ADVANCED SCIENCE
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Supporting Information

for *Adv. Sci.*, DOI: 10.1002/advs.201903366

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Figure and Figure legends

Figure S1

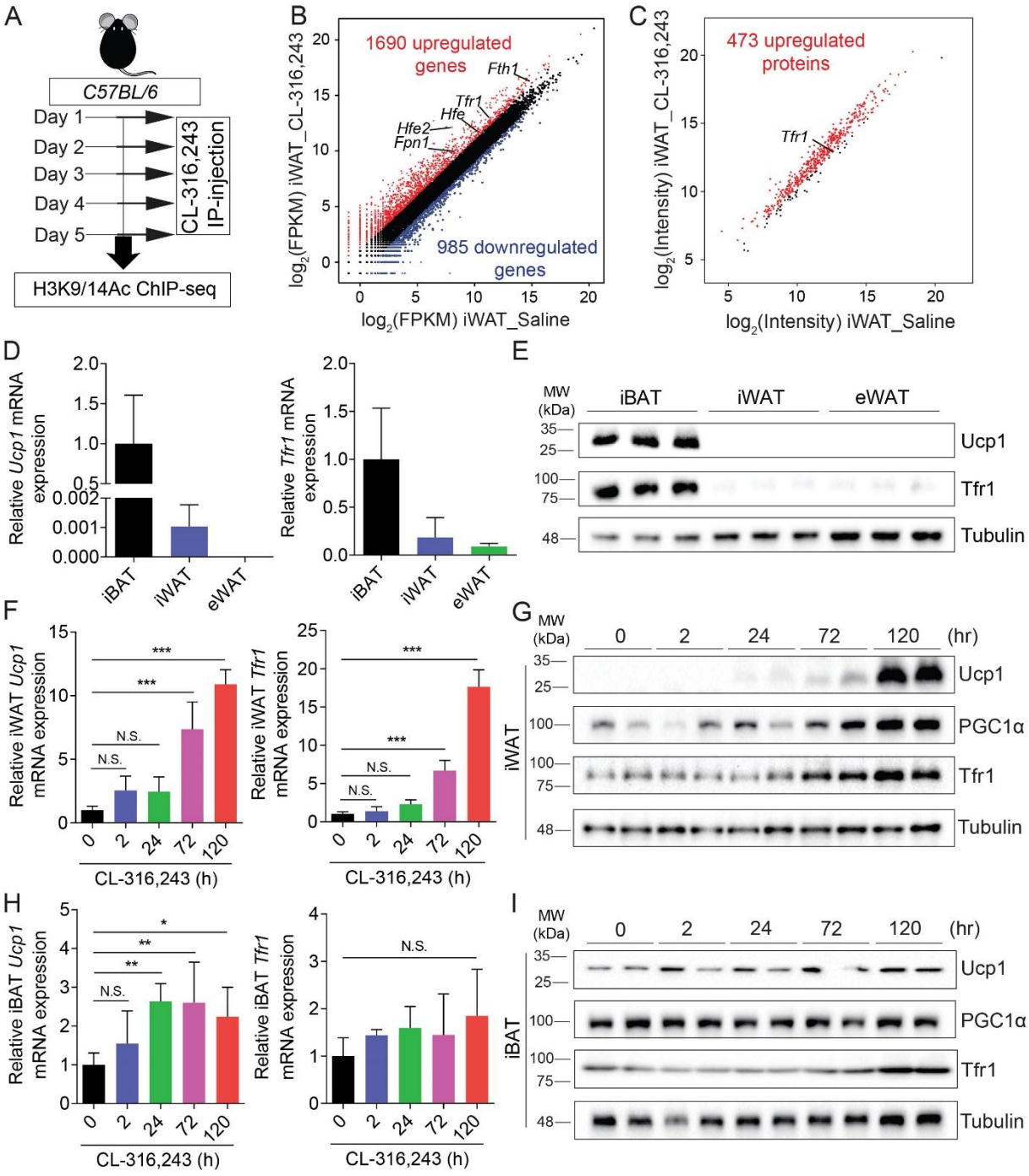

Figure S1. Related to Figure 1. CL-316,243 increases Tfr1 expression primarily in beige adipocytes.

(**A**) Timeline for CL-316,243 injections to induce beigeing of white adipocyte in C57BL/6J mice and

H3K9/14Ac ChIP-seq analysis. (**B**) Scatter plot of differentially expressed genes in iWAT following CL-316,243 injections. (**C**) Scatter plot of membrane-enriched proteins following CL-316,243 injections. (**D and E**) *Ucp1* and *Tfr1* mRNA and protein measured in iBAT, iWAT, and eWAT of *C57BL/6J* mice at steady state (n=6-8 mice/group), presented as mean ± SD, pooled from 3 independent experiments. (**F and H**) qRT-PCR analysis of *Ucp1* and *Tfr1* mRNA in iWAT and iBAT of *C57BL/6J* mice 0, 2, 24, 72, and 120 hr after CL-316,243 injections (n=6 mice/group), presented as mean ± SD, pooled from 3 independent experiments. (**G and I**) Ucp1, PGC1α, Tfr1, and Tubulin protein in iWAT and iBAT of *C57BL/6J* mice 0, 2, 24, 72, and 120 hr after CL-316,243 injections (n=6 mice/group), presented as mean ± SD, pooled from 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001, and N.S., not significant. One-way ANOVA with a Bonferroni post-hoc analysis was used for comparison among multiple groups.

Figure S2

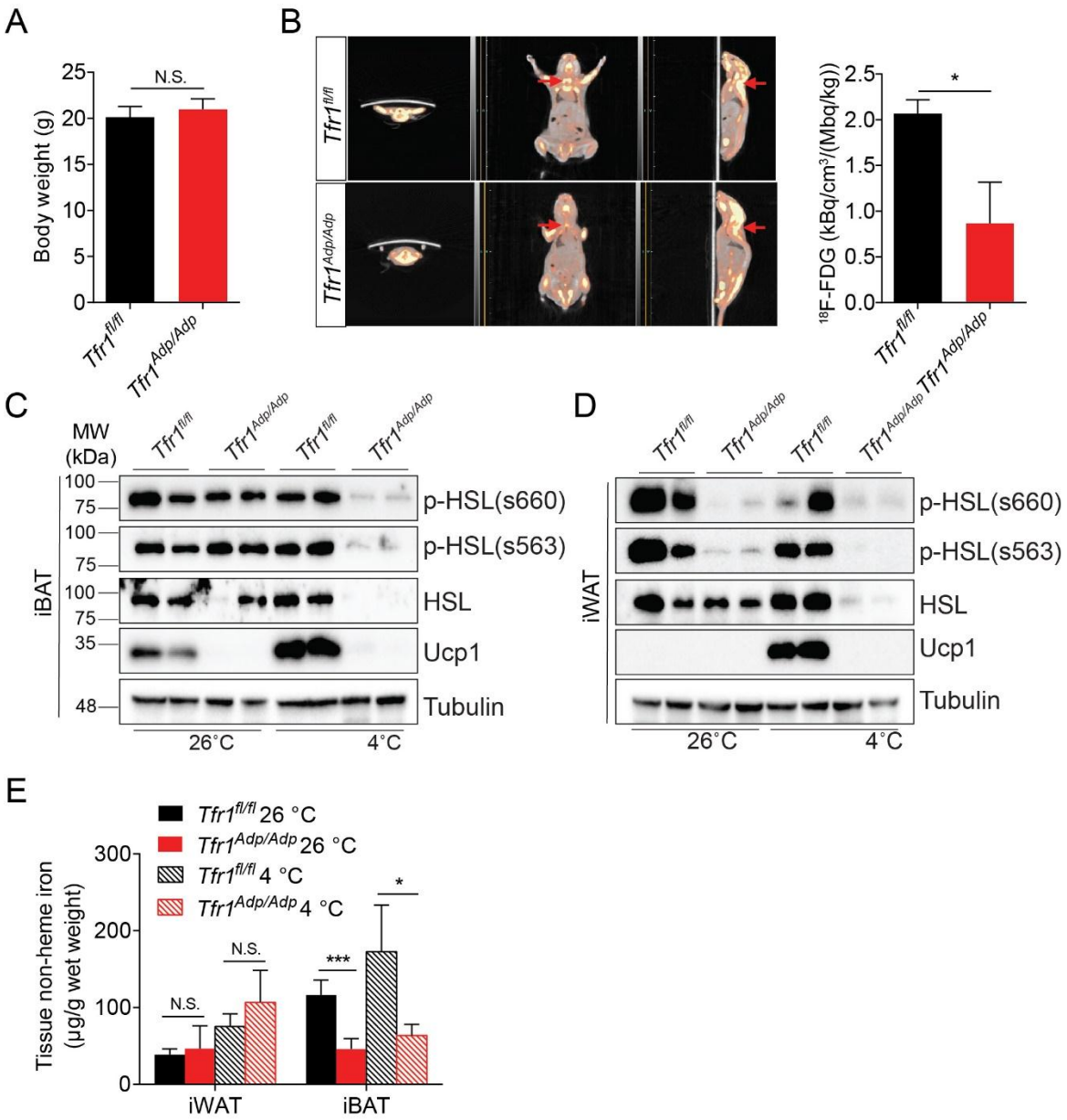

Figure S2. Related to Figure 2. Metabolic phenotype of control and *Tfr1*$^{Adp/Adp}$ mice. (**A**) Body weight of 8-week-old control (*Tfr1*$^{fl/fl}$) and *Tfr1*$^{Adp/Adp}$ mice (n=4-5 mice/group), presented as mean ± SD, pooled from 2 independent experiments. (**B**) Example ^{18}F-FDG micro-PET/CT images of *Tfr1*$^{fl/fl}$ and *Tfr1*$^{Adp/Adp}$ mice after exposure to cold. The quantitative summary of FDG uptake is shown at the right (n=3 mice/group), presented as mean ± SD. (**C**) Representative western blot analysis of HSL, pHSL(s660), p-HSL(s563), Ucp1, and Tubulin proteins in iBAT of *Tfr1*$^{fl/fl}$ and *Tfr1*$^{Adp/Adp}$ housed at 26°C

or 4°C (n=4 mice/group). (**D**) Representative western blot analysis of HSL, pHSL(s660), p-HSL(s563), Ucp1, and Tubulin proteins in iWAT of *Tfr1*$^{fl/fl}$ and *Tfr1*$^{Adp/Adp}$ housed at 26°C or 4°C (n=4 mice/group). (**E**) The non-heme iron levels were detected in iBAT and iWAT of *Tfr1*$^{fl/fl}$ and *Tfr1*$^{Adp/Adp}$ mice housed for 7 days at 26°C or 4°C (n=4-6 mice/group), presented as mean ± SD, pooled from 2 independent experiments. *P<0.05, ***P<0.001, and N.S., not significant. Unpaired Student's *t*-test was used for comparison between two groups. One-way ANOVA with a Bonferroni post-hoc analysis was used for comparison among multiple groups.

Figure S3

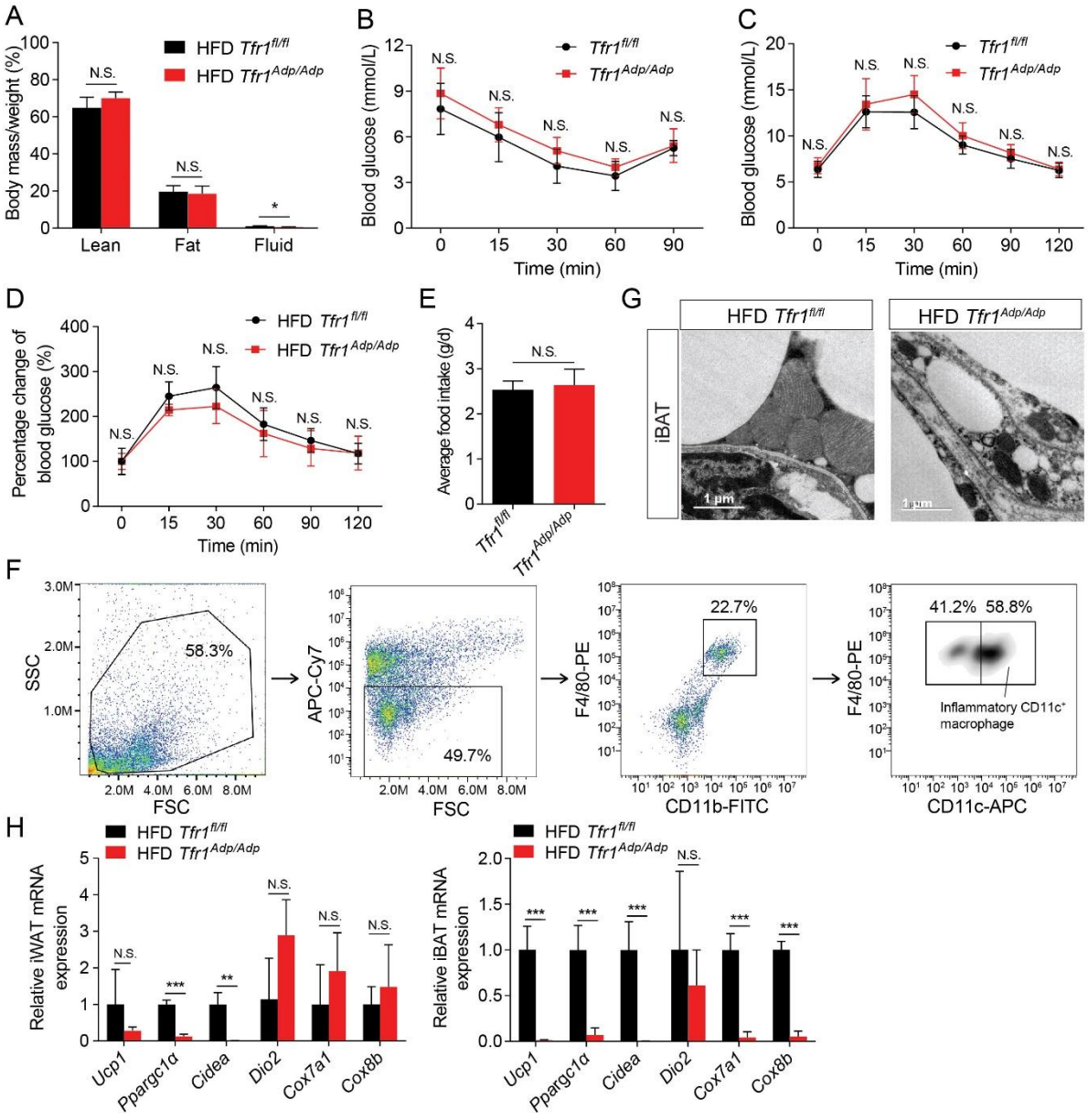

Figure S3. Related to Figure 3. Metabolic phenotype of HFD-fed control and *Tfr1*$^{Adp/Adp}$ mice. (**A**) Quantification of lean mass, fat mass and fluid of HFD-fed *Tfr1*$^{Adp/Adp}$ and control mice by Low-field NMR instrument (QMR06-090H, Suzhou Niumag Analytical Instrument Corporation, China) (n=4-6 mice/group), presented as mean ± SD**.** (**B and C**) Blood glucose was measured during the glucose tolerance test (GTT, B) and insulin tolerance test (ITT, C) in 2-month-old *Tfr1*$^{fl/fl}$ and *Tfr1*$^{Adp/Adp}$ mice (n=7 mice/group), presented as mean ± SD, pooled from 2 independent experiments. (**D**) The

percentage change of blood glucose of HFD-fed *Tfr1*$^{Adp/Adp}$ and control mice in GTT (n=7-8 mice/group). presented as mean ± SD, pooled from 2 independent experiments. (**E**) Average food intake of HFD-fed *Tfr1*$^{Adp/Adp}$ and control mice were measured for 5 days (n=6 mice/group), presented as mean ± SD, pooled from 2 independent experiments. (**F**) Flow cytometry results of antibody labeling and gating. FSC, forward scatter; SSC, side scatter. (**G**) Representative transmission electron microscopy images of iBAT in an HFD-fed *Tfr1*$^{fl/fl}$ mouse and an HFD-fed *Tfr1*$^{Adp/Adp}$ mouse (n=3 mice/group). (**H**) qRT-PCR analysis of *Ucp1, Ppargc1α, Cidea, Dio2, Cox7a1* and *Cox8b* mRNA in iWAT (left) and iBAT (right) of HFD-fed *Tfr1*$^{Adp/Adp}$ and control mice (n=3-4 mice/group), presented as mean ± SD, pooled from 2 independent experiments. *P<0.05, ***P<0.001, and N.S., not significant. Unpaired Student's *t*-test was used for comparison between two groups. One-way ANOVA with a Bonferroni post-hoc analysis was used for comparison among multiple groups.

Figure S4

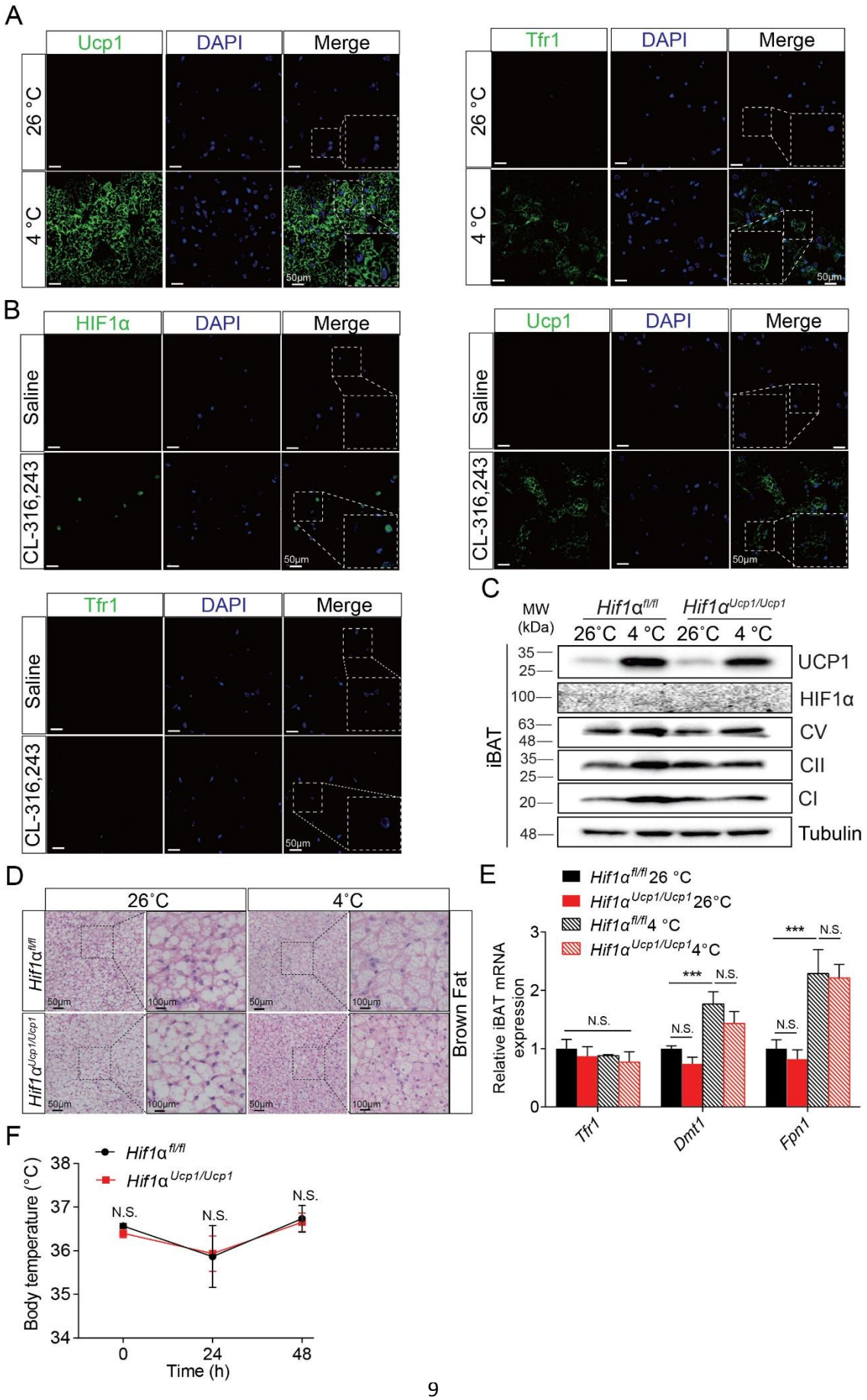
A
Ucp1
DAPI
Merge
26 °C
4 °C
50μm
Tfr1
DAPI
Merge
26 °C
4 °C
50μm
B
HIF1α
DAPI
Merge
Saline
CL-316,243
50μm
Ucp1
DAPI
Merge
Saline
CL-316,243
50μm
Tfr1
DAPI
Merge
Saline
CL-316,243
50μm
C
MW (kDa)
Hif1α fl/fl
Hif1α Ucp1/Ucp1
26°C 4 °C 26°C 4 °C
35
25
100
63
48
35
25
20
48
iBAT
UCP1
HIF1α
CV
CII
CI
Tubulin
D
26°C
4°C
Hif1α fl/fl
Hif1α Ucp1/Ucp1
50μm
100μm
Brown Fat
E
Hif1α fl/fl 26 °C
Hif1α Ucp1/Ucp1 26°C
Hif1α fl/fl 4 °C
Hif1α Ucp1/Ucp1 4°C
Relative iBAT mRNA expression
3
2
1
0
N.S.
***
N.S.
Tfr1
Dmt1
Fpn1
F
Hif1α fl/fl
Hif1α Ucp1/Ucp1
Body temperature (°C)
38
37
36
35
34
N.S.
0
24
48
Time (h)

Figure S4. Related to Figure 4. Loss of Hif1α in iBAT does not cause impaired thermogenesis. (**A**) Representative images of white adipocytes obtained from C57BL/6J mice housed at 26°C or 4°C and immunostained for Tfr1 (left) or Ucp1 (right); the nuclei were counterstained with DAPI (n=6 mice/group). (**B**) Representative images of white adipocytes obtained from control-treated (saline) and CL-316,243–treated C57BL/6J mice and immunostained for HIF1α (top left), Tfr1 (top right), or Ucp1 (bottom left); the nuclei were counterstained with DAPI (n=6 mice/group). (**C**) Representative western blot analysis of Ucp1, HIF1α, Tubulin, and mitochondrial complex I, II, and V proteins in iBAT of *Hif1α*$^{fl/fl}$ *and Hif1α*$^{Ucp1/Ucp1}$ mice housed at 26°C or 4°C (n=4 mice/group). (**D**) Representative images of H&E-stained iBAT sections from *Hif1α*$^{fl/fl}$ *and Hif1α*$^{Ucp1/Ucp1}$ mice housed at 26°C or 4°C (n=4 mice/group). (**E**) qRT-PCR analysis of *Tfr1*, *Dmt1*, and *Fpn1* expression in iBAT of *Hif1α*$^{fl/fl}$ and *Hif1α*^{Ucp1Ucp1} mice housed at 26°C or 4°C for 7 days (n=4 mice/group), presented as mean ± SD, pooled from 2 independent experiments. (**F**) Time course of rectal temperature of *Hif1α*$^{fl/fl}$ *and Hif1α*$^{Ucp1/Ucp1}$ mice mice at 4°C (n=3 mice/group), presented as mean ± SD.****P*<0.001, and N.S., not significant. One-way ANOVA with a Bonferroni post-hoc analysis was used for comparison among multiple groups.

Figure S5

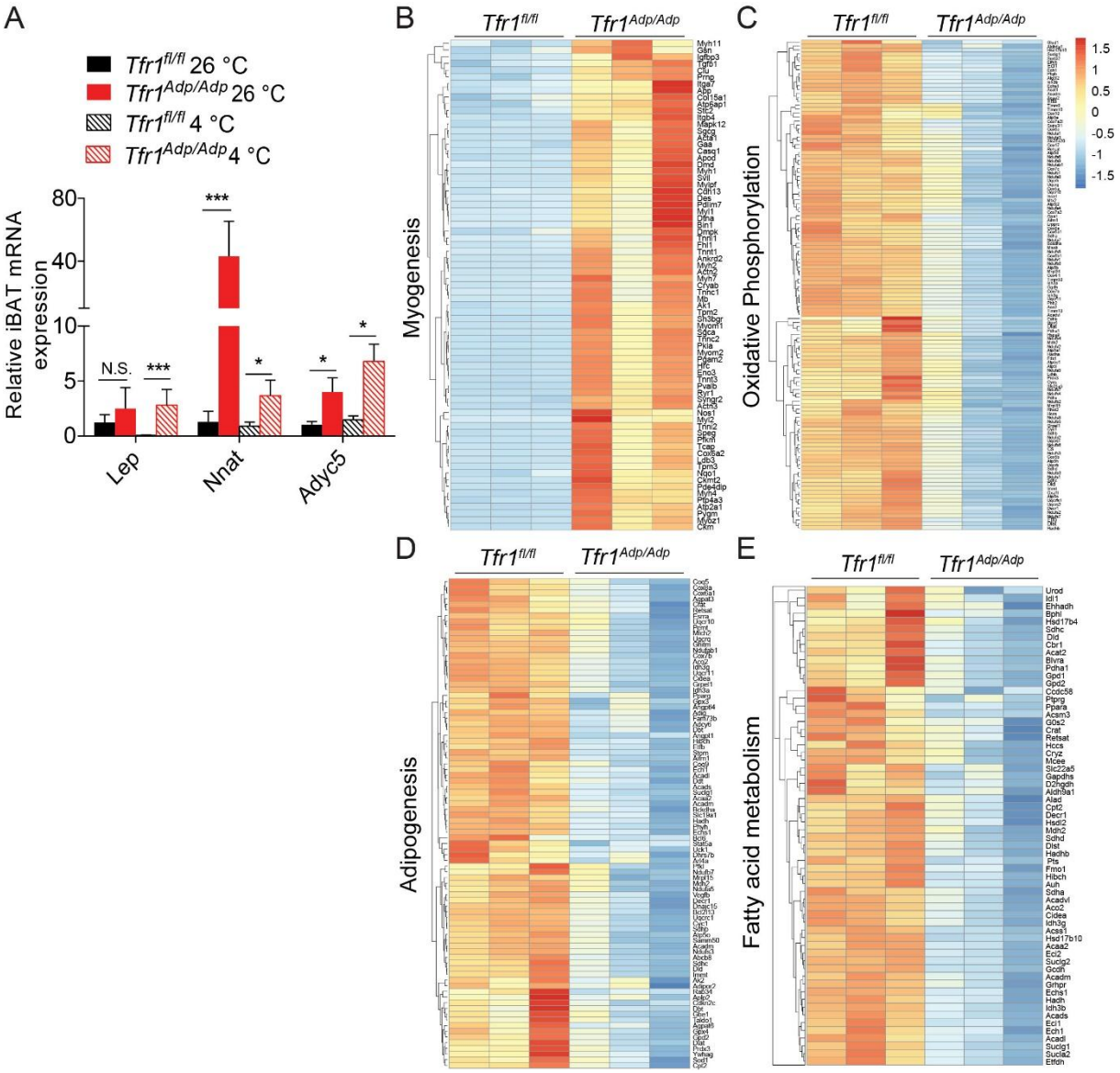

Figure S5. Related to Figure 5. White adipocyte marker genes are differentially expressed between *Tfr1*$^{Adp/Adp}$ and *Tfr1*$^{fl/fl}$ mice. (**A**) qRT-PCR analysis of the white adipocyte–related genes *Lep*, *Nnat*, and *Adcy5* in iBAT of *Tfr1*$^{fl/fl}$ *and Tfr1*$^{Adp/Adp}$ mice housed for 7 days at 26°C or 4°C n=6-8 mice/group, presented as mean ± SD, pooled from 2 independent experiments. (**B-E**) Heatmaps of differentially expressed genes associated with myogenesis, oxidative phosphorylation, adipogenesis, and fatty acid metabolism in iBAT of *Tfr1*$^{fl/fl}$ and *Tfr1*$^{Adp/Adp}$ mice; *P<0.05, ***P<0.001, and N.S., not significant.

One-way ANOVA with a Bonferroni post-hoc analysis was used for comparison among multiple groups.

Figure S6

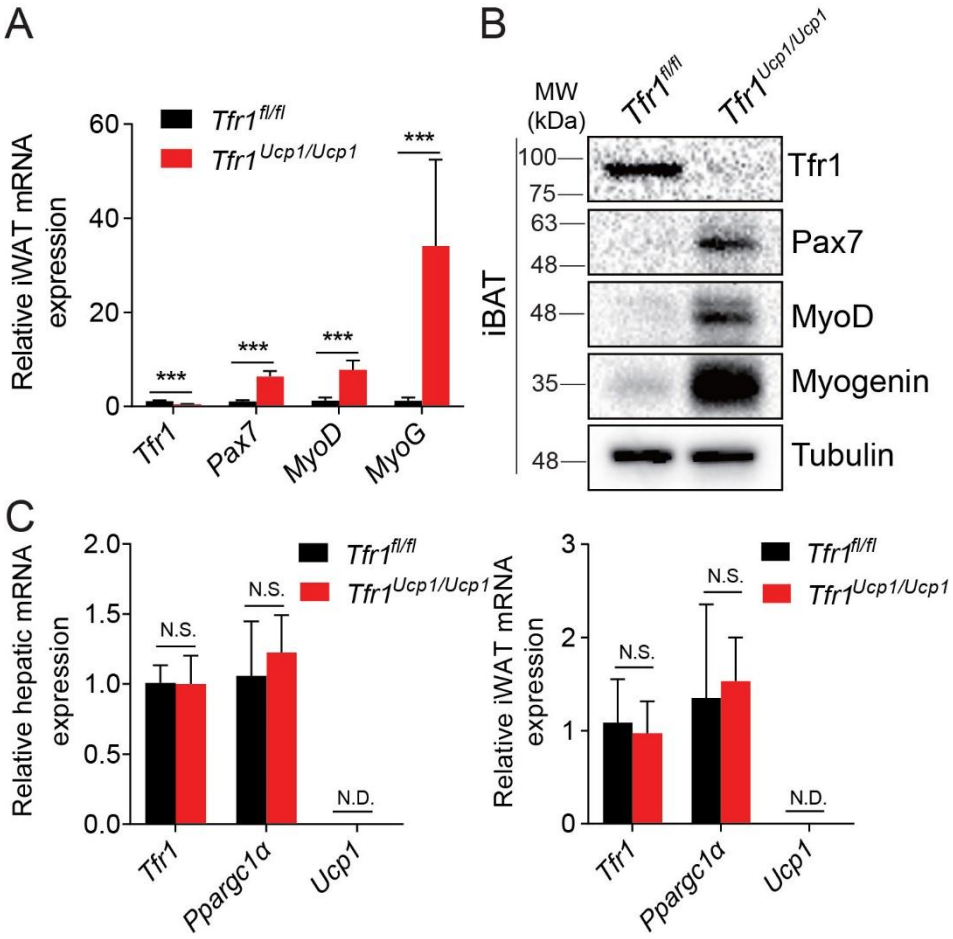

Figure S6. Related to Figure 5. Validation of thermogenic cell–specific *Tfr1* knockout and muscle cell marker gene expression. (**A**) qRT-PCR analysis of skeletal muscle–related gene expression in iBAT of $Tfr1^{fl/fl}$ and $Tfr1^{Ucp1/Ucp1}$ mice (n=6 mice/group), presented as mean ± SD, pooled from 2 independent experiments. (**B**) Representative western blot analysis of Tfr1, Pax7, MyoD, Myogenin, and Tubulin protein in iBAT of $Tfr1^{fl/fl}$ and $Tfr1^{Ucp1/Ucp1}$ mice (n=6 mice/group). (**C**) qRT-PCR analysis of *Tfr1*, *Ppargc1α*, and *Ucp1* mRNA in the liver (left) and iWAT (right) of $Tfr1^{fl/fl}$ and $Tfr1^{Ucp1/Ucp1}$ mice

(n=6 mice/group), presented as mean ± SD. ***P<0.001, N.S., not significant, and N.D., not detectable. Unpaired Student's t-test was used for comparison between two groups.

Figure S7

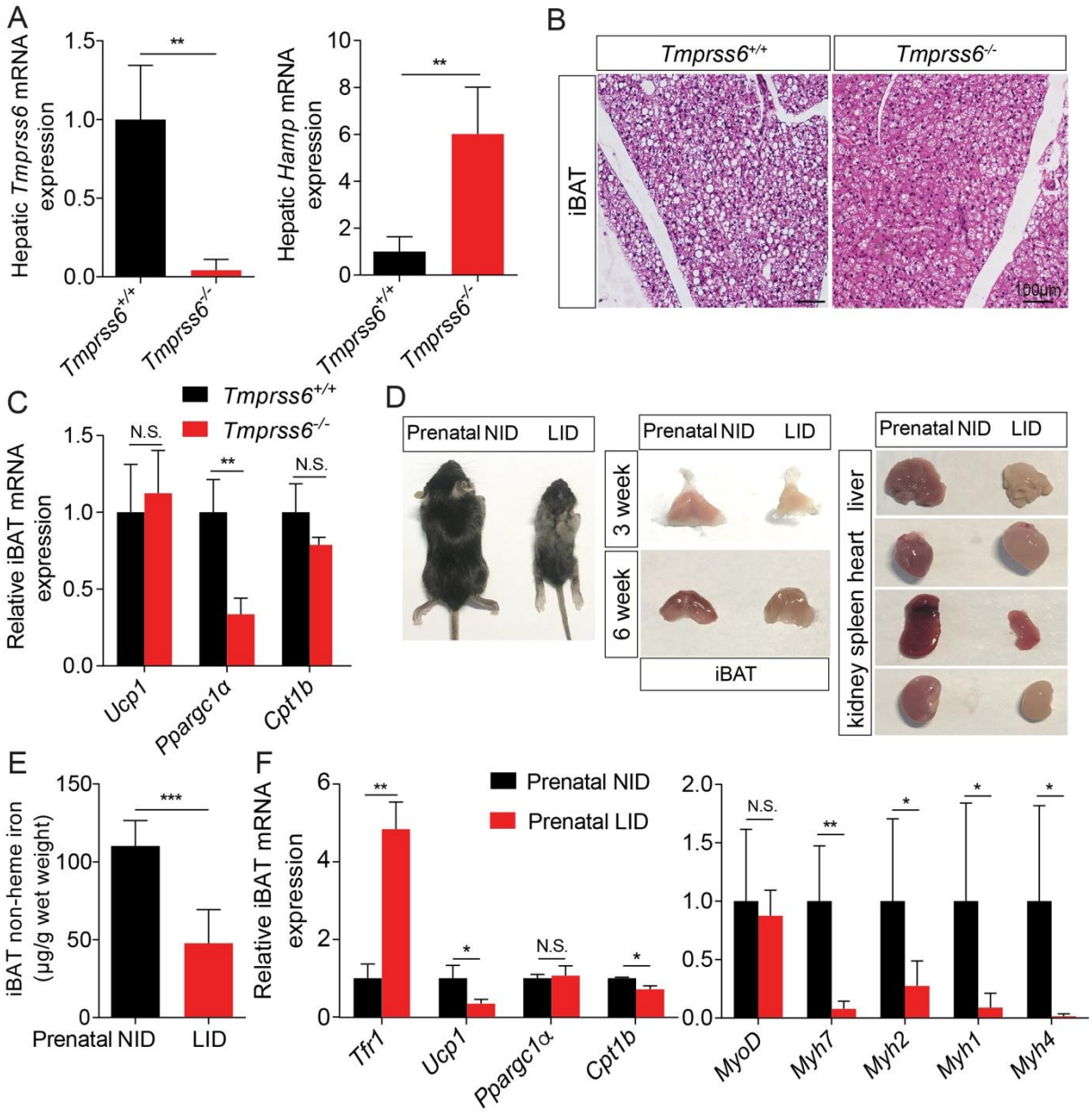

Figure S7. Related to Figure 6. Characterization of iron-deficient *Tmprss6* knockout mice and offspring exposed *in utero* and postnatally to a maternal low-iron diet. (**A**) qRT-PCR analysis of hepatic *Tmprss6* and *Hamp* mRNA in liver in control (*Tmprss6*$^{+/+}$) and *Tmprss6*$^{-/-}$ mice (n=4-6 mice/group), presented as mean ± SD, pooled from 2 independent experiments. (**B**) Representative images of H&E-stained iBAT from *Tmprss6*$^{+/+}$ and *Tmprss6*$^{-/-}$ mice (n=3 mice/group). (**C**) Representative images of iBAT, heart, liver, spleen, and kidney obtained from 3-week-old and 6-

week-old C57BL/6J mice that were exposed to either a normal-iron diet (NID) or a low-iron diet (LID) since conception and then maintained on that diet (n=6 mice/group), presented as mean ± SD, pooled from 2 independent experiments. (**D**) Representative images of H&E-stained iBAT in prenatal NID-fed and LID-fed mice (n=3 mice/group). (**E**) Non-heme iron level was measured in iBAT of prenatal NID-fed and LID-fed mice (n=6 mice/group), presented as mean ± SD, pooled from 2 independent experiments. (**F**) qRT-PCR analysis of thermogenic (left) and myogenic (right) genes in iBAT of prenatal NID-fed and LID-fed mice (n=6 mice/group), presented as mean ± SD, pooled from 2 independent experiments $*P<0.05$, $**P<0.01$, $***P<0.001$, and N.S., not significant. Unpaired Student's *t*-test was used for comparison between two groups.

Table S1. Primers used for genotyping the knockout mice used in this study

Knockout mouse line	Forward (5'→3')	Reverse (5'→3')
Tfr1$^{fl/fl}$	CAGTAATCCCAGAGGAATCATTAG	CTAAACCGGGTGTATGACAATG
Hif1α$^{fl/fl}$	TGCATGTGTATGGGTGTTTTG	GAAAACTGTCTGTAACTTCATTTCC
Tmprss6$^{fl/fl}$	CAAGGATCCCCTCAAACCCC	CACCTGGCTTACGGTCACTT
Ucp1-Cre	TGCTGTTTCACTGGTTATGCGG	TTGCCCCTGTTTCACTATCCAG
Adipoq-Cre	GGATGTGCCATGTGAGTCTG	ACGGACAGAAGCATTTTCCA
Cmv-Cre	ATTTGCCTGCATTACCGGTC	ATCAACGTTTTCTTTTCGG

Table S2. Primers used for qRT-PCR and ChIP-qPCR

Stem cell markers		
Gene	**Forward (5'→3')**	**Reverse (5'→3')**
Dclk1	TGAGCATCCCTGGGTTAATGAT	GAAACTCCTGCTGCAGTGC
Cd44	TTCGATGGACCGGTTACCATAA	AGCTTTCTGGGGTGCTCTT
Ly6a	TCAATTACCTGCCCCTACCC	CAGAGGTCTTCCTGGCAACA
Cd34	CCAGGGTATCTGCCTGGAAC	TCAGCCTCCTCCTTTTCACA
Kit	AGAGATTTGGCAGCCAGGA	TCTCTGGTGCCATCCACTTC
EN1	ACAGCAACCCCTAGTGTGG	TAGCGGTTTGCCTGGAACT
Skeletal muscle markers		
Gene	**Forward (5'→3')**	**Reverse (5'→3')**
Pax7	TGCCCTCAGTGAGTTCGATT	GAGGTCGGGTTCTGATTCCA
MyoD	CGCCACTCCGGGACATAG	GAAGTCGTCTGCTGTCTCAAAGG
MyoG	ATCCAGTACATTGAGCGCCT	GCAAATGATCTCCTGGGTTGG
Mstn	CCAGGACCAGGAGAAGATGG	AGTCCCATCCAAAGGCTTCA
Myh7(MYH I)	GCTGCAGCAGTTCTTCAACC	GGAACATGCACTCCTCCTCA
Myh2(MYH_IIA)	CCAAGGCCATGTATGAGAAGATGTT	AGCTGTTCCAGGCTGTTGA
Myh1(MYH_IIX)	CGCTGGCTTTGAGATCTTTGATT	CAGGTCCATCCCAAAGTCAATG

Myh4(MYH_IIB)	CCCTGGCCAAGTCCATGTAT	ACAGCTGCTCCAAGGTGT
	Iron metabolism	
Gene	**Forward (5'→3')**	**Reverse (5'→3')**
Dmt1	CCAGGATGTGGAGCACCTA	GCTTGTGAACGTGAGGATGG
Fpn1	TTGTGGCAGGAGAAAACAGG	GCCAATGACTGGAGAACCAA
Tfr1	TCGTACAGCAGCGGAAGT	TCTCCACGAGCGGAATACAG
Tmprss6	CATGGCTCCAGGCCATTGAT	GGGCATCCTTCAAGAGTGGAA
Hif1a	CAGAATGCTCAGAGGAAGCG	CTGCATGCTAAATCGGAGGG
	Thermogenic genes	
Gene	**Forward (5'→3')**	**Reverse (5'→3')**
Ppargc1a	CTCTGGAACTGCAGGCCTAA	TGCCTTGGGTACCAGAACA
Ucp1	ATACTGGCAGATGACGTCCC	CGAGTCGCAGAAAAGAAGCC
Cidea	ATACATCCAGCTCGCCCTTT	ACTTACTACCCGGTGTCCAT
Dio2	CAGTGTGGTGCACGTCTC	TGAACCAAAGTTGACCACCAG
Cox7a1	AGCTGCTGAGGACGCA	GCTTCTGCTTCTCTGCCAC
Cox8b	TTCCCAAAGCCCATGTCTCT	GGCTAAGACCCATCCTGCT
Cpt1b	GAATCCTCGACGACCCTTCC	TGAAGAAGGTCTGACGTGCC
	White adipocyte markers	

Gene	Forward (5'→3')	Reverse (5'→3')
Lep	GTGTCGGTTCCTGTGGCT	AGCCCAGGAATGAAGTCCAA
Nnat	TGCTGCAGACCCTGTCC	GCGTTGGTCGCGATCG
Adcy5	TCTTCGTGCTGGCTCTGT	CTGCAGCTCCTCCATCTCC
ChIP-qPCR		
Gene	**Forward (5'→3')**	**Reverse (5'→3')**
Tfr1_HRE	TACTCCGCGCACGCACTGG	CGGCCCTCTGGATCTACG
Cav1_HRE	CCTTGGGGATGTGCCTAGA	AGGGGTTTGTTCTGCTCTCA
Pdk3_HRE	CCGCGACACCTACACAAG	TACCGGGGCTTTAAGGAAGC
H3K9Ac14 binding site		
Gene	**Forward (5'→3')**	**Reverse (5'→3')**
Int_Ctrl_Chr5	CCCGTCACTCAACCATTTCA	CTTATCAATGGGGGCTCTGG
Tfr1	CTCCAAAGACACGGCGG	TAGAAAAGGCGCCAAGGC
Dmt1	CTGATCCGGAGGCGTGAT	GGGGATGGGAGTTGCCA
Fpn1	AGCGGCTTATAGGGAGCC	CGGACCTGGACGTCCAG