SUPPLEMENTARY APPENDIX

Reversal of hemochromatosis by apotransferrin in non-transfused and transfused Hbb$^{th3/+}$ (heterozygous *b1/b2* globin gene deletion) mice

Monique P. Gelderman,[1]* Jin Hyen Baek,[2]* Ayla Yalamanoglu,[2,4] Michele Puglia,[3,4] Florence Vallelian,[4] Bo Burla,[4] Jaroslav Vostal,[1] Dominik J. Schaer,[4,5] and Paul W. Buehler[2]

[1]Laboratory of Biochemistry and Vascular Biology, Center of Biologics Evaluation and Research (CBER), FDA, Silver Spring, MD, USA; [2]Laboratory of Cellular Hematology, Center of Biologics Evaluation and Research (CBER), FDA, Silver Spring, MD, USA; [3]Functional Genomics Center Zurich, Swiss Federal Institute of Technology Zurich / University of Zurich, Switzerland, [4]Division of Internal Medicine, University of Zurich, Switzerland; and [5]Center of Evolutionary Medicine, University of Zurich, Switzerland

**MPG and JHB contributed equally to this work*

 doi:10.3324/haematol.2014.117325
Manuscript received on September 13, 2014. Manuscript accepted January 20, 2015.
Correspondence: paul.buehler@fda.hhs.gov or dominik.schaer@usz.ch

SUPPLEMENTAL MATERIALS AND METHODS

Mouse models

Mice were maintained in the animal facility of the FDA's Center for Biologics Research and Review (Bethesda and Silver Spring, MD). Animal protocols were approved by the FDA CBER Institutional Animal Care and Use Committee with all experimental procedures performed in adherence to the National Institutes of Health guidelines on the use of experimental animals. *Study 1: Non-transfused mice* - Age and sex matched (3 month old equal number of male (n=5) and female (n=5) animals per group) wild type (WT, C57BL/6J-B6) and heterozygous $Hbb^{th3/+}$ mice on a C57BL/6J-B6 background were dosed with 300 mg/kg (0.3 ml) apo-transferrin (apoTf) daily via the intra-peritoneal (i.p.) route for 60 days. Control groups included 0.9% NaCl, 300 mg/kg (0.3 ml) hemopexin (Hpx) and 300 mg/kg (0.3 ml) haptoglobin (Hp). We evaluate Hp and Hpx as controls for anemic correction in this study based on previous studies that suggest Hp and perhaps related proteins can influence ferroportin and iron regulation in mice. [20, 21] Blood (75 μl) was obtained bi-weekly via the tail vein for hematocrit and reticulocyte counts. Urine was collected using metabolic cages at baseline and at every 2 weeks during the studies duration. Satellite groups of animals (sex matched (3 month old equal number of male (n=5) and female (n=5) animals per group)) were dosed parallel to the primary study groups for the purpose of terminal blood collection (0.5-1.0 ml) from the vena cava at baseline, 2, 4, 6 and 8 weeks. Plasma samples were stored under liquid nitrogen and later evaluated for plasma iron (total and NTBI) and plasma mouse/human transferrin. *Study 2: Transfused mice* – Age and sex matched (3 month old equal number of male (n=5) and female (n=5) animals per group) heterozygous $Hbb^{th3/+}$ mice were dosed i.p. once daily for 15 days with 300 mg/kg (0.3 ml) apo-Tf or 0.9% NaCl (0.3 ml). Both groups were transfused every 3 days (5 transfusions) over a 15 day period with 0.2 ml of packed red blood cells (pRBCs). This accounted for approximately 2.5 times each animals RBC volume. The control groups for this study included Age matched (3 month old) wild type (WT, C57BL/6J-B6) and $Hbb^{th3/+}$ mice dosed i.p. with 0.9% NaCl (0.3 ml). Male mice were kindly provided to FDA by Michel Sadelain, Sloan-Kettering Cancer Center, New York, NY. [12, 19] These mice were crossed with female C57BL/6J-B6 and their offspring was subsequently screened for the heterozygous phenotype.

Blood collection and transfusion

Whole blood was collected in CP2D from the thoracic aorta of anesthetized donor WT mice. After bleeding animals were euthanized with CO_2. Whole blood was pooled and centrifuged at 2000 rpm for 10 minutes, plasma was removed and pRBCs were stored overnight in 14% AS-3 at 6^{o}C. pRBCs were administered after 1 day of storage via tail vein injection over 1-2 minutes.

Reagents

Transferrin (<2% iron saturation, apoTf), hemopexin (Hpx) and haptoglobin (Hp) were purified from human plasma and provided by CSL Berhring (Kankakee, Illinois). All preparations contained less than 0.5 EU of lipopolysaccharide on the limulus amebocyte lysate (LAL) assay (Lonza, Walkersville, MD).

Plasma analysis of human/murine transferrin, human Hpx and Hp

Plasma from Hbb$^{th3/+}$ mice was evaluated for human hemopexin, human haptoglobin and human and murine transferrin using mouse and human ELISA kits purchased from Abcam (Cambridge, MA). Plasma samples were diluted in sample dilution buffer and loaded into the wells of a microplate and the ELISA assay was performed according to manufacturer's instructions. Absorbance changes were measured at 450 nm using a microplate reader (Synergy 4 Hybrid Multi-Mode microplate reader (Biotek, Winooski, VT)) and quantitative analysis was performed from a calibration curve, which was obtained by determining immunoactivity of each standard. Each sample was tested in duplicate.

Plasma NTBI measurement

Plasma from Hbb$^{th3/+}$ mice was evaluated for non-transferrin bound iron (NTBI) by using a direct colorimetric NTBI detection assay as previously described (see supplemental material). [22] Plasma samples were mixed with 800 mM nitrilotriacetic acid solution pH 7.0 and samples incubated for 30min at room temperature. The solution was then ultra-filtered using Microcon®-10 centrifugal filters (Millipore Corporation, Billerica, MA). The micro-filterates were mixed with 1 mM HCl and 10% Trichloroacetic acid (TCA) at a ratio of 1:0.5 and incubated for 5 min at room temperature. Ascorbic acid (5 mg/ml) and ferrozine (0.85% wt/vol in hydroxylamine hydrochloride) were then added to the mixtures. The samples were allowed to develop for 30

minutes and the absorbance was measured at 560 nm using the Synergy 4 Hybrid Multi-Mode microplate reader (Biotek, Winooski, VT). A standard curve was generated using a stock iron standard solution (500 μg/dl).

Tissue iron measurements

Kidney, liver, spleen and heart tissue (100 mg) were homogenized in double deionized H_2O at 1:10 wt/vol. Homogenates were mixed with 500 μl of an acid mixture containing 1mM HCl and 10% Trichloroacetic acid (TCA), and incubated at 60°C for 20 h with intermittent shaking. The samples were then centrifuged at 15,000 × *g* for 15 min at room temperature. The clear supernatant (750 μl) was mixed with 250 μl of 20 mg/ml ascorbic acid followed by 200 μl of ferrozine (0.85% wt/vol in hydroxylamine hydrochloride). The samples were allowed to completely develop for 30 min. The absorbance was measured at 560 nm using the Synergy 4 Hybrid Multi-Mode microplate reader (Biotek, Winooski, VT). A standard curve was generated using an iron standard (500 μg/dl).

Western Blot Analyses

Tissue lysates were resolved on 4–12% Bis-Tris gels, transferred to nitrocellulase membranes, and blocked for 1 h in Tris-buffered saline and Tween-20 (TBS-T) with 5% nonfat dry milk. Membranes were incubated overnight at 4°C with an antibody to Ferroportin-1 (1:200) Abcam (cat# ab85370, Cambridge, MA). Rabbit polyclonal anti- Ferroportin-1 antibody washed and then incubated with a relevant horseradish peroxidase–conjugated secondary antibody for 1 h. The signal was developed using an ECL Plus kit and detected with the KODAK image station 4000MM pro (Carestream Health, Inc. Rochester, NY). Membranes were stripped and re-probed for β-actin. Densitometry analysis was performed using KODAK Molecular Imaging Software (Carestream Health, Inc. Rochester, NY) with normalization to actin.

Quantitative Real-time PCR

Total RNA isolated from mouse liver and spleen was used for cDNA synthesis. Real-time PCR reactions were performed using the Applied Biosystems® ViiA™ 7 Real-Time PCR System and its TaqMan Fast Universal PCR Master Mix (Applied Biosystems,Foster City, CA), according to the manufacturer's protocol. The specific TaqMan® gene probes for HAMP (Mm 00519025-

m1), Fam132b (Mm 00557748-m1), and 18S rRNA (Mm 3928990-g1) were purchased from Applied Biosystems. Real-time PCR for each gene were performed in triplicate and normalized to 18S values. Results are presented as fold change and expressed as means ± S.E.M. Statistical analysis real-time PCR was performed on raw $^{\triangle}C_t$ data. Within-group differences for all comparisons were determined by ANOVA with a post hoc analysis for determination of differences between groups. Significance was set at $P \leq 0.05$.

LC-MS/MS analysis for tissue ferritin

Synthetic mouse hepcidin-25 and stable isotope labeled [13C18,15N3]-human hepcidin-25, were manufactured by Peptide Institute, Inc. (Osaka, Japan) and purchased via PeptaNova GmbH (Sandhausen, Germany). LC-MS/MS analysis was performed using a single ion monitoring (SIM) MS1 quantification method. Tissue protein lysates were prepared and digested with trypsin as described. [23] Lyophilized peptide mixtures were cleaned using Zip-Tip C18 columns and analyzed by reversed-phase liquid chromatography nanoLC−MS/MS, performed on an LTQ-Velos mass spectrometer (Thermo Scientific, Bremen, Germany) coupled to an Eksigent NanoLC-Ultra® System (Eksigent Technologies, Dublin, CA). Samples were separated on a 15 cm-long fused silica frit column (75 μm i.d.; BGB Analytik, Boeckten, Switzlerand) and in-house packed with 3 μm, 200 Å pore size C18 resin (Michrom BioResources, Auburn, CA). The chromatographic separation was performed using an ACN/water solvent system containing 0.1% formic acid with a flow rate of 250 nL/min. Elution was achieved using a step-gradient from 5 to 30% ACN in 62 min. The total run time was 96 min, which also included sample injection and column equilibration. Samples were acquired using a method whereby precursor m/z have been monitored in 4 targeted SIM windows (between 70 and 170 amu) over the mass range of 360-900m/z. Targeted MS/MS spectra were acquired in the linear ion trap using a global scheduled Inclusion Lists (IL). The LC applied gradient allowed us to obtain an optimal number of data point (> 10) along the peak elution profile for the quantification. Details of the measured peptides for ferritin light (UniProt Q9CPX4), ferritin heavy chain (UniProt P09528) and actin (UniProt P60710) are provided in supplementary Table 1. This method was used based on its mass accuracy and identification of peptides specific to ferritin H and L chains.

Hematological analyses

Blood was obtained by tail vein nick at baseline and at weeks 2, 4, 6, 8. At study termination blood was obtained from the abdominal aorta of anesthetized mice. At each blood collection reticulocyte count, hematocrit (Hct), and red blood cell (RBC) morphology was evaluated following Wright-Giemsa staining using the HEMA 3® stain kit (Fisher diagnostics, Middletown, VA). Transferrin saturation was determined from the concentration of total plasma iron, the total iron binding capacity of the plasma and unsaturated iron binding capacity according to the method of Yamanishi H *et.al*.. [24] Complete blood counts were measured and compared for all groups at the end of saline or apo-Tf dosing using a Cell-Dyn 3700 hematology analyzer (Abbott Diagnostics, Abbott Park, Il, USA) in veterinary mode for C57 black mice.

Hepcidin LC-MS/MS analysis

Synthetic mouse hepcidin-25 and stable isotope labeled [13C18,15N3]-human hepcidin-25, were manufactured by Peptide Institute, Inc. (Osaka, Japan) and purchased via PeptaNova GmbH (Sandhausen, Germany). The internal standard solution (ISTD) was prepared by diluting [13C18,15N3]-human hepcidin-25 to a final concentration of 1 µM in 20% acetonitrile containing 0.1% (v/v) TFA. Protein precipitation and purification from plasma samples was based on the method described by Rochat et al. (2013)[25], but was combined with a subsequent solid-phase purification (SPE) step, using 96-well Oasis® µElution HLB plates from Waters (Milford, USA). Briefly, 30 µL of plasma was mixed with 2.5 µL ISTD (1 µM) solution. For calibration standards, pooled wild-type plasma samples (30 µL) were spiked with 2.5 µL ISTD (1 µM) solution and with 0.63, 1.25 or 2.5 µL mouse hepcidin solution (2 µM). After SPE, eluates were diluted by adding 60 µL 0.1% (v/v) TFA and transferred to polypropylene autosampler vials. The Hepcidin SIM-target-MS^2 approach was performed on the same nanoLC-MS/MS system described for the liver and kidney ferritin measurements with the following method modifications. Elution was achieved using a step-gradient from 10 to 60% acetonitrile in 22 min with a total run time of 56 min. Blank runs were performed among every sample. In the MS method one scan cycle consisted of a survey full scan from m/z 300 to 1700, followed by 2 targeted SIM windows (50amu) centered around m/z 697.7637 (4+) and m/z 918.3460 (3+) corresponding to the human (DTHFPICIFCCGCCHRSKCGMCCKT) and mouse

(DTNFPICIFCCKCCNNSQCGICCKT) hepcidin-25. MS/MS spectra were acquired in the linear ion trap where the 2 hepcidin-25 peptides were fragmented in CID mode (CE=35). The total cycle time was 0.03 min with more than 10 data points along the peak elution profile.

LC-MS/MS data analysis

Raw SIM spectra of ferritin measurements in kidney and liver were processed with Mascot Distiller 2.4.3.3 (Matrix Science, London, UK), and subsequent peptide and protein identification was performed using Mascot Version 2.4.1 (Matrix Science) as the search engine. Analysis of the RAW files from ferritin and hepcidin analyses was carried out with Skyline™ 2.1.0 software (MacCoss Lab Software, Lab Genome Sciences, UW). Using the Skyline internal scripts we exported the area under the curve (AUC) relative to the MS1 scan of the precursor and the 3 most intense product ions per peptide. For quantitative analyses of ferritin and actin expression the AUC of the individual peptides per protein per sample were summed before normalization was performed. Normalization was performed using the AUC values of actin (for ferritin analyses) and human hepcidin-25 (for mouse hepcidin-25 analyses) that were measured within the same LC-MSMS run.

Histopathology

Spleens, livers, kidneys and hearts were fixed in 10% formalin for 24 hours then stored in 100% isopropanol, embedded in paraffin, and 5 μm sections were prepared. *Perls iron* - Non-heme ferric iron deposition was detected using Perls method with diaminobenzidine (DAB) intensification. All sections were then rinsed in 0.1 M phosphate buffer, pH 7.4, incubated with DAB for three minutes, washed in deionized water, and lightly counterstained with Gill's II hematoxylin. *Hematoxylin and Eosin* – Slides were stained with alum hematoxylin, rinsed with deionized water, differentiated with 0.3% acid alcohol and stained with eosin for 2 minutes. *Masson's Trichrome* – tissue was re-fixed in Bouin's solution, washed in deionized water, stained in Weigert's iron hematoxylin, Biebrich scarlet-acid fuchsin solution and finally phosphomolybdic-phosphotungtic acid differentiation. After dehydrating in graded ethanol and Safeclear, slides were mounted in Permount and cover-slipped. Images were obtained using an Olympus IX71 inverted microscope (Olympus America Inc., Center Valley, Pa).

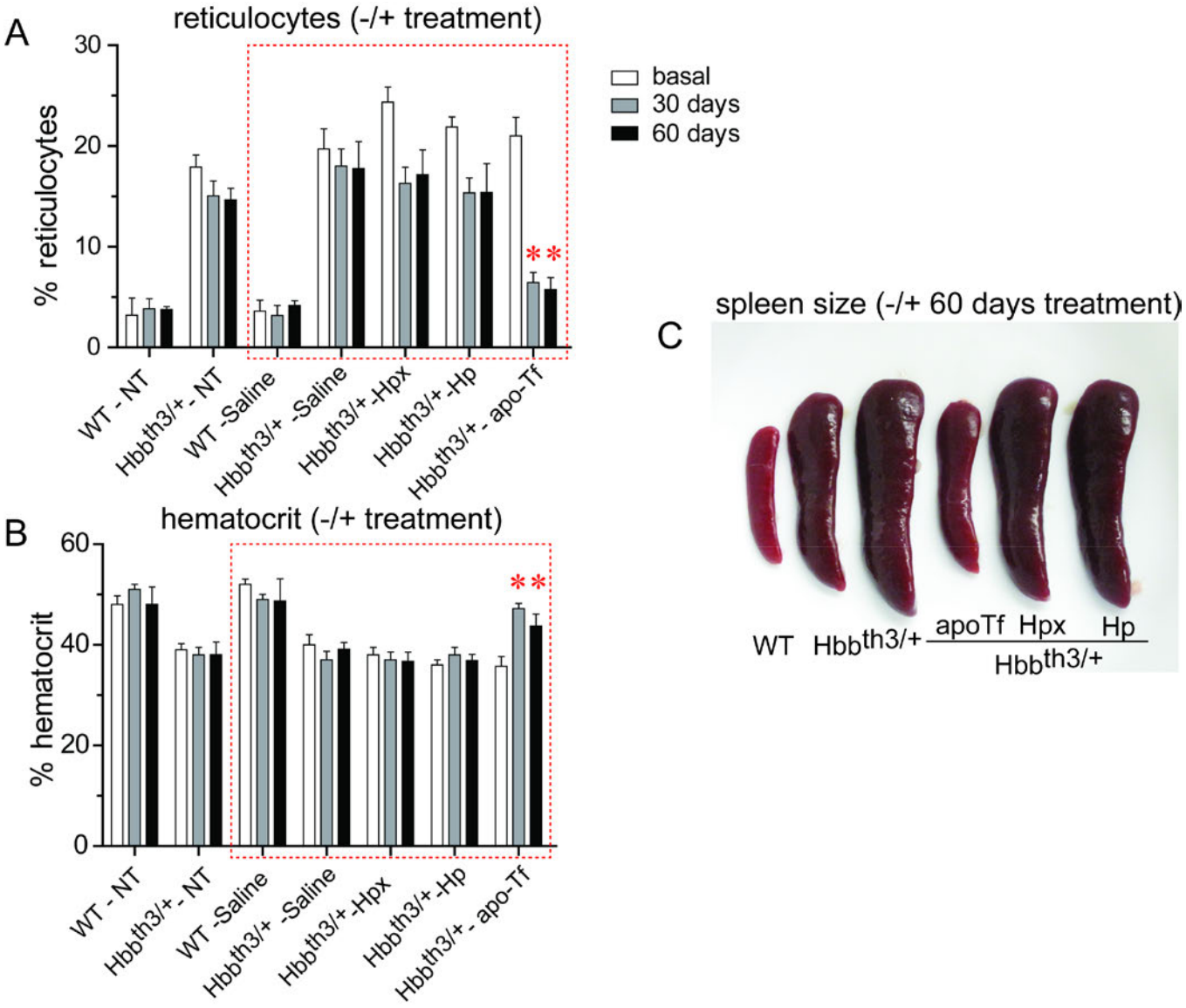
Morphometric/pathology evaluation

All images were acquired using an Olympus IX71 inverted microscope equipped with an Olympus DP70 digital camera). Images of liver sections stained with the Masson's Trichrome captured at 40x magnification were used for analysis. Analysis was performed on images obtained at 40x magnification and processing was carried out using NIH Image J software (NIH, Bethesda, MD). The area of collagen stained tissue was divided by the total area (stained + non-stained) and then divided by 100 to give the percent staining per image. Data are represented as mean ± SEM percent collagen stained area. Images of collagen stained tissue sections from the liver tissue sections were taken at 20x, 40x and 60x magnification and 1200 x 1600 resolution, the whole tissue section (10 tissue sections per animal, n=10 per group) was captured in 20 non-overlapping pictures. Images were consecutively and non- overlapping. When taking Images representing all datasets, the camera and microscope settings remained unchanged to ensure image consistency.

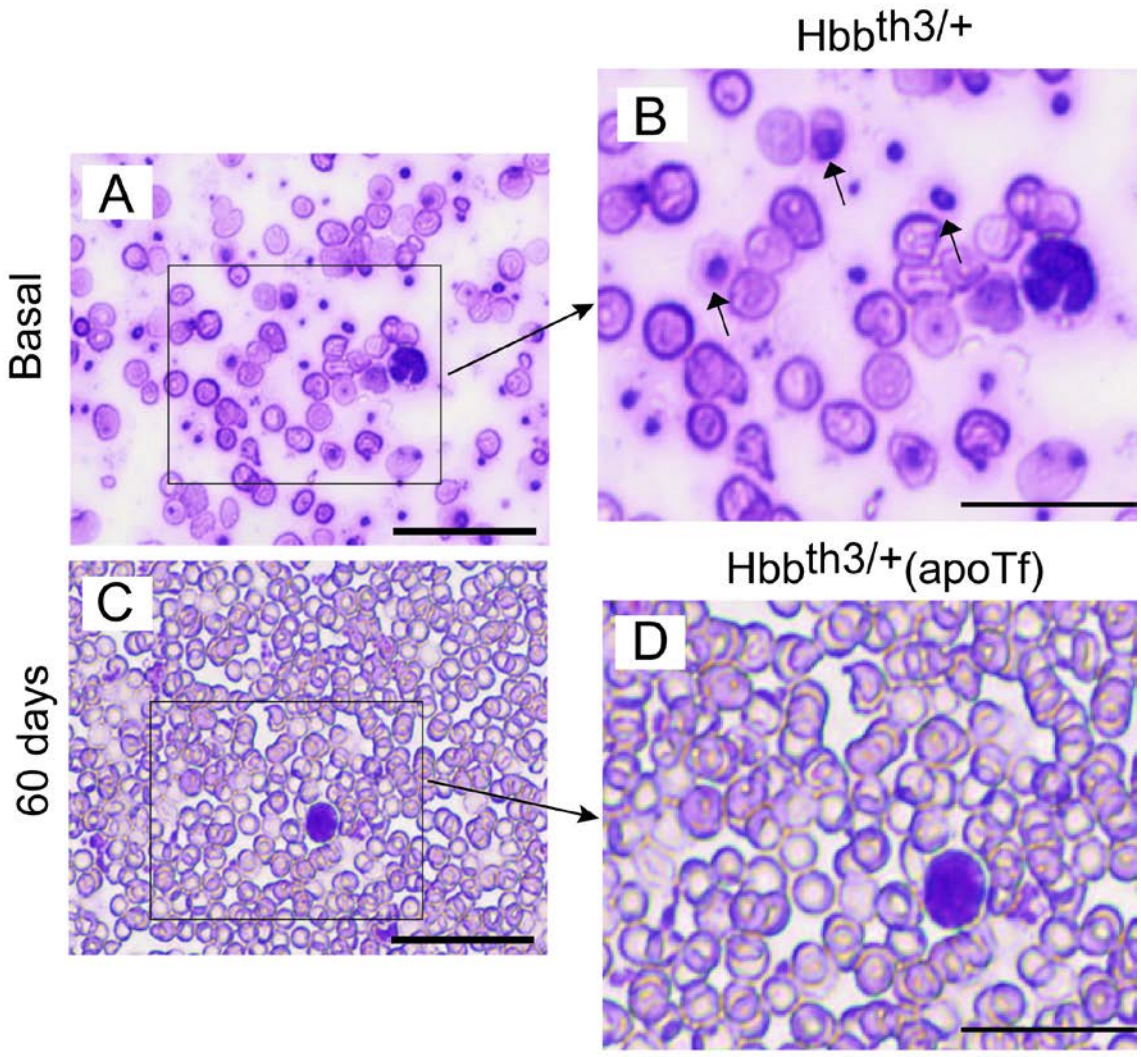
Images were captured for H&E stained liver sections at 60x magnification. Images from 10 tissue sections per animal, with n=10 animals per group were captured in 20 non-overlapping pictures. The numbers of granulocytes per field were counted and data represented as mean ± SEM granulocytes per image field.

Images of H&E stained kidney were captured in the same manner. For analysis of injury, tissue pathology was scored using a semi-quantitative grading system from images captured at 40x magnification as follows: 1, minimal; 2, mild; 3, moderate; and 4, severe (minimal indicates a detectable process but barely present, mild indicates small aggregates of inflammatory or necrotic cells, moderate indicates large aggregated inflammatory or necrotic cells making up 20 % of the tissue area, and severe indicates large multifocal aggregates making up greater than 50% of the tissue area). All analyses were performed blinded to treatment groups.

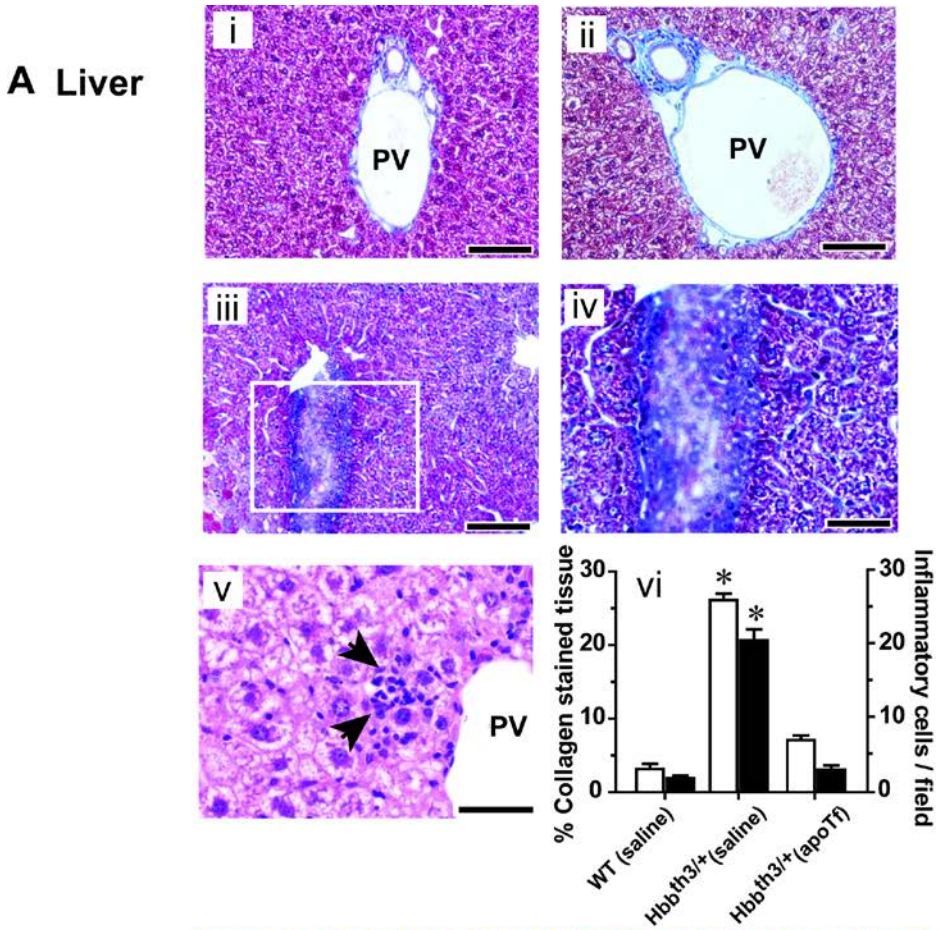
SUPPLEMENTAL FIGURES



Supplemental Figure 1: Hematological changes in apoTf, Hpx and Hp treated Hbb$^{th3/+}$ mice. Data are graphed for percent reticulocyte count (A); percent hematocrit (B). Mean values include a total of 10 animals per group and error bars represent the SEM. Statistical significance is set at a P value < 0.05 indicated in panel A and B for comparisons between all Hbb$^{th3/+}$ and Hbb$^{th3/+}$ (apoTf) mice.



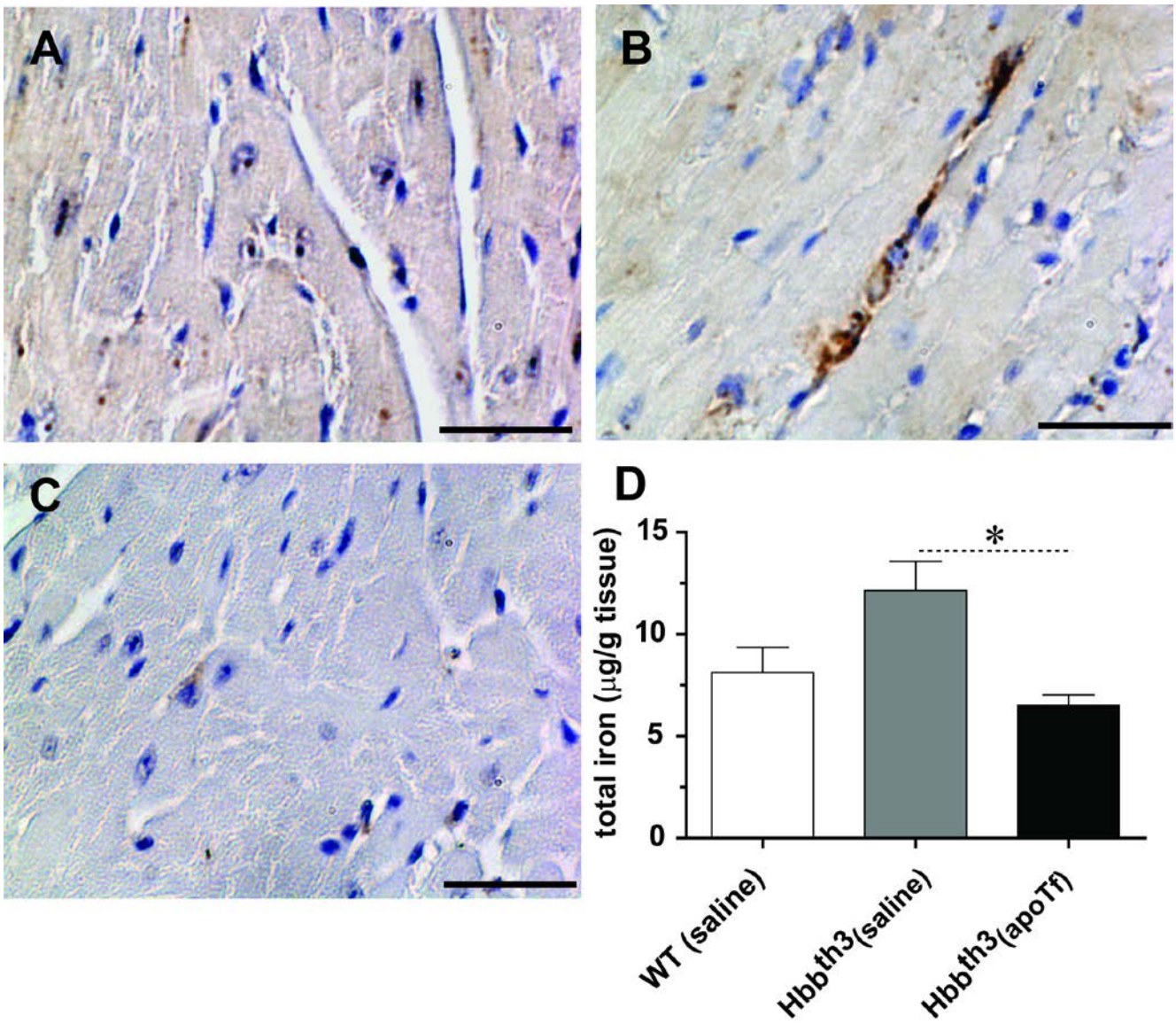
Supplemental Figure 2: Effects of 60 day apoTf treatment on RBC morphology in Hbb$^{th3/+}$ mice. Light microscopy images of Giemsa stained RBCs from Hbb$^{th3/+}$ mice at baseline 40x magnification (A); Hbb$^{th3/+}$ at baseline 64x magnification (B); RBCs from Hbb$^{th3/+}$ (apoTf) mice at 60 days, 40x magnification (C); Hbb$^{th3/+}$ (apoTf) at 60 days, 64x magnification (D). Scale bars at 40x and 64x represent 100 micron and 50 micron, respectively.


A Liver
i
PV
ii
PV
iii
iv
v
PV
vi
% Collagen stained tissue
Inflammatory cells / field
30
20
10
0
*
*
WT (saline)
Hbbth3/+(saline)
Hbbth3/+(apoTf)
B Kidney
i
ii
iii
iv
Severity score
4
3
2
1
0
*
*
WT (saline)
Hbbth3/+(saline)
Hbbth3/+(apoTf)

Supplemental Figure 3: Effects of apoTf on hepatic collagen deposition and renal tubular changes in $Hbb^{th3/+}$ mice.

Liver: Light microscopy images of the hepatic portal and periportal spaces (A); collagen staining (Masson's Trichrome) of WT (saline) liver tissue sections under 20x magnification (i), collagen staining (Masson's Trichrome) of $Hbb^{th3/+}$ (apo-Tf) liver tissue sections under 20x magnification (ii). Collagen staining (Masson's Trichrome) of $Hbb^{th3/+}$ (saline) liver tissue sections under 20x magnification (iii), 60x magnification (white boxed region) (iv); hematoxylin and eosin staining of $Hbb^{th3/+}$ (saline) liver tissue sections under high magnification (60x) showing granulocyte accumulation (black arrows). Scale bars represent 200 micron for 20x magnification images and 50 micron for 60x magnification images. PV (portal vein) is identified for anatomical orientation. (vi) Morphometric analysis of mean % collagen stained tissue ± SEM (left Y axis, open bars) and mean number of granulocytes per image field ± SEM (right Y axis, solid bars).

Kidney: tissue sections are shown for wild type WT (saline) mouse kidney sections following 60 days dosing (B). For hematoxylin and eosin stained tissue the inset represents an image obtained at 96x magnification from the region designated by the smaller white lined box. Insets associated with WT (saline) (i) and $Hbb^{th3/+}$ (apoTf) (ii) show normal renal cortical tubules, while the inset from $Hbb^{th3/+}$ (saline) (iii) shows abnormal distribution of nuclei, vacuolation and necrosis. Pathology assessment of mean tissue severity score ± SEM (iv).



Supplemental Figure 4: Effects of apoTf on myocardial distribution and content of non-heme iron in Hbb$^{th3/+}$ mice. Light microscopy images of Perls DAB non-heme iron staining of myocardial tissue sections under for WT mice following 60 days of saline dosing (A). The same tissue histopathology is shown for Hbb$^{th3/+}$ saline dosed mice (C) and for Hbb$^{th3/+}$ (apoTf) dosed mice (C). Scale bars represent 100 micron at 64x magnification. Group comparisons for tissue iron at 60 days of treatment are shown (D Mean values include a total of 10 animals per group and errors bars represent the standard SEM. Statistical significance is set at a P value < 0.05 indicated as a * for comparisons between Hbb$^{th3/+}$ (saline) and Hbb$^{th3/+}$ (apoTf) mice.

Supplemental Table 1 - Ferritin H and L chains

Peptide details used for SIM LC-MS/MS analysis of ferritin light chain and ferritin heavy chain expression in liver and kidney samples.

Protein (UniProt ID)	Peptide sequence	m/z	charge state
Ferritin light	QNYSTEVEAAVNR	740.854969	2
Q9CPX4	LVNLHLR	432.774337	2
	ALFQDVQKPSQDEWGK	625.979368	3
	TQEAMEAALAMEK	711.833802	2
	NLNQALLDLHALGSAR	569.316315	3
	VAGPQPAQTGAPQGSLGEYLFER	791.732634	3
Ferritin heavy	YFLHQSHEER	449.214309	3
P09528	SVNQSLLELHK	423.238343	3
	ELGDHVTNLR	577.301644	2
	MGAPEAGMAEYLFDK	815.368009	2
Actin (beta)	AGFAGDDAPR	488.72778	2
P60710	GYSFTTTAER	566.767102	2
	SYELPDGQVITIGNER	895.949597	2
	QNYHQDAEAAINR	510.574565	3