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

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ABSTRACT

Feline leukemia virus subgroup C receptor 1 (*Flvcr1*) encodes two heme exporters: FLVCR1a, which localizes to the plasma membrane, and FLVCR1b, which 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/1b*-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* contributes to control the size of the 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 in *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 to normal erythroid development and results in pathological conditions.[6]

FLVCR1a is a heme exporter initially identified as the membrane receptor for feline leukemia virus subgroup C (FeLV-C).[7,8] A second, mitochondrial isoform was later identified.[9] FLVCR1a is a 12 transmembrane domain protein of the major facilitator superfamily 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 came from the observation that its overexpression or silencing in HeLa cells results in heme accumulation in the 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 pro-erythroblast stage and die in 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 *Flvcr1*, specific for the *Flvcr1a* isoform, display normal erythropoiesis.[9] Together, these findings suggest that *Flvcr1b* is required for the differentiation of fetal erythroid progenitors. However, they do not exclude a role for *Flvcr1a* in some other phase of erythroid progenitor maturation.

To get insights into the specific functions of *Flvcr1a* and *Flvcr1b* in erythropoiesis, we used animal and cultured cell models that differentially express the two isoforms. Our data indicate that *Flvcr1a* is required for the expansion of commit-

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ted erythroid progenitors, whereas *Flvcr1b* is crucial for terminal differentiation.

Methods

Mice and zebrafish

Flvcr1a$^{-/-}$ mice have been described previously.[9] To generate *Flvcr1a*$^{fl/fl}$; *Mx-cre* mice, *Flvcr1a*$^{fl/fl}$ mice[12] were crossed to mice expressing *cre* recombinase under control of an interferon-responsive *Mx* promoter (*Mx-cre*).[13] To induce *Mx-cre* expression, 8-day old neonatal *Flvcr1a*$^{fl/fl}$; *Mx-cre* pups were administered three doses of 50 μg of poly(I)-poly(C) (Amersham/GE Lifesciences, Piscataway, NJ, USA) intraperitoneally every other day for three doses. Control *Flvcr1a*$^{fl/fl}$ animals were treated in the same way. Mice were sacrificed 6 to 8 weeks after treatment for analysis.

Zebrafish embryos were maintained according to standard procedures.[14] Embryos were staged using hours post-fertilization (hpf) and morphological criteria.[15] Zygotes were collected at one-cell stage and injected with 4 ng of oligomorpholino, in the presence of phenol red for subsequent selection. The sequences of oligomorpholinos are reported in the *Online Supplementary Methods*.

For rescue experiments, murine *Flvcr1a* and *Flvcr1b* cDNA were cloned into the pCS2^{+} expression vector and cRNA was synthesized using the SP mMachine RNA transcription kit (Ambion, Austin, TX, USA). Eighty pg of cRNA were co-injected with oligomorpholino. The morpholino targeting the AUG start codon of *Flvcr1a* mRNA (MoATG) sequence did not perfectly match *Flvcr1a* cRNA.

In some experiments, zebrafish were grown in 50 μM hemin and 400 μM L-arginine (Sigma-Aldrich, Milan, Italy), dissolved in sterile water, from 24 hpf to 48 hpf, or in 1 mM dioxoheptanoic acid (succinylacetone) (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 with Italian regulations for protecting animals used in research, including Legislative Decree 116/92. The ethics committee of the University of Turin approved this study.

Quantification of dsRed^{+} cells from zebrafish embryos

Transgenic *kdrl:GFP*^{s843}/*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 elsewhere.[18]

Erythropoiesis

To obtain burst-forming units-erythroid (BFU-E) and colony-forming units-erythroid (CFU-E), 3x10^{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, Milan, 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 down-regulated 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, Milan, Italy).

Quantitative real-time polymerase chain reaction

Total RNA was extracted and quantitative real-time polymerase chain reaction (qRT-PCR) was performed as previously reported.[9,19]

Statistics

Results were expressed as mean ± standard error of mean. Statistical analyses were performed using one-way or two-way ANOVA followed by a Bonferroni post-test or Student *t* test (GraphPad Software). A *P* value of less than 0.05 was considered statistically 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*$^{-/-}$ mice and *Flvcr1a*$^{fl/fl}$; *Mx-cre* mice. *Flvcr1a*$^{-/-}$ mice have been described previously:[9] they carry a null allele, obtained by inserting a neomycin resistance cassette in the first exon of the *Flvcr1* gene, and do not express *Flvcr1a*. *Flvcr1a*$^{fl/fl}$; *Mx-cre* mice carry a floxed allele with loxP sites flanking the first exon of the *Flvcr1* gene (*Online Supplementary 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*$^{fl/fl}$; *Mx-cre* mice, while the floxed allele was detected in all tissues except bone marrow (*Online Supplementary Figure S1B*), indicating that *cre* recombinase excision was complete only in the bone marrow compartment. *Flvcr1a*$^{fl/fl}$; *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 (*Online Supplementary Figure S1B*). Loss of *Flvcr1a* and *Flvcr1b* expression occurred in both Ter119^{+} (erythroid) and Ter119^{-} (non-erythroid) cells of *Flvcr1a*$^{fl/fl}$; *Mx-cre* mice (*Online Supplementary Figure S1C*). Thus, we referred to *Flvcr1a*$^{fl/fl}$; *Mx-cre* mice as double knockout for both *Flvcr1a* and *Flvcr1b* in the hematopoietic lineage.

Flvcr1a$^{-/-}$ mice died in mid-gestation. We previously showed that fetal erythroid progenitors of *Flvcr1a*$^{-/-}$ mice undergo 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 about 50% lower in *Flvcr1a*$^{-/-}$ mice than in *Flvcr1a*$^{+/+}$ 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 analyzing primitive erythropoiesis (*Online Supplementary Figure S2*).

Flvcr1a$^{fl/fl}$; *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] (*Online Supplementary Figure S3*). The number of BFU-E and CFU-E derived from the bone marrow of *Flvcr1a*$^{fl/fl}$; *Mx-cre* mice was reduced by about 75% compared to that obtained from *Flvcr1a*$^{fl/fl}$ mice (Figure 1C). Moreover, *Flvcr1a*$^{fl/fl}$; *Mx-cre* bone marrow colonies were smaller than those derived from *Flvcr1a*$^{fl/fl}$ animals (Figure 1D). Analysis of erythroid differentiation demonstrated that *Flvcr1a*$^{fl/fl}$; *Mx-cre* mice had a block of erythroid differentiation at the pro-erythroblast 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 that 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*

The FLVCR1 protein sequence is highly conserved among fish and mammals (*Online Supplementary Figure S4*). Analysis of the expression of *Flvcr1* isoforms during zebrafish development demonstrated that *Flvcr1a* transcript was detectable as a maternal mRNA in unfertilized eggs and then increased from 8 to 96 hpf (*Online Supplementary Figure S5A*). The *Flvcr1b* transcript was detectable by 24 hpf and increased throughout development (*Online Supplementary 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 (MoI3Ex4), successfully down-regulated the expression of both *Flvcr1a* and *Flvcr1b* (*Online Supplementary Figure S5B*). *Flvcr1a/1b* morphants displayed a delay in development by 24 hpf, and had a shorter body than controls, a ventrally bent tail and smaller heads by 48 hpf. More than 90% of *Flvcr1a/1b* morphants developed hydrocephalus and lacked yolk extension (*Online Supplementary Figure S5C*). All morphants died within 5 days post-fertilization (dpf) (*Online Supplementary Figure S5D*), thereby confirming a crucial role for *Flvcr1* during development.

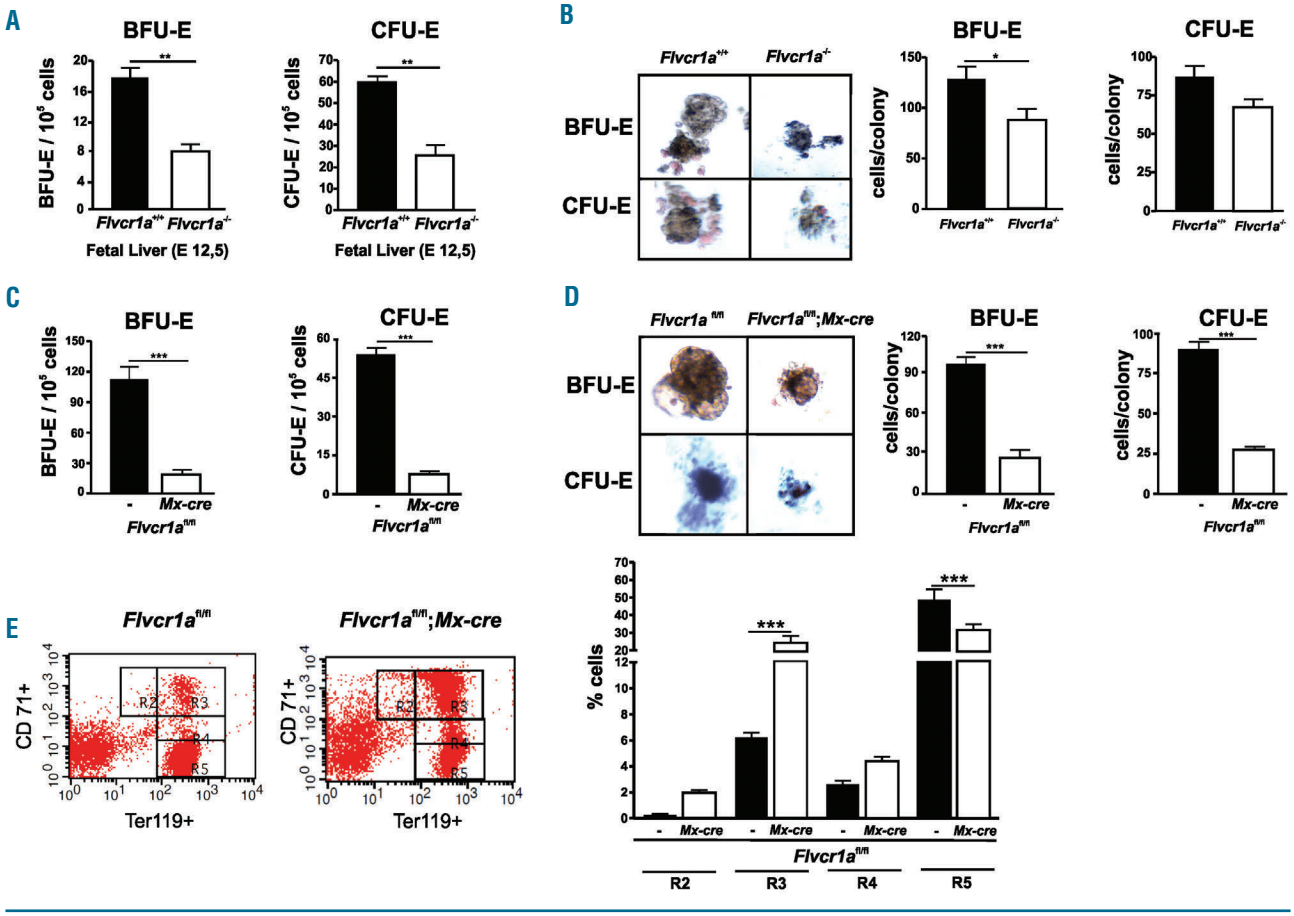

Figure 1. *Flvcr1a* and *Flvcr1b* deficiency affects the expansion and differentiation of murine committed erythroid progenitors. (A) Number and (B) dimension of BFU-E and CFU-E isolated at E12.5 from fetal liver of *Flvcr1a*$^{+/+}$ and *Flvcr1a*$^{-/-}$ embryos (n=6). Values represent mean ± SEM, **P*<0.05; ***P*<0.005, t-test. (C) Number and (D) dimension of BFU-E and CFU-E isolated from bone marrow of 7 to 9-week *Flvcr1a*$^{fl/fl}$ and *Flvcr1a*$^{fl/fl}$; *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 *Flvcr1a*$^{fl/fl}$ and *Flvcr1a*$^{fl/fl}$; *Mx-cre* mice immunostained with antibodies to CD71 and Ter119. Regions R2-R5, corresponding to different stages of maturation, are indicated. The percentage of cells in each population is reported. Values represent mean ± SEM ****P*<0.001, n=4, two-way ANOVA.

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 MoI3Ex4 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 fewer circulating erythroid cells than had controls (Figure 2D). Thus, *Flvcr1a/1b* morphants show an impairment of erythropoiesis similar to that observed in *Flvcr1a*$^{fl/fl}$*;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^{+} cells. O-dianisidine staining, heme content and globin expression were used as markers of differentiation. Co-injection of MoI3Ex4 and *Flvcr1a* cRNA rescued the number of circulating erythroid cells but had no effects on heme content or globin expression (Figure 3A-E). Co-injection of MoI3Ex4 and *Flvcr1b* cRNA had a negligible effect on both the number of circulating erythroid cells and their differentiation (Figure 3A-E). Co-injection of MoI3Ex4 and *Flvcr1a* and *Flvcr1b* cRNA fully rescued the erythroid defect: the number of circulating dsRed+ cells was comparable to that of controls and heme content and globin expression were normal (Figure 3A-E).

These findings 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 (*Online Supplementary 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*).

Table 1. Hematologic parameters of *Flvcr1a*$^{fl/fl}$; *Mx-cre* mice.

Parameter	*Flvcr1a*$^{fl/fl}$	*Flvcr1a*$^{fl/fl}$*; Mx-cre*	*P* value
WBC (x10^{9}/L)	2.658±0.3127	2.084±0.3601	0.235
RBC (x10^{9}/L)	8.047±0.1681	5.110±0.5328	<0.0001
Hemoglobin (g/dL)	11.02±0.2312	7.418±0.8215	0.0001
Hematocrit (%)	39.23±0.8358	27.03±2.506	<0.0001
MCH (pg)	13.67±0.1159	13.81±0.5931	0.8168
MCV (fL)	48.75±0.3456	54.49±1.438	0.0004
MCHC (g/dL)	28.08±0.2278	25.50±1.141	0.0325
Platelets (x10^{9}/L)	997.4±80.32	1838±180.2	0.0001

WBC: white blood cell count; RBC: red blood cell count; MCH: mean corpuscular hemoglobin; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin content.

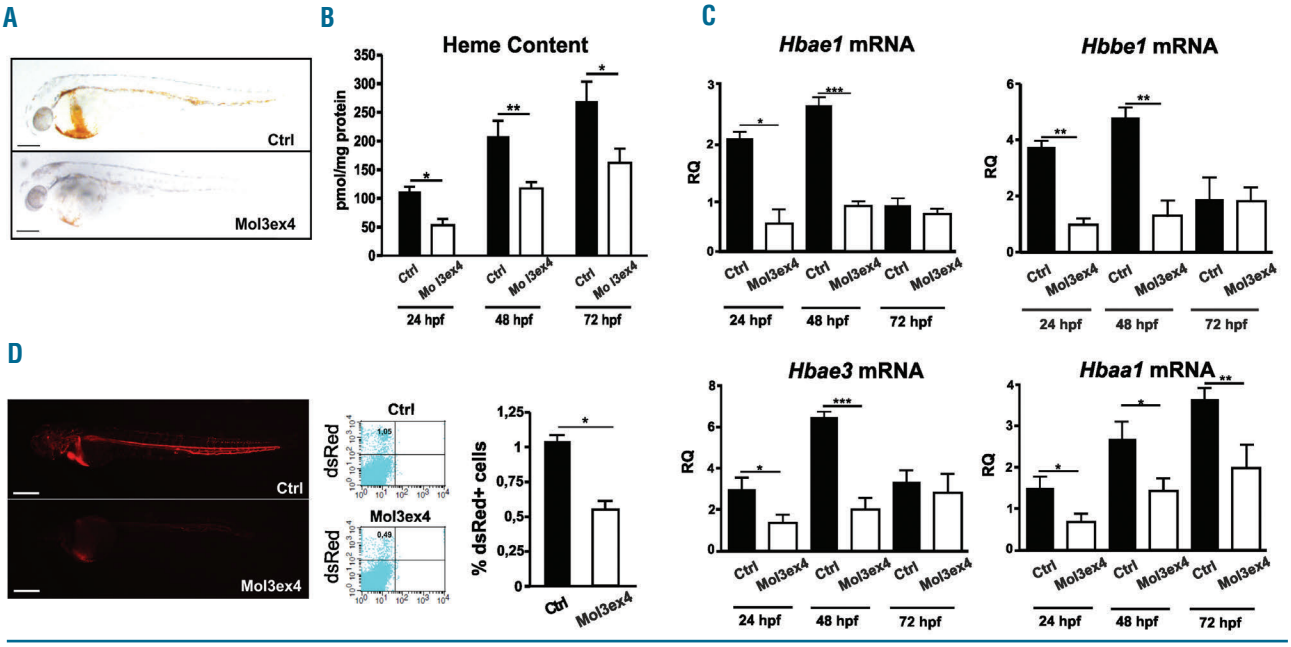

Figure 2. *Flvcr1a/1b* morphants have defective erythropoiesis. (A) O-dianisidine staining of control and MoI3ex4 morphants at 48 hpf. Eighty-six percent of morphants were anemic (n = 102); 100% of control embryos were unaffected (n = 123). Bar=200 μm. (B) Heme content in control and MoI3ex4 morphants at 24, 48 and 72 hpf. Values represent mean ± SEM. **P*<0.05; ***P*<0.005, n=3, two-way ANOVA. (C) qRT-PCR analysis of *Hbae1*, *Hbbe1*, *Hbae3* and *Hbaa1* 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^{+} cells is reported on the right. Values represent mean ± SEM. **P*<0.05, n=3, t-test. Bar=200 μm.

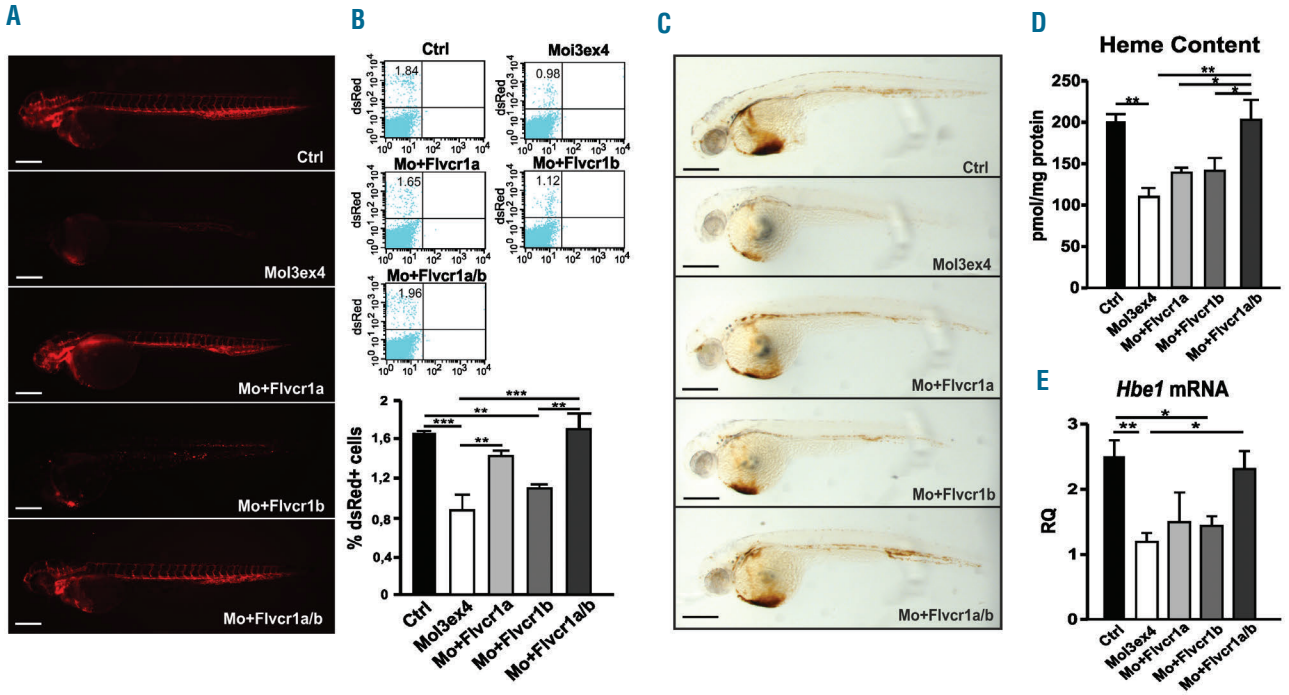

Figure 3. *Flvcr1a* and *Flvcr1b* are required to rescue erythropoiesis in *Flvcr1a/1b* morphants. (A) Photographs of transgenic *gata-1:dsRed* embryos injected with control or Mol3ex4 morpholino alone or with *Flvcr1a* cRNA and/or *Flvcr1b* cRNA. Bar=200 μm. (B) Representative flow cytometry analyses of dsRed fluorescence. The percentage of dsRed$^+$ 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 Mol3ex4 morphants injected with *Flvcr1a* cRNA and/or *Flvcr1b* cRNA. Anemia was evident in 85% of morphants (n = 60), 82% of embryos injected with Mol3Ex4 and *Flvcr1a* (n = 92), 86% of embryos injected with Mol3Ex4 and *Flvcr1b* (n = 99) and 29% of embryos injected with Mol3Ex4 and both *Flvcr1a* and *Flvcr1b* (n = 85). Bar=200 μm. (D) Heme content in control and Mol3ex4 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 *Hbae1* mRNA in control and Mol3ex4 morphants injected with *Flvcr1a* cRNA and/or *Flvcr1b* cRNA. Values represent mean ± SEM. $^{*}P< 0.05$; $^{**}P<0.005$, n=4, one-way ANOVA.

FLVCR1a *and* FLVCR1b *control the size of the cytosolic heme pool required for proliferation and differentiation of K562 cells*

Data from 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 the 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 down-regulated 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 *Flvcr1a*$^{fl/fl}$; *Mx-cre* mice and *Flvcr1a/1b* morphants, respectively.

Heme accumulated to a greater extent in the cytosolic fraction of *FLVCR1a*-down-regulated K562 cells compared with *FLVCR1a/1b*-down-regulated 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 (*Online Supplementary Figure S7*). Consistently with *in vitro* results, HO-1 up-regulation was observed in zebrafish and mice lacking *Flvcr1a* or both *Flvcr1a* and *Flvcr1b* (*Online Supplementary 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 with that in 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 with defective proliferation and/or differentiation, and differential heme accumulation in subcellular compartments. These data suggest that cytosolic heme accumulation, due to *FLVCR1a* deficiency, is detrimental to cell proliferation and promotes erythroid differentiation. On the other hand, mitochondrial heme accumulation is deleterious for both cell proliferation and differentiation. Thus, balanced expression of *FLVCR1a* and *FLVCR1b* is required for proper cell expansion and differentiation.

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

Data from cellular models established a correlation

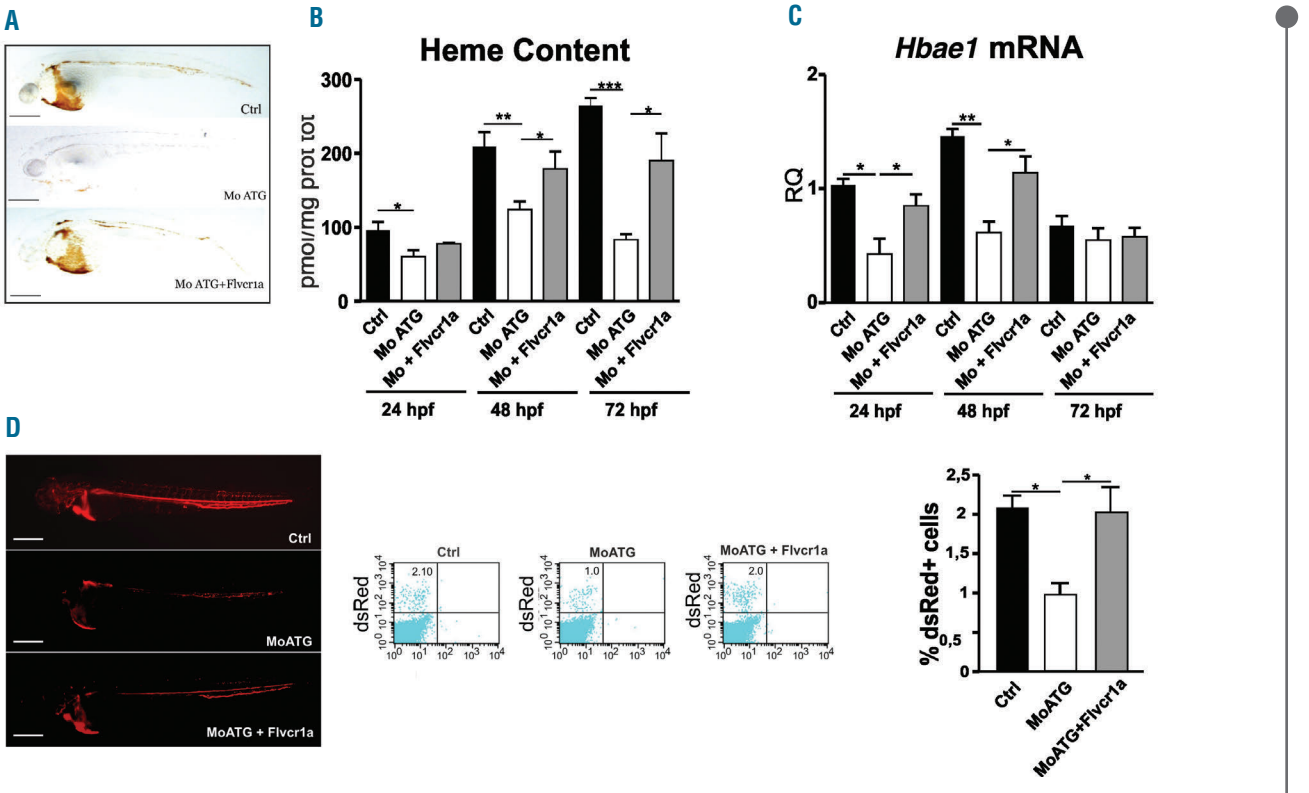

Figure 4. ***Flvcr1a*** **morphants are anemic. (A) O-dianisidine staining of control, MoATG morphants and MoATG morphants injected with** ***Flvcr1a*** **cRNA at 48 hpf. All control embryos were normal (n=119), 90% of morphants were anemic (n=110) in comparison to 28% of morphants injected with** ***Flvcr1a*** **cRNA (n=88). (B) Heme content in control, MoATG morphants and MoATG morphants injected with** ***Flvcr1a*** **cRNA at 24, 48 and 72 hpf. 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$^+$ cells is reported on the right. Values represent mean ± SEM. ****P*<0.05, n=3, one-way ANOVA. Bar=200 μm.

between defective proliferation and/or differentiation of cells lacking *FLVCR1a* or *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 *Flvcr1a* deficiency. On the other hand, heme supplementation is expected to bypass the mitochondrial block to heme export caused by *Flvcr1b* loss and thus it should rescue the differentiation defects associated with *Flvcr1b* deficiency. We performed the rescue experiments in zebrafish. In particular, we used the inhibitor of heme synthesis succinylacetone to reduce the cytosolic heme pool in *Flvcr1a* morphants and we supplemented the water with heme to increase the size of the cytosolic heme pool in *Flvcr1a/1b* morphants. As shown in Figure 6A, the inhibition of heme synthesis in zebrafish deficient in *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 *Flvcr1a/1b* morphants resulted in heme loading and death of the embryos (*Online Supplementary Figure S8*), further demonstrating that embryos cannot tolerate heme overload if the plasma membrane *Flvcr1a* is lost. Nevertheless, if *Flvcr1a/1b* morphants were injected with *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 *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 *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. In contrast, 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.

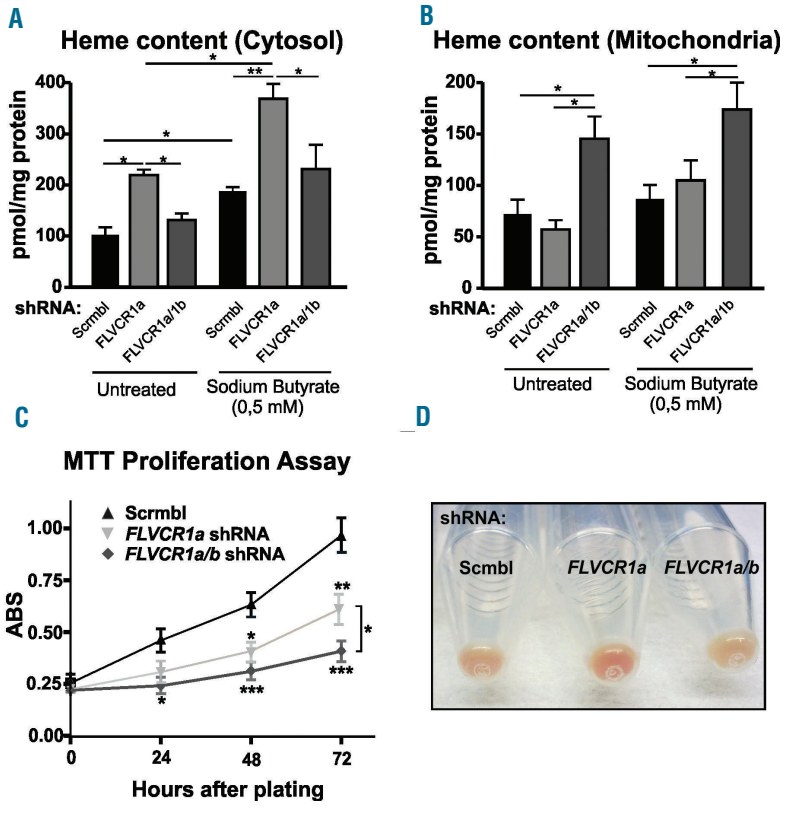

Figure 5. Silencing of *FLVCR1a* or *FLVCR1a/1b* in K562 cells differentially affects proliferation and differentiation. Heme content in the (A) cytosolic and (B) mitochondrial fractions of *FLVCR1a*- or *FLVCR1a/1b*-down-regulated K562 cells. Erythroid differentiation was induced with sodium butyrate (0.5 mM, for 72 h). 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 shRNA 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.

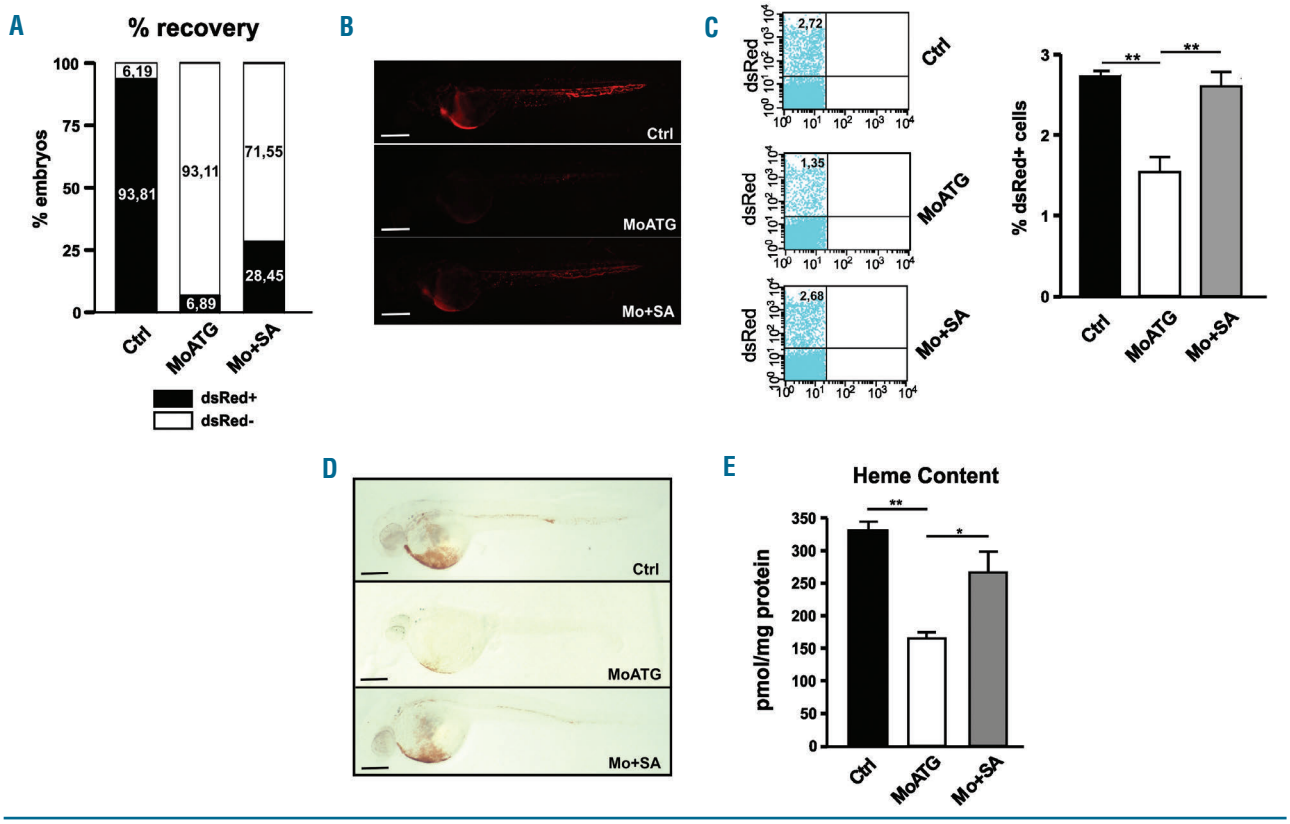

Figure 6. Inhibition of heme synthesis rescues anemia in *Flvcr1a* morphants. (A) Percentage of normal (non-anemic, dsRed$^+$) embryos when zebrafish were injected with a control morpholino, MoATG morpholino or MoATG morpholino in the presence of succinylacetone (SA). A representative experiment is shown. (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$^+$ cells is reported on the right. Values represent mean ± SEM. ***P*<0.005, n=3, one-way ANOVA. (D) O-dianisidine staining of control, MoATG morphants and MoATG morphants grown in the presence of SA, at 48 hpf. Bar=200 μm (E) Heme content in control, MoATG morphants and MoATG morphants grown in the presence of SA, at 48 hpf. Values represent mean ± SEM. **P*<0.05, ***P*<0.005, n=3, one-way ANOVA.

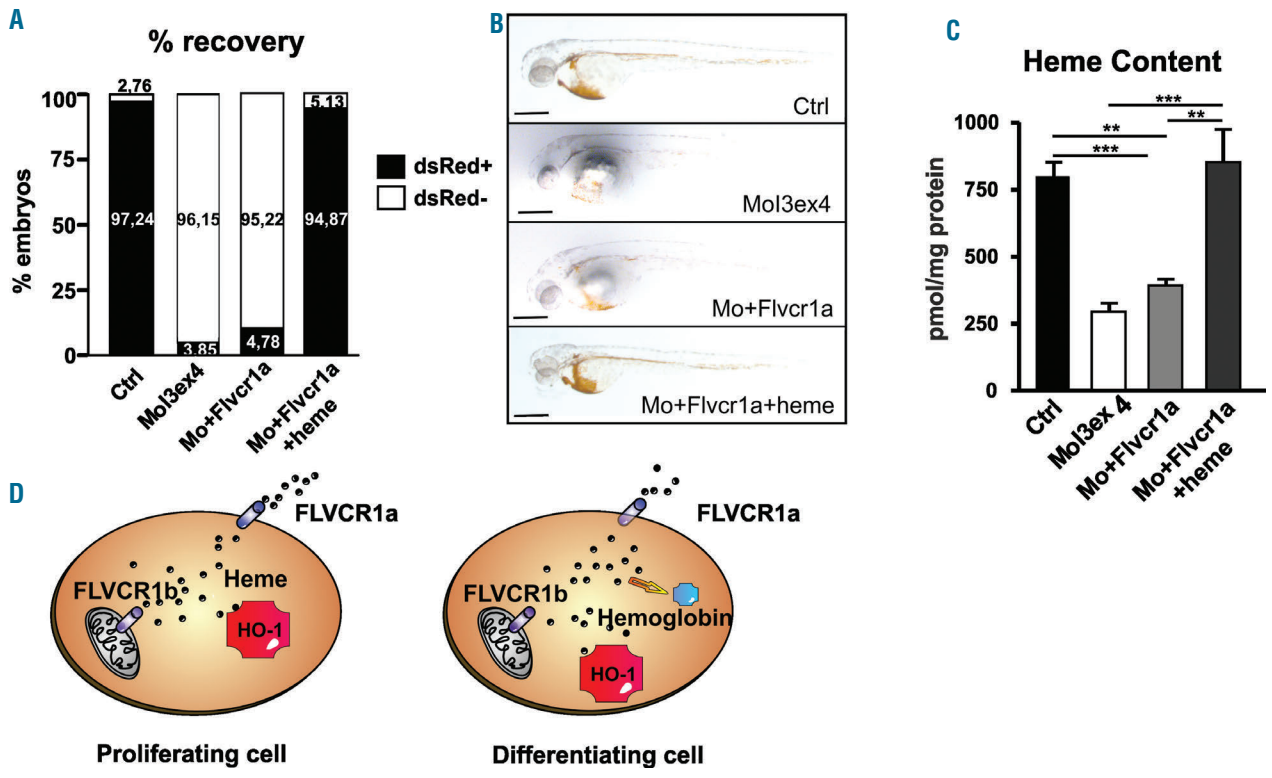

Figure 7. Supplementation with heme rescues anemia in *Flvcr1a/1b* morphants. (A) Percentage of normal (non-anemic, dsRed$^+$) embryos when zebrafish were injected with a control morpholino, Mo3ex4 morpholino, Mo3ex4 morpholino and *Flvcr1a* cRNA or Mo3ex4 morpholino and *Flvcr1a* cRNA, in the presence of heme. A representative experiment is shown. (B) O-dianisidine staining of zebrafish injected with a control morpholino, Mo3ex4 morpholino, Mo3ex4 morpholino and *Flvcr1a* cRNA or Mo3ex4 morpholino and *Flvcr1a* cRNA, in the presence of heme, at 48 hpf. Bar=200 μm. (C) Heme content in zebrafish injected with a control morpholino, Mo3ex4 morpholino, Mo3ex4 morpholino and *Flvcr1a* cRNA or Mo3ex4 morpholino and *Flvcr1a* cRNA, in the presence of heme, at 48 hpf. Values represent mean ± SEM. $^{**}P<0.005$, $^{***}P<0.001$, n=3, one-way ANOVA. (D) A model of the fuctions of the *Flvcr1* isoforms. 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 a high rate of proliferation. In differentiating cells, the 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]

Moreover, in both species, *Flvcr1b* alone is not sufficient for proper embryo development since *Flvcr1a*$^{-/-}$ embryos die in mid-gestation 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 hypothesized that their coordinated expression might contribute to control the size of the cytosolic free heme pool. The concept of a "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.[1,21,22] Recently, Garcia-Santos *et al.* introduced the concept of a 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, 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, contrariwise, a contraction of the heme pool should result in inappropriate hemoglobin production. The control of the size of the regulatory heme pool is, therefore, 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 the cytosol and mitochondria, respectively. *FLVCR1a*-silenced K562 cells accumulate heme in the cytosol, show defective proliferation and are more prone to differentiate compared to control cells. *FLVCR1a/1b*-silenced cells accumulate 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 an 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, the 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 from mice and zebrafish indicate that *Flvcr1b*, which exports heme from mitochondria to the cytosol, is required along all the phases of maturation of committed erythroid progenitors. In fact, heme has to be exported out from mitochondria for incorporation into the 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,[23] our data from 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 the *Flvcr1a* gene. Alternatively, other mechanisms may have 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 and it is sequentially reduced and oxidized to transfer electrons that ultimately reduce O_2.[24] Thus, it is conceivable that cytosolic heme availability must be regulated to allow the electron transport chain function. We think that *Flvcr1a* contributes to maintain a pool of *de novo* synthesized 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.[25] Concomitantly, the rate of heme synthesis increases to sustain hemoglobin production. At this time, the expression of FLVCR1a at the plasma membrane becomes dispensable, as indicated by our data from *Flvcr1a*$^{-/-}$ embryos. It is likely that during differentiation most of the heme is incorporated into the hemoglobin molecule whereas the rate of incorporation into the electron transport chain 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 cells 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 the HO-1 promoter.[26] 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.[27] HO-1 up-regulation 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, which is an endoplasmic reticulum 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 an adequate heme supply to sustain metabolic activity for cell cycling and hemoglobin production.

We previously showed that fetal liver cells of *Flvcr1a*$^{-/-}$ mice can reconstitute the bone marrow of lethally irradiated adult mice.[9] Nevertheless, we followed the mice for only 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 pathological conditions characterized by chronic stress erythropoiesis. Consistently, limitation of hemoglobin production has been shown to be a viable therapeutic strategy in mouse models of β-thalassemia and polycythemia vera.[6,28,29]

In conclusion, we demonstrate that the *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. Pharmacological modulation of intracellular heme content, likely through interference with heme exporters, may represent a new approach to disorders characterized by ineffective erythropoiesis.

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Authorship and Disclosures

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