SUPPLEMENTARY APPENDIX

A competitive enzyme-linked immunosorbent assay specific for murine hepcidin-1: correlation with hepatic mRNA expression in established and novel models of dysregulated iron homeostasis

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Supplementary Materials and Methods

Mouse Hepcidin-1 ELISA

Briefly, rabbits were immunized with synthetic Hepc-1 (Peptides International, Louisville, KY) conjugated to keyhole limpet hemocyanin (KLH; Imject mcKLH and EDC Conjugation Kit, Thermo Scientific, Rockford, IL). Antibodies were purified on staphylococcal protein A columns (Thermo Scientific) according to the manufacturer's protocol. Microwell plates (96-well; Nalge Nunc International, Rochester, NY) coated with antibody were incubated with 100µl/well (standard or sample) using a 1:20 dilution of serum or urine (5µl) in a Tris-buffered saline containing 0.05% Tween-20 (TBST) with 5 ng/ml of biotinylated Hepc-1 (Peptides International) acting as a tracer. Standard curves were prepared by serial 2-fold (12-point) or 3-fold (8-point) dilutions of synthetic Hepc-1 starting at 1000 ng/ml. Following a wash step, the captured biotin conjugate was detected with streptavidin-horseradish peroxidase (SA-HRP; Thermo Scientific) and tetramethyl benzidine (TMB; Moss Inc., Pasadena, MD). The enzymatic reaction was stopped by sulfuric acid, and the absorbance measured at 450nm on a DTX 880 microplate reader (Beckman Coulter, Fullerton, CA). To generate standard curves, we plotted the concentration of the standard versus the absorbance, employing four-parameter logistic curve-fitting software (Prism, GraphPad Software, San Diego, CA). The standard curve was then used to convert sample absorbance readings to Hepc-1 concentrations. Mouse Hepc-2 (supplied by Tomas Ganz, UCLA), rat hepcidin-25 and human hepcidin-25 (Peptides International) were assessed for cross-reactivity. Urinary creatinine concentrations were measured by the Jaffe reaction using the Creatinine Parameter Assay (R&D Systems, Minneapolis, MN).

Western Blotting

Hepc-1 and a Hepc-1-biotin conjugate were electrophoresed on Novex 10-20% Tricine Gel (Life Technologies, Carlsbad, CA) and then either stained with SimplyBlue SafeStain (Life Technologies) or transferred to an Immobilon-P PVDF Membrane (Millipore Corp., Bedford, Mass). Following transfer, blots were fixed in 0.05% glutaraldehyde (Sigma, St Louis, MO) for 20 minutes and blocked for 2 hours at room temperature (RT) with Blocker A/B (Life Technologies) in TRIS buffered saline (TBS; Sigma). The blots were then probed with anti-Hepc-1 antibodies overnight at 4°C. After washing with

WesternBreeze wash solution (Life Technologies, Grand Island, NY), the blots were incubated for 1 hour at RT with horseradish peroxidase conjugated to anti-rabbit (H+L) (Sigma) or to streptavidin (Thermo Scientific, Waltham, MA) at 340 ng/ml or 200 ng/ml, respectively, in Diluent A/B (Life Technologies) in TBS. Following another wash step, the bands were visualized using an insoluble TMB substrate (Moss Inc., Pasadena, MD).

Animal Husbandry and Transgenic mouse models

All wild type C57BL/6 mice employed in physiology experiments were housed either at the UCLA David Geffen School of Medicine animal facilities or at Intrinsic LifeSciences, maintained in a temperature- and light-controlled environment, and given free range to water and diets containing 4ppm, 20ppm, 300ppm, or 30,000ppm iron (Teklad Custom Research Diets, Harlan Laboratories, Madison, WI). All genetically modified mice were born and housed in the barrier facility at Children's Hospital Boston. There, animals were maintained on Prolab RMH 3000 diet (380 ppm iron; LabDiet, St. Louis, MO). Only females were analyzed in studies of transgenic animals.

A full-length mouse Hfe transgene (*Hfe*^{WT}) under the control of the transthyretin (TTR) promoter (pPJS095) has been described previously[1] and is available from the Mouse Mutant Resource Center (MMRC, #030621-UNC). Mice expressing full-length mouse *Hfe* on a C57BL6/J background were bred to *Tfr2*$^{Y245X/Y245X}$ (heretofore referred to as *Tfr2*$^{-/-}$) animals[2] (a gift of Robert Fleming) on an FVB/J background. All mice analyzed in this cross were littermates on a mixed C57BL6/J-FVB/J background. *Hbb*$^{th3/+}$ (B6; 129P-*Hbb-b1*^{tm1Unc} *Hbb-b2*^{tm1Unc}/J) and *Hfe*$^{-/-}$ mice have been described previously,[3,4] and bred onto a C57BL/6J genetic background (>N_{10}) and treated as previously described.[5] *Hjv*$^{-/-}$ animals were generated previously,[6] bred onto a C57BL6/J background (>N_{10}), and then mated with (*Tfr2*$^{-/-}$) animals. All mice analyzed in this cross were littermates on a mixed C57BL6/J;FVB/J background. 129S6/SvEvTac-*Tfrc*$^{+/-}$ animals have been described previously[7] and bred to *Hfe*$^{-/-}$ mice also on a 129S6/SvEvTac background. *Hjv*$^{-/-}$ animals were bred to *Hfe*$^{-/-}$ mice on a 129S6/SvEvTac background. All mice analyzed in both of these isogenic crosses were littermates. C57BL6/J-*Tmprss6*$^{hem8/hem8}$,[8] *Trf*$^{hpx/hpx}$,[9] and *Tmprss6*$^{-/-}$,[10] on C57BL6/J, BALB/cJ, and C57BL6/J backgrounds, respectively, were employed. Samples were frozen at -80°C until analysis.

Blood and tissue iron analyses

Whole blood for complete blood counts was collected by retro-orbital vein into EDTA-coated microtainer tubes (Becton Dickinson, Franklin Lakes, NJ) from animals anesthetized with 1.0% tribromoethanol in isoamyl alcohol (Avertin). Samples were analyzed on an Avida 120 analyzer (Bayer, Whippany, NJ) in the Children's Hospital Boston Department of Laboratory Medicine Clinical Core Laboratories. Whole blood for other purposes was collected by retro-orbital or sub mandibular bleeding into serum separator tubes (Becton Dickinson), and serum was prepared according to the manufacturer's instructions. Serum iron values were determined with the Serum Iron/UIBC kit (Thermo Fisher, Waltham, Mass.) or by using the Ferene reagent in a photometric assay (Genzyme, Cambridge, Mass.) according to the manufacturer's instructions. Liver and spleen tissues were collected and tissue non-heme iron concentrations were determined as described previously.[11] Serum ferritin was measured using a sandwich ELISA (Genway Biotech, San Diego, CA). Serum hepcidin was analyzed in all murine model serum samples in this study at a 1:20 dilution (5% serum) unless noted otherwise using the murine C-ELISA described and validated herein.

RNA isolation and qRT-PCR

In wild-type animals ~50mg of freshly frozen liver was homogenized in TRIzol reagent (Life Technologies) according to the manufacturer's protocol. Single-pass cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using iQ SYBR Green Supermix (Bio-Rad). Murine Hepc-1 mRNA concentrations were normalized to murine β-actin. The following primers were used in qRT-PCR: murine Hepc-1: forward, 5'-TTGCGATACCAATGCAGAAGA-3'; reverse, 5'-GATGTGGCTCTAGGCTATGTT-3';[12] murine β-actin: forward, 5'-ACCCACACTGTGCCCATCTA-3'; reverse, 5'-CACGCTCGGTCAGGATCTTC-3'. One male animal on the 300ppm replete iron diet was chosen as the calibrator sample and all other data is presented in relation to this sample. Relative hepcidin expression was plotted as $2^{-\Delta\Delta Ct}$.

cDNA from transgenic animals was prepared and analyzed by real-time PCR quantification for Hepc-1 (*Hamp*) and *β-actin* mRNA transcript levels as described previously.[13] Total liver RNA was isolated from flash-frozen tissue with TRIzol

(Invitrogen) as per manufacturer's instructions. Total RNA was treated with DNase I (Roche) to remove contaminating genomic DNA. Single-pass cDNA was synthesized from 1μg total mRNA using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using iQ SYBR Green Supermix (Bio-Rad).

Relative hepcidin expression was calculated using the relative standard curve method. cDNA from within each data set was employed to generate a standard curve. Assuming complete cDNA synthesis from the mRNA, 150, 15, 1.5, 0.15, and 0.015ng total cDNA were analyzed for the standard curve. Each sample in the data set was fit to the resulting linear equation and a concentration (ng) was generated. Relative hepcidin expression from 25ng cDNA was calculated from a ratio of *Hamp1* (ng) to *β-actin* concentration (ng) yielding a unit-less relative amount. Data was graphed with WT, or untreated WT, animals normalized to 1.

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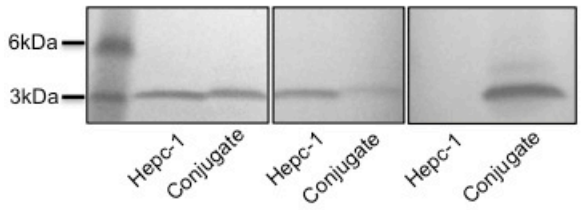
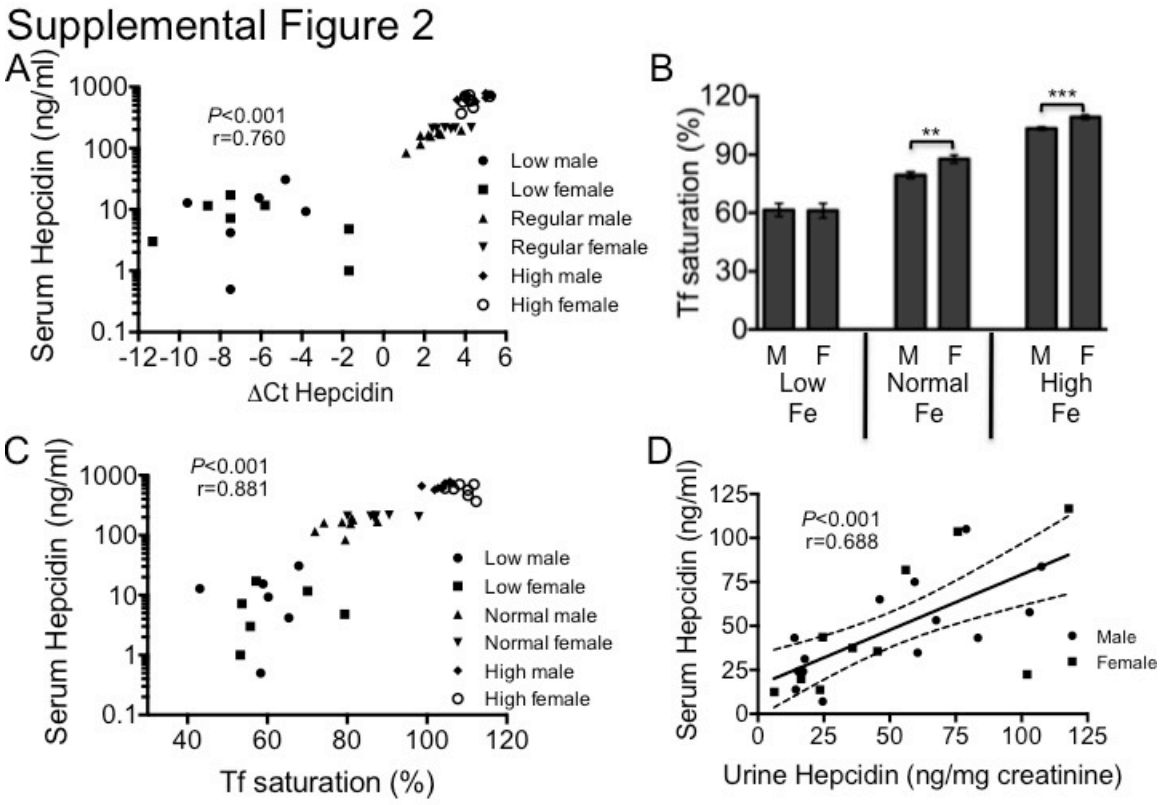
Supplemental Figure 1



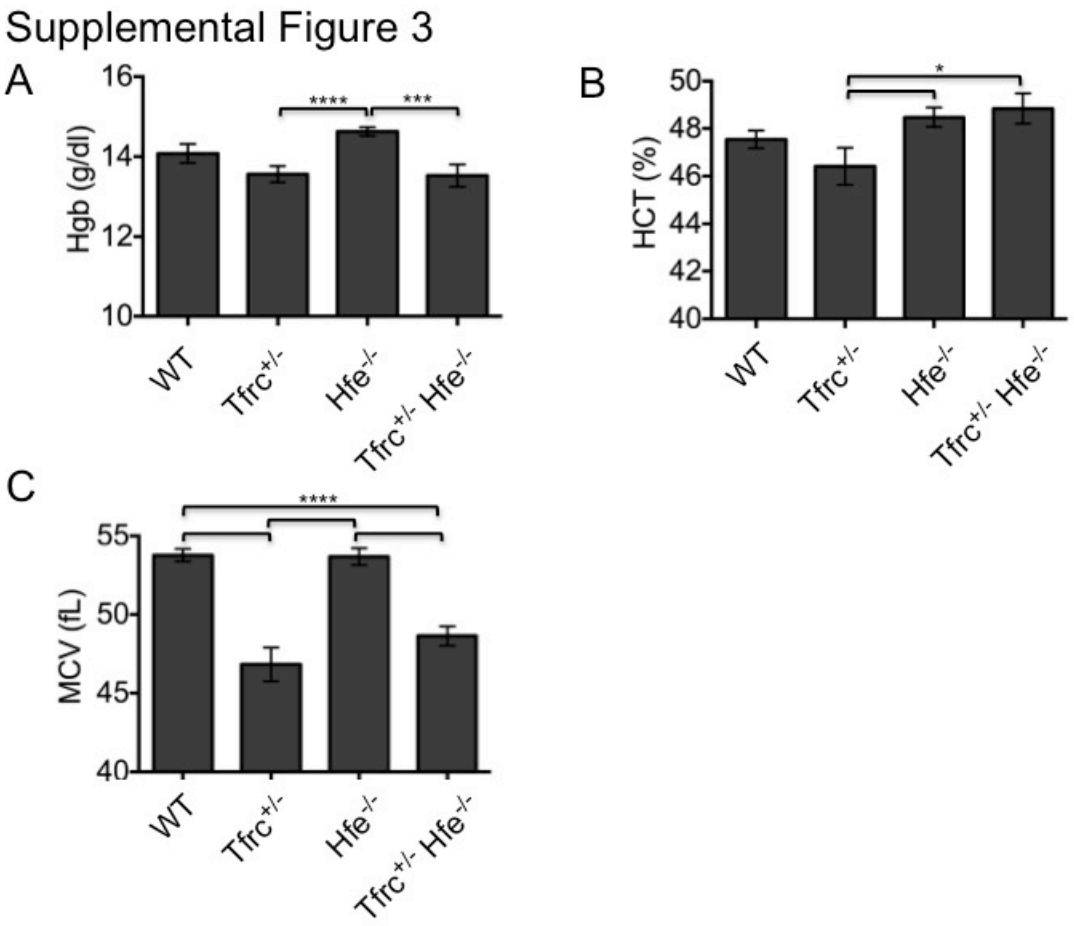
Supplemental Figure 1. Binding characterization of Hepc-1 C-ELISA.

Hepc-1 and Hepc-1-biotin conjugate was run on a non-reducing 10-20% tricine gel and Coomasie stained to show protein loading (left panel). Western blot of Hepc-1 and Hepc-1 conjugate detected by the anti-Hepc-1 antibody and visualized by goat-anti-rabbit-HRP. Both peptides are bound by the anti-Hepc-1 antibody (center panel). Western blot of Hepc-1 and Hepc-1-biotin conjugate detected by streptavidin – horseradish peroxidase, the signal enhancing protein in the C-ELISA. Only the Hepc-1 conjugate is bound by SA-HRP (right panel).



Supplemental Figure 2. Serum Hepc-1 response to dietary iron.

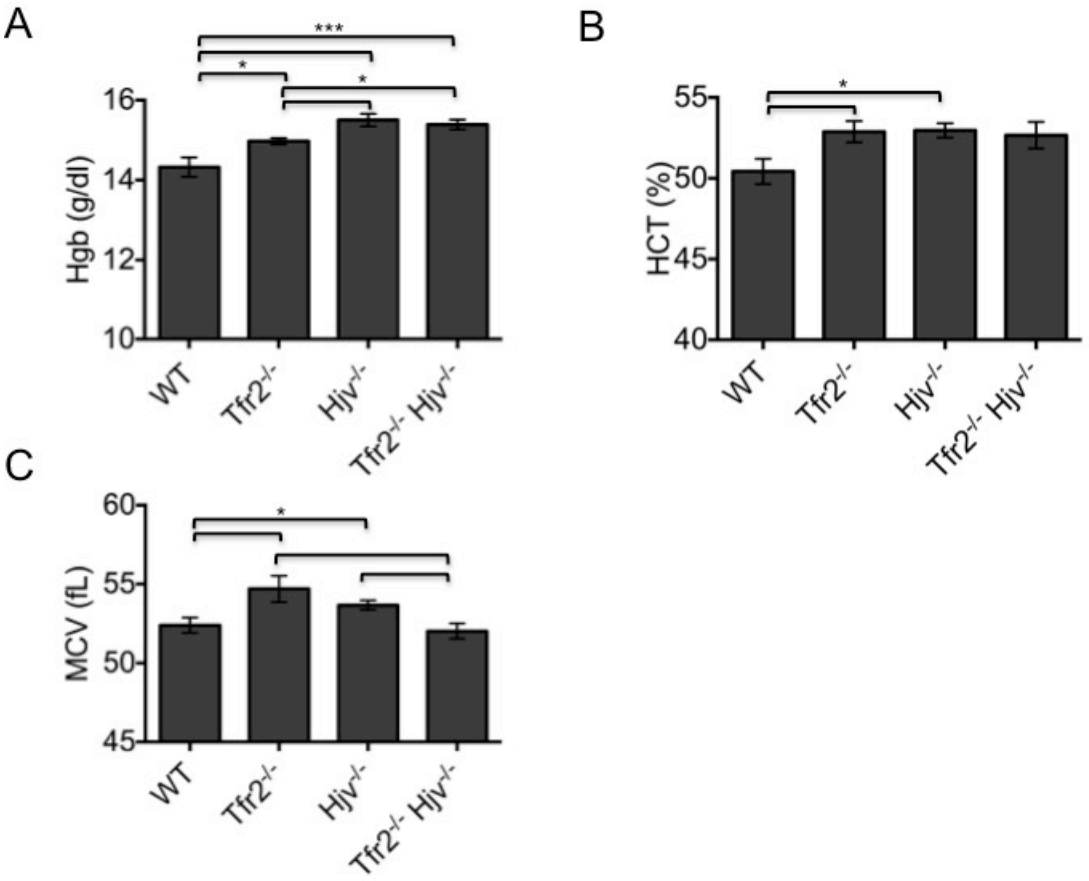
(A) Correlation of serum Hepc-1 by C-ELISA with Hepc-1 mRNA from liver. Hepc-1 mRNA values were normalized to β-actin controls. (B) Transferrin saturation (%) response to varying iron diets. Male and female mice were separately grouped (n = 8) and fed low (4 ppm Fe), normal (300 ppm Fe), or high (30,000 ppm Fe) iron diets (n = 48 total). (C) Serum Hepc-1 were compared to serum transferrin saturation (%). (D) Correlation between serum and urine Hepc-1 concentrations in male and female mice under conditions of steady state and induced anemia**.** Matched serum and urine from male (*n*=6) and female (*n*=6) mice were collected over the course of acclimation to a low iron diet (20 ppm). Samples were taken 0, 9, 12, 13, and 14 days after diet initiation. Urine Hepc-1 values were normalized to creatinine. Linear regression and 95% confidence limits are shown. Ratios are expressed ± SEM. p-values were calculated using Student's t-test. . ***P < 0.005 and **P < 0.01.



Supplemental Figure 3. Hematological Features of *Tfrc*$^{+/-}$ *Hfe*$^{-/-}$mice

The red blood cell parameters hemoglobin (A, Hgb), hematocrit (B, Hct) and mean cell volume (C, MCV) were measured in 8-week-old female WT (*n*=5), *Tfrc*$^{+/-}$ (*n*=9), *Hfe*$^{-/-}$ (*n*=11), and *Tfrc*$^{+/-}$ *Hfe*$^{-/-}$ (*n*=11). Data are presented as mean ± SEM. p values were calculated using Student's t test. ****P < 0.001, ***P < 0.005, and *P < 0.05.

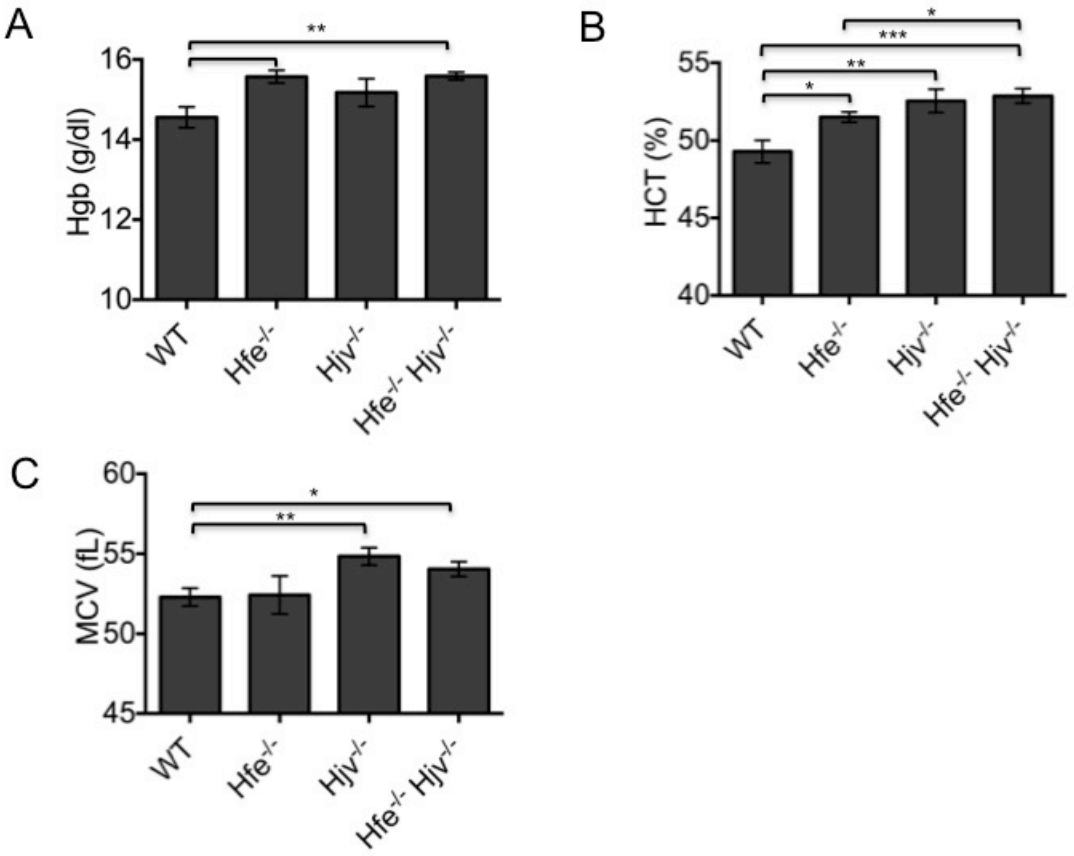
Supplemental Figure 4



Supplemental Figure 4. Hematological features of *Tfr2-/- Hjv*$^{-/-}$ animals

The red blood cell parameters hemoglobin (A, Hgb), hematocrit (B, Hct) and mean cell volume (C, MCV) were measured in 8-week-old female WT (*n*=8), *Tfr2*$^{-/-}$ (*n*=7), *Hjv*$^{-/-}$ (*n*=8) and *Tfr2*$^{-/-}$ *Hjv*$^{-/-}$ (*n*=8). Data are presented as mean ± SEM. p values were calculated using Student's t test. ***P < 0.005 and *P < 0.05.

Supplemental Figure 5



Supplemental Figure 5. Hematological features of *Hfe*$^{-/-}$ *Hjv*$^{-/-}$ mice

The red blood cell parameters hemoglobin (A, Hgb), hematocrit (B, Hct) and mean cell volume (C, MCV) were measured in 8-week-old female WT (*n*=7), *Hfe*$^{-/-}$ (*n*=8), *Hjv*$^{-/-}$ (*n*=8) and *Hfe*$^{-/-}$ *Hjv*$^{-/-}$ (*n*=10). Data are presented as mean ± SEM. p values were calculated using Student's t test. ***P < 0.005, **P < 0.01 and *P < 0.05.

Supplemental Table 1. Pearson correlation (r) coefficient between relative hepcidin mRNA and serum Hepc-1 values.

WT	-0.156
$Trf^{hpx/hpx}$	-0.720
WT	0.406
$Tmprss6^{+/-}$	0.405
$Tmprss6^{-/-}$	0.494
WT	0.164
$Tmprss6^{hem8/hem8}$	-0.120
WT	0.718
$Tmprss6^{hem8/+}$	0.973
$Tmprss6^{+/-}$	0.189
$Tmprss6^{hem8/-}$	-0.218

WT LNP-Luc	0.470
WT LNP-Tmprss6	0.431
th3/+ LNP-Luc	-0.185
th3/+ LNP-Tmprss6	0.806
WT LNP-Luc	0.772
WT LNP-Tmprss6	0.097
$Hfe^{-/-}$ LNP-Luc	0.826
$Hfe^{-/-}$ LNP-Tmprss6	0.875
WT	0.184
$Tfr2^{-/-}$	0.140
Hfe tg	-0.392
Hfe tg $Tfr2^{-/-}$	0.485

WT	-0.204
$Tfrc^{+/-}$	0.218
$Hfe^{-/-}$	0.459
$Tfrc^{+/-}$ $Hfe^{-/-}$	0.011
WT	0.671
$Tfr2^{-/-}$	0.531
$Hjv^{-/-}$	0.656
$Tfr2^{-/-}$ $Hjv^{-/-}$	0.773
WT	0.919
$Hfe^{-/-}$	0.394
Hjv-/-	0.550
Hfe-/- Hjv-/-	0.576