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Supplemental information

Downregulation of hepatic ceruloplasmin ameliorates NAFLD via SCO1-AMPK-LKB1 complex

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Supplemental Information

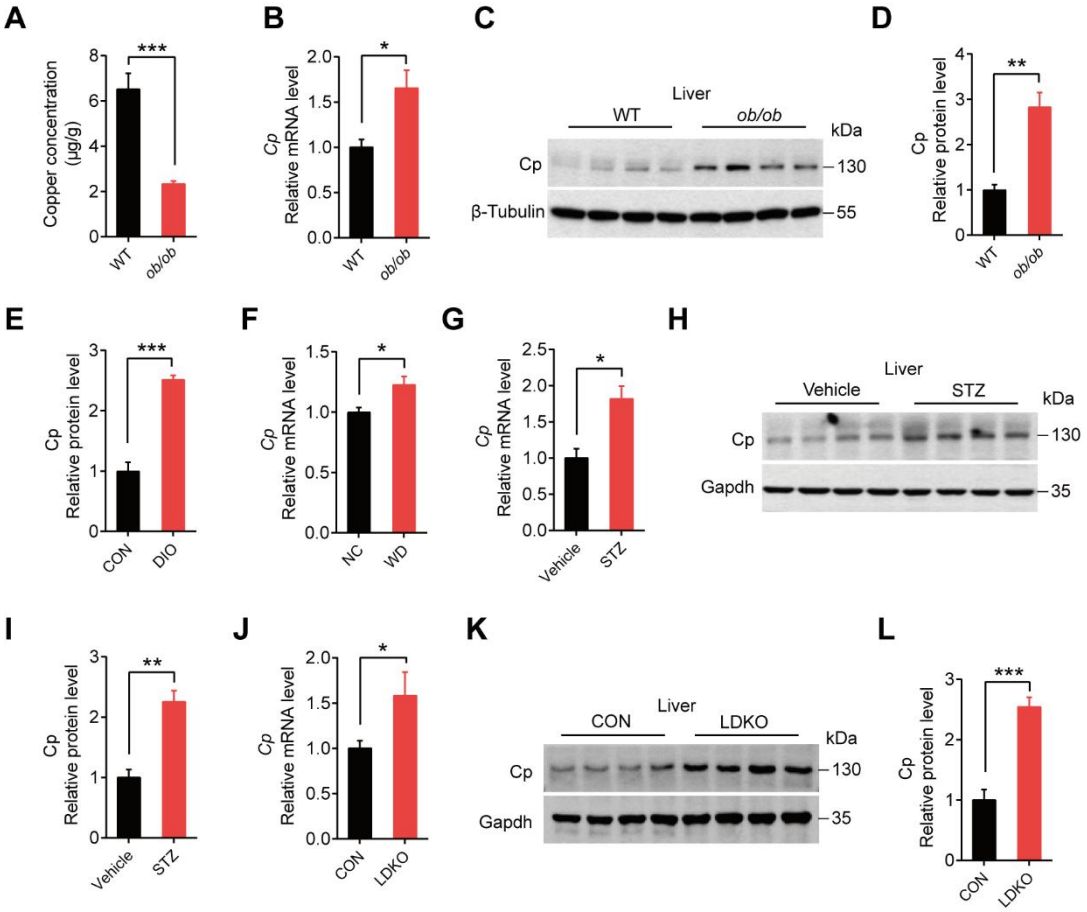

Figure S1. Hepatic copper concentrations are downregulated and Cp expression is upregulated in fatty liver and diabetes, related to Figure 1.

(A) Hepatic copper concentrations in *ob/ob* and wild-type (WT) mice (n=6 in each group). (B) *Cp* mRNA levels in livers of *ob/ob* and WT mice. (C-D) Cp protein levels (C) and their quantified signal intensity (D) in livers of *ob/ob* and WT mice. (E) Quantification of Cp protein in Figure 1F. (F) *Cp* mRNA levels in livers of western diet-fed (WD) and normal chow-fed mice (NC). (G) *Cp* mRNA levels in livers of vehicle and STZ-injected mice. (H-I) Immunoblots of Cp

protein (H) and their quantification (I) in the livers of vehicle and STZ-injected mice. (J) *Cp* mRNA levels in the livers of LDKO and CON mice. (K-L) Immunoblots of Cp protein (K) and their quantification (L) in the livers of LDKO and CON mice.

Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the control group.

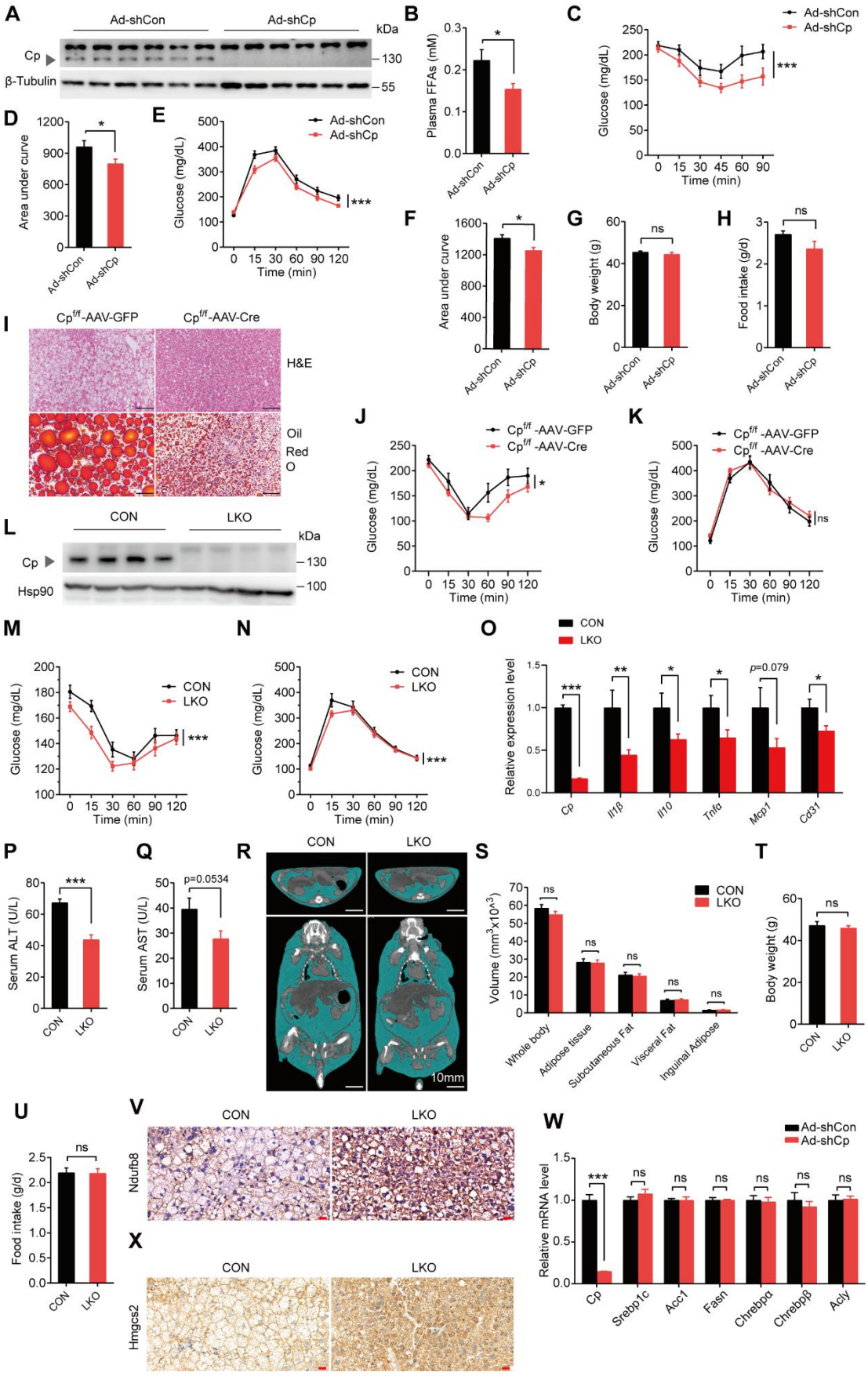

Figure S2. Cp-knockdown attenuates HFD-induced hepatic

steatosis, related to Figure 1 and Figure 2.

(A-H) DIO mice (fed with a HFD for 13 weeks) were injected with Ad-shCp or Ad-shCon (n=10 in each group). (A) Immunoblots of Cp protein in mouse livers. (B) Free fatty acid (FFA) concentrations in plasma. (C) Insulin tolerance tests (ITT) analysis on day 7 post-injection. (D) Area under curve analysis of ITT in (C). (E) Glucose tolerance tests (GTT) analysis on day 15 post-injection. (F) Area under curve analysis of GTT in (E). (G) Body weight (g) of the mice. (H) Average daily food intake (g/d) of the mice. (I-K) $Cp^{flox/flox}$ Mice (fed with a HFD for 8 weeks) were injected with AAV-Cre ($Cp^{f/f}$-AAV-GFP) or AAV-GFP ($Cp^{f/f}$-AAV-Cre) (n=6 in each group). (I) Representative images of Oil Red O- and H&E-stained liver sections; H&E staining scale bar: 100 μm, Oil Red O staining scale bar: 50 μm. (J-K) ITT analysis(J) and GTT analysis(K) were performed after HFD feeding for 12 weeks. (L-V) liver-specific Cp knockout (LKO) and control littermates ($Cp^{f/f}$, CON) fed with a HFD for 20 weeks. (L) Immunoblot of Cp in mouse liver. (M-N) ITT analysis (M) and GTT analysis (N) after 12 weeks of HFD feeding. (O) mRNA levels of inflammatory factors in the liver of mice. (P-Q) Serum ALT (P) and AST (Q) level of mice. (R-S) The micro-CT analysis of mice (R) and their quantification (S), scale bar: 10 mm. (T) Body weight (g) of the mice. (U) Average daily food intake (g/d)

of the mice. (V) The immumohistochemical staining of Ndufb8 in the liver of mice, scale bar: 20 μm. (W) mRNA levels of lipogenic genes in primary hepatocytes treated with Ad-shCon or Ad-shCp. (X) The immumohistochemical staining of Hmgcs2 in the liver of mice, scale bar: 20 μm.

Data shown in (O, W) are from one representative experiment of at least three independent experiments. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the control group. ns, not significant.

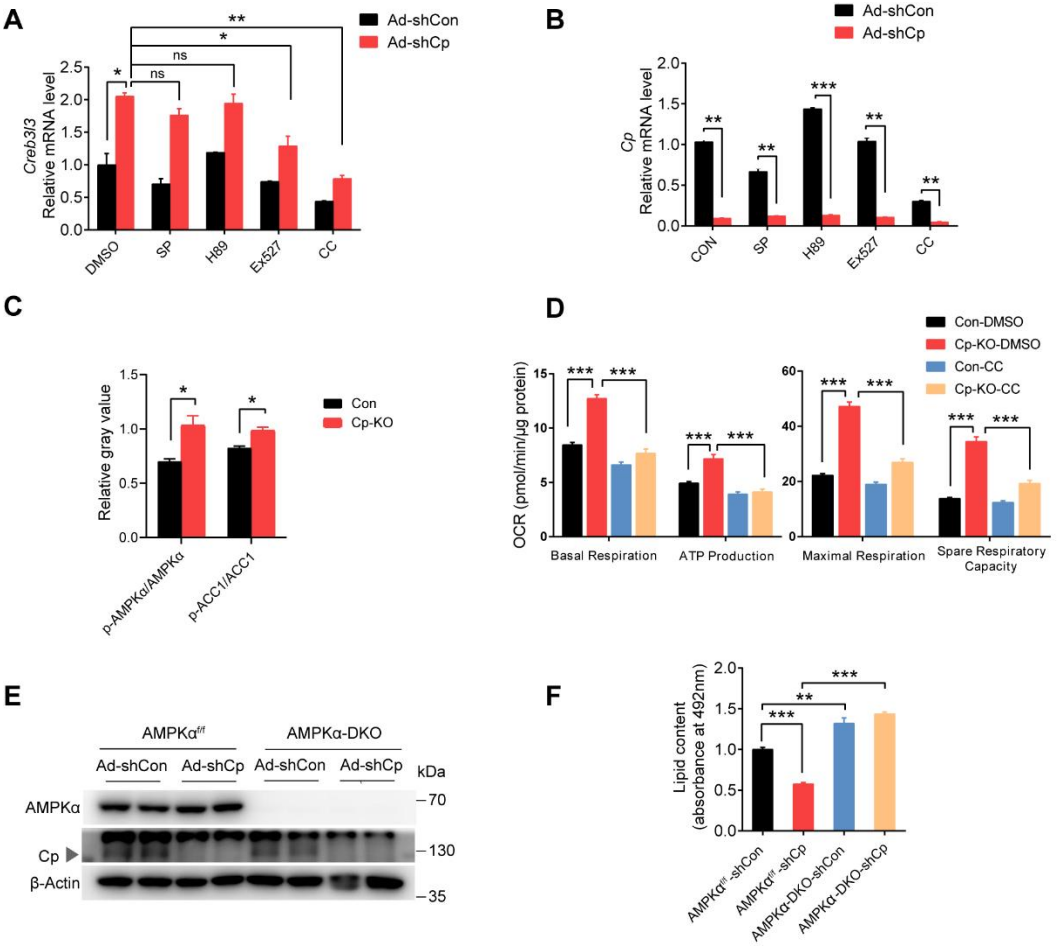

Figure S3. Cp regulates fatty acid oxidation genes through AMPK pathway, related to Figure 3.

(A-B) *Creb3l3* (A) and *Cp* (B) mRNA levels in Cp-knockdown primary hepatocytes treated with JNK inhibitor (SP), PKA inhibitor (H89), SIRT1 inhibitor (Ex527) and AMPK inhibitor (CC). (C) Quantitative analysis of p-AMPKα/AMPKα and p-ACC1/ACC1 ratios in Figure 3C. (D) OCR in primary hepatocytes from CON and LKO mice and treated with CC for 24 h. (E) Immunoblot analysis of Cp and AMPKα in primary hepatocytes. The primary hepatocytes were isolated from AMPKα1/α2 DKO (AMPKα-DKO) or AMPKα1$^{flox/flox}$ and α2$^{flox/flox}$ (AMPKα$^{f/f}$) mice, followed by the infection with Ad-shCp or Ad-shCon. Triangle indicates Cp. (F) Quantification of Oil Red O staining in Figure 3F.

Data shown in (A-B) are from one representative experiment of at least three independent experiments. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the control group.

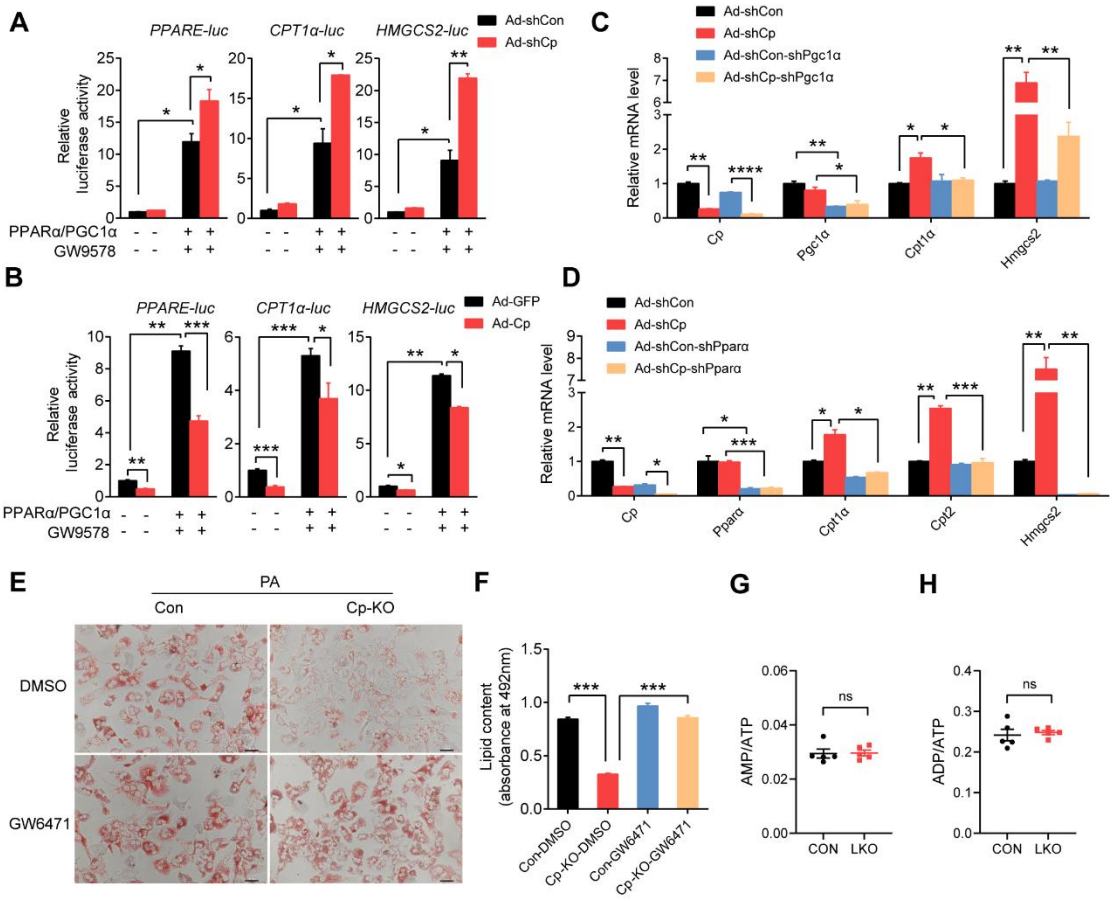

Figure S4. Cp regulates fatty acid oxidation genes via the AMPK-PGC1α axis, related to Figure 3.

(A-B) Luciferase activity of reporters containing PPARα response element (PPARE) (*PPARE-Luc*) and the promoter regions of *CPT1α* (*CPT1α-Luc*) and *HMGCS2* (*HMGCS2-Luc*) in Cp-knockdown (A) and Cp-overexpression (B) AML12 cells. (C-D) mRNA levels of FAO genes in Cp and Pgc1α double-knockdown (C)

or Cp and Pparα double-knockdown (D) AML12 cells. (E-F) Representative images of Oil Red O staining (E) and their quantification (F) in primary hepatocytes from CON and LKO mice treated with a PPARα inhibitor (GW6471), (scale bar: 50 μm). (G-H) Analysis of AMP/ATP (G) and ADP/ATP (H) ratio in the livers of CON and LKO mice.

Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the control group.

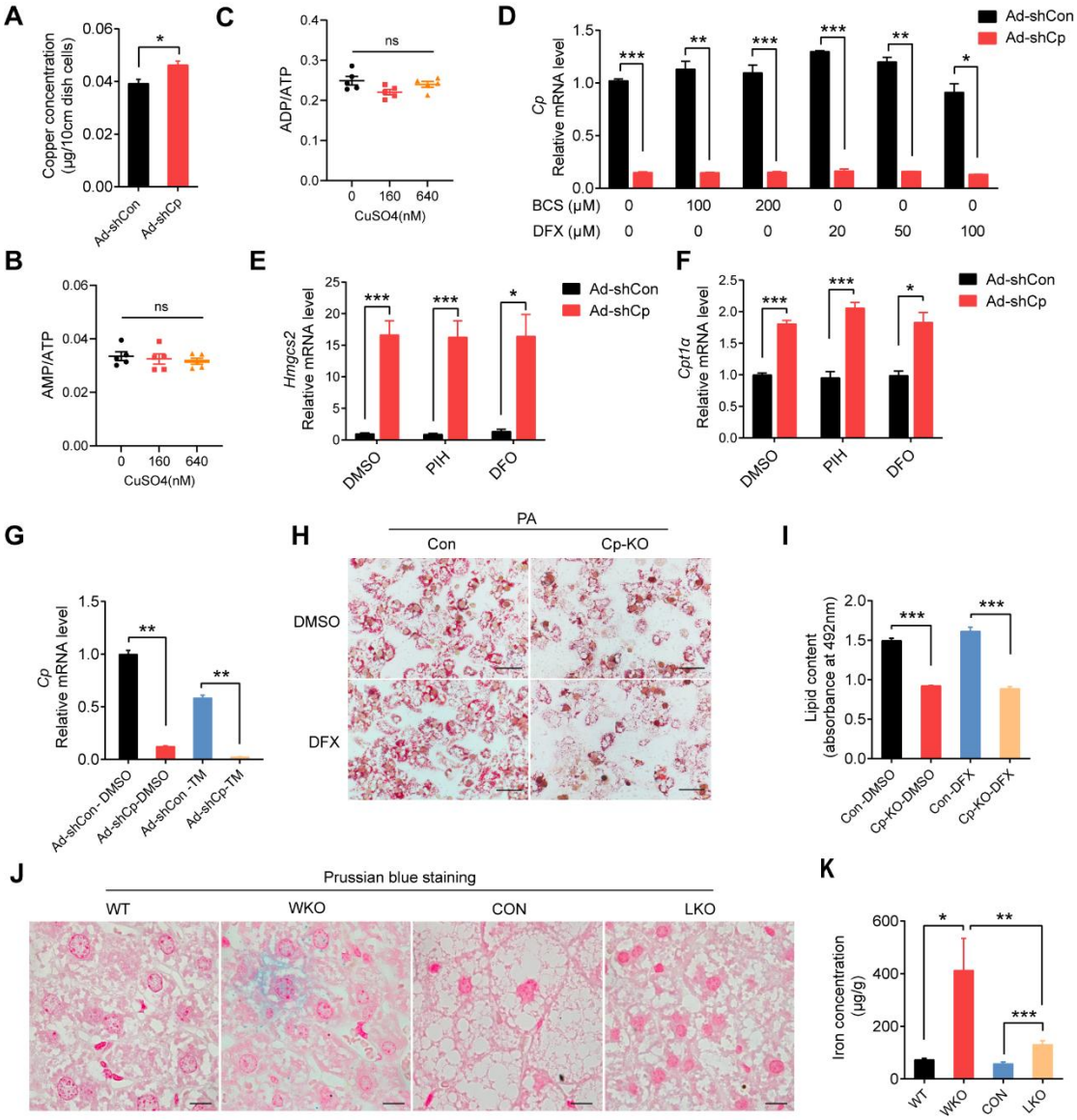

Figure S5. Cp regulates AMPK signaling by changing intracellular copper concentrations, related to Figure 4.

(A-G) Primary hepatocytes treated with Ad-shCp or Ad-shCon. (A) Cellular copper concentrations. (B-C) Analysis of AMP/ATP and ADP/ATP ratio in primary hepatocytes treated with $CuSO_4$ for 4h. (D) mRNA level of *Cp* in primary hepatocytes treated with a copper chelator (BCS) or an iron chelator (DFX). (E-F) mRNA levels of *Hmgcs2* (E) and *Cpt1α* (F) in primary hepatocytes treated with iron

chelators (PIH and DFO). (G) mRNA levels of *Cp* in primary hepatocytes treated with a copper chelator (TM). (H-I) Representative images of Oil Red O staining (H) and their quantification (I) of Con and Cp-KO primary hepatocytes treated with DFX and PA (scale bar: 50 μm). (J-K) Prussian blue staining of iron (J) and ICP-MS detection of iron (K) in the livers from thirteen-month-old Cp-WKO mice and Cp-LKO mice fed five-month-HFD along with their control (scale bar: 10 μm).

Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the control group.

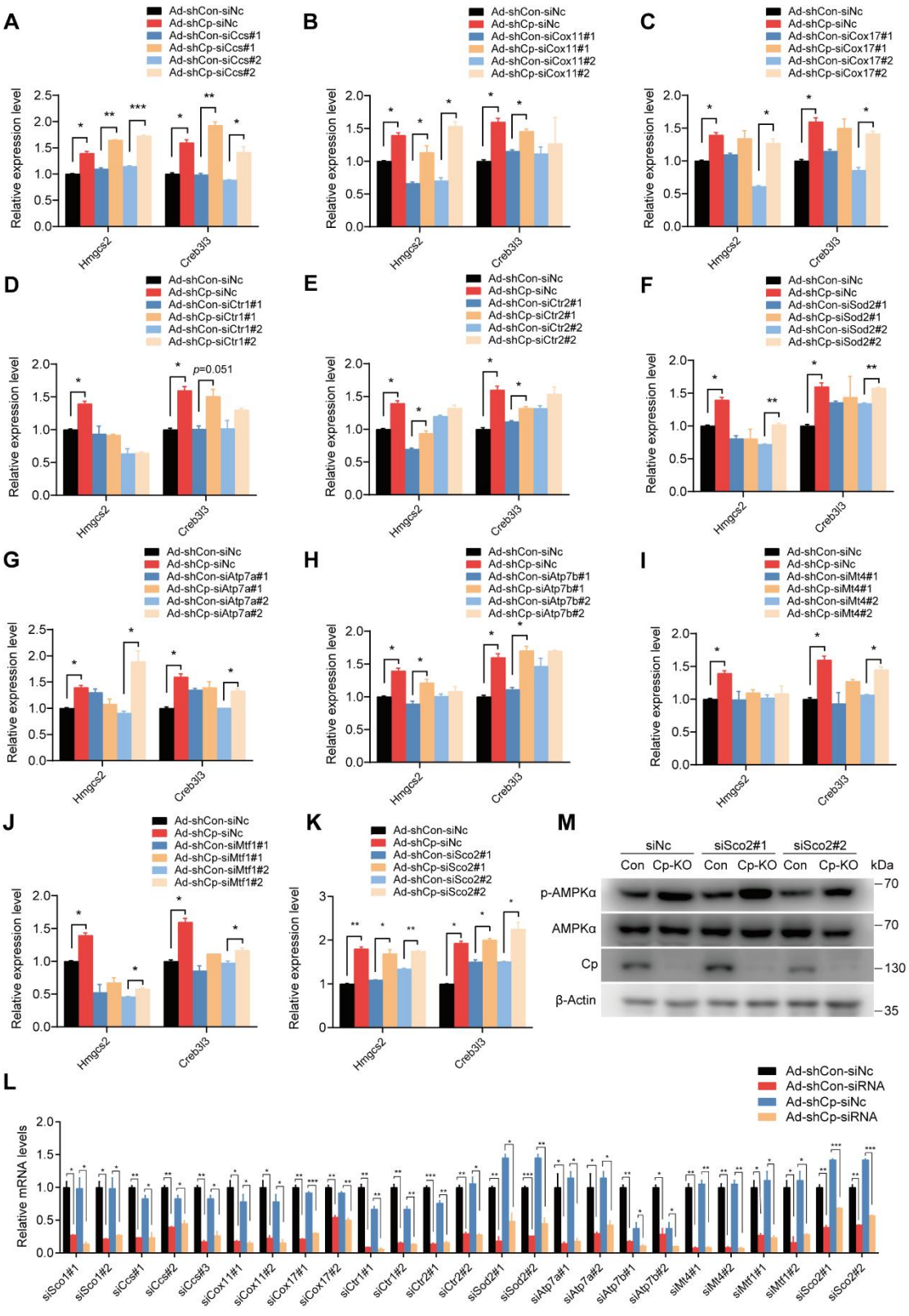

Figure S6. Copper upregulates hepatic FAO via SCO1, related to Figure 4.

(A-K) mRNA levels of *Hmgcs2* (A) and *Creb3l3* (B) in primary

hepatocytes isolated from Ad-shCp- and Ad-shCon-injected C57BL/6J mice and treated with indicated siRNAs. (L) mRNA levels of siRNAs targeted genes in (A-K). (M) Immunoblotting for p-AMPKα, AMPK and Cp in LKO primary hepatocytes (Cp-KO) treated with siRNA targeting Sco2 (siSco2#1, siSco2#2).

Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the control group.

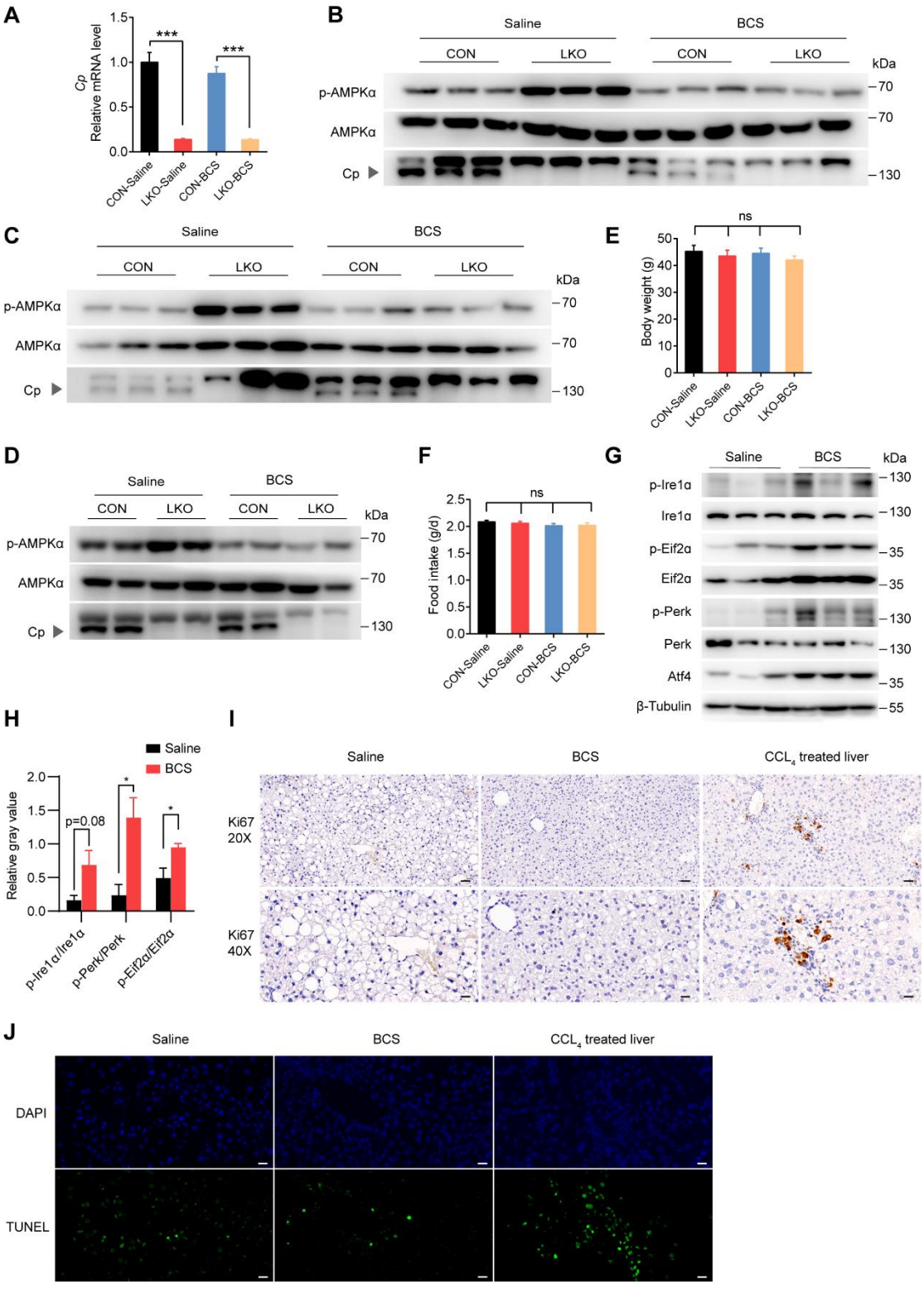

Figure S7. Copper chelation aggravates hepatic steatosis, related to Figure 7.

(A-J) LKO and control mice were treated as described in Figure 7A.

(A) mRNA level of *Cp* in mouse livers. (B-D) Immunoblots of p-AMPKα, AMPKα and Cp in in mouse livers. Triangles indicate Cp band. (E) Body weight (g) of the mice. (F) Average daily food intake (g/d) of the mice. (G-H) Immunoblots of phosphorylated Ire1α, Eif2α, Perk and the level of Atf4 in the liver of control mice treated with BCS or Saline (G) and the quantitative analysis of p-Ire1α/Ire1α, p-Eif2α/Eif2α, p-Perk/Perk ratios (H). (I-J) The representative image of Ki67 (I) and TUNEL staining (J) in the liver of control mice treated with BCS or Saline, Ki67: 20X: scale bar, 50 μm; 40X: scale bar, 20 μm; TUNEL staining: scale bar, 20 μm.

Data shown in (A) are from one representative experiment of at least three independent experiments. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the control group. ns, not significant.

Table S1. Information of Human Samples, related to STAR Methods.

NO.	Gender	Age	Steatosis (%)
1	M	55	0
2	F	45	0
3	F	32	0
4	M	57	0
5	F	36	0
6	M	28	0
7	M	29	0
8	M	28	0
9	M	41	0

10	M	36	0
11	M	47	0
12	M	34	0
13	F	53	0
14	F	47	0
15	F	29	5
16	M	35	5
17	M	57	5
18	M	31	5
19	M	32	5
20	F	45	5
21	F	40	5
22	F	55	5
23	F	55	10
24	M	39	10
25	M	43	10
26	M	29	10
27	M	45	10
28	M	40	10
29	M	39	15
30	M	58	15
31	M	56	15
32	M	34	15
33	M	29	15
34	M	28	15
35	M	35	15
36	F	33	20
37	M	31	20
38	M	45	30
39	M	35	30
40	M	54	30
41	M	53	35

Table S2. Information of lipidomics, related to Figure 1.

NO.	ID	fold change (ShCp VS shCON)	P-value
1	DAG 14:0/18:2	0.203179	0.00505
2	DAG 16:0/16:0	0.335805	0.014843
3	DAG 16:0/18:0	0.651595	0.016338
4	DAG 16:0/18:1	0.148825	0.009722
5	DAG 16:0/18:2	0.142379	0.010293
6	DAG 16:0/20:4	0.178932	0.006072

7	DAG 16:0/22:5	0.181946	0.03114
8	DAG 16:0/22:6	0.050415	0.044015
9	DAG 16:1/16:0	0.131867	0.015356
10	DAG 16:1/18:2	0.193039	0.001997
11	DAG 17:1/18:1	0.24864	0.002951
12	DAG 18:0/18:0	0.693817	0.019554
13	DAG 18:0/18:1	0.401737	0.006887
14	DAG 18:1/18:1	0.190529	0.010619
15	DAG 18:1/18:2	0.273773	0.004599
16	DAG 18:1/20:3	0.315891	0.013765
17	DAG 18:1/22:6	0.21095	0.004348
18	DAG 18:2/20:1	0.242298	0.005977
19	FFA 12:0	0.431744	6.66E-06
20	FFA 14:0	0.531987	1.45E-06
21	FFA 14:1	0.400239	1.36E-07
22	FFA 15:0	0.550607	3.62E-09
23	FFA 16:0	0.712516	0.0019
24	FFA 16:1	0.379435	2.07E-05
25	FFA 17:0	0.815127	0.012047
26	FFA 17:1	0.476851	7.37E-05
27	FFA 18:0	0.882111	0.064643
28	FFA 18:1	0.557908	0.000312
29	FFA 18:2	0.681812	0.005801
30	FFA 18:3	0.700244	0.016515
31	FFA 19:0	0.887949	0.041527
32	FFA 20:0	0.886782	0.112484
33	FFA 20:1	0.514584	0.000109
34	FFA 20:2	0.642481	0.002911
35	FFA 20:4	0.675421	0.006337
36	FFA 22:3	0.544562	0.002284
37	FFA 22:4	0.575477	0.001089
38	FFA 22:5	0.615834	0.001407
39	FFA 22:6	0.451489	5.42E-05
40	FFA 23:0	0.81223	0.008478
41	TAG 10:0/16:0/18:1	0.175741	0.000377
42	TAG 10:0/16:0/18:2	0.219829	0.000792
43	TAG 12:0/16:1/18:2	0.210866	0.000157
44	TAG 14:0/14:0/16:0	0.336743	0.000267
45	TAG 14:0/16:0/16:0	0.322	0.000199
46	TAG 14:0/16:0/16:1	0.204175	0.000129
47	TAG 14:0/16:0/18:1	0.255234	8.68E-05
48	TAG 14:0/16:1/16:1	0.17706	0.000412
49	TAG 14:0/16:1/17:1	0.230074	9.47E-05

50	TAG 14:0/16:1/18:2	0.172002	0.000301
51	TAG 14:0/18:2/22:6	0.217273	0.000618
52	TAG 15:0/16:0/16:1	0.330914	7.01E-05
53	TAG 15:0/16:0/18:1	0.34106	3.08E-05
54	TAG 16:0/16:0/16:0	0.373871	0.000246
55	TAG 16:0/16:0/17:0	0.594759	0.004503
56	TAG 16:0/16:0/18:0	0.438551	0.003694
57	TAG 16:0/16:0/18:1	0.331956	9.92E-05
58	TAG 16:0/17:0/18:0	0.54892	0.041553
59	TAG 16:0/17:0/18:1	0.375125	8.03E-05
60	TAG 16:0/17:1/18:1	0.313881	6.62E-05
61	TAG 16:0/17:1/18:2	0.309259	0.000151
62	TAG 16:0/18:0/18:0	0.519471	0.040591
63	TAG 16:0/18:0/18:1	0.36072	0.000787
64	TAG 16:0/18:1/18:1	0.442939	0.000415
65	TAG 16:0/18:1/18:2	0.456923	0.000669
66	TAG 16:0/18:1/19:0	0.365011	0.002655
67	TAG 16:0/18:1/20:0	0.398746	0.02791
68	TAG 16:0/18:1/20:1	0.339675	0.000675
69	TAG 16:0/18:1/20:2	0.418473	0.000151
70	TAG 16:0/18:1/20:3	0.444619	0.000462
71	TAG 16:0/18:1/22:0	0.548186	0.121562
72	TAG 16:0/18:1/22:1	0.390536	0.023651
73	TAG 16:0/18:1/22:4	0.380091	0.000326
74	TAG 16:0/18:1/22:5	0.356483	0.000431
75	TAG 16:0/18:1/22:6	0.275288	9.82E-05
76	TAG 16:0/18:2/18:2	0.387978	0.000553
77	TAG 16:0/18:2/20:3	0.44159	0.001823
78	TAG 16:0/18:2/20:4	0.284178	0.000135
79	TAG 16:0/18:2/20:5	0.351232	0.008167
80	TAG 16:0/18:2/22:6	0.288102	0.000574
81	TAG 16:0/20:4/22:6	0.165419	9.01E-05
82	TAG 16:0/20:5/22:6	0.155254	0.000158
83	TAG 16:0/22:4/22:5	0.424561	0.000745
84	TAG 16:0/22:4/22:6	0.216182	6.05E-05
85	TAG 16:0/22:6/22:6	0.136051	0.000285
86	TAG 16:1/16:0/16:1	0.208135	0.000139
87	TAG 16:1/16:0/17:1	0.270558	5.76E-05
88	TAG 16:1/16:0/18:1	0.301881	0.00015
89	TAG 16:1/16:0/18:2	0.244964	0.000129
90	TAG 16:1/16:1/17:1	0.225473	0.00014
91	TAG 16:1/16:1/18:2	0.221624	0.000397
92	TAG 16:1/16:1/18:3	0.238949	0.001181

93	TAG 16:1/17:1/18:2	0.287303	0.000332
94	TAG 16:1/18:2/18:2	0.266578	0.00051
95	TAG 16:1/18:2/22:6	0.232136	0.000624
96	TAG 17:0/18:1/18:1	0.333675	0.000285
97	TAG 17:0/18:1/18:2	0.361684	0.000238
98	TAG 17:0/18:1/20:4	0.371808	3.64E-05
99	TAG 17:1/18:1/18:2	0.367938	0.000568
100	TAG 17:1/18:1/22:4	0.395809	0.001597
101	TAG 18:1/18:1/19:0	0.33393	0.001306
102	TAG 18:1/18:1/19:1	0.328988	0.000263
103	TAG 18:1/18:1/20:1	0.348914	0.0009
104	TAG 18:1/18:1/22:4	0.374304	0.000148
105	TAG 18:1/18:1/22:6	0.358374	0.000154
106	TAG 18:1/18:2/19:1	0.411503	0.000353
107	TAG 18:1/18:2/20:1	0.439837	0.000583
108	TAG 18:1/18:2/22:4	0.402705	0.000331
109	TAG 18:1/20:1/20:1	0.448971	0.032627
110	TAG 18:1/20:1/20:2	0.471922	0.017026
111	TAG 18:1/20:1/20:3	0.422835	0.001225
112	TAG 18:1/20:1/22:4	0.395572	0.000937
113	TAG 18:1/20:1/22:5	0.433163	0.007402
114	TAG 18:1/20:3/22:4	0.400049	0.000917
115	TAG 18:2/18:1/22:6	0.364218	0.000671
116	TAG 42:1	0.27355	0.00166
117	TAG 55:1	0.448081	0.036021
118	TAG 57:2	0.449781	0.026737
119	TAG 57:3	0.377408	0.00597
120	TAG 60:5	0.5577	0.04277

Table S4. The siRNA sequences, related to Figure 1, Figure 4, Figure 5 and Figure S6.

NO.	Genes	siRNA oligo
1	Cox11 #1	TCAAGGACCGGGTCATTAA
2	Cox11 #2	GAACTTCAGACCTCAGCAA
3	Cox17 #1	GCGTGATGCGTGCATCATT
4	Cox17 #2	GCGTGCATCATTGAGAAAG
5	Ctr1 #1	CCACACGGACGACAACATT
6	Ctr1 #2	CCAAATGGAACCATCCTAA
7	Ctr2 #1	CCGTGCTTCTCTTTGATTT
8	Ctr2 #2	CCAGATCAACTTCAGACAA
9	Sod2 #1	GCCAAGGGAGATGTTACAA

10	Sod2 #2	GCTCTAATCAGGACCCATT
11	Atp7a #1	GCTCTTCTCCACAATGCTA
12	Atp7a #2	GCAGCCGAAGTACCTCAAA
13	Atp7b #1	CCTTAAGGCTGGGACCAAT
14	Atp7b #2	CCCATCACTTGGACCACAA
15	Mt4 #1	GCGGAGATAATTGCAAATG
16	Mt4 #2	TCTGCATCTGCGGAGATAA
17	Mtf1 #1	GCAGACAGCTGCCTTGATT
18	Mtf1 #2	CCAACTCTGTCCTAACTAA
19	Sco1 #1	CCTGTTTCTTGGAAGTCTT
20	Sco1 #2	GCAGCATTGGGAAGCCTTT
21	Ccs #1	CCTTCCGGATAGAGGATAA
22	Ccs #2	GCCATCCCTTATCCAAGAT
23	Ccs #3	CCTTACGAGGGACTGCAAT
24	Nfkb1	GCAGGCCACCAACTACAAT
25	Atox1	TCTCCAGAGTCCTCAACAA
26	Pfkp	GCAATGTAGCCGTCATCAA
27	Creb3l3 #1	GGACATGTGGAGCACAGAT
28	Creb3l3 #2	GCTGCCTCTCACCAAGTAT
29	Gys1	GCACCTGGACTTCAACCTA
30	Vcam1	GCTGTGACCTGTCTGCAAA
31	Hk2	GCTGCTGTTCCAAGGGAAA
32	Akr1d1	GCACATTGATGGCGCCTAT
33	Cyp7a1	GCACATAAAGCCCGGGAAA
34	Acsbg1	GGGTGTGATGCTGAGTCAA
35	Aldh1l2	GCAGAGATGGTGGTGGATT
36	Slc10a1	GCTCCAATATCCTGGCCTA
37	Bbox1	CCATCTGGAAGGAGCGTAT
38	Cp	CGGAACAGTCCAATTTCTATCTTCA
39	Sco2 #1	CAGCCUACUAGACCACAAATT
40	Sco2 #2	GCAGAUCGUAGAGAGUAUATT
41	Lkb1 #1	GATTGCCAATGGACTGGACACCTTT
42	Lkb1 #2	CAGTTTGCCCAAGGCTGTTTGTGTG