FULL METHODS

Animals

Tsc2$^{fl/fl}$*Lyz2*$^{cre/+}$ (denoted as cre/+) mice were utilized as previously described[20]. Briefly, *Tsc2*$^{fl/fl}$ mice were crossed to *Lyz2*$^{cre/+}$ mice to obtain *Tsc2*$^{fl/fl}$*Lyz2*$^{cre/+}$ mice. *Tsc2*$^{fl/fl}$*Lyz2*$^{+/+}$ (denoted *+/+*) littermates were used as controls and were on a C57BL/6J background. All mice were kept and bred at the Anna Spiegel Center for Translational Research (ASCTR) under SPF conditions. Mice were housed with constant temperature and a 12 h day/night cycle. All animal experiments were approved by the official Austrian institutional ethics and animal welfare committees and the national authority according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012-TVG2012 (BMWF-66.009/0116/II/3b/2014, BMBWF-2020-0.547.514 and BMBWF-68.205/0173-V/3b/2019).
All animals were used for experiments between 7 and 16 weeks of age and groups in individual experiments were sex- and age-matched. Mice were sacrificed by cardiac puncture to collect serum and blood samples or by cervical dislocation.

Red blood cell (RBC) lifespan determination

Blood was collected in heparinized microhematocrit tubes (Microvette CB 300 LH, Sarstedt) and erythrocytes were collected by centrifuging at 400 x g for 5 min. The cells were separated and washed twice in phosphate buffered saline (1 x PBS) supplemented with 2 % fetal calf serum (FCS). RBCs (1 x 10^8/ml) were labelled with 0.1 mM sulfo-NHS-biotin reagent (EZ-Link, NHS-Biotin Reagents, 20217, Thermo Scientific, Waltham, MA, USA) in 1x PBS (pH 8.0) for 30 min at room temperature. RBCs were washed in a mixture of 1x PBS containing 100 mM glycine. 1 x 10^9 labelled RBCs were injected intravenously (*i.v.*) in 100 µl 1x PBS into the tail veins of +/+ and cre/+ mice. For serial blood collection, ~ 50 µL blood was collected from facial vein into heparin tubes at regular time points for 10 consecutive weeks. Collected RBCs were washed in 1 x PBS with 2 % FCS and stained with Streptavidin-APC antibody (BD Biosciences, 554067, Franklin Lakes, NJ, USA). Data were analyzed using a FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA).

Everolimus treatment

To inhibit mTORC1 signaling, mice were treated with Everolimus by oral gavage (RAD001, Selleckchem, S1120, Houston, TX, USA) as previously described[20]. In brief, 7 to 16 weeks old

cre/+ mice were treated by oral gavage with either 5 mg/kg everolimus or sterile 1 x PBS daily for five consecutive days.

^{55}Fe absorption

For measuring whole body iron distribution, ^{55}Fe was used (NEZ043, Perkin Elmer, Waltham, MA, USA). Mice were fasted overnight but had access to water *ad libitum*. A single dose of 2.5 μCi (9.25 x 10^4 Bq) $^{55}FeCl_3$/animal was administered to the mice by oral gavage with an olive-tipped gavage needle in 100 μl 1 x PBS supplemented with 0.5 M ascorbic acid, 0.15 M NaCl, $FeSO_4$ for a final total iron of 5 μg Fe^{2+} per mouse. The animals were sacrificed 1h after ^{55}Fe administration. Blood and tissues were collected and weighed. Duodenal segments were opened longitudinally and rinsed in sterile 1 x PBS. Isolated BM, spleen, liver, and duodenum were digested in SOLVABLE (6NE9100, Perkin Elmer, Waltham, MA, USA) according to the manufacturer's instruction. ^{55}Fe retention in the tissues in Ultima Gold XR (6012119, Perkin Elmer) liquid scintillation medium and the radioactivity was determined using TRI-CARB 2800TR Liquid Scintillation Analyzer (Perkin Elmer Instruments, Shelton, CT, USA).

***In vivo* Fe dextran treatment**

9-12 weeks old control (+/+) and cre/+ mice were injected intravenously (*i.v.*) with a bolus of 1g/kg iron dextran (D8517, Sigma Aldrich, Saint Louis, MO, USA) in 1 x PBS. To maintain iron loading, mice received three further injections (*i.v.*) of 0.2 g/kg iron dextran during 4 weeks. Mice in the control group (+/+) received 1 x PBS during the treatment period.

Intestinal macrophage depletion

To induce intestinal macrophage depletion, 8 to 12 weeks-old mice were injected intraperitoneally (*i.p.*) with 1.2 mg M279 anti-muCfms ralgG1, mAb (P14168.16, Amgen, Thousand Oaks, CA, USA) in 1x PBS three times a week for 6 weeks. Control mice (+/+) received 1.2 mg rat IgG (I4131, Sigma Aldrich, Darmstadt, Germany).

Starving and re-feeding of mice

Three groups of 9-16 weeks old control (+/+) and cre/+ mice were starved over-night. Mice in the first group were sacrificed after starvation (starve). Mice in the second and third

group were fed for 1h and 3h respectively before sacrificing (1h feed and 3h feed). Duodenum cryo-samples were collected for immunostainings.

Protease inhibition *in vivo with nafamostat*

To inhibit proteases *in vivo* 9 to 16 weeks-old mice were injected intraperitoneally (*i.p.*) with 10 mg/kg nafamostat mesylate (FUT-175, Futhan, 552035, BD Biosciences, Franklin Lakes, NJ, USA) every other day for one week. Mice in the control group (+/+) received sterile 1 x PBS.

***Citrobacter rodentium* infection**

Mice infection with *Citrobacter rodentium* (*C. rodentium)* was performed at the Institute of Animal Breeding and Genetics & University Center Biomodels Austria, University of Veterinary Medicine Vienna. 5 x 10^8 pfu *C. rodentium* in a volume of 200 µL were administered to 7-9 weeks old C57BL/6J mice via oral gavage, control mice received sterile 1 x PBS. On day 7 post-infection mice were sacrificed and duodenum samples were flash-frozen in liquid nitrogen for histology.

Preparation of bone marrow (BM) smear

BM was isolated freshly by flushing the femur and tibia of mice with 1 x PBS supplemented with 2 % FCS and cells were collected by centrifugation at 350 x g for 7 min. BM smear were prepared directly after BM isolation and prepared on a glass slide, smeared, and air-dried over-night.

Mouse whole blood count analysis

Mouse blood was collected from facial vein into a heparinized microhematocrit tube. Hematological values were determined by Complete blood cell (CBC) analysis on scil Vet Abc Hematology Analyzer (scil animal care, Grayslake, IL, USA).

Serum collection

Serum samples were collected, after short coagulation at room temperature by centrifuging whole blood at 350 x g for 10 min. The collected serum samples were stored at -80°C.

RBC osmotic fragility test

Red blood cell osmotic fragility test was performed as previously described[21]. 5 µL blood was added to each of a series of 200 µL NaCl solution (pH 7.7) with concentrations ranging from 0 to 0.9 % (0.9 %, 0.85 %, 0.75 %, 0.65 %, 0.6 %, 0.55 %, 0.5 %, 0.45 %, 0.4 %, 0.35 %, 0.3 %, 0 %). The samples were incubated at room temperature for 30 min. After centrifugation at 3000 x g for 10 min, supernatants were collected, and absorption was determined at 540 nm with dH_2O as blank.

Prussian Blue staining

3-6 µm paraffin embedded tissue sections were deparaffinized (3x for 7 min in Neo-Clear, 109843, Merck, Darmstadt, Germany) and dehydrated (2x 1 min 100 % EtOH, 2x 1 min 96 % EtOH and 2x 10 min in dH2O). The sections were incubated 20 min in a mixture of 1 part Perls' Prussian Blue solution (Potassium hexacyanoferrate II – trihydrate, 31254, Sigma Aldrich/Merck, Darmstadt, Germany) and 1 part 2 % hydrochloric acid followed by counterstaining with nuclear fast red solution (N3020, Sigma Aldrich/ Merck, Darmstadt, Germany). After mounting using Eukitt (03989, Sigma Aldrich/ Merck, Darmstadt, Germany), tissue sections were visualized using Olympus BX63 microscope and images were acquired using Olympus cellSens Dimension 2.3 software (Olympus). Images were quantified by Fiji/ImageJ[22].

Total iron concentration measurements with AAS

20 - 30 mg liver, spleen, small intestine tissues (duodenum and ileum) were digested in 1.5 ml Nitric acid Rotipuran Supra 69 % (HN50.1, Carl Roth, Karlsruhe, Germany) using a microwave oven (Mars (CEM) one touch technology). Total iron levels in serum and digested tissues were determined using flame atomic absorption spectrometry (AAS) (Hitachi Z-8200 Polarized Zeeman Atomic Absorption Spectrometer, SpectraLab). The limit of detection was set at the concentration equivalent to the threefold of standard deviation of the blank.

Measurements of serum heme

Serum mouse heme levels were measured as previously described[23]. Briefly, 50 µL serum sample aliquots and Hemin standards (H9039, Sigma-Aldrich, Saint Louis, MO, USA) were mixed with 750 µL 1.5 M Oxalic acid. Samples were boiled at 120°C for 30 min. After cooling

at room temperature, 200 µL samples were placed in a standard 96 well plate in duplets and the fluorescence was recorded on Synergy HT BioTek microplate reader (Szabo scandic, Vienna, Austria) at excitation and emission wavelengths of 405 nm and 662 nm, respectively.

Transferrin saturation

Transferrin saturation in mouse serum was measured using Iron/TIBC kit (I7504, POINTE Scientific, Canton, Michigan, USA) according to manufacturer's protocol. Total iron-binding capacity (TIBC), serum iron and Unsaturated Iron-Binding Capacity (UIBC) were determined based on absorbance at 570 nm, and transferrin saturation was calculated from serum iron level compared to TIBC. The iron standard curve was prepared using iron solution provided by the kit.

Visualization of transferrin saturation by urea-PAGE

Visualization of transferrin saturation by urea-polyacrylamide gel electrophoresis (PAGE) was performed as previously described[24]. In brief, mouse serum was collected as described above. Most abundant serum proteins were precipitated and removed with 0.6 % Rivanol in Tris-Borat-electrophoresis buffer (100 mmol/L Tris, 10 mmol/L boric acid, pH 8.4) and 10 % glycerine. The supernatant was separated on 6 % polyacrylamide gels containing 6M urea in Tris-borate-electrophoresis buffer. The density of the bands representing apo-, holo - and monoferric-transferrin were visualized with 0.1 % Amidoblack in a Methanol and acetic acid mixture (4:1).

Quantitative measurement of transferrin concentration in serum

Total transferrin concentration in serum was determined using ELISA Kit according to manufacturer's protocol (ab187391, abcam, Cambridge, UK).

Quantitative measurement of ferritin in serum

Serum ferritin levels were measured using the Mouse Ferritin ELISA Kit (ab157713, abcam, Cambridge, UK) according to manufacturer's instructions. Absorbance measurements were performed on a Peqlab Biotek Synergy HT Plate Reader at 450 nm using the Gen5 1.10 Software.

Modified colorimetric ferrozine-assay for quantification of total and Fe^{2+} in tissues

Flash-frozen small tissue pieces were weighed and homogenized using a Precellys Lysing Kit (KT0396, Bertin Instruments, Montigny-le-Bretonneux, France). Quantification of iron concentration in tissues were determined by colorimetric ferrozine assay as described before with minor modifications [25,26]. Briefly, 100 µL cell lysates were mixed with 100 µL of 10 mM HCl and 100 µL of freshly mixed iron-releasing agent (equal volume of 1.4 M HCl and 4.5 % (w/v) $KMnO_4$ in H_2O) and incubated for 2h at 60°C in a fume hood. After cooling at room temperature, 30 µL of ferrozine iron-detection reagent (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 M ammonium acetate) was added to a reducing agent (1 M ascorbic acid). After 30 min, absorbance was measured at 540 nm. To determine Fe^{2+} content 200 µL tissue lysates were mixed with 200 µL 50 mM NaCl, 200 µL 10 mM HCl and 200 µL iron-releasing agent. The mixture was incubated for 2h at 60°C in a fume hood. After cooling, 60 µL of ferrozine iron-detection reagent was added without reducing agent. After 30 min, absorbance was measured at 540 nm. The iron content was compared to the absorbance of standard, a mixture of 20 µg/ml $FeCl_2$ and 10 mM FAS (Ferric-III-ammonium sulphate).

Immunoblot analysis

Tissue homogenization was prepared using Precellys Lysing Kit (Bertin Instruments, Montigny-le-Bretonneux, France) according to the manufacturer's instruction. Liver homogenates were prepared in RIPA buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dedocyl sulfate 1 mM ethylenediaminetetraacetic acid and 50 mM Tris, pH 7.4). Spleen was prepared in lysis buffer (20 mM Hepes pH 7.9, 0.4 M NaCl, 25 % (v/v) Glycerin, 1 mM ethylenediaminetetraacetic acid, 0.5 mM Na_3VO_4, 0.5 mM DTT, 1 % Triton X-100). Cell homogenates from duodenum were prepared in modified RIPA buffer (150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholic acid, 0.1 % sodium dedocyl sulfate, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride and 50 mM Tris, pH 7.4). All lysis buffers were supplemented with protease and phosphatase inhibitors (11836170001, Roche, Basel, Switzerland) and 4 µg/ml aprotinin, 4 µg/ml leupeptin, 0.6 µg/ml benzamidinchloride, 20 µg/ml trypsin inhibitor and 2 mM PMSF (all from Sigma Aldrich, Saint Louis, MO, USA). Protein concentration was measured with Bio-

Rad protein assay (5000001, Bio-Rad, Hercules, CA, USA) and equal amounts of denaturated lysate were resolved on 10 % SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in Intercept blocking buffer TBS (927-60001, LI-COR Biosciences, Lincoln, NE, USA) for 1 hour at room temperature and incubated with primary antibodies prepared in Intercept T20 (TBS) Antibody Diluent (927-65001, LI-COR Biosciences) at 4° C overnight.

Antibodies were diluted in blocking solution as follows: mouse anti-alphaTubulin mAb (1:1000, AC012, ABclonal, Woburn, MA, USA), mouse anti-alphaTubulin (1:1000, DM1A, Merck, Darmstadt, Germany), goat anti- Tf (1:1000, AF3987, R&D, NE, Minneapolis, USA), rabbit anti-FPN- MTP1/IREG1/Ferroprotein (1:4000, MTP11, Alpha Diagnostics, San Antonio, TX); rabbit anti-Ferritin (1:4000, F5012, Sigma-Aldrich, St Louis MO, USA); rabbit anti-Ceruloplasmin (Cp) (1:100, MAK177, Sigma Aldrich, St Louis, MO); rabbit anti- IRP1/Aco1 (1:1000, RN036PW, MBL, Sunnyvale, CA, USA). Fluorophore-conjugated secondary antibodies were prepared 1:20000 in Intercept T20 (TBS) Antibody Diluent (927-65001, LI-COR Biosciences): IRDye 800CW goat anti-mouse IgG secondary antibody (926-32210), IRDye 680RD goat anti-rabbit IgG (926-68071), IRDye 800CW donkey anti-goat IgG (926-32214) secondary antibodies (LI-COR, Lincoln, NE, USA). Proteins were detected by the Odyssey-CLX imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Real-time quantitative PCR

Total RNA from liver was extracted using the RNeasy Micro Kit by Qiagen (74004, Qiagen, Germantown, MD, USA) combined with RNase-free DNase Set (79254, Qiagen, Germantown, MD, USA) to eliminate gDNA. 100 to 500 ng RNA was reverse transcribed into cDNA using GoScript™ Reverse Transcriptase kit (A2791, Promega, Madison, WI, USA) according to the manufacturer's instruction. cDNA was diluted 1:10 in nuclease-free dH_2O and amplified via RT-qPCR using GoTaq™ Master Mix (M7132, Promega, Madison, WI, USA). Real-time PCR was then performed using a StepOnePlus™ Real-Time PCR-System (4376600, Thermo Fisher, Waltham, MA, USA) with the *Hamp* primers (Hamp forward: 5´- GCAGGGCAGACATTGCGATAC - 3´. Hamp reverse: 5´- GCAGGGCAGACATTGCGATAC - 3´). Expression was normalized to the housekeeping-gene β-actin and the values were calculated using the comparative threshold cycle method ($2^{-\Delta Ct}$).

Hepcidin ELISA

~ 20 mg Tissues (duodenum, liver) were minced and homogenized in 1 ml cold 1 x PBS using Precellys Lysing Kit (KT0396, Bertin Instruments, Montigny-le-Bretonneux, France) according to the manufacturer's instruction. Homogenates were centrifuged at 15000 x rpm for 15 min at 4°C and supernatants were collected for assaying. Serum samples were prepared as described above. Hepcidin levels were determined by the mouse HAMP/Hepcidin Elisa Kit (LS-F5905, LSBio, Seattle, WA). The analysis was performed according to manufacturer's instructions. Absorbance was read at 450 nm by Synergy HT BioTek microplate reader (Szabo scandic, Vienna, Austria).

Immunofluorescence (IF) staining of mouse cryo-sections

All mouse tissues were immediately flash-frozen in liquid nitrogen for IF staining. 3-6 μm cryosections were blocked with 2.5 % horse serum and 0.1 % Triton X-100 for 1h at room temperature. After blocking, tissue sections were incubated with primary antibodies in appropriate concentration overnight at 4°C. Antibodies were diluted in blocking solution as follows: goat anti-Tf (1:100, AF3987, R&D, NE, Minneapolis, USA); rabbit anti-Tf (1:100, PA3-913, Invitrogen, Rockford, IL, USA); rabbit anti-FPN- MTP1/IREG1/Ferroprotein (1:600, MTP11, Alpha Diagnostics, San Antonio, TX); rabbit anti-Ferritin (1:500, F5012, Sigma-Aldrich, St Louis MO, USA); rabbit anti-Ceruloplasmin (Cp) (1:50, MAK177, Sigma Aldrich, St Louis, MO); rabbit anti-Tfrc1 (CD71, 1:100, 13-6800, Thermo Fischer, Waltham, MA, USA); rabbit anti-ATPV0D2 (1:1000, SAB2103221, Sigma Aldrich/Merck, Darmstadt, Germany), rat anti-F4/80 (1:600, BM8, BioLegend, San Diego, CA, USA), rat anti-CD68 (1:200, FA-11, Biolegend, San Diego, CA, USA), goat anti-E-cadherin (1:50, AF648, R&D, NE, Minneapolis, USA). After washing with 1 x PBS, secondary antibodies were applied for 45 min at room temperature with following secondary antibodies: donkey anti-rabbit-Alexa Fluor 555, donkey anti-rabbit-Alexa Fluor 488, donkey anti-rabbit-Alexa Fluor647, donkey anti-goat-Alexa Fluor488, donkey anti-goat-Alexa Fluor647, donkey anti-rat-Alexa Fluor488, donkey anti-rat-Alexa Fluor555. All Alexa Fluor secondary antibodies were from ThermoFisher (Waltham, MA, USA) and used at a dilution of 1:500. DAPI (D9542, Sigma Aldrich/Merck, Darmstadt, Germany) was used for nuclear counterstaining. After washing, sections were mounted with Fluoromount (F4680, Sigma Aldrich/Merck, Darmstadt, Germany) and visualized using Leica TCS SP8 Confocal microscope (Mannheim, Germany). Images were

acquired using the Leica LasX software. For the quantitative analysis images Fiji/ImageJ software was used [22]. The area fraction of the epithelial cells and the lamina propria fraction were set and the mean intensity in the set areas were measured after thresholding.

Enzyme activity test on mouse cryo-sections

3-6 μm duodenum flash-frozen, non-fixed fresh cryo- sections were used for enzyme activity assay with activity-based probes (ABP) [27]. 1 μM ABPs for elastase-like and chymotrypsin proteases were incubated on tissue sections for 30 min at 37°C. After washing DAPI (D9542, Sigma Aldrich/Merck, Darmstadt, Germany) was used for nuclear counterstaining. As negative controls tissue-sections were incubated with ABPs at 4°C and with 0.1 mM protease inhibitor Phenylmethylsulfonylfluorid (PMSF). Active proteases were visualized by using Leica TCS SP8 Confocal microscope (Mannheim, Germany) after mounting with Fluoromount (F4680, Sigma Aldrich/Merck, Darmstadt, Germany). The mean intensity in whole villus and in the lamina propria fraction were measured after thresholding using Fiji/ImageJ software [22].

Immunohistochemistry (IHC) of formalin-fixed, paraffin-embedded mouse tissues

Mouse tissues were fixed in 4 % buffered paraformaldehyde overnight at RT and subsequently dehydrated through a series of isopropanol baths (80 %, 90 %, 100 % isopropanol). Finally, isopropanol was replaced by Xylol twice and tissue were infiltrated with paraffin prior to embedding. 3-6 μm paraffin- embedded tissue sections were prepared with a microtome (Micros). After clearing in Neo-Clear (109843, Merck, Darmstadt, Germany) and rehydrating (100 % EtOH, 96 % EtOH, dH2O), epitope retrieval was performed in autoclave using Antigen Retrieval solution pH6 (S1699, Agilent/DAKO, Carpinteria, CA, USA). The sections were permeabilized (0.1 % Triton X in 1 x PBS), treated with 3 % H_2O_2 for 10 min at room temperature and blocked with 2.5 % horse serum for 1h at room temperature. After washing, the primary antibodies were added to the slides at predefined concentrations and incubated overnight at 4°C. Antibodies were diluted in the blocking solution as follows: rabbit anti-phospho-S6 Ribosomal Protein (Ser240/244), (1:200, D68F8, Cell Signaling, Danvers, MA, USA), rabbit anti-Heph (Hephaestin) (1:250, PA5-34844, ThermoFisher/Invitrogen, Waltham, MA, USA). On next day, after washing with 1 x PBS, horse biotinylated anti rabbit IgG antibody (1:500, BA-100, Vector Laboratories, Burlingame,

CA, USA) was applied for 45 min at room temperature. After washing in 1 x PBS, Novocastra streptavidin-HRP (Leica) and AEC-high sensitivity substrate chromogen (K3469, DAKO, Glostrup, Denmark) were subsequently added to the tissue sections for detection. Nuclei were counterstained with Mayer's hematoxylin (105174, Sigma Aldrich/Merck, Darmstadt, Germany), Eosin (HT110116, Sigma Aldrich/Merck, Darmstadt, Germany) and mounted with Aquatex (1.08562.0050, Merck, Darmstadt, Germany). Slides were mounted using Fluoromount (F4680, Sigma Aldrich/Merck, Darmstadt, Germany) and visualized using Olympus BX63 microscope and Olympus cellSens Dimension 2.3 software (Olympus). Fiji/ImageJ software was used for quantifying[22].

Ceruloplasmin ferroxidase activity test

Duodenum tissue homogenates were prepared as described above in 1 ml 1 x PBS using Precellys Lysing Kit (KT0396, Bertin Instruments, Montigny-le-Bretonneux, France) according to the manufacturer's instruction. Homogenates were centrifuged at 15000 x rpm for 15 min at 4°C and supernatants were collected. Ceruloplasmin activity was determined using Ceruloplasmin Activity Colorimetric Kit (MAK177, Sigma Aldrich, St. Louis, MO, USA) according to manufacturer´s instructions. Briefly, 100 µL of samples were mixed with 100 µL saturated ammonium sulfate solution and incubated for 5 min on ice to remove chloride. Samples were centrifuged at 10,000 rpm at room temperature for 5 min and the supernatants were collected. 5 µL sample was diluted with Ceruloplasmin Assay Buffer to a final volume of 100 µL. A reaction mix of 10 µL Ceruloplasmin Substrate and 90 µL Ceruloplasmin Assay Buffer was added to each sample and the absorption was determined after 15 min at 560 nm on Synergy HT BioTek microplate reader (Szabo scandic, Vienna, Austria). Serum samples were used as positive controls and H_2O was used as negative control.

Flow cytometry analysis of erythropoiesis

BM was isolated freshly by flushing from the femur and tibia of mice and cells were collected by centrifuging at 350 x g for 7 min. Spleen was minced through a 70 µm cell strainer. Thereafter red blood cells were lysed with RBC lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and the flow-through of a 40 µm cell strainer was collected in FACS buffer (2% FCS, 1mM EDTA in 1x PBS).

Erythroid cells in the cell suspensions were stained with CD71-PE (1:200, R17217, BioLegend, San Diego, CA, USA) and Ter119-APC (1:200, BioLegend, San Diego, CA, USA) antibodies in in FACS buffer (2 % FCS, 1mM EDTA in 1x PBS) and incubated for 30 min on ice. After washing in FACS buffer cells were acquired using CytoFLEXS (Beckman Coulter, Brea, CA, USA). The analysis was performed using Software CytExpert V.2.3.1.22.

Erythrophagocytosis assay

Erythrocytes were obtained from C57BL/6J and incubated 15 min at 37°C after washing three times. The artificially aged erythrocytes were labeled with CellVue Claret Far Red Fluorescent Cell Linker Mini Kit (Merck/Sigma Aldrich, MinClaret) according to the manufacturer´s protocol. Differentiated BMDMs were treated with labelled erythrocytes (1:10) for 1h at 37°C. BMDMs were washed thoroughly in FACS buffer (2 % FCS, 1mM EDTA in 1x PBS) and stained with macrophage marker CD11b- PerCP as described analyzed using CytoFLEXS (Beckman Coulter, Brea, CA, USA). The analysis was performed using Software CytExpert V.2.3.1.22.

Flow cytometry analysis of duodenum epithelial cells

First 3-4 cm of the small intestine was used as duodenum sample. The samples were cut longitudinally and rinsed in ice-cold HBSS and immediately transferred to ice cold HBSS. Cell suspension from duodenum was prepared using mouse Lamina Propria Dissociation Kit (130-097-410, MACS Miltenyi Biotec, Auburn, CA) according to the manufacturer´s protocol. Epithelial cells from the duodenum were isolated by shaking tissue pieces in fresh digestion solution (1x HBSS containing 5 mM EDTA, 5 % fetal bovine serum (FBS), 1 mM DTT) from mouse Lamina Propria Dissociation Kit (130-097-410, Miltenyi Biotec, Auburn, CA, USA). Samples were incubated for 20 min at 37°C under continuous rotation. This step was repeated three times and the cell suspension was applied through 100 µm cell strainer. The flow through containing epithelial cells was combined and washed in FACS buffer (2 % FCS, 1mM EDTA in 1x PBS). FcγIII/II receptors were blocked by incubating 1x10^6 cells with 1 µL TruStain fcX™ (Biolegend, San Diego, CA, USA) for 20 minutes. Epithelial cells were stained with CD45.2 – PE (1:200, 104, BioLegend, 10980, San Diego, CA, USA) and EpCAM (CD326) – FITC (1:200, G8.8, BioLegend, 118207, San Diego, CA, USA) combined with life-dead cell exclusion with 7-amino-actinomycin D (7-AAD). For life-dead cell exclusion 7-amino-

actinomycin D (7-AAD, 1 µL/ml, A1310, Thermo Fisher/Invitrogen, Carlsbad, CA, USA) was used. Cells were identified by flow cytometry using CytofLEXS (Beckman Coulter). Data were analysed using Software CytExpert V.2.3.1.22 and FlowJo X10 software, v10.8 (BD, Franklin Lakes, NJ, USA).

Flow cytometry analysis of duodenum macrophages

Cell suspension from duodenum was prepared using mouse Lamina Propria Dissociation Kit (130-097-410, MACS Miltenyi Biotec, Auburn, CA) according to the manufacturer´s protocol. Cell suspension was prepared using gentleMACS Dissociator (130-093-135, Miltenyi Biotec, Auburn, CA). Isolated cell suspensions were further used for flow cytometry. FcγIII/II receptors were blocked by incubating $1x10^6$ cells with 1 µL TruStain fcX™ (Biolegend, San Diego, CA, USA) for 20 minutes. Subsequently the cells were stained with the indicated antibodies for 25 min on ice, light protected (Table 1). Pellets were washed twice and resuspended in fresh FACS buffer (2 % FCS, 1mM EDTA in 1x PBS). For life-dead cell exclusion 7-amino-actinomycin D (7-AAD) was used. Cells were identified by flow cytometry using CytoflexS (Beckman Coulter). Data were analyzed using Software CytExpert V.2.3.1.22 and FlowJo X10 software, v10.8 (BD, Franklin Lakes, NJ, USA).

Transferrin uptake of duodenum macrophages

To analyze pH-sensitive Tf-iron endocytosis, cells suspension from duodenum was prepared and LP macrophages ($Cd45^+$, $Cd11c^+$, $Cd11b^+$, $Ly6C^-$, $Cd64^{+-}$, $Cd68^+$) were identified as described above. First, isolated LP cells were incubated with pHrodo-Red Transferrin conjugate (P35376, Invitrogen, Carlsbad, CA, USA) in live cell imaging solution (LCIS buffer) (1X PBS with 20 mM Glucose and 1 % BSA) for 20 min at 37 °C. In parallel, cells were treated with 50 µM hydro-chloroquine (CQ) for 30 min and the CQ treatment continued during the 20 min-incubation with pHrodo-Red Transferrin conjugate. After washing cells with LCIS buffer, cells were stained with the following surface antibodies for flow cytometry: CD45-PE, CD11c-PE-Cy7, CD11b- eFluor450, Ly6c- APC Cy-7, CD64 – APC, CD68– AF488. The uptake of pHrodo-Tf in macrophages was measured by flow cytometry using CytoFLEX S (Beckman Coulter). Data were analyzed as described.

Bulk RNA-Sequencing

Duodenal LP macrophages ($Cd45^+$, $Ly6G^-$, $Cd11c^+$, $Cd11b^+$, $Ly6C^-$, $Cd64^+$) and $Cd45^-EpCAM^+$ epithelial cells were isolated as described above and sorted at the FACS Core Unit of the Children's Cancer Research Institute of Vienna (CCRI, Vienna, Austria). Freshly sorted cells were harvested into RLT-Buffer containing ß-mercaptoethanol and stored at -80°C until RNA isolation via the RNeasy Micro Kit (74004, Qiagen, Valencia, CA). RNA was further processed for the small RNA-Seq libraries and sequenced with the low input (smart-seq) protocol at the Biomedical Sequencing Facility of the CeMM, Vienna[28].

The subsequent NGS library preparation from the amplified cDNA was performed using the Nextera XT DNA library prep kit (Illumina, San Diego, CA, USA). Library concentrations were quantified with the Qubit 2.0 Fluorometric Quantitation system (Life Technologies, Carlsbad, CA, USA) and the size distribution was assessed using the Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA, USA). For sequencing, samples were diluted and pooled in equimolar amounts. Expression profiling libraries were sequenced on HiSeq 3000/4000 instruments (Illumina, San Diego, CA, USA) following a 50-base-pair, single-end recipe. Raw data acquisition (HiSeq Control Software, HCS, HD 3.4.0.38) and base calling (Real-Time Analysis Software, RTA, 2.7.7) was performed on-instrument, while the subsequent raw data processing off the instruments involved two custom programs based on Picard tools (2.19.2). In a first step, base calls were converted into lane-specific, multiplexed, unaligned BAM files suitable for long-term archival (IlluminaBasecallsToMultiplexSam, 2.19.2-CeMM). In a second step, archive BAM files were demultiplexed into sample-specific, unaligned BAM files (IlluminaSamDemux, 2.19.2-CeMM). NGS reads were mapped to the Genome Reference Consortium GRCm38 assembly via "Spliced Transcripts Alignment to a Reference" (STAR)[29] utilizing the "basic" Ensembl transcript annotation from version e96 (April 2019) as reference transcriptome. Since the mm10 assembly flavor of the UCSC Genome Browser was preferred for downstream data processing with Bioconductor packages for entirely technical reasons, Ensembl transcript annotation had to be adjusted to UCSC Genome Browser sequence region names. STAR was run with options recommended by the ENCODE project. Aligned NGS reads overlapping Ensembl transcript features were counted with the Bioconductor (3.9) GenomicAlignments (1.20.1) package via the summarizeOverlaps function in Union mode, taking into account that Smart-seq2 does not yield strand-specific data. Transcript-level counts were aggregated to gene-level counts. Differentially expressed genes were identified using Bioconductor

package 'edgeR'[30]. All heatmaps were created by CRAN package 'pheatmap' v1.0.12 (Raivo Kolde, 2019). Volcano plots were generated by 'Enhanced volcano' Package v3.14[31]. KEGG gene set enrichment analysis was performed by Bioconductor package 'ClusterProfiler' v4.2.1[32]. All analysis were performed using R statistics v4.0.4. Sequencing data has been deposited on NCBI GEO (GSE175992).

Single-cell RNA-sequencing (scRNA-Seq)

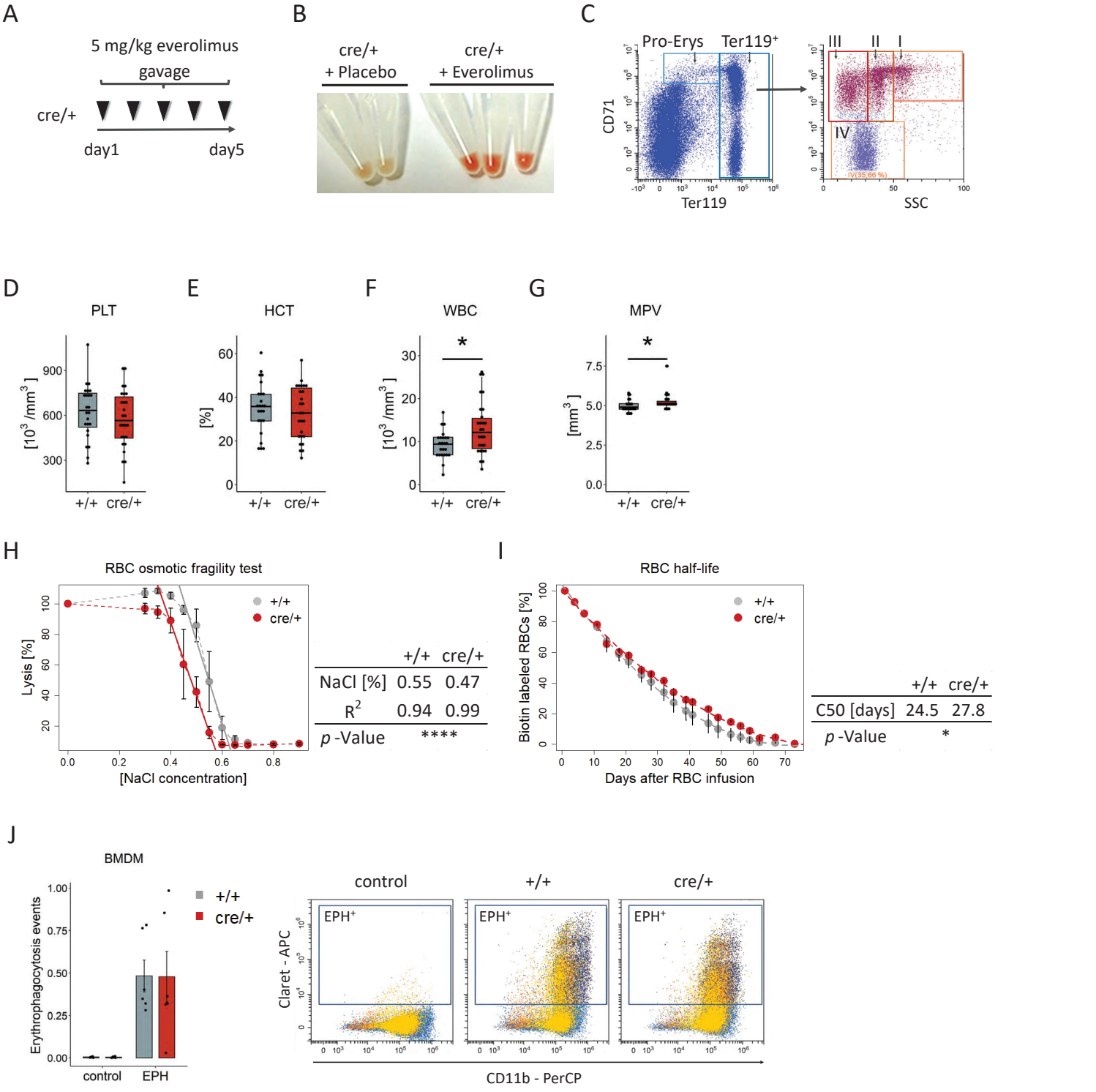
$CD45^+$ (clone 104, CD45.2 – PE, 1:200, BioLegend, 10980) cells (30,000) were sorted into PBS containing 0.5 % bovine serum albumin (BSA) after live-dead exclusion with 7AAD, using Sony MA900 cell sorter (Sony, Tokyo, Japan). Single-cell next-generation sequencing libraries were generated using the 10x Genomic Single Cell 3' v3 workflow (10x Genomics, San Francisco, CA) and sequenced on a NovaSeq 6000 instrument (Illumina, San Diego, CA, USA) in paired-end configuration as recommended by the manufacturer of the single cell kit. Demultiplexing, alignment, feature counting, and filtering steps of the raw sequencing data were performed with the Cell Ranger pipeline version 5.0.1 (10x Genomics, San Francisco, CA) against reference transcriptome mm10-2020-A, resulting in files subsequently explored via Loupe Browser version 4.0.0 (10x Genomics, San Francisco, CA). The single cell library preparation, sequencing and initial data analysis were performed by the Biomedical Sequencing Facility at the CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria.

The 'c-loupe' files were generated by the Cell Ranger software. sc-RNA-Seq data were explored by Seurat v3.5.3[33]. In Seurat cowplot was used to normalize data, "JackStrawPlot" and "ElbowPlot" and 'RunUMAP' functions were used to identify principal components and to identify clusters. All CD68-positive cells were further clustered by Seurat subset function and analysed by Seurat default functions. Expression of interested genes were identified by "FindAllMarkers" function. DoHeatmap" function was used to show top genes in each cluster and gene expression associated with iron regulation.

All genes identified in cluster 1 were used for GO (CC) and KEGG gene enrichment analysis using Bioconductor package 'ClusterProfiler' v4.2.1[32]. The processed scRNA-seq data are available from NCBI Gene Expression Omnibus under the accession number GSE197175.

Statistical analysis

Mice or samples were randomly assigned to experimental, or processing groups and experiments were performed in an age- and sex matched manner. Data were represented as means ± standard deviation (SD), unless otherwise presented. Groups were compared using unpaired two-tailed Student's *t* test. Differences with *P* value < 0.05 were considered statistically significant and *P* values were provided in the figures as follows: (* P < 0.05, ** P < 0.01, *** P < 0.001, ****P < 0.0001). Correlations were analyzed by Pearson´s correlation and correlation coefficient (r) and P values were depicted. Correlations were considered statistically significant at *P* values < 0.05. All statistical analysis were performed using R statistics v3.6.3 - v4.0.4.

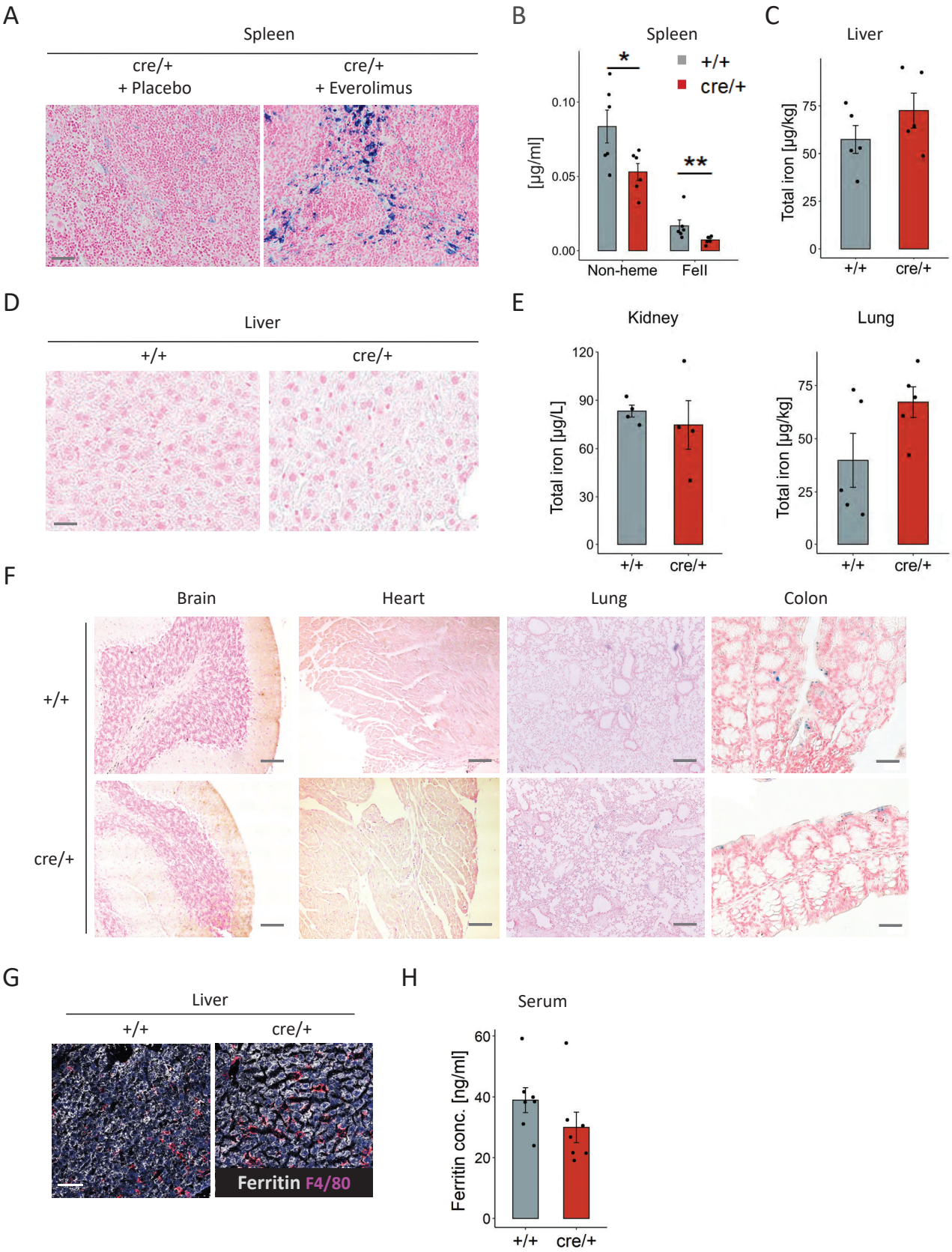


	+/+	cre/+
NaCl [%]	0.55	0.47
R^2	0.94	0.99
p -Value		****

	+/+	cre/+
C50 [days]	24.5	27.8
p -Value		*

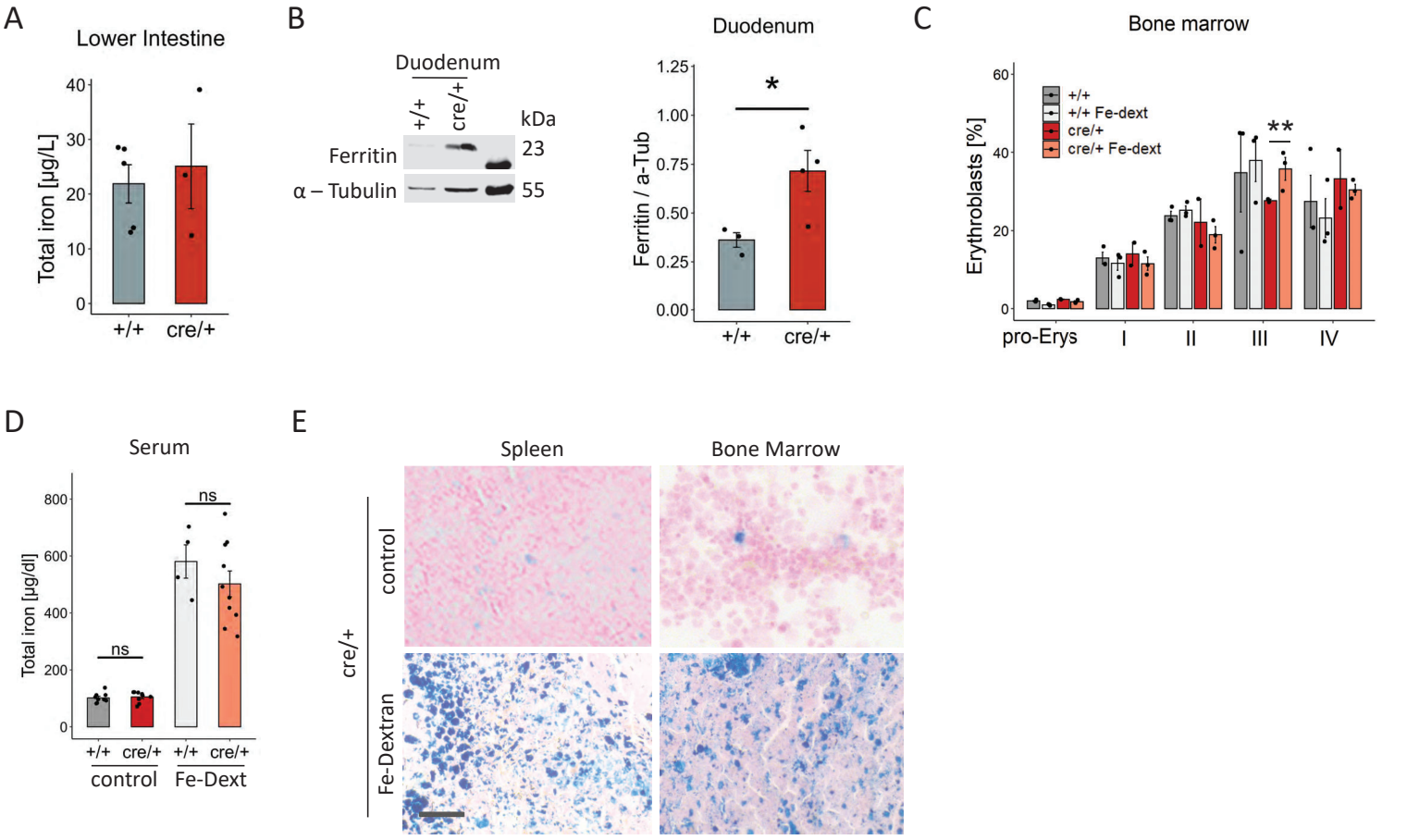
Supplementary Figure. 1. TSC2-KO mice have impaired erythropoiesis.

(A) Graphical presentation of 5 mg/kg everolimus treatment of 9-15 weeks old cre/+ mice to inhibit activated mTORC1 signaling in the macrophages; Everolimus was administered by oral gavage for five consecutive days. **(B)** Representative image showing color of bone marrow (BM) from cre/+ mice treated with everolimus (inhibitor mTORC1 signal) compared to control cre/+ mice treated with 1 x PBS. **(C)** A representative example of gating strategy of flow cytometry analysis to identify erythroblasts in different maturation stages (stages: pro-Erys, I, II, III, IV) in BM and spleen based on CD71 (transferrin receptor), Ter119 (erythrocyte marker) expression and cell size (SSC). **(D-G)** Complete blood cell count (CBC) analysis showing hematological parameters in whole blood from control (+/+) and cre/+ mice: Platelet count (PLT) (D), Hematocrit count (HCT) (E), White blood cell count (WBC) (* $P < 0.05$) (F), Mean platelet volume (MPV) (* $P < 0.05$) (G). **(H)** Red blood cell osmotic fragility test. Osmotic fragility is increased in cre/+ animals (right shift) with increased stability of RBCs at lower saline concentration of 0.47 compared to the controls (+/+) (0.55). Slope of the curves were compared. **** $P < 0.0001$. **(I)** Half-life of RBCs (C50) in control (+/+) and cre/+ mice. Decrease of frequencies of with biotin labeled red cells after *i.v.* injection of 0.1 mM sulfo-NHS-biotin was monitored. Areas under curves were compared. * $P < 0.05$. (J) Flow cytometry quantitative analysis of erythrophagocytosis (EPH) assay of $CD11b^+$ bone marrow-derived macrophages (BMDMs). Mean fluorescence intensity of labeled cells were measured. A representative example of gating strategy to detect EPH^+ BMDMs.


A
Spleen
cre/+
+ Placebo
cre/+
+ Everolimus
B
Spleen
+/+
cre/+
*
**
[µg/ml]
0.10
0.05
0.00
Non-heme
FeII
C
Liver
Total iron [µg/kg]
75
50
25
0
+/+
cre/+
D
Liver
+/+
cre/+
E
Kidney
Total iron [µg/L]
120
90
60
30
0
+/+
cre/+
Lung
Total iron [µg/kg]
75
50
25
0
+/+
cre/+
F
Brain
Heart
Lung
Colon
+/+
cre/+
G
Liver
+/+
cre/+
Ferritin F4/80
H
Serum
Ferritin conc. [ng/ml]
60
40
20
0
+/+
cre/+

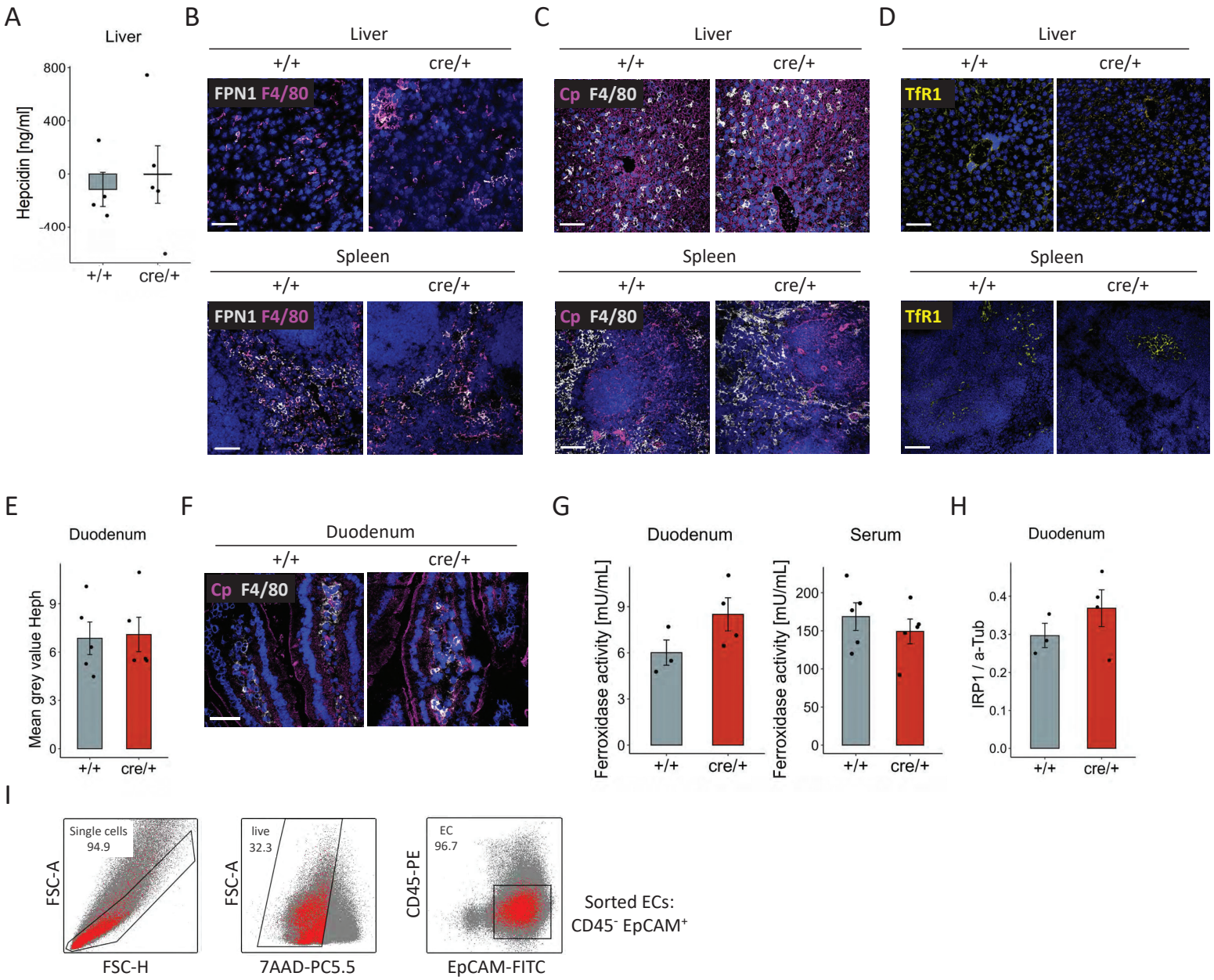
Supplementary Figure. 2. mTORC1 activation in macrophages causes iron deficiency in cre/+ mice.

(A) Representative image of ferric iron Prussian Blue staining on splenic sections of cre/+ mice compared to cre/+ mice after everolimus treatment, counterstained with nuclear fast red. Scale bar 100 μm. **(B)** Non-heme iron levels and free ferrous iron (Fe^{2+}) levels were measured using modified Ferrozine assay in spleen of 8-12 weeks old control (+/+) and cre/+ mice. Total iron levels were normalized to protein concentration of the tissues (*$P < 0.05$, **$P < 0.01$). **(C)** Analysis of total iron concentration using atomic absorption spectrometry (AAS) in the liver of 8-12 weeks old +/+ and cre/+ mice. Total iron levels were normalized to tissue weight. **(D)** Representative image of ferric iron Prussian Blue, counterstained with nuclear fast red on mouse liver FFPE-sections obtained from control (+/+) and cre/+ mice. Scale bar 50 μm. **(E)** Total iron concentration was measured using AAS in kidney and lung of 8-12 weeks old control (+/+) and cre/+ mice. Total iron levels were normalized to tissue weight. **(F)** Representative image of Prussian Blue, counterstained with nuclear fast red on mouse tissue FFPE-sections (brain, heart, lung, colon) obtained from control (+/+) and cre/+ mice. Scale bar 100 μm. **(G)** Representative image of immunofluorescent staining for Ferritin (iron storage protein) and F4/80 (macrophage marker) on mouse liver cryo-sections. Scale bar 50 μm. **(H)** Ferritin concentration was measured in serum using ELISA.


A
Lower Intestine
Total iron [µg/L]
40
30
20
10
0
+/+
cre/+
B
Duodenum
+/+
cre/+
kDa
Ferritin
23
α – Tubulin
55
Duodenum
Ferritin / a-Tub
1.25
1.00
0.75
0.50
0.25
0.00
*
+/+
cre/+
C
Bone marrow
Erythroblasts [%]
60
40
20
0
+/+
+/+ Fe-dext
cre/+
cre/+ Fe-dext
**
pro-Erys
I
II
III
IV
D
Serum
Total iron [µg/dl]
800
600
400
200
0
ns
ns
+/+
cre/+
+/+
cre/+
control
Fe-Dext
E
Spleen
Bone Marrow
cre/+
control
Fe-Dextran

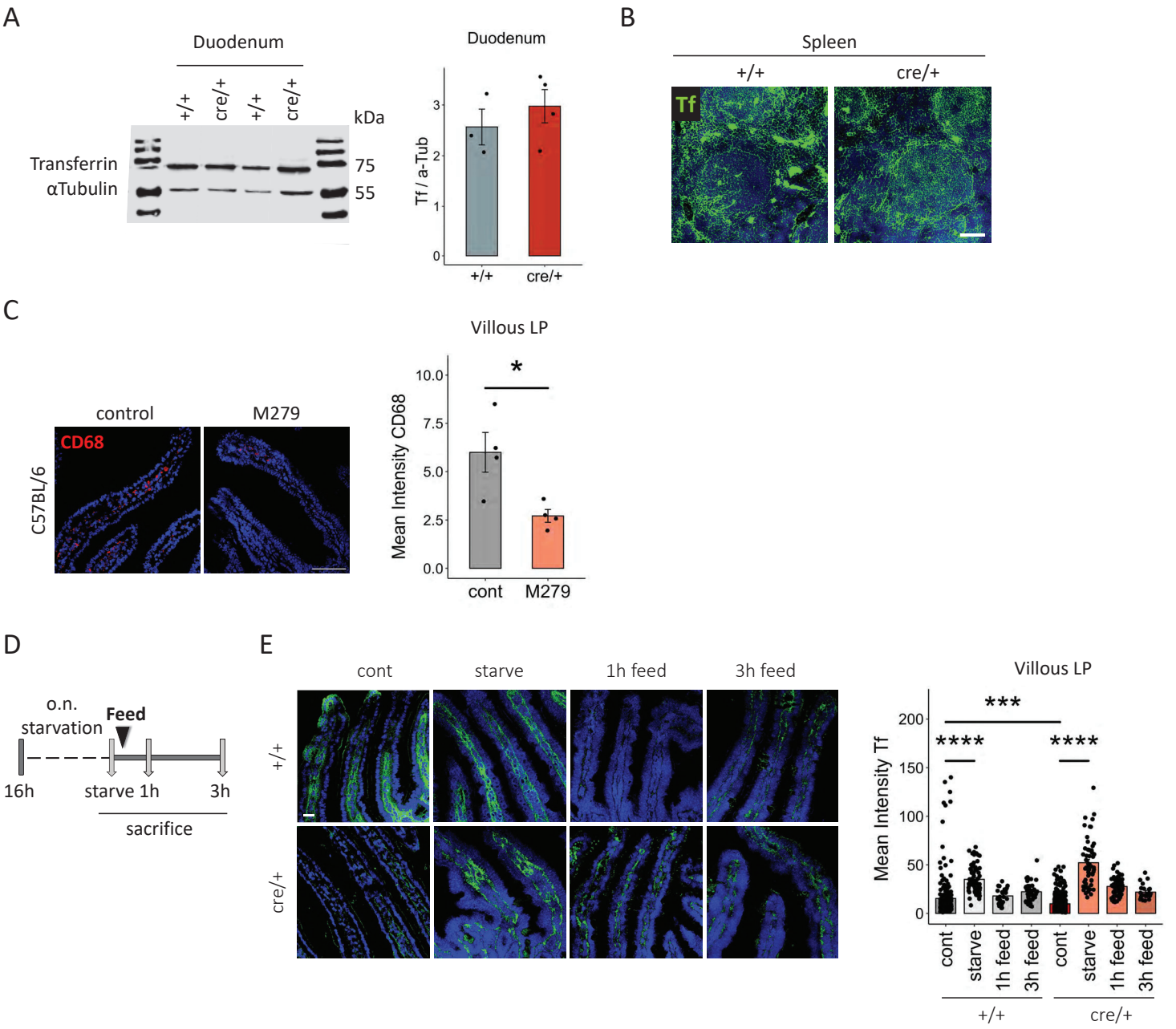
Supplementary Figure. 3. Iron transport from duodenal epithelial cells is impaired in cre/+ mice

(A) Total iron concentration was measured using AAS in the lower part of the small intestine including duodenum of control (+/+) and cre/+ mice. Total iron levels were normalized to tissue weight. **(B)** Representative western blot analysis of total Ferritin (23 kDa) in duodenum lysates from control (+/+) and cre/+ mice. Quantification of Ferritin normalized to α-Tubulin (55 kDa) (*$P < 0.05$). **(C)** Bar graph of flow cytometric analysis showing erythroblast counts in bone marrow (BM) in mice after intravenous (*i.v.*) Fe-dextran treatment compared to controls (+/+) *i.v.* treated with 1 x PBS (**$P < 0.01$). Different maturation stages of erythroblasts (pro-Erys = Proerythroblasts, I = basophilic erythroblasts, II = orthochromatic erythroblasts, III = polychromatic erythroblasts, IV = acidophilic erythroblasts) were identified based on CD71 (transferrin receptor) and Ter119 (erythrocyte marker) staining and cell size (SSD). **(D)** Total iron concentration in serum was determined in cre/+ mice treated intravenously (*i.v.*) with Fe-dextran and compared to controls (+/+) treated with 1 x PBS (**$P < 0.01$, ****$P < 0.0001$). Bars mean ± SD. **(E)** Representative image of Prussian Blue staining of ferric iron counterstained with nuclear fast red on spleen FFPE-sections and BM smears of cre/+ controls, treated *in vivo* with 1x PBS and cre/+ mice, treated *in vivo* with Fe-dextran for four weeks. Scale bar 100 µm.


A
Liver
Hepcidin [ng/ml]
800
400
0
-400
+/+
cre/+
B
Liver
+/+
cre/+
FPN1 F4/80
Spleen
+/+
cre/+
FPN1 F4/80
C
Liver
+/+
cre/+
Cp F4/80
Spleen
+/+
cre/+
Cp F4/80
D
Liver
+/+
cre/+
TfR1
Spleen
+/+
cre/+
TfR1
E
Duodenum
Mean grey value Heph
9
6
3
0
+/+
cre/+
F
Duodenum
+/+
cre/+
Cp F4/80
G
Duodenum
Ferroxidase activity [mU/mL]
9
6
3
0
+/+
cre/+
Serum
Ferroxidase activity [mU/mL]
200
150
100
50
0
+/+
cre/+
H
Duodenum
IRP1 / a-Tub
0.4
0.3
0.2
0.1
0.0
+/+
cre/+
I
Single cells
94.9
FSC-A
FSC-H
live
32.3
FSC-A
7AAD-PC5.5
EC
96.7
CD45-PE
EpCAM-FITC
Sorted ECs:
CD45- EpCAM+

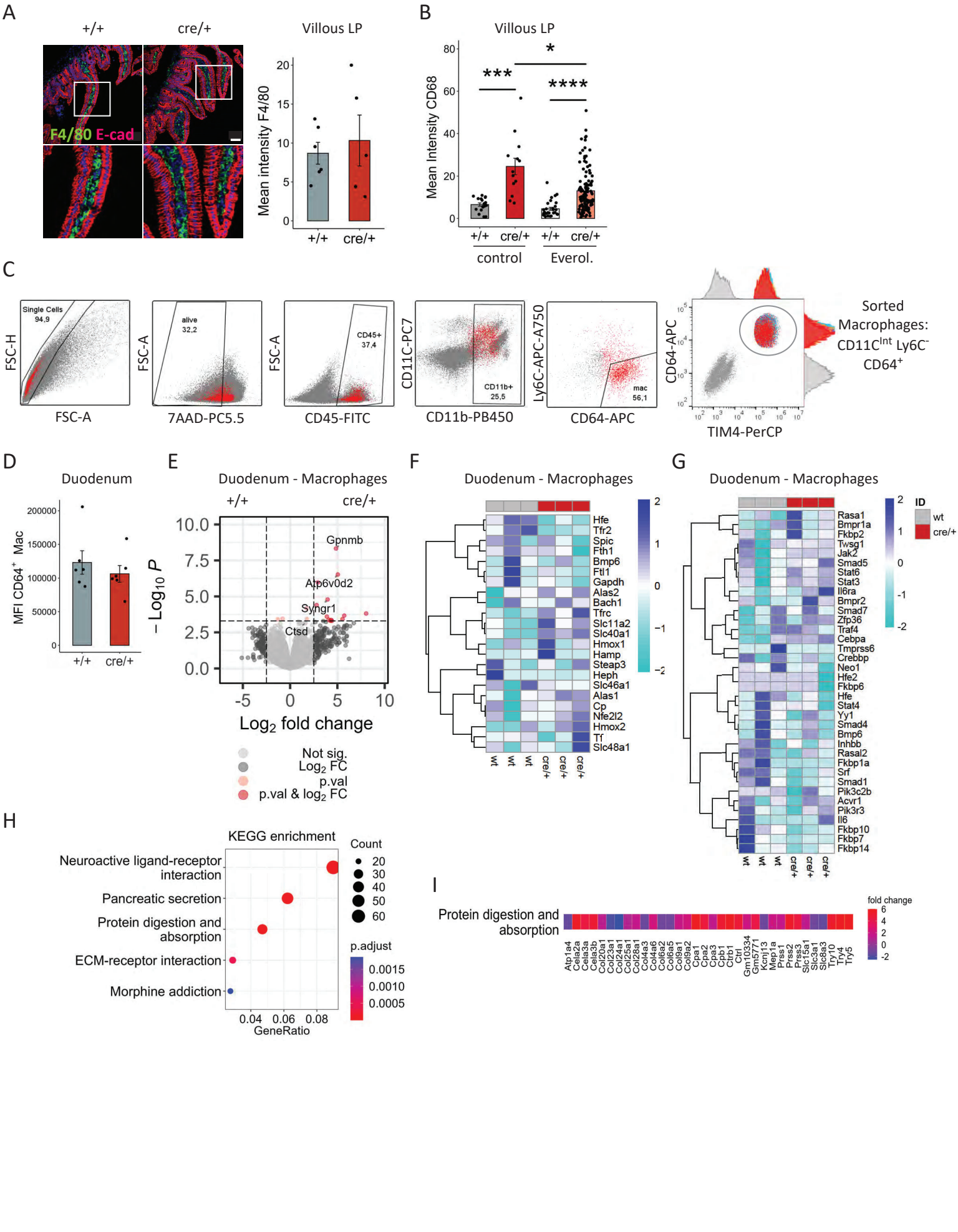
Supplementary Figure. 4. Impact of mTORC1 activation in macrophages in systemic and cellular iron regulation in cre/+ mice

(A) Hepcidin concentration measured in liver of control (+/+) and cre/+ mice via ELISA. **(B-D)** Representative image of immunofluorescent staining for FPN1 (Ferroportin1- cellular iron exporter), F4/80 (macrophage marker) and Transferrin receptor (TfR1); (B) and Cp (Ceruloplasmin - ferroxidase), F4/80 (C) and TfR1 (D) on mouse liver and spleen cryo-sections obtained from control (+/+) and cre/+ mice. Scale bar 50 μm. **(E)** Bar plot represents quantification of immunohistochemistry staining of Heph (Hephaestin) area in duodenal villi (Fig. 4H). **(F)** Representative image of immunofluorescent staining for Cp and F4/80 on duodenum cryo-sections obtained from control (+/+) and cre/+ mice. Scale bar 50 μm. **(G)** Ferroxidase activity of Cp measured in duodenum lysates and in serum. **(H)** Quantification of western blot expression of Iron regulatory protein IRP1/ Aco1 (Iron regulatory protein/Aconitase1, 98 kDa) in duodenum lysates from control (+/+) and cre/+ mice calibrated to α-Tubulin (55 kDa). **(I)** A representative example of gating strategy of flow cytometry analysis for the identification of $CD45^-$ $EpCAM^+$ epithelial cells (EC) in duodenum after live/dead cell exclusion ($7AAD^-$).


A
Duodenum
+/+
cre/+
+/+
cre/+
kDa
Transferrin
αTubulin
75
55
Duodenum
Tf / a-Tub
+/+
cre/+
B
Spleen
+/+
cre/+
Tf
C
control
M279
CD68
C57BL/6
Villous LP
Mean Intensity CD68
cont
M279
D
o.n.
starvation
Feed
16h
starve 1h
3h
sacrifice
E
cont
starve
1h feed
3h feed
+/+
cre/+
Villous LP
Mean Intensity Tf
cont
starve
1h feed
3h feed
+/+
cre/+

Supplementary Figure. 5. Local depletion of transferrin in duodenum by macrophages

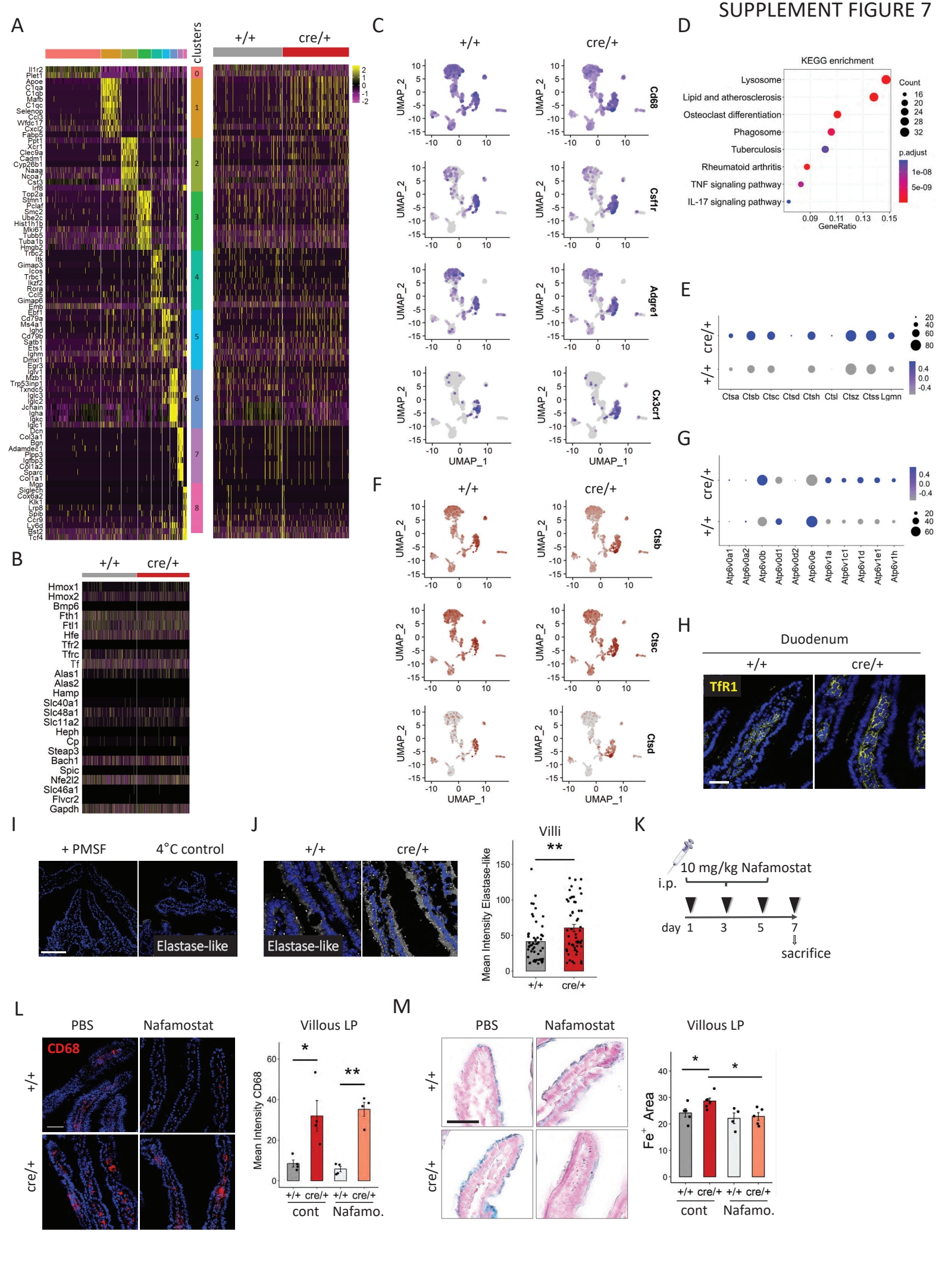
(A) Representative western blot analysis Tf (Transferrin, 75 kDa) and the house-keeping protein α-Tubulin (55 kDa) in duodenum lysates from control (+/+) and cre/+ mice. Quantification of Tf normalized to α-Tubulin (55 kDa). **(B)** Representative image of immunofluorescent staining for Tf on spleen cryo-sections obtained from control (+/+) and cre/+ mice. Scale bar 100 μm. **(C)** Representative image of immunofluorescent staining for CD68 (macrophage activation marker) on mouse cryo-duodenum sections obtained from C57BL/6 wt mice treated with CSFR1 receptor inhibitor (M279) to deplete LP macrophages. Scale bar 100 μm. Quantification of CD68 fluorescence intensity in lamina propria of duodenum. (*$P < 0.05$). **(D)** Graphical depiction of starvation and re-feeding of 9-16 weeks old +/+ and cre/+ mice. One next day, duodenum tissue samples were collected and analyzed. **(E**) Representative image of immunofluorescent staining for Tf on +/+ and cre/+ mice starved or fed for 1h and 3h after starvation compared to +/+ and cre/+ controls, scale bar 50 μm. Quantification of Tf fluorescence intensity in duodenum lamina propria (****$P < 0.0001$).


A
+/+
cre/+
F4/80 E-cad
Villous LP
Mean intensity F4/80
+/+
cre/+
B
Villous LP
Mean Intensity CD68
***
*
****
+/+
cre/+
+/+
cre/+
control
Everol.
C
Single Cells
94,9
FSC-H
FSC-A
alive
32,2
FSC-A
7AAD-PC5.5
CD45+
37,4
FSC-A
CD45-FITC
CD11C-PC7
CD11b+
25,5
CD11b-PB450
Ly6C-APC-A750
mac
56,1
CD64-APC
CD64-APC
TIM4-PerCP
Sorted
Macrophages:
CD11C^Int Ly6C^-
CD64^+
D
Duodenum
MFI CD64^+ Mac
+/+
cre/+
E
Duodenum - Macrophages
+/+
cre/+
Gpnmb
Atp6v0d2
Syngr1
Ctsd
– Log10 P
Log2 fold change
Not sig.
Log2 FC
p.val
p.val & log2 FC
F
Duodenum - Macrophages
Hfe
Tfr2
Spic
Fth1
Bmp6
Ftl1
Gapdh
Alas2
Bach1
Tfrc
Slc11a2
Slc40a1
Hmox1
Hamp
Steap3
Heph
Slc46a1
Alas1
Cp
Nfe2l2
Hmox2
Tf
Slc48a1
wt
wt
wt
cre/+
cre/+
cre/+
G
Duodenum - Macrophages
ID
wt
cre/+
Rasa1
Bmpr1a
Fkbp2
Twsg1
Jak2
Smad5
Stat6
Stat3
Il6ra
Bmpr2
Smad7
Zfp36
Traf4
Cebpa
Tmprss6
Crebbp
Neo1
Hfe2
Fkbp6
Hfe
Stat4
Yy1
Smad4
Bmp6
Inhbb
Rasal2
Fkbp1a
Srf
Smad1
Pik3c2b
Acvr1
Pik3r3
Il6
Fkbp10
Fkbp7
Fkbp14
wt
wt
wt
cre/+
cre/+
cre/+
H
KEGG enrichment
Count
Neuroactive ligand-receptor interaction
Pancreatic secretion
Protein digestion and absorption
ECM-receptor interaction
Morphine addiction
p.adjust
GeneRatio
I
Protein digestion and absorption
fold change
Atp1a4
Cela2a
Cela3a
Cela3b
Col20a1
Col23a1
Col24a1
Col25a1
Col28a1
Col4a3
Col4a6
Col6a2
Col6a5
Col9a1
Col9a2
Cpa1
Cpa2
Cpa3
Cpb1
Ctrb1
Ctrl
Gm10334
Gm5771
Kcnj13
Mep1a
Prss1
Prss2
Prss3
Slc15a1
Slc3a1
Slc8a3
Try10
Try4
Try5

Supplementary Figure. 6. Bulk RNA sequencing identifies active digestive proteases in LP macrophages in cre/+ mice

(A) Representative image of immunofluorescent staining for F4/80 (macrophage marker) and E-cad (E-cadherin) on mouse duodenum FFPE-sections obtained from control (+/+) and cre/+ mice. Quantification of F4/80 fluorescence intensity in the duodenal LP. Scale bar 50 μm. **(B)** Quantification of CD68 fluorescence intensity in lamina propria of duodenum on +/+ and cre/+ mice treated with everolimus compared to control +/+ and cre/+ mice treated with 1 x PBS starved (*P < 0.05, ***P < 0.001, ***P < 0.0001). **(C)** A representative example of gating strategy of flow cytometry analysis for the identification of lamina propria macrophages ($CD64^+$ $Ly6C^-$ $CD11b^+$ $CD11c^{int}$ LP macrophages). Cells were isolated from pre-gating of CD45+ after live-dead exclusion ($7AAD^-$). **(D)** Flow cytometry quantitative analysis of CD64 mean fluorescence intensity of LP macrophages ($CD64^+$ $Ly6C^-$ $CD11b^+$ $CD11c^{int}$). **(E)** Volcano-plot of gene expression profiles of duodenum LP macrophages ($CD64^+$ $Ly6C^-$ $CD11b^+$ $CD11c^{int}$) in control (+/+) and cre/+ mice. **(F)** Heatmap for genes associated with iron regulation in LP macrophages obtained from bulk RNA-Seq analysis. **(G)** Heatmap for gene expression associated with hepcidin regulation by BMP-SMAD signaling in LP macrophages obtained from bulk RNA-Seq analysis. **(H)** KEGG enrichment analysis was performed with genes differentially expressed (fold change ≥1.5 and P value ≤ 0.05) between +/+ and cre/+ mice in bulk RNA-Seq. **(I)** Heatmap of identified genes in KEGG enriched pathways.

SUPPLEMENT FIGURE 7



Supplementary Figure. 7. scRNA-Seq identifies upregulated phagocytosis with active digestive proteases in LP macrophages in cre/+ mice

(A-B) Heatmaps of top 10 signature genes of $CD68^+$ macrophage clusters (cluster 0-8) (A) and of genes associated with iron regulation in $CD68^+$ macrophages from scRNA-Seq analysis (B), comparing +/+ and cre/+ mice. **(C)** Umap plots of expression levels of macrophage markers: Cd68, Adgre1 (F4/80), Csf1r and Cx3cr1 in +/+ and cre/+ mice. **(D)** KEGG enrichment analysis with differentially expressed genes from cluster 1. **(E)** Dot plots of different cathepsins encoding genes compared between +/+ and cre/+ mice. Count numbers in dots and expression levels in color bar are shown. **(F)** Umap plots of expression levels of Ctsb (Cathepsin b), Ctsc (Cathepsin c) and Ctsd (Cathepsin d) compared between +/+ and cre/+ mice. **(G)** Dot plot of differentially expressed vATPases (vacuolar-type of H^+ -ATPase) encoding genes compared between +/+ and cre/+ mice. **(H)** Representative image of immunofluorescent staining for Transferrin receptor (TfR1) on mouse duodenum cryo-sections obtained from control (+/+) and cre/+ mice. Scale bar 50 μm. **(I-J)** Representative images of Elastase-like enzyme activity and the controls on duodenum cryo-sections with ABP (Fluorophore labeled covalent inhibitor). Scale bar 50-100 μm. Representative images of Elastase-like enzyme activity on duodenum controls with ABP (Fluorophore labeled covalent inhibitor). (I) As control the assay was performed at 4°C and by adding protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Scale bar 100 μm. (J) Representative images of Elastase-like enzyme activity on duodenum cryo-sections with ABP (Fluorophore labeled covalent inhibitor). Scale bar 50 μm. Quantification of elastase like enzyme activity bases on mean fluorescence intensity in villi (**$P < 0.01$). **(K)** Graphical presentation of protease inhibitor nafamostat (FUT-175, Futhan) intraperitoneal (*i.p.*) treatment of 9-15 weeks old cre/+ mice. **(L)** Representative immunofluorescent staining image of CD68 in LP of control (+/+) and cre/+ mice after nafamostat treatment. Scale bar 50 μm. Quantification of CD68 mean fluorescence intensity in lamina propria of duodenum (*$P < 0.05$). **(M)** Representative image of ferric iron Prussian Blue staining, counterstained with nuclear fast red on duodenum FFPE-sections obtained from +/+ and cre/+ mice treated with nafamostat compared to controls treated with 1x PBS. Quantification of the Blue stained area in the duodenal lamina propria (*$P < 0.05$). Scale bar 50 μm.