SUPPLEMENTARY APPENDIX

Transferrin receptor 1-mediated iron uptake plays an essential role in hematopoiesis

Shufen Wang,[1,2*] Xuyan He,[1*] Qian Wu,[1] Li Jiang,[1] Liyun Chen,[1] Yingying Yu,[1] Pan Zhang,[1] Xin Huang,[3] Jia Wang,[3] Zhenyu Ju,[4] Junxia Min[1] and Fudi Wang[1,2,3]

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[#] Shufen Wang and Xuyan He contributed equally to this work.

Supplementary information

Transferrin receptor 1-mediated iron uptake plays an essential role in hematopoiesis

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Supplementary Methods

Histological analysis

Femur and tibia were collected from control and cKO pups. The long bones were fixed in 4% paraformaldehyde and embedded in paraffin, and 5 μm sections were prepared on a Microtome (Leica, RM2235). Paraffin sections were dewaxed and stained with hematoxylin and eosin for histological examination.

Hematological parameters

Peripheral blood was obtained via left ventricle puncture in adult mice, and neonates were decapitated for blood collection from the thoracic cavity using Sarstedt microvette tubes. Hematological parameters were measured using ADVIA 2120i hematology analyzer (Siemens) at the Center for Drug Safety Evaluation and Research, Zhejiang University.

Blood smears

Blood smears were prepared by using the wedge technique followed by air drying and Wright-Giemsa staining.

Embryo dissection and single-cell isolation

Embryonic developmental time was determined by considering the day of vaginal plug observation as E0.5. Dissection of the fetal liver were performed as previously described (1). Total bone marrow cells were flushed out of femurs and tibias with DMEM containing 2% FBS and penicillin/streptomycin (Gibco). Spleen, thymus, and fetal liver tissues were minced in the same medium. Single-cell suspensions were prepared by passing them through 40-μm cell strainers (FALCON). Red blood cells were lysed with Red Blood Cell (RBC) Lysis Buffer (eBioscience). The cell number was counted on ACEA NovoCyteTM flow cytometer (ACEA Biosciences).

Flow cytometric analysis

For lineage labeling, fetal liver, neonatal bone marrow were stained with biotin-conjugated lineage marker antibodies (CD3e (145–2C11), CD4 (GK1.5), CD5 (53–7.3), CD8a (53–6.7), B220 (RA3–6B2), Gr-1 (RB6–8C5) and Ter119 (TER119)) and followed by Apc-Cy7 conjugated streptavidin. Whereas for adult bone marrow cells, the lineage cocktail was composed of AlexaFluor700-conjugated antibodies against CD3e (145–2C11), B220 (RA3–6B2), Gr-1 (RB6–8C5), CD11b (M1/70), and Ter119 (TER119) were used. For detection of HSCs and progenitors, cells were stained with respective combinations of fluorophore-conjugated monoclonal antibodies. See Table S2 for a complete list of antibodies. For analysis of HSCs, antibodies except those to lineage markers were used at a 1:50 dilution and those to lineage markers were used at a 1:60 dilution. For analysis of differentiated cells, all antibodies were used at a 1:100 dilution. Cells incubated with the indicated antibodies for 30 mins on ice. After washing, labeled cells were performed with the BD Fortessa

instruments (BD Biosciences, San Jose, CA). FACS data were analyzed with Flowjo software (Tree Star). Sorting of HSCs, LSK cells, and other hematopoietic cells was performed on the BD FACS S ORP ARIA II.

To determine expression of Ferritin, cells were first cytofixed and permeabilized (BD Bioscience), stained with rabbit monoclonal anti-Ferritin antibodies (clone EPR3004Y, Abcam) followed by an FITC-conjugated anti-Rabbit IgG (Life Tech).

Evaluation of intracellular iron status

BM mononuclear cells were stained with 0.25 μM calcein-AM (Invitrogen) in PBS for 15 min at 37℃ and then washed with PBS. The calcein-loaded cells were stained with antibodies further to surface markers for 30 min on ice, washed, and analyzed with the BD Fortessa instruments.

Iron parameters

Serum iron and tissue non-heme iron were measured as described [2-3].

Measurement of serum hepcidin

Hepcidin level in mouse sera was measured by Hepcidin-Murine Compete™ ELISA (Intrinsic Lifesciences, SKU# HMC-001) according to the manufacturer's instructions.

Colony formation assays

Cells were harvested from the fetal livers of control and cKO embryos. The cells were filtered through 40 μm cell strainers (StemCell Technologies) to obtain single-cell suspension, and red blood cells were lysed with an RBC lysis buffer (eBioscience). Colony formation assays were performed in MethoCult M3434 (Stem Cell Technologies, Durham, NC, USA) with 2×10^4 BM-MNCs and scored for CFU-GEMM, -GM and BFU-E colony formation at 12 days. Fe (III)/8-hydroxyquinoline complex contained 10 mM iron / 20 mM 8HQ in DMSO as previous described [4]. 0.25 μM Fe (III)/8-hydroxyquinoline complex, 10 μM ferric ammonium citrate (FAC), 5 μM Holo-transferrin or 10 μM hemin was applied to the MethoCult M3434.

Lentivirus infection

Tfr1 cDNA from mice with or without the L622A mutation, R654A mutation were cloned into pCDH-EF1-MCS-T2A-copGFP. For lentivirus package, the plasmid constructs were transfected into 293T cells with pSPAX2 and pMD.2G, and the supernatant was collected after 48 hours and centrifuged at 60,000g for 2 hours at 4℃, then lentivirus pellet was re-suspended in sterile IMEM. For lentivirus infection, E16.5 fetal liver cells were cultured in 24-well plate (Corning) with stemspan SFEM (Stemcell) containing cytokines cocktail (100 ng/mL SCF, 50 ng/mL TPO and 50 ng/mL FLT3, all from PeproTech). Then lentivirus (MOI:100) were added into the cells plus with 8 mg/mL polybrene, 6 hours later cells were changed to fresh stemspan medium containing cytokines cocktail for another 12 hours, then the cells can be collected for CFU assay.

Transplantation assays

About 8-week old CD45.1^{+} recipient mice were irradiated at 9 Gy (split into 2 doses of 4.5 Gy, 3 h apart) with an X-ray irradiator (Rad Source, RS-2000Pro). 1×10^5 nucleated cells (CD45.2^{+}) from E16.5 control and cKO fetal liver were mixed with 1×10^5 freshly isolated bone marrow nucleated cells (BMNCs) (in the medullary space of bilateral femurs, tibias) (CD45.1^{+}), and the mixture was injected intravenously via the caudal vein into lethally-irradiated recipient mice. The peripheral blood samples were collected at 4, 8, 12 and 16 weeks after transplantation, and stained with multilineage markers, CD45.1-PE and CD45.2-APC for analysis of the engraftment. The bone marrow was collected at week 16 for engraftment analysis by multicolor flow cytometry on BD Fortessa instruments (BD Biosciences, San Jose, CA).

HSC homing

2×10^5 fetal liver cells (CD45.2) were obtained from E14.5 control and cKO embryos and were intravenously injected into lethally-irradiated recipients (CD45.1). 40 hours later, recipients were sacrificed, and BMNCs were collected and analyzed by FACS.

Apoptosis analyses

For apoptotic analysis, cells were first labeled with surface markers, and then stained with Annexin-V antibody (Multi sciences) and 7-AAD according to the manufacturer's instructions (Multi sciences) for flow cytometry. The FACS data were analyzed using FlowJo software (Tree Star).

Real-Time qRT-PCR

Total RNA was isolated from cells or tissues using TRIzol (Invitrogen) and reverse-transcribed into cDNA using the PrimeScript RT kit (Takara). Real-time PCR was performed using LightCycler® 480 Instrument (Roche). The level of each amplified target gene was normalized to the respective *Gapdh* mRNA level. We quantified expression levels of critical transcription factors required for the determination of hematopoietic cell fate, as previous studies suggested [5-12]. The sequences of the various primers were shown in Table S3.

Western blot analysis

Samples from lysed cells (20μg total protein) were resolved on 10–12% SDS-PAGE gels, transferred to nitrocellulose membranes and probed with rabbit anti-mouse Vav1(1:1000 dilution, Invitrogen), or mouse anti-mouse Gapdh (1:10000 dilution, Bioworld), followed by HRP goat anti-rabbit IgG secondary antibodies (1:4000 dilution, ABclone) and detection using the ECL System (Thermo Scientific).

Supplementary Table S1. Hematological Parameters in 3-Day-Old *Tfr1*$^{fl/fl}$ and *Tfr1*$^{fl/fl}$*;Vav-Cre* Mice

Parameter	*Tfr1*$^{fl/fl}$	*Tfr1*$^{fl/fl}$ *;Vav-cre*	*P* value
WBC ($10^{\wedge 9}$/L)	8.4±2.2	5.1±0.9	0.006
RBC ($10^{\wedge 12}$/L)	3.6±0.4	2.3±0.7	0.002
Hemoglobin (g/L)	11.5±1.6	3.8±0.8	0.000
Hematocrit (%)	36.0±5.1	15.2±3.2	0.000
MCV (fl)	99.0±9.2	66.6±5.8	0.000
MCH (pg)	31.6±2.3	17.2±3.9	0.000
MCHC (g/L)	32.0±2.0	25.5±3.8	0.004
RETI ($10^{\wedge 9}$/L)	414.0±46.7	141.5±7.8	0.015
Platelet count ($10^{\wedge 9}$/L)	870.0±193.9	1993.3±426.9	0.000

WBC, white blood cell; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RETI, Reticulocyte. Data are reported as mean ± SD. (n = 4 for each group).

Supplementary Table S2. Antibodies for flow cytometry

Antigen	Fluorochrome	Company	Clone
Biotin-conjugated lineage antibodies			
B220(CD45R)	Biotin	BioLegend	RA3-6B2
CD3e	Biotin	BioLegend	145-2C11
CD5(Ly-1)	Biotin	BioLegend	53-7.3
CD4	Biotin	BioLegend	GK1.5
CD8a	Biotin	BioLegend	53-6.7
Gr-1(Ly-6G)	Biotin	BioLegend	RB6-C5
TER-119	Biotin	BioLegend	TER-119
CD135	Biotin	BioLegend	A2F10
Streptavidin	APC-Cy7	BioLegend	
Lineage cocktail antibodies			
B220(CD45R)	AlexaFluor700	BioLegend	RA3-6B2
CD3e	AlexaFluor700	BioLegend	145-2C11
Gr-1(Ly-6G)	AlexaFluor700	BioLegend	RB6-C5
TER-119	AlexaFluor700	BioLegend	TER-119
CD11b(Mac-1)	AlexaFluor700	BioLegend	M1/70
Bone marrow, fetal liver, spleen, liver , thymus and endothelium staining			
Sca-1	PE-Cy7	BD Biosciences	D7
CD117(c-Kit)	APC	BioLegend	2B8
CD117(c-Kit)	BV510	BioLegend	2B8
CD115	APC	eBioscience	AFS98
CD135(Flt3)	PE	BioLegend	A2F10
CD135(Flt3)	APC	BioLegend	A2F10
CD34	PE	BioLegend	RAM34
CD34	FITC	BD Biosciences	RAM34
CD127(IL-7R)	BV421	BioLegend	A7R34
CD16/32	PerCP-Cy5.5	BioLegend	93
CD71	PerCP-Cy5.5	BD Biosciences	C2

CD71	PE	BioLegend	RI7217
Ter119	APC	BioLegend	TER-119
CD44	PE-Cy7	BioLegend	IM7
B220	FITC	BioLegend	RA3-6B2
B220	APC	BioLegend	RA3-6B2
CD19	PE-Cy7	BioLegend	1D3
CD11b(Mac-1)	PE	BioLegend	M1/70
CD11b(Mac-1)	APC	BioLegend	M1/70
CD11b(Mac-1)	FITC	BioLegend	M1/70
CD11b(Mac-1)	PerCP-Cy5.5	BioLegend	M1/70
Gr-1	PE	BioLegend	RB6-8C5
Gr-1	APC	BioLegend	RB6-8C5
CD3e	AlexaFluor700	BioLegend	145-2C11
CD31	PE-Cy7	BioLegend	390
Rat IgG2a,k	PE	BioLegend	RTK2758
Transplantation staining			
CD45.2	APC	BioLegend	104
CD45.2	BV650	BioLegend	104
CD45.2	PerCP-Cy5.5	eBioscience	104
CD45.2	Biotin	eBioscience	104
CD45.1	PE	eBioscience	A20
CD45.1	Biotin	eBioscience	A20

Supplementary Table S3. Primers used for real-time PCR

Gene	Forward primer	Reverse primer
Gapdh	CTACCCCCAATGTGTCCGTCGTG	GATGGAAATTGTGAGGGAGATGC
Tfr1 (KO)	GCCAGATCAGCATTCTCTAACT	CACTAGCCTTCATGTTATTGTC
Fpn1	GTCGGCCAGATTATGACATTTG	ATTCCAACCGGAAATAAAACCA
Dmt1	GCGGCCAGTGATGAGTGAGT	ATGCCACCGGCAATCCT
Fth	AAGATGGGTGCCCCTGAAG	CCAGGGTGTGCTTGTCAAAGA
Ftl	CGGGCCTCCTACACCTACCT	CCCTCCAGAGCCACGTCAT
Tfr2	AGACGTGGTTCTCAGGCACATCGGC	GGGTCAGCCCGCGCTCCTTGAG
Slc39a14	ATTGTCAACTCCATGTCTGTGCAGG	CTGTCGTTCTTCTCATCCTCCTGG
Flvcr1a	GTTTGGACCCAAGGAGGTGTC	AGGCACTAAAACAGGTGGCAA
Flvcr1b	ATGGTTTGGACCCAAGGAGG	TTGGAGGCAACGGAGGTTTC
Flvcr2	TCCATCCATCCTAGTGTCTCCA	CACAAGGAGTAGCAACTGAACA
Abcg2	CCATAGCCACAGGCCAAAGT	GGGCCACATGATTCTTCCAC
Hrg1	CTTCGTGGGTGCTCTCTTCTC	GACTCTGATGCTGGGTGATGG
Alas2	GATCCAAGGCATTCGCAACA	GATGGCCTGCACATAGATGC
C/EBPa	GACCATTAGCCTTGTGTGTACTGTATG	TGGATCGATTGTGCTTCAAGTT
Mll	GCAGATTGTAAGACGGCGAG	GAGAGGGGGTGTTCCTTCCTT
Tel	AGCAGGAACGAATTTCATACACG	GGCAGGTGGATCGAGTCTTC
Tcf1	AGCTTTCTCCACTCTACGAACA	AATCCAGAGAGATCGGGGGTC
EBF	GCATCCAACGGAGTGGAAG	GATTTCCGCAGGTTAGAAGGC
Pax5	CCATCAGGACAGGACATGGAG	GGCAAGTTCCACTATCCTTTGG

Gata3	CTCGGCCATTCGTACATGGAA	GGATACCTCTGCACCGTAGC
Xbp1	AGCAGCAAGTGGTGGATTTG	GAGTTTTCTCCCGTAAAAGCTGA
E2A	TTTGACCCTAGCCGGACATAC	GCATAGGCATTCCGCTCAC
Gata2	GCAGAGAAGCAAGGCTCGC	CAGTTGACACACTCCCGGC
Gata1	TGGGGACCTCAGAACCCTTG	GGCTGCATTTGGGGAAGTG
Fli1	ACTTGGCCAAATGGACGGGACTAT	CCCGTAGTCAGGACTCCCG
Eklf	TCTGAGGAGACGCAGGATTT	CTCGGAACCTGGAAAGTTTG
Gfi1	CAGCTTACCGAGGCTCCCGACAGG	CAAGACCGCTCCATGCATAGGGCTT
PU.1	ATGTTACAGGCGTGCAAAATGG	TGATCGCTATGGCTTTCTCCA
Scl	ATTGCACACACGGGATTCTG	GAATTCAGGGTCTTCCTTAG
Tal1	CCAACAACAACCGGGTGAAG	GCCGCACTACTTTGGTGTGAG
Lmo2	ACGGAAATTGTGCAGGAGAG	ACCCGCATCGTCATCTCATA
Ldb1	TGCTGACCATCACTTTCTGC	GGCTGAGGCTGTAGGTCTTG
Fog1	CCAACTGTGAACGCCATCTC	GATCTCACCCTTGGAGCCTG
Bcl1a	AACCCCAGCACTTAAGCAAA	ACAGGTGAGAAGGTCGTGGT
Bmi1	GGCTCGCATTCATTTTATGCTG	TGTTCGATGCATTTCTGCTTGA
Runx1	TTTCAAGGTACTCCTGCCTGA	CAGTGAGAAGGACCAGAGACT
Notch1	GATGGCCTCAATGGGTACAAG	TCGTTGTTGTTGATGTCACAGT

Legends to Supplementary Figures

Supplementary Figures S1. Additional Tfr1 expression in hematopoietic stem/progenitor cells from database. (A) *Tfr1* expression retrieved from Gene Expression Omnibus dataset GSE59636. (B) *Tfr1* expression retrieved from the mouse hematopoietic system shown as output from BloodSpot (GSE14833 and GSE6506). (C) Heat map of *Tfr1* expression levels in hematopoietic hierarchy from BloodSpot server using mouse normal hematopoietic system (GSE14833 and GSE6506).

Supplementary Figures S2. Morphological analysis of peripheral blood. Peripheral blood smears prepared from a control mouse and a cKO mouse at postnatal 3 days (P3) and stained with Wright-Giemsa.

Supplementary Figures S3. Impaired erythroblasts in cKO Spleen and decreased HSPCs propotion in cKO bone marrow of P3 mice. (A) The absolute number of erythrocytes at indicated differentiation stages measured in the spleen of control and cKO mice at P3 (n = 5 per group). (B) Propotion of LSK and HPC cells in the bone marrow of control and cKO mice at P3 (n = 4 for each group). *P<0.05; **P<0.01; ***P<0.001.

Supplementary Figure S4. Postnatal cKO mice have decrease organ size. Liver weight (A), and Spleen weight (B) were obtained from control and cKO pups at P3 (n= 5 per group). *P<0.05; ***P<0.001.

Supplementary Figure S5. Tfr1 deletion didn't influence intracellular iron and membrane iron transporters in HPCs in E16.5 fetal liver. (A) Ferritin expression was determined by intracellular staining in HPCs of E16.5 fetal liver. Data shown are representative (left) or relative mean fluorescence intensity (MFI) of Ferritin (right). (B) Quantitative RT-PCR analysis of the indicated iron-related genes measured in HPCs obtained from control and cKO fetal livers at E16.5 (n = 5 for each group). (C) Quantitative RT-PCR analysis of the indicated transcription factor in hematopoieis(n = 5 for each group). *P<0.05; **P<0.01; ***P<0.001.

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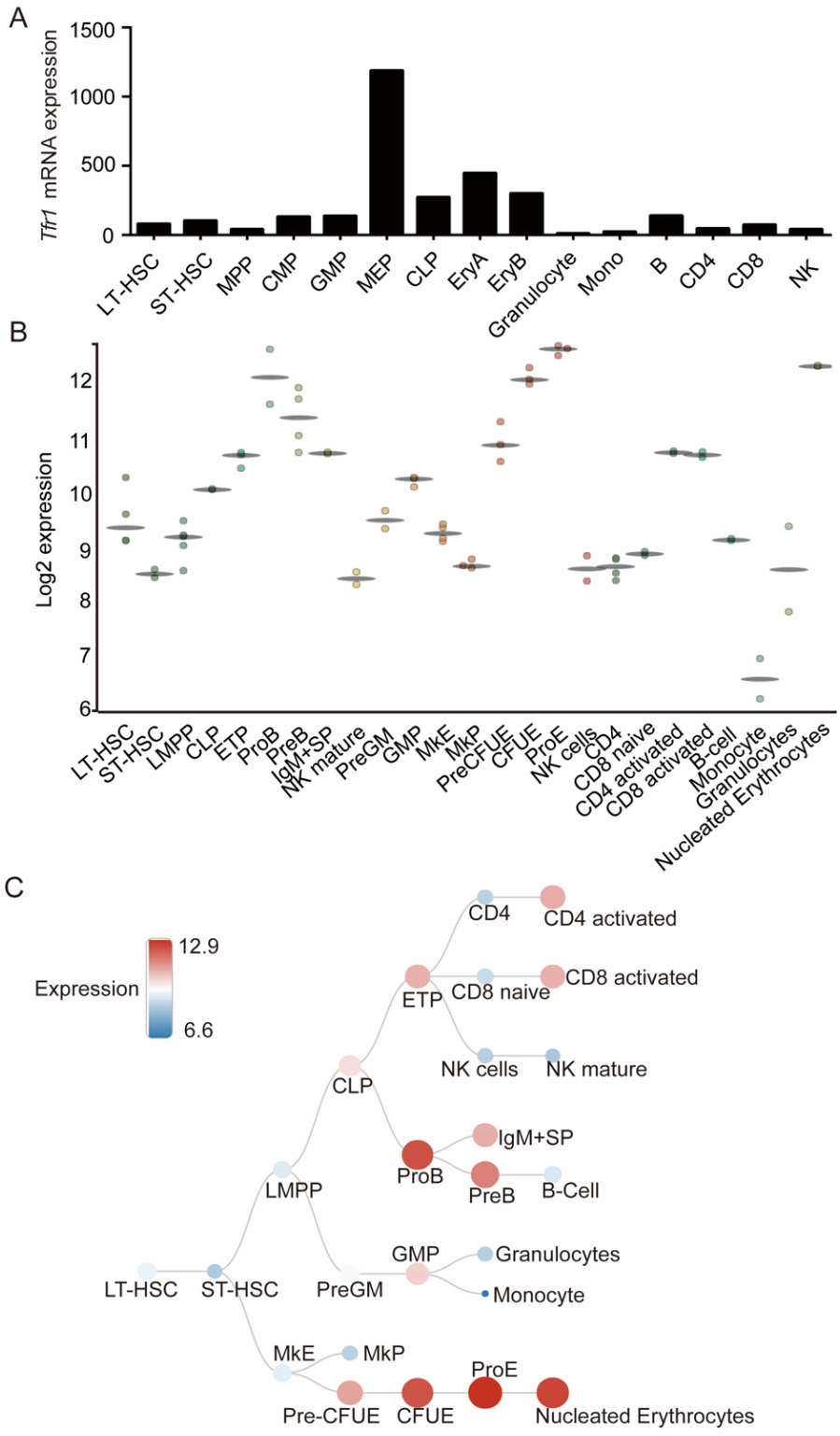
Figure S1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Mono
B
CD4
CD8
NK
B
Log2 expression
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10
9
8
7
6
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ST-HSC
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Figure S2

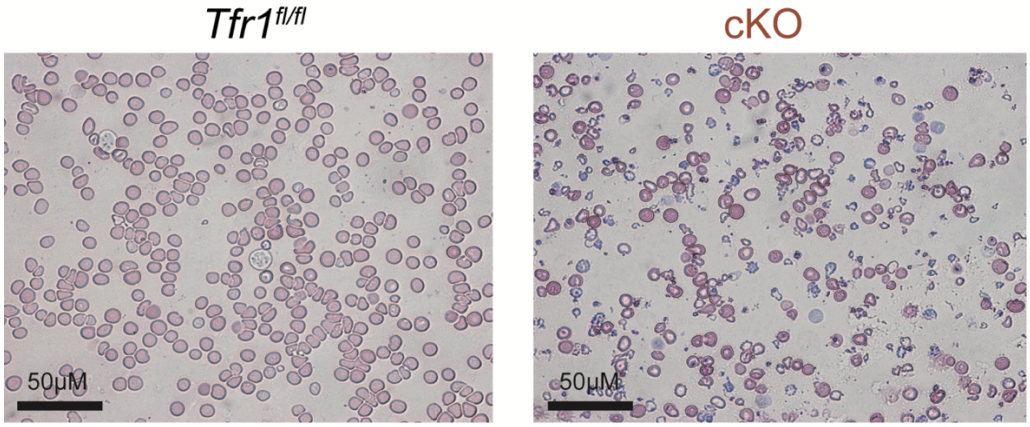
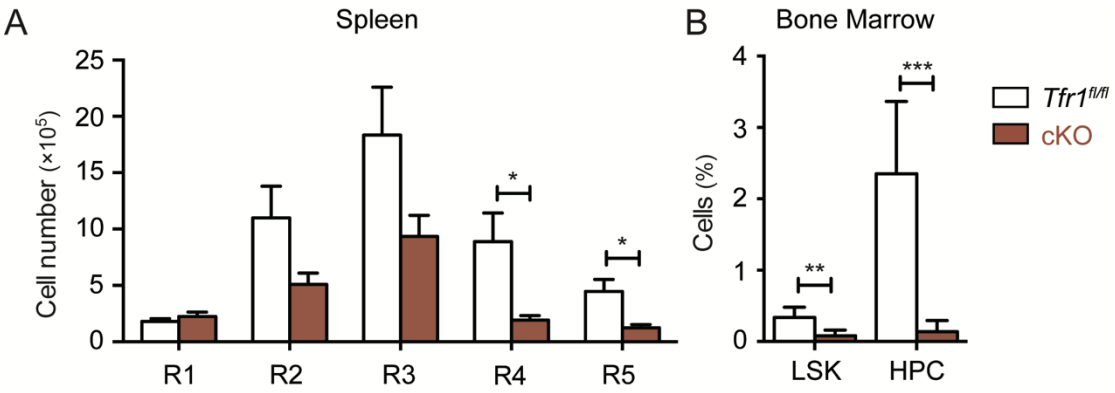


Figure S3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R1
R2
R3
R4
R5
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*
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Bone Marrow
Cells (%)
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2
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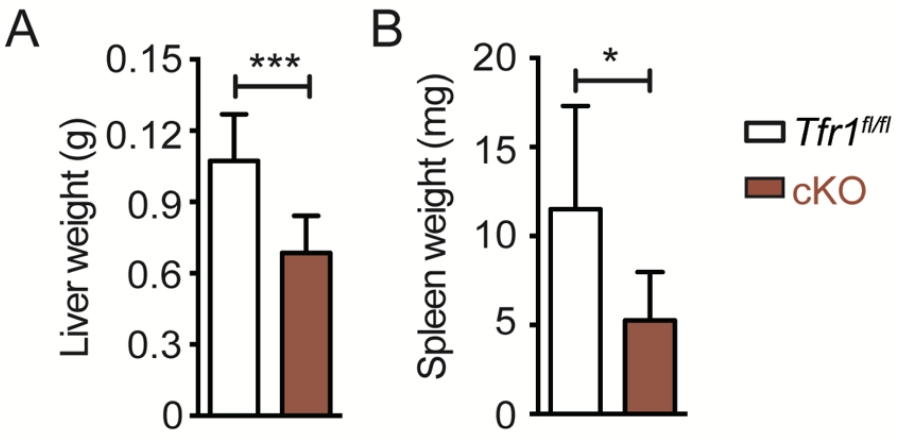
LSK
HPC
**
***
Tfr1^fl/fl
cKO

Figure S4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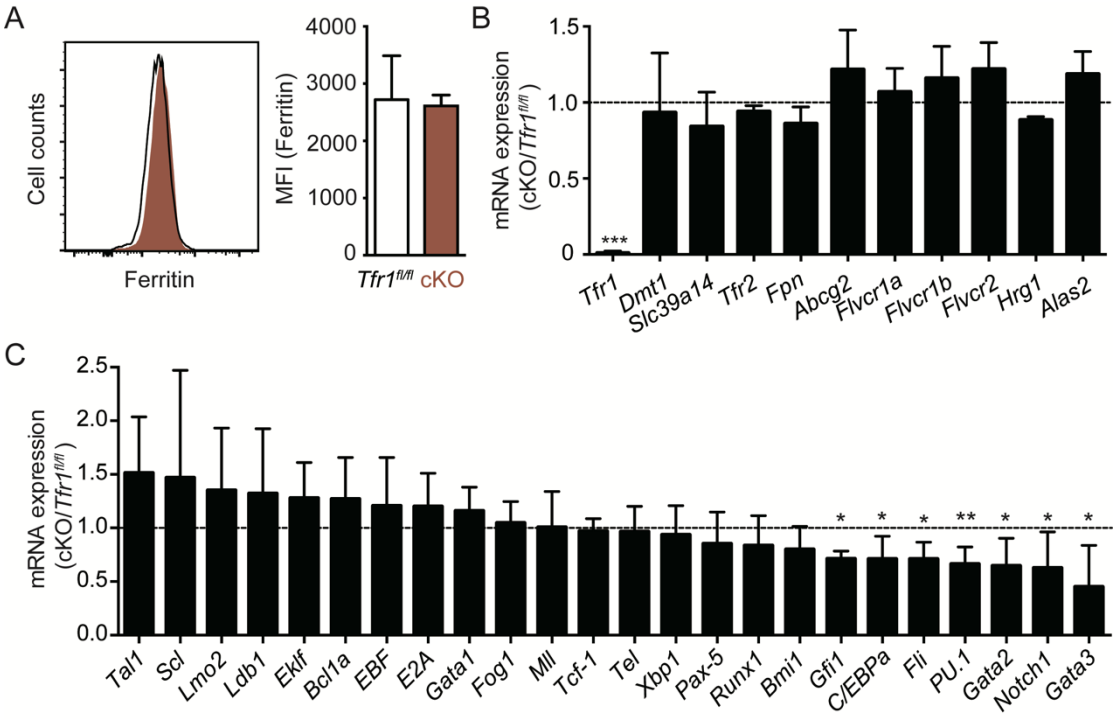
cKO

Figure S5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