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Hypoxia Inducible Factor-2α Is Translationally Repressed in Response to Dietary Iron Deficiency in Sprague-Dawley Rats[1–3]

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Abstract

Iron regulatory proteins (IRP) regulate cellular iron metabolism by binding to iron-responsive elements (IRE) located in untranslated regions of mRNA-encoding proteins of iron metabolism. Recently, IRE have been identified in mRNA-encoding proteins with previously uncharacterized roles in iron metabolism, thus expanding the role of IRP beyond the regulation of cellular iron homeostasis. The mRNA for HIF 2-α contains an IRE and undergoes iron-dependent regulation in vitro, though the translational regulation of HIF-2α in vivo remains unknown. To examine HIF-2α translational regulation in vivo, we evaluated the effects of iron deficiency on the regulation of hepatic IRP activity and HIF-2α translation. Rats were fed either a control (C; 50 mg Fe/kg diet) or iron-deficient (ID; <5 mg Fe/kg diet) diet or were pair-fed (PF) the C diet for 21 d. In ID rats, there was a 2-fold increase in IRP activity compared to the PF group ($P < 0.05$), which was reflected by a 30–40% increase in HIF-2α repression ($P < 0.05$). In agreement with a decrease in translation, the levels of HIF-2α proteins were also decreased. The relative abundance of HIF-2α mRNA did not differ between treatment groups. Taken together, these results suggest that the translation of HIF-2α in the liver is regulated in part by the action of IRP in response to dietary iron deficiency and provide evidence that IRP may assist in coordinating the cellular response to alterations in iron and oxygen status associated with iron deficiency anemia. J. Nutr. 141: 1590–1596, 2011.

Introduction

Iron is required for DNA synthesis, key metabolic reactions, and cellular respiration, yet alterations in iron status continue to be a major health concern. In fact, iron deficiency remains the world's most common nutrient deficiency, affecting an estimated 2 billion individuals worldwide (1,2). Inadequate dietary iron can lead to the development of iron deficiency anemia characterized by symptoms such as weakness or fatigue, reduced work capacity, and a reduced capacity to transport oxygen due to impaired erythropoiesis and hemoglobin production. In contrast, there is considerable potential for damage due to excess iron by promoting tissue and organ damage through the production of free radicals (3).

Because iron is an essential yet potentially toxic nutrient, iron homeostasis is controlled at both systemic and cellular levels. The expression, synthesis, and secretion of the liver peptide hormone hepcidin are increased due to elevated hepatic iron stores or in response to inflammation (4–6). Hepcidin represses cellular iron export by binding to the iron export protein FPN[4] and promoting its degradation (7). Conversely, a depletion in iron stores, or hypoxia, decreases hepcidin expression and enhances cellular iron export to support increased iron demands and erythropoiesis (7–11). Thus, the level of iron stores or demand for iron to support erythropoiesis contributes to the regulation of iron uptake or recycling through the role of hepcidin and emphasizes the central role that the liver plays in the maintenance of systemic iron homeostasis.

Cellular iron metabolism is controlled by a family of RNA binding proteins known as iron regulatory protein (IRP) that are essential for the maintenance of iron homeostasis in vertebrates (12,13). IRP function as RNA binding proteins and/or enzymes that interconvert citrate and isocitrate. IRP bind stem-loop structures termed IRE in the UTR of mRNA-encoding proteins involved in iron uptake, storage, and utilization. IRP1 and IRP2 differ in the mechanism through which iron regulates their RNA binding function. Iron inactivates IRP1 RNA binding activity

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[3] Supplemental Table 1 and Figures 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.
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[4] Abbreviations used: C, control; EPO, erythropoietin; FPN, ferroportin; FTL, light-chin ferritin; GLUT, glucose transporter; HIF, hypoxia inducible factor; HRE, hypoxia response element; ID, iron-deficient; IRE, iron-responsive element; IRP, iron regulatory protein; PB, polysome buffer; PF, pair-fed; PMS, postmitochondrial supernatant; RNP, ribonucleoprotein; TFRC, transferrin receptor; UTR, untranslated region.

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through the formation of an iron-sulfur cluster converting it to the cytoplasmic isoform of aconitase, whereas IRP2 undergoes iron-mediated proteasomal degradation (11,14–17). Classic targets of IRP action include both heavy and light subunits of the iron storage protein ferritin (Fth and Ftl) and the iron uptake TFRC. In the case of Ferritin mRNA, IRP bind to an IRE in the 5′UTR, blocking the initiation of translation (18). IRP binding to an IRE in the 3′UTR of *Tfrc* mRNA blocks the access of a ribonuclease system, resulting in the stabilization of the mRNA (19). IRP also regulate the synthesis of additional proteins with roles in the maintenance of iron homeostasis, including erythroid 5-aminolevulinate synthase, divalent metal transporter-1 (DMT1), and FPN (20–24).

Severe iron deficiency impairs oxygen transport due to decreased hemoglobin synthesis. Cells respond to hypoxic conditions by altering the expression of genes aimed at limiting the effects of decreased oxygen availability. This response to hypoxia is mediated by a family of oxygen-regulated transcription factors known as HIF. To date, 3 HIF regulatory subunits (1α, 2α, and 3α) have been identified that heterodimerize with the constitutively expressed oxygen-insensitive HIF-1β subunit (25,26). HIF function by binding to HRE located in the promoters of target genes involved in glucose metabolism, cell growth, apoptosis, and angiogenesis (27,28). Under normoxic conditions, HIF regulatory subunits are targeted for proteasomal degradation by prolyl 4-hydroxylases, a family of iron-, oxygen-, and 2-oxoglutarate-dependent dioxygenases that promote prolyl hydroxylation and interaction with the von Hippel-Lindau protein (29–31). Thus, in the absence of oxygen or iron, HIF proteins are stabilized and capable of regulating target gene expression.

HIF are of particular interest in terms of iron metabolism, because both hepcidin and EPO may be regulated by this family of transcription factors (8,32). The hepcidin promoter contains an HRE that upon HIF-1α binding results in a repression of gene expression, potentially increasing iron export to support erythropoiesis (8). The EPO promoter also contains an HRE, though it appears to be somewhat specific for HIF-2α (32,33). In the liver, HIF-2α binding to the EPO promoter increases EPO expression to support enhanced erythropoiesis. Thus, HIF proteins are capable of coordinating both iron and oxygen metabolism. In the absence of adequate iron stores, the EPO-dependent stimulation of erythropoiesis may be regulated to prevent the formation of immature RBC (34).

The discovery of an IRE in the 5′UTR of HIF-2α mRNA has led to the speculation that IRP-dependent regulation of HIF-2α could serve to modulate EPO levels, thereby adjusting the rate of erythropoiesis to iron stores (34–36). To date, these suggestions have largely been limited to results from in vitro studies (34,37). The focus of this study was to examine the extent to which HIF-2α is translationally regulated in an IRP-dependent manner in vivo using an animal model of dietary iron deficiency. We focused on the response to iron deficiency in the liver given its role in contributing to the regulation of systemic iron homeostasis. Additionally, we sought to compare the regulation of HIF-2α translation with established targets of IRP action such as *Ftl and Tfrc*.

Methods

Animal care and diets. Weanling male Sprague-Dawley rats (n = 24 Harlan Sprague Dawley) weighing 47 ± 0.6 g were housed in individual stainless-steel, wire-bottomed cages in a humidity- and temperature-controlled room with a 12-h-light/-dark cycle at the Laboratory Animal Resource Facility at Oklahoma State University. Rats were provided either purified control (C; 50 mg Fe/kg diet, TD.89300) or iron-deficient (ID; <5 mg Fe/kg diet, TD.80396) diets from Harlan Teklad (38). Upon arrival at the facility, rats were fed the C diet for 3 d to acclimate to laboratory conditions. After acclimation, rats were randomly assigned to 1 of 3 treatment groups (n = 8/group): C and ID groups were allowed ad libitum access to diet, whereas the pair-fed (PF) group was fed the C diet restricted to the mean amount consumed by the ID group. All groups were allowed ad libitum access to deionized water throughout the treatment period. Food intake and body weight for animals were recorded daily. Rats received their respective diets for the experimental period of 21 d. After 21 d, rats were killed between 0800 and 1000 h by exsanguination following anesthetization with a ketamine/xylazine cocktail. Tissues were snap-frozen, but fresh liver tissue was prepared for polysome analysis (n = 6/group) and nuclear or cytosolic extracts (n = 2/group). All protocols were approved by the Institutional Animal Care and Use Committee of Oklahoma State University.

Blood and liver iron analyses. Whole blood from the abdominal aorta was collected into EDTA-coated tubes and assayed for hemoglobin concentration and hematocrit using a hematology analyzer (Stillwater Medical Center). To collect plasma, whole blood collected into EDTA-coated tubes was centrifuged at 800 × g at 4°C for 15 min. Plasma was removed and stored at −80°C for further analyses. Liver iron content was assessed on an ELAN 9000 inductively-coupled plasma mass spectrophotometer (PerkinElmer).

Sample collection and polysome preparation. Following the modification of Anthony et al., livers were excised, washed in ice-cold PB (40 mmol/L HEPES, pH 7.4, 100 mmol/L KCl, 5 mmol/L $MgCl_2$, 2 mmol/L citrate, and 1 mmol/L DTT), minced, and placed in a Potter-Elvehjem homogenizer (39). Samples were homogenized in 3 volumes of PB using a fitted Teflon pestle and centrifuged at 5000 × g at 4°C for 20 min. One volume of detergent (10% deoxycholate and 10% Triton X-100) was added to 9 volumes of the PMS and mixed gently. The PMS was supplemented with the protease inhibitors pepstatin (100 mg/L), leupeptin (100 mg/L), soybean trypsin inhibitor (250 mg/L), PMSF (1 mmol/L), and the proteasome inhibitor MG-132 (10 μmol/L). Next, 500 μL of each PMS sample was layered onto an ice-cold, 11-mL, linear 10–50% sucrose gradient in PB. Gradients were then centrifuged at 180,000 × g at 4°C for 2 h. Immediately following centrifugation, the gradients were fractionated using an ISCO gradient fractionator (Teldyne Isco). The absorbance at 254 nm was continuously monitored and 11 × 1-mL fractions were collected and stored at −80°C.

RNA isolation. Total RNA was isolated from each gradient fraction or from PMS using STAT-60 according to the manufacturer's directions (Teltest). The concentration of RNA from each fraction or PMS sample was determined using a Nanodrop spectrophotometer (Thermo Scientific). The integrity of isolated RNA was determined by examining 18S and 28S rRNA by agarose gel electrophoresis.

Determination of mRNA abundance in gradient fractions by qPCR. One-tenth (2 μL) of total RNA isolated from each fraction, or 2 μg of total RNA from PMS samples, was treated with DNase I (Roche) and reverse-transcribed using Superscript II (Invitrogen) following the manufacturer's directions for first-strand synthesis. For the purposes of these studies, mRNA localized in fractions 1–4 represents a translationally repressed RNP pool of mRNA. In contrast, fractions 5–11 represent the polysomal pool of RNA (**Supplemental Fig. 1**). The relative abundance of *HIF-2α, Ftl, Tfrc,* cyclophilin *(Cyclo)* mRNA and 18S rRNA in each gradient fraction and PMS sample were determined by qPCR using SYBR green chemistry on an ABI 7900HT Real-Time PCR system (Applied Biosystems). Primers for each gene were designed using Primer Express v3.0 software (Applied Biosystems) and validated by both template titration and dissociation curves in addition to exhibiting an efficiency slope of −3.3. Whenever possible, primers were designed such that the amplicon spanned at least one intron (**Supplemental Table 1**). All qPCR results were analyzed using the comparative cycle number at threshold method (User Bulletin no. 2, Applied Biosystems). Relative

abundance of mRNA in gradient fractions and PMS samples were normalized to the abundance of 18S rRNA and Cyclo mRNA, respectively.

Electrophoretic mobility shift assay. IRP RNA binding activity from homogenized liver was determined by electrophoretic mobility shift assay as previously described (40) using a plasmid containing the entire rat L-ferritin cDNA for IRE synthesis (41). Cytosolic fractions from the PMS samples were obtained by adding Triton X-100 (to a 1% final concentration) followed by centrifugation at 16,000 × *g* at 4°C for 25 min. The protein concentration of the supernatants was determined by bicinchoninic acid assay (Sigma). Spontaneous IRP1 and IRP2 RNA binding activities were assessed by incubating 5 μg cytosolic protein with saturating levels of [^{32}P]-labeled RNA. Total IRP1 RNA binding activity was measured by adding 1 μg of cytosolic protein in the presence of 2% β-mercaptoethanol to saturating levels of RNA. RNA binding activity was quantified using Optiquant Acquisition and Analysis software (Packard Bioscience). Spontaneous and total RNA binding activities are expressed as fmol or pmol RNA bound/mg protein, respectively.

Western-blot analyses. Cytosolic and nuclear proteins from liver were prepared as previously described (42). Proteins were separated by SDS-PAGE and transferred to PVDF membranes to determine abundance of HIF-2α, Lamin-A/C, ferritin, and γ-tubulin. Blots were blocked in a Blotto-Tween solution for at least 1 h and then incubated overnight at 4°C in Blotto-Tween with anti-HIF-2α polyclonal (Novus Biologicals), anti-Lamin-A/C polyclonal (Cell Signaling Technology), anti-ferritin polyclonal (Santa Cruz Biotechnologies), or anti-γ-tubulin monoclonal (Sigma-Aldrich) antibodies. The abundance of IRP2 was assessed using an anti-IRP2 polyclonal antibody (provided by Richard Eisenstein). HRP-coupled secondary antibodies at 1:10,000 dilutions in Blotto-Tween were used to detect primary antibody binding using chemiluminescence (SuperSignal West Pico) and analyzed using Optiquant software.

Statistical analysis. Statistical analyses using 1-way ANOVA and Student's *t* test techniques were performed to determine treatment effects using SPSS v17.0 software (IBM-SPSS). All tests were done at the 95% confidence level (α = 0.05). Descriptive statistics were calculated for all variables and include mean ± SEM. Data for percentage distribution of mRNA in hepatic gradient fractions were log-transformed prior to statistical analysis.

Results

Effects of dietary iron deficiency on growth and hematologic variables. Others have shown that animals consuming a severely iron-restricted diet exhibit decreased growth rates (43,44). At the end of the study, rats in the ID and PF groups weighed 20% less than those in the C group (**Supplemental Fig. 2**) ($P < 0.05$). Body weights in the PF and ID groups were significantly lower than the C group at d 14 and remained reduced for the duration of the study. Final body weight did not differ between the ID and PF groups.

Both hemoglobin and hematocrit were reduced by >50% in the ID group (66 ± 2 g/L and 0.168 ± 0.007%, respectively) compared to the PF group (133 ± 2 g/L and 0.381 ± 0.005%, respectively) ($P < 0.05$). Hematocrit and hemoglobin did not differ between the C and PF groups. In addition to lower hemoglobin and hematocrit, the liver iron concentration was lower in the ID group (430 ± 18 nmol/g) compared to the PF group (1330 ± 90 nmol/g) ($P < 0.001$). To control for any nonspecific effects as a result of an overall decrease in food intake, subsequent analyses examined differences between the PF and ID groups.

Effects of dietary iron deficiency on hepatic IRP RNA binding activity. Iron deficiency increased liver IRP1 RNA binding activity by 70% compared to the PF group (**Fig. 1***A* and *C*) ($P < 0.05$). IRP2 RNA binding activity was 248% higher in the ID group than in the PF group (Fig. 1*A* and *C*) ($P < 0.05$). The activation of IRP RNA binding activity is consistent with a depletion in the cytosolic iron pool in the ID group. Total IRP1 RNA binding activity did not differ between the PF and ID groups, indicating that differences in spontaneous binding activity could be accounted for by a change in distribution between the RNA binding and enzymatic (cytoplasmic aconitase) forms of IRP1 (Fig. 1*B* and *D*).

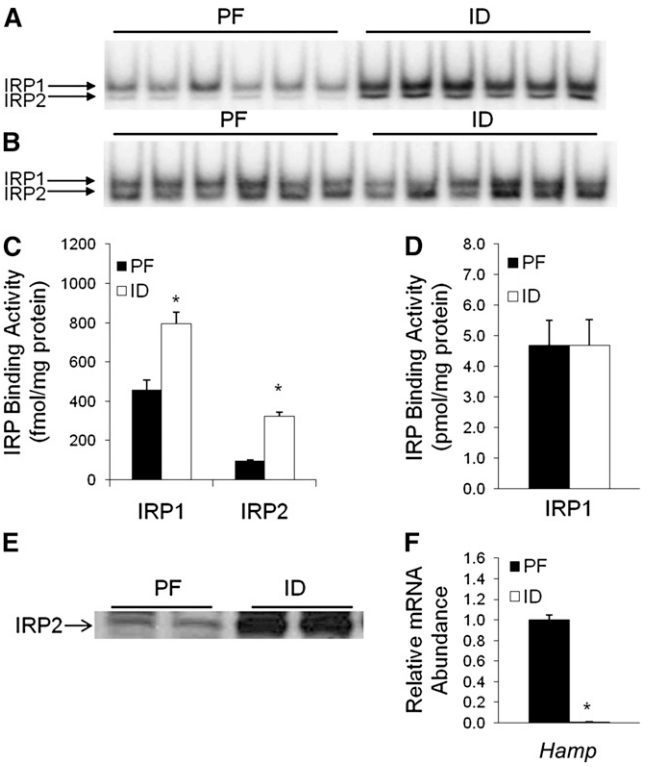

FIGURE 1 Spontaneous (*A*) and total (*B*) iron regulatory protein (IRP) RNA binding activity in livers of pair-fed (PF) and iron-deficient (ID) rats. Quantitative analysis of (*C*) spontaneous IRP1 and IRP2 RNA binding activity and (*D*) total IRP1 RNA binding activity. (*E*) Representative immunoblot of IRP2 from livers. (*F*) Expression of *Hamp* mRNA in response to dietary iron intake. Values are means ± SEM, n = 6. *Different from PF, $P < 0.05$.

Regulation of IRP2 protein abundance and hepcidin expression in iron deficiency. In agreement with changes in IRP2 RNA binding activity, IRP2 protein abundance in the liver increased 2-fold in the ID group compared to the PF group (Fig. 1*E*) ($P < 0.05$). The decrease in total liver iron, increase in IRP RNA binding activity, and accumulation of IRP2 protein indicates a depletion of liver iron in response to lack of adequate dietary iron. Furthermore, compared to the PF group, the expression of *Hamp* mRNA was repressed in the ID group (Fig. 1*F*) ($P < 0.05$).

Iron-dependent translational regulation of IRP target mRNA. In the ID group, *Hif-2α* mRNA moved from a polysomal pool of mRNA to a translationally repressed pool compared to the PF group (**Fig. 2***A*) ($P < 0.05$). These results are consistent with previous cell-based studies wherein iron chelation decreased polysomal *Hif-2α* mRNA (34) and indicate that the IRE in *Hif-2α* is sufficient to confer IRP-dependent regulation in response to iron deficiency.

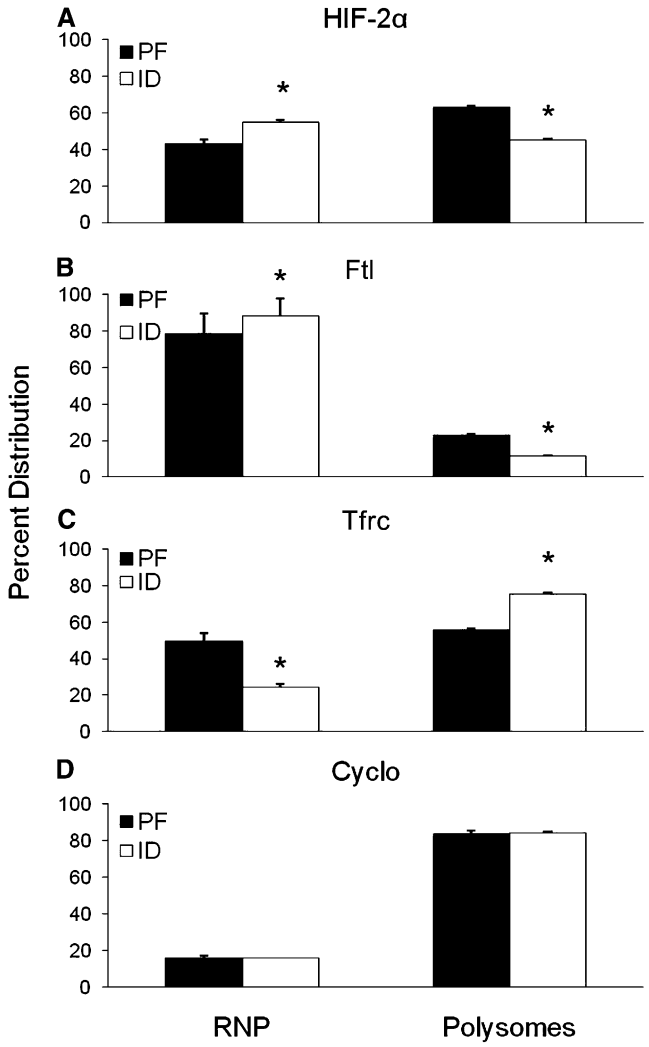

FIGURE 2 Relative mRNA distribution of hypoxia inducible factor (HIF)-2α (*A*), light-chain ferritin (*Ftl*) (*B*), transferrin receptor 1 (*Tfrc*) (*C*), and Cyclo (*D*) in the liver ribonucleoprotein (RNP) or polysome pools of pair-fed (PF) and iron-deficient (ID) rats. Values are mean percent distribution across the gradient ± SEM, $n = 6$. *Different from PF, $P < 0.05$.

The ~1.3-fold increase in repression was further reflected by a ~1.4-fold decrease in polysome-associated *Hif-2α* mRNA in the ID group compared to the PF group (Fig. 2*A*) ($P < 0.05$). These results provide evidence that the translation of *Hif-2α* in the liver is controlled at least in part by the action of IRP in response to iron deficiency.

The polysomal distribution of other known IRP targets was also examined. The iron storage protein *Ftl* contains an IRE in its 5′UTR and is translationally repressed by IRP RNA binding activity (45,46). In the livers of these rapidly growing animals, regardless of dietary iron, the majority of *Ftl* mRNA was localized to a repressed pool of mRNA and indicates the relatively low level of iron storage needs during growth (Fig. 2*B*). Despite the majority of *Ftl* mRNA being repressed, the polysome-associated *Ftl* mRNA was further reduced by 50% in the ID group compared to the PF group (Fig. 2*C*) ($P < 0.05$). In agreement with a role for IRP in regulating the stability of TfR mRNA in response to iron deficiency, polysome-associated TfR mRNA increased 35% in the ID group compared to the PF group (Fig. 2*C*) ($P < 0.05$). Taken together, these results indicate that the regulation of *Hif-2α* translation observed in these studies is consistent with a role of IRP in modulating *Hif-2α* translation.

Lastly, to examine if translational repression of *Hif-2α* and other 5′UTR IRE-containing mRNA in response to iron deficiency was due to nonspecific effects on translation, the polysomal distribution of *Cyclo* mRNA was assessed. The polysomal distribution of *Cyclo* mRNA was unaffected by iron deficiency (Fig. 2*D*), indicating that the translational repression of *Hif-2α* mRNA was unlikely due to a repression in global protein synthesis in the liver.

IRP-dependent translational repression alters HIF-2α protein abundance. The movement of *Hif-2α* mRNA into the RNP fraction may be predicted to decrease steady-state levels of protein. Indeed, when normalized to Lamin A/C as a loading control, liver HIF-2α protein in the ID group decreased >50% compared to the PF group (**Fig. 3***A*,*B*). The abundance of FTL protein was also assessed and, consistent with the repression of FTL translation in the ID group, FTL protein was essentially undetectable (Fig. 3*A*,*C*), though there were no changes in the abundance of γ-tubulin (Fig. 3*A*). A decrease in *Hif-2α* translation resulting in a diminution of HIF-2α protein in iron deficiency indicates that IRP partially contribute to the in vivo regulation of hepatic HIF-2α protein expression, providing evidence that IRP play a role in coordinating both cellular iron and oxygen signaling.

Regulation of IRP-target gene expression in the liver. Because a change in total mRNA abundance could alter the inter-

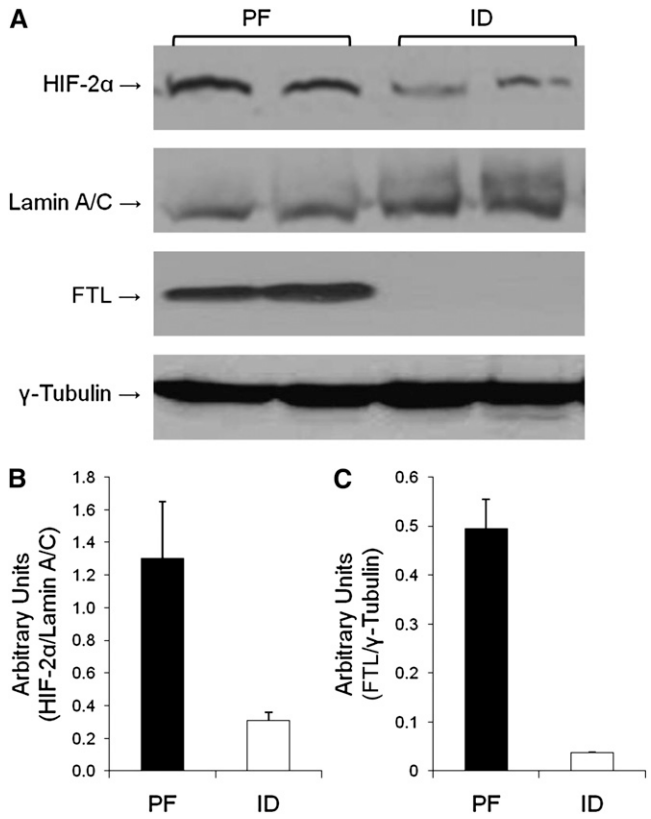

FIGURE 3 Protein abundance of hepatic hypoxia inducible factor (HIF)-2α, Lamin A/C, light-chain ferritin (FTL), and γ-tubulin in pair-fed (PF) and iron-deficient (ID) rats. Representative Western blots are in *A*. Relative HIF-2α (*B*) and FTL (*C*) protein levels are shown normalized to the appropriate loading control. Values are mean ± SD, $n = 2$–4.

pretation of the polysome profile analyses, the relative expression of *Hif-2α*, *Ftl*, and *Tfrc* mRNA was assessed in samples of the PMS loaded on the sucrose gradients. There was a 6-fold increase in *Tfrc* mRNA in the ID group compared to the PF group (**Fig. 4***A*) ($P < 0.05$), though the expression of *Hif-2α* and *Ftl* mRNA did not differ between the 2 groups (Fig. 4*A*). These results indicate that changes in polysomal distribution of *Hif-2α* and *Ftl* were unlikely to be a result of changes in mRNA abundance. The expression of HIF-1*β*, the obligate heterodimeric partner for HIF-1*α* and HIF-2*α*, was unchanged regardless of dietary iron (Fig. 4*A*). Interestingly, the total abundance of *Hif-1α* mRNA decreased ~50% in the ID group compared to the PF group (Fig. 4*A*) ($P < 0.05$), suggesting that either *Hif-1α* transcription or mRNA stability is decreased in response to iron deficiency, though similar observations are reported elsewhere (47).

Modulation of the hepatic HIF transcriptional network by dietary iron. Using qPCR, we examined expression of HIF target genes in the liver of rats in the PF and ID groups. Although not the primary GLUT in the liver, compared to the PF group, the expression of the HIF target gene *Glut1* decreased 50% in the ID group (Fig. 4*B*) ($P < 0.05$). The expression of other HIF target genes, including *Hmox*, *Ldhα*, and *Pparα* all decreased in response to dietary iron deficiency (Fig. 4*B*) ($P < 0.05$). Despite a decrease in HIF-2*α* protein abundance, the expression of hepatic *Epo* mRNA increased to detectable levels in the ID group, though no expression was detected in rats in the PF group (data not shown). Although *EPO* expression was induced, the expression of *Vegfα*, another HIF-2*α* target gene, was unaffected by iron deficiency. The extent to which translational control of HIF-2*α* by IRP modulates hypoxia signaling remains unknown and is beyond the scope of the studies presented here.

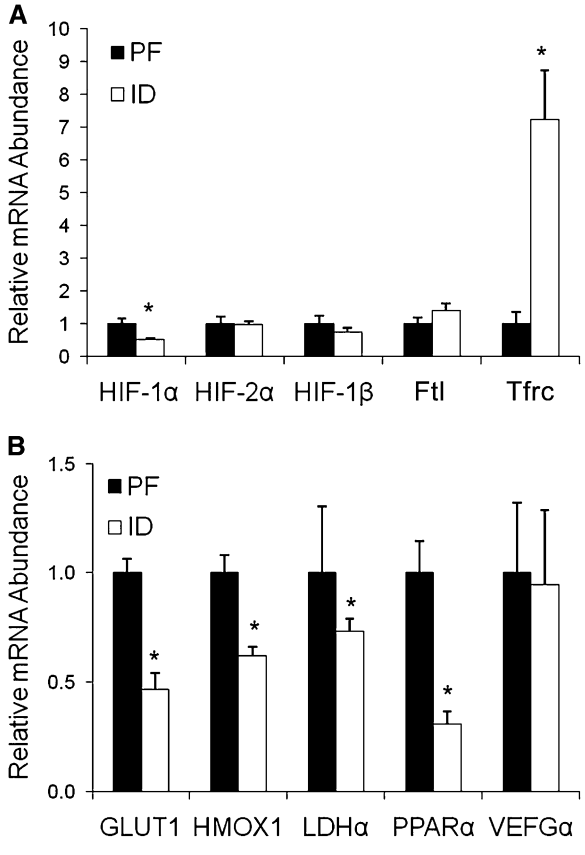

FIGURE 4 Expression of (*A*) hypoxia inducible factor (HIF)-1*α*, HIF-2*α*, HIF-1*β*, light-chain ferritin (*Ftl*), and transferrin receptor *(Tfrc)*-1 mRNA and (*B*) of the HIF target genes, including glucose transporter (GLUT)1, heme oxgenase (*Hmox*)1, lactate dehydrogenase (Ldh*α*), Ppar*α*, and Vegf*α* in the liver of pair-fed (PF) and iron-deficient (ID) rats. Gene expression is presented as fold of the PF group. Values are mean ± SEM, $n = 6$. *Different from PF, $P < 0.05$.

Discussion

Due to the essential yet potentially toxic nature of iron, cellular and organismal iron homeostasis are tightly controlled to prevent iron deficiency or iron toxicity. Whereas the peptide hormone hepcidin is a critical mediator of organismal iron homeostasis, IRP may be considered as the key regulators of cellular iron homeostasis due to their role in regulating the expression of proteins involved in iron storage (e.g. ferritin), acquisition (e.g. TFRC or DMT1), and utilization [e.g. erythroid 5-aminolevulinate synthase or mitochondrial aconitase (ACO2)]. More recently, targets of IRP have been expanded beyond those encoding iron-related proteins and now include proteins involved in regulating the cell cycle (CDC14a) and perhaps even proteins implicated in the etiology of neurodegenerative disease (*α*-synuclein and amyloid beta A4 precursor protein) (48–50). The current study confirms the findings of initial cell-culture based studies indicating that the oxygen-regulated transcription factor HIF-2*α* is also a target of IRP (34) and extends these findings by demonstrating that *HIF-2α* translation is regulated in vivo in the liver in response to dietary iron deficiency.

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 flanking region sequences, may contribute to the extent of IRP-dependent regulation (43,51–53). The identification of a functional IRE in the 5′UTR of *Hif-2α* prompted us to examine the extent to which *HIF-2α* was translationally regulated, presumably in an IRP-dependent manner, in response to dietary iron deficiency. The presence of an IRE in *Hif-2α* is particularly intriguing given that the erythropoiesis-stimulating hormone EPO has been characterized as an extra-renal target gene of Hif-2*α* (32,33). In fact, hepatic EPO expression may account for nearly 30% of total EPO produced in response to hypoxic conditions (54). Thus, based on the functions of IRP, others have proposed that IRP-dependent regulation of HIF-2*α* synthesis may provide an adaptive mechanism to coordinate iron stores with the production of RBC (34). Under the low oxygen conditions that could result from impaired erythropoiesis in iron deficiency anemia, the IRP-mediated repression of *Hif-2α* translation may attenuate increased RBC production, thereby decreasing the overall production of microcytic hypochromic erythrocytes resulting from inadequate iron stores.

In this present study, we have established a role for IRP in regulating hepatic *Hif-2α* translation in response to a dietary iron deficiency. By examining the effects of iron deficiency on other well-characterized targets of IRP, we demonstrate that the translational regulation of *Hif-2α* mRNA is consistent with the induction of liver IRP RNA binding activity. In fact, in this animal model of iron deficiency, the extent of *Hif-2α* translational regulation appears to be as robust as *Ftl*, a canonical target of IRP, though this may be partially explained by the relatively rapid growth of the animals. Given the role of ferritin as the major iron storage protein and the requirement of iron to support cell growth and differentiation, the expression of *Ftl*

would be predicted to be tightly controlled and therefore not actively translated under the physiological conditions imposed by the use of the current animal model. Indeed, we found that the majority of *Ftl* mRNA is repressed in these rapidly growing animals and that a greater proportion of *Hif-2α* mRNA is translationally active, perhaps underscoring the relative importance of this constitutively expressed transcription factor that allows cells to rapidly adapt to changes in oxygen supply. The absence of duodenal *Hif-2α* expression leads to the development of an iron-deficient phenotype and indicates the critical role that HIF-2α plays in coordinating iron absorption (36,55). Activation of HIF-2α expression in response to a low-iron diet in the small intestine is associated with increased expression of HIF target genes in addition to increased DMT1 and duodenal cytochrome b protein abundance, presumably in the presence of a functional IRP-IRE system (55). In fact, IRP are critical for the expression of both DMT1 and FPN and indicate the role of IRP in contributing to the maintenance of systemic iron homeostasis (56). Differences in HIF-2α expression in the response to a lack of dietary iron between the intestine and the liver may be indicative of tissue-specific differences in relative oxygen concentrations (57). Our results support the findings of others indicating that the regulation of *Hif-2α* gene and protein expression in the intestine and liver is an important component in the maintenance of systemic iron homeostasis (34,36,55,56).

Although oxygen- and iron-dependent regulation of protein stability represents the primary means through which HIF-2α protein abundance is modulated (58,59), we have provided in vivo evidence that *Hif-2α* is indeed subject to translational control through an IRP-mediated mechanism in the liver. Given the critical role of this transcription factor in allowing an organism to adapt to hypoxic conditions, translation is unlikely to be fully repressed, even when iron deficiency or hypoxia is most severe. In fact, a similar mode of regulation has been demonstrated for the tricarboxylic acid cycle enzyme m-acon (43,51). Despite both *Ftl* and *Aco2* containing an IRE in their 5′UTR, iron deficiency differentially affects the translation of these 2 targets of IRP with only a ~50% maximal repression in m-acon compared with a >90% reduction in *Ftl* translation (43). Thus, the IRP-dependent regulation of *Hif-2α* likely serves as an additional mechanism to the regulation of prolyl 4-hydroxylases proteins to coordinate iron and oxygen sensing, providing a molecular mechanism that promotes the greatest degree of iron conservation in severe iron deficiency. Under iron-replete but hypoxic conditions, the translation of *Hif-2α* would be predicted to be unaffected by IRP, thereby increasing the expression of HIF-2α–dependent target genes (e.g. *Epo*) and promoting the biological adaptation to hypoxia. We have shown that *Hif-2α* translation and protein in the liver is decreased in response to a dietary iron deficiency. These findings provide additional insight into the regulation of iron metabolism and utilization of iron stores under hypoxic conditions, though the extent to which hypoxia or iron status alone affects the HIF-2α transcriptional network remains to be assessed.

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