Hepatic mitochondrial NAD^+ transporter SLC25A47 activates AMPKα mediating lipid metabolism and tumorigenesis

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Table of Contents

Supplementary materials and methods

Cell culture

HEK293, L02, HepG2, and Huh7 cells were all cultured in DMEM containing 10% FBS and 100 U/mL penicillin/streptomycin. The cells were transfected using lipofectamine 2000 according to the manufacturer's instructions.

For metformin-treated hepatocytes, the cAMP analog dibutyryl-cAMP (Bt_2-cAMP) was added during the treatment in order for metformin to have a stronger activation of AMPKα phosphorylation. After hepatocytes adhered, hepatocytes were cultured in glucose-free DMEM containing dimethyl sulfoxide (DMSO, Sigma-Aldrich, Cat# 67-68-5) alone or with Bt_2-cAMP (100 μM, Selleck, Cat# 16980-89-5), with or without metformin (500 μM, Sigma-Aldrich, Cat# 1115-70-4) for 6 hours before harvesting. For NAD^+-treated hepatocytes, hepatocytes were cultured in HBSS (with Ca^{2+} and Mg^{2+}) with or without NAD^+ (1 mM, Sigma-Aldrich, CAS#53-84-9) for 24 hours before harvesting.

Animals

For *Slc25a47*-KO mice genotyping, genomic DNA was extracted from the tail tips of the newborn pups and digested by proteinase K. The genomic sequences around the guide RNA target sites were PCR amplified using the following primers: forward primer TTGTTCTAGAAGGAGCTCGC and reverse primer TTAGCCTGTGGACACAGTAAC. The PCR products were purified and then sequenced.

For *Slc25a47* or *Slc25a51*-overexpressing mice, the His-Tag was ligated to the C-terminus of *Slc25a47* (NM_001012310.2) or *Slc25a51* (NM_001009949.3), and then the *Slc25a47* or *Slc25a51* gene with 6*His-Tag was cloned into the pAdTrack-CMV vector. The method of adenovirus preparation was the same as described previously(1). Adenoviruses (1×10^8 plaque forming units, pfu) were delivered to male C57BL/6J mice fed with HFD for 4 weeks by tail vein injection. Seven days after injection, the mice were sacrificed and the tissues and serum were collected for analysis.

For AMPKα and SIRT3 knockdown experiment, male C57BL/6J mice were injected with lentiviruses that express control shRNA (shCtrl, Sigma-Aldrich), AMPKα shRNA (shAMPKα, CloneID: TRCN0000024000, Sigma-Aldrich) or SIRT3 shRNA (shSIRT3, CloneID**:** TRCN0000332649, Sigma-Aldrich) through tail vein (2). Seven days after injection, the primary hepatocytes were isolated for analysis.

The C57BL/6J mice were purchased from the Laboratory Animal Research Center of Tsinghua University (Beijing, China).

Dual-luciferase reporter assays

Genomic DNA was isolated from L02 cells. The promoter region (from -2000 to 0 bp) of SLC25A47 was cloned into the pGL3-Basic firefly luciferase reporter vector. HepG2 and Huh7 cells were co-transfected in duplicate with the pGL3-Basic or pGL3-SLC25A47 and PRL-TK (Renilla luciferase plasmid) plasmids. After 24 hours of transfection, cells were treated with or without metformin (500 μM) for 6 hours. Then,

the cell lysates were analyzed using Dual-luciferase reporter assays (Promega) according to the manufacturer's instructions.

TAG, CHO, HDL and LDL measurements

The TAG and CHO content of serum, liver tissues or primary hepatocytes was measured using quantification kits (Jiancheng Bioengineering, Nanjing, China) according to the manufacturer's instructions, and the absorbances were determined by a microplate reader (PerkinElmer, EnSpire^{TM}) at a wavelength of 510 nm. The HDL and LDL content of serum was measured with an automatic biochemical analyzer (Roch, COBAS C702).

ELISA measurement

Malonyl-CoA content in primary hepatocytes was quantified using a mouse Malonyl-CoA ELISA kit (MEIMIAN, MM-44888M1) according to the manufacturer's instructions.

Histology and immunofluorescence analysis

Liver tissues were fixed with 4% paraformaldehyde (PFA) overnight at 4 °C, and then divided into two portions for each sample. A portion of the sections were frozen and embedded, and then coupled for ORO staining to determine the lipid accumulation. Another portion was embedded in paraffin and sectioned for H&E, Masson's, IHC and immunofluorescence staining. The antibodies used in this study were as follows: Flag-Tag antibody (1:500, Proteintech, 66008-3-Ig), TOMM20 antibody (1:200, Abcam, ab186735), COX IV antibody (1:200, Abcam, ab202554), SREBP1 antibody (1:200, Abcam, ab191857), SREBP2 antibody (1:200, Abcam, ab28482 and ab30682),

SLC25A47 antibody (1:200, Abcam, ab77196), α-SMA antibody (1:200, CST, #19245), Collagen I antibody (1:200, BOSTER, BA0325), FN1 antibody (1:200, BOSTER, BA1771), Alexa Fluor 555 donkey anti-rabbit IgG H&L (1:1000, Abcam, ab150074), Alexa Fluor 488 goat anti-mouse IgG H&L (1:1000, ThermoFisher, A11001). Images were acquired using light (Olympus, BX63F) or fluorescence (Zeiss, LSM980) microscopy and quantified by Image Pro Plus software.

Transmission electron microscopy (TEM)

Cells were washed with ice-cold PBS, and then chemically fixed with 2.5% glutaraldehyde. Sections were inspected with TEM (Hitachi, H-7650) after staining.

BODIPY staining

Hepatocytes were incubated with the BODIPY-C_{12} dye at a final concentration of 20 μg/mL in PBS for overnight at 37 °C, and then hepatocytes were fixed with 4% PFA at 4 °C. After 30 min, the fixative was removed and the nuclei were stained with DAPI for 10 min, washed 3 times with PBS, and mounted with an anti-fluorescent quencher. Samples were analyzed using a confocal microscope (Zeiss, LSM980).

Measurement of mitochondria respiration

Mitochondrial respiration was measured using a Seahorse XF96 extracellular flux analyzer (Seahorse Bioscience). Briefly, primary hepatocytes were plated onto XF96-well microplates at a density of 1×10^4 cells/well, and the medium was replaced after 4-6 hours with XF assay medium (Seahorse Bioscience 103334-100, supplemented with 10 mM glucose, 2 mM pyruvate and 4 mM glutamine). Inhibitors were used at the

following concentrations: DMSO (0.1%), etomoxir (40 μM, MCE, Cat# HY-50202), UK5099 (40 μM, MCE, Cat# HY-15475), and aminooxyacetate (AOA, 40 μM, Macklin, Cat# 0805087). The oxygen consumption rate (OCR) was measured by an XF96 extracellular flux analyzer according to the manufacturer's recommendations.

[U-^{13}C] Glucose and [U-^{13}C,^{15}N] Glutamine labeled metabolic flux analysis

Hepatocytes were seeded in 100 mm plates at a density of 3×10^6 cells per plate, respectively, and after 4 hours, hepatocytes were washed with PBS and pre-incubated in a base medium without glucose or amino acids for 1 hour. The base medium was replaced with fresh base medium supplemented with the isotopic tracers, 10 mM [U-^{13}C] glucose (CLM, Cat#CLM-1396-PK) or 2.5 mM [U-^{13}C,^{15}N] glutamine (CLM, Cat#CNLM-1275-H-PK). After 2 hours, hepatocytes were washed with PBS for 3 times, and 1 mL of 80% (vol/vol) methanol (precooled at -80 °C) was added to each 100 mm plate. The cells were then transferred from the plate to a new tube and then placed at -80 °C overnight. The cells were then centrifuged at 12,000 rpm at 4 °C for 10 minutes. The supernatant was transferred to a new tube and dried under N_2 flow. The protein concentrations of the pellets were determined by BCA assay for quantification. The supernatants were measured by liquid chromatography and mass spectrometry (LC-MS).

To label newly synthesized fatty acids, hepatocytes were seeded in 100 mm plates at a density of 3×10^6 cells per plate, respectively, and after 4 hours, hepatocytes were washed with PBS and pre-incubated in M199 medium supplemented with 100 nM

insulin for lipogenesis activation, and then incubated in the same media containing 2.5 mM [U-^{13}C,^{15}N] glutamine for 6 hours. The hepatocytes were scraped down using a 50% (vol/vol) methanol solution with 0.1 M HCl and transferred into 10 mL glass tubes, followed by the addition of 1.5 mL chloroform was added into each tube and mixed with vortex for 1 minutes. The samples were centrifuge at 3,000 rpm for 15 minutes to achieve phase separation, carefully transferred the bottom layer to a new glass tube, and dried under N_2 flow. The fatty acids from the total lipids were then released by saponification, and the samples were acidified with formic acid, then extracted with N-hexane. After vortexing, the mixture settled naturally and the upper layer was transferred to a new glass tube, while the lower layer was re-extracted with N-hexane, combined and dried under N_2 flow. The samples were measured by LC-MS.

Lipidomics

Dichloromethane: methanol (2:1) was used to homogenize liver tissue, and then a water phase was added to achieve two layers based on a ratio of water phase to organic phase of 1:4. The mixture was vortexed and allowed to stand. This process was repeated three times, followed by centrifugation for 15 min at 8,000rpm. The organic phase was removed in an equal volume and dried under N_2 flow. The samples were measured by LC-MS.

RNA-Seq and data processing

Total RNA from liver tissues was extracted by the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA quality was assessed on an Agilent 2100, and the qualified RNA was detected by a BGISEQ-5003.

Real-time quantitative PCR (qPCR) analysis

Total RNA was extracted from cells or liver tissues with ultrapure TRIzol reagent (Tiangen) and was reverse-transcribed with a TIANScript First Stand cDNA synthesis kit (Tiangen). qPCR was performed on cDNA samples using SYBR Green Master Mix (Transgen) and gene-specific primers were used on an ABI QuantStudio 7 Real-time System (Life Technologies). Primers for mouse *Slc25a47* were as follows: forward primer GGGGCCATTGGAGGAGTCTG and reverse primer TCTTGACGATACGTGT-CCCG; human *Slc25a47* were as follows: forward primer ACTGTATGGTGA-CCAGCGTTC and reverse primer CTGCCTCATAGGCGACGAAG; mouse *G6pc* were as follows: forward primer ACTGTGGGCATCAATCTCCTC and reverse primer CGGGACAGACAGACGTTCAGC; mouse *β-actin* were as follows: forward primer GGCTGTATTCCCCTCCATCG and reverse primer CCAGTTGGTAACAATGCCATGT; human *β-actin* were as follows: forward primer TATAAAACCCAGCGGCGCGA and reverse primer TATCATCATCCATGGTGAGCTGG. Each sample was run in triplicate, and the comparative threshold cycle (*C*t) method was used to quantify fold increase ($2^{-\Delta\Delta Ct}$) compared with controls.

Immunoblot

Cells or liver tissues were lysed with RIPA buffer containing Halt Protease/ Phosphatase inhibitors (ThermoFisher Scientific). The antibodies used in this study were as follows: AMPKα antibody (1:1000, CST, #5831), p-AMPKα (Thr172) antibody (1:1000, CST, #2535), ACC antibody (1:1000, CST, #3676), p-ACC (Ser79) antibody (1:1000, CST, #11818), FASN antibody (1:1000, CST, #3180), His-Tag antibody (1:1000, CST, #12698), mTOR substrate antibody sampler kit (1:1000, CST, #9862), S6K antibody (1:1000, CST, #9202), 4EBP1 antibody (1:1000, CST, #9644), α-SMA antibody (1:1000, CST, #19245), HMGCR antibody (1:1000, Abcam, ab174830), LDLR antibody (1:1000, Abcam, ab52818), PCSK9 antibody (1:1000, Abcam, ab181142), SREBP1 antibody (1:1000, Abcam, ab191857), SREBP2 antibody (1:1000, Abcam, ab30682), SIRT3 antibody (1:1000, Abcam, ab246522), Collagen I antibody (1:1000, BOSTER, BA0325), FN1 antibody (1:1000, BOSTER, BA1771), CYP7A1 antibody (1:1000, Beyotime, AF6657), β-actin antibody (1:1000, Proteintech,66009-1-Ig), rabbit IgG HRP-linked antibody (1:10000, CST, #7074), mouse IgG HRP-linked antibody (1:10000, CST, #7076). Protein expression was detected using the HRP Chemiluminescent Substrate Kit (Merck Millipore) and quantified with ImageJ software.

Co-immunoprecipitation

Cell lysates derived from HEK293T transiently transfected with plasmids were prepared using a lysis buffer (50 mM Tris, pH7.4, 150 mM sodium chloride, 0.25% NP-40, and protease Inhibitor cocktail (Roche, 04693116001)). The anti-flag magnetic beads (Bimake, B26101) or anti-HA magnetic beads (Bimake, B26201) were incubated with cell lysates at 4 °C overnight on a rotary shaker and subsequently

washed five times with a washing buffer (PBS buffer containing 0.1% Tween-20). The immunoprecipitated proteins were eluted with 1× SDS loading buffer.

SLC25A47 structural modelling

The human SLC25A47 (Uniprot ID: Q6Q0C1) sequence showed the presence of three solute carrier (SOLCAR; Prosite ID: PS50920) repeats, a characteristic feature of the mitochondrial carrier family (TC 2.A.29). Using protein BLAST and considering sequence homology as well as characteristic sequence and structural motifs we identified the bovine mitochondrial ADP/ATP transporter structure (Gene: SLC25A4; Uniprot ID: P02722; PDB ID: 1OKC) as a suitable template for modelling the 3D structure of the SLC25A47 protein using Modeller v9.25(3, 4). The query and template sequences shared sequence identity of nearly 21%. During the modelling process, the bound ligand carboxyatractyloside (CXT) from the template structure was retained in the modelled structure. 500 homology models were generated and the top model was selected based on DOPE score for subsequent docking analysis.

Virtual screening of human metabolites

To identify potential substrates of SLC25A47, a chemical library comprising 3739 human metabolites(5) was extracted from the ZINC database(6, 7). Virtual screening of this chemical library against the homology model of human SLC25A47 was performed using DOCK 3.6(8, 9). The docking poses were scored using the DOCK energy that was the sum of van der Waals, Poisson−Boltzmann electrostatic, and ligand-desolvation penalty terms. Thereafter, substrate candidates were selected from

the 300 top ranked compounds, taking into consideration both docking ranks by DOCK energy scores, and docking poses by human inspection(10, 11).

Transport substrate assay

Transport substrate assay was performed in mitochondria isolated from mice livers using a tissue mitochondria isolation kit (Beyotime). Briefly, mitochondria were incubated in HBSS containing candidate substrates such as NAD^+, acetyl-CoA (Sigma-Aldrich, CAS#102029-73-2), adenosine (Sigma-Aldrich, CAS#58-61-7), FAD (Sigma-Aldrich, CAS#84366-81-4) or a ^{13}C-labbeled amino acid mixture (CIL, Cat#CNLM-452) at 37 °C. After a centrifugation at 12,000 rpm and 4 °C for 10 min, mitochondria were resuspended in 80% (vol/vol) methanol to extract candidate substrates. After a centrifugation at 12,000 rpm and 4 °C for 10 min, the supernatant was removed to a new tube for vacuum concentration, dried with N_2 flow and then measured by LC-MS, and normalized to the protein concentration measured by BCA assay.

SIRT3 deacetylase activity assay

SIRT3 deacetylase activity was examined using a SIRT3 Activity Assay Kit (Fluorometric) (Abcam, ab156067) following the manufacturer's protocol. Briefly, mitochondrial extracts were incubated with SIRT3 assay buffer, and then co-incubated with Fluoro-Substrate Peptide, NAD, and Developer at 37 °C for 1 hour(12, 13). Fluorescent intensity was measured using a microplate reader (PerkinElmer, EnSpire™) at 355/460 nm.

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Author names in bold designate shared co-first authorship.

Supplementary Figures and Figure Legends S1-S6

Supplemental Figure 1. Generate *Slc25a47*-KO model. (A) Tissue distribution of mouse *Slc25a47* mRNA (8-week-old male C57BL/67 mice, *n*=*3*). (B) Tissue distribution of human SLC25A47 mRNA (*n*=*3*). (C) mRNA expression of the SLC family in mouse liver (*n*=2). (D) SLC25A47 subcellular co-localization with TOMM20 and COX IV in Hela cells overexpressing the Flag-Slc25a47 fusion protein. (E) Plot profile of the Flag-Slc25a47 and TOMM20/COX IV fluorescence intensity along the axis. (F) DNA sequencing revealed the *Slc25a47* knockout site. (G) Map of *Slc25a47* KO mice. (H) *Slc25a47* expression in mice liver (*n*=6). Data are presented as Mean ± SEM. ****p<0.0001.

Supplemental Figure 2. Slc25a47 deficiency increase the lipid synthesis in mice liver. (A) Liver H&E staining of WT and *Slc25a47*-KO mice with MCD and HFD treatment. (B) Growth curve of *ob/ob* (WT) and *ob/ob* (*Slc25a47*-KO) mice (*n*=6). (C) Appearance and weight of epididymal white adipose tissue (eWAT) in *ob/ob* (WT) and *ob/ob* (*Slc25a47*-KO) mice (*n*=6). (D) HDL, LDL, and LDL/HDL (*ob/ob* (WT) *n*=5; *ob/ob* (*Slc25a47*-KO) *n*=4) contents in serum of 12-week-old *ob/ob* (WT) and *ob/ob* (*Slc25a47*-KO) mice. (E) Quantitative analysis of lipid droplets in H&E staining images of 12-week-old *ob/ob* (WT) and *ob/ob* (*Slc25a47*-KO) mice. (F) Immunofluorescence of SREBPs in hepatocytes of WT and *Slc25a47*-KO mice. Data are presented as Mean ± SEM. **p<0.01, ***p<0.001, ****p<0.0001.

Supplemental Figure 3. *Slc25a47* deficiency imbalance the homeostasis of lipid metabolism in the primary hepatocytes. (A) BODIPY staining shows lipid droplets formation in hepatocytes of WT and *Slc25a47*-KO mice. (B) Representative TEM shows lipid droplets in primary hepatocytes of WT and *Slc25a47*-KO mice. (C) TAG and CHO contents in primary hepatocytes of WT and *Slc25a47*-KO mice (*n*=4). (D) AMPKα was markedly reduced in primary hepatocytes of WT and *Slc25a47*-KO mice infected with lentiviruses AMPKα shRNA by immunoblots. (E) TAG and CHO contents in primary hepatocytes of WT and *Slc25a47*-KO mice infected with shCtrl and shAMPKα, respectively (*n*=3). (F) The specific contributions of individual substrates toward mitochondrial OCR were measured by specific chemical inhibitors. (G) Schematic illustration of isotope tracing experiments with [U-^{13}C] Glucose in mouse primary hepatocytes. (H) Accumulation kinetics of ^{13}C isotope-labeled glucose and its metabolites in primary hepatocytes from WT and *Slc25a47*-KO mice during [U-^{13}C] Glucose tracking (*n*=3). Data are presented as Mean ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001, *ns*, not significant.

Supplemental Figure 4. Overexpression of *Slc25a47* inhibits lipid accumulation in primary hepatocytes. (A) The body weight of Ad-*GFP* and Ad-*Slc25a47* mice (*n*=5). (B) Liver H&E staining of overexpressing Ad-*GFP* and Ad-*Slc25a47 in ob/ob* (WT) and *ob/ob* (*Slc25a47*-KO) mice, respectively. (C) TAG content in liver of overexpressing Ad-*GFP* and Ad-*Slc25a47 in ob/ob* (WT) and *ob/ob* (*Slc25a47*-KO) mice, respectively.

(*n*=3). (D) BODIPY staining shows lipid droplets formation in primary hepatocytes of Ad-*GFP* and Ad-*Slc25a47* mice. (E) TAG and CHO contents in primary hepatocytes of Ad-*GFP* and Ad-*Slc25a47* mice (*n*=3). Data are presented as Mean ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001, *ns*, not significant.

Supplemental Figure 5. Spontaneous HCC occurred in *Slc25a47*-deficient mice. (A) The liver appearance of 20-month-old WT and *Slc25a47*-KO mice. (B) Statistics of HCC formation rate in 20-month-old WT and *Slc25a47*-KO mice. (C) H&E and ORO staining and IHC staining of Ki67 in liver of 20-month-old WT and *Slc25a47*-KO mice. (D) IHC staining of SREBPs expression in liver of WT and *Slc25a47*-KO mice with DEN-treatment. (E) The liver appearance of DEN-induced WT and *Slc25a47*-KO HCC mice treated with rapamycin.

Supplemental Figure 6. The candidate of SLC25A47. (A) Uptake of ^{13}C-isotope-labeled amino acids by liver mitochondria of WT and *Slc25a47*-KO mice (*n*=3). (B) Uptake of non-isotopically labeled amino acids by liver mitochondria of WT and *Slc25a47*-KO mice (*n*=3). (C) Uptake of acetyl-CoA, adenosine and FAD by liver mitochondria of WT and *Slc25a47* KO mice (*n*=3). Ctrl, Control. (D) NAD^+ uptake measured in isolated mitochondria from WT, *Slc25a47* KO and *Slc25a47* KO with overexpression of *Slc25a47* mice (*n*=3). (E) AMPKα phosphorylation was down-regulated as SIRT3 was knockdown by SIRT3 shRNA in primary hepatocytes of WT and *Slc25a47*-KO mice. Ctrl, Control. (F) Co-IP of overexpressed Flag-Slc25a47 and

HA-Sirt3 fusion proteins in HEK293T cells. A total of 5% of the cell lysate was used as the input. (G) Single-cell RNA-seq analysis the expression of *Slc25a47*, *51,* and *52* in hepatocytes of mouse liver. (H) NAD^+ uptake measured in isolated mitochondria from WT, *Slc25a47* KO and *Slc25a47* KO liver with exogenous expression of *Slc25a51* mice (*n*=3). (I) Degree of residue conservation in human SLC25A47 structural model calculated using Consurf and illustrated using maroon-cyan color gradient scale. Data are presented as Mean ± SEM. *nd* means not detected. **p*<0.05, ***p*<0.01, ****p*<0.001, *ns*, not significant.

Supplementary Tables S1. Substrate candidates predicted by virtual screening against the homology model of human SLC25A47

No.	HMDB ID	Compound name
1	HMDB0003419	Formyl-CoA
2	HMDB0001206	Acetyl-CoA
3	HMDB0001423	CoA
4	HMDB0001275	Propionyl-CoA
5	HMDB0001248	FAD
6	HMDB0006557	ADP-glucose
7	HMDB0000902	NAD
8	HMDB0001491	PLP
9	HMDB0001058	Fructose 1,6-bisphosphate
10	HMDB0000061	Adenosine 3',5'-diphosphate
11	HMDB0000050	Adenosine
12	HMDB0000045	AMP
13	HMDB0001138	N-Acetyl-L-glutamic acid
14	HMDB0000148	Glutamic acid
15	HMDB0001341	ADP