Supplementary Figures and Legends

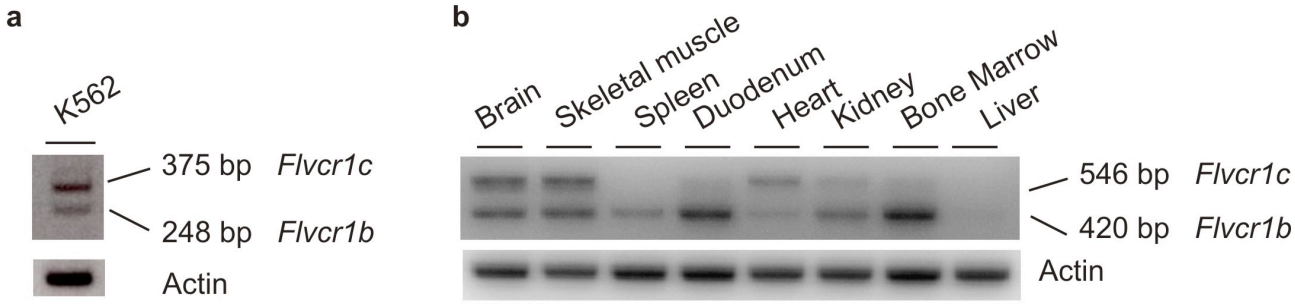

Figure S1

Identification of *Flvcr1b* transcript. (**a**) To experimentally validate the existence of *Flvcr1b* transcript, we analyzed RNA extracted from K562 cells by RT-PCR using a forward primer designed on the 5'UTR of this transcript and a reverse primer on exon 3. As shown in the figure two bands could be amplified, 248 and 375 bp long. Sequencing of these bands showed that the 248 bp band corresponds to a mRNA containing the end of the first intron, exon 2 and exon 3 of *Flvcr1*; the 375 bp band corresponds to a mRNA composed by the end of the first intron, exon 2, an additional exon that we called 2b, and exon 3 of *Flvcr1*. We called the first transcript *Flvcr1b* and the other *Flvcr1c* to distinguish them from the canonical *Flvcr1* mRNA that we designed as *Flvcr1a*. (**b**) To further confirm the existence of these novel isoforms, we performed RT-PCR analyses on RNA extracted from different mouse tissues with a forward primer on the end of first intron of the orthologous mouse gene major facilitator superfamily domain containing 7b (*Mfsd7b*) and a reverse primer on exon 3. Two bands could be detected in almost all mouse tissues. Sequencing of these bands demonstrated the existence of *Flvcr1b* and *Flvcr1c* mRNAs also in the mouse. Importantly, the same experiments were performed using a reverse primer located on exon 10, demonstrating the existence of full length *Flvcr1b* and *Flvcr1c* in both mouse tissues and in human cell lines (data not shown). Of note, no significant open reading frames were identified in *Flvcr1c* inducing us to mainly focus on *Flvcr1b*.

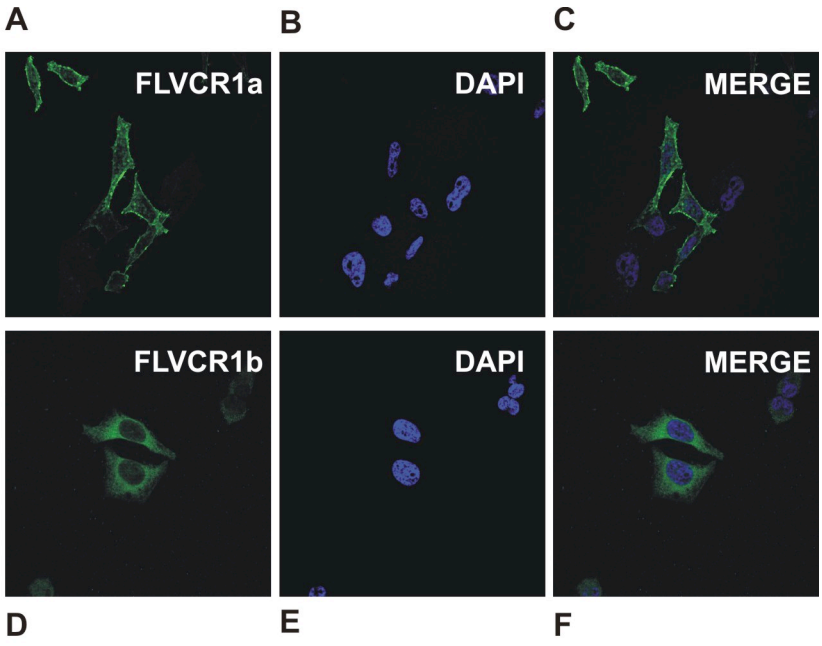

Figure S2

Different subcellular localization of FLVCR1a and FLVCR1b. Immunofluorescence analysis of HeLa cells overexpressing FLVCR1a-myc (**A-C**) or FLVCR1b-myc (**D-F**) showing different sub-cellular localization of the two isoforms. FLVCR1a was mainly expressed at the cell membrane whereas FLVCR1b was an intracellular protein. An anti-myc antibody was used to detect the overexpressed proteins.

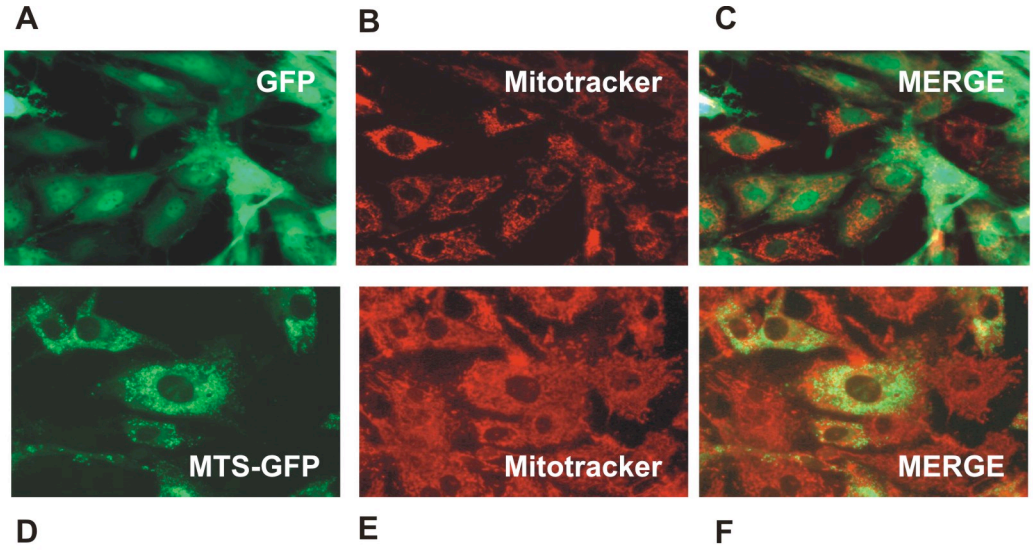

Figure S3

The mitochondrial targeting sequence of FLVCR1b directs GFP expression in the mitochondrion. The putative mitochondrial targeting sequence of FLVCR1b was fused at the N-terminus of GFP (MTS-GFP), overexpressed in HEK293 cells and immunofluorescence was performed. (**A-C**) HEK293 cells overexpressing GFP alone; (**D-F**) HEK293 cells overexpressing MTS-GFP. The colocalization of MTS-GFP with Mitotracker is shown, indicating that this sequence is able to specifically guide GFP expression into the mitochondrion.

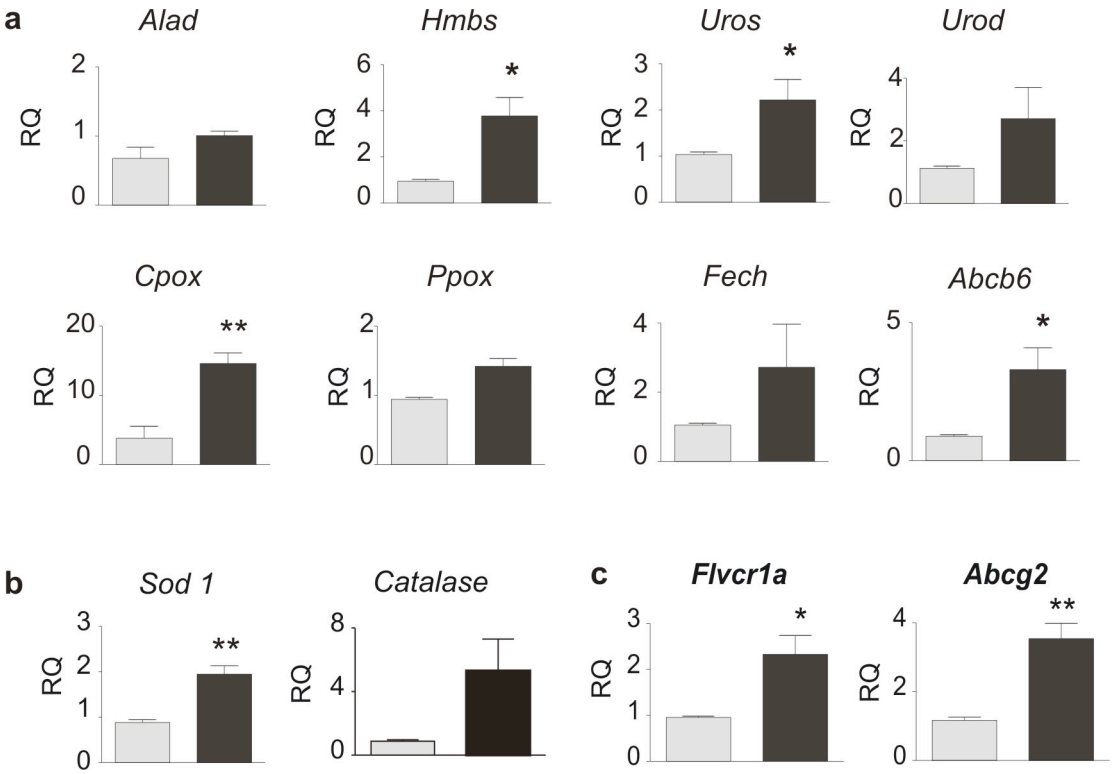

Figure S4.

The overexpression of FLVCR1b potentiates heme biosynthesis and activates heme detoxifying pathways. (**a**) qRT-PCR analysis of mRNA levels of the enzymes and transporters involved in heme biosynthesis in HeLa cells overexpressing FLVCR1b compared to controls. (**b**) Oxidative stress in HeLa cells overexpressing FLVCR1b is evidentiated by the up-regulation of *Sod1* and *Catalase* mRNA. (**c**) Activation of heme export pathway at the cell membrane is evinced by the enhanced expression of *Flvcr1a* and *Abcg2* in FLVCR1b overexpressing-HeLa cells. Values represent mean ± SEM. n=6. Statistical analyses were performed using t-test (*=P<0.05; **=P<0.01).

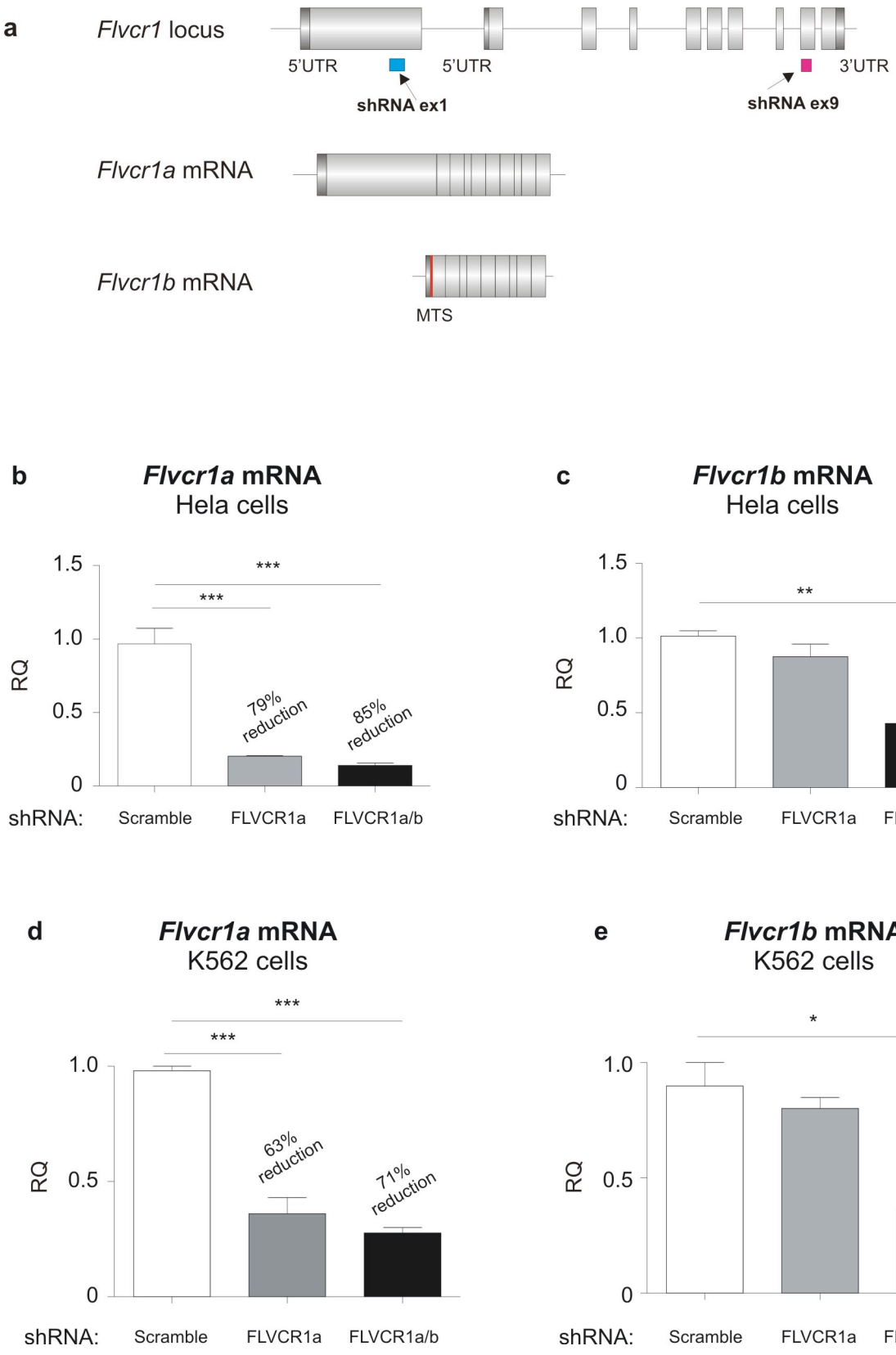
a
Flvcr1 locus
5'UTR
5'UTR
3'UTR
shRNA ex1
shRNA ex9
Flvcr1a mRNA
Flvcr1b mRNA
MTS
b
Flvcr1a mRNA
Hela cells
RQ
1.5
1.0
0.5
0
***
***
79% reduction
85% reduction
shRNA:
Scramble
FLVCR1a
FLVCR1a/b
c
Flvcr1b mRNA
Hela cells
**
58% reduction
d
Flvcr1a mRNA
K562 cells
63% reduction
71% reduction
e
Flvcr1b mRNA
K562 cells
*
60% reduction

Figure S5.

Silencing strategy. (**a**) Schematic representation of the silencing strategy. *Flvcr1b* mRNA is completely identical to *Flvcr1a*, with the exception of the first exon. Using a shRNA against the first exon (blue) we generate FLVCR1a-silenced cells while using a shRNA against exon 9 (red) we generate FLVCR1a- and 1b-silenced cells. Real-time PCR analysis of *Flvcr1a* (**b-d**) and *Flvcr1b* (**c-e**) mRNA in HeLa (**b-c**) or K562 (**d-e**) cells expressing a shFLVCR1a, a shFLVCR1a/1b or a scrambled sequence. Values represent mean ± SEM. n=6. Statistical analyses were performed using one-way analysis of variance (*=P<0.05; **=P<0.01; ***=P<0.001).

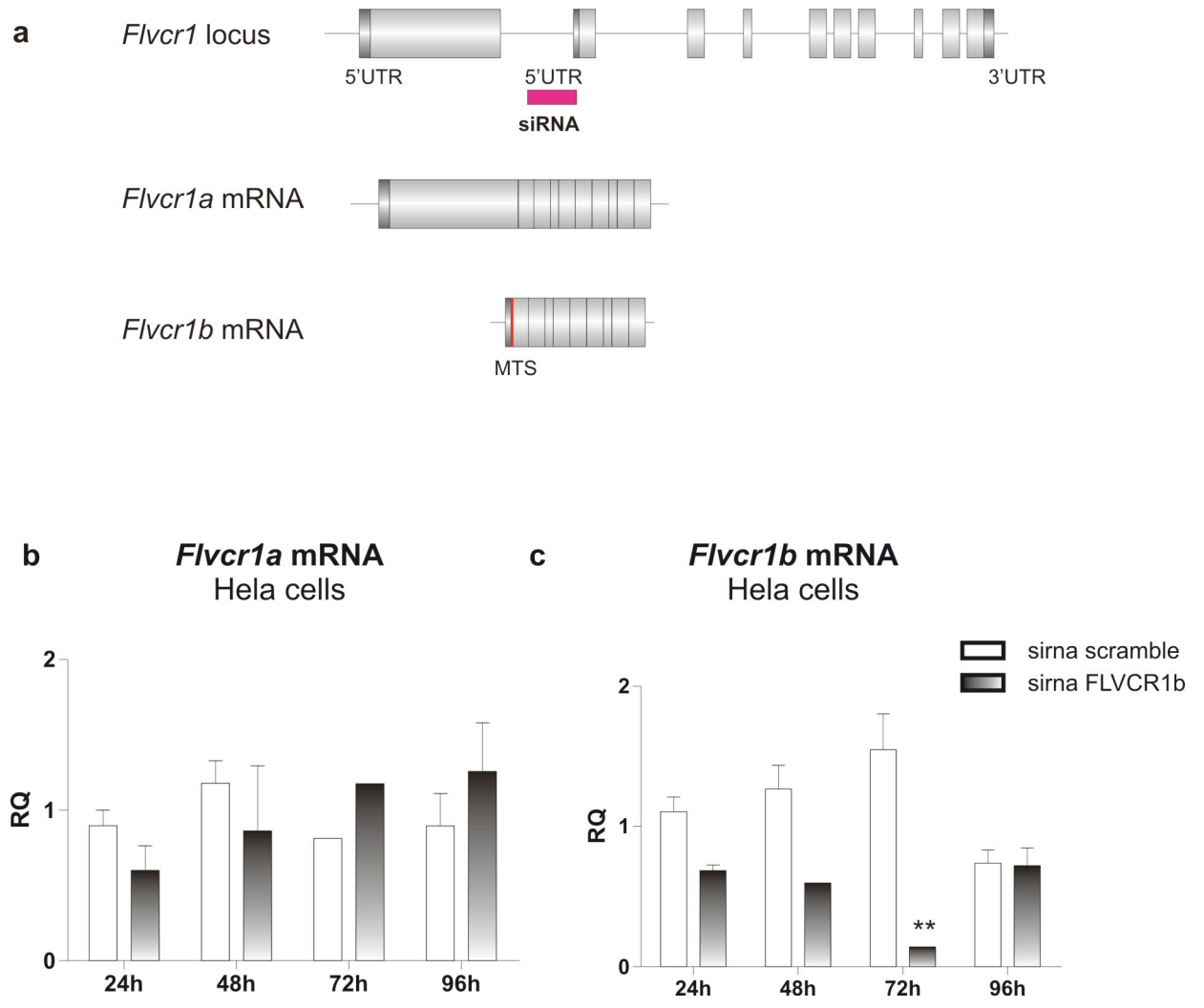

Figure S6.

FLVCR1b specific silencing. (**a**) Schematic representation of the silencing strategy. *Flvcr1b* mRNA is completely identical to *Flvcr1a*, with the exception of the first exon. To specifically interfere with the expression of *Flvcr1b* without altering that of *Flvcr1a*, we designed a siRNA against the 5'UTR of *Flvcr1b*. As a control we used a scramble siRNA. Real-time PCR analysis of *Flvcr1a* (**b**) and *Flvcr1b* (**c**) mRNA in HeLa cells at different time points following the transfection. Transcript abundance, normalized to 18S RNA expression, is expressed as a fold increase over a calibrator sample. Values represent mean ± SEM. n=6. Statistical analyses were performed using two-way analysis of variance (**=P<0.01). Experiments were performed at 72hours following the transfection when only the expression of *Flvcr1b* was significantly down-regulated.

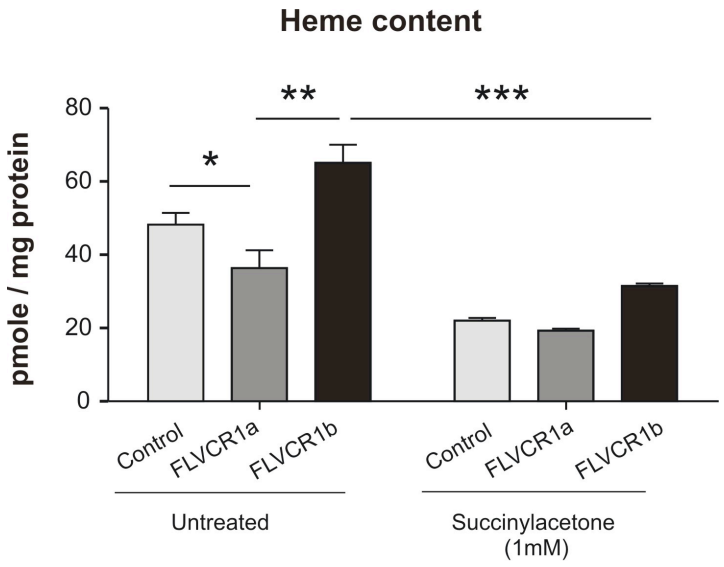

Figure S7

Figure S7.

Increased intracellular heme content in K562 cells overexpressing FLVCR1b. Total intracellular heme content was measured in K562 cells overexpressing FLVCR1a-myc, FLVCR1b-myc or a control vector. The increase of heme amount in FLVCR1b overexpressing cells is completely prevented by the treatment with Succynilacetone. Values represent mean ± SEM. n=6. Statistical analyses were performed using two-way analysis of variance (*=P<0.05; **=P<0.01; ***=P<0.001).

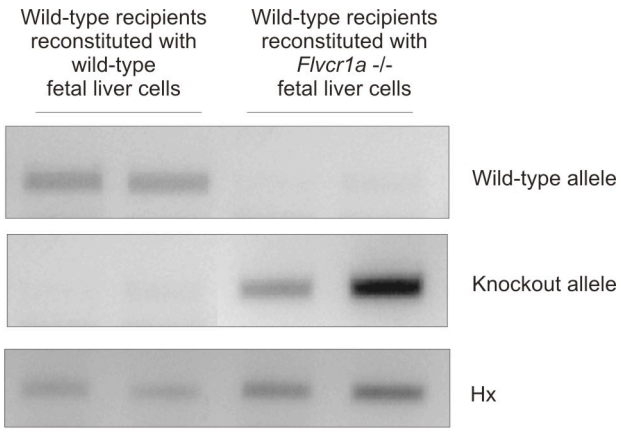

Figure S8

Efficiency of transplantation assay. The transplantation rate was assessed on blood genomic DNA by PCR using specific primers to amplify the wild-type or the *Flvcr1a* knockout allele. Hemopexin (Hx) gene was amplified as an internal control.

	+/+	+/-	-/-	**Ratio**
10,5 ÷ 12,5	50 (24,04%)	118 (56,73%)	40 (19,23)	1 : 2,3 : 0,8
13,5 ÷ 15,5	56 (28,57%)	96 (48,98%)	44 (22,24%)	1 : 1,7 : 0,8
16,5 ÷ 18,5	9 (23,08%)	22 (56,41%)	8 (20,51%)	1 : 2,4 : 0,8
Tot	115 (25,96%)	236 (53,27%)	92 (20,77%)	1 : 2 : 0,8
4 Wks	156 (35,86%)	279 (64,14%)	0	1 : 1,8 : 0

Table S1. *Flvcr1a*$^{-/-}$ embryos die during embryonic development. Genotyping of embryos and pups derived from F1 to F4 intercrosses.

Oligo Name	**Sequence 5' to 3'**
mouseFLVCR1a Fw	CCGTCGCCTCGGTATGG
mouseFLVCR1b Fw	TCGCTTCCTATTGACAGCTATTAACA
mouseFLVCR1ab Rev	CACTAAAACAGGTGGCAACAAAAA
humanFLVCR1a Fw	TTGGGCCCAAAGAGGTGTC
humanFLVCR1b Fw	TCCTCTTTATGTTCTGTTAATTGCCA
humanFLVCR1ab Rev	GCCAGGAGATTTGTGTCATTCTG
Probes FAM	**Sequence 5' to 3'**
mouseFLVCR1 probe	TTGGAACTGCAGTTGGT
humanFLVCR1 probe	ACCACCAGTTTTAGTACCCAA

Table S2. Primers and probes used for Real-time PCR analysis.