FAM210B is dispensable for erythroid differentiation in adult mice

Mark Perfetto, PhD[1], Catherine M. Rondelli, PhD[2], Samantha Gillis[2], Amber N. Stratman, PhD[3], Yvette Y. Yien, PhD[1*]

[1]Pittsburgh Heart Lung and Blood Vascular Medicine Institute and Department of Medicine, Division of Hematology/Oncology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA.
[2]Department of Biological Sciences, University of Delaware, Newark, Delaware, USA.
[3]Department of Cell Biology and Physiology, Washington University in St Louis School of Medicine, St Louis, Missouri, USA.
*To whom correspondence may be addressed: yieny@pitt.edu

Abstract

Iron plays a central role in cellular redox processes, but its ability to adopt multiple oxidation states also enables it to catalyze deleterious reactions. The requirement for iron in erythropoiesis has necessitated the evolution of mechanisms with which to handle the iron required for hemoglobinization. FAM210B was identified as a regulator of mitochondrial iron import and heme synthesis in erythroid cell culture and zebrafish models. In this manuscript, we demonstrate that while FAM210B is required for erythroid differentiation and heme synthesis under standard cell culture conditions, holotransferrin supplementation was sufficient to chemically complement the iron-deficient phenotype. As the biology of FAM210B is complex and context specific, and whole-organism studies on FAM210 proteins have been limited, we sought to unravel the role of FAM210B in erythropoiesis using knockout mice. We were surprised to discover that *Fam210b*$^{-/-}$ mice were viable and the adults did not have erythropoietic defects in the bone marrow. In contrast to studies in *C. elegans*, *Fam210b*$^{-/-}$ mice were also fertile. There were some modest phenotypes, such as a slight increase in lymphocytes and white cell count in *Fam210b*$^{-/-}$ females, as well as an increase in body weight in *Fam210b*$^{-/-}$ males. However, our findings suggest that FAM210B may play a more important role in cellular iron homeostasis under iron deficient conditions. Here, we will discuss the cell culture conditions used in iron metabolism studies that can account for the disparate finding on FAM210B function. Moving forward, resolving these discrepancies will be important in identifying novel iron homeostasis genes.

Introduction

Iron plays an essential role as a redox center in iron-sulfur clusters, heme and iron-binding proteins (1,2). Most iron in vertebrate organisms is used by developing erythroid cells for hemoglobin production, but iron metabolism also regulates processes related to terminal erythropoiesis, such as proliferation and nuclear condensation (3-5). Iron-containing proteins are also required for numerous other cell-specific processes such as dopamine production (6,7), adipocyte function (8,9) and proliferation of hepatocytes in response to injury (10), as well as essential “housekeeping” processes such as mitochondrial respiration and DNA synthesis (11,12). Despite the diversity of

cellular processes which require iron, the mechanisms that control tissue iron allocation in the body and the intracellular regulation of iron fate are not well understood.

Fam210b was previously identified to be a transcriptional target of EPO and GATA1 whose expression was enriched in erythroid cells (5,13). Erythroid FAM210B is an inner mitochondrial membrane protein that facilitates the mitochondrial import of iron and its incorporation into heme (5,13). FAM210B regulates metabolism in several tissues (5,13-15) and its biology is complex. In ovarian cancer cells, FAM210B promotes glycolysis (14). In *C. elegans*, the FAM210 homolog is required for reproduction via its regulation of oogenesis (16). More recently, Suzuki et al. demonstrated that when HiDEP cells-- fetal globin expressing erythroid cells derived from induced pluripotent cells (iPS)-- are differentiated in the presence of holotransferrin and 20% FBS, the absence of FAM210B actually *promotes* erythroid terminal differentiation (15). These divergent findings suggested that our knowledge of FAM210B function is context specific and dependent on tissue type, source of tissue, and cell culture conditions.

Currently published studies on FAM210B have largely been performed on cells in culture. We previously determined that FAM210B was required for maximal heme synthesis, proliferation, and maintenance of cell number in *Fam210b* knockdown primary murine fetal liver erythroid cells in which and zebrafish embryos (5). While we completed this work to describe the role of FAM210B in fetal/embryonic tissue, no work had addressed the role of FAM210B in adult erythroid niches. To perform a more extensive *in vivo* study and to investigate the erythropoietic phenotype of adult *Fam210b*$^{-/-}$ mice, we took advantage of the whole body *C57BL6N/6N-*^{Atm1Brd}*Fam210b*$^{tm1b(KOMP)/Mbp}$ /MbpMmucd mouse line established by the Mutant Mouse Resource and Research Center (MMRRC) at University of California at Davis. We found that FAM210B is dispensable for erythroid differentiation and heme synthesis in the adult bone marrow. Interestingly, *Fam210b*$^{-/-}$ mice are viable and reproduce normally, unlike their *C. elegans* counterparts. Mice do not require maternal *Fam210b* mRNA for the initial stages of embryognesis as the *Fam210b*$^{-/-}$ offspring of a *Fam210b*$^{-/-}$ homozygote incross are viable and survived until they were sacrificed at around 1 year of age. In light of previous findings, we discuss several possibilities to reconcile the seeming discrepancies in the role of FAM210B in erythroid biology.

Results

To determine the extent to which immortalized erythroid cells model the role of FAM210B in erythropoiesis, we first verified that differentiated *Fam210b*$^{-/-}$ murine erythroleukemia (MEL) cells are hemoglobin deficient (Figure 1A, B). Both non-differentiated and differentiated *Fam210b*$^{-/-}$ MEL cells are larger than wild-type cells, indicating a defect in erythroid terminal differentiation (Figure 1C). We previously described FAM210B's main erythroid phenotype as a defect in mitochondrial iron metabolism under cell culture conditions where the only iron sources were DMEM and 10% FBS (5). Recent work by Suzuki *et al.* (15) reported that their HiDEP erythroid cells were differentiated in serum containing 20% FBS and a transferrin supplement, which provides significantly more bioavailable iron than what was used in our previous work. Therefore, we postulated that increased iron content in the HiDEP cell

differentiation media could have chemically complemented the iron deficiency and hemoglobinization defects in *Fam210b*$^{-/-}$ cells, masking the phenotypes which we had previously observed (5).

To determine if we could replicate the findings of Suzuki *et al.*, we cultured *Fam210b*$^{-/-}$ MEL cells in 10% FBS with transferrin supplement, 20% FBS alone, or 20% FBS with transferrin supplement and quantitated benzidine staining as a metric of hemoglobinization. For this experiment, we decreased the staining time to 5 min to remain in the linear range to allow us to observe increases in hemoglobin content caused by iron treatment (Figure 1D, E). Consistent with our previous results using fetal murine cells, there was a significant decrease in hemoglobinization in *Fam210b*$^{-/-}$ cells relative to wild-type cells when grown in either 10% or 20% FBS. However, addition of a holotransferrin supplement increased the hemoglobinization in *Fam210b*$^{-/-}$ cells such that their benzidine staining signal does not significantly differ from wild-type cells. These data indicate that the elevated levels of transferrin-bound iron in HiDEP cell culture media may have masked phenotypes that manifest during differentiation under iron-deficient conditions, where FAM210B's role is likely to be most important. While the work on *Fam210b* deficient HiDEP cells does support that FAM210B is required for mitochondrial function and metabolism, it is interesting to note that other mitochondrial respiratory complex proteins were upregulated in *Fam210b* deficient HiDEP cells, suggesting a level of genetic compensation is occurring within these cells to allow for erythroid cell survival and differentiation. Finally, because *Fam210b*$^{-/-}$ primary erythroid cells experienced proliferation defects, we were curious to determine if this defect could be recapitulated in *Fam210b*$^{-/-}$ MEL cells. MEL cells did not recapitulate the cell cycle defects in primary cells, indicating the limits of immortalized cell lines as experimental models (Figure 1F). Together, these findings—that addition of holotransferrin is sufficient to rescue hemoglobinization in *Fam210b*$^{-/-}$ cells to wild-type levels—should be considered particularly important to note when studying erythroid cells, which predominantly rely on transferrin bound iron for hemoglobin synthesis (17).

There are currently few published studies on the role of FAM210B in intact organisms, as most work has been performed on cell lines. We previously attempted to generate zebrafish *fam210b* knockout mutants using CRISPR/Cas9 with no success. The genomic region was too poorly annotated and contains extensive repetitive regions, making gRNA design and genotyping challenging. Our zebrafish experiments therefore used morpholino-mediated gene suppression (5) to investigate the role of *Fam210b* in zebrafish development. In this work, we showed that *fam210b* was required for embryonic erythropoiesis and erythroid heme synthesis and that iron supplementation could restore erythropoiesis in *fam210b*-deficient zebrafish (5). However, we were not able to follow the full course of development to adulthood because of the transient nature of morpholino-mediated knockdown.

To address this gap, we took advantage of *C57BL6N/6N-*^{Atm1Brd} *Fam210b*$^{tm1b(KOMP)/Mbp}$/MbpMmucd (from here on *Fam210b*$^{-/-}$) full body knockout mice generated by the Mutant Mouse Resource and Research Center (MMRRC) at the University of California at Davis. Crosses of *Fam210b*$^{+/-}$ mice resulted in 25% of pups with the *Fam210b*$^{-/-}$ genotype, indicating that they were viable. To determine if FAM210B was required for fertility in mice, we crossed *Fam210b*$^{+/-}$ to *Fam210b*$^{-/-}$ mice which resulted in 48.4% *Fam210b*$^{-/-}$ offspring, indicating that *Fam210b*$^{-/-}$ did not have fertility

defects, a difference from what Kang *et al.* see with FAM210 loss in *C. elegans* where it is required for oogenesis and reproduction (16). Our previous studies showed that zebrafish *Fam210b* is maternally expressed (5). To determine if maternal *Fam210b* was required for the survival of *Fam210b*$^{-/-}$ mice, we bred *Fam210b*$^{-/-}$ mice to one another. We obtained 100% of *Fam210b*$^{-/-}$ pups, indicating that maternal FAM210B is not required for survival of *Fam210b*$^{-/-}$ embryos (Table 1). As *in vivo* erythropoiesis in *Fam210b*$^{-/-}$ fetuses was sufficient for them to survive to birth, we did not carry out studies on fetal liver erythropoiesis. *Fam210b*$^{-/-}$ mice survived to adulthood until they were sacrificed at about 1 year of age.

Globally verified knock-out of *Fam210b* expression by semi-quantitative RT-PCR of *LacZ* that was analyzed on an agarose gel (Figure 2A) and suppression of the *Fam210b* transcript (Figure 2B) using Taqman probes. Male *Fam210b*$^{-/-}$ mice were heavier than wild-type and heterozygote males, although we did not observe any difference in the weights of female *Fam210b*$^{-/-}$ mice relative to wild-type controls (Figure 2C). Because of our previous studies suggesting that *Fam210b* is required for erythroid terminal differentiation, we measured splenic weights, as mouse spleens are an extramedullary site of stress erythropoiesis (18). To our surprise, there was no significant difference in the weights (Figure 2D) or gross morphology (Figure 2E) between wild-type and *Fam210b*$^{-/-}$ spleens. There were a few *Fam210b*$^{-/-}$ females that had enlarged spleens (Figure 2E), but the high variance in spleen weight decreased the level of statistical significance. Peripheral blood smears from adult wild-type and *Fam210b*$^{-/-}$ mice did not reveal any gross abnormalities in erythroid cell morphology (Figure 2F).

We conducted complete blood count (CBC) analysis to determine if there were any hematological defects in adult *Fam210b*$^{-/-}$ mice. We observed an increase in white blood cell (Figure 3A) and lymphocyte (Figure 3B) counts in *Fam210b*$^{-/-}$ females. Monocyte counts were decreased in *Fam210b*$^{+/-}$ females, while there was no statistical difference between wild-type and *Fam210b*$^{-/-}$ mice (Figure 3C). There was no difference in neutrophil counts (Figure 3D). Of interest, there were no significant differences in red cell counts, hemoglobin, and hematocrit between WT, *Fam210b*$^{+/-}$ and *Fam210b*$^{-/-}$ mice of either sex (Figure 3E-G). Consistent with the HiDEP cell findings reported by Suzuki et al., we observed a modest increase in mean corpuscular volume and mean cellular hemoglobin in *Fam210b*$^{-/-}$ mice (Figure 3H-I). The are no significant differences in mean cellular hemoglobin concentration or red cell distribution width (3J, K). We did not observe changes in platelet count or platelet volume (3L-M) though there were slight changes in platelet distribution width (Figure 3N) which were unlikely to be biologically significant.

To determine if the modest changes in mean corpuscular volume and cellular hemoglobin were caused by alterations in erythropoiesis, we analyzed erythropoietic populations in the bone marrow. Bone marrow smears did not reveal any gross abnormalities between *Fam210b*$^{+/-}$ and *Fam210b*$^{-/-}$ animals (Figure 4A). We followed up by quantitating erythroid progenitor populations in the bone marrow as described by Liu (19). Briefly, we depleted the bone marrow of lymphocyte populations using anti-CD45 beads. The remaining cells were sorted by their expression of CD44 and Ter119, and forward scattered into progenitor populations (representative FACS plots in Figure 4B). These populations were denoted as populations I-VI on the leftmost panels in

Figure 4B, corresponding to erythroid cells at progressively mature stages of differentiation. Population I corresponds to proerythroblasts, while population VI corresponds to the terminally differentiated red cell population. We quantitated the proportion of each progenitor population in the adult bone marrow in Figure 4C. Consistent with the findings described by Suzuki et al., we observed that there was a trend towards decreased numbers of erythroid progenitors (populations I-III—corresponding to the proerythroblast, basophilic erythroblast and polychromatic erythroblast populations), but a significant increase in *Fam210b*$^{+/-}$ reticulocytes (population V). There was an approximately 50% increase in *Fam210b*$^{-/-}$ reticulocytes relative to wild-type mice, but statistical significance was blunted due to experimental variance. The percentage of terminally differentiated red cells was similar across all 3 genotypes, consistent with the CBC data (Figure 4C). The gross morphology of sorted erythroid cell populations was also similar across all 3 genotypes, suggesting that adult erythroid cells are largely refractory to FAM210B deficiency (Figure 4D).

Collectively, our data indicate that FAM210B is not required for terminal erythroid differentiation or hemoglobinization within the context of the adult mouse bone marrow. We observed a slight but significant increase in lymphocyte counts in female *Fam210b*$^{-/-}$ mice, suggesting that FAM210B may play a role in regulating other adult hematopoietic lineages.

Discussion

This manuscript describes the first study of FAM210B's role in the regulation of adult erythropoiesis *in vivo*. Our previous studies reported that Fam210b is required for erythroid heme synthesis and terminal erythropoiesis in zebrafish embryos and primary murine fetal liver erythroid cells (5). Our results in zebrafish embryos and *shFam210b* primary fetal liver cells that were grown *ex vivo* correlate with the requirement of FAM210B for heme synthesis in the K562, HiDEP, and MEL erythroid cell lines. Compellingly, expression of *Fam210b* mRNA was able to restore cell proliferation and enucleation of primary murine fetal liver erythroid cells, and we were able to restore the number of erythroid cell progenitors in *fam210b* morphant zebrafish embryos by supplementing them with iron (5). Collectively, this previously published work shows that Fam210b is required for iron metabolism and erythroid differentiation in embryonic/fetal tissues, it is expressed in tissues that have high heme demand (particularly in terminally differentiating erythroid cells and liver during development), and it plays a functional role in regulating biology at these sites (5,13). Additionally, the *C. elegans Fam210* ortholog is required for reproduction, primarily by maintenance of mitochondrial biology in oocytes. Published work therefore shows strong evidence for the importance of FAM210B in metabolism and early development (5,16). We therefore used *Fam210b*$^{-/-}$ mice to examine the role of FAM210B within the context of adult mammalian biology.

Based on the preponderance of the above evidence, we expected that the *Fam210b*$^{-/-}$ mouse phenotype would be pre-embryonically lethal from anemia, due to its requirement for fetal liver erythroid cell hemoglobinization and differentiation, as we had observed in primary cell culture (5) . We were therefore surprised that murine FAM210B was dispensable for reproduction and embryonic viability in mice. The survival of

Fam210b$^{-/-}$ mice to adulthood suggested that any erythropoietic defects caused by *Fam210b* deficiency were compensated by genetic or environmental factors. Suzuki et al. demonstrated that differentiating *Fam210b*$^{-/-}$ HiDEP erythroid cells hemoglobinized normally, even though non-differentiating cells were hemoglobin deficient. An important note is that HiDEP cells were differentiated in the presence of 20% FBS used for OP9 feeder cell co-culture. In addition, a holotransferrin supplement was added to provide bioavailable iron. We predicted that the 20% FBS concentration, with holotransferrin supplementation was likely sufficient to chemically complement the iron transport defect present in *Fam210b*$^{-/-}$ HiDEP cells (15). To support this hypothesis, we replicated the hemoglobinization of *Fam210b*$^{-/-}$ MEL cells when treating them with the same holotransferrin supplement, even when cells were grown in 10% FBS. These data are consistent with our previous results demonstrating that supplemental iron, in the presence of hinokitiol (an iron chelator that can transport iron across membranes down a concentration gradient), restores the erythroid differentiation and hemoglobinization defects in *Fam210b*$^{-/-}$ erythroid cells that were cultured in 10% FBS, restoring both hemoglobinization. Iron-hinokitiol also restores erythroid cell number *in vivo,* in *fam210b* morphant zebrafish (5). In HiDEP cell culture, transferrin supplement was added at a concentration of 0.06uM. As one transferrin molecule carries 2 Fe^{3+} ions, this is an effective concentration of 0.12uM of supplemental bioavailable iron. We used 10uM iron citrate in our experiments as a supplement, which is effectively impermeable to cellular membranes unless chelated with hinokitiol. In our previous work, 1 uM of hinokitiol was used. As it binds to iron as a tridendate complex, the maximum potential concentration of iron transported by hinokitiol is approximately 0.3uM (20), which is within a similar concentration ranged used by Suzuki et al (15). The anemia observed in *fam210b* morphant zebrafish embryos (which rely on iron stores in the yolk as their only bioavailable source) was rescued by 10 uM iron-citrate complexed with 1 uM hinokitiol dissolved in egg water to rescue the *fam210b* defects. These conditions supplied to the zebrafish for these studies are far more iron-deficient than cell culture serum, yet clearly supports the idea that you can bypass the need for Fam210b in embryos by supplying bioavailable iron.

The access to bioavailable iron, in the form of holotransferrin or other iron chelates, is very likely why *Fam210b*$^{-/-}$ mice are viable and able to develop mature erythroid cells. In vertebrates, bone marrow and fetal liver erythroid cells predominantly obtain iron from holo-transferrin *in vivo*, in niches known as erythroblastic/erythromyeloblastic islands (21,22). Erythroblastic islands consist of a central macrophage surrounded by differentiating erythroid and myeloid cells. Erythroid cells remain in close spatial proximity to the central macrophage from the proerythroblast stage to enucleation, where they proliferate, differentiate and hemoglobinize. The central macrophage of the erythroblastic island is a major source of iron for differentiating erythroid cells (23-25), expressing high levels of iron transport proteins such as transferrin, ferroportin and ferritin (26). Ferritin-bound iron from the erythroblastic macrophage is known to be a major source of iron for the differentiating erythroid cell (3), with the erythroblastic island and its surrounding extracellular matrix creates localized regions of highly concentrated bioavailable iron. This local “sink” of iron is recapitulated by the culture conditions of differentiating HiDEP employed by Suzuki et al. (15). Therefore, it is probable that high local concentrations of iron are

able to circumvent hemoglobinization defects in *Fam210b*$^{-/-}$ erythroid cells. We note that the holotransferrin concentration in fetal human serum is approximately 5uM, far higher than the concentrations adopted in cell culture experiments described across research groups (which generally contain 10-20% serum) (27). *In vivo* conditions may therefore be completely adequate to compensate for genetic deficiencies in iron metabolism that can be observed in cell culture experiments.

Finally, the fetal liver and bone marrow environments are hypoxic (28,29), which increases intracellular iron uptake relative to normoxic conditions (such as in standard tissue culture) via the upregulation of genes such as *Dmt1* and *TfR1* (30-32). In combination with high local holotransferrin levels, this is likely able to compensate for *Fam210b* deficiency *in vivo*. In comparison, zebrafish erythroid cells do not enucleate and are not known to form erythroblastic islands. Further, zebrafish embryos grow under normoxic conditions where passive diffusion is initially sufficient for tissue oxygenation in the absence of blood (33). Zebrafish obtain iron from their yolk stores, before they begin eating at 5-6 dpf. In contrast, mammalian embryos obtain their iron from maternal circulation (34). These factors underlie why *fam210b*-deficient zebrafish erythrocytes appeared severely anemic, while *Fam210b*$^{-/-}$ fetuses were able to produce sufficient numbers of erythrocytes for viability to the point of birth.

Suzuki et al. reported that FAM210B may play a role in ATP generation in erythroid cells via its interaction with mitochondrial proteins and metabolic reprogramming. However, we did not observe any obvious developmental or functional defects in *Fam210b*$^{-/-}$ mice. Male *Fam210b*$^{-/-}$ were heavier than their wild-type and female counterparts, which warrants closer investigation of body composition and cellular oxygen consumption. Of note, substrate concentration in anatomical and developmental niches (such as iron in the erythroblastic island) can sometimes mask defects in metabolic genes. For example, ATPIF1/IF1, initially observed to be important for erythropoiesis in zebrafish embryos (35), was shown to be dispensable for development and reproduction in mice (36). Another hypothesis is that there may be other uncharacterized compensatory mechanisms that exist in mammals that have not been fully appreciated.

Our studies demonstrate the importance of combining whole animal studies with *ex vivo* studies on cultured primary cells and cell lines to fully dissect the mechanistic roles of proteins regulating iron transport and metabolism. FAM210B's role in cellular homeostasis is highly complex. Our collective data indicates that careful control of cell culture conditions is essential for carrying out and interpreting studies on nutrient metabolism *ex vivo*. Our current studies indicate that FAM210B is dispensable for erythropoiesis and iron uptake in the context of normal mammalian physiology, but may play a role in metabolic and erythropoietic regulation under iron-deficient conditions. Further studies on the role of FAM210B under conditions where erythroblastic islands are disrupted, or in iron deficient mice will be informative moving forward. The ability of mice to function in the absence of FAM210B also raises the possibility that human FAM210B mutations may function as modifiers of disease severity in patient disorders of iron metabolism or erythropoiesis.

Methods and materials

Cell culture

Murine erythroleukemia (MEL) cells were a gift of Dr. Arthur Skoultchi at the Albert Einstein College of Medicine. The Fam210b-/- MEL cells were previously generated in this study (5). Cells were cultured in DMEM with high glucose, supplemented with 1% glutamine and 10% FBS unless otherwise indicated.

Heme staining

o-Dianisidine staining was performed as previously described (37).
Cells were stained in the dark for 10 minutes at room temperature unless otherwise indicated. Imaging was conducted using a Zeiss Axio Imager.A2 and ZEN Blue software (version 3.1). Heme staining was quantitated using ImageJ.

Cell size analysis

$2.5*10^5$ MEL cells were mounted onto a slide and imaged with a Zeiss Axio Imager.A2 and ZEN Blue software (version 3.1). Cell size was calculated in ImageJ by measuring the area of individual cells.

Mice

C57BL6N/6N-^{Atm1Brd} Fam210b$^{tm1b(KOMP)/Mbp}$/MbpMmucd (*Fam210b$^{-/-}$*) whole body knockout mice were generated by UC Davis and purchased from MMRRC. *Fam210b* was genotyped by Transnetyx using primers flanking exon 2 with the following sequences: forward: CTCTGGGTCAGCGAGACAAC, reverse: AGGACTGAGACCCAGGCAAT. All mice were maintained at the University of Delaware Animal Facility and according to institutional guidelines. Mice used in these studies were between 4 to 6 months of age.

RNA Isolation and cDNA generation from mice

RNA was isolated from mouse tissue using the RNAeasy kit (Qiagen). cDNA was then immediately generated using the high-capacity cDNA reverse transcription kit (Applied Biosystems).

RT-PCR analysis

The following Taqman probes from Applied Biosciences were used for qRT-PCR analysis: *Actb*-VIC (Mm02619580_g1) and *fam210b*-FAM (Mm01248184_m1).

RT-PCR for *lacz* was then conducted using the HOTSTAR protocol (Thermo) with forward primer sequence: 5' CTCCACAAGGATAACAGTTGA and reverse primer sequence: 5' ACTACCATCAATCCGGTAGGT. Electrophoresis of PCR products were done on a 1% agarose gel and analysis was conducted on a LI-COR Odyssey Fc and Image Studio software (version 5.2; LI-COR Biosciences).

Blood and bone marrow extractions

Blood was extracted by doing a cardiac puncture using EDTA coated needles and syringes. At least 100uL of blood was gathered and slowly added to an EDTA coated

blood collection tube (K3 EDTA micro500; SAI Infusion Technologies). The blood was kept at 4°C and was analyzed and processed further within 2 hours of extraction.

Bone marrow was extracted from the long bones of the mice. In brief, tissue surrounding the bone was removed with sterile scissors and scalpels. The bone caps were then cut off using scissors and the marrow was removed by gently passing ice-cold 1mL 1X PBS with 0.5% BSA and 2mM EDTA through the bone cavity and into a microcentrifuge tube using a 1mL EDTA coated syringe capped with a 27G EDTA coated needle. The bone marrow was kept at 4°C and was analyzed and processed further withing 6 hours of extraction.

Blood and bone marrow smears

Thin blood and bone marrow smears were made by placing a 10uL of blood or isolated bone marrow solution (see above) was placed on a charged slide near the frosted end. Another slide was brought up to the drop at a 30-45° angle and the drop was allowed to spread along the contact line between the slides. The angled slide was then quickly dragged toward the unfrosted end. The blood and bone marrow were allowed to dry overnight at room temperature and then stained using May-Grunwald solution (Sigma-Aldrich) for 5 minutes followed by 3 washes in 40mM Tris buffer (pH=7.2) and then 1:20 Giemsa solution (Sigma-Aldrich). The blood and bone marrow were then imaged on a Zeiss Axio Imager.A2 and Zen Blue software (version 3.1).

Complete blood count

A complete blood count was conducted using an Abaxis Vetscan HM5 Hematology Analyzer (version 2.3) according to the manufacturer's protocol.

Erythroid progenitor flow cytometry

Erythroid progenitor cells were sorted as described previously (19). In brief, single-cell bone marrow suspensions (see above) were centrifuged at 4°C, 300g for 10 minutes. CD45 was depleted by resuspending 10^7 cells with 100uL of a 10% CD45 MicroBead Slurry (MiltenyiBiotec) in 1X PBS with 0.5% BSA and 2mM EDTA. CD45 depleted bone marrow suspensions were blocked with rat anti-mouse CD16/CD32 (BD Bioscience) and stained using BD Bioscience antibodies: TER-119-FITC, CD44-APC, CD45-APC-Cy7, CD11b-APC-Cy7, GR1-APC-Cy7, and 7AAD.
The bone marrow cells obtained after bead depletion were imaged in Figure 4A.

For flow cytometry, stained bone marrow suspensions removed dead cells by gating out 7AAD positive cells (Cy5). From this population, erythroid progenitor cells were selected by gating out APC-Cy7 positive immune cells cells (CD45, GR1, CD11b) and cells with high internal complexity by SSC-A. The remaining cells were then selected for TER-119 expression (FITC) and then this population was sorted by CD44 (APC) and TER119 (FITC). Erythroid progenitor groups I-VI were then selected and sorted from this population. For imaging, cells were sorted directly onto a slide and allowed to dry overnight. The cells were then stained using May-Grunwald solution and Giemsa as described above and imaged on a Zeiss Axio Imager.A1 and Zen Blue Software (version 3.1).

Cell cycle analysis of MEL cells
Cells were harvested and then washed three times with PBS followed by fixation with ice-cold 70% ethanol for 30 minutes on ice. The cells were then rehydrated and washed 3 times with 1X PBS. The RNA was digested using 100ug/mL RNase and DNA was stained using 40ug/mL propidium iodide. The cells were then analyzed for cell cycle phase by flow cytometry.

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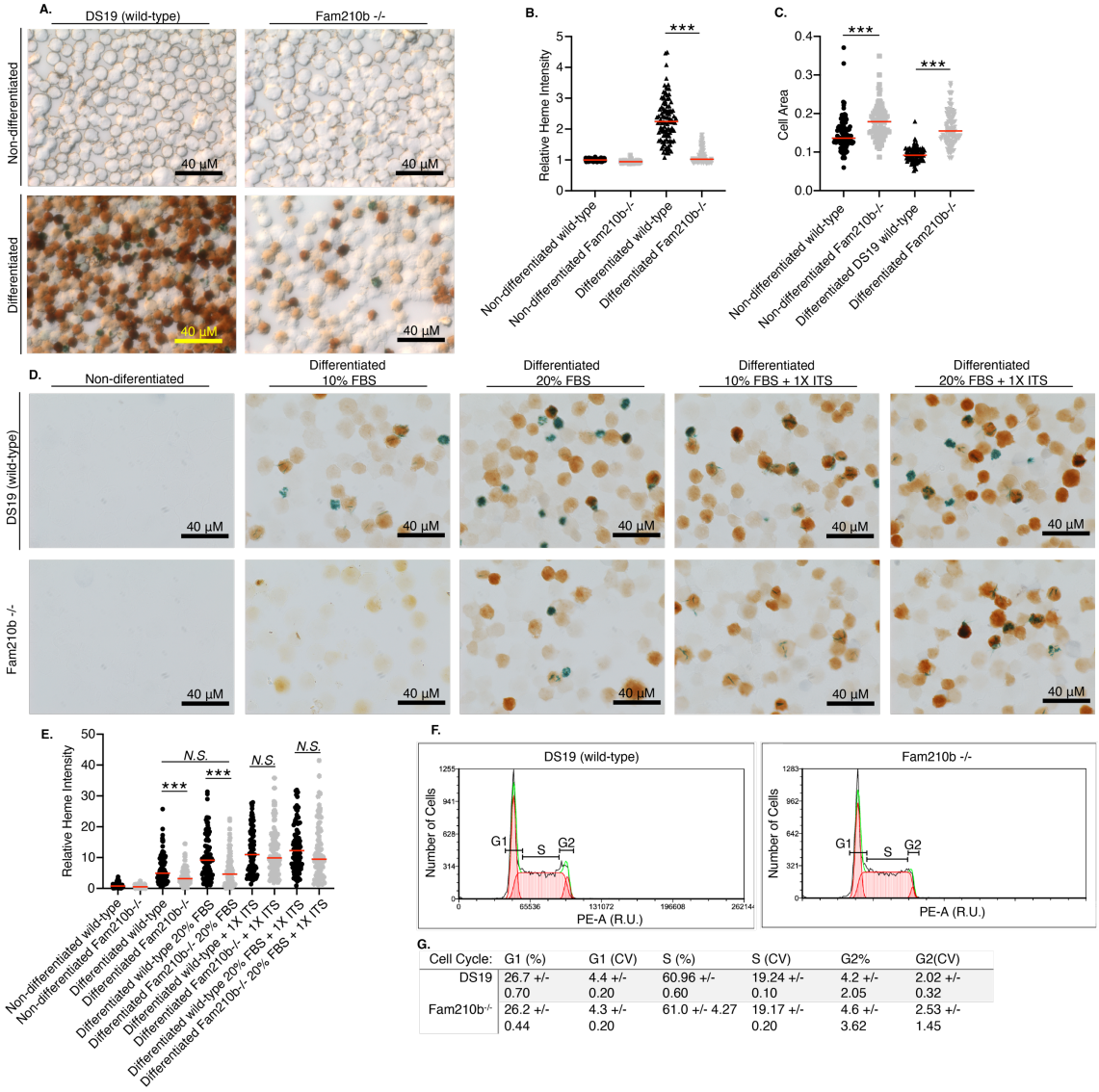


Cell Cycle:	G1 (%)	G1 (CV)	S (%)	S (CV)	G2%	G2(CV)
DS19	26.7 +/- 0.70	4.4 +/- 0.20	60.96 +/- 0.60	19.24 +/- 0.10	4.2 +/- 2.05	2.02 +/- 0.32
Fam210b$^{-/-}$	26.2 +/- 0.44	4.3 +/- 0.20	61.0 +/- 4.27	19.17 +/- 0.20	4.6 +/- 3.62	2.53 +/- 1.45

Figure 1. Fam210b is required for heme synthesis and normal cell size in MEL cells. A., 63X brightfield image of non-differentiated and differentiated MEL cells stained for heme (brown color), N=3 from 3 experiments. **B.**, Relative heme stain intensity of the MEL cells from A. Each point represents one cell, n=102 cells from 3 separate biological replicates. **C.**, 63X brightfield image of non-differentiated and differentiated MEL cells stained for heme (brown color), N=3. **D.**, 63X brightfield image of non-differentiated and differentiated MEL cells incubated with fetal bovine serum (FBS) and insulin-transferrin-selenium (ITS) and stained for heme (brown/green color). **E.**, Relative heme intensity of MEL cells from D (n=100). **F.**, Cell cycle analysis of WT and Fam210b$^{-/-}$ MEL cells, N=3. **G.**, Quantitation of cell cycle stage progression. ***, $p<0.001$; N.S., not significant.

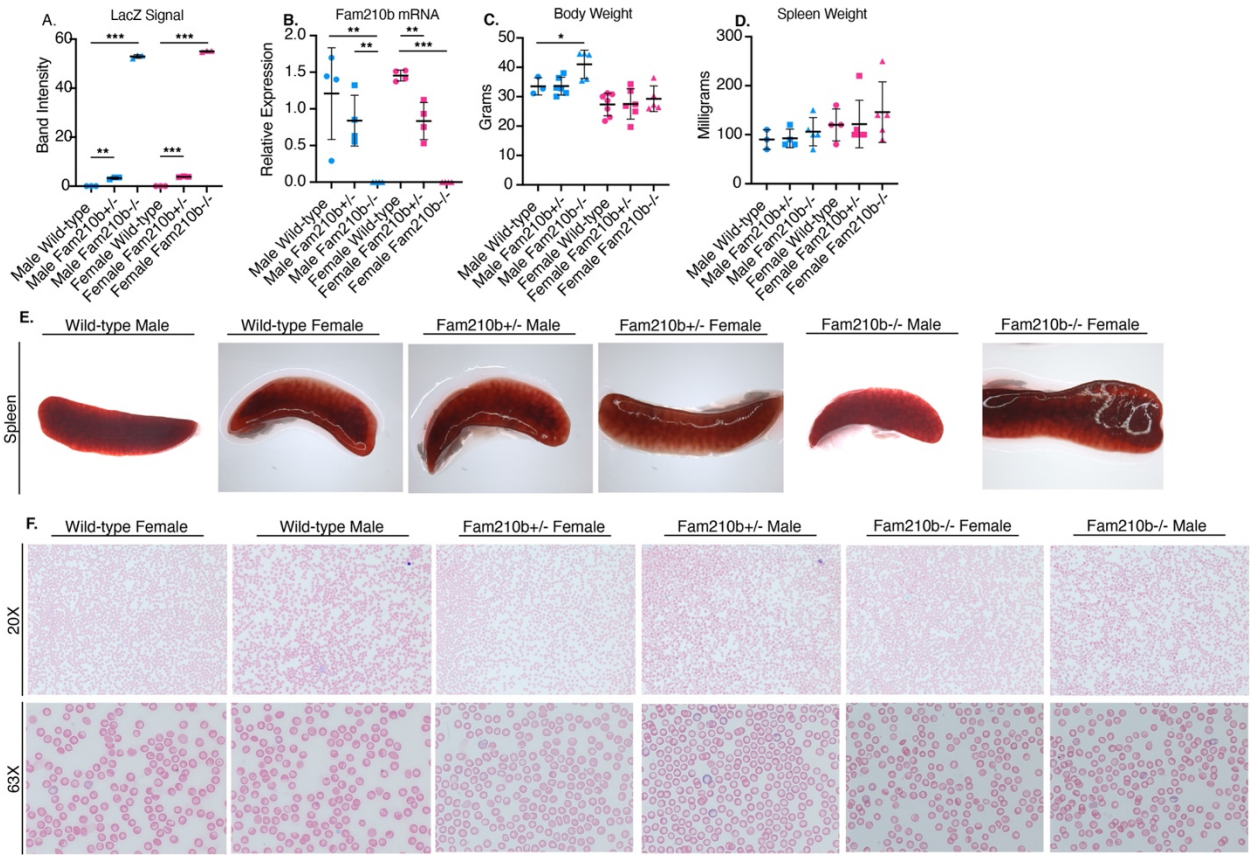


Figure 2. Fam210b deficiency causes a slight increase in male body weight, but has no effect on the gross morphology of the spleen or peripheral blood cells. A., *LacZ* semi-quantitative RTPCR from liver, n=3 per group. **B.**, qRTPCR for *Fam210b* mRNA from liver, n=4. **C.**, whole body weight of the mice taken just before dissection, n≥3. **D.**, Weights of mouse spleens, n≥3. **E.**, 4X brightfield image of mouse spleens. **F.**, 20X and 63X brightfield images of mouse blood smears stained with May-Grunwald and Giemsa.

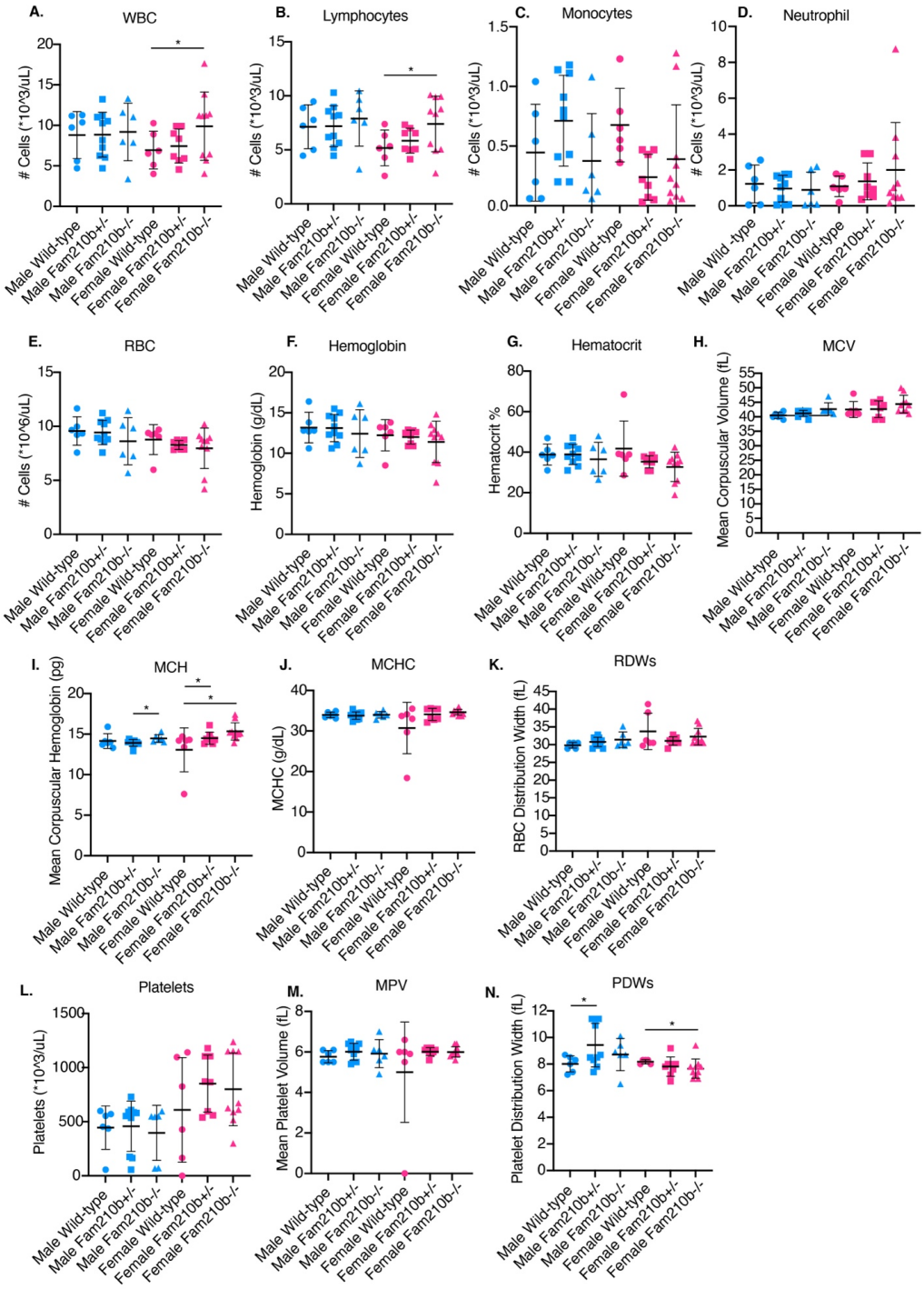
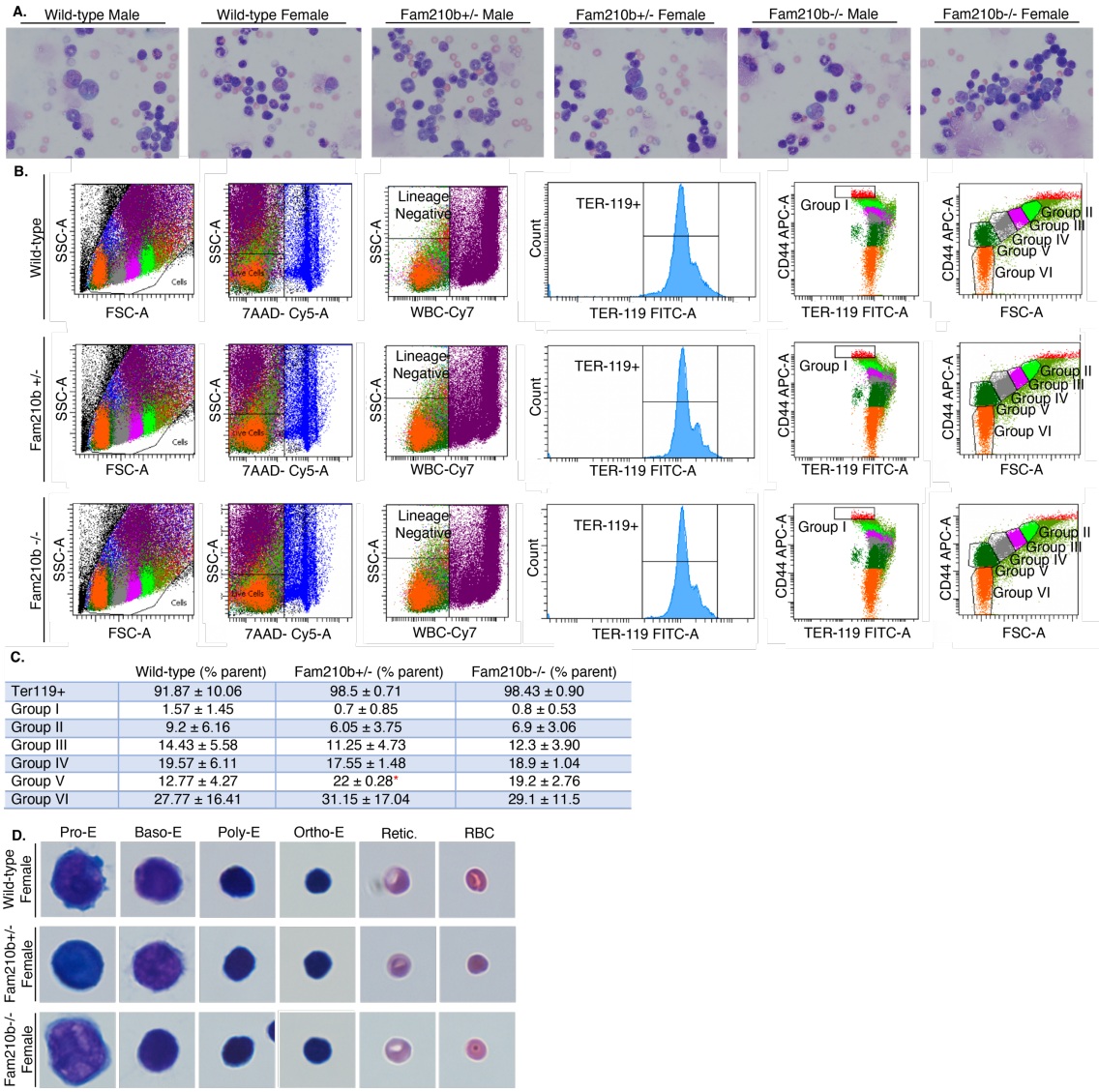


Figure 3. Complete Blood Count of wild-type, Fam210b$^{+/-}$ and Fam210b$^{-/-}$ mice. A., white blood cell (WBC) count, **B.**, lymphocyte count, **C.**, monocyte count, **D.**, neutrophil count, **E.**, red blood cell (RBC) count, **F.**, hemoglobin concentration, **G.**, hematocrit, **H.**, mean corpuscular volume (MCV), **I.**, mean corpuscular hemoglobin (MCH), **J.**, mean corpuscular hemoglobin concentration (MCHC), **K.**, red cell distribution width (RDWs), **L.**, platelet count, **M.**, mean platelet volume (MPV), **N.**, platelet distribution width (PDWs). n≥6 for all values; *, $p<0.05$.



C.

	Wild-type (% parent)	Fam210b+/- (% parent)	Fam210b-/- (% parent)
Ter119+	91.87 ± 10.06	98.5 ± 0.71	98.43 ± 0.90
Group I	1.57 ± 1.45	0.7 ± 0.85	0.8 ± 0.53
Group II	9.2 ± 6.16	6.05 ± 3.75	6.9 ± 3.06
Group III	14.43 ± 5.58	11.25 ± 4.73	12.3 ± 3.90
Group IV	19.57 ± 6.11	17.55 ± 1.48	18.9 ± 1.04
Group V	12.77 ± 4.27	22 ± 0.28*	19.2 ± 2.76
Group VI	27.77 ± 16.41	31.15 ± 17.04	29.1 ± 11.5

Figure 4. Fam210b deficiency does not affect bone marrow gross morphology but increases the numbers of reticulocytes. A., 20x brightfield images of Grunwald/Giemsa stained mouse bone marrow cells. **B.**, Flow cytometry gating for quantification of erythroid progenitor populations; Group I: Pro-E, Group 2: Baso-E, Group III: Poly-E, Group IV: Ortho-E, Group V: Reticulocytes, Group VI: RBC. **C.**, Table quantifying erythroid progenitor cell frequency. n=3 for wild-type and Fam210b$^{-/-}$ mice, n=2 for Fam210b$^{+/-}$. *, $p<0.05$.**D.**, 63X brightfield images of Grunwald/Giemsa stained sorted erythroid progenitors.

Parental Genotype	*Offspring Genotype*		
	Wild-type	*Fam210b$^{+/-}$*	*Fam210b$^{-/-}$*
Wild-type x Fam210b$^{+/-}$	48.39% (15/31)	51.61% (16/31)	0%
Fam210b$^{+/-}$ x Fam210b$^{+/-}$	24.29% (34/140)	50% (70/140)	25.71% (36/140)
Fam210b$^{+/-}$ x Fam210b$^{-/-}$	0%	51.52% (68/132)	48.48% (64/132)
Fam210b$^{-/-}$ x Fam210b$^{-/-}$	0%	0%	100% (6/6)

Table 1. Proportions of offspring genotypes obtained from matings of *Fam210b$^{+/-}$* or *Fam210b$^{+/-}$* mice.

References

1. Chen, C., and Paw, B. H. (2012) Cellular and mitochondrial iron homeostasis in vertebrates. *Biochim Biophys Acta* **1823**, 1459-1467
2. Yien, Y. Y., and Perfetto, M. (2022) Regulation of Heme Synthesis by Mitochondrial Homeostasis Proteins. *Front Cell Dev Biol* **10**, 895521
3. Muckenthaler, M. U., Rivella, S., Hentze, M. W., and Galy, B. (2017) A Red Carpet for Iron Metabolism. *Cell* **168**, 344-361
4. Shaw, G. C., Cope, J. J., Li, L., Corson, K., Hersey, C., Ackermann, G. E., Gwynn, B., Lambert, A. J., Wingert, R. A., Traver, D., Trede, N. S., Barut, B. A., Zhou, Y., Minet, E., Donovan, A., Brownlie, A., Balzan, R., Weiss, M. J., Peters, L. L., Kaplan, J., Zon, L. I., and Paw, B. H. (2006) Mitoferrin is essential for erythroid iron assimilation. *Nature* **440**, 96-100
5. Yien, Y. Y., Shi, J., Chen, C., Cheung, J. T. M., Grillo, A. S., Shrestha, R., Li, L., Zhang, X., Kafina, M. D., Kingsley, P. D., King, M. J., Ablain, J., Li, H., Zon, L. I., Palis, J., Burke, M. D., Bauer, D. E., Orkin, S. H., Koehler, C. M., Phillips, J. D., Kaplan, J., Ward, D. M., Lodish, H. F., and Paw, B. H. (2018) FAM210B is an erythropoietin target and regulates erythroid heme synthesis by controlling mitochondrial iron import and ferrochelatase activity. *J Biol Chem* **293**, 19797-19811
6. Matak, P., Matak, A., Moustafa, S., Aryal, D. K., Benner, E. J., Wetsel, W., and Andrews, N. C. (2016) Disrupted iron homeostasis causes dopaminergic neurodegeneration in mice. *Proc Natl Acad Sci U S A* **113**, 3428-3435
7. Carlson, E. S., Tkac, I., Magid, R., O'Connor, M. B., Andrews, N. C., Schallert, T., Gunshin, H., Georgieff, M. K., and Petryk, A. (2009) Iron is essential for neuron development and memory function in mouse hippocampus. *J Nutr* **139**, 672-679
8. Galmozzi, A., Kok, B. P., Kim, A. S., Montenegro-Burke, J. R., Lee, J. Y., Spreafico, R., Mosure, S., Albert, V., Cintron-Colon, R., Godio, C., Webb, W. R., Conti, B., Solt, L. A., Kojetin, D., Parker, C. G., Peluso, J. J., Pru, J. K., Siuzdak, G., Cravatt, B. F., and Saez, E. (2019) PGRMC2 is an intracellular haem chaperone critical for adipocyte function. *Nature* **576**, 138-142
9. Joffin, N., Gliniak, C. M., Funcke, J. B., Paschoal, V. A., Crewe, C., Chen, S., Gordillo, R., Kusminski, C. M., Oh, D. Y., Geldenhuys, W. J., and Scherer, P. E. (2022) Adipose tissue macrophages exert systemic metabolic control by manipulating local iron concentrations. *Nat Metab* **4**, 1474-1494
10. Seguin, A., Jia, X., Earl, A. M., Li, L., Wallace, J., Qiu, A., Bradley, T., Shrestha, R., Troadec, M. B., Hockin, M., Titen, S., Warner, D. E., Dowdle, P. T., Wohlfahrt, M. E., Hillas, E., Firpo, M. A., Phillips, J. D., Kaplan, J., Paw, B. H., Barasch, J., and Ward, D. M. (2020) The mitochondrial metal transporters mitoferrin1 and mitoferrin2 are required for liver regeneration and cell proliferation in mice. *J Biol Chem* **295**, 11002-11020
11. Puig, S., Ramos-Alonso, L., Romero, A. M., and Martínez-Pastor, M. T. (2017) The elemental role of iron in DNA synthesis and repair. *Metallomics* **9**, 1483-1500
12. Acín-Pérez, R., Fernández-Silva, P., Peleato, M. L., Pérez-Martos, A., and Enriquez, J. A. (2008) Respiratory active mitochondrial supercomplexes. *Mol Cell* **32**, 529-539
13. Kondo, A., Fujiwara, T., Okitsu, Y., Fukuhara, N., Onishi, Y., Nakamura, Y., Sawada, K., and Harigae, H. (2016) Identification of a novel putative mitochondrial protein FAM210B associated with erythroid differentiation. *Int J Hematol* **103**, 387-395

14. Sun, S., Liu, J., Zhao, M., Han, Y., Chen, P., Mo, Q., Wang, B., Chen, G., Fang, Y., Tian, Y., Zhou, J., Ma, D., Gao, Q., and Wu, P. (2017) Loss of the novel mitochondrial protein FAM210B promotes metastasis via PDK4-dependent metabolic reprogramming. *Cell Death Dis* **8**, e2870
15. Suzuki, C., Fujiwara, T., Shima, H., Ono, K., Saito, K., Kato, H., Onodera, K., Ichikawa, S., Fukuhara, N., Onishi, Y., Yokoyama, H., Nakamura, Y., Igarashi, K., and Harigae, H. (2022) Elucidation of the Role of FAM210B in Mitochondrial Metabolism and Erythropoiesis. *Mol Cell Biol* **42**, e0014322
16. Kang, J., Zhou, H., Sun, F., Chen, Y., Zhao, J., Yang, W. J., Xu, S., and Chen, C. (2020) Caenorhabditis elegans homologue of Fam210 is required for oogenesis and reproduction. *J Genet Genomics* **47**, 694-704
17. Levy, J. E., Jin, O., Fujiwara, Y., Kuo, F., and Andrews, N. C. (1999) Transferrin receptor is necessary for development of erythrocytes and the nervous system. *Nat Genet* **21**, 396-399
18. Ruan, B., and Paulson, R. F. (2022) Metabolic regulation of stress erythropoiesis, outstanding questions, and possible paradigms. *Front Physiol* **13**, 1063294
19. Liu, J., Zhang, J., Ginzburg, Y., Li, H., Xue, F., De Franceschi, L., Chasis, J. A., Mohandas, N., and An, X. (2013) Quantitative analysis of murine terminal erythroid differentiation in vivo: novel method to study normal and disordered erythropoiesis. *Blood* **121**, e43-49
20. Grillo, A. S., SantaMaria, A. M., Kafina, M. D., Cioffi, A. G., Huston, N. C., Han, M., Seo, Y. A., Yien, Y. Y., Nardone, C., Menon, A. V., Fan, J., Svoboda, D. C., Anderson, J. B., Hong, J. D., Nicolau, B. G., Subedi, K., Gewirth, A. A., Wessling-Resnick, M., Kim, J., Paw, B. H., and Burke, M. D. (2017) Restored iron transport by a small molecule promotes absorption and hemoglobinization in animals. *Science* **356**, 608-616
21. Romano, L., Seu, K. G., Papoin, J., Muench, D. E., Konstantinidis, D., Olsson, A., Schlum, K., Chetal, K., Chasis, J. A., Mohandas, N., Barnes, B. J., Zheng, Y., Grimes, H. L., Salomonis, N., Blanc, L., and Kalfa, T. A. (2022) Erythroblastic islands foster granulopoiesis in parallel to terminal erythropoiesis. *Blood* **140**, 1621-1634
22. Seu, K. G., Papoin, J., Fessler, R., Hom, J., Huang, G., Mohandas, N., Blanc, L., and Kalfa, T. A. (2017) Unraveling Macrophage Heterogeneity in Erythroblastic Islands. *Front Immunol* **8**, 1140
23. Korolnek, T., and Hamza, I. (2015) Macrophages and iron trafficking at the birth and death of red cells. *Blood* **125**, 2893-2897
24. Lévesque, J. P., Summers, K. M., Bisht, K., Millard, S. M., Winkler, I. G., and Pettit, A. R. (2021) Macrophages form erythropoietic niches and regulate iron homeostasis to adapt erythropoiesis in response to infections and inflammation. *Exp Hematol* **103**, 1-14
25. Li, W., Wang, Y., Zhao, H., Zhang, H., Xu, Y., Wang, S., Guo, X., Huang, Y., Zhang, S., Han, Y., Wu, X., Rice, C. M., Huang, G., Gallagher, P. G., Mendelson, A., Yazdanbakhsh, K., Liu, J., Chen, L., and An, X. (2019) Identification and transcriptome analysis of erythroblastic island macrophages. *Blood* **134**, 480-491
26. Ginzburg, Y., An, X., Rivella, S., and Goldfarb, A. (2023) Normal and dysregulated crosstalk between iron metabolism and erythropoiesis. *Elife* **12**

27. Evans, P., Cindrova-Davies, T., Muttukrishna, S., Burton, G. J., Porter, J., and Jauniaux, E. (2011) Hepcidin and iron species distribution inside the first-trimester human gestational sac. *Mol Hum Reprod* **17**, 227-232
28. Mohyeldin, A., Garzón-Muvdi, T., and Quiñones-Hinojosa, A. (2010) Oxygen in stem cell biology: a critical component of the stem cell niche. *Cell Stem Cell* **7**, 150-161
29. Torres-Cuevas, I., Parra-Llorca, A., Sánchez-Illana, A., Nuñez-Ramiro, A., Kuligowski, J., Cháfer-Pericás, C., Cernada, M., Escobar, J., and Vento, M. (2017) Oxygen and oxidative stress in the perinatal period. *Redox Biol* **12**, 674-681
30. Robach, P., Cairo, G., Gelfi, C., Bernuzzi, F., Pilegaard, H., Viganò, A., Santambrogio, P., Cerretelli, P., Calbet, J. A., Moutereau, S., and Lundby, C. (2007) Strong iron demand during hypoxia-induced erythropoiesis is associated with down-regulation of iron-related proteins and myoglobin in human skeletal muscle. *Blood* **109**, 4724-4731
31. Yoon, D., Pastore, Y. D., Divoky, V., Liu, E., Mlodnicka, A. E., Rainey, K., Ponka, P., Semenza, G. L., Schumacher, A., and Prchal, J. T. (2006) Hypoxia-inducible factor-1 deficiency results in dysregulated erythropoiesis signaling and iron homeostasis in mouse development. *J Biol Chem* **281**, 25703-25711
32. Anderson, S. A., Nizzi, C. P., Chang, Y. I., Deck, K. M., Schmidt, P. J., Galy, B., Damnernsawad, A., Broman, A. T., Kendziorski, C., Hentze, M. W., Fleming, M. D., Zhang, J., and Eisenstein, R. S. (2013) The IRP1-HIF-2α axis coordinates iron and oxygen sensing with erythropoiesis and iron absorption. *Cell Metab* **17**, 282-290
33. Lyons, S. E., Lawson, N. D., Lei, L., Bennett, P. E., Weinstein, B. M., and Liu, P. P. (2002) A nonsense mutation in zebrafish gata1 causes the bloodless phenotype in vlad tepes. *Proc Natl Acad Sci U S A* **99**, 5454-5459
34. Zhang, J., and Hamza, I. (2019) Zebrafish as a model system to delineate the role of heme and iron metabolism during erythropoiesis. *Mol Genet Metab* **128**, 204-212
35. Shah, D. I., Takahashi-Makise, N., Cooney, J. D., Li, L., Schultz, I. J., Pierce, E. L., Narla, A., Seguin, A., Hattangadi, S. M., Medlock, A. E., Langer, N. B., Dailey, T. A., Hurst, S. N., Faccenda, D., Wiwczar, J. M., Heggers, S. K., Vogin, G., Chen, W., Chen, C., Campagna, D. R., Brugnara, C., Zhou, Y., Ebert, B. L., Danial, N. N., Fleming, M. D., Ward, D. M., Campanella, M., Dailey, H. A., Kaplan, J., and Paw, B. H. (2012) Mitochondrial Atpif1 regulates haem synthesis in developing erythroblasts. *Nature* **491**, 608-612
36. Nakamura, J., Fujikawa, M., and Yoshida, M. (2013) IF1, a natural inhibitor of mitochondrial ATP synthase, is not essential for the normal growth and breeding of mice. *Biosci Rep* **33**
37. Rondelli, C. M., Perfetto, M., Danoff, A., Bergonia, H., Gillis, S., O'Neill, L., Jackson, L., Nicolas, G., Puy, H., West, R., Phillips, J. D., and Yien, Y. Y. (2021) The ubiquitous mitochondrial protein unfoldase CLPX regulates erythroid heme synthesis by control of iron utilization and heme synthesis enzyme activation and turnover. *J Biol Chem* **297**, 100972