Supplementary Material

SIRT3 Acts As a Negative Regulator of Autophagy Dictating Hepatocyte Susceptibility to Lipotoxicity

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Supplementary Methods

Animal Model and Experimental Protocol

All animal studies were performed in accordance with the animal care committee at the University of Illinois at Chicago. All mice were fed *ad libitum*, and housed on a 12:12 h light: dark cycle at 25°C. Male SIRT3$^{-/-}$ mice and WT 129Sv mice (8-week of age) were purchased from Jackson Laboratory (Bar Harbor, ME), and maintained on a standard chow diet. Male C57BL/6 mice (8-week old, Jackson Laboratory, Bar Harbor, ME) were randomly assigned to normal diet, palm coil high-fat diet, and corn oil high-fat diet (Supplementary data Table 1). The liver-specific SIRT3 overexpression mice and corresponding control mice were generated via the injection of recombinant adeno-associated viral (AAV) serotype 8 gene transfer vectors containing a liver-specific promoter combination (albumin promoter) with either mouse SIRT3 sequence or empty as vector control (Cyagen Biosciences Inc., Guangzhou, China). Finally, there are 6 groups in total (n = 8 mice/group) and the animals were fed for 8 weeks. AAV8 vectors were administered by tail vein injection at a dose of 1×10^{12} viral titer/mL in a total volume of 100 μl/mice at the beginning of the experiment. Recombinant AAV8 vectors encoding mouse SIRT3 (NM_022433.2) under the control of albumin promoter (a liver-specific promoter) were generated by Cyagen Biosciences Inc. (Guangzhou, China). A noncoding plasmid carrying only the albumin promoter was used to produce vector control particles. The overexpression efficiency was shown in Fig. 1D and Supplementary data, Fig. 30. Mice were sacrificed after 8-week of feeding. Plasma and liver were collected for analysis. Plasma alanine aminotransferase (ALT) content was determined by a commercial ALT kit (Thermo Fisher, Waltham, MA). Triglyceride was assayed using a

Triglycerides Assay Kit (Thermo Fisher, Waltham, MA). Liver redox state was detected using a commercial TBARS Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Small pieces of liver were fixed immediately in 10% buffered formalin. After paraffin embedding, 5 μm sections were deparaffinized in xylene and were rehydrated through a series of decreasing concentrations of ethanol. Sections were stained with hematoxylin and eosin (H&E).

Chemicals

All chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO), unless otherwise specified. Actinomycin D was obtained from Solarbio (Beijing, China). PA- and SA-BSA conjugates were prepared as described previously [(1)]. Briefly, PA or SA was dissolved in ethanol and saponified with sodium hydroxide. The sodium salt was dried, re-suspended in saline and heated at 80°C until completely dissolved. While the solution was still warm, isovolumetric 20% (w/v) BSA was added and the mixture was stirred at 50°C for 4 h to allow PA or SA to bind to BSA. The PA- or SA-BSA complex (3 mmol/L fatty acid: 1.5 mmol/L BSA; molar ratio, 2:1) was then sterilized by filtering, and aliquoted for future use. In all the experiments, the control group was exposed to an equal amount of solvent (e.g. BSA, ethanol, DMSO).

Cell Culture

The HepG2 cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum, 100 U /ml penicillin, and 100 μg /ml streptomycin at 37°C in a humidified O_2/CO_2 (19:1) atmosphere.

Alpha mouse liver (AML)-12 hepatocyte culture was established from a mouse transgenic for human transforming growth factor α, and was obtained from the American Type Culture Collection (ATCC, CRL-2254), and was cultured in Dulbecco's Modified Eagle Medium /Ham's Nutrient Mixture F-12, 1:1 (DMEM /F-12, Sigma-Aldrich, 051M8322) containing 10% (v/v) fetal bovine serum (PAA Laboratories, A15-701), 5 mg/ml insulin (Sigma-Aldrich, I9278), 5 μg/ml transferrin (Sigma-Aldrich, T8158), 5 ng/ml selenium (Sigma-Aldrich, 229865), 40 ng/ml dexamethasone (Sigma-Aldrich, D4902), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life technologies, 15140-122) at 37°C in a humidified O_2/CO_2 (95:5) atmosphere.

Establishment of stable human SIRT3 over-expression cell lines

The eukaryotic expression vector plasmid pcDNA3.1$^+$/hSIRT3 containing the human SIRT3 gene sequence was kindly provided by Dr. Yu Wang (University of Hong Kong) [(2)]. HepG2 cells grown to 80–90% confluence were transfected with 0.8 μg/well of the expression vector pcDNA3.1$^+$/hSIRT3 or empty vector control pcDNA3.1$^+$ (Invitrogen, Grand Island, NY) in 24-well plate using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY) following the manufacturer's guidelines. After 48 hours transfection, cells were trypsinized into 24-well plates at a ratio of 1:100 for monoclonal cell selection using 800 μg/ml G418. The stable cell line was established and confirmed by both western blot analysis and Real Time PCR. SIRT3 over-expression cells and vector control cells were cultured in DMEM containing 10% (v/v) fetal bovine serum, 400 μg/ml G418, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified O_2/CO2 (19:1) atmosphere.

MnSOD Overexpression

Recombinant lentivirus vector (pLV[Exp]-EGFP:T2A:Neo) containing human MnSOD (NM_001024465.1) under the control of EF-1alpha promoter was generated by Cyagen Biosciences Inc. (Guangzhou, China). A noncoding vector carrying the EF1alpha promoter was used to produce vector control particles. The optimal virus titer used for cell transfection was screened according to the manufacturer's instructions. HepG2 cells were transfected with either pLV[Exp]-EGFP:T2A:Neo-EF1alpha-hMnSOD to overexpress MnSOD or pLV[Exp]-EGFP:T2A:Neo-EF1alpha-null as a vector control.

Cell Death Assays

2×10^5 /ml cells were seeded in 24-well plates and, after the indicated treatments, cell death was determined by measurement of LDH release, propidium iodide (PI) staining, or Hoechst staining. For LDH assay, culture medium was collected and detected using an LDH assay kit (Thermo Scientific Inc, VA) according to the manufacturer's instructions. For PI staining, cells were trypsinized and stained with PI staining solution (BD Pharmingen, CA) according to the manufacturer's instructions. Fluorescence was measured by flow cytometry (Accuri c6, BD, CA). For Hoechst staining, cells were stained with Hoechst staining solution (Beyotime Biotechnology, Nantong, China) according to the manufacturer's instructions and imaged by Nikon eclipse Ti-S fluorescence microscope (Nikon, Tokyo, Japan).

Analysis of GFP-LC3 puncta

The recombinant adenovirus GFP-LC3 (1×10^{10} viral titer/mL) was obtained from Hanbio Biotechnology Co. Ltd. (Shanghai, China). Cells were transiently transfected with recombinant adenovirus GFP-LC3 according to the manufacturer's instruction. Puncta was detected by a laser scanning confocal microscope (Nikon A1R, Japan) from at least 50 cells

for each individual experiment after different treatment.

Analysis of autophagic flux

The autophagic flux was measured according to methods described previously [3]. The cells were pretreated with chloroquine (CQ), inhibitor of lysosome acidification, after genetically overexpressing or knocking-down SIRT3. The autophagic flux was determined via detecting GFP-LC3 puncta and LC3-II expression using laser scanning confocal microscope and Western blot, respectively. Additionally, cells were transiently transfected with recombinant adenovirus mRFP-GFP-LC3 (Hanbio Biotechnology Co. Ltd., Shanghai, China). Yellow or red puncta was detected by a laser scanning confocal microscope (Nikon A1R, Japan) from at least 50 cells for each individual experiment after different treatment.

Real Time-PCR

Total RNA isolation, reverse transcription, and real time PCR were performed as described previously [4]. Briefly, total RNA from cultured cells was isolated with a phenol-chloroform extraction. For each sample, 1 μg total RNA was reverse-transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). The cDNA was amplified in MicroAmp Optical 96-well reaction plates with a SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an Applied Biosystems Prism 7000 sequence detection system. Relative gene expression was calculated after normalization by a house-keeping gene (18S rRNA).

Western-blot Analysis

Western-blot was performed as described previously [4] and the following antibodies were used: Anti-Sirt3, anti-phospho-AMPK, anti-AMPK, anti-phospho-Akt, anti-Akt,

anti-phospho-Erk1/2, anti-Erk1/2, anti-phospho-p70S6K, anti-p70S6K, anti-LC3B, anti-p62, anti-phospho-ACC, anti-ACC, anti-MnSOD, anti-acetylation, anti-COX Ⅳ, anti-caspase-3, anti-parp-1, anti-Atg5, and anti-beta-actin antibodies (Cell Signaling Technology, Danvers, MA).

Immunoprecipitation

Cells were lysed in an immunoprecipitation (IP) buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P-40, pH 7.8, and a mammalian cell-specific protease inhibitor cocktail. Total cellular extracts (200 μg of protein) were incubated with anti-MnSOD antibody (1 μg/ml) in IP buffer overnight at 4 °C on a rocker. The antibody-protein mixture was agitated at 150 rpm with Protein A/G agarose (Santa Cruz Biotechnology) for 1 hr at 4°C. The immunoprecipitates were washed four times with IP buffer. The washed immunoprecipitates were incubated in 50 μl of 1 × electrophoresis loading buffer and heated at 100 °C for 5 min. The beads were spun out and the supernatant was resolved by SDS-PAGE, and the modification of MnSOD by Sirt3 was analyzed by Western blot.

RNA interference

Cultured cells were transfected with human SIRT3 siRNA, human AMPK siRNA, human or Atg5 siRNA (Santa Cruz Biotechnology) using Lipofectamine 2000 according to the manufacturer's instructions. In the control group, cells were transfected with scrambled siRNA (Santa Cruz Biotechnology).

Reactive oxygen species (ROS) detection

The intracellular superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) were detected as described previously [5]. Briefly, hepatocytes were stained with DCFH-DA for the

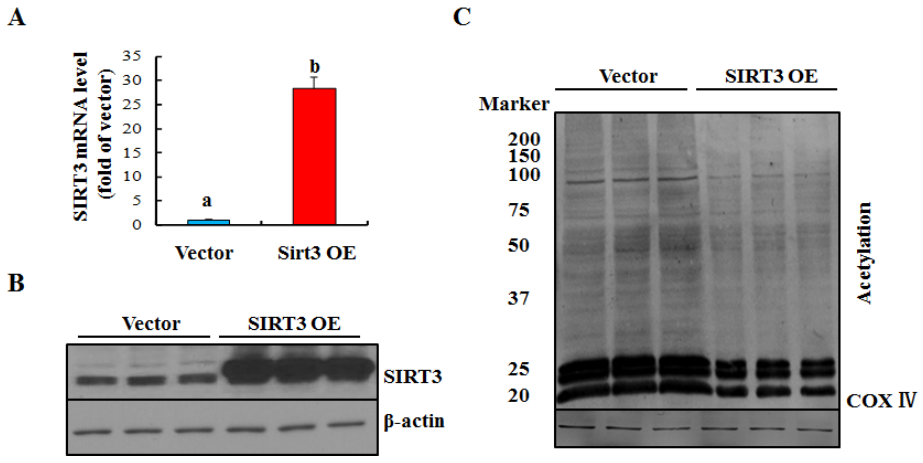
158 measurement of H_2O_2 after the indicated treatments. Intracellular $O_2^{\cdot -}$ was detected using a
159 commercial Superoxide Detection kit (Enzo Life Science, NY) according to manufacturer's
160 instructions. The fluorescent signal of listed ROS was collected by fluorescence microscope
161 and flow cytometry.

Supplemental Table

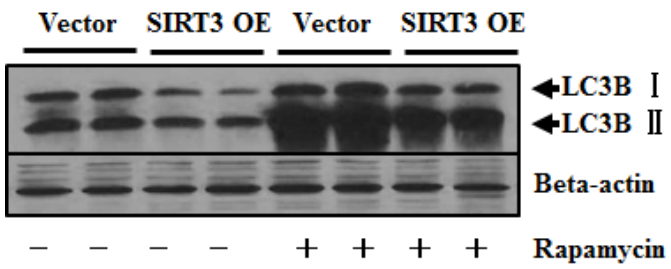
Supplementary Table 1

	Normal diet	High-fat diet	
		Palm oil	Corn oil
Corn Starch (g/kg)	417.5	227.5	227.5
Casein (g/kg)	200	200	200
Dextrinized cornstarch (g/kg)	132	132	132
Sucrose (g/kg)	100	100	100
Palm oil (g/kg)	-	190	-
Corn oil (g/kg)	-	-	190
Soybean oil (g/kg)	50	50	50
Fiber (g/kg)	50	50	50
Mineral mix(AIN-93G-MX, g/kg)	35	35	35
Vitamin mix(AIN-93G-MX, g/kg)	10	10	10
L-cystine (g/kg)	3	3	3
Choline bitartrate (g/kg)	2.5	2.5	2.5
Fat energy (kcal%)	11.7	45.0	45.0
Carbohydrates energy (kcal%)	67.5	38.3	38.3
Protein energy (kcal%)	20.8	16.7	16.7
Total energy (kcal/kg)	3848	4798	4798

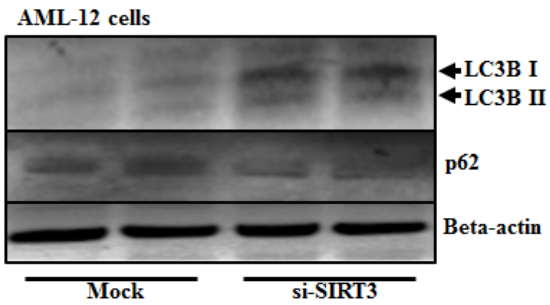
Supplemental Figures



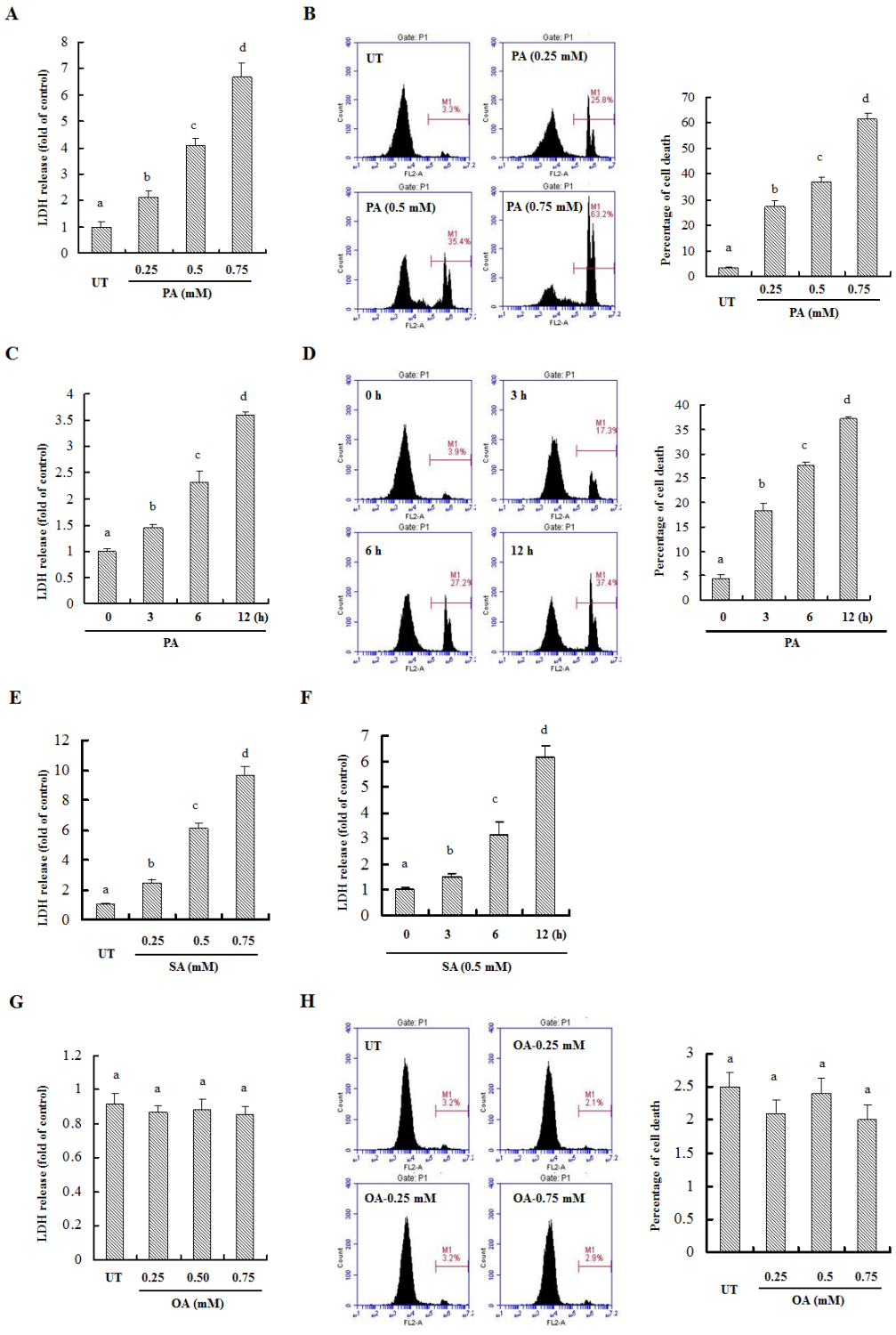
Supplementary Fig. 1 Stable SIRT3-overexpressing (SIRT3 OE) hepatocyte cell line was established in HepG2 cells. (A) SIRT3 mRNA level. (B) SIRT3 protein level. (C) SIRT3 activity was detected by the assay of acetylated proteins abundance in the mitochondrial fractions. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.



Supplementary Fig. 2 SIRT3-overexpressing inhibites LC3-II formation with or without autophagy activation. SIRT3 OE and vector control HepG2 cells were treated with rapamycin for 12 h. LC3-II level was detected by Western-blotting.



Supplementary Fig. 3 Knocking-down SIRT3 stimulates autophagy in mouse hepatocytes. AML-12 mouse hepatocytes were transfected with siSIRT3 or scramble siRNA. Total lysates were subjected to immunoblotting assay for LC3 and p62.



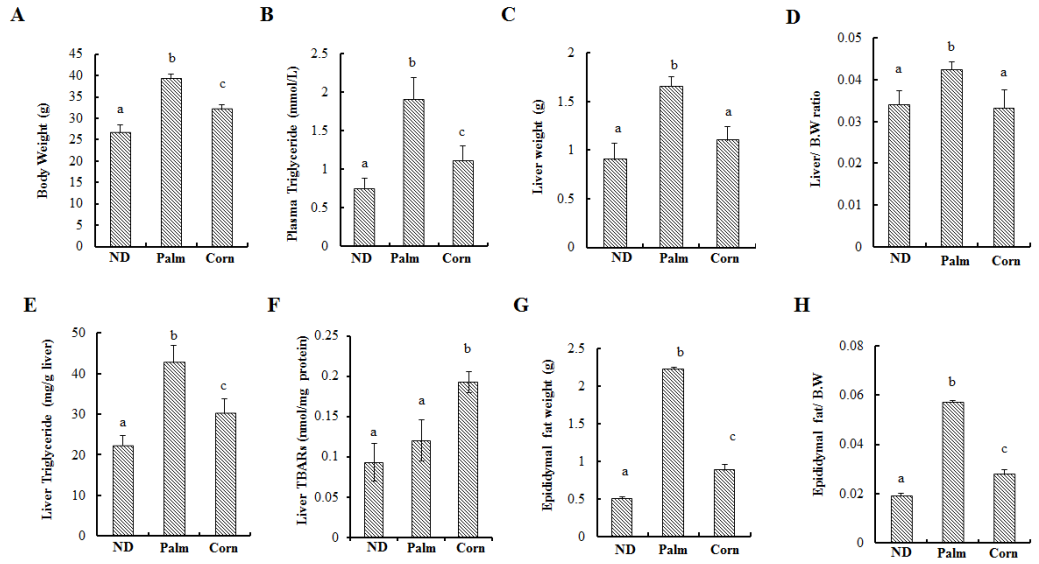
182

183 **Supplementary Fig. 4** SFAs induces cell death in hepatocytes. (A) & (B) HepG2 cells

184 were treated with 0.25, 0.5, 0.75 mM palmitic acid (PA) for 12 h. LDH release in the

185 culture medium and propidium iodide (PI) staining were detected according to the

description in the Material and methods, respectively. (C) & (D) HepG2 cells were treated with 0.5 mM (PA) for the indicated duration. LDH release in the culture medium and propidium iodide (PI) staining were detected according to the description in the Material and methods, respectively. (E) & (F) HepG2 cells were exposed to 0.5 mM stearic acid (SA) for 12 h or as indicated in the figure. LDH release in the culture medium was detected according to the description in the Material and methods. (G) & (H) HepG2 cells were treated with 0.25, 0.5, 0.75 mM oleic acid (OA) for 12 h. LDH release in the culture medium and propidium iodide (PI) staining were detected according to the description in the Material and methods, respectively. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.



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198 **Supplementary Fig. 5** SFAs-rich (palm oil) high-fat diet induces more severe
199 metabolic impairments than unsaturated fatty acids-rich (corn oil) high-fat diet in
200 C57BL/6 mice. Male C57BL/6 mice (8-week of age) were fed with normal or high-fat
201 diet using either palm oil or corn oil as fat source. Mice were sacrificed after 8-week
202 of feeding. Plasma and liver were collected for analysis. (A) Body weight. (B) Plasma
203 triglyceride was determined by a Triglycerides Assay Kit (Thermo Fisher, TR22421).
204 (C) Liver weight. (D) Liver weight/body weight ratio. (E) Liver triglyceride content.
205 (F) Liver TBARS. (G) Epididymal fat weight. (H) Epididymal fat/ body weight ratio.
206 All values are denoted as means ± SD (n = 8). Bars with different characters differ
207 significantly, $p < 0.05$.

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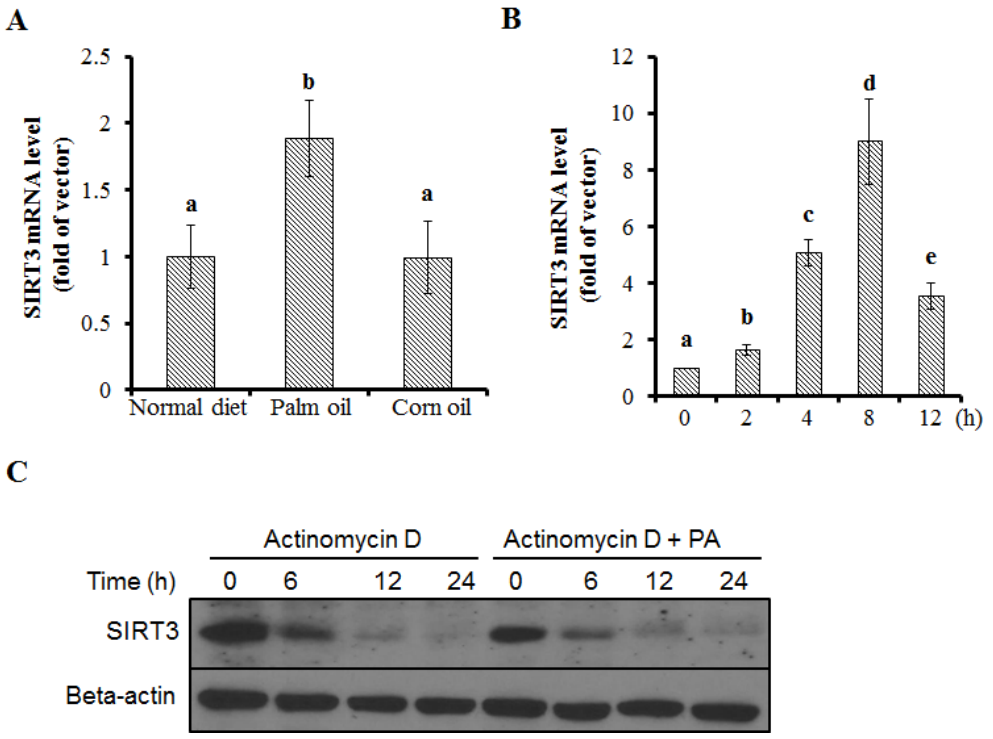

209

210 **Supplementary Fig. 6** SFAs up-regulates SIRT3 mRNA expression in the liver and
211 HepG2 cells. (A) Male C57BL/6 mice (8-week of age) were fed with normal or
212 high-fat diet using either palm oil or corn oil as fat source. Mice were sacrificed after
213 8-week of feeding. Liver mRNA was extracted for the analysis of SIRT3 mRNA level.
214 (B) HepG2 cells were treated with 0.5 mM PA for the indicated duration. The mRNA
215 expression of SIRT3 was detected by RT-PCR. (C) HepG2 cells treated with or
216 without 0.5 mM PA for the indicated duration in the presence of actinomycin D (5 μg/
217 ml). Immunoblotting assay was performed for SIRT3 expressions. Each *in vitro* test
218 was performed at least three times (n = 8 for mice), and a representative blot was
219 shown. All values are denoted as means ± SD. Bars with different characters differ
220 significantly, $p < 0.05$.

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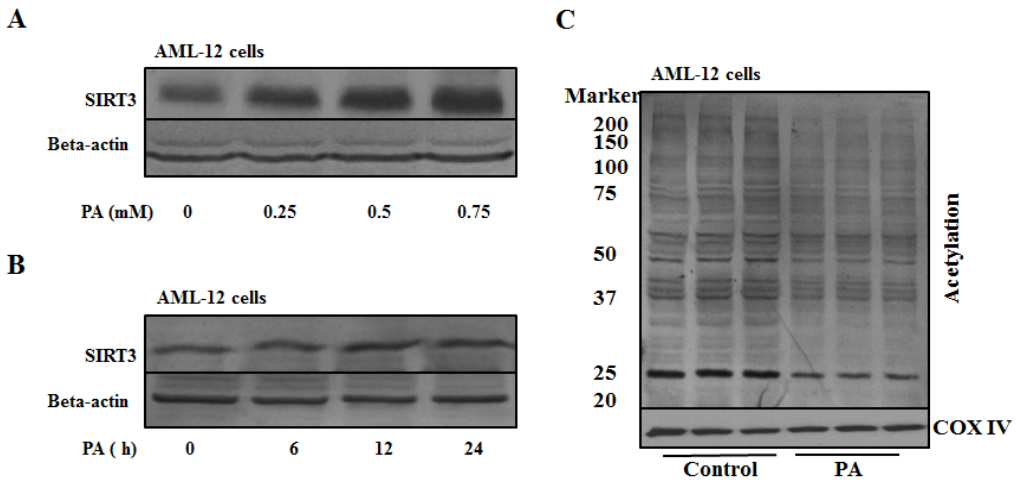
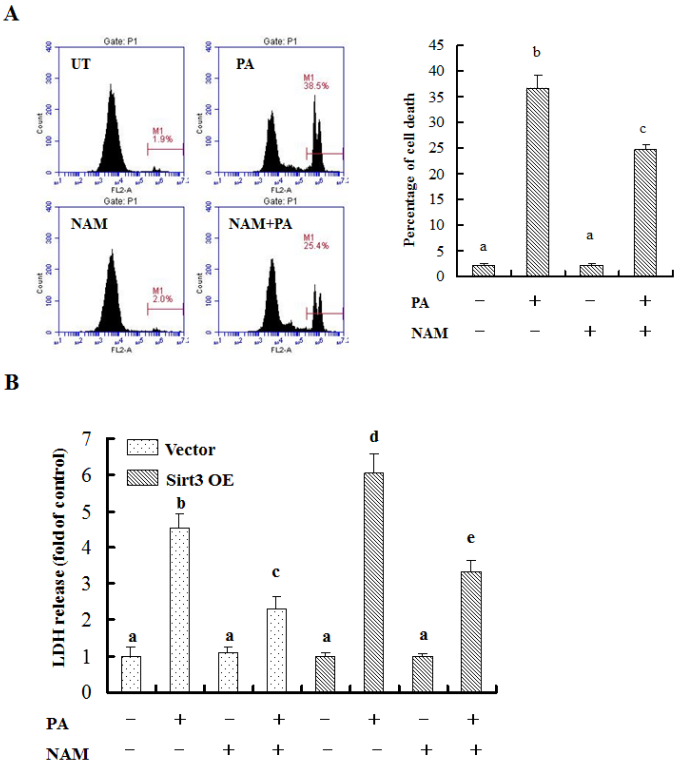
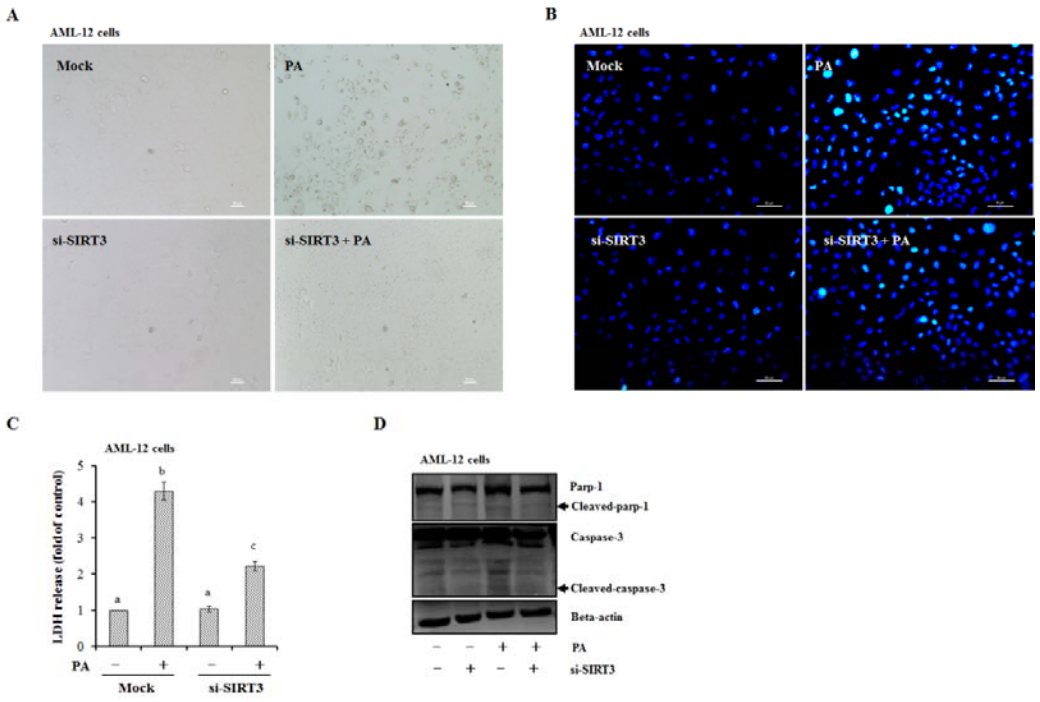


Supplementary Fig. 7 Palmitate increases SIRT3 expression and activity in mouse hepatocytes. (A) AML-12 cells were exposed to 0.25, 0.5, 0.75 mM palmitic acid (PA) for 12 h. Total cellular lysates were subjected to immunoblotting assay for SIRT3. (B) AML-12 cells were treated with 0.5 mM PA for the indicated duration. SIRT3 expression was detected. (C) AML-12 cells were incubated with 0.5 mM PA for 12 h. Mitochondrial proteins were extracted using a commercial Mitochondria Isolation kit (Beyotime, China) for the measurement of acetylizad proteins degree.

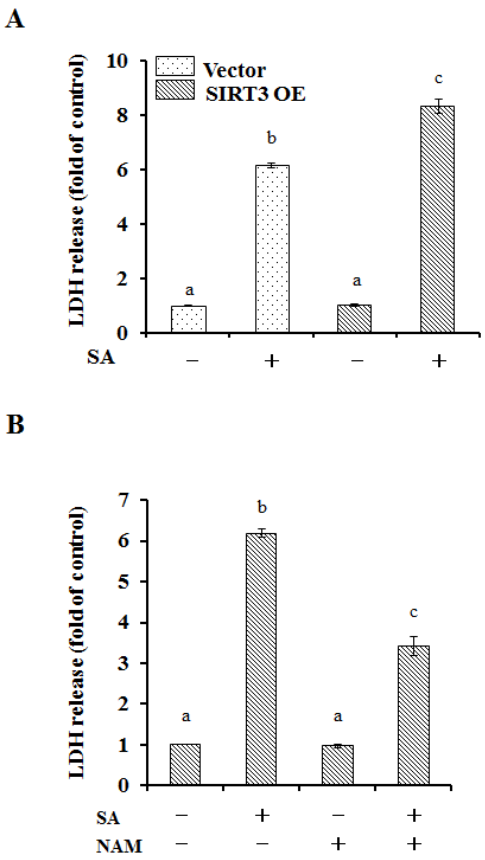


230

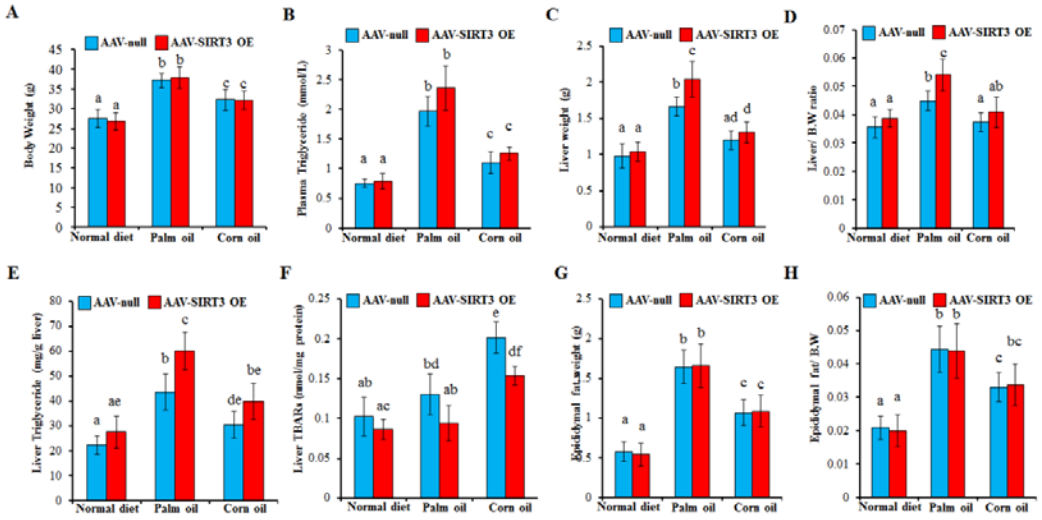
231 **Supplementary Fig. 8** Nicotinamide (NAM) protects hepatocytes against
232 palmitate-induced cell death. (A) HepG2 cells were treated with 0.5 mM palmitic acid
233 (PA) for 12 h with or without 1 h pre-incubation of 5 mM NAM. Cell death was
234 detected via propidium iodide (PI) staining using flow cytometry. (B) SIRT3 OE and
235 vector control HepG2 cells were treated with 0.5 mM PA for 12 h with or without 1 h
236 pre-incubation of NAM. Cell death was detected by the measurements of LDH release.
237 All values are denoted as means ± SD from three or more independent batches of cells.
238 Bars with different characters differ significantly, $p < 0.05$.



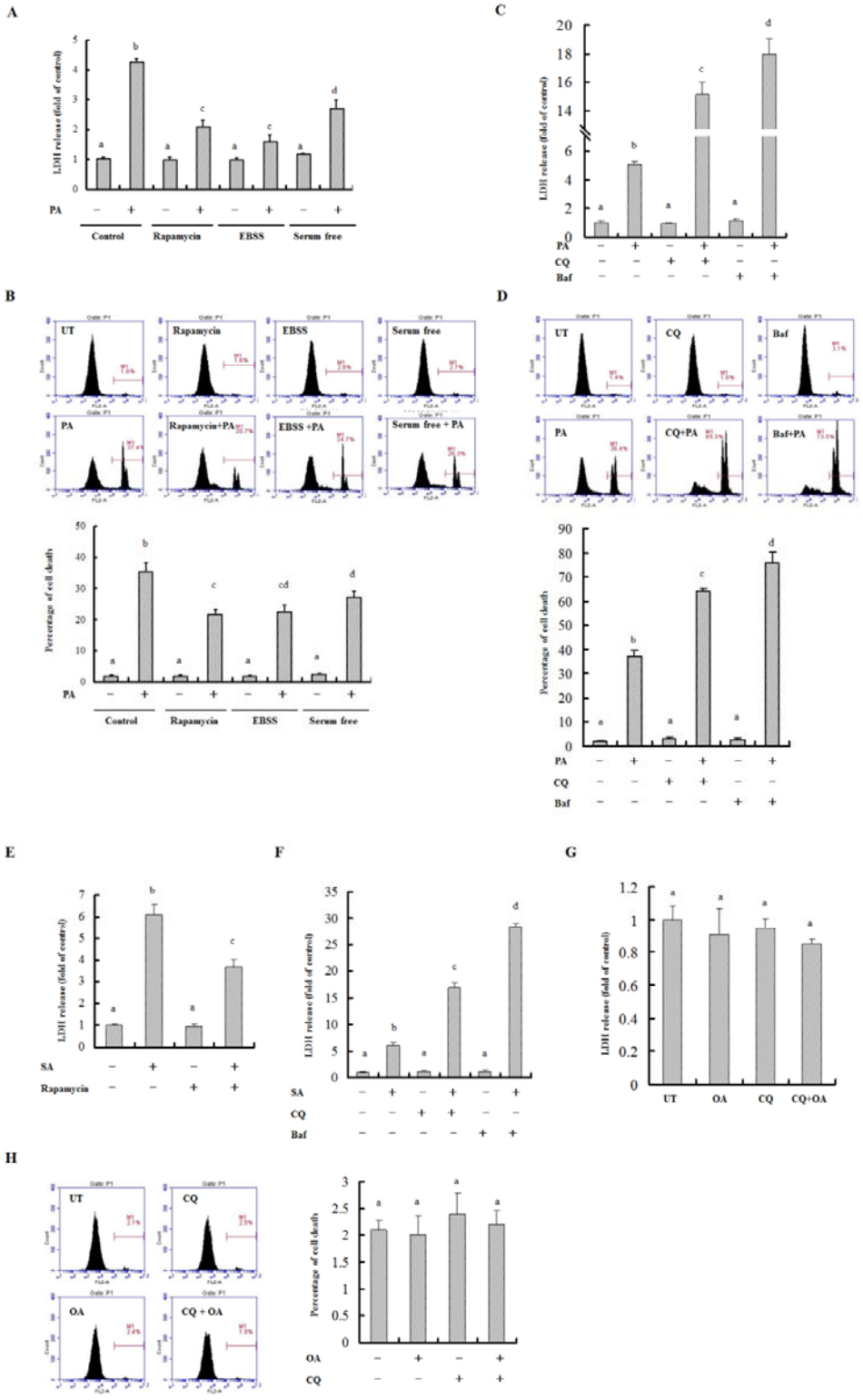
Supplementary Fig. 9 Knocking-down SIRT3 protects palmitate-induced cell death in mouse hepatocytes. AML-12 mouse hepatocytes were transfected with siSIRT3, and exposed to palmitic acid (0.5 mM) treatment for 12 h. Cell death was determined by the following assays. (A) Cellular morphological changes were examined by inverted phase contrast microscope at a scope of 100 ×. (B) Nuclear morphology was detected by Hoechst staining using fluorescence microscopy at a magnification of 200 ×. (C) LDH release was detected as described in the Methods. (D) Total lysates were subjected to immunoblotting assay for Caspase-3 and Parp-1. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.



Supplementary Fig. 10 SIRT3 overexpression aggravates stearic acid-induced cell death. (A) Vector control or SIRT3 OE HepG2 cells were suffered to stearic acid (SA, 0.5 mM) exposure for 12 h. Cell death was detected by the measurements of LDH release. (B) HepG2 cells were treated with 0.5 mM SA for 12 h with or without 1 h pre-incubation of 5 mM NAM. Cell death was detected by the measurements of LDH release. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.

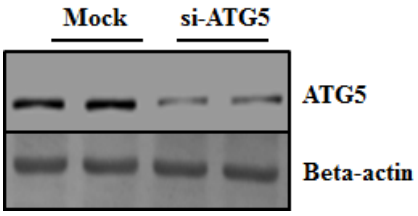


Supplementary Fig. 11 SIRT3 overexpression aggravates SFAs-rich (palm oil) high-fat diet induces metabolic impairments in C57BL/6 mice. Liver specific SIRT3 OE mice model was established and fed with normal or high-fat diet using either palm oil or corn oil as described in the Methods. Plasma and liver were collected for analysis (n = 8). (A) Body weight. (B) Plasma triglyceride was determined by a Triglycerides Assay Kit (Thermo Fisher, TR22421). (C) Liver weight. (D) Liver weight/body weight ratio. (E) Liver triglyceride content. (F) Liver TBARS. (G) Epididymal fat weight. (H) Epididymal fat/ body weight ratio. All values are denoted as means ± SD (n = 8). Bars with different characters differ significantly, $p < 0.05$.

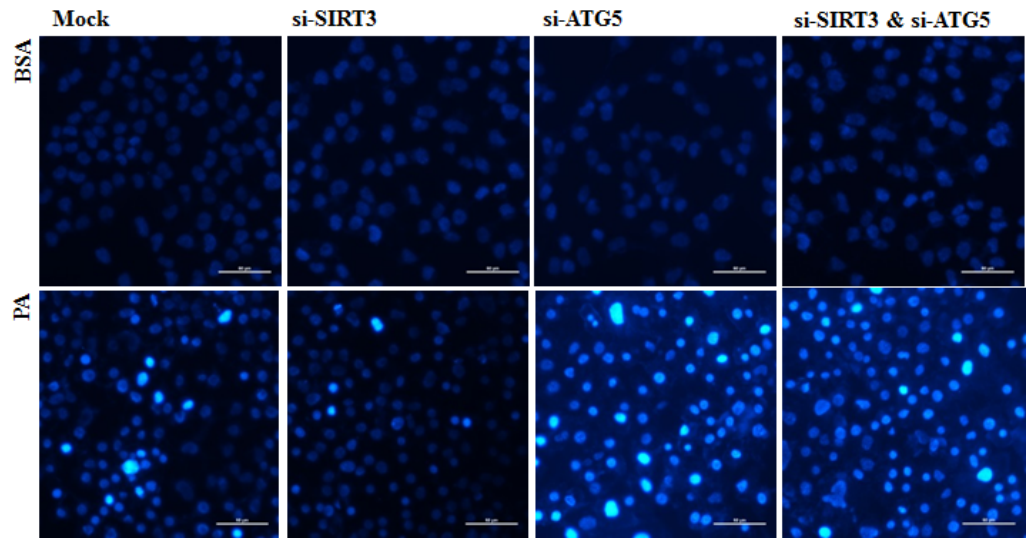


Supplementary Fig. 12 Autophagy regulates SFAs-induced lipotoxicity in HepG2 cells. (A) & (B) HepG2 cells were treated with 0.5 mM PA for 12 h in normal DMEM,

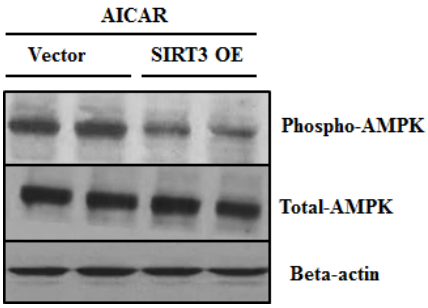
EBSS, or serum free DMEM culture medium, respectively. Rapamycin (50 nM) was added 1 h before PA treatment. Cell death was detected by the measurements of LDH release and propidium iodide (PI) staining using flow cytometry. (C) & (D) HepG2 cells were treated with 0.5 mM PA for 12 h. Autophagy inhibitor, chloroquine (CQ, 20 μM) or bafilomycin A1 (Baf, 100 nM), was added 1 h before PA treatment, respectively. Cell death was detected by the measurements of LDH release and propidium iodide (PI) staining using flow cytometry. (E) & (F) HepG2 cells were treated with 0.5 mM SA for 12 h. Rapamycin, CQ, and Baf were added 1 h before SA treatment, respectively. Cell death was detected by the measurements of LDH release in the culture medium. (G) & (H) HepG2 cells were treated with 0.5 mM OA for 12 h. CQ was added 1 h before OA treatment. Cell death was detected by the measurements of LDH release and propidium iodide (PI) staining using flow cytometry. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.



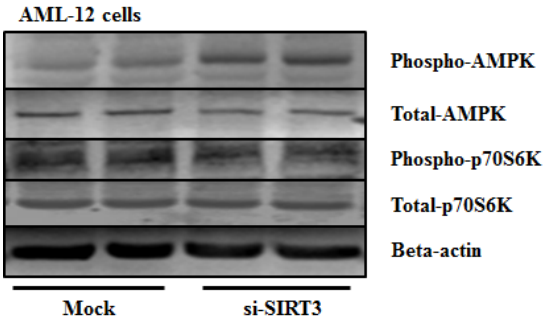
Supplementary Fig. 13 Knocking-down efficiency of siRNA for Atg5. Special siRNA for Atg5 was transfected into HepG2 cells as described in the Methods. Protein expressions of Atg5 were detected for testing the silencing efficiency.



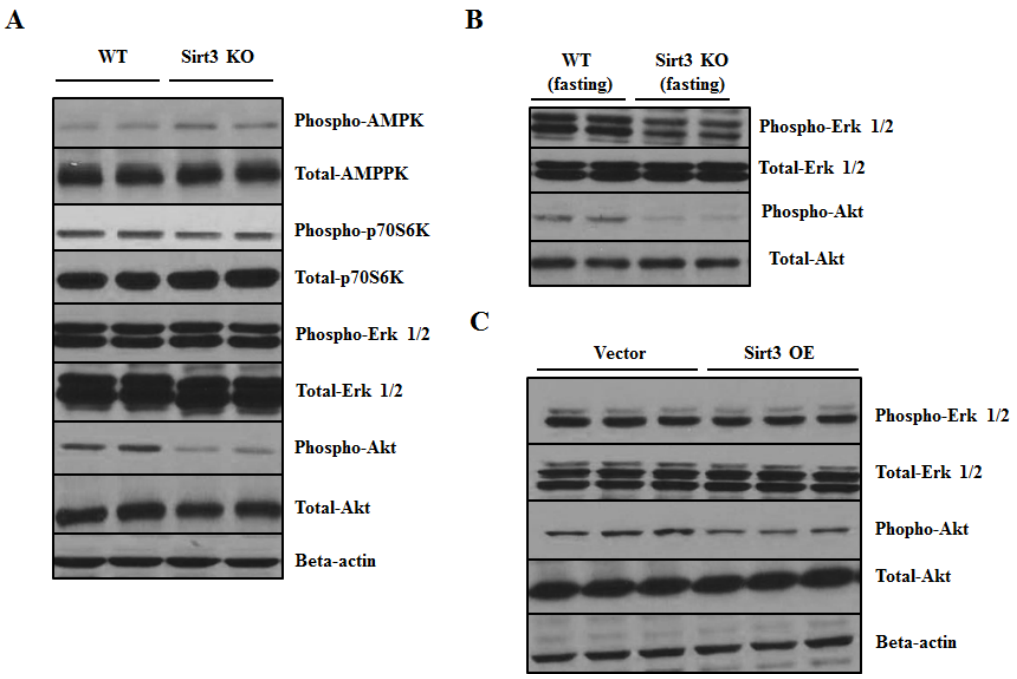
Supplementary Fig. 14 Inhibiting autophagy blocks knocking-down SIRT3-protected lipotoxicity in HepG2 cells. HepG2 cells were co-transfected with siRNA for SIRT3 and Atg5 siRNA, followed with 0.5 mM PA exposure for 12 h. Cell death was detected by Hoechst staining.



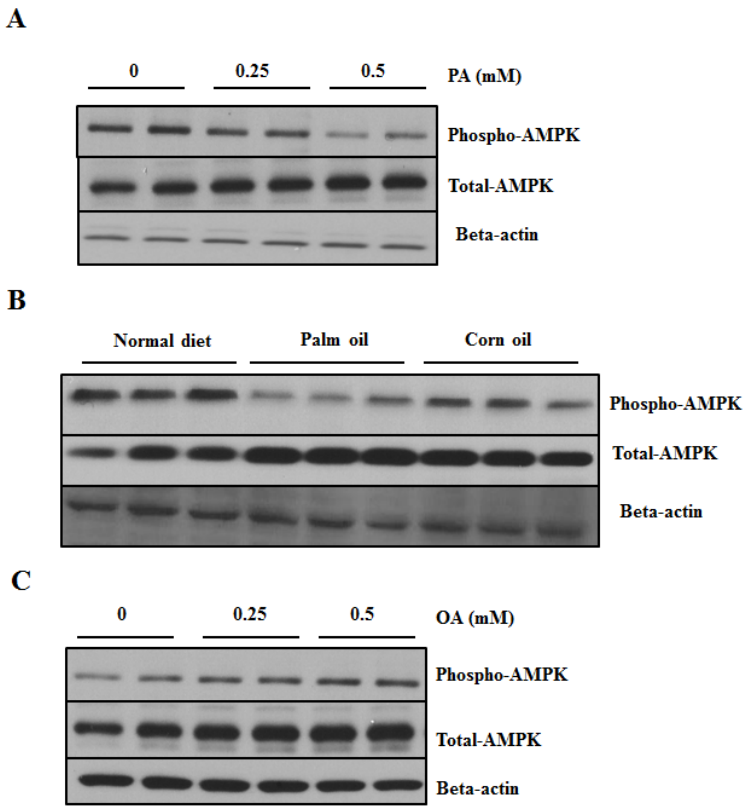
Supplementary Fig. 15 SIRT3-overexpressing decreases AICAR-activated AMPK phosphorylation. SIRT3 OE and vector control HepG2 cells were treated with AMPK agonist AICAR (2.5 mM) for 4 h. Immunoblotting assay was performed for phospho-AMPK.



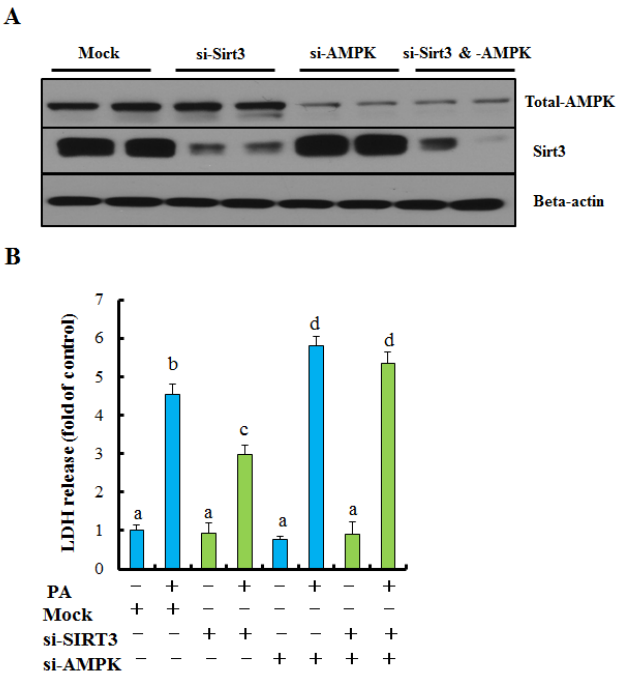
Supplementary Fig. 16 Knocking-down SIRT3 activates AMPK/mTOR axis in mouse hepatocytes. AML-12 mouse hepatocytes were transfected with siSIRT3 or scramble siRNA. Total lysates were subjected to immunoblotting assay for phosphorylated-AMPK and -p70S6K.



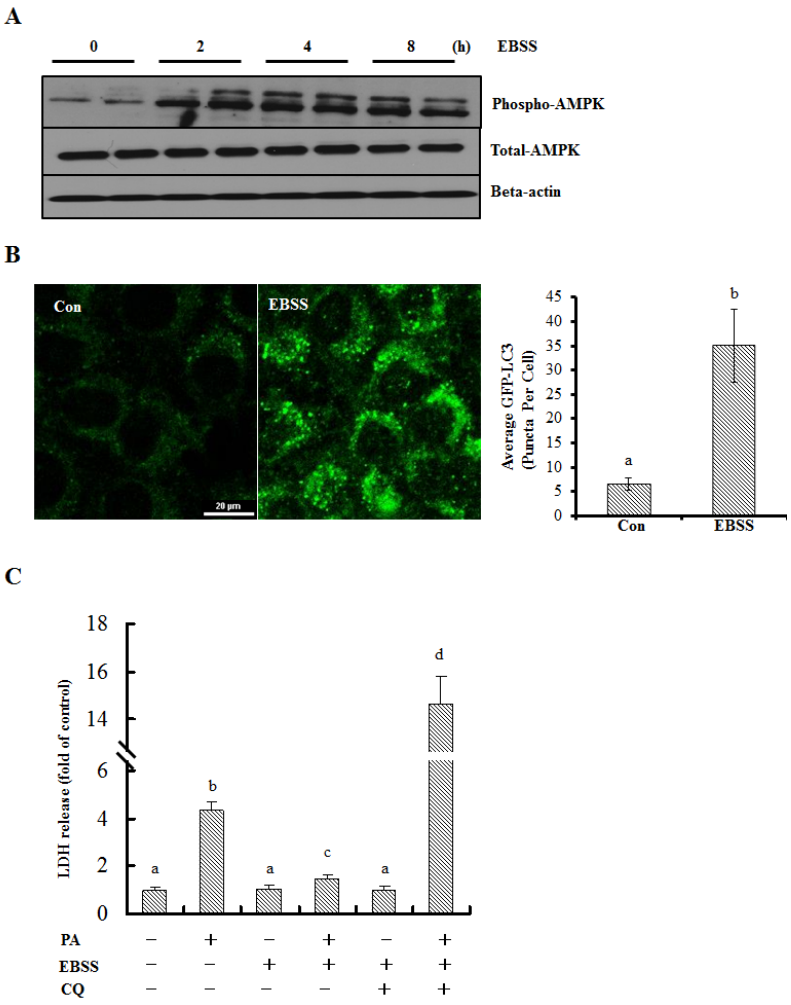
Supplementary Fig. 17 Neither ERK1/2 nor Akt is regulated by SIRT3. Liver tissue was collected from SIRT3 knock-out (KO) or wild type mice with or without 12 h fasting (n = 5). (A) Phosphorylated-AMPK, -p70S6K, -ERK1/2, and -Akt expressions were detected. (B) Phosphorylated-ERK1/2 and -Akt expressions were detected. (C) Immunoblotting assay for phosphorylated-ERK1/2 and -Akt in SIRT3 OE and vector control HepG2 cells.



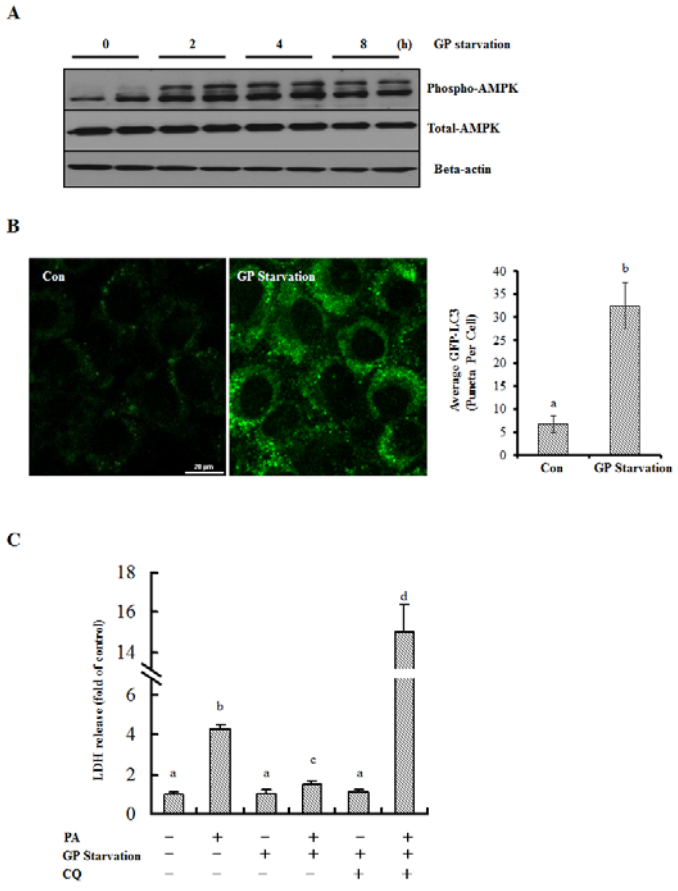
Supplementary Fig. 18 SFAs down-regulates AMPK phosphorylation in the liver and hepatocytes. Male C57BL/6 mice (8-week of age) were fed with normal or high-fat diet using either palm oil or corn oil as fat source. Mice were sacrificed after 8-week of feeding. Liver was collected for analysis. HepG2 cells were treated with PA or OA for 12 h. Total cellular lysates from hepatocytes and liver tissues (n = 8) were subjected to immunoblotting assay for phosphor-AMPK. (A) PA inhibits AMPK phosphorylation in HepG2 cells. (B) Palm oil feeding inhibits AMPK phosphorylation. (C) OA induces AMPK phosphorylation in HepG2 cells.



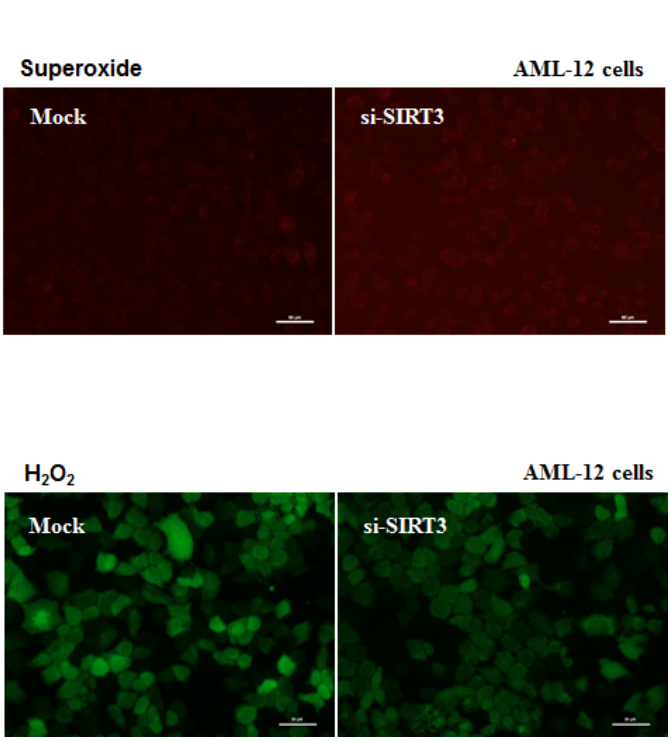
Supplementary Fig. 19 Silencing AMPK inhibits knocking-down SIRT3-protected lipotoxicity in HepG2 cells. HepG2 cells were co-transfected with siSIRT3 and siAMPK, and were exposed to 0.5 mM PA for 12 h. (A) The transfected efficiency was detected using Western-blotting. (B) Cell death was detected by LDH release. Values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.



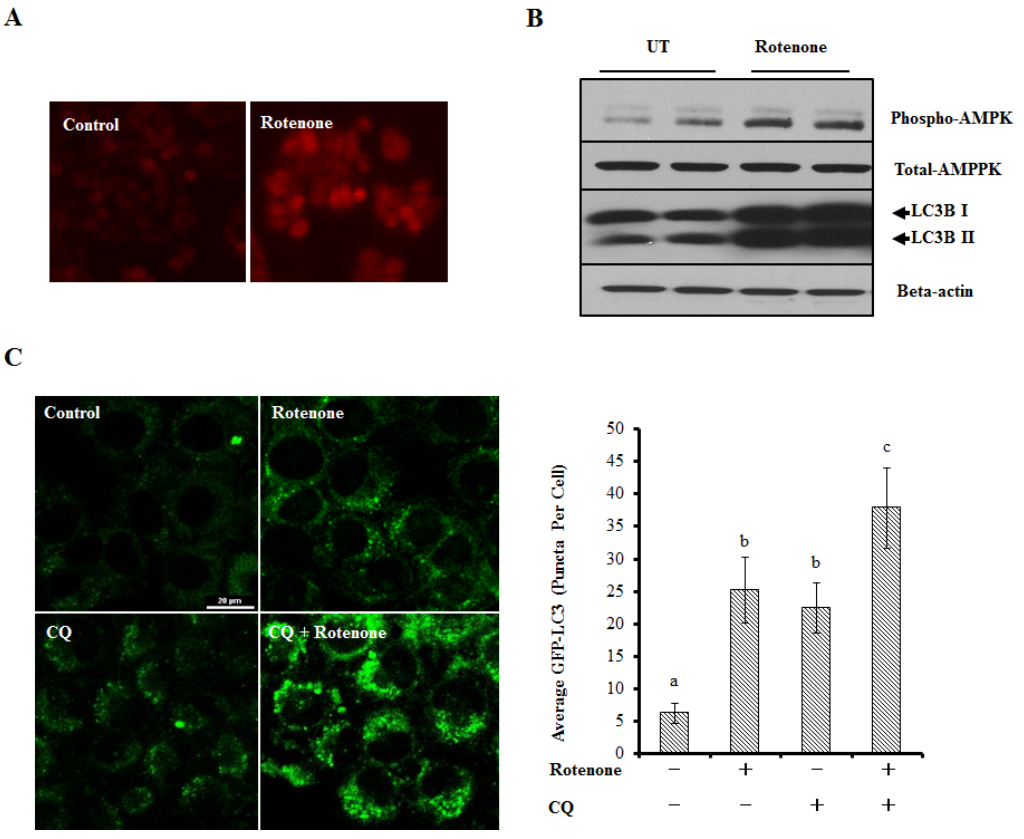
Supplementary Fig. 20 EBSS protects against PA-induced lipotoxicity via activating AMPK-regulated autophagy. (A) HepG2 cells were incubated in EBSS medium for the indicated duration. Phosphorylated-AMPK expression was detected. (B) HepG2 cells were transfected with recombinant adenovirus GFP-LC3 and treated with EBSS for 12 h. Puncta was detected. (C) HepG2 cells were treated with 0.5 mM PA for 12 h with or without EBSS incubation. CQ (20 μM) was added 1 h before PA treatment. Cell death was detected by the measurements of LDH release. Values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.



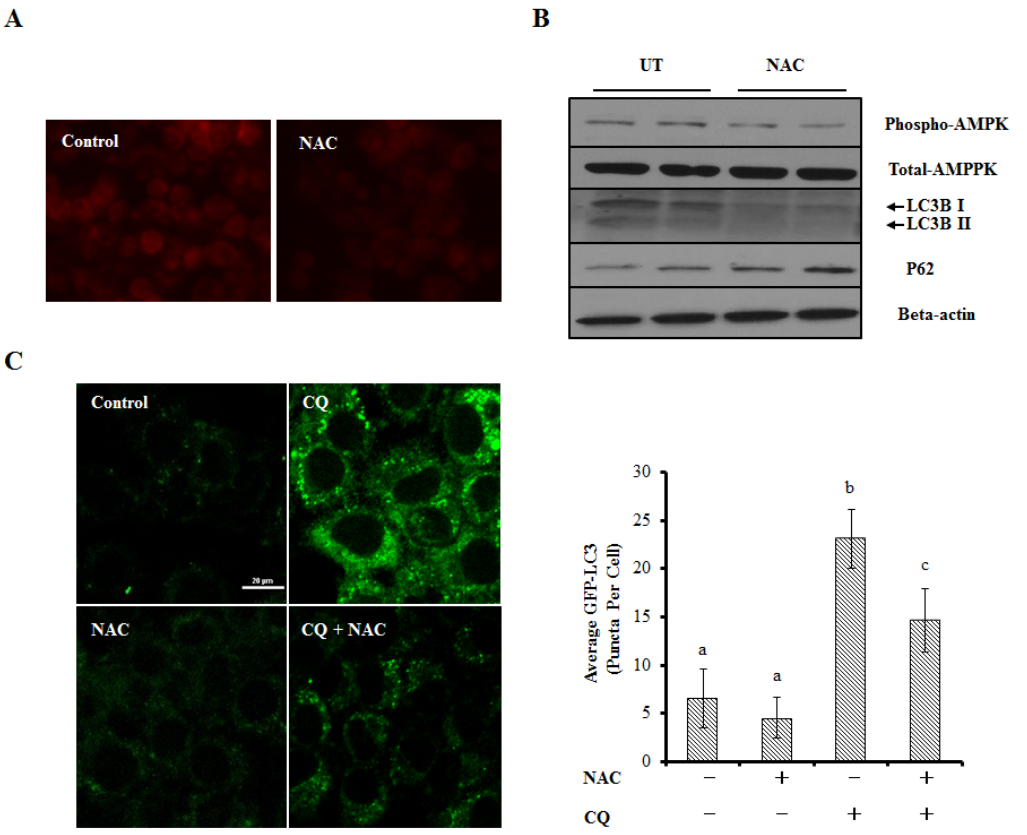
Supplementary Fig. 21 GP starvation protects against PA-induced lipotoxicity via activating AMPK-regulated autophagy. (A) HepG2 cells were incubated in GP starvation (glucose, L-glutamine, pyruvate, and serum-depleted) medium for the indicated duration. Phosphorylated-AMPK expression was detected. (B) HepG2 cells were transfected with recombinant adenovirus GFP-LC3 and treated with GP starvation medium for 12 h. Puncta was detected. (C) HepG2 cells were treated with 0.5 mM PA for 12 h with or without GP starvation incubation. CQ (20 μM) was added 1 h before PA treatment. Cell death was detected by the measurements of LDH release. Values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.



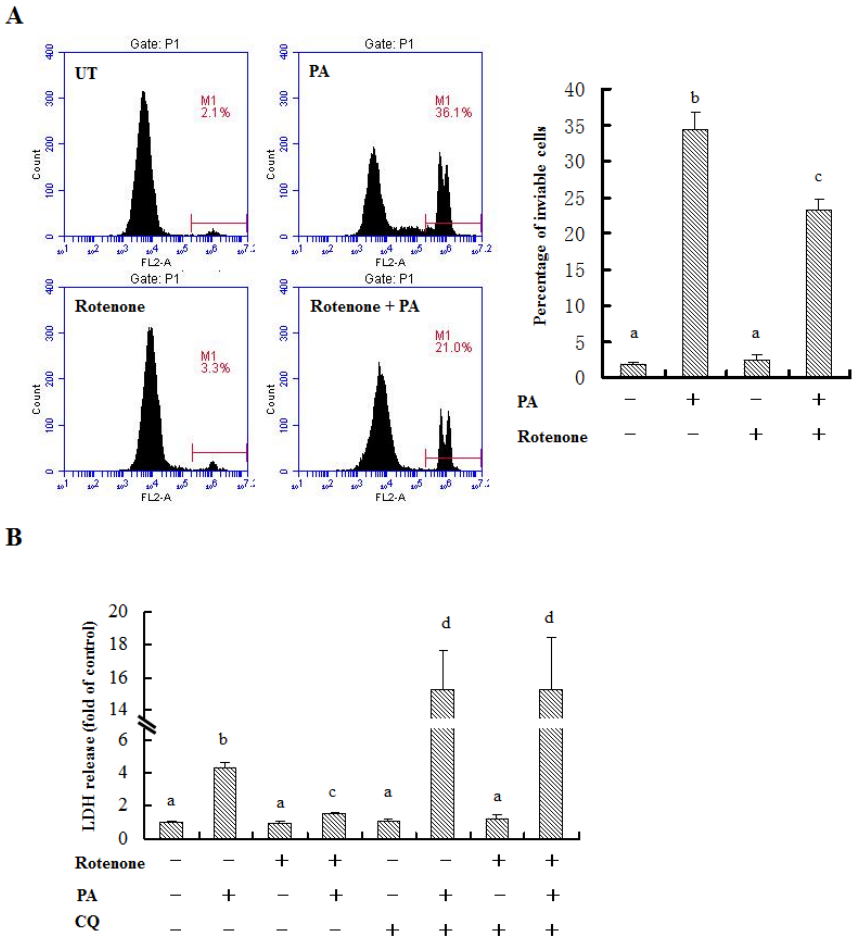
Supplementary Fig. 22 Knocking-down SIRT3 regulates intracellular redox in mouse hepatocytes. AML-12 mouse hepatocytes were transfected with siSIRT3 or scramble siRNA. (A) Intracellular superoxide level was detected as described in the Material and methods. (B) Intracellular H_2O_2 level was detected as described in the Material and methods.



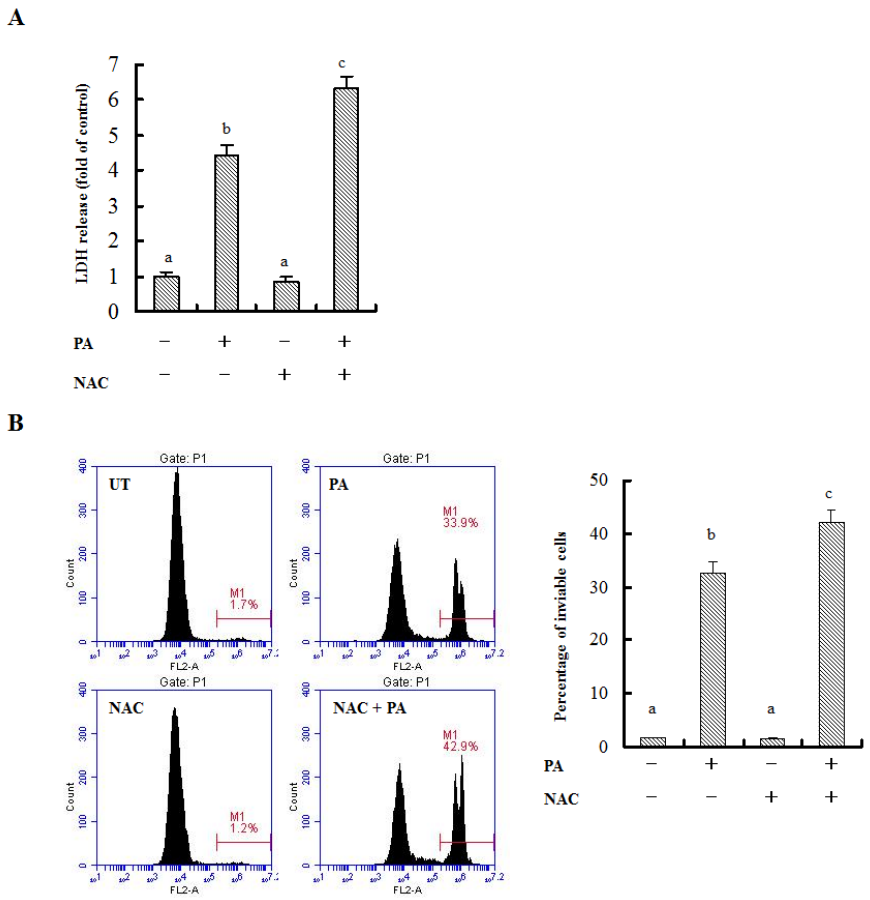
Supplementary Fig. 23 Rotenone increases intracellular superoxide and stimulates AMPK phosphorylation and autophagy. (A) HepG2 cells were treated with rotenone (10 nM) for 12 h. Intracellular superoxide level was detected as described in the Material and methods. (B) The expressions of phosphor-AMPK and LC3-II were detected after 12 h rotenone (10 nM) treatment in HepG2 cells. (C) HepG2 cells were transfected with recombinant adenovirus GFP-LC3 and treated with rotenone for 12 h. CQ (20 μM) was added 1 h before rotenone treatment. Puncta was detected. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.



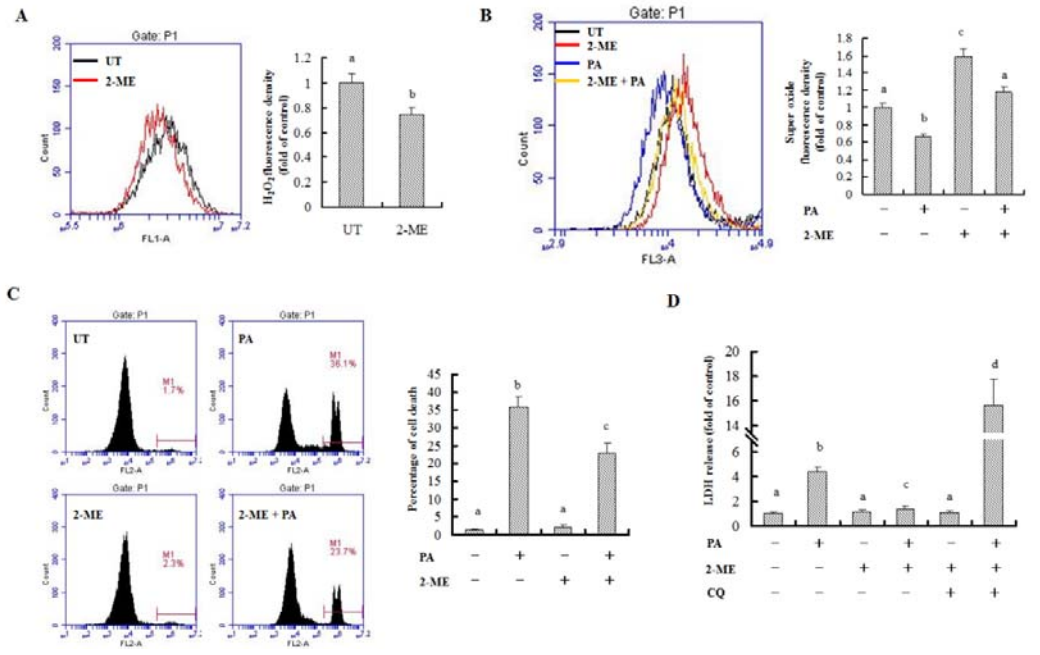
Supplementary Fig. 24 NAC decreases intracellular superoxide and inhibits AMPK phosphorylation and autophagy. (A) HepG2 cells were treated with NAC (5 mM) for 12 h. Intracellular superoxide level was detected as described in the Material and methods. (B) The expressions of phosphor-AMPK, LC3-II, and p62 were detected after 12 h NAC (5 mM) treatment in HepG2 cells. (C) HepG2 cells were transfected with recombinant adenovirus GFP-LC3. NAC was added for 12 h with or without CQ (20 μM) incubation. Puncta was detected. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.



Supplementary Fig. 25 Rotenone protects against PA-induced lipotoxicity via activating autophagy. (A) HepG2 cells were treated with 0.5 mM PA for 12 h with or without rotenone (10 nM) pre-treatment. Cell death was detected by propidium iodide (PI) staining using flow cytometry. (B) HepG2 cells were treated with 0.5 mM PA for 12 h with or without rotenone (10 nM) pre-treatment. CQ (20 μM) was added 1 h before rotenone treatment. Cell death was detected by the measurements of LDH release. Values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.



Supplementary Fig. 26 NAC aggravates PA-induced lipotoxicity. HepG2 cells were treated with 0.5 mM PA for 12 h with or without NAC (5 mM) pre-treatment. (A) Cell death was detected by propidium iodide (PI) staining using flow cytometry. (B) Cell death was detected by the measurements of LDH release. Values are denoted as means ± SD from three or more independent batches of cells. Bars with different characters differ significantly, $p < 0.05$.



394

395 **Supplementary Fig. 27** Inhibiting MnSOD protects against PA-induced lipotoxicity
396 via activating autophagy. (A) HepG2 cells were treated with MnSOD inhibitor, 2-ME
397 (100 μM), for 12 h. Intracellular hydrogen peroxide (H_2O_2) level was detected using
398 the method as described in the Material and methods. (B) HepG2 cells were treated
399 with 0.5 mM PA for 12 h with or without 2-ME pre-treatment. Intracellular
400 superoxide level was detected using the method as described in the Material and
401 methods. (C) HepG2 cells were treated with 0.5 mM PA for 12 h with or without
402 2-ME pre-treatment. Cell death was detected by propidium iodide (PI) staining using
403 flow cytometry. (D) HepG2 cells were treated with 0.5 mM PA for 12 h with or
404 without 2-ME pre-treatment. CQ (20 μM) was added 1 h before 2-ME treatment. Cell
405 death was detected by the measurements of LDH release. All values are denoted as
406 means ± SD from three or more independent batches of cells. Bars with different
407 characters differ significantly, $p < 0.05$.

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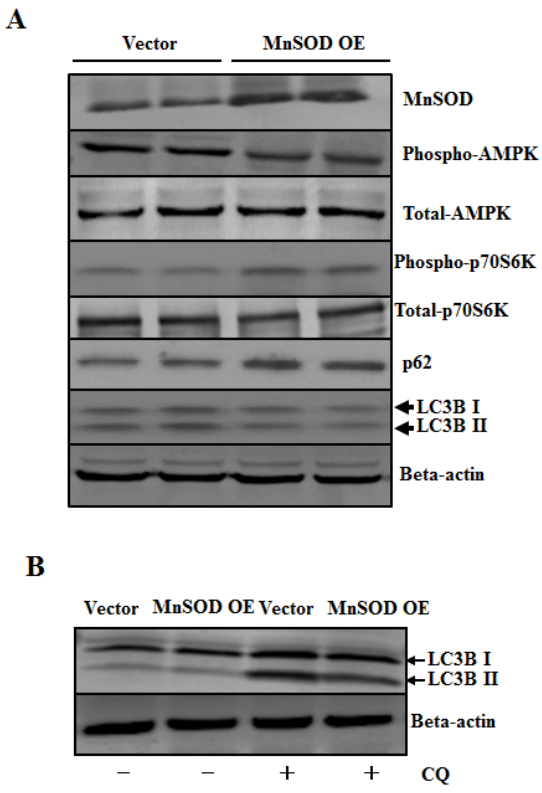


409

410 **Supplementary Fig. 28** Over-expressing MnSOD inhibits autophygic flux in HepG2

411 cells. HepG2 cells were transfected with recombinant lentivirus containing human

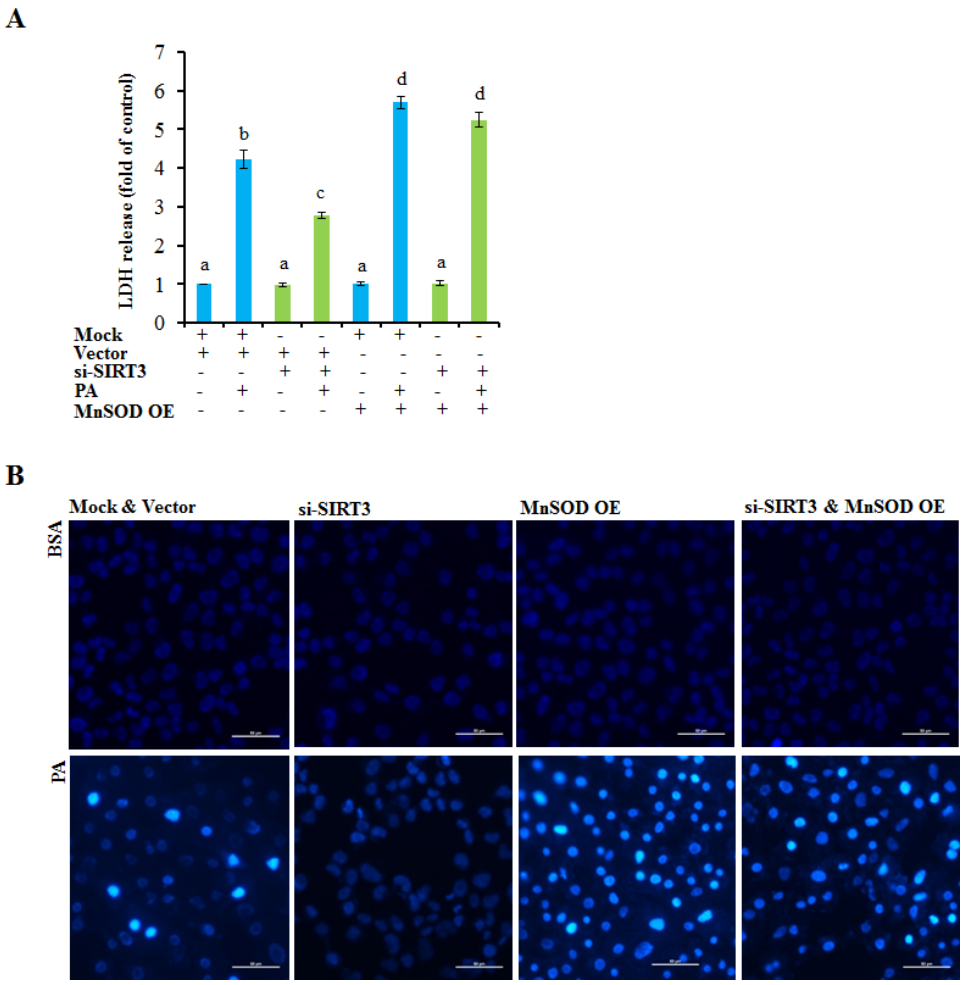
412 MnSOD or empty vector as control, and treated with or without CQ (20 μM) for 12 h.

413 (A) Immunoblotting assay was performed for MnSOD, phospho-AMPK, -p70s6k,

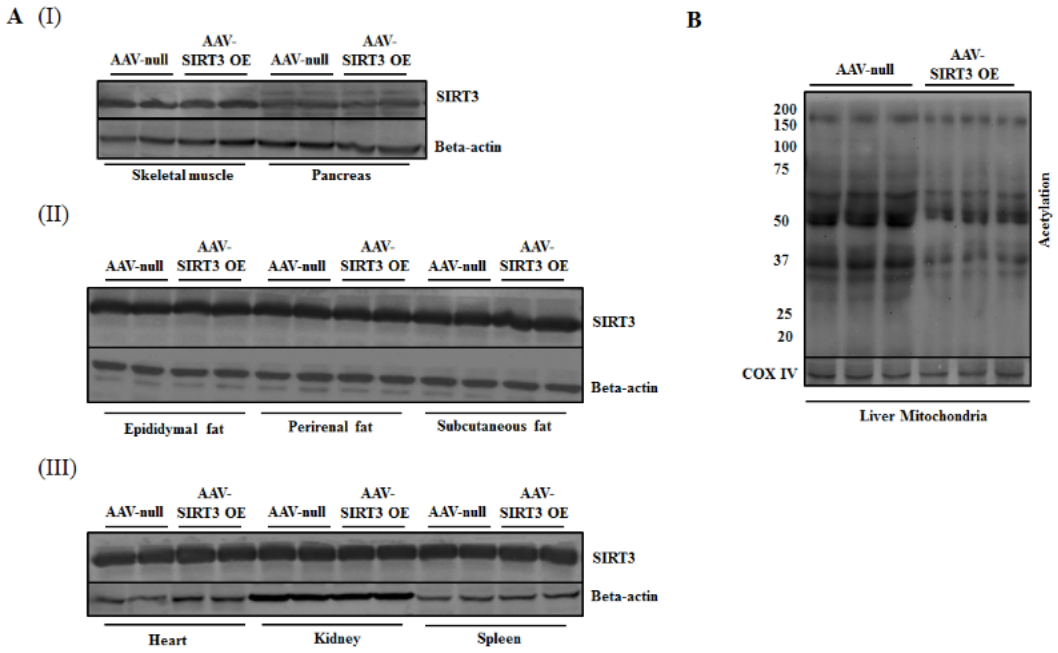
414 p62, and LC3 expressions in MnSOD OE or vector control cells. (B)

415 Immunoblotting assay was performed for LC3-II expression.

416



Supplementary Fig. 29 Over-expressing MnSOD blocks knocking-down SIRT3-protected lipotoxicity in HepG2 cells. HepG2 cells were co-transfected with siRNA for SIRT3 and recombinant lentivirus containing human MnSOD or empty vector as control, followed with 0.5 mM PA exposure for 12 h. Cell death was detected by LDH release (A) and Hoechst staining (B), respectively. All values are denoted as means ± SD. Bars with different characters differ significantly, $p < 0.05$.



426

427 **Supplementary Fig. 30** SIRT3 protein expressions in different tissues from liver

428 specific SIRT3 over-expression mice. (A) Total lysates from different tissues (n = 8)

429 were subjected to immunoblotting assay for SIRT3. (B) Mitochondrial proteins were

430 extracted from liver tissues using a commercial Mitochondria Isolation kit (Beyotime,

431 China) for the measurement of acetylated proteins abundance.

432

Reference

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