NCOA4-mediated ferritinophagy in macrophages is crucial to sustain erythropoiesis in mice

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SUPPLEMENTAL MATERIAL

Supplementary methods

Sv129/J *Ncoa4-ko* mouse model generation

Several overlapping genomic clones were isolated from a λ FIX II phage library of Sv129/J mouse strain (Stratagene, Cedar Creek, TX, USA) using standard procedures. Exons II-VII of the murine *Ncoa4* gene were replaced by a neomycin-resistant (neo) cassette through homologous recombination. The targeting construct (pKO Scrambler NTKV-1906) contained the neo-cassette flanked by a BamHI-EcoRI 2800 bp fragment containing *Ncoa4* gene intron 1 sequences (left arm) and by a BamHI-KpnI 2600 bp fragment containing *Ncoa4* gene exon 8-exon 9 sequences (right arm). *Ncoa4*-pKO vector was transfected by electroporation into embryonic stem (ES) cells. Two correctly targeted ES cell lines were injected into C57BL/6 blastocysts at BIOGEM facility (Ariano Irpino, Italy). Both blastocysts gave rise to germ line chimeric animals that were then crossed to Sv129/J animals.

Bone Marrow Transplantation

BM cells were isolated from a subset of 3-month-old SV129J *Ncoa4-ko* and wt littermate male mice and $5*10^6$ cells were used for BMT in each recipient. Lethally irradiated 8-week-old *Ncoa4-ko* (Sv129/J) animals were used as recipients for the experiments reported in figure 4 and supplemental figure S5. Lethally irradiated 8-week-old C57BL/6-Ly5.1 wild-type animals were transplanted with BM cells from SV129J *Ncoa4-ko* and wt male mice for the experiments reported in supplemental figure S3.

Hematological analysis

CBC was measured on a IDEXX Procyte dx automated blood cell analyzer (Idexx Laboratories). Transferrin saturation was calculated as the ratio between serum iron and total iron binding capacity, using The Total Iron Binding Capacity Kit (Randox Laboratories Ltd.), according to the manufacturer's instructions.

Flow cytometry

Erythroid progenitors were analyzed by labeling total BM or spleen cells with rat-anti-mouse CD16/CD32 (BD Biosciences) in order to block unspecific Ig binding, and subsequently stained with BV421 rat anti-mouse Ter119 (BD Horizon, BD Biosciences) and APC rat anti-mouse CD44 (BD Pharmingen) for 30 min in the dark at 4°C. Flow cytometry analyses were performed using the FACS Canto™ II (BD Biosciences). Data were analyzed with FCS express 6 Flow (De Novo Software).

Colony-forming unit assay

Bone marrow cells from tibiae and femurs were harvested into Miltenyi Biotec buffer (autoMACS Running Buffer – MACS Separation Buffer; Miltenyi Biotec GmbH), $1*10^4$ cells were seeded in methylcellulose medium (MethoCult M3434; STEMCELL Technologies) and colonies were scored on day 10 ± 2. BFU-E were visualized by staining with benzidinehydrochloride (Sigma).

Tissue iron content

To measure total iron concentration, tissue samples were dried at 65°C for 1 week, weighed, and digested in 1 mL of acid solution (3M HCl, 0.6M trichloroacetic acid) for 20 hours at 65°C. The acid extract (20 µL) was added 1 mL of working chromogen reagent (1 volume of 0.1% bathophenanthroline sulfate and 1% thioglycolic acid solution, 5 volumes of water, and 5 volumes of saturated sodium acetate). The absorbance of the solutions was measured at 535 nm after 30 minutes of incubation at room temperature. A standard curve was generated, using an acid solution containing increasing amounts of iron sulfate.

Perl's blue staining

Formalin-fixed paraffin embedded (FFPE) sections (5 µm thickness) were deparaffinized and rehydrated by passages through xylene and alcohol series. Slides were incubated for 30 min with a solution 1:1 ferrocyanide and hydrochloric acid 2%, then washed in water

and counter stained with RED staining and mounted.

^{57}Fe solution preparation and Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

The solution of the stable iron isotope ^{57}Fe was prepared dissolving ^{57}Fe (22.85g/L) into 0.4M H_2SO_4. The day of the treatment 0.83 mg of ascorbic acid and 437 mg of saccarose per mg of ^{57}Fe were added to the solution and pH was adjusted to 7 by using 1M NaOH.

^{56}Fe and ^{57}Fe isotopes content into tissue samples was determined using inductively coupled plasma mass spectrometry (ICP-MS) (Element-2; Thermo-Finnigan, Rodano (MI), Italy) at medium mass resolution (M/ΔM ~ 4,000). Sample digestion was performed by means of microwave heating for 10 min at 160 °C in 1 mL of concentrated HNO_3 (70%) (Milestone, Ethos Up Microwave Digestion System, Bergamo, Italy). A natural abundance iron standard solution was analysed during sample runs in order to check changes in the systematic bias. The calibration curve was obtained using four iron absorption standard solutions (Sigma-Aldrich) in the range 0.2–0.005 μg/mL. Natural abundance of 91.72% and 2.2% for ^{56}Fe and ^{57}Fe, respectively, were used for isotopes calibration curve calculation. For each tissue, the amount of ^{57}Fe was expressed as μg of iron per g of wet tissue. In order to measure the amount of ^{57}Fe retained by the tissue upon the treatment with ^{57}Fe-labelled solution (Figure 2), the amount of naturally occurring ^{57}Fe was subtracted from the total measured value. The amount of naturally occurring ^{57}Fe was calculated considering an average ^{57}Fe/^{56}Fe percentage of 2.4 calculated by their natural abundance. This percentage is in agreement with the values measured under basal conditions in wild-type mice.

Western-Blot

Tissues were lysed in a buffer containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.5), 1% (vol/vol) Triton X-100, 150 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM phenylmethylsulphonyl fluoride (PMSF) and 1 μg/ml aprotinin. Lysates were clarified by centrifugation at 10,000 x g for 20 min. Lysates containing comparable amounts of proteins, estimated by a modified Bradford assay (Bio-Rad, Munchen, Germany), were subjected to direct Western blot. Immune complexes were detected with the enhanced

chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfort, UK). Immunoblotting was carried out with specific antibodies. Anti-FTH1 was from Cell Signalling Technology (Danvers, MA, USA). Anti-tubulin was from SIGMA-Aldrich. Secondary antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology. Densitometric analysis was performed using Image Processing and Analysis in Java (Image J) program, available online.

Quantitative RT-PCR

RNA was extracted using the UPzol reagent (Biotechrabbit) for liver and spleen samples and the mini RNeasy kit (Qiagen) for BM cells. RNA (2 µg) was used for synthesis of cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to manufacturer's instructions. For real-time PCR analysis, specific murine Assays-on-Demand products (20x) and TaqMan Master Mix (2x) (Applied Biosystems) or specific murine oligos (designed using the Universal ProbeLibrary Assay Design Center by Roche and generated by Eurofins Italy) and SYBRgreen Master Mix (2x) (Applied Biosystems) were used. The reactions were run on 7900HT Fast Real-Time PCR System (Applied Biosystems) in a final volume of 20 or 15 µL respectively. Each cDNA sample was amplified in duplicate and the RNA level was normalized to the corresponding level of *Hprt1* or *Gapdh* mRNA. Primers used for qRT-PCR are in Supplementary Tables S1 and S2.

Supplementary tables

Table S1. Oligonucleotide primers used for qRT-PCR by TaqMan

Transcript	**Assay Id**
Hprt1	Mm01318743_m1
Hamp	Mm00519025_m1
Id1	Mm00775963_g1
Bmp6	Mm01332882_m1
Tfr1	Mm00441941_m1
Epo	Mm01202755_m1

Table S2. Oligonucleotide primers used for qRT-PCR by SybrGreen

Transcript	**Forward primer**	**Reverse primer**
Gapdh	5'-tccactcacggcaaattcaa-3'	5'-tttgatgttagtggggtctcg-3'
Erfe	5'-atggggctggagaacagc-3'	5'-tggcattgtccaagaagaca-3'
Tfr1	5'-cccaagtattctcagatatgatttca-3'	5'- cagtccagctggcaaagattat-3'
Bmp2	5'-cggactgcggtctcctaa-3'	5'-ggggaagcagcaacactaga-3'

Table S3. Complete statistical analysis relative to Figure 1C

wt *vs Ncoa4-ko*		
	BM	SP
I	0.1382	0.6702
II	0.5175	0.9081
III	0.5063	0.5369
IV	0.0648	0.6714
V	0.9474	0.1627

Table S4. Complete statistical analysis relative to Figure 1D

	wt *vs Ncoa4-ko*	
	3 months	9 months
BFU-e	p=0.2029	0.2868
CFU-GM	p=0.2914	0.3395

Table S5. Complete statistical analysis relative to Figure 3B

wt *vs Ncoa4-ko*		
	BM	SP
I	0.2834	0.2895
II	0.2435	0.2999
III	0.3629	0.3425
IV	0.6548	0.4449
V	0.5636	0.8072

Table S6. Complete statistical analysis relative to Figure 4C

$ko^{wt\ BM}$ *vs* $ko^{ko\ BM}$		
	BM	SP
I	0.9477	0.1740
II	0.5081	0.8045
III	0.1023	0.9589
IV	0.4692	0.9258
V	0.1599	0.8658

Table S7. Complete statistical analysis relative to Figure 5B

wt *vs Ncoa4-ko*		
	BM	SP
I	0.6511	0.8278
II	0.9467	0.7304
III	0.9538	0.8601
IV	0.0208	0.6690
V	0.3849	0.9773

Legend to supplementary figures

Figure S1: Erythropoietin and iron gene expression analysis in different tissues of wt *and Ncoa4-ko* mice fed a standard diet

Ncoa4-ko and wild-type (wt) mice (of both gender) on Sv129/J background were fed a standard diet until sacrifice when 9-month-old. In the figure are graphed quantitative real-time PCR to measure mRNA levels of: **A)** Erythropoietin *(Epo)* relative to Hypoxanthine phosphoribosyltransferase 1 (*Hprt1)* in the kidney; **B)** Bone Morphogenetic Protein 6 (*Bmp6*) relative to Hypoxanthine phosphoribosyltransferase 1 (*Hprt1)* and **C)** Bone Morphogenetic Protein 2 (*Bmp2*) relative to Glyceraldehyde 3-Phosphate dehydrogenase (*Gapdh)* in the liver*;* Erythroferrone *(Erfe)* in: **D)** the bone marrow (BM) and **E)** the spleen relative to Glyceraldehyde 3-Phosphate dehydrogenase (*Gapdh)**;*** **F)** Transferrin Receptor 1 *(Tfr1)* relative to Hypoxanthine phosphoribosyltransferase 1 (*Hprt1)* in the kidney. Mean values of 5-6 animals for genotype are graphed. **P*<0.05.

Figure S2: Iron phenotype and gene expression analysis in tissues of wt *and Ncoa4-ko* mice fed an iron-deficient diet

Ncoa4-ko and wild-type (wt) mice (of both gender) on Sv129/J background were fed an iron-deficient (ID) diet for 6 months starting at 3 months of age. In the figure are graphed: **A)** representative pictures of Perl's staining performed on duodenal sections (thickness of section 4 μm; magnification 20X, 40X in the inset); **B)** western blot and relative densitometric analysis of ferritin H (FtH) protein levels in the liver. Tubulin was used as loading control; quantitative real-time PCR of **C)** Hepcidin (*Hamp*), **D)** Inhibitor of differentiation 1 (*Id1*), **E)** Bone Morphogenetic Protein 6 (*Bmp6*) to measure mRNA levels relative to Hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) and **F)** real-time PCR of Transferrin Receptor 1 (*Tfr1*) to measure mRNA levels relative to Glyceraldehyde 3-Phosphate dehydrogenase (*Gapdh)* in the liver***;*** **G)** real-time PCR of Transferrin Receptor 1 *(Tfr1)* and **H)** Erythropoietin *(Epo)* to measure mRNA levels relative to Hypoxanthine phosphoribosyltransferase 1 (*Hprt1)* in the kidney. Mean values of 4-5 animals for genotype are graphed. Error bars indicate standard error. Asterisks refer to statistically significant differences between age-matched wt and *Ncoa4-ko* mice. **P*<0.05; ***P*<0.01; ****P*<0.005. P<0.001.

Figure S3: Duodenal iron absorption of wt *and Ncoa4-ko* mice

Nine-month-old wt and *Ncoa4-ko* mice (of both gender) were administered a solution containing the stable iron isotope ^{57}Fe by oral gavage and sacrificed one hour later. ^{57}Fe concentration was determined via ICP-MS in **A)** duodenum, **B)** serum and **C)** liver.

Figure S4: Hematological parameters of *wt*$^{wt\ BM}$ and *wt*$^{Ncoa4\text{-}ko\ BM}$ mice fed an iron-deficient diet

C57BL/6-Ly5.1 wild-type (wt) mice were transplanted with Sv129/J wt (*wt*$^{wt\ BM}$) or *Ncoa4-ko* (*wt*$^{Ncoa4\text{-}ko\ BM}$) bone marrow (BM). Complete blood count was determined 2 months after BM transplantation. In the figure are graphed: **A)** a scheme of BMT procedures, **B)** Red Blood Cells count (RBC), **C)** Hemoglobin levels (Hb), **D)** Mean Corpuscular Volume (MCV) and **E)** Mean Corpuscular Hemoglobin (MCH). Mean values of 9-20 animals for genotype are graphed. Asterisks refer to statistically significant differences. ***P<0.005.

Figure S5: Iron phenotype of *Ncoa4-ko*$^{wt\ BM}$ and *Ncoa4-ko*$^{ko\ BM}$ mice fed an iron-poor diet

Ncoa4-ko mice on Sv129/J background were transplanted with wt (*Ncoa4-ko*$^{wt\ BM}$) or *Ncoa4-ko* (*Ncoa4-ko*$^{ko\ BM}$) bone marrow (BM). Animals were fed an iron-deficient diet for 3 months starting at 2 months after BM transplantation (BMT). In the figure are graphed: **A)** Transferrin Saturation; **B)** Serum Iron and **C)** real-time PCR to measure hepatic mRNA levels of hepcidin (*Hamp*) relative to Hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) of *Ncoa4-ko*$^{wt\ BM}$ and *Ncoa4-ko*$^{ko\ BM}$ mice 5 months after BMT. Mean values of 4-5 animals for genotype are graphed.

Figure S6: Gene expression analysis of wt *and Ncoa4-ko* mice after an acute erythropoietin challenge

Three-month-old *Ncoa4-ko* and wild-type (wt) mice on Sv129/J background were treated with a single i.p. injection of erythropoietin (EPO, 8UI/g) or saline as a control and sacrificed 15 hours later. In the figure are graphed **A)** percentage of Ter119^{+} cells on alive cells and subpopulation composition (determined as in Figure 1) both in the bone marrow (BM) and in the spleen (SP) of saline (data from Figure 1B) and EPO (data from Figure 4B) treated mice; real-time PCR for **B)** Inhibitor of differentiation 1 (*Id1*), **C)** Bone Morphogenetic Protein 6 (*Bmp6*) and **D)** Bone Morphogenetic Protein 2 (*Bmp2*) to measure mRNA levels relative to Hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) in the

liver. Real-time data are expressed as the difference (Δ) between EPO and saline (dotted grey line) treated mice of the same genotype. Mean values of 5 animals for genotype are graphed. Error bars indicate standard error. Asterisks refer to statistically significant differences between age-matched EPO-treated wt and *Ncoa4-ko* mice. ***P*<0.01. Hashtag refers to statistically significant differences between EPO- and saline-treated mice of the same genotype. #*P*<0.05.

Supplemental Figures

Figure S1

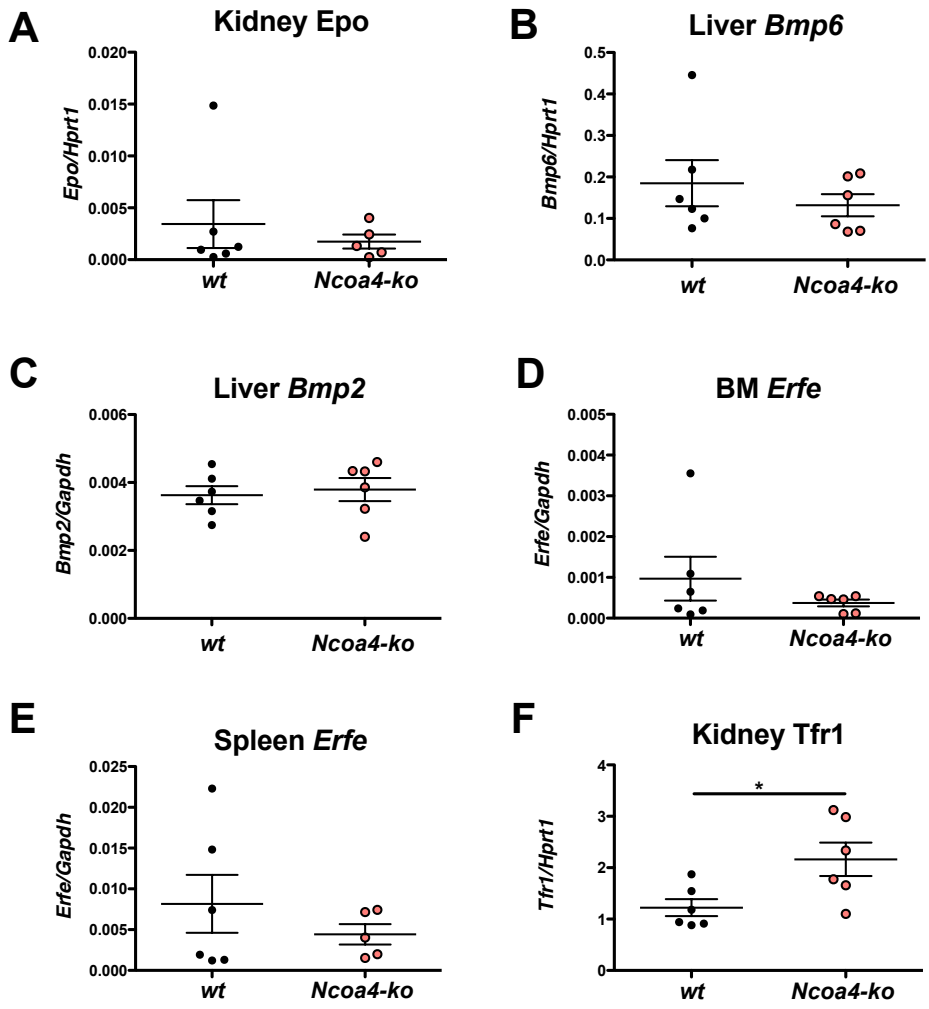


Figure S2

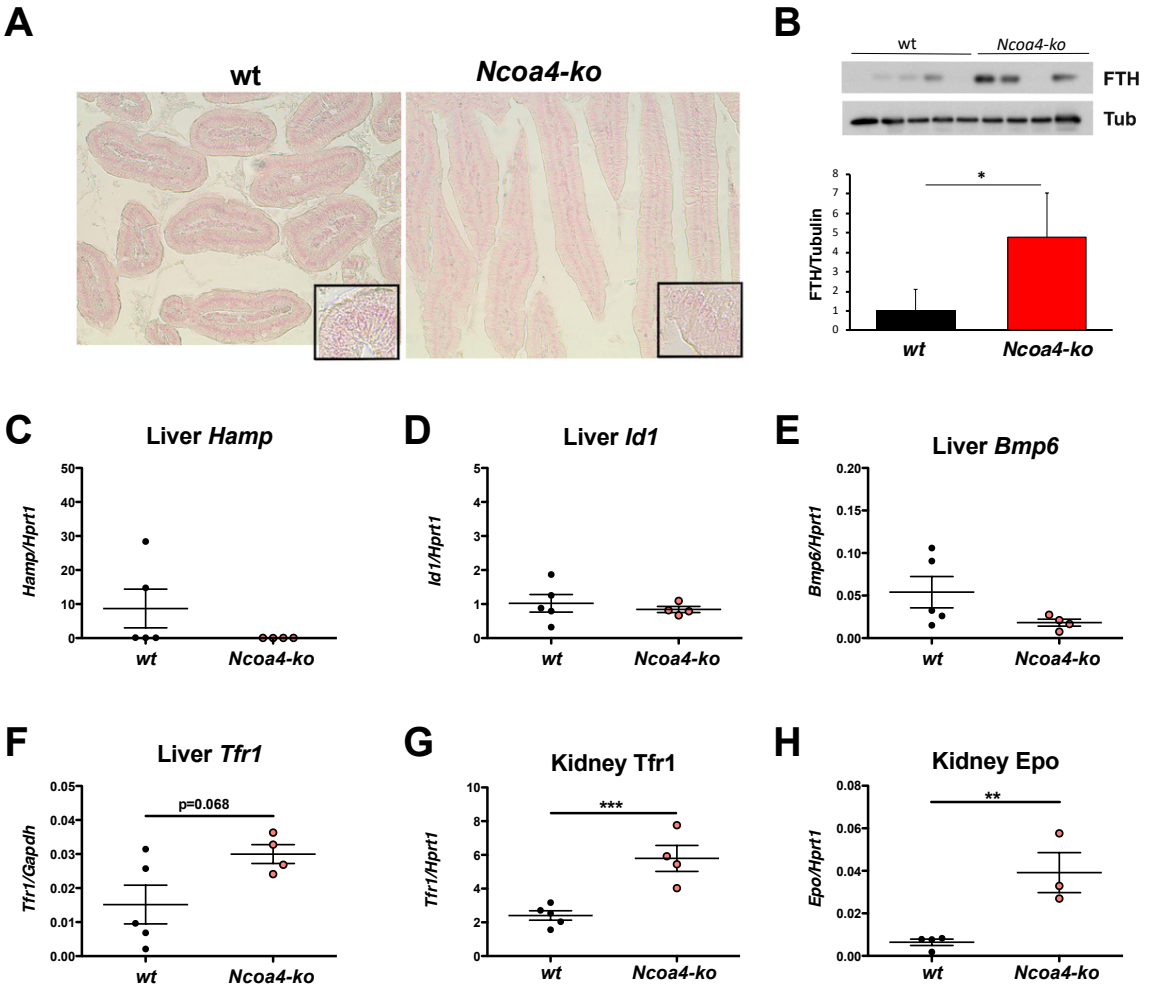


Figure S3

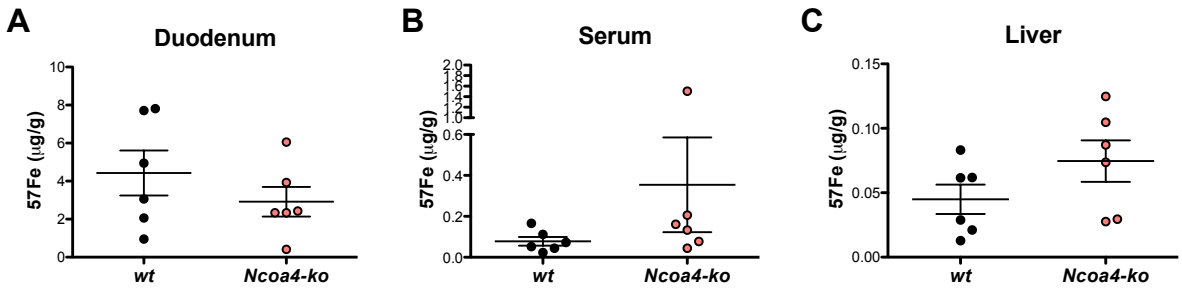


Figure S4

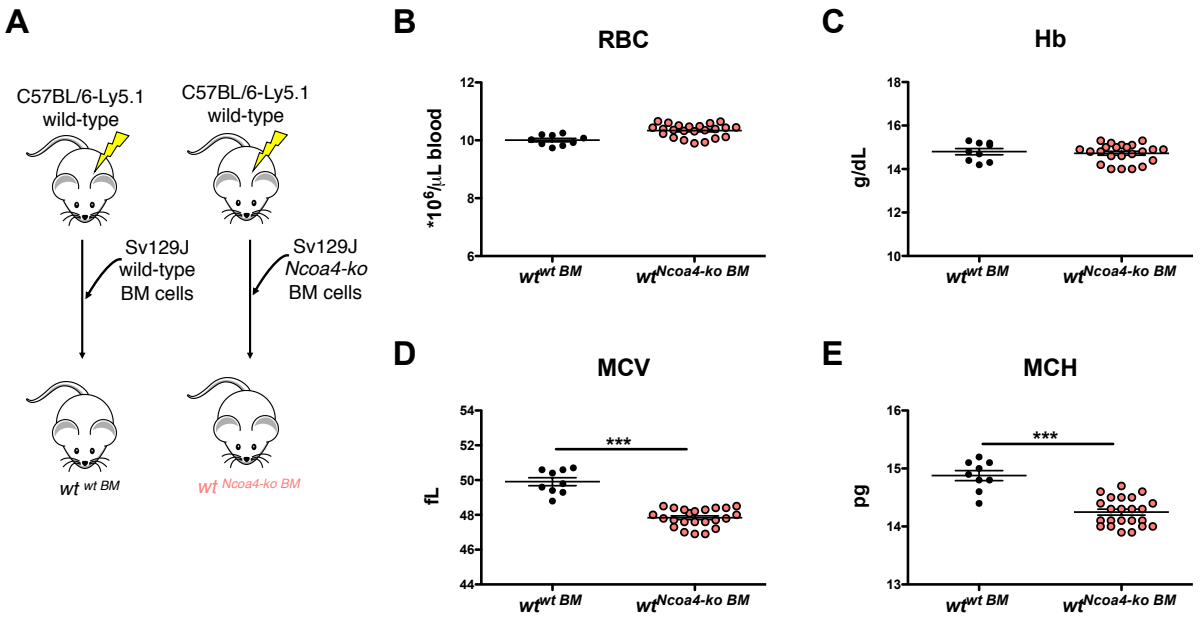

A
C57BL/6-Ly5.1 wild-type
C57BL/6-Ly5.1 wild-type
Sv129J wild-type BM cells
Sv129J Ncoa4-ko BM cells
wt wt BM
wt Ncoa4-ko BM
B
RBC
*10^6/µL blood
wtwt BM
wtNcoa4-ko BM
C
Hb
g/dL
wtwt BM
wtNcoa4-ko BM
D
MCV
fL
***
wtwt BM
wtNcoa4-ko BM
E
MCH
pg
***
wtwt BM
wtNcoa4-ko BM

Figure S5

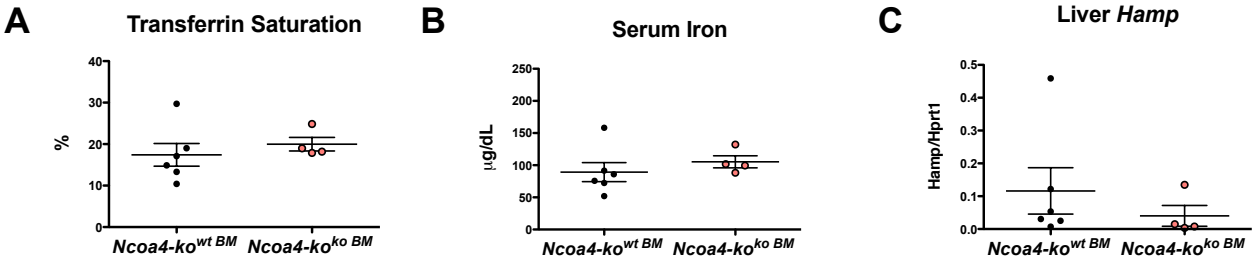
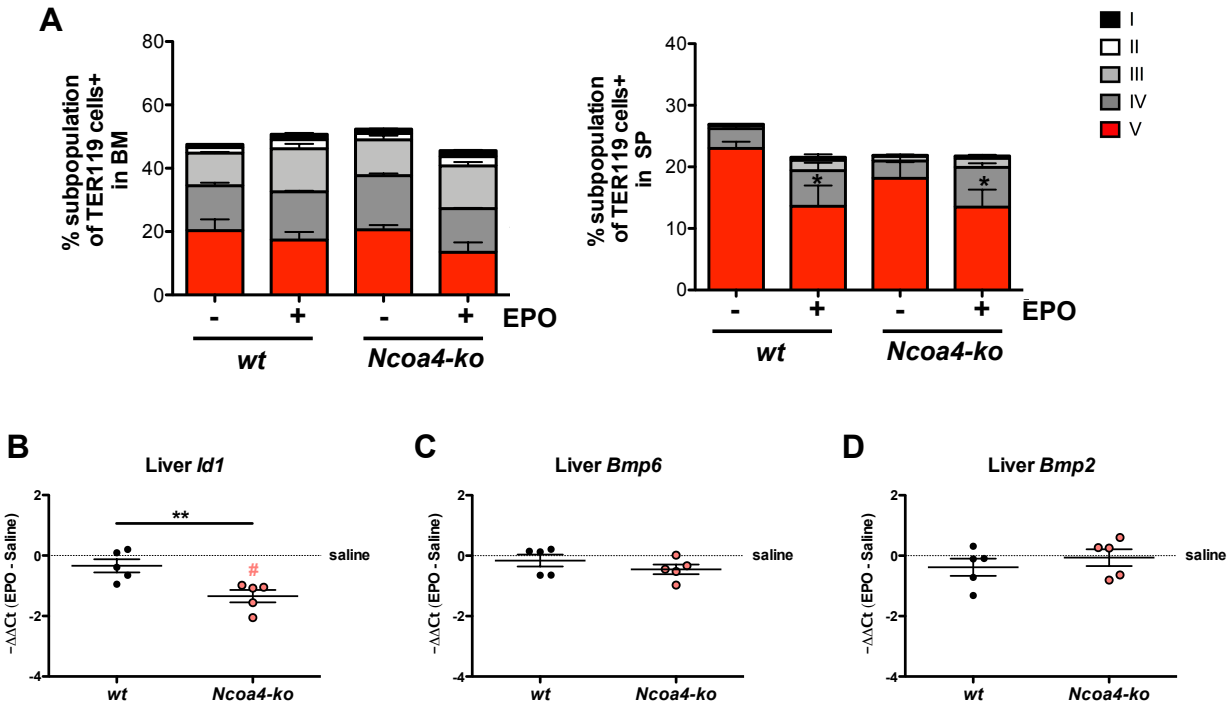

A
Transferrin Saturation
%
Ncoa4-kowt BM
Ncoa4-koko BM
B
Serum Iron
µg/dL
Ncoa4-kowt BM
Ncoa4-koko BM
C
Liver Hamp
Hamp/Hprt1
Ncoa4-kowt BM
Ncoa4-koko BM

Figure S6


A
% subpopulation of TER119 cells+ in BM
80
60
40
20
0
- + - +
EPO
wt
Ncoa4-ko
% subpopulation of TER119 cells+ in SP
40
30
20
10
0
- + - +
EPO
wt
Ncoa4-ko
I
II
III
IV
V
B
Liver Id1
**
#
saline
-ΔΔCt (EPO - Saline)
2
0
-2
-4
wt
Ncoa4-ko
C
Liver Bmp6
saline
-ΔΔCt (EPO - Saline)
wt
Ncoa4-ko
D
Liver Bmp2
saline
-ΔΔCt (EPO - Saline)
wt
Ncoa4-ko