Supporting Information

Mechanistic insights into dietary CuO nanoparticles (CuO NPs)-induced hepatic lipotoxicity: The critical role of the Ccs/Mek1/Erk1/2/Pparα pathway and mitochondrial oxidative stress

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Text S1

Characterization of CuO NPs. The primary particle sizes and morphology were analyzed using a transmission electron microscope (TEM, Tecnai G2 20 TWIN, FEI, Oregon). The average hydrodynamic size and zeta potential in water were measured using a Nano ZS Zetasizer (Malvern Instruments, Worcestershire, U.K.). To determine the release of Cu^{2+} ions from CuO NPs in various media, CuO NPs at different concentrations were dispersed in deionized water and culture media at different pH levels, followed by incubation for 24 h. The solutions were then ultracentrifuged at 100,000×g, and the supernatants were analyzed using inductively coupled plasma-optical emission spectrometry (ICP-OES, Thermo Jarrel Ash Corporation, MA, USA)

Text S2

Cu^{2+} release behavior of CuO NPs in biological simulated fluids. To simulate the dissolution behavior of Cu^{2+} ions from CuO NPs in the gastrointestinal tract and lysosomal environment of fish, we prepared three types of biological simulated fluids: simulated gastric fluid (SGF, pH 2.0), simulated intestinal fluid (SIF, pH 8.0), and artificial lysosomal fluid (ALF, pH 4.5). CuO NPs at a concentration of 10 μg/mL were dispersed in each of these media and incubated at 28°C for 2, 6, 12, 24, and 48 h. Subsequently, the solutions were ultracentrifuged at 100,000×g, and the supernatants were analyzed by ICP-OES to determine the release efficiency of Cu^{2+} at different time points.

Text S3

Animal sampling. At the end of the experiment, yellow catfish were fasted for 24 h, and then euthanized with MS-222 solution (100 mg/L). 6 fish were selected randomly from each tank, and their livers were isolated on ice, and fixed in 2.5% glutaraldehyde for ultrastructural observation or 4% buffered formalin solution for histological analysis. The remaining hepatic tissue samples were immediately frozen in the liquid nitrogen and then kept in -80℃ freezer for analysis of Cu content, enzymatic activities, mRNA and protein expression.

Text S4

Isolation and culture of yellow catfish hepatocytes. Yellow catfish were euthanized and killed by spinal cord section for liver removal. The liver was kept in phosphate buffered saline (PBS, pH 7.6, 4℃) supplemented with amphotericin-B (25 μg/L), streptomycin (100 μg/mL) and penicillin (100 U/mL) during 10 min for antibiotic shock and perfused through the portal vein and arterial system with ice-cold PBS-EDTA solution (2 mM EDTA, 1.0 g/L D-glucose in phosphate buffered saline-PBS, pH

7.6) for blood removal. After perfusion, the liver was aseptically minced with stainless steel blades in PBS containing dispase (1.0 U/L) and 1.0 g/L d-glucose. Subsequently, it was incubated with pancreatic enzyme (0.25% in PBS) at room temperature for 5 min four times, allowing the liver cells to dissociate in pancreatic enzyme. The cell suspension was forced through a stainless-steel mesh (70 and 100 mesh) for additional mechanical disruption. Cells were collected and centrifuged at low speed (1000-1200 rpm, 5 min), washed four times with PBS to remove debris. Then, the cells were diluted with M199 medium (10% fetal bovine serum, 40 mg/L gentamycin, 10 μg/mL streptomycin, and 10 U/mL penicillin; 11150059; Thermo Fisher Scientific). Finally, 2.0×10^5 and 1.0×10^6 cells (viability ≥ 95%) were, respectively, seeded onto 12-, 24- and 96- well microplates and kept at 28°C in a CO_2 incubator (5% of CO_2). For each cell culture, a pool of cells from three fish was utilized.

Text S5

Real-time quantitative PCR (qPCR) analysis. Total RNA was obtained with RNAiso Plus reagent (9109; Takara Biomedical Technology, Beijing, China) and transcribed to the cDNA using a Reverse Transcription Kit (CW2582; Cwbio IT Group, Beijing, China). Gene expressions were determined using the Hieff qPCR SYBR Green Master Mix (11201ES03; Yeasen, Shanghai, China). 10 housekeeping genes (*β-actin*, *rpl7*, *hprt*, *tuba*, *b2m*, *ubce*, *tbp*, *gapdh*, *elfa* and *18s rRNA*) were chosen to evaluate their transcription stability. We normalized them to the geometric mean of the best combination of two genes as analyzed by geNorm (https://genorm.cmgg.be/), and calculated the relative expression of genes by the $2^{-\Delta\Delta Ct}$ method.

Text S6

Small interference RNA transfection. The siRNAs (50 nM/each-well of the 12-well plate) were transfected into yellow catfish hepatocytes using EntransterTM-R4000 Transfection Reagent (Engreen Corp., Beijing, China) for 48 h, based on the manufacturer's instructions. The knockdown efficiency was measured by the change of mRNA and protein expressions using qPCR and western blot analysis, respectively. The siRNA with the highest efficiency of knockdown was used for our experiments. A non-silencing control siRNA was used as a control.

Text S7

Plasmid construction and transient transfection. The open reading frame of Ccs, Mek1, Erk1/2, and Pparα sequences were subcloned into pcDNA3.1 (+) vector with Myc-tag, eGFP-tag, Flag-tag or eGFP-tag, and Myc-tag sequences, respectively, using ClonExpress II One Step Cloning Kit (C112;

Vazyme, Piscataway, NJ, USA). Mutations of Ccs sequence were constructed with Mut Express II Fastmutagensis Kit (C214; Vazyme). Different fragment-deletion Ccs mutants were produced in the Myc-Ccs plasmids, named M1, M2, M3 and M4. Site-mutations of Erk1/2 and Pparα sequences were constructed with Mut Express II Fast Mutagensis Kit (C214; Vazyme). We mutated the threonine 202 (T202) site of Erk1 and threonine 185 (T185) site of Erk2 to alanine (T202A and T185A) and tyrosine 204 (Y204) site of Erk1 and tyrosine 187 (Y187) site of Erk2 to phenylalanine (Y204F and Y187F). These two-site mutations are named $Erk1^{2M}$ and $Erk2^{2M}$, respectively. We also mutated the predicted 5 Erk1/2-specific serine phosphorylation sites of Pparα to alanine and named S34A, S51A, S71A, S77A, and S84A. HEK-293T cells were transfected with each plasmid by using Lipofectamine 2000 (11668019; Thermo Fisher Scientific, Waltham, MA, USA). The transfection protocols were carried out based on the manufacturer's instructions. After 24 h, the cells were collected for immunoprecipitation and immunoblotting assays.

In order to carry out the analysis of promoters, the *cptIa1b* and *acadl* promoter sequences were amplified and subcloned into pGL3-basic vector (1751; Promega, Madison, WI, USA) using ClonExpress II One Step Cloning Kit (C112; Vazyme).

Text S8

Western blotting (WB) analysis. Liver tissues and cell lysates were lysed with RIPA buffer (BL504A; Biosharp, Hefei, China) on ice for 30 min. The samples were then separated by SDS-PAGE, and transferred to PVDF membranes (88518; Thermo Fisher Scientific). Then the membranes were blocked with 8% (w/v) nonfat milk in TBST buffer for 2 h at room temperature, and then washed three times with TBST buffer for 5 min each time. They were then incubated with specific primary antibodies, such as anti-Ccs (1:1000, prepared in our laboratory), anti-Mek1 (1:1000, A19565; Abclonal, Wuhan, China), anti-Erk1/2 (1:2000, T40071; Abmart, Shanghai, China), anti-Phospho-Erk1 (Thr202/Tyr204) + Erk2 (Thr185/Tyr187) (1:1000, TA8208; Abmart), anti-Pparα (1:1000, 15540-1-AP; Proteintech Group), anti-Gapdh (1:10000, 60004-1-Ig; Proteintech Group), anti-pan phosphor-serine/threonine (1:1000, #AP1067, Abclonal), anti-Myc-tag (1:5000, 16286-1-AP; Proteintech Group), anti-Flag-tag (1:5000, 66008-4-Ig; Proteintech Group), and anti-eGFP-tag (1:2000, AE012; Abclonal) overnight at 4°C. Subsequently, they were incubated with HRP-conjugated anti-rabbit or mouse IgG antibody (1:10000, 7074; Cell Signaling Technology). Finally, the protein bands were recorded by a Fusion FX6 Spectra imaging system (Vilber, Paris, France), and the densities of protein were

quantified by Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Text S9

Immunoprecipitation and co-immunoprecipitation. In order to conduct the immunoprecipitation and co-immunoprecipitation analysis, the liver and cells samples were lysed in NP-40 lysis buffer (P0013F; Beyotime, Beijing, China) with the addition of protease and phosphatase inhibitor cocktail (P1045; Beyotime). The lysate was mixed with corresponding antibodies at 4℃ overnight followed by the addition of protein A/G magnetic beads (HY-K0202; Med Chem Express, New Jersey, USA) for 4 hours at 4℃. NP-40 lysis buffer was used to wash the immunocomplexes. Finally, immunoblot assays were carried out.

Text S10

Immunofluorescence analysis. After transfection, cells were collected and washed in PBS. Then, cells were fixed in 4% paraformaldehyde for 15 min at room temperature, and then blocked in 5% BSA for 2 h, followed by incubation with primary antibodies rabbit anti-Myc-tag (1:200, 16286-1-AP; Proteintech Group) overnight at 4°C. The samples were washed three times with PBST, 5 min each time, then incubated with a Goat Anti-Rabbit IgG H&L (Alexa Fluor® 647, 1:500, ab150079; Abcam, Cambridge, MA, USA) or Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488, 1:500, ab150077; Abcam) secondary antibody for 1 h at room temperature in the dark. Hoechst 33342 (1:1000, ab228551; Abcam) was used to stain the nucleus of cells. The images were acquired with a TCS SP8 LSCM (Leica, Wetzlar, Germany).

Table S1 Formulation and proximate composition analysis (of dry weight basis) of three experimental diets

Ingredients (g/kg)	Low CuO NPs	Middle CuO NPs	High CuO NPs
Fish meal	50	50	50
Corn gluten meal	475	475	475
Corn flour	50	50	50
Wheat gluten	100	100	100
Wheat flour	150	150	150
Fish oil	25	25	25
Soybean oil	25	25	25
Choline chloride	5	5	5
Lecithin	1	1	1
Vitamin premix[1]	5	5	5
Mineral premix (Cu-free)[2]	5	5	5
$Ca(H_2PO_4)_2 \cdot H_2O$	10	10	10
CuO NPs	0	0.009	0.015
Cellulose	99	98.991	98.985
Proximate analysis (% of dry matter basis)			
Moisture (%)	7.69	7.90	7.58
Crude protein (%)	41.16	41.40	42.15
Lipid (%)	9.37	9.36	9.41
Ash (%)	3.24	2.98	3.34
Cu content (mg kg^{-1})	2.07	9.19	13.96

[1]Vitamin premix (mg or IU per kg diet): retinylacetate, 10000IU; cholecalciferol, 1000IU; all-rac-a-tocopheryl acetate, 30IU; menadione nicotinamide bisulfite, 7; thiamine hydrochloride, 6; riboflavin, 3; pyridoxine hydrochloride, 12; D-calcium pantothenate, 30; niacin, 50; biotin, 1; folic acid, 6; cyanocobalamine, 0.03.

[2]Mineral mixture (mg per kg diet): $FeSO_4 \cdot 7H_2O$, 40; $ZnSO_4 \cdot 7H_2O$, 40; $MnSO_4 \cdot H_2O$, 40; $CaIO_3 \cdot 6H_2O$, 3; Na_2SeO_3, 0.05; $CoSO_4$, 0.05.

Table S2 Primers used for quantitative real-time PCR analysis, plasmid construction, siRNA sequences, and promoter cloning

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Accession no.
qPCR analysis			
g6pd	CAGGAATGAACGCTGGGATG	TCTGCTACGGTAGGTCAGGTCC	JX992744
6pgd	GCTCTGATGTGGCGAGGTGG	CGTAGAAGGACAGTGCAGTGG	JX992745
fas	AACTAAAGGCTGCTGGTTGCTA	CACCTTCCCGTCACAAACCTC	JN579124
acca	GGGGTTTTCACGCTGCTTC	GGTTCTGATTGGGTCGTCCTG	JX992746
hsl	GAAGGACAGGACAATGAGAAGC	TGTACCACCAGCCAAGGAGA	KJ588765
atgl	AACACGTACACACGCTGGTC	GGAGGGAACAGACCACAAAA	KF614123
cptIa1b	ATTTGAAGAAGCACCCAGAGTATGT	CCCTTTTATGGACGGAGACAGA	XM_027172959
fabp1	CCCTGGAAAATCGGTCACTA	GGTGGTTCCTTCCAGAGTCA	XM_027170676
pparα	CGAGGATGGGATGCTGGTG	CGTCTGGGTGGTTCGTCTGC	JX992740
pparγ	ACGCCCCGTTCGTTATCC	TGAGCAGAGTCACCTGGTCATTG	JX992741
srebp1	CTGGGTCATCGCTTCTTTGTG	TCCTTCGTTGGAGCTTTTGTCT	JX992742
acads	AAGTAGGCTGCTTTGCTTTGAGTGAG	AATTGGTGATCCAGGCTTTGGTG	MG599806
acadsb	ACGGTCGGGAGTTTCTGT	GATGTGAAGCCCCTCAG	MG599810

	TTATCTG	TGTCGC	
acadl	TAACGGCTGGATGAGTGACCTGG	GCTATTATCAACCGCTCCTGTGGC	MG599805
acadvl	CGAGACCTGCGAATCTTCCGTAT	CGAGACCTGCGAATCTTCCGTAT	MG599809
acad8	ACCATTACATCCTGAACGGCTCCA	GCGAGTTCCAACCAACCTTCTTCT	MG599807
acad9	TGTGAGCGAGGCATTACAAG	GCTGACATTCCCCTTTTTCA	XM_027143977
acox1	ACACACATCCGTGGACTTGA	GAGGCTGGTGGGTTTTCATA	XM_027133527
acox3	GGCTGTTTTGCACTGACAGA	ACAACTGGGCAAACACAACA	XM_027134490
atp8	TTTGCAATCCTTGTGTTCTCA	GCGCTTAGTGCTGTGACCTC	NC_015888
cox1	GAGCCCTTCTAGGGGATGAC	GGAATGCCATATCTGGTGCT	NC_015888
cox2	GGCCAATTCCGACTACTTGA	GGTTTAATCGCCCTGGTACA	NC_015888
nd1	AGGCTGAGCTTCCAATTCAA	GAGATAGTCTGGGCGACTGC	NC_015888
nd3	GCCTTTTTCCCTACGCTTCT	GGGCAGGGGTAGTAGGAGAG	NC_015888
ctr1	TCAACACACCTGGAGAGATG	CACCTGGATGACGTGAAGTA	KY646156
ctr2	CAAGGTGTGGAAGAACGTTC	GAACAATGTGCAGTCCTGTC	KY646157
dmt1	CTGATCAGAACGGTGGGATT	AACCCTGCTACAGCTCCTGA	KY660275

atox1	TGACATGTGAAGGATGCTCT	AGGACCAATATACGTGACAGT	KY660270
ccs	GGTGCAGATGAGCTGTGAGA	CTCCGATCCCCTTCAGTACA	KY660271
cox17	GCTTAGCTGCTGCAAGTGTG	TTGAAACCAAGTGCCCTCAT	KY660272
atp7b	CTCATGTGTACGTGCCATCC	CCTCCACCCATTCAGACTGT	KY660274
mt	CGAGTGCTCCAAGACTGGAT	GGAATCGCCCTTACACACAC	FJ418583
cav1	GTGGACTTCGAGGACGTGAT	TAGACCTGGCTGATGCAGTG	XM_027160261
cav2	GAGACATTCACGCGCACTTA	GCGAAAACAAGGCCTGATAC	XM_027160228
dnm1	TGTCCAGCAAGCACATCTTC	GTCTCCACCTGCCTCTCAAG	XM_047809295
dnm2	TTCAGGAGCTCATCAACACG	AATGGGCCTTTTCTTGTGTG	XM_047808178
dnm3	CACACACATTCGGGAACAAG	CACAATTTGACTGGGACGTG	XM_027171245
cltc	GAGCAAGATGAAGGCACACA	CGGAAATCCCAATCAGAAGA	XM_047820649
eps15	ATGTCCTCAGCCAGCACTCT	TGGGATGACTCCTCCTTTTG	XM_027166552
keap1	TTGCGACACAGGAGGAGTTC	GCTGAAGGAAGTGTGGTGGA	XM_027133477
nrf2	CCAAAGACAGAGCAGGCAGA	TGCCTCGTTCAACTGATGCT	KX455917
cat	TCCCACACCTTCAAGCTG	GATGGCGTTGTACAGGT	KX455919

	AT	CAC	
sod1	TGGGTAATGTGACTGCCGAT	CCTCCTGCATTGCCTGTTTT	KX455916
sod2	TCATGCAGCTTCACCATAGC	CTGTGGTTCTCCTCCACCAT	XM_027166181
gpx1	AAGTATGTGCGTCCTGGGAA	ATGAACTTGGGGTCGGTCAT	XM_027155810
mek1	GGGCCTTCTACAGTGATGGA	GCCAAAGTCACACAGCTTGA	XM_027161872
rpl7	GGCAAATGTACAGGAGCGAG	GCCTTGTTGAGCTTGACGAA	KP938522
hprt	ATGCTTCTGACCTGGAACGT	TTGCGGTTCAGTGCTTTGAT	KP938523
tuba	TCAAAGCTGGAGTTCTCGGT	AATGGCCTCGTTATCCACCA	KP938526
b2m	GCTGATCTGCCATGTGAGTG	TGTCTGACACTGCAGCTGTA	KP938520
ubce	TCAAGAAGAGCCAGTGGAGG	TAGGGGTAGTCGATGGGGAA	KP938524
tbp	AGCAAAGAGTGAGGAGCAGT	ACTGCTGATGGGTGAGAACA	KP938525
gapdh	TTTCAGCGAGAGAGACCCAG	ATGACTCTCTTGGCACCTCC	KP938521
18s rRNA	AGCTCGTAGTTGGATCTCGG	CGGGTATTCAGGCGAGTTTG	KP938527
β-actin	GCACAGTAAAGGCGTTGTGA	ACATCTGCTGGAAGGTGGAC	EU161066
elfa	GTCTGGAGATGCTGCCAT	AGCCTTCTTCTCAACGCT	KU886307

	TG	CT

Plasmid construction

Myc-Ccs-round1	GCAAGCTAAATAAGACTCGTA	ACATTTAATGGCTTTTTTCCC
Myc-Ccs-round2	GCAAGCTAAATAAGACTCGTA	GGTCACAGATCCTCTTCAGAGATGAGTTTCTGCTCTAA
Myc-Ccs-round3	ctagcgtttaaacttaagcttATGGAAACAAGTCGACTTGCTAAA	tgctggatatctgcagaattcTCACAGATCCTCTTCAGAGATGAGTT
Myc-Ccs-M1	CAGAACCAGATGAGCAGAAACTCATCTCTGAAGAGG	TCTGCTCATCTGGTTCTGATCCTCCGATCCCCT
Myc-Ccs-M2	CACTGCAGAGCAGAAACTCATCTCTGAAGAGGA	AGTTTCTGCTCTGCAGTGCGAGCGATGATCCCA
Myc-Ccs-M3	CTTAAGCTTCTGGGTGCTGCCGTGGCTATGTTG	AGCACCCAGAAGCTTAAGTTTAAACGCTAGCCAG
Myc-Ccs-M4	CAGAACCAGATGGACTCTTTCAGAACCCCAAACA	AGAGTCCATCTGGTTCTGATCCTCCGATCCCCT
eGFP-Mek1-round1	TGCCTTATCAGATCGCGCTCC	GGGGGTCTTGATATGATTCGT
eGFP-Mek1-round2	ctagcgtttaaacttaagcttATGCAAAAACGGAGGAAGCC	tgctggatatctgcagaattcTATCCCCACACTGTGAGTTGGGGTTGG
Flag-Erk1-	TGTCAGTTGAAGTGCGTT	TTGTTCTTAGCCTAAAC

round1	GT	GTG
Flag-Erk1-round2	GAACGGATTACAAGGATGACGACGATAAGATGGAG	TTGTTCTTAGCCTAAACGTG
Flag-Erk1-round3	ctagcgtttaaacttaagcttGATTACAAGGATGACGACGATAAGAT	tgctggatatctgcagaattcTCAGGAGCCTTGGTAGTTGGC
eGFP-Erk1-round1	TGTCAGTTGAAGTGCGTTGT	TTGTTCTTAGCCTAAACGTG
eGFP-Erk1-round2	ctagcgtttaaacttaagcttATGTCCACAGCAGCAGCTGC	tgctggatatctgcagaattcTGGTCTGTAGCCTGGTTGGAA
Flag-Erk2-round1	GACTCTGCTCTGTTAACACC	TAGTGATAACGATGACCTAA
Flag-Erk2-round2	AGAGGATTACAAGGATGACGACGATAAGATGTCCA	TAGTGATAACGATGACCTAA
Flag-Erk2-round3	ctagcgtttaaacttaagcttGATTACAAGGATGACGACGATAAGAT	tgctggatatctgcagaattcTTATGGTCTGTAGCCTGGTTGGA
Myc-Pparα-round1	ATGGGTTTGACAGGAAGTGG	TTATATCAGAGCAGGTTAAT
Myc-Pparα-round2	ATGGGTTTGACAGGAAGTGG	AGTTTACAGATCCTCTTCAGAGATGAGTTTCTGCTCGT
Myc-Pparα-round3	ctagcgtttaaacttaagcttATGAGTGATTCAGTGCTTGCAGTAC	tgctggatatctgcagaattcTTACAGATCCTCTTCAGAGATGAGTTTC

eGFP-Erk1-T202A	TTgggttgcgacggataGCTGACCCAGAGCATGACCA	tatccgtcgcaacccAAAGTCACAGATCTTGAGGTCACAGG
eGFP-Erk1-Y204F	TTGgcgcggtttgctgACCCAGAGCATGACCACACTG	TcagcaaaccgcgcCAACCCAAAGTCACAGATCTTGAG
eGFP-Erk2-T185A	ATTTtgggcgagcgCGTGTAGCCGACCCGGACC	CACGcgctcgcccaAAATCGCAGATCTTGAGATCACAG
eGFP-Erk2-Y187F	TGGGctcgcgtttgtagCCGACCCGGACCACGATC	ctacaaacgcgagCCCAAAATCGCAGATCTTGAGA
Myc-Pparα-S34A	ATTGgaccgacctCTGTGTGGAGATCTGATCAAGGAA	ACAGaggtcggtcCAATCCTATTTCCCCCACTGAA
Myc-Pparα-S51A	GACatccgacgaTCCTTCAGCGATGACCCGTT	AAGGAtcgtcggatGTCCTCAAGCTCCTCCATTTCC
Myc-Pparα-S71A	GGAatcccgaacaaGTTCCAACAGCTCTGCTACTCTCG	AACttgttcgggatTCCTGGATGTCAGGAAGGTATATCA
Myc-Pparα-S77A	AAcagccgagctaCTCTCGATGTTTTGAGCCCG	AGAGtagctcggctgTTGGAACTTGTGCTGGATTCCT
Myc-Pparα-S84A	TGTtttgcgaccggCCTCTAGTCCGTCTTCAGCAACC	AGGccggtcgcaaaACATCGAGAGTAGCAGAGCTGTTG

siRNA sequences

si-*mek1*-1	CUGCUUGACUACAUUGUUA	UAACAAUGUAGUCAAGCAG
si-*mek1*-2	GAGAGAUCAAGCUGUGUGA	UCACACAGCUUGAUCUCUC

si-*mek1*-3	GGAAGCUGAUCCACCUAGA	UCUAGGUGGAUCAGCUUCC

Promoter cloning

pGL3-*acadl*-round1	GGTGTGTAATTTCAGTAACA	AGTGTAAATGTTAAATCTGT
pGL3-*acadl*-round2	ctatcgataggtaccgagctcGGTGTGTAATTTCAGTAACATACAAATTATG	cagtaccggaatgccaagcttAGTGTAAATGTTAAATCTGTGTTATATGTAACTAC

Abbreviations: 6pgd, 6-phosphogluconate dehydrogenase; Acad8/9, Acyl-CoA dehydrogenase 8/9; Acadl, Long-chain acyl-CoA dehydrogenase; Acads, Short-chain acyl-CoA dehydrogenase; Acadsb, Short/branched-chain acyl-CoA dehydrogenase; Acadvl, Very long-chain acyl-CoA dehydrogenase; Acca, Acetyl-CoA carboxylase α; Accb, Acetyl-CoA carboxylase β; Acox1/3, Acyl-CoA oxidase 1/3; Atgl, Adipose triglyceride lipase; Atox1, Antioxidant protein 1; Atp7b, ATPase Cu transporting β; Atp8, ATP synthase F0 subunit 8; B2m, Beta-2-microglobulin; Cat, Catalase; Cav1/2; Caveolin 1/2, Ccs, Cu chaperone for superoxide dismutase; Cltc, Clathrin heavy chain; Cox1/2, Cytochrome c oxidase subunit I/II; Cox17, Cytochrome c oxidase Cu chaperone; CptIa1b, Carnitine palmitoyl transferase Ia1b; Ctr1/2, Cu transporter 1/2; Dmt1, Divalent metal-ion transporter-1; Dnm1/2/3, Dynamin1/2/3; Elfa, Elongation factor 1-alpha; Eps15, Epidermal growth factor receptor pathway substrate clone 15; Erk1/2, Extracellular regulated protein kinase 1/2; Fabp1, Fatty acid binding protein 1; Fas, Fatty acid synthase; G6pd, Glucose 6-phospate dehydrogenase; Gapdh, Glyceraldehyde-3-phosphate dehydrogenase; Gpx1, Glutathione peroxidase 1; Hprt, Hypoxanthine-guanine phosphoribosyltransferase; Hsl, Hormone-sensitive triglyceride lipase; Keap1, Kelch-like ECH-associated protein-1; Mek1, Mitogen-activated protein kinase kinase 1; Mt, Metallothionein; Nd1/2, NADH dehydrogenase subunit 1/2; Nrf2, Nuclear factor erythroid2-related factor 2; Pparα/γ, Peroxisome proliferator-activated receptor α/γ; Rpl7, Ribosomal protein L7; Sod1/2, Superoxide dismutase 1/2; Srebp1, Sterol regulatory element binding proteins-1; Tbp, TATA-box-binding protein; Tuba, Tubulin alpha chain; Ubce, Ubiquitin-conjugating enzyme.

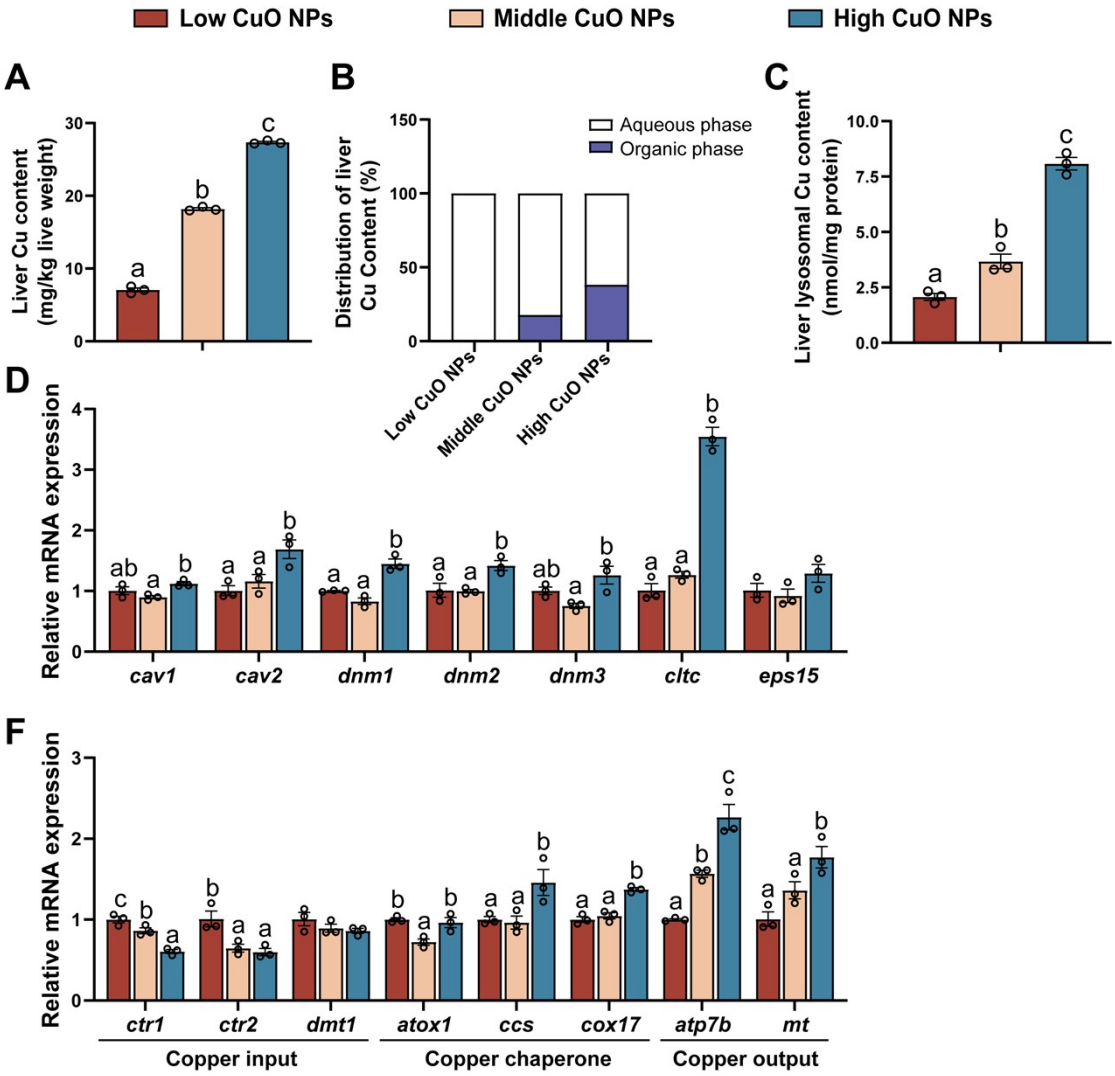

Fig. S1 Effects of dietary CuO NPs levels on hepatic Cu metabolism of yellow catfish. (A) Cu content in the liver; (B) The percentages of ionic Cu and nanoparticulate cu relative to the total copper content in liver tissue; (C) Cu content in the lysosomes of liver tissue; (D) mRNA expressions of endocytosis related genes; (F) mRNA expressions of Cu transport related genes. Relative mRNA expression values were normalized to housekeeping genes (*β-actin* and *b2m*) expressed as a ratio of the low CuO NPs group. Data are expressed as mean ± SEM (n = 3). Each data point (n) represents an independent fish tank, and its value was derived from the analysis of pooled tissue samples from 6 fish within that tank. *P* value was calculated by one-way ANOVA and Duncan's multiple range test. Values without the same letter indicate significant difference among three treatments ($P< 0.05$).

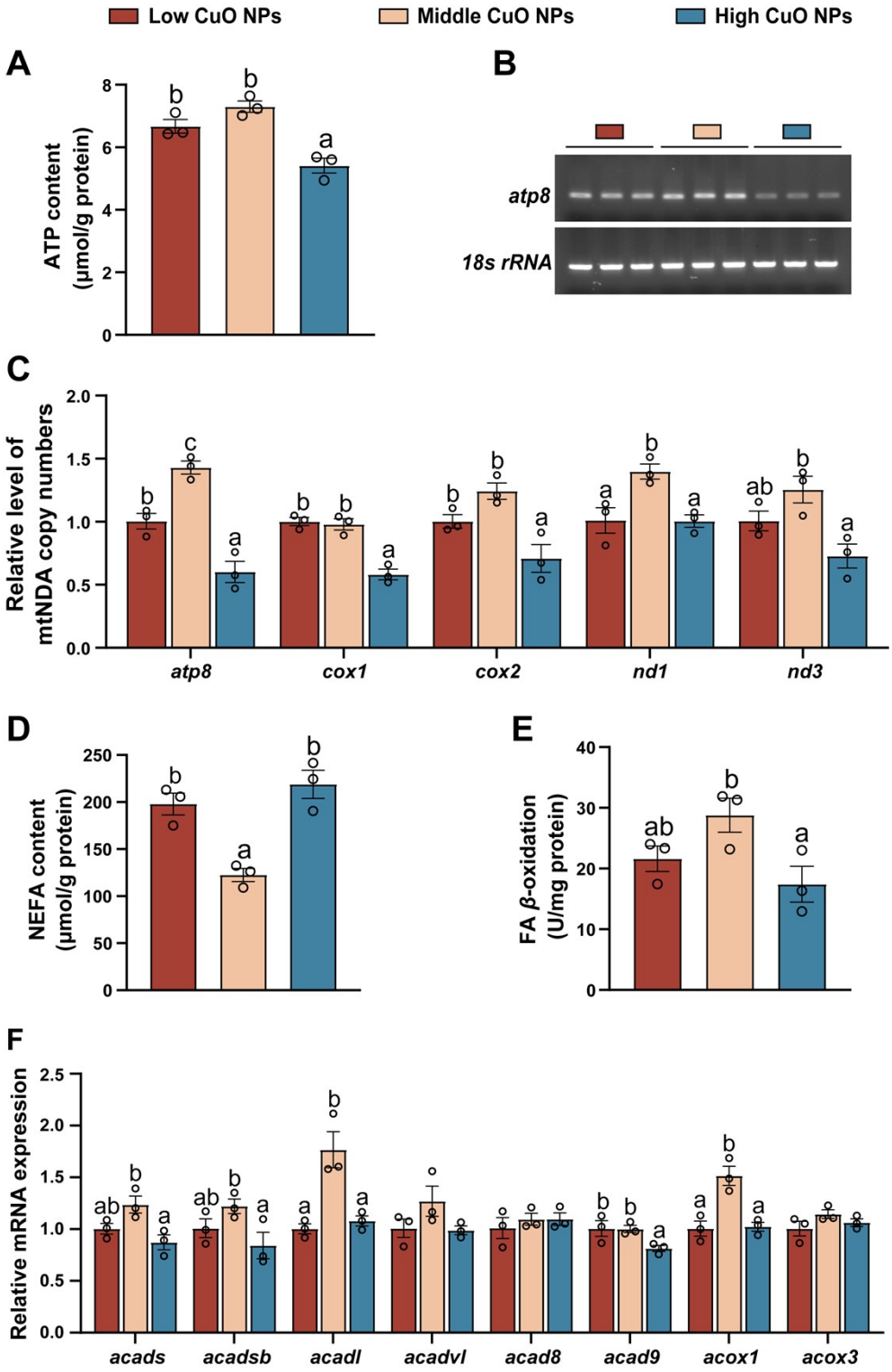

Fig. S2 Effects of dietary CuO NPs levels on hepatic mitochondrial function of yellow catfish. (A) ATP content; (B) Semi-quantification of mitochondrial-encoded gene *atp8*; (C) Relative levels of mitochondrial copy number; (D) NEFA content; (E) FA *β*-oxidation rate; (F) mRNA expressions of fatty acid *β*-oxidation related genes. Relative mRNA expression values were normalized to housekeeping genes (*β-actin* and *b2m*) expressed as a ratio of the low CuO NPs group. Data are

expressed as mean ± SEM (n = 3). Each data point (n) represents an independent fish tank, and its value was derived from the analysis of pooled tissue samples from 6 fish within that tank. *P* value was calculated by one-way ANOVA and Duncan's multiple range test. Values without the same letter indicate significant difference among three treatments ($P< 0.05$).

A

Erk1 T202/Y204

Homo_sapiens	ARIADPEHDHTGFLTEYVATRWYRAPEIML	217
Mus_musculus	ARIADPEHDHTGFLTEYVATRWYRAPEIML	218
Sus_scrofa	ARIADPEHDHTGFLTEYVATRWYRAPEIML	218
Danio_rerio	ARIADPEHDHTGFLTEYVATRWYRAPEIML	231
Pelteobagrus_fulvidraco	ARIADPEHDHTGFLTEYVATRWYRAPEIML	231

B

Erk2 T185/Y187

Homo_sapiens	ARVADPDHDHTGFLTEYVATRWYRAPEIML	200
Mus_musculus	ARVADPDHDHTGFLTEYVATRWYRAPEIML	198
Sus_scrofa	ARVADPDHDHTGFLTEYVATRWYRAPEIML	199
Danio_rerio	ARVADPDHDHTGFLTEYVATRWYRAPEIML	209
Pelteobagrus_fulvidraco	ARVADPDHDHTGFLTEYVATRWYRAPEIML	215

Fig. S3 Alignment of Erk1 and Erk2 amino acid sequences among five species. The phosphorylation sites were marked in red. (A) Erk1 threonine 202 and tyrosine 204; (B) Erk2 threonine 185 and tyrosine 187.

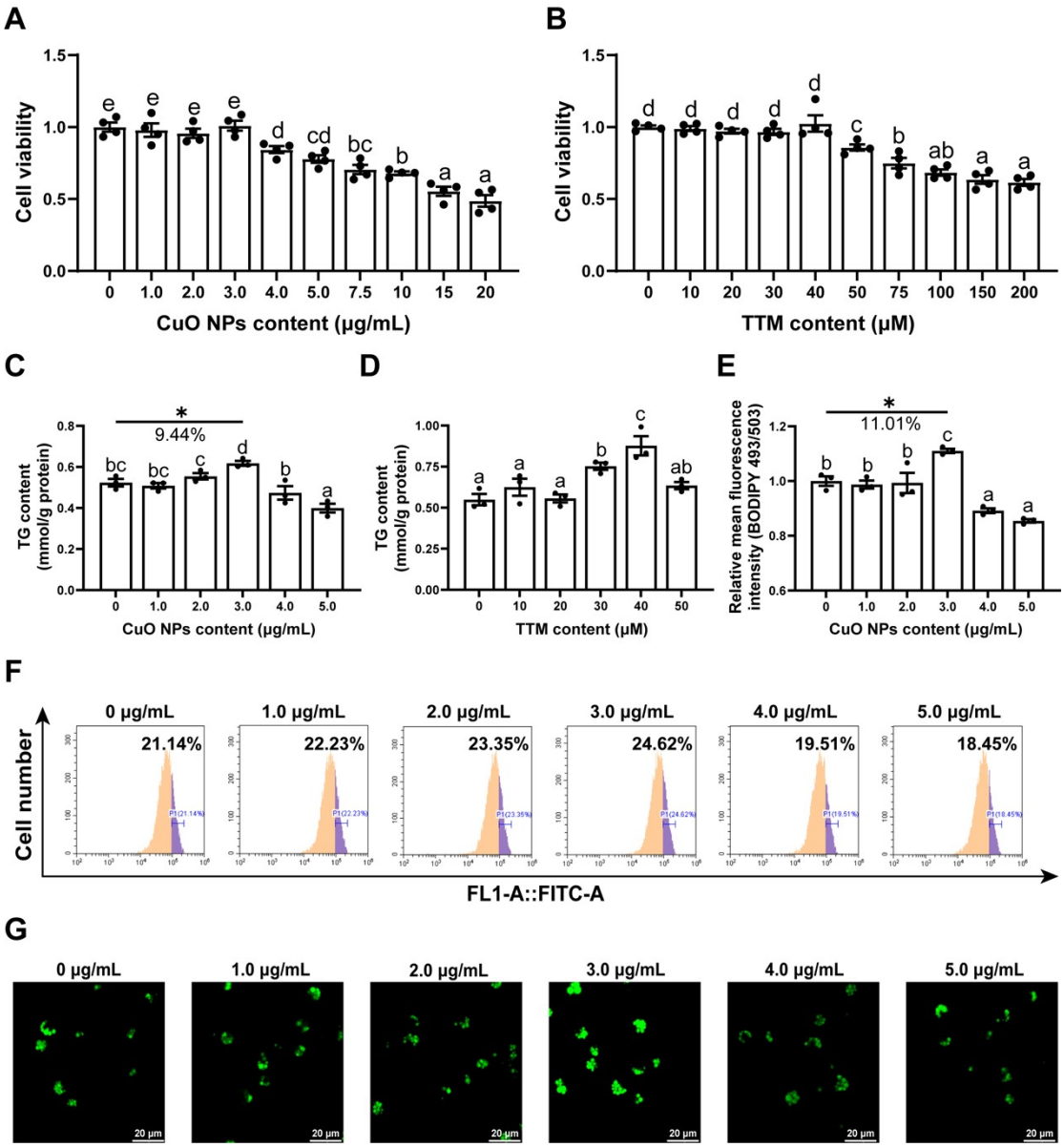

Fig. S4 CuO NPs and TTM concentration screening. (A) Cell viability of the hepatocytes incubated with CuO NPs for 48 h; (B) Cell viability of the hepatocytes incubated with TTM for 48 h; (C) TG content of the hepatocytes incubated with CuO NPs for 48 h; (D) TG content of the hepatocytes incubated with TTM for 48 h; (E) The lipid content was quantified by flow cytometric analysis of FL1 (green) mean fluorescence intensity with BODIPY$^{493/503}$ staining; (F) The presence of BODIPY$^{493/503}$-stained LDs were demonstrated by flow cytometry; (G) Representative confocal microscopy image of hepatocytes with BODIPY$^{493/503}$ staining. Scale bars: 20 μm. Values are mean ± SEM (n = 4 independently biological experiments in Fig. S4A-B; n = 3 independently biological experiments in Fig. S4C-G). *P* value was calculated by one-way ANOVA and Duncan's multiple range test. Letters indicate significant differences among the groups ($P< 0.05$).

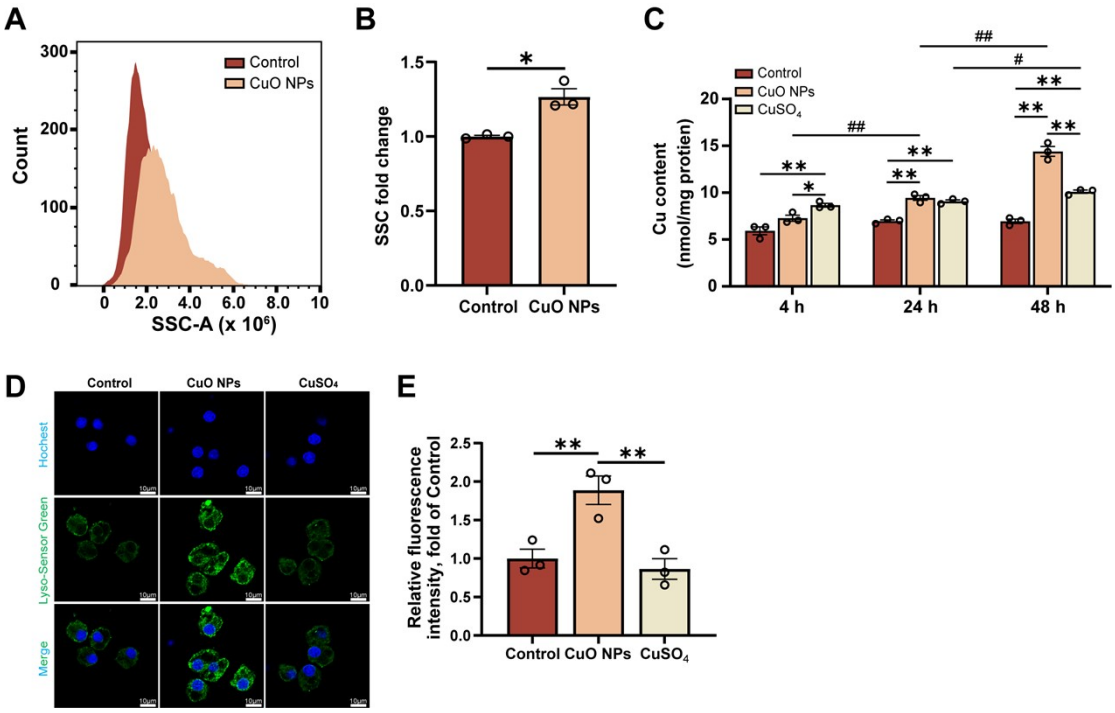

Fig. S5 Uptake of CuO NPs in hepatocytes and effects of equivalent doses of CuO NPs and $CuSO_4$ on intracellular Cu content and lysosomal acidification level. (A) Representative flow cytometry data for SSC intensity of hepatocytes treated with 3 μg/mL CuONPs for 48 h; (B) Quantitative analysis of SSC intensity; (C) Intracellular Cu content after exposure to 3 μg/mL CuO NPs and 6 μg/mL $CuSO_4$ for 4, 24, and 48 h; (D) Hepatocytes were labeled with Lyso-Sensor Green after 24 h of exposure to CuO NPs (3 μg/mL) and $CuSO_4$ (6 μg/mL), and visualized by confocal microscopy. Scale bar: 10 μm. (E) Quantitative analysis of Lyso-Sensor Green mean fluorescence intensity. Values are mean ± SEM (n = 3 independently biological experiments). *P* value was calculated by Student's *t* tests. $^{*}P< 0.05$, $^{**}P< 0.01$ indicate a significant difference between two treatments.

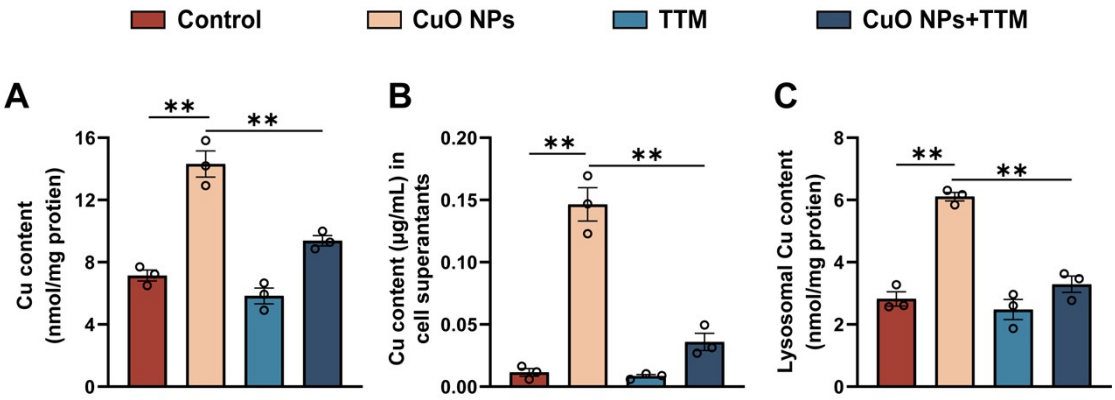

Fig. S6 Effects of CuO NPs and TTM treatments on Cu content in yellow catfish hepatocytes. (A) The intracellular concentration of Cu; (B) The concentration of Cu in cell supernatants; (C) The concentration of Cu in lysosomes. Values are mean ± SEM (n = 3 independently biological experiments). *P* value was calculated by Student's *t* tests. **P*< 0.05, ***P*< 0.01 compared with CuO NPs-treated group.

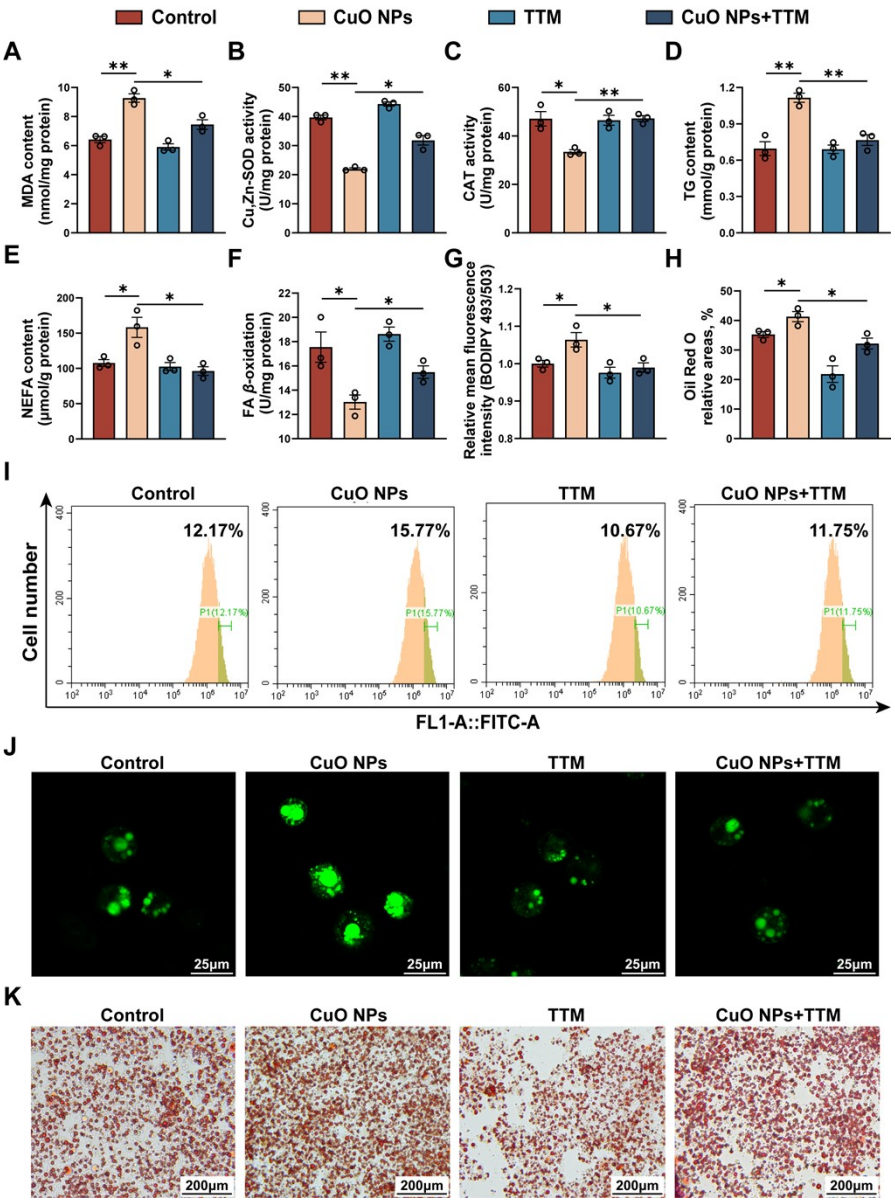

Fig. S7 CuO NPs incubation induced oxidative stress and lipid accumulation in yellow catfish hepatocytes. (A) MDA content; (B) Cu,Zn-SOD activity; (C) CAT activity; (D) TG content; (E) NEFA content; (F) Fatty acid *β*-oxidation rate; (G) FL1 (green) average fluorescence intensity (BODIPY$^{493/503}$ fluorescence staining) was calculated to quantify LDs; (H) Relative areas for lipid droplets after oil red O staining; (I) The green fluorescence intensity of LDs was measured by flow cytometry; (J) Representative confocal microscopy image of hepatocytes stained with BODIPY$^{493/503}$. Scale bars: 25 μm; (K) Representative microphotograph of hepatocytes stained by oil-red O. Scale bars: 200 μm. Values are mean ± SEM (n = 3 independently biological experiments). *P* value was calculated by Student's *t* tests. $^{*}P< 0.05$, $^{**}P< 0.01$ compared with CuO NPs-treated group.

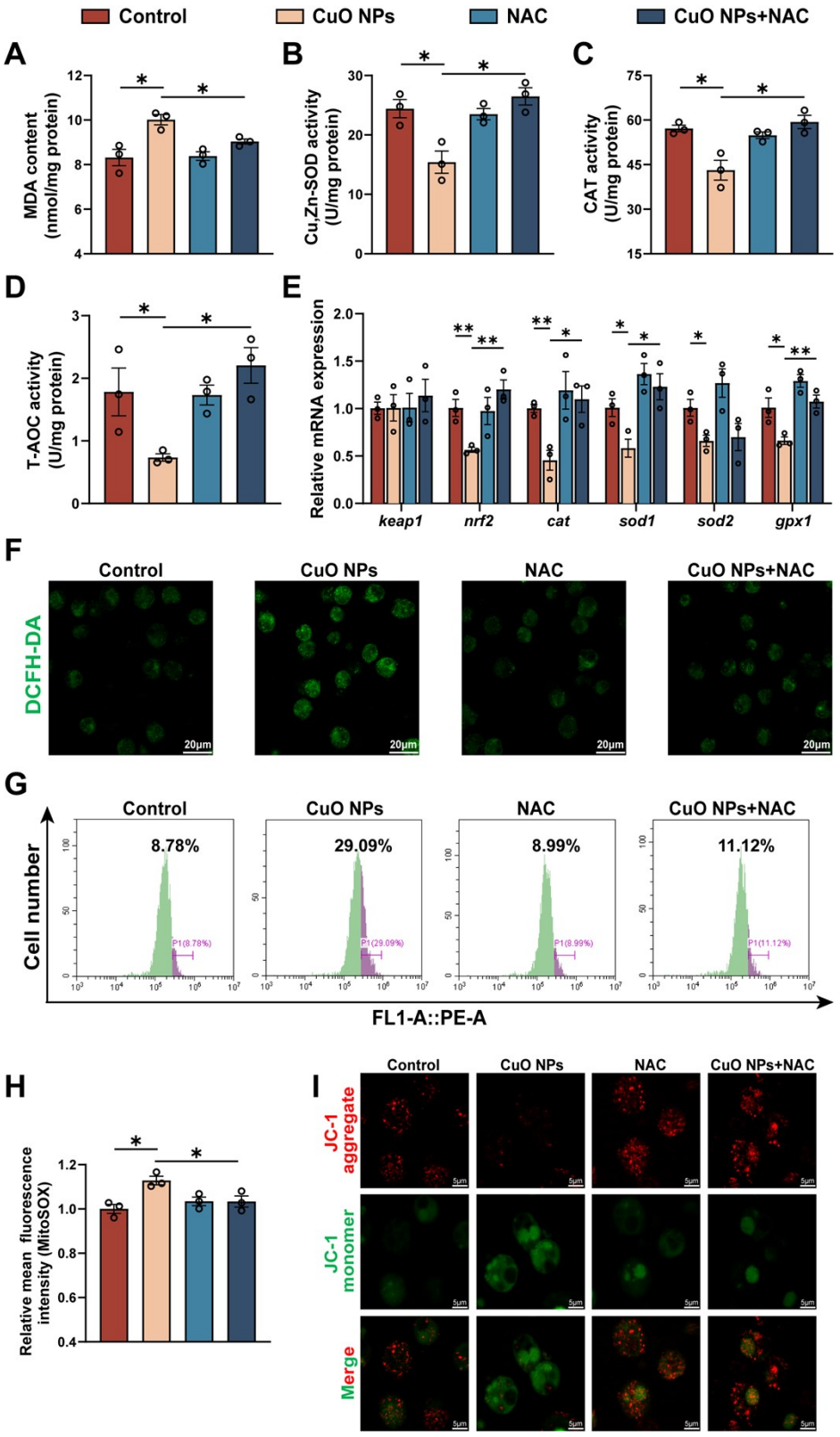

Fig. S8 Effects of NAC (ROS scavenger) pretreatment on CuO NPs-induced changes of oxidative stress and mitochondrial damage in hepatocytes of yellow catfish. The hepatocytes of yellow catfish were incubated in the control and CuO NPs (3.0 μg/mL CuO NPs)-containing medium for 48 h, with or without 2-h pretreatment with ROS scavenger (0.5 mM NAC); (A) MDA content; (B) Cu,Zn-SOD

activity; (C) CAT activity; (D) T-AOC activity; (E) mRNA expressions of oxidative stress related genes. Relative mRNA expression values were normalized to housekeeping genes (*ubce* and *β-actin*) expressed as a ratio of the control. (F) Representative confocal microscopy image of hepatocytes stained with DCFH-DA, Scale bars: 20 μm. (G) The fluorescence intensity of mtROS was measured by flow cytometry; (H) The mtROS was quantified by flow cytometric analysis with MitoSOX staining. (I) Representative confocal microscopic image of hepatocytes stained with JC-1. Red fluorescence indicates normal ΔΨm with JC-1 aggregates in mitochondria and green fluorescence reflects cytosolic JC-1 monomer indicative of ΔΨm loss. Scale bar: 5 μm. Values are mean ± SEM (n = 3 independently biological experiments). *P* value was calculated by Student's *t* tests. **P*< 0.05, ***P*< 0.01 compared with CuO NPs-treated group.

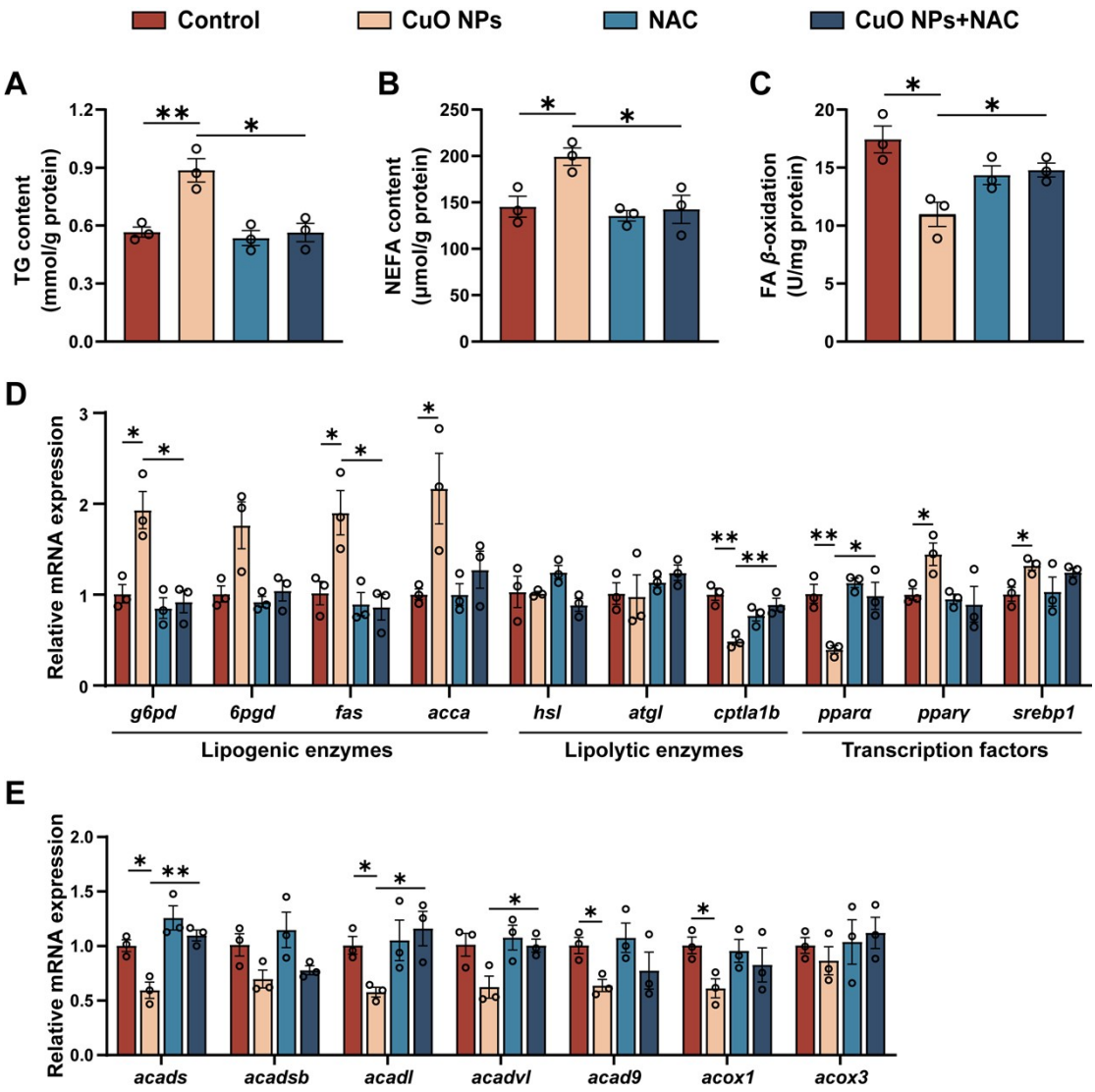

Fig. S9 NAC pretreatment alleviated CuO NPs-induced lipid deposition in hepatocytes of yellow catfish. (A) TG content; (B) NEFA content; (C) Fatty acid *β*-oxidation rate; (D) mRNA expressions of lipid metabolism related genes; (E) mRNA expressions of fatty acid *β*-oxidation related genes. Relative mRNA expression values were normalized to housekeeping genes (*ubce* and *β-actin*) expressed as a ratio of the control. Values are mean ± SEM (n = 3 independently biological experiments). *P* value was calculated by Student's *t* tests. **P*< 0.05, ***P*< 0.01 compared with CuO NPs-treated group.

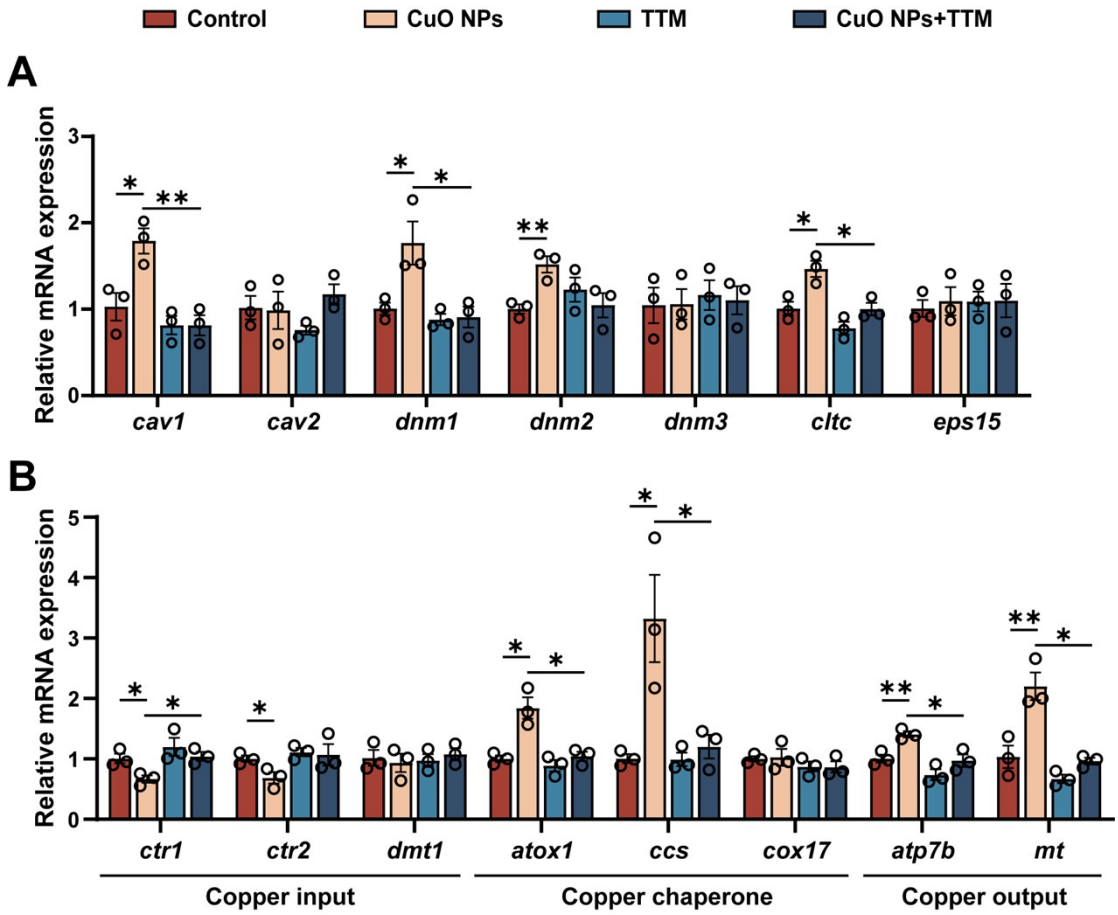

Fig. S10 Effects of CuO NPs treatment on Cu metabolism in hepatocytes of yellow catfish. (A) mRNA expressions of endocytosis related genes; (B) mRNA expressions of Cu transport related genes. Relative mRNA expression values were normalized to housekeeping genes (*gapdh* and *18s rRNA*) expressed as a ratio of the control. Values are mean ± SEM (n = 3 independently biological experiments). *P* value was calculated by Student's *t* tests. $^{*}P< 0.05$, $^{**}P< 0.01$ compared with CuO NPs-treated group.

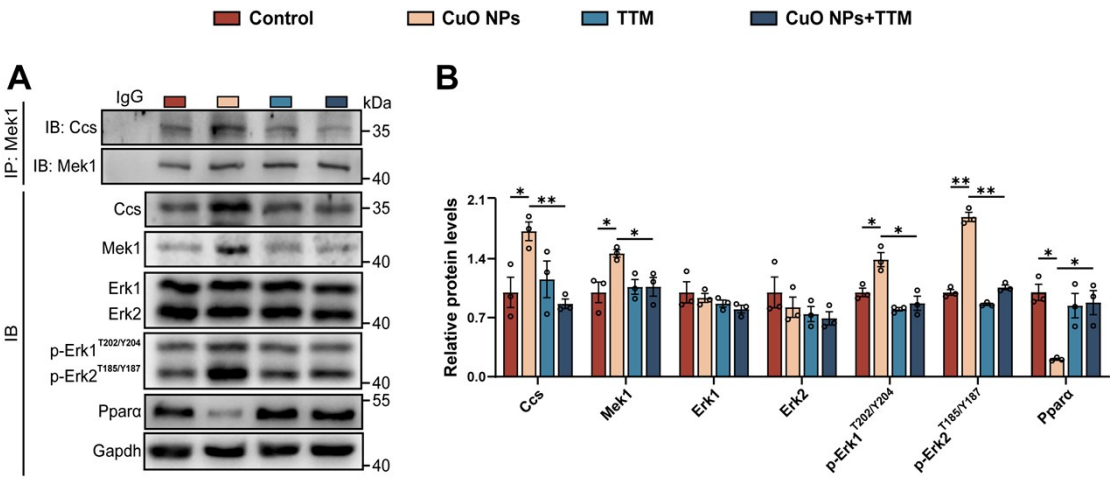

Fig. S11 Effects of CuO NPs and TTM treatment on the interaction of Ccs and Mek1 and the protein expression of Ccs, Mek1, Erk1/2, p-Erk1/2 and Pparα. (A) Co-IP of Ccs with Mek1 from yellow catfish hepatocytes were incubated in control, CuO NPs (3.0 μg/mL), TTM (20 μM) or CuO NPs+TTM for 48 h in M199 medium and western blot analysis of Ccs, Mek1, Erk1/2, p-Erk1$^{T202/Y204}$, p-Erk2$^{T185/Y187}$ and Pparα; (B) Relative quantification of Ccs, Mek1, Erk1/2, p-Erk1$^{T202/Y204}$, p-Erk2$^{T185/Y187}$ and Pparα. Values are mean ± SEM (n = 3 independently biological experiments). *P* value was calculated by Student's *t* tests. $^{*}P< 0.05$, $^{**}P< 0.01$ compared with CuO NPs-treated group.

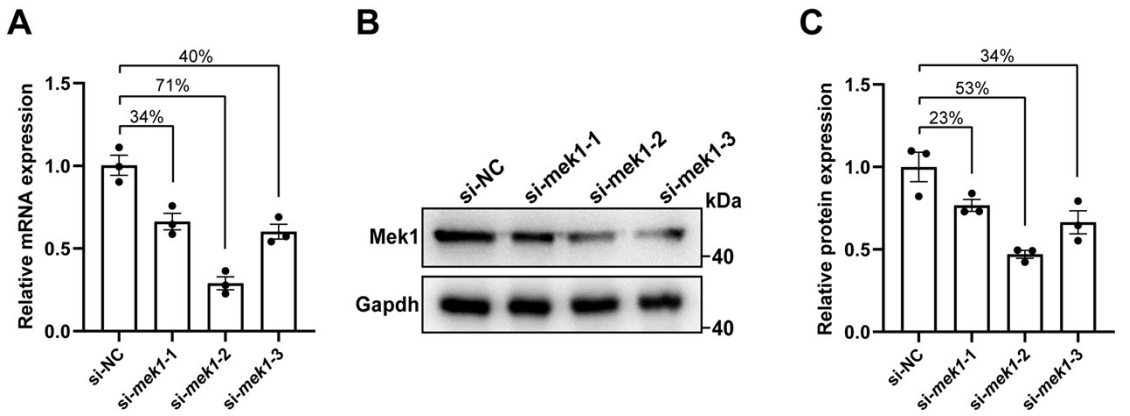

Fig. S12 The siRNA knockdown efficiency of *mek1* gene in hepatocytes of yellow catfish. The yellow catfish hepatocytes were incubated in M199 medium for 48 h. (A) The mRNA levels of *mek1* in yellow catfish hepatocytes treated with different small interfering RNA fragments of *mek1* for 48h; (B and C) The protein levels of Mek1 in yellow catfish hepatocytes treated with different small interfering RNA fragments of *mek1* for 48h. Relative mRNA expression values were normalized to housekeeping genes (*β-actin* and *elfa*) expressed as a ratio of the si-NC. All data was expressed as mean ± SEM (n = 3 independently biological experiments). The value in the figure represents the gene silence efficiency compared to the si-NC group.

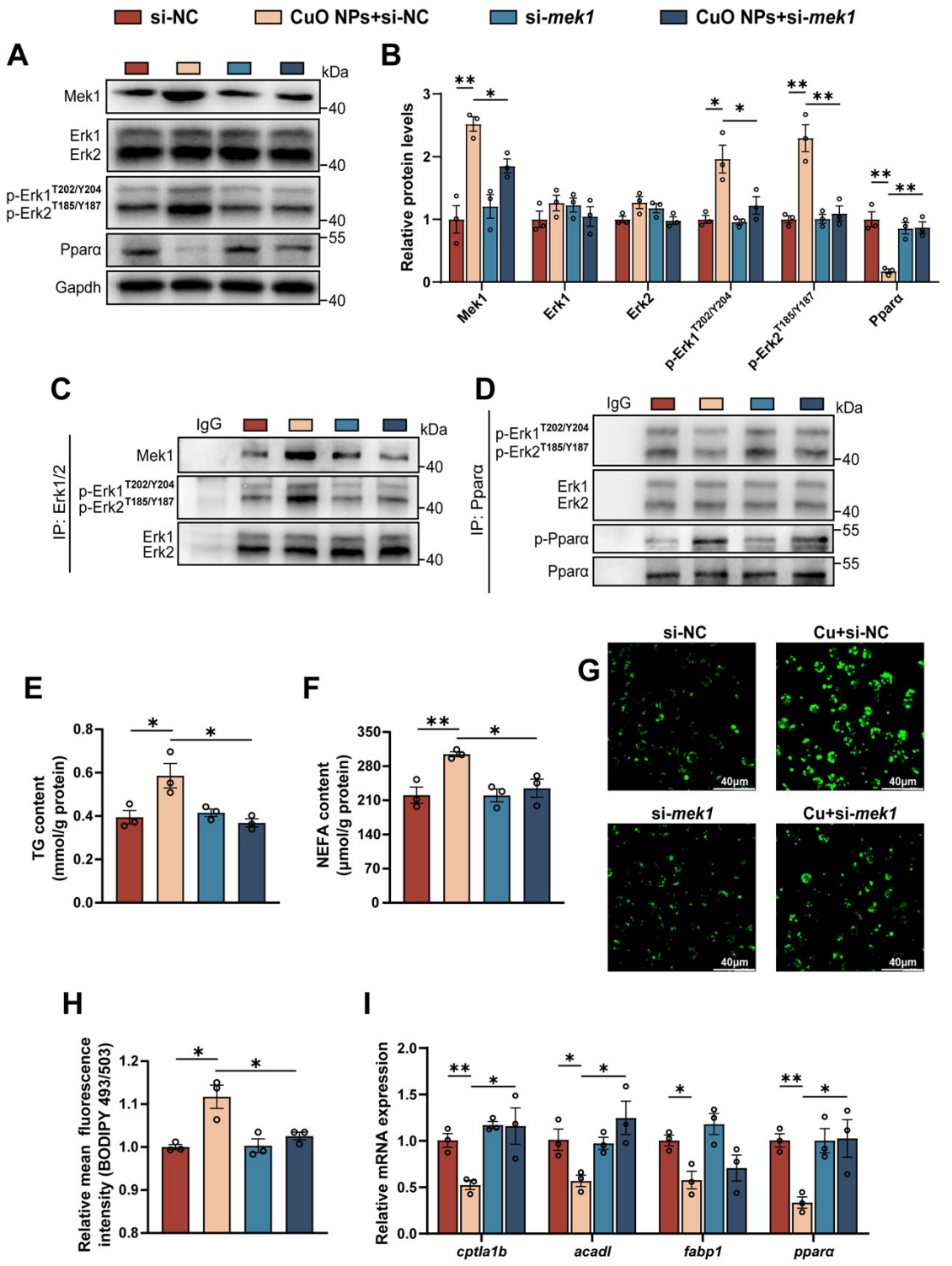

Fig. S13 Effects of *mek1* knockdown and CuO NPs incubation on Mek1, Erk1/2, p-Erk1/2 and Pparα expressions, TG and NEFA contents, and lipid metabolism in yellow catfish hepatocytes. (A and B) western blot and statistical analyses of Mek1, Erk1/2, p-Erk1$^{T202/Y204}$, p-Erk2$^{T185/Y187}$ and Pparα expression in hepatocytes after treatment with *mek1* knockdown with or without CuO NPs incubation for 48 h; (C) Co-IP of Mek1 with Erk1/2 and phosphorylation level of p-Erk1$^{T202/Y204}$ and p-Erk2$^{T185/Y187}$ in hepatocytes; (D) Co-IP of Erk1/2 with Pparα and phosphorylation level of Pparα in

hepatocytes; (E and F) TG and NEFA contents measured in hepatocytes after treatment with *mek1* knockdown with or without CuO NPs incubation for 48 h; (G) Representative confocal microscopy image of hepatocytes stained with BODIPY$^{493/503}$ after treatment with *mek1* knockdown with or without CuO NPs incubation for 48 h. Scale bars: 40 μm. (H) FL1 (green) average fluorescence intensity (BODIPY$^{493/503}$ fluorescence staining) was calculated to quantify LDs; (I) mRNA levels of *pparα* target genes determined in hepatocytes after treatment with *mek1* knockdown with or without CuO NPs incubation for 48 h. Values are means ± SEM (n = 3 independently biological experiments). *P* value was calculated by Student's *t* tests. $^{*}P< 0.05$, $^{**}P< 0.01$ compared with si-NC-treated group.

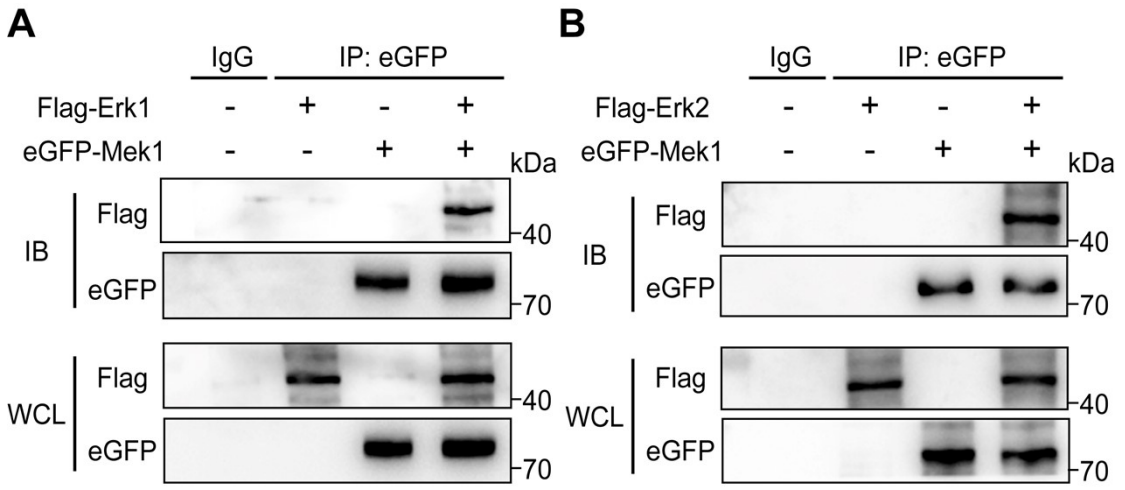

Fig. S14 The interaction between Mek1 and Erk1/2 in HEK-293T cells. (A) Co-IP of Flag-Erk1 with eGFP-Mek1. HEK-293T cells were co-transfected with indicated plasmids for 24 h, followed by immunoprecipitation and immunoblotting with antibodies as indicated; (B) Co-IP of Flag-Erk2 with eGFP-Mek1. HEK-293T cells were co-transfected with indicated plasmids for 24 h, followed by immunoprecipitation and immunoblotting with antibodies as indicated.

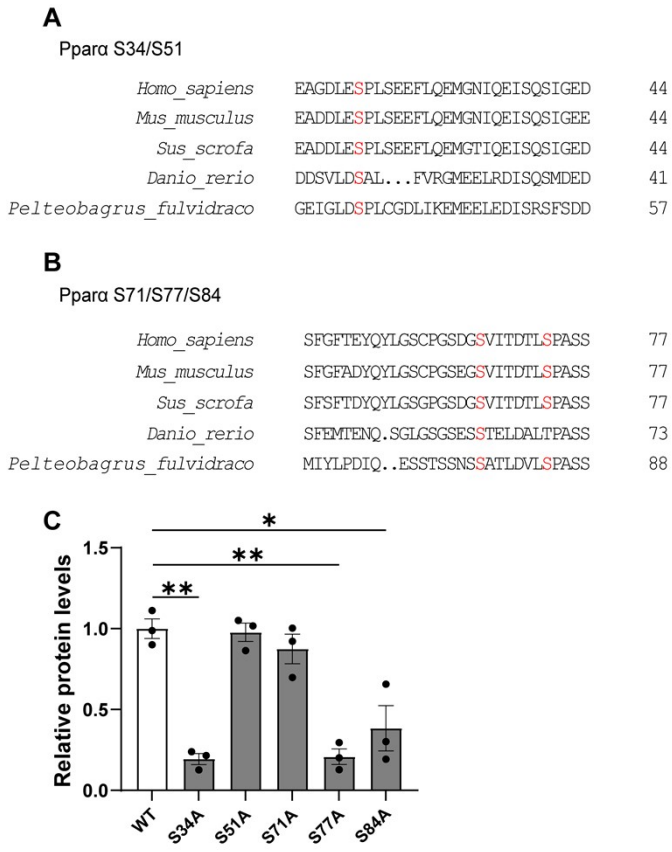

Fig. S15 Alignment of Pparα amino acid sequences among five species and statistical analysis of Pparα phosphorylation levels. (A) Pparα serine 34/51; (B) Pparα serine 71/77/84; (C) Statistical analysis of Pparα phosphorylation levels, related to Fig. 7D in the main text. Values are mean ± SEM (n = 3 independently biological experiments). Results were analyzed using Student's *t* tests ($^{*}P< 0.05$, $^{**}P< 0.01$).

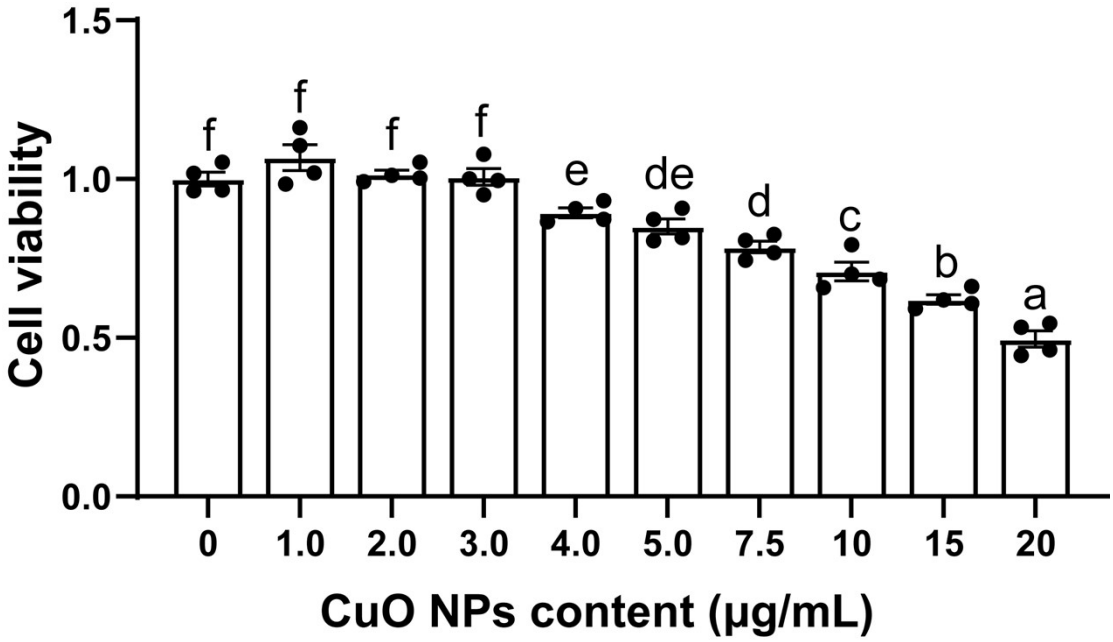

Fig. S16 Cell viability of the HEK-293T cells incubated with CuO NPs for 48 h. All data were expressed as mean ± SEM (n = 4 independently biological experiments). *P* value was calculated by one-way ANOVA and Duncan's multiple range test. Letters indicate significant differences among the groups ($P < 0.05$).