Supplementary information

Methods

**Mouse models of iron overload**

C57BL6/J wild-type and *Slco4a1*<sup>Fpn(C326S)</sup> congenic mice[1] (backcrossed for at least 10 times in C57BL6/J) were housed in EMBL's Laboratory Animal Resources (LAR), provided with a constant light-dark cycle and maintained on a standard mouse diet containing 200 part per million (ppm) iron with *ad libitum* access to food and water. Animal experiments were approved by and conducted following the guidelines of the EMBL Institutional Animal Care and Use Committee. Seven week-old C57BL6/J wild-type mice were maintained on a custom control (E15510 supplemented with 200 ppm iron; sniff S8624-E710) or high iron diet (E15510 supplemented with 2% carbonyl iron; sniff S8624-E712) for 4 weeks[2]. All mice were sacrificed at 10-11 weeks of age. Five to six mice per group were analysed. Sex and number of mice used are indicated in figure legends.

**Mice for primary liver cell culture preparation**

C57BL6/N wild-type mice were housed in the specific-pathogen free (SPF) barrier at the Interfakultäre Biomedizinische Forschungseinrichtung (IBF) animal facility at the University of Heidelberg (Germany). Mice were provided a constant light-dark cycle and maintained on a standard mouse diet containing 200 ppm iron with *ad libitum* access to food and water. Animal experiments were approved by the Regierungspräsidium Karlsruhe (T58/21, T51/20, T60/19, T84/18, T75/17). Male and female mice were used.

**Cell culture conditions**

Primary mouse liver sinusoidal endothelial cells (LSEC) were isolated from C57BL6/N wild-type mice (12-18 weeks old) and maintained in William’s E medium (ThermoFisher - #32551-020) supplemented with 4% FBS, 1% penicillin/streptomycin and 10 ng/ml VEGF (Peprotech - #450-32). LSECs were seeded in collagen I coated plates at a concentration of 5 x 10<sup>5</sup> cells/cm<sup>2</sup> and collected within 30 hours after plating. To obtain a sufficient number of LSECs, a minimum of 4 mice per experiment were used. Primary mouse hepatocytes (HC) were isolated from C57BL6/N wild-type mice (8-11 weeks old), resuspended in William’s E medium supplemented with 4% FBS, 1% penicillin/streptomycin and cultivated in collagen I coated plates (6.5 x 10<sup>4</sup> cells/cm<sup>2</sup>). Experiments were performed with both male and female animals. After a gradual transition from Dulbecco's Modified Eagle's Medium (DMEM) + 10% FBS, Hepa1-6 were maintained in William’s E medium supplemented with 4% FBS, 1% penicillin/streptomycin and seeded at 30 x 10<sup>3</sup> cells/cm<sup>2</sup>. VEGF (10 ng/ml) was added in hepatocyte medium when hepatocytes were co-cultured.
with LSECs. Half of the cell number indicated above was used when LSECs and hepatocytes were cultured together. All cells were maintained at 37°C, 5% CO₂, 20% oxygen tension.

**Liver perfusion for hepatocyte and LSEC isolation**

A two-step liver perfusion was performed through the vena cava inferior with 20 ml of liver perfusion medium (ThermoFisher - #17701-038), to remove infiltrating blood, and with 20 ml of liver digest medium (ThermoFisher - #17703-034), to dissociate the extracellular matrix. The cava vein was cannulated with a 27G x 3/4" needle with a constant perfusion speed of 5 ml/min. The digested liver was maintained in cold hepatocyte medium (ThermoFisher - #17704-024) and its capsule was mechanically disrupted. The liver homogenate was filtered through 100 µm and 70 µm strainers to obtain a single cell suspension of liver cells. Liver cell suspension was then centrifuged for 5 minutes at 50 g (4°C) to separate hepatocytes and non-parenchymal cells (NPC).

Hepatocytes were further cleaned by NPC contaminants by resuspending the pellet in cold PBS and centrifugation at 50 g, 5 minutes, 4°C. Hepatocytes were either snap-frozen in liquid nitrogen for total RNA extraction or resuspended in cell culture medium and cultivated as described in ‘Cell culture conditions’.

For the preparation of LSECs, the NPC suspension was centrifuged at 50 g, 5 minutes, 4°C to remove hepatocyte contaminants and then pelleted at 400 g, 10 minutes, 4°C. NPCs were resuspended in 5 ml of Optiprep 17.6%, then 5 ml of Optiprep 8.2% and 2 ml of B-PBS (PBS + 0.1% BSA) were carefully loaded on top of cell suspensions to create an Optiprep (Sigma-Aldrich - # D1556) gradient for cellular separation by density. The Optiprep gradient was centrifuged at 1400 g for 17 minutes without brake, room temperature. The cell fraction between the interface of Optiprep 8.2% and 17.6%, containing a mixed suspension of LSECs and Kupffer cells (KCs) was collected and washed with 10 ml of B-PBS (PBS + 0.1% BSA). For primary LSEC culture preparation, the LSEC-KC pellet was resuspended in LSEC cell culture medium and subjected to two consecutive adherence steps that allow for the separation of LSECs and KCs: LSECs/KCs were incubated at 37°C for 15 min and non-adherent cells (LSECs) were recovered and plated as indicated under ‘Cell culture conditions’. For LSECs purification from mouse models of iron overload, the LSEC-KC pellet was resuspended in MACS buffer (PBS, 0.5% bovine serum albumin, 2 mM EDTA) and incubated with FcR-block (Miltenyi Biotec - #130-110-443) and anti-F4/80 microbeads (Miltenyi Biotec - #130-092-575). The negative F4/80 fraction was then immunoselected with CD146 microbeads (Miltenyi Biotec - #130-092-007) and LSECs were snap-frozen in liquid nitrogen. MACS separation was performed following the manufacturer’s protocol.

**Cell culture treatments**

Primary mouse LSECs, primary mouse hepatocytes and the Hepa1-6 cell line were incubated with 50 µM FeNTA (Iron(III) chloride and nitrilotriacetic acid in a molar ratio 1:4) for 1, 3, 6 and 15 hours or left untreated. Primary mouse LSECs were subjected to treatment with ML334 (Cayman
Chemical - # Cay31439-1)(20 µM, 8 hours), apo- and holo-transferrin (Sigma-Aldrich - # T2036 and # T0665)(30 µM, 18 hours) and ferritin (Sigma-Aldrich - # 341482)(0.5, 1, 5, 10, 100 µg/ml, 6 hours). These experiments were performed in serum free medium after 2 hours of cell starvation.

For experiments with conditioned medium, primary mouse hepatocytes or Hepa1-6 cells were serum starved for 18 hours. Hepatocyte medium was collected and centrifuged two times for 5 minutes at maximum speed to remove cellular debris. Hepatocyte medium was then added to primary mouse LSECs that were previously serum starved for 1 hour. Fifty µM FeNTA, 30µM of holo-Tf or vehicle was then added for 1, 3 and 6 hours.

**Protein inactivation of hepatocyte-conditioned medium**

Mouse primary hepatocyte conditioned medium was heat-inactivated at 95°C for 30 minutes. Insoluble molecules were eliminated by 4 consecutive high-speed centrifugations. Cell culture medium was then used for LSEC treatment or concentrated with centrifugal filters (3 kDa cutoff) and loaded onto a 10% SDS-PAGE and subjected to Coomassie Blue staining. LSEC treatment follows the protocol described above.

**Protein degradation of hepatocyte-conditioned medium**

Twenty mg of proteinase K were immobilized on an agarose resin (column) using AminoLink™ Plus Immobilization Kit (ThermoFisher - # 20394) following the manufacturer's instructions. Conditioned medium of primary mouse hepatocytes that were serum starved for 18 hours was incubated for 3 hours, at RT, with immobilized proteinase K. Digested hepatocyte conditioned medium was eluted via low speed centrifugation and either concentrated and loaded onto a 10% SDS-PAGE or used to treat LSECs. LSEC treatment follows the protocol described above.

**Western blot**

Snap-frozen isolated hepatocytes and LSECs were homogenised in RIPA buffer (10 mM Tris/HCl, 0,15 M NaCl, 1 mM EDTA, 1% NP40, 0,1% SDS) supplemented with protease and phosphatase inhibitors. Protein concentration of hepatocytes was detected using the Pierce BCA Protein Assay, following manufacturer's instructions. LSEC protein concentration could not be determined due to sample contamination with BSA. Fifty µg of total hepatocyte lysate and an equal volume of total LSEC lysate were loaded in 10 % SDS-PAGE and analyzed by western blot. Anti-Ferritin L (FtL) antibody (abcam - # ab69090) was diluted 1:2500 in 5% milk and normalized to vinculin levels (Sigma-Aldrich - # V4505)(anti-vinculin: 1:1000 in 5% milk). Images were acquired with the Vilber Lourmat Fusion-FX system and quantified with ImageJ software.

**Coomassie Blue staining**

Gel separated proteins were stained for 15 hours with Blue Comassie R250 (0.05% Comassie Brilliant Blue in CH3COOH / H2O / CH3OH (ratio 1:4:5)) and destained for 24 hours (a) H2O /
CH₃CH₂OH (ratio 3:1); b) 10% CH₃COOH). Images were acquired with the Vilber Lourmat Fusion-FX system.

**RNA Extraction, Reverse Transcription, and qRT-PCR**

Total RNA was isolated using TRIzol (ThermoFisher - #15596018)(liver tissues) or RNeasy Micro Kit (QIAGEN GmbH - #74004)(cell culture) and reverse transcribed according to manufacturer's protocol. SYBR green real-time PCR was performed using the ABI StepONE Plus real-time PCR system (Applied Biosystems) for gene expression analysis. Primer sequences are listed in the key resource table. mRNA expression of the gene of interest was normalized to the housekeeping gene *Rpl19* and data were analyzed using the ΔΔCt method. Primer sequences are here listed:

- **Mouse Bmp6** FW: ATGGCAGGACTGGATCATTGC;
- **Mouse Bmp6** Rev: CCATCACAGTAGTTGGCAGCG;
- **Mouse Ho1** FW: AGGCTAAGACCGCCTTCT;
- **Mouse Ho1** Rev: TGTGTTCCTCTGTCAGCATCA;
- **Mouse Nqo1** FW: AGCGTTCGGTATTACGATCC;
- **Mouse Nqo1** Rev: AGTACAATCAGGGCTTCTTCT;
- **Mouse Rpl19** FW: AGGCATATGGGCATAGGGAAGAG;
- **Mouse Rpl19** Rev: TTGACCTTCAGGTACAGGC;
- **Mouse Tfrc** FW: CCCATGACGTTGAATTGAACCT;
- **Mouse Tfrc** Rev: GTAGTCTCCACGAGCGGAATA.

**Serum iron measurement**

Blood was collected via cardiac puncture in gel clot activator tubes for serum separation. Serum iron content was then measured with the SFBC kit (Biolabo - #80008), following manufacturer’s instructions.

**Tissue iron measurement**

Tissue non-heme iron content was measured using the bathophenanthroline (BDA) method and calculated against dry weight tissue[3]. To solubilize tissue iron, dried liver (drying conditions: 45°C for 3 days) was suspended in 10% Trichloroacetic acid (TCA) / 10% Hydrochloric acid (HCl) solution for 2 days at 65°C, shaking at 500 rpm. The ratio of dried tissue (mg) and TCA/HCl (µl) is 1:2. Solubilized tissue iron was measured with a colorimetric assay by using the colorimetric solution (0.1% of BDA10%, 1% thioglycolic acid, 3M NaAcetate) and quantifying the absorbance at 535 nm.

**Statistical analysis**

Statistical analyses were performed using Prism v7 (GraphPad Software). Data are shown as mean ± SEM and two-tailed Student’s t test and 1 or 2-way ANOVA were calculated when shown. p-values < 0.05 (*), < 0.01 (**), < 0.001 (***)< 0.0001 (****) are indicated.
Supplementary references


Supplementary figure legends

Figure S1 – Liver and circulating iron parameters in iron-loaded wild-type mice. (A-D) Wild-type female mice were maintained on an iron balanced (n=5) (200ppm) or iron-loaded (Fe\textsuperscript{high}) (n=5) (2% carbonyl iron) diet for 4 weeks and sacrificed at 11 weeks of age. (E-H) Six female Fpn(C326S) mice and 6 wild-type control mice were sacrificed at 11 weeks of age. Total liver was purified and mRNA expression of (A,E) Hepcidin and (B,F) Bmp6 was analyzed by qRT-PCR and normalized to the housekeeping gene Rpl19. Data are reported as mean ± SEM and represented as expression relative to the mean of control mice. (C,G) Serum and (D,H) liver iron content are shown. (I-N) Hepatocytes (HC) and liver sinusoidal endothelial cells (LSEC) were isolated from male wild-type (wt) mice maintained on an iron balanced or iron-loaded diet or from male Fpn(C326S) mice and wt controls (Fpn(wt))(n = 4-5 per group). All groups were sacrificed at 11 weeks of age. (I-J) Ferritin L (FtL) protein levels in (I) HCs and (J) LSECs were assessed by western blot. Vinculin was used as loading control. (K-N) Ferritin L quantification normalized to vinculin expression is shown. Two-tailed Student’s t-test was calculated: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, ns: not significant. RQ: relative quantification.

Figure S2 – Different sources of ‘free’ iron do not induce Bmp6 mRNA levels in LSECs. Primary mouse LSECs were serum starved for 2 hours and then incubated with 50 µM of FeNTA or 50 µM FAC for 6 hours. mRNA expression of (A) Tfr1 (Tfrc), (B) Ho1 and (C) Bmp6 was analyzed by qRT-PCR and normalized to the housekeeping gene Rpl19. A minimum of three biological replicates per condition is shown. A minimum of two independent experiments with at least three biological replicates was performed. Data are reported as mean ± SEM and represented as relative expression to untreated cells. One-way ANOVA was calculated: *p < 0.05; ****p < 0.0001, ns: not significant. RQ: relative quantification.

Figure S3 – Hepa1-6 cells are required for the iron-dependent Bmp6 induction in primary LSECs. Primary mouse LSECs were co-cultivated with the Hepa1-6 cell line or individually and treated with 50 µM of FeNTA for (A-B) 6 and (C-D) 15 hours. (E-F) LSECs were incubated with Hepa1-6 conditioned medium (med(Hepa1-6)) or unconditioned medium (med), as control, and treated with 50 µM of FeNTA for 6 hours. (G) Primary mouse LSECs were cultivated in HC derived medium (med(HC)) or left untreated; two hours later 30 µM of holo-transferrin (Holo-TF) was supplemented for 6 hours. Total RNA was retrotranscribed and mRNA expression of (A,C,E) Tfr1 (Tfrc gene) and (B,D,F,G) Bmp6 was analyzed by qRT-PCR. Data are represented as relative quantification to vehicle treated cells and reported as mean ± SEM. Two-way ANOVA was calculated: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, ns: not significant. RQ: relative quantification.
Figure S1

A. Hepcidin mRNA

B. Bmp6 mRNA

C. Serum Iron

D. Liver Iron Content

E. Hepcidin mRNA

F. Bmp6 mRNA

G. Serum Iron

H. Liver Iron Content

I. Hepatocytes (HC)

J. Liver sinusoidal endothelial cells (LSEC)

K. Ferritin protein

L. Ferritin protein

M. Ferritin protein

N. Ferritin protein

- Hepcidin mRNA
- Bmp6 mRNA
- Serum Iron
- Liver Iron Content
- Hepatocytes (HC)
- Liver sinusoidal endothelial cells (LSEC)
- Ferritin protein

** ns
Figure S2

A  
**Tfr1 mRNA**

B  
**HO1 mRNA**

C  
**Bmp6 mRNA**
Figure S3

A  
Transferrin receptor 1 mRNA

B  
Bmp6 mRNA

C  
Transferrin receptor 1 mRNA

D  
Bmp6 mRNA

E  
Transferrin receptor 1 mRNA

F  
Bmp6 mRNA

G  
Bmp6 mRNA

**Figure S3**

A  
Transferrin receptor 1 mRNA

B  
Bmp6 mRNA

C  
Transferrin receptor 1 mRNA

D  
Bmp6 mRNA

E  
Transferrin receptor 1 mRNA

F  
Bmp6 mRNA

G  
Bmp6 mRNA