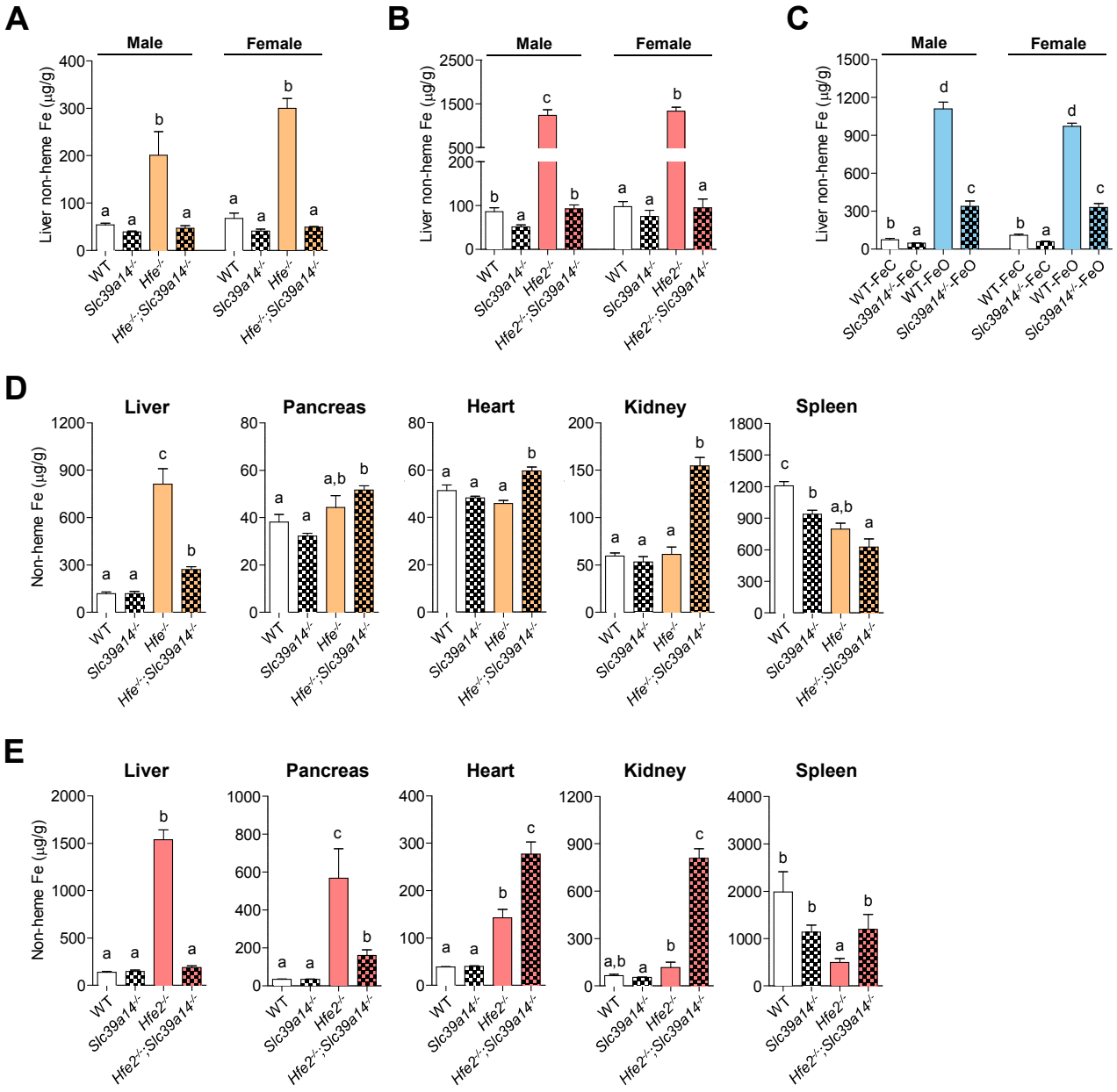

Figure S1. Liver Non-Heme Iron Concentrations by Sex and in Older Mice, Related to Figure 2, Figure 3 and Figure 5

(A) Liver non-heme iron concentrations in male and female WT, *Slc39a14*$^{-/-}$, *Hfe*$^{-/-}$, and *Hfe*$^{-/-}$*;Slc39a14*$^{-/-}$ mice at 4 wk of age (n = 4–6 per group). (B) Liver non-heme iron concentrations in male and female WT, *Slc39a14*$^{-/-}$, *Hfe2*$^{-/-}$, and *Hfe2*$^{-/-}$*; Slc39a14*$^{-/-}$ mice at 6 wk of age (n = 3–8 per group). (C) Liver non-heme iron concentrations in male and female WT-FeC, *Slc39a14*$^{-/-}$-FeC, WT-FeO, and *Slc39a14*$^{-/-}$-FeO mice at 7 wk of age (n = 3–6 per group). (D) Tissue non-heme iron concentrations in WT, *Slc39a14*$^{-/-}$, *Hfe*$^{-/-}$, and *Hfe*$^{-/-}$*;Slc39a14*$^{-/-}$ mice at 16 wk of age (n = 4–5 females per group). (E) Tissue non-heme iron concentrations in WT, *Slc39a14*$^{-/-}$, *Hfe2*$^{-/-}$, and *Hfe2*$^{-/-}$*;Slc39a14*$^{-/-}$ mice at 12 wk of age (n = 5–7 females per group). All data are shown as the mean ± SEM. Means without a common superscript differ significantly ($p < 0.01$).

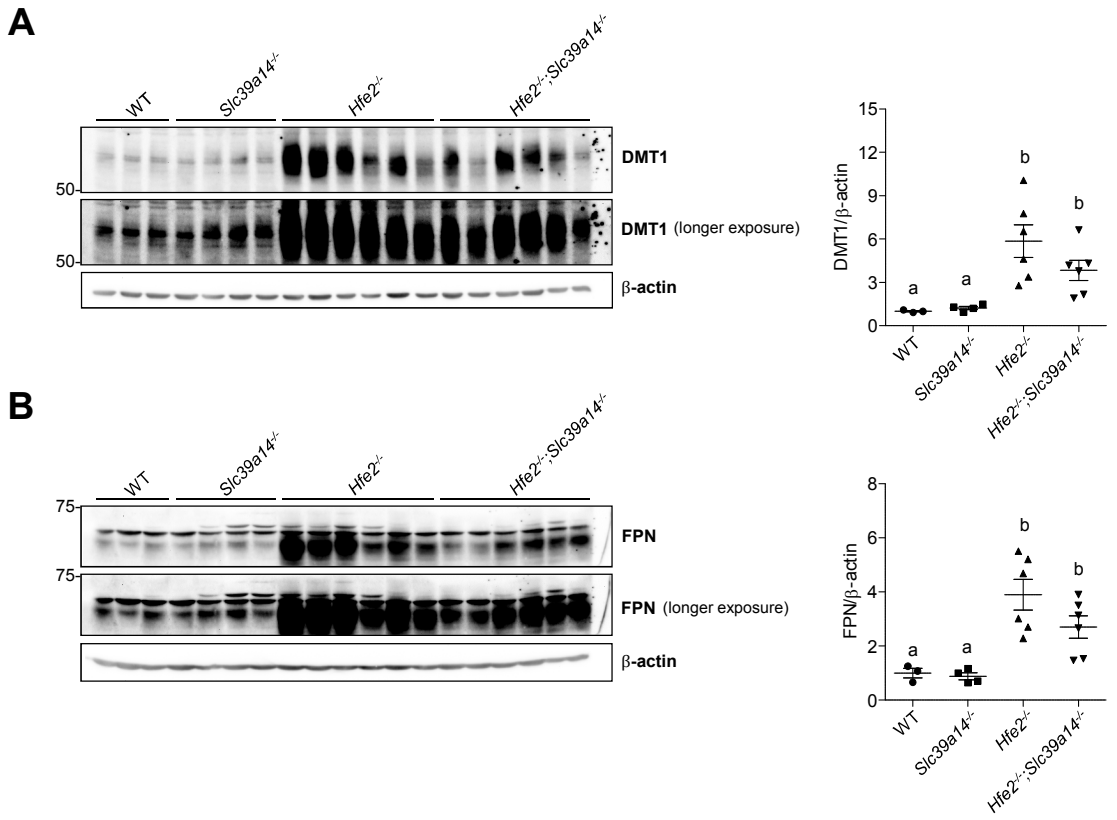

Figure S2. Immunoblot Analysis of Intestinal DMT1 and FPN Protein Levels, Related to Figure 3B
Enterocytes from the proximal small intestine were isolated from WT, *Slc39a14*$^{-/-}$, *Hfe2*$^{-/-}$, and *Hfe2*$^{-/-}$*;Slc39a14*$^{-/-}$ mice at 5 wk of age and analyzed for DMT1, FPN, and β-actin protein levels by immunoblotting. Representative immunoblots are shown for (A) DMT1 and (B) FPN. Densitometric analyses of normalized protein levels (n = 3–6 per group) are shown at right. All data are shown as the mean ± SEM. Means without a common superscript differ significantly ($p < 0.0001$).

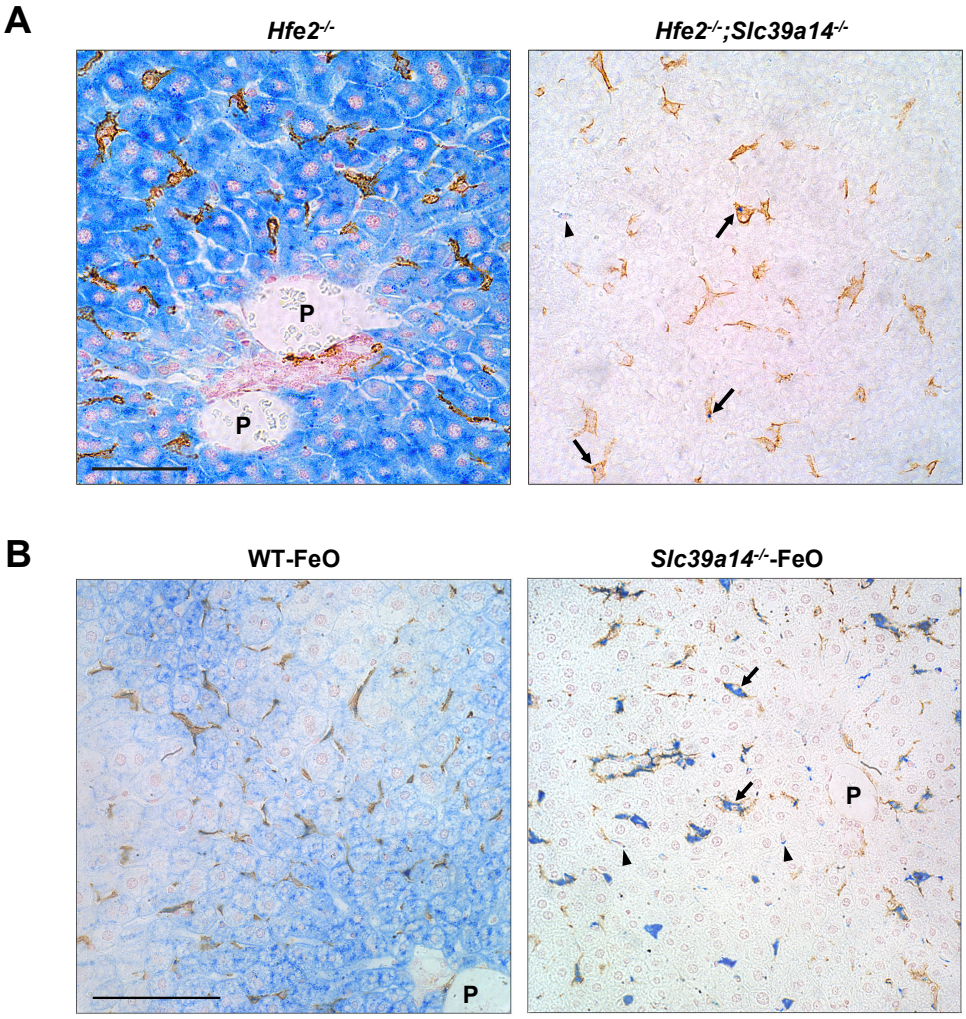

Figure S3. *Slc39a14*$^{-/-}$ Mice Load Hepatic Iron in Kupffer Cells, but not Hepatocytes, in Response to Iron Overload, Related to Figure 3 and Figure 5

Representative images of co-staining for iron deposits (Perls' stain, blue) and F4/80 (brown stain) as a marker for Kupffer cells in paraffin-embedded liver sections of (A) *Hfe2*$^{-/-}$ and *Hfe2*$^{-/-}$*;Slc39a14*$^{-/-}$ at 6 wk of age (n = 2 per group), and (B) WT-FeO (n = 1) and *Slc39a14*$^{-/-}$-FeO (n = 2) mice at 7 wk of age. Branches of the portal (P) veins are indicated. Arrows indicate iron-loaded Kupffer cells. Arrowheads indicate iron-loaded cells that are stained negative for F4/80. Scale bars, 50 μm (A) and 100 μm (B).

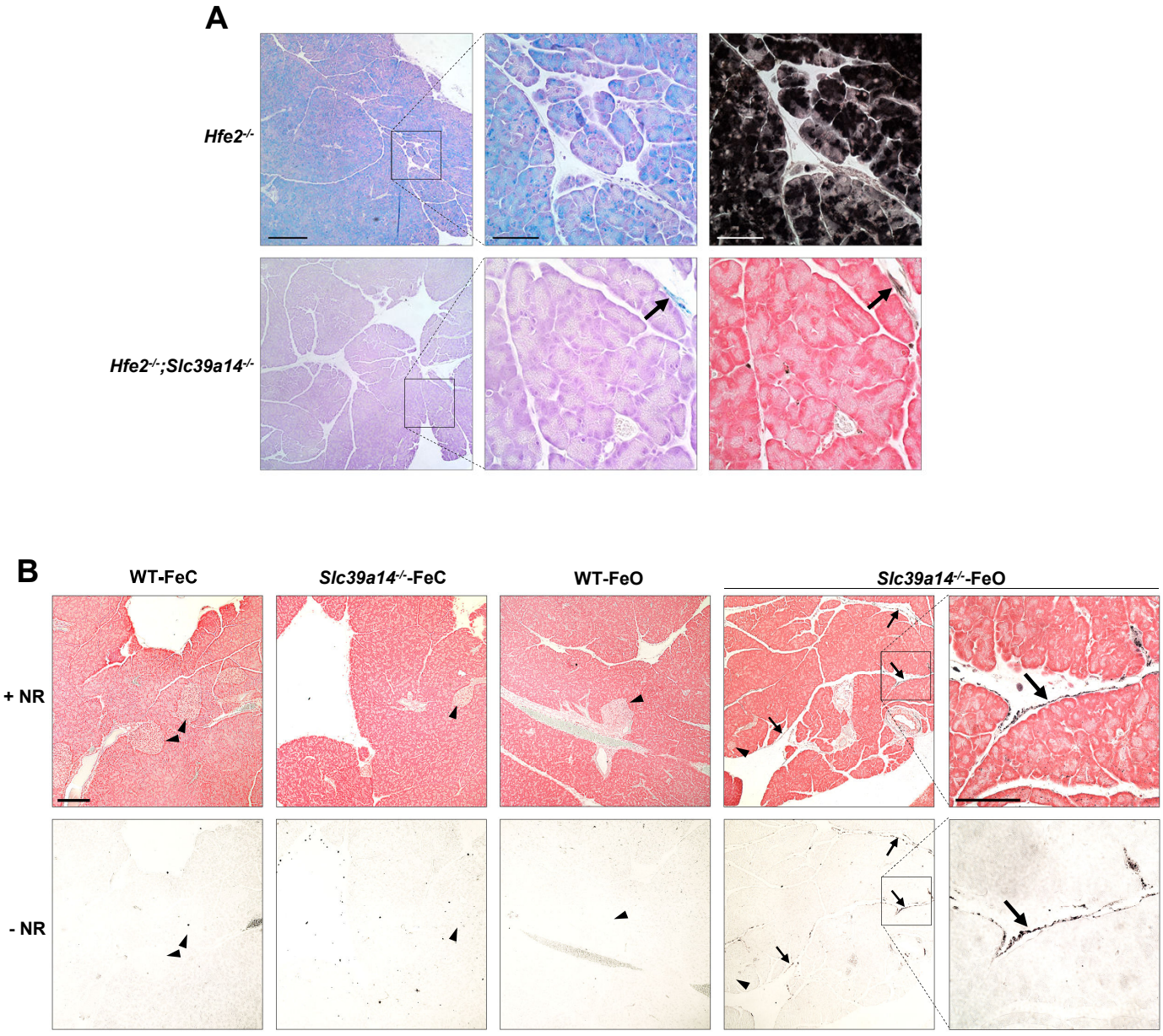

Figure S4. *Slc39a14*$^{-/-}$ Mice Display Altered Pancreatic Iron Loading in *Hfe2* Deficiency and in Response to Dietary Iron Overload, Related to Figure 4A and Figure S1E

(A) Representative image of Perls' iron stain in paraffin-embedded pancreas sections of *Hfe2*$^{-/-}$ and *Hfe2*$^{-/-}$*;Slc39a14*$^{-/-}$ mice at 12 wk of age (n=2 per group). (Far right panel) Serial section of pancreas processed in parallel by using DAB-enhanced Perls' iron stain (black stain) with neutral red counterstain. Arrows indicate iron deposits. Scale bars, left panel 250 μm; middle and right panels, 50 μm. (B) Representative images of DAB-enhanced Perls' iron stain in paraffin-embedded pancreas sections of WT-FeC (n = 2), *Slc39a14*$^{-/-}$-FeC (n = 2), WT-FeO (n = 4), and *Slc39a14*$^{-/-}$-FeO (n = 4) mice at 7 wk of age. Serial sections of pancreas processed in parallel by using DAB-enhanced Perls' iron stain with or without neutral red counterstain (+NR and –NR, respectively). Arrowheads indicate pancreatic islets. Arrows indicate iron deposits in *Slc39a14*$^{-/-}$-FeO mice. Scale bars, 200 μm; far right panel, 100 μm.

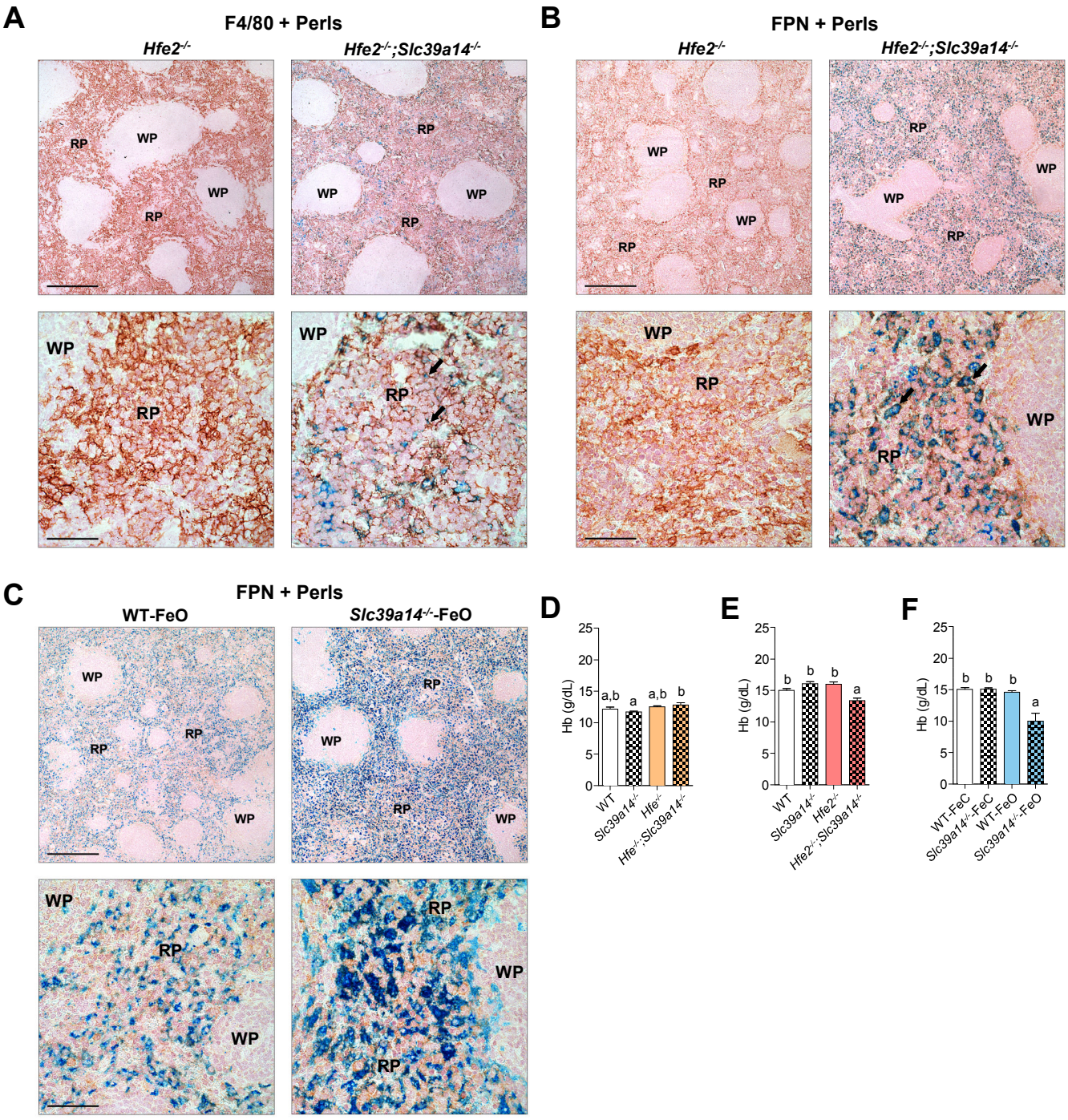

Figure S5. *Slc39a14*$^{-/-}$ Mice Load Iron in Splenic Red Pulp Macrophages, Related to Figure 2A, Figure 3A and Figure 5A

(A) Representative images of co-staining for iron deposits (Perls' stain, blue) and F4/80 (brown stain) as a marker for splenic red pulp macrophages in paraffin-embedded spleen sections of *Hfe2*$^{-/-}$ and *Hfe2*$^{-/-}$;*Slc39a14*$^{-/-}$ mice (n = 2 per group) at 6 wk of age. Arrows in the lower-right panel indicate iron-loaded F4/80-positive macrophages in the spleen of *Hfe2*$^{-/-}$;*Slc39a14*$^{-/-}$ mice. (B and C) Representative images of co-staining for iron deposits (Perls' stain, blue) and FPN (brown stain) in paraffin-embedded spleen sections. (B) *Hfe2*$^{-/-}$ and *Hfe2*$^{-/-}$;*Slc39a14*$^{-/-}$ at 6 wk of age and (n=2 per group). Arrows in the lower right panel indicate iron-loaded FPN-positive macrophages in the spleen of *Hfe2*$^{-/-}$;*Slc39a14*$^{-/-}$ mice. (C) WT-FeO and *Slc39a14*$^{-/-}$-FeO mice at 7 wk of age (n = 2 per group). Splenic white pulp (WP) and red pulp (RP) are indicated. Scale bars in A, B, and C: top panel 300 µm, bottom panel 50 µm. (D) Hemoglobin levels in WT, *Slc39a14*$^{-/-}$, *Hfe*$^{-/-}$, and *Hfe*$^{-/-}$;*Slc39a14*$^{-/-}$ mice at 4 wk of age (n = 6 per group) (E) Hemoglobin levels in WT, *Slc39a14*$^{-/-}$, *Hfe2*$^{-/-}$, and *Hfe2*$^{-/-}$;*Slc39a14*$^{-/-}$ mice at 6 wk of age (n = 7-13 per group), and (F) Hemoglobin levels in WT-FeC, *Slc39a14*$^{-/-}$-FeC, WT-FeO, and *Slc39a14*$^{-/-}$-FeO mice at 7 wk of age (n = 4-8 per group). All data are shown as the mean ± SEM (mixed sex). Means without a common superscript differ significantly ($p < 0.05$) as analyzed by one-way ANOVA.

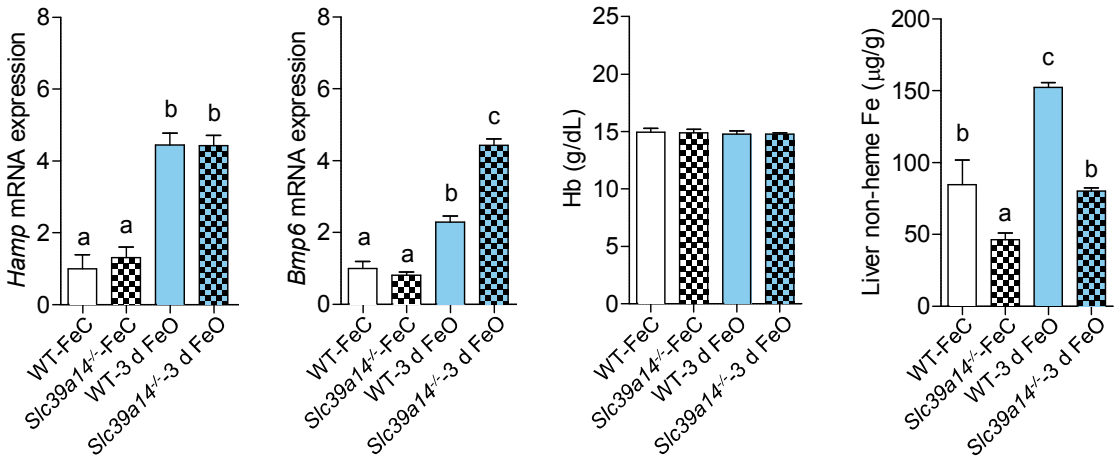

Figure S6. Hepatic Expression of *Hamp* and *Bmp6* mRNA in *Slc39a14*$^{-/-}$ Mice with Short-Term Dietary Iron Overload, Related to Figure 6C

WT and *Slc39a14*$^{-/-}$ male mice (n=6 per group) were fed a control diet containing 240 ppm iron (FeC) from weaning. Three days before sacrifice at 7 wk of age, half of the WT and *Slc39a14*$^{-/-}$ mice were switched to an iron-overloaded diet containing 1% carbonyl iron (designated as WT- and *Slc39a14*$^{-/-}$ -3 d FeO). Livers were analyzed for relative *Hamp* and *Bmp6* mRNA levels normalized to *Rpl13a*. Hemoglobin and liver non-heme iron concentrations were also measured. All data are shown as the mean ± SEM. Means without a common superscript differ significantly ($p < 0.0001$).

Table S1. Hematological Parameters in 6-Week-Old *Hfe2*$^{-/-}$;*Slc39a14*$^{-/-}$ Mice, Related to Figure 3 and Figure S5

Parameter	WT	n	*Slc39a14*$^{-/-}$	n	*Hfe2*$^{-/-}$	n	*Hfe2*$^{-/-}$;*Slc39a14*$^{-/-}$	n
RBC (10^{12}/l)	9.93 ± 0.17^{b}	8	9.40 ± 0.24^{a,b}	10	9.80 ± 0.18^{b}	13	8.46 ± 0.45^{a}	6
Hb (g/dl)	15.14 ± 0.19^{a,b}	8	14.59 ± 0.44^{b}	10	15.65 ± 0.21^{b}	13	14.07 ± 0.17^{a}	6
Hct (%)	50.64 ± 0.81^{b}	8	49.54 ± 1.04^{b}	10	52.65 ± 0.82^{b}	13	43.51 ± 2.20^{a}	6
MCV (fl)	51.06 ± 0.72	8	52.76 ± 0.67	10	53.82 ± 0.67	13	51.35 ± 1.06	6
MCH (pg)	15.28 ± 0.24	8	15.51 ± 0.28	10	16.02 ± 0.19	13	16.82 ± 0.92	6
MCHC (g/dl)	29.91 ± 0.18	8	29.46 ± 0.69	10	29.76 ± 0.23	13	32.97 ± 2.32	6
RDW (%)	12.56 ± 0.18^{a}	8	13.20 ± 0.19^{a}	10	13.02 ± 0.19^{a}	13	14.50 ± 0.23^{b}	6
HDW (g/dl)	1.56 ± 0.01^{a}	8	1.57 ± 0.03^{a}	10	1.60 ± 0.02^{a}	13	2.47 ± 0.12^{b}	6
Reticulocytes (%)	2.33 ± 0.13^{a}	7	3.17 ± 0.44^{a}	9	2.65 ± 0.34^{a}	10	5.58 ± 0.79^{b}	5
Reticulocyte counts (10^{3}/µl)	233.2 ± 16.07^{a}	6	286.0 ± 34.87^{a}	9	253.3 ± 30.87^{a}	10	469.2 ± 71.65^{b}	5

RBC, red blood cell; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; HDW, hemoglobin distribution width. Data are reported as mean ± SEM and analyzed by using one-way ANOVA. Means without common superscript differ significantly ($p < 0.01$).

Table S2. Hematological Parameters in *Slc39a14*$^{-/-}$ Mice Subjected to Dietary Iron Overload for 4 Weeks, Related to Figure 5 and Figure S5

Parameter	WT-FeO	n	*Slc39a14*$^{-/-}$-FeO	n	p-value
RBC (10^{12}/l)	9.81 ± 0.24	6	7.21 ± 0.29***	3	0.0003
Hb (g/dl)	15.88 ± 0.23	6	12.20 ± 0.74***	3	0.0004
Hct (%)	53.32 ± 0.91	6	41.40 ± 1.30***	3	0.0001
MCV (fl)	54.42 ± 0.79	6	57.53 ± 0.75*	3	0.0422
MCH (pg)	16.23 ± 0.18	6	16.87 ± 0.32	3	0.0996
MCHC (g/dl)	29.78 ± 0.25	6	29.40 ± 0.81	3	0.5692
RDW (%)	16.00 ± 0.38	6	17.50 ± 0.55	3	0.0588
HDW (g/dl)	1.85 ± 0.12	6	3.13 ± 0.30**	3	0.0019
Reticulocytes (%)	2.80 ± 0.47	6	20.60 ± 6.80	2	ND
Reticulocyte counts (10^3/µl)	274.3 ± 37.09	6	1422 ± 452.0	2	ND

RBC, red blood cell; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; HDW, hemoglobin distribution width. Data are reported as mean ± SEM and analyzed by using unpaired Student's *t*-test. ***p < 0.001, **p < 0.01, *p < 0.05. ND, not determined.

Table S3. Numbers of Male (M) and Female (F) Mice Represented in Each Figure, Related to Experimental Procedures

	WT		*Slc39a14*$^{-/-}$		*Hfe(2)*$^{-/-}$		*Hfe(2)*$^{-/-}$ *;Slc39a14*$^{-/-}$		WT-FeC		*Slc39a14*$^{-/-}$-FeC		WT-FeO		*Slc39a14*$^{-/-}$-FeO		*Slc39a14*$^{+/-}$	
Figure	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1A	2	3	4	6														
1B	4	0	4	0													4	0
1C	4	6	9	6													5	4
1D	1	2	1	2														
1E	2	3	1	3														
2A	4	5	5	2	5	3-4	3	2										
2B	2	6	2	3	6	7	3	4										
2C	2	3	2	3	4	2	2	3										
2D-F	2	1	1	2	1	2	1	2										
2G-I	3	3	3	3	3	2	3	3										
2J	6	3	5	4	7	6	5	7										
2K	6	6	5	3	5	7	4	2										
3A	8	3	3	1	5-6	3	6	1										
3B-E	5	5	3	3	3	5	3	1										
3F-H	2	1	2	1	2	1	2	1										
3I-K	4	1	3	1	4	1	5	1										
3L	7	2	4	1	3	2	6	1										
3M	8	3	5	2	6	3	6	1										
4A-B	2	2	3	1	3	1	3	1										
5A									4	3	2	5	3-5	3-5	3-5	3-5		
5B-D									2	1	2	1	2	1	2	2		
5E-G									4	3	2	4	7	4	4	4		
5H									2	4	2	3	3	3	3	3		
5I									5	6	4	7	10	6	7	5		
6A	3	4	4	3	4	4	4	5										
6B	5	3	3	1	6	4	6	1										
6C									3	3	3	3	3	3	3	3		

Table S4. Numbers of Male (M) and Female (F) Mice Represented in Each Supplemental Figure or Table, Related to Experimental Procedures

	WT		*Slc39a14*$^{-/-}$		*Hfe(2)*$^{-/-}$		*Hfe(2)*$^{-/-}$ *;Slc39a14*$^{-/-}$		WT-FeC		*Slc39a14*$^{-/-}$-FeC		WT-FeO		*Slc39a14*$^{-/-}$-FeO		*Slc39a14*$^{+/-}$	
Figure	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
S1A	4	6	5	4	5	4	4	5										
S1B	8	4	5	5	6	5	6	3										
S1C									4	5	4	5	6	6	3	4		
S1D	0	4-5	0	4-5	0	4-5	0	4-5										
S1E	0	5	0	5	0	5-7	0	5										
S2A-B	2	1	2	2	3	3	5	1										
S3A					1	1	1	1										
S3B													1	0	1	1		
S4A					1	1	1	1										
S4B									1	1	1	1	2	2	2	2		
S5A-B					1	1	2	0										
S5C													1	1	1	1		
S5D	3	3	3	3	2	4	3	3										
S5E	9	4	4	3	6	5	7	3										
S5F									4	3	2	5	5	3	3	1		
S6									3	0	3	0	3	0	3	0		
Table S1	4-5	2-4	3	6-7	8-10	2-3	4	1-2										
Table S2													4	2	1	1-2		

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Primary Hepatocyte Isolation and Hepatocyte NTBI Uptake

Mouse livers were perfused via the portal vein with a calcium-free buffer (140 mM NaCl, 7 mM KCl, 10 mM HEPES, pH 7.3) 8 ml/min for 10 min. Subsequently, the livers were perfused with collagenase solution (67 mM NaCl, 7 mM KCl, 5 mM $CaCl_2$, 100 mM HEPES (pH 9.3), 0.4% collagenase) via portal vein, 7 ml/min for 14 min. The viable hepatocyte population was filtered through a nylon mesh followed by two centrifugations to remove debris. Hepatocytes were subsequently resuspended in Waymouth's medium (Invitrogen) containing 100 units/mL penicillin, 100 µg/mL streptomycin, 10% fetal calf serum, 100 nmol/L insulin, and 100 nmol/L dexamethasone. The purified viable hepatocytes, as judged by trypan blue, were seeded in collagen-coated plates at 1×10^6 cells per well in a 6-well plate in Waymouth's medium as noted above. Hepatocytes were used after an overnight incubation in humidified 5% CO_2 -95% air at 37 °C. For NTBI uptake, hepatocytes were washed 3 times with warm HBSS medium and incubated with NTBI uptake medium (DMEM supplemented with 2% bovine serum albumin and 2 µM ^{59}Fe-labeled ferric citrate) for 1 h. Cells were then washed twice with cold HBSS medium following by 3 washes with cold iron chelator solution (1 mM bathophenanthroline disulfonate/1 mM diethylenetriaminepentaacetate in PBS) and lysed in SDS lysis buffer (0.2 N NaOH/0.2% SDS). Cell lysates were subsequently subjected to gamma counting and protein concentrations were determined by using the *RC DC* assay (Bio-Rad).

Cobalt, DAB-enhanced Perls' Iron Staining

After incubating tissue-section slides with Perls' stain, slides were washed with distilled water and incubated in 0.3% (v/v) H_2O_2 in methanol for 20 min to quench endogenous peroxidase activity. Slides were then incubated with intensifying solution (0.05% (w/v) DAB, 0.015% (v/v) H_2O_2 and 0.025% (w/v) $CoCl_2$ in PBS) for 15 min. The reaction was stopped by rinsing with distilled water. Slides were counterstained with 1% neutral red solution or hematoxylin QS (Vector Labs) and visualized with an Olympus IX70 inverted microscope and QImaging QCapture Pro 6.0 Software.

Immunoblot Analyses

SLC39A14 protein levels in liver. Liver samples were homogenized in RIPA buffer containing 1× Complete Mini Protease Inhibitor Cocktail (Roche). Protein concentrations of the lysates were determined by using the *RC DC* Protein Assay (Bio-Rad). Samples were mixed with Laemmli buffer without heating and electrophoretically separated on an SDS-polyacrylamide gel (7.5% acrylamide), transferred to nitrocellulose, and incubated for 1 h in blocking buffer (5% nonfat dry milk in Tris-buffered saline-Tween 20, TBS-T). Blots were then incubated overnight at 4 °C in blocking buffer containing affinity-purified rabbit anti-ZIP14 antibody (1 μg/mL; (Nam et al., 2013)). After four washes in TBS-T, blots were incubated 40 min with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG secondary antibody (1:2000; Amersham Biosciences). Blots were then washed with TBS-T and TBS, and immunoreactivity was visualized by using enhanced chemiluminescence (SuperSignal West Pico, Pierce) and the FluorChem E digital darkroom (ProteinSimple). To indicate lane loading, blots were stripped and reprobed

with mouse anti-tubulin (1:5000; Sigma-Aldrich) followed by goat HRP-conjugated anti-mouse (1:10000; Invitrogen) secondary antibody.

DMT1 and FPN protein levels in enterocytes. Intestinal enterocytes were isolated from 5-week-old WT, *Slc39a14*$^{-/-}$, *Hfe2*$^{-/-}$, and *Hfe2*$^{-/-}$*;Slc39a14*$^{-/-}$ mice after an overnight fast as described elsewhere (Ranganathan et al., 2012). Immediately after sacrificing mice, approximately 10 cm of proximal intestine was removed, cut into two segments, and everted on individual wooden sticks. After two washes in ice-cold PBS, segments were incubated in ice-cold PBS containing 1.5 mM EDTA for 3 min with gentle rotation to release enterocytes, and were subsequently pelleted at 500 × *g* for 3 min at 4 °C. Enterocytes were washed three times with ice-cold PBS and lysed in ice-cold RIPA buffer containing 3× Complete Mini Protease Inhibitor Cocktail (Roche). Enterocyte lysates were sonicated on ice and centrifuged at 10,000 × *g* for 15 min at 4 °C to remove nuclei and cell debris. Supernatants were collected for subsequent analyses or stored at -80 °C. Protein concentrations of the lysates were determined by using the *RC DC* Protein Assay (Bio-Rad). Samples were mixed with Laemmli and incubated for 30 min at 37 °C for DMT1 samples or without heating for FPN samples and eletrophoretically separated on an SDS-polyacrylamide gel (7.5% acrylamide), transferred to nitrocellulose, and incubated for 1 h in blocking buffer (5% nonfat dry milk in Tris-buffered saline-Tween 20, TBS-T). Blots were then incubated overnight at 4 °C in blocking buffer containing rabbit anti-DMT1 antibody (1:2000, kind gift from Dr. Francois Canonne-Hergaux, INSERM, Toulouse, France) or rabbit anti-FPN antibody (2.5 µg/mL, (Knutson et al., 2005)). After four washes in TBS-T, blots were incubated 40 min with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG secondary antibody (Amersham Biosciences). Blots were then washed with TBS-T and TBS, and

immunoreactivity was visualized by using enhanced chemiluminescence (SuperSignal West Pico, Pierce) and the FluorChem E digital darkroom (ProteinSimple). To indicate lane loading, blots were stripped and reprobed with mouse anti-β-actin (1:2000, AM4302, Ambion) followed by goat HRP-conjugated anti-mouse (1:10000; Invitrogen) secondary antibody.

Primers for Quantitative Reverse-Transcriptase PCR

The following primers were used:

Hepcidin *(Hamp)*

forward: 5'-AGCCCCTCAACCCCATTATT-3'

reverse: 5'-TCGTCTTTATTTCAAGGTCATTGG-3'

Bone morphogenetic protein 6 *(Bmp6)*

forward: 5'-AGCACAGAGACTCTGACCTATTTTT-3'

reverse: 5'-CCACAGATTCTAGTTGC-TGTGA-3'

Ribosomal protein L13a (*Rpl13a*):

forward: 5'-GCAAGTTCACAGAGGTCCTCAA-3'

reverse: 5'-GGCATGAGGCAAACAGTCTTTA-3'

Iron Staining and Immunohistochemistry for F4/80 and FPN

Paraffin-embedded tissue sections were processed for antigen retrieval in 0.1 % trypsin in HBSS buffer for 30 min at 37 °C. Slides were then incubated for 30 min with rat anti-F4/80 antibody (1:250; sc-59171, Santa Cruz) or rabbit anti-FPN antibody (1 μg/ml; (Knutson et al., 2005)) followed by a 30 min incubation with biotinylated species-specific secondary antibodies (1:200). Slides were then incubated with streptavidin-HRP for 30

min (Vectastain, ABC kit; Vector Labs) and the immunoperoxidase reaction was visualized by using the VECTOR NovaRED Peroxidase HRP Substrate Kit (Vector Labs). After the immunohistochemistry procedure, the same slides were subsequently processed for Perls' Prussian blue iron staining and were not counterstained. Slides were visualized with an Olympus IX70 inverted microscope and QImaging QCapture Pro 6.0 Software.

Short-Term Dietary Iron Overload Study

Slc39a14$^{-/-}$ mice on a mixed 129+Ter/SvJcl x C57BL/6 x Balb/cJ background and their WT control littermates (n=6 per group) were weaned at 3 wk of age and fed a standard diet (Teklad 7912, Harlan Laboratories) containing 240 ppm iron for 4 wk. Three days before sacrifice at 7 wk of age, half of the WT and *Slc39a14*$^{-/-}$ mice were switched to a modified AIN-93G rodent diet formulated to contain 10,000 ppm (1%) carbonyl iron (Research Diets, diet D08090805i).

Hematological Parameters

Hematological parameters were determined by using the Advia 120 Hematology System (Siemens) at the University of Florida Veterinary Diagnostics Laboratories (Gainesville, FL).

SUPPLEMENTAL REFERENCES

Knutson, M.D., Oukka, M., Koss, L.M., Aydemir, F., and Wessling-Resnick, M. (2005). Iron release from macrophages after erythrophagocytosis is up-regulated by ferroportin 1 overexpression and down-regulated by hepcidin. Proc. Natl. Acad. Sci. U. S. A. 102, 1324-1328.

Nam, H., Wang, C.Y., Zhang, L., Zhang, W., Hojyo, S., Fukada, T., and Knutson, M.D. (2013). ZIP14 and DMT1 in the liver, pancreas, and heart are differentially regulated by iron deficiency and overload: implications for tissue iron uptake in iron-related disorders. Haematologica 98, 1049-1057.

Ranganathan, P.N., Lu, Y., Fuqua, B.K., and Collins, J.F. (2012). Discovery of a cytosolic/soluble ferroxidase in rodent enterocytes. Proc. Natl. Acad. Sci. U. S. A. 109, 3564-3569.