Iron Regulatory Protein-1 Protects against Mitoferrin-1-deficient Porphyria*

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Background: Heme and [Fe-S] cluster assembly are tightly regulated processes that require mitochondrial iron.

Results: Loss of mitochondrial iron activates the [Fe-S]-dependent RNA-binding activity of IRP1 that inhibits protoporphyrin biosynthesis.

Conclusion: IRP1 forms a critical feedback mechanism, preventing protoporphyrin accumulation under limiting mitochondrial iron conditions.

Significance: This study provides evidence linking heme biogenesis to that of [Fe-S] clusters synthesis.

Mitochondrial iron is essential for the biosynthesis of heme and iron-sulfur ([Fe-S]) clusters in mammalian cells. In developing erythrocytes, iron is imported into the mitochondria by MFRN1 (mitoferrin-1, SLC25A37). Although loss of MFRN1 in zebrafish and mice leads to profound anemia, mutant animals showed no overt signs of porphyria, suggesting that mitochondrial iron deficiency does not result in an accumulation of protoporphyrins. Here, we developed a gene trap model to provide in vitro and in vivo evidence that iron regulatory protein-1 (IRP1) inhibits protoporphyrin accumulation. Mfrn1+/-+/Mfrn1gt/gt; Irp1-/-/- erythroid cells exhibit a significant increase in protoporphyrin levels. IRP1 attenuates protoporphyrin biosynthesis by binding to the 5' iron response element (IRE) of alas2 mRNA, inhibiting its translation. Ectopic expression of alas2 harboring a mutant IRE, preventing IRP1 binding, in Mfrn1gt/gt cells mimics Irp1 deficiency. Together, our data support a model whereby impaired mitochondrial [Fe-S] cluster biogenesis in Mfrn1gt/gt cells results in elevated IRP1 RNA-binding that attenuates ALAS2 mRNA translation and protoporphyrin accumulation.

Iron is an essential element that is incorporated into prosthetic groups that function in a multitude of biochemical processes. Two of these iron-containing prosthetic groups are heme and iron-sulfur ([Fe-S]) clusters. Heme is a moiety with diverse functions in many cell types and is an integral component of hemoglobin in erythroid cells (1). [Fe-S] clusters are requisite cofactors for many enzymes, including aconitases and succinate dehydrogenase (2, 3). Although functionally distinct, assembly of both cofactors require mitochondrial function and mitochondrial iron assimilation (4).

Iron is imported into mitochondria via two transporters: mitoferrin-1 (MFRN1, SLC25A37) and mitoferrin-2 (MFRN2, SLC25A28). MFRN2 is expressed ubiquitously, whereas MFRN1 is specifically induced in differentiating erythroid cells (5–7). Previous work has shown that loss of Mfrn1 in frascati zebrafish and mice disrupts heme and [Fe-S] cluster synthesis, owing to a severe reduction in mitochondrial iron in erythroid progenitors (7, 8). However, neither Mfrn1-/-/- mice nor...
zebrafish, frascati embryos deficient in Mfrn1 develop porphyria. In fact, conditional deletion of Mfrn1 in hepatocytes only causes porphyria and hepatobiliary stasis when the animals are fed a δ-aminolevulinic acid (ALA)-rich diet.

Recent evidence suggests that cross-talk between [Fe-S] cluster assembly and protoporphyrin synthesis pathways may explain the absence of porphyria in Mfrn1-/- animals. Cellular iron homeostasis is largely regulated by iron regulatory proteins-1 (IRP1) and -2 (IRP2) that can directly influence protein expression via two mechanisms (10–12). IRPs can bind to iron responsive elements (IREs) in the 5′-UTRs of mRNAs, blocking translation initiation. Alternatively, mRNAs with IRE(s) in the 3′-UTR have increased stability when bound by IRPs. As a result, this latter class of transcripts will have increased accumulation when IRPs are activated by iron deficiency. Although IRP1 and IRP2 exhibit some functional redundancy and can be regulated by common mechanisms (13–15), IRP1 is unique in that its expression is negatively regulated by [Fe-S] cluster biogenesis. Insertion of an [Fe-S] cluster into IRP1 converts IRP1 from an IRE-RNA binding protein to cytosolic aconitase (ACO1). A decline in [Fe-S] cluster synthesis would, therefore, trigger increased IRP1 RNA-binding activity (16–19).

One mRNA harboring a 5′-IRE is erythroid-specific aminolevulinate synthase 2 (Alas2), which encodes the enzyme that catalyzes the first step in the heme biosynthetic pathway (20, 21). Wingert and colleagues (9) previously demonstrated that inhibition of glutaredoxin 5 (Glrx5), a gene required for mitochondrial [Fe-S] cluster assembly, resulted in anemia in zebrafish via increased IRP1-mediated inhibition of ALAS2 translation. Thus, a reduction in [Fe-S] cluster assembly in response to iron deficiency can feedback to attenuate the very proximal step of heme assembly (9, 22). Here, we examined whether loss of Mfrn1 can also trigger IRP1 activity in erythroid cells. We generated a model to show that in the absence of MFRN1, IRP1 blocks ALAS2 translation, preventing the accumulation of protoporphyrins in vitro and in vivo. Loss of IRP1 or the 5′-IRE in Alas2 mRNA resulted in porphyria. Our work indicates that IRP1 functions as a critical link coupling heme and [Fe-S] cluster biosynthetic pathways and that in the absence of MFRN1-mediated mitochondrial iron import, IRP1 protects erythroid cells against porphyria.

EXPERIMENTAL PROCEDURES

Cell Culture—Friend murine erythroleukemia cells were cultured and differentiated as described previously (23). Mouse embryonic stem (mES) cells containing a gene trap insertion in Mfrn1 (Mfrn1*/* cell line, XB454, derived from strain 129P2) were obtained from the University of California, San Francisco BayGenomics (24). Insertion of the gene trap vector (containing the coding sequence for β-geo) into Mfrn1 was verified by direct sequencing of cDNA obtained by 5′-rapid amplification of cDNA ends (25). mES cells were maintained on gelatin-coated 100-mm dishes in mES cell medium (7). Null Mfrn1*/* mES clones were derived using G418 selection as described previously (7).

Mouse Blastocyst Injections and Knock-out Mouse Creation—ES cells were injected into C57BL/6 blastocysts and transferred to pseudopregnant C57BL/6 hosts using standard techniques (26). Male chimeras identified by agouti coat color were mated to C57BL/6 females to generate heterozygotes for interbreeding to produce homozygous knock-out progeny. Progeny were genotyped as described below. All mice were maintained at The Jackson Laboratory in a climate-controlled room with a 12-h light cycle and provided acidified water and NIH 5K52 chow ad libitum. The Jackson Laboratory Animal Care and Use Committee approved all protocols. In accordance with the International Committee on Standardized Genetic Nomenclature for Mice, the official genetic designation for this Mfrn1 strain is B6.129P2-Slc25a37<gt(XB454)Byg>/Lip.

Mouse Genotyping—for analysis by Southern blotting, HindIII-digested mouse genomic or ES cell DNA was transferred onto Hybond Nylon (Amersham Biosciences), and probed with random primed labeled [α-32P]dCTP (3000 Ci/mmol; New England Nuclear) murine Mfrn1 or Escherichia coli LacZ cDNA under standard hybridization and washing conditions. Genotyping by PCR on genomic DNA was performed with the following primer pairs and resolved on a 5% native polyacrylamide gel:

- The following were used in this work for the wild-type allele (269-bp fragment), 5′-GCTTATGGAAAGGACCCAGC-3′ (F1 primer) and 5′-ACAAGGAAGGCAAGACTGTAC-3′ (R1 primer); mutant gt allele (350-bp fragment) as shown above (F1 primer) and 5′-GCGGATACGTCTCCATCATC-3′ (R2 primer); LacZ, 5′-TTATCGTGACGGGTTATGTCG-3′ (F primer) and 5′-GGCCCAGCGCGAGAATAATC-3′ (R1 primer). Primers for Irp1 mutant mice were as described previously (27).

LacZ Staining—β-Galactosidase staining of mouse embryos was performed as described previously (7).

Mixed Chimera Analysis—Mfrn1*/* ES cell clones were injected into C57BL/6 mouse blastocysts and transferred to C57BL/6 recipients. The degree of mosaicism in offspring was estimated by coat color. The contributions of donor (129P2) versus recipient (C57BL/6) ES cells to the red cell and leukocyte compartments were determined by cellulose acetate electrophoresis to detect strain-specific hemoglobin and glucose-6-isomerase alleles as described previously (28) in three chimeric mice (one female with moderate chimerism, two males with moderate to high chimerism) at 10 weeks of age.

o-Dianisidine Staining, CFU-E, and Cytospin Assays—Wild-type E14 and Mfrn1 null ES clones were generally split 1 day after thawing with 106 mES cells plated onto gelatinized 100-mm plates in mES media as described previously (7). The following day, mES cells were maintained in “switch media” (7). Two days after the split, the cells were collected by trypsinization and replated on untreated 100-mm dishes at a density of 3 × 105 cells/ml (total 6 × 104 cells) in embryoid body (EB) media with murine VEGF (10 ng/ml) on days 1 and 5. ALA (0.25

12 The abbreviations used are: ALA, δ-aminolevulinic acid; ES, embryonic stem; PPIX, protoporphyrin IX; [Fe-S], iron-sulfur; IRE, iron-response element; IRP1, iron regulatory protein-1; Alas2, aminolevulinate synthase 2; E1, embryonic day 1; EB, embryoid body; FECH, ferrochelatase.
pressure liquid chromatography system that consisted of a 996 Photodiode Array Detector, a 474 scanning fluorescence detector, an Alliance HT 2795 separations module, and a μBondapak C18 3.9 × 300 mm column with a guard column attached. Bovine Type 1 crystalline heme (Sigma, H-2250) or Protoporphyrin IX (Sigma, P-8293) was used as the standard. The absorbance values were normalized by cell number or protein concentration. EBs were supplemented with 0.25 mm ALA (Sigma) for 24 h prior to cell harvest and biochemical analysis.

**Western Analysis—**Immunoblotting was performed as described previously (32). Custom, affinity-purified anti-mouse MFRN1 rabbit polyclonal antibody was generated using the following peptide: C-HESHVQEVSHTSPT (Genemed Synthesis, San Antonio, TX). Goat polyclonal anti-HSP60 (K-19) was purchased from Santa Cruz Biotechnology. Anti-ALAS2 was a generous gift of Dr. Makoto Murakami (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Quantitative densitometry was performed using ImageJ software (NIH, Bethesda, MD) and normalized to HSP60 expression levels.

**Zebrafish alas2 Expression Constructs—**Wild-type zebrafish alas2 cDNA with the 5'-IRE (+IRE; GenBank™ accession no. NM_131682.2) was subcloned into the BamHI/Xhol sites of pCS2+ as described previously (33). The alas2 cDNA lacking the 5'-IRE (−IRE; 5'-GTTCCGTCTCAGTGACGGTGACAC-3') was amplified from this construct by PCR and subcloned into pCS2+. The alas2 cDNA lacking the 5'-IRE with a premature nonsense mutation at amino acid residue 26 (Y62X), (−IRE/Stop), was generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). These clones were subsequently subcloned into MluI/Xhol sites in the pMMP-HA-ires-eGFP retroviral vector (34) for subsequent lentivirus production.

**Lentivirus Infection—**Lentivirus production and infection was performed as described previously (35). Briefly, 293T cells were plated at 6 × 10^6 cells per 100 mm dish and incubated overnight. Cells were transduced with retrovectors together with psPax2 and pMD2G. Forty-eight hours after transfection, the supernatants were collected and ultra-centrifuged at 28,000 rpm for 2 h. Supernatants from three to ten 10-cm dishes were concentrated into 100 μl of PBS. Murine ES cells in 24-well plates were transduced using various amounts of concentrated virus supplemented with 8 μg/ml polybrene. After 24 h, the cells were changed back to standard medium. Three days after transduction, the cells were dissociated by 0.05% trypsin and sorted by FACS to obtain the GFP-positive cells.

**Mouse Matings between Mfrn1 and Irp1 Mice—**Mfrn1+/+ mice were crossed with Irp1−/− mice (27, 36). Mfrn1+/+Irp1+/+ offspring were intercrossed to produce Mfrn1+/+Irp1+/+ and Mfrn1+/−Irp1+/+ sires and dams. Embryos from mating Mfrn1+/−Irp1−/− sires and dams, and embryos from mating Mfrn1+/+Irp1−/− sires and dams were harvested at E10.5. After removing heads for genotyping, the remaining tissue was homogenized in 20 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) pH 7.5, buffer for heme and protoporphyrin analysis. All use of animals met the requirements of the University of Wisconsin-Madison Institutional Animal Care and Use Committee.
Mitoferrin-1 and Cellular [Fe-S] Homeostasis

**RESULTS**

*Mfrn1*<sup>gt/gt</sup> ES Cells Are Defective at Erythroid Differentiation—*Mfrn1* has been studied previously in mice using tissue-specific Cre/Lox technology (8). Although global loss of *Mfrn1* in this model leads to embryonic lethality due to a profound embryonic anemia, a cell-autonomous role for *MFRN1* in adult hematopoiesis has never been described. Thus, to address this and to study the underlying molecular mechanisms, we developed a model where we could manipulate *MFRN1* function in vitro and in vivo. Gene trap mutagenesis was used to disrupt the *Mfrn1* locus between introns 1 and 2 (Fig. 1A). Proper integration in murine ES cells was confirmed using PCR and Southern blot analysis (Fig. 1, B and C). Wild-type (*Mfrn1<sup>+/+</sup>), heterozygote (*Mfrn1<sup>+/gt</sup>), and null (*Mfrn1<sup>gt/gt</sup>) ES cells were then cultured in vitro under conditions that facilitate primitive or definitive erythropoiesis. *Mfrn1<sup>gt/gt</sup>* cells, lacking *MFRN1* protein expression, showed defective hemoglobinization, failed to form both primitive and definitive CFU-E colonies and were morphologically distinct from wild-type differentiated erythroid cells (Fig. 2, A–E). Semi-quantitative RT-PCR analysis also revealed that expression of erythroid markers, α-globin, βH1-globin, β-major-globin, and *Alas2* were diminished in differentiating *Mfrn1<sup>gt/gt</sup>* cells compared with wild-type (Fig. 2F), indicating that erythroid maturation in mutant cells was blocked. In contrast, the early erythroid marker, Gata-1, and ubiquitously expressed genes such as *Mfn2*, *Hprt*, and *GAPDH* were normal compared with wild-type and *Mfrn1<sup>+/+</sup>* cells. Total intracellular iron, mitochondrial iron, and intracellular heme content were all decreased without protoporphyrin accumulation (Fig. 2, G–I, and data not shown). We also examined both endogenous aconitase 1 activity and IRP1 RNA-binding (data not shown). However, we were unable to detect significant changes using either assays with this embryoid culture system. We believe that this is due to a heterogeneous population of cells in our differentiation cultures. Under optimal conditions, only 30% of ES cells undergo proper erythroid maturation (Fig. 2C). RNA-binding and aconitase assays may lack sufficient sensitivity to detect moderate changes.

*Mfrn1* Is Required for Primitive and Definitive Erythropoiesis in Vivo—We next examined whether the primitive erythropoietic defects in ES cells can be recapitulated in vivo by injecting wild-type or *Mfrn1<sup>gt/gt</sup>* ES cells into early-stage embryos and generating mixed-chimeric mice. *Mfrn1<sup>+/gt</sup>* and *Mfrn1<sup>gt/gt</sup>* offspring were obtained following germ line transmission of the mutant allele. Surrogate lacZ-staining for *Mfrn1* expression in heterozygote and gene-trapped embryos detected staining in the yolk sac blood islands and fetal liver at E8.5 and E11.5, respectively (Fig. 3, A and B). At E11.5, *Mfrn1<sup>gt/gt</sup>* embryos were severely anemic compared with wild-type littermates (Fig. 3B), and no mutant embryos survived past E11.5 (Table 1). *Mfrn1* is also required *in vivo* for definitive erythropoiesis. In mixed chimeras, only the host recipient C57BL/6J-derived hemoglobin isoform is detected by cellulose acetate electrophoresis of adult red cells (Fig. 3C). In contrast, genetic contributions from both recipient and donor (mutant, 129P2) glucose-6-isomerase isoforms were detected in leukocytes, indicating the essential role of *MFRN1* in the development of the definitive erythroid lineage and its nonessential role in non-erythroid lineages in adult mice. These data confirm that our gene trap model provides an accurate representation of *MFRN1* function *in vitro* and *in vivo*.

IRP1 Attenuates Protoporphyrin Production—Because disruption of [Fe-S] cluster synthesis has previously been linked to *Alas2* mRNA translational repression by IRP1 (9), we next used our model to ask whether ALAS2 translation had any influence on protoporphyrin production in the absence of *MFRN1*. *Mfrn1<sup>gt/gt</sup>* EBs had lower expression of ALAS2 protein compared with *Mfrn1<sup>+/+</sup>* EBs (Fig. 4A), suggesting that ALAS2 translation is indeed compromised in the absence of *MFRN1*. To examine the contribution of the 5′-IRE in *Mfrn1* regulation of ALAS2 function and expression, *Mfrn1<sup>gt/gt</sup>* ES cells were transduced with lentivirus expressing either empty vector (mock) or zebrafish *alas2* cDNA constructs harboring a wild-type IRE (WT) or mutant IRE (–IRE) in the 5′-UTR. An additional mutant *alas2* cDNA with a premature transcriptional stop codon (–IRE/Stop) was also included as a negative control (Fig. 4, B and C). These cells were then differentiated into EBs. As expected, ectopic expression of WT-*alas2* resulted in a modest increase in protoporphyrin levels above mock-transduced control cells (Fig. 4, C and D). However, there was almost a 2-fold increase in protoporphyrin levels in cells expressing the −IRE mutant construct due to increased ALAS2 expression (Fig. 4, C and D). The level of protoporphyrin IX (PPIX) dropped to basal levels in cells expressing the double mutant *alas2* construct (–IRE/Stop) (Fig. 4, C and D). Furthermore, *Mfrn1<sup>gt/gt</sup>* EBs supplemented with exogenous ALA to by-pass the block in protoporphyrin synthesis resulting from diminished ALAS2 protein expression fully restored PPIX but not heme production to levels comparable with *Mfrn1<sup>+/+</sup>* cells (Fig. 4E). Thus, inhibition of protoporphyrin synthesis through action of the 5′-IRE in *Alas2* mRNA is substantially increased when mitochondrial
FIGURE 2. Defect in hemoglobinization and maturation of primitive and definitive erythroblasts with Mfn1 deficiency. A, wild-type (+/+), heterozygote (+/gt), or Mfn1 gene-trap (gt/gt) ES cells undergoing primitive erythropoiesis were lysed and immunoblotted with anti-MFRN1 or anti-HSP60 antibodies, confirming the loss of Mfn1 protein expression in gt/gt cells. B and C, EBs cultured under primitive (EryP) or definitive (EryD) conditions were analyzed for hemoglobinization by o-dianisidine staining using phase contrast microscopy (B) and quantified, showing a defect in hemoglobinization (C). The arrows denote cell clusters. D, quantitation of erythroid colonies (CFU-E) that formed under primitive or definitive conditions, showing defects on erythroid colony formation. E, primitive or definitive wild-type and gene trap cells were stained with Wright-Giemsa dye, showing maturation arrest of erythroblasts from Mfrn1gt/gt ES cells. Arrows denote representative erythroid cells. Mfrn1+/H11001/H11001 cells are morphologically normal, whereas Mfrn1gt/gt cells have a larger nuclei. F, [α-32P]dCTP-radiolabeled semi-quantitative RT-PCR analysis was performed on wild-type (+/+), heterozygote (+/gt), or two mutant (gt/gt) Mfn1 ES cell clones undergoing primitive differentiation using the indicated gene-specific primers. G–I, total intracellular iron (Fe; G), mitochondrial iron (H), and heme (I) content of Mfrn1+/H11001/H11001 and Mfrn1gt/gt cells were examined, showing a consistent decrease in MFRN1-deficient embryoid bodies induced to undergo erythroid differentiation. Mean ± S.E. is shown, and all experiments were performed at least twice. IB, immunoblot.
[Fe-S] cluster assembly is compromised. A deficiency of MFRN1 should result in elevated cytosolic IRP1 RNA-binding activity that would attenuate translation of ALAS2 and lower PPIX production and its intracellular accumulation.

To address whether this was the case in vivo and to confirm whether IRP1 was responsible for negatively regulating ALAS2 expression, we examined heme and protoporphyrin concentrations in four cohorts of murine embryos, Mfrn1/H11001/H11001; Irp1/H11001/H11001, Mfrn1/H11001/H11001; Irp1/gt/gt, Mfrn1/H11001/H11001; Irp1/H11002/H11002, and Mfrn1/gt/gt; Irp1/H11002/H11002, at E10.5. Mfrn1 heterozygosity has little effect on heme levels in Irp1+/+ background (Fig. 4F). Protoporphyrin levels decreased, consistent with the notion that a reduction in mitochondrial iron import, while having little effect on heme biosynthesis, activates IRP1 due to a mitochondrial [Fe-S] cluster biogenesis defect (7) to inhibit ALAS2 translation. It should be noted that ferrochelatase (FECH), the terminal and rate-limiting enzyme in heme biosynthesis, is a [Fe-S] cluster protein and would also be influenced by the ability to form [Fe-S] clusters (37). However, the decreased FECH activity is unlikely to be responsible for the decline in protoporphyrin levels because diminished FECH function is predicted to exacerbate protoporphyrin accumulation. In contrast, in the absence of IRP1, loss of one copy of Mfrn1 causes a sharp rise in cellular protoporphyrin content, providing in vivo evidence that IRP1 prevents porphyria when MFRN1 is deficient. Taken together, our data strongly suggest that IRP1-mediated translational inhibition of ALAS2 in erythroid cells constitutes a feedback mechanism to attenuate protoporphyrin synthesis and accumulation when mitochondrial iron import through MFRN1 is compromised (Fig. 5).

**DISCUSSION**

The role of MFRN1 in primitive erythropoiesis and heme synthesis has been established in zebrafish and murine models (7, 8). Although these genetic studies have been highly informative, a cell-autonomous in vivo role for MFRN1 has never been described. It was also unclear as to why porphyria was never observed with MFRN1 deficiency. In this work, we developed a genetic and biochemical model to address these questions. We provide evidence that MFRN1 is essential for primitive and definitive erythropoiesis in vivo. These defects could be recapitulated in ES cell cultures, which allowed us to study the underlying

[FIGURE 3. MFRN1 is required for primitive and definitive erythropoiesis in vivo. A, LacZ staining of gene trap Mfrn1+/gtr E8.5 yolk sac, showing erythroid-specific expression in sites of hematopoiesis. B, gross morphology of E11.5 wild-type (+/+) or gene trap (gt/gt) embryos. The mutant embryos were also stained for lacZ and have a profound anemia due to a defect in primitive erythropoiesis. C, erythrocytes and leukocytes from recipient, donor, or chimeric mice with medium or light penetrance (agouti coat color) were isolated and lysed, and isoforms were resolved on starch-cellulose gel electrophoresis and Ponceaured stained for hemoglobin or glucose-6-isomerase (GPI). MFRN1-deficient donor-derived cells do not contribute to adult erythrocytes but do contribute to the myeloid lineage, indicating that MFRN1 is dispensable for myelopoiesis.]

**TABLE 1**

| Developmental stage | Total no. of embryos | Mfrn1+/+ | Mfrn1+/gt | Mfrn1gt/gt |
|---------------------|----------------------|---------|-----------|-----------|
| E8.5                | 30                   | 8       | 12        | 10        |
| E10.5               | 39                   | 10      | 19        | 10        |
| E11.5               | 9                    | 1       | 6         | 2         |
| E12.5               | 8                    | 4       | 4         | 0         |
| E14.5               | 24                   | 8       | 16        | 0         |
| E15.5               | 7                    | 2       | 5         | 0         |
molecular mechanisms that couple mitochondrial iron homeostasis, heme metabolism, and [Fe-S] assembly. We demonstrate that IRP1 is a critical negative regulator of protoporphyrin synthesis in Mfrn1gt/gt erythroid cells via IRE-mediated repression of ALAS2 translation (Fig. 5). Mfrn1 mutant embryos only developed porphyria in the absence of IRP1 (Fig. 4).

Although our studies have focused on IRP1, both IRP1 and IRP2 function in red blood cells to regulate ALAS2 translation (9, 17, 38). Studies of translational regulation of 5’-IRE-containing mRNA in a cell culture model of erythropoiesis found that unlike ferritin mRNA, which was almost totally translationally repressed, Alas2 mRNA was partially repressed with significant amounts of the mRNA present on polyribosomes as well as in the translationally silent ribonucleoprotein pool (39). Thus, an erythropoietic phenotype tied to altered ALAS2 expression is predicted in response to either a reduction or an increase in IRP RNA-binding activity. Irp2/H11002/H11002 mice develop erythropoietic protoporphyria resulting from translational derepression of Alas2 mRNA and subsequent overproduction of protoporphyrin intermediates (16, 17). In contrast, impairment of [Fe-S] cluster biogenesis when ABCB7 (40–42) or GLRX5 (9, 22) are disrupted specifically activates IRP1 RNA-
Mitoferrin-1 and Cellular [Fe-S] Homeostasis

FIGURE 5. Schematic model of mitochondrial heme and Fe/S cluster co-regulation. A, MFRN1 imports iron into the mitochondria for heme and [Fe-S] cluster biosynthesis. When mitochondrial iron is readily available, sufficient [Fe-S] cluster synthesis converts IRP1 from an IRE-binding protein to cytosolic aconitase. B, however, when mitochondrial iron stores are compromised by MFRN1 mutation, both heme and [Fe-S] cluster syntheses are disrupted. Deficient [Fe-S] cluster availability results in activation of IRP1 that subsequently binds to IREs on target transcripts. IRP1 binding to the 5′-IRE of Alas2 mRNA inhibits Alas2 translation, thereby forming a feedback loop that attenuates protoporphyrin accumulation in the absence of mitochondrial iron.

binding activity leading to repression of Alas2 mRNA translation. The observed increase in IRP1-mediated inhibition of Alas2 via its 5′-IRE suggests that [Fe-S] cluster insertion into AC01 is also compromised in the absence of MFRN1. Thus, by further supporting the concept that cytosolic [Fe-S] biogenesis is downstream of mitochondrial [Fe-S] formation that is fueled, in part, by the action of MFRN1, our findings also illustrate the unique role of IRP1 in linking mitochondrial iron metabolism with protoporphyrin synthesis in erythroid cells (9, 40, 43, 44). Taken together, these studies highlight differing roles of IRP1 and IRP2 in regulating Alas2 expression and mitochondrial function, whereas IRP2 may have a more general “housekeeping” role in iron metabolism.

Our work presented here has important clinical implications. They suggest that although Mfrn1 mutations may contribute to human porphyrias, Mfrn1 inactivation alone is not likely sufficient for development of erythropoietic protoporphyria. In accord, expression of aberrant Mfrn1 and a concomitant decrease in Fech expression is associated with human erythropoietic protoporphyria (45). Interestingly, C-terminal ALAS2-activating mutations (45–47) were also found in these erythropoietic porphyria patients with abnormal Mfrn1 transcripts. Also, Alas2 has been shown to be a genetic modifier in patients with congenital erythropoietic porphyria (48–50). This indicates that the IRP1/ALAS2 negative feedback loop also exists in humans and that disruption of multiple regulators in the heme and [Fe-S] cluster synthesis pathways are required for protoporphyrin accumulation (45). In Mfrn1−/− conditional knock-out mice, this porphyria effect can be recapitulated by exogenous chemical supplementation of ALA in the diets (8). Given the cytotoxic effects of protoporphyrin and its biosynthetic intermediates, the IRP1/ALAS2 feedback mechanism may have evolved in mammals to tightly link protoporphyrin synthesis with iron availability to guard against their deleterious overaccumulation.

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REFERENCES

1. Schultz, I. J., Chen, C., Paw, B. H., and Hamza, I. (2010) Iron and porphyrin trafficking in heme biogenesis. J. Biol. Chem. 285, 26753–26759
2. Rouault, T. A., and Tong, W. H. (2008) Iron-sulfur cluster biogenesis and human disease. Trends Genet. 24, 398–407
3. Lill, R. (2009) Function and biogenesis of iron-sulphur proteins. Nature 460, 831–838
4. Chung, J., Cope, J. J., Li, L., Corson, K., Hersey, C., Ackermann, G. E., Gwynn, B., Bann, L. A., Wingert, R. A., Traver, D., Tred, N. S., Barut, B. A., Zhou, Y., Mimet, E., Donovon, A., Brownie, A., Balzan, R., Weiss, M. J., Peters, L. L., Kaplan, J., Zon, L. I., and Paw, B. H. (2006) MFRN1 is essential for erythroid iron assimilation. Nature 440, 96–100
5. Amigo, J. D., Yu, M., Troadec, M. B., Gwynn, B., Dooley, K., Davidson, A. J., Schmidt, B., Schmidt, B., Paw, C. W., Gwyne, B., Kingsley, P., Palis, J., Schubert, H., Chen, O., Kaplan, J., Zon, L. I., and Tübingen 2000 Screen Consortium (2005) Deficiency of glutaredoxin 5 reveals Fe-S clusters are required for vertebrate haem synthesis. Nature 436, 1035–1039
6. Paradkar, P. N., Zumbrennen, K. B., Paw, B. H., Ward, D. M., and Kaplan, J. (2009) Regulation of mitochondrial iron import through differential turnover of mitoferrin 1 and mitoferrin 2. Mol. Cell. Biol. 29, 1007–1016
7. Shaw, G. C., Cope, J. J., Li, L., Corson, K., Hersey, C., Ackermann, G. E., Gwynn, B., Bann, L. A., Wingert, R. A., Traver, D., Tred, N. S., Barut, B. A., Zhou, Y., Mimet, E., Donovon, A., Brownie, A., Balzan, R., Weiss, M. J., Peters, L. L., Kaplan, J., Zon, L. I., and Paw, B. H. (2006) Mitoferrin is essential for erythroid iron assimilation. Nature 440, 96–100
8. Troade, M. B., Warner, D., Wallace, J., Thomas, K., Spangrude, G. J., Phillips, J., Khalimchouk, O., Paw, B. H., Ward, D. M., and Kaplan, J. (2010) Targeted deletion of the mouse Mitoferrin1 gene: from anemia to protoporphyria. Blood 117, 5494–5502
9. Wingert, R. A., Galloway, J. L., Barut, B., Footh, H., Frauenkel, P., Axe, J. L., Weber, G. J., Dooley, K., Davidson, A. J., Schmidt, B., Schmidt, B., Paw, C. W., Gwyne, B., Kingsley, P., Palis, J., Schubert, H., Chen, O., Kaplan, J., Zon, L. I., and Tübingen 2000 Screen Consortium (2005) Deficiency of glutaredoxin 5 reveals Fe-S clusters are required for vertebrate haem synthesis. Nature 436, 1035–1039
10. Rouault, T. A. (2006) The role of iron regulatory proteins in mammalian iron homeostasis and disease. Nat. Chem. Biol. 2, 406–414
11. Anderson, C. P., Shen, M., Eisenstein, R. S., and Leibold, E. A. (2012) Mammalian iron metabolism and its control by iron regulatory proteins. Biochim. Biophys. Acta 1823, 1468–1483
12. Hentze, M. W., Muckenhalter, M. U., Galby, B., and Camachela, C. (2010) Two to tango: regulation of mammalian iron metabolism. Cell 142, 23–38
13. Rouault, T. A. (2009) Cell biology. An ancient gauge for iron. Science 326, 676–677
14. Salahudeen, A. A., Thompson, J. W., Ruiz, J. C., Ma, H. W., Kinch, I. N., Li, Q., Grishin, N. V., and Bruck, R. K. (2009) An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis. Science 326, 722–726
15. Vashisht, A. A., Zumbrennen, K. B., Huang, X., Powers, D. N., Durazo, A., Sun, D., Bhaskaran, N., Persson, A., Uhlen, M., Sangfelt, 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
16. Galby, B., Ferring, D., Minana, B., Bell, O., Janser, H. G., Muckenthaler, M., Schümann, K., and Hentze, M. W. (2005) Altered body iron distribution by an iron-regulated ubiquitin ligase. Science 326, 718–721
17. Cooperman, S. S., Meyron-Holtz, E. G., Oliviere-Wilson, H., Ghosh, M. C., McConnell, J. P., and Rouault, T. A. (2005) Microacytin amemia, erythropoietic protoporphyria, and neurodegeneration in mice with targeted deletion of iron-regulatory protein 2 (IRP2). Blood 106, 2580–2589
18. Meyron-Holtz, E. G., Ghosh, M. C., Iwai, K., LaVauette, T., Brazzolotto, X., Berger, U. V., Land, W., Oliviere-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
19. Clarke, S. L., Vasanthakumar, A., Anderson, S. A., Pondarreé, C., Koh, C. M., Deck, K. M., Pittala, 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
20. Hamza, I., and Dailey, H. A. (2012) One ring to rule them all: trafficking of heme and heme synthesis intermediates in the metabolons. Biochim. Bio-
21. Dandeker, T., Stripecek, R., Gray, N. K., Goossens, B., Constable, A., Johansson, H. E., and Hentze, M. W. (1991) Identification of a novel iron-responsive element in murine and human erythroid 6-aminolevulinic acid synthase mRNA. *EMBO J.* **10**, 1903–1909

22. Ye, H., Jeong, S. Y., Ghosh, M. C., Kvetonovych, G., Silvestri, L., Ortizlo, D., Uchida, N., Tsidale, J., Camauschella, C., and Rouault, T. A. (2010) Glutaredoxin 5 deficiency causes sideroblastic anemia by specifically impairing heme biosynthesis and depleting cytosolic iron in human erythroblasts. *J. Clin. Invest.* **120**, 1749–1761

23. Chen, C., Garcia-Santos, D., Ishikawa, Y., Seguin, A., Li, L., Fegan, K. H., Hildick-Smith, G. J., Shah, D. I., Cooney, J. D., Chen, W., King, M. J., Yien, Y. Y., Schultz, I. J., Anderson, H., Dalton, A. J., Freedman, M. L., Kingsley, P. D., Palis, J., Hattangadi, S. M., Lodish, H. F., Ward, D. M., Kaplan, I., Maeda, T., Ponka, P., and Paw, B. H. (2013) Snx3 regulates recycling of the transferrin receptor and iron assimilation. *Cell Metab.* **17**, 343–352

24. Styke, D., Kawamoto, M., Huang, C. C., Johns, S. J., King, L. A., Harper, C. A., Meng, E. C., Lee, R. E., Yee, A., L’Italien, L., Chuan, P. T., Young, S. G., Skarnes, W. C., Babbi, P. C., and Ferrin, T. E. (2003) BayGenomics: a resource of insertional mutations in mouse embryonic stem cells. *Nucleic Acids Res.* **31**, 278–281

25. Townley, D. J., Avery, B. J., Rosen, B., and Skarnes, W. C. (1997) Rapid sequence analysis of gene trap integrations to generate a resource of insertional mutations in mice. *Genome Res.* **7**, 293–298

26. Robertson, E. J. (1987) Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. Oxford University Press, New York

27. Anderson, S. A., Nizzi, C. P., Chang, Y. I., Deck, K. M., Schmidt, P. J., Galy, B., Damennsawad, A., Broman, A. T., Kendzierski, C., Hentze, M. W., Fleming, M. D., Zhang, J., and Eisenstein, R. S. (2013) The IRP1-HIF-2α axis coordinates iron and oxygen sensing with erythropoiesis and iron absorption. *Cell Metab.* **17**, 282–290

28. Chen, J., Astle, C. M., and Harrison, D. E. (2000) Genetic regulation of heme synthesis in developing erythroblasts. *Exp. Hematol.* **28**, 46–50

29. Stachura, D. L., Reyes, J. R., Bartunek, P., Paw, B. H., Zon, L. L., and Traver, D. (2009) Zebrafish kidney stromal cell lines support multilineage hematopoiesis. *Blood* **114**, 279–289

30. Li, L., Chen, O. S., McVey Ward, D., and Kaplan, J. (2001) CCCI is a transporter that mediates vacuolar iron storage in yeast. *J. Biol. Chem.* **276**, 29515–29519

31. Cook, J. D. (1980) Iron, Churchill Livingstone, New York

32. Shah, D. I., Takahashi-Makise, N., Cooney, J. D., Li, L., Schultz, I. J., Pierce, E. I., Narla, A., Seguin, A., Hattangadi, S. M., Medlock, A. E., Langer, N. B., Dailey, T. A., Hurst, S. N., Facenda, D., Wiwczar, J. M., Heggars, S. K., Vogen, G., Chen, W., Chen, C., Campagna, D. R., Brugnara, C., Zhou, Y., Ebert, B. L., Danial, N. N., Fleming, M. D., Ward, D. M., Campanella, M., Dailey, H. A., Kaplan, J., and Paw, B. H. (2012) Mitochondrial Atfpi1 regulates haem synthesis in developing erythroblasts. *Nature Genet.* **491**, 608–612

33. Brownlie, A., Donovan, A., Pratt, S. J., Paw, B. H., Oates, A. C., Brugnara, C., Wittkowski, H. E., Sassa, S., and Zon, L. I. (1998) Positional cloning of the zebrafish sauternes gene: a model for congenital sideroblastic anemia. *Blood* **90**, 491–497

34. Krip, G., Cser, P., Rohr, C., and Lill, R. (1999) The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. *EMBO J.* **18**, 3981–3989

35. Wang, Y., Langer, N. B., Shaw, G. C., Yang, G., Li, L., Kaplan, J., Paw, B. H., and Bloomer, J. R. (2011) Abnormal mitoferrin-1 expression in patients with erythropoietic protoporphyria. *Exp. Hematol.* **39**, 784–794

36. Whatley, S. D., Ducamp, S., Gouya, L., Grandchamp, B., Beaumont, C., Badminton, M. N., Elder, G. H., Holme, S. A., Anstey, A. V., Parker, M., Corrigall, A. V., Meissner, P. N., Hift, R. J., Marsden, J. T., Ma, Y., Mieli-Vergani, G., Deybach, J. C., and Puy, H. (2008) C-terminal deletions in the ALAS2 gene lead to gain of function and cause X-linked dominant protoporphyria without anaemia or iron overload. *Am. J. Hum. Genet.* **83**, 408–414

37. Bishop, D. F., Tchaikovskii, V., Hoffbrand, A. V., Fraser, M. E., and Margolis, S. (2012) X-linked sideroblastic anaemia due to carboxyl-terminal ALAS2 mutations that cause loss of binding to the β-subunit of succinyl-CoA synthetase (SUCLA2). *J. Biol. Chem.* **287**, 28943–28955

38. To-Figueras, J., Ducamp, S., Clayton, J., Badenas, C., Delaby, C., Gouya, S., Gouya, L., de Verneuil, H., Beaumont, C., Ferreira, G. C., Deybach, J. C., Herrero, C., and Puy, H. (2011) ALAS2 acts as a modifier gene in patients with congenital erythropoietic porphyria. *Blood* **118**, 1443–1451

39. Kadirvel, S., Furuyama, K., Harigae, H., Kaneko, K., Tamai, Y., Ishida, Y., and Shibahara, S. (2012) The carboxy-terminal region of erythroid-specific 5-aminolevulinic acid synthase acts as an intrinsic modifier for its catalytic activity and protein stability. *Exp. Hematol.* **40**, 477–486

40. Balwani, M., Dohey, D., Bishop, D. F., Nazarenko, I., Yasuda, M., Dailey, H. A., Anderson, K. E., Bissell, D. M., Bloomer, J., Bonkowsky, H. L., Phillips, J. D., Liu, L., Desnick, R. J., and Porphyrias Consortium of the National Institutes of Health Rare Diseases Clinical Research Network (2013) Loss-of-function ferrochelatase and gain-of-function erythropoietic-specific 5-aminolevulinic acid synthase mutations cause erythropoietic protoporphyria and x-linked protoporphyria in North American patients reveal novel mutations and high prevalence of X-linked protoporphyria. *Mol. Med.* **19**, 26–35