Coordinate expression of heme and globin is essential for effective erythropoiesis

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Introduction

The synthesis of heme is uniquely regulated in developing erythroblasts. The first, and rate-limiting, enzymatic step is mediated by 5-aminolevulinate synthase 2 (ALAS2) in erythroblasts and ALAS1 in all other cells. While ALAS1 is transcriptionally, translationally, and posttranslationally regulated by heme (1–3), ALAS2 expression is dependent on adequate iron to relieve IRE/IRP-mediated inhibition but lacks this direct feedback inhibition by heme (4–6). Thus, the regulation of heme synthesis via feedback inhibition is active in all tissues except developing erythroblasts wherein iron availability drives heme synthesis without feedback regulation. This differential regulation likely reflects the fact that 95% of rbc protein content is hemoglobin, and thus developing erythroblasts need to produce high levels of heme. Heme itself is necessary to induce globin transcription (7) and translation (8); therefore, heme production must precede and may exceed the levels of heme utilization in hemoglobin during early erythropoiesis. Excess heme will inhibit transferrin-bound iron utilization in erythroblasts (9) and result in the generation of heme-mediated ROS, leading to cell damage and death (10). Therefore, any excess heme must be detoxified either by degradation or elimination.

Heme degradation via heme oxygenase (HMOX) is the primary mechanism that most cells employ to handle excess heme. While recent evidence suggests HMOX is present and active in late-stage fetal erythroblasts and erythroid cell lines (11), it is unclear whether HMOX is present and functional throughout erythroid cell differentiation postnatally. Furthermore, it is not known how heme degradation versus utilization are prioritized at the various stages of rbc development. Direct export of heme via FLVCR1 (12) provides an alternative cellular mechanism to handle excess heme when its supply exceeds globin availability.

In this study, we show erythroblasts from mice lacking the heme export protein FLVCR1 (13) have elevated heme, ROS, and apoptosis, and that adult erythropoiesis requires the cell surface isoform of FLVCR1, FLVCR1a, but not the mitochondrial isoform, FLVCR1b (14). In addition, we demonstrate that decreasing heme synthesis in mice with induced deletion of both FLVCR1a and FLVCR1b (Flvcr1-deleted mice) improves their rbc production, which implies that excess heme causes the erythroid marrow failure. These data may provide new pathophysiological insight into why human erythroblasts die in disease states where heme supply likely exceeds its utilization, such as Diamond Blackfan anemia and myelodysplasia (13, 15).

Results

The loss of FLVCR1 results in ineffective erythropoiesis and macrocytic anemia. Flvcr1-deleted mice have a block in erythroid differentiation at the CFU-E/proerythroblast stage, resulting in severe macrocytic anemia (13). Complete blood count (CBC) analysis reveals a 50% decrease of rbc numbers and hemoglobin levels by 4 weeks after deletion (Figure 1), consistent with a near complete developmental block based on mouse erythrocyte lifespan (16, 17). The anemia in Flvcr1-deleted mice stabilizes after 8 weeks postdeletion, when the rbc become macrocytic with a mean corpuscular volume of 64.3 ± 11.0 fl vs. 44.1 ± 3.7 fl and hemoglobin of 6.1 ± 2.7 g/dl vs. 14.3 ± 0.6 g/dl (Figure 1). Up to 60% of Flvcr1-deleted mice need to be euthanized by 16 weeks after deletion due to severe anemia, with rbc counts below 2.0 M/μl (Supplemental Figure 1; sup-
The loss of FLVCR1 results in ineffective erythropoiesis with a CFU-E/proerythroblast block. (A) Peripheral rbc, hemoglobin concentration (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), hematocrit (HCT), and wbc counts from control (solid squares, n = 6) and Flvcr1-deleted (open squares, n = 9) mice were measured before and every 4 weeks after induced deletion for up to 16 weeks. The rbc, HGB, MCV, MCH, and HCT values in Flvcr1-deleted mice significantly differ from the values in control mice. Student’s t test: P < 0.003. (B) Representative flow plots from control (++) Mx) and Flvcr1-deleted (FF Mx) cells from femurs and tibias were isolated either 4 or 12 weeks after deletion, as indicated. Lin– cells were analyzed for CD71 and Ter119 to identify committed erythroid cells and LNPC, which include stem cells and progenitors of all hematopoietic lineages. Marrow erythroid cells were gated, as shown in the first 3 columns, to identify each developing erythroid cell population (I, CFU-E/proerythroblasts; II, basophilic erythroblasts; III, polychromatic erythroblasts; IV, orthochromatic erythroblasts and reticulocytes; V, reticulocytes and rbc). The right 3 columns are overlays of all gated erythroid cells presented as CD44 by size (forward scatter, FSC), dot plots, and histograms of CD44 and FSC to show their size and distribution. (C–F) The frequency of developing erythroblasts as a percent of lin– cells 4 (C) or 12 weeks (D) after deletion, and the absolute numbers of erythroblasts in femur and tibias 4 (E) and 12 weeks (F) after deletion from control (solid bars) and Flvcr1-deleted (open bars) mice. The 4-week data is from 6 mice each, and the 12-week data is from 4 mice each. Student’s t test: *P ≤ 0.05. All P values are listed in Supplemental Tables 2 and 3.
the Flvcr1-deleted mice, demonstrating a developmental block at the CFU-E/proerythroblast to basophilic erythroblast stage (Figure 1 and Supplemental Tables 2 and 3). This block is much more evident at or after 12 weeks postdeletion, as there is nearly a 3-fold increase in population I erythroblasts compared with control mice. Serum erythropoietin (EPO) levels increase over time with the severity of the anemia (control: 384 ± 181 pg/ml; 4 weeks: 10,552 ± 1,896 pg/ml; 12 weeks: 51,269 ± 22,560 pg/ml; \( n = 6, 3, 3 \), respectively; all pairwise \( P \) values < 0.03). Thus, EPO is the most likely cause of increased numbers of CFU-E/proerythroblasts. Even with this significant increase in lin–CD71 +Ter119–/lo precursors entering the erythroid lineage under EPO drive, significantly fewer erythroblasts survive through the basophilic erythroblast stage (Figure 1). This developmental block in Flvcr1-deleted mice implies that FLVCR1 is necessary for erythroid differentiation beyond the CFU-E/pro-erythroblast stage. Marrow culture studies confirm these findings, as the CFU-E/proerythroblasts from Flvcr1-deleted mice generate too few hemoglobinizing cells to be scored morphologically as erythroid colonies; thus, CFU-E are not detectable (Supplemental Table 1 and ref. 13). Identical marrow culture findings are observed in cats viremic with feline leukemia virus subgroup C (FeLV-C) where FLVCR is targeted for destruction (25, 26).

Heme synthesis is upregulated prior to substantial globin production. To further characterize the role of FLVCR1 in erythroid differentiation in control and Flvcr1-deleted mice under acute conditions (4 weeks after deletion) and under chronic, steady-state conditions (12 weeks or longer after deletion). At both times, there is an increase in the frequency and absolute numbers of CFU-E/proerythroblasts (population I), along with a subsequent reduction in the frequency and absolute numbers of erythroblasts in later stages of development in the Flvcr1-deleted mice, demonstrating a developmental block at the CFU-E/proerythroblast to basophilic erythroblast stage (Figure 1 and Supplemental Tables 2 and 3). This block is much more evident at or after 12 weeks postdeletion, as there is nearly a 3-fold increase in population I erythroblasts compared with control mice. Serum erythropoietin (EPO) levels increase over time with the severity of the anemia (control: 384 ± 181 pg/ml; 4 weeks: 10,552 ± 1,896 pg/ml; 12 weeks: 51,269 ± 22,560 pg/ml; \( n = 6, 3, 3 \), respectively; all pairwise \( P \) values < 0.03). Thus, EPO is the most likely cause of increased numbers of CFU-E/proerythroblasts. Even with this significant increase in lin–CD71 +Ter119–/lo precursors entering the erythroid lineage under EPO drive, significantly fewer erythroblasts survive through the basophilic erythroblast stage (Figure 1). This developmental block in Flvcr1-deleted mice implies that FLVCR1 is necessary for erythroid differentiation beyond the CFU-E/pro-erythroblast stage. Marrow culture studies confirm these findings, as the CFU-E/proerythroblasts from Flvcr1-deleted mice generate too few hemoglobinizing cells to be scored morphologically as erythroid colonies; thus, CFU-E are not detectable (Supplemental Table 1 and ref. 13). Identical marrow culture findings are observed in cats viremic with feline leukemia virus subgroup C (FeLV-C) where FLVCR is targeted for destruction (25, 26).
this, we evaluated gene expression of ALAS, globin, HMOX, and FLVCR1 throughout erythroid differentiation. Peak expression of Alas2 and β-globin (Hbb-bs) occurs at the orthochromatic and reticulocyte stage (population IV) of erythroid differentiation when Hmox1, Hmox2, and Flvcr1 expression are at their lowest (Figure 2). However, we are interested in what is occurring around the CFU-E/proerythroblast stage. As cells differentiate from a lin precursor cell (LNPC) to CFU-E/proerythroblast stage (population I), Alas2 is significantly upregulated (≥10-fold), while Alas1 is still highly expressed and β-globin transcription initiates (Figure 2). Thus, the CFU-E/proerythroblast stage is when erythroid heme synthesis initiates. Since heme is required for both globin transcription and translation (7, 8), CFU-E/proerythroblasts would be subject to heme-mediated damage until adequate globin protein is produced, unless excess heme is degraded or eliminated. During this same transition, Hmox1 and Hmox2 are both downregulated about 50%, significantly reducing that capacity of heme degradation, while Flvcr1 expression is not significantly reduced (Figure 2). Therefore, CFU-E/proerythroblasts preferentially utilize FLVCR-mediated heme export to eliminate excess heme. Since FLVCR1 has a relatively long half-life (27) — and during human erythroid differentiation, the FLVCR1 mRNA levels peak before protein levels (12) — the measured Flvcr1 mRNA levels may underestimate the actual levels of FLVCR1 protein in subsequent developmental stages. The lack of a specific antibody for FLVCR1 in mouse samples prevents direct analysis of FLVCR1 protein. During the CFU-E/proerythroblast (population I) to basophilic erythroblast (population II) transition, Alas2 is further upregulated, along with significant upregulation of β-globin mRNA and complete loss of Hmox2 expression (Figure 2). Thus, heme synthesis is substantially upregulated during the CFU-E/proerythroblast stage, when HMOXs are low or absent and Flvcr1 is still highly expressed. Expression of Flvcr1b-specific transcripts encoding the mitochondrial isoform peak in population II and are mostly gone in population IV, when Alas2 and β-globin are expressed the highest. This suggests that other mitochondrial heme exporters may play an important role in erythroid differentiation during peak heme production.

In Flvcr1-deleted mice, there are alterations in expression of both heme synthesis and degradation genes during erythroid differentiation compared with control mice. While the expression of functional Flvcr1 isoforms (those containing exon 3) are ablated in the cell populations that normally express them as expected, there are no apparent alterations in Flvcr1b-specific transcript levels in any population using a probe directed at its unique 5' untranslated region (14). However, these Flvcr1b transcripts will lack exon 3 and result in nonfunctional proteins. Hmox1 is slightly elevated in CFU-E/proerythroblasts (population I) and significantly increased in the late stages, where it is normally not expressed. The most likely reason for this is elevated heme levels. However, it could be in response to other oxidative stresses. There is also a substantial reduction in Alas1 expression in population I erythroblasts of Flvcr1-deleted mice, which would be consistent with heme-mediated inhibition of Alas1 expression in these cells (3). Both Alas2 and β-globin expression are significantly reduced in populations IV and V, when they are normally expressed at high levels (Figure 2). Since Alas2 transcription is regulated by iron but not heme (4, 5), the reduction of Alas2 transcription most likely reflects selection for cells that synthesize less heme and are, thus, capable of surviving in the absence of FLVCR1. The reduction in Alas2 and reduced heme synthesis would be expected to result in less β-globin transcription, since β-globin transcription is at least in part regulated by heme levels (7). Thus, these data suggest that the loss of FLVCR1 results in increased heme levels, increased heme degradation in CFU-E/proerythroblasts, and subsequently, preferential survival of cells that make less heme to later stages of differentiation.

FLVCR1-deficient erythroblasts die from excess heme. Since mature rbc in Flvcr1-deleted mice are macrocytic with elevated mean corpuscular heme, we wanted to determine if these alterations are concomitant with the developmental block observed in erythroblasts. There is a significant increase in erythroblast size in all Flvcr1-deleted erythroblasts, including marrow reticulocytes and mature erythroid cells (Figure 3A), consistent with the macrocytosis observed in peripheral rbc. The increased cell size...
Figure 4. Excess cytoplasmic heme, but not mitochondrial heme, causes erythroid failure in Flvcr1-deleted mice. (A) Peripheral rbc counts, hemoglobin (HGB), and hematocrit (HCT) levels in transplanted mice that received normal marrow (NL Control, n = 9) or Flvcr1-deleted marrow that had been transduced with empty vector (n = 9) or vectors expressing Flvcr1a (n = 15), Flvcr1b (n = 8), or Hmox1 (n = 10). (B and C) The frequency of the rbc, platelets (PLT), and the donor-derived peripheral granulocytes (Gr1), B cells (B220), and T cells (CD3), which are positive for the GFP transduction marker at 4 weeks (B) and 7 weeks (C) after transplant with Flvcr1-deleted marrow transduced with empty vector, Flvcr1a, Flvcr1b, or Hmox1 as indicated. Student’s t test: *P < 0.001.

is limited to the erythroid lineage, as neither granulocytes nor B lineage cells from Flvcr1-deleted mice are larger, and the morphology of sorted erythroblasts is not altered, other than being slightly larger (Supplemental Figure 3). While the presence of macrocytic erythroblasts is consistent with elevated heme levels as observed in mature rbc, it does not directly demonstrate that Flvcr1-deleted erythroblasts have elevated heme.

To directly determine whether the erythroid failure in Flvcr1-deleted mice is due to a loss of heme export via FLVCR1 and, consequently, an increase in intracellular heme, we sorted developing erythroblasts and measured total cellular heme content from pooled marrow samples. The heme content in Flvcr1-deleted erythroblasts is significantly elevated compared with control erythroblasts in populations II and III, but not in LNPC, which includes progenitor and stem cells of all hematopoietic lineages (Figure 3B). In normal control erythroblasts, heme content increases at the orthochromatic stage (population IV) when Alas2 and β-globin are most highly expressed and Flvcr1 and Hmox1 are both expressed (least time). We completed additional studies using marrow samples from individual mice by combining populations I and II into a single collection gate, and we obtained essentially identical results with a larger sample number (Supplemental Figure 4), consistent with the CFU-E/proerythroblast to basophilic erythroblast block observed in these mice. Since FLVCR1 exports planar porphyrins (late heme precursors) in addition to heme (28), we evaluated protoporphyrin IX (PPIX) content in Flvcr1-deleted erythroblasts and found that PPIX content was slightly elevated in all erythroblast populations (Supplemental Figure 5). Thus, FLVCR-deficient erythroblasts accumulate PPIX, in addition to heme. This result may reflect that PPIX is routinely exported by FLVCR (28), or alternatively, excess heme in Flvcr1-deleted erythroblasts exceeds apo-hemoprotein capacity, thus, interfering with ferrochelatase activity (29) and resulting in increased PPIX levels.

Since heme is a powerful driver of oxidative damage, we next evaluated ROS levels in developing erythroblasts from control and Flvcr1-deleted mice using the ROS sensor CM-H2DCFCA. In Flvcr1-deleted marrow, there are significant increases of ROS in all erythroblast populations (Figure 3C), concordant with the elevated heme levels observed. Even CFU-E/proerythroblasts had a significant elevation in ROS, even though heme was only marginally elevated. As CFU-E/proerythroblasts express very low levels of globin (Figure 2), they would be most sensitive to elevated heme levels, which is consistent with these observations. As an alternative ROS sensor, we utilized CellROX Deep Red, which did not detect any alteration in ROS levels in Flvcr1-deleted erythroblasts relative to control erythroblasts. Furthermore, it did not indicate increased ROS in response to 10 μM H2O2, while CM-H2DCFCA provided a 7-fold increase in fluorescence intensity in marrow cells. This would suggest that the cellular ROS signal we detected is dependent upon H2O2, or other peroxides. This is consistent with published results indicating heme sensitizes cells to H2O2 and peroxide damage (10).

Since the FLVCR1b isoform is a mitochondrial heme exporter, we evaluated mitochondrial ROS in Flvcr1-deleted erythroblasts to determine the role of mitochondrial heme in the erythroid failure of Flvcr1-deleted mice using MitosOX Red. There was no elevation in mitochondrial ROS detected at any stage, and all erythroblasts from Flvcr1-deleted mice have reduced mitochondrial ROS staining with MitosOX Red (Figure 3D), suggesting there may be a loss of mitochondrial integrity. The loss of mitochondrial integrity may be a primary defect or secondary to elevated cytoplasmic heme and cytoplasmic ROS. Treatment of marrow cells with 10 μM H2O2 resulted in
an average 10% reduction in MitoSOX Red fluorescence, similar to what we observed in Flvcr1-deleted erythroblasts. This is consistent with elevated cytoplasmic heme and cytoplasmic ROS, resulting in reduced mitochondrial integrity. Since heme sensitizes cells to H$_2$O$_2$ and peroxide damage (10), the elevated heme levels in Flvcr1-deleted erythroblasts may lead to peroxide damage and cell death. We previously demonstrated that Flvcr1-deleted CD71$^+$ erythroblasts die via apoptosis (30), and with further delineation of erythroblast development in Flvcr1-deleted mice, we found increased cell death at all stages of erythroid differentiation (Supplemental Figure 5).

The erythroid defect in Flvcr1-deleted mice is caused by the absence of cytoplasmic heme export. The Flvcr1-deletion disrupts both FLVCR1a and FLVCR1b transcripts, resulting in nonfunctional proteins (13, 14). The low expression of Flvcr1 during peak heme production and the presence of elevated cytoplasmic but not mitochondrial ROS suggests it is not the loss of mitochondrial heme export, but rather cellular heme export, that drives the erythroid cell death in Flvcr1-deleted mice. To directly test this, we reconstituted mice with Flvcr1-deleted marrow transduced with a retroviral vector encoding either FLVCR1a (expressed on the cell membrane) or FLVCR1b (expressed on the mitochondria) to determine the role each isoform plays during erythropoiesis. Mice transplanted with marrow cells expressing FLVCR1a remained alive and healthy with normal hematocrits and hemoglobin levels at least 6 months after transplantation and had a median survival of more than 35 weeks. Conversely, mice transplanted with marrow cells expressing FLVCR1b die with severe anemia by 10 weeks after transplant and have a median survival time of 7 weeks, which is comparable to mice that receive marrow cells transduced with a control vector encoding only GFP (Figure 4 and Supplemental Figure 6). There were no differences in the marking frequency of white cell populations between mice that received either FLVCR1a- or FLVCR1b-expressing marrow (Figure 4), and there were no differences in the levels of GFP protein, reflecting equivalent mRNA levels (Supplemental Table 4). However, FLVCR1a-expressing cells contribute to the rbc compartment, while the FLVCR1b-expressing cells do not. This

Figure 5. The role of FLVCR1 in the coordination of heme and globin during erythroid differentiation. (A) When erythroid cells reach the CFU-E/proerythroblast stage, transferrin receptor 1 (Tfrc, CD71) is upregulated, transferrin-iron enters the cells, and heme synthesis initiates as ALAS2, the erythroid-specific initial step of the heme synthetic pathway, has a 5' iron-responsive element (IRE) preventing translation in the absence of sufficient iron. Heme then induces globin production, since it is a transcriptional and translational regulator of globin synthesis. Thus, there is an obligate time when heme is present but globin protein is insufficient. During this interval, FLVCR1a is required to export surplus heme. (B) Deletion of both isoforms of FLVCR1 results in the accumulation of intracellular heme in excess of cellular needs, resulting in cytoplasmic ROS and erythroblast death, which leads to anemia. (C) Subsequent expression of FLVCR1a on the cell surface restores normal cytoplasmic heme export and erythroblast differentiation, while expression of the mitochondrial isoform FLVCR1b does not (see Figure 4). Thus, the anemia in Flvcr1-deleted mice results from proerythroblast death caused by excessive cytoplasmic heme and ROS.
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Tfrc+/+
throblasts, we sorted lin−CD71 + erythroblasts (populations I–IV) to determine if overexpressed HMOX1 was capable of functioning in erythroblasts. To determine whether HMOX1 expression could overcome the need for FLVCR1 during erythroid differentiation, we overexpressed Hmox1 in Flvcr1−/− deleted marrow and then transplanted these cells into irradiated recipient mice. We found that Hmox1 expression did not overcome the need for functional FLVCR1a in erythroblasts despite having marking frequency in early erythroblasts or if newly synthesized heme is not being degraded heme sufficiently to overcome the need for FLVCR1a in early erythroblasts. It is unclear whether HMOX1 is not functional in early erythroblasts or if newly synthesized heme is not being delivered to HMOX1 for degradation in these cells.

demonstrates that cytoplasmic heme export by FLVCR1a, but not mitochondrial heme export by FLVCR1b, is able to support erythropoiesis in mice lacking both FLVCR1 isoforms (Figure 5).

To directly test whether heme degradation via HMOX1 could overcome the need for FLVCR1a during erythroid differentiation, we overexpressed Hmox1 in Flvcr1−/− deleted marrow and then transplanted these cells into irradiated recipient mice. We found that Hmox1 expression did not overcome the need for functional FLVCR1a in erythroblasts despite having marking frequency in granulocytes comparable with that of FLVCR1a-transduced marrow (Figure 4 and Supplemental Figure 6) and comparable levels of mRNA, as reflected by equivalent GFP levels (Supplemental Table 4). Furthermore, we demonstrated the vector produces high levels of HMOX1 protein in mouse cells (Supplemental Figure 7). The lack of marking among rbc indicates HMOX1 is not able to replace the loss of FLVCR1 in developing erythroblasts. To determine if overexpressed HMOX1 was capable of functioning in erythroblasts, we sorted lin−CD71 + erythroblasts (populations I–IV) from these mice into HMOX1 (GFP−) and HMOX1+ (GFP+) populations. The level of heme in these cells was 0.31 ± 0.1 μg per 10^6 cells and 0.14 ± 0.1 μg per 10^6 cells, respectively: an approximate 2-fold reduction in cellular heme in cells expressing HMOX1. While we were unable to further delineate which stage of erythroblasts had functional HMOX1 in these transplant studies because of the paucity of erythroblasts, it is clear that overexpressed HMOX1 can function in erythroblasts; however, HMOX1 is not capable of degrading heme sufficiently to overcome the need for FLVCR1a in early erythroblasts. It is unclear whether HMOX1 is not functional in early erythroblasts or if newly synthesized heme is not being delivered to HMOX1 for degradation in these cells.

The reduction of heme synthesis improves erythropoiesis in Flvcr1−/− mice. Since direct heme degradation was ineffective, we took an alternative approach and tested whether interference with the heme biosynthetic pathway could ameliorate the anemia in Flvcr1−/− deficient mice. We first bred Flvcr fl/fl; Mxcre Mxcre mutants to ferrochelatase mutant mice to make them functionally heme deficient. However, ferrochelatase (Fech) mutant mice have increased levels of reactive porphyrins, which build up in Flvcr1−/− deleted mice (28, 31). Consequently, rbc parameters in double mutant mice did not significantly differ from rbc parameters in Flvcr1−/− deleted mice (Supplemental Figure 8).

As a preferable strategy to reduce iron availability for heme synthesis, we bred Flvcr fl/fl; Mxcre mice to transferrin receptor heterozygous (Tfrc+/−) mice. Similar to Fech-deficient mice, Tfrc+/− mice have a mild microcytic, hypochromic anemia consistent with iron/heme deficiency (32, 33). As iron availability is a key factor in initiating erythroid heme synthesis (29), we tested whether iron restriction in Tfrc+/− mice was sufficient to improve erythropoiesis in Flvcr1−/− deleted mice. While Tfrc+/−; Mxcre mice start with a higher rbc count and lower hemoglobin and hematocrit than Mxcre control mice, the Tfrc+/−; Flvcr1−/−; Mxcre−/− deleted mice have a higher rbc count and also higher hemoglobin and hematocrits than the Flvcr1−/− deleted mice by 12 weeks after deletion (Figure 6 and Supplemental Figure 9). Thus, the iron restriction mediated by reduced transferrin receptor improves the anemia caused by Flvcr1 deletion during erythropoiesis. We further restricted iron by feeding mice an iron-deficient diet (<6 ppm iron vs. 220 ppm iron) after deleting Flvcr1. This iron-deficient diet reduced the hemoglobin and hematocrit in Mxcre control mice while increasing the hemoglobin and hematocrit in Flvcr1−/− deleted mice (Figure 6 and

Figure 6. Iron restriction improves the anemia in mice with erythroblast heme overload. (A–C) The difference in peripheral rbc counts (A), hemoglobin levels (HGB) (B), and hematocrits (HCT) (C) in Tfrc−/−; Mxcre mice compared with the mean value of Tfrc−/−; Mxcre mice (solid bars, n = 10) and Tfrc−/−; Flvcr1−/− deleted mice compared with the mean value of Tfrc−/−; Flvcr1−/− deleted mice (open bars, n = 7). (D–F) The difference in peripheral rbc counts (D), HGB (E), and HCT (F) in Mxcre mice fed an iron-deficient diet compared with the mean value of Mxcre mice fed a normal diet (solid bars, n = 10) and in Flvcr1−/− deleted mice fed an iron-deficient diet compared with the mean value of Flvcr1−/− deleted mice fed a normal diet (open bars, n = 11). CBC measurements are presented in Supplemental Figures 9 and 10. One-way ANOVA Tukey-Kramer test: **P < 0.01, ***P < 0.001.
Supplemental Figure 10). Thus, restricting iron delivery to the erythron via reduced transferrin receptor expression or an iron-deficient diet improves rbc development in mice with elevated erythroblast heme mediated by the loss of FLVCR1.

In addition to altering iron availability for heme synthesis, we treated mice with isonicotinylhydrazine (INH) to inhibit the first step in the heme synthesis pathway: the conversion of succinyl CoA and glycine to 5-aminolevulinic acid by ALAS (34). After 8 weeks of INH treatment, Mxcre mice had a slight reduction in their rbc numbers, hematocrits, and hemoglobin levels compared with control-treated Mxcre mice, while the INH-treated Flvcr1-deleted mice showed increased rbc numbers, hematocrits, and hemoglobin levels compared with control-treated Flvcr1-deleted mice (Figure 7 and Supplemental Figure 11). This improvement in rbc parameters was also accompanied by a significant improvement in survival (Supplemental Figure 11).

Discussion
Erythroblasts from Flvcr1-deleted mice die at the CFU-E/proerythroblast-to-basophilic erythroblast transition, resulting in severe anemia. The cell death is associated with elevated intracellular heme and elevated cytoplasmic ROS in developing erythroblasts at all stages. Both restricting iron availability and impairing a heme biosynthetic enzyme as 2 experimental approaches to inhibit heme synthesis improve the anemia in Flvcr1-deleted mice, proving that heme excess causes the erythroid marrow failure and that FLVCR1-mediated heme export is required for successful erythroid differentiation prior to heme-dependent upregulation of globin levels.

The major developmental block between the CFU-E/proerythroblast and basophilic erythroblast stages in Flvcr1-deleted mice (Figure 1) suggests these early erythroblasts are particularly sensitive to excess heme and ROS, which correlates to the stages when globin production is absent or just beginning. Additionally, the elevated cytoplasmic ROS and apoptosis throughout all subsequent erythroblast stages in Flvcr1-deleted mice is accompanied with high levels of heme, suggesting ongoing ROS damage, leading to cell death throughout erythroid differentiation. Thus, the imbalance of heme synthesis to heme utilization, degradation, or export at any erythroblast stage may lead to cell death. Recent studies have identified a novel form of iron-dependent cell death in tumors termed ferroptosis (35–37), which may, along with apoptosis, contribute to the demise of Flvcr1-deleted erythroblasts. Ferroptosis is an iron-dependent cell death resulting from elevation of cytosolic and lipid (but not mitochondrial) ROS, which does not include traditional markers of apoptosis such as mitochondrial cytochrome c release or caspase activation (35). While ferroptosis is not well characterized, it is a relatively slow death occurring over many hours, as opposed to the rapid cell death observed with traditional apoptosis. Since erythropoiesis is a very rapid process in mice, and if the elevated heme-induced ROS causes a slow cell death via ferroptosis, then cell death could occur very early (e.g., CFU-E/proerythroblast stage), accounting for the drop in cell numbers between stages I and II. However, cells continue to die during later stages of erythropoiesis. These kinetics and the presence of sustained cytosolic ROS are supported by our observations.

There have been 2 isoforms of FLVCR1 identified; the long isoform, FLVCR1a, is a broadly expressed cell surface heme exporter, while the short isoform, FLVCR1b, is a mitochondrial heme exporter (12, 14). Both the FLVCR1a/b-null and the FLVCR1a–specific null mice fail to survive to birth and display craniofacial and digit abnormalities during development. However, the FLVCR1a-null mice appear to have intact fetal erythropoiesis (13, 14), suggesting that FLVCR1a may not be essential for erythropoiesis. This was tested in a short-term transplant model that failed to observe any defect in mice transplanted with FLVCR1a-deficient fetal liver cells 4 weeks after transplant using CD71 and Ter119 flow analysis (14). Hematocrit and hemoglobin were not reported. There are subtle differences that can be identified by flow cytometry in deleted mice; however, they are not easily observed by CD71 and Ter119 staining 4 weeks after deletion (Figure 1B). Without quantitative CBC measurements from the FLVCR1a-null transplant, it is not possible to determine if the FLVCR1a-specific null has a unique developmental defect or is completely recapitulated with the combined FLVCR1a/b deletion observed here. The ability of FLVCR1a to completely rescue erythropoiesis in a combined FLVCR1a/b-deleted mouse (Figure 4 and Supplemental Figure 6), however, clearly demonstrates that erythropoiesis in adult mice requires FLVCR1a and not FLVCR1b. Therefore, the cell surface isoform (FLVCR1a), which exports heme from the cell, is essential, while the mitochondrial isoform (FLVCR1b) is not essential (Figure 5) and suggests other mitochondrial heme exporters are functioning during erythropoiesis. This is directly supported by the lack...
of elevated mitochondrial ROS within any erythroid population in Flvcr1-deleted mice (Figure 3D) and low levels of Flvcr1b transcripts during times of peak heme production in normal control mice (Figure 2 and Figure 3B). Since FLVCR1a-deleted mice die in utero with apparently intact fetal erythropoiesis (14), the FLVCR1b isoform could be needed during fetal erythropoiesis.

Overexpression of HMOX1 was unable to compensate for the loss of FLVCR1 (Figure 4 and Supplemental Figure 6), indicating that heme export and heme degradation have distinct roles in handling excess heme during erythropoiesis. Potentially, HMOX1 does not function in early erythroblasts or endogenously synthesized heme does not traffic directly to cytoplasmic HMOX1 in erythroid cells. Our results are consistent with the late onset of anemia observed in HMOX1-deficient mice (38), where the anemia is attributed to poor iron recycling as opposed to ineffective erythropoiesis from heme excess. Alternatively, HMOX1 may degrade heme and release iron, which is then recycled for heme synthesis by rerouting the iron within the cell or by its reuptake (via the transferrin receptor) after ferroportin-mediated export (39, 40). Thus, it is possible that heme degradation and subsequent iron export may be a way to maintain the balance between available heme and globin production (11) only during the late stages of erythropoiesis, when the transferrin receptor (CD71) is no longer expressed.

Heme synthesis requires both porphyrin synthesis and iron delivery. We therefore tested ways to interfere with these processes in Flvcr1-deleted mice with the goal of reducing heme synthesis and improving anemia, and we learned that heme and iron regulation is complex. Since transferrin receptor heterozygous mice have reduced iron delivery to the erythron for erythropoiesis (32), we initially hypothesized that Flvcr1-deleted mice that were also haploinsufficient for the transferrin receptor would have reduced heme toxicity and improved erythropoiesis. While these mice still developed severe anemia, suggesting that a modest reduction of heme synthesis is inadequate to overcome the loss of FLVCR1 in early erythroblasts, there was a consistent improvement in the hemoglobin and hematocrit levels (Figure 6, B and C). This contrasted the reduction in hemoglobin and hematocrit seen in control mice (intact Flvcr) that were haploinsufficient for the transferrin receptor. Since EPO regulates transferrin receptor expression (33, 41), the increasing levels of EPO observed as the anemia progresses in Flvcr1-deleted mice may counteract the mild iron restriction by increasing transferrin receptor levels in the anemic mice, allowing better scavenging of transferrin-iron. Further restriction of iron delivery to the erythron with an iron-deficient diet also resulted in improved hemoglobin and hematocrits (Figure 6, E and F). Finally, using INH to interfere with ALAS function also improved rbc parameters and led to a significant improvement in viability (Figure 7 and Supplemental Figure 11). Thus, interfering with heme synthesis either via reducing porphyrin synthesis or restricting iron availability for erythropoiesis significantly improves, but does not completely correct, the anemia of mice lacking FLVCR1. This further validates the concept that FLVCR1 is essential for effective erythropoiesis because it reduces early erythroblast heme levels when alternative methods to detoxify heme are either not present or nonfunctional.

Our studies imply that the production of hemoglobin requires the balance between the enzymatic synthesis of heme and the transcription and translation of globin to assure that heme is always available but never in excess. By extension, normal (unbridled) heme production coupled with a slowed initiation of globin transcription or translation could result in a clinical outcome similar to that seen in Flvcr1-deleted mice. The finding that Diamond Blackfan anemia and the macrocytic anemia that characterizes myelodysplasia with an isolated deletion of chromosome 5q (del[5q] MDS) result from haptinsufficiencies of ribosomal proteins and poor ribosome assembly (42, 43) may be a relevant human pathophysiology. When ribosome function is impaired, erythroblast globin production would be impaired, resulting in quantities of free heme that exceed the export capacity of FLVCR1, leading to erythroblast apoptosis and anemia similar to Flvcr1-deleted mice.

Thalassemia is also associated with ineffective erythropoiesis, but its pathogenesis is more complex. Although globin synthesis should initiate normally, globin transcription is reduced, which could lead to heme excess. This early erythropoiesis insult, however, would be greatly compounded by the imbalance of α and β globin chains and later erythropoiesis cell death. Interventions such as apotransferrin administration, increasing hepcidin, and reducing Tmprss6 (all of which could restrict iron delivery to the erythron) improve rbc production in β-thalassemia (Hbbαβ+) mice (44–47), which supports the concept that heme excess plays a role and suggests that our observations in Flvcr1-deleted mice may be relevant to this disorder, as well. Finding a way to reduce heme synthesis or improve FLVCR1 function may improve the ineffective erythropoiesis of these disorders.

**Methods**

**Animals and transplant studies.** Flvcr1+/+, Flvcr1a+/+, Flvcr1b+/+, Mxcre mice with a conditional deletion of both FLVCR1 isoforms were previously described (13). Tfrc−/− (32) were obtained from Nancy Andrews (Duke University, Durham, North Carolina, USA) and Fech mutant mice (48), Fech−/−/Flvcr1+/+, Fech−/−/Flvcr1−/−/Flvcr1b−/− were obtained from Xavier Montagutelli (Institute Pasteur, Paris, France). Mice were backcrossed at least 10 generations on the C57BL/6j background (The Jackson Laboratory). C57BL/6J (CD45.2) and B6–CD45.1 congenic (stock 002014, The Jackson Laboratory) were crossed to generate CD45.1/2 F1 mice as transplant recipients. Adult control Flvcr1+/−; Flvcr1+/− × Flvcr1+/−; Flvcr1+/− × Flvcr1+/− were treated with 3 i.p. injections of 7.5 mg/kg polyI-polyC (GE Healthcare) 48 hours apart as described (13) to ablate Flvcr1a and Flvcr1b expression. Any Flvcr1−/− deleted mice with rbc counts below 2 M/μl were euthanized and excluded from subsequent analysis. Donor mice for transduction studies were treated with 3 mg fluorouracil (APP Pharmaceuticals) 11 days after deletion with polyI-polyC. Total marrow was harvested 3 days later and cultured overnight in IMDM with 10% FBS, 20 ng/ml murine IL-3, 50 ng/ml murine SCF, and 100 ng/ml human IL-6 (PeproTech). Marrow was transduced with bicistronic retroviral vectors expressing GFP in the second position, and either Flvcr1a (13), Flvcr1b (14), or Hmox1 (OriGene), in the first position, at a MOI of 5 as described previously (49). Three million transduced cells were transplanted i.v. into each myeloablated (11 Gy) recipient. Mice transplanted with transduced marrow cells were treated with 2 mg/ml neomycin in drinking water for 3 weeks to prevent opportunistic infections prior to reconstitution of the granulocyte lineage. All mice were maintained under specific pathogen-free conditions with free access to food and water. Iron-deficient diet (TD 99397; <6 ppm iron) was from Harlan Teklad. INH (Sigma-Aldrich) or carrier control (water)
was given by oral gavage at 50 mg/kg 5 days a week for 8 weeks. Serum EPO concentrations were determined by ELISA (R&D Systems).


