Supporting Information

Two Zn sources (ZnO nanoparticles and $ZnSO_4$) differentially affect lipid metabolism via ferroptosis pathway and $SLC7A11^{K23}$ acetylation by HDAC8 and HDAC6 in a freshwater teleost

Yi-Chuang Xu,‡[a] Hua Zheng,‡[a] Xiao-Ying Tan,[a] Christer Hogstrand,[c] Tao Zhao,[a] Xiao-Lei Wei[a] and Zhi Luo*[ab]

[a]Hubei Hongshan Laboratory, Fishery College, Huazhong Agricultural University, Wuhan 430070, China

[b]Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China

[c]Diabetes and Nutritional Sciences Division, School of Medicine, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, UK

*Corresponding author at: Zhi Luo, Hubei Hongshan Laboratory, Fishery College, Huazhong Agricultural University, Wuhan 430070, China, Tel.: +86-27-8728-2113; Fax: +86-27-8728-2114;

E-mail address: luozhi99@mail.hzau.edu.cn; luozhi99@aliyun.com (Z. Luo).

‡ These two authors contributed equally.

Abbreviations:

ACCa, acetyl-CoA carboxylase a; acad, acyl-CoA dehydrogenase; ACSL, acyl-CoA synthetase long chain family member; ATGL, adipose triglyceride lipase; CBS, cystathionine beta-synthase; CF, Condition factor; CPT 1, carnitine palmitoyltransferase 1; CTH, cystathionine gamma-lyase; ELOVL6, ELOVL fatty acid elongase 6; DMEM, Dulbecco's Modified Eagle's medium; FAS, fatty acid synthase; FBS, fetal bovine serum; FBW, final mean body weight; FCR, feed conversion rate; FSP1, Ferroptosis suppressor protein 1; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase regulator subunit; GPX, glutathione peroxidase; GSR, glutathione reductase; GSS, Glutathione synthetase; GSSG, glutathione disulfide; GST, glutathione S-transferase; H&E, Hematoxylin and eosin; HDAC, histone deacetylase; HEK-293, human embryonic kidney 293 cell; HSL, hormone-sensitive lipase; LDs, lipid droplets; MTF-1, metal response element binding transcription factor 1; NAFLD, non-alcoholic fatty liver disease; NEFA, non-esterified free fatty acids; ORO, Oil red O; ORFs, open reading frames; PPARA, peroxisome proliferator activated receptor alpha; PTGS2, prostaglandin G/H synthase 2; qRT-PCR, Real-time quantitative PCR; RPMI, Roswell Park Memorial Institute; SGR, specific growth rate; SDS, sodium dodecyl sulfate; siRNA, small interference RNA; SLC7A11, solute carrier family 7 member 11; VSI, viscerosomatic index; WG, weight gain.

Text S1

Animals feeding, management, and sampling

In this study, a total of 180 juvenile grass carp with a uniform size (initial mean weight: 3.54 ± 0.00g, mean ± S.E.M.) were randomly allocated to nine 300-L circular aquariums, with 20 fish per tank. Each experimental diet was assigned to three replicate tanks, and the fish were fed to satiation twice daily over a period of 8 weeks. Throughout the feeding experiment, water temperature, dissolved oxygen, NH4-N, nitrite, and Zn levels were regularly monitored. The water temperature was maintained

within the range of 28.7 - 29.3°C, dissolved oxygen levels were kept at ≥5.8 mg L^{-1}, NH_4-N levels remained below 0.09 mg L^{-1}, nitrite levels were below 0.01 mg L^{-1}, and Zn concentration in the water was 0.04 ± 0.00 mg L^{-1}.

Feeding experiment lasted for 8 weeks. Then, 12 fish were selected randomly from each tank, and their liver was isolated on ice, and fixed in 4% buffered formalin solution for histological analysis or 2.5% glutaraldehyde solution for ultrastructural observation. The liver samples from other fish were frozen quickly in liquid nitrogen and then kept in a -80°C freezer for analysis of Zn levels, TG and NEFA contents, enzymatic activities, mRNA and protein abundance.

Text S2

Histological and ultrastructural observation

To perform ORO staining, liver tissues were frozen at -25°C and sectioned into 5 μm thick slices after sucrose-induced dehydration. The sections were then stained with oil-saturated O solutions and subsequently washed twice with 60% isopropanol. Being washed twice in distilled water and sealed with glycerin jelly, the sections were imaged under a light microscope (Olympus BX53, Tokyo, Japan). Image J software (NIH, Bethesda, MD, USA) was utilized to analyze the relative areas of lipid droplets (LDs) in the ORO staining.

For H&E staining, the samples were sectioned into 5-μm thick slices. The sections were then stained with H&E dye, undergoing a process of dehydration in graded ethanol concentrations, and finally embedded in paraffin. The sections were imaged under a light microscope (Olympus BX53, Tokyo, Japan).

For TEM observation, the samples underwent a series of preparation steps. Firstly, they were postfixed in a solution of 1% osmium tetroxide in sodium cacodylate. Then, the samples were treated with 1% uranyl acetate, dehydrated using ethanol, and embedded in resin. Subsequently, the samples were stained with uranyl acetate and lead citrate. The prepared grids were examined using a FEI Tecnai G^2 20 TWIN

transmission electron microscope operating at an acceleration voltage of 200 kV.

Text S3

Immunoblot and immunoprecipitation

For immunoblot analysis, the samples were lysed in RIPA buffer (#P0013B, Beyotime Biotechnology) supplemented with a protease, phosphatase inhibitor cocktail (#P1045, Beyotime Biotechnology), and deacetylase inhibitor cocktail (#P1112, Beyotime Biotechnology). Protein samples were loaded onto SDS-polyacrylamide gels and subsequently transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 4% bovine serum albumin (BSA, #ST023, Beyotime Biotechnology) for 1 hour at room temperature. Primary antibodies were incubated with the membranes overnight at 4 °C. Subsequently, the membranes were treated with the appropriate secondary antibodies. After thorough washing, the membranes were visualized using ECL (#P0018FS, Beyotime Biotechnology).

For immunoprecipitation assays, the samples were lysed using NP-40 lysis solution (#P0013F, Beyotime Biotechnology) supplemented with a protease and deacetylase inhibitor cocktail (#P1112, Beyotime Biotechnology). After incubation with the required antibodies overnight at 4 °C, protein A/G magnetic beads (#HY-K0202, Med Chem Express, New Jersey, USA) were added. The immunocomplexes were then washed using NP-40 lysis buffer, and subsequent immunoblot experiments were performed.

Text S4

Plasmid construction

ClonExpress^{TM} II One Step Cloning Kit (#C112, Vazyme, Piscataway, NJ, USA) was employed to subclone the open reading frames (ORFs) of SLC7A11, HDAC6, and HDAC8 sequences into pcDNA3.1 (+) vector with GFP-tag, HA-tag, and 6×His -tag sequences, respectively. Mutations in SLC7A11 were conducted using the Mut Express

II Fastmutagensis Kit (#C214, Vazyme). The ORFs of HDAC3 and HDAC8 were subcloned into the pGEX-6P-1 vector for prokaryotic expression.

Text S5

Cystine uptake assay

Cells were seeded in 6-well plates at an optimal confluency. After the respective treatment, the cells were washed three times with PBS at room temperature. Subsequently, the cells were pre-incubated in 1 ml cystine uptake buffer (0.4 mM $MgSO_4$, 1.8 mM KCl, 1.3 mM $CaCl_2$, 25 mM triethylammonium bicarbonate, 122 mM choline chloride, 1.2 mM potassium phosphate, 10 mM glucose, pH7.4) for 15 min. Following the pre-incubation, 1 μM L-cystine was added, and the cells were further incubated at 37 °C for 1 h. Next, 500 μl of the uptake buffer was collected from each well and centrifuged at 14,000 rpm for 2 minutes. The cystine concentration was determined using a customized kit from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China), and the values were normalized to cell counts from each well.

Table S1. Feed formulation and proximate analysis (% dry matter).

Ingredients (g kg^{-1})	Control	ZnO NPs	$ZnSO_4$
Fish meal	80	80	80
casein	35	35	35
Rice bran	110	110	110
Soybean meal	200	200	200
Rapeseed meal	250	250	250
Wheat Starch	250	250	250
Fish oil	20	20	20
Soybean oil	10	10	10
Choline chloride	1.5	1.5	1.5
Lecithin	1	1	1
Vitamin premix	4	4	4
Mineral premix	7.5	7.5	7.5
DL-methionine	0.15	0.15	0.15
L-lysine	0.4	0.4	0.4
L-threonine	0.4	0.4	0.4
$Ca(H_2PO_4)_2 \cdot H_2O$	20	20	20
Cellulose	10.05	10.008	9.9
ZnO NPs		0.042	
$ZnSO_4 \cdot 7H_2O$			0.150
Proximate analysis %, dry weight			
Moisture	8.65	8.43	8.57
Crude protein	30.79	31.09	30.82
Crude lipid	6.80	6.63	6.31
Ash	7.59	7.64	7.67
Zn (mg kg^{-1})	57.36	81.12	81.09

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.

Mineral mixture (mg per kg diet): NaCl, 500; $FeSO_4 \cdot 7H_2O$, 400; $MnSO_4 \cdot H_2O$, 40; $CuSO_4 \cdot 5H_2O$, 12; $CaIO_3 \cdot 6H_2O$, 1.5; Na_2SeO_3, 0.45; $CoSO_4$, 1.

Table S2. Primers used for quantitative real-time PCR (qRT-PCR) analysis.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Accession no.
acad11	CGAACTGCTCTGTATGTGGC	TCATTGGTTGGCAGGTTGTG	XM051882736
acadsb	GAAGTTTGCTCAGGAACGCA	GAGGGTGTTCTGAATGTCGC	XM051913664
acca	GCTTGCGGCGGTTATTACTG	AGCTGCCTCTCCAACCATTC	GU908474
acsl1a	TACAGCTATTGCTCGCCCAC	CCAGTGCCTCTGACCTTGAA	MK929568
acsl4	TGGACCTGAGTACCGTTGTC	AGGGTGTCTTTTCCAGGGAG	MK929567
atgl	CGAGCTGTCTTCTGGAGCAA	AGGGCCCAGATTACGCTTTC	HQ845211
atp8	CCTCAACTAAACCCAAACCCC	ATCATGGTCAGTCTCAGGGC	EU391390
β-actin	ACCCTGAAGTACCCCATCGA	CAGAGGCATACAGGGACAGC	DQ211096
cbsa	GGTGGAGGACATCATGGTGA	AAGCAAGCATGTTTCCCAGG	XM051889812
cbsb	GGATTCGACCAGATTTGCCC	GGATCTTGTTCATGCGGACC	XM051906005
cpt1a1b	ACCATCCCCATGTGTTCGTC	CTGCTGCTCTATCTCCCGTG	KJ816748
cth	AACGCCCACAAACCCTACTA	TCAGCCGTTCATAGAGGTCC	XM051897977
elovl6	TTTCTACAACGGACCGGTCA	CGGCATGCACCAGATAGTTC	XM051860058
eef1a1	GCCGATCTCACCAACCAATC	TAACCTGTCCTGACCAACCC	NC067232
fsp1	ATTGTCGGTGGTGGTTTTGG	AAGCACAACTGTCTGCGTTT	XM051917950
gapdh	GGGAAACTGTGGAG	TGCAGCCTTGACCAC	GQ245759

	GGATGG	TTTCT	
gclc	CACGGCATCCTTCAG TTCCT	GAGTAGGGTGGTTGG GGTTG	XM051916883
gclm	TGTGGGAAGAGCTGG AGAGT	TACTGCTCCCCTGTA CTGCT	XM051901926
gpx4	GGGGTTACGTGTGCA TCATC	AACTCCTTGATCTCC GCCTC	XM051912906
gsr	GAACACTACACGTCC AGGCA	TCACGTTGTCCTTTCC CCAG	XM051860462
hsla	TGCTGAGCGTGTTTG TTTGG	GGCAGCAGAGGGTC GATTAA	HQ446238
ppara	CGAGCCTGTACAGTC CCTTG	TGGACTAGATGCCGG GGTTA	MZ727200
ptgs2	GCATTGCAGCCGAAT TCAAC	AGCATTAACAGCCAC TCCCT	XM051861202
slc7a11	GAGCCCATCTTCATG CCATG	AGTCTGCTGTGTTGA CCTCA	XM051860495
srebp1	ACGGGGATGGGGAA TACTCA	TTATCACCACGGCCA CTGAC	KJ162572
zip1	CTAGATGAGGGCTTC CCGCT	CATTCAACGAGTGCC GTCCA	OP106599
zip4	TCTTGCGGGCGTCTA TTTCT	AAGTTATGGATGCCG TCGCC	OP106600
zip5	TTAGGTGATTTGGCG GTGCT	TGGAGCATTTCGGGC ATCAT	OP106601
zip6	GAAGGTTTGTCCAGC GGTCT	CATACCCGCCTTCAG CAAGA	OP106602
zip7	CCAGTGCAGTCAAAC ACGGA	AGAGTGAGAGTGCG AGTGTC	OP106603
zip8	AAAACGGCTCTCTGT CCACG	ATTACCCAACCCAAA GGCGG	OP106604
zip9	TCCAGAAGCAGCAAG GACTG	AGCAGGAGCCTTATG CAACA	OP106605

zip10	TTCTGGTGCCCATCC TCAAC	CCTGCTAGTGCAGTC AGTCC	OP106606
zip13	ATCAGCCCTTACACA GAGCC	TACGAGTGGCTTTCC AACGG	OP106608
zip14	GCGCTATGCCAACTC CAATG	TAACATCCACCCGTT CCAGC	OP106609
znt1	GCAACCCGATGTGGT GATTG	GGCTGTTGTTGTGAT TCCCG	OP106610
znt4	CTCAATCAGTCCGGG CATCT	CACACCCTCCAGCAG AATGA	OP106612
znt5	ATGTGCTGTCTGGTG GAGTG	TGATGAAGCGAGGCA GAGAC	OP106613
znt6	CGAGAGGAAAGTGTC GCCTT	ACACCAGATTCAACA CGCCA	OP106614
znt7	CGCTAGAACCCCCTG ATGTG	TGTCCTCCATGTGAA TGCCC	OP106615
znt8	GCCCAGAACGAGCA GAAACT	CTGTAAAGATCGCCG GAGGA	OP106616
znt9	TTGGCGTGGGTTTAT ACGGG	GGCGATGTAACGCAT GTTGG	OP106617
znt10	ACTGGCTAAAGGACG GAACG	CCACCAGGACAACAG GAGAG	OP106618

Abbreviation: *acca*, acetyl-CoA carboxylase a; *acad*, acyl-CoA dehydrogenase; acsl, acyl-CoA synthetase long chain family member; *atgl*, adipose triglyceride lipase; *atp8*, ATP synthase F0 subunit 8; cbs, cystathionine beta-synthase; *cpt 1*, carnitine palmitoyl transferase 1; *cth*, cystathionine gamma-lyase; *dgat*, diacylglycerol O-acyltransferase; *elovl6*, ELOVL fatty acid elongase 6; *fas*, fatty acid synthase; *fsp1*, Ferroptosis suppressor protein 1; *gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *gclc*, glutamate-cysteine ligase, catalytic subunit; *gclm*, glutamate-cysteine ligase, modifier subunit; *gpx*, glutathione peroxidase; *gsr*, glutathione reductase; *hsl*, hormone-sensitive lipase; *ppara*, peroxisome proliferator activated receptor alpha; *ptgs2*, prostaglandin G/H synthase 2; *slc7a11*, solute carrier family 7 member 11.

Table S3. siRNA sequences.

	sense	antisense
si-*hdac6*	GAGCUUCGCUGAUCUUUC UTT	AGAAAGAUCAGCGAAGCU CTT
si-*hdac8*	GCACCAUCAAAGGGAAUC UTT	AGAUUCCCUUUGAUGGUG CTT

Table S4. Primers used for plasmid construction of expression vector.

	Forward primer (5'-3')	Reverse primer (5'-3')
pcDNA3.1-FL-SLC7A11	ctagcgtttaaacttaagcttATGCCCAGAAGGACTGTGTCA	aacgggccctctagactcgagTCACAGATCCTCTTCAGAGATGAGTT
pcDNA3.1-HDAC6	cccctgggatccccggaattcATGGATCCTGTTCCAGTTTCCA	gtcacgatgcggccgctcgagTTAAGCGTAGTCTGGGACGTCG
pcDNA3.1-HDAC8	ctagcgtttaaacttaagcttGCCACCATGGTGCATCATCA	aacgggccctctagactcgagCTAATGGTGATGGTGATGATGAACT
pcDNA3.1-SLC7A11 K12R	AGTTCCACATCCCagaGCGGATCTGACTCCAAGTAATTTT	CtctGGGATGTGGAACTGACACAGTCCTTCTG
pcDNA3.1-SLC7A11 K23R	TTGGAGAGagaGACCCTCTCAAATCAAATGGAGA	AGGGTCtctCTCTCCAAAATTACTTGGAGTCAGATC
pcDNA3.1-SLC7A11 K27R	CCCTCTCagaTCAAATGGAGACTCCCACGCCG	CATTTGAtctGAGAGGGTCTTTCTCTCCAAAATTAC
pcDNA3.1-SLC7A11 K45R	ACTGCGAagaAAGGTGACCCTGCTCCGAGGAA	TCACCTTtctTCGCAGTTCCACCTTCTCCTCG
pcDNA3.1-SLC7A11 2-46aa mutant	TGAAGAGGATCTGGTGACCCTGCTCCGAGGAAT	GTCACCAGATCCTCTTCAGAGATGAGTTTCTGC
pcDNA3.1-SLC7A11 47-472 mutant	GTGGAACTGCGAAAGAAGATCGAAAGAAAACCCAAATGGTT	CTTCTTTCGCAGTTCCACCTTCTCCTCGGGGGC

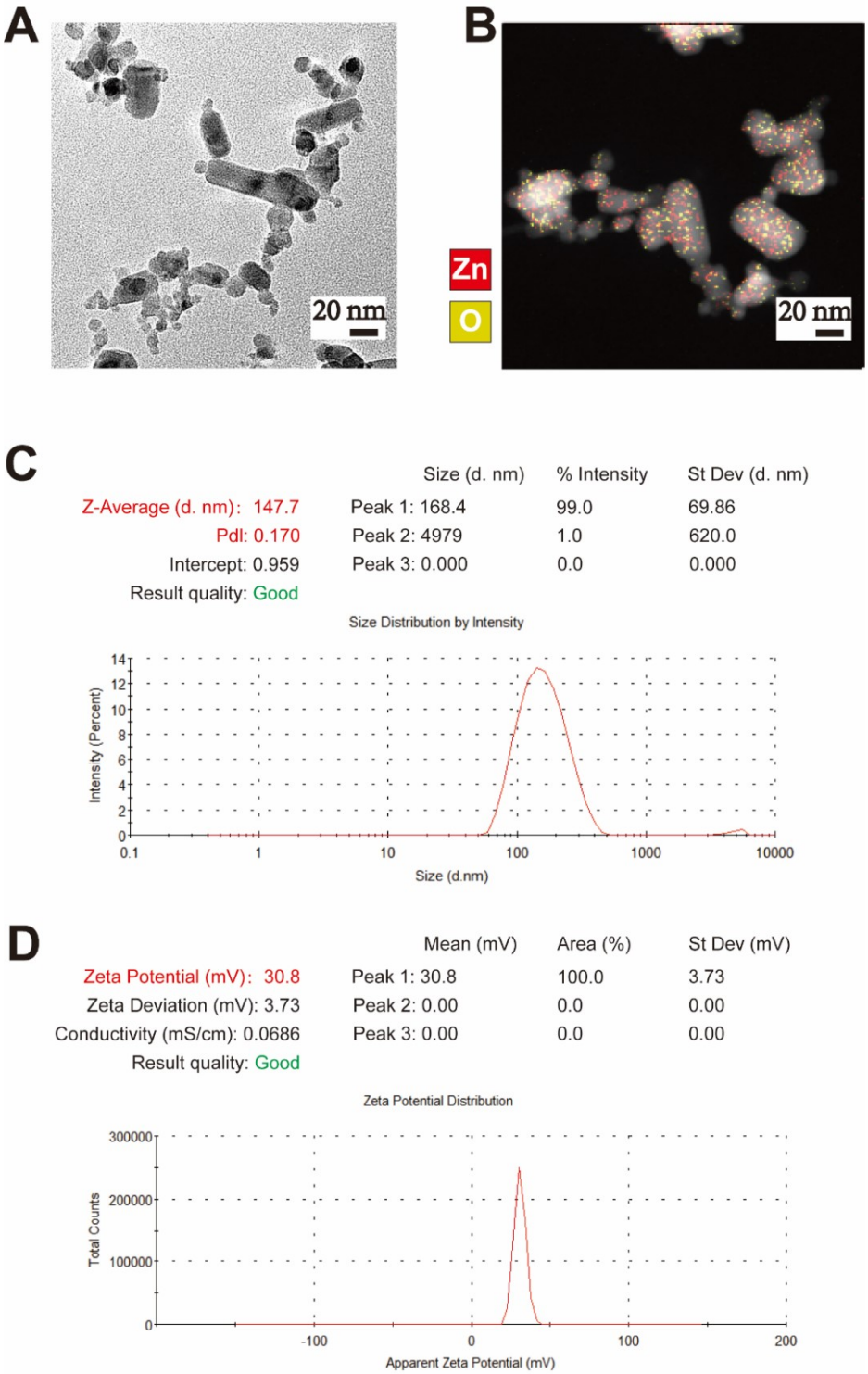

Fig S1. Particle characterization and physicochemical properties of ZnO NP. (A) Transmission electron microscopy (TEM) micrograph of the ZnO NP; (B) TEM elemental mapping image of the ZnO NP; (C) The intensity-weighted average hydrodynamic diameter in the distilled water of ZnO NP; (D) Zeta potential in the distilled water of ZnO NP.

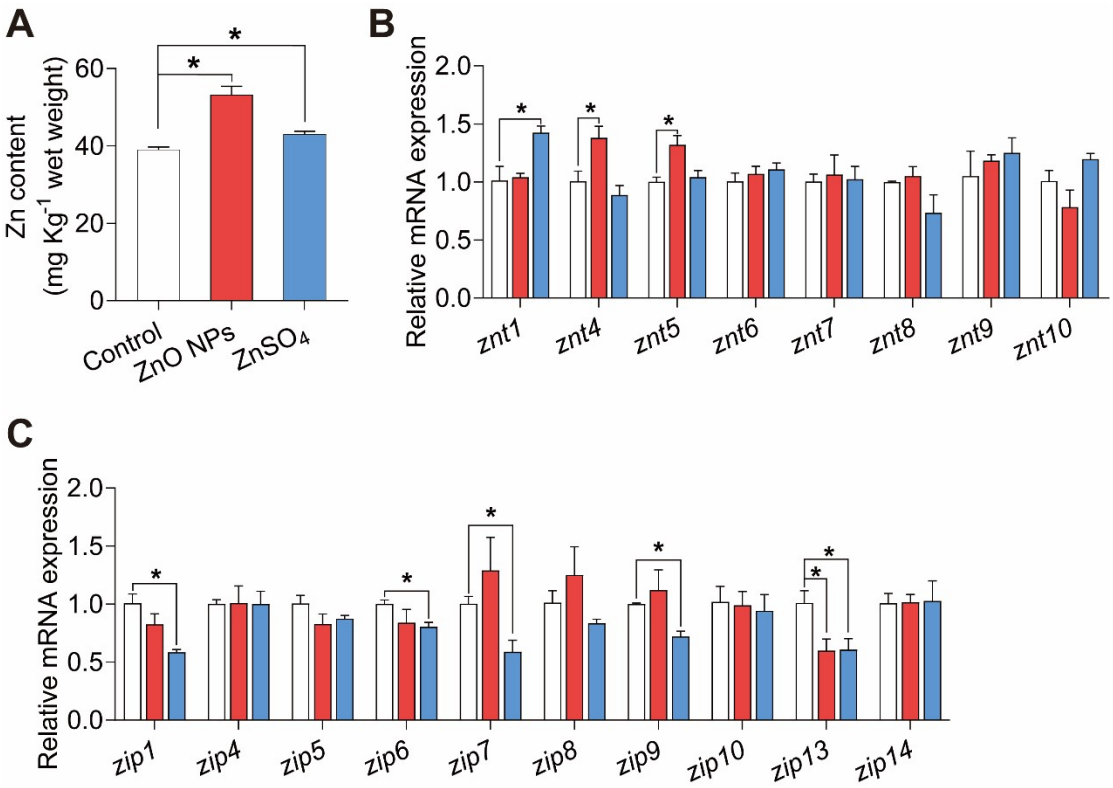

Fig S2. The effects of dietary ZnO NPs and $ZnSO_4$ on hepatic Zn metabolism. (A) Zn content; (B) The mRNA levels of ZnT family proteins; (C) The mRNA levels of ZIP family proteins. Results are presented as means ± S.E.M. (n=3). Statistical analysis was performed by Student' *t*-test, $*P < 0.05$.

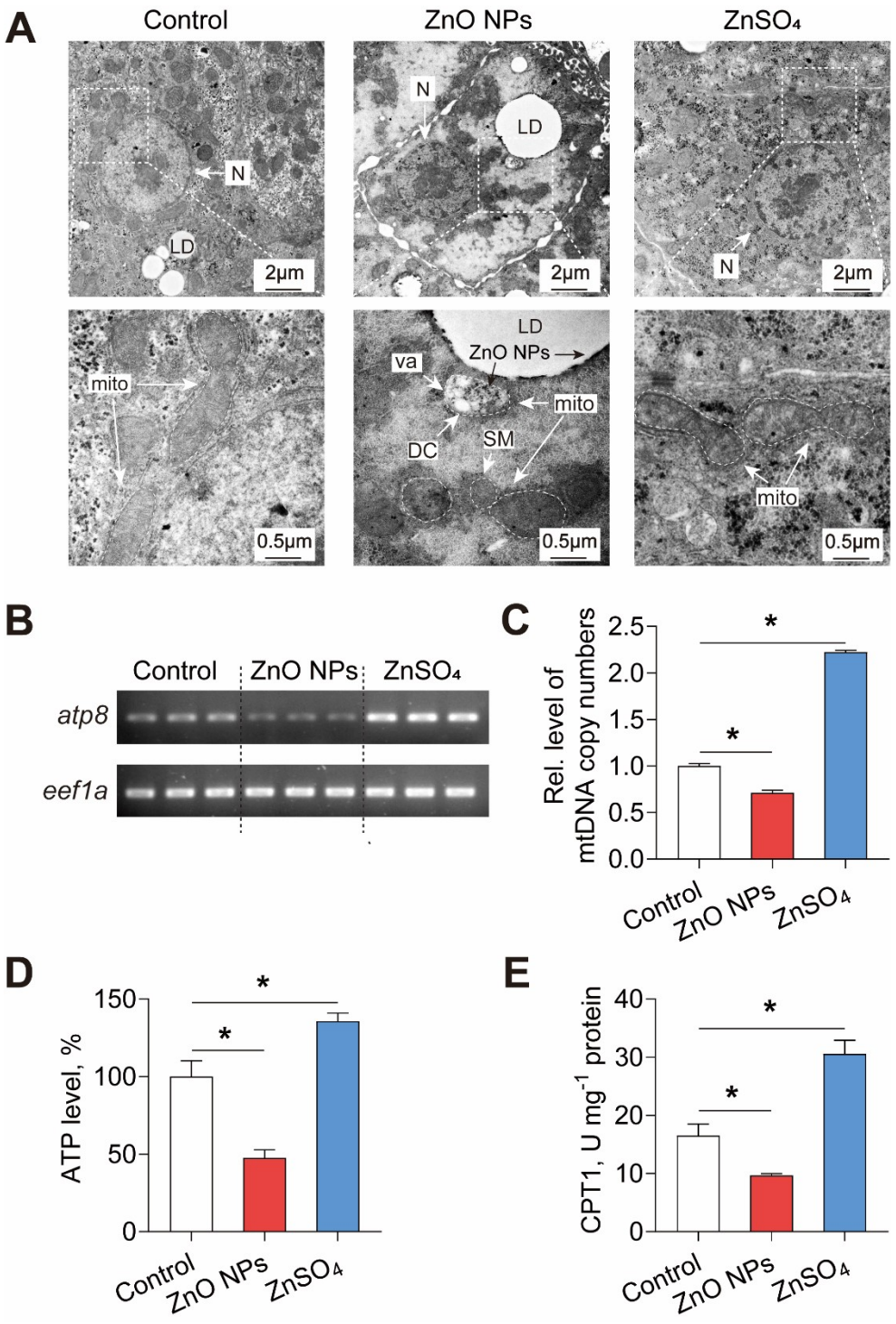

Fig. S3. Dietary ZnO NPs and $ZnSO_4$ influenced hepatic mitochondrial structure. (A) Representative hepatic TEM images. DC, disrupted cristae. LD, lipid droplet. mito, mitochondria. N, nucleus. SM, smaller size. va, vacuolation; (B) Semi-quantitative of mitochondrial-encoded gene *atp8*; (C) Relative level of mitochondrial copy number. Results are presented as means ± S.E.M. (n=3). Statistical analysis was performed by Student' *t*-test, $*P < 0.05$.

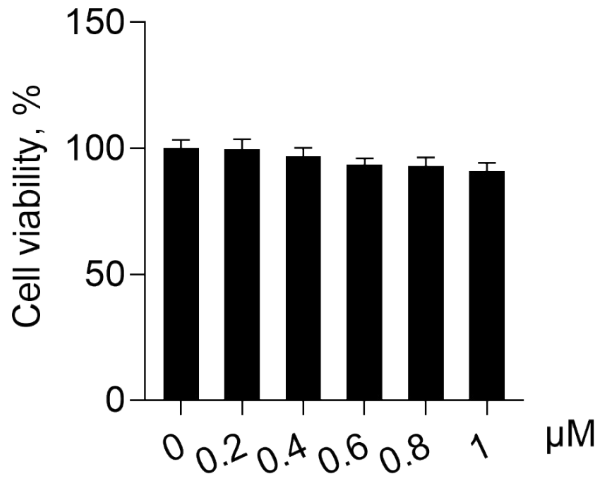

Fig. S4. Effects of Fer-1 concentration on the viability of L8824 cells. Results are presented as means ± S.E.M. (n≥3). Statistical analysis was performed by Student' *t*-test, **P* < 0.05.

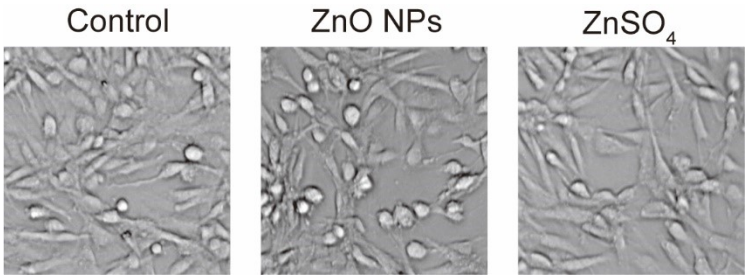

Fig. S5. Effects of ZnO NPs and $ZnSO_4$ exposure on the viability of L8824 cells.

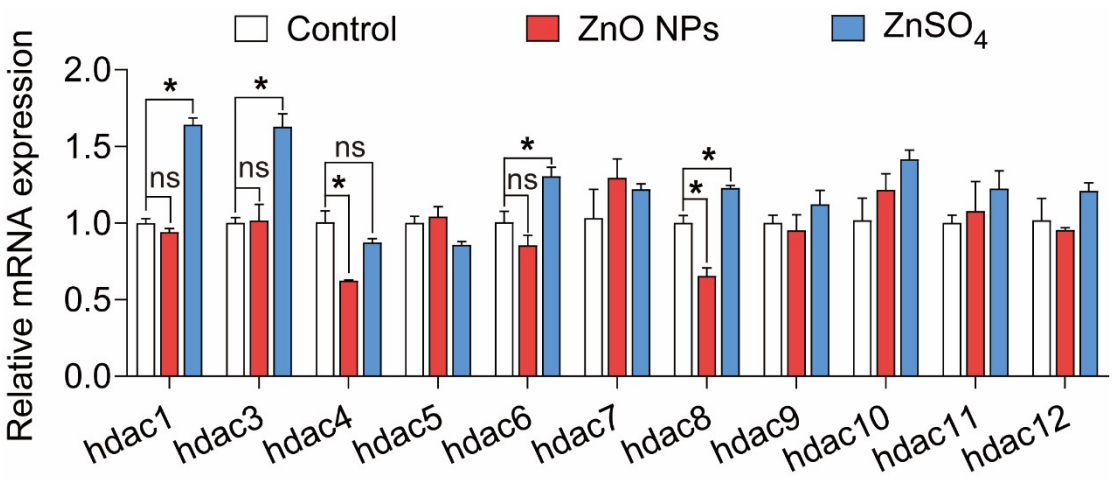

Fig. S6. Effects of ZnO NPs and $ZnSO_4$ on the mRNA expression of hepatic HDACs family genes. Results are presented as means ± S.E.M. (n≥3). Statistical analysis was performed by Student' *t*-test, $*P < 0.05$.

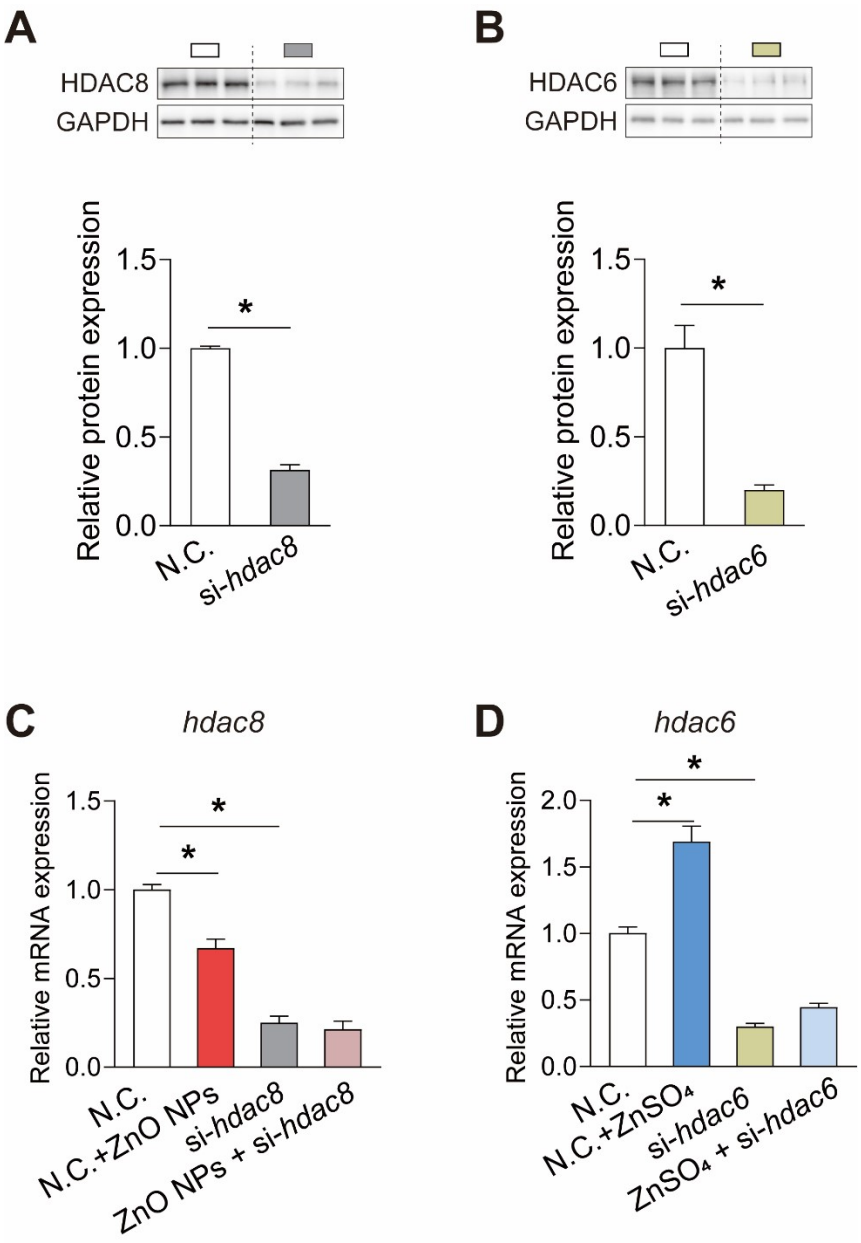

Fig. S7. The effect of si-*hdac8* and si-*hdac6* on the expression of HDAC8 and HDAC6, respectively. (A) Western blot and statistical analysis of HDAC8. L8824 cells were treated with or without si-*hdac8* for 48 h; (B) Western blot and statistical analysis of HDAC6, L8824 cells were treated with or without si-*hdac6* for 48 h; (C) The *hdac8* mRNA expression. L8824 cells were treated with ZnO NPs and/or si-*hdac8* for 48 h; (D) The *hdac6* mRNA expression. L8824 cells were treated with $ZnSO_4$ and/or si-*hdac6* for 48 h.Results are presented as means ± S.E.M. (n≥3 independent biological replicates). Statistical analysis was performed by Student' *t*-test, **P* < 0.05.