Supplementary materials for:

Coordinate expression of heme and globin is essential for effective erythropoiesis.

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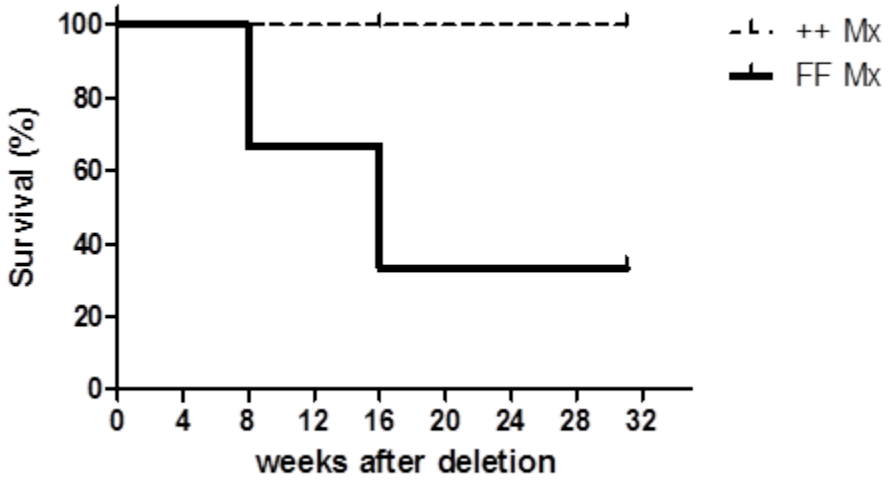
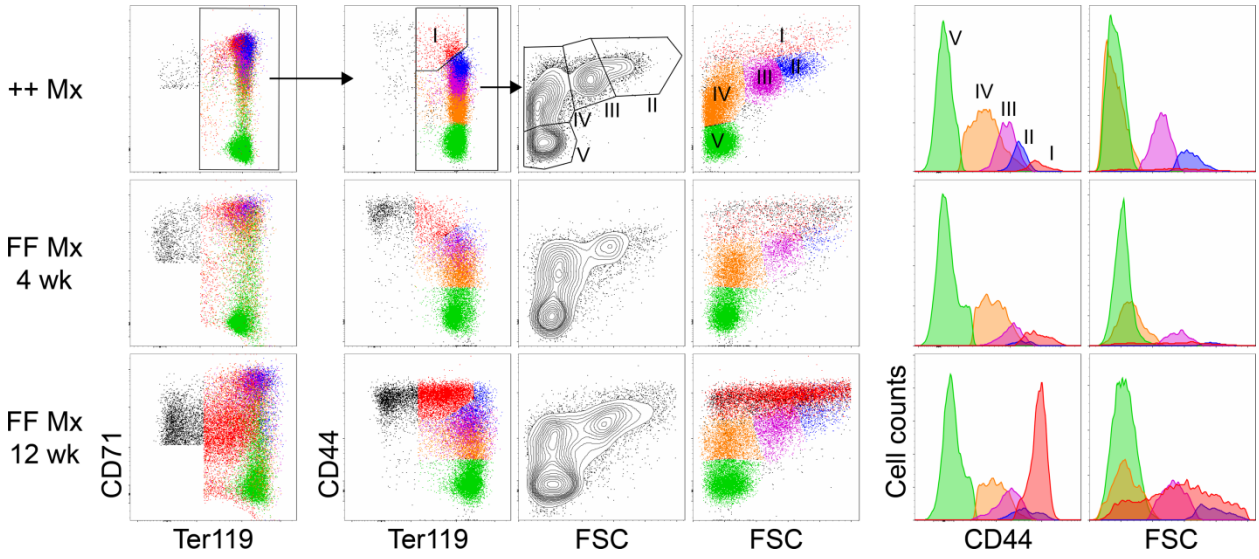
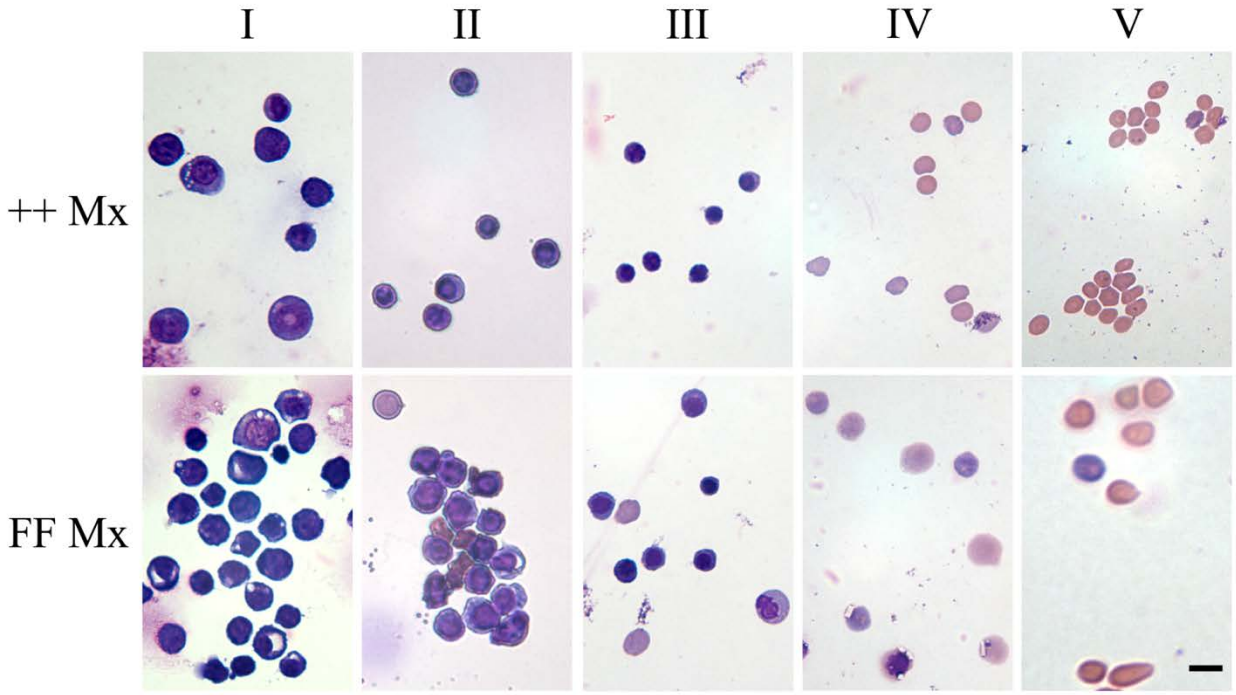


Supplementary Figure 1.
Kaplan-Meier survival curve of control (dashed line, N=6) and *Flvcr1*-deleted (solid line, N=9) mice after polyI-polyC treatment. Tick marks indicate when mice were removed from further analysis in the study for reasons other than anemia, usually for flow analysis of marrow samples. Median survival of *Flvcr1*-deleted mice is 16 wk. Mantel-Cox log rank test: $p < 0.02$.



Supplementary Figure 2. Flow cytometry analysis of control and *Flvcr1*-deleted mice. Representative flow cytometry analysis of lineage negative marrow cells which were stained and gated according to Chen et al. (20). Lin^- cells were analyzed with CD71 and Ter119 with $Ter119^+$ cells used for subsequent gating of populations I-V as diagrammed in the first 3 columns (I=proerythroblasts, II=basophilic erythroblasts, III=polychromatic erythroblasts, IV=orthochromatic erythroblasts & reticulocytes, V=reticulocytes & rbc). The black dots in the dot plot panels represent erythroid cells which are excluded when using the strict $Ter119^+$ gating above but included when $CD71^+Ter119^-$ cells are included in erythroid population I as in Figure 1. The three panel columns on the right include overlays of the gated populations to show CD44 and size (FSC) distribution of each population.



Supplementary Figure 3. Morphology of sorted control and *Flvcr1*-deleted erythroblasts. Photomicrographs of sorted erythroblast populations I-V from control (++ Mx) and *Flvcr1*-deleted (FF Mx) mice are presented. A 10 μM scale bar is in the lower right panel. Cytospins of each sorted population were Wright-Giemsa stained and photographed with a Nikon DS-Fi1 camera mounted on a Leica DM 1000 microscope. The resulting images were adjusted for optimal brightness levels and then cropped and assembled into the final figure in Adobe Photoshop CS4.

Supplementary Table 1. Erythroid colony forming cells in sorted erythroid populations.

	CFU-E[A]		BFU-E[A]	
population[B]	control	deleted	control	deleted
whole marrow	105±20.5	1.0±1.4	15±3.5	11±2.1
LNPC	0	0	820±198	360±0.0
I	4640±1018	0	340±141	720±170
II	0	NT	0	NT

[A] per 10^5 cells, NT = not tested.
[B] whole marrow, N=2; LNPC, I, II, N=2 technical replicates from a pool of marrow from 2 mice.

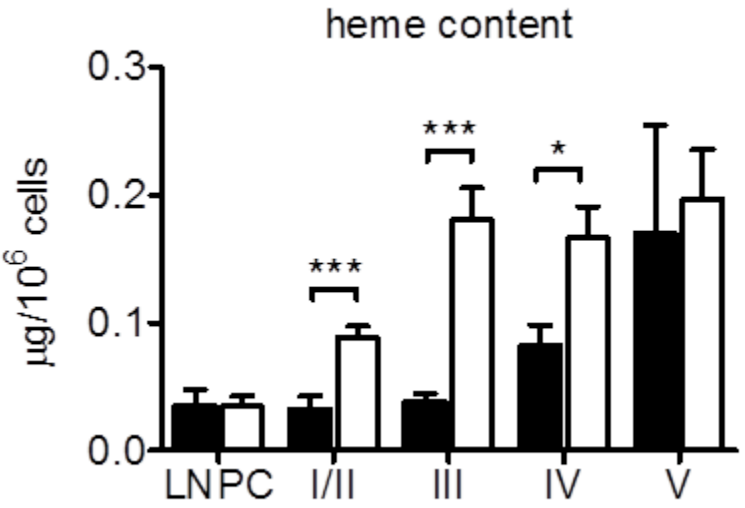
Supplementary Table 2. Frequency of lineage negative cells in each erythroblast population

	4 wk post deletion, N=6			12 wk post deletion, N=5		
population	control, %	deleted, %	p value	control, %	deleted, %	p value
I	4.6±1.7	12.4±7.7	0.04	4.1±1.3	22.1±11.5	0.009
II	6.1±1.4	1.6±1.2	<0.001	4.4±1.4	2.6±0.5	0.03
III	14.7±2.6	5.3±3.9	0.001	13.6±2.8	8.5±1.8	0.008
IV	28.2±5.5	15.5±4.3	0.001	31.3±3.8	22.3±6.1	0.02
V	36.6±5.7	44.7±11.4	0.15	34.2±2.8	23.3±9.6	0.04

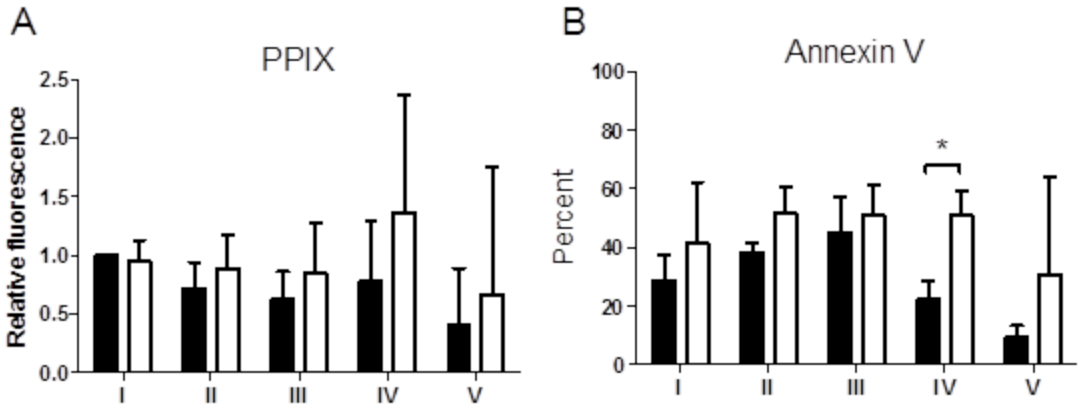
Supplementary Table 3. Absolute erythroblast counts from femurs and tibias

	4 wk post deletion, N=6			12 wk post deletion, N=4		
Population	control[A]	deleted[A]	p value	control[A]	deleted[A]	p value
I	1.7±1.1	2.4±1.8	0.42	1.3±0.39	3.1±1.4	0.05
II	2.1±0.86	0.35±0.35	0.001	1.6±0.81	0.44±0.14	0.03
III	5.1±1.4	1.1±1.0	<0.001	4.6±1.4	1.6±0.5	0.007
IV	9.9±3.0	2.9±1.2	<0.001	11.6±1.2	4.5±1.3	<0.001
V	13.0±4.5	8.2±2.6	0.05	11.9±0.68	4.4±3.1	0.003
Abs EB[B]	32.2±9.2	15.3±55	0.003	31.5±3.7	14.6±3.8	<0.001

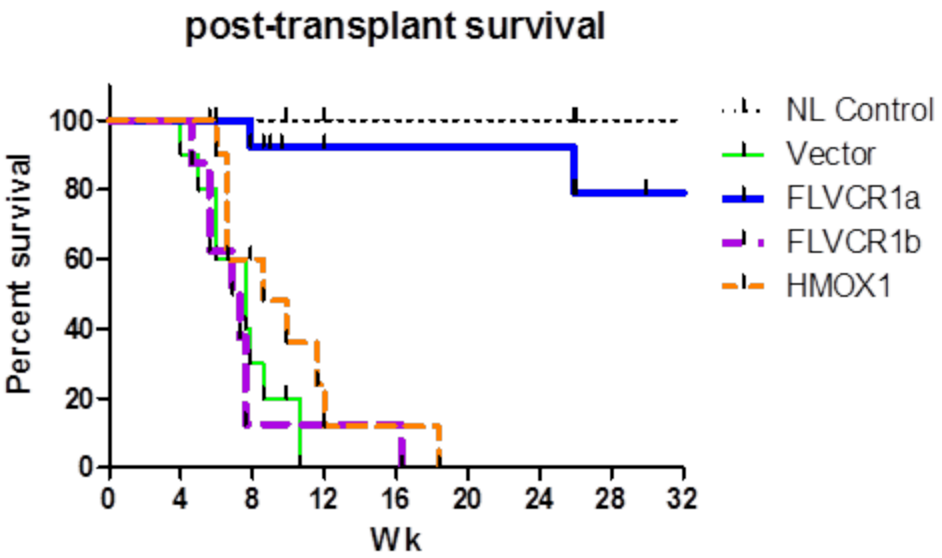
[A] × 10^6
[B] absolute number of erythroblasts in populations I-V combined.



Supplementary Figure 4. Heme content in *Flvcr1*-deleted erythroblasts does not increase further after the polychromatic erythroblast stage unlike control erythroblasts.
Heme content of sorted lineage negative precursor cells (LNPC) and committed erythroid progenitors (populations I-V) from control (solid bars, N=5) and *Flvcr1*-deleted mice (open bars, N=8). Mean ± SD is shown. T test: *$p < 0.05$, ***$p < 0.001$.



Supplementary Figure 5. Protoporphyrin IX and apoptosis are elevated in *Flvcr1*-deleted erythroblasts. (A) Relative fluorescence intensity of PPIX (N=10 each) in differentiating erythroblasts and (B) the frequency of Annexin V stained erythroblasts (N=3 each) from control (solid bars) and *Flvcr1*-deleted (open bars) mice. To combine data from multiple independent flow studies, the PPIX fluorescence of each sample was normalized to the mean PPIX fluorescence of population I from control samples. Normalized data from multiple studies were combined for this analysis. T test: *$p < 0.03$.

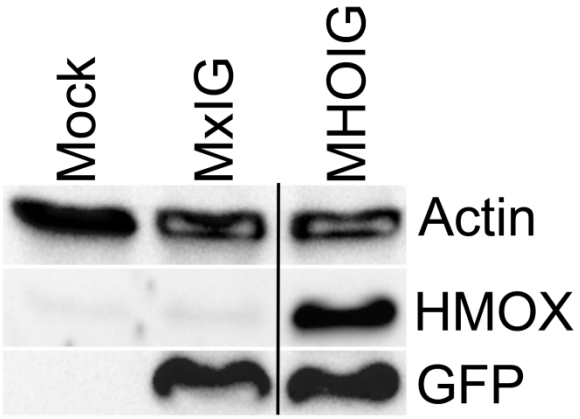


Supplementary Figure 6. The ability of FLVCR1a, FLVCR1b, or HMOX1 to replace FLVCR1 during erythropoiesis.
Survival curves of mice transplanted with normal marrow (NL control) or *Flvcr1*-deleted marrow transduced with vector, *Flvcr1a*, *Flvcr1b*, or *Hmox1* as indicated. Median survival times: vector, 7.6 wk; *Flvcr1a*, >35 wk; *Flvcr1b*, 7.1 wk; *Hmox1*, 8.6 wk. Tick marks indicate when mice were removed from further analysis in the study for reasons other than anemia, usually for flow analysis of marrow samples. Mantel-Cox log rank test: $p < 0.001$.

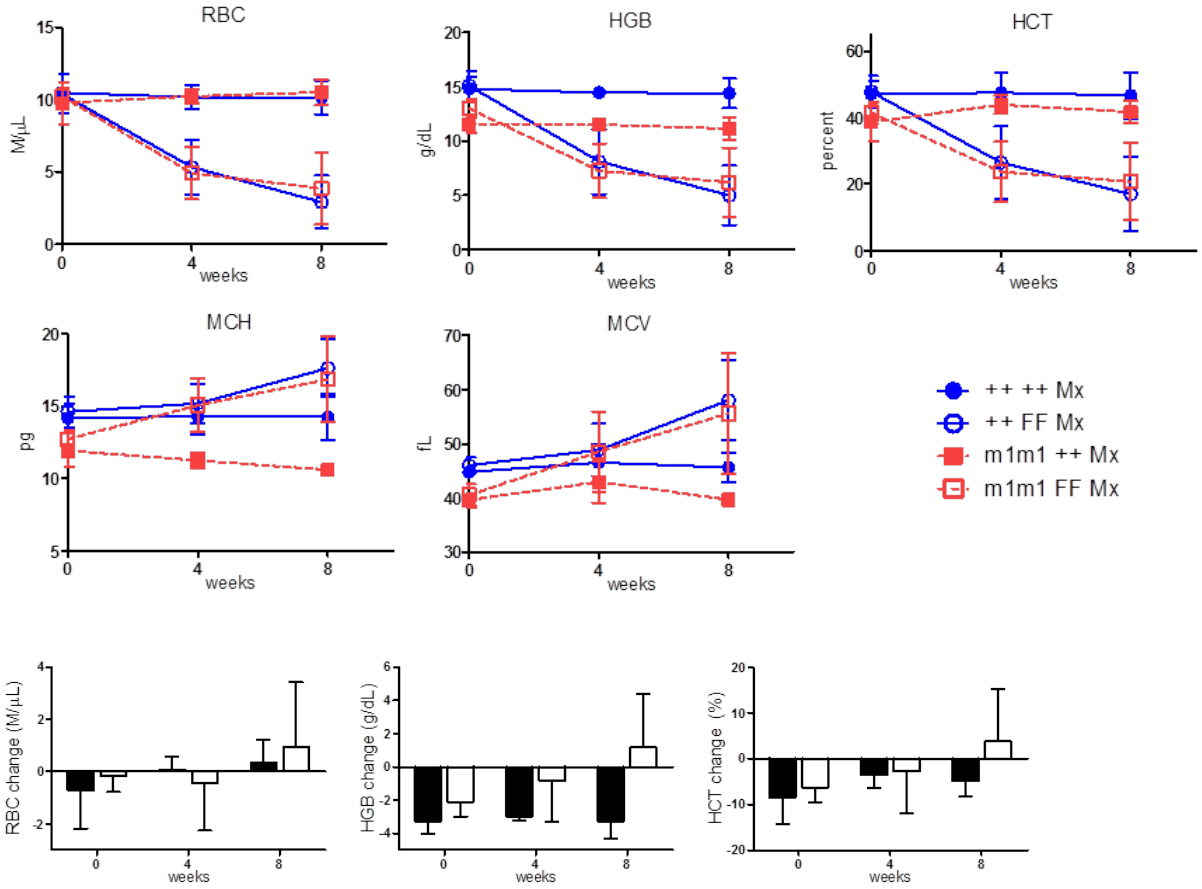
Supplementary Table 4. GFP expression levels in peripheral blood cells 4 weeks after transplant.

	WBC[A]	GR-1[A]	B220[A]	CD3[A]	N
Vector	43.6±2.4	24.3±1.8	41.3±1.7	50.5±0.6	3
FLVCR1a	22.0±2.4	16.4±2.2	21.6±2.0	25.7±1.9	4
FLVCR1b	25.6±4.2	18.2±2.8	20.9±5.7	27.8±6.6	4
HMOX1	19.1±1.5	16.5±1.0	18.3±1.8	21.1±2.1	8

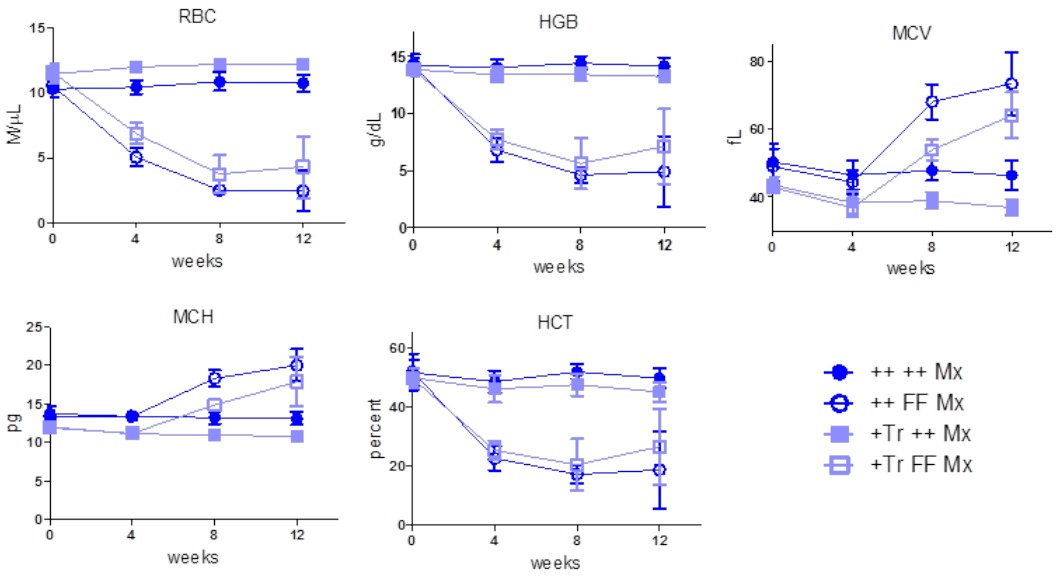
[A] fold over GFP^- cells; calculated by dividing the geometric mean fluorescence of the GFP^+ population by that of the GFP^- cells.



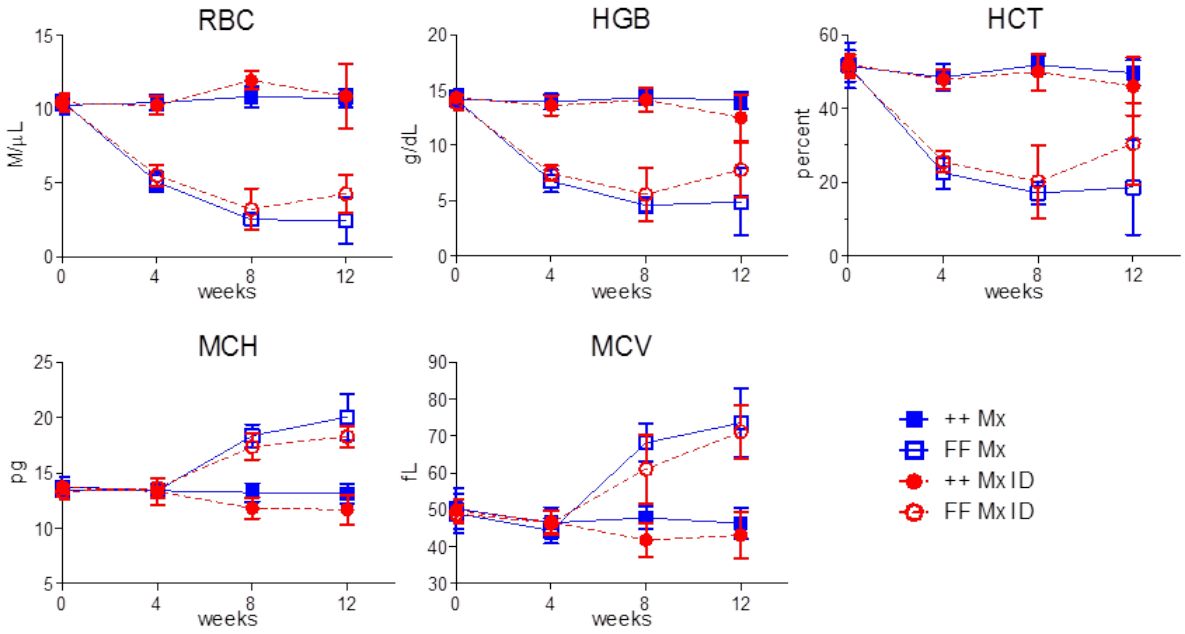
Supplementary Figure 7. HMOX1 is highly expressed in transduced 3T3 cells.
3T3 cells were transduced with either no vector (Mock), or the bi-cistronic vectors expressing only GFP or *Hmox1* and GFP. After a week in culture, cells were collected, lysed, and analyzed for β-Actin, GFP, and Hmox1 expression levels by western blot. All samples were run on the same gel. The blots were imaged on a Chemi-Doc running Image Lab 5.1 (BioRad) and subject to automatic scaling. High resolution TIFF images were transferred to Adobe Photoshop CS4 to assemble the final figure. The vertical line indicates lanes which were omitted from the figure.



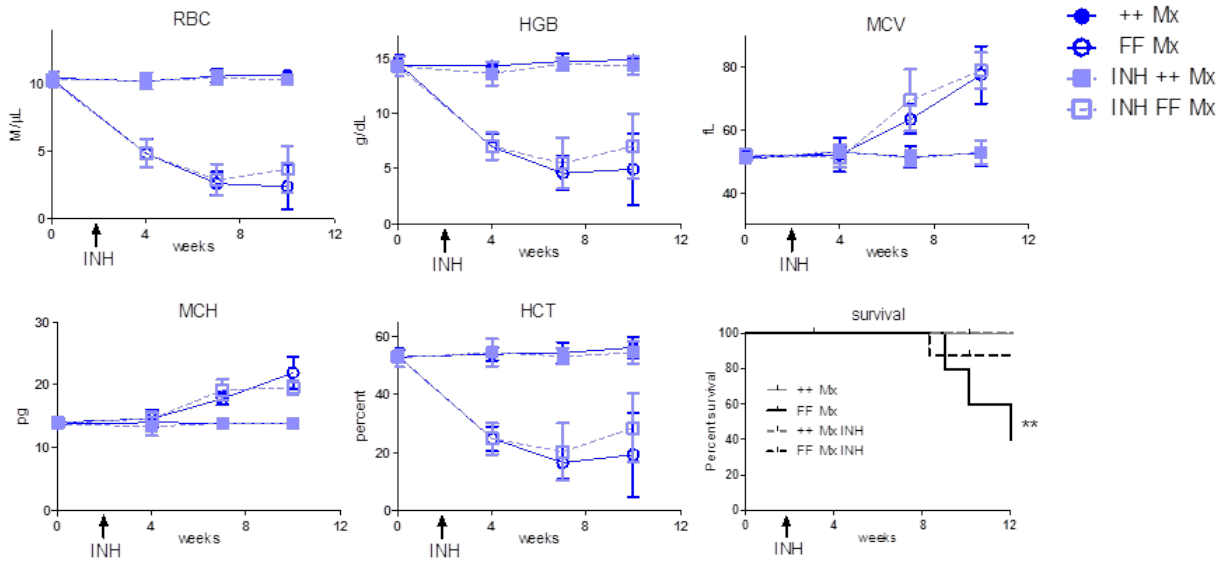
Supplementary Figure 8. The effect of ferrocheletase deficiency on anemia in *Flvcr1*-deleted mice. Peripheral blood red cell counts (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) from control (++ ++ Mx, N=8), *Flvcr1*-deleted (++ FF Mx, N=11), ferrocheletase mutant (m1m1 ++ Mx, N=4) and ferrochelatase mutant, *Flvcr1*-deleted (m1m1 FF Mx, N=15) mice for up to 8 weeks after deletion. The difference in peripheral RBC counts, hemoglobin levels, and hematocrits in m1m1 ++ Mx mice compared to the mean values of ++ ++ Mx mice (solid bars) and m1m1 FF Mx mice compared to the mean values of ++ FF Mx mice (open bars).



Supplementary Figure 9. The effect of *Tfrc* haploinsufficiency on anemia in *Flvcr1*-deleted mice. Peripheral blood red cell counts (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) from control (++ ++ Mx, N=16), *Flvcr1*-deleted (++ FF Mx, N=11), *Tfrc*$^{+/-}$ (+Tr ++ Mx, N=10) and *Tfrc*$^{+/-}$; *Flvcr1*-deleted (+Tr FF Mx, N=7) mice for up to 12 weeks after deletion.



Supplementary Figure 10. The effect of iron restriction on anemia in *Flvcr1*-deleted mice. Peripheral blood red cell counts (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) from control (++ Mx, N=16), *Flvcr1*-deleted (FF Mx, N=11), or control (++ Mx ID, N=10) and *Flvcr1*-deleted (FF Mx ID, N=11) mice fed an iron deficient diet for up to 12 weeks after deletion.



Supplementary Figure 11. The effect of INH on anemia in *Flvcr1*-deleted mice.
Peripheral blood red cell counts (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and a Kaplan-Meier survival curve from control (++ Mx, N=9), *Flvcr1*-deleted (FF Mx, N=8) mice treated with water or control (INH ++ Mx, N=10) and *Flvcr1*-deleted (INH FF Mx, N=8) mice treated with INH for 8 weeks. The start of INH treatment is indicated by an arrow at 2 weeks after deletion. Mantel-Cox log rank test: **p < 0.01.

Supplementary Table 5. Primers and probes used for quantitative PCR

Flvcr1	P1	ATCTGGAACCTGTGCAGAAACA
	P2	ATTGAATAAAATGCTCCAGTCATGAT
	Probe	CCCCTTTGTTCTCCTGCTGGTCAGTTATG
Flvcr1b	P1	TCGCTTCCTATTGACAGCTATTAACA
	P2	CACTAAAACAGGTGGCAACAAAAA
	Probe	TTTGGAACTGCAGTTGGT
Act-b	P1	ACGGCCAGGTCATCACTATTG
	P2	CAAGAAGGAAGGCTGGAAAAGA
	Probe	CAACGAGCGGTTCCGATGCCC
Hmox1	P1	CTGCTAGCCTGGTGCAAGATACT
	P2	GTCTGGGATGAGCTAGTGCTGAT
	Probe	AGACACCCCGAGGGAAACCCCA
Hmox2	P1	GAATGCCTTGGACCTGAATTTG
	P2	CTCCAGGGTTTCTCTTGCTAG
	Probe	TGGCCTCCTCCACAATCCTCTCT
Alas1	P1	TGGTCGGTTTAGCGTCCTC
	P2	GGGATAAGAATGGGCATCGG
	Probe	CGAGTGCCTACCGCCGCTTC
Alas2	P1	TTTAGTATTGGACGCTGCCC
	P2	CTTCCTGTCTTGGAGTTCTGAC
	Probe	AGCCTTGGTTGCCTTAAGATGGATTTGA
Hbb-bs	P1	TGAATCACTTGGACAGCCTC
	P2	ATCACGATCATATTGCCCAGG
	Probe	TGAAGTTCTCAGGATCCACATGCAGC