Mitochondrial iron import is essential for iron–sulfur cluster formation and heme biosynthesis. Two nuclear-encoded vertebrate mitochondrial high-affinity iron importers, mitoferrin1 (Mfrn1) and Mfrn2, have been identified in mammals. In mice, the gene encoding Mfrn1, solute carrier family 25 member 37 (Slc25a37), is highly expressed in sites of erythropoiesis, and whole-body Slc25a37 deletion leads to lethality. Here, we report that mice with a deletion of Slc25a28 (encoding Mfrn2) are born at expected Mendelian ratios, but show decreased male fertility due to reduced sperm numbers and sperm motility. Mfrn2−/− mice placed on a low-iron diet exhibited reduced mitochondrial manganese, cobalt, and zinc levels, but not reduced iron. Hepatocyte-specific loss of Slc25a37 (encoding Mfrn1) in Mfrn2−/− mice did not affect animal viability, but resulted in a 40% reduction in mitochondrial iron and reduced levels of oxidative phosphorylation proteins. Placing animals on a low-iron diet exaggerated the reduction in mitochondrial iron observed in liver-specific Mfrn1/2-knockout animals. Mfrn1−/−/Mfrn2−/− bone marrow–derived macrophages or skin fibroblasts in vitro were unable to proliferate, and overexpression of Mfrn1-GFP or Mfrn2-GFP prevented this proliferation defect. Loss of both mitoferrins in hepatocytes dramatically reduced regeneration in the adult mouse liver, further supporting the notion that both mitoferrins transport iron and that their absence limits proliferative capacity of mammalian cells. We conclude that Mfrn1 and Mfrn2 contribute to mitochondrial iron homeostasis and are required for high-affinity iron import during active proliferation of mammalian cells.

Iron is essential for the formation of iron-sulfur (Fe-S) clusters and heme, both of which are prosthetic groups in proteins involved in metabolic processes including redox reactions, electron transport, endoplasmic reticulum stress and oxygen transport, and DNA synthesis (DNA helicases such as XPD, polymerases, primases, ribonucleotide reductases) (1–3). The synthesis of Fe-S clusters and heme occurs in the mitochondria, therefore, iron must be imported to generate Fe-S and heme. Excess iron import into the mitochondria without efficient Fe-S cluster formation or in conditions of mitochondrial dysfunction lead to diseases including Friedreich’s ataxia, X-linked and congenital sideroblastic anemias, and infantile mitochondrial complex deficiency (1–3). Two nuclear-encoded mammalian mitochondrial iron transporters, Slc25a37 (hereafter referred to as Mitoferrin1 (Mfrn1)) and Slc25a28 (hereafter referred to as Mitoferrin2 (Mfrn2)) have been implicated in iron import into mitochondria (4–8). Paralogous genes are found in all eukaryotes. Plants, Caenorhabditis elegans, and Drosophila melanogaster have only one Mfrn gene (9–12), whereas fungi and vertebrates have two Mfrns. In Saccharomyces cerevisiae, the Mfrn genes are named MRS3 and MRS4 (13, 14). S. cerevisiae MRS3/MRS4 and its plant and vertebrate homologues (Mfrn1/Mfrn2) are members of the solute carrier (SLC) family of mitochondrial transporters, which have been shown to be important in the transport of metabolites such as inorganic anions, cofactors, amino acids, and nucleotides into the mitochondrial matrix (15). Conserved functions for the homologous proteins was demonstrated by the fact that overexpression of murine Mfrn1 rescued defects seen in the mutant zebrafish Mfrn (frascati) and overexpression of zebrafish Mfrn (zMfrn) complemented the poor growth on low-iron media of Δmrs3Δmrs4 yeast cells (7). Genetic and biochemical studies have shown that MRS3/MRS4 are high-affinity iron importers that are only essential under iron-limiting conditions (13, 14). The vertebrate homologue Mfrn1 has been established as the iron transporter important in erythroid development and its loss is embryonic lethal with embryonic stem cells showing maturation arrest (10) and loss of Mfrn1 in adult mice leads to severe anemia (7, 8). Deletion of Mfrn1 in liver, heart, or muscle is not essential for development (8). Organisms with only one Mfrn such as C. elegans, D. melanogaster, and plants show...
introduced by electroporation into mouse G4 embryonic stem (ES) cells (C57BL/6/129 hybrid). ES cell clones resistant to G418 were analyzed for correct homologous recombination by Southern blotting and PCR analyses. Southern blotting analysis showed several ES clones positive for the insert (Fig. S1B). PCR analyses confirmed the presence of the Loxp–FRT–neomycin–FRT cassette flanking exon 4, and the second Loxp site in the recombinant Mfrn2 locus. Cells from a single heterozygous clone (#8) were injected into C57BL/6J-derived blastocysts that were implanted into foster mothers. Chimeric animals were identified by coat color, and males were mated with C57BL/6J females to produce mice heterozygous at the Mfrn2 locus (Mfrn2flooxneo/+). The neomycin-resistance cassette was subsequently excised by recombination of FRT sites by breeding Mfrn2floox/+ mice with flp/flp mice that express Flp recombinase under the Rosa26 promoter generating heterozygous Mfrn2floox/+ animals. Mfrn2floox/+ animals were mated with CMV-Cre recombinase mice to generate Mfrn2−/− animals. Mfrn2−/+ animals were mated to generate Mfrn2−/− animals. PCR genotyping demonstrated the generation of Mfrn2−/+, Mfrn2+/−, and Mfrn2+/+ animals (Fig. 1C) and RT-PCR confirmed the loss of Mfrn2 mRNA (Fig. 1D). Mfrn2−/− animals were born in the expected Mendelian ratios. Tissue-specific RT-qPCR demonstrated a 50% reduction in Mfrn2 mRNA in heterozygotes and complete loss of Mfrn2 mRNA in all tissues of Mfrn2−/− animals (Fig. 2A). Unfortunately, we were unable to determine Mfrn2 protein levels as available antibodies did not work in mouse tissues. There was a slight increase in liver nonheme iron levels in the absence of Mfrn2 (Fig. 2B) but no differences in mitochondrial nonheme iron levels as measured by the bathophenanthroline chromogen assay (Fig. 2C). Animals placed on a low-iron diet, however, showed changes in mitochondrial metals as assessed by inductively coupled plasma mass spectrometry (ICP-MS). Loss of Mfrn2 resulted in a slight but significant increase in total mitochondrial iron (Fe) levels and significantly decreased manganese (Mn), cobalt (Co), and zinc (Zn) with no difference in copper (Cu) levels, which was seen in both Mfrn2+/− and Mfrn2−/− liver mitochondria (Fig. 2D) suggesting haploinsufficiency.

We hypothesized that Mfrn1 might be up-regulated to compensate for the loss of Mfrn2, however, qPCR of different organs showed no significant changes in Mfrn1 transcript levels, with the exception of kidney where Mfrn1 transcripts were increased 2-fold as a consequence of the loss of Mfrn2 (Fig. 2A). Western blotting analysis, however, did not show an increase in kidney Mfrn1 protein levels (Fig. S2).

Loss of Mfrn2 results in decreased male fertility

Mfrn2−/− animals did not show any overt phenotypes in hematologic parameters (Fig. S3A). Breeder pairs of Mfrn2−/− mice revealed that the Mfrn2−/− males showed a decreased ability to reproduce, which worsened over time (Fig. 3A). The decreased fertility phenotype in mice was not fully penetrant as some Mfrn2−/− male mice were able to reproduce normally. The decreased male fertility could be attributed to reduced numbers of sperm (Fig. 3B) and significantly reduced sperm motility (Fig. 3C). These results confirm reports that mutations

lethality when deleted (9, 10). Mutations in the D. melanogaster homologue dmfrn result in male sterility, which can be rescued by providing high iron in the diet (12), suggesting other transporters can transport iron into the mitochondria under high iron conditions. It has been speculated that mammalian Mfrn2 is the mitochondrial iron importer for all other tissues, however, one study suggests that Mfrn1 and Mfrn2 are both needed in adipogenic differentiation (16).

Here, we show that Mfrn2 is not essential for viability but is important in reproduction and contributes to effective cell proliferation. Mfrn2−/− male mice show decreased sperm counts in seminal fluid and decreased sperm motility. Mfrn2−/− mice do not show reductions in mitochondrial iron levels in liver but show reduced mitochondrial manganese, cobalt, and zinc levels when animals are placed on a low-iron diet. Loss of both Mfrn1 and Mfrn2 in primary macrophages and skin fibroblasts resulted in a proliferation defect with corresponding reductions in mitochondrial oxidative phosphorylation proteins. Indeed, feeding animals a low-iron diet revealed dramatic reductions in iron conditions. It has been speculated that mammalian Mfrn2 in seminal fluid and decreased sperm motility.

Results

Targeting for disruption of the murine Mfrn2 (Slc25a28) gene

The murine Mfrn2 (Slc25a28) gene consists of 4 exons spread over 11 kb on the reverse strand of mouse chromosome 19. We originally designed a strategy to conditionally disrupt the Mfrn2 gene by knocking out exon 1, however, we never obtained mouse embryonic stem cell G418-resistant clones that showed the correct homologous recombination. We attributed this result to poor recombination due to the high level of repeats in the 5′-upstream region of exon 1. Mfrn2 has six predicted transmembrane domains. We hypothesized that loss of the final two transmembrane domains would disrupt function. To test this hypothesis, we generated a yeast-expressed truncated zMfrn2 construct where the last two transmembrane domains were deleted. Although full-length zMfrn2 complemented the loss of Mfrn3 and Mfrn4 (Δmrs3Δmrs4), a truncated zMfrn2 (truncZMfrn2) did not complement the loss (Fig. 1A). To determine whether loss of the last two transmembrane domains affected protein expression or mitochondrial localization we generate mammalian constructs of full-length and truncated mouse Mfrn2 with a carboxyl GFP tag expressed under the cytomegalovirus (CMV) promoter. We transfected those constructs along with mouse Mfrn1-mCherry into mouse fibroblasts and examined expression by microscopy. Full-length Mfrn2-GFP localized with Mfrn1-mCherry, whereas, truncating the last two transmembrane domains of mouse Mfrn2 (truncMfrn2-GFP) resulted in the loss of mitochondrial localization (Fig. 1B). Based upon these results, we generated a “knock-out” construct that targeted deletion of exon4 in mouse Mfrn2, which would eliminate the last two transmembrane domains. Two Loxp sites and a neomycin-resistance gene flanked with FRT sites (FNP) were cloned on either side of exon 4 to permit excision of exon 4 (Fig. S1A). This construct was...
in the *D. melanogaster dmfrn* gene result in loss of male reproduction with unorganized, small or immature sperm (12, 17).

**Mfrn1 and Mfrn2 are not necessary in the adult liver**

Previously, we demonstrated that liver-specific loss of Mfrn1 resulted in increased PPIX accumulation in the livers of animals fed aminolevulenic acid (ALA), a condition that bypasses the rate-limiting step in heme biosynthesis resulting in increased heme synthesis (8). We performed similar experiments in total body *Mfrn2* \(^{-/-}\) mice. While feeding ALA did increase porphyrin levels in liver of all animals (note the scale), there were no statistically significant genotypic differences in PPIX levels (Fig. S3B). These results support that there are no differences in mitochondrial iron in the liver of *Mfrn2* \(^{-/-}\) mice (if iron was decreased PPIX would accumulate) and the iron present is available and sufficient to populate PPIX when heme synthesis is increased. We did not see a significant increase in PPIX in contrast to our previous report in hepatocyte-specific *Mfrn1* \(^{-/-}\) mice (8). We also did not observe the histologic changes reported previously in the hepatocyte-specific *Mfrn1* \(^{-/-}\) mice. The differences with previously reported data may be due to different experimental factors such as age of the mice (6 versus 12 weeks) when the ALA feeding was started or a different environment in the mouse environment. No changes in liver mitochondrial oxygen consumption were observed in the absence of Mfrn2 (Fig. S4A) supporting that mitochondrial function was not compromised.

Studies have demonstrated that Mfrn1 was necessary for red cell formation, however, Mfrn1 loss was not reported to affect heart, liver, or muscle development (8). RNA-Seq of WT mouse livers revealed that transcript levels for Mfrn1 and Mfrn2 were similar (FPKM *Mfrn1*: 705 and *Mfrn2*: 690). To determine whether the absence of both Mfrn1 and Mfrn2 affected liver development we generated animals with a tissue-specific deletion (hepatocyte-*Alb-Cre*) of *Mfrn1* on a *Mfrn2* \(^{-/-}\) background. Surprisingly, *Alb-Cre Mfrn1*\(^{f/f}\)*Mfrn2*\(^{-/-}\) animals were born in expected Mendelian ratios. RT-qPCR analysis of livers from 3-month-old mice demonstrated an 80% reduction in *Mfrn1* transcripts in whole liver in *Alb-Cre Mfrn1*\(^{f/f}\)*Mfrn2*\(^{-/-}\) mice and an even greater reduction in *Mfrn1* in a crude isolation of hepatocytes confirming the ability to delete *Mfrn1* in the *Mfrn2*\(^{-/-}\) background (Fig. 4A). Corresponding to the reduction in *Mfrn1* transcripts, we observed a dramatic reduction in Mfrn1 protein. We also successfully generated hepatocyte-specific deletion of *Mfrn2* using floxed mice and these animals were born at Mendelian ratios as expected. We noted that loss of Mfrn1 or Mfrn2 in hepatocytes reduced most oxidative

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**Figure 1. Strategy and generation of conditional deletion of mouse *Slc25a28 (Mfrn2).* A, WT (WT) and *Δmrs2Δmrs4* cells transformed with empty vector, pZebrafish full-length Mfrn2 (pMfrn2) or pZebrafish-truncated Mfrn2 (trunZMfrn2) were grown on CM-ura bathophenanthroline sulfonate supplemented with 5 μM FeSO₄ for 2 days. B, mouse fibroblasts were transfected with plasmid containing full-length mouse Mfrn2-GFP or truncated mouse Mfrn2-GFP and Mfrn1-mCherry. Images were captured using an Olympus BX51 upright epifluorescence microscope with a ×60 oil immersion objective and QuantOne software (Olympus, Melville, NY). C, PCR analysis of Cre recombinase–mediated excision of exon 4 in *Slc25a28* \(^{lox/lox}\) offspring. D, Mfrn2 RT-PCR analysis of the offspring from the breeding of *Mfrn2*\(^{-/-}\) mice using actin as a loading control.
Mfrn1 and Mfrn2 are necessary for cell proliferation
Mfrn1 and Mfrn2 are necessary for cell proliferation

Figure 2. Tissue-specific RT-qPCR in Mfrn2+/+, Mfrn2−/−, and Mfrn2−/+ mice confirmed loss of Mfrn2 mRNA in all tissues and no changes in mitochondrial iron. A, RT-qPCR of Mfrn1 (dark gray bars), Mfrn2 (light gray bars) and actin in liver, muscle, heart, kidney, spleen and testes using primers listed in Table 3. Error bars represent mean ± S.E., n = 3-5. B, livers were isolated from Mfrn2−/+ and Mfrn2−/− mice. Nonheme iron was measured as described under “Materials and methods.” Error bars represent the mean ± S.E., n = 8-10. C, mitochondria were isolated from livers of Mfrn2−/+ and Mfrn2−/− mice and nonheme iron measured as described under “Materials and methods.” Data are normalized to sample weight (mg). Error bars represent mean ± S.E., n = 11-19. D, 3-month-old animals (4-6 genotype) were fed a low-iron diet (5-6 ppm Fe/kg, Harland Teklad) for 10 weeks and liver mitochondria were isolated. Mitochondrial metals (Fe, Mn, Co, Cu, and Zn) were detected using ICP-MS as described under “Materials and methods.” Mfrn2−/+, dark gray bars; Mfrn2−/, gray bars; and Mfrn2−/+, white bars. *, p value ≤ 0.05; **, p ≤ 0.01. Error bars represent the mean ± S.E.

Figure 3. The absence of Mfrn2 results in decreased male fertility. A, the ability to successfully breed was measured over a 3-4 month period in Mfrn2−/+ (n = 9) and Mfrn2−/− (n = 13) male mice starting breeding at 2 months of age. The data are expressed as the percent of successful males breeding two (2×) or three (3×) times. B, sperm counts, and C, motility were assessed in 4-month-old Mfrn2−/+ and Mfrn2−/− mice (n = 4). *, p value ≤ 0.05. Error bars represent mean ± S.E.

phosphorylation protein levels (OXPHOS) from 50 to >90% (Fig. 4B). Quantification revealed the most dramatic changes associated with CI-NDUF88 protein when Mfrn1 was absent either in the single or double hepatocyte-specific knockout (Fig. 4C, column 1 versus columns 2-4). Although the decrease in protein levels was most significant when Mfrn1 was absent, CI complex activity was reduced by ~40% in all deletion strains (Fig. 4D). CI-NDUF88 is the first protein in the electron transport chain that catalyzes the two electron oxidation of NADH by coenzyme Q10. CII levels were also dramatically decreased in the absence of Mfrns suggesting that Fe-S cluster synthesis may be compromised. These data also suggest that both Mfrn1 and Mfrn2 contribute to iron delivery to the mitochondria for sustaining Fe-S cluster formation. Another Fe-S cluster protein whose activity is highly dependent upon the presence of the Fe-S cluster is mitochondrial aconitase. Indeed, loss of Mfrn1 or Mfrn2 reduced aconitase activity (Fig. 5A). Placing animals on low iron chow for 10 weeks resulted in a decrease in aconitase activity in WT animals but no statistically significant differences in aconitase activity were observed upon Mfrn1 and/or Mfrn2 hepatocyte-specific loss (Fig. 5A, white bars). We did not observe differences in the activity of the cytosolic Fe-S cluster protein xanthine oxidase (Fig. S4B). We also did not observe differences in the heme-containing enzyme catalase in most strains of mice fed normal chow. We did observe genotypic reductions in catalase activity when animals were placed on the low-iron diet where whole body loss of Mfrn2 showed the most dramatic reductions in catalase activity (Fig. S4C). These differences were not explained by changes in catalase protein levels (Fig. S4D).

We next examined total liver and mitochondrial iron levels in WT and hepatocyte-specific Mfrn1−/−, Mfrn2−/−, Mfrn1−/−/Mfrn2−/−, and total body Mfrn2−/− mice. No statistically significant differences in liver nonheme iron levels were
Mfrn1 and Mfrn2 are necessary for cell proliferation

A. qPCR Whole Liver and qPCR Hepatocytes

B. Western Blot Analysis

C. Graphs showing fold change in various mitochondrial complex activities

D. Mitochondrial Complex Activity (Arbitrary units/µg protein)
**Mfrn1 and Mfrn2 are necessary for cell proliferation**

detected between animals fed normal chow and all animals showed reduced nonheme iron levels when placed on low iron chow (Fig. 5B, blue bars). Liver mitochondrial isolation revealed that loss of Mfrn1 (Alb-CreMfrn1f/f (M1)) in hepatocytes showed a trend toward reduced mitochondrial nonheme iron on normal chow, which was further decreased (approximately 50%) with loss of both Mfrn1 and Mfrn2 (Alb-CreMfrn1f/fMfrn2f/f (M1/M2)) even on normal chow (Fig. 5C, black bars, Albcre M1/M2). Importantly, placing animals on the low-iron diet showed significant reductions in mitochondrial nonheme iron in all animals and revealed genotype-specific differences in Alb-CreM1, Alb-CreM2, and Alb-CreM1/M2 compared with WT mice (Fig. 5C, blue bars). Liver-specific loss of Mfrn2 mimicked the total body loss of Mfrn2 supporting that the mitochondrial iron changes were specific to hepatocyte loss of Mfrn2 and were not due to iron changes in other liver cell types such as Kupffer cells or fibroblasts. Furthermore, the loss of both Mfrn1 and Mfrn2 was additive resulting in the largest reduction in mitochondrial nonheme iron levels in animals fed a low iron diet (Fig. 5C, blue bars, Albcre M1/M2 low).

To determine whether there were transcriptional changes associated with the loss of Mfrn1 and Mfrn2 in hepatocytes, we performed RNA-Seq on livers from WT and Alb-CreMfrn1f/f Mfrn2−/− (Mfrn1−/−Mfrn2−/−) mice. A heat map of the top 100 differentially expressed genes is shown (Fig. 6A). Slc25a28 (Mfrn2) and Slc25a37 (Mfrn1) were identified in the top 50 genes with Slc25a28 as the most dramatically changed (~3.7046 log2 ratio KO/WT) as it was a total body deletion, whereas Slc25a37 was hepatocyte-specific deletion and the analysis was done on whole liver. Transcript changes were confirmed using RT-qPCR for a small subset of genes (Fig. S5A). We note that many cytochrome P450 family members transcripts were altered with eight Cyp genes in the top 40 transcripts up-regulated in Alb-CreMfrn1f/fMfrn2−/− livers representing 20% of the top up-regulated transcripts. We note that many of these enzymes are heme-containing proteins. We do not know the mechanism of up-regulation of these P450 genes but speculate that they may be up-regulated in response to liver stress. Ingenuity Pathway Analysis of the RNA-Seq data identified several pathways that were significantly affected by the absence of Mfrn1 and Mfrn2 in liver including amino acid metabolism, small molecule biochemistry, cellular movement, function and maintenance, molecular transport (Fig. 6B, black bars), as well as liver proliferation, inflammation, fibrosis, hepatocellular carcinoma, and hyperproliferation (Fig. 6B, gray bars). The most significantly (p < 0.0001) altered pathway was amino acid metabolism with nine genes transcript levels changed. We confirmed that amino acid metabolism was affected using LC–MS metabolomic analysis. Loss of Mfrn1 and Mfrn2 in the liver significantly changed amino acid levels, specifically tryptophan and arginine were elevated and valine, glutamine, glutamic acid, and asparagine were significantly reduced in the Alb-CreMfrn1f/fMfrn2−/− mouse livers compared with WT livers (Fig. 6C). In addition, AMP levels were dramatically reduced in the livers of Mfrn1−/−Mfrn2−/− mice. The pathway with the most transcript level alterations was the liver hyperplasia/hyperproliferation pathway, however, we did not observe changes in liver size in the Mfrn1−/−Mfrn2−/− mice compared with WT mice (Fig. S5B). Together, these results support that the livers of Mfrn1−/−Mfrn2−/− mice are metabolically altered, which may affect cellular proliferation and differentiation (21).

**Mfrn1 and Mfrn2 are necessary for in vitro cell proliferation**

To determine whether Mfrn1 and Mfrn2 were necessary for cell proliferation we isolated bone marrow-derived macrophages (BMDM) from Mfrn1f/fMfrn2−/+ and Mfrn1f/fMfrn2−/− mice. Dividing BMDMs were treated with recombinant Tat-Cre to excise the floxed allele and cells genotyped for deletion of the floxed allele of Mfrn1. RT-qPCR confirmed that we were able to delete Mfrn1 (Fig. 7A). Loss of Mfrn1 but retention of Mfrn2 (Mfrn1−/−Mfrn2−/+ + Tat-Cre) appeared to reduce cell numbers (Fig. 7B, left upper and lower panel for comparison) by ~50%. Loss of Mfrn1 and Mfrn2 (Mfrn1−/−Mfrn2−/−) dramatically reduced cell numbers (>90%) and cells died within 72 h of Tat-Cre treatment (Fig. 7B, arrowheads). This suggests that bone marrow-derived macrophages are highly dependent upon iron transport through Mfrns for proliferation and survival in cell culture conditions. To determine whether other highly proliferative cells needed Mfrn1 and Mfrn2, we generated immortalized fibroblasts from Mfrn1f/f, Mfrn2−/−, and Mfrn1f/fMfrn2−/− mice and exposed those cells to Tat-Cre recombinase to excise the floxed alleles of Mfrn1 (8). We were able to successfully delete Mfrn1 in fibroblasts as shown by genomic PCR (Fig. 7C, upper band, + Tat-Cre), however, genomic PCR revealed that the deletion was not 100% in the cell population. We predicted that deletion may be complete in some of the Tat-Cre–treated cells but incomplete in other cells, so we cloned out single cell colonies treated with Tat-Cre. We successfully generated stable Mfrn1−/− cell lines, but we were unable to clone out double null Mfrn1−/−Mfrn2−/− cells (Table 1). We used the cell proliferation dye carboxyfluorescein succinimidyl ester (CFSE) to determine whether the Tat-Cre–treated fibroblasts showed reduced proliferation. Although the Tat-Cre–treated cells showed a diminution in CFSE staining, the levels remained significantly higher than control cells (Fig. 7D) suggesting either reduced proliferation or increased replication time.
Mfrn1 and Mfrn2 are necessary for cell proliferation

Furthermore, Tat-Cre was not 100% efficient, therefore, we speculated that the diminution in CFSE observed may be ascribed to those cells that still retained a floxed allele of Mfrn1 and were capable of proliferating. The Tat-Cre–treated fibroblast population (80–90% Mfrn1<sup>−/−</sup>/Mfrn2<sup>−/−</sup> cells) also showed significant increases in mitochondrial oxidants (Fig. 7E), suggesting that mitochondria are stressed due to loss of Mfrn1 in Mfrn2<sup>−/−</sup> cells. Supplementing growth media with iron (ferric ammonium citrate (FAC)) or Tf(Fe)<sub>2</sub> before and after treatment with Tat-Cre did not enhance the ability to generate proliferating Mfrn1<sup>−/−</sup>/Mfrn2<sup>−/−</sup> cells (Table 1). Furthermore, treatment of cells with iron-containing hinokitiol, which has been shown to promote iron transport into and within cells in the absence of membrane transporters such as Divalent Metal Transporter1 (18), before and after Tat-Cre treatment did not result in the ability to obtain Mfrn1<sup>−/−</sup>/Mfrn2<sup>−/−</sup> stable cell lines. To confirm that the Mfrn1 allele could be deleted in the absence of Mfrn2, we transfected Mfrn<sup>F</sup>/Mfrn2<sup>−/−</sup> fibroblasts with a plasmid containing mouse Mfrn2-GFP and then treated those cells with Tat-Cre. We successfully deleted the floxed alleles of Mfrn1 with ~85% efficiency. We sorted for single GFP-positive cells, cloned out those cells, confirmed deletion of the floxed alleles of Mfrn1 and that those clones that showed loss of Mfrn1 but retention of the Mfrn2-GFP plasmid remained viable and capable of proliferating. Similar results were obtained if we transfected cells with a plasmid containing Mfrn1-GFP (data not shown). Importantly, Mfrn1<sup>−/−</sup>/Mfrn2<sup>−/−</sup> pMfrn2-GFP cells were unable to lose the pMfrn2-GFP when antibiotic selection was removed, whereas Mfrn1<sup>−/−</sup>/Mfrn2<sup>−/−</sup> cells containing pMfrn2-GFP were able to lose the plasmid when selection was removed (Fig. 7F). These results demonstrate that both Mfrn1 and Mfrn2 are necessary for fibroblast proliferation. Attempts to measure changes in mitochondrial iron levels in the Tat-Cre cell culture experiments were unsuccessful using either fluorescent dyes or ICP-MS (data not shown). We do not know why the mitochondrial fluorescent dyes (RPA or MitoFerroGreen) did not work. We believe that the inability to use ICP-MS was due to the limited amounts of mitochondria we were able to obtain from cells after Tat-Cre treatment. Together, these data show that other mitochondrial metal transporters are sufficient for the majority of heme and Fe-S cluster synthesis but they cannot compensate for the loss of Mfrns during increased proliferation demand.

To identify consequences of loss of Mfrn1 and Mfrn2 that might lead to an inability to proliferate, we generated a Mfrn1<sup>−/−</sup>/Mfrn2<sup>−/−</sup> fibroblast cell line that harbored a TET-regulated human MFRN1-GFP expression vector, which was turned off in the presence of doxycycline (Fig. 8A). We noted that some cells expressed higher levels of Mfrn1-GFP as micrograms of liver nonheme iron/mg of protein or liver mitochondrial nonheme iron/mg of protein, n = 5-9 animals/group. Dots represent individual animals. Error bars represent the mean ± S.E. Blue bars represent liver of animals fed a low-iron diet for 10 weeks. Blue bars show statistical significance, *, p = 0.05 for all genotypes compared with WT low. For mitochondria, the data are expressed as micrograms of mitochondrial nonheme iron/mg of dry weight. Error bars represent the mean ± S.E., n = 5-18 animals/group. Blue bars represent liver mitochondria from animals fed a low-iron diet for 10 weeks. Student’s t tests were done as described under “Materials and methods” with all compared with WT control. *, p = 0.05; **, p = 0.01; ***, p = 0.001.

Figure 5. A low-iron diet exaggerates the changes in liver and mitochondrial nonheme iron levels seen in hepatocyte-specific loss of Mfrn1 and Mfrn2 mice. A, mitochondrial aconitase activity was determined from livers of 3-month-old WT (WT), Alb-Cre Mfrn1<sup>−/−</sup> (M1), Alb-Cre Mfrn2<sup>−/−</sup> (M2), and Alb-Cre Mfrn1<sup>−/−</sup> Mfrn2<sup>−/−</sup> (M1/M2) female mice fed normal (black border bars) or low-iron chow (blue border bars) for 10 weeks. n = 4-11 animals/genotype. Error bars represent the mean ± S.E. B, livers (left panel) and C, liver mitochondria (right panel) from mice as in A and total body Mfrn2<sup>−/−</sup> (null) mice were isolated and nonheme cellular or mitochondrial nonheme iron measured as described under “Materials and methods.” Data are expressed as micrograms of liver nonheme iron/mg of protein or liver mitochondrial nonheme iron/mg of protein, n = 5-18 animals/group. Dots represent individual animals. Error bars represent the mean ± S.E. Blue bars represent liver of animals fed a low-iron diet for 10 weeks. Blue bars show statistical significance, *, p = 0.05 for all genotypes compared with WT low. For mitochondria, the data are expressed as micrograms of mitochondrial nonheme iron/mg of dry weight. Error bars represent the mean ± S.E., n = 5-18 animals/group. Blue bars represent liver mitochondria from animals fed a low-iron diet for 10 weeks. Student’s t tests were done as described under “Materials and methods” with all compared with WT control. *, p = 0.05; **, p = 0.01; ***, p = 0.001.
Mfrn1 and Mfrn2 are necessary for cell proliferation
compared with others. We generated clonal population of these cells and the analyses were done on two separate clones. Images were quantified for Mfrn1-GFP fluorescence and we noted that expression of Mfrn1-GFP was long lived with some protein still detected even after 96 h growth in doxycycline (Fig. B8). This may reflect expression under the tetracycline promoter or that the t1/2 of Mfrn1 is long. Previous studies have suggested that mitochondrial inner membrane proteins have a t1/2 of ~36 h (19). We isolated mitochondria from the two clones (1 and 2) incubated in the presence or absence doxycycline and examined the levels of Mfrn1-GFP, mitochondrial VDAC, and oxidative phosphorylation proteins by Western blotting. As expected, Mfrn1-GFP was dramatically reduced in cells grown in doxycycline (Fig. 8, C and D). Decreases in Mfrn1-GFP levels in Mfrn1+/−/Mfrn2−/− fibroblasts resulted in significant reductions in OXPHOS complex protein levels (Fig. 8E, +Dox). The change in OXPHOS complex proteins was specific to loss of hMfrn1-GFP and was not the result of doxycycline toxicity as in Mfrn1+/−/Mfrn2−/− we did not observe changes in GAPDH (a cytosolic marker) or reductions in VDAC (a mitochondrial marker) upon exposure to doxycycline (Fig. 8C, Con − versus + Dox). These results support that a consequence of loss of Mfrns is reduced levels of mitochondrial oxidative phosphorylation proteins, most dramatically those containing Fe-S clusters such as CIII and CII. We note that addition of doxycycline induced a slight decrease in complex II of the OXPHOS proteins in control cells compared with control cells without Dox. Previous studies have suggested that tetracyclines can disturb mitochondrial function (20), however, the decrease was only seen in CII and was much more dramatic in the “shut-off” system further underscoring that the reduction/loss of Mfrn1-GFP contributes to reduced OXPHOS proteins.

Mfrn1 and Mfrn2 are necessary for liver regeneration

To determine whether the mitochondrial oxidative phosphorylation changes and metabolic changes implicated in the RNA-Seq and metabolomic results affected proliferation, we took advantage of the fact that the liver can regenerate in vivo after tissue loss (21). We performed 2/3 partial hepatectomies on 3-month-old WT Alb-Cre, Alb-Cre Mfrn1f/fMfrn2+/−, Alb-Cre Mfrn1+/−/Mfrn2−/−, and Alb-Cre Mfrn1f/fMfrn2−/− mice. We examined cell proliferation pre- and post-hepatectomy using the cell proliferation marker Ki67 (22). Histologic examination of liver samples at the time of hepatectomy showed minimal Ki67 staining in all genotypes (Fig. 9A, pre). Forty-eight hours post-hepatectomy, WT Alb-Cre and Alb-Cre Mfrn1+/−/Mfrn2−/− liver samples showed increased Ki67 staining, whereas, both Alb-Cre Mfrn1f/f and Alb-Cre Mfrn1f/fMfrn2−/− mice showed decreased Ki67 staining compared with WT (Fig. 9A, post). Quantification of the Ki67 staining revealed a trend toward decreased Ki67 staining in Alb-Cre Mfrn1f/fMfrn2−/− animals that was reduced further when both Mfrns were lost (Fig. 9B). We noted some evidence of steatosis in histologic analysis predominantly in the livers after partial hepatectomy (WT 3/7, Alb-Cre Mfrn1f/f 3/8, Alb-Cre Mfrn1+/−/Mfrn2−/− 2/7, and Alb-Cre Mfrn1f/fMfrn2−/− 6/9) with the Alb-Cre Mfrn1f/fMfrn2−/− livers showing the highest incidence (66.7%). This suggest that liver lipid metabolism under the stress of regeneration is compromised by the loss of Mfrn1 and Mfrn2. Our previous RNA-Seq (Fig. 5C) revealed dramatic reductions in both Cebp and Hist4h4 transcripts in Alb-CreMfrn1f/fMfrn2−/− livers compared with WT livers (Fig. 9, C and D), whereas other Cebp and histone family members did not show significant changes in transcript levels. We were unable to detect these proteins using conventional Western blots (data not shown). Cebp is a member of the CCAAT enhancer-binding protein family of transcription factors that regulate the expression of genes involved in mitotic expansion and a role for Cebp has been shown for granulocyte differentiation (23–25) as well as in acute lymphoblastic leukemia proliferation (26). One target gene of the Cebp family, specifically Cebpb, is Hist4h4, which contains Cebp-binding sites in its promoter region and is activated during mitotic clonal expansion (27). Hist4h4 encodes for histone H4, which is important in nucleosome structure and increased expression is associated with some types of highly proliferative cancer (28). It may be that in the absence of Cebp in hepatocytes, Hist4h4 does not get activated and thus limits mitotic expansion. Together with our metabolomic results that show significant reductions in essential amino acids and the loss of Cebp expression and reduced Hist4h4 transcripts could be contributing to reduced proliferative capacity in Alb-CreMfrn1f/fMfrn2−/− livers.

Figure 6. Loss of Mfrn1 and Mfrn2 results in significant transcriptional and metabolic changes in the liver. A, RNA-Seq was performed on three WT and five Alb-Cre Mfrn1+/−/Mfrn2−/− (DKO) mouse livers. A heat map showing gene expression differences in 100 protein-coding genes 3 WT and 5 Alb-Cre Mfrn1+/−/Mfrn2−/− animals is provided. B, Ingenuity Pathway Analysis (Qiagen) of RNA-Seq results from A showing the number of genes significantly changed in particular pathways in Alb-Cre Mfrn1+/−/Mfrn2−/− compared with WT for cellular function and metabolism (black bars) and hepatotoxicity (gray bars) and the corresponding p values for those gene pathway changes. C, liver metabolomics was performed on four WT and four Alb-Cre Mfrn1+/−/Mfrn2−/− mouse livers. LC–MS and statistical analyses were done as described under “Materials and methods.” Specific amino acid changes are shown. The data are presented as the fold-change Alb-Cre Mfrn1+/−/Mfrn2−/− versus WT.

Discussion

Mitoferrins have been identified in all eukaryotes (7, 10, 12–14, 29). Mfrn1 is essential and is necessary for red cell development (7, 8). In contrast, very little is known about Mfrn2 and its role in mitochondrial iron homeostasis (4, 6, 16). In our current study, we determined that total body loss of Mfrn2 did not affect viability and showed no overt phenotypes. However, when combined with deletion of Mfrn1 in the liver, it resulted in reduced mitochondrial iron and reduced OXPHOS proteins including Fe-S cluster proteins CI-NDUF8B and CII-SDHB. Loss of Mfrn1 and Mfrn2 also resulted in a dramatic reduction in liver regeneration capacity. We also showed that primary and immortalized cells lost proliferative capacity in the absence of Mfrn1 and Mfrn2. Previous studies have shown that altering OXPHOS protein activity affects cell amino acid levels and proliferation (30, 31). Our RNA-Seq data suggested that there are significant transcriptional shifts in the liver in response to loss of both Mfrns with the most significant changes in amino acid metabolism. Furthermore, we determined that several amino
Mfrn1 and Mfrn2 are necessary for cell proliferation

A

B

C

D

E

F
Mfrn1 and Mfrn2 are necessary for cell proliferation

Table 1
Percentage of clones showing Mfrn1<sup>−/−</sup> genotype after Tat-Cre

| Pre-Tat-Cre fibroblast genotype | Post-Tat-Cre | Percentage | Number of clones |
|---------------------------------|-------------|------------|-----------------|
| Mfrn1<sup>+/+</sup>             | Mfrn1<sup>−/−</sup> | 43.1%      | 9/20            |
| Mfrn1<sup>+/+</sup>Mfrn2-GFP    | Mfrn1<sup>−/−</sup> | 50.0%      | 5/10            |
| Mfrn1<sup>−/−</sup>Mfrn2        | Mfrn1<sup>−/−</sup>Mfrn2<sup>−/−</sup> | 0.00%  | 0/100           |
| Mfrn1<sup>−/−</sup>Mfrn2<sup>−/−</sup>/pMfrn2-GFP | Mfrn1<sup>−/−</sup>Mfrn2<sup>−/−</sup> | 87.0%      | 47/54           |
| 10 μM FAC Mfrn1<sup>−/−</sup>Mfrn2<sup>−/−</sup> | Mfrn1<sup>−/−</sup>Mfrn2<sup>−/−</sup> | 0.00%      | 0/26            |
| 10 μM TF(Fe<sub>2</sub>)Mfrn1<sup>−/−</sup>Mfrn2<sup>−/−</sup> | Mfrn1<sup>−/−</sup>Mfrn2<sup>−/−</sup> | 0.00%      | 0/15            |
| 2 μM Fe-hinokitiol Mfrn1<sup>−/−</sup>Mfrn2<sup>−/−</sup> | Mfrn1<sup>−/−</sup>Mfrn2<sup>−/−</sup> | 0.00%      | 0/63            |

Our results also show that male fertility is affected by the loss of Mfrn2 with decreased sperm numbers and sperm motility although the phenotype was not fully penetrant. We hypothesize that this may be due to the presence of Mfrn1. Our results in cell proliferation demonstrate that there is an additive effect to the loss of Mfrn1 and Mfrn2 and that these proteins both transport iron into mitochondria. We anticipate that the loss of Mfrn1 would also affect male fertility, however, further investigation into the role of Mfrn1 in male fertility is beyond the scope of this study. It may also be that Mfrn2 is the predominant mitochondrial iron transporter in testes and Mfrn1 does not play a role but that iron can be imported through other low-affinity divalent metal transporters. One prediction of this would be a diminution in other mitochondrial metals due to iron competition, which was seen in livers of low-iron-fed mice in response to loss of Mfrn2. We did not measure mitochondrial metal levels in testes due to limited reagents, however, if this were found to be the case in testes then one could conclude that Mfrn2 is the homologue of dMfrn, as mutations in dMfrn result in male infertility in D. melanogaster (17).

Loss of Mfrn2 alone did not affect mitochondria nonheme iron levels in animals fed normal chow, however, when animals were placed on low-iron chow total mitochondrial iron (Fe and heme-Fe) was slightly increased in Mfrn2<sup>−/−</sup> mouse livers, whereas, mitochondrial cobalt (Co), manganese (Mn), and zinc (Zn) levels were reduced. This suggests that either Mfrn2 transports these metals and does not transport Fe or the loss of Mfrn2 drives Fe import through other mitochondrial metal transporters at the consequence of reduced Co, Mn, and Zn. We speculate that the essential nature of mitochondrial iron would provide the demand to alter metal transport specificity. We did not see significant changes in Cu levels supporting that

Figure 7. Mfrn1 and Mfrn2 are necessary for cell proliferation. A, bone marrow-derived macrophages were isolated from Mfrn1<sup>−/−</sup>Mfrn2<sup>−/−</sup> and Mfrn1<sup>+/+</sup>Mfrn2<sup>−/−</sup> mice, cells were cultured as described previously until actively proliferating (48), treated with or without recombinant Tat-Cre, and 48 h post-Tat-Cre treatment deletion of the floxed Mfrn1 allele was confirmed by RT-qPCR using the primers with β-actin primers as control (Table 3). Error bars represent mean ± S.E., n = 3. B, as cells in A were examined by microscopy 72 h post-Tat-Cre treatment. Representative whole fields are shown. Whole field images were captured with an Olympus tissue culture microscope with a 10× objective. Rounded luminescent Tat-Cre cells represent actively dividing cells and are noted by an asterisk (*). Arrowheads represent regions of cell death. C, skin fibroblasts isolated and immortalized from 3-month old Mfrn1<sup>−/−</sup>Mfrn2<sup>−/−</sup> mice were treated with or without recombinant Tat-Cre and 48 h post-treatment genomic DNA was harvested and PCR for Mfrn1 and Mfrn2 performed using the primers described in Table 2. (†) WT Mfrn1 allele; (f) floxed Mfrn1 allele; and (−) Mfrn1 null allele and Mfrn2 background was always null (−). D, cells in C were labeled with CFSE at time 0 and CFSE levels in the cell populations after 24 and 48 h quantified using flow cytometry. Black bars, Tat-Cre; white bars, + Tat-Cre. The data are expressed as the percentage of time 0 CFSE for 2-3 × 10<sup>6</sup> cells (n = 3). Error bars represent mean ± S.E. ***, p < 0.0003 compared with the control at 24 or 48 h. E, mitochondrial oxidants were measured by flow cytometry using MitoSOX in Mfrn1<sup>−/−</sup>Mfrn2<sup>−/−</sup> cells treated without (Control) or with Tat-Cre. The data are expressed as the mean fluorescence with 1-3 × 10<sup>6</sup> cells per sample (n = 12 replicates). Error bars represent mean ± S.E., ***, p < 0.0001. F, Mfrn1<sup>−/−</sup> and Mfrn1<sup>+/+</sup>Mfrn2<sup>−/−</sup> cells were transfected with a CMV-based plasmid containing Mfrn2-GFP or Mfrn1-GFP (data not shown) and cells grown in G418 to maintain the plasmid. Cells were then treated with Tat-Cre as in A, deletion of the floxed Mfrn1 allele was confirmed and cells split onto glass coverslips and grown in DMEM with or without G418 selection for 14 days. Images were captured using an Olympus BX51 upright epifluorescence microscope with a ×60 oil immersion objective and QuantOne software (Olympus, Melville, NY). Representative fields are shown. n = 10-20 fields/group.
Cu is also essential and would not be outcompeted by Fe in the absence of Mfrn2. Divalent metal transporters have been shown to have different affinities for several metals. Recent studies using an in vitro system have shown that Mfrn1 transports Fe as well as Mn, Co, Zn, and Cu (34). Metal specificity for Mfrn2 has not yet been determined. It may be that in the absence of Mfrn2, Mfrn1 increases Fe transport at the consequence of other metal import. In vitro reconstitution assays are necessary to distinguish between these possibilities. Our liver Mfrn1−/− Mfrn2−/− mitochondrial iron measurements support that Mfrn1 and Mfrn2 transport iron as their loss is additive to the reduction in mitochondrial iron observed.

We were extremely surprised to find viable animals when Mfrn1 was deleted in the livers of whole body Mfrn2−/− mice. We anticipated that animals would not be viable due to the essential nature of iron import into mitochondria for heme and Fe-S cluster formation. Hepatic stipulation during development occurs at day e8.5 to e9.0 and albumin is expressed at this time in the liver bud (35, 36), suggesting that deletion of Mfrn1 should occur before birth. We did not, however, examine Mfrn mRNA in the developing fetus. Our RT-qPCR and Western blotting results showed loss of Mfrn1 mRNA and protein in 3-month-old mice. Studies by Hopkinson and colleagues (37) suggest that during in vitro stem cell to hepatic lineage differentiation, hepatic progenitor cells are primarily dependent upon glycolysis and hepatic maturation depends on mitochondrial respiration, but that both stages of development show some oxidative phosphorylation. Our results support that hepatic lineage is not compromised due to loss of Mfrns, but both Mfrns are necessary when the demand for mitochondrial respiration is increased (e.g. regeneration).
support this conclusion as knockout cells can be generated but loss of both Mfrn1 and Mfrn2 results in an inability to proliferate. We observed that the in vitro loss of proliferative capacity could not be corrected by the addition of exogenous iron (Tf (Fe)₃) or ferric/ferrous iron nor the iron-binding molecule hinokitiol (18). This suggests that simply increasing cellular

Figure 9. Mfrn1 and Mfrn2 are necessary for liver regeneration. A, two-thirds partial hepatectomy were performed on 3-month-old WT (WT), Alb-Cre Mfrn1f/f, Mfrn2−/−, and Alb-Cre Mfrn1f/fMfrn2−/− female mice. Liver sections were fixed in formalin and processed for Ki67 staining immediately after hepatectomy (pre) and 48 h post-hepatectomy as described under “Materials and methods.” Images were captured using an Olympus BX51 upright microscope and QuantOne software (Olympus, Melville, NY) with a ×20 objective and five fields capture per liver. Representative images are shown. B, quantification of Ki67 staining (n = 7–9 animals/genotype with ~200–250 cells/field/five fields) were performed on slides coded by animal ear tag numbers and genotypes were not revealed until after quantification. Student’s t tests were performed and p values were determined compared with the WT (Alb-Cre Mfrn1f/fMfrn2−/−) Ki67 staining. C, Cebp and D, Hist4h4 RNA sequencing reads/transcripts (FPKM) from WT and Alb-Cre Mfrn1f/fMfrn2−/− female mice (Fig. 6A). ****, p < 0.0001 by Student’s t test.
Mfrn1 and Mfrn2 are necessary for cell proliferation

Iron is not sufficient, but that mitochondrial iron transport through Mfrns is necessary during cell proliferation.

The results of the low-iron diet suggest that Mfrns are more important for iron transport under iron-limited conditions. This is also true in yeast where loss of Mrs3 and Mrs4 does not result in a phenotype until cells are grown under low-iron conditions (13, 14, 38). It is interesting to speculate regarding transporters that might compensate for the loss of Mfrns. Candidates suggested from the literature include the mitochondrial calcium uniporter MCU1 (39–41) and pyrimidine transporters Slc25a33 and Slc25a36 (mammalian Rim2 homologues) (42–44). It is also possible that there are other unidentified transporters that can transport Fe under the stress conditions provided by the loss of Mfrns. Further studies are needed to identify other transporters or mechanisms that provide iron during liver development. In conclusion, this study demonstrates that both Mfrns contribute to mitochondrial iron homeostasis and that when proliferation is increased both Mfrn1 and Mfrn2 high-affinity iron transport is necessary.

Materials and methods

Generation of the Slc25a28 targeting vector

To generate the Slc25a28-floxed mouse, exon 4 of mouse Slc25a28 was floxed using standard BAC recombineering techniques in SW106 Escherichia coli cells. C57BL/6 mouse BAC DNA clone (RP23-181M12) was obtained from BACPAC Resources (RRID:SCR_001520) (Oakland, CA) and a Loxp-Neo-Loxp (LNL) cassette was introduced 250 bp upstream of the exon 4 followed by Cre-based recombination resulting in a single Loxp sequence. Next, a Frt-Neo-Frt-Loxp (FNFL) cassette was introduced into the 3′-UTR within exon 4 (339 bp downstream of the stop codon). The targeting construct was then completed by a homologous recombination-based retrieval procedure using a diphteria toxin A (DTA)-Amp′ cassette to include a short arm (2582 bp) at the upstream of the single Loxp and a long arm (4430 bp) at the downstream of the FNFL cassette, respectively. The construct was authenticated by activating Cre recombinase and by DNA sequencing.

Screening of the ES cell clone and genotyping of animals

ES cell clones resistant to G418 and ganciclovir were analyzed for correct homologous recombination by Southern blotting and PCR analyses. For Southern blotting analysis, DNA was purified, digested by SpeI, and probed with a 5′-flank-probe generated as a PCR product using the forward primers M2probeF1 or M2probeF2 and the reverse primers M2probeR1 or M2probeR2 (Table 2). Cells from a single heterozygous clone (Np. 8) were injected into C57BL/6J-derived blastocysts that were implanted into foster mothers. Chimeric animals were identified by coat color, and males were mated with C57BL/6J females to produce mice heterozygous at the Mfrn2 locus (Mfrn2fllox/neos/+). The neomycin-resistance cassette was subsequently excised by recombination of FRT sites by breeding Mfrn2fllox/neos/+ mice with FLP/FLP mice that express FLP recombinase under the Rosa26 promoter generating heterozygous Mfrn2flxox/+ animals. Mfrn2floxox/+ animals were mated with CMV-Cre recombinase mice to generate Mfrn2floxox/−/− animals. Mfrn2floxox/−/− animals were mated to generate Mfrn2floxox/−/− animals. All animals were subsequently genotyped using primers for Mfrn1 and Mfrn2 as listed in Table 2 (Mito1-R7, Mito1-F4, Mito1-R9, Mito2-R, Mito2-F, and EXN4-100).

Animals

Mfrn1flox/flox mice were bred with Mfrn2floxox/−/− animals to generate Mfrn1flox/flox Mfrn2floxox/−/− homozygous animals, which were subsequently bred to Alb-Cre (Jackson Laboratory) to selectively inactivate Mfrn1 in hepatocytes of Mfrn2floxox/−/− animals. In addition, Mfrn2flxox/−/− mice were bred to Alb-Cre (Jackson Laboratory) to selectively inactivate Mfrn2 in hepatocytes. Tail DNA was subjected to PCR for genotyping along with specific tissues. All procedures involving animals were approved by the University of Utah Animal Care Committee. All mice were born and housed in the University of Utah transgenic facility or mouse house facility. Mice were maintained on a standard rodent chow of 350 mg/kg or low-iron chow 2–6 mg/kg (Harland, Teklad). For ALA experiments animals fed ALA in the drinking water for 6 weeks prior to liver porphyrin analyses (8).

Protoporphyrin IX, heme, and nonheme iron measurements

Liver was weighted and 100 mg mixed with water to ~200 μl in a microcentrifuge tube and sonicated for 12 cycles of 5-s intervals at 50% duty (~2.5 s on, 2.5 s off) using a microtip. A 50-μl aliquot was mixed vigorously with 200 μl of an extraction mixture of ethyl acetate (4 volumes) and glyacial acetic acid (1 volume). The phases were separated by microcentrifugation for 1 min at maximum speed. The upper organic layer was immediately analyzed simultaneously for protoporphyrin IX and heme in the HPLC. For porphyrins, 80 μl of the sonicated cell homogenate was mixed with 80 μl of 3 M HCl, incubated at 37 °C for 1 h, and then microcentrifuged at maximum speed for 10 min. The supernatant was analyzed for porphyrins in the HPLC (45). For tissue and mitochondrial nonheme iron, tissues were weighed, 300 μl of TCA–HCl solution added, tissue was homogenized, and incubated at 65 °C for 20 h. Samples were centrifuged at 12,000 × g for 10 min, 10 μl of supernatant was transferred to 96-well–plates, 200 μl of Chromogen reagent (1% bathophenanthroline sulfonate/thioglycolic acid) was added, samples incubated 10 min and read at OD535 nm. Iron levels in samples were calculated using an iron reference (1 mg/ml, Fisher) standard curve.

Table 2

Mouse genotyping primers

| Name              | Sequence                        |
|-------------------|---------------------------------|
| M2probeF1         | ATTTCTCCAGTCTACACTCTCGAG        |
| M2probeF2         | TTTCCTCCGCTTTTAAAGAAGG         |
| M2probeR1         | ACCCGGAGAGTCTGGCCAGTTCC        |
| M2probeR2         | TCTCTGTCCTCCAGTCCAGCTAG        |
| Mito1-R7          | AACCCTCTCAAAAACTTGGG           |
| Mito1-F4          | CCACAACCCTTCTTGTTTCAT          |
| Mito1-R9          | GTTCTCGGCAGCTTTAAAAGT          |
| Mito2-R           | TCGAGTCGTTGAGAAACAACTTCT       |
| Mito2-F           | AAGCTTTCTCATTTTCACAGAGAC       |
| EXN4-100          | CTGGGTGTGGAGACAGGTGTTTT        |
Metabolomics

Metabolite extraction for LC–MS analysis—25-50 mg of each sample was suspended in 450 μl of cold ACN + 0.1 μg/ml of d<sub>9</sub>-carnitine, d<sub>9</sub>-tmam, and d<sub>4</sub>-succinate internal standards and transferred into ceramic bead mill tubes (Omni International, Kennesaw, GA). Distilled water was added to each sample according to 50 μl/sample weight (mg). A process blank was prepared using only ACN and internal standards and 50 μl of water. Samples were processed in a bead mill for 30 s, then chilled at −20°C for 1 h. Samples were then vortexed for 30 s. Samples were centrifuged at 20,000 × g for 10 min and the pellet discarded. The supernatant was transferred to fresh micro-centrifuge tubes.

LC–MS analysis—50 μl of each sample, QC, and process blank solution was added to individual PTFE autosampler vials and randomized prior to analysis. An Agilent 6545 UPLC-QToF (Agilent Technologies, Inc., Santa Clara, CA) run in the positive mode was used for analysis. Separation was achieved using a Waters BEH amide 2.1 × 100-mm column with BEH amide pre-column (Waters Corporation, Milford, MA). An initial concentration of 99% ACN with 5% 10 mM NH₄OAc (A) and 1% 10 mM NH₄OAc in H₂O (pH 7, B) was held for 1.33 min. B was increased linearly to 30% at 5.13 min and again to 60% at 6.33 min. Finally, B was increased to 70% at 6.87 min and held for 2 min. Eluents were returned to starting conditions over 2 min. The system was allowed to re-equilibrate for 4 min between runs.

LC–MS data analysis—Data were collected using MassHunter software (Agilent). Metabolites were identified and their peak area was recorded using MassHunter Quant. These data were transferred to an Excel spread sheet (Microsoft, Redmond, WA). Metabolite identity was established using a combination of an in-house metabolite library developed using pure purchased standards and the METLIN library (RRID:SCR_010500). Further analysis was performed using Metaboanalyst4.0 (RRID:SCR_015539). To determine whether known metabolites were altered we used fold-change threshold = 1.5, p value threshold = 0.05; FDR-adjusted off.

ICP-MS

Mitochondria were isolated from livers of animals fed a low-iron diet for 10 weeks. Isolated mitochondria were subject to ICP-MS. Briefly, purified mitochondria were digested over-night in Optima 68% HNO₃ at room temperature, heated at 95°C for 5 h until dry, and resuspended in 2% HNO₃ for analysis using an Agilent 7900 ICP-MS. Calibration standard solutions for determination of Fe, Zn, Co, Cu, and Mn were prepared from Agilent multielement calibration standard-2A. Protein concentrations of homogenates were determined by BCA Protein Assay (Thermo Fisher Scientific) for normalization.

Partial hepatectomy

Three-month–old female mice were subject to 2/3 partial hepatectomy as described (22). Forty-eight hours post-hepatectomy animals were sacrificed, livers were harvested and histologic examination was performed on pre- and posthepatectomized liver samples using Ki67 staining as a measure of cell proliferation.

Histology

Tissues were isolated from 3-month–old mouse skin (tail or armpit), grown in culture in Dulbecco’s minimal essential medium (DMEM) with penicillin/streptomycin and 10% fetal bovine serum (FBS). Cells were immortalized by force through a senescence crisis. For localization studies, WT fibroblasts were transfected with pmfrn2-GFP or truncated Mfrn2-GFP and Mfrn1-mCherry. Twenty-four hours post-transfection cells were imaged using an Olympus BX51 microscope (×60 oil immersion objective with a 1.3 aperature) and QuantOne software. For deletion of the Mfrn1<sup>flkox/flkox</sup> allele cells were plated on 35-mm tissue culture plates, incubated with recombinant Tat-Cre for 1 h, growth medium was replaced, and cells were grown for 48 h. Genomic DNA or mRNA was prepared and PCR or qPCR were performed to confirm deletion of the Mfrn1<sup>flkox/flkox</sup> allele. Mouse hepatocyte isolation was performed as described (46, 47). Bone marrow–derived macrophages were isolated and grown as described (48). Tissue mitochondria were lysed in 1% octylphenoxypolyethoxyethanol, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.2, 0.5 mM EDTA with 2× protease inhibitor mixture (Roche) and isolated as described (49). Cell culture mitochondria were isolated using the mitochondrial isolation kit from ThermoFisher (catalog number 89874).

Generation of Mfrn1<sup>−/−</sup>/Mfrn2<sup>/−</sup> TET-LV-Tight.HuMFRN1-GFP fibroblasts

Mfrn1<sup>−/−</sup>/Mfrn2<sup>/−</sup> fibroblasts were transduced with two vectors, LV-TetOff and pLV-Tight.HuMFRN1-GFP. The plasmids and viral vectors for the TET-regulated human mitoferrin1-GFP lentiviral system were generated and provided by the CCEH Vector Core at the Fred Hutchinson, Seattle, WA, essentially as described previously (50). Transduced cells were sorted for GFP expression and a stable line generated by selection in puromycin. HuMfrn1-GFP mitochondrial localization was confirmed by epifluorescence microscopy. Cells were treated with recombinant Tat-Cre to delete the chromosomal floxed allele of mouse Mfrn1, clones were isolated (No. 44 and 50) and deletion of Mfrn1 confirmed by PCR.

RNA isolation, RNA-Seq, and RT-qPCR

For RT-qPCR, tissues were homogenized in TRIzol reagent and stored at −80°C prior to RNA isolation. mRNA was extracted using the RNeasy kit from Qiagen. Two μg of total mRNA was used to synthesize cDNA using the High Capacity cDNA Reverse Transcription kit (AB Biosystems). Power SYBR Green Master mix (Life Technologies) was used on a Realplex2
**Mfrn1 and Mfrn2 are necessary for cell proliferation**

**Table 3**

| Gene    | Primer Sequence          | Reference  |
|---------|--------------------------|------------|
| B-Actin | Forward                  | GACGGCCAGTCTACATATTG | This study |
| Slc30a1 | Forward                  | CCACTCCGATCTTGAGGCACGA | This study |
| Hif2a   | Forward                  | GTTACCAAGGAGAATGCGG  | This study |
| Slc39a5 | Forward                  | CAGTGTGGTCTCAGCTCATA | This study |
| Cebpe   | Forward                  | CTGGGGAAGAACAGCTACTG | This study |
| Cyp3a44 | Forward                  | GGTGCTCACGTGTTAAACCTGGC | This study |
| Enpp6   | Forward                  | CAGAGAGATTGTGAACAGAGG | PrimerBank |

Primers for qPCR validated by cloning and sequencing the PCR products. Primers were checked before using this method. Primers were used in this study are listed in Table 3. RNA-Seq was performed to quantify the variation of transcripts among samples. Specificity and efficiency were checked before using this method.

**Other procedures**

Blood was collected from anesthetized animals by retro-orbital eye bleed into heparinized tubes containing anticoagulant citrate dextrose. Hematocrit, hemoglobin, RBC numbers, and mean corpuscular volume were measured. Protein determinations were performed with BCA kit (Pierce). 50 μg of whole cell lysate or mitochondrial lysate with Laemmli buffer was heated to 65°C for 20 min and run on 4–20% Tris glycene SDS-PAGE. Western blotting was performed using the following primary antibodies: rabbit anti-VDAC (1:1000, Abcam), mouse anti-OXPHOS (1:1000, Abcam), rabbit anti-Mfrn1 (1:2000), rabbit anti-GAPDH (1:1000, Thermo Fisher), rabbit anti-GFP (1:2000, GeneTex), rabbit anti-catalase (1:1000, Abcam), and either horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (1:10,000, Jackson ImmunoResearch Laboratory). Western blots were developed using Western Lightning reagent (PerkinElmer Life Sciences). Aconitase activity was assayed as previously described (29). Xanthine oxidase was performed using the Amplex Red xanthine oxidase assay kit from Thermo Fisher. Complex I activity was assayed using Cayman MitoCheck activity kits (Cayman Chemicals).

**Statistical analysis**

Results were analyzed comparing single variables of either genotype or diet using a paired Student’s t test. Significance was noted at: *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. Data are presented as mean ± S.D. or S.E. where stated in the legends.

**Data availability**

All data are contained within the manuscript with the exception of the complete RNA Sequencing data set, which is deposited in the National Center for Biotechnology Information (GSE143925).

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