MATERIALS AND METHODS

Animal studies

Constitutive ASB3 knockout mice were generated by the PiggyBac Transposase System in the FVB/N mouse strain and were kindly provided by the Chinese Academy of Medical Science (Shanghai, China). Mice containing PiggyBac transposon were mated with mice inserted with transposable enzyme and after mating the transposon could be integrated into the chromosome under the effects of transposable enzyme ASB3 floxed mice (*ASB3*$^{fl/fl}$) were generated by CRISPR–Cas9 with loxP sites flanking the exon in the C57BL/6J mouse strain. Albumin-Cre mice with the C57BL/6J genetic background were provided by Cyagen Biosciences Incorporation (Suzhou, China). Liver-specific ASB3 knockout mice (*ASB3*^{LKO}) are generated by breeding the *ASB3*$^{fl/fl}$ with Albumin-Cre mice. The genotypes of the mice were identified by PCR techniques. Animals were housed in an animal facility with a 12-hour light/12-hour dark cycle at a room temperature of 22–24℃ and humidity of 40–60% with free access to food and water. Six-week-old males were fed with a normal chow diet (NCD), high-fat diet (HFD) containing 60% fat, 20% carbohydrates, and 20% protein (D12492, Research Diets) or methionine-choline deficiency (MCD, A02082002B, Research Diets) for the indicated weeks as previously described.[1,2] Additionally, *ASB3*$^{fl/fl}$ mice and *ASB3*^{LKO} mice of the same age were equally divided into two groups and fed with the Gubra amylin NASH diet (GAN, 09100310, Research Diets) starting at 12 weeks old for 3 months. All animal experimental procedures were conducted in accordance with the ARRIVE guidelines and National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals, and were approved by the Bioethics Committee of Fudan University.

Cell lines

Human HCC cell line Huh7 was obtained from the Cell Bank of the Chinese Academy of Medical Science (Shanghai, China). Human HCC cell lines HepG2 and human embryonic kidney cell line HEK293T were obtained from the ATCC (Virginia, USA). Cells were cultured in Dulbecco's modified Eagle's medium (11966025, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (12484010, Thermo Fisher Scientific, USA) and 1% penicillin-streptomycin at 37℃ with 5% CO_2. High-fat medium with 1 mM FFA was prepared with 1/10 volume of 6.66mM OA (Oleic acid) (O7501, Sigma-Aldrich, USA) and 3.33 mM PA (palmitic acid) (P9767, Sigma-Aldrich, USA) added into DMEM with 10% FBS. Lipid droplets were inducted into cells by the high-fat medium for hours. A vehicle or etomoxir was added into the medium 2 hours in advance.

Plasmid construction

HA-ASB3 and Flag-CPT1A expression plasmids were constructed as previously reported.[3] The deleted ASB3 (ASB3^{ANK1-4}, ASB3$^{\Delta SOCS}$), the deleted CPT1A (CPT1A$^{\Delta 1-122}$, CPT1A$^{\Delta 1-350}$, CPT1A$^{\Delta 351-773}$) and the mutants of CPT1A constructs (CPT1A^{180A}, CPT1A^{639A} and CPT1A^{180A,639A}) were subcloned in pcDNA3.1 vector.

Plasmid transfection

The cells were distributed in the 60-mm plate with a concentration of 0.5×10^6/5 mL and 18–24 hours later they were transfected with Lipofectamine RNAimax (Invitrogen, California, USA).[3,4] Separately, shRNA and Lipofectamine RNAimax were incubated with Opti-MEM (Invitrogen) for 5 minutes and then mixed for 20 minutes at room temperature. The mixture was applied to the cells. The shRNA and scramble shRNA (shNC) were purchased from RiboBio (Guangzhou, China). The sequences of shASB3 and shNC are as previously described.[5]

Co-immunoprecipitation and Western blotting

Cells were collected and lysed in lysis buffer for 10 minutes on ice containing 1% NP-40 (P0013F, Beyotime, Shanghai, China), phenylmethanesulfonyl fluoride (PMSF) (ST506, Beyotime, Shanghai, China) and protease cocktail (B15002, Bimake, Texas, USA). For denaturing conditions, cells were lysed in buffer containing 1% SDS, sonicated (5 s on/5 s off for 1 min), heated at 95℃ for 5 minutes, then supplemented with 900 μl SDS-free lysis buffer (1% NP-40, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, protease cocktail) to dilute SDS before co-IP. The samples were centrifuged at 12,000 rpm for 15 minutes. The supernatant was collected and Flag-beads (P2115, Beyotime, Shang-

 https://doi.org/10.3350/cmh.2024.1041

hai, China) or HA-beads (P2121, Beyotime, Shanghai, China) were added for 4-hour incubation at 4℃ followed by centrifugation at 3,000 rpm for 3 minutes to get the precipitate. The beads were then washed with NP-40 buffer 3 times. Protein samples were extracted from cultured cells using RIPA buffer (P0013C, Beyotime, Shanghai, China) with PMSF and protease cocktail. Sodium dodecyl sulfate (SDS) lysis buffer was added into protein samples or the precipitated components with beads and heated at 100℃ for 5 minutes for denaturation. The samples were electrophoresed and separated on SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes, and detected by antibodies listed in Supplementary Table 1.

Histological staining

For Hematoxylin and eosin staining (H&E), tissues were kept in 4% paraformaldehyde (P0099, Beyotime, Shanghai, China) and then embedded with paraffin followed by being sectioned serially at 4 μm (adipose tissues were 7 μm). H&E was performed after the deparaffinization and rehydration. For Oil Red O Staining (BA4081, BASO, China), tissues were embedded in OCT and frozen in liquid nitrogen followed by sectioning at 12 μm. Oil Red O staining was performed for 10 minutes and the color was separated by 60% ethanol.[4] After being washed with water tissues were stained by hematoxylin to visualize the cell nuclei. Images were captured by a microscope and images were acquired. All stained sections were determined, respectively, by two pathologists who did not know the research contents.

Bodipy, DAPI, and Oil Red O Staining

Cells were washed by 1X PBS 3 times. The cells were stained by 2 μM BODIPY dye (D2184, Thermofisher Scientific, USA) and incubated in a 37℃ incubator for 15 minutes. Then the cells were washed with 1X PBS 3 times, and fixed with 4℃ 4% formaldehyde (P0099, Beyotime, Shanghai, China) for 30 minutes. After being washed with 1X PBS 3 times, the cells were stained with 0.4 μg/mL DAPI (C1002, Beyotime, Shanghai, China) for 10 minutes. After being washed with 1X PBS 3 times, the cells were photographed under the microscope, and the mean fluorescence intensity of BODIPY staining was measured using ImageJ. Cells were stained with Oil Red O solution for 20 minutes after 30 minutes of fixation by 4% formaldehyde (P0099, Beyotime, Shanghai, China).[4] Cells were washed by ddH_2O for 3 times and separated by color by 75% ethanol for 2 minutes. After being washed by ddH_2O 3 times, the cells were photographed under the microscope and the Oil Red O staining area was measured using ImageJ. The experiment was repeated at least 3 times.

Mitochondrial function and respiration

Mitochondrial OCRs in HepG2 cells were measured using a Seahorse XFe96 Analyzer (Agilent).[4] The tests were performed according to the recommended protocol of the XF Palmitate Oxidation Stress Test kit (103693-100, Agilent). HepG2 cells were seeded onto an XFe96 cell culture microplate (3,000 cells per well). The cells were equilibrated in XF DMEM for 1 hour in a 37℃ incubator without CO_2. The XFe96 plate was then transferred to the Seahorse XFe96 Analyzer to be estimated. Oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and rotenone/actinomycin A were then sequentially added into the cell culture microplate automatically (the final concentrations of the reagents were 1.5 μM, 2 μM, and 0.5 μM, respectively) followed by the measurement of OCR. The results were then analyzed by Seahorse Wave Desktop Software (Agilent).

Mitochondrial membrane potential testing

Mitochondrial membrane potential in Huh7 cells was measured according to the recommended protocol JC-1 Staining Kit (C2006, Beyotime, Shanghai, China). Cells were seeded onto a cell culture plate (10,000 cells per well). Carbonyl cyanide 3-chlorophenylhydrazone was added into the cell culture plate (the final concentrations of the reagents were 10 μM) for mitochondrial depolarization. JC-1 staining working solution was added into the cell culture plate 100 μl per pore at 37℃ for 20 minutes and the cells were then washed by JC-1 staining buffer solution 3 times. Images were captured and quantified by an Agilent BioTek Cytation microscope.

Protein-mitochondria colocalization assay

Huh 7 Cells were seeded onto a cell culture plate (10,000 cells per well) and were stained by Mito-Tracker Red CMXRos (C1035, Beyotime, Shanghai, China) for 30 minutes. After being washed by PBS for 3 times, cells were fixed by 4% formaldehyde (P0099, Beyotime, Shanghai, China) for 10 minutes and Triton X-100 solution (ST797, Beyotime, Shanghai, China) for 5 minutes. The cells were blocked by 5% BSA solution (ST025, Beyotime, Shanghai, China). After being washed by PBS for 3 times, primary antibodies (diluted by 1:600) were used to incubate the cells at 4℃ overnight. Secondary antibody AF488-labeled Goat Anti-Rabbit IgG (diluted by 1:1,000) (A0423, Beyotime, Shanghai, China) was used to incubate the cells at room temperature for 1 hour. After being washed by PBS for 3 times, the cells were stained by 0.4 μg/mL DAPI (C1002, Beyotime, Shanghai, China) for 10 minutes. After being washed by PBS 3 times, the cells were photographed under the Agilent BioTek Cytation microscope.

Glucose and insulin tolerance tests

Mice were intraperitoneally injected with 20% glucose solution (2 mg glucose/g) after fasting for 16 hours. The blood glucose was measured at 0, 30, 60, 90, and 120 minutes after injection for glucose tolerance test. Mice were intraperitoneally injected with human insulin (0.75 mU/g) after fasting for 4 hours. The blood glucose was measured at 0, 15, 30, 45, 60, and 90 minutes after injection for an insulin tolerance test. All the mice used in the tests were male.

Small animal MRI and metabolic cages analysis

Mice were weighed and the data was input into the connected computer of the minispec Live Mice Analyzer. Mice were fixed and placed into the instrument to detect fat mass, lean mass, and fluid mass. The Metabolic Measurement System was calibrated by standard samples and then mice were put into the metabolic cage separately to detect the metabolism. The data was stored and analyzed.

Triglyceride extraction

Cells were collected and the intracellular triglycerides were estimated using a triglyceride assay kit (E1013-50, Applygen Technologies Inc., Beijing, China) according to the manufacturer's recommended protocol. Add lysate buffer and let stand for 10 minutes. Heat the supernatant at 70℃ for 10 minutes. Collect the supernatant after centrifugation at 20,000 g at 20℃ for 10 minutes. Mix R1 and R2 working solutions according to the proportion of 4:1. Dilute standard triglyceride samples and set a blank tube as control. Mix 10 μl supernatant and 190 μl working solution to react for 15 minutes at 37℃. Measure the absorbance at 550 nm.

Ubiquitination assays

The *in vitro* protein ubiquitination assay was performed as previously described.[6] Cells were collected and lysed in sodium dodecyl sulfate (SDS) lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, and 1% SDS) and heated at 95℃ for 5 minutes to denature. Supernatants were added to the IP buffer and then analyzed by immunoblotting procedures.

SILAC labeling, affinity enrichment, and LC-MS/MS analysis

Stable isotope labeling by amino acids in cell culture (SILAC labeling)

The Huh7 cells were grown to 80% confluence in high glucose Dulbecco's modified Eagle's medium (Pierce) containing 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin at 37℃ with 5% CO_2. The cells of control were labeled with "heavy isotopic lysine", and the cells of treatment were labeled with "light isotopic lysine and arginine" using a SILAC Protein Quantitation Kit (Pierce, Thermo) according to the manufacturer's instructions. The cell line was grown for more than six generations to achieve more than 97% labeling efficiency. After the "light" labeled cells were transfected with the shRNA plasmid and the "heavy" labeled cells were transfected with the control plasmid, the cells were cultured in SILAC media for another 48 hours. The cells were collected and washed twice with cold PBS.

Protein extraction and trypsin digestion

The collected cells were sonicated three times on ice using a high-intensity ultrasonic processor (Scientz) in lysis

 https://doi.org/10.3350/cmh.2024.1041

buffer (8M urea, 2 mM EDTA, 50 μM PR-619, 10 mM dithiothreitol and 1% Protease Inhibitor Cocktail Ⅲ). The remaining debris was removed after centrifugation at 20,000 g at 4℃ for 10 minutes. The samples were precipitated with 15% TCA for 2 hours at –20℃. The remaining precipitate was washed with cold acetone after centrifugation. The protein was redissolved in buffer (8M urea, 100 mM TEAB, pH 8.0) and the protein concentration was determined with a 2-D Quant kit (GE Healthcare) according to the manufacturer's instructions. Trypsin (Promega) was added with the ratio of trypsin to protein at 1:50 (w/w) at 37℃ for 16 hours. Dithiothreitol (DTT) (Sigma) was added to the final concentration of 5 mM and was incubated at 50℃ for 30 minutes. Iodoacetamide (IAA) (Sigma) was added to alkylate proteins with a final concentration of 15 mM and was incubated for 30 minutes. The alkylation reaction was quenched by cysteine with the final concentration of 30 mM for 30 minutes. Trypsin was added with the ratio of trypsin to protein at 1:100 (w/w) for digestion at 37℃ for 4 hours.

High-performance liquid chromatography (HPLC) and affinity enrichment

The samples were fractionated by high pH reverse-phase HPLC using Agilent 300Extend C18 column. The samples were dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0) and incubated with antibody beads (PTM Biolabs) at 4℃ overnight with shaking. The beads were washed with NETN buffer and ddH_2O 4 times and 3 times, respectively. 0.1% trifluoroacetic acid (Sigma) was used to elute fractions. The fractions were combined and vacuum-dried. The peptides were cleaned with C18 ZipTips (Millipore) according to the manufacturer's instructions.

LC-MS/MS analysis

The results of mass spectrometry were processed using MaxQuant with integrated Andromeda search engine (v.1.4.1.2). Tandem mass spectra were searched against SwissProt_human database concatenated with reverse decoy database and finally performed by bioinformatic analysis.

Untargeted metabolomics analysis of samples

Liver tissues were collected from $ASB3^{LKO}$ mice and $ASB3^{fl/fl}$ mice fed with HFD and harvested in tubes containing the chelating agent EDTA. Then centrifuge the samples at 1,500×g for 15 minutes at 4℃. The supernatants were thawed at 4℃ and homogenized with cold methanol/acetonitrile/water solution (2:2:1, v/v). The mixture was sonicated for 30 minutes at 4℃, followed by centrifugation at 14,000×g for 15 minutes at 10℃. The organic layer was obtained and dried under nitrogen, and the collected samples were redissolved in 100 μl 90% isopropanol/acetonitrile for LC-MS analysis. Analysis was performed using a Hydrophilic Interaction Chromatography of Agilent 1290 Infinity LC Ultra High-Performance Liquid Chr. Analysis was performed using a Hydrophilic Interaction Chromatography of Agilent 1290 Infinity LC Ultra High-Performance Liquid Chromatography. For data processing, XCMS software was used to identify and quantify the metabolite species. Multivariate data analysis, including principal component analysis (PCA) and volcano plot visualization of differentially abundant metabolites was applied to the processed data.

Hepatocyte isolation

ASB3 knockout mice and WT mice in the FVB/N background were anesthetized. Collagenase IV (C5138, Sigma) was added into Hanks Balanced Salt Solution (H1025, Solarbio, Beijing, China) and the terminal concentration was 0.25 g/L. D-Hanks Balanced Salt Solution (H1045, Solarbio, Beijing, China) and Hanks Balanced Salt Solution were injected into the portal vein sequentially. Thirty minutes after injection, the liver was removed into the F12 medium (Gibco) with 10% FBS. The F12 medium with hepatocytes was filtered through a 100 μm cell strainer into collection tubes. The cell suspension was centrifuged at 50 g for 3 minutes at 4℃. The supernatant was removed and the precipitate was resuspended with 35% percoll solution and the suspension was centrifuged at 150 g for 5 minutes at 4℃. The cells were suspended in F12 medium with 10% FBS. The primary liver cells were maintained at 37℃ with 5% CO_2 in a humidified environment.

Quantitative reverse transcription-PCR

Total RNA was extracted from tissues using TRIzol reagent (T9424, Sigma). 1 μg RNA was used to synthesize cDNA by PrimeScriptTM RT reagent Kit (RR047A, Takara). Real-time quantitative PCR was performed using qPCR SYBR Green master mix (Q111-02, Vazyme) and gene-specific primers (Genewiz). Results were normalized against β-actin expression. The primer sequences of the human and mouse genes are shown in Supplementary Table 2.

RNA sequencing

Huh7 cells were seeded in 60-mm plates (0.5×10^6 cells/5 mL) and transfected 18–24 hours later with shASB3 or scramble shRNA (shNC) using Lipofectamine RNAiMAX (Invitrogen) per the manufacturer's protocol. At 48 hours post-transfection, cells were treated with 1 mM FFA (PA:oleate=1:2) for 8 hours, followed by RNA extraction using TRIzol and RNeasy Mini Kit (Qiagen). RNA quality (RIN>8.0) was verified via Agilent Bioanalyzer. Stranded mRNA libraries (TruSeq Kit, Illumina) were prepared from 1 μg RNA and sequenced on an Illumina NovaSeq 6000 (150 bp paired-end). Raw reads were aligned to GRCh38 (HISAT2), and quantified (featureCounts). Differential gene expression (|log2FC|>1, FDR P<0.05) was analyzed using DESeq2.

Fatty acid oxidase testing

Liver tissues or primary liver cells were collected and the fatty acid oxidase was estimated using an ELISA Test Kit Fatty Acid Oxidase Assay Kit (CB11345-Mu, Coibo Biology, Shanghai, China) according to the manufacturer's recommended protocol. 1X PBS was added into the EP tube with samples according to 1.8 mL/mg, and ground for 3 minutes according to 60 HZ with a grinder. The supernatant was obtained by centrifugation for 5 minutes at 5,000 g. Then add 50 μl 16, 8, 4, 2, 1, 0.5 ng/mL standards and samples to the plate. Add 100 μl horseradish peroxidase-labeled antibody. Incubate it for 60 minutes at 37℃. Clear the plate with 350 μl detergent, and rest it for 1 minute, then keep it until it turns dry. Repeat 5 times. Add 50 μl solution A and 50 μl solution B into the plate to react for 15 minutes at 37℃. Add 50 μl terminator, and the absorbance was detected at 450 nm.

Liver-directed AAV virus infection

A liver-directed AAV Serotype 8 vector was used to interfere with the expression of CPT1A (AAV8-CPT1A-RNAi) in mouse liver[7-10] High titer of the virus was generated in 1.32×10^{13} genome copies/mL for AAV8-CPT1A-RNAi and 1×10^{13} genome copies/mL AAV8-Control (Genechem, Shanghai, China). CPT1A knockdown was accomplished by tail vein injection of 1.5×10^{11} viral genomes/mouse (200 μl) of AAV8-CPT1A-RNAi vs. AAV8-Control, respectively in the mice at the age of 6 weeks (two weeks ahead of HFD feeding).

Availability of data and materials

The accession number for Proteomics reported in this paper are GSE135251. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Statistical analysis

A two-tailed Student's *t*-test was used for comparisons between two groups in statistical analysis by the GraphPad Prism 8 software. All data were expressed as the mean±SEM. Three levels of significance (**P*<0.05, ***P*<0.01, ****P*<0.001, ns=not significant) were used for comparisons. The differences were considered statistically significant at *P*<0.05. All biochemical experiments were independently repeated at least twice.

SUPPLEMENTARY REFERENCES

1. Yang T, Qu X, Wang X, Xu D, Sheng M, Lin Y, et al. The macrophage STING-YAP axis controls hepatic steatosis by promoting the autophagic degradation of lipid droplets. Hepatology 2024;80:1169-1183.
2. Hwang S, Wang X, Rodrigues RM, Ma J, He Y, Seo W, et al. Protective and Detrimental Roles of p38α Mitogen-Activated Protein Kinase in Different Stages of Nonalcoholic Fatty Liver Disease. Hepatology 2020;72:873-891.

3. Lv B, Pan Y, Hou D, Chen P, Zhang J, Chu Y, et al. RNF4 silencing induces cell growth arrest and DNA damage by promoting nuclear targeting of p62 in hepatocellular carcinoma. Oncogene 2022;41:2275-2286.
4. Ge M, Huang L, Ma Y, Sun S, Wu L, Xu W, et al. MLN4924 Treatment Diminishes Excessive Lipid Storage in High-Fat Diet-Induced Non-Alcoholic Fatty Liver Disease (NAFLD) by Stimulating Hepatic Mitochondrial Fatty Acid Oxidation and Lipid Metabolites. Pharmaceutics 2022;14.
5. Zhang W, Liu F, Che Z, Wu M, Tang Z, Liu J, et al. ASB3 knockdown promotes mitochondrial apoptosis via activating the interdependent cleavage of Beclin1 and caspase-8 in hepatocellular carcinoma. Sci China Life Sci 2019;62:1692-1702.
6. Chen SY, Zhang HP, Li J, Shi JH, Tang HW, Zhang Y, et al. Tripartite Motif-Containing 27 Attenuates Liver Ischemia/Reperfusion Injury by Suppressing Transforming Growth Factor β-Activated Kinase 1 (TAK1) by TAK1 Binding Protein 2/3 Degradation. Hepatology 2021;73:738-758.
7. Zhang Z, Yuan Y, Hu L, Tang J, Meng Z, Dai L, et al. ANGPTL8 accelerates liver fibrosis mediated by HFD-induced inflammatory activity via LILRB2/ERK signaling pathways. J Adv Res 2023;47:41-56.
8. Zhang H, Li Y, Zhang C, Huang K, Zhao J, Le S, et al. B-cell lymphoma 6 alleviates nonalcoholic fatty liver disease in mice through suppression of fatty acid transporter CD36. Cell Death Dis 2022;13:359.
9. Ge C, Tan J, Dai X, Kuang Q, Zhong S, Lai L, et al. Hepatocyte phosphatase DUSP22 mitigates NASH-HCC progression by targeting FAK. Nat Commun 2022;13:5945.
10. Lu H, Fan L, Zhang W, Chen G, Xiang A, Wang L, et al. The mitochondrial genome-encoded peptide MOTS-c interacts with Bcl-2 to alleviate nonalcoholic steatohepatitis progression. Cell Rep 2024;43:113587.