Supplementary information

Supplementary figure legends

Figure S1 – Characterization of the iron-related parameters in *Stab2*-Cre mice. 20-week old male C57BL/6 *Stab2*-CRE mice (n=5 per group) were analyzed for: **(A)** serum iron levels, **(B)** transferrin saturation, **(C)** spleen and **(D)** liver non-heme iron content and for the hepatic mRNA expression of **(E)** *hepcidin,* **(F)** *Bmp2* and **(G)** *Bmp6.* qRT-PCR data were normalized to the housekeeping gene *Rpl19.* Tissue non-heme iron content was calculated as µg of non-heme iron (Fe) on grams (g) of dry tissue. Data are reported as mean ± SEM. ns: non-significant.

Figure S2 – Iron parameters are not affected in female *Hfe*^LSEC-KO mice. 20-week old female *Hfe*^flx (n=5) and *Hfe*^LSEC-KO (n=5) mice were analysed for: **(A)** serum iron levels, **(B)** transferrin saturation, **(C)** spleen and **(D)** liver non-heme iron content, **(E)** serum hepcidin levels and **(F)** liver *hepcidin* mRNA expression. qRT-PCR data were normalized to the housekeeping gene *Rpl19.* Tissue non-heme iron content was calculated as µg of non-heme iron (Fe) on grams (g) of dry tissue. Data are reported as mean ± SEM. ns: non-significant.

Figure S3 – Iron parameters of aged *Hfe*^LSEC-KO mice are unchanged. 31-week old male *Hfe*^flx (n=5) and *Hfe*^LSEC-KO (n=5) male mice were analyzed for: **(A)** serum iron levels, **(B)** transferrin saturation, **(C)** spleen and **(D)** liver non-heme iron content and **(E)** liver *hepcidin* mRNA expression. qRT-PCR data were normalized to the housekeeping gene *Rpl19.* Tissue non-heme iron content was calculated as µg of non-heme iron (Fe) on grams (g) of dry tissue. Data are reported as mean ± SEM. ns: non-significant.

Material and Methods

RNA-sequencing analysis

RNA-sequencing data from mouse liver sinusoidal endothelial cells (LSEC) and hepatocytes (HC) were obtained from the NCBI GEO repository (Dataset GSE135789, samples GSM4030172-5 for the LSECs and GSM4030184-7 for the HCs) (1). Prefetch tool (NCBI) and fasterq-dump tool (NCBI) were used to download Sequence Read Archive (SRA) files and extract fastq files, respectively. Fastq files were mapped against the mm10 mouse genome with the STAR package to generate bam files. Reads were annotated against the GRCm38.p5 reference gene set (ENSEMBL) and counted using the htseq-count tool. TPM (Transcripts Per Million) were calculated with the software R as described in (2).

Isolation of liver sinusoidal endothelial cells (LSECs) and hepatocytes

Liver perfusion of male C57BL/6 *wild-type* mice (14-15 week-old) was performed following a standard two-step perfusion method (3–5) with liver perfusion and liver digest mediums (Life Technologies). The liver capsule was mechanically disrupted and passed through 100 μm and 70 μm strainers. The single cell suspension of liver cells were resuspended in hepatocyte wash medium (Life Technologies). Hepatocytes and non-parenchymal cells (NPC) were separated with low speed centrifugation (5 mins, 50g (4°C)). A pellet of hepatocytes was then immediately snap-frozen. NPCs were separated through density gradient isolation and immunoselection with magnetic beads (Miltenyi Biotec) as described in (6), with minor modifications. Briefly, Optiprep (Sigma Aldrich) gradient was made by carefully loading 5 ml of Optiprep 8.2% and 2 ml of B-PBS (PBS + 0.1 % Bovine Serum Albumin) on top of NPCs resuspendend in 5 ml of Optiprep 17.6% and centrifuged at 1400 g, 17 min, without brake, room temperature (RT). The cell fraction between Optiprep 17.6% and 8.2%, containing LSECs and Kupffer cells, was subjected to immunoselection: 1) cells were incubated with Fc-block reagent (Miltenyi Biotec); 2) KCs were depleted by using antibody conjugated with magnetic microbeads recognizing the F4/80 macrophage specific marker (Miltenyi Biotec), following the manufacturer's protocol; 3) CD146 positive cells (LSECs) were selected and isolated (Miltenyi Biotec).

Mouse models and genotyping

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. All mouse breeding and animal experiments were approved by the Regierungspräsidium Karlsruhe (T-60/19, T-84/18). C57BL/6 *Hfe^{fl/fl}* were crossed with C57BL/6 *Stab2*-CRE mice to generate *Hfe^{fl/fl}*;Stab2^{Cre-} (*Hfe^{flx}*) and *Hfe^{fl/fl}*;Stab2^{Cre+} (*Hfe^{LSEC-KO}*) animals. Male and female mice were sacrificed at 20- and 31-weeks of age, as indicated in figure legends. Genotyping was performed by polymerase chain reaction (PCR) analysis of genomic DNA extracted from a small ear part, isolated hepatocytes and isolated LSECs with primers indicated in Table S1. The genotyping strategy allowed to discriminate the Hfe-flx allele that produces a product of 591 bp (corresponding to amplification of primers Fw and Rev1) from the Hfe-ko allele that generates an amplicon of 798 bp (primers Fw and Rev2) (Figure 1C,D). The presence of the Stab2Cre allele was identified using the primers Cre-fw and Cre-rev (Table S1).

Haematological parameters, serum and tissue iron quantification

Haematological parameters were determined using the Scil Vet ABC plus analyser (Scil Vet GmbH). Serum iron concentration was measured using the SFBC kit (Biolabo). Unsaturated iron binding capacity was assessed with the UIBC kit (Biolabo) and transferrin saturation was

calculated using the formula SFBC / (SFBC + UIBC) x 100. Tissue non-heme iron content was measured using the bathophenanthroline method and calculated against dry weight tissue (7).

Serum hepcidin quantification

Serum hepcidin was measured in duplicate on 12 µl of serum using the Hepcidin-Murine Compete enzyme-linked immunosorbent assay kit (Intrinsic Lifescience), according to the manufacturer's protocol and calculated against a standard curve using the 4-parameter logistic model (GraphPad Prism v7).

RNA Extraction, Reverse Transcription, and qRT-PCR

Total RNA was isolated using TRIzol (Life Technology) or RNeasy Micro Kit (Qiagen), and reverse transcribed with RevertAid H Minus Reverse Transcriptase (Life Technology) according to manufacturer's instructions. SYBR green real-time PCR was performed by using the ABI StepONE Plus real-time PCR system (Applied Biosystems) for gene expression analysis. Primers used are listed in Table S1.

Statistical analysis

Data are shown as mean values ± SEM. Statistical analysis was performed using GraphPad Prism v7 and two-tailed Student's t-test was calculated. ns: not significant; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ are indicated.

References

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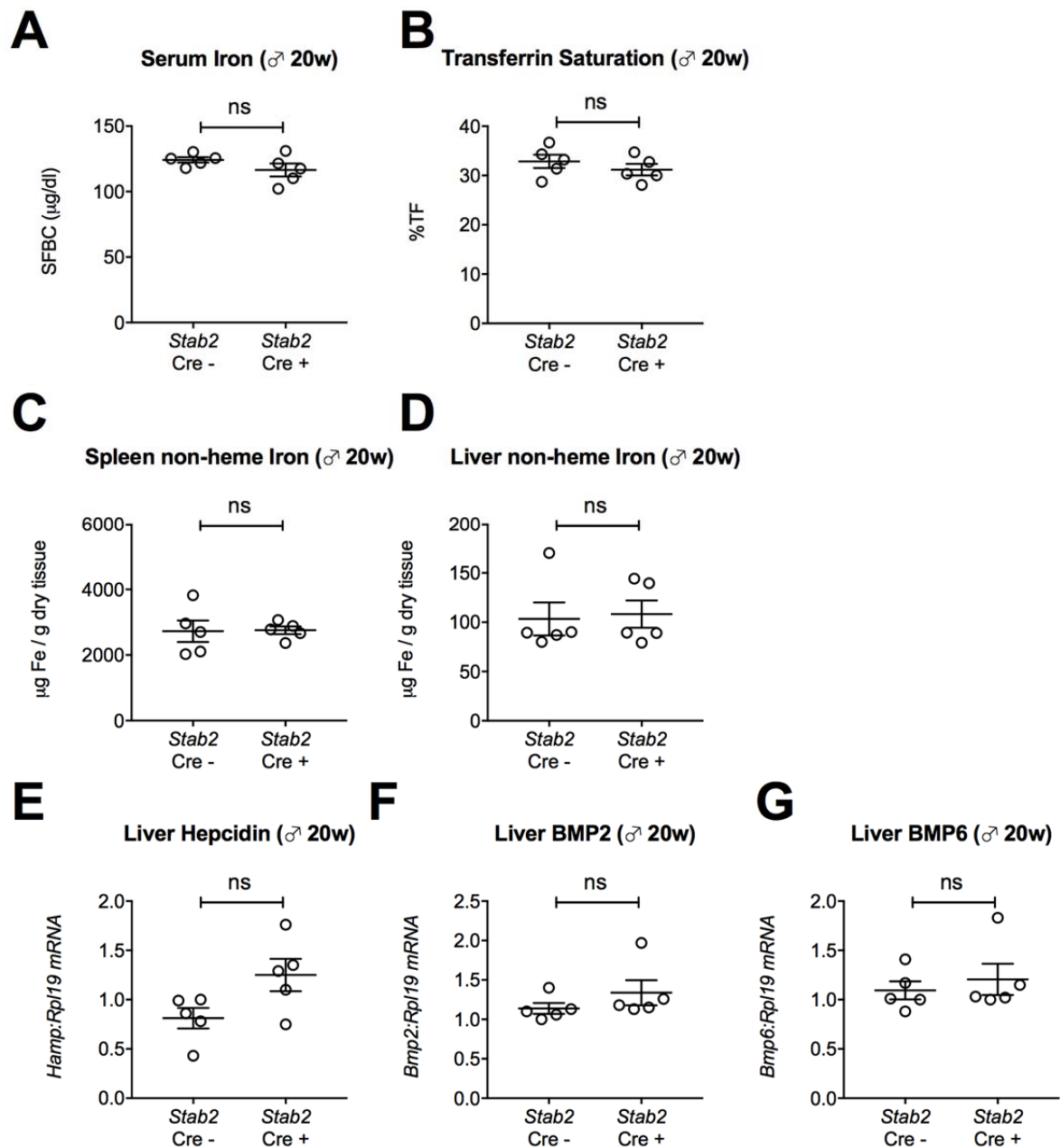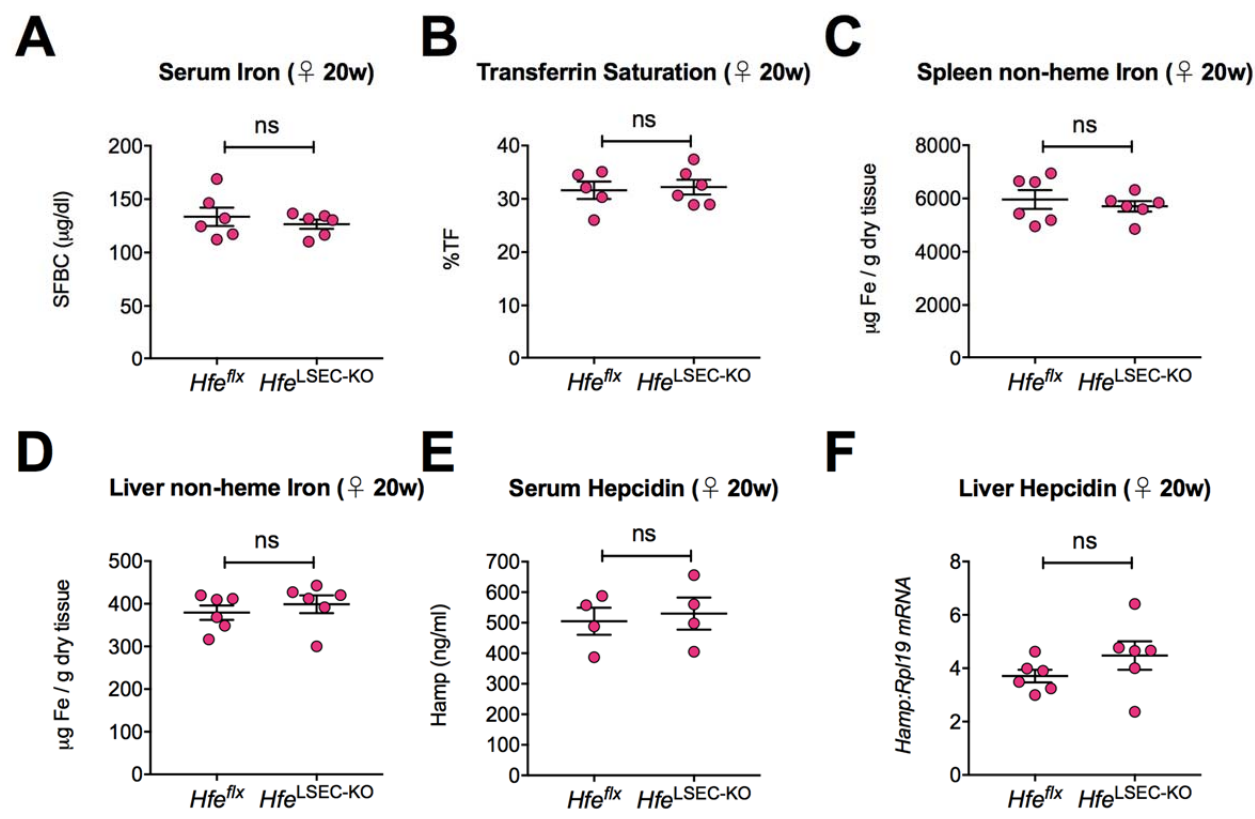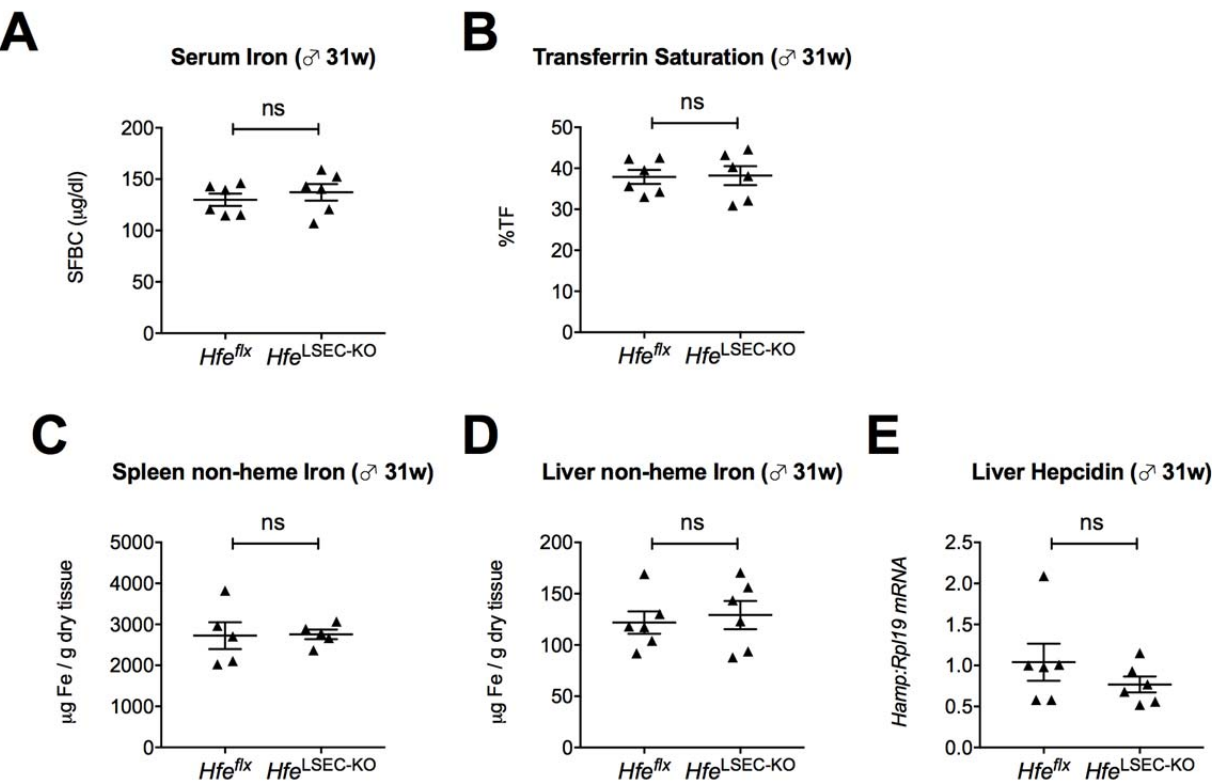
Figure S1

Figure S2

A

Serum Iron (♀ 20w)

B

Transferrin Saturation (♀ 20w)

C

Spleen non-heme Iron (♀ 20w)

D

Liver non-heme Iron (♀ 20w)

E

Serum Hepcidin (♀ 20w)

F

Liver Hepcidin (♀ 20w)

Figure S3

A

Serum Iron (♂ 31w)

B

Transferrin Saturation (♂ 31w)

C

Spleen non-heme Iron (♂ 31w)

D

Liver non-heme Iron (♂ 31w)

E

Liver Hepcidin (♂ 31w)

Table S1

PCR	
	Sequence (5'-> 3')
Fw	CACAGTAAGGGTACGTGGAG
Rev1	TGGAGACAGTGCAGTAGAGC
Rev2	AGGGTCACAAACAGCCATAC
CRE-fw	GAACTGATGGACATGTTCAGG
CRE-rev	AGTGCGTTCGAACGCTAGAGCCTGT

qRT-PCR		
Gene name	**Fw (5'-> 3')**	**Rev (5'-> 3')**
Rpl19	AGGCATATGGGCATAGGGAAGAG	TTGACCTTCAGGTACAGGCTGTG
Hamp	ATACCAATGCAGAAGAGAAGG	AACAGATACCACACTGGGAA
Bmp2	GCGCAGCTTCCATCACGA	CCCACTCATCTCTGGAAGTTCCT
Bmp6	ATGGCAGGACTGGATCATTGC	CCATCACAGTAGTTGGCAGCG
Id1	TCGTCGGCTGGAACACATG	ACCCTGAACGGCGAGATCA
Smad6	GTTGCAACCCCTACCACTTC	GGAGGAGACAGCCGAGAATA
Smad7	GCAGGCTGTCCAGATGCTGT	GATCCCCAGGCTCCAGAAGA
Tfrc	CCCATGACGTTGAATTGAACCT	GTAGTCTCCACGAGCGGAATA
Tfr2	GGAGGTCAATTCCCATACCCT	CGACCACCAACACGGAGTC
Hfe	CACCGTCTGTGCCATCTTCTT	ACATAGCCACCCATGGTTCCT
Fpn	TGTCAGCCTGCTGTTTGCAGGA	TCTTGCAGCAACTGTGTCACCG