

Gastrointestinal iron excretion and reversal of iron excess in a mouse model of inherited iron excess

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Supplementary Methods

Animals and transferrin treatment

Human transferrin at 30% saturation was used in this study. We judged this acceptable for two reasons. First, injections of human or mouse transferrin decrease liver iron concentrations in $Trf^{hpx/hpx}$ mice.¹³ Second, our efforts to purchase or purify sufficient amounts of mouse transferrin for this study were complicated by the discovery that many commercial sources of mouse transferrin or serum are contaminated with murine pathogens (data not shown). We also rationalized that 30%-saturated transferrin was unlikely to impact iron levels in $Trf^{hpx/hpx}$ mice. Our calculations estimated that treatment of $Trf^{hpx/hpx}$ pups three times prior to weaning introduced ~3.5 μ g iron, while treatment of adult $Trf^{hpx/hpx}$ mice three times a week for two weeks introduced ~7 μ g iron. These levels are minimal compared to body iron levels in adult $Trf^{hpx/hpx}$ mice.

Non-radioactive sample harvest and analysis

For data shown in Figures 1 and 2, mice were anesthetized by intraperitoneal injection with ketamine/xylazine. Blood was collected by retro-orbital puncture and serum and plasma isolated and stored at -80 °C. Mice were then euthanized by cervical dislocation. Tissues were collected and flash-frozen and stored at -80 °C or fixed in 10% phosphate-buffered formalin overnight then stored in 70% ethanol. Transferrin levels were analyzed by immunoblot using a transferrin-specific antibody (Proteintech) or stained protein gel using Simply Blue Safe Stain (Life Technologies). Hemoglobin levels were measured in anticoagulated blood using a Vet abc Plus+ (Scil). Hepcidin levels were measured by Hepcidin Murine-Compete ELISA (Intrinsic LifeSciences). RNA levels were measured by tissue RNA isolation using Trizol, cDNA synthesis using High Capacity cDNA Reverse Transcription Kit, and RNA level measurement using Taqman Gene Expression Assays (Life Technologies). For histology, fixed tissues were embedded, sectioned, stained with Iron Stain Kit (Sigma), and scanned using Aperio ScanScope (Leica Biosystems). To measure iron levels in tissue samples, 25-100 mg tissue were digested in 1 mL trace metal-grade 70% nitric acid (Fisher) at 65 °C for two hours, then diluted 25-fold in MilliQ water and analyzed by ICP-AES. Mock digests were included to control for background metal contamination. Standards were included to ensure consistency throughout and between each run.

For data shown in Figure 3, mice were euthanized by cervical dislocation. Blood was not removed from mice. Gastrointestinal tracts were removed and cleaned of contents. After pelt removal, carcasses were frozen in liquid nitrogen and pulverized using a mortar and pestle. All samples were stored at -80 °C until analysis. Gastrointestinal tracts and five samples of pulverized carcasses were digested in nitric acid, diluted in water, and analyzed by ICP-AES as above. Pelts were digested by slow addition of a 2:1 mix of nitric acid and hydrogen peroxide (Fisher), heating to 70 °C until dry, then re-digested again. Samples were then diluted in water, syringe-filtered, and analyzed by ICP-AES.

To analyze liver and feces lysates for ferritin levels, mouse livers were lysed in RIPA buffer with protease inhibitors then centrifuged to pellet debris. Supernatants were electrophoresed under non-denaturing, non-reducing conditions using precast 4-20% polyacrylamide gels (Invitrogen). Gels were then rinsed with MilliQ water for five minutes four times then stained using the Iron Stain Kit (Sigma). Duplicate gels were also transferred to nitrocellulose membranes and immunoblotted using anti-ferritin heavy (Cell Signaling) and light chain (Proteintech) antibodies. Antibodies were detected using chemiluminescence.

Estimate of amount of iron mobilized from liver and pancreas after two-week transferrin treatment of $Trf^{hpx/hpx}$ mice

To estimate the amount of iron liberated from the liver and pancreas of mutant mice during transferrin treatment from two to 2.5 months of age, we employed the following calculations:

- Untreated 2.5-month-old $Trf^{hpx/hpx}$ mice:
 - 2.34 mg iron/g liver (Fig. 1E); liver mass: 0.88 g; 2.06 mg iron in liver
 - 1.92 mg iron/g pancreas (Fig. 1E); pancreas mass: 0.20 g; 0.38 mg iron in pancreas
 - Total amount of iron in liver and pancreas: 2.44 mg
- Treated 2.5-month-old $Trf^{hpx/hpx}$ mice:
 - 1.75 mg iron/g liver (Fig. 1E); liver mass: 1.06 g; 1.86 mg iron in liver
 - 1.21 mg iron/g pancreas (Fig. 1E); pancreas mass: 0.23 g; 0.28 mg iron in pancreas
 - Total amount of iron in liver and pancreas: 2.14 mg
- Difference in liver and pancreas totals between untreated and treated mice: 0.30 mg

Estimate of amount of iron required to correct hemoglobin deficit in $Trf^{hpx/hpx}$ mice during two-week transferrin treatment

To estimate the difference in hemoglobin iron content between 2.5-month-old old $Trf^{+/+}$ and treated 2.5-month-old $Trf^{hpx/hpx}$ mice, we relied on the published factor of 5.9 mL total blood/100 g body mass in BALB/c mice (Vácha J. Blood volume in inbred strain BALB/c, CBA/J and C57BL/10 mice determined by means of ^{59}Fe -labelled red cells and ^{59}Fe bound to transferrin. *Physiol Bohemoslov* 1975;24(5):413–419). (Estimates of blood volume are not published for $Trf^{hpx/hpx}$ mice.) We employed the following calculations:

- For 2.5-month-old $Trf^{+/+}$ mice:
 - 15.55 g hemoglobin/dL blood (Fig. 1B)
 - Body mass of 23.24 g (Fig. 3A)
 - 1.37 mL blood (assuming 5.9 mL blood/100 g body mass)
 - 0.21 g hemoglobin
- For 2.5-month-old treated $Trf^{hpx/hpx}$ mice:
 - 6.14 g hemoglobin/dL blood (Fig. 1B)
 - Body mass of 15.29 g (Fig. 3A)

- 0.90 mL blood (assuming 5.9 mL blood/100 g body mass)
- 0.06 g hemoglobin.
- Difference in mass of hemoglobin between mice: 0.15 g
- Assuming four atoms of iron per hemoglobin and 65 kD mass of hemoglobin, 0.15 g hemoglobin would contain ~475 ug iron.

⁵⁹Fe treatments and sample harvest and analysis

To convert body ⁵⁹Fe counts to ⁵⁹Fe half-lives and excretion rates, ⁵⁹Fe counts were decay-corrected to the date of gavage. Body ⁵⁹Fe counts were expressed as a percent of body counts from the day after gavage ('day 0'), then plotted versus time. Data was fit to an exponential decay curve with equation $y=ae^{-bx}$, where b is a decay constant and the fraction of body ⁵⁹Fe excreted per day. Biological ⁵⁹Fe half-lives ($t_{1/2}$) were calculated as natural log of 2 divided by b . Day 0 counts were excluded from analysis as it was assumed that ⁵⁹Fe had not yet fully equilibrated within each mouse at this time.

To process fecal and urinary ⁵⁹Fe levels, ⁵⁹Fe levels were extrapolated to a twenty-four-hour collection time and expressed as a percent of body ⁵⁹Fe levels measured on the day of collection. Values were then multiplied by correction factors to control for the fact that feces and urine samples could be placed closer to the detector than could live mice. Correction factors were established at the end of the excretion study by dividing the sum of ⁵⁹Fe levels in organs, pelts, and carcasses by whole body ⁵⁹Fe counts taken prior to dissection. These factors were: (mean ± SEM) 2.64±0.02 (male *Trf*^{+/+}), 3.20±0.07 (male *Trf*^{hpx/hpx}), 3.31±0.27 (male transferrin-treated *Trf*^{hpx/hpx}), 2.63±0.20 (female *Trf*^{+/+}), 3.19±0.15 (female *Trf*^{hpx/hpx}), and 3.29±0.12 (female transferrin-treated *Trf*^{hpx/hpx}).

To measure iron levels in mice at the end of the excretion study, mice were euthanized and organs, pelts, and carcasses were isolated, weighed, and counted. Iron levels were measured in nitric acid-digested tissues by bathophenanthroline sulfonate (BPS)-based assay.¹² (ICP-AES could not be used given that this instrument is shared and samples were radioactive.) While the BPS-based assay typically involves digestion of tissue with trichloroacetic acid and hydrochloric acid for measurement of non-heme iron levels, nitric acid digestion of tissues permits measurement of total iron levels. This was confirmed by measuring iron levels in fifteen nitric acid-digested liver samples by both ICP-AES and BPS-based assay, which indicated that the ratio of BPS- to ICP-AES-derived values was 1.19 +/- 0.04. This ratio was used to convert BPS-derived values to ICP-equivalent values.

For analysis of feces, samples were thawed, then rehydrated overnight at 4 °C in 10 volumes of MilliQ water. Samples were then vortexed aggressively. Aliquots were analyzed for iron levels using ICP-AES as described above for non-radioactive tissues. To measure fecal ferritin levels, fecal homogenates were diluted with two volumes of RIPA buffer and mechanically homogenized on ice using a PowerGen 125 homogenizer (Fisher). Samples were incubated on ice for 10 minutes, then centrifuged at 14,000 g for 10 minutes. Supernatants were then analyzed for

mouse ferritin levels using an Ftl-specific ELISA (Abcam, catalog #ab157713) using instructions provided with the kit.

Mathematical modeling

A mathematical model of mouse iron homeostasis using differential equations was modified from a previous version¹⁷ to include uptake of non-transferrin-bound iron from plasma into liver and other compartments and the effect of erythropoietin on iron incorporation into red blood cells and hepcidin expression (manuscript under review). Since this study covers ages where mice are still growing significantly, the mathematical model included variable compartment sizes, which depend on the measured body mass and are interpolated by a third-order polynomial. All simulations and parameter estimations were carried out with COPASI software⁴⁹ as previously performed.¹⁷ Parameter estimation was carried out with the particle swarm algorithm then values were refined with the Hooke-Jeeves algorithm. The value for six-month-old untreated $Tr^{hpx/hpx}$ mice was not used—most mice did not survive to this age. Full models in COPASI and SBML formats⁵⁰ are available at:

<https://data.mendeley.com/datasets/yw85m85mpz/draft?a=5a4874ef-7398-4a9b-88aa-863ee3a70ddd>

Supplementary Figures

Figure S1. Body iron (Fe) levels measured in 2.5- and 4.5-month-old male and female mice for Figure 5A are consistent with body Fe content measured in one- to six-month-old mice from Figure 3B. Body Fe levels from Figure 3B are reproduced here in both left and right panels for $Trf^{+/+}$ ('+/+', orange) and untreated $Trf^{hpx/hpx}$ mice ('hpx/hpx', green) from one to six months of age and in $Trf^{hpx/hpx}$ mice treated with transferrin (TF) from two to six months of age ('hpx/hpx +TF', blue); each point represents mean \pm SEM and five mice, with males and females grouped together. Body Fe levels from Figure 5A are also shown for $Trf^{+/+}$ and untreated and treated $Trf^{hpx/hpx}$ mice at 2.5 and 4.5 months of age; each point represents mean \pm SEM and at least five mice, with males depicted on left panel and females depicted on right panel.

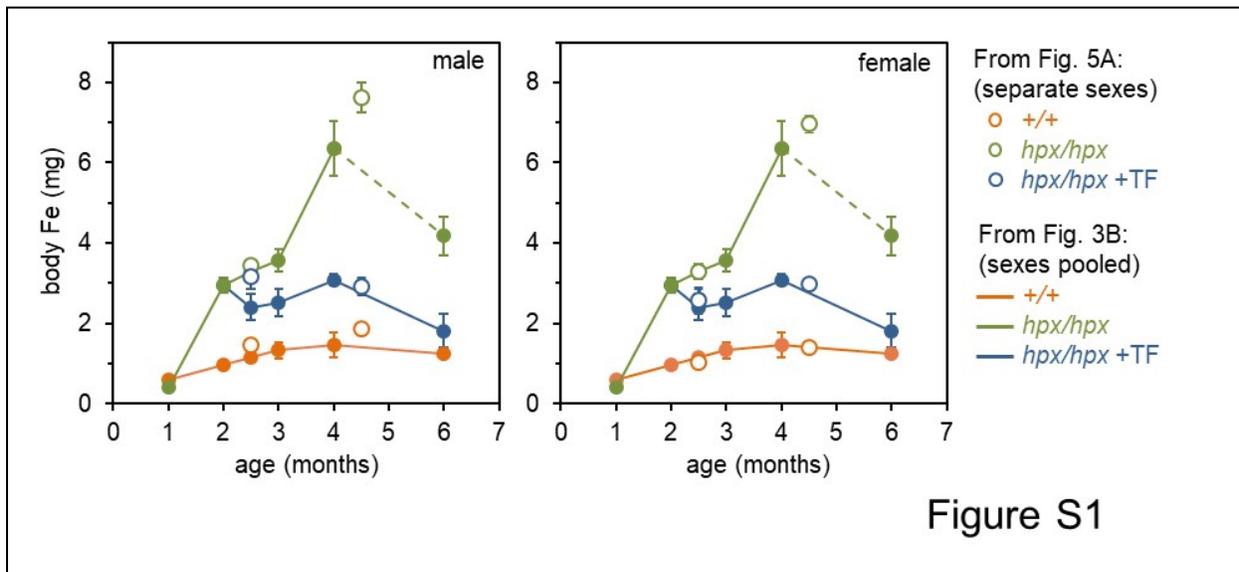


Figure S1

Figure S2. ^{59}Fe is distributed differently between $\text{Trf}^{+/+}$ and untreated $\text{Trf}^{\text{hpx/hpx}}$ mice. (A) Percent total cpm ^{59}Fe plotted versus percent total iron for all organs/compartments from male and female $\text{Trf}^{+/+}$ ('+/+'), untreated $\text{Trf}^{\text{hpx/hpx}}$ ('hpx/hpx'), and treated $\text{Trf}^{\text{hpx/hpx}}$ ('hpx/hpx +TF') mice harvested at the end of the excretion study. (B) Percent total cpm ^{59}Fe for $\text{Trf}^{+/+}$ mice plotted versus untreated and treated $\text{Trf}^{\text{hpx/hpx}}$ mice. Organs/compartments with markedly dissimilar x-axis and y-axis values are labeled. For all panels, each value represents the average value for a different organ/compartment and bars indicate SEM. Both axes are logarithmic. Dashed lines indicate identical values for x- and y-axis. Pearson correlation coefficients ('r') and P values are indicated.

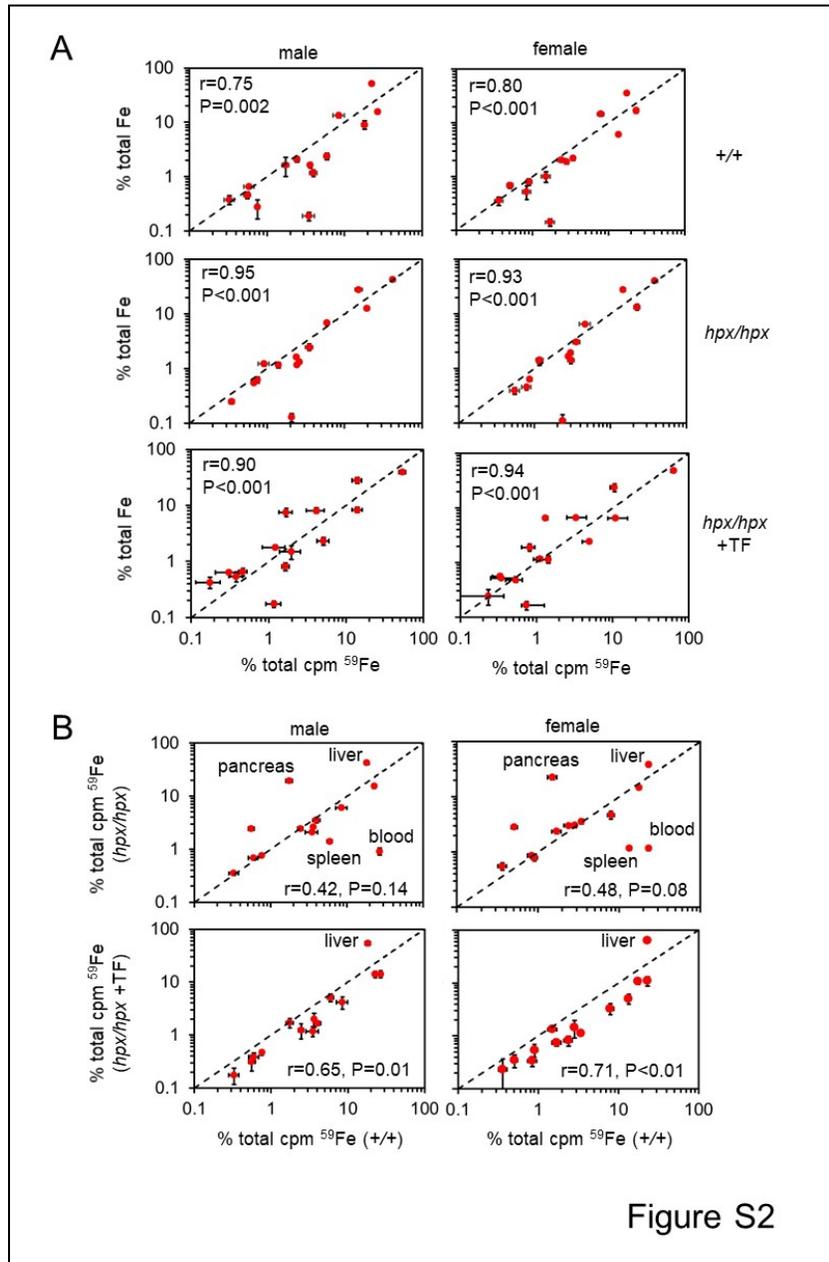


Figure S3. Urine iron levels differ between *Trf*^{+/+} and *Trf*^{hpx/hpx} mice. Urinary iron (Fe) levels in μg Fe excreted per day plotted versus day of collection in male and female *Trf*^{+/+} ('+/+'), untreated *Trf*^{hpx/hpx} ('hpx/hpx'), and treated *Trf*^{hpx/hpx} ('hpx/hpx +TF') mice. 'Day 0' indicates day after ⁵⁹Fe gavage. Each value represents one overnight collection from one mouse using a metabolic cage. Pearson correlations are indicated as 'r' with P value.

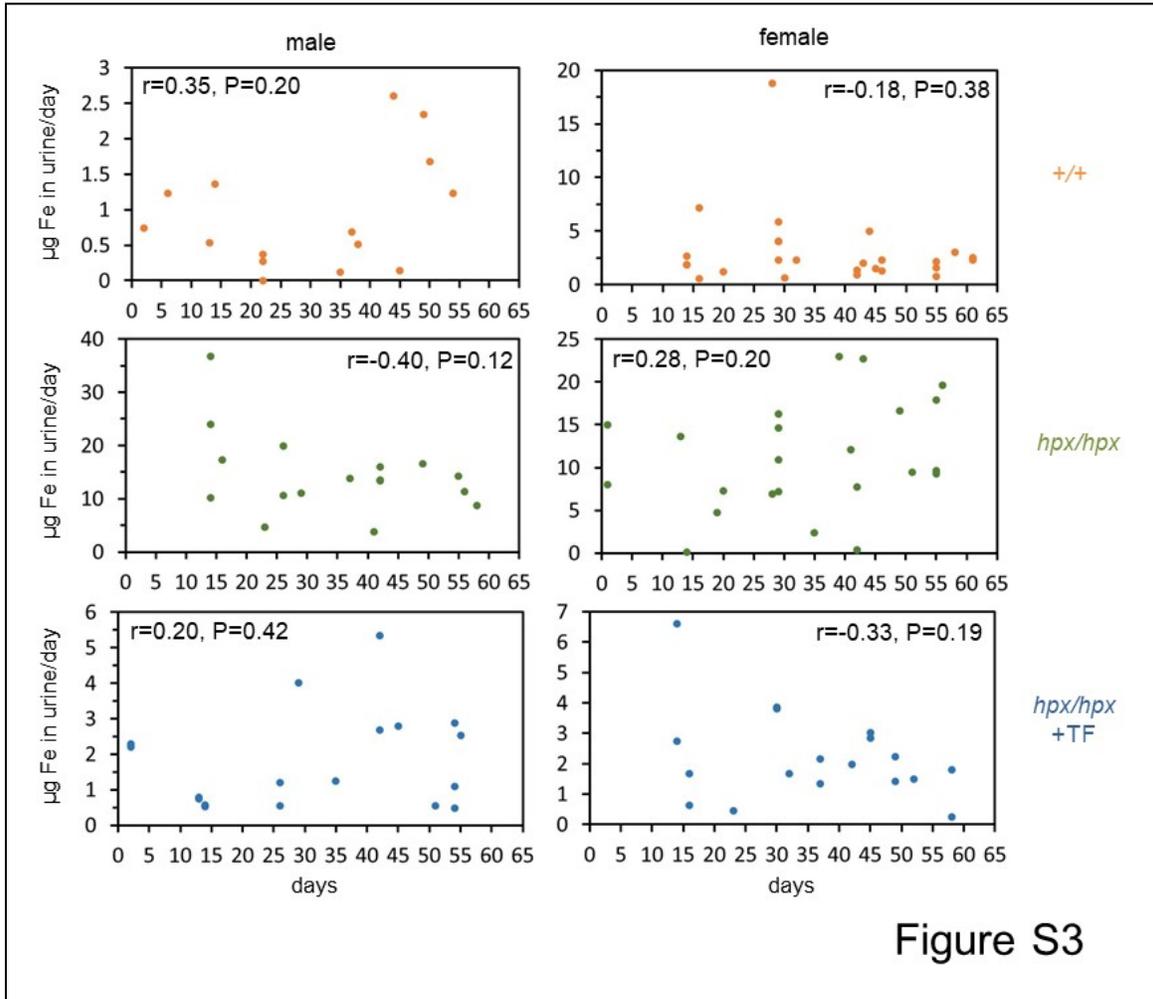


Figure S3

Figure S4. Total fecal iron levels do not differ between *Trf*^{+/+} and *Trf*^{hpx/hpx} mice. (A) Fecal iron (Fe) levels in $\mu\text{g Fe}$ per day plotted versus day of collection in male and female *Trf*^{+/+} ('+/+', orange), untreated *Trf*^{hpx/hpx} ('hpx/hpx', green), and treated *Trf*^{hpx/hpx} ('hpx/hpx +TF', blue) mice. 'Day 0' indicates day after ⁵⁹Fe gavage. Each value represents one overnight collection from one mouse using a metabolic cage. Pearson correlations ('r') and P values are indicated. (B) Average fecal Fe levels for male and female mice in each group. Bars indicate standard error.

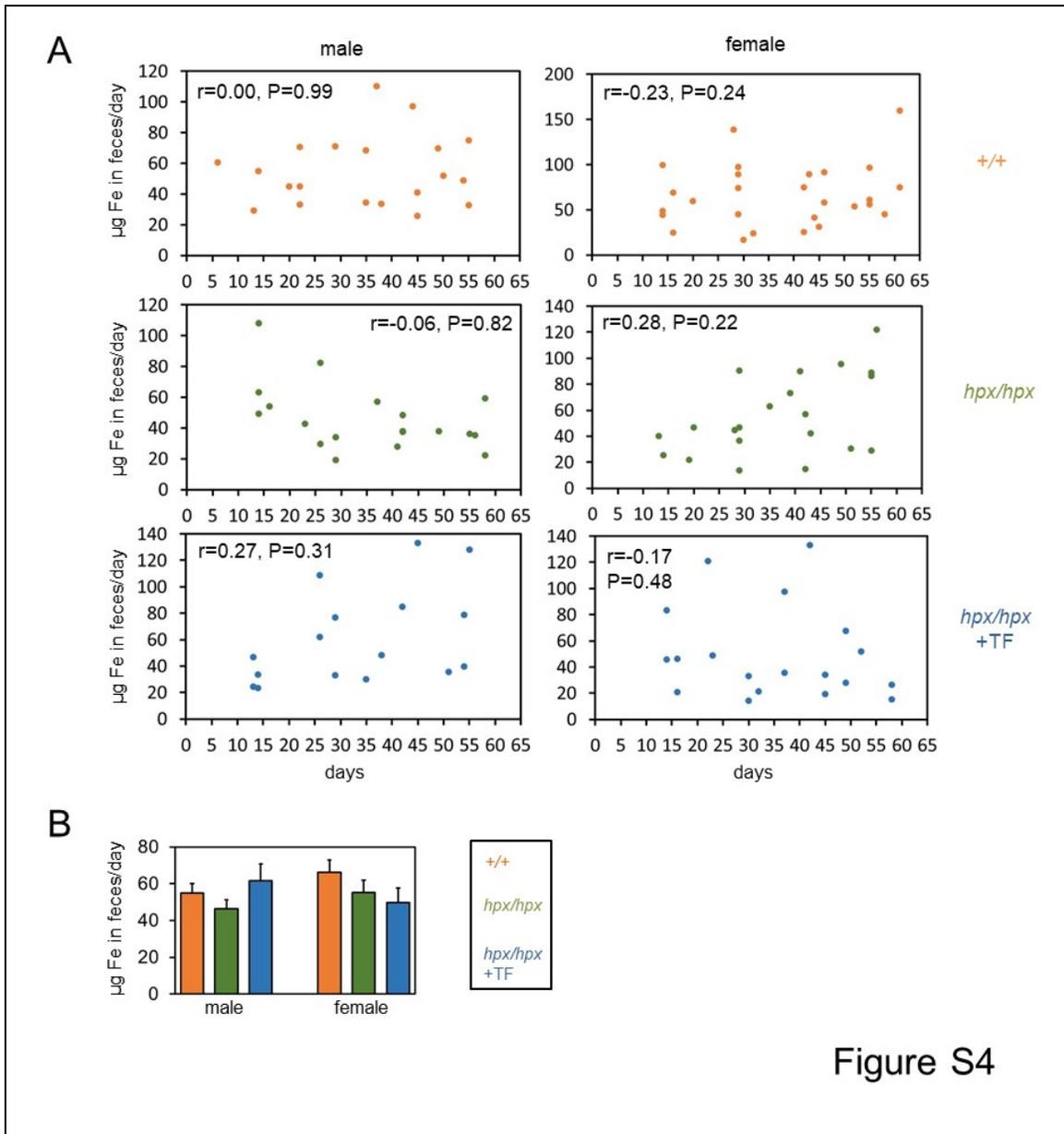


Figure S4

Figure S5. Fecal ferritin levels differ between $Trf^{+/+}$ and $Trf^{hpx/hpx}$ mice. Fecal ferritin levels in μg excreted per day plotted versus day of collection in male and female $Trf^{+/+}$ ('+/+'), untreated $Trf^{hpx/hpx}$ ('hpx/hpx'), and treated $Trf^{hpx/hpx}$ ('hpx/hpx +TF') mice. 'Day 0' indicates day after ^{59}Fe gavage. Each value represents one overnight collection from one mouse using a metabolic cage. Pearson correlations ('r') and P values are indicated.

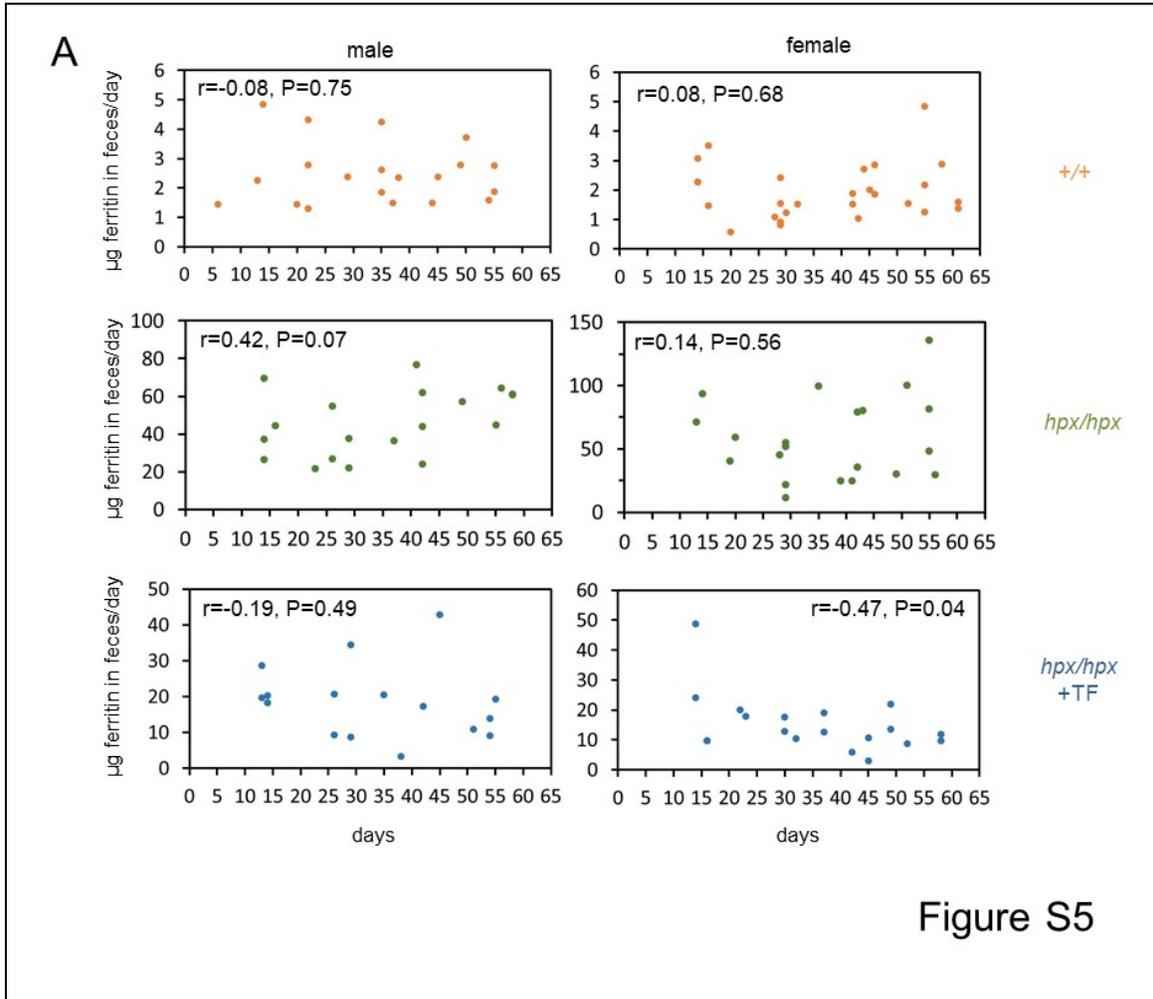


Figure S6. Iron-rich ferritin can be detected using native PAGE. 100 μg $\text{Trf}^{+/+}$ and $\text{Trf}^{\text{hpx/hpx}}$ liver lysates were electrophoresed under non-denaturing, non-reducing conditions on 4-20% polyacrylamide gels. Gels were then stained using the Iron Stain Kit (Sigma) ('Fe stain') or immunoblotted with anti-ferritin heavy chain (Fth) or light chain (Ftl) antibodies.

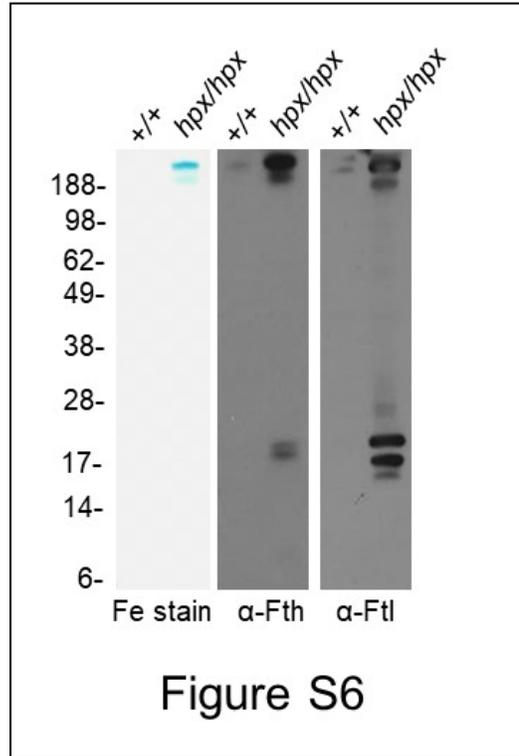


Figure S7. Biliary iron levels are increased in untreated *Trf^{hpx/hpx}* mice. Bile was extracted from gallbladders of 2.5-month-old *Trf^{+/+}* (n=7) and untreated *Trf^{hpx/hpx}* (n=6) mice, then analyzed by graphite furnace atomic absorption spectroscopy for iron (Fe) levels. Male and female mice of the same genotype were pooled. Data are represented as mean \pm SEM. Bracket indicates $P < 0.05$ between values as measured by two-tailed t-test.

