SUPPLEMENTARY APPENDIX

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

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

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Supplemental Methods

Mouse genotyping

For genotyping, genomic DNA was amplified with the following primers: IloxFw: 5'- TCTAAGGCCCAGTAGGACCC-3', IIloxFw: 5'- GGCCTCAACTGCCTGGGAGC-3', IIloxRev: 5'- AGAGGGCAACCTCGGTGTCC-3'.

Oligomorpholino

Oligomorpholinos (MOs) were designed (GeneTool® oligo design) either to block splicing or to anneal to the ATG start codon and inhibit translation initiation.

MO sequences were as follows:

Control (Flvcr1 mism), 5′- CATAATACATAAAAAATGAACAAAT -3';

MoATG, 5' CCTGGAGAAACTCACCTGCCACCAT-3';

MoIn1Ex2, 5'-ACCAAGCTGACGGGAAATAAAGAGA -3';

MoIn3Ex4, 5'CATGATACCTAAAACATGAGCAGAT -3'.

In vitro colony assays

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

digested with 0,1% type I collagenase (200 U/mg) in calcium/magnesium free Hank's balanced salt solution, with 20% bovine serum (Life Technologies Italia, Monza MB; Italy) for 3 hours at 37°C and 10^3 cells were cultured in MethoCult M3334 (Stemcell Technologies). Colonies were analysed with an optical microscope (Zeiss, Germany) using an objective with a 10x magnification.

Erythroid differentiation

Single-cell suspensions were prepared from freshly isolated adult bone marrows and were immunostained with anti–TER119-PE and anti–CD71-FITC (BD Italia, Milano, Italy), then subjected to flow cytometry.

Blood analysis

Blood was collected by retrorbital bleeding and analyzed using an ADVIA120 Hematology System (Siemens Diagnostics).

Histology

Embryos were fixed in 4% PFA, embedded in paraffin and cut at microtome. Three μm-thick sections were stained with hematoxylin-eosin. Blood islands were analyzed by Metamorph software (Universal Imaging Corporation, Downingtown, PA, USA).

Western blotting

Tissue and cell proteins were extracted with 1%Triton, 150 mM NaCl, 50 mM TrisHCl (pH 8), 5 mM EDTA plus protease inhibitor (Roche Diagnostics Corp., Milano, Italy) and protein concentration was determined using the Bio-Rad protein assay system (Bio-Rad, Germany). Fifty μg of total protein extracts were separated on 12% SDS-PAGE and analyzed by Western blotting using antibodies against H-Ft (1) and a monoclonal antibody to vinculin (2).

Heme content

Ten µg of proteins were added to 500 µl of 2M Oxalic acid. The samples were then shaken vigorously and boiled at 95°C for 30 min in the dark. Standards were prepared by adding known concentrations of heme to 500 µl of 2M Oxalic acid and treated as indicated above. After cooling, the fluorescence of the samples was measured in a spectrofluorometer (Glomax Promega Italia, Milano, Italy): excitation and emission wavelengths were set to 405 nm and 662 nm, respectively. Heme content for each sample was recorded in double. The background was evaluated by measuring fluorescence in non boiled samples.

O-dianisidine staining

A staining solution containing benzidine 1.5 mg/ml (Sigma-Aldrich, Milano, Italy), sodium acetate (0.01 M, pH 4.5) and ethanol (40%) was prepared (3). Ten embryos/condition were collected in 2 ml eppendorf tubes and stained with 200 µl of staining solution for 15 min at room temperature. The embryos were analyzed under a Leica MZ9.5 stereo-microscope.

Flvcr1 gene silencing

To downregulate *Flvcr1a* or *Flvcr1a/1b* expression in K562 cells, two shRNAs against exon 1 (5'-CCAGTACAGCATCATTAGCAA-3') or exon 9 (5'-CTGCGAAGACACAACATAAAT-3'), respectively, of human *Flvcr1* gene were used (4). The lentiviruses pLKO.1.Flvcr1a and pLKO.1.Flvcr1a/1b (Open Biosystems, Thermo Scientific, Milano, Italy), expressing the shRNAs, were produced in HEK293FT cells as previously described (5, 6). K562 cells were infected with the lentiviruses in the presence of Sequa-brene. Following infection, cells were selected with 0.02 µg/ml puromycin. To induce erythroid differentiation, cells were treated with 0.5mM sodium butyrate for 72 hours.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from mouse tissues or from pools of at least 20 zebrafish embryos using TRIzol reagent according to the manufacturer's protocol (Life Technologies). One μg of total RNA was treated with DNAse (Promega Italia) and reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega Italia) and random primers. cDNA was then amplified by PCR using the following primer pairs:

zebrafish *Flvcr1a*, 5'-CCTGTGCCTTTGCTGTGTT-3' and 5'-AGTAGAACGATCCGGTCATG-3';

zebrafish *Flvcr1b*, 5'-TATGGTCTCTTTATTTCCCGTC-3' and 5'-GGAGGTCTGTCCTTAATGACAA-3';

mouse *Flvcr1a*:5'- AACGCCTTCCAGTGGATCCAGTAC-3' and 5'-AATAAGCTTGAGTGAAGATTCCGACTGTATAGACACC-3';

mouse Flvcr1b: 5'-CCGGAATTCATGTTTTACGGAACAGCATTTATC-3' and 5'-AATAAGCTTGAGTGAAGATTCCGACTGTATAGA-3';

zebrafish/mouse Actin, 5'-TTCCTTCTTGGGTATGGAAT-3' and 5'-GAGCAATGATCTTGATCTTC-3'.

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

Total RNA was extracted using Pure Link RNA Mini Kit (Life Technologies Italia) and reverse transcribed using Superscript II reverse transcriptase (Life Technologies Italia).

qRT-PCR was performed on a 7700 Real Time PCR System (Life Technologies Italia). Primers and probes were designed using the ProbeFinder software (www.roche-applied-science.com). For amplification of *Flvcr1a* and *Flvcr1b* specific primers and probes were used (4).

Supplemental Figures

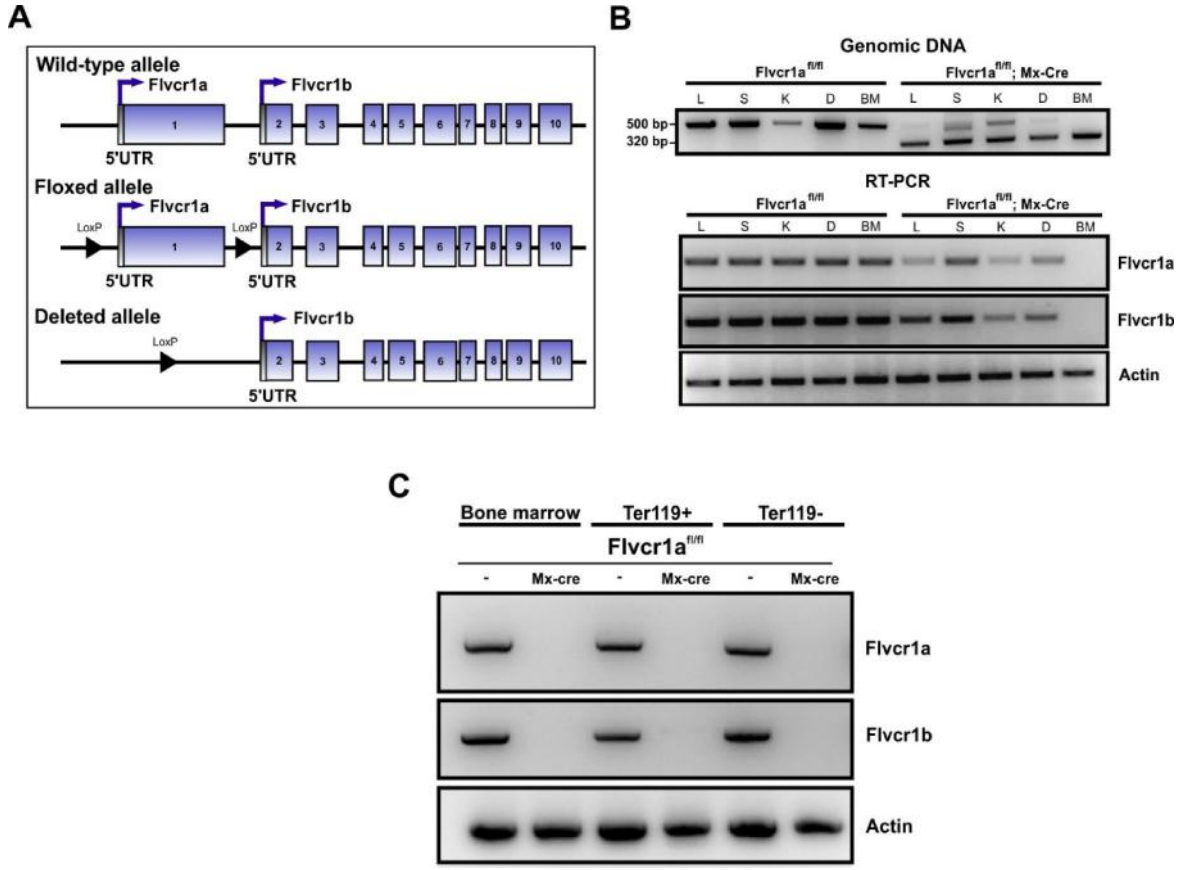

Figure S1.
(A)Schematic representation of *Flvcr1* locus showing the loxP sites flanking the first exon of the gene. The deleted allele is shown below.
(B)Top: representative PCR analysis on genomic DNA isolated from liver (L), spleen (S), kidney (K), duodenum (D) and bone marrow (BM) of *Flvcr1a*$^{f/fl}$ and *Flvcr1a*$^{fl/fl}$*;Mx-cre* mice. 500 bp band corresponds to the floxed allele, 320 bp band to the deleted allele. Bottom: representative RT-PCR analysis of *Flvcr1a* and *Flvcr1b* expression in the same tissues.
(C)Representative RT-PCR analysis of *Flvcr1a* and *Flvcr1b* expression in total bone marrow and in Ter119+ and Ter119- cell populations isolated from bone marrow of *Flvcr1a*$^{f/fl}$ and *Flvcr1a*$^{fl/fl}$*;Mx-cre* mice.

These data demonstrate that deletion of exon 1 results in the loss of expression not only of *Flvcr1a* as expected, but also of *Flvcr1b*. This is specific for the hematopoietic lineage since in other models, *Flvcr1a*$^{fl/fl}$*;Alb-cre* (7) and *Flvcr1a*$^{fl/fl}$*;Villin-cre* mice (unpublished), carrying the same deleted allele in hepatocytes and enterocytes, respectively, *Flvcr1b* expression is maintained. Thus, the deleted region might contain some regulatory elements required for *Flvcr1b* expression in the hematopoietic lineage. These data indirectly support our conclusion that *Flvcr1a* and *Flvcr1b* are co-regulated to maintain an adequate heme content in cytosol. Alternatively, a strong selective pressure against *Flvcr1a*-deficient cells that still express *Flvcr1b* can exist in a tissue with a high proliferation rate like the bone marrow.

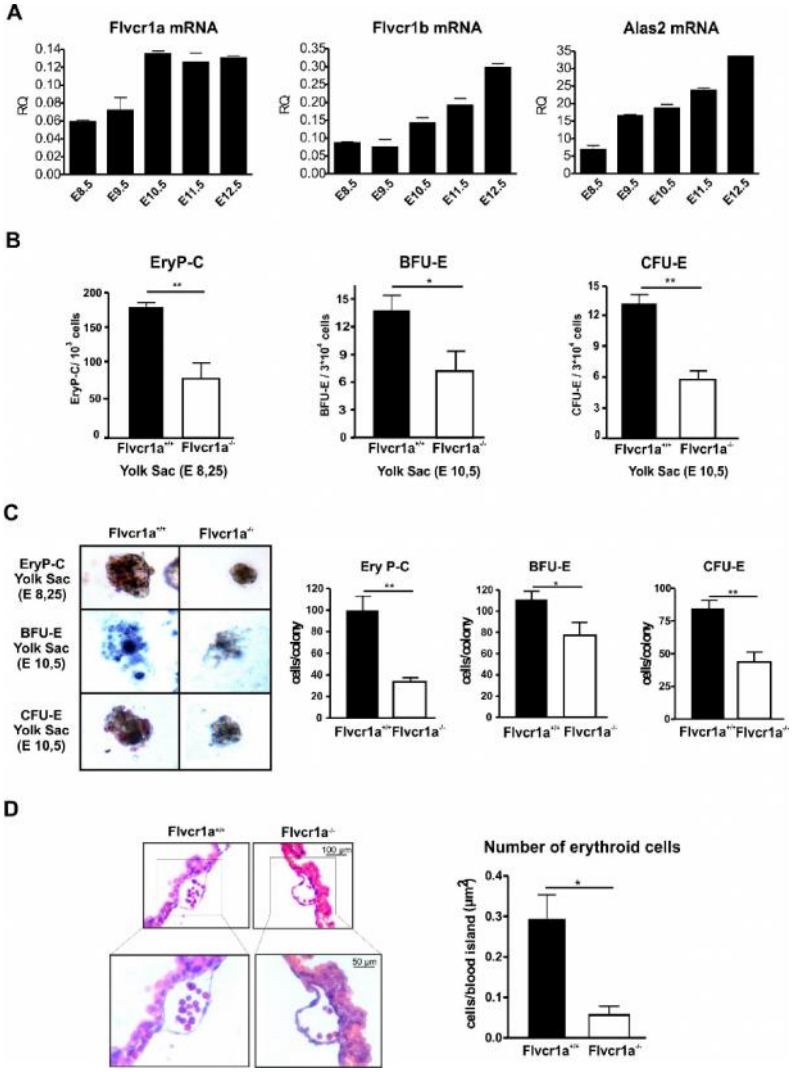

Figure S2. Analysis of primitive erythropoiesis in *Flvcr1a*$^{-/-}$ mice.
(A)qRT-PCR analysis of *Flvcr1a*, *Flvcr1b* and *Alas2* mRNA levels in primitive erythroid cells. To study the expression of *Flvcr1* isoforms during the expansion and differentiation of primitive erythroid progenitors, we took advantage of an *ε-globin*-H2B-GFP transgenic mouse line in which the expression of a nuclear GFP reporter is driven by human *ε-globin* regulatory elements specifically expressed within the primitive erythrocyte lineage (EryP) (8). EryP at different stages of maturation were isolated from *ε-globin*-H2B-GFP embryos and *Flvcr1a*, *Flvcr1b* and *Alas2* mRNA levels were evaluated. *Flvcr1a* and *Flvcr1b* expression increased from E8.5 to E12.5, along with expression of *Alas2*. Transcript abundance was normalized to Rn18s RNA expression. Relative mRNA expression levels were calculated using the dCt formula: Relative expression = A * 2^ (control gene Ct - target gene Ct) where A is an arbitrary value, here set at 100,000. Values represent mean ± SEM. n=3.
(B)Number of EryP-C isolated from yolk sac of *Flvcr1a*$^{+/+}$ and *Flvcr1a*$^{-/-}$ mice at E8.25 (n= 4) and BFU-E and CFU-E isolated from yolk sac at E10.5 (n=4).
(C)Dimension of EryP-C, BFU-E and CFU-E derived from yolk sac of *Flvcr1a*$^{+/+}$ and *Flvcr1a*$^{-/-}$ embryos (n=8). Representative images are shown on the left.
(D)Sections of yolk sac from *Flvcr1a*$^{+/+}$ and *Flvcr1a*$^{-/-}$ embryos at E10.5 stained with hematoxylin and eosin. The number of erythroid cells per blood island is reported on the right. n = 4, 6 sections/mouse were counted.
Values represent mean ± SEM. **P* < 0.05; ***P* < 0.005, t-test.

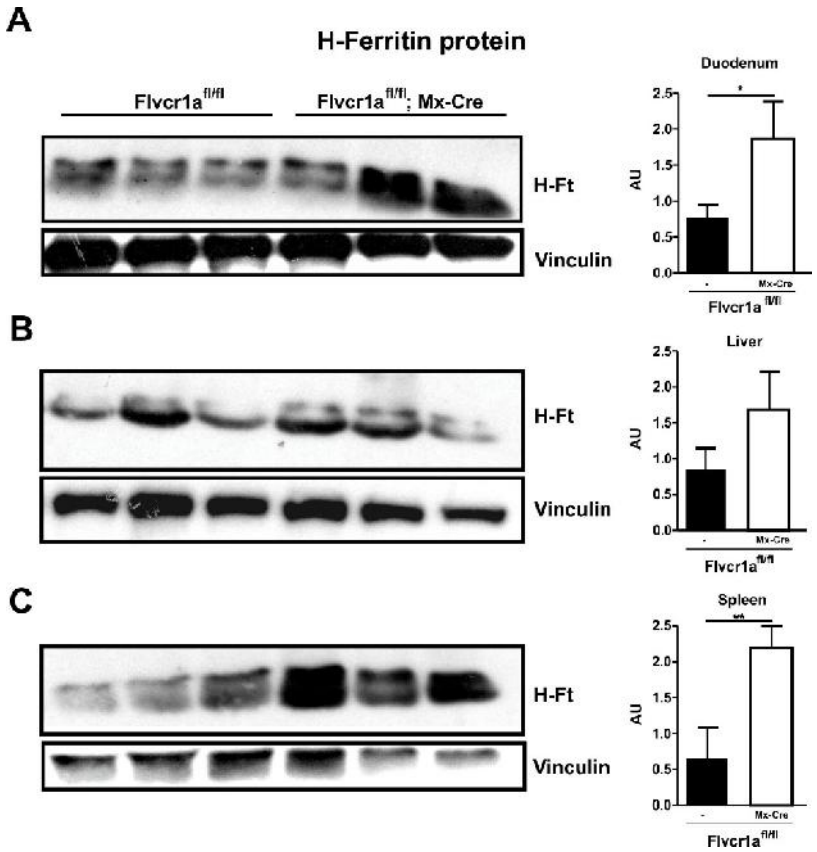

Figure S3. H-Ferritin expression in *Flvcr1a*$^{fl/fl}$*;Mx-cre* mice.
Representative Western blotting of H-Ferritin (H-Ft) in duodenum, liver and spleen of *Flvcr1a*$^{f/fl}$ and *Flvcr1a*$^{fl/fl}$*;Mx-cre* mice. AU: Arbitrary Units. Values represent mean ± SEM. **P* < 0.05; ***P* < 0.005, t-test; n=4

Figure S4. *Flvcr1* gene is highly conserved among fish and mammals.
Protein sequence alignment and the dendogram for a hierarchical clustering for *M. musculus, H. sapiens* and *D. rerio Flvcr1* gene. The blue colour in the protein sequences represents the identity between the three species. In the dendogram, the horizontal axis represents the distance or dissimilarity between clusters.

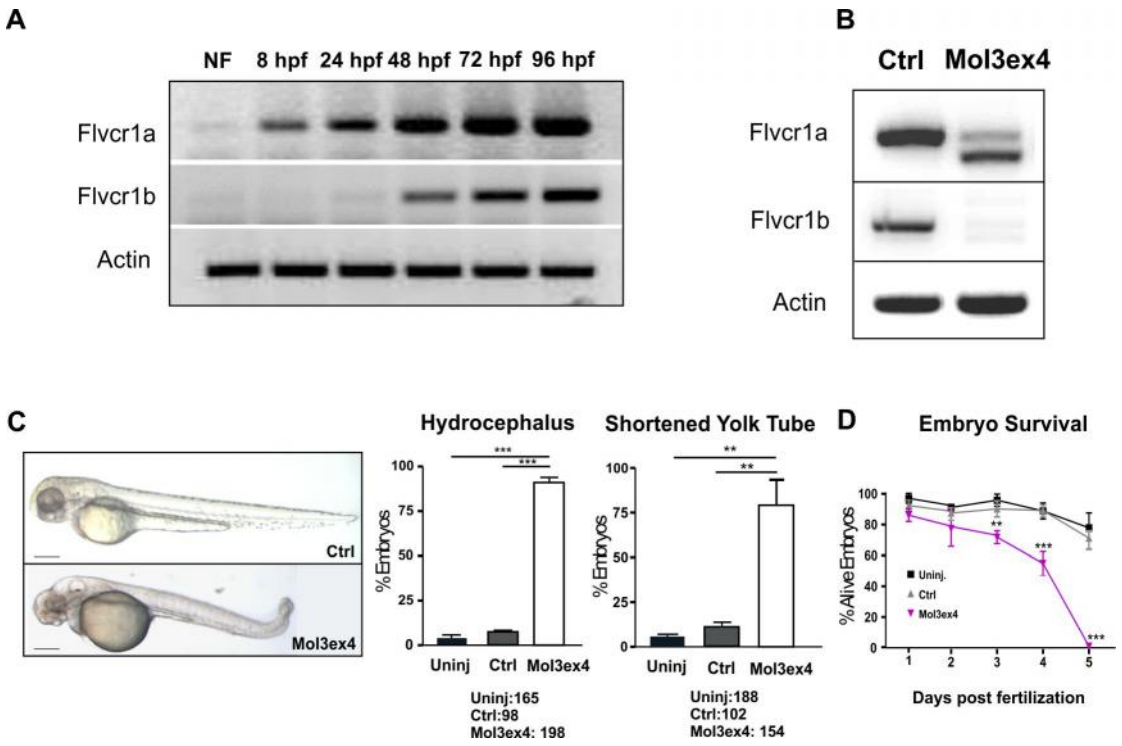

Figure S5- Knockdown of *Flvcr1a/1b* in zebrafish disrupts normal embryonic development.
(A)RT-PCR analysis of *Flvcr1a* and *Flvcr1b* expression during zebrafish development.
(B)RT-PCR analysis of *Flvcr1a* and *Flvcr1b* expression in zebrafish injected with a control morpholino (Ctrl) or an *Flvcr1a*/*1b* splicing morpholino (MoI3ex4). Actin RNA was analyzed as control.
(C)Embryos injected with MoI3ex4 are defective. A representative photograph is shown and the percentage of embryos showing developmental defects (hydrocephalus and shortend yolk tube) is reported. ***P* < 0.005; ***P< 0.001. Bar=200 µm.
(D)Embryo survival after injection of control or MoI3ex4 morpholino. ***P* < 0.005; ***P< 0.001. The results were obtained from several independent experiments with n > 150.

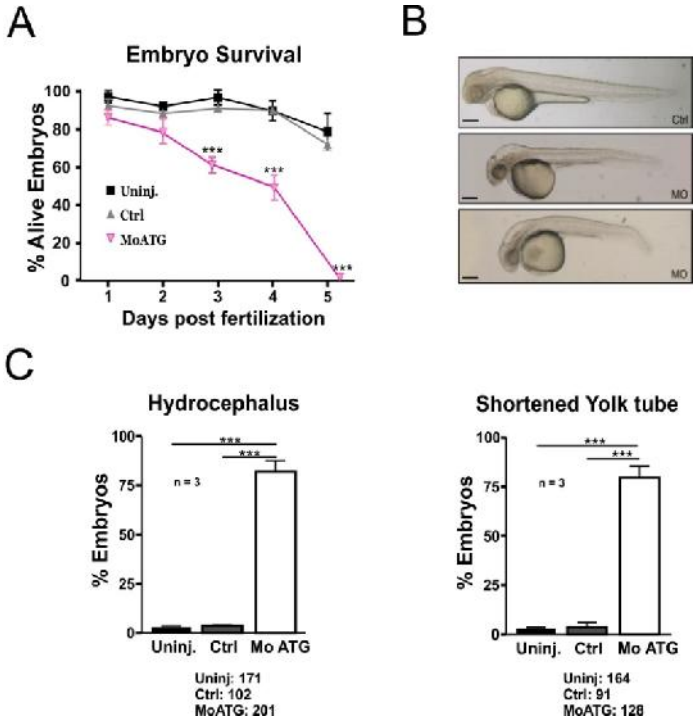

Figure S6. *Flvcr1a* morphants show abnormal embryo development.
(A)embryo survival after injection of control or MoATG morpholino. ***P< 0.001.
(B) Embryos injected with MoATG are defective. A representative photograph at 48 hpf is shown. Bar=200 μm
(C)The percentage of embryos showing developmental defects (hydrocephalus and shortend yolk tube) is reported. ***P< 0.001.

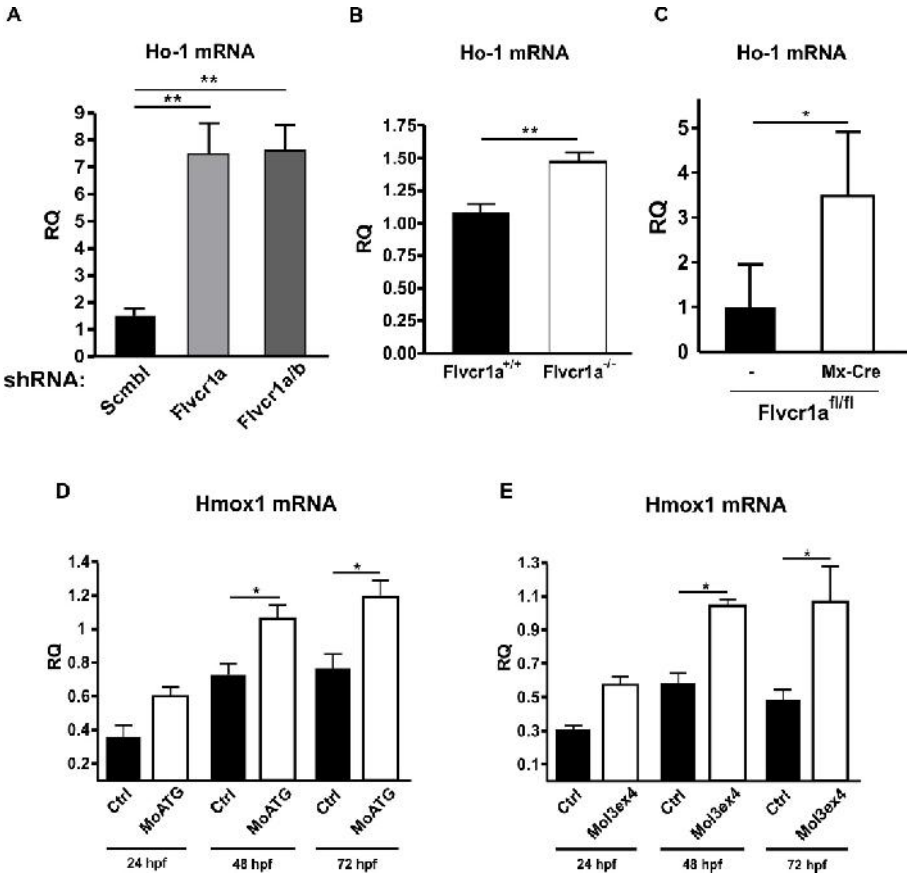

Figure S7. HO-1 gene expression.
The expression of the gene coding for HO-1 was analysed by qRT-PCR on:
(A)K562 cells infected with a control vector or with vectors carrying a specific shRNA for *FLVCR1a* or *FLVCR1a*/*1b*,
(B)Ter+ cells isolated from the fetal liver of *Flvcr1a*$^{+/+}$ or *Flvcr1a*$^{-/-}$ embryos at E 14,5,
(C)Ter+ cells isolated from bone marrow of *Flvcr1a*$^{f/fl}$ or *Flvcr1a*$^{fl/fl}$*;Mx-cre* mice,
(D)control or *Flvcr1a* morphants,
(E)control or *Flvcr1a/1b* morphants.
Values represent mean ± SEM. **P* < 0.05; **P< 0.005, one-way ANOVA (A), t-test (B, C), and two-way ANOVA (C, D), n=5.

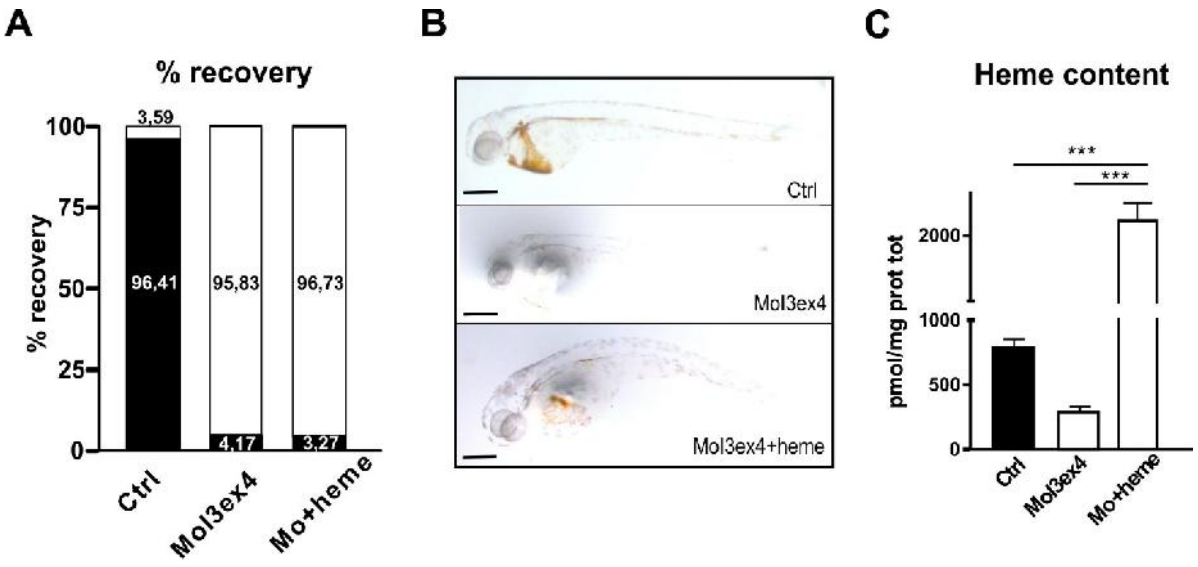

Figure S8. *Flvcr1a/1b* morphants do not tolerate heme supplementation.
(A)Percentage of normal (non anemic) embryos when zebrafish were injected with a control morpholino, MoI3ex4 morpholino, or MoI3ex4 morpholino in the presence of heme.
(B)O-dianisidine staining of zebrafish injected with a control morpholino, MoI3ex4 morpholino, or MoI3ex4 morpholino in the presence of heme, at 48hpf. Bar=200 µm
(C)Heme content in zebrafish injected with a control morpholino, MoI3ex4 morpholino, or MoI3ex4 morpholino in the presence of heme, at 48hpf . Values represent mean ± SEM. ****P* < 0.001, n=5, one-way ANOVA.

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