Supplemental Materials and Methods

Materials and Methods

Phlebotomy and PHZ-induced hemolytic anemia

For **PHZ treatment**, mice were given two doses of phenylhydrazine hydrochloride solution (60 mg/kg body weight) via i.p. injection on two consecutive days, and 50 μL blood was then collected on days 1, 3, 6, 10 for analysis of blood indices. For **Phlebotomy experiments**, 300 μL blood was collected from the facial veins on day 0, 1, 3, 5, 7 and 10 to induce anemia and for analysis of blood indices.

ELISA to measure hepcidin and ferritin

ELISA kits were used to measure hepcidin (Intrinsic LifeSciences) and ferritin (Abcam) levels according to manufacturer's protocols.

Antioxidant enzyme activity assay

The enzyme activities of glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD) were measured with the activity kits from Abcam according to manufacturer's protocols.

Non-heme iron and serum iron measurements

Non-heme iron of tissues and cells was measured according to the previous description.[1] **Serum iron** was measured with Stanbio Iron and Total Iron Binding Capacity kit (Stanbio Laboratory) following the manufacturer's protocol.

Urine iron measurement

Total iron concentrations in the urine samples were measured by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent model 7900). For each sample, 200 μL of concentrated trace-metal-grade nitric acid (Fisher) was added to 75-210 μL of sample taken in a 15 mL Falcon tube. Tubes were sealed with electrical tape to prevent evaporation, taken inside a 1L glass beaker, and then placed at 90 ^{0}C oven. After

overnight digestion, each sample was diluted with 4 mL deionized water, and then analyzed by ICP-MS.

LC-MS/MS analysis

Peripheral bloods from WT mice were washed with cold PBS for 3 times to remove the buffy coat, then RBC ghost membranes were prepared as we described previously.[2] Briefly, RBCs were lysed in 10 volumes of 5 mM sodium phosphate buffer, pH8.0, and then RBC ghosts were pelleted by centrifugation at 10000g for 5 min at 4^{o}C. After washing with PBS for five times, RBC ghosts were lysed in 3 volumes of RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 0.1% SDS, 0.5% Na.Deoxycholate, 1% Triton X-100, 5mM EDTA, Halt protease inhibitor). For mass spectrometry analysis, the protein samples were reduced with TCEP (Sigma-Aldrich) and alkylated with NEM (Sigma-Aldrich), followed by trypsin digestion overnight at 37 °C. A 2nd digestion was performed at 37 °C for 8 hr. Tryptic digests were acidified and desalted with Oasis HLB μElution plate (Waters). Samples were analyzed using an UltiMate 3000 Nano LC system coupled to an Orbitrap Fusion Lumos mass spectrometer equipped with an EasySpray Source (Thermo Fisher Scientific). Peptides were separated over a 60-min gradient from 2-24% MPB (0.1% formic acid, 98% acetonitrile, 1.9% water) on a ES802 column (Thermo Fisher Scientific) at a flow rate of 300 nL/min. The LC-MS/MS data were acquired in data-dependent mode. The resolution of the survey scan (375-1500 m/z) was set at 120k. The automated gain control target was set to 2×10^5. Precursors with charge states 2−6 and intensity higher than 1×10^4 were selected for HCD fragmentation with an exclusion window of 20 sec. The quadrupole isolation window was 1.6 m/z. MS/MS data were acquired in ion trap with Rapid scan rate. Raw data were processed with Mascot Distiller, and database search was performed using Mascot Daemon (2.6.0) (Matrix Science) against Sprot Mouse database with a decoy search as well. The error tolerances for precursor and product ions were ±5 ppm and ±0.6 Da, respectively. Cysteine Nethylmaleimide and Methionine Oxidation were set as fixed and variable modification respectively. The value of max missed cleavages of the trypsin is set at 1. The false discovery rate of PSMs above homology was adjusted to 1%. For analyses of protein abundance, the exponentially modified protein abundance

index (emPAI) of proteins were normalized with emPAI of Band 3 (set as 100%), one of the most abundant RBC membrane proteins, and expressed as percentage of Band 3.

Isolation of splenic macrophages and bone marrow erythroblasts

Splenic macrophages and bone marrow erythroblasts were isolated with Dynabead® Biotin Binder (Thermo Fisher Scientific) with biotinylated CD11b or biotinylated Ter119 antibodies (R&D systems), respectively, according to our protocol described previously.[3]

RT-PCR

Total RNAs were prepared with TRIzol® reagent (Ambion), RNAs (2 μg) were then reverse transcribed into cDNA with High-Capacity cDNA Reverse Transcription kit (Applied Biosystems), and quantitative Real-Time PCRs were performed with Fast SYBR® Green PCR master mixture (Applied Biosystems) on a StepOnePlus™ Real-Time PCR System. The primers were shown in Supplemental Table S4. The mRNA expression levels were normalized against actin levels.

Statistical analysis

Significances for two-group comparisons were determined using two-tail unpaired t-tests. Significances for two-group comparisons with apparently unequal variances were determined using Welch's t-test. Significances for multi-group comparisons were analyzed with two-way ANOVA followed by Sidak's multiple comparisons test. All tests were performed with GraphPad Prism 7, and data were expressed as Mean ± 95% confidence interval (CI) except where indicated otherwise. The data were taken as significant when p values were <0.05 (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Reference

1. Grundy MA, Gorman N, Sinclair PR, Chorney MJ, Gerhard GS. High-throughput non-heme iron assay for animal tissues. *J Biochem Biophys Methods*. 2004;59(2):195-200.
2. Zhang DL, Wu J, Shah BN, et al. Erythrocytic ferroportin reduces intracellular iron accumulation, hemolysis and malaria risk. *Science*. 2018;359(6383):1520-1523.

3. Zhang DL, Hughes RM, Ollivierre-Wilson H, Ghosh MC, Rouault TA. A ferroportin transcript that lacks an iron-responsive element enables duodenal and erythroid precursor cells to evade translational repression. *Cell Metab*. 2009;9(5):461-473.

Figure S1

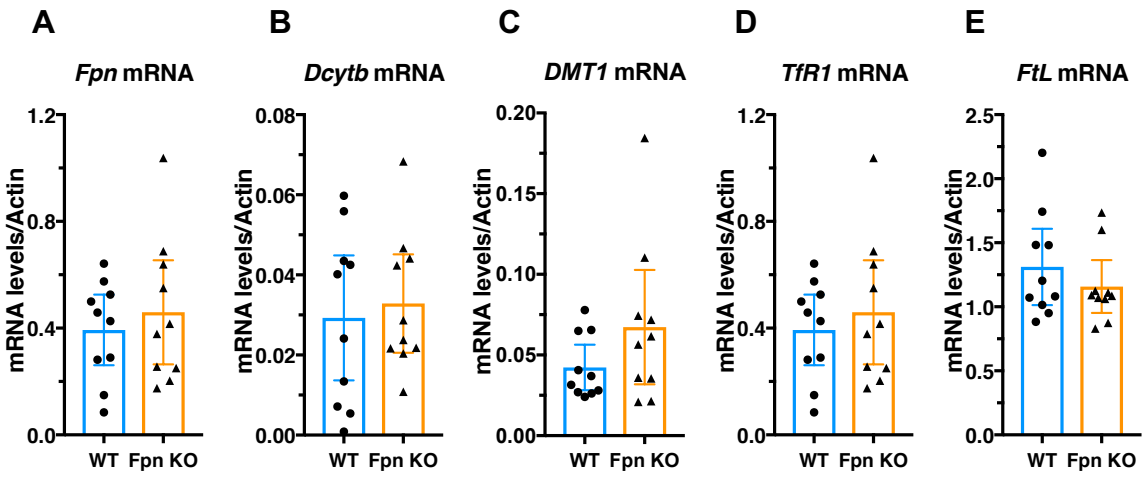

Figure S1 mRNA expression in duodenum of WT vs *Fpn* KO mice. The mRNA levels of (**A**) *Fpn*, (**B**) *Dcytb*, (**C**) *DMT1*, (**D**) *TfR1,* (**E**) L-ferritin (*FtL*) in duodenums of WT and *Fpn* KO mice. Data are presented as mean ± 95% confidence interval.

Figure S2

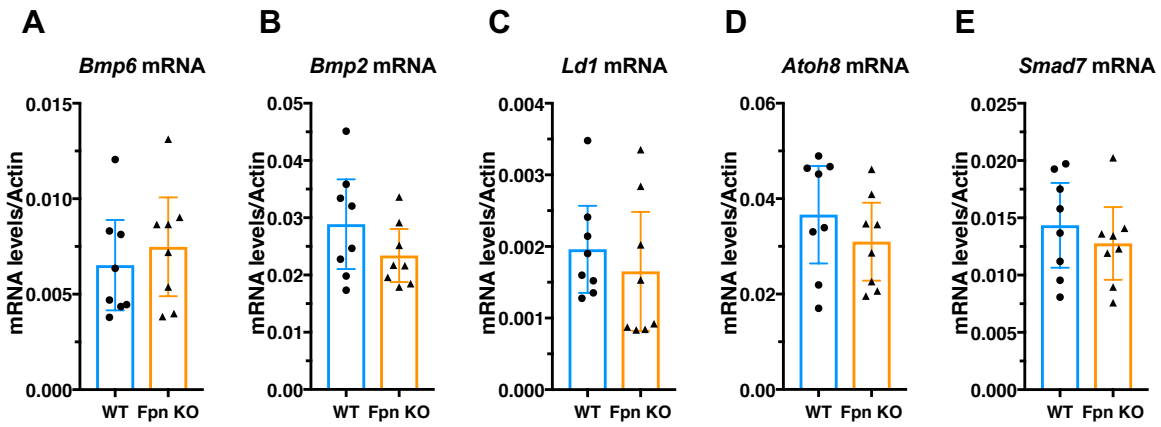

Figure S2 mRNA expression in livers of WT vs *Fpn* KO mice. The mRNA levels of (**A**) *Bmp6*, (**B**) *Bmp2*, (**C**) *Ld1*, (**D**) *Atoh8,* (**E**) *Smad7* in the livers of WT and *Fpn* KO mice. Data are presented as mean ± 95% confidence interval.

Figure S3

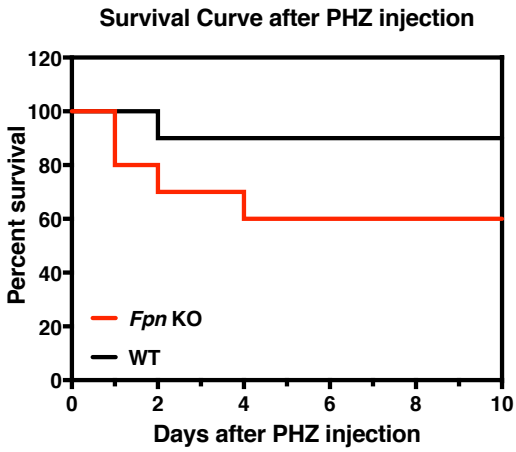

Figure S3 The survival curve of WT and *Fpn* KO mice after phenylhydrazine treatment. N=10 for each group.

Figure S4

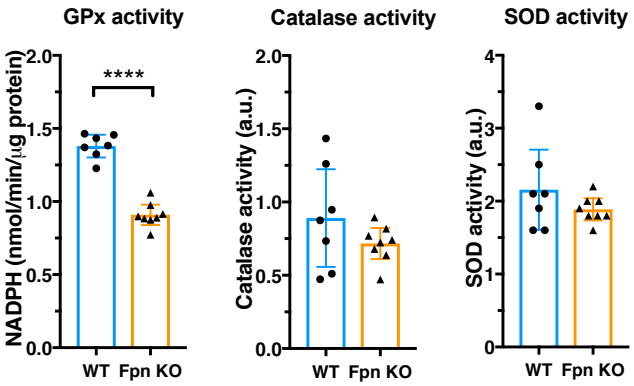

Figure S4 The enzyme activities of glutathione peroxidase (GPx), catalase and superoxide dismutase (SOD) in the RBCs of WT and *Fpn* KO mice.

Figure S5

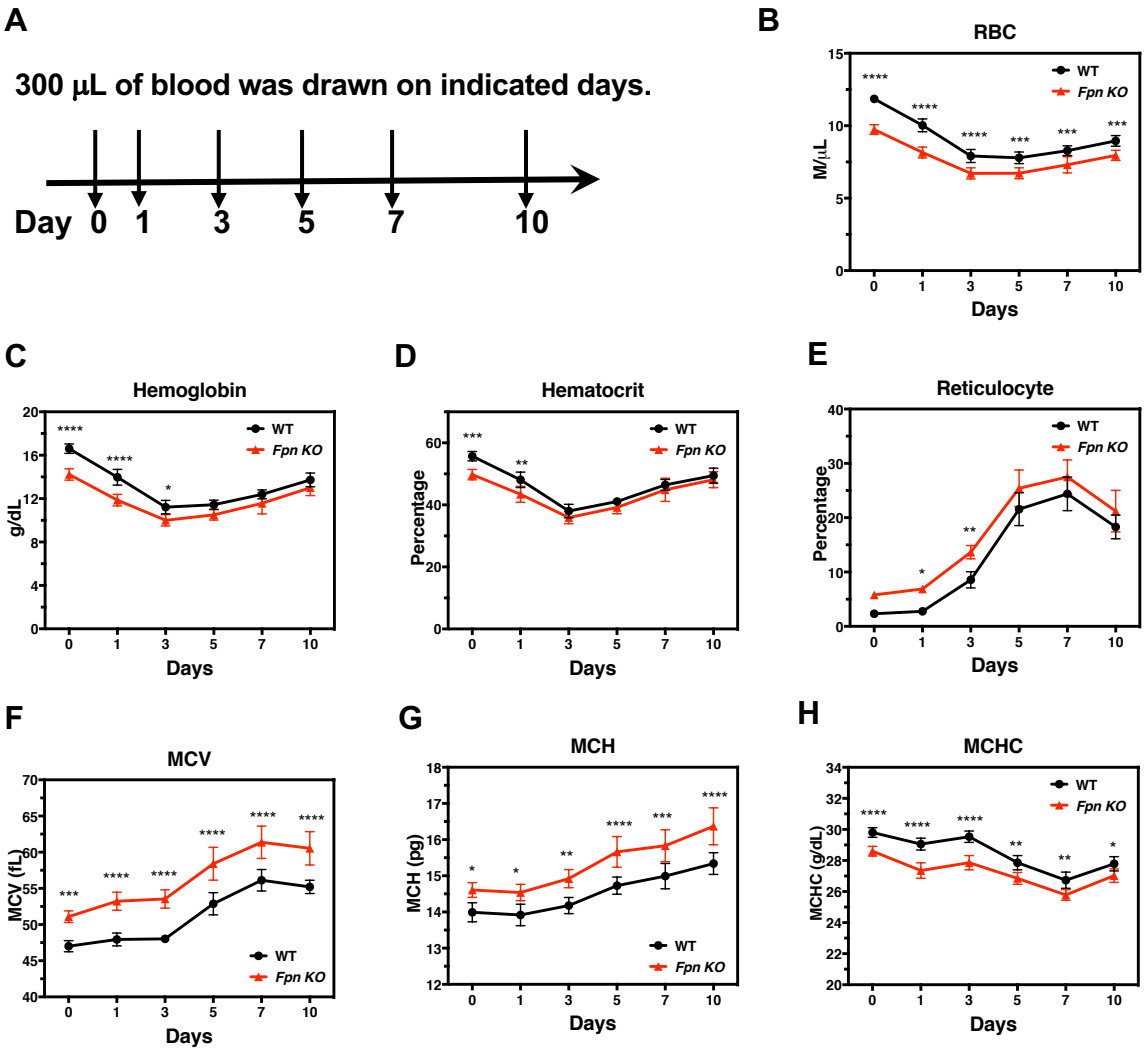
A
300 µL of blood was drawn on indicated days.
Day 0 1 3 5 7 10
B
RBC
M/µL
Days
C
Hemoglobin
g/dL
Days
D
Hematocrit
Percentage
Days
E
Reticulocyte
Percentage
Days
F
MCV
MCV (fL)
Days
G
MCH
MCH (pg)
Days
H
MCHC
MCHC (g/dL)
Days
WT
Fpn KO

Figure S5 *Fpn* conditional KO mice were able to tolerate phlebotomy-induced anemia. (**A**) Scheme of a phlebotomy procedures designed to induce anemia. Blood samples (300 mL) were collected from the facial vein on days 0, 1, 3, 5, 7 and 10 to induce anemia and provide samples for blood analysis. **(B**) RBCs, (**C**) hemoglobin, (**D**) hematocrit, (**E**) mean corpuscular volume (MCV), (**F**) mean corpuscular hemoglobin (MCH), (**G**) mean corpuscular hemoglobin contents (MCHC), and (**H**) reticulocytes. Repeated phlebotomy significantly decreased RBCs, hemoglobin and hematocrit in both WT and *Fpn* KO mice. Anemia induced by phlebotomy stimulated RBC production, and reticulocytes increased significantly at every time point, whereas RBCs, hemoglobins and hematocrits did not start to recover until after the fifth day of phlebotomy treatments. When *Fpn* KO mice were compared to WT mice, there were consistently more reticulocytes generated and the difference in hemoglobin and hematocrit values disappeared on days 7 and 10, suggesting that the ability of *Fpn* KO mice to produce RBCs was fully comparable to WT mice. Mean ± 95% CI, n=10 for each group; analyzed by two-way ANOVA and Sidak's multiple comparisons tests.

Table S2. Blood indices of *Fpn* KO mice

Hematological Parameters of 4-6 months old mice

Parameter	Males			Females		
	FPN$^{fl/fl}$/*ErCre* $^{-/-}$ (WT, n=13)	*FPN*$^{fl/fl}$/*ErCre* $^{+/-}$ (KO, n=11)	p Value	*FPN*$^{fl/fl}$/*ErCre* $^{-/-}$ (WT, n=8)	*FPN*$^{fl/fl}$/*ErCre* $^{+/-}$ (KO, n=10)	p Value
RBC ($10^6/\mu L$)	11.8±0.1	10.0±0.1	<0.0001	11.8±0.2	10.2±0.1	<0.0001
Hb (g/dL)	16.7±0.2	14.6±0.2	<0.0001	16.5±0.2	14.9±0.3	<0.001
HCT (%)	51.3±0.6	45.8±0.3	<0.0001	51.4±0.7	47.2±0.7	<0.0001
MCV (fL)	47.3±0.3	51.4±0.3	<0.0001	46.6±0.3	50.7±0.4	<0.0001
MCH (pg)	14.2±0.1	14.6±0.2	<0.05	14.1±0.1	14.6±0.1	<0.05
MCHC (g/dL)	29.9±0.1	28.3±0.2	<0.0001	30.2±0.2	28.9±0.1	<0.0001
RDW-SD (fL)	29.9±0.6	34.1±0.2	<0.0001	28.7±0.3	33.3±0.7	<0.0001
RET (%)	2.9±0.2	5.2±0.2	<0.0001	2.7±0.2	5.2±0.5	<0.001

RBC, red blood cell; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin content; RDW-SD, stand deviation of red blood cell distribution; RET, reticulocyte. Data are reported as Mean ± SEM.

Table S4.

Table S4. Primers for RT-PCR

Primers for RT-PCR

Gene	Primer F	Primer R
Fpn	ATGGGAACTGTGGCCTTCAC	TCCAGGCATGAATACGGAGA
TfR1	TTGCGGCGAAGTCCAGTGTG	CCTGCAGTCCAGCTGGCAAA
Hepcidin	TTGCGATACCAATGCAGAAG	TGCAACAGATACCACACTGG
Erfe	ATGGGGCTGGAGAACAGC	TGGCATTGTCCAAGAAGACA
Dcytb	ACGGTTCTCATGGGAGTGAC	GAAGGCCCAGCGTATTTGTA
DMT1	TCTGGGCAGTGGGGATCCTG	GACGAGCAGGGTGGGGATGA
FtL	CACCTACCTCTCTCTGGGCT	CGCGATCGTTCTGAAACTCG
Bmp6	AGGAGCATCAGCACAGAGAC	GTCACCACCCACAGATTGCT
Bmp2	CAGAAGCCCAGTTGCTGCTC	ACACTAGAAGACAGCGGGTC
Atoh8	GAGCCAAGAAACGGAAGGAGT	GGAATGAGGCTGGTCGTCTC
Smad7	GGAAGATCAACCCCGAGCTG	CAGCCTGCAGTTGGTTTGAG
Ld1	GAGTCTGAAGTCGGGACCAC	GGAACACATGCCGCCTCA
Actin	GCCACTGCCGCATCCTCTTC	AGCCTCAGGGCATCGGAACC

Note: Table S1 and S3 were in separate files.

Table S1. Abundance of FPN in the mouse RBC membrane compared with some well known RBC membrane proteins. Ghost membrane fractions from six individual mice were digested and measured with mass spectrometry. Then the exponentially modified protein abundance index (emPAI) of each protein was normalized to the emPAI of Band 3, one of the most abundant RBC membrane protein, and expressed as percentage. N/A, not applicable. Results showed that FPN was about 2.54% of Band 3, which is comparable to its abundance of 5.4% of Band 3 in human RBC membrane, confirming that FPN is an abundant RBC membrane protein. Of note, Glut1 and Cybrd1 could not be detected, consistent with previous findings of low abundance in the mouse RBCs, supporting previously observed species differences between human and mouse RBCs.

Table S3. The mean cell volume and serum transferrin saturation of patients with *FPN* mutations. These data were summarized from previous literature indicated in the first column.