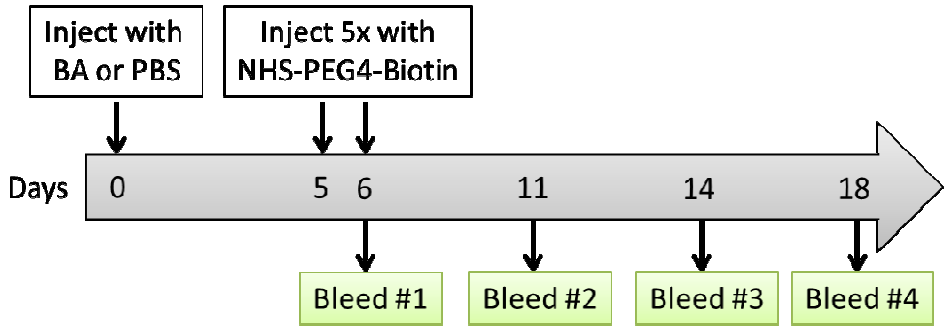
Supplemental Data

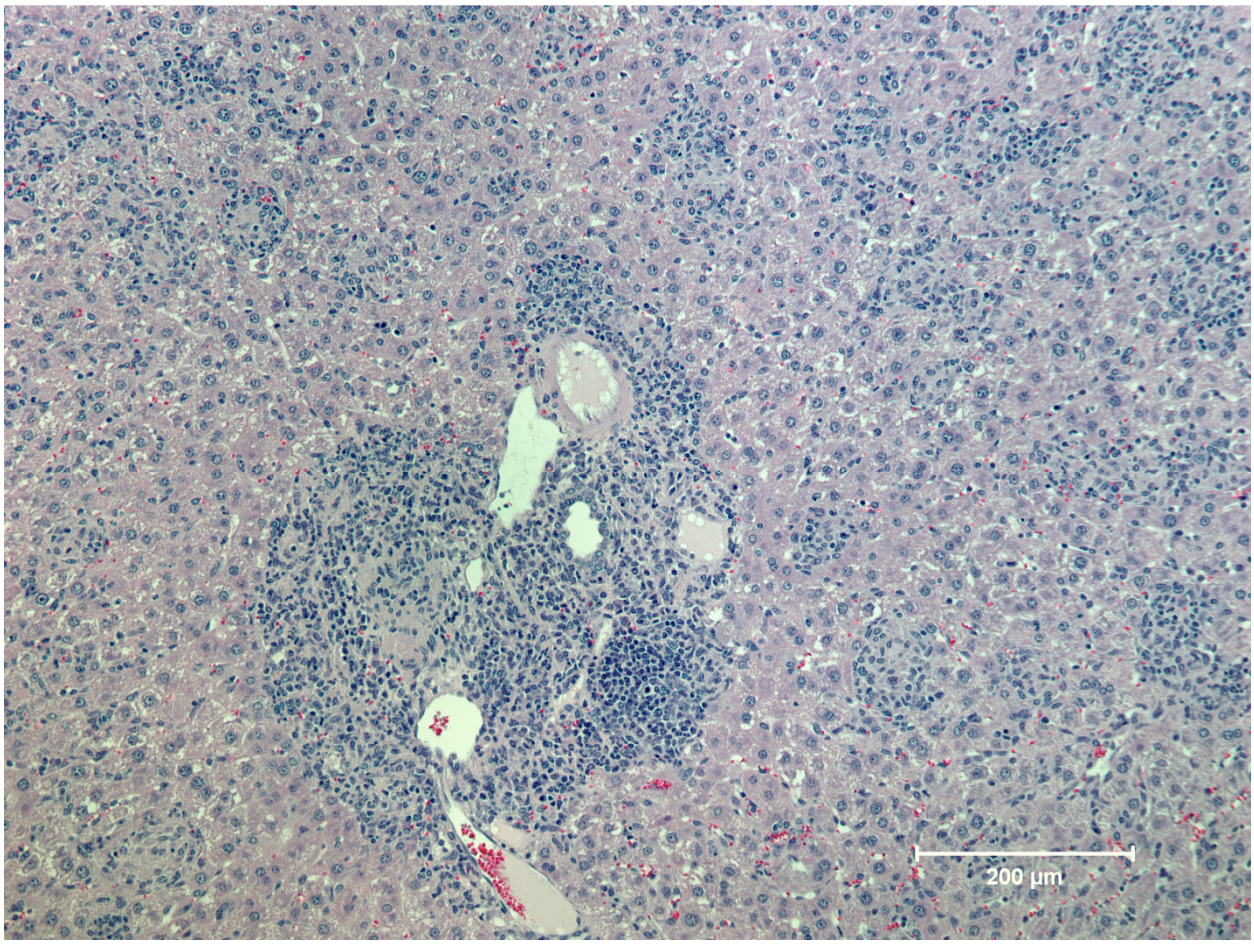
Supplemental Table 1: PCR Primers

Gene target	Forward Primer	Reverse Primer
Murine HAMP1	5'-CCTATCTCCATCAACAGATG-3'	5'-AACAGATACCACACTGGGAA-3'
Murine β-Actin	5'-ACCCACACTGTGCCCATCTA-3'	5'-CACGCTCGGTCAGGATCTTC-3'
Murine SAA-1	5'-TGACCAGGAAGCCAACAG-3'	5'-GTAGGAAGACCAGACC-3'

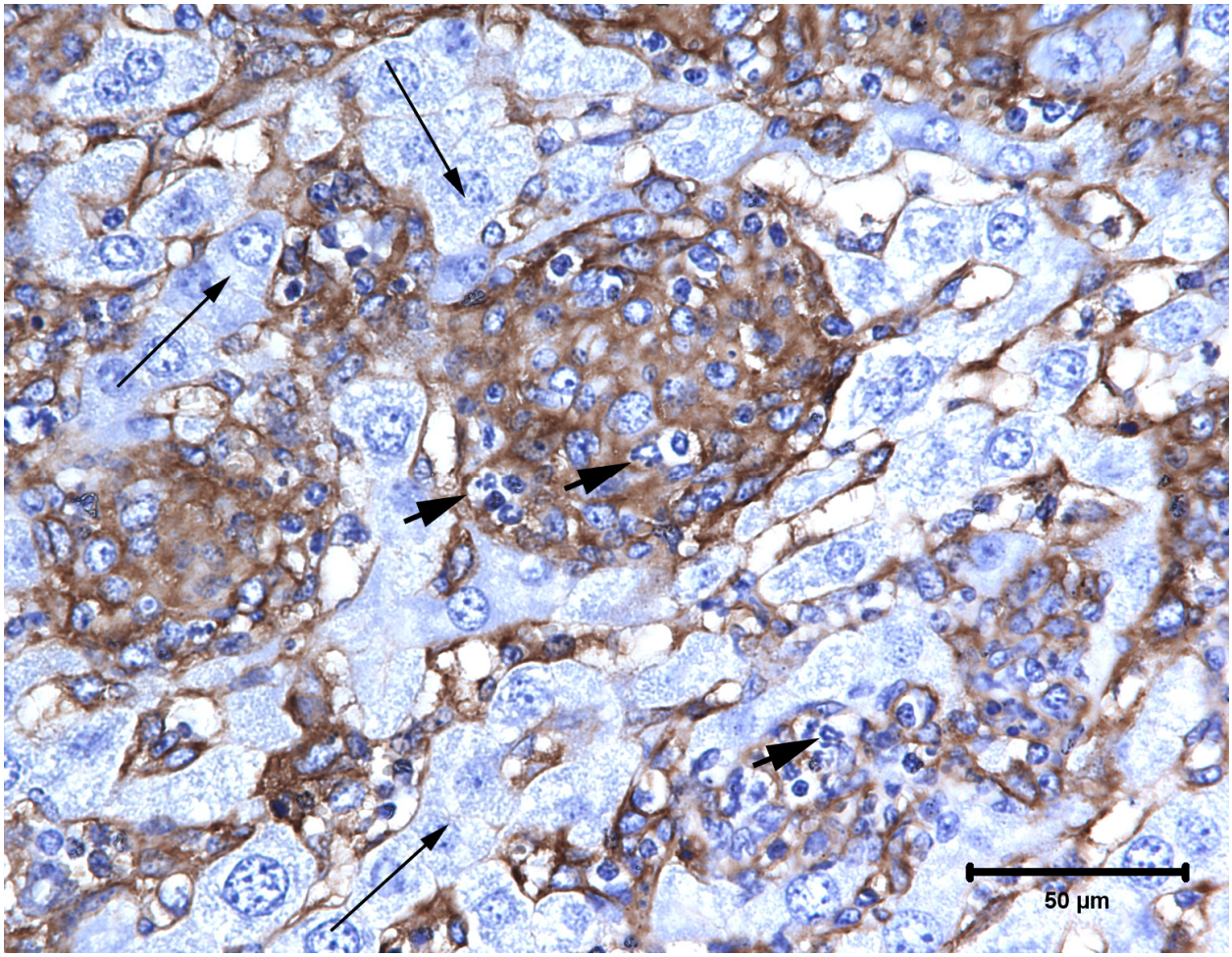


Supplemental Figure 1: Erythrocyte biotinylation timeline

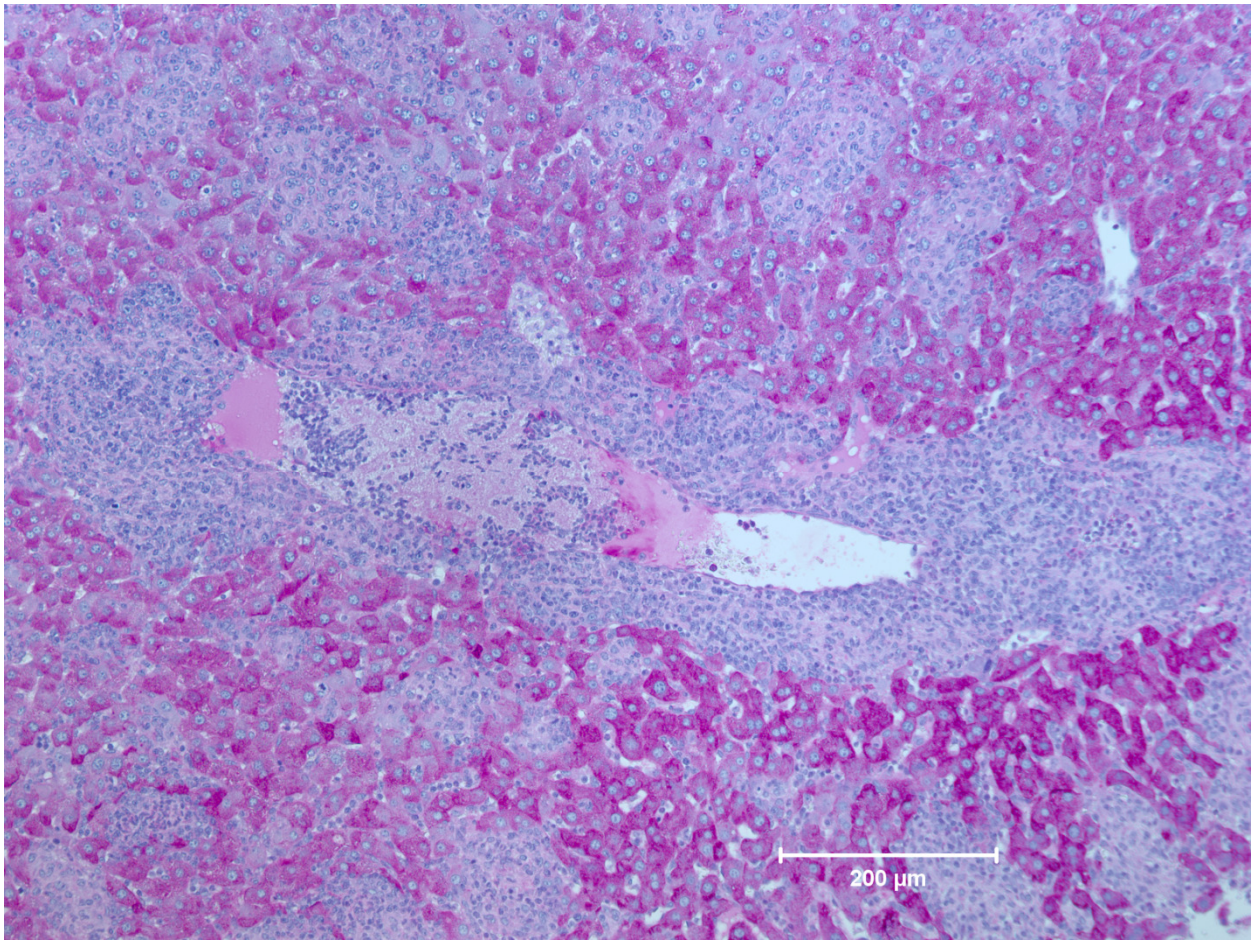
Supplemental Figure 2: BA treatment causes perivascular granulomatoid inflammation with thrombosis



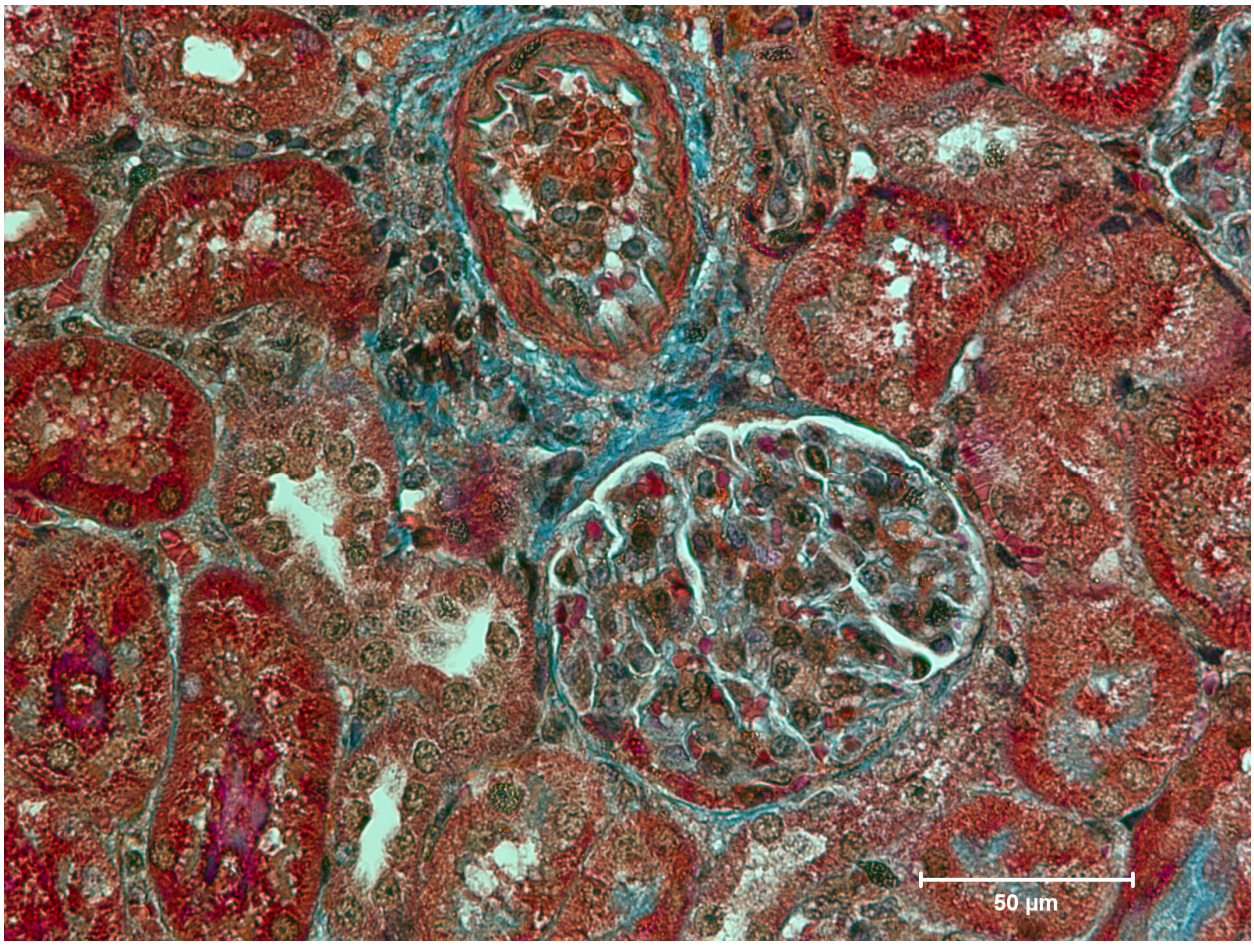
Panel A: An H&E stained liver section from BA-treated mouse at 2 wks shows granulomatoid accumulations of inflammatory cells predominantly in the periportal area.



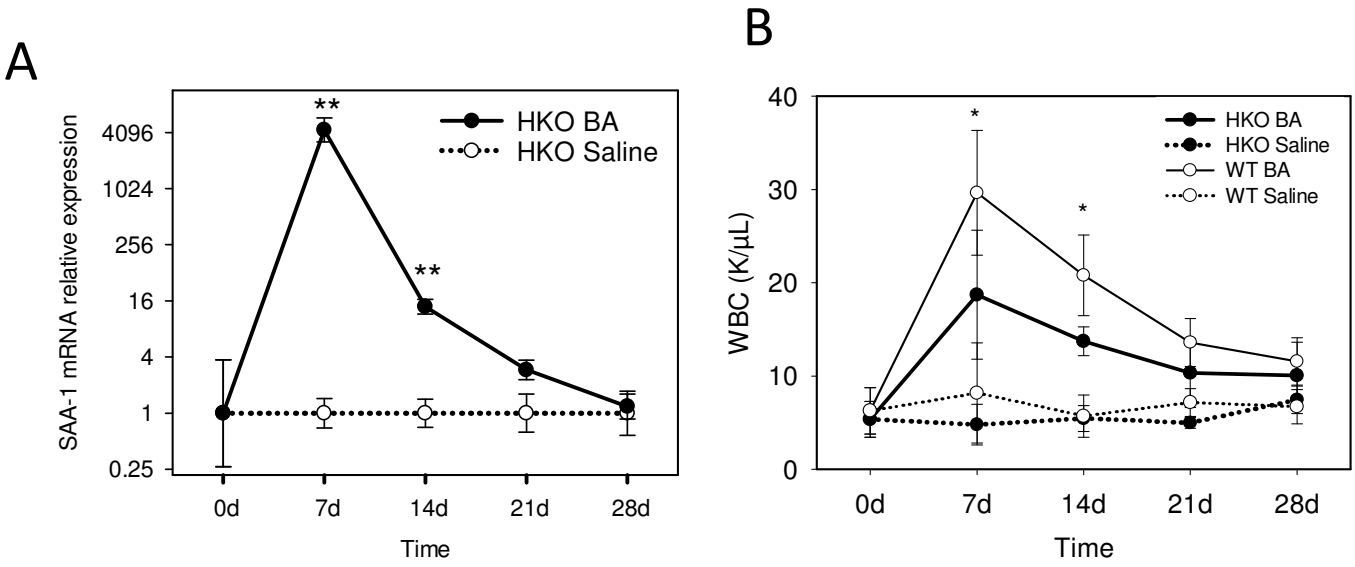
Panel B: Liver section from BA-treated mouse at 2 wks stained with a monoclonal antibody against the F4/80 macrophage marker (brown) shows granulomatoid accumulations of macrophages (brown staining predominantly on cell membranes) and granulocytes (short arrows, unstained white with multilobed blue nuclei) surrounded by cords of hepatocytes (long arrows, hematoxylin blue staining with large nuclei and reticulated cytoplasm).



Panel C: A PAS-stained liver section from BA-treated mouse at 2 wks shows perivascular inflammation and thrombosis.

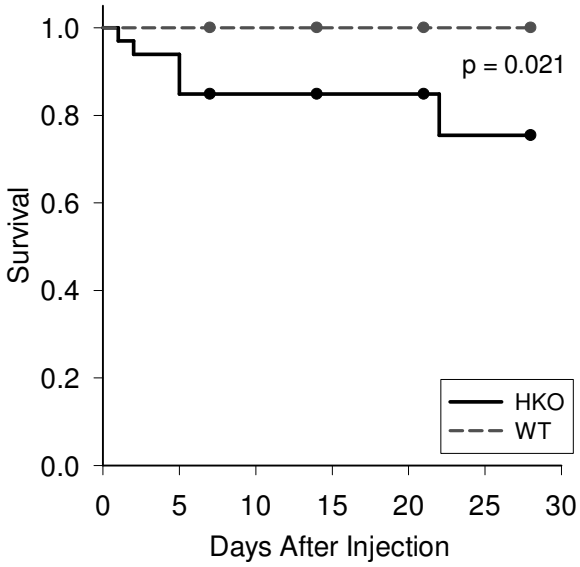


Panel D: A Masson-trichrome stained kidney section from a BA-treated mouse at 2 wks shows periarteriolar inflammatory cells with arteriolar thrombosis and a hypoperfused glomerulus.



Supplemental Figure 3: Inflammatory markers in HKO mice. A: Hepatic SAA-1 mRNA concentrations in BA-treated HKO mice relative to those of contemporaneous saline-treated HKO mice. HKO mice have increased SAA-1 mRNA, with a peak at 7 d and a gradual return to normal by day 21. Means ± SE are shown, p by t-test or Mann-Whitney rank sum test. **p<0.001.

B: WBC (means ± SD) of HKO and WT mice, *p<0.05 by t-test, comparing BA-treated HKO to BA-treated WT. The data shown for WT mice are a subset of those in Figure 2D.



Supplemental Figure 4: HKO mice have significantly decreased survival compared to their WT counterparts. Kaplan-Meier survival analysis with p by log-rank test.

Experimental validation of the Amgen sandwich ELISA for mouse hepcidin-1

We tested whether the hepcidin-1 ELISA appropriately detected the predicted effect of dietary iron on serum hepcidin-1 concentrations. Serum hepcidin-1 concentrations were also correlated with hepatic hepcidin-1 mRNA, the current gold standard for studies of hepcidin responses in mice.

Materials and Methods

Animals

Four week old C57BL/6 mice (8 males and 8 females per diet group) were placed on 3 different iron-containing diets for one month. One set of mice was put on a low iron diet containing 4ppm iron (Harland Laboratories, Indianapolis, IN, TD80396) for 4 weeks, a second set was kept on the regular mouse chow which contained approximately 300 ppm iron. The third set of mice was maintained on the regular diet for one week then changed to a high iron (carbonyl) diet containing 10,000 ppm iron (Harland Labs, TD08043) for the remaining three weeks.

Sample collection

Approximately 100 µl blood was collected in microtubes containing 3 µl of K_3-EDTA and mixed immediately. Complete blood count was performed by a hematology analyzer (Hemavet HV950FS, Drew Scientific, Dallas, Texas).

Additional blood was allowed to clot in a serum separator. The serum was collected after centrifugation at 5000 RPM for 5 minutes, and stored at -20C until used for the ELISA. Liver tissue was collected and analyzed for HAMP mRNA using qRT-PCR.

ELISA

Mouse hepcidin-1 monoclonal antibodies, Ab2B10 (capture) and AB2H4-HRP (detection), as well as synthetic mouse hepcidin-25, were a generous gift from Amgen (Thousand Oaks, CA). A protocol[1] that was designed by Dr. Keegan Cooke and Dr. Barbara Sasu at Amgen was used as the starting point for further development and validation of this assay.

High binding 96-well EIA plates (Corning Costar, Tewksbury MA, #3590) were coated overnight at room temperature with 50 µl/well of 3.6 µg/ml Ab2B10 in 0.2 M carbonate-bicarbonate buffer pH 9.4 (Pierce - Thermo Scientific, Rockford, IL). Plates were washed two times with wash buffer (PBS, 0.5% Tween-20) and then blocked for 45 minutes with 200 µl/well blocking buffer (PBS, 1% BSA, 1% normal goat serum, 0.5% Tween-20). Samples and standards were then placed in the wells in duplicate. A standard curve was generated by diluting the stock mouse hepcidin peptide (50 ng/ul) to a final concentration of 4 ng/ml for the highest standard followed by two-fold dilutions in blocking buffer, thereby generating an 8-point standard curve. Serum samples from mice on a high iron diet were diluted approximately 1:5000, whereas those on a low iron diet, as well as the hepcidin knock-out control serum were diluted 1:100. After a one hour incubation period at room temperature (with mixing), the wells were washed four times with wash buffer then incubated for an additional hour with 50 µl/well of 130 ng/ml Ab2H4-HRP, washed 4 times then developed with 100 µl/well Ultra-TMB substrate (Thermo Scientific) for 30 minutes in the dark at room temperature. The reaction was stopped by adding 50 µl 2 M sulfuric

acid and the absorbance was measured at 450 nm using a 96-well plate reader (Molecular Devices, Sunnyvale, CA).

qRT-PCR:

Total RNA was extracted from mouse liver tissue using the Trizol method (Invitrogen, Grand Island, NY) according to manufacturer's instructions. cDNA was made using the BioRad iScript cDNA kit (Hercules, CA) using approximately 250 ng total RNA using the following conditions; 25C for 5 min, 42C for 30 min, 85C for 5 min. BioRad IQ SyberGreen supermix was used for qRT-PCR reactions with a two-step program set at 95C for 10 sec and 60C for 30 sec for 35 cycles. The following primer sets were used:

Mouse Hepcidin:	FWD: 5'-AAGCAGGGCAGACATTGCGAT-3'
	REV: 5'-CAGGATGTGGCTCTAGGCTAT -3'
Mouse Actin:	FWD: 5'-ACCCACACTGTGCCCATCTA-3'
	REV: 5'-CACGCTCGGTCAGGATCTTC-3'

Results

There was no significant difference in the blood hemoglobin concentration among the three groups (12.9±3.0, 13.3±3.1, 12.9±3.3 g/dl for low, standard and high iron diets respectively). As expected for iron-restricted erythropoiesis, the MCV of the low iron group was lower (35.7±1.3, mean ± SD, $p=10^{-10}$, t-test compared to standard diet) than that of standard and high iron groups (40.6±1.6 and 41.6+1.6 respectively).

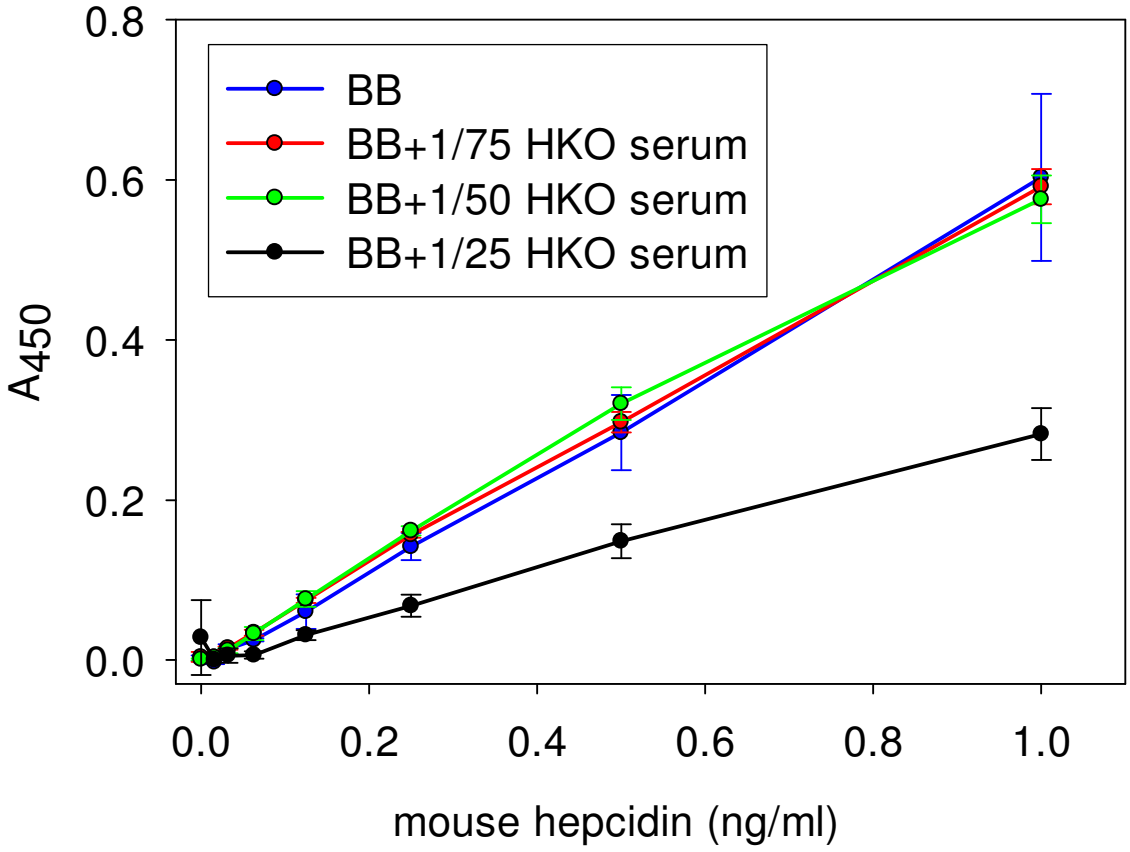
Hepcidin concentrations in the serum of mice on the low iron diet were mostly below the lower limit of detection (3 ng/ml) in 1:100 dilution of serum. This minimum serum dilution was determined by comparing hepcidin-1 peptide standard curves generated in various dilutions of hepcidin knock-out (HKO) mouse serum to those generated in blocking buffer (BB) alone (Supplemental Figure 5), showing nonspecific interference at serum dilutions below 1:50.

Increasing concentrations of hepcidin were seen with increasing amounts of iron in the diet (Supplemental Figure 6). Dietary iron ranged over a 10,000 fold range and hepcidin also increased over a similar range (3-7000 ng/ml). On the 300 ppm (standard mouse chow) diet, females had roughly two-times higher serum hepcidin concentrations than males, median (interquartile range) 1825 (1705, 1988) ng/ml vs. 938 (700, 1275) ng/ml, $p<0.001$ by Mann-Whitney, whereas females and males had approximately the same concentrations of hepcidin on the high iron diet, 4863 (4541, 5224) ng/ml and 5055 (4406, 6200) ng/ml. Replicate analysis for this assay showed a coefficient of variation of 9% in the same plate and 19% for replicates in separate plates over the range of the assay (3-7000 ng/ml).

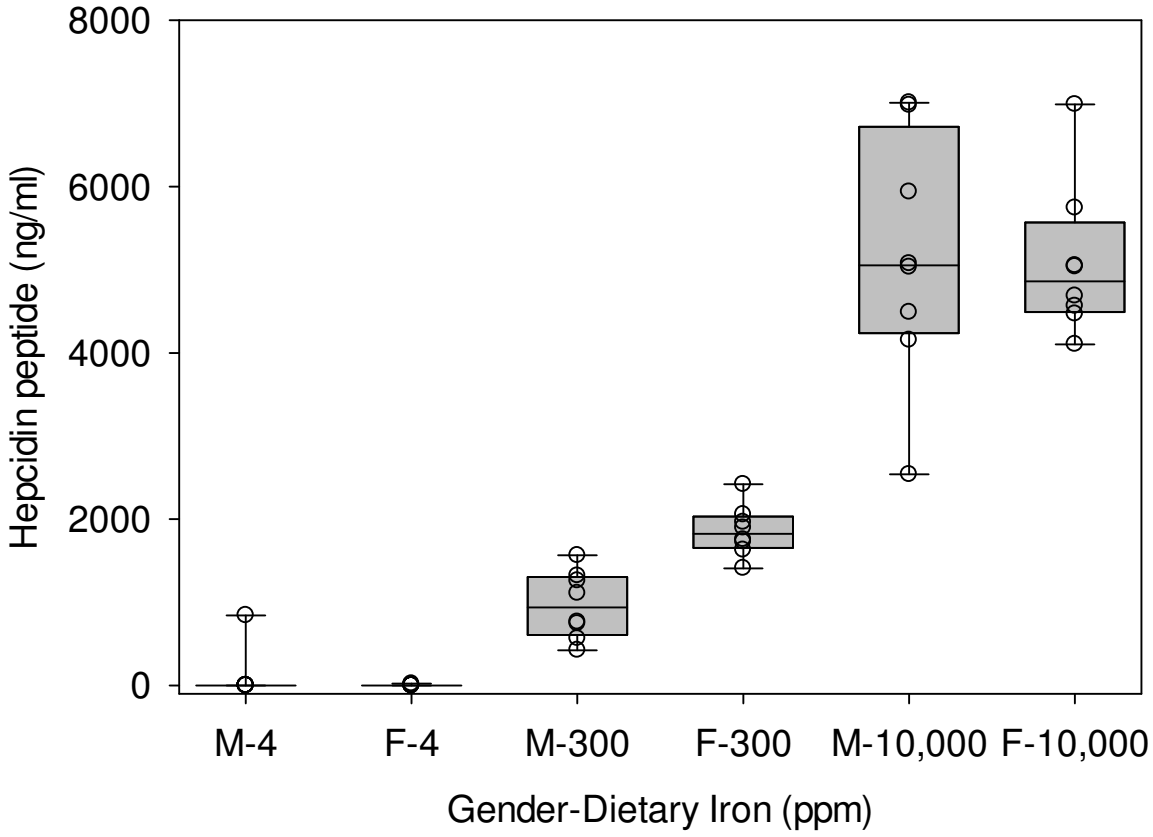
As shown in Supplemental Figure 7, liver hepcidin mRNA (normalized to actin mRNA) was very low in the low iron diet mice dCt median (interquartile range) females -7.5 (-7.8, -4.7) and males -6.1 (-7.5, -4.5), $p=0.65$ by Mann-Whitney test, whereas on the 300 ppm diet, females had 2-fold more hepcidin mRNA compared to male mice dCt 3.3 (2.7, 4.1) and 2.3 (1.8, 2.8)

respectively, p=0.02 by Mann-Whitney. Like hepcidin peptide concentrations, the hepcidin mRNA concentrations were very similar between the female and male mice on the high iron diet 4.2 (3.9, 4.4) and 4.3 (3.9, 5.0), p= 0.72 by Mann-Whitney test.

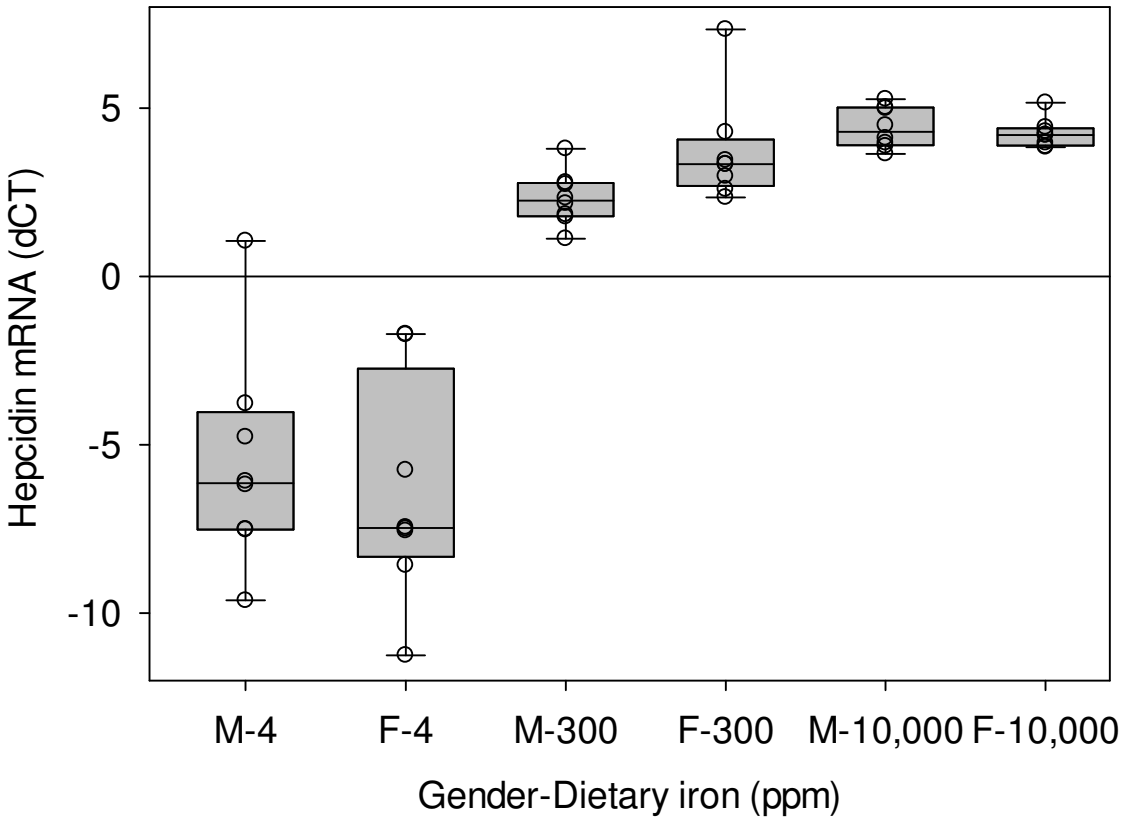
There was a very strong correlation (r^2 = 0.91, Pearson) between log serum hepcidin concentration and liver mRNA levels (Supplemental Figure 8), supporting the validity of the new serum hepcidin assay, and reinforcing the validity of hepatic hepcidin mRNA concentrations as a proxy for the less accessible serum hepcidin peptide measurements.



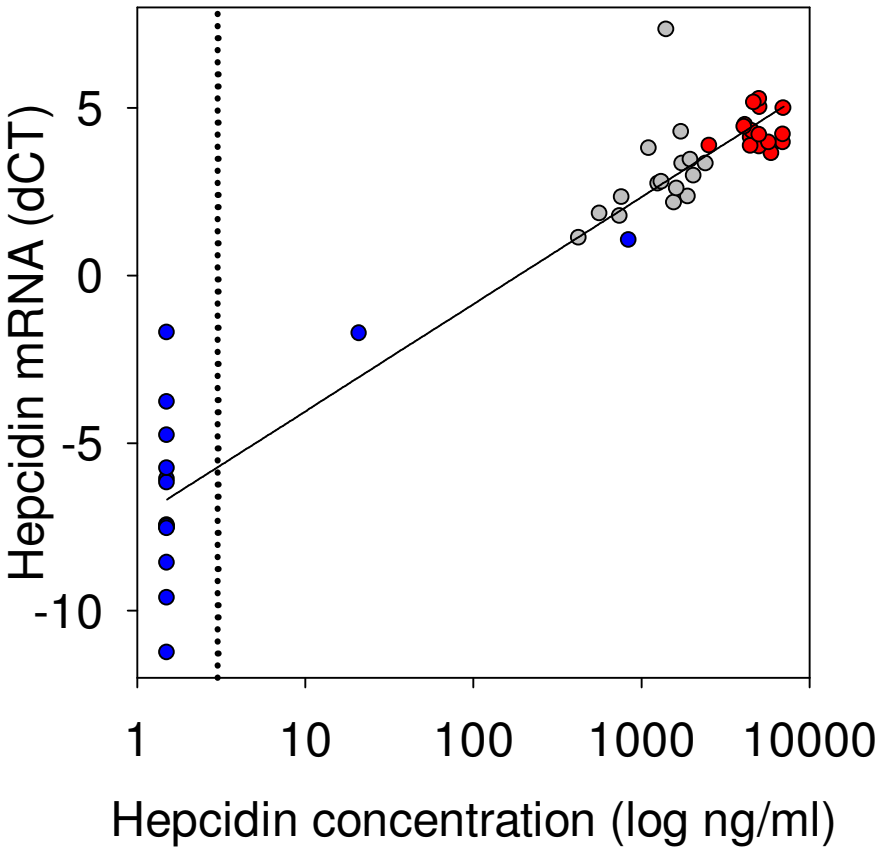
Supplemental Figure 5. Mouse hepcidin standard curves in dilutions of hepcidin-knock-out mouse serum (HKO). Standard curves of mouse hepcidin peptide in different concentrations of hepcidin-knockout (HKO) mouse serum diluted in blocking buffer (BB) were tested to determine the lowest concentration of serum that does not cause interference (1:50). Standard curves are shown with means ± SD of replicate measurements.



Supplemental Figure 6. Hepcidin peptide concentrations (ng/ml) in sera of male (M) and female (F) mice fed diets with different iron contents (4 ppm, 300 ppm or 10,000 ppm) for one month. Median and interquartile range are shown in the box plot and individual data points are shown as hollow circles.



Supplemental Figure 7. Hepcidin Liver mRNA (dCT) in Male (M) and Female (F) mice on different iron containing diets (4ppm, 300ppm or 10,000ppm) for one month. Hepcidin and actin cDNA was amplified using qRT-PCR and Hepcidin mRNA normalized to actin. Median and interquartile range are shown in the box plot and individual data points are shown in the hollow circles.



Supplemental Figure 8. Correlation of liver hepcidin-1 mRNA concentrations with serum hepcidin-1 peptide concentrations. Value for individual mice on different iron containing diets are indicated by the colors: blue = 4 ppm , gray = 300 ppm and red =10,000 ppm. Serum hepcidin concentrations not detectable by the EIA were assigned a concentration of 1.5 ng/ml, representing 50% of the lower limit of detection (dotted vertical line).

Reference

1. Hod EA, Francis RO, Spitalnik SL, Winters A, Cooke KS, Sasu BJ. Validation and Preclinical Correlation of a New Sandwich ELISA for Measuring Murine Hepcidin. *ASH Annual Meeting Abstracts*. 2012;120(21):2100.