Online Supplemental Data

Hepatic Transferrin Plays a Role in Systemic Iron Homeostasis and Liver Ferroptosis

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Supplemental Methods

Genotyping of *Trf*-LKO, *Slc39a14*-LKO, and DKO mice

Genomic DNA was extracted from mouse tail biopsies using the TIANamp Genomic DNA Kit (DP304, Tiangen), and the primer pairs used for PCR-based genotyping are listed in **supplemental Table 2**.

PHZ-induced hemolysis

Eight-week-old male control ($Trf^{fl/fl}$) and *Trf*-LKO mice were given intraperitoneal injections of PHZ (40 μg/g body weight) for two consecutive days.[1] The first day of PHZ injection was designated as day 0. The mice were then sacrificed either 4 or 8 days later.

Iron dextran injection

Male wild-type mice (2-month-old) were injected intraperitoneally with iron dextran(250μg/g).[1] One week post injection, the treated mice were sacrificed.

Dietary high iron treatment

At 4 weeks of age, $Trf^{fl/fl}$ (control) and *Trf*-LKO mice were fed a high-iron diet (HID) containing 8.3 g carbonyl iron/kg (Research Diets, Inc.) or a standard iron diet (SID) containing 50 mg iron/kg for 8 weeks.[2]

Ferrostatin-1 (Fer-1) treatment

Fer-1 (S7243, Selleck Chemicals) was administered as previously described.[2] In brief, 4-week-old control ($Trf^{fl/fl}$), *Trf*-LKO, and DKO mice were fed a HID for 8 weeks; during the last 3 weeks of HID feeding, the mice received daily intraperitoneal injections of either Fer-1 (1 mg/kg) or vehicle (saline).

CCl_4-induced fibrosis murine model

Liver fibrosis was induced in mice at 8-9 weeks of age by intraperitoneal injections of carbon tetrachloride (CCl_4) three times/week for 4 weeks. CCl_4 solution (6 μl/mouse) was dissolved in olive oil (300 μl/mouse), and each mouse was injected with 2% (v/v) of CCl_4 or 300 μl oil as previously described.[3] For the ferroptosis inhibitor experiments, the following six groups of mice (n=5 mice/group) were used in this experiment: control ($Trf^{fl/fl}$) + oil; *Trf*-LKO + oil; control + CCl_4; *Trf*-LKO + CCl_4; *Trf*-LKO + CCl_4 + Fer-1; and DKO + CCl_4. The mice in the Fer-1 group also received injections of Fer-1 (1 mg/kg) three times/week for 4 weeks at the interval time as the CCl_4 injections. For supplementation with apo-transferrin (Apo-TF), the following six groups of mice (n=4-

6 mice/group) were used in this experiment: control; *Trf*-LKO; control + CCl_4; *Trf*-LKO + CCl_4; control + CCl_4 + Apo-TF; and *Trf*-LKO + CCl_4 + Apo-TF. The mice in the Apo-TF group also received injections of Apo-TF (500 mg/kg) three times/week for 4 weeks at the interval time as the CCl_4 injections. The mice were sacrificed 48 hours after the last injection in order to avoid inducing an -acute response to chronic liver injury.[4]

Flow cytometry analysis

Cells were isolated from the bone marrow and spleen, and then analyzed by using flow cytometry as previously described.[5] For evaluation of erythropoiesis, the erythroid cells were stained with anti-CD44 (103007, Biolegend) and anti-TER-119 (116212, Biolegend) antibodies for 30 min at 4°C. For evaluation of intracellular iron status, the cells were incubated either with or without the high-affinity iron chelator 2,2'-bipyridyl, washed, and then loaded with calcein-AM (final concentration: 0.5 mM) for 10 min at 37°C. After washing, the erythroid cell population was identified by staining with anti-CD44 and anti-TER-119 antibodies for 30 min at 4°C, followed by flow cytometry using an LSRFortessa cell analyzer (BD Biosciences, San Jose, CA).

Measurements of serum iron, tissue non-heme iron, and serum non-transferrin-bound iron (NTBI)

Serum iron concentration was measured using a serum iron-unsaturated iron-binding capacity kit (Pointe Scientific, Inc.) in accordance with the manufacturer's instructions. Quantitative measurements of tissue non-heme iron were performed as previously described.[1, 6] The data as presented as micrograms of iron per gram wet weight of tissue. Serum NTBI was measured using a colorimetric method as previously described.[7]

Perls' blue, ALT, Sirius red, Masson's trichrome staining, and 4-HNE staining

Perls' blue staining was performed as previously described.[1] ALT measurements, Sirius red staining, and Masson's trichrome staining were performed as described previously.[2, 8]. 4-hydroxynonenal (4-HNE) staining was performed using an anti-4 hydroxynonenal antibody (ab46545, Abcam).

Measurements of MDA, Thiol, and SOD enzyme activity

Hepatic malondialdehyde (MDA) content was measured using a kit (S0131, Beyotime) in accordance with the manufacturer's instructions. Thiol content was measured using a Thiol Quantification Assay kit (ab112158, Abcam). Hepatic total (superoxide dismutase) SOD enzyme activity was measured using a kit (S0101, Beyotime) in accordance with the manufacturer's instructions.

Western blot analysis

The following primary antibodies were used for western blot analysis: anti-Transferrin (ab82411, Abcam), anti-Ferritin Light Chain (ab69090, Abcam), anti-Ferritin Heavy Chain (ab65080, Abcam), anti-Transferrin receptor 1 (13-6800, Invitrogen), anti-Slc39a14 (TB2519862D, Invitrogen), and anti-Gapdh (AC001, ABclonal). Western blot analysis of Ferroportin-1 was performed as previously described.[1]

Immunohistochemistry

The anti-Transferrin and anti-Slc39a14 antibodies used for immunohistochemistry (IHC) were obtained from Abcam (ab82411) and Invitrogen (TB2519862D), respectively. Ferroportin IHC was performed as previously described.[6] Image J software was used for processing and quantifying the positive areas from those images. In detail, we firstly set the area fraction for the measurement using the software, and then converted the scanned color images to grayscale, followed by setting a threshold using manual sliders to include the positive area in red (applied the same threshold values to indicated groups).

Measurements of serum Trf using enzyme-linked immunosorbent assay

Serum mouse transferrin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Alpha Diagnostic International, San Antonio, TX) in accordance with the manufacturer's instructions.[9]

Measurements of serum Erythropoietin (Epo) using enzyme-linked immunosorbent assay

Serum mouse transferrin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (E-EL-M0027c, Elabscience) in accordance with the manufacturer's instructions.

Quantitative real-time PCR analysis

Total RNA was extracted using TRIzol (Invitrogen), then reverse-transcribed into cDNA using the PrimeScript RT kit (Takara). Real-time PCR was then performed using the two-step real-time quantitative RT-PCR method (Bio-Rad) with the primers listed in **supplemental Table 2**.

Supplemental Table 1. Baseline demographics and clinical characteristics of patients with liver cirrhosis and healthy controls

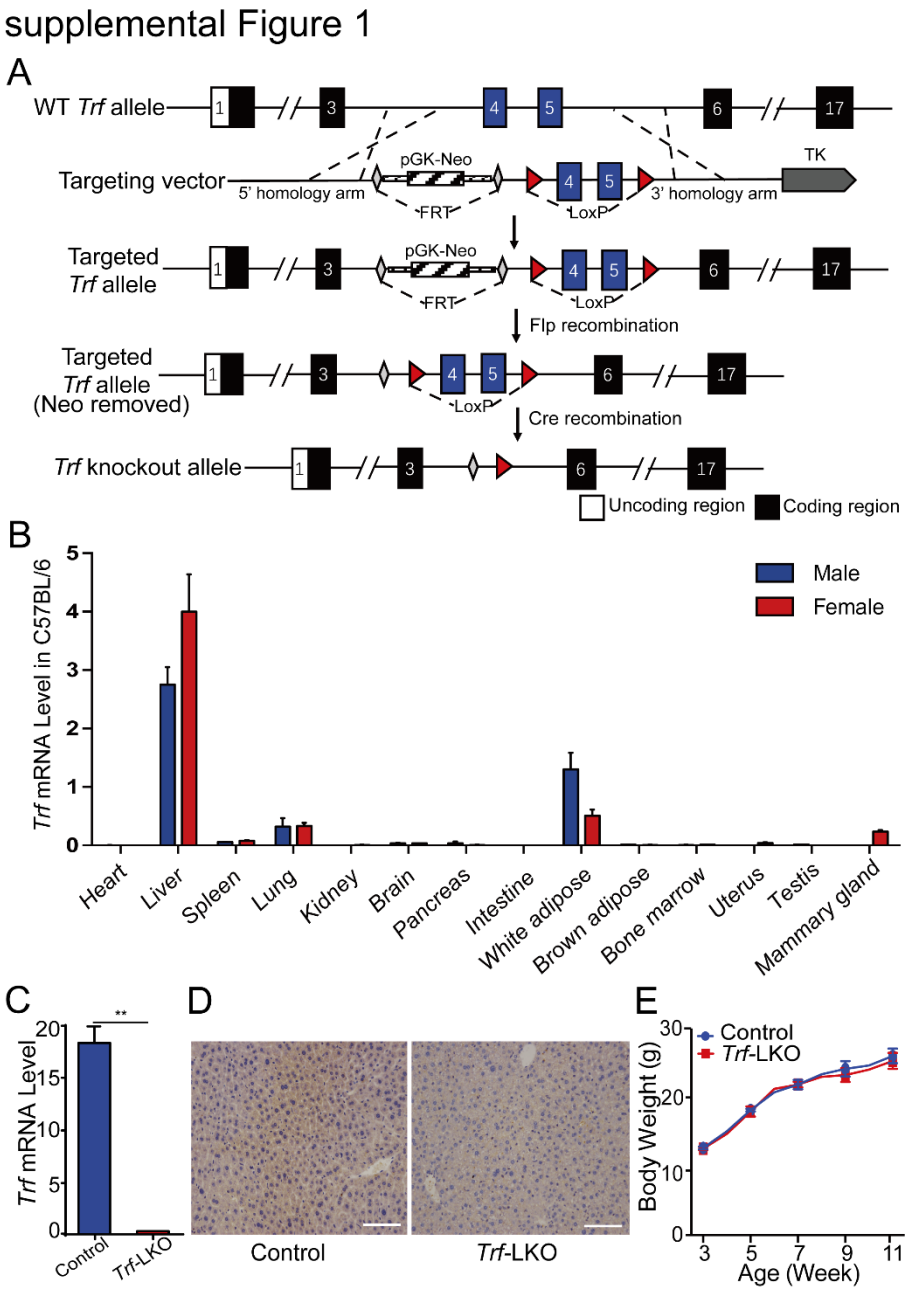
	Cirrhosis patients (n=48)	Healthy controls (n=52)	*P*-value
Sex (male/female)	36/13	39/13	0.86
Age (years)	60.4±10.2	56.9±8.7	0.064
ALT (U/L)	34 (5-200)	19 (8-38)	0.002
Transferrin (mg/dl)	154.8±60.8	212.5±32.73	<0.001
HA (ng/ml)	409.3±380.1	ND	NA
LN (ng/ml)	53.0±78.1	ND	NA
PCIII (ng/ml)	125.4±107.1	ND	NA
IV-C (ng/ml)	118.2±103.4	ND	NA

ALT, alanine aminotransferase; HA, hyaluronic acid, LN, laminin; PCIII, procollagen III; IV-C, collagen IV; ND, not determined; NA, not applicable.
Data are expressed as the mean ± SD or the median (interquartile range). Chi-squared test and Student's t-test were used to statistical analyses.

Supplemental Table 2. Sequences of the primers used for RT-qPCR analyses and PCR-based genotyping

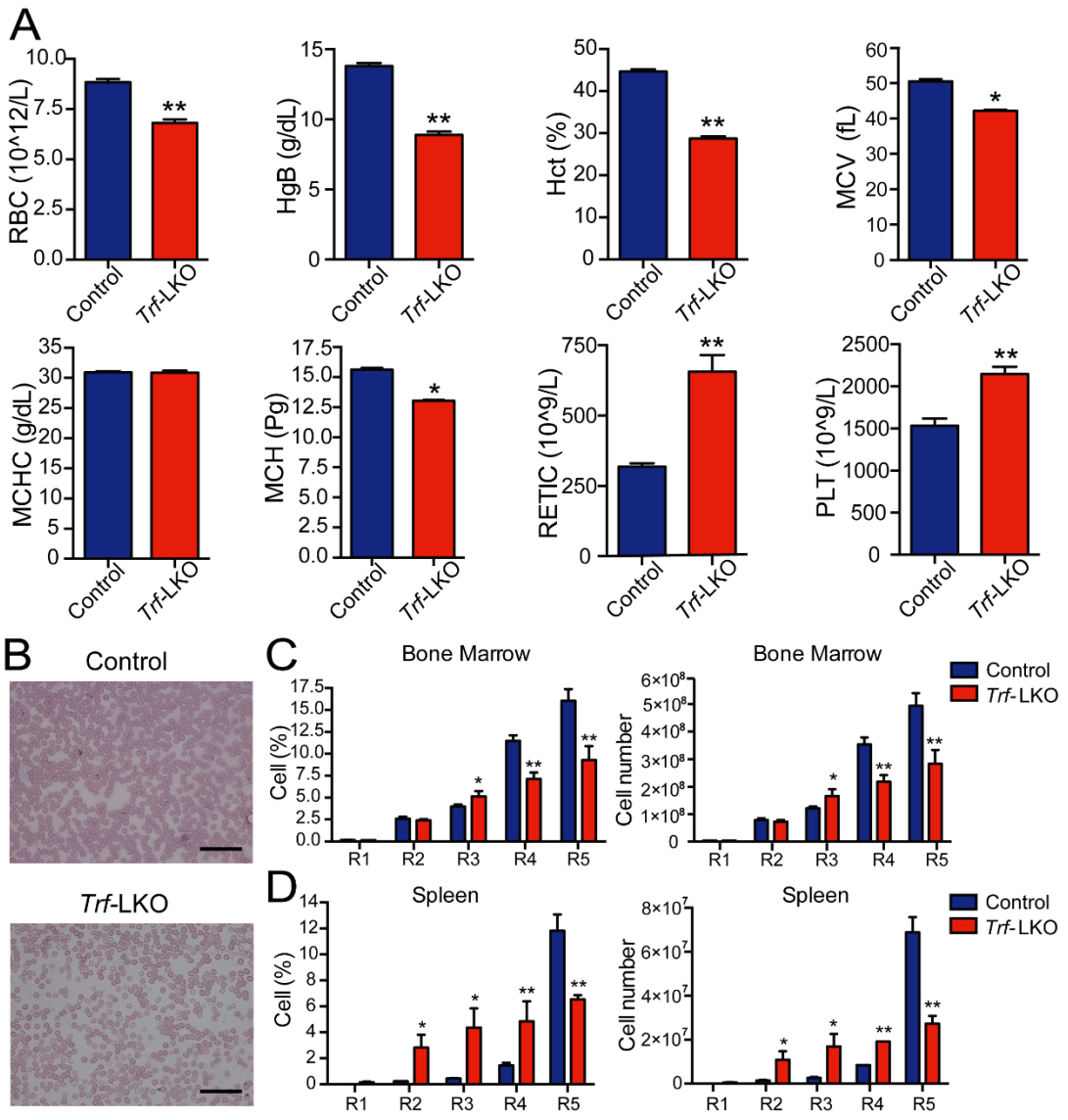
Real-time PCR Primers		
Gene	Forward primer (5'→3')	Reverse primer (5'→3')
β-actin	AAATCGTGCGTGACATCAAAGA	GCCATCTCCTGCTCGAAGTC
Hamp1	GCACCACCTATCTCCATCAACA	TTCTTCCCCGTGCAAAGG
Gpx4	CCTCCCCAGTACTGCAACAG	GGCTGAGAATTCGTGCATGG
Tfr1	TGGCTGAAACGGAGGAGACAGA	TGGCTCAGCTGCTTGATGGTGT
Slc7a11	TGGCGGTGACCTTCTCTGA	ACAAAGATCGGGACTGCTAATGA
Fth	AAGATGGGTGCCCCTGAAG	CCAGGGTGTGCTTGTCAAAGA
Ftl	CGGGCCTCCTACACCTACCT	CCCTCCAGAGCCACGTCAT
Slc39a14	TTTCCCAGCCCAAGGAAG	CAAAGAGGTCTCCAGAGCTAAA
Trf	TTCTGTAAGCTGTCGGAGCC	GACACAACTGCCCGAGAAGA
PDGF	CATCCGCTCCTTTGATGATCTT	GTGCTCGGGTCATGTTCAAGT
Col1a1	GTGCTCGGGTCATGTTCAAGT	CCCGGTGACACACAAAGACA
Epo	GCCTCACTTCACTGCTTCGG	GGAGGCGACATCAATTCCTTC
Erfe	ATGGGGCTGGAGAACAGC	TGGCATTGTCCAAGAAGACA

Genotyping PCR Primers		
Trf	TTATGGATCAGTGGAACGTGAG	GCTTACAGAAGAGCAAGCCAAT
Alb-Cre	GCAAACATACGCAAGGGATT	AGGCAAATTTTGGTGTACGG
Slc39a14	CTGTGGTCTTCCTGCCTTGG	TACCCTGCCCTACACGACTC



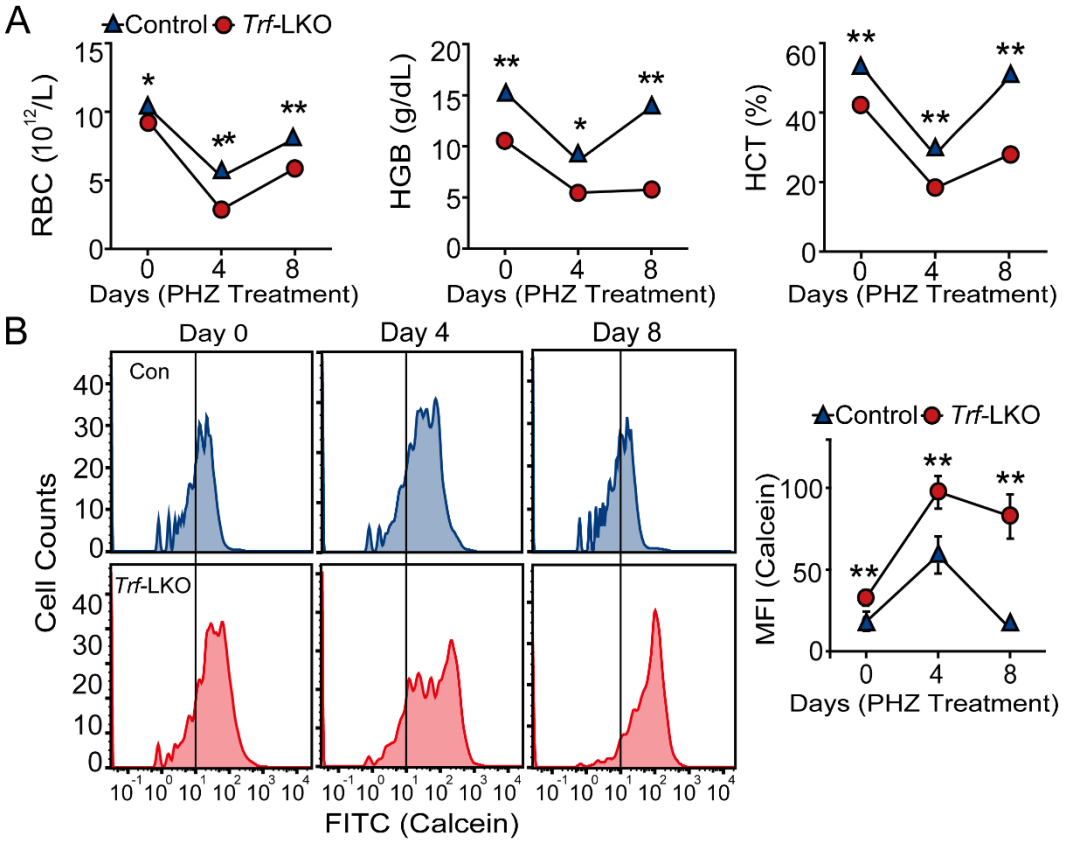
supplemental Figure 1. Generation and characterization of hepatocyte-specific transferrin knockout (*Trf*-LKO) mice. (A) Schematic diagram depicting the targeting strategy used to generate the conditional *Trf* knockout allele. **(B)** *Trf* mRNA levels were measured in the indicated tissues of male and female wild-type C57BL/6J mice (n=3 mice/group). **(C)** Hepatic *Trf* mRNA levels were measured in control ($Trf^{fl/fl}$) and *Trf*-LKO mice (n=6 mice/group). **(D)** Trf IHC staining of liver sections obtained from control and *Trf*-LKO mice; the scale bars represent 100 μm. **(E)** Summary of body weight measured from 3 weeks of age until 11 weeks of age (n=6 mice/group). ***P*<0.01, Student's *t*-test.

supplemental Figure 2

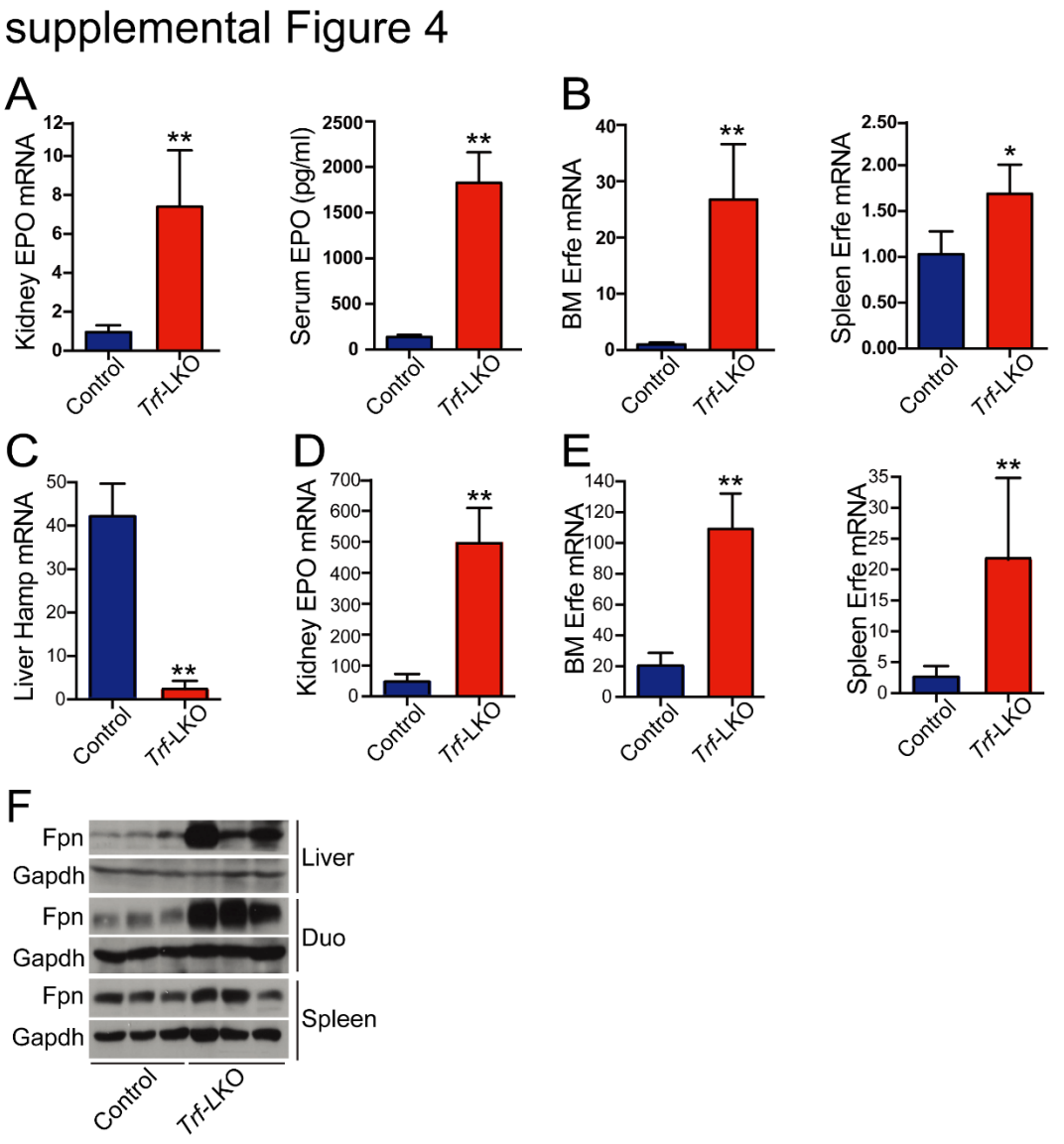


supplemental Figure 2. *Trf*-LKO mice showed microcytic and hypochromic anemia. (A) Complete blood count parameters including red blood cell count (RBC), total hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean cellular hemoglobin levels (MCH), mean corpuscular hemoglobin concentrations (MCHC), reticulocyte count (RET) and platelet (PLT) values were measured in 8-week-old control and *Trf*-LKO mice (n=6-8 mice/group). **(B)** Example blood smears prepared from an 8-week-old control and *Trf*-LKO mouse are shown at the right, the scale bars represent 100 μm. **(C)** Flow cytometry analysis of erythroid cell populations (R1-R5) in bone marrow and spleen stained with anti-CD44 and anti-TER-119 in two-month-old control and *Trf*-LKO mice (n=5 mice/group). ***P*<0.01, Student's *t*-test.

supplemental Figure 3

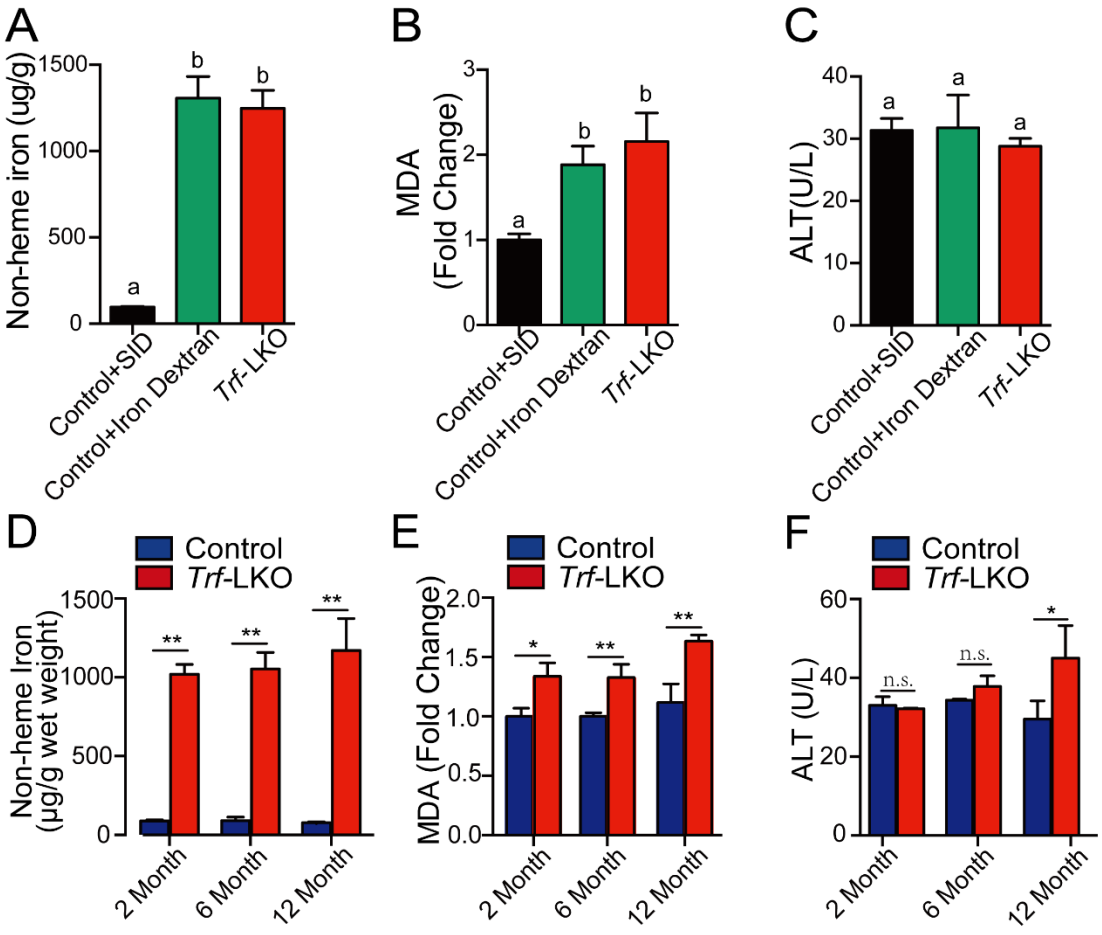


supplemental Figure 3. *Trf*-LKO mice have increased phenylhydrazine-induced hemolytic anemia. (A) RBC, HGB, and HCT values were measured in control and *Trf*-LKO mice (n=3-4 mice/group) at 0, 4, and 8 days after phenylhydrazine (PHZ) injection. **(B)** Flow cytometry analysis and summary of calcein mean fluorescence intensity (MFI) measured in control and *Trf*-LKO bone marrow cells at 0, 4, and 8 days after PHZ injection (n=3 mice/group). **P*<0.05, ***P*<0.01, Student's *t*-test.

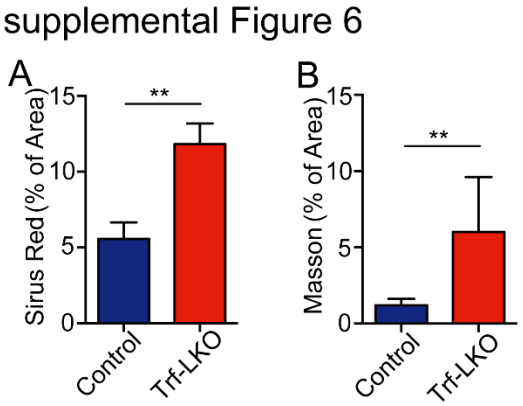


supplemental Figure 4. *Trf*-LKO mice have increased EPO and ERFE levels fed either a standard-iron or high-iron diet. (A, B) *Epo* mRNA levels in kidney and Epo protein levels in serum **(A)**, and *Erfe* mRNA levels in the bone marrow and spleen **(B)** were measured in the control and *Trf*-LKO mice fed a standard diet (n=5-6 mice/group). **(C-E)** *Hamp* mRNA levels in the liver **(C)**, *Epo* mRNA levels in the kidney **(D)**, and *Erfe* mRNA levels in the bone marrow and spleen **(E)** were measured in the control and *Trf*-LKO mice fed a high-iron diet (n=4-5 mice/group).*P<0.05, **P<0.01, Student's *t*-test. **(F)** Western blot analysis for Fpn proteins of liver, duodenum and spleen in control and *Trf*-LKO (n=3 mice/group)

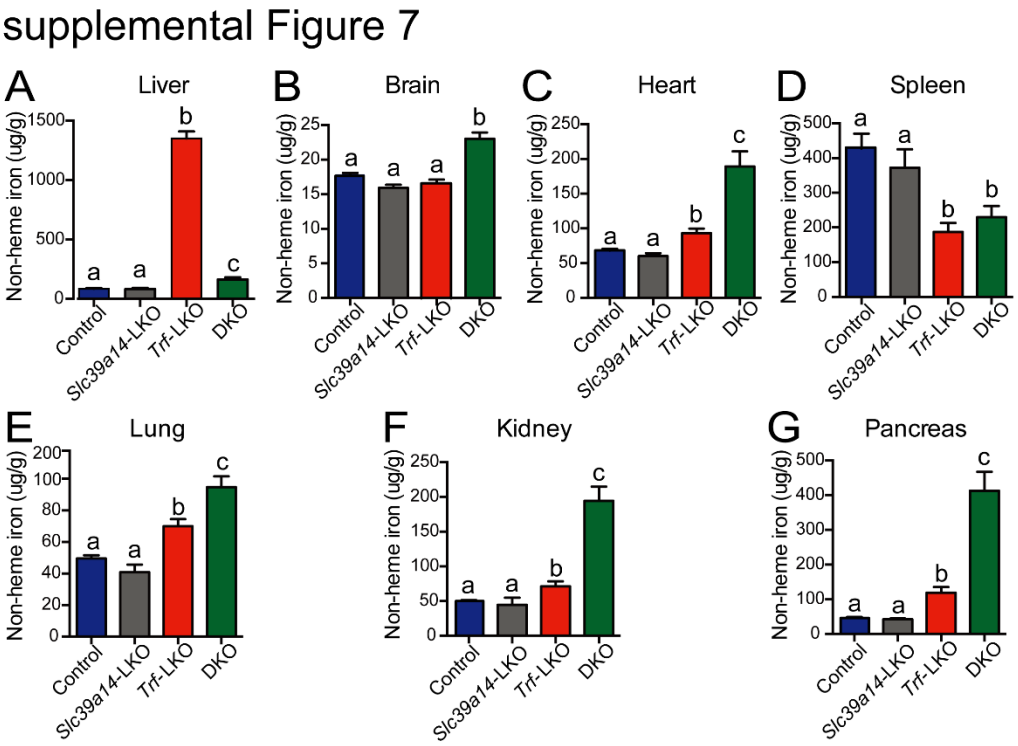
supplemental Figure 5



supplemental Figure 5. Characterization of iron overloaded control and *Trf*-LKO mice. (A-C) Hepatic non-heme iron **(A)**, hepatic MDA **(B)**, and serum ALT **(C)** were measured in 2-month-old control mice fed a standard iron diet (SID group) or *i.p.* injected with iron dextran, and *Trf*-LKO mice (n=6 mice/group). **(D-F)** Hepatic non-heme iron **(D)**, hepatic MDA **(E)**, and serum ALT **(F)** were measured in 2-month-old, 6-month-old and 12-month-old control ($Trf^{fl/fl}$) and *Trf*-LKO mice (n=4 mice/group). The data in A-C were analyzed using a one-way ANOVA with Tukey's post hoc test, groups labeled without a common letter were significantly different ($P<0.05$); the data in D-E were analyzed using Student's *t*-test. *$P<0.05$ and **$P<0.01$, n.s. stands for not statistically significant.

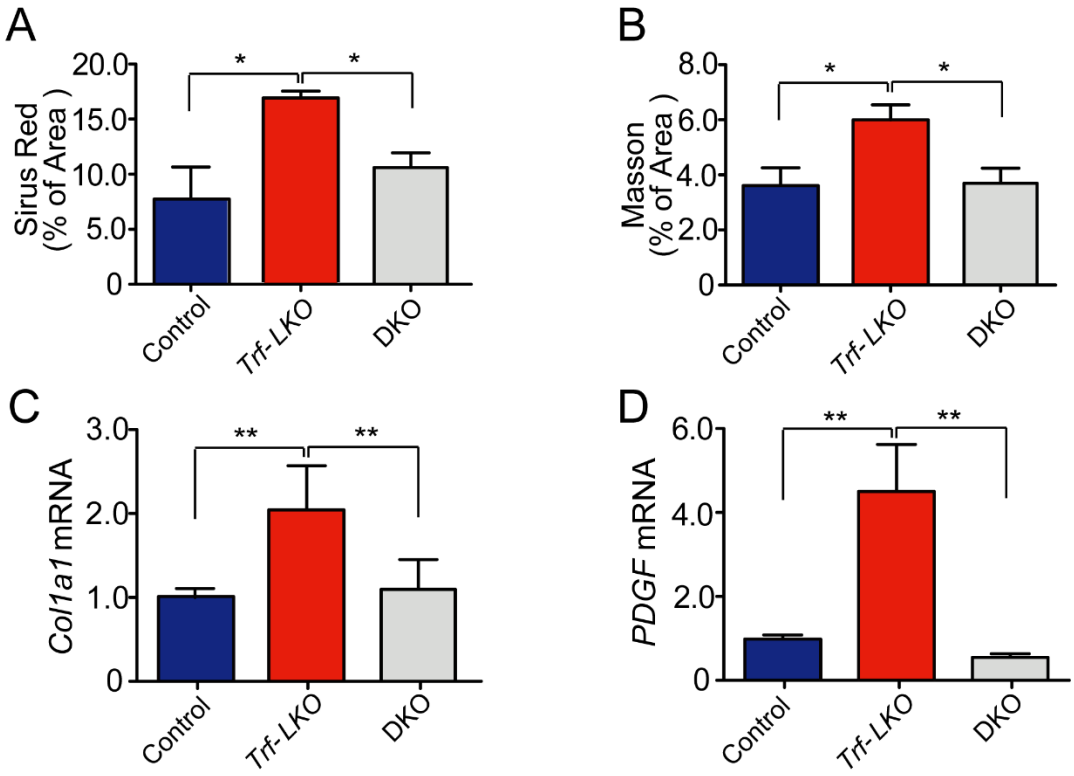


supplemental Figure 6. Quantification of Sirus Red and Masson staining in WT and *Trf*-LKO mice upon HID diet. (A, B) Quantitative analyses for Sirius red **(A)** and Masson's trichrome staining **(B)** in Control and *Trf*-LKO mice fed with HID (n=4 mice/group). The data presented in A-B were analyzed by Student's *t*-test, **P*<0.05 and ***P*<0.01.



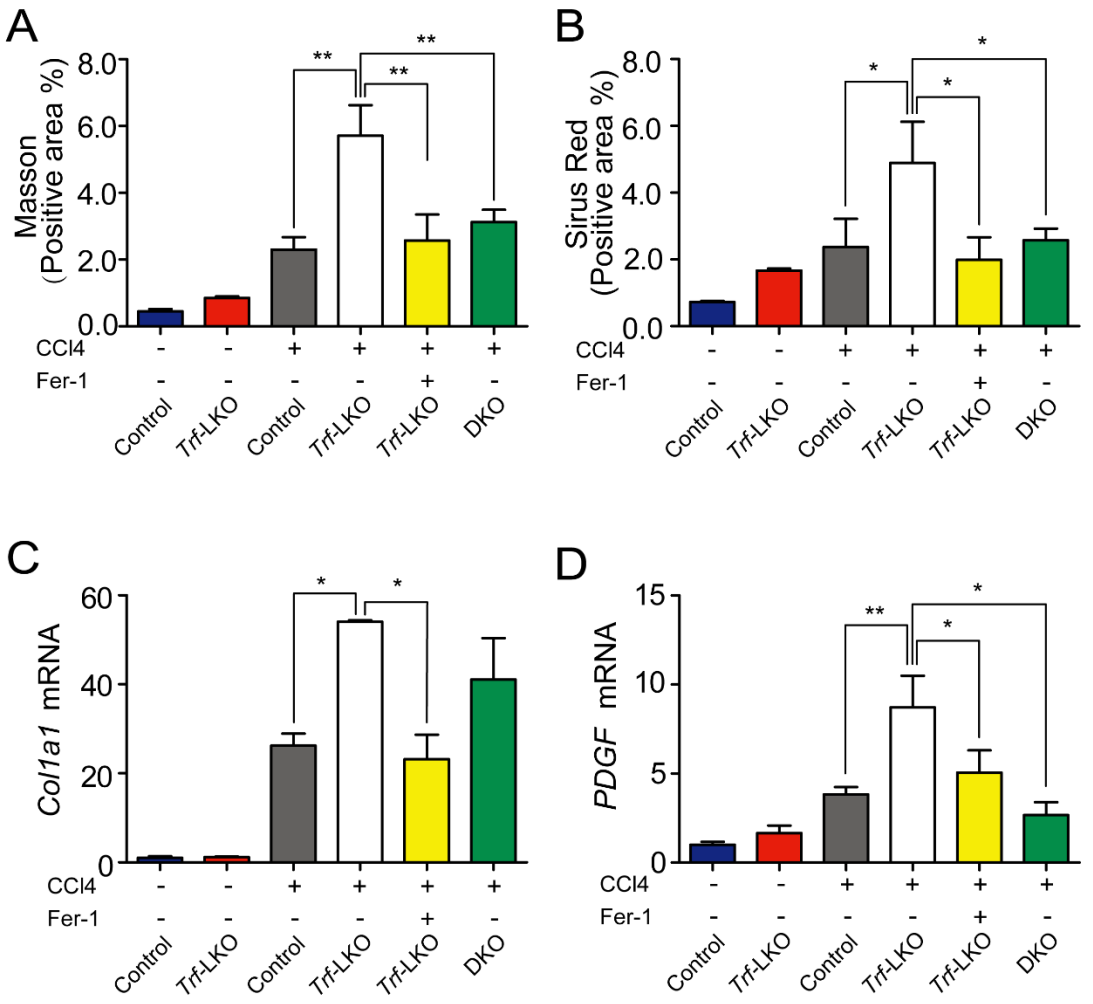
supplemental Figure 7. Tissue non-heme iron content in *Slc39a14*-LKO, *Trf*-LKO and DKO mice fed a standard-iron diet. (A-F) Non-heme iron contents were measured in the liver **(A)**, brain **(B)**, heart **(C)**, spleen **(D)**, lung **(E)**, kidney **(F)** and pancreas **(G)** tissues in indicated group (Control, *Slc39a14*-LKO, *Trf*-LKO and DKO) fed a standard iron diet (n=7-8 mice/group). The data in A-G were analyzed using a one-way ANOVA with Tukey's post hoc test, groups labeled without a common letter were significantly different (*P*<0.05)

supplemental Figure 8



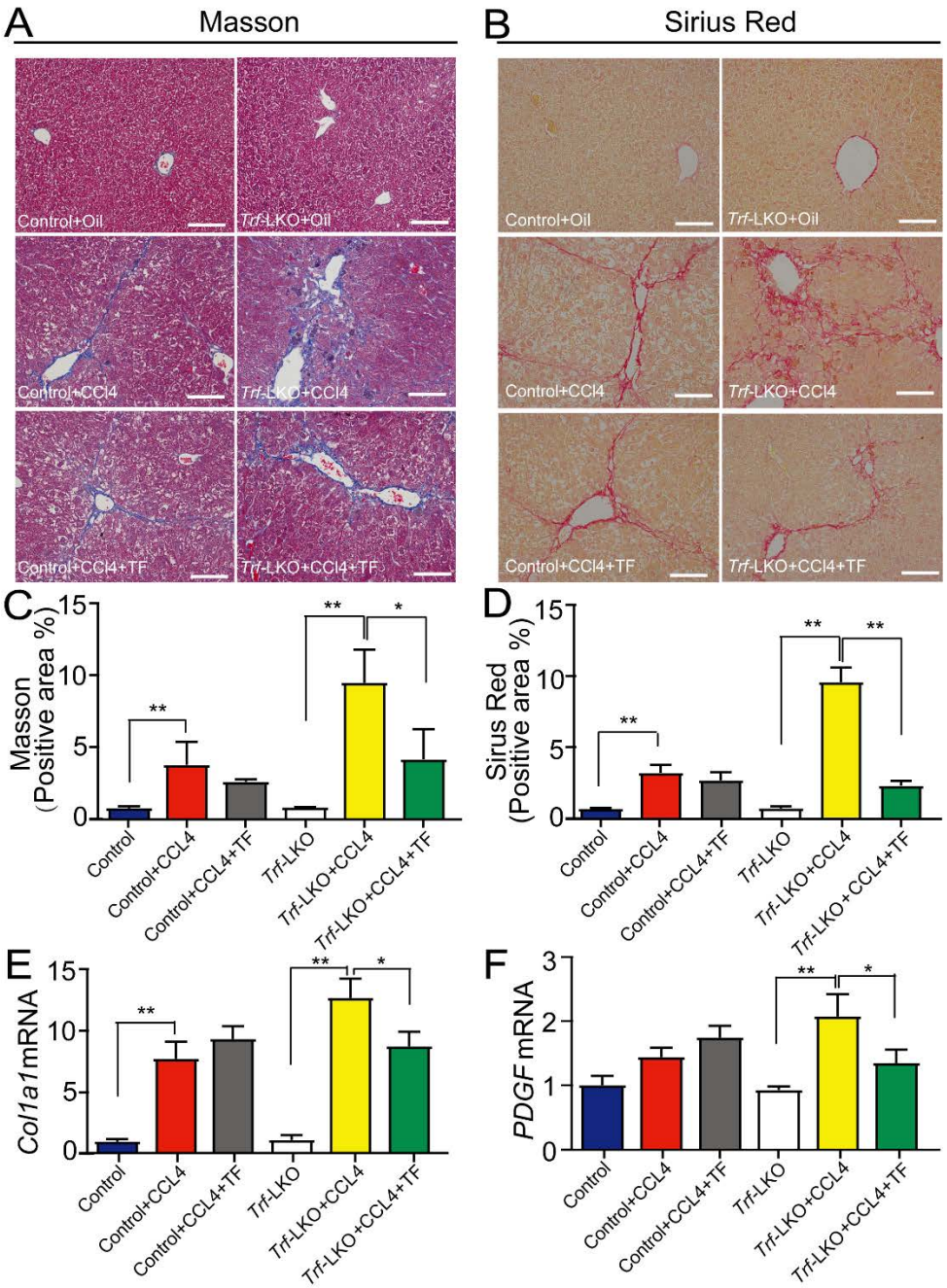
supplemental Figure 8. Expression levels of hepatic fibrotic genes (*Col1a1* and *PDGF*) in *Trf*-LKO and DKO mice induced by high-iron diet. (A, B) Quantitative analyses for Sirius red **(A)** and Masson's trichrome staining **(B)** in indicated three groups fed with high-iron diet (Control, *Trf*-LKO and DKO). **(C, D)** Hepatic mRNA levels of fibrotic genes *Col1a1* **(C)** and *PDGF* **(D)** were measured in indicated group (Control, *Trf*-LKO and DKO). The data in A-D were analyzed using a one-way ANOVA with Tukey's post hoc test; **P*<0.05 and ***P*<0.01.

supplemental Figure 9



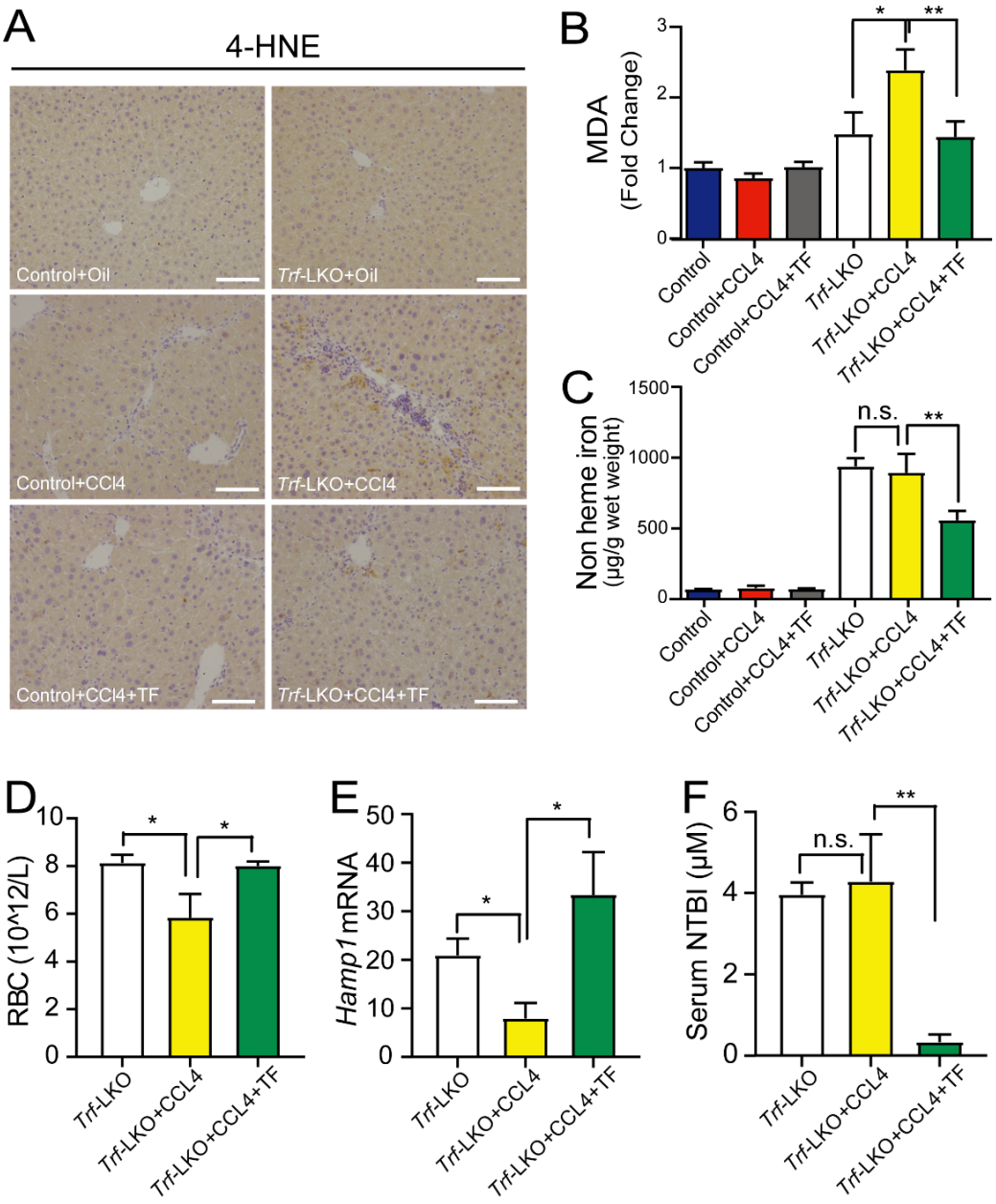
supplemental Figure 9. Quantification of Sirus Red, Masson staining and expression levels of hepatic fibrotic genes (*Col1a1* and *PDGF*) in *Trf*-LKO and DKO mice induced by CCL4. (A, B) Quantitative analyses for Masson's trichrome **(A)** and Sirius red staining **(B)** in the indicated mice (n=4-5 mice/group) (Control, *Trf*-LKO, or DKO) were treated with vehicle (oil) or CCl_4. **(C, D)** Hepatic mRNA levels of fibrotic genes *Col1a1* **(C)** and *PDGF* **(D)** were measured in indicated group. The data in A-D were analyzed using a one-way ANOVA with Tukey's post hoc test; **P*<0.05 and ***P*<0.01.

supplemental Figure 10



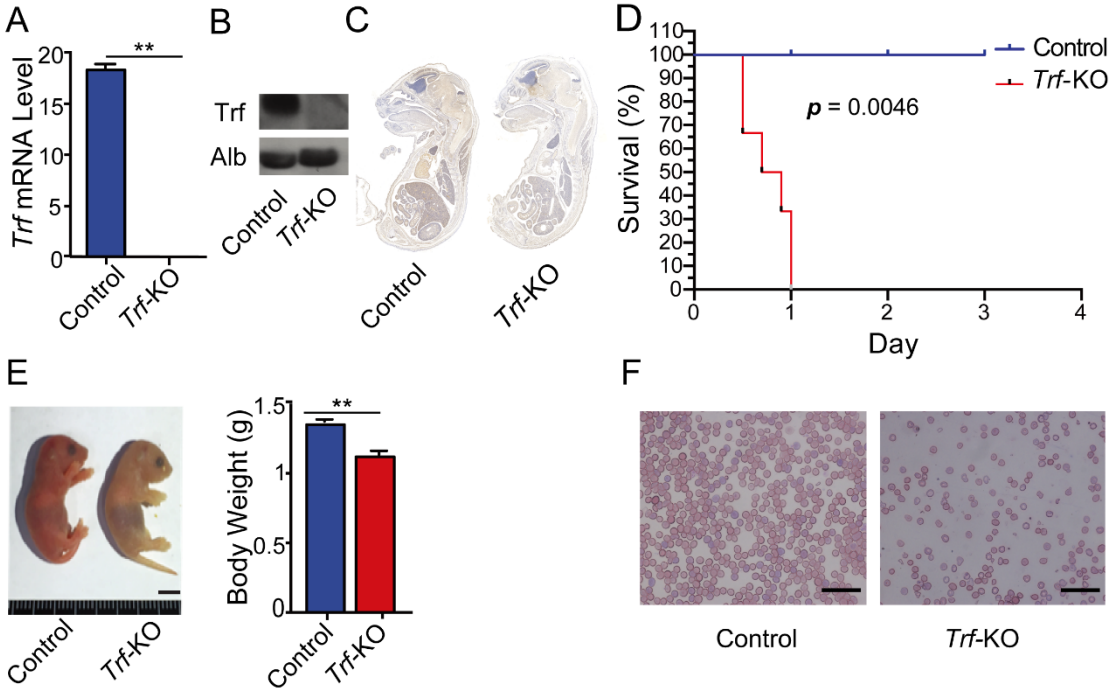
supplemental Figure 10. Apo-TF treatment alleviates CCl4-induced fibrosis in *Trf*-LKO mice. Control and *Trf*-LKO mice were treated with vehicle (oil) or CCl4, or CCL4+Apo-TF. **(A, B)** The liver sections were obtained and stained with Masson's trichrome **(A)**, Sirius red **(B)**, the scale bars represent 100 μm. **(C, D)** Quantitative analyses for Masson's trichrome **(C)** and Sirius red staining **(D)** in the indicated mice (n=4-6 mice/group). **(E, F)** Hepatic mRNA levels of fibrotic genes *Col1a1* **(E)** and *PDGF* **(F)** were measured in indicated group. The data in C-F were analyzed using a one-way ANOVA with Tukey's post hoc test; **P*<0.05 and ***P*<0.01.

supplemental Figure11



supplemental Figure 11. Apo-TF treatment alleviates hepatic MDA, non heme iron and serum NTBI in *Trf*-LKO mice. Control and *Trf*-LKO mice were treated with vehicle (oil) or CCl4, or CCL4+Apo-TF. **(A)** The liver sections were obtained and stained with 4-HNE, the scale bars represent 100 μm. **(B, C)** Hepatic MDA **(B)** and non-heme iron **(C)** were measured in the indicated mice (n=4-6 mice/group). **(D-F)** RBC counts **(D)**, hepatic *Hamp* mRNA levels **(E)**, and serum NTBI levels **(F)** were measured in the *Trf*-LKO mice (n=4-6 mice/group) treated with vehicle (oil), CCl_4, or CCL4+Apo-TF. The data in B-F were analyzed using a one-way ANOVA with Tukey's post hoc test; **P*<0.05 and ***P*<0.01.

supplemental Figure 12



supplemental Figure 12. Characterization of global *Trf* knockout (*Trf*-KO) mice. **(A, B)** Hepatic *Trf* mRNA **(A)** and serum Trf protein **(B)** levels were measured in newborn (P1) *Trf*-KO mice and control littermates. **(C)** Whole-body Transferrin IHC of an E16.5 control embryo and *Trf*-KO embryo. **(D)** Kaplan-Meier survival curves of control and *Trf*-KO mice (n=6 mice in *Trf*-KO mice group), the log-rank test was used to analyze the survival curve. **(E)** Example images of a newborn (P1) control and *Trf*-KO mouse; body weight at P1 is summarized at the right (n=4 mice/group). **(F)** Example blood smears from a control and *Trf* -KO mouse at P1, the scale bars represent 100 μm. ***P*<0.01, Student's *t*-test.

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