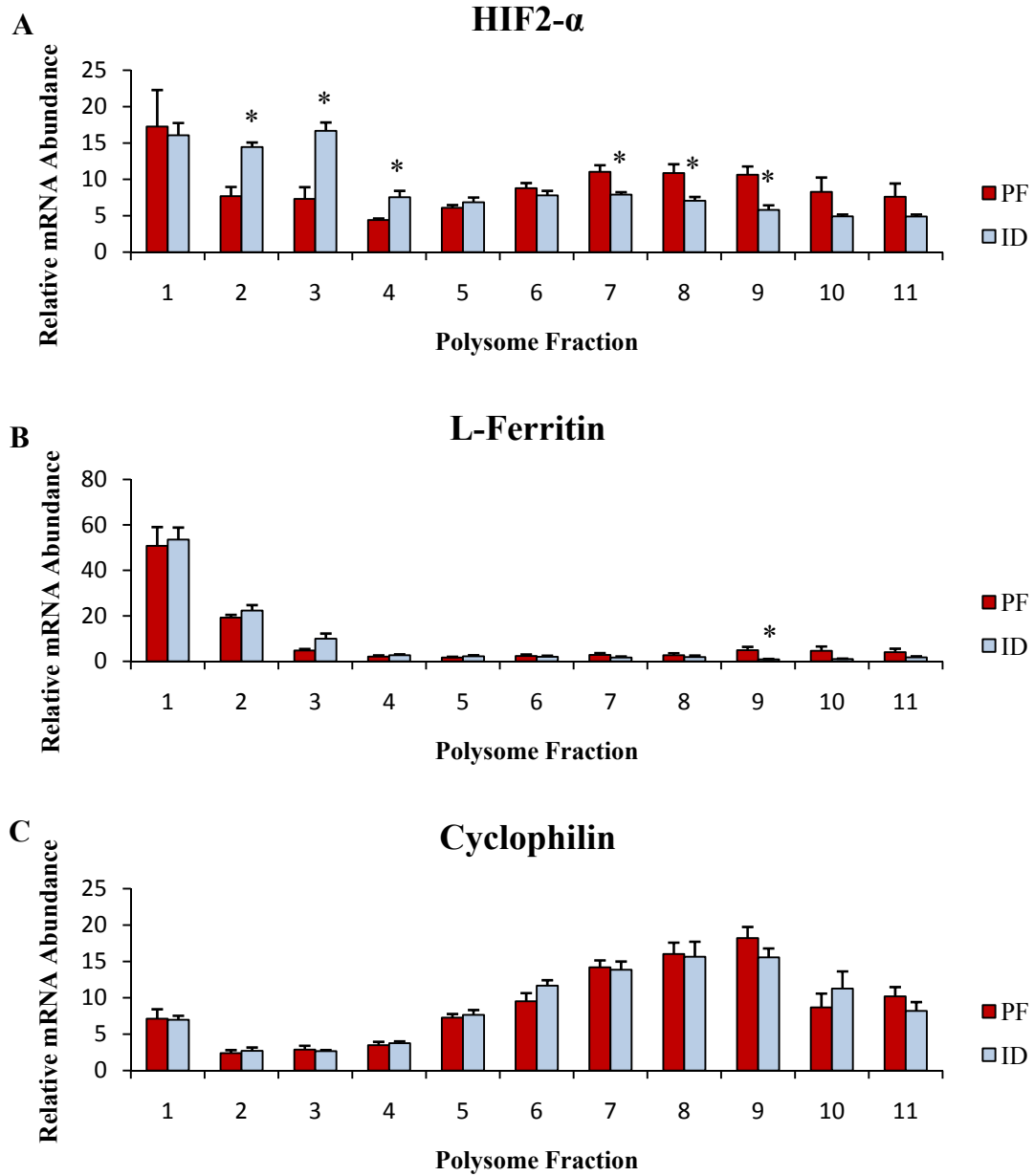
Figure 9 Polysomal distributions of (**A**) HIF-2α, (**B**) L-Ferritin, and (**C**) Cyclophilin B. Fractions 1-5 represent RNPs and ribosomal subunits, and fractions 6-11 correspond to polysomes. Total 18S rRNA in each fraction was used as a control. [*]Statistical significance between PF and ID animals ($p < 0.01$). Error bars show SEM.
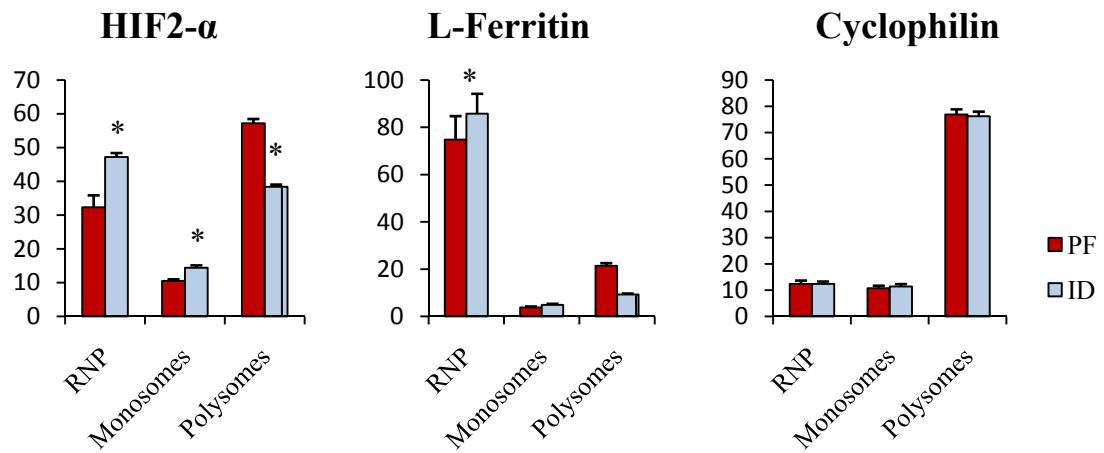
Figure 10 Total mRNA Distribution in RNP, Subunits, and Polysome fractions.
*Statistical significance between PF and ID animals ($p < 0.05$). Error bars show SEM.

Table 5 Fractional distribution of total mRNA in gradient fractions.

mRNA	Diet	mRNP	40S/60S/80S	Polysomes
HIF-2α	PF	32.3	10.5	57.2
	ID	47.2*	14.4*	38.2*
L-Ferritin	PF	74.8	3.8	21.4
	ID	85.8*	4.9	9.3
Cyclophilin	PF	12.4	10.7	76.9
	ID	12.4	10.4	76.2

*Statistical significance between PF and ID animals ($p < 0.05$).

HIF-dependent gene expression is decreased in dietary iron deficiency. Upon

determining that HIF-2α translation is repressed in dietary iron deficiency, we were

interested in examining the HIF-1α expression as well as the expression of HIF target

genes in response to dietary iron deficiency. To do this we compared the relative mRNA

abundance of HIF-1α and HIF targets in total RNA isolated from the post-mitochondrial

supernatant from the ID and PF animals. Interestingly, and in contrast to HIF-2α, HIF-1α

mRNA expression is decreased in livers of iron deficient animals (**Figure 11**).

Next, we investigated the expression of HIF target genes

(Phosphofructokinase/Fructose Bishpohpatase 3 - PFKFB3, Glucose-6-Phosphatase –

G6Pase, Glucokinase – GK, and Glucose Transporter 1 – GLUT1), which are typically

induced in response to hypoxia (**Figure 11**). Hepatic expression of PFKFB3, G6Pase,

and GK did not appreciably change in response to dietary iron deficiency. In agreement

with the decreased expression of HIF-1α expression however, GLUT1 expression

significantly decreased in response to dietary iron deficiency ($p = 0.02$). The changes in

HIF-dependent gene expression were contrary to what was predicted as *in vitro* treatment

with iron chelators observed by others, as well as in our own lab, typically results in an

increase in HIF-dependent gene expression [116, 123].

Iron dependent changes in gene expression. Lastly, we wanted to examine the

expression of genes with known responses to dietary iron deficiency to serve as positive

and negative controls. We examined HAMP1 and TfR1, both of which are expected to

change in response to dietary iron deficiency, and the post-transcriptionally regulated L-

Ft [3, 4, 42]. As anticipated, hepcidin (HAMP1) mRNA expression is greatly reduced in

response to iron deficiency ($p < 0.001$) [3, 55]. The relative abundance of transferrin
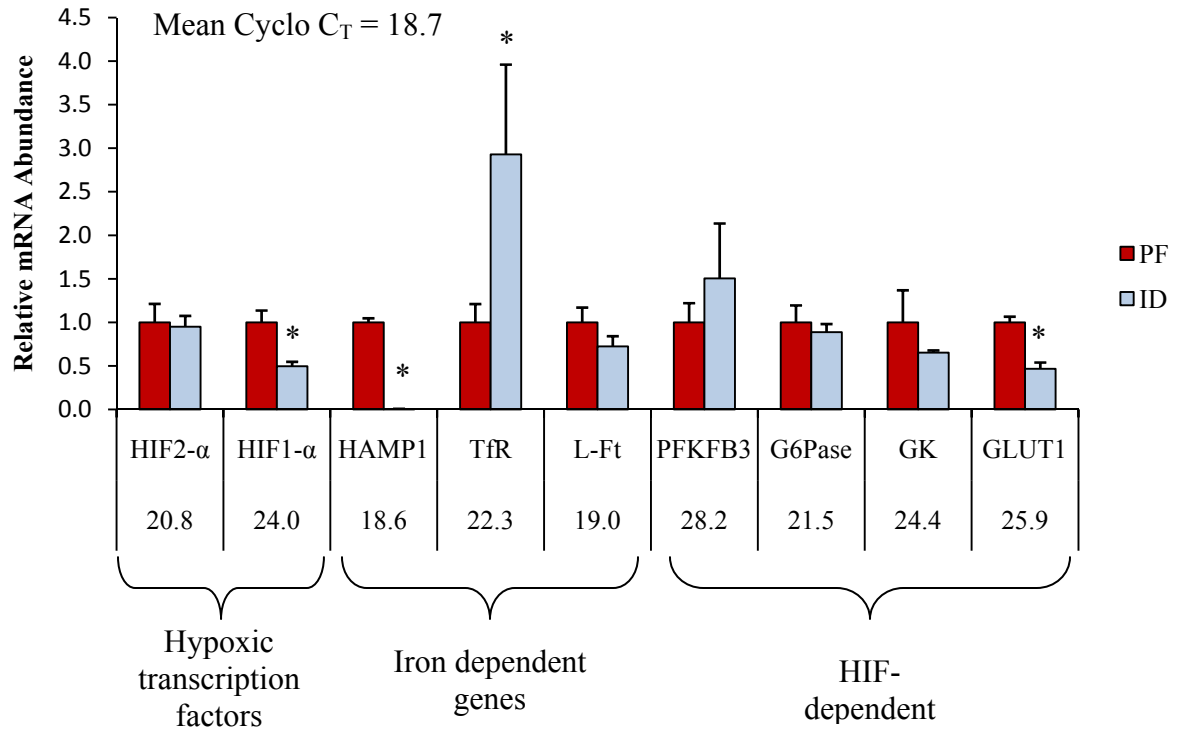
Figure 11 Real-time PCR analyses HIF-1α and HIF-2α regulatory subunits, iron regulated targets, and previously irentified HIF targets. mRNA levels were normalized to Cyclo mRNA as the invariant control. Numbers beneath gene names indicate C_T value obtained by real-time PCR for the pair-fed group. *Statistical significance between PF and ID groups ($p < 0.05$). Error bars shown SEM.

receptor (TfR) is increased in ID animals (p = 0.006); largely as a result of enhanced

mRNA stability due to an increase in IRP RNA binding activity. L-Ft mRNA abundance

is not significantly altered by iron status, in agreement with previous results indicating

that the IRP-dependent regulation of L-Ft occurs post-transcriptionally (p = 0.234)

(**Figure 11**).

CHAPTER V

DISCUSSION

Due to the essential, yet potentially toxic nature of iron, cellular and organismal

iron homeostasis must be tightly regulated to prevent iron deficiency or iron toxicity.

The iron stores regulator peptide hormone, hepcidin, is a critical mediator of organismal

iron homeostasis, as evidenced by results demonstrating that mutations in either hepcidin,

or its molecular target Fpn1, are associated with dysregulation of iron metabolism [3,

124, 125]. While hepcidin is considered a systemic regulator of iron metabolism, IRPs

are generally considered to be the global regulators of cellular iron metabolism due to

their role in regulating the expression of proteins involved in iron storage, transport, and

utilization. More recently, the targets of IRPs are being expanded beyond mRNAs

encoding iron-related proteins to include proteins involved in cell cycle

progression/cellular proliferation (Cell division cycle 14A – cdc14a), oxygen

sensing/hypoxic adaptation (HIF-2α), and perhaps even proteins implicated in the

etiology of neurodegenerative disease (α-Synuclein and Amyloid beta A4 precursor

protein) [8, 126, 127]. Although relatively little is known about the hierarchical

regulation of IRE-containing transcripts, not all IRE- containing transcripts appear to be

regulated identically *in vivo* supporting the concept that both IRE and non-IRE

sequences, including proximal flanking regions, can contribute to the extent of IRP-

dependent regulation (S.L. Clarke, K.L. Ross, R.S. Eisenstein, unpublished observations) [19, 84].

The relatively recent discovery of an apparently functional IRE in the 5'UTR of HIF-2α prompted us to examine the extent to which HIF-2α is translationally regulated, presumably by IRPs, in response to dietary iron deficiency. The presence of an IRE in HIF-2α is particularly intriguing given that one of the key HIF-2α target genes is the erythropoiesis-stimulating hormone erythropoietin [14]. Although the kidney is the primary site of EPO expression under normal conditions, hepatic expression of EPO may account for approximately 30% of total EPO in response to hypoxic conditions [128]. Based on the function of IRPs, we and others have proposed that IRP-dependent regulation of HIF-2α expression is an adaptive mechanism to coordinate iron stores (availability) with the production of red blood cells [8]. Thus, under low-oxygen conditions in the absence of adequate iron stores, the increased rate of erythropoiesis is attenuated, decreasing the overall production of microcytic hypochromic erythrocytes.

In this present study, we have established a role for IRPs in regulating hepatic HIF-2α translation in response to dietary iron deficiency. By examining the effects of dietary iron deficiency on well-characterized targets of IRPs (i.e., L-Ft) we demonstrate that the translational regulation of HIF-2α mRNA is consistent with the alterations in IRP RNA binding activity in response to dietary iron deficiency. In fact, the degree of regulation of HIF-2α appears to be more robust than L-Ft, a canonical target of IRPs. We have demonstrated in rapidly growing, but presumably iron replete animals, that only ~20% of L-Ft mRNA is on polysomes, whereas ~57% of HIF-2α mRNA is in the translationally active state. In response to iron deficiency, about 85% of total L-Ft

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mRNA is found in the RNP (15% increase relative to control). For HIF-2α, a fractionally

greater proportion of mRNA (46% increase relative to control) is shifted into the

repressed pool. Given the role of ferritin as the major storage protein and the requirement

of iron for cell growth and proliferation, the expression of ferritin would be expected to

be tightly controlled and therefore not actively translated under most physiological

conditions. In fact, we found that the majority of L-Ft mRNA is repressed in these

rapidly growing animals. Conversely, a greater proportion of HIF-2α mRNA is

translationally active regardless of iron status, perhaps underscoring the relative

importance of this constitutively expressed transcription factor in allowing cells to adapt

to rapidly changing oxygen conditions. Although oxygen- and iron-dependent changes in

protein stability are primary mechanisms regulating HIF-2α activity, we have

demonstrated *in vivo* that HIF-2α is also regulated at the level of protein translation.

Again, given the important role of this protein in allowing the organism to adapt to

hypoxic conditions, translation would not likely be fully blocked, even when iron

deficiency or hypoxia is most severe. Instead, the IRP-dependent regulation of HIF-2α

likely serves as a means to coordinate iron and oxygen sensing, providing the greatest

degree of conservation of iron in severe iron deficiency. Under iron-replete but hypoxic

conditions, the translation of HIF-2α would likely be unaffected by IRPs, thereby

increasing the expression of HIF-2α-dependent target genes promoting the biological

adaptation to hypoxia. However, the extent to which hypoxia or iron deficiency alone

affects the HIF-2α-dependent transcriptional network remains unclear.

In an effort to characterize the effects of iron deficiency on HIF-dependent target

expression, we examined both iron-regulated and HIF-related genes. Consistent with the

role of IRPs in regulating TfR mRNA stability, TfR expression was increased 3 fold in the livers of iron deficient animals. Furthermore, expression of the gene encoding the iron peptide hormone hepcidin (HAMP1) is repressed in iron deficient animals. In the absence of hepcidin both intestinal iron uptake and reticuloendothelial iron export is increased to provide sufficient iron to promote heme synthesis and erythropoiesis. Hepcidin expression is controlled in part through a Bone Morphogenetic Protein (BMP) signaling pathway [129]. Hemojuvelin (HJV), expressed primarily by hepatocytes and skeletal muscle, is a glycosylphosphatidyl inositol-linked cell surface protein that functions as a co-receptor for BMPs [130]. Hemojuvelin acts as an antagonist to BMP signaling and can reduce the level of HAMP1 expression by inhibiting the BMP-dependent signaling cascade [56]. Interestingly, HJV is shed from skeletal muscle in response to iron deficiency and is capable of moderately suppressing hepatic HAMP1 expression [131]. In addition to regulation of expression through a BMP-dependent pathway, HAMP1 is also regulated by hypoxia [3]. Although less well-characterized, HIFs can also function as transcriptional repressors [105, 132, 133]. Thus, under relatively severe iron deficiency or hypoxia, HAMP1 expression is repressed by HIFs leading to an enhanced iron uptake by enterocytes and iron release from macrophages. In our study, it is unclear if HAMP1 expression is being negatively regulated through an HJV-dependent or HIF-dependent pathway. Despite a significant repression of HIF-1α, mRNA and given the enhanced stability of HIF1-α in iron deficiency and hypoxic conditions, it is possible that HAMP1 expression is still, at least in part, being regulated by HIFs.

In support of the HIF-dependent regulation of HAMP1 is the significant down-regulation of the HIF target gene, Glucose Transporter 1 (GLUT1), a glucose transporter expressed at low levels in the liver. With its low-affinity and high capacity for glucose transport, GLUT2 is a significantly more important glucose transporter, however, the regulation of GLUT1 in the liver may be indicative of HIF-dependent repression in other tissues (i.e., erythrocytes). Interestingly, not all GLUTs appeared to be regulated in a similar manner. For example, HIFs increase the expression of GLUT1 and GLUT3, but decrease the expression of GLUT2 [134]. It remains unclear how the regulation of GLUT1 affects hepatic glucose metabolism, however, down-regulation of GLUT1 in the CNS (a major site for glucose uptake) would result in decreased glucose uptake by the brain and may contribute to alterations in systemic glucose utilization [135]. Others have previously shown that severe iron deficiency is associated with an increased reliance on glucose as a metabolic fuel for peripheral tissues [117, 136, 137].

Even with moderate iron deficiency, blood glucose levels are significantly elevated consistent with increased oxidation of glucose [117]. It remains unclear, especially in iron deficiency, if this hyperglycemia is a result of decreased insulin sensitivity or perhaps a diminution of postprandial hepatic glucose uptake. With a significant repression of GLUT1 expression, it is possible that this is one of the mechanisms contributing to iron deficient induced hyperglycemia. Additionally, insulin secretion by the pancreatic β-cells may be negatively regulated by iron deficiency and could further contribute to hyperglycemia (Eisenstein and Clarke, unpublished observations). A few critical questions still remain. First, to what extent does a decrease in HIF-2α translation affect either hepatic or renal erythropoietin expression? Second, is

there a fundamentally different response to a hypoxic situation in the absence of iron deficiency? Third, are alterations in whole body fuel metabolism related to alterations in HIF-dependent signaling pathways? The extent to which alterations in iron status are associated with changes in oxygen sensing and fuel oxidation remains unknown. Nonetheless, to the best of our knowledge, the results herein are the first to demonstrate in an animal that the translation of HIF-2α is regulated in an iron-dependent manner. Furthermore, we found that HIF-dependent gene expression is repressed, which may begin to provide a mechanistic basis for one of the contributing factors to hyperglycemia observed in moderate to severe iron deficiency. Clearly, more studies need to be conducted to further ascertain ontological changes in oxygen sensing in response to iron deficiency and how these changes are related to both hepatic and peripheral tissue glucose utilization and oxidation.

CHAPTER VI

CONCLUSIONS

In this study, we have established a role for IRPs in regulating hepatic HIF-2α translation in response to dietary iron deficiency utilizing a weanling rat model of iron deficiency. A severely iron deficient diet has been shown to decrease total food intake leading to decreased body weight [19, 84, 121]. To control for this, we included a group that was pair fed a control diet to the amount of deficiency diet consumed by the ID group to ensure our findings were not the result of dietary factors other than the absence of iron. Biochemical measures of systemic iron status indicated that only animals consuming the iron-deficient diet were anemic as evidenced by both the significantly lower hemoglobin and hematocrit levels in addition to an elevation of the number of reticulocytes. This affirms that observed changes in IRP binding activity and translational control were more likely the result of dietary iron deficiency than simply a decrease in food intake.

To examine the extent to which dietary iron deficiency alters hepatic IRP1 and IRP2 RNA binding activity, we assayed both spontaneous and total IRP binding activity. In agreement with previous studies, spontaneous liver RNA binding activity of both IRP1 and IRP2 was increased 0.6- and 3.5-fold, respectively, in the livers of the ID animals [19, 74, 84, 138]. Next, in order to attribute changes in binding activity to the post-translational regulation of activity through the recruitment of IRP to its RNA binding form through the loss of the [4Fe-4S] cluster, we examined the total RNA binding activity of IRP1. Addition of the strong reductant β-ME allowed us to measure total

IRP1 RNA binding, reflecting the total pool of IRP1 (i.e., IRP1 and c-acon) protein present by recruiting inactive IRPs to their active binding forms [84, 98, 122]. Total RNA binding activity remained unchanged in response to dietary iron deficiency. Thus, the increase in spontaneous RNA binding activity observed in the livers of ID animals was not a function of increased IRP1 protein abundance, but reflected a shift in IRP1 to its active binding form. The iron-dependent regulation of IRP1 and IRP2 RNA binding activity is consistent with other studies demonstrating a role for IRPs in iron sensing in response to iron deficiency [19, 84, 89].

In accordance with increases in IRP RNA binding activity, we hypothesized that IRE-containing transcripts L-Ft and HIF-2α would be regulated in an IRP-dependent manner. In response to our model of dietary iron deficiency, we expected that both L-Ft and HIF-2α mRNA would be translationally repressed. We used sucrose density centrifugation to separate mRNA into inactively (RNP) and actively (polysome) translating fractions, then used real-time PCR to examine mRNA distribution across the sucrose gradient. As predicted, the translation of the canonical IRP target L-Ft was repressed in the livers of ID rats. We also show for the first time that translation of HIF-2α, a newly identified potential IRP target, was also repressed *in vivo* in response to dietary iron deficiency. Importantly, these results are not due to a global repression in translation as polysomal distribution of cyclophilin mRNA was unaffected by iron status. This affirmed our hypothesis that the iron-dependent regulation of HIF-2α translation likely occurs through direct action of IRPs in repressing translational activity.

Polysomal analysis may not accurately reflect changes in the total pool of a given mRNA, thus it was necessary to examine total mRNA expression to establish that

repression of HIF-2α mRNA was due to a repression in translation and not simply due to a decrease in the total pool size of mRNA via transcriptional regulation. To examine HIF-2α mRNA abundance, RNA was isolated from an equal amount of post-mitochondrial supernatant separated on the sucrose gradients, reverse transcribed, and subjected to real-time PCR to examine alterations in gene expression. In the liver, the relative abundance of HIF-2α mRNA was unaffected by diet, further confirmation of the translational, rather than transcriptional, regulation of this IRE-containing transcript. Likewise, the relative abundance of the well-characterized IRP target L-Ft was unchanged in response to dietary iron deficiency. These results demonstrate that HIF-2α is translationally controlled by IRPs in response to dietary iron availability.

Interestingly, suppression of HIF2-α translation was coordinated with a decrease in HIF-1α expression, suggesting possible cross-talk between HIF-1α and HIF-2α in the rodent in response to iron deficiency. To further explore this possibility, we determined the extent to which gene expression of known HIF targets (PFKFB3, G6Pase, GK, and GLUT1) is altered in response to dietary iron deficiency. Although PFKFB3, G6Pase and GK gene expression remained unchanged in response to iron deficiency, GLUT1 expression was actually decreased. This decrease in GKUT1 expression may be a result of decreased HIF-2α translation or perhaps through the down-regulation of HIF-1α expression. The attenuation of HIF-dependent gene expression suggests that the iron sensing pathway may play a more prominent role than the oxygen sensing pathway in response to iron deficiency anemia.

Although HIF-1α and HIF-2α transcriptionally control an overlapping set of target genes, it does appear that these isoforms exhibit a differential preference for

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hypoxia responsive elements (HREs) present in the promoters of HIF target genes [17, 113]. Concordantly, ablation of HIF-1α results in a very different phenotype than that of HIF-2α knock-out models. HIF-1α deficient mice die *in utero* as a result of insufficient vascularization [103].   On the other hand, HIF-2α deficient mice, depending on genetic background, exhibit phenotypic abnormalities ranging from impaired oxygen homeostasis for those that survive the perinatal period to embryonic or perinatal lethality because of defects in vascular modeling or lung maturation [139-141]. HIF-2α also regulates hematopoiesis in mice in an EPO dependent manner [141]. Mutations in HIF-2α also promote retinal degeneration in association with increased oxidative stress [141-143]. These data, and results from short interfering RNA knockdown experiments, demonstrate that the EPO gene is preferentially targeted by HIF-2α, thus making the iron-dependent regulation of HIF-2α of particular interest with respect to the integration of iron and oxygen sensing.

The discovery of a conserved IRE in the 5'UTR of HIF-2α mRNA revealed a previously unknown mechanism for feedback between iron and oxygen sensing, such that when iron is scarce, translational repression of HIF-2α could serve to modulate EPO production and thereby iron utilization [8]. As previous studies had been limited primarily to *in vitro* work, we demonstrate for the first time the iron-dependent regulation of HIF-2α mRNA translation in relation to dietary iron availability. Our results confirm that in dietary iron deficiency, HIF-2α is translationally repressed though direct action of IRP RNA binding activity, thus providing evidence that IRPs may serve to coordinate iron and oxygen sensing.

Findings from this study have provided a foundation for future studies in the coordination of iron and oxygen signaling stimulated an interest further characterizing the molecular mechanism associated with altered fuel metabolism in iron deficiency. Measurement of serum glucose levels in ID rats from this study and real-time PCR analyses of other glucose transporters will allow us to investigate possible mechanisms contributing to iron deficiency-related hyperglycemia. An understanding of the factors associated with hyperglycemia in conjunction with iron deficiency has significant implications because of the high prevalence of iron deficiency throughout the world and how alterations in iron status can affect organismal reliance on glucose as a metabolic substrate.

The results presented herein reveal a translational repression of HIF-2α mRNA in response to dietary iron deficiency. To further assess the consequences of translational repression, we plan to perform western blots on hepatic nuclear and cytosolic protein extracts to assess the steady-state levels of HIF1-α and HIF-2α . We expect to find that HIF-1α protein is stabilized leading to an increased abundance in the ID animals. Of particular interest is determining the relationship between HIF-2α protein abundance as a function of both translational repression and PHD-dependent stabilization. A decrease in HIF-2α protein abundance will confirm the translational repression observed in the current study, and, combined with the results from HIF-1α westerns, will indicate the extent to which oxygen-dependent and iron-dependent mechanisms control HIF-2α protein expression. Future studies will focus on changes in HIF-2α gene expression in response to dietary iron availability. Using a weanling rat model of iron deficiency we will assay time- and iron-dependent changes in IRP activity, in HIF-2α target gene

expression, and HIF-2α protein abundance to determine the extent to which changes in

IRP activity are associated with changes in HIF-2α target gene expression. These studies

will help us to define the relative importance of the repression of HIF-2α gene expression

in response to dietary iron availability and in the coordination of the physiological

response to iron deficiency and hypoxia.

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VITA

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Candidate for the Degree of

Master of Science

Thesis: COORDINATION OF IRON AND OXYGEN SENSING THROUGH
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Pages in Study: 78   Candidate for the Degree of Master of Science

Major Field: Nutritional Sciences

Scope and Method of Study: Examining the regulation of HIF-2α in response to iron
          availability using a weanling rat model of iron deficiency.

Findings and Conclusions:

Iron Regulatory Proteins (IRPs) regulate iron metabolism by binding to Iron Responsive

Elements (IREs) located in mRNAs encoding proteins of iron metabolism. The

discovery of an IRE in the 5' untranslated region (UTR) of Hypoxia Inducible Factor 2-

alpha (HIF-2α) mRNA suggests that translation of HIF-2α mRNA may be iron regulated.

We examined the extent to which iron deficiency alters translation of HIF-2α mRNA.

Weanling Sprague –Dawley rats were fed either an iron deficient diet (ID, <5 mg Fe/kg

diet) or a control (C, 50 mg Fe/kg diet) diet for 21 days. A third group was pair-fed (PF)

to receive the same amount of diet consumed by the ID animals. Translational control

was assessed by a combination of sucrose density centrifugation and real-time PCR. Our

results indicate both HIF-2α and L-ferritin (a known IRP target) translation were

repressed in ID animals. To our knowledge, these results provide the first whole –animal

evidence that HIF-2α is a target of IRP-mediated translational regulation.

ADVISER'S APPROVAL: Dr. Stephen Clarke