FAM210B is an erythropoietin target and regulates erythroid heme synthesis by controlling mitochondrial iron import and ferrochelatase activity

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Abstract
Erythropoietin (EPO) signaling is critical to many processes essential to terminal erythropoiesis. Despite the centrality of iron metabolism to erythropoiesis, the mechanisms by which EPO regulates iron status are not well understood. To this end, here we profiled gene expression in a EPO-treated pro-B cell line to identify additional iron regulatory genes. We determined that FAM210B, a mitochondrial inner-membrane protein, is essential for hemoglobinization, proliferation, and enucleation during terminal erythroid maturation. *Fam210b* deficiency led to defects in mitochondrial iron uptake, heme synthesis, and iron–sulfur cluster formation. These defects were corrected with a lipid-soluble, small-molecule iron transporter, hinokitiol, in *Fam210b*-deficient murine erythroid cells and zebrafish morphants. Genetic complementation experiments revealed that FAM210B is not a mitochondrial iron transporter, but is required for adequate mitochondrial iron import to sustain heme synthesis and iron-sulfur cluster formation during erythroid differentiation. FAM210B was also required for maximal ferrochelatase activity in differentiating erythroid cells. We propose that FAM210B functions as an adaptor protein that facilitates the formation of an oligomeric mitochondrial iron transport complex, required for the increase in iron acquisition for heme synthesis during terminal erythropoiesis. Collectively, our results reveal a critical mechanism by which EPO signaling regulates terminal erythropoiesis and iron metabolism.

Introduction
The glycoprotein cytokine erythropoietin (EPO) plays a major role in regulation of erythroid survival and differentiation by activation of signaling pathways via EPO receptor (EPOR) binding (1). Among its roles is the up-regulation of erythroferrone to suppress hepcidin to increase availability of iron during stress erythropoiesis (2, 3). However, the physiological functions of EPO in the regulation of iron metabolism are largely uncharacterized.

Iron, as an ion, and in the forms of iron-sulfur [Fe-S] clusters and heme, is an essential cofactor for redox reactions in diverse cellular processes, such as dopamine synthesis, oxygen transport, mitochondrial respiration, xenobiotic metabolism and maintenance of the circadian rhythm (4–9). However, the majority of iron in the body is used by erythroid cells to synthesize hemoglobin (10, 11). Free iron is highly toxic because of its ability to generate free radicals through the Fenton reaction. Although iron is obtained from outside the cell and is required for reactions that take place in membrane-bound organelles, cellular membranes are not permeable to iron. Organisms have therefore evolved sophisticated mechanisms to efficiently transport iron across membranes (11, 12). In the majority of vertebrate cells, iron is mainly obtained through the transferrin-mediated pathway (13, 14), which is SNX3- and SEC15L1-dependent for efficient transferrin receptor recycling in erythroid cells (15, 16). In the plasma, each transferrin molecule binds two Fe^{3+} ions. Fe-bound transferrin interacts with transferrin receptor on the cell surface. This complex is internalized via clathrin-mediated endocytosis. Within the acidified endosome, Fe^{3+} is reduced to Fe^{2+} by STEAP proteins (17). Fe^{2+} is exported from the endosome by endosomal DMT1 (18–20), and may enter the mitochondrial intermembrane space by DMT1, which is localized on the mitochondrial outer membrane (21). Iron that is used for heme synthesis or iron-sulfur [Fe-S] clusters is imported into the mitochondrial matrix by the mitochondrial iron importer mitoferrins 1 and/or 2 (MFRN1/SLC25A37 or MFRN2/SLC25A28) (22–24). In developing red cells, the stabilization of MFRN1 by ABCB10 (25) is a mechanism by which the cell ensures efficient iron import for its utilization in hemoglobin synthesis (26). However, the ∆mrs3∆mrs4 yeast strain that is deficient in the mitoferrins exhibited a partial defect in mitochondrial iron import. This suggests the existence of other mitochondrial
iron importers (27, 28) or of auxiliary mechanisms that augment iron import to compensate for the genetic insufficiency of an iron transporter.

We identified potential EPO-regulated iron transport genes by microarray analysis of EPO-treated EpoR expressing, pro-B cell line, 32D, and comparing EPO responsive genes to genes that were upregulated during terminal erythropoiesis. One candidate gene, Fam210b (C20orf120), encoded a protein containing an N-terminal mitochondrial-targeting sequence. Fam210b was previously described to be a target of the erythroid transcription factor, GATA1, which encoded a mitochondrial localized protein (29) that regulates mitochondrial metabolism in non-erythroid cells (30). In this study, we decided to interrogate the role of Fam210b in erythroid physiology. Fam210b expression is highly up-regulated during terminal erythropoiesis, which requires large quantities of iron. Loss of function studies in zebrafish embryos, primary murine fetal liver cells and Friend murine erythroleukemia (MEL) cell lines show that FAM210B is required specifically to maintain the massive quantities of mitochondrial iron necessary for heme synthesis during terminal erythropoiesis. While FAM210B is not an iron transporter per se, it interacts with terminal heme synthesis enzymes to form an oligomeric complex. Collectively, our data reveal that FAM210B plays a critical role as an adaptor protein in a mitochondrial iron import oligomeric complex. FAM210B is essential for integration of the rapid iron import with high rate of porphyrin synthesis that supports hemoglobinization during terminal erythropoiesis. This study describes a novel mechanism by EPO governs enucleation and cellular proliferation—processes underlying terminal erythropoiesis, by its control of mitochondrial iron uptake and heme biosynthesis.

**Results**

Fam210b is an immediate early target of EPO during terminal erythroid differentiation.

Maturing erythroid cells acquire large quantities of exogenous iron to keep pace with the enormous demands of hemoglobin production (31, 32). To determine how EPO regulates iron transport, we identified genes upregulated by EPO in the EpoR expressing, pro-B cell line, 32D (33). Cells were cultured in media without erythropoietin for 6 hrs prior to treatment with Epo (0.5U/ml) for 2 hrs before harvesting. We analyzed global gene expression in control and EPO-treated murine 32D cells by microarray analysis. To determine which EPO targets could also be required for erythroid terminal differentiation, we compared the EPO responsive genes in 32D cells, with genes that were enriched in TER119+ cells (34), which comprise the terminally differentiating erythroid cell population. We found that Fam210b expression was enriched in both datasets (Figure 1A). To determine if Fam210b was an EPO early response gene, a parallel experiment was set up in which 32D cells were treated with cycloheximide (10 µg/ml) 30 min prior to EPO stimulation to inhibit protein translation (35, 36). We observed that Fam210b expression was induced by EPO treatment, suggesting that Fam210b is an EPO target. The upregulation of Fam210b expression persisted during cycloheximide treatment, indicating that Fam210b is an EPO target. The upregulation of Fam210b expression persisted during cycloheximide treatment, indicating that Fam210b is an EPO early response gene (Figure 1B). The increase in Fam210b mRNA levels in response to EPO stimulation and during terminal differentiation translated to increases in FAM210B protein. Interestingly, FAM210B protein was also upregulated in the presence of cycloheximide, indicating that EPO increases the post-translational stability of FAM210B (Figure 1C).

To determine how Fam210b is developmentally regulated, we performed RNA sequencing (RNaseq) on transcripts from murine fetal liver erythroid cells (34) that were sorted into fractions corresponding to their stage of maturation (R1–R5) (15, 37–39). Fam210b expression was up-regulated during maturation from R2 to R3, which corresponds to the developmental shift from TER119− to TER119+ expression. This
expression pattern was similar to that of genes involved in heme synthesis and iron import, such as Fech and Tfrc (Figure 1D), and is consistent with the enrichment of Fam210b in the TER119⁺ population (Figure 1A). Fam210B protein expression in the R2-R3 transition was recapitulated at the protein level (Figure 1E). Consistent with this, Fam210B protein levels increase during in vitro differentiation of primary fetal liver erythroid cells (Figure 1F) and in DMSO-induced terminal differentiation of MEL cells (Figure 1G). The increases in protein expression are coordinated with α-globin (HBA) (Figure 1F), TFRC and FECH (Figure 1G), which are critical to erythroid differentiation, heme and hemoglobin synthesis, suggesting that Fam210b plays an essential role in erythroid development.

Although the expression of iron homeostasis genes is sometimes regulated by intracellular iron status via iron-regulatory proteins, IRP1 and IRP2 (13), we found that this was not the case for Fam210B (Supplemental Figure 1). In situ hybridization of E12.5 murine embryos (performed as described (40)) indicated that Fam210b mRNA is enriched in the murine fetal liver at E12.5, the site of definitive erythropoiesis (Figure 2A). In the adult mouse, Fam210b is enriched in the bone marrow, liver, skeletal muscle, which are tissues which require large amounts of heme for synthesis of hemoproteins, and the testis, where iron plays an essential role in spermatogenesis (41)(Figure 2B). In contrast to gata1, fam210b mRNA is maternally expressed in the developing zebrafish embryo. At 24 hpf, expression of fam210b mRNA is restricted to neuronal tissue and the intermediate cell mass (ICM), which is the site of primitive erythropoiesis in teleosts. fam210b is not expressed in cloche embryos (42, 43), which have defective blood and vascular progenitors. Conversely, fam210b expression is expanded in dino embryos. In dino embryos, the tail is expanded at the expense of the head (44). The tail is also the site of the intermediate cell mass (ICM), which is the site of primitive hematopoiesis in the zebrafish. These zebrafish hence have expanded blood due to their ventralized phenotype. The enrichment of fam210b in early hematopoietic tissues in zebrafish embryos, and its absence in mutants that do not make hematopoietic progenitors, and the expansion of its expression in the dino mutants which have an expanded ICM, indicates that fam210b expression is dependent on hematopoietic specification (Figure 2C). At 72 hpf, fam210b expression persists in neural tissue and the liver (Figure 2D), which are tissues which require large amounts of iron for their function (8, 13).

Fam210b is required for erythropoiesis in vivo.

To determine the biological function of fam210b, we injected anti-sense morpholinos to knock down its expression in developing zebrafish embryos. As fam210b is highly expressed in terminally differentiating erythroid cells (Figure 1A, B), we assayed heme synthesis in morphant embryos by o-dianisidine staining as a measure of terminal erythropoiesis. Compared to the controls, fam210b morphants have significantly decreased heme staining, indicative of their anemia (Figure 3A). We quantitated the erythropoietic defect by flow cytometry analysis of GFP⁺ cells in morphant Tg(globin-LCR:eGFP) transgenic zebrafish, which expresses GFP under the control of a globin locus control region (LCR) enhancer, restricting GFP expression to the erythroid lineage (45). Morphant embryos exhibited decreased erythropoiesis at 24- and 48-hpf, highlighting the importance of fam210b in erythropoiesis (Figure 3B).

To determine if Fam210b was required for mammalian erythropoiesis, we knocked down Fam210b in primary fetal liver erythroid cells with shRNA (Figure 3C). Fam210b knockdown caused defects both in cell proliferation (Figure 3F) and in enucleation (Figure 3G), indicating its additional function in erythroid maturation. The independent
validations of the erythroid defect in Fam210b knockdown zebrafish and murine models indicate that the role of FAM210B in terminal erythroid maturation is highly conserved in vertebrates. In higher vertebrates, continuous FAM210B expression in the erythron is required for optimal erythroid cell proliferation, maturation, hemoglobinization, and enucleation.

Fam210b is localized to the mitochondrial inner membrane.

To delineate the cellular mechanism by which FAM210B regulates erythropoiesis, we transfected fam210b-GFP into HEK293T cells and examined its subcellular localization. Fam210b contains three hydrophobic stretches of ~21 amino acids, suggesting that FAM210B is an integral membrane protein. Confocal immunofluorescence microscopy revealed that FAM210B-GFP co-localized with COX4, a mitochondrial resident protein (Figure 4A). Subcellular fractionation and western analysis of primary fetal liver cells confirmed that FAM210B specifically co-localized with HSP60, a mitochondrial resident protein (Figure 4B).

We carried out further localization studies in MEL cells stably expressing Fam210b to determine the sub-mitochondrial localization of FAM210B. To determine the localization of FAM210B in the mitochondrial outer membrane, purified mitochondria were treated with proteinase K, followed by centrifugation to separate the intact mitochondria (P) from soluble proteins (S) (Fig. 4C). Immunoblotting showed that TOMM20, an outer membrane protein with a large cytosolic domain, was degraded upon protease treatment, whereas FAM210B and control proteins YME1 (intermembrane space) and PreP (matrix) were intact. FAM210B is therefore not an outer mitochondrial membrane protein.

The mitochondria were then subjected to osmotic shock to determine if FAM210B was in the intermembrane space or matrix (Figure 4C). Mitoplasts, which contain the inner membrane and matrix components and intermembrane space/outer membrane components that stick to the mitoplasts were recovered by centrifugation (P), while soluble proteins (S) remained in the supernatant. The mitoplasts were subsequently treated with a low dose of proteinase K to degrade proteins that were in the intermembrane space. FAM210B and PreP were resistant to protease digestion, whereas intermembrane space protein YME1 was degraded as expected. As a control for proteinase K activity, the mitoplast were treated with Triton X-100, thereby releasing membrane bound proteins into the supernatant and exposing digestion sites to enzymatic digestion. The addition of proteinase K to the Triton X-100 detergent-treated samples resulted in complete degradation, with the exception of PreP that has a tightly folded domain that is resistant to the low doses of proteinase K. These data suggested that FAM210B was bound to the mitochondrial inner membrane.

To rigorously confirm that this was the case, mitochondria were subjected to sodium carbonate extraction (46), followed by centrifugation, to separate membrane proteins (pellet, P) from soluble proteins (S) (Fig. 4D). FAM210B and membrane-bound TIMM23 were recovered in the pellet, whereas soluble matrix-localized HSPA9 (mortalin) was released in the supernatant. Thus, FAM210B is an integral membrane protein residing in the inner membrane. The collective data indicate that FAM210B is integrated in the inner membrane, and any soluble domains are likely facing the matrix, because protease addition during osmotic shock did not markedly degrade the full-length protein. A smaller fragment, which was sub-stoichiometric, was detected detected in the pellet (Figure 4C, marked with a triangle), which may indicate that the N- or C-terminus is facing the intermembrane space and is digested by protease that may have leaked into the intermembrane space. The smaller fragment sediments with the pellet, suggesting that the segment of FAM210B that is exposed to the protease is tightly associated with the membrane.
FAM210B facilitates mitochondrial iron import during terminal erythropoiesis

To determine the biochemical function of FAM210B in heme synthesis, we generated Fam210b deficient MEL cell lines by CRISPR/Cas9 genome editing (Figure 5A). Metabolic 55Fe-labeling revealed that undifferentiated Fam210b<sup>-/-</sup> MEL cells had normal rates of heme synthesis and iron uptake compared to wildtype controls (Figure 5B, C). However, when MEL cells were chemically induced to differentiate, heme synthesis and iron uptake in Fam210b<sup>-/-</sup> MEL cells was significantly impaired (Figure 5D, E). The heme synthesis defect in Fam210b<sup>-/-</sup> MEL cells was due to a deficiency in mitochondrial iron acquisition (Figure 5F, G). As FAM210B is required for optimal mitochondrial iron uptake, we assayed the activity of mitochondrial aconitase (EC4.2.1.3) in FAM210B<sup>+/+</sup> cells. Mitochondrial aconitase activity is sensitive to mitochondrial iron deficiency (47) caused by defects in mitochondrial iron transport, and this is reflected by the aconitase defect in Mfn1<sup>-/-</sup> MEL cells (Figure 5H) (23, 28). Fam210b<sup>-/-</sup> cells had a decrease in mitochondrial aconitase activity that is comparable to Mfn1<sup>-/-</sup> cells, indicating that it plays a critical role in mitochondrial iron metabolism.

The heme synthesis (Figure 6A) and iron transport (Figure 6B) defects in Fam210b<sup>-/-</sup> MEL cells were functionally complemented to the level of wild-type cells by hinokitiol (CAS 499-44-5; ChEBI:10447), a natural product that binds iron and autonomously transports both Fe(II) and Fe(III) across lipid membranes to bypass defects in iron transport (48). Fe-hinokitiol functionally complemented the erythropoietic defect in fam210b morphant zebrafish embryos (Figure 6C), demonstrating that the primary role of FAM210B is to facilitate mitochondrial delivery of iron for the synthesis of heme and [Fe-S] clusters in the developing erythroid cell. C2-deoxy hinokitiol, which does not bind or transport iron, did not chemically complement the anemia in Fam210b<sup>-/-</sup> cells (Figure 6A and 6B), indicating that the ability of the Fe-hinokitiol to rescue heme synthesis was directly dependent on the ability of hinokitiol to chelate and transport iron across membranes (48). The specificity of the functional complementation is further demonstrated by the restoration of both 55Fe and 55Fe-heme only in cells defective for iron transport, such as the Dmt1 knockdown cell line (19, 20) but not in cells with a porphyrin transport defect, such as the Tmem14c CRISPR cell line (38).

HPLC analysis of protoporphyrinogen IX (PPIX) content in differentiating Fam210b<sup> +/-</sup> cells indicated that FAM210B was not required for synthesis of the tetrapyrrole ring in the mitochondria (Supplemental Figure 2A). This was further confirmed by the inability of deuteroporphyrin IX (DP), a PPIX analog that can chemically complement porphyrin synthesis defects (38), to rescue heme synthesis in Fam210b<sup>-/-</sup> cells (Supplemental Figure 2B).

To exclude the possibility that the heme defect in FAM210B deficiency was caused by a lesion in mitochondrial biogenesis or physiology, we stained control and Fam210b<sup>-/-</sup> cells with Mitotracker dye and analyzed mitochondrial staining by flow-cytometry. The percentage of Mitotracker positive cells and average Mitotracker-dye staining did not differ between control and Fam210b<sup>-/-</sup> cells, indicating that Fam210b deficiency did not cause defects in cell survival (Supplemental Figure 2C), mitochondrial membrane potential, and mitochondrial mass (Supplemental Figure 2D).

FAM210b is not a direct mitochondrial iron carrier in vertebrate erythroblasts.

To determine if FAM210B functions as a mitochondrial iron importer or if it played a role in regulation of FECH, we carried out an in vitro heme synthesis assay to compare the amount of iron transported across the mitochondrial membrane to synthesize deuteroheme (49). 55Fe and DP were added to isolated mitochondria from wild-type and Fam210b<sup> +/-</sup> MEL cells. DP was added in excess. As DP is lipid soluble, any
differences in the synthesis of 55Fe-DP are caused by alterations in FECH activity or iron transport across the mitochondrial membrane. In the absence of FAM210B, in vitro heme synthesis decreased to background levels comparable with cells treated with NMMP, a PPIX analog that inhibits FECH and cannot be metallated (50)(Figure 6D). These data suggested that FAM210B is required for either iron import or heme synthesis. To determine which was the case, we performed a FECH activity assay as described (http://cihd.cores.utah.edu/wp-content/uploads/2016/06/FECH_Activity_Assay.pdf). Using this method, cells are disrupted by sonication and treatment with Tween 20 detergent, allowing the substrates, mesoporphyrin IX and zinc access to FECH without the need to cross membranes. FECH activity is measured by quantification of the product Zn-mesoporphyrin IX by HPLC. In the absence of a mitochondrial membrane, we observed that Fam210b-- cells exhibited decrease in FECH activity (Figure 6E, left). This was not attributable to a decrease in FECH protein levels (Figure 6E, right). Based on Figures 6D and 6E, FAM210B is required for optimal FECH activity in erythroid cells. However, we observed that the decrease in FECH activity was far less significant when membranes were disrupted (Figure 6E) compared to when the mitochondrial membrane was intact (Figure 6D). We concluded that FAM210B is required both for full activation of FECH, but also plays a role in facilitating iron transport across the mitochondrial membrane for heme synthesis.

To determine if FAM210B is a bona fide iron transporter, we expressed epitope-tagged Fam210 or Mfrn in the Δmrs3Δmrs4 yeast strain (denoted as Δmrs3/4), deficient in the Mfrn orthologs (23), or in Mfrn2 deficient primary murine fibroblasts (Ward DM and Kaplan J, unpublished), which are deficient in mitochondrial iron transporter Slc25a28 (22). Mouse FAM210B, with the N-terminal mitochondrial targeting propiece from Mrs3, properly targeted to the mitochondrial compartment in yeast (Figure 6F), Supplemental Figure 3). Δmrs3/4 yeast exhibited Fe-auxotrophy when grown on Fe-deficient media, which was genetically complemented by vertebrate Mfrn1 but not Fam210b (Figure 6G), suggesting that FAM210B does not directly function as a Fe-solute carrier.

We next treated mouse embryonic fibroblasts from Mfrn2-- with varying concentrations of desferrioxamine (DFO), an iron chelator, for 48h. As cells require iron for basic cellular processes such as respiration, defects in cellular iron uptake and utilization would decrease cellular viability with DFO treatment. Conversely, increasing the expression of iron transporters would increase the efficiency of iron uptake and utilization in cells, resulting in increased survival. As expected, the expression of Mfrn2-GFP in Mfrn2-- fibroblasts significantly improved cellular viability in DFO-treated cells (Figure 6H). In contrast, Fam210b co-expression did not significantly enhance fibroblast viability above the basal control, demonstrating that Fam210b could not genetically complement Mfrn2 deficiency (Figure 6H). However, FAM210B significantly increases the survival of wild-type fibroblasts in a wide range of DFO concentrations beyond that of Mfrn2-GFP expression (Figure 6I). As FAM210B augments iron import in wild-type but not in Mfrn2-- MEFs, we propose that FAM210B increases the efficiency of mitochondrial iron import by a MFRN-dependent mechanism.

FAM210b is in an iron-import oligomeric complex to enhance heme synthesis.

To further characterize the biochemical function of FAM210B in an oligomeric complex, we examined MFRN1 levels in Fam210b-- MEL cells to determine if Fam210b plays a role in Mfrn1 stability analogous to ABCB10, as MFRN1 is the predominant erythroid mitochondrial iron transporter (22, 23, 25). MFRN1 protein levels were similar in control and Fam210b-- mitochondria, indicating that FAM210B does not play a role in the maintenance of MFRN1 stability (Supplemental Figure 2E).
Immunoprecipitation of FAM210B that was ectopically co-expressed with MFRN1 in HEK293T cells showed that FAM210B and MFRN1 do not interact (Figure 7A). In contrast, we co-immunoprecipitated ectopically expressed FAM210B with PPOX and FECH (Figure 7B), which are terminal enzymes in the heme synthesis pathway. As the ectopically expressed proteins are expressed at levels far higher than endogenous FAM210B, PPOX and FECH or potential bridging proteins that can mediate indirect interactions, it is likely that the physical interactions between FAM210B, PPOX and FECH are direct. Notably, PPOX and FECH are in the same complex as MFRN1 (Chen et al., Blood 2010; Medlock et al., 2015). The specificity of these protein-protein interactions is assured by the lack of COPX co-immunoprecipitation with FAM210B (Figure 7B). To further determine if FAM210B can interact with FECH at endogenous levels, we generated a MEL cell line that stably expressed FAM210B-FLAG. We immunoprecipitated FAM210B with FLAG antibodies in differentiating cells. Western blot analysis of the immunoprecipitated protein showed that FAM210B FLAG interacted with endogenous FECH in vivo (Figure 7C). FAM210B does not homo-oligomerize, as would be expected if FAM210B were a membrane embedded solute carrier (Figure 7C). Collectively, these results are consistent with a role for FAM210B as an auxiliary factor in an iron transport oligomeric complex that includes the terminal heme biosynthesis enzymes in the mitochondria. The intact oligomeric complex is essential for maintaining the high rate of mitochondrial iron import that is required to fulfill the demands for heme synthesis in maturing red cells.

**Discussion**

Our study demonstrates that FAM210B, a protein of previously unknown function, is required for mitochondrial iron import to support sustained synthesis of heme, iron-sulphur cluster and hemoglobin during erythroid maturation. In addition, FAM210B regulates FECH enzyme activity. Our results are consistent with, and extend the findings of a recent study identifying \textit{Fam210b} as a GATA1 transcriptional target that is required for erythroid differentiation (29). Mitochondrial mass, membrane potential and porphyrin levels are normal in \textit{Fam210b–/–} cells, precluding a role for FAM210B in housekeeping mitochondrial homeostasis or tetrapyrrole synthesis, respectively. Although heme synthesis and iron import was decreased in \textit{Fam210b} deficient cells, we did not observe an accumulation in porphyrin, similar to the phenotype in \textit{Mfrn1} deficiency (51, 52). This is attributable to impaired [Fe-S] cluster biosynthesis in \textit{Fam210b} deficient cells (Figure 5H), which elevates IRP1 RNA binding and suppresses \textit{Alas2} mRNA translation and porphyrin accumulation (52, 53).

FAM210B deficiency can be functionally complemented by the addition of hinokitiol, which acts as a small molecule mimetic of iron transporters (48). However, \textit{Fam210b} overexpression cannot genetically complement the iron transport defect in \textit{Δmrs3/4} yeast or \textit{Mfrn2–/–} fibroblasts. We therefore conclude that the primary function of FAM210B is to facilitate mitochondrial iron import in differentiating erythroid cells by functioning as an auxiliary factor in promoting formation of an oligomeric iron-transport complex with terminal heme synthesis enzymes, thereby increasing the kinetics of mitochondrial iron import to keep pace with its cellular demands for heme. Our studies on FAM210B are the first to describe an EPO-induced mitochondrial chaperone that is tissue- and differentiation stage-specific, revealing a novel mechanism by which cells regulate intracellular iron metabolism. We previously identified a mitochondrial chaperone, CLPX, which facilitates the delivery of the vitamin B6 cofactor required for the catalytic activity of ALAS, the enzyme that catalyzes the committed step of protoporphyrin synthesis (54). The oligomerization of MFRN1, ABCB10, and FECH integrates the efficient import of iron with its metallation to form
heme during erythroid maturation (26). We further note that the use of small molecules that autonomously perform protein-like functions as a distinct type of biological probe, as demonstrated herein with the transmembrane iron transporter hinokitiol, may be more widely applicable and have complementary strengths to other experimental approaches (48, 55).

Fam210b is transcriptionally activated by GATA-1 (29, 56), consistent with its expression in erythropoietic tissues. Iron chelation by DFO, or iron supplementation by iron citrate did not cause changes in its steady-state protein expression, indicating that Fam210b is not regulated by cellular iron status (Supplemental Figure 1). EPO regulates Fam210b expression both at the levels of mRNA transcription and protein stability, suggesting that Fam210b is an essential component of EPO’s function and signaling in erythropoiesis. The upregulation of Fam210b by EPO in EpoR expressing 32D pro-B cells suggests that Fam210b is direct target of EPO. Although Fam210b is localized in the mitochondria, it is required for cellular processes that take place during terminal erythroid differentiation, such as proliferation, and finally, enucleation. These data suggest a link between nuclear processes in terminal erythroid differentiation and mitochondrial iron metabolism, and also demonstrate a novel mechanism by which EPO regulates the biology of terminal erythropoiesis via its regulation of cellular iron uptake.

While published GWAS studies did not reveal any extant association between Fam210b mutations and hematologic traits (57–61), the profoundly anemic phenotype of the Fam210b morphant embryos and the heme defects in primary murine fetal liver and in MEL cells predict that mutations in Fam210b may lead to hematologic and iron metabolism defects in humans. As Mfrn1 genetic or splicing defects have been shown to be modifiers of porphyria (62) or sideroblastic anemia (63), we predict that detailed genetic studies will reveal the function of Fam210b mutations in patients suffering from anemias and porphyrias of unknown etiology. As Fam210b−/− cells only exhibited heme and iron metabolism defects in differentiating erythroid cells, it is expected that Fam210b mutations will exhibit a phenotype specifically in the terminally differentiating erythroid cell population.

To adapt to their role as oxygen carriers in vertebrates, erythroid cells have evolved several mechanisms to efficiently transport and utilize iron within the cell for adequate heme synthesis. This is critical as mitochondrial iron import is tightly coupled with heme synthesis and cells cannot preload iron in the mitochondria (64). In addition to the housekeeping mitochondrial iron importer Mfrn2, erythroid cells express Mfrn1 during their terminal maturation (22, 23). The tissue-restricted regulation at the transcriptional level for Mfrn1 at “erythroid super-enhancer” by GATA-factors (65, 66), is reminiscent of the transcriptional regulation of 5-aminolevulinate synthase (ALAS), the first and rate-limiting enzyme in the heme biosynthetic pathway (67). Like the Mfrn genes, these ALAS genes are encoded by two separate genes, encoding distinct ALAS isoenzymes. The ubiquitous ALAS1 is regulated by heme via the peroxisome proliferator-activated 1α (PGC-1α) (68). The erythroid-enriched ALAS2 and Mfrn1 genes are not regulated by heme. The post-translational regulation of ALAS2 is mediated by the intracellular levels of iron and iron-responsive protein 1 (IRP1) binding to its cognate iron-responsive element (IRE) in the 5′ untranslated region (UTR) of the ALAS2 mRNA (69); however, no such regulation by iron on IRE have been reported and ascribed to the Mfrn genes.

During terminal erythropoiesis, Mfrn1 protein stability is also enhanced by oligomerization with ABCB10 (25, 26). Mfrn1 and ABCB10 are in a physical complex with Fech, which kinetically favors the coupling of iron transport and heme synthesis. The addition of Fam210b, which forms an oligomer with Ppox and Fech to enhance mitochondrial iron import and heme synthesis, fits into this experimental model where Fam210b can
nucleate or stabilize the formation of a complex between PPOX and FECH. In this manner, it can facilitate the transfer of PPIX to FECH and promote iron import coupled with heme synthesis (Figure 7D). While PPOX and FECH physically interact, they do not form a tight complex. This supports the idea that bridging molecules, such as FAM210B, may mediate the strength of the interaction, hence modulating the rate of iron import and heme synthesis (70).

FAM210B plays a second role in heme synthesis by activating FECH enzymatic activity (Figure 6D, E). It is well established that red cells increase the expression of ferrochelatase protein during erythropoiesis. This occurs either by increase in FECH mRNA transcription(71) or stabilization of FECH protein by binding of iron-sulfur clusters (72). In contrast, FAM210B deficiency does not alter FECH protein levels (Figure 6E), suggesting that its effects on FECH activity may be mediated by the requirement for FAM210B for efficient iron delivery to FECH, and/or by playing a role in allosteric activation of the FECH enzyme. These data add to our understanding of the mechanisms by which Epo signaling regulates heme synthesis by post-translational regulation of FECH(49) and suggest mechanisms by which the activity of heme synthetic enzymes; in this case, FECH, may be rapidly modulated in response to metabolic requirements.

As Fam210b is also expressed in non-differentiating erythroid cells, neural and hepatic tissues, it may also play a role in these tissues that is independent of heme synthesis and iron metabolism. Since FAM210B is a mitochondrial protein, detailed sequencing studies may reveal mutations in FAM210B that play a role in modifying the severity of mitochondriopathy in these tissues. Our identification of Fam210b as a novel mitochondrial protein that is required for optimal mitochondrial iron uptake during terminal erythropoiesis thus provides a novel genetic tool for further studies on vertebrate erythropoiesis and mitochondrial biology.
Materials and Methods

Cell lines.
MEL DS19 subclone cells were obtained from Arthur Skoultchi (Albert Einstein College of Medicine, New York, NY, USA). Human HEK293T cells were used for transient transfections.

GenBank accession numbers.
Zebrafish fam210b (c20orf108), NM_001033751; mouse Fam210b, NM_025912.

Knockdown of Dmt1 and Fam210b by shRNA hairpins in mouse cells.
Dmt1 (GenBank NM_008732.2) stable knockdown MEL clones were obtained by stable transfection of an shRNA hairpin, TRCN0000079535. Fam210b (Genbank NM_025912) stable knockdown MEL clones were obtained by transfection of shRNA hairpins: shFam210b-1, TRCN0000103732; shFam210b-2 TRCN0000103734. shRNA hairpins were obtained from Sigma-Aldrich. MEL clones were electroporated with shRNA plasmids by electroporation and stable clones were obtained by limiting dilutions and selection with G418 (Dmt1).

CRISPR design and cloning.
CRISPR guide sequences were designed to direct cleavages at genetic loci to generate chromosomal deletions (73, 74). CRISPR guide sequences were designed to have a unique 12 bp seed sequence 5’-NNNNNNNNNNNNGG-3’ in the mouse genome (http://www.genome-engineering.org) to minimize off-target cleavages. Exons 1 and 3 of the Fam210b locus were targeted. The exon 1 tgt1 targeting sequence was: 5’-GCTCGCGGCGCGCATGGCC-3’; exon 1 tgt2 targeting sequence was 5’-TCGGGCGTCGCCAACCAGGAT-3’ and the exon 3 targeting sequence was 5’-TACCGGGAGCAGGACCTATGGAC-3’. Exons 2 and 4 of the Mfrn1 locus were targeted. The exon 2 targeting sequence was: 5’-GATGCTTTGTATACCGGGCTT-3’; the exon 4 targeting sequence was 5’-GAAGAACTCATACACGGGACC-3’.

CRISPR guides were cloned into pX330 plasmid (Addgene) with BbsI ligation as previously described (75).

CRISPR/Cas9 genome editing in MEL cells.
CRISPR/Cas9 constructs were delivered to MEL DS19 cells by electroporation. CRISPR/Cas9 constructs were co-electroporated with pEF1α at a 9:1 ratio. Two days later cells were resuspended in puromycin media (5 µg/ml). Cells were plated by limiting dilution at 0.3 cells per well in 96-well plates and cultured at 37°C. Clones were screened by dilution at 0.3 cells per well in 96-well plates and cultured at 37°C. Clones were validated by western analysis for FAM210B protein; Mfrn1 gene expression was assayed by qRT-PCR (48). The PCR primers to genotype Fam210b CRISPR 1 were: mFam210b_F 5’-CTATAGAGCCCCGCCCTA-3’ and mFam210b_R 5’-TCGGTCAGGTGCAATGTAAA-3’. The PCR primers used to genotype Fam210b CRISPR 2 and 3 were: E3F 5’-GCATAGACATGTCTGCAATCC-3’ and E3R 5’-AGCCACCUUGCACAACAGCC-3’. Fe radiolabeling and radio Fe-heme measurements.

55FeCl3 (specific activity: 1.28 Ci/mmol) (Perkin Elmer) were loaded onto transferrin as described previously (Roy et al., 1999). Metabolic labeling with 55Fe-Transferrin and quantitation of the incorporation of Fe into heme were carried out as described previously (38).

ICP analysis of mitochondrial iron content.
Mitochondrial iron was measured as previously described (76). Isolated mitochondria were treated with nitric acid.
and sample iron content was determined by using a Perkin-Elmer Inductively Coupled Plasma (ICP) Optical Emission Spectrometer. The results were normalized to mitochondrial protein content.

**HPLC analysis of heme and porphyrins.**
HPLC analysis was carried out with MEL cells that were differentiated for 72h with 1.5% DMSO. A cell pellet spun down from a 30-50 mL culture was mixed with water to about 200μL in a microfuge tube and sonicated for 12 cycles of 5-s intervals at 50% duty (about 2.5 sec on, 2.5 sec off) using a microtip. A 50 μL aliquot was mixed vigorously with 200 μL of an extraction mixture of ethyl acetate (4 volumes) and glacial 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 (38, 51).

**Silencing of Fam210b in mouse primary fetal liver cells (MPFL).**
Silencing of *Fam210b* and hemoglobin quantification in MPFL was carried out as described (77). The retroviral plasmids expressing shRNAs for *Fam210b* were transfected in a packaging HEK293T cell line. The collected retroviral supernatants were added to erythroid precursor cells purified from E14.5 mouse fetal liver (C57BL/6J). The retrovirally-transduced cells were sorted for GFP expression using a FACSria machine (BD Biosciences), followed by *ex vivo* erythroid terminal development in Epo-only medium for 48 hours. After culture, cells were collected for analysis on enucleation and hemoglobin content. The hemoglobin content was quantified with Drabkin’s reagent used human hemoglobin as standards.

**Fetal liver proliferation and enucleation assays**
To measure proliferation rates, GFP positive cells were plated in 24-well plates with a density at 100,000 cells/per ml, cultured in Epo-only erythroid differentiation medium. The number of cells was counted at the 24th hour and 48th hour using a hemocytometer. In each time point, the cell numbers were the average of three biological samples, which were the average of two technical duplicates. To measure the rate of enucleations, after 48 hours of culture, one million cells were spun down and re-suspended with 100 μL FACS buffer (PBS+2%FBS+100 μM EDTA). Anti-Ter119 antibodies conjugated with APC (eBioscience) and Hoechst 33342 (Sigma) were added to stain the cells for 10 minutes at room temperature following manufacturer protocols. After staining, cells were washed with cold PBS twice, and re-suspended with the FACS buffer containing propidium iodide (PI, Sigma) to distinguish between live and dead cells, and further analyzed on a FACS Fortessa machine. The details of protocols were previously described (78).

**Functional complementation assays with chemicals in cultured cells.**
MEL cells were differentiated chemically with 2% DMSO for 72 hours. For functional complementation assays with hinokitiol, cells were concurrently treated with 10μM iron citrate and Hinokitiol (1μM) or C2dOHino (1μM) (48). Chemical complementation with DP was carried out by treating cells with 5 μM DP (Frontier Scientific) as described (38).

**Sub-mitochondrial localization studies.**
Crude mitochondria were isolated from MEL or primary fetal liver cells using a mitochondrial isolation kit (Pierce). For mitochondrial sublocalization studies, MEL cells stably expressing mouse Fam210b were harvested at confluency and lysed in homogenization buffer (20 mM HEPES pH 7.4, 220 mM mannitol, 70 mM sucrose) supplemented with 2 mg/ml BSA, and 0.5 mM PMSF using a Teflon-dounce homogenizer to release mitochondria. The lysates were centrifuged at 100 x g at 4°C for 5 minutes. The post-nuclear supernatant was centrifuged at 10,000 x g for 10 minutes to obtain mitochondria pellets. The pellets were
subsequently washed with homogenization buffer without BSA and PMSF and mitochondria were collected by centrifugation. The protein concentration was measured using BCA assay according to the vendor’s protocols (Thermo Scientific). For mitochondrial fractionation, mitochondria (equivalent of 50 μg mitochondrial protein per reaction) were suspended in homogenization buffer, 20 mM HEPES (for osmotic shock) or 20 mM HEPES with 1% TX-100 in the presence or absence of 5 μg/ml Proteinase K for 30 min at 4°C. Proteinase K was inactivated with the addition of 1 mM PMSF. Samples were centrifuged at 10,000 x g at 4°C to obtain the pellet that contained mitoplasts (the matrix and inner membrane). The supernatant was collected and precipitated with trichloroacetic acid (TCA, final concentration of 15% TCA) to obtain soluble proteins. The proteins (50 μg per lane) were separated by SDS-PAGE followed by immunoblot analysis. Mitochondria (equivalent of 50 μg mitochondrial protein per reaction) were suspended in homogenization buffer or 0.1 M sodium carbonate (pH 11.5) for 30 min at 4°C. Following, the samples were centrifuged at 28,000 x g for 30 min at 4°C to obtain the pellet of precipitated membrane proteins. The supernatant with soluble proteins was TCA-precipitated. The proteins (50 μg per lane) were separated by SDS-PAGE followed by immunoblot analysis.

**Western analysis.**

The following antibodies were used in this study: TIMM23 (BD Biosciences #611222), TOMM20 (Santa Cruz Bioscience clone FL-145), Mortalin (Proteintech #1488-14-AP), and PreP and Yme1L polyclonal antibodies were raised against recombinant proteins (Pacific Immunology). Immunoblotting of FAM210B was performed using a custom anti-mouse FAM210B polyclonal antibody generated against three peptides, (C-LSHPVPDARLLRTARGDC, C-TGTEKKLSRTQQLKKVC and C-KLGFKESLVQSKMAC), and immune-affinity purified against these antigenic peptides (Genemed Synthesis, Inc., San Antonio, TX). Anti-FECH polyclonal (C-20), HA (Y-11), Hemoglobin α (H-80) and HSP60 (K-19) antibodies were obtained from Santa Cruz (Athens, GA). Anti-TFRC (H68.4) was obtained from Invitrogen. Anti-GAPDH was obtained from Pierce (GA1R). Anti-FLAG M2-HRP was obtained from Sigma-Aldrich (Cat #A8592). HSP90 antibody was obtained from Cell Signaling (C45G5). For Fig 7C, we used anti-FECH polyclonal antibodies from Kerafast (EGA191).

**qRT-PCR**

qRT-PCR probes were obtained from Invitrogen. Murine Fam210b probe, Mm00508881_m1; Hprt probe: Mm01545399_m1. Zebrafish fam210b probe, Dr03426307_m1; hprt probe: Dr03138604_m1. The mouse tissue cDNA array was purchased from Clontech (Mountain View, CA).

**Chemical hinokitiol and functional complementation in zebrafish.**

24 hpf morphant embryos in the transgenic Tg(globinLCR:eGFP) line were dechorionated with pronase as described (54) and incubated in the presence of 1 μM Hinokitiol + 10 μM FeCitrate for another 48 h (48). For specificity, the control, inactive C2deoxy hinokitiol was 1 μM final concentration with Fe citrate. Vehicle treated embryos were exposed to 0.01 mM DMSO. Control or morphant embryos at roughly 72 hpf were mechanically homogenized as previously described for flow cytometry (45, 54).

**Genetic complementation assays.**

Genetic complementation of the Δmrs3/4 yeast strain with chimeric vertebrate Fam210b and Mfrn1, containing the first N-terminal 23 amino acid residues from yeast Msr3 subcloned in the pRS426-ADH vector, was performed as previously described (23). Western analysis of the total, mitochondrial and cytosolic fraction was as previously described with anti-FLAG and anti-porin antibodies.
Control and Mfrn2−/− mouse embryonic fibroblasts (MEF) were isolated from the skin of 3-month old mice and immortalized by forcing through a senescence crisis. Mfrn2-GFP or Fam210b-HA was transiently expressed in these MEF cells. Transfected MEF cells were treated with DFO (10–50 μM). Cellular viability was then assayed using a cell titer blue assay, which measures conversion of resazurin to resorufin on a 96 well format (Promega).

**Immunoprecipitation of FAM210B and its interacting proteins**

CPOX-FLAG, PPOX-FLAG, FECH-FLAG and FAM210B-HA were cloned into the pEF1 vector and expressed under the control of the EF1 promoter. pEF1/FAM210B-HA was co-transfected with pEF1 empty vector, pEF1/CPOX-FLAG, pEF1/PPOX-FLAG, or pEF1/FECH-FLAG into 10 cm³ dishes of HEK 293T cells using a Lipofectamine 2000 reagent based method (Invitrogen). Cells were harvested 48h post-transfection and lysed in a buffer containing 1% Nonidet P-40, 300 mM NaCl, 50 mM Tris, pH 8.0 and protease inhibitor cocktail (Pierce). Cell lysates were precleared with mouse IgG-agarose (Sigma) for 60 min at 4°C. Precleared lysates were then incubated with anti-FLAG or anti-HA agarose beads (Sigma) for 2h at 4°C. Beads were washed 3x 15 min in lysis buffer. Immunoprecipitated proteins were subsequently eluted with Laemmli buffer and resolved by SDS-PAGE.

To determine if FAM210B interacted with endogenous FECH, we electroporated pEF1/FAM210B-FLAG into MEL cells and selected FAM210B-FLAG expressing single cell clones by limiting dilution. pEF1/FAM210B-HA and pEF1 empty vector expressing cell lines were differentiated with 1.5% DMSO for 72h. For each immunoprecipitation experiment, we lysed 100 million differentiated cells with 1 ml of lysis buffer (77). 2 mg of protein was used as the starting material for each immunoprecipitation sample. Samples were precleared with mouse IgG beads and immunoprecipitated with anti-FLAG M2-agarose for 4h at 4°C. Beads were washed for 3x15 min at 4°C. Proteins were eluted with Laemmli buffer, resolved by SDS-PAGE and analyzed by western blot.

**Statistics**

Statistical analysis was carried out using 2-tailed paired or unpaired Student’s t test. Significance was set at P < 0.05. All data points were plotted as scatter plots, representing all replicates of each experiment.

**Study approval**

All zebrafish experiments were performed in accordance with the Brigham and Women’s Hospital’s Institutional Animal Care and Use Committee (IACUC) regulations. Wild-type AB zebrafish were used for all zebrafish experiments. All mouse experiments were performed in accordance with IACUC regulations at the Massachusetts Institute of Technology and University of Utah.

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Conflicts of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
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Figure 1. *Fam210b* is an EPO early response gene and is induced in terminally differentiating erythroid cells. (A) Microarray analysis of EPO-treated 32D pro B cells shows that *Fam210b* is an EPO responsive gene that is also highly enriched in the terminally differentiating, TER119⁺ population of fetal liver erythroid cells (34). (B) qRT-PCR demonstrates that Epo treatment of the EpoR expressing pro-B cell line, 32D, upregulated expression of *Fam210b* mRNA. This upregulation persists in the presence of cycloheximide (CHX), an inhibitor of protein translation, demonstrating that *Fam210b* is an EPO early response gene \( n = 6 \). * \( p < 0.05 \), Student t-test. (C) FAM210B protein levels are up-regulated in response to EPO treatment of the EpoR expressing 32D pro-B cell line. The increase in protein levels persist with CHX treatment, indicating increased stability. Changes in FAM210B protein expression normalized to GAPDH are quantitated relative to control levels. (D) RNAseq analysis of primary murine fetal liver cells sorted according to TER119 and CD71 (R1-R5) expression demonstrates upregulation of *Fam210b* during the R2-R3 transition. (E) This up-regulation is recapitulated by western analysis of FAM210B protein expression. (F) FAM210B protein expression is up-regulated during *in vitro* differentiation of primary fetal liver cells. (G) FAM210B protein is induced upon terminal differentiation of MEL cells in parallel with genes required for heme synthesis, TFRC and FECH.
Figure 2. Expression of Fam210b is enriched in vertebrate tissues with high heme content. (A) Fam210b expression (red pseudocolor) is enriched in the murine fetal liver (FL) at E12.5. (B) In murine tissues, Fam210b expression is enriched in the bone marrow and fetal liver (erythroid), adult liver, skeletal muscle and testis, all of which require high levels of iron for their function (n=3). (C) Zebrafish fam210b is maternally expressed during early embryonic development. At 24 hpf, fam210b expression is enriched in neural tissue and the intermediate cell mass, the site of primitive hematopoiesis (top). The expression of fam210b in the ICM is abolished in cloche embryos, which do not form hematopoietic or vascular tissue. In contrast, fam210b expression in the intermediate cell mass is expanded in dino embryos, which exhibit a ventralized phenotype (bottom), paralleling the increased expression of erythroid gata1. (D) At 72 hpf, fam210b expression persists in the neural tissue and is enriched in the liver, delineated by sid4 expression.
Figure 3. *Fam210b* is continuously required for terminal erythroid differentiation. (A) Knockdown of *fam210b* in zebrafish embryos with 2 independent antisense morpholinos caused a defect in erythroid hemoglobinization. (B) *Tg(globin LCR:eGFP)*, *fam210b* morphant zebrafish exhibited a decrease in GFP+ erythroid cells, indicating defective erythropoiesis n=4, * p-value < 0.05 Student t-test. (C) shRNA mediated knockdown of *Fam210b* in primary fetal liver cells decreased FAM210B protein expression. (D) *Fam210b* knockdown primary fetal liver cells had a decrease in hemoglobin content. (E) The hemoglobinization defect in murine *Fam210b* knockdown cells is complemented by human *FAM210B*. (F) *Fam210b* knockdown in primary fetal liver cells caused a cell proliferation defect. (G) *Fam210b* knockdown caused an enucleation defect, indicative of erythroid maturation arrest. n=3, * p-value < 0.05 Student t-test.
Figure 4. FAM210B localizes to the mitochondrial inner membrane. (A) Confocal fluorescence microscopy of exogenously expressed FAM210B-GFP (green) in HEK293T cells indicated that FAM210B co-localized with COXIV (red), a mitochondrial resident protein. The overlap is indicated in the merged panel (yellow). (B) Subcellular fractionation of primary fetal liver cells showed that FAM210B co-sedimented with HSP60 (mitochondrial) but not with HBA and GAPDH (cytoplasmic), indicating its mitochondrial localization. (C) Isolated mitochondria (Mito, T; lanes 1-3) purified from a MEL cell line stably expressing mouse Fam210b, was treated with Proteinase K, which degraded outer membrane proteins (TOMM20) but not intermembrane space proteins (YME1L) or matrix (PreP) proteins (lane 2). Inner mitochondrial proteins, which were impervious to proteinase K treatment, (P) were separated from soluble (S) proteins by centrifugation. FAM210B
co-sedimented with the pellet fraction (lane 2). Mitoplasts (lanes 4-11) were generated by subjecting intact mitochondria to osmotic shock, exposing inner membrane and intermembrane space proteins to proteinase K digestion. Mitoplasts were centrifuged to separate the soluble proteins (S) from the mitoplast pellet (P). Proteinase K treatment degraded a fraction of FAM210B (lane 6) and completely degraded YME1L but not PreP, suggesting that at least a fraction of FAM210B was situated in the mitochondrial inner membrane or intermembrane space. Triton X-100 treatment liberated FAM210B, PreP and YME1L from the pellet into the soluble fraction (lane 9). These solubilized proteins were digested by proteinase K, demonstrating specificity of assay (lane 10). The asterisk marks a non-specific band that was detected with the mouse FAM210B antibody; the triangle marks a likely FAM210B degradation product; and the square marks a core of PreP that is tightly folded and resistant to protease treatment. (D) To determine if FAM210B was an integral inner membrane protein, isolated mitochondria (T) were analyzed by carbonate extraction. Pellet (P) and supernatant (S) fractions were analyzed by immunoblotting for FAM210B. The blot was probed for TIMM23 as an integral membrane protein control, and mortalin as a soluble protein control. An asterisk marks non-specific bands detected by the FAM210B antibody.
Figure 5. *Fam210b* is required for heme synthesis by facilitating mitochondrial iron import. (A) *Fam210b*<sup>-/-</sup> MEL cell lines generated by CRISPR/Cas9 expressed no detectable FAM210B protein. (B) <sup>55</sup>Fe metabolic labeling showed that undifferentiated *Fam210b*<sup>-/-</sup> MEL cell clones did not cause changes in heme synthesis (n=3) or (C) iron uptake (n=3) (right). (D) Differentiated *Fam210b* knockout MEL cells exhibited defective heme synthesis (n=6) and (E) iron uptake (n=5). (F) Mitochondria from differentiated *Fam210b* knockout cells had decreased <sup>55</sup>Fe-Tf uptake (n=5). (G) ICP-MS demonstrated that mitochondria from *Fam210b* knockout cells had a decrease in total iron (n=6). (H) Mitochondrial aconitase activity, which is dependent on [Fe-S] cluster synthesis, is decreased in *Fam210b*<sup>-/-</sup> cells indicating a defect in mitochondrial iron acquisition. *Mfrn1*<sup>-/-</sup> MEL clones served as controls for mitochondrial iron deficiency (n=4). * p < 0.05; § p < 0.1 Student t-test. All data are normalized to wild-type controls.
Figure 6. *Fam210B* increases the kinetics of mitochondrial iron import in differentiating MEL cells by functioning as an auxiliary factor. (A) $^{55}$Fe metabolic labeling confirmed a decrease in heme synthesis in differentiating *Fam210b* (CRISPR1-3), *Tmem14c* and *Dmt1* deficient MEL cells (black bars). The addition of hinokitiol, a lipid soluble iron carrier (white bars) restored heme synthesis in *Fam210b* and *Dmt1* deficient cells, but not *Tmem14c* deficient cells. C2-deoxy hinokitiol, which does not chelate iron, did not complement heme synthesis in the *Fam210b* knockout cells (gray bars) (n=6). (B) $^{55}$Fe metabolic labeling confirmed an iron uptake defect in differentiating *Fam210b*, *Tmem14c* and *Dmt1* deficient cells (black bars). The iron uptake defect in *Fam210b* and *Dmt1* deficient cells was chemically complemented by hinokitiol (white bars). Hinokitiol did not rescue iron uptake in *Tmem14c* deficient cells, which have a porphyrin synthesis defect. C2-deoxy hinokitiol did not rescue the Fe deficiency in *Fam210b* deficient cells (gray bars) (n=6). (C) Treatment of *fam210b* morphant zebrafish embryos (MO) with iron citrate and hinokitiol (MO+ Hino/Fe) rescued their anemia (n=8). (D) FECH activity was measured in intact mitochondria isolated from wild-type and *Fam210b* deficient MEL cells, using $^{55}$Fe and deuteroporphyrin (DP) as substrates. FECH activity in *Fam210b* deficient mitochondria was approximately 1/3 that of wild-type controls. Both wild-type and *Fam210b* deficient mitochondria had very little measurable FECH activity when NMMP was used as a
substrate (n=4). (E) FECH activity was measured in isolated mitochondria from wild-type and Fam210b -/- MEL cells that were treated with detergent and disrupted by sonication, allowing access of reaction substrates to FECH in the absence of intact membranes. FECH activity was decreased in Fam210b -/- mitochondria, but not to the extent of intact mitochondria (n=3). (F) FLAG-tagged MFRN1 and FAM210B colocalized with porin in Δmrs3/4 yeast, indicating correct mitochondrial localization. (G) Mfrn1 expression complemented the growth defect of Δmrs3/4 yeast in low-iron media, while Fam210b expression did not. (H) Survival of Mfrn2-/- fibroblasts in DFO is significantly complemented by expression of Mfrn2-GFP, but less so by expression of Fam210b-HA (n=3). (I) Expression of Fam210b-HA and Mfrn2-GFP in wildtype fibroblasts increase cell survival in the presence of DFO, with Fam210b overexpression possessing a protective effect over a larger dose range than Mfrn2-GFP. n = 3, * p < 0.05; § p < 0.1 Student t-test.
Figure 7. FAM210B directly interacts with terminal heme synthesis enzymes but does not with MFRN1 (Slc25a37). (A) Immunoprecipitation of co-expressed FAM210B-HA and FLAG-MFRN1 demonstrates FAM210B does not directly interact with MFRN1. (B) FAM210B-HA interacts with the terminal enzymes of the heme synthesis pathway, PPOX and FECH, but not CPOX. MFRN1, CPOX, PPOX and FECH are all localized to the mitochondrial inner membrane. (C) Immunoprecipitation of FAM-210B FLAG stably expressed in differentiating MEL cells shows that ectopically expressed FAM210B interacts with endogenous FECH. (D) FAM210B-HA and FAM210B-FLAG do not interact, demonstrating that FAM210B does not homo-oligomerize. (E) Model of the role of FAM210B as an adaptor protein in an oligomeric complex with terminal heme enzymes, required for terminal erythropoiesis and mitochondrial iron importation.
FAM210B is an erythropoietin target and regulates erythroid heme synthesis by controlling mitochondrial iron import and ferrochelatase activity


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