Heme exporter Flvcr1 regulates expansion and differentiation of committed erythroid progenitors by controlling intracellular heme accumulation

by Sonia Mercurio, Sara Petrillo, Deborah Chiabrando, Zuni Irma Bassi, Dafne Gays, Annalisa Camporeale, Andrei Vacaru, Barbara Miniscalco, Giulio Valperga, Lorenzo Silengo, Fiorella Altruda, Margaret H. Baron, Massimo Mattia Santoro, and Emanuela Tolosano

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Abstract

Feline Leukemia Virus subgroup C Receptor 1 (Flvcr1) encodes two heme exporters: Flvcr1a, that localizes to the plasma membrane, and Flvcr1b, that localizes to mitochondria. Here, we investigated the role of the two Flvcr1 isoforms during erythropoiesis. We showed that, in mice and zebrafish, Flvcr1a is required for the expansion of committed erythroid progenitors but cannot drive their terminal differentiation, while Flvcr1b contributes to the expansion phase and is required for differentiation. FLVCR1a-down-regulated K562 cells have defective proliferation, enhanced differentiation, and heme loading in the cytosol, while FLVCR1a/b deficient K562 cells show impairment in both proliferation and differentiation, and accumulate heme in mitochondria. These data support a model in which the coordinated expression of Flvcr1a and Flvcr1b is contributes to control the size of cytosolic heme pool required to sustain metabolic activity during the expansion of erythroid progenitors and to allow hemoglobinization during their terminal maturation.

Consistently, reduction or increase of the cytosolic heme rescued the erythroid defects in zebrafish deficient for Flvcr1a or Flvcr1b, respectively.

Thus, heme export represents a tightly regulated process that controls erythropoiesis.
Introduction

Heme is critical for many biological processes, including cellular respiration, oxygen storage and transport, drug metabolism, and resistance to oxidative stress. Within the cell, heme regulates transcription, translation, miRNA processing and post-translational modifications (1). Heme synthesis is highest in differentiating erythroblasts, in which hemoglobin production continues to increase. Moreover, in erythroid cells, heme regulates the expression of globin chains, thereby ensuring a balanced production of these two components of the assembled hemoglobin protein (2-5). This is important because an excess of either heme or globin peptide is detrimental for normal erythroid development and results in pathologic conditions (6).

Flvcr1a is a heme exporter initially identified as the membrane receptor for Feline Leukemia Virus subgroup C (7, 8). A second, mitochondrial isoform was later identified (9). Flvcr1a is a 12 transmembrane domain protein of the Major Facilitator Superfamily (MFS) of transporters. Flvcr1b is a shorter, six transmembrane domain protein that is thought to homo/heterodimerize to form a functional transporter. Flvcr1a was shown to function as a heme exporter and to prevent heme accumulation in several cell lines and primary cells, including NRK, HeLa, mouse macrophages and hepatocytes (9-12). Liver specific deletion of Flvcr1a resulted in heme accumulation in the liver and enhanced heme catabolism (12). Evidence that Flvcr1b is involved in mitochondrial heme export comes from the observation that its overexpression or silencing in HeLa cells results in heme accumulation in cytosol or mitochondria, respectively (9).

A role for Flvcr1 in erythropoiesis has long been recognized: cats infected with FeLV-C develop a severe red blood cell aplasia (7). Moreover, FeLV-C-infected-K562 cells cannot differentiate (10). Nevertheless, the specific role of Flvcr1 isoforms in erythropoiesis is still debated. Mice carrying a Flvcr1 allele deleted in the third exon, common to both Flvcr1a and Flvcr1b, have a block in erythroid differentiation at the proerythroblast stage and die at mid-gestation (11). Adult mice with
the same mutant allele develop a macrocytic anemia (11). Mouse fetuses carrying a deletion in the first exon of \textit{Flvcr1}, specific for the \textit{Flvcr1a} isoform, display normal erythropoiesis (9). Together, these findings suggest that \textit{Flvcr1b} is required for the differentiation of fetal erythroid progenitors. However, they do not exclude a role for \textit{Flvcr1a} in some other phase of erythroid progenitor maturation.

To get insights into the specific functions of \textit{Flvcr1a} and \textit{Flvcr1b} in erythropoiesis, we used animal and cultured cell models that differentially express the two isoforms. Our data indicate that \textit{Flvcr1a} is required for the expansion of committed erythroid progenitors, whereas \textit{Flvcr1b} is crucial for terminal differentiation.

\textbf{Methods}

\textit{Mice and Zebrafish}

\textit{Flvcr1a}\textsuperscript{+/−} mice were previously described (9). To generate \textit{Flvcr1a}\textsuperscript{−/−}; \textit{Mx-cre} mice, \textit{Flvcr1a}\textsuperscript{fl/fl; Mx-cre} mice (12) were crossed to mice expressing the Cre recombinase under the control of the interferon-responsive Mx promoter (\textit{Mx-cre}) (13). To induce \textit{Mx-cre} expression, 8 day-old neonatal \textit{Flvcr1a}\textsuperscript{−/−}; \textit{Mx-cre} pups were treated with 50 \(μg\) of poly(I)-poly(C) (Amersham/GE Lifesciences, Piscataway, NJ) IP every other day for three doses. Control \textit{Flvcr1a}\textsuperscript{−/−} animals were treated in the same way. Mice were sacrificed 6-8 weeks post treatment for analysis. Zebrafish embryos were maintained according to standard procedures (14). Embryos were staged using hours post fertilization (hpf) and morphologic criteria (15). Zygotes were collected at one-cell stage and injected with 4 ng of oligomorpholino (MO), in presence of phenol red for subsequent selection. The sequences of oligomorpholinos are reported in Supplemental Methods. For rescue experiments, murine \textit{Flvcr1a} and \textit{Flvcr1b} cDNAs were cloned into the pCS2+ expression vector and cRNA was synthetized using the SP mMachine RNA transcription kit.
Eighty pg of cRNA were co-injected with MOs. MoATG sequence did not perfectly match Flvcr1a cRNA.

In some experiments, zebrafish were grown in 50 µM hemin, 400 µM L-Arginin (Sigma-Aldrich, Milano, Italy), dissolved in sterile water, from 24 hpf to 48 hpf, or in 1mM Dioxoheptanoic acid (succynilacetone, SA) (Frontier Scientific, Logan, USA), dissolved in 0,1%DMSO, from 16,5 hpf to 48 hpf, after they were deyolked with forceps.

Experimental procedures related to mouse and fish manipulation followed previously reported recommendations and conformed to the Italian regulation for protecting animals used in research, including DL 116/92. The Ethics committee of the University of Torino approved this study.

Quantification of dsRed+ cells from zebrafish embryos

Transgenic kdrl:GFP$^{843}$/gata-1:dsRed$^{sd2}$ embryos, expressing green fluorescent protein (GFP) under the control of the kdrl promoter and red fluorescent protein (dsRed) under the gata-1 promoter (16), were dissociated into single cells and analyzed by flow cytometry (14).

O-dianisidine staining and Heme content

For O-dianisidine staining, ten embryos/condition were collected and stained according to standard procedure (17). Heme content was measured as described (18).

Erythropoiesis

To obtain BFU-E and CFU-E, 3 x 10$^4$ cells from fetal liver or adult bone marrow were cultured in MethoCult M3334 (Stemcell Technologies, Vancouver, Canada).

For erythroid differentiation, single-cell suspensions were immunostained with anti–TER119-PE and anti–CD71-FITC (BD Italia, Milano, Italy) and analyzed by flow cytometry.
Cell culture and gene silencing

The human lymphoblast K562 cell line (ATTC number CCL-243™) was propagated in RPMI medium (Life Technologies) with 10% Fetal Bovine Serum (Life Technologies). Flvcr1a or Flvcr1a/1b expression was downregulated as previously described (9). Cell proliferation was analyzed by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Roche Italia, Milano, Italy).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA extraction and qRT-PCR were performed as previously reported (9, 19).

Statistics

Results were expressed as mean ± SEM. Statistical analyses were performed using 1-way or 2-way ANOVA followed by Bonferroni post test or Student’s t test (GraphPad Software). A P value of less than 0.05 was considered significant.

Results

Flvcr1a and Flvcr1b have specific roles during the expansion and differentiation of murine committed erythroid progenitors

To address the specific roles of Flvcr1a and Flvcr1b during the expansion and differentiation of erythroid progenitors, we compared two mouse models that differentially express Flvcr1a and Flvcr1b. Flvcr1a<sup>−/−</sup> mice and Flvcr1a<sup>−/−</sup>; Mx-cre mice. Flvcr1a<sup>−/−</sup> mice were previously described (9): they carry a null allele, obtained by inserting a neomycin resistance cassette in the first exon of Flvcr1 gene, and do not express Flvcr1a. Flvcr1a<sup>+/−</sup>; Mx-cre mice carry a floxed allele with loxP sites flanking the first exon of Flvcr1 gene (Figure S1A), and express the cre recombinase under the control of the inducible Mx promoter. After cre recombinase induction, the deleted allele was observed in all tissues of Flvcr1a<sup>+/−</sup>; Mx-cre mice, while the floxed allele was detected in all tissues.
except bone marrow (Figure S1B), indicating that cre recombinase excision was complete only in bone marrow compartment. Flvcr1a<sup>0/0</sup>; Mx-cre mice did not express Flvcr1a and Flvcr1b in bone marrow and showed only a slight reduction of Flvcr1a and Flvcr1b mRNA level in all other tissues (Figure S1B). Loss of Flvcr1a and Flvcr1b expression occurred in both Ter119+ (erythroid) and Ter119- (non-erythroid) cells of Flvcr1a<sup>0/0</sup>; Mx-cre mice (Figure S1C). Thus, we referred to Flvcr1a<sup>0/0</sup>; Mx-cre mice as double knockout for both Flvcr1a and Flvcr1b in the hematopoietic lineage.

Flvcr1a<sup>−/−</sup> mice died at midgestation. We have previously shown that fetal erythroid progenitors of Flvcr1a<sup>−/−</sup> mice underwent normal terminal differentiation (9). To evaluate whether Flvcr1a deficiency may affect the expansion of committed erythroid progenitors, we counted BFU-E and CFU-E from the fetal liver at E12.5. The number of BFU-E and CFU-E was reduced by about 50% in Flvcr1a<sup>−/−</sup> mice compared to Flvcr1a<sup>+/+</sup> controls (Figure 1A). Moreover, the colonies derived from mutant mice were smaller than the colonies obtained from wild-type animals (Figure 1B). The same results were obtained by analysing primitive erythropoiesis (Figure S2).

Flvcr1a<sup>0/0</sup>; Mx-cre mice suffered from a severe macrocytic anemia (Table 1). They showed splenomegaly and accumulated iron in duodenum, liver and spleen as reported in another model of Flvcr1 deficiency (11) (Figure S3). The number of BFU-E and CFU-E derived from the bone marrow of Flvcr1a<sup>0/0</sup>; Mx-cre mice was reduced by about 75% compared to that obtained from Flvcr1a<sup>0/0</sup> mice (Figure 1C). Moreover, Flvcr1a<sup>0/0</sup>; Mx-cre bone marrow colonies were smaller than those derived from Flvcr1a<sup>0/0</sup> animals (Figure 1D). Analysis of erythroid differentiation demonstrated that Flvcr1a<sup>0/0</sup>; Mx-cre mice had a block of erythroid differentiation at the proerythroblast stage (Figure 1E).

Putting together these data and those obtained from our previous work (9), we conclude that Flvcr1a plays a role in the expansion of committed erythroid progenitors, but it is dispensable for terminal differentiation, whereas Flvcr1b contributes to the expansion of committed erythroid progenitors and is indispensable for terminal erythroid differentiation.
Flvcr1a and Flvcr1b are required for the expansion and differentiation of erythroid progenitors during zebrafish development

Flvcr1 protein sequence is highly conserved among fish and mammals (Figure S4). Analysis of Flvcr1 isoforms expression during zebrafish development demonstrated that Flvcr1a transcript was detectable as a maternal mRNA in unfertilized eggs and then increased from 8 to 96 hpf (Figure S5A). Flvcr1b transcript was detectable by 24 hpf and increased throughout development (Figure S5A). To further address the role of Flvcr1a and Flvcr1b in erythropoiesis, we analyzed erythropoiesis in zebrafish embryos in which the expression of both Flvcr1a and Flvcr1b was down-regulated by morpholino injection.

A splicing-blocking morpholino targeting the boundary of intron 3 and exon 4 (MoIn3Ex4), successfully down-regulated the expression of both Flvcr1a and Flvcr1b (Figure S5B). Flvcr1a/1b morphants displayed a delay in development by 24 hpf, and presented a shorter body than controls, a ventrally bent tail and smaller heads by 48 hpf. More than 90% of Flvcr1a/1b morphants showed hydrocephalus and lack yolk extension (Figure S5C). All morphants died within 5 days postfertilization (dpf) (Figure S5D), thereby confirming a crucial role for Flvcr1 during development.

Flvcr1a/1b morphants were anemic as demonstrated by O-dianisidine staining, measurement of heme content and expression of embryonic (Hbae1, Hbbe1, Hbae3) and adult (Hbaa1) globin genes (Figure 2A-C). Consistently, injection of MoIn3Ex4 in the transgenic zebrafish line gata-1:dsRed, carrying dsRed under the control of the erythroid specific gata1 transcription factor, demonstrated that Flvcr1a/1b morphants had less circulating erythroid cells than controls (Figure 2D). Thus, Flvcr1a/1b morphants show an impairment of erythropoiesis similar to that observed in Flvcr1a\textsuperscript{fl/fl};Mx-Cre mice.
To assess the specific contribution of *Flvcr1a* and *Flvcr1b* to erythropoiesis in zebrafish, we performed rescue experiments by co-injecting the morpholino with *Flvcr1a* or *Flvcr1b* cRNA or both.

Expansion of the erythroid population was evaluated by measuring the number of dsRed-positive cells. O-dianisidine staining, heme content and globin expression were used as markers of differentiation. Co-injection of MoIn3Ex4 and *Flvcr1a* cRNA rescued the number of circulating erythroid cells but had no effects on heme content and globin expression (Figure 3A-E). Co-injection of MoIn3Ex4 and *Flvcr1b* cRNA had a negligible effect on both the number of circulating erythroid cells and their differentiation (Figure 3A-E). Co-injection of MoIn3Ex4 and *Flvcr1a* and *Flvcr1b* cRNAs fully rescued the erythroid defect: the number of circulating dsRed-positive cells was comparable to that of controls and heme content and globin expression were normal (Figure 3A-E).

These data demonstrate that *Flvcr1a* is required for the expansion of erythroid progenitors but it is not sufficient to drive their terminal maturation, while *Flvcr1b* alone cannot sustain the expansion but is required for differentiation. This was further strengthened by the analysis of zebrafish injected with a morpholino targeting the AUG start codon of *Flvcr1a* mRNA (MoATG) and thus lacking only the *Flvcr1a* isoform. *Flvcr1a* morphants showed developmental abnormalities and were anemic (Figure S6 and Figure 4). Injection of *Flvcr1a* cRNA in *Flvcr1a* morphants rescued anemia indicating that, if *Flvcr1b* is present, the expansion of erythroid progenitors allowed by *Flvcr1a* is sufficient to recover the anemic phenotype (Figure 4). The same results were obtained with a morpholino targeting the boundary of intron 1 and exon 2 (data not shown).

*Flvcr1a and Flvcr1b control the size of cytosolic heme pool required for proliferation and differentiation of K562 cells*

Data in animal models demonstrated that *Flvcr1a* and *Flvcr1b* differentially affect the proliferation and differentiation of committed erythroid progenitors. Since *Flvcr1a* and *Flvcr1b* are heme
exporters localized at the plasma membrane and in mitochondria (9, 10), respectively, we hypothesized that their coordinated expression controls the size of cytosolic free heme pool required for proper erythropoiesis (20). We tested our hypothesis in K562 cells, in which the expression of FLVCR1a alone or both FLVCR1a and FLVCR1b was downregulated using specific shRNA (9). FLVCR1a - or FLVCR1a/1b-down-regulated K562 cells were expected to mimic what occurred in Flvcr1a−/− mice and Flvcr1a morphants or in Flvcr1a10; Mx-cre mice and Flvcr1a/1b morphants, respectively.

Heme accumulated to a higher extent in the cytosolic fraction of FLVCR1a -downregulated K562 cells compared with FLVCR1a/1b-downregulated or control cells and heme overload further increased after differentiation (Figure 5A). On the other hand, heme content was significantly higher in the mitochondrial fraction of FLVCR1a/1b-down-regulated cells than in the corresponding fraction of FLVCR1a-down-regulated or control cells (Figure 5B). These data demonstrate that FLVCR1 isoforms play a crucial role in controlling heme loading in subcellular compartments. The gene coding for the heme-degrading enzyme heme oxygenase 1 (HO-1) was induced in both FLVCR1a-down-regulated and FLVCR1a/1b-down-regulated cells (Figure S7). Consistently with in vitro results, HO-1 up-regulation was observed in zebrafish and mice lacking Flvcr1a or both Flvcr1a and Flvcr1b (Figure S7).

We next evaluated whether the loss of a specific FLVCR1 isoform could affect in vitro erythroid proliferation and/or differentiation. FLVCR1a-down-regulated K562 cells showed reduced proliferation compared to control cells. The lack of both FLVCR1a and FLVCR1b was associated with a worse defect in cell proliferation compared to control cells (Figure 5C). In agreement with heme content data, hemoglobinization, a marker of erythroid differentiation, was more pronounced in FLVCR1a-down-regulated K562 cells than in control cells and clearly deficient in FLVCR1a/1b-down-regulated cells (Figure 5D).

Our in vitro data established a correlation between FLVCR1a or FLVCR1a/1b deficiency, associated to defective proliferation and/or differentiation, and differential heme accumulation in
subcellular compartments. These data suggest that cytosolic heme accumulation, due to $\text{FLVCR1a}$ deficiency, is detrimental for cell proliferation and promotes erythroid differentiation. On the other hand, mitochondrial heme accumulation is deleterious for both cell proliferation and differentiation. Thus, the balanced expression of $\text{FLVCR1a}$ and $\text{FLVCR1b}$ is required for proper cell expansion and differentiation.

*Restoration of the cytosolic heme pool rescues the erythropoietic defects in zebrafish morphants*

Data on cellular models established a correlation between defective proliferation and/or differentiation of cells lacking $\text{FLVCR1a}$ or $\text{FLVCR1a/1b}$ and differential intracellular heme accumulation. We reasoned that stimuli able to reduce heme loading in the cytosol should rescue the proliferative defect caused by $\text{Flvcr1a}$ deficiency. On the other hand, heme supplementation is expected to bypass the mitochondrial block to heme export caused by $\text{Flvcr1b}$ loss and thus it should rescue the differentiation defects associated to $\text{Flvcr1b}$ deficiency. We performed the rescue experiments in zebrafish. In particular, we used the inhibitor of heme synthesis succynilacetone to reduce the cytosolic heme pool in $\text{Flvcr1a}$ morphants and we supplemented the water with heme to increase the size of cytosolic heme pool in $\text{Flvcr1a/1b}$ animals. As shown in Figure 6A, the inhibition of heme synthesis in zebrafish deficient for $\text{Flvcr1a}$ rescued the anemic phenotype in a significant percentage of embryos: in these animals, the number of circulating erythroid cells was comparable to that of controls and hemoglobinization was normal (Figure 6B-E).

Heme supplementation in $\text{Flvcr1a/1b}$ morphants resulted in heme loading and death of the embryos (Figure S8), further demonstrating that animals cannot tolerate heme overload if the plasma membrane $\text{Flvcr1a}$ is lost. Nevertheless, if $\text{Flvcr1a/1b}$ morphants were injected with $\text{Flvcr1a}$ cRNA and supplemented with heme, they showed normal erythropoiesis as demonstrated by O-dianisidine staining and heme content (Figure 7A-C).

We conclude that $\text{Flvcr1}$ isoforms work in close association with heme synthesis to regulate the size of the cytosolic heme pool required for proper erythropoiesis.
Discussion

Here we addressed the specific role of the Flvcr1 isoforms during the expansion and differentiation of committed erythroid progenitors. In mice, Flvcr1a has a mild impact on the proliferation of committed erythroid progenitors and is dispensable for their differentiation, while Flvcr1b is required for both proliferation and differentiation. In zebrafish, Flvcr1a deficiency has a stronger impact on the expansion of erythroid precursors as demonstrated by the anemic phenotype of Flvcr1a morphants. Nevertheless, the re-expression of Flvcr1a in Flvcr1a morphants that maintain Flvcr1b expression is sufficient to recover anemia. On the contrary, re-expression of Flvcr1a alone in Flvcr1a/1b morphants is able to rescue the defective expansion of erythroid precursors, but cannot drive their terminal maturation. Collectively, these data indicate that Flvcr1b is required along all phases of erythropoiesis while Flvcr1a is important to sustain proliferation of erythroid precursors but becomes dispensable when they differentiate. Moreover, in both species, Flvcr1b alone is not sufficient for proper embryo development since Flvcr1a/- embryos die at midgestation and Flvcr1a/1b morphants in which Flvcr1b is re-expressed do not survive. These data further indicate that Flvcr1 isoforms work together.

Since Flvcr1a and Flvcr1b export heme through the plasma and the mitochondrial membrane respectively, we hypothesised that their coordinated expression might contribute to control the size of the cytosolic free heme pool. The concept of “free” or “regulatory” heme pool has been well established through studies in hepatocytes (21). In these cells, the free heme pool depends on a balance between heme synthesis, heme degradation and heme export through the plasma membrane, and because of its small size, dynamic properties and ability to readily exchange with hemoproteins, reflects the overall status of cellular heme content (21-23). Recently, Garcia-Santos et al. introduced the concept of free heme pool also for erythroid cells and demonstrated that HO-1 is involved in its control thus regulating hemoglobinization (20). In fact, in erythroid cells, the free heme is thought
to increase during terminal maturation and control the expression of globin genes. Premature expansion of the regulatory heme pool is expected to result in premature erythroid differentiation while, on the contrary, a contraction of the heme pool should result in inappropriate hemoglobin production. Therefore, the control of the size of regulatory heme is crucial for proper hemoglobinization.

The experiments on K562 cells allowed us to highlight a correlation between Flvcr1a or Flvcr1a/1b deficiency and differential heme loading in cytosol and mitochondria, respectively. FLVCR1a-silenced K562 cells accumulated heme in the cytosol, show defective proliferation and are more prone to differentiate compared to control cells. FLVCR1a/1b-silenced cells accumulated heme in mitochondria, show defective proliferation and cannot differentiate. Even if not conclusive, these results support our hypothesis that Flvcr1a and Flvcr1b have to be expressed together in order to maintain adequate intracellular heme level. More importantly, rescue experiments in zebrafish further support our conclusion, and provide the first, strong in vivo evidence of the existence of the regulatory heme pool in erythroid cells. In fact, the reduction of cytosolic heme caused by the inhibition of heme synthesis, rescued the defective proliferation in Flvcr1a morphants whereas the increase in cytosolic heme due to heme supplementation rescued the differentiation defect in Flvcr1a/1b morphants in which Flvcr1a was re-expressed. Based on these results, we propose the model shown in Figure 7D. Flvcr1 is involved, together with heme synthesis and HO-1-mediated heme degradation, in the control of the size of the cytosolic free heme pool. In proliferating erythroid precursors, heme synthesis, HO-1 activity and Flvcr1a-, and Flvcr1b-mediated heme export are set to maintain a restricted heme pool required to sustain metabolic activity for cell proliferation. In differentiating erythroid cells, heme pool has to be increased to allow hemoglobinization. This could be achieved by enhancing δ-Aminolevulinic acid synthase 2 (ALAS2) and Flvcr1b expression/activity and/or by reducing HO-1 and Flvcr1a expression/activity.
Our data in mice and zebrafish indicate that Flvcr1b that exports heme from mitochondria to cytosol is required along all the phases of maturation of committed erythroid progenitors. In fact, heme has to be exported out from mitochondria for the incorporation in hemoproteins required for cell metabolism and, at later stages of maturation, for hemoglobin production. This conclusion is supported by our experiment showing that the administration of exogenous heme may rescue the differentiation defect in zebrafish lacking Flvcr1b. Even if other mitochondrial proteins with porphyrin transport capacity have been reported (24), our data in animal models demonstrate that no other mitochondrial transporter can compensate for the loss of Flvcr1b function.

On the other hand, we showed that Flvcr1a is required in the initial phase of erythropoiesis and dispensable during terminal maturation. This was confirmed in both mice and fish even if in the latter the effect of Flvcr1a deficiency on the expansion of erythroid precursors was stronger. This may be due to species-specific differences and/or to the different ways used to knock-out/knock-down Flvcr1a gene. Alternatively, other mechanisms may be evolved in mammals to preserve erythroid cell proliferation. Proliferating erythroid progenitors use oxidative metabolism. In eukaryotic cells, heme is embedded in the proteins of the electron transport chain (ETC) and it is sequentially reduced and oxidized to transfer electrons that ultimately reduce O2 (25). Thus, it is conceivable that cytosolic heme availability has to be regulated to allow ETC function. We think that Flvcr1a contributes to maintain a pool of de novo synthetized heme required to sustain the activity of hemoproteins as previously demonstrated in hepatocytes (12). Interestingly, the switch from proliferation to differentiation in the erythroid lineage is obtained by restraining oxidative metabolism (26). Concomitantly, heme synthesis rate increases to sustain hemoglobin production. At this time, the expression of Flvcr1a at the plasma membrane becomes dispensable as indicated by our data on Flvcr1a−/− embryos. It is likely that during differentiation most of heme is incorporated into the hemoglobin molecule whereas the rate of incorporation in ETC complexes declines. At this time, HO-1-mediated heme degradation could play a major role in controlling intracellular heme content.
As stated before, HO-1 was reported to play a role in controlling intracellular heme content in erythroid cells during terminal maturation (20). HO-1 overexpression in MEL cells impaired hemoglobin synthesis, while fetal liver cell from HO-1 knock-out embryos contained more hemoglobin. We showed that HO-1 is induced in both Flvcr1a- and Flvcr1a/1b-deficient cells and animals. HO-1 up-regulation when Flvcr1a is lost is likely mediated by heme control on HO-1 promoter (27). On the other hand, HO-1 induction in conditions of Flvcr1a and Flvcr1b deficiency might be related to oxidative stress resulting from heme accumulation in mitochondria since HO-1 is a well-known stress responsive gene (28). HO-1 upregulation when Flvcr1a is lost was already observed in the liver. In fact, liver specific Flvcr1a knockout mice show high HO-1 expression and HO activity in hepatocytes (12). These data suggest that heme export and heme catabolism control different pools of heme. In particular, we think that Flvcr1 regulates heme coming from de novo synthesis since heme accumulation in Flvcr1a-deficient cells can be prevented by inhibiting heme synthesis (9, 12). HO-1, that is an endoplasmic reticulum (ER) enzyme, could be involved in the control of heme that fails to be incorporated into apo-hemoproteins.

Together these data indicate that, during the expansion and differentiation of committed erythroid progenitors, the rate of heme synthesis, degradation and Flvcr1-mediated heme export is set to ensure adequate heme supply to sustain metabolic activity for cell cycling and hemoglobin production.

We have previously shown that fetal liver cells of Flvcr1a− mice can reconstitute the bone marrow of lethally irradiated adult mice (9). Nevertheless, we had followed the mice only for 4 weeks after transplantation. Thus, we cannot rule out the possibility that the ability of transplanted bone marrow cells to sustain erythropoiesis declines with time. Data shown here suggest that under conditions of stress erythropoiesis, the proliferative defect due to Flvcr1a deficiency might compromise the performance of bone marrow cells. On the other hand, the pharmacological inhibition of Flvcr1 function could be used to control erythroid progenitor proliferation and differentiation in pathologic
conditions characterized by chronic stress erythropoiesis. Consistently, the limitation of hemoglobin production has been shown to be a viable therapeutic strategy in mouse models of β-thalassemia and polycythemia vera (6, 29, 30).

In conclusion, we demonstrate that Flvcr1 gene plays a crucial role in erythropoiesis by contributing to control the size of the intracellular heme pool required to sustain proliferation and differentiation of committed erythroid progenitors. The pharmacological modulation of intracellular heme content, likely through the interference with heme exporters, may represent a new approach to disorders characterized by ineffective erythropoiesis.

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Authorship

S.M. performed research and analyzed data, S.P. performed some experiments on mouse models, D.C. performed some experiments on mouse models, analyzed and discussed data, Z.I.B. and D.G. performed some experiments on zebrafish model, A.C. performed flow cytometry analyses, A.V. and G.V. performed some experiments, B.M. performed blood analysis, L.S., F.A., M.H.B. and M.M.S. analyzed and discussed data, E.T. designed research, analyzed data and wrote the paper. The authors declare no competing financial interests.
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<td>RBC (M/µl)</td>
<td>8.047 ± 0.1681</td>
<td>5.110 ± 0.5328</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>HGB (g/dl)</td>
<td>11.02 ± 0.2312</td>
<td>7.418 ± 0.8215</td>
<td>0.0001</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>39.23 ± 0.8358</td>
<td>27.03 ± 2.506</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>13.67 ± 0.1159</td>
<td>13.81 ± 0.5931</td>
<td>0.8168</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>48.75 ± 0.3456</td>
<td>54.49 ± 1.438</td>
<td>0.0004</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>28.08 ± 0.2278</td>
<td>25.50 ± 1.141</td>
<td>0.0325</td>
</tr>
<tr>
<td>platelets (k/ul)</td>
<td>997.4 ± 80.32</td>
<td>1838 ± 180.2</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Table 1. Hematological parameters of $Flvcr1a^{fl/fl}; Mx$-cre mice
Legends for Figures

Figure 1-Flvcr1a and Flvcr1b deficiency affects the expansion and differentiation of murine committed erythroid progenitors.

(A, B) Number (A) and dimension (B) of BFU-E and CFU-E isolated at E12.5 from fetal liver of Flvcr1a+/+ and Flvcr1a-/- embryos (n=6). Values represent mean ± SEM, *P < 0.05; **P < 0.005, t-test. (C, D) Number (C) and dimension (D) of BFU-E and CFU-E isolated from bone marrow of 7-9 week-old Flvcr1a0/0 and Flvcr1a0/0;Mx-cre mice (n=4). Representative images of colonies are shown in B and D. Values represent mean ± SEM, ***P< 0.001, t-test.

(E) Representative flow cytometry analysis of spleen cells from Flvcr1a0/0 and Flvcr1a0/0;Mx-cre mice immunostained with antibodies to CD71 and Ter119. Regions R2-R5 corresponding to different maturational stages are indicated. The percentage of cells in each population is reported. Values represent mean ± SEM ***P< 0.001, n=4, two-way ANOVA.

Figure 2-Flvcr1a/1b morphants have defective erythropoiesis.

(A) O-dianisidine staining of control and MoI3ex4 morphants at 48hpf. 86% of morphants was anemic (n = 102); 100% of control embryos was unaffected (n = 123). Bar=200 μm.

(B) Heme content in control and MoI3ex4 morphants at 24, 48 and 72hpf. Values represent mean ± SEM. *P < 0.05; **P< 0.005, n=3, two-way ANOVA.

(C) qRT-PCR analysis of HbAlexander, HbBell, HbEpsilon and HbAlpha mRNA level in control and MoI3ex4 morphants at 24, 48 and 72 hpf. Values represent mean ± SEM. *P < 0.05; **P < 0.005; ***P< 0.001, n=6, two-way ANOVA. RQ: Relative Quantity.

(D) Photographs of transgenic gata-1:dsRed embryos injected with control or MoI3ex4 morpholino. Representative flow cytometry analysis of dsRed fluorescence is shown. The percentage of dsRed-positive cells is reported on the right. Values represent mean ± SEM. *P < 0.05, n=3, t-test. Bar=200 μm.
Figure 3. Flvcr1a and Flvcr1b are required to rescue erythropoiesis in Flvcr1a/1b morphants.

(A) Photographs of transgenic gata-1:dsRed embryos injected with control or MoI3ex4 morpholino alone or with Flvcr1a cRNA and/or Flvcr1b cRNA. Bar=200 μm.

(B) Representative flow cytometry analyses of dsRed fluorescence. The percentage of dsRed-positive cells is reported. Values represent mean ± SEM. **P < 0.005; ***P< 0.001, n=6, one-way ANOVA.

(C) O-dianisidine staining of control and MoI3ex4 morphants injected with Flvcr1a cRNA and/or Flvcr1b cRNA. Anemia was evident in 85% of morphants (n = 60), 82% of embryos injected with MoI3Ex4 and Flvcr1a (n = 92), 86% of embryos injected with MoI3Ex4 and Flvcr1b (n = 99) and 29% of embryos injected with MoI3Ex4 and both Flvcr1a and Flvcr1b (n = 85). Bar=200 μm.

(D) Heme content in control and MoI3ex4 morphants injected with Flvcr1a cRNA and/or Flvcr1b cRNA. Values represent mean ± SEM. *P < 0.05; **P< 0.005, n=3, one-way ANOVA.

(E) qRT-PCR analysis of Hbαα1 mRNA in control and MoI3ex4 morphants injected with Flvcr1a cRNA and/or Flvcr1b cRNA. Values represent mean ± SEM. *P < 0.05; **P< 0.005, n=4, one-way ANOVA.

Figure 4. Flvcr1a morphants are anemic.

(A) O-dianisidine staining of control, MoATG morphants and MoATG morphants injected with Flvcr1a cRNA at 48hpf. All control embryos were normal (n = 119), 90% of morphants was anemic (n = 110) in comparison to 28% of morphants injected with Flvcr1a cRNA (n=88).

(B) Heme content in control, MoATG morphants and MoATG morphants injected with Flvcr1a cRNA at 24, 48 and 72hpf. Values represent mean ± SEM. *P < 0.05; **P< 0.005, ***P< 0.001, n=3, two-way ANOVA.
(C) qRT-PCR analysis of Hbae1 mRNA level in control, MoATG morphants and MoATG morphants injected with Flvcr1a cRNA at 24, 48 and 72 hpf. Values represent mean ± SEM. *P < 0.05; **P < 0.005; n=6, two-way ANOVA.

(D) Photographs of transgenic gata-1:dsRed embryos injected with control, MoATG morpholino or MoATG morpholino and Flvcr1a cRNA. Representative flow cytometry analyses of dsRed fluorescence. The percentage of dsRed-positive cells is reported on the right. Values represent mean ± SEM. *P < 0.05, n=3, t-test. Bar=200 μm

Figure 5. Silencing of FLVCR1a or FLVCR1a/1b in K562 cells differentially affects proliferation and differentiation.

(A, B) Heme content in the cytosolic (A) and mitochondrial (B) fractions of FLVCR1a- or FLVCR1a/1b-down-regulated K562 cells. Erythroid differentiation was induced with sodium butyrate (0.5mM, for 72 hours). Values represent mean ± SEM. *P < 0.05; **P < 0.005; n=4, two-way ANOVA.

(C) MTT assay on K562 cells infected with a control vector or with vectors carrying a specific shRNAs for FLVCR1a or FLVCR1a/1b. Values represent mean ± SEM. *P < 0.05; **P < 0.005; ***P< 0.001, n=3, two-way ANOVA.

(D) Redness of cell pellets of control, FLVCR1a- or FLVCR1a/1b-down-regulated K562 cells.

Figure 6. Inhibition of heme synthesis rescues anemia in Flvcr1a morphants.

(A) Percentage of normal (non anemic) embryos when zebrafish were injected with a control morpholino, MoATG morpholino or MoATG morpholino in the presence of SA. A representative experiment is shown.

(B) Photographs of transgenic gata-1:dsRed embryos injected with control, MoATG morpholino or MoATG morpholino in the presence of SA. Bar=200 μm
(C) Representative flow cytometry analyses of dsRed fluorescence. The percentage of dsRed-positive cells is reported on the right. Values represent mean ± SEM. **P < 0.005, n=3, t-test.

(D) O-dianisidine staining of control, MoATG morphants and MoATG morphants grown in the presence of SA, at 48hpf. Bar=200 μm

(E) Heme content in control, MoATG morphants and MoATG morphants grown in the presence of SA, at 48hpf. Values represent mean ± SEM. *P < 0.05, **P < 0.005, n=3, one way ANOVA.

**Figure 7. Supplementation with heme rescues anemia in Flvcr1a/1b morphants.**

(A) Percentage of normal (non anemic) embryos when zebrafish were injected with a control morpholino, MoI3ex4 morpholino, MoI3ex4 morpholino and Flvcr1a cRNA or MoI3ex4 morpholino and Flvcr1a cRNA, in the presence of heme. A representative experiment is shown.

(B) O-dianisidine staining of zebrafish injected with a control morpholino, MoI3ex4 morpholino, MoI3ex4 morpholino and Flvcr1a cRNA or MoI3ex4 morpholino and Flvcr1a cRNA, in the presence of heme, at 48hpf. Bar=200 μm

(C) Heme content in zebrafish injected with a control morpholino, MoI3ex4 morpholino, MoI3ex4 morpholino and Flvcr1a cRNA or MoI3ex4 morpholino and Flvcr1a cRNA, in the presence of heme, at 48hpf. Values represent mean ± SEM. **P < 0.005, ***P < 0.001, n=3, one way ANOVA.

(D) A model for Flvcr1 isoforms function. Flvcr1a and Flvcr1b control the size of a cytosolic heme pool required for proper erythropoiesis. During expansion of committed erythroid progenitors, Flvcr1a and Flvcr1b expression is set to ensure heme for metabolic activity of cycling cells. Export of heme out of the cell by Flvcr1a is critical to avoid heme accumulation and maintain high proliferating rate. In differentiating cells, heme pool increases to sustain hemoglobin production. At this time, the expression of Flvcr1a at the plasma membrane becomes dispensable. Other than by heme export, the size of heme pool is controlled by heme synthesis and HO-1-mediated heme degradation (20).