SYVN1-mediated ubiquitylation directs the localization of MCT4 in plasma membrane to promote the progression of lung adenocarcinoma

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Article

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Abstract

Tumor cells mainly generate energy from glycolysis that is commonly coupled with lactate production even under normoxic conditions. As one critical lactate transporter, monocarboxylate transporter 4 (MCT4) is highly expressed in glycolytic tissues like muscles and tumors. Overexpression of MCT4 is associated with poor prognosis for patients with various tumors. However, it remains largely unknown how MCT4 function is post-translationally regulated. Taking advantage of human lung adenocarcinoma (LUAD) cells, this study reveals MCT4 can be polyubiquitylated in a non-proteolytic manner by SYVN1 E3 ubiquitin ligase. The polyubiquitylation facilitates the localization of MCT4 into the plasma membrane that enhances the activity of MCT4 to export lactate; in accordance, SYVN1 knockdown effectively reprograms metabolism that is characterized by reduced glycolysis and lactate production, which can be reversed by ectopic MCT4 expression. Biologically, SYVN1 knockdown successfully compromises cell proliferation and tumor xenograft growth in mouse models. Clinicopathologically, overexpression of SYVN1 is associated with poor prognosis in patients with LUAD, highlighting the importance of SYVN1-MCT4 axis contributes to the progression of LUAD through metabolic reprogramming.

1. Introduction

Metabolic reprogramming plays critical roles in tumorigenesis and tumor progression[1]. There are two major characteristics of tumor metabolism: the Warburg effect and active glutaminolysis[2]. The Warburg effect defines tumor cells prefer for glycolysis that is commonly coupled to lactic acid production even in the presence of oxygen. Although being considered as a “waste product” of glycolysis, lactate and the relevant acidosis as a result of increased lactate levels in carcinogenesis are gaining more and more attention, for example, the importance of lactate functioning as a tumor-promoting metabolite. Increased lactate levels in tumor microenvironment facilitate multiple oncogenic, lactate-stimulated processes, such as hypoxic response, neoangiogenesis, invasion and metastasis[3–6]. Lactate-induced acidification of the microenvironment also leads to the evasion of immune response in tumors[7]. Moreover, lactate can act as an extracellular ligand through binding to its receptor, GPR81, at the cell surface. GPR81, as a G<sub>i</sub>-coupled receptor for lactate, is commonly expressed in various tumor cells. The activation of GPR81 signaling tunes the lactate-sensitive machinery to maintain tumor growth and metastasis by activating the PD-L1/PD-1 immune checkpoint, which finally compromises immune surveillance[8, 9]. Given the involvement of lactate in supporting tumor initiation and progression, it is considered as a promising approach to treat cancers through targeting aberrant lactate metabolism in tumor cells. Therefore, to delineate the detailed mechanisms of how lactate metabolism is subtly regulated is a prerequisite to develop strategies through targeting lactate metabolism and relevant signaling.

Tumor cells resort to the export of lactate to survive the accumulation of lactate and to maintain the intracellular acid-base homeostasis. In most mammalian cells, the transport of lactate is mediated by members of the monocarboxylate transporter (MCT) family. These passive transporters are localized at the plasma membrane to convey monocarboxylate ions together with protons bi-directionally depending on the concentration gradient of their substrates[10]. Among the four MCTs, MCT4 is mainly expressed in
glycolytic tissues such as muscles and tumor tissues[10–13], and overexpression of MCT4 is associated with poor prognosis in patients with a plethora of tumors[14–16], including non-small cell lung cancer (NSCLC), breast cancer, cervical cancer, colorectal cancer, esophageal adenocarcinoma, hepatocellular carcinoma, melanoma, and so forth[17]. Mechanistically, MCT4 is upregulated by hypoxia-inducible factor-1 (HIF-1) to enhance the cellular efflux of lactic acid/H⁺[10, 18]. Therefore, these facts suggest it is promising to inhibit the expression or to block the functions of MCT4 for a wide variety of neoplasms[11, 19, 20]. However, it remains elusive whether MCT4 function is regulated at the post-translational levels. Thus, to clarify post-translational modifications may provide new insights to target MCT4.

Protein ubiquitylation regulates a plethora of biological functions that depends on the specific target residues in the modified proteins and the nature of the ubiquitin chain linkages[21, 22]. Ubiquitylation can target proteins to proteasome or lysosome for degradation, or regulate protein interactions, activity and localization through non-proteolytic mechanisms[23]. Ubiquitylation is catalyzed by a cascade of enzymes including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3)[24]. Synoviolin 1 (SYVN1), also known as 3-Hydroxy-3-methylglutaryl reductase degradation (HRD1), is a RING domain E3 ubiquitin ligase. As a component of ER-associated degradation (ERAD) system, SYVN1 resides in the ER membrane, and catalyzes the polyubiquitylation and degradation of a subset of ERAD targeted proteins when unfolded/misfolded, such as, p53, AMFR/Gp78, NRF2, NRF3, etc. [25]. Other than protein degradation, it remains elusive whether SYVN1 can also mediate non-proteolytic ubiquitylation of its substrates. Notably, several studies reveal SYVN1 can mediate the ubiquitylation of multiple metabolic enzymes to maintain energy and metabolic homeostasis[26–28], for example, PFKP, CPTII, GLUT1, CD147, indicating SYVN1 is a critical regulator of metabolic reprogramming.

In this study, MCT4 is found to undergo polyubiquitylation that promotes the localization of MCT4 in plasma membrane, where MCT4 performs its function to export lactate. Importantly, SYVN1 is identified as an E3 ubiquitin ligase to catalyze the non-proteolytic ubiquitylation of MCT4 that regulates glycolytic metabolism, thereafter, controlling cell proliferation and the progression of lung adenocarcinoma (LUAD).

2. Materials And Methods

2.1 Antibodies and reagents

The following antibodies were used for immunoblot (IB) and immunofluorescent (IF) analyses: Mouse anti-Flag (IB, 1:1000; IF, 1:200; F1804, Sigma-Aldrich), rabbit anti-HA (IB, 1:1000; IF, 1:200; 3724S, CST), rabbit anti-His (IB,1:1000; IF, 1:200; 12698S, CST), mouse anti-ubiquitin (IB, 1:1000; 3936S, CST), rabbit anti-ubiquitin (IF, 1:50; R26024, Zenbio), mouse anti-MCT4 (IB, 1:500; IF,1:50; sc-376140, Santa Cruz Biotechnology), rabbit anti-SYVN1 (IB, 1:1000; IF,1:50; 121294, Zenbio), mouse anti-β-actin (IB, 1:1000; A1978, Sigma-Aldrich), rabbit anti-Na+/K + ATPase 1 (IB, 1:1000; 380790, Zenbio). Peroxidase-conjugated goat anti-mouse IgG (IB, 1:10000; ab205719) or goat anti-rabbit IgG (IB, 1:10000; ab6721), Alexa Fluor 488-conjugated anti-mouse IgG (IF, 1:200; ab150113), and Texas red-conjugated anti-rabbit IgG (IF, 1:1000; ab6719) were purchased from Abcam. Anti-Flag M2 affinity gel (A2220) and 3×Flag peptide
(F4799) were purchased from Sigma-Aldrich, NI-NTA Beads 6FF (SA004100) was obtained from Smart Lifescience, and cycloheximide (HY-12320) was purchased from MedChemExpress.

### 2.2 Plasmids and transfection

Flag-tagged MCT4 and HA-tagged SYVN1 were purchased from Genechem. MCT4-ΔC (aa1-399) and MCT4-C only mutant (aa400-465) were constructed into GV141 C-terminal Flag-tagged vector. N-terminal His-tagged ubiquitin was cloned into pCDNA3.1 vector. shSYVN1s (Target sequences, shSYVN1-1#: 5’-GCTCACGC CTACTACCTCAAA-3’, shSYVN1-2#: 5’-GACCGTGTGGACTTTATGGAA-3’) were cloned into pLKO.1-puro vector. His-tagged CD147 were purchased from Genechem. All of the constructs were confirmed via DNA sequencing. Transfections were performed using polyethylenimine (PEI) reagent (Sigma) or Lipofectamine 3000 (Invitrogen).

### 2.3 Cell culture and viral infection

All cell lines were obtained from National Collection of Authenticated Cell Cultures unless otherwise specified. HEK293T, HepG2, Hep3B, SK, HLE, HCT116, and DU145 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. A549, H1752, H460, H1299, OVCAR3 and 786-O cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. Cell lines where the SYVN1 gene was stably silenced (A549/H1752-shSYVN1) were generated from A549 and H1752 cells. To prepare retrovirus for the knockdown experiments, HEK293T cells were transfected with the pLKO.1-SYVN1-shRNA vector and the packaging vectors PSPAX2 and pMD2G using PEI reagent. Medium containing the virus was collected 48h after transfection. A549 and H1752 cells were incubated with collected virus supernatants for 12h with 8µg/mL polybrene (Solarbio). Infected cells were selected with puromycin (Sigma-Aldrich).

### 2.4 Tumor xenografts

The animal studies were reviewed and approved by the Ethics Committee for Animal Studies at Tianjin Medical University Cancer Institute & Hospital (No.: NSFC-AE-2021195). Thirty female BALB/c Nude mice were purchased from Beijing Vital River Laboratory Animal Technologies. All mouse studies were approved by the Animal Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. All mice were 5–6 weeks of age at the time of injection. A549-scramble, A549-shSYVN1-1, A549-shSYVN1-2 were trypsinized into single cell suspensions and resuspended in PBS. Approximately 5×10⁶ cells in 100µl were injected into the right flanks subcutaneously. Mice were euthanized 3–4 weeks after inoculation. Then, the weight of the subcutaneous tumors was recorded and used to determine tumor growth.

### 2.5 Nuclear Magnetic Resonance Analysis

NMR analysis was performed with human NSCLC tissues and mice tumor tissues from the A549-scramble and A549-shSYVN1 groups by Protein T (Tianjin). The tissue samples were cut into small pieces on dry ice and 50 ± 5 mg of each sample was weighed for further extraction. 0.6 mL extraction buffer (methanol: water 2:1, precooling at -20°C overnight) was mixed with sample and 5 min (2s-on, 3s-off) of ultrasonication was processed to release the metabolism. The samples were then centrifuged
(4°C, 12,000g, 10 min) to collect the supernatant. The sediments were further ultrasonicated for 5 min (2s-on, 3s-off) with 0.6 mL extraction buffer and centrifuged (4°C, 12,000g, 10 min) to collect the supernatant for two times. Combine the supernatant and centrifuge at 4°C, 16,099 g for 10 min to collect the supernatant. Dry the supernatant samples with lyophilization and weigh the dried pellets. The pellets were dissolved into 0.6 mL detection buffer (75 mM Na₂HPO₄, 2 mM NaN₃, 4.6 mM sodium trimethylsilyl propionate-[2,2,3,3-2H₄] (TSP) in 80% D₂O, pH 7.4 ± 0.1) and centrifuged at 4°C, 16,099 g for 10 min. 550 µL of the mixture was transferred into a Bruker SampleJetTM NMR tube (5 mm), sealed with POM balls added to the caps.

NMR measurements were performed on Bruker 600 MHz Avance III HD spectrometers (IVDr) equipped with a BBI probes and fitted with Bruker SampleJetTM robots with the cooling system set to 5°C. A quantitative calibration was completed prior to the analysis. All samples were analyzed with 12.5 minute-method using the Bruker in vitro Diagnostics research (IVDr) methods and the report data were generated using Bruker IVDr Lipoprotein Subclass Analysis (B.I.LISATM) method. The standards of the target metabolites were dissolved in detection buffer separately and detected with the same method of the samples to normalize the reported quantification value.

2.6 Glucose consumption, lactate production and LDH Activity

A549/H1752-scramble and A549/H1752-shSYVN1 cells were cultured in RPMI-1640 (no phenol red) supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. Cell supernatants at various time intervals were analyzed with the glucose assay reagent (1707801, VITROS) and the lactic acid assay kit reagent (8433880, VITROS) by VITROS 5600 automatic biochemical analyzer (Ortho Clinical Diagnostics). The LDH activity kit (A020-1, Jianchengbio) was used according to the manufacturer’s instructions.

2.7 Cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurement

OCR and ECAR were measured using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience) as previously described[29]. In brief, pretreated SCLC cells were seeded in 24-well plates at a density of 1,000 cells/well and cultured overnight. Then, cells were washed with either OCR medium (containing 4.5 g/L glucose, 2 mM glutamine and 1 mM pyruvate) or ECAR medium (containing 2 mM glutamine and no pyruvate or glucose) and incubated in a CO₂-free incubator at 37°C for 1 h to allow for temperature and pH equilibration prior to loading into the XF24 apparatus. XF assays consisted of 3 cycles of: Mix (3 min), Wait (2 min), and Measure (3 min), including 3 basal rate measurements prior to the first injection and 3 rate measurements after each injection. ECAR was measured under baseline conditions and after treatment with glucose (100 mM), oligomycin (100 µM) and 2-deoxy glucose (2-DG; 500 mM). OCR was measured under baseline conditions and after treatment with Oligomycin (100 µM), FCCP (100 µM) and Rotenone/Antimycin (50 µM). Values were normalized to 1 x 10 4 cell counts. Values are presented as the mean ± standard error.
2.8 In Situ Proximity Ligation Assay

The Duolink In Situ Red Starter Kit (DUO92101, Sigma-Aldrich) was used according to the manufacturer’s instructions. The cells were cultured on coverslips in 24-well plates. For ubiquitin detection, the cells were transfected with Flag-MCT4-WT, Flag-MCT4-ΔC or Flag-MCT4-C only, fixed with 4% paraformaldehyde for 15min, and permeabilized with 0.2% Triton X-100. After being blocked with the Duolink blocking solution for 1h at 37°C, the cells were incubated with primary antibodies against Flag (dilution 1:200) and ubiquitin (dilution 1:50) at 4°C overnight. After three washes in Buffer A, the PLA probe solution was applied and incubated for 1h at 37°C. Subsequently, cells were incubated in ligation buffer for 30min at 37°C, and the amplification solution was added for 100min at 37°C. Then, the slides were mounted using Duolink in situ mounting medium containing DAPI for 15min. The images were obtained in laser scanning confocal microscopy (Zeiss LSM-880) and analyzed using the ImageJ. For interaction with MCT4 and SYVN1, A549/H1752 cells were fixed, washed, permeabilized and blocked similarly, then incubating with anti-MCT4 (dilution 1:50) and anti-SYVN1 (dilution 1:50) at 4°C overnight. Subsequent steps are as described above.

2.9 Immunoprecipitation

For non-denaturing IP, cells were lysed with native lysis buffer (Solarbio) containing 1mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Sigma-Aldrich) for 1h at 4°C. Lysates were clarified by centrifugation at 12,000 rpm for 10min at 4°C and then incubated with anti-Flag M2 beads (Sigma-Aldrich) overnight at 4°C. For denaturing conditions, cells were lysed in EBC lysis buffer (50mM Tris-HCL, pH 7.5, 120mM NaCl, 1mM EDTA, 0.5% NP40) containing 4% SDS and protease inhibitor cocktail, and heated at 95°C for 12 min to disrupt noncovalent interactions. After sonication, lysates were centrifuged at 13,000 rpm for 10 min at room temperature to remove precipitates, the resultant supernatant was diluted to 0.4% SDS with the above-mentioned lysis buffer. Lysates were incubated with anti-Flag M2 beads (Sigma) overnight at 4°C, followed by five washes with lysis buffer. The proteins were eluted with 3×Flag peptide and analyzed via immunoblotting.

2.10 In vivo ubiquitylation assay

For the in vivo ubiquitylation assay using Ni-NTA beads, the cells were transfected with His-ubiquitin and Flag-MCT4. Then, the transfected cells were lysed in denaturing buffer A (6M guanidine-HCl, 0.1M Na$_2$HPO$_4$/NaH$_2$PO$_4$, 10mM imidazole, pH 8.0), and the ubiquitylated proteins were pulled down by Ni-NTA beads. Beads were then collected and sequentially washed with buffer A, buffer B (8M urea, 0.1M Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 8.0, 0.01M Tris-HCl, pH 8.0, 25mM imidazole) five times. The proteins were incubated with 60µl elution buffer (8M urea, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, 0.15M Tris-HCl, pH 6.7, 200mM imidazole). After elution, ubiquitylation of MCT4 was detected by immunoblotting using anti-Flag antibody. For using anti-Flag M2 beads, the cells transfected with Flag-MCT4 were lysed in EBC buffer containing 4%SDS. Then, IP and western blot were performed as previously described. Ubiquitylation of MCT4 was detected by immunoblotting using an anti-ubiquitin antibody.
2.11 Immunopurification and silver staining

The cells transfected with vector/Flag-MCT4 were immunopurified by anti-Flag M2 beads. Isolated proteins were separated in 8% SDS-PAGE gel by electrophoresis. Silver staining was performed with the Pierce™ Silver Stain Kit (Thermo scientific) following the manufacturer’s recommendations.

2.12 Western blot

Cells were treated with EBC lysis buffer containing 4% SDS, phenylmethylsulfonyl fluoride, protease inhibitor cocktail and heated at 95°C for 12min. The BCA Protein Assay Kit (Solarbio) was used to quantify total protein amounts. Lysates were subjected to SDS/PAGE gel electrophoresis followed by transferring to PVDF membranes (Millipore). After blocking with 5% non-fat milk for 1h, the membrane was incubated at 4°C overnight with the relevant primary antibodies. The images were obtained with a Gel Imager (Tanon) and analyzed with the Gel Image System (Tanon).

2.13 Immunohistochemistry analysis

The studies involving human specimens were reviewed and approved by the Tianjin Medical University Cancer Institute & Hospital (No.: bc2019038). All participants had signed an informed consent. Immunohistochemistry was performed on serial tissue microarrays of NSCLC purchased from Shanghai Outdo Biotech and on tumor tissues from the mouse model. Immunohistochemistry and histological analysis of animal tissues were carried out at Bioss Biotechnology Co., Ltd. The tissue microarrays were evaluated by two independent pathologists, who were blinded to the experiment. The intensity and density of positive cells were two important evaluation parameters used for the scoring. The intensity of positive cells was evaluated based on the color of the positive cells, which was classified as 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong). The density of positive cells was sorted into four levels: 0 (staining ≤ 5%), 1 (5% < staining ≤ 25%), 2 (25% < staining ≤ 50%), 3 (50% < staining ≤ 75%) and 4 (staining 75%). According to the total scores, generated by adding the scores for the intensity and density of positive cells, the levels of staining were graded as ‘–’ (score 0), ‘+’ (score 1–4), ‘++’ (score 5–8), and ‘+++’ (score 9–12). Cases of ‘–’ and ‘+’ were assigned to the group of low expression levels, whereas cases of ‘++’ and ‘+++’ were assigned to the group of high expression levels.

2.14 Immunofluorescence Staining

The cells were cultured on coverslips in 24-well plates, washed three times with PBS, fixed for 15min at room temperature with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 for 10min. Following permeabilization, the cells was blocked by incubation for 30min with 0.5% goat serum in PBS and then cells were incubated overnight with specific primary antibodies. After washing with PBS for three times, cells were incubated in 0.5% goat serum with secondary antibodies for 1h at room temperature. After washing, DAPI was used to stain the cell nuclei. The slides were mounted with mounting solution and observed under laser scanning confocal microscope (Zeiss LSM-880).

2.15 qRT-PCR
Total mRNA was isolated using Total RNA Extraction Kit (Solarbio), and 2 µg of RNA was used to synthesize cDNA using the ReverAid First Strand cDNA synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Real-time PCR was performed using 2×SYBR Green qPCR Master Mix (bimake) in an ABI 7500 Real-Time PCR system (Applied Biosystems). Primer sequences are provided in Table 1. All gene expression levels were normalized against the corresponding levels of GAPDH. Sequences of primers used in this study: MCT4 forward, 5’-CGGCTTTGTGCTTTACGCC-3’, MCT4 reverse, 5’-GCTGAAGAGGTAGACGGAGTA-3’; SYVN1 forward, 5’-AGCCTGCGTAACATCCACAC-3’, SYVN1 reverse, 5’-AGTTGACTGAAGTGGCAGGC-3’; GAPDH forward, 5’-GTCTCCTCTGACTTCAACAGCG-3’, GAPDH reverse, 5’-ACCACCCCTGGTTGCTGTAGCCAA-3’.

2.16 Colony formation assay

A549/H1752 scramble and shSYVN1 cells were seeded into 6-well plates at a density of 500 cells per well using RPMI-1640 supplemented with 10% FBS. The medium was replaced every two days. When most cell clumps achieved > 50 cells, as observed under a microscope (Olympus), the colonies were then fixed with 4% paraformaldehyde for 1h, stained with crystal violet (Solarbio), and counted.

2.17 Cell viability assay

Cell viability was analyzed via Cell Counting Kit (ZETA). Briefly, the A549/H1752 scramble and shSYVN1 cells were plated in a 96-well plate at a density of 1000 cells per well and cultured overnight. 10µl CCK-8 solution was added to each well of the plate, cells were incubated at 37°C for 2h, and optical density at 450nm was measured. Similar assays were performed after 24, 48, and 72 hours.

2.18 EdU Cell Proliferation assay

EdU staining was carried out with an EdU Cell Proliferation Image Kit (KTA2030, Abbkine) according to the manufacturer’s instruction. Briefly, A549/H1752 scramble and shSYVN1 cells were fixed and then permeabilized before staining. A Click-iT reaction mixture containing fluorescently labeled EdU was added, and the cells were incubated for 30 min. The stained samples were observed and analyzed under a fluorescence microscope (Olympus), the density of positive cells was analyzed using ImageJ.

2.19 Plasma Membrane Protein Isolation

Plasma membrane and cell fractionation were isolated using Minute™ Plasma Membrane Protein Isolation and Cell Fractionation Kit (SM-005, inventbiotech). All procedures were performed on ice and followed the manufacturer’s recommended protocols. Then, the plasma membrane isolation was dissolved in Minute™ Denaturing Protein Solubilization Reagent (WA-009, inventbiotech). Proteins were detected by immunoblotting using an anti-MCT4 antibody.

2.20 Statistical analysis.
Quantiﬁcation and statistical analysis GraphPad Prism 8 software was used for data analysis. All experiments were repeated at least three times. Data were shown as mean ± SD. P An unpaired, 2-tailed Student’s t test was used for 2-group comparisons. ANOVA with Bonferroni’s correction was used to compare multiple groups. A P value of less than 0.05 was considered statistically signiﬁcant. In the graphed data *P values < 0.05 and **P values < 0.01, respectively. ns not significant.

3. Results

3.1 MCT4 is ubiquitylated in LUAD cells

There are smear patterned bands detected when blotting with MCT4 antibody in a panel of tested tumor cell lines (Fig. 1A). The smear patterned bands suggest the existence of post-translational modiﬁcations, such as protein ubiquitylation. To identify the post-translational modiﬁcation that contributes to this smear, in vivo ubiquitylation assay was performed by overexpressing MCT4 in HEK293T cells. This assay conﬁrmed there is protein ubiquitylation when pulling-down MCT4 (Fig. 1B); in accordance, MCT4 is also found in the pool of ubiquitylated proteins (Fig. 1C). To further verify the ubiquitylation of endogenous MCT4, in situ proximity ligation assay (PLA) was performed using both MCT4 and ubiquitin antibodies in human LUAD cells. The ubiquitylation of MCT4 was visualized by the strong PLA signals shown in red “spots” (Fig. 1D). In addition, MCT4 ubiquitylation was also observed in lung tissues in patients with LUAD (Fig. 1E).

Structurally, MCT4 protein comprises intracellular N-termini, 12 transmembrane (TM) helices, intracellular C-termini, and a cytosolic loop between the sixth and seventh TM domains (Fig. 1F). To gain insights into the molecular details involved in MCT4 ubiquitylation, Flag-tagged C-termini (C only) and its deletion mutant (ΔC) were generated for further analysis (Fig. 1F). In vivo ubiquitylation assay revealed comparable levels of ubiquitylation for puriﬁed MCT4-full length (FL) and ΔC mutant, but not for the C only mutant (Fig. 1G). Consistently, pretty similar levels of MCT4-FL and ΔC mutant were also found in the pool of ubiquitylated proteins, while not for the C only mutant (Fig. 1H). Moreover, the PLA assay showed signiﬁcantly weaker signals of ubiquitylation of the C only mutant than that of MCT4-FL and ΔC mutant (Fig. 1I & J). Taken together, these results indicate MCT4 can be ubiquitylated mainly in the transmembrane domain rather than in C-terminal intracellular domain.

3.2 SYVN1 is one speciﬁc E3 ubiquitin ligase for MCT4

Immunoprecipitation combined with mass spectrometry analysis were employed to identify the speciﬁc E3 ubiquitin ligase of MCT4. The LC-MS/MS analysis revealed SYVN1 is identiﬁed in MCT4 immunoprecipitates (Fig. 2A). SYVN1, also termed as HRD1, function as a RING domain E3 ubiquitin ligase that catalyzes the polyubiquitylation of ERAD targeted proteins. As such, SYVN1 is pickup for further analysis because it might function as a potential E3 ubiquitin ligase for MCT4. To conﬁrm the MS results, HEK293T cells were co-transfected with HA-SYVN1 and Flag-MCT4 and used for non-denaturing co-immunoprecipitation. The results showed MCT4 is efﬁciently co-immunoprecipitated with SYVN1 (Fig. 2B); reciprocally, SYVN1 can also efﬁciently pull down MCT4 (Fig. 2C). To further validate their
physical association, the co-localization of MCT4 and SYVN1 was examined using immunofluorescent staining in LUAD cells. The staining revealed that MCT4, as a monocarboxylate transporter, is predominantly localized in plasma membrane, while SYVN1, as a component of the endoplasmic reticulum quality control (ERQC) system, is mainly observed in cytoplasm; interestingly, there are some signal overlay between MCT4 and SYVN1 in cytoplasm (Fig. 2D). In accordance, the PLA assay also found the co-localization of MCT4 and SYVN1 in the cytoplasm (Fig. 2E). Importantly, immunoprecipitation also revealed the interaction between endogenous SYVN1 and MCT4 (Fig. 2F).

Next, the association of MCT4 with SYVN1 was analyzed to further consolidate their interaction and to gain insights into the molecular details involved in the interaction between these two proteins. Co-immunoprecipitation analysis revealed the transmembrane domain is indispensable for the interaction between MCT4 and SYVN1 (Fig. 2G & H). Consistently, both intensity and positive rate of the PLA signals originating from SYVN1 with the C only mutant are significantly lower than those with MCT4-FL and ΔC mutant (Fig. 2I-J). Collectively, these data suggest the molecular interface between MCT4 and SYVN1 in which MCT4 mainly binds to SYVN1 through its transmembrane domain that is consistent with the ubiquitylation sites assay.

### 3.3 SYVN1 catalyzes the ubiquitylation of MCT4

To test whether SYVN1 can catalyze the ubiquitylation of MCT4, in vivo ubiquitylation assay was performed in HEK293T cells with overexpression of SYVN1. The results showed SYVN1 enhances MCT4 ubiquitylation when using total MCT4 amount as a control for normalization (Fig. 3A & B). Accordingly, increased MCT4 was also found in the pool of ubiquitylated proteins (Fig. 3C & D). To consolidate these observations, lentiviral shRNAs were utilized to knockdown SYVN1 in LUAD cells (Fig. 3E). Upon SYVN1 knockdown, reduced MCT4-ubiquitin signal was demonstrated when being analyzed by the PLA assay, highlighting SYVN1 is required for MCT4 ubiquitylation (Fig. 3F-H). To further investigate the region that determines the ubiquitylation of MCT4 by SYVN1, in vivo ubiquitylation assay was performed using Flag-tagged MCT4-FL or deletion mutants (ΔC and the C only). Overexpression of SYVN1 leads to increased ubiquitylation of MCT4-FL and ΔC mutant, but not for the C only mutant (Fig. 3I-L). Accordingly, the PLA assay also demonstrated enhanced PLA signals for MCT4-FL and ΔC mutant but not for the C only mutant (Fig. 3M & N). Taken together, these results indicate that SYVN1 catalyzes MCT4 ubiquitylation in the transmembrane domain of MCT4.

### 3.4 Ubiquitylation of MCT4 determines its localization in plasma membrane

To further dissect the functional importance of MCT4 ubiquitylation, the effects of SYVN1 on MCT4 protein levels were first examined. Cychloheximide (CHX) chase assay showed knockdown of SYVN1 doesn’t alter the half-life of MCT4 protein (Fig. 4A & B), suggesting SYVN1 has no effects on the protein stability of MCT4 and SYVN1 catalyzes a non-proteolytic ubiquitylation of MCT4. In consistency, no negative correlation was detected for SYVN1 and MCT4 protein amount (Supplementary Fig. 1).
Normally, non-proteolytic ubiquitylation controls the status of signaling pathways, for example, K63-linked ubiquitylation regulates the activity of YAP and Akt signaling. It is well-known that CD147 functions as a molecular chaperone of MCT4, and the interaction between these two proteins is critical for lactate export. To address whether SYVN1-mediated ubiquitylation affects the interaction between MCT4 and CD147, HEK293T cells were co-transfected with Flag-MCT4 and HA-CD147, and co-immunoprecipitation assay was performed to investigate the status of the physical interaction between these two proteins. Surprisingly, SYVN1 overexpression doesn’t alter their interaction (Fig. 4C & D). In accordance, the PLA assay found either ectopic expression or knockdown of SYVN1 also doesn’t change the co-localization signal of these two proteins (Fig. 4E & G), further confirming MCT4 ubiquitylation doesn’t affect the formation of heterodimeric complex with CD147. However, immunofluorescent staining revealed knockdown of SYVN1 effectively attenuates the localization of MCT4 in plasma membrane while enhances its localization in cytoplasm (Fig. 4H). Consistently, immunoblot demonstrated that SYVN1 knockdown also compromises MCT4 expression in plasma membrane (Fig. 4I-K), suggesting the existence of an unknown mechanism that facilitates the localization of MCT4 in plasma membrane upon ubiquitylation.

3.5 SYVN1 enhances glycolysis and lactate export

Increased glucose consumption and lactate production are the major characteristics of Warburg effects. The cellular efflux of lactic acid/H⁺ is mainly controlled by MCT4[12]. It remains elusive whether SYVN1-mediated ubiquitylation affects metabolic phenotype of LUAD cells through regulating the activity of MCT4. The enzymatic assay showed SYVN1 knockdown efficiently reduces lactate export compared with control group (Fig. 5A & B). Importantly, SYVN1 knockdown cells have a significant decrease in glucose consumption (Fig. 5C & D), suggesting the existence of reduced aerobic glycolysis. Lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate. In accordance with reduced lactate export, SYVN1 knockdown cells also have lower LDH activity relative to control cells (Fig. 5E & F). To obtain a detailed metabolic profile of SYVN1 knockdown cells, Seahorse analysis was performed to monitor the extracellular acidification rate (ECAR, indicative of aerobic glycolysis), and oxygen consumption rate (OCR, indicative of mitochondrial respiration). A significant decrease of ECAR was observed upon SYVN1 knockdown when glucose was supplemented to enable glycolysis, suggesting SYVN1 is a critical factor controlling basal glycolysis (Fig. 5G-J). However, upon oligomycin treatment, no difference of ECAR was revealed in both SYVN1 knockdown and control cells, indicating the cells have equivalent maximum glycolysis capacity (Fig. 5G-J). SYVN1 knockdown cells demonstrated decreased OCR compared with control cells, indicating impaired basal respiration (Fig. 5K-N). While same level of OCR was reached by both SYVN1 knockdown and control cells upon the treatment of FCCP, an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential (Fig. 5K-N), demonstrating comparable maximal respiration in both cells. Importantly, ectopic expression of MCT4 successfully reverses lactate production (Fig. 5O & P) and glucose consumption (Fig. 5Q & R) upon the SYVN1 knockdown, highlighting the importance of SYVN1-MCT4 axis in regulating metabolic reprogramming.
3.6 SYVN1 promotes LUAD progression through metabolic reprogramming

Since SYVN1 is strongly linked to tumor metabolic reprogramming and, in particular, to increase the lactate efflux from LUAD cells, it is with importance to check the role of SYVN1 in tumor progression. Colony formation assay showed that knockdown of SYVN1 significantly decreases colony formation of LUAD cells (Fig. 6A & B) that can be rescued by ectopic expression of MCT4 (Supplementary Fig. 2). In addition, SYVN1 knockdown also effectively compromises cell proliferation (Fig. 6C), and the incorporation of EdU (Fig. 6D & E), highlighting SYVN1 function is critical for DNA replication. To further verify the role of SYVN1 in LUAD progression, nude mouse xenografts were established. Notably, SYVN1 knockdown greatly reduces tumor volume (Fig. 6F-H), and tumor growth rate, indicated by reduced Ki-67 staining (Fig. 6I & J). Interestingly, there is a dramatic reduction of microvessel density in xenografts with SYVN1 knockdown as indicated by decreased CD31 (+) capillary structures (Fig. 6I & J). These results evidence a tumor progressive role of SYVN1.

Since SYVN1-mediated ubiquitylation regulates MCT4 activity, nuclear magnetic resonance (NMR) analysis was performed to examine the metabolic impacts of SYVN1 on xenograft tumor samples. Partial least-square-discriminant analysis (PLS-DA) was performed to show an obvious separate metabolic trend in tumor samples when comparing the SYVN1 knockdown and control samples (Fig. 6K). The PLS-DA load diagram represents the contribution of metabolites to discriminate the sample groups. A higher distance from the center point suggests a greater contribution to distinguishing the sample groups. The results revealed that lactate makes an important contribution to discriminate all tested samples (Fig. 6L). Consistent with in vitro data, the variable importance in projection (VIP) value of lactate is the largest among the top metabolites (Fig. 6M). Compared with the control group, the SYVN1 knockdown group has a significant reduction of lactate production (Fig. 6N). The above data suggests SYVN1 can promote LUAD progression by enhancing lactate export.

3.7 SYVN1-MCT4 axis drives tumor progression in human tumors

To define the expression of SYVN1 in LUAD tissues, IHC analysis was performed in LUAD tissue microarrays, which included lung tumor tissues and their matched adjacent normal tissues. The positive rate of SYVN1 expression in both LUAD and lung squamous cell carcinoma was significantly higher than that in adjacent normal tissues (Fig. 7A & B). Then, the correlation between the expression of SYVN1 and pathological grading of LUAD was assessed using the Mann-Whitney test. According to the intensity and density of positive cells, the IHC staining results indicate the expression of SYVN1 is associated with pathological grading in LUAD, but not in lung squamous cell carcinoma (Fig. 7C & D). Consistently, IHC staining demonstrated that LUAD tissues with high pathological grade have much higher SYVN1 levels (Fig. 7E); moreover, SYVN1 is also overexpressed in lung squamous cell carcinoma compared with adjacent normal tissues (Fig. 7F). Kaplan-Meier analysis indicated that high SYVN1 expression is correlated with poor overall survival in LUAD and lung squamous cell carcinoma based on our samples.
and the data from KM plotter website (Fig. 7G-J). Next, the overall survival rate of LUAD and lung squamous cell carcinoma were evaluated according to SYVN1 and MCT4 expression independently using the cancer genome atlas (TCGA) data sets (Fig. 7K & L). LUAD fresh specimens were collected for nuclear magnetic resonance (NMR) analysis. First, IHC analysis was performed to classify these tumors into two groups with low and high SYVN1 levels (Fig. 7M). In accordance, elevated SYVN1 expression is correlated with lactate production (Fig. 7N), suggesting SYVN1 can promote the progression of LUAD through enhancing lactate export.

To further investigate the correlation of SYVN1 and ubiquitylation of MCT4 in tumor progression, in situ proximity ligation assay of ubiquitylation of MCT4 and IHC staining of SYVN1 were performed in LUAD. Compared with the tumors with high SYVN1 expression, the PLA signal of ubiquitylation of MCT4 was significantly decreased in tissues with low SYVN1 expression (Fig. 7M). These findings are in consistent with the results from cell culture that demonstrates SYVN1 regulates MCT4 ubiquitylation. Furthermore, NMR analysis was also performed on tumors with different levels of MCT4 ubiquitylation (Fig. 7O). The results showed that tumors with higher MCT4 ubiquitylation have more lactate production. Taken together, SYVN1 may promote the progression of LUAD by increasing MCT4 ubiquitylation, finally leading to lactate export.

4. Discussion

Lactate performs dual roles in metabolic pathways as it is both a final product of glycolysis and an energy-rich oxidative fuel. There are two major reasons of the production and efflux of lactate: to avoid intracellular acidification and to supplement NAD$^+$ production. Intercellularly, lactate functions as a crosstalk nutrient that supports metabolic cooperation between cancer and host cells and between cancer cells with different metabolic activities[30]. It is not difficult to infer that precise adjustment of lactate efflux is of great importance for tumor cells to survive, proliferate and migrate. The transport of lactate across cellular membrane requires transporters like MCT4. To date, there is no detailed report on post-translational modification of MCT4. As such this study identified MCT4 can be ubiquitylated in established cell lines and samples from LUAD patients. This modification promotes the localization of MCT4 into plasma membrane; therefore, enhancing the lactate export from LUAD cells. Mechanistically, as a membrane-integrated ubiquitin E3 ligase, SYVN1 is found to orchestrate MCT4 shuttling to plasma membrane by catalyzing non-proteolytic ubiquitylation of MCT4. MCT4 ubiquitylation by SYVN1 promotes glycolysis and proliferation of LUAD cells in vitro, tumor growth in vivo, finally leading to tumor progression. This work reveals a previously uncharacterized modification of MCT4 in LUAD and undescribed functions of SYVN1 in cancer metabolism.

Although extensive attention was given to MCT4 in the field of tumor metabolism, our knowledge of how MCT4 is regulated is confined at the transcriptional level. For instance, hypoxia induces $SLC16A3$ (MCT4) gene expression directly via the activation of HIF-1 that can bind to hypoxia response elements (HREs) in $SLC16A3$ promoter[12, 31]. Hyper-methylation of $SLC16A3$ promoter leads to reduced MCT4 expression in colorectal carcinoma[32]. This study focuses on post-translational modification of MCT4, particularly,
MCT4 ubiquitylation is revealed in both established cell lines and NSCLC tumor samples. Notably, this modification is non-proteolytic as it does not alter the expression levels, and half-life of MCT4 protein, but it efficiently affects the localization of MCT4 in plasma membrane. Previous studies demonstrate that CD147 can facilitate the localization of MCT4 into the plasma membrane[33]; however, our data revealed that ubiquitylation of MCT4 did not affect the interaction between CD147 and MCT4. Therefore, our findings provide a new insight in the regulation of MCT4 subcellular localization and establish a firm foundation for further investigation. Importantly, it is interesting to find out whether subcellular localization of other MCTs is also regulated by protein ubiquitylation.

This study identified SYVN1 is a potential E3 ubiquitin ligase of MCT4 that is evidenced by mass spectrometric analysis of the interacting partners of MCT4. Ubiquitylation of MCT4 is compromised after SYVN1 knockdown and increased after SYVN1 overexpression, suggesting SYVN1 as a bona fide E3 ubiquitin ligase for MCT4. SYVN1, belonging to the RING domain E3 ubiquitin ligase family, is well known for mediating the degradation of unfolded/misfolded proteins in ER, a process termed as ERAD[34]. Previous studies focus on the roles of SYVN1 as a component E3 ubiquitin ligase complex to promote proteolytic ubiquitylation of its substrates[35]. However, our data provide a non-proteolytic function of SYVN1 that suggests SYVN1 mediated ubiquitylation of MCT4 does not affect the expression and half-life of MCT4 but enhances the translocation of MCT4 from cytoplasm to plasma membrane. Accordingly, knockdown of SYVN1 suppresses glycolysis and lactate export, which is supported by the histological data showing concurrence of SYVN1 expression with MCT4 ubiquitylation and lactate concentrations in tumor samples from NSCLC patients. SYVN1 acts as a membrane hub that organizes a large set of proteins around it to form the main structural component of the channel through which ERAD substrates are transported across the ER membrane[25]. Whether SYVN1 utilizes the ERAD system to mediate non-proteolytic ubiquitylation of MCT4 warrants further investigation.

Tumor cells regulate lactate flow through modulating the expression and activity of key enzymes that control lactate production and consumption, such as LDH and pyruvate dehydrogenase complex (PDH). Another way is to alter the expression of membrane transporters for lactate, such as MCT1 and MCT4[10, 36]. This study provides an emerging role of post-translational modification of membrane transporters in regulating lactate efflux. Consistently, SYVN1 induces a Warburg phenotype, enhances glycolysis and lactate production through ubiquitylating MCT4 in NSCLC cells. Interestingly, the LDH activity is also compromised upon SYVN1 knockdown. Not like inhibitors targeting MCT1, for example, AZD3965 is currently undergoing clinical trials for several types of cancers (ClinicalTrials.gov NCT01791595)[37, 38], the MCT4 inhibitors are still in the developing phase[10, 39]. Nevertheless, the findings in this study suggest inhibiting MCT4 ubiquitylation can be a new strategy to selectively target MCT4 and to treat metabolism-addicted tumors.

More and more evidences suggest SYVN1 is involved in the progression of various malignancies, such as tumors from liver, breast, and prostate as well as lymphoma[28, 40–42]. This study adds LUAD to the growing list of human cancers with SYVN1 getting involved. Importantly, SYVN1 is found to play a role in mediating Warburg effect in LUAD. A recent study showed that deletion of SYVN1 in liver resulted in a
significant reduction in serum glucose levels after refeeding. There are dramatically lower serum TG levels and significant changes in the expression of a large number of genes critical for lipid and fatty acid metabolism in SYVN1<sup>Alb</sup> mice compared to control ones[28]. Another study revealed that SYVN1 ubiquitylating CPT2 to inhibit fatty acid oxidation and tumorigenesis in triple-negative breast cancer[43]. Together with our study, these studies suggest SYVN1 functions as a potential metabolic regulator.

In summary, we found a previously undescribed mechanism by which polyubiquitylation of MCT4 promotes its plasma localization, lactate export, and cell proliferation. Moreover, we identified the E3 ubiquitin ligase SYVN1 regulates the non-proteolytic ubiquitylation of MCT4 without altering its protein levels, but promotes metabolic reprogramming and progression of LUAD.

**Abbreviations**

Akt, protein kinase B; AMFR, Autocrine Motility Factor Receptor; CD147, Basigin; CPTII, Carnitine palmitoyltransferase II; ER, Endoplasmic Reticulum; GLUT1, Glucose transporter type 1; GPR81, G-Protein Coupled Receptor 81; LS-MS/MS, Liquid Chromatograph Mass Spectrometer/Mass Spectrometer; NAD<sup>+</sup>, Nicotinamide adenine dinucleotide; NRF2, Nuclear factor erythroid 2-related factor 2; NRF3, Nuclear factor erythroid 2-related factor 3; p53, Cellular tumor antigen p53; PD-1, Programmed Cell Death 1; PD-L1, Programmed Death Ligand 1; PFKP, ATP-dependent 6-phosphofructokinase-platelet type; RING, Really Interesting New Gene; YAP, Yes-associated protein.

**Declarations**

**Consent for Publication**

Informed consents were received from patients who participated in this study.

**Author contributions**

LR, MZ and SQ conceived and designed the study. MZ, LXY, CH, JC, YJ, GZ and DSY collected the data. LXY, CH, JC and YJ performed the experiments. MZ, LXY, CH and JC analyzed the data. LR, MZ and SQ wrote the draft and reviewed and edited the manuscript. All authors read and approved the final manuscript.

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**Conflict of Interest**

No potential conflicts of interest were disclosed.

**Data Availability Statement**
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**Figures**
Figure 1

MCT4 is post-translationally modified by ubiquitylation in LUAD cells.

(A) MCT4 expression pattern is detected by western blotting. Smeared bands were observed.
(B) MCT4 ubiquitylation is detected in in vivo ubiquitylation assay. Flag-tagged MCT4 was transfected into HEK293T cells. Cell lysates were immunoprecipitated with anti-Flag antibody under denaturing conditions. The immunoprecipitates were resolved and analyzed by western blot.

(C) Reverse ubiquitylation assay demonstrates the ubiquitylation of MCT4. Flag-tagged MCT4 was transfected into HEK293T cells along with His-tagged ubiquitin. The ubiquitylated proteins were purified under denaturing conditions via Ni-NTA agarose beads and were resolved and analyzed.

(D) The ubiquitylation of MCT4 is visualized by an in situ proximity ligation (PLA) assay with anti-MCT4 and anti-Ub antibodies. The PLA signal (red) represents the intensity of ubiquitylated MCT4. Blue, nucleus. Scale bar: 20μm.

(E) The PLA assay identifies the ubiquitylation of MCT4 in LUAD tissues. Scale bar: 50μm.

(F) The schematic diagram of MCT4 molecule and its deletion mutants.

(G) In vivo ubiquitylation assay using MCT4 deletion mutants. Flag-tagged MCT4 or its mutants were transfected into HEK293T cells. Cell lysates were subjected to a denaturing immunoprecipitation, and immunoblotted with the indicated antibodies.

(H) Reverse ubiquitylation assay using MCT4 deletion mutants. Flag-tagged MCT4 or its mutants were transfected into HEK293T cells along with His-tagged ubiquitin. The ubiquitylated proteins were purified under the denaturing conditions via Ni-NTA agarose beads and were resolved and analyzed.

(I) The ubiquitylation of Flag-MCT4 and its mutants in HEK293T is detected by PLA assay. Scale bar: 20μm.

(J) The quantification of the PLA signals measured in (I) is performed using the ImageJ software. Data are presented as mean ± SD (**, p < 0.01, n = 5).
Figure 2

SYVN1 is physically associated with MCT4.

(A) Immunopurification and mass spectrometry analysis of proteins associated with MCT4. Cellular extracts from Flag-MCT4-expressing HEK293T cells were purified with anti-Flag affinity beads and eluted
with Flag peptide. The elutes were resolved by SDS-PAGE and silver-stained. The protein bands were retrieved and analyzed by mass spectrometry.

(B & C) HEK293T cells were co-transfected with Flag-tagged MCT4 and HA-tagged SYVN1. 48 h post transfection, cell lysates were immunoprecipitated with anti-Flag antibody (B) or anti-HA antibody (C), and then immunoblotted with the indicated antibodies.

(D) The co-localization of MCT4 and SYVN1 was determined by an immunofluorescence assay. Blue, nucleus; green, MCT4; red, SYVN1. Scale bar: 20μm.

(E) The interaction between MCT4 and SYVN1 is detected by the PLA assay. The PLA signal represents the intensity of the interaction between MCT4 and SYVN1. Blue, nucleus; red, PLA signal. Scale bar: 50μm.

(F) Immunoprecipitation analysis confirms the interaction between endogenous SYVN1 and MCT4 in A549 cell.

(G & H) HA-tagged MCT4 or its deletion mutants were transfected into HEK293T cells along with HA-tagged SYVN1. The cell lysates were immunoprecipitated with anti-Flag antibody (G) or anti-HA antibody (H), and then immunoblotted with the indicated antibodies.

(I) The interaction between HA-tagged SYVN1 and Flag-tagged MCT4 or its deletion mutants is detected by the PLA assay in HEK293T cells co-transfected with HA-tagged SYVN1 and Flag-tagged MCT4 or its deletion mutants. The PLA signal represents the intensity of the interaction. Scale bar: 20μm.

(J) The positive rate and intensity of the PLA signals measured in (I) is performed using the ImageJ software. Data are presented as mean ± SD. (**, p < 0.01, n = 8).
Figure 3

SYVN1 catalyzes the ubiquitylation of MCT4.

(A & B) In vivo ubiquitylation assay of MCT4 catalyzed by SYVN1. Flag-tagged MCT4 were transfected into HEK293T cells along with HA-tagged SYVN1. 48 h post transfection, cell lysates were immunoprecipitated with an anti-Flag antibody under denaturing conditions. The immunoprecipitates...
were analyzed by immunoblotting with the indicated antibodies (A). (B) Amounts of ubiquitylated MCT4 in (A) were determined by densitometry of protein bands. Flag-MCT4 is used as the loading control. Data are presented as mean ± SD. (**, p < 0.01, n = 3).

(C & D) Reverse ubiquitylation assay of MCT4 catalyzed by SYVN1. HEK293T cells were transfected with the indicated plasmids. 48 h post transfection, the ubiquitylated proteins were purified under denaturing conditions via Ni-NTA agarose beads and were analyzed by immunoblotting with indicated antibodies (C). (D) Amounts of ubiquitylated MCT4 in (C) were determined by densitometry of protein bands. PolyUb is used as the loading control. Data are presented as mean ± SD. (**, p < 0.01, n = 3).

(E) Western blots show he knockdown of SYVN1. A549 and H1752 cells were infected with lentivirus carrying scramble control shRNA (scramble) or shRNAs targeting SYVN1.

(F) The PLA assay demonstrates MCT4 ubiquitylation in cells with SYVN1 knockdown. Scale bar: 20μm.

(G & H) The quantification of the PLA signals measured in (F). Data are presented as mean ± SD (**, p < 0.05, n = 6).

(I) HEK293T cells were transfected with indicated plasmid. 48 h post transfection, cell lysates were immunoprecipitated with an anti-Flag antibody under denaturing conditions. The immunoprecipitates were analyzed by immunoblotting with indicated antibodies.

(J) Amounts of ubiquitylated MCT4 and its mutants in (I) were quantified by densitometry of protein bands. Flag-MCT4 is used as the loading control. Data are presented as mean ± SD. (**, p < 0.01, n = 3).

(K) HEK293T cells were transfected with the indicated plasmids. 48 h post transfection, the ubiquitylated proteins were purified under denaturing conditions via Ni-NTA agarose beads and were analyzed by immunoblotting with indicated antibodies.

(L) Amounts of ubiquitylated MCT4 and its mutants in (K) were quantified by densitometry of protein bands. PolyUb is used as the loading control. Data are presented as mean ± SD. (**, p < 0.01, n = 3).

(M) The PLA assay demonstrates the ubiquitylation of MCT4 and its deletion mutants upon SYVN1 overexpression. Scale bar: 20μm.

(N) The quantification of the PLA signals measured in (M). Data are presented as mean ± SD (**, p <0.01, n = 6).
Figure 4

Ubiquitylation of MCT4 determines its localization in plasma membrane.

(A & B) SYVN1 doesn't affect the protein stability of MCT4. A549 and H1752 cells infected with lentiviruses carrying the indicated shRNAs were treated with CHX (500 μg/ml) and harvested at the indicated time followed by western blotting analysis.

(C & D) HEK293T cells were transfected with indicated plasmids. 48h post transfection, cell lysates were immunoprecipitated with anti-Flag antibody (C) or pulled-down by Ni-NTA agarose beads (D), and then immunoblotted with the indicated antibodies.
(E) HEK293T cells were transfected with indicated plasmids. The interaction between MCT4 and CD147 was detected by the PLA assay with anti-Flag and anti-His antibodies. The PLA signal represents the intensity of the interaction. Scale bar: 20μm.

(F) The positive rate (left) and intensity (right) of the PLA signals measured in (E) was performed using the ImageJ software. Data are presented as mean ± SD. (n.s., no significance, n = 4).

(G) The PLA assay demonstrates no obvious alteration of MCT4 and CD147 interaction. Blue, nucleus. Scale bar: 20μm.

(H) The subcellular localization of MCT4 (green) in cells infected with lentivirus carrying scramble control shRNA (scramble) or shRNAs targeting SYVN1 was determined by an immunofluorescence assay. Blue, nucleus. Scale bar: 20μm.

(I) A549 and H1752 cells were infected with lentivirus carrying scramble control shRNA (scramble) or shRNAs targeting SYVN1. Total cell lysate (T), cytosol fraction (C) and plasma membrane fraction (PM) were analyzed by immunoblotting with indicated antibodies.

(J & K) The quantification of MCT4 signals in different cellular fractions. (**, p < 0.01, n = 3 two-tailed unpaired t-test).
Figure 5

SYVN1 enhances glycolysis and lactate export in LUAD Cells.

(A-D) Lactate production and glucose consumption are monitored at indicated time points in A549 and H1752 cells infected with lentiviruses carrying scramble shRNA (scramble) or shRNAs targeting SYVN1. Data are presented as mean ± SD (**P < 0.01, two-way ANOVA).
(E & F) LDH activity is compromised in A549 (E) and H1752 (F) cells upon SYVN1 knockdown. Data are presented as mean ± SD (*p < 0.05, **p < 0.01, n = 3, two-tailed unpaired t-test).

(G-J) SYVN1 knockdown alters extracellular acidification rate (ECAR). A549 and H1752 cells were infected with lentiviruses carrying indicated shRNA. ECAR was assessed after addition of glucose, oligomycin (oligo), and 2-deoxyglucose (2DG). G & I: time course of a representative experiment. H & J: determination of glycolysis rate and glycolytic capacity. Data are presented as mean ± SD (*p < 0.05, **p < 0.01, n = 3, two-tailed unpaired t-test).

(K-N) SYVN1 knockdown alters Oxygen consumption rate (OCR). A549 and H1752 cells were infected with lentiviruses carrying indicated shRNA. OCR was measured after addition of oligomycin, FCCP, and rotenone. K & M: time course of a representative experiment. L & N: determination of the OCR used for basal respiration and maximal respiration. Data are presented as mean ± SD (*p < 0.05, **p < 0.01, n = 3, two-tailed unpaired t-test).

(O-R) Ectopic expression of MCT4 reverses the lactate production (O & P) and glucose consumption (Q & R) in A549 and H1752 cells infected with scramble or shRNAs targeting SYVN1. OE indicates overexpression. Data are presented as mean ± SD (*P<0.05, **P<0.01, ***P<0.001, two-tailed unpaired t-tests.)
Figure 6

SYVN1 promotes LUAD progression through metabolic reprogramming.

(A & B) SYVN1 knockdown reduces colony formation. A549 and H1752 cells were infected with lentiviruses carrying scramble control shRNA (scramble) or shRNAs targeting SYVN1. (B) Data are quantified and presented as mean ± SD (*p < 0.05, **p < 0.01, n = 3, two-tailed unpaired t-test).
(C) SYVN1 knockdown compromises cell proliferation. CCK8 assays for the growth of A549 and H1752 cells infected with lentiviruses carrying scramble control shRNA (scramble) or shRNAs targeting SYVN1. Data are presented as mean ± SD (**p < 0.01, n = 3, two-way ANOVA.)

(D & E) SYVN1 knockdown suppresses cell proliferation. EdU (5-ethynyl-2'-deoxyuridine) incorporation assays of A549 and H1752 cells infected with lentiviruses carrying scramble control shRNA (scramble) or shRNAs targeting SYVN1. (E) Data are quantified and presented as mean ± SD (*p < 0.05, **p < 0.01, n = 3, two-tailed unpaired t-test). Scale bar: 50μm.

(F-H) A tumor formation assay was performed by injecting A549 cells infected with lentiviruses carrying scramble control shRNA (scramble) or shRNAs targeting SYVN1. Tumor volumes and weight were measured. Data are presented as mean ± SD (**p < 0.01, n = 10, two-tailed unpaired t-test for tumor weight analysis and two-way ANOVA for tumor volume analysis).

(I & J) Representative images of IHC of the cell proliferation marker Ki67 and capillary marker CD31 in tumor sections from the xenograft models at endpoint. The histogram shows the quantification of Ki67-positive cells and CD31+ capillary area density. Data are presented as mean ± SD (*p < 0.05, **p < 0.01, n= 10 mice, two-tailed unpaired t-test). Scale bar: Ki67, 100μm; CD31, 250μm.

(K and L) Metabolites present in tumor tissues isolated from the A549 cells infected with lentiviruses carrying scramble control shRNA (scramble) or shRNAs targeting SYVN1 were detected by NMR analysis. The PLS-DA score plot (K) shows the distinct trend followed by the two groups, and the PLS-DA load diagram (L) shows the contribution of the different metabolites to the discrimination between the two groups.

(M) VIP values of different metabolites between the A549 cells infected with lentiviruses carrying scramble control shRNA (scramble) or shRNAs targeting SYVN1 were detected by NMR analysis.

(N) Lactate export in tumor tissues from the the A549 cells infected with lentiviruses carrying scramble control shRNA (scramble) or shRNAs targeting SYVN1 were detected by NMR analysis. Data are presented as mean ± SD (**p < 0.01, n = 10 mice).
Figure 7

SYVN1 contributes to the progression of LUAD.

(A & B) Comparison of SYVN1 expression in tumor and matched adjacent normal tissues in LUAD (E) and lung squamous cell carcinoma (F) by paired T test (**p < 0.001).
(C & D) Correlation analysis between SYVN1 expression and pathological grading of patients with LUAD (C) and lung squamous cell carcinoma (D) by Mann-Whitney test.

(E) The expression of SYVN1 in adjacent normal tissues and different pathological grading of LUAD tissues are determined by IHC staining; scale bar, 50μm.

(F) The expression of SYVN1 in adjacent normal tissues and lung squamous cell carcinoma tissues is determined by IHC staining; scale bar, 50μm.

(G & H) Overall survival rates of patients with low and high SYVN1 expression are determined in LUAD (G) and lung squamous cell carcinoma (H) by Kaplan-Meier analysis.

(I & J) KM plot analysis indicates the importance of SYVN1 expression in determining the prognosis of patients with LUAD (I) and lung squamous cell carcinoma (J) using KM plotter (https://kmplot.com/).

(K & L) Overall survival rates of individuals with low and high SYVN1 (K) and MCT4 (L) expression levels are determined using TCGA datasets.

(M) IHC staining determines LUAD tissues with low and high SYVN1 expression. The ubiquitylation of MCT4 is detected by an in situ proximity ligation assay (PLA) in LUAD tissues. Scale bar, 50μm.

(N) Lactate levels are determined by NMR analysis in tumor tissues with low and high SYVN1 expression. Data are presented as mean ± SEM. *p < 0.05.

(O) The PLA signal represents the intensity of the ubiquitylation of MCT4 in LUAD tissues. **p <0.01.

**Supplementary Files**

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