Maintenance of cellular iron homeostasis requires post-transcriptional regulation of iron metabolism genes by iron regulatory protein 2 (IRP2). The hemerythrin-like domain of F-box and leucine-rich repeat protein 5 (FBXL5), an E3 ubiquitin ligase subunit, senses iron and oxygen availability and facilitates IRP2 degradation in iron replete cells. Disruption of the ubiquitously expressed murine Fbxl5 gene results in a failure to sense increased cellular iron availability, accompanied by constitutive IRP2 accumulation and misexpression of IRP2 target genes. FBXL5-null mice die during embryogenesis, whereas Fbxl5 heterozygotes perform better than wild type littermates when fed a low iron diet due to enhanced iron absorption.

**Results:** FBXL5-null mice die during embryogenesis, whereas Fbxl5 heterozygotes perform better than wild type littermates when fed a low iron diet due to enhanced iron absorption.

**Conclusion:** FBXL5 plays an essential role in the in vivo maintenance of cellular and systemic iron homeostasis.

**Significance:** FBXL5 is an essential physiological iron sensor.

Iron is widely employed throughout biology. Failure to maintain bioavailable iron concentrations within appropriate levels may result in deleterious consequences ranging from anemia to iron overload disease (1, 2). IRPs3 regulate the post-transcriptional expression of several iron metabolism genes upon binding iron responsive elements (IREs) within the 5′ or 3′ untranslated regions (UTRs) of their mRNAs in iron-depleted cells (3, 4). For example, synthesis of the iron storage protein ferritin heavy chain 1 is reduced upon binding of IRPs to its 5′ IRE (5).

Conversely, iron uptake is promoted by stabilization of the mRNAs encoding the iron import factors transferrin receptor 1 (TfR1) and DMT-1 upon IRP binding to IREs within their 3′ UTRs (4, 6). As iron bioavailability increases, IRPs lose their RNA binding capacity either through conformational changes resulting from the enhanced FeS cluster assembly within IRP1 (3) or enhanced proteasomal degradation of IRP2 (7).

Selective degradation of IRP2 is preceded by its iron-dependent polyubiquitination via a Skp1/Cul1/Rbx1 (SCF) E3 ubiquitin ligase complex containing FBXL5 (8, 9). FBXL5 contains an N-terminal hemerythrin-like domain characterized by a helical bundle held together by a di-iron center (8–11). When iron and oxygen are abundant, this domain resides in a compact conformation that masks a degron within the hemerythrin-like domain itself, promoting FBXL5 accumulation and subsequent IRP2 degradation (11, 12). When low levels of bioavailable iron limit assembly of the di-iron center, the hemerythrin-like degron becomes accessible and FBXL5 is degraded by the proteasome (11–13). These properties of the FBXL5 hemerythrin-like domain suggest that it is a key sensor of bioavailable ferrous iron within cells (14). The importance of FBXL5 to the maintenance of cellular iron homeostasis was initially confirmed through siRNA-mediated knockdown of FBXL5 expression, which resulted in inappropriate stabilization of IRP2 in iron-replete cells (8). Such cells aberrantly repress ferritin expression while promoting continued iron uptake through stabilization of the TfR1 mRNA (8, 9, 15), exacerbating the metabolic stress of excessive iron.

To investigate the in vivo role for FBXL5 in the maintenance of iron homeostasis, we generated mice in which the Fbxl5 gene locus has been disrupted. FBXL5-null mice die during embryonic development.
FBXL5 Regulates Iron Metabolism in Vivo

Iron Metabolism Genes are Aberrantly Regulated in FBXL5GT/GT Mouse Embryonic Cells—Given the proposed role of FBXL5 in mediating cellular iron homeostasis, we suspected that FBXL5GT/GT embryos would fail to properly regulate IRE-containing mRNAs. Due to the early embryonic lethality of the FBXL5GT/GT mice, we generated mouse embryonic cell lines from FBXL5+/+, FBXL5+/GT, and FBXL5GT/GT embryos (Fig. 4A) harvested on day E8. FBXL5+/+ and FBXL5+/GT cells depleted of bioavailable iron with the metal chelator deferoxamine mesylate (DFO) strongly accumulate IRP2 compared with iron-replete cells incubated with ferric ammonium citrate. However, FBXL5GT/GT cells aberrantly accumulate IRP2 under both conditions (Fig. 4C). Total IRP1 protein levels remained constant under all conditions and in all cell types (Fig. 4C). This inappropriately accumulated IRP2 is competent for IRE binding (Fig. 4D) and FBXL5GT/GT cells exhibit increased TR1 expression and decreased ferritin expression under iron replete conditions (Fig. 4, B and C). An increase in iron uptake, coupled with a decrease in iron storage capacity, could result in the overaccumulation of iron accompanied by increased oxidative stress in these FBXL5-null cells.

To ascertain whether embryonic lethality was due to deregulated IRP2 expression, we crossed FBXL5+/GT mice with IRP2

Additional “Materials and Methods”—See supplemental “Materials and Methods” for supporting information.

RESULTS

FBXL5 Is Ubiquitously Expressed—As all mammalian cells have a requirement for iron, yet are susceptible to damage when bioavailable iron accumulates, a bona fide cellular iron sensor should be ubiquitously expressed. FBXL5 mRNA levels were quantitated by quantitative PCR across panels of human and murine tissue extracts. As predicted, FBXL5 expression was detected in all samples with the highest relative levels present in the brain, particularly the metal-rich cerebellum (19), eye, and testis (Fig. 1, A and B). These same FBXL5 mRNA-abundant tissues also tend to express relatively high levels of IRP1 and IRP2 mRNAs (Fig. 1, A and B, and Ref. 20). In the mouse, FBXL5 expression is high at embryonic day (E) 7.5 (Fig. 1B), suggesting a role in early embryogenesis, although its expression decreases as the embryo progresses through development (E11 and E15).

Mice Lacking a Functional FBXL5 Gene Die during Embryogenesis—Insertion of a gene-trapping (GT) vector downstream of the second exon of FBXL5 generated a disrupted allele (FBXL5GT; Fig. 2). Progeny from mice containing one copy of this nonfunctional allele were genotyped at various developmental time points. Although wild type (FBXL5+/+), heterozygous (FBXL5+/GT), and homozygous null (FBXL5GT/GT) embryos were initially observed in a Mendelian 1:2:1 ratio, no FBXL5GT/GT pups were born (Table 1). In the mixed genetic background of our mice, E9.5 FBXL5GT/GT embryos exhibited growth retardation as compared with wild type and heterozygous littersmates (Fig. 3A). Although developmentally delayed, histopathological analysis revealed that E9.5 FBXL5GT/GT embryos had undergone normal placentation (Fig. 3, B and C), gastrulation, and cardiovascular development (Fig. 3D). However, at E10.5 these embryos appeared very distorted and were completely absorbed by day E12.5.

EXPERIMENTAL PROCEDURES

Animals—Murine 129 Sv/Ev embryonic stem cells heterozygous for the gene-trapped FBXL5 allele (clone OST386421) were obtained from Texas A&M Institute for Genomic Medicine and injected into C57Bl/6j blastocysts at the University of Texas Southwestern (UTSW) Transgenic Technology Center. The resulting chimeric mice were crossed to C57Bl/6j mice (Jackson Laboratories) to produce heterozygous animals. Backcrossing FBXL5 heterozygous mice of mixed background to wild type 129 Sv/Ev mice resulted in the generation of syngeneic FBXL5GT/+ mice that were 99.99% of 129 Sv/Ev genetic background as determined by speed congenics (Taconic). FBXL5 wild type and FBXL5 heterozygous mice were weaned onto a ad libitum iron-sufficient (50 ppm) or low iron (5 ppm) diet (Harlan-Teklad) for 3 weeks. For the 59Fe feeding experiments, mice fed a low iron diet for 3 weeks were then fasted for 24 h and gavaged with an olive-tipped needle containing 200 µl of PBS supplemented with 2.5 µCi of 59FeCl3 (PerkinElmer Life Sciences) and 0.5 M ascorbic acid. At the indicated time points, animals were exsanguinated and 59Fe accumulation in tissues was measured in a Packard Cobra Gamma Counter. Blood samples were collected from the tail vein or via cardiac puncture following intraperitoneal administration of anesthetic rodent mixture (ketamine/xylazine/acepromazine). Complete Blood Count analysis was performed by the UTSW Diagnostic Lab. Serum iron concentration, iron saturation, and total iron binding capacity were measured by Idexx Laboratories, Cornell University. The Irp1+/− and Irp2−/− (16, 17) mice were generously provided by Matthias Hentze. All animal experiments were performed with the approval of the UTSW Institutional Animal Care and Use Committee.

Isolation, Culture, and Characterization of Mouse Embryonic Cells—Mouse embryonic cells were prepared as described (18). Briefly, E8 embryos were digested with trypsin at 37 °C for 30 min. Cells were cultured on a layer of mitotic inactivated mouse embryonic fibroblast feeder cells in Dulbecco’s modified high glucose Eagle’s medium (HyClone) supplemented with 20% fetal bovine serum (Atlanta Biological), 1× nonessential amino acids (HyClone), 1× penicillin and streptomycin (HyClone), 2 mM glutamine (HyClone), 55 µM β-mercaptopethanol (Sigma), and 110 mg/liter of pyruvate (HyClone). After 4 weeks, cells were grown without feeder cells and immortalized upon transfection of the SV40 large T antigen (pSV3-Neo, ATCC) with Lipofectamine 2000 (Invitrogen). Stably transfected cells were selected in the presence of 500 µg/ml of G418.

Iron Metabolism Genes are Aberrantly Regulated in FBXL5GT/GT Mouse Embryonic Cells—Given the proposed role of FBXL5 in mediating cellular iron homeostasis, we suspected that FBXL5GT/GT embryos would fail to properly regulate IRE-containing mRNAs. Due to the early embryonic lethality of the FBXL5GT/GT mice, we generated mouse embryonic cell lines from FBXL5+/+, FBXL5+/GT, and FBXL5GT/GT embryos (Fig. 4A) harvested on day E8. FBXL5+/+ and FBXL5+/GT cells depleted of bioavailable iron with the metal chelator deferoxamine mesylate (DFO) strongly accumulate IRP2 compared with iron-replete cells incubated with ferric ammonium citrate. However, FBXL5GT/GT cells aberrantly accumulate IRP2 under both conditions (Fig. 4C). Total IRP1 protein levels remained constant under all conditions and in all cell types (Fig. 4C). This inappropriately accumulated IRP2 is competent for IRE binding (Fig. 4D) and FBXL5GT/GT cells exhibit increased TR1 expression and decreased ferritin expression under iron replete conditions (Fig. 4, B and C). An increase in iron uptake, coupled with a decrease in iron storage capacity, could result in the overaccumulation of iron accompanied by increased oxidative stress in these FBXL5-null cells.

To ascertain whether embryonic lethality was due to deregulated IRP2 expression, we crossed FBXL5+/GT mice with IRP2

Additional “Materials and Methods”—See supplemental “Materials and Methods” for supporting information.

RESULTS

FBXL5 Is Ubiquitously Expressed—As all mammalian cells have a requirement for iron, yet are susceptible to damage when bioavailable iron accumulates, a bona fide cellular iron sensor should be ubiquitously expressed. FBXL5 mRNA levels were quantitated by quantitative PCR across panels of human and murine tissue extracts. As predicted, FBXL5 expression was detected in all samples with the highest relative levels present in the brain, particularly the metal-rich cerebellum (19), eye, and testis (Fig. 1, A and B). These same FBXL5 mRNA-abundant tissues also tend to express relatively high levels of IRP1 and IRP2 mRNAs (Fig. 1, A and B, and Ref. 20). In the mouse, FBXL5 expression is high at embryonic day (E) 7.5 (Fig. 1B), suggesting a role in early embryogenesis, although its expression decreases as the embryo progresses through development (E11 and E15).

Mice Lacking a Functional FBXL5 Gene Die during Embryogenesis—Insertion of a gene-trapping (GT) vector downstream of the second exon of FBXL5 generated a disrupted allele (FBXL5GT; Fig. 2). Progeny from mice containing one copy of this nonfunctional allele were genotyped at various developmental time points. Although wild type (FBXL5+/+), heterozygous (FBXL5+/GT), and homozygous null (FBXL5GT/GT) embryos were initially observed in a Mendelian 1:2:1 ratio, no FBXL5GT/GT pups were born (Table 1). In the mixed genetic background of our mice, E9.5 FBXL5GT/GT embryos exhibited growth retardation as compared with wild type and heterozygous littersmates (Fig. 3A). Although developmentally delayed, histopathological analysis revealed that E9.5 FBXL5GT/GT embryos had undergone normal placentation (Fig. 3, B and C), gastrulation, and cardiovascular development (Fig. 3D). However, at E10.5 these embryos appeared very distorted and were completely absorbed by day E12.5.

Iron Metabolism Genes are Aberrantly Regulated in FBXL5GT/GT Mouse Embryonic Cells—Given the proposed role of FBXL5 in mediating cellular iron homeostasis, we suspected that FBXL5GT/GT embryos would fail to properly regulate IRE-containing mRNAs. Due to the early embryonic lethality of the FBXL5GT/GT mice, we generated mouse embryonic cell lines from FBXL5+/+, FBXL5+/GT, and FBXL5GT/GT embryos (Fig. 4A) harvested on day E8. FBXL5+/+ and FBXL5+/GT cells depleted of bioavailable iron with the metal chelator deferoxamine mesylate (DFO) strongly accumulate IRP2 compared with iron-replete cells incubated with ferric ammonium citrate. However, FBXL5GT/GT cells aberrantly accumulate IRP2 under both conditions (Fig. 4C). Total IRP1 protein levels remained constant under all conditions and in all cell types (Fig. 4C). This inappropriately accumulated IRP2 is competent for IRE binding (Fig. 4D) and FBXL5GT/GT cells exhibit increased TR1 expression and decreased ferritin expression under iron replete conditions (Fig. 4, B and C). An increase in iron uptake, coupled with a decrease in iron storage capacity, could result in the overaccumulation of iron accompanied by increased oxidative stress in these FBXL5-null cells.

To ascertain whether embryonic lethality was due to deregulated IRP2 expression, we crossed FBXL5+/GT mice with IRP2
knock-out mice (21). Viable Irp2−/−; Fbxl5GT/GT mice were born from Irp2+/−; Fbxl5+/+ intercrosses, although no Irp2−/−; Fbxl5GT/GT mice were observed (Table 2). Like their littermates, Irp2−/−; Fbxl5GT/GT mice grow normally and are fertile. This result suggests that the early embryonic mortality of FBXL5-null animals is due to the constitutive accumulation of IRP2 with concomitant dysregulation of its target genes. Interestingly, when Irp1−/−; Fbxl5+/- mice were crossed, no Irp1−/−; Fbxl5GT/GT pups were observed at birth (Table 3), despite the significant redundancy in IRP1 and IRP2 function (22, 23).

**Fbxl5−/GT Mice Differ from Fbxl5−/+ Littermates When Fed a Low Iron Diet**—To determine whether Fbxl5−/− mice manifest a non-overt iron-related phenotype(s), we challenged wild type and heterozygous mice with a low iron (5 ppm) diet and compared their results to mice weaned onto an iron-sufficient (50 ppm) diet (23, 24). After 3 weeks ingesting the specified diets, complete blood counts and serum iron levels were determined. Wild type mice fed a low iron diet exhibited an expected 77% decrease in serum iron levels despite a compensatory 149% increase in the total iron binding capacity of transferrin (Table 4). Consistent with the low dietary iron availability, wild type animals report significant reductions in hematocrit and hemoglobin levels, and a modest reduction in the number of red blood cells (RBC) (Table 4). All measurements taken from Fbxl5−/GT mice fed an iron-sufficient (50 ppm) diet were indistinguishable from control Fbxl5−/+ mice. Even Fbxl5−/GT mice fed a low iron diet exhibit similar changes in serum iron and total iron binding capacity values to those of their wild type counterparts (Table 4). However, this reduced iron availability does not result in any corresponding reductions in hematocrit, hemoglobin, or erythrocyte levels in the Fbxl5 heterozygotes (Table 4). To guard against the possibility that this unexpected phenotype was due to the mixed genetic background of these mice, Fbxl5−/GT mice were backcrossed to 129 Sv/Ev mice to generate syngeneic Fbxl5−/GT mice. Just like their mixed background Fbxl5−/GT counterparts, syngeneic Fbxl5−/GT mice still maintained normal hematologic values when fed a low iron diet (Table 5). These data suggest that Fbxl5−/GT mice have altered their systemic iron homeostasis so as to make iron preferen-

---

**FIGURE 1.** FBXL5 is ubiquitously expressed in humans and mice. Relative mRNA levels of FBXL5, IRP1, and IRP2 were quantitated by quantitative PCR from human (A) and murine (B) tissue samples. Each column represents the average of three experiments ± S.D.
FBXL5 Regulates Iron Metabolism in Vivo

A

Wild-Type Allele (+)

Targeted Allele (GT)

B

Fbxl5

+/+

+/GT

GT/GT

GT/GT

No. of Progeny

Stage

+/+

+/GT

GT/GT

Total

E9.5

20

44

18

82

E10.5

11

22

9

42

E11.5

12

28

10\* 

50

E12.5

10

22

0

32

Postnatal

85

155

0

240

\* Partially absorbed embryos.

![Figure 2](image)

**FIGURE 2. Disruption of the murine Fbxl5 gene.** A, embryonic stem cells containing a gene-trapped (GT) Fbxl5 allele were used to generate FBXL5-null mice. A trapping vector containing a promoter-less neomycin (Neo) resistance cassette flanked by a splicing acceptor (SA) site and a polyadenylation signal sequence (pA) was inserted 3′ of Fbxl5 exon 2. Utilization of the SA site generates a truncated FBXL5 mRNA transcript competent for Neo expression. The trapping vector also contains the first exon of the Bruton’s tyrosine kinase (Btk) gene flanked by the PGK promoter sequence and a splicing donor site (SD). The resultant chimeric fusion transcript was used to generate a sequence tag of the trapped gene by 3′ RACE. B, genotypic analysis by PCR using genomic DNA isolated from wild type mice (Fbxl5+/+), heterozygous mice (Fbxl5+/−), or FBXL5-null mice (Fbxl5−/−).

**TABLE 1**

| Stage     | +/+  | +/GT | GT/GT | Total |
|-----------|------|------|-------|-------|
| E9.5      | 20   | 44   | 18    | 82    |
| E10.5     | 11   | 22   | 9     | 42    |
| E11.5     | 12   | 28   | 10*   | 50    |
| E12.5     | 10   | 22   | 0     | 32    |
| Postnatal | 85   | 155  | 0     | 240   |

- Partially absorbed embryos.

Iron Absorption and Systemic Distribution Are Altered in Fbxl5+/− Mice Fed a Low Iron Diet—We hypothesized that the requisite iron needed to maintain normal hematocrit and hemoglobin values in Fbxl5+/− mice could be made available through depletion of iron stores or through increased intestinal absorption. The metal content of the liver, a major site of iron storage (4), was determined by inductively coupled plasma mass spectrometry (supplemental Fig. S1 and Fig. 5). As shown in Fig. 5, total iron content in the liver was reduced in an equivalent amount in all mice fed the low iron diet for 3 weeks, providing no indication that additional iron was mobilized from Fbxl5+/− liver stores.

To determine whether Fbxl5+/− mice are more efficient than wild type mice at absorbing limiting dietary iron, 59Fe was directly introduced via gastric gavage into the stomachs of mice that had been fed a low iron diet for 3 weeks and the distribution of 59Fe in various tissues was measured over time. After 1 h, almost 80-fold more 59Fe was incorporated within the duodena of Fbxl5+/− mice as compared with wild type animals (Fig. 6A). This increased efficiency in intestinal uptake was accompanied by a corresponding 3-fold increase in serum 59Fe levels (Fig. 6B). The whole body distribution of 59Fe is also markedly different in the Fbxl5−/− mice, as this newly absorbed iron is made preferentially available to the spleen (Fig. 6C) and the bone marrow (Fig. 6D, femur) for rapid incorporation into red blood cells (Fig. 6E), rather than sites of storage (Fig. 6F, liver).

Fbxl5 Heterozygosity Specifically Alters the Iron Responsiveness of the Duodenum—Although Fbxl5+/− mice are indistinguishable from Fbxl5+/+ mice when fed an iron-sufficient diet, the heterozygous mice take up iron more efficiently when dietary availability is limiting. To determine the underlying cause of this difference, immunoblot analysis was used to examine the expression of iron metabolism genes. In wild type mice fed a low iron diet, intestinal iron absorption is typically promoted in multiple ways. At the cell autonomous level (25, 26), IRP activity is induced within iron-depleted intestinal epithelial cells (supplemental Fig. S2), stabilizing an IRE-containing DMT-1 transcript (supplemental Table S1) and subsequently promoting iron uptake through increased DMT-1 expression (Fig. 7A). At the same time, reduced serum iron levels attenuate hepcidin (HAMP) transcription in the liver (supplemental Table S1) (27). A reduction in circulating hepcidin levels stabilizes the iron export protein ferroportin (Fig. 7A) to facilitate iron absorption through the intestine (27, 28). In the Fbxl5+/− animals, the systemic, ferroportin-dependent, response is identical to Fbxl5+/+ mice, both at the level of hepcidin expression in the liver (supplemental Table S1) and ferroportin accumulation in the duodenum (Fig. 7A). However, the cell autonomous response to low iron is dramatically altered in the duodena of Fbxl5+/− mice fed a low iron diet. IRP2 protein levels are 7-fold higher than in the corresponding wild type mice (Fig. 7A and supplemental Table S2). The accompanying 2-fold increase in IRP-binding activity (Fig. 7C) mimics the 2-fold additional increase in the IRE-containing DMT-1 mRNA (but not the IRE-independent DMT-1 mRNA isoform; supplemental Table S1) and DMT-1 protein levels (Fig. 7A and supplemental Table S2)
FBXL5 Regulates Iron Metabolism in Vivo

in the Fbxl5<sup>+/</sup> GT duodenum samples. Interestingly, this enhanced IRP responsiveness appears to be primarily limited to the intestine as the expression of iron metabolism genes was identical between wild type and heterozygous livers (Fig. 7B), spleens, and brains (supplemental Tables S1 and S2). Similar results were observed in syngeneic Fbxl5<sup>/</sup>/H11001 GT mice (supplemental Fig. S3). Interestingly, through Irp2<sup>−/−</sup>; Fbxl5<sup>GT/GT</sup> mice are viable, the lack of FBXL5 was not sufficient to reverse the anemia that accompanies Irp2 deletion. This result further supports a role for IRP2 in mediating the erythropoietic phenotypes observed in Fbxl5 heterozygotes.

DISCUSSION

FBXL5 plays a critical role in the maintenance of cellular iron homeostasis (8, 9). Here we show that previous in vitro findings extend to a required in vivo role for FBXL5. Global inactivation of the Fbxl5 gene results in embryonic lethality, with growth defects readily apparent prior to day E9 despite normal placentaion, gastrulation, and cardiovascular development. Cells derived from FBXL5-null embryos are unable to sufficiently degrade IRP2 when incubated in the presence of excess iron and are apt to import iron that cannot be appropriately sequestered within the limiting amount of ferritin available. Using an independently generated Fbxl5 knock-out mouse, it was recently shown that FBXL5-null embryos accumulate excess ferrous iron and are exposed to damaging levels of oxidative stress (15). Although many E3 ligases ubiquitinate multiple substrates (29), simultaneous inactivation of both the Fbxl5 and Irp2 genes is sufficient to rescue embryonic lethality, also consistent with the prior report (15).

Despite sharing >70% identity (30), the mechanisms by which the IRE-binding activity of IRP1 and IRP2 are inactivated in iron-replete cells largely differ (31). Both FBXL5-mediated degradation (8, 9) and IRE recognition of IRP1 are inactivated by iron upon insertion of an iron-sulfur cluster (31–33), although the contribution of protein degradation to in vivo
FBXL5 Regulates Iron Metabolism in Vivo

IRP1 regulation is not completely understood (8, 9, 32, 34). In extracts from both mouse embryonic cells and the duodenum, IRE binding by IRP1 is enhanced upon partial or complete FBXL5 inactivation (Fig. 4D), even though total IRP1 protein accumulation remains relatively constant (Fig. 4C). However, if only a small percentage of IRP1 protein were competent for RNA binding, as is the case in liver and perhaps other tissues (23, 32, 35), significant changes in the accumulation of this apo form may be difficult to detect over the background of bulk holo-IRP1. Alternatively, iron-sulfur cluster assembly on IRP1

FIGURE 4. Iron metabolism genes are aberrantly regulated in FBXL5GT/GT mouse embryonic cells. A, mouse embryonic cells genotypes assessed by PCR assay (Fig. 2), B, relative TR1 mRNA accumulation measured by quantitative PCR (supplemental Table S3). Each column represents the average of three experiments ± S.D. C, immunoblot analysis of IRPs and their targets (supplemental Table S4) from mouse embryonic cells treated with FAC and DFO. Actin levels were assessed as a loading control. D, assessment of RNA binding activity from mouse embryonic cells treated with ferric ammonium citrate (FAC) or deferoxamine mesylate (DFO).

TABLE 2
Genotypes of mice produced from Fbxl5+/GT, Irp2+/− intercrosses

| No. of animals | Fbxl5+/+ | Fbxl5+/GT | Fbxl5GT/GT | Total |
|----------------|---------|------------|------------|-------|
| Fbxl5+/+       | 11      | 20         | 7          | 38    |
| Fbxl5+/GT      | 24      | 36         | 7          | 67    |
| Fbxl5GT/GT     | 0       | 0          | 5          | 5     |
| Total          | 35      | 56         | 19         | 110   |

TABLE 3
Genotypes of mice produced from Fbxl5+/GT, Irp1+/- intercrosses

| No. of animals | Fbxl5+/+ | Fbxl5+/GT | Fbxl5GT/GT | Total |
|----------------|---------|------------|------------|-------|
| Fbxl5+/+       | 37      | 54         | 21         | 112   |
| Fbxl5+/GT      | 39      | 124        | 37         | 200   |
| Fbxl5GT/GT     | 0       | 0          | 0          | 0     |
| Total          | 76      | 178        | 58         | 312   |

TABLE 4
Hematological parameters and serum values from Fbxl5+/+ and Fbxl5GT/GT mice fed either an iron sufficient or low iron diet

| Values expressed as mean ± S.D. of 3 replicates. In the rows, differences between paired values denoted by superscript letters are statistically significant as determined by t test. |

| Fbxl5+/+ | Fbxl5GT/GT |
|----------|------------|
| Fe (μg of tissue) | Fe (μg of tissue) |
| Sufficient Fe | Low Fe | Sufficient Fe | Low Fe |
| RBC (M/μl) | 9.9 ± 0.3 | 7.0 ± 0.5 | 8.9 ± 1.1 | 8.7 ± 0.2 |
| Hematocrit (%) | 51.4 ± 1.3 | 28.3 ± 1.8 | 49 ± 2 | 45 ± 3 |
| Hemoglobin (g/dl) | 15.2 ± 0.3 | 9.2 ± 0.6 | 15 ± 1 | 12.6 ± 0.3 |
| Iron (μg/dl) | 285 ± 36 | 57 ± 13 | 298 ± 48 | 51 ± 10 |
| TIBC (μg/dl) | 343 ± 20 | 257 ± 13 | 333 ± 28 | 579 ± 14 |

* p < 0.005.
** p < 0.003.
*** p < 0.001.

FIGURE 5. Total iron concentration in Fbxl5+/+ and Fbxl5GT/GT mice. The iron (Fe) content in perfused livers from Fbxl5+/+ and Fbxl5GT/GT mice fed either an iron-sufficient (S; 5 ppm) or a low (L; 5 ppm) iron diet for 3 weeks was determined by inductively coupled plasma mass spectrometry. Each column represents the average of 4 mice ± S.D. and statistically significant differences were determined by t test: ***, p < 0.001; **, p < 0.003; *, p < 0.05.
may be compromised in FBXL5-deficient cells as a result of increased oxidative stress (31, 36) or some other mechanism.

Nevertheless, although IRP1 and IRP2 are thought to bind many of the same mRNA targets in vivo and are thought to be largely redundant (16, 17, 22, 23, 37), simultaneous inactivation of the IRP1 gene product did not rescue the embryonic lethality in FXBL5-null mice (Table 3). If rescue of embryonic lethality upon IRP2 ablation were solely due to a reduction in total IRE-binding capacity, IRP1 inactivation might have been expected to result in similar compensatory changes given that IRP1 and IRP2 have comparable RNA binding capacities in both the mouse embryonic cells and duodenum. Although indicating that IRP1 and IRP2 have distinct physiological roles, these results cannot distinguish between possible underlying mechanisms including IRP2-selective IRE targets (38), differences in temporal and spatial expression patterns (Fig. 1), or differential IRP responsiveness in the context of stresses such as hypoxia (39).

In addition to the similar results observed upon global FBXL5 inactivation, Moroishi and colleagues (15) also constructed a mouse model in which FBXL5 was selectively ablated in the liver. Although viable, these mice exhibited both cell-autonomous effects, including hallmarks of liver damage, and systemic effects, consisting of increased serum iron levels likely due to decreased hepcidin expression in the liver. When challenged with a high iron diet, severe iron overload was restricted to the liver and proved fatal within a day (15). In this context, the distinct phenotype we observed with the heterozygous Fbxl5/H11001/GT mice was of particular interest in at least two key respects: 1) the phenotype was manifested with a low iron diet and 2) the phenotype suggests that the intestine has distinct iron-sensing characteristics that may underlie a more privileged role in the maintenance of systemic iron homeostasis in response to iron deficiency than previously appreciated.
**FBXL5 Regulates Iron Metabolism in Vivo**

Fbxl5 heterozygosity had no observed effect on the behavior of most tissues we examined, including the liver, either from animals fed a low iron (5 ppm) or iron-sufficient (50 ppm) diet (Fig. 7B and supplemental Table S2). We also saw no difference in animals fed standard chow that contains excess iron (250 ppm, data not shown), although it remains possible that new phenotypes could emerge in the Fbxl5+/GT mice when challenged with supraphysiological dietary iron. Nevertheless, Fbxl5+/GT mice fed a low iron diet were more effective than their wild type counterparts. In livers of mice replete with iron, there is a set point in different cell types. Despite sharing this same FBXL5 iron-sensor, the iron “set point” of the duodenum appears distinct from most other tissues, conferring the capacity to influence systemic iron homeostasis in a previously unappreciated manner. It will be of great interest to investigate those responsible factors that work in conjunction with the FBXL5 iron sensor to establish the homeostatic iron set point in different cell types.

**Acknowledgments**—We thank Sean McCormick and Paul A. Lindahl for inductively coupled plasma mass spectrometry analysis, Sandi Jo Estill, James Richardson, and the UT Southwest Molecular Pathology Core for assistance with the animal studies, C. Nizzi and N. Keeler for statistical analysis, and members of the Bruick lab for helpful advice. This investigation was conducted in a facility constructed with support from the Research Facilities Improvement Program Grant C06 RR 15437-01 from the National Center for Research Resources.

**REFERENCES**

1. Camaschella, C., and Poggioli, E. (2011) Inherited disorders of iron metabolism. *Curr. Opin. Pediatr.* 23, 14–20

2. Grosbois, B., Decaux, O., Cador, B., Cazalets, C., and Jego, P. (2005) Human iron deficiency. *Bull. Acad. Natl. Med.* 189, 1649–1663; discussion 1663–1664

3. Rouault, T. A. (2006) The role of iron regulatory proteins in mammalian iron homeostasis and disease. *Nat. Chem. Biol.* 2, 406–414

4. Hentze, M. W., Muckenthaler, M. U., Galy, B., and Camaschella, C. (2010) Two to tango. Regulation of mammalian iron metabolism. *Cell* 142, 24–38

5. Thomson, A. M., Rogers, J. T., and Leedman, P. J. (1999) Iron-regulatory proteins, iron-responsive elements and ferritin mRNA translation. *Int. J. Biochem. Cell Biol.* 31, 1139–1152

6. Koehler, D. M., Casey, J. L., Hentze, M. W., Gerhardt, E. M., Chan, L. N., Klausner, R. D., and Harford, J. B. (1989) A cytosolic protein binds to structural elements within the iron regulatory region of the transferrin receptor mRNA. *Proc. Natl. Acad. Sci. U.S.A.* 86, 3574–3578

7. Guo, R., Phillips, J. D., Yu, Y., and Leibold, E. A. (1995) Iron regulates the intracellular degradation of iron regulatory protein 2 by the proteasome. *J. Biol. Chem.* 270, 21645–21651

8. Salahudeen, A. A., Thompson, J. W., Ruiz, J. C., Ma, H. W., Kinch, L. N., Li, Q., Grishin, N. V., and Bruick, R. K. (2009) An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis. *Science.* 326, 722–726

9. Vasishth, A. A., Zumbrengen, K. B., Huang, X., Powers, D. N., Durazo, A., Sun, D., Bhaskaran, N., Persson, A., Uhlen, M., Sallengt, O., Spruck, C., Leibold, E. A., and Wohlschlegel, J. A. (2009) Control of iron homeostasis by an iron-regulated ubiquitin ligase. *Science.* 326, 718–721

10. Shu, C., Sung, M. W., Stewart, M. D., Igunomenma, T. I., Tan, X., and Li, P. (2012) The structural basis of iron sensing by the human F-box protein FBXL5. *Chembiochem.* 788–791

11. Thompson, J. W., Salahudeen, A. A., Chollangi, S., Ruiz, J. C., Brautigam, C. A., Makris, T. M., Lipscomb, J. D., Tomchick, D. R., and Bruick, R. K. (2012) Structural and molecular characterization of iron-sensing hemerythrin-like domain within F-box and leucine-rich repeat protein 5 (FBXL5). *J. Biol. Chem.* 287, 7357–7365

12. Chollangi, S., Thompson, J. W., Ruiz, J. C., Gardner, K. H., and Bruick, R. K. (2012) Hemerythrin-like domain within F-box and leucine-rich repeat protein 5 (FBXL5) communicates cellular iron and oxygen availability by distinct mechanisms. *J. Biol. Chem.* 287, 23710–23717

13. Thompson, J. W., and Bruick, R. K. (2012) Protein degradation and iron homeostasis. *Biochim. Biophys. Acta.* 1823, 1484–1490

14. Salahudeen, A. A., and Bruick, R. K. (2009) Maintaining mammalian iron and oxygen homeostasis. Sensors, regulation, and cross-talk. *Ann. N.Y. Acad. Sci.* 1177, 30–38

---

5 J. C. Ruiz and R. K. Bruick, unpublished data.
FBXL5 Regulates Iron Metabolism in Vivo

15. Moroiishi, T., Nishiyama, M., Takeda, Y., Iwai, K., and Nakayama, K. I. (2011) The FBXL5-IRP2 axis is integral to control of iron metabolism in vivo. Cell Metab. 14, 339–351
16. Galy, B., Ferring, D., and Hentze, M. W. (2005) Generation of conditional alleles of the murine iron regulatory protein (IRP)-1 and -2 genes. Genesis 43, 181–188
17. Galy, B., Ferring, D., Minana, B., Bell, O., Janser, H. G., Muckenholzer, M., Schümann, K., and Hentze, M. W. (2005) Altered body iron distribution and microcytosis in mice deficient in iron regulatory protein 2 (IRP2). Blood 106, 2580–2589
18. Lee, J., Petris, M. J., and Thiele, D. J. (2002) Characterization of mouse embryonic cells deficient in the Ctrl1 high affinity copper transporter. Identification of a Ctrl1-independent copper transport system. J. Biol. Chem. 277, 40253–40259
19. Popescu, B. F., Robinson, C. A., Rajput, A., Rajput, A. H., Harder, S. L., and Nichol, H. (2009) Iron, copper, and zinc distribution of the cerebellum. Cerebellum. 8, 74–79
20. Guo, B., Brown, F. M., Phillips, J. D., Yu, Y., and Leibold, E. A. (1995) Characterization and expression of iron regulatory protein 2 (IRP2). Presence of multiple IRP2 transcripts regulated by intracellular iron levels. J. Biol. Chem. 270, 16529–16535
21. Galy, B., Ferring, D., Benesova, M., Benes, V., and Hentze, M. W. (2004) Targeted mutagenesis of the murine IRP1 and IRP2 genes reveals context-dependent RNA processing differences in vivo. RNA 10, 1019–1025
22. LaVaute, T., Smith, S., Cooperman, S., Iwai, K., Land, W., Meyron-Holtz, E., Drake, S. K., Miller, G., Abu-Asab, M., Tsokos, M., Switzer, R., 3rd, Grinberg, A., Love, P., Tresser, N., and Rouault, T. A. (2001) Targeted deletion of the gene encoding iron regulatory protein-2 causes misregulation of iron metabolism and neurodegenerative disease in mice. Nat. Genet. 27, 209–214
23. Meyron-Holtz, E. G., Ghosh, M. C., Iwai, K., LaVaute, T., Brazzolotto, X., Berger, U. V., Land, W., Ollivierre-Wilson, H., Grinberg, A., Love, P., and Rouault, T. A. (2004) Genetic ablations of iron regulatory proteins 1 and 2 reveal why iron regulatory protein 2 dominates iron homeostasis. EMBO J. 23, 386–395
24. Ross, K. L., and Eisenstein, R. S. (2002) Iron deficiency decreases mitochondrial aconitase abundance and citrate concentration without affecting tricarboxylic acid cycle capacity in rat liver. J. Nutr. 132, 643–651
25. Galy, B., Ferring-Appel, D., Kaden, S., Gröne, H. J., and Hentze, M. W. (2008) Iron regulatory proteins are essential for intestinal function and control key iron absorption molecules in the duodenum. Cell Metab. 7, 79–85
26. Mastrogiannaki, M., Matakas, P., Keith, B., Simon, M. C., Vaulont, S., and Peyssonnaux, C. (2009) HIF-2α, but not HIF-1α, promotes iron absorption in mice. J. Clin. Invest. 119, 1159–1166
27. Ganz, T., and Nemeth, E. (2012) Hepcidin and iron homeostasis. Bioclin. Biophys. Acta. 1823, 1434–1443
28. De Domenico, I., Ward, D. M., and Kaplan, J. (2011) Hepcidin and ferroportin. The new players in iron metabolism. Semin. Liver Dis. 31, 272–279
29. Ho, M. S., Ou, C., Chan, Y. R., Chien, C. T., and Pi, H. (2008) The utility of F-box for protein destruction. Cell Mol. Life Sci. 65, 1977–2000
30. Pantopoulos, K. (2004) Iron metabolism and the IRE/IRP regulatory system. An update. Annu. N.Y. Acad. Sci. 1012, 1–13
31. Eisenstein, R. S. (2000) Iron regulatory proteins and the molecular control of mammalian iron metabolism. Annu. Rev. Nutr. 20, 627–662
32. Clarke, S. L., Vasanthakumar, A., Anderson, S. A., Pondarré, C., Koh, C. M., Deck, K. M., Pitula, J. S., Epstein, C. J., Fleming, M. D., and Eisenstein, R. S. (2006) Iron-responsive degradation of iron-regulatory protein 1 does not require the Fe-S cluster. EMBO J. 25, 544–553
33. Pantopoulos, K., Gray, N. K., and Hentze, M. W. (1995) Differential regulation of two related RNA-binding proteins, iron regulatory protein (IRP), and IRPB. RNA 1, 155–163
34. Wang, J., Fillebeen, C., Chen, G., Biederbick, A., Lill, R., and Pantopoulos, K. (2007) Iron-dependent degradation of apo-IRP1 by the ubiquitin-proteasome pathway. Mol. Cell. Biol. 27, 2423–2430
35. Chen, O. S., Schalinske, K. L., and Eisenstein, R. S. (1997) Dietary iron intake modulates the activity of iron regulatory proteins and the abundance of ferritin and mitochondrial aconitase in rat liver. J. Nutr. 127, 238–248
36. Mueller, S., Pantopoulos, K., Hübner, C. A., Stremmel, W., and Hentze, M. W. (2001) IRP1 activation by extracellular oxidative stress in the perfused rat liver. J. Biol. Chem. 276, 23192–23196
37. Smith, S. R., Ghosh, M. C., Ollivierre-Wilson, H., Hang Tong, W., and Rouault, T. A. (2006) Complete loss of iron regulatory proteins 1 and 2 prevents viability of murine zygotes beyond the blastocyst stage of embryonic development. Blood Cells Mol. Dis. 36, 283–287
38. Butt, J., Kim, H. Y., Basilion, J. P., Cohen, S., Iwai, K., Philpott, C. C., Altschul, S., Klausner, R. D., and Rouault, T. A. (1996) Differences in the RNA binding sites of iron regulatory proteins and potential target diversity. Proc. Natl. Acad. Sci. U.S.A. 93, 4435–4439
39. Schneider, B. D., and Leibold, E. A. (2003) Effects of iron regulatory protein regulation on iron homeostasis during hypoxia. Blood 102, 3404–3411
40. Leibold, E. A., and Guo, B. (1992) Iron-dependent regulation of ferritin and transferrin receptor expression by the iron-responsive element binding protein. Annu. Rev. Nutr. 12, 345–368
41. Ponka, P., and Lok, C. N. (1999) The transferrin receptor: role in health and disease. Int. J. Biochem. Cell Biol. 31, 1111–1137