

Knockdown of ACC1 promotes migration and invasion of U251 glioma cells by epigenetically suppressing SDH

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Abstract. Glioma is a common and aggressive malignant brain tumor. Despite advances in research, the mechanisms driving glioma initiation and progression remain incompletely understood. The present study aimed to assess the role of acetyl-CoA carboxylase 1 (ACC1) in glioma, focusing on its mechanistic function in U251 cells and its clinical significance. ACC1 expression was first assessed in four glioma cell lines and then the effects on cellular functions were evaluated. Based on the finding that ACC1 knockdown altered the phenotype of U251 cells, potentially through modulation of succinate dehydrogenase (SDH) activity, further mechanistic assessments were performed. Finally, the association between ACC1 expression and patient prognosis was analyzed. The results demonstrated that ACC1 overexpression inhibited proliferation, migration and invasion in U87 cells. Conversely, ACC1 knockdown promoted these processes in U251, T98G and LN229 cells. Mechanistically, in U251 cells, ACC1 knockdown increased acetyl-CoA levels, enhancing substrate availability for P300. This led to upregulation of DNA methyltransferase 1 (DNMT1), hypermethylation of the SDH promoter and subsequent SDH downregulation. The resulting increase in reactive oxygen species (ROS) levels promoted U251 cell migration and invasion. Analysis of clinical data revealed a significant correlation between low ACC1 expression and poor survival outcomes in patients with glioma. These findings suggest that ACC1 functions as a tumor suppressor in glioma. Its down-regulation promotes a pro-tumorigenic phenotype via the acetyl-CoA/P300/DNMT1/SDH/ROS pathway, highlighting

its potential as a prognostic marker and therapeutic target. This underscores the importance of developing personalized treatment strategies targeting ACC1 in glioma.

Introduction

Glioma is the most common malignant tumor within the central nervous system, constituting ~80% of primary brain tumors (1). Patients with glioblastoma multiforme have a median survival of ≤15 months (2). Current treatment modalities for glioma include conventional approaches such as surgical resection, pharmacological interventions and chemotherapy, as well as emerging therapies such as immunotherapy and proton therapy. However, despite technical advancements in these methods, they have not markedly improved patient survival periods (3-5). Notably, the propensity of gliomas for local invasion and metastasis within brain tissue complicates radical resection and effective local radiotherapy, consequently yielding suboptimal treatment outcome. Hence, there is an urgent need to elucidate the molecular mechanisms driving glioma invasion and local metastasis whilst developing effective therapeutic interventions.

Acetyl-CoA carboxylase 1 (ACC1), the rate-limiting enzyme in fatty acid synthesis, catalyzes the conversion of acetyl-CoA to malonyl-CoA (6). As acetyl-CoA is also a key cofactor for histone acetyltransferases, ACC1 indirectly influences protein acetylation (7). Elevated ACC1 expression in liver cancer has been associated with enhanced tumor metastasis via increased fatty acid synthesis (8). Conversely, ACC1 deficiency in breast cancer has been associated with invasion and metastasis, attributed to elevated acetyl-CoA levels that promote protein acetylation and the upregulation of metastasis-associated gene transcription (7). These findings suggest that the role of ACC1 in tumor development exhibits tissue heterogeneity, likely influenced by signaling pathways triggered by its regulated metabolites within distinct tissue environments. Thus, unraveling the underlying regulatory mechanisms of ACC1 may provide valuable insights into glioma progression.

Our unpublished data demonstrated that ACC1 knockdown promoted U251 glioma cell proliferation, as revealed by cell density assessment based on phase-contrast images,

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cell counting and real-time cell analysis (RTCA). However, MTT assays revealed lower readouts in ACC1-knockdown cells compared with controls (Wang *et al.*, unpublished data), suggesting a discrepancy between assays. Therefore, we hypothesized that MTT may not accurately reflect ACC1-knockdown cell proliferation. Furthermore, as MTT relies on succinate dehydrogenase (SDH) activity to reduce MTT to formazan (9), we hypothesized that ACC1 knockdown may inhibit SDH activity in glioma cells.

SDH, a mitochondrial complex II enzyme crucial for electron transport, serves a role in regulating reactive oxygen species (ROS) levels. Decreased SDH activity has been associated with elevated ROS and tumor progression in several cancers (10-12). Increased ROS can activate signaling pathways that promote cell proliferation, migration and invasion (13-16). Redox imbalance is also implicated in glioma progression (17).

The present study aimed to comprehensively investigate the role of ACC1 in modulating proliferation, migration and invasion of glioma cells and its clinical relevance. To achieve this, ACC1 overexpression and knockdown models were employed across four glioma cell lines (U87, U251, T98G and LN229), and phenotypes were assessed via functional assays. Based on the observed cell line-specific responses, U251 cells were selected for mechanistic studies focusing on regulation of the SDH-ROS axis, DNA methyltransferase (DNMT)-mediated epigenetic modifications, and P300-acetyl-CoA crosstalk, validated by inhibitors and small interfering RNA (siRNA). Clinical correlation was analyzed using tissue microarray immunohistochemistry and public datasets.

Materials and methods

Cell culture and reagents. The human glioma cell line, U87 MG ATCC (GCPC096821, glioblastoma of unknown origin), was purchased from Shanghai Genechem Co., Ltd; U251 (SCSP-559) and T98G (SCSP-5274) cells were purchased from the China Infrastructure of Cell Line Resources, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences; and LN229 (CBP60302) cells were purchased from Nanjing Cobioer Biotechnology Co., Ltd. All the cell lines used in the present study were verified by short tandem repeat analysis, confirming their identity and purity. U87, U251 and T98G cells were cultured in MEM (cat. no. SH30024.01; HyClone™; Cytiva) supplemented with 10% fetal bovine serum (FBS; Shanghai XP BioMed Ltd.) and 1% penicillin-streptomycin (cat. no. 15140122; Gibco; Thermo Fisher Scientific, Inc.). LN229 cells were cultured in DMEM (cat. no. 10-013-CV; Corning, Inc.) with the same supplements. All cells were maintained at 37°C in a humidified 5% CO₂ incubator and passaged at 80-90% confluence. N-acetyl-cysteine (NAC) was purchased from Beyotime Institute of Biotechnology (cat. no. S0077). Azacitidine (Aza) (cat. no. S1782) and C646 (cat. no. S7152) were purchased from Selleck Chemicals. Drug treatments were performed as follows: NAC (5 mM), Aza (4 μM) and C646 (5 μM) were applied to cells for 48 h at 37°C under standard culture conditions.

Transfection. The human ACACA plasmid and vector plasmid were purchased from GeneCopeia, Inc. Pre-packaged lentiviral particles for human ACACA knockdown [short hairpin

(sh)ACC1] and its scramble negative control (NC), as well as siRNA targeting P300 and its siNC, were purchased from Shanghai GenePharma Co., Ltd. siRNA and shRNA sequences are listed in Table S1.

Plasmid and siRNA transfections were performed using Lipofectamine® 2000 (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, for U87 cells, 1 μg ACACA or vector plasmid DNA was complexed with the transfection reagent, whereas U251 cells were transfected with 20 μM siP300 or siNC. The complexes were applied to respective cells cultured in 6-well plates. Transfection mixtures were incubated with cells at 37°C for 6 h, after which the medium was replaced with fresh complete medium. Functional assays were conducted 48 h post-transfection to assess phenotypic effects.

For lentiviral transduction, commercially provided lentiviral particles (third generation system, titer: 1x10⁸ TU/ml) were thawed on ice. U251, T98G and LN229 cells were incubated with lentiviruses at a multiplicity of infection of 4 in the presence of 5 μg/ml polybrene for 24 h at 37°C. The viral suspension was then replaced with fresh complete medium. To establish stable cell lines, transduced cells underwent selection with 2 μg/ml puromycin starting 72 h post-transduction for 3 days. Surviving cells were maintained in medium containing 1 μg/ml puromycin. All subsequent experiments were performed post-selection.

Western blotting. U87, U251, T98G and LN229 cells were lysed in RIPA buffer (cat. no. AR0105; Wuhan Boster Biological Technology, Ltd.) containing 1% protease inhibitor cocktail (cat. no. AR1182; Wuhan Boster Biological Technology, Ltd.), 1% PMSF (cat. no. P0100, Beijing Solarbio Science & Technology Co., Ltd.) and 5% phosphatase inhibitor cocktail (cat. no. 4906845001; Roche Diagnostics). Protein concentration was quantified using a BCA assay (cat. no. PC0020; Beijing Solarbio Science & Technology Co., Ltd.). A total of 30 μg protein per sample was separated by SDS-PAGE on 10% gels and transferred to PVDF membranes (cat. no. IPVH00010; MilliporeSigma; Merck KGaA). Membranes were blocked with 5% nonfat dry milk (cat. no. D8340; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 1 h and incubated overnight at 4°C with primary antibodies against the following targets: ACC1 (1:1,000; cat. no. 4190), cyclin D1 (1:1,000; cat. no. 2922), p21 (1:1,000; cat. no. 2947), DNMT1 (1:1,000; cat. no. 5032), histone H3 acetylation at lysine 9 (H3K9ac; 1:1,000; cat. no. 9649) and H3 (1:1,000; cat. no. 4499) purchased from Cell Signaling Technology, Inc.; cyclin B1 (1:1,000; cat. no. 55004-1-AP), SDHA (1:2,000; cat. no. 14865-1-AP), SDHB (1:1,000; cat. no. 10620-1-AP), fatty acid synthase (FASN; 1:2,000; cat. no. 10624-2-AP) and β-actin (1:2,000; cat. no. 20536-1-AP) purchased from Proteintech Group, Inc.; and fibronectin (1:1,000; cat. no. WL03677), vimentin (1:500; cat. no. WL01960), plasminogen activator inhibitor-1 (PAI-1; 1:1,000; cat. no. WL01486), P300 (1:1,000; cat. no. WL01307), sterol regulatory element-binding protein 1 (SREBP1; 1:1,000; cat. no. WL02093) and GAPDH (1:1,000; cat. no. WL01114) from Wanleibio Co., Ltd. Membranes were then incubated with HRP-conjugated anti-rabbit IgG secondary antibodies (1:5,000; cat. no. bs-0295G-HRP; BIOSS) at room temperature for 1 h. Signals were detected

using an ECL substrate (cat. no. PE0010; Beijing Solarbio Science & Technology Co., Ltd.) and images were captured using an Amersham Imager 600 system (Cytiva). Band density semi-quantification was performed using ImageJ software (version v1.8.0.345; National Institutes of Health).

Transwell migration and invasion assays. For migration assays, U87, U251, T98G and LN229 cells (2×10^4 cells) in 200 μ l serum-free MEM (U87, U251 and T98G cells) or DMEM (LN229 cells) were seeded into the upper chambers of Transwell inserts. The lower chambers contained 600 μ l corresponding medium with 10% FBS. For invasion assays, inserts were pre-coated with 100 μ l Matrigel (cat. no. 354262; Corning, Inc.) diluted 1:3 in corresponding serum-free medium. To ensure consistency in Matrigel coating, standardized protocols recommended by the manufacturer were followed, including pre-cooling plates to 4°C, dilution with ice-cold medium and incubation at 37°C for 1 h (18). Additionally, when adding Matrigel, the culture plate was placed in a tray equipped with a spirit level to ensure a level surface for sample addition. After 24 h incubation at 37°C in a 5% CO₂ incubator, non-migrated/non-invaded cells remaining on the upper surface of the membrane were removed. Migrated/invaded cells that adhered to the lower surface of the membrane were fixed with 4% para-formaldehyde for 10 min and stained with crystal violet (cat. no. C0121; Beyotime Institute of Biotechnology) for 5 min at room temperature. Cells were counted using an inverted optical microscope (Nikon Eclipse Ts2; Nikon Corporation) equipped with a digital camera (DS-Fi1c; Nikon Corporation).

Wound-healing assay. U87, U251, T98G and LN229 cells were seeded in a 6-well plate until ~90% confluency was reached in complete medium containing 10% FBS. Uniform linear scratches were created by gently dragging a sterile 200 μ l pipette tip across the cell monolayer in a single, consistent motion. Detached cells and debris were then removed by washing the monolayer three times with PBS. The medium was then replaced with low-serum medium (2% FBS in MEM for U87/U251/T98G or DMEM for LN229). Immediately after scratching, the initial scratch width was visualized under a microscope (Nikon Eclipse Ts2; Nikon Corporation) to ensure consistency. After 24 and 48 h of culture in low-serum conditions, images of the cells were captured under a microscope (Nikon Eclipse Ts2; Nikon Corporation). Cell migration was assessed by calculating the proportion of the area occupied by migrated cells within the scratch region relative to the initial scratch area.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA from U87, U251, T98G and LN229 cells was extracted using TRIzol™ reagent (cat. no. 15596018; Invitrogen™; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the All-In-One 5X RT MasterMix with AccuRT Genomic DNA Removal Kit (cat. no. G492; Applied Biological Materials Inc.) under the following conditions: 42°C for 2 min (gDNA removal), followed by 25°C for 10 min, 42°C for 15 min and 85°C for 5 min (cDNA synthesis). qPCR was performed using the BlasTaq™

2X qPCR MasterMix with SYBR-like dye (cat. no. G891; Applied Biological Materials Inc.) on a QuantStudio™ Dx Real-Time PCR Instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: 95°C for 10 min; followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min; and final dissociation at 95°C for 15 sec and 60°C for 1 min. Relative mRNA expression was calculated using the $2^{-\Delta\Delta C_q}$ method (19). Primer sequences are listed in Table SII. Primers for DNMT1/3A/3B, SDHA/B/C/D, P300 and GAPDH were purchased from GenScript Biotech Corporation.

SDH enzyme activity assay. SDH activity was measured using the Complex II Enzyme Activity Microplate Assay Kit (cat. no. ab109908; Abcam), according to the manufacturer's instructions. A total of 8×10^6 U251 cells were used per assay. Samples were normalized to protein content determined using a BCA assay. Absorbance was measured at 600 nm using a microplate reader (Multiskan Spectrum; Thermo Fisher Scientific, Inc.).

Cellular ROS measurement. U251 cells were harvested and stained with 10 μ M dihydroethidium (cat. no. KGA7502; Jiangsu KeyGen Biotech Co., Ltd.) in serum-free medium for 30 min at 37°C in the dark, with gentle mixing every 5 min. Cells were washed with PBS and fluorescence of the oxidized DHE-nucleic acid adduct was measured using the FL2 channel (585/42 nm bandpass filter) on a flow cytometer (BD FACSCalibur™; BD Biosciences). Data were analyzed using FlowJo software (version v10.8; BD Biosciences).

Acetyl-CoA measurement. Cellular acetyl-CoA levels were measured using the Acetyl-Coenzyme A Assay Kit (cat. no. MAK039; Sigma-Aldrich; Merck KGaA), according to the manufacturer's instructions. A total of 8×10^6 U251 cells were used per assay. Cell lysates were deproteinized with perchloric acid, neutralized with potassium bicarbonate and centrifuged at 1,000 x g for 10 min at 4°C. Acetyl-CoA levels in the supernatant were determined using fluorescence (excitation 535 nm, emission 587 nm) using the SpectraMax Gemini EM Microplate Reader (Molecular Devices, LLC).

Methylation-specific PCR. Genomic DNA was extracted using the EasyPure® Genomic DNA Kit (cat. no. EE101; TransGen Biotech Co., Ltd.) and bisulfite-converted using the EpiArt DNA Methylation Bisulfite Kit (cat. no. EM101; Vazyme Biotech Co., Ltd.). The SDHB promoter region was amplified using methylation-specific primers and the BlasTaq™ 2X qPCR Master Mix with SYBR-like dye (cat. no. G891; Applied Biological Materials Inc.) under the following thermocycling conditions: 95°C for 5 min; followed by 45 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec; and final extension at 72°C for 10 min. Unconverted DNA was used as a loading control. PCR products were separated on a 2% agarose gel and visualized using a UViPro gel imaging system with UViBand software v2.9.0 (UViTec Ltd.). Primers for the methylated/unmethylated SDHB promoter regions were purchased from GenScript Biotech Corporation. Primer sequences are listed in Table SII.

Immunohistochemical staining. A commercially pre-fixed glioma tissue microarray (cat. no. HBraG149Su01; Shanghai Outdo Biotech Co., Ltd.), containing 123 evaluable samples from an original cohort of 149 samples, was used. Samples were excluded due to incomplete tissue integrity during microarray production or detachment during staining. Sections were deparaffinized, rehydrated through xylene twice (10 min each) and graded alcohols (100, 90, 80 and 60%; 5 min each), then subjected to heat-induced antigen retrieval in citrate buffer (pH 6.0) at 100°C for 10 min using a water bath. Retrieval was followed by washing in PBS. Endogenous peroxidase activity was blocked with 3% H₂O₂ for 10 min at room temperature, and cell membranes were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. Sections were blocked with 10% goat serum (cat. no. C0265; Beyotime Institute of Biotechnology) at room temperature for 1 h, then incubated with ACC1 antibodies (1:300; cat. no. 67373-1-Ig; Proteintech Group, Inc.) at 4°C overnight. The ready-to-use HRP-conjugated universal anti-rabbit/mouse IgG secondary antibody [cat. no. GK500705; Genetech (Shanghai) Co., Ltd.] was applied and incubated at room temperature for 1 h. Subsequently, antigen detection was performed using the DAB chromogen substrate (components A and B of the GK500705 kit mixed at a 1:1 ratio) for 1 min. Nuclei were counterstained with hematoxylin (cat. no. C0107; Beyotime Institute of Biotechnology) at room temperature for 2 min. Whole-slide scanning of the tissue microarray was conducted using the Panoramic DESK scanner (3DHISTECH, Ltd.), with the results viewed using CaseViewer 2.4 software (3DHISTECH, Ltd.), and semi-quantitative analysis of ACC1 expression was performed using Aipathwell v2 software (Wuhan Servicebio Technology Co., Ltd.).

Public database analysis. Transcriptomics data from The Cancer Genome Atlas (TCGA; <https://portal.gdc.cancer.gov/>) glioblastoma cohort (TCGA-GBM) were analyzed via the UALCAN portal (<http://ualcan.path.uab.edu>). Proteomics data were obtained from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) dataset PDC000204 (<https://pdc.cancer.gov/pdc>). Additionally, transcriptomics and clinical data from three Chinese Glioma Genome Atlas (CGGA) datasets (mRNA_array_301, mRNAseq_325, mRNAseq_693) were accessed through the CGGA portal (<http://www.cgga.org.cn>).

Additional experimental methods. Additional experimental methods associated with the supplementary figures of the present study are detailed in Data S1.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 8.0 (Dotmatics). Data from three independent experimental repeats are presented as mean ± standard deviation. Unpaired Student's t-test and one-way ANOVA followed by Tukey's post hoc test were used for group comparisons. Spearman's correlation was applied for correlation analyses. For recurrence risk assessment of CGGA datasets, univariate Cox proportional hazards regression analysis was performed to determine the association between ACC1 expression levels (dichotomized as high/low) and recurrence risk. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ACC1 knockdown promotes proliferation, migration and invasion of glioma cells and selectively inhibits SDH in U251 cells. To assess the role of ACC1 in glioma, its protein expression in four glioma cell lines was first evaluated (Fig. S1A). U87 cells, demonstrating the lowest ACC1 expression compared with the other cell lines, were selected for plasmid-mediated overexpression (Fig. 1A). U251, T98G and LN229 cells, with comparably higher ACC1, underwent lentiviral knockdown (Fig. 1F, K and P). ACC1 overexpression in U87 cells significantly decreased cell migration and invasion compared with the control (Fig. 1B-E), whilst ACC1 knockdown significantly increased these behaviors in U251 (Fig. 1G-J), T98G (Fig. 1L-O) and LN229 (Fig. 1Q-T) cells.

Proliferation analyses revealed a cell line-specific pattern. MTT assays demonstrated that, compared with controls, ACC1 overexpression significantly inhibited U87 cell proliferation (Fig. S1B) and ACC1 knockdown significantly increased proliferation in T98G and LN229 cells (Fig. S1D and E). By contrast, ACC1 knockdown in U251 was associated with a significant reduction in cell proliferation (Fig. S1C), initially suggesting growth suppression. However, this finding contradicted multiple independent lines of evidence. Specifically, microscopic observation of cell density, manual cell counts and RTCA (detailed methods in Data S1; Fig. S1F-H), together with colony formation assays (Fig. S1I and J), cell cycle progression assays (Fig. S1K-N) and 3D tumor-sphere formation assays (Fig. S1O and P) consistently demonstrated that ACC1 knockdown significantly promoted U251 cells proliferation in comparison with controls. Supporting this, RT-qPCR revealed that ACC1 modulation influenced SDH subunit transcript levels specifically in U251 cells (Fig. S2B), with no significant changes observed in the other glioma cell lines (Fig. S2A, C and D). Moreover, western blot analysis demonstrated that the protein levels of SDHA and SDHB, the most abundantly expressed subunits of the SDH complex (20), were significantly reduced in U251 cells following ACC1 knockdown compared with controls (Fig. S2E-L), indicating a cell line-specific regulatory effect.

Taken together, the aforementioned findings indicate that the reduced proliferation rate observed in U251 cells upon ACC1 knockdown was due to SDH suppression rather than impaired proliferation. Given the consistent phenotype of enhanced proliferation, migration and invasion, along with the unique metabolic response to ACC1 silencing, U251 cells were selected for further mechanistic studies. However, additional studies are warranted to elucidate ACC1-related pathways in other glioma cell lines.

ACC1 knockdown promotes migration and invasion of U251 cells by increasing ROS levels. Due to the well-established association between SDH inhibition and ROS generation (21), the present study aimed to assess whether the downregulation of ACC1 facilitates glioma cell migration and invasion by stimulating ROS production through SDH inhibition. To address this, a series of experiments were performed, and the experimental results demonstrated that, compared with controls, ACC1 knockdown significantly decreased SDHA and SDHB protein levels (Fig. 2A and B) and SDH enzyme

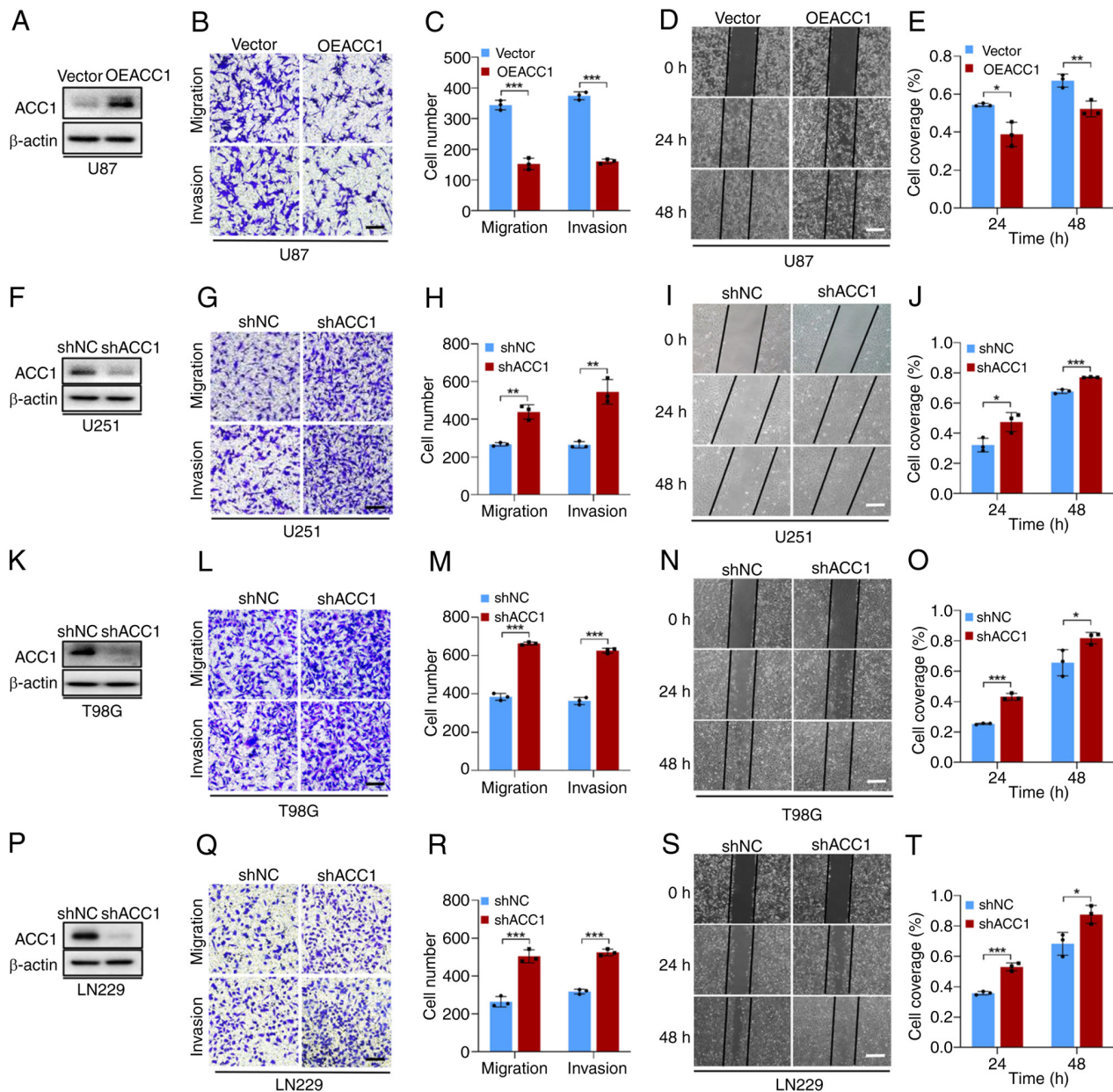


Figure 1. Effects of ACC1 modulation on glioma cell migration/invasion. (A) WB of ACC1 in U87-OE cells. (B) Images of Transwell migration and invasion assays in U87-OE cells. (C) Quantification of Transwell assay findings in U87-OE cells. (D) Wound-healing images of U87-OE cells. (E) Quantification of wound-healing assay findings in U87-OE cells. (F) WB of ACC1 in U251-KD cells. (G) Images of Transwell migration and invasion assays in U251-KD cells. (H) Quantification of Transwell assay findings in U251-KD cells. (I) Wound-healing images of U251-KD cells. (J) Quantification of wound-healing assay findings in U251-KD cells. (K) WB of ACC1 in T98G-KD cells. (L) Images of Transwell migration and invasion assays in T98G-KD cells. (M) Quantification of Transwell assay findings in T98G-KD cells. (N) Wound-healing images of T98G-KD cells. (O) Quantification of wound-healing assay findings in T98G-KD cells. (P) WB of ACC1 in LN229-KD cells. (Q) Images of Transwell migration and invasion assays in LN229-KD cells. (R) Quantification of Transwell assay findings in LN229-KD cells. (S) Wound-healing images of LN229-KD cells. (T) Quantification of wound-healing assay findings in LN229-KD cells. Scale bars, 100 μ m. Error bars represent the mean $\pm$ standard deviation from three independent experiments. * P <0.05; ** P <0.01; *** P <0.001. ACC1, acetyl-CoA carboxylase 1; WB, western blotting; OE, overexpression; KD, knockdown; sh, short hairpin; NC, negative control.

activity (Fig. 2C), leading to significantly elevated intracellular ROS levels (Fig. 2D and E). Application of 5 mM NAC (Fig. S3A), a free radical scavenger (22), effectively prevented the ACC1 knockdown-induced upregulation of intracellular ROS (Fig. 2F and G). Moreover, Transwell (Fig. 2H and I) and wound healing assays (Fig. 2J and K) demonstrated that NAC at least partially reversed ACC1 knockdown-induced cell migration and invasion. Western blot results also revealed that NAC application prevented ACC1 knockdown-induced upregulation of migration-related markers, fibronectin and

vimentin, and the invasion-related marker PAI-1 (Fig. 2L and M). Moreover, rescue experiments were performed by reintroducing ACC1 into ACC1 knockdown cells (shACC1 + OEACC1). The findings revealed that, compared with in the shACC1 + vector group, ACC1 overexpression significantly attenuated the enhanced cell migration, invasion and ROS elevation (Fig. S4). These findings suggest that knockdown of ACC1 promotes the migration and invasion of U251 cells by increasing intracellular ROS levels induced by SDH inhibition.

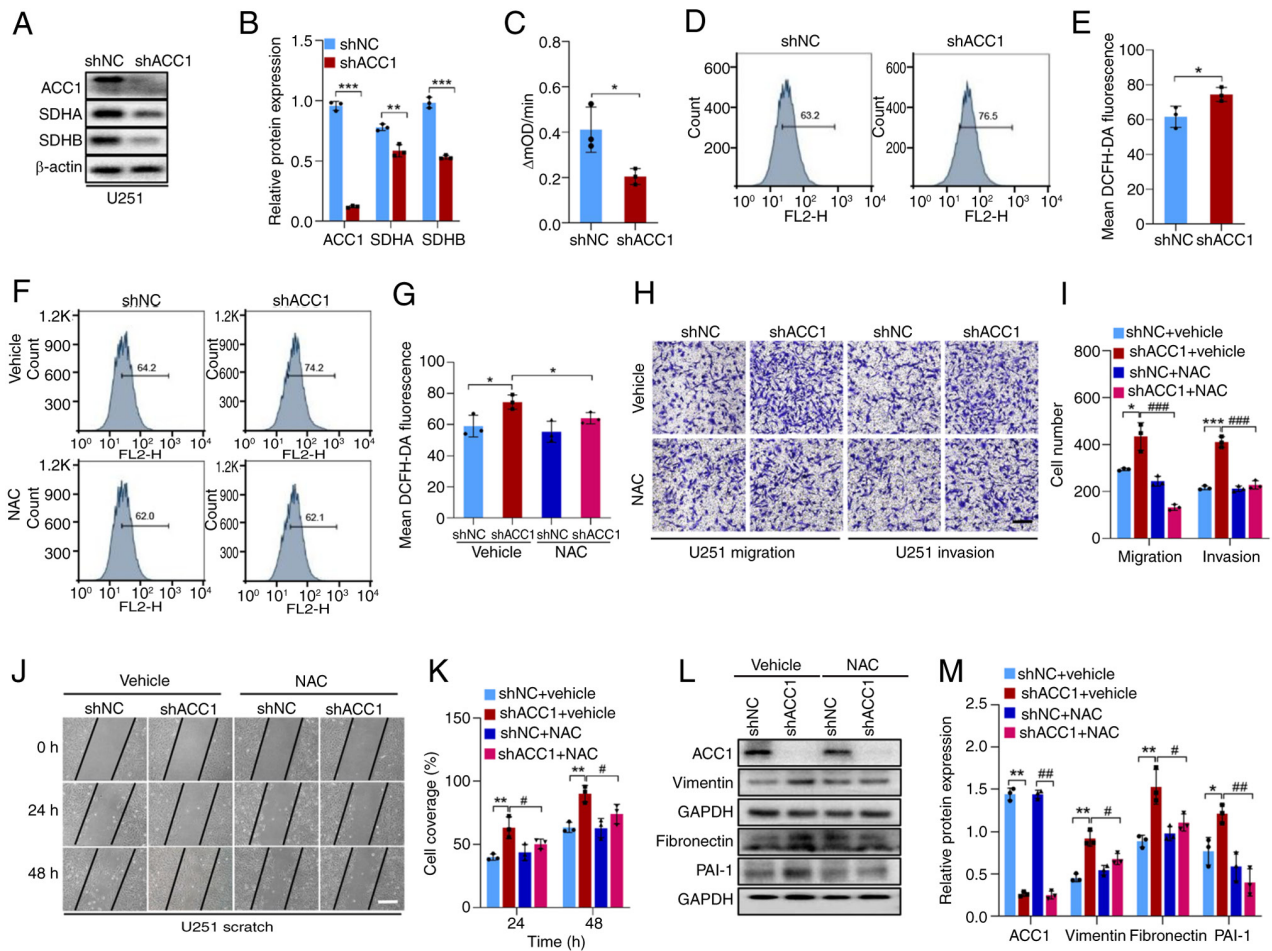


Figure 2. KD of ACC1 in U251 cells promotes cell migration and invasion by down-regulating SDH and thus elevating ROS level. (A) WB of ACC1, SDHA and SDHB after ACC1 KD. (B) Semi-quantification of ACC1, SDHA and SDHB protein levels after ACC1 KD. (C) Quantification of SDH activity following ACC1 KD. (D) Flow cytometric analysis of ROS after ACC1 KD. (E) Quantification of cellular ROS fluorescence signals after ACC1 KD. (F) Flow cytometric analysis of ROS after NAC treatment. (G) Quantification of cellular ROS fluorescence signals after NAC treatment. (H) Images of Transwell migration and invasion assays after NAC treatment. (I) Quantification of Transwell migration/invasion assay findings after NAC treatment. (J) Wound-healing images of cells after NAC treatment. (K) Quantification of wound-healing assay after NAC treatment. (L) WB of ACC1, vimentin, fibronectin and PAI-1 after NAC treatment. (M) Semi-quantification of ACC1, fibronectin, vimentin and PAI-1 protein levels after NAC treatment. Scale bars, 100 μ m. Error bars represent the mean $\pm$ standard deviation from three independent experiments. * P <0.05; ** P <0.01; *** P <0.001. # P <0.05; ## P <0.01; ### P <0.001. ACC1, acetyl-CoA carboxylase 1; SDH, succinate dehydrogenase; ROS, reactive oxygen species; NAC, N-acetyl-cysteine; PAI-1, plasminogen activator inhibitor-1; sh, short hairpin; NC, negative control; OD, optical density; WB, western blotting; KD, knockdown.

Increased DNMT1 expression leads to SDH hypermethylation and ROS upregulation. The primary mechanism underlying gene transcription inhibition involves DNA methylation, which is mediated by DNMTs (23). The DNMT family consists of DNMT1/2/3A/3B/3L (24), with DNMT2 primarily targeting transfer RNA methylation (25) and DNMT3L lacking catalytic activity (26). DNMT1/3A/3B are the main mediators of DNA methyltransferase activity *in vivo* (27). Analysis of TCGA database revealed upregulation of DNMT1/3A/3B mRNA levels in glioma tissues, compared with in normal tissues (Fig. S5A-C). This trend is consistent with the elevated protein expression of DNMT1 and DNMT3A observed in the CPTAC database (28), although DNMT3B data was unavailable (Fig. S5D and E). CPTAC data also revealed that all four subunits of SDH (SDHA-D) are downregulated in glioma tissue compared with in normal tissue (Fig. S5F-I), which is by contrast to the upregulation of DNMTs. This opposing expression pattern suggests a possible inverse association between DNMT and SDH levels in gliomas.

RT-qPCR analysis revealed that, in U251 cells, DNMT1 was predominantly expressed, whereas DNMT2 and DNMT3 exhibited very low expression levels. Moreover, ACC1 knockdown significantly increased DNMT1 expression compared with that of controls (Fig. 3A-C). To further evaluate whether ACC1 knockdown reduces SDH levels by promoting its methylation, thereby increasing ROS levels to promote cell migration and invasion, the DNMT inhibitor Aza was utilized to inhibit DNMT activity and reduce methylation levels. In comparison with controls, treatment with 4 μ M Aza, a concentration that was demonstrated to not affect cell viability (Fig. S3B), significantly reduced SDH downregulation caused by ACC1 knockdown (Fig. 3D-F) and hypermethylation of the SDHB promoter (Fig. 3G and H), and significantly attenuated the ACC1 knockdown-induced increase in ROS and cell migration/invasion (Fig. 3I-P). These results collectively indicate that ACC1 knockdown upregulates DNMT1, leading to the downregulation of SDH, the increase of ROS levels, and ultimately the promotion of U251 cell migration and invasion.

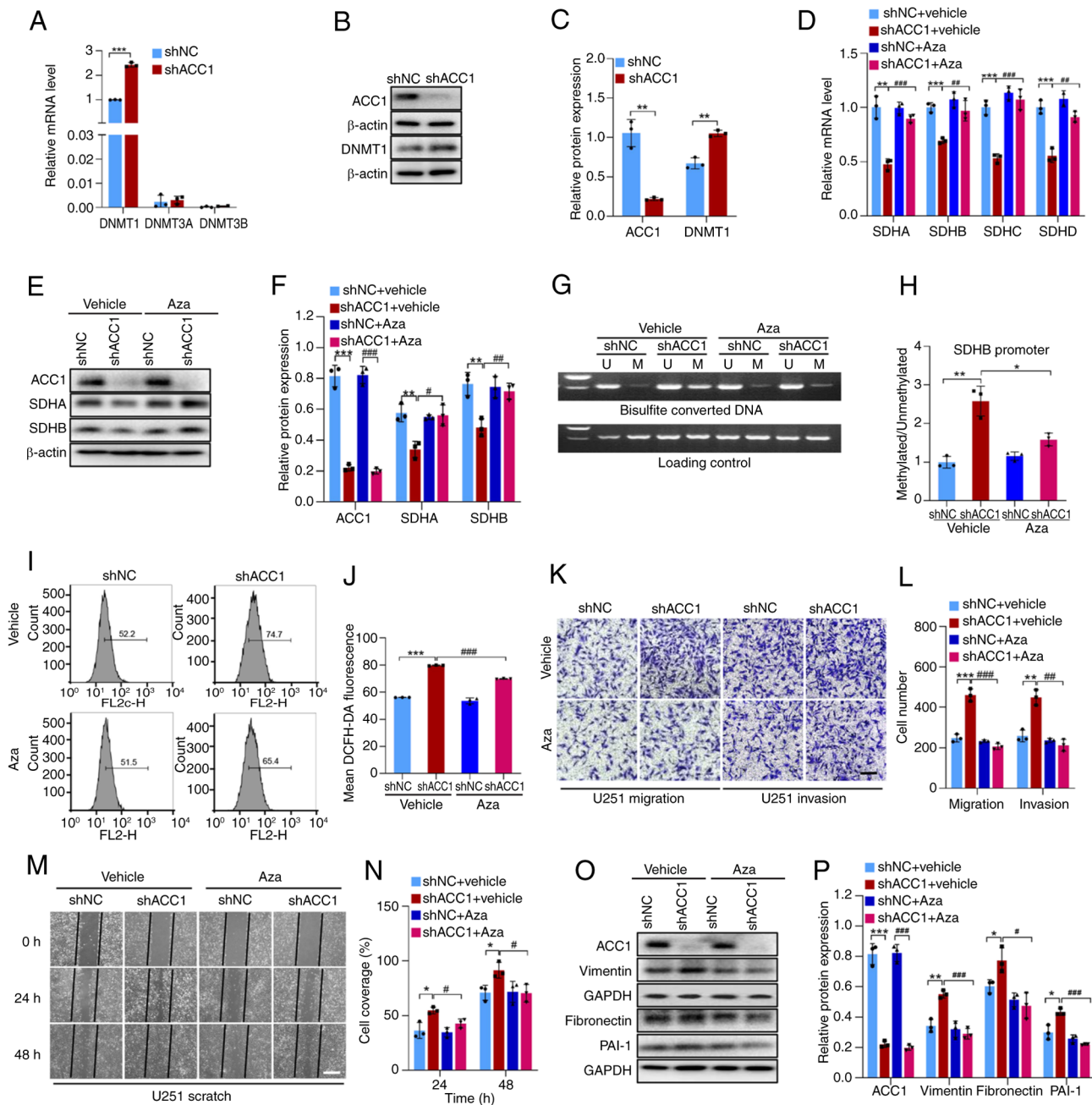


Figure 3. KD of ACC1 in U251 cells increases DNMT1 expression, leading to hypermethylation of SDH and subsequent upregulation of ROS levels. (A) RT-qPCR of DNMT1, DNMT3A and DNMT3B mRNA levels after ACC1 KD. (B) WB of ACC1 and DNMT1 after ACC1 KD. (C) Semi-quantification of ACC1 and DNMT1 protein levels after ACC1 KD. (D) RT-qPCR of SDHA, SDHB, SDHC and SDHD mRNA levels after treatment with Aza. (E) WB of ACC1, SDHA and SDHB after Aza treatment. (F) Semi-quantification of ACC1, SDHA and SDHB protein levels after Aza treatment. (G) Methylation-specific PCR of SDHB promoter methylation after Aza treatment. (H) Semi-quantification of SDHB promoter methylation levels after Aza treatment. (I) Flow cytometric analysis of ROS after Aza treatment. (J) Quantification of cellular ROS fluorescence signals after Aza treatment. (K) Images of Transwell migration and invasion assays after Aza treatment. (L) Quantification of Transwell migration/invasion assays after Aza treatment. (M) Wound-healing images after Aza treatment. (N) Wound-healing assay quantification after Aza treatment. (O) WB of ACC1, vimentin, fibronectin and PAI-1 after Aza treatment. (P) Semi-quantification of ACC1, vimentin, fibronectin and PAI-1 protein levels after Aza treatment. Scale bars, 100 μ m. Error bars represent the mean $\pm$ standard deviation from three independent experiments. * P <0.05; ** P <0.01; *** P <0.001. ACC1, acetyl-CoA carboxylase 1; DNMT, DNA methyltransferase; SDH, succinate dehydrogenase; ROS, reactive oxygen species; RT-qPCR, reverse transcription-quantitative PCR; Aza, azacitidine; PAI-1, plasminogen activator inhibitor-1; U, unmethylated; M, methylated; WB, western blotting; KD, knockdown; sh, short hairpin; NC, negative control.

ACC1 knockdown increases histone acetylation by elevating Acetyl-CoA, leading to DNMT1 upregulation. As ACC1 influences protein acetylation by modulating acetyl-CoA levels (29), we hypothesized that the upregulation of DNMT1 upon ACC1 knockdown may be associated with this phenomenon. The results of the present study revealed that, compared

with controls, knockdown of ACC1 significantly increased acetyl-CoA levels (Fig. 4A) and promoted H3K9ac expression (Fig. 4B and C). Notably, the knockdown of ACC1 did not significantly reduce the intracellular level of fatty acids compared with the control (Fig. S6A). SREBP1, encoded by the sterol regulatory element-binding transcription factor 1

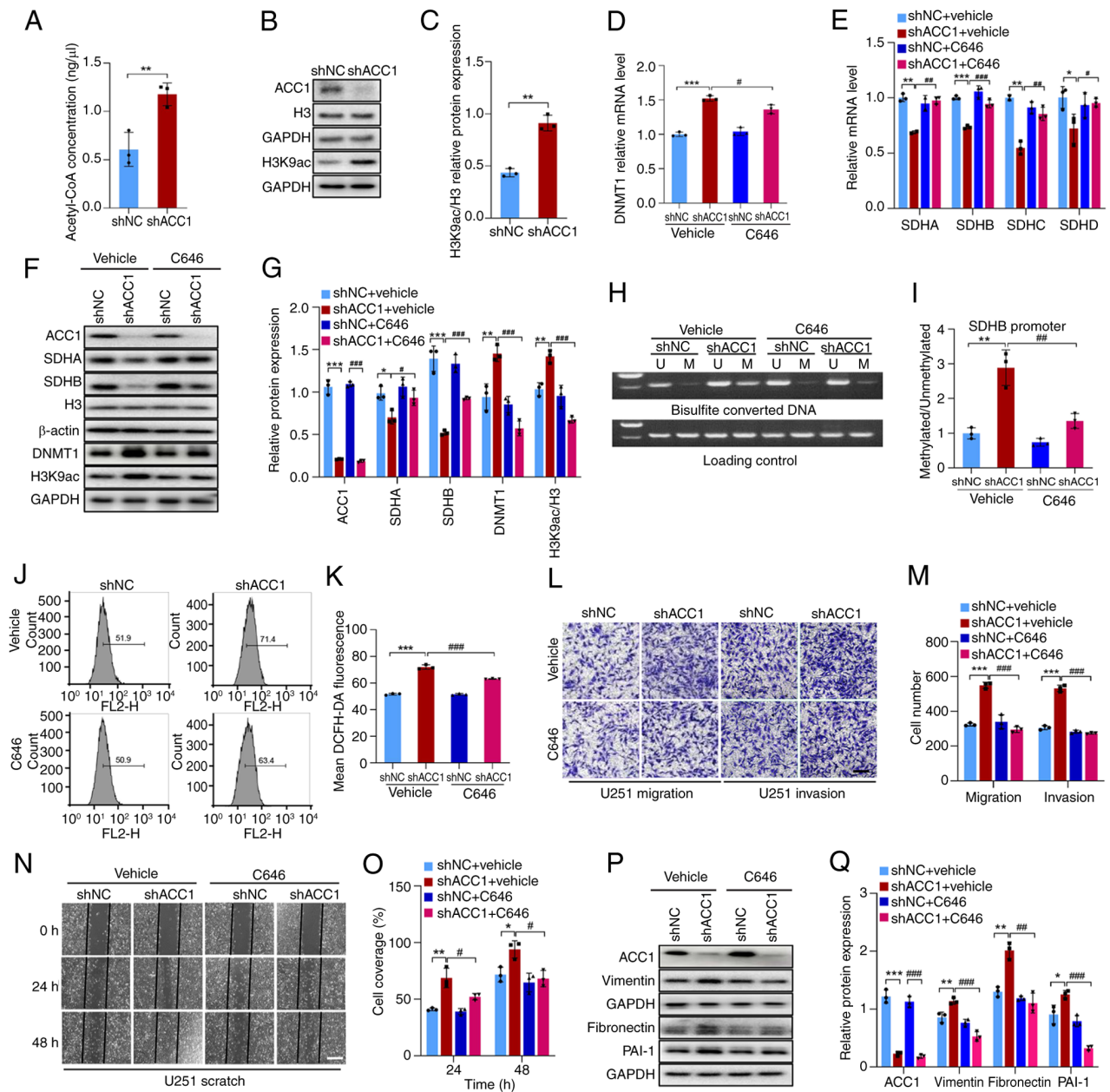


Figure 4. KD of ACC1 induces histone acetylation via acetyl-CoA elevation, resulting in DNMT1 upregulation. (A) Fluorometric assay of acetyl-CoA levels after ACC1 KD. (B) WB of ACC1, H3K9ac and H3 after ACC1 KD. (C) Semi-quantification of H3K9ac/H3 protein levels after ACC1 KD. (D) RT-qPCR of DNMT1 mRNA levels after treatment with C646. (E) RT-qPCR of SDHA, SDHB, SDHC and SDHD mRNA levels after treatment with C646. (F) WB of ACC1, SDHA, SDHB, H3, DNMT1 and H3K9ac after treatment with C646. (G) Semi-quantification of ACC1, SDHA, SDHB, DNMT1 and H3K9ac/H3 protein levels after treatment with C646. (H) Methylation-specific PCR of SDHB promoter methylation after treatment with C646. (I) Semi-quantification of SDHB promoter methylation level after treatment with C646. (J) Flow cytometric analysis of ROS after treatment with C646. (K) Quantification of cellular ROS fluorescence signals after treatment with C646. (L) Images of Transwell migration/invasion assays after treatment with C646. (M) Quantification of Transwell migration/invasion assays after treatment with C646. (N) Wound-healing images after treatment with C646. (O) Wound-healing assay quantification after treatment with C646. (P) WB of ACC1, vimentin, fibronectin and PAI-1 after treatment with C646. (Q) Semi-quantification of ACC1, vimentin, fibronectin and PAI-1 protein levels after treatment with C646. Scale bars, 100 μ m. Error bars represent the mean $\pm$ standard from three independent experiments. * P <0.05; ** P <0.01; *** P <0.001. # P <0.05; ## P <0.01; ### P <0.001. ACC1, acetyl-CoA carboxylase 1; DNMT, DNA methyltransferase; H3K9ac, histone H3 acetylation at lysine 9; SDH, succinate dehydrogenase; PAI-1, plasminogen activator inhibitor-1; U, unmethylated; M, methylated; WB, western blotting; KD, knockdown; sh, short hairpin; NC, negative control.

(SREBF1) gene, is a key regulator of lipid metabolism that can activate the transcription of fatty acid synthesis-related genes such as FASN, thereby maintaining cellular fatty acid homeostasis (30). The results demonstrated that, compared with controls, ACC1 knockdown significantly upregulated both SREBF1 (Fig. S6B and C) and FASN mRNA levels (Fig. S6D), as well as SREBP1 and FASN protein levels

(Fig. S6E and F). This suggests a compensatory enhancement of fatty acid synthesis to counteract the reduction caused by ACC1 depletion, ultimately preserving cellular fatty acid balance. As this compensatory mechanism maintains fatty acid levels, the phenotypic changes observed after ACC1 knockdown may be driven by alterations in histone acetylation rather than disruptions in fatty acid metabolism.

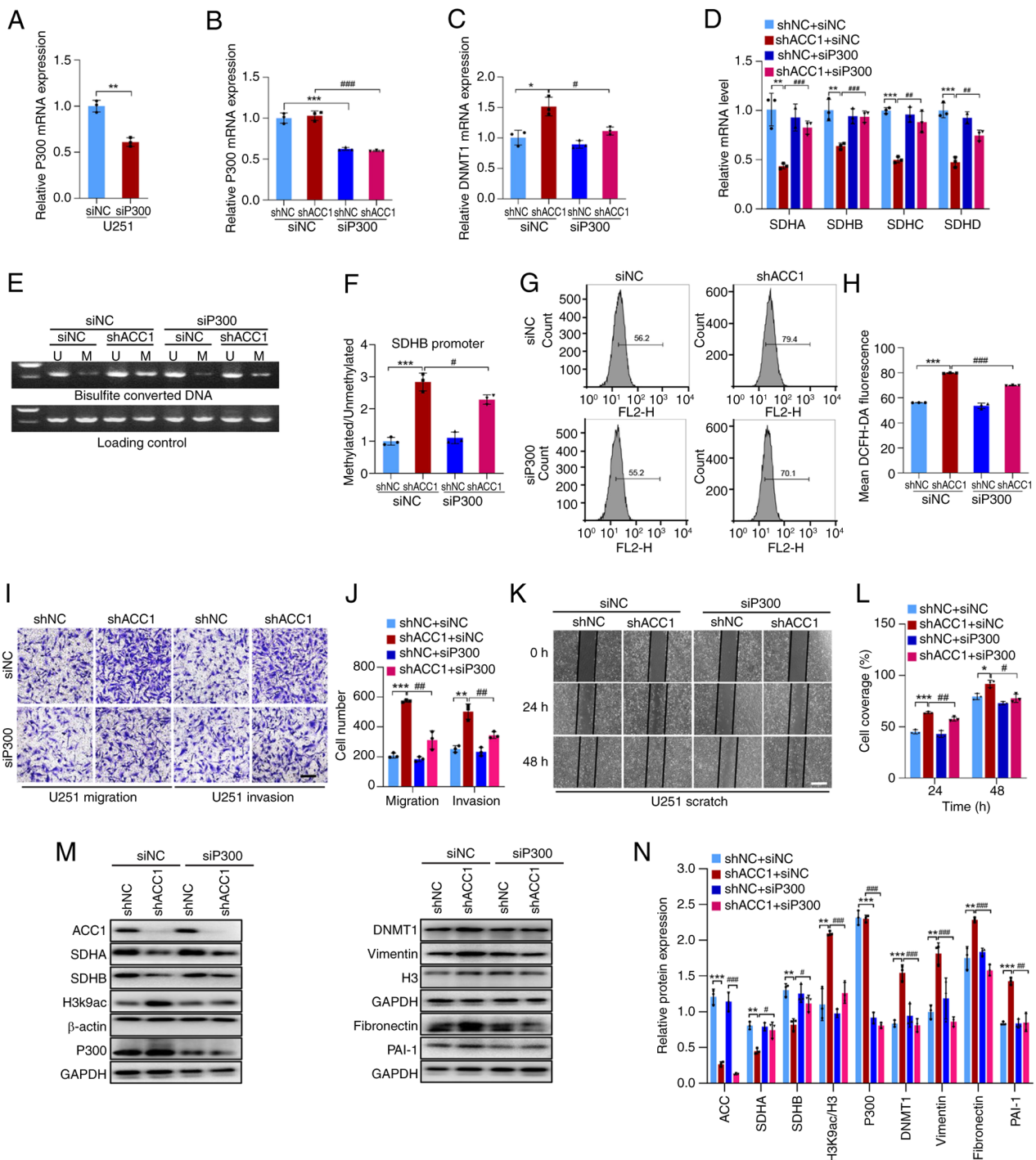


Figure 5. KD of ACC1 in U251 cells elevates DNMT1 expression via P300. (A) RT-qPCR of P300 mRNA after siP300 transfection in wild-type U251 cells. (B) RT-qPCR of P300 mRNA after siP300 transfection in ACC1 KD cells. (C) RT-qPCR of DNMT1 mRNA after siP300 transfection. (D) RT-qPCR of SDHA, SDHB, SDHC and SDHD mRNA after siP300 transfection. (E) Methylation-specific PCR of SDHB promoter methylation after siP300 transfection. (F) Semi-quantification of SDHB promoter methylation levels after siP300 transfection. (G) Flow cytometric analysis of ROS after siP300 transfection. (H) Quantification of cellular ROS fluorescence signals after siP300 transfection. (I) Images of Transwell migration/invasion assays after siP300 transfection. (J) Quantification of Transwell migration/invasion assays after siP300 transfection. (K) Wound-healing images after siP300 transfection. (L) Wound-healing assay quantification after siP300 transfection. (M) WB of ACC1, SDHA, SDHB, H3K9ac, P300, DNMT1, vimentin, H3, fibronectin and PAI-1 after siP300 transfection. (N) Semi-quantification of ACC1, SDHA, SDHB, H3K9ac/H3, P300, DNMT1, vimentin, fibronectin and PAI-1 protein levels after siP300 transfection. Scale bars, 100 μ m. Error bars represent the mean $\pm$ standard deviation from three independent experiments. * P <0.05; ** P <0.01; *** P <0.001. # P <0.05; ## P <0.01; ### P <0.001. ACC1, acetyl-CoA carboxylase 1; DNMT, DNA methyltransferase; SDH, succinate dehydrogenase; si, small interfering; U, unmethylated; M, methylated; ROS, reactive oxygen species; H3K9ac, histone H3 acetylation at lysine 9; PAI-1, plasminogen activator inhibitor-1; WB, western blotting; KD, knockdown; RT-qPCR, reverse transcription-quantitative PCR; sh, short hairpin; NC, negative control.

To further evaluate whether increased histone acetylation leads to the upregulation of DNMT1, C646 was utilized to inhibit histone acetylation. A total of 5 μ M C646 (Fig. S3C) effectively

reduced histone acetylation levels (Fig. 4F and G), prevented the upregulation of DNMT1 induced by ACC1 knockdown (Fig. 4D, F and G) and subsequently reversed the effects of

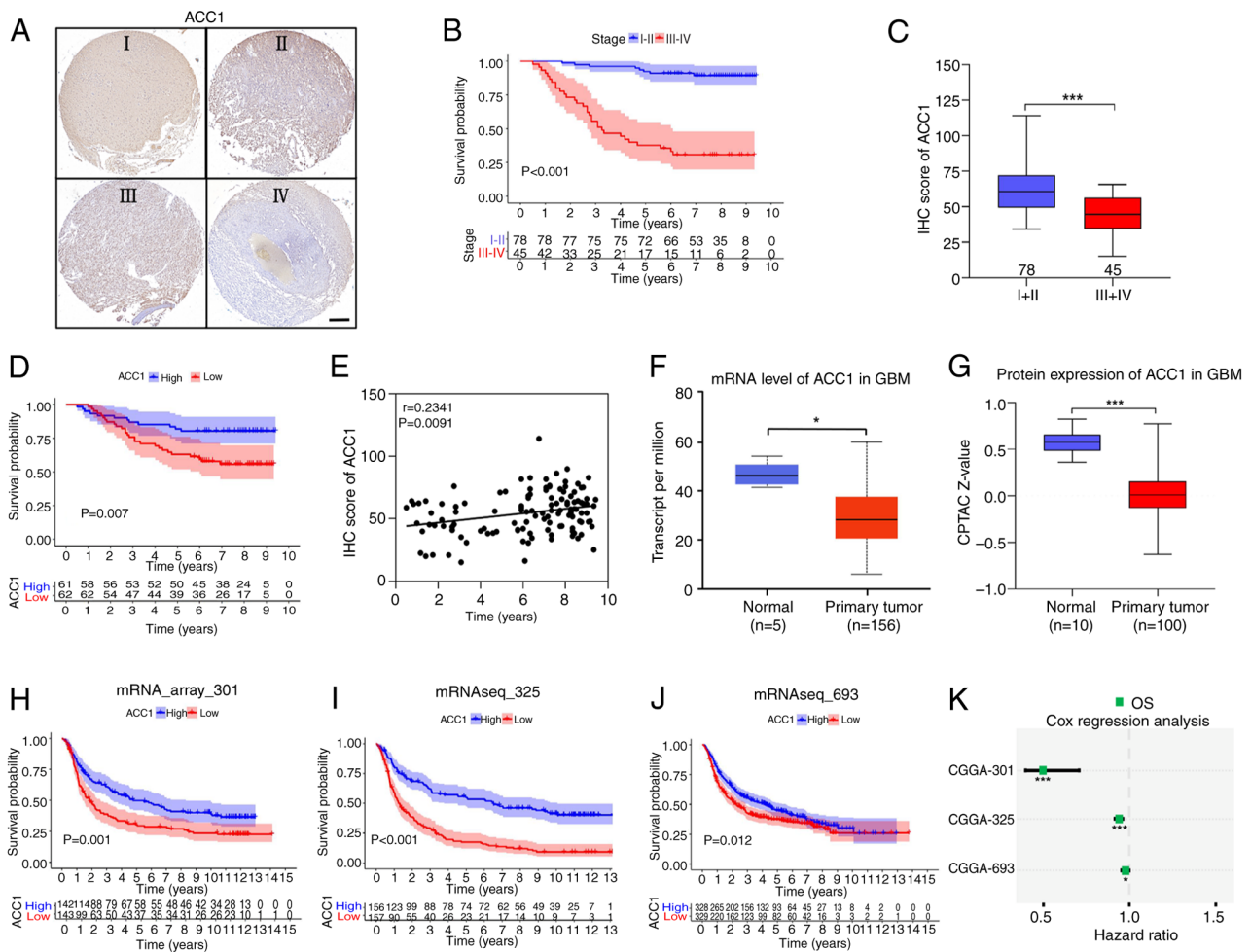


Figure 6. Patients with glioma with low ACC1 expression exhibit poor prognosis. (A) Representative IHC staining of ACC1 on glioma tissue microarray (n=123). (B) Patients with grade III-IV glioma demonstrated poor OS. (C) Protein expression of ACC1 in glioma specimens of different grades. (D) Patients with glioma with low ACC1 expression exhibited poor OS. (E) Correlation analysis of survival time and ACC1 expression in glioma samples. (F) TCGA analysis shows downregulated ACC1 mRNA in glioma tissues. (G) CPTAC analysis shows downregulated ACC1 protein in glioma tissues. (H) Analysis of CGGA mRNA_array_301 links low ACC1 expression to poor glioma prognosis. (I) Analysis of CGGA mRNAseq_325 links low ACC1 expression to poor glioma prognosis. (J) Analysis of CGGA mRNAseq_693 links low ACC1 expression to poor glioma prognosis. (K) Patients with glioma with low ACC1 expression demonstrated a high risk of recurrence. Scale bars, 200 μ m. * $P<0.05$; *** $P<0.001$. ACC1, acetyl-CoA carboxylase 1; IHC, immunohistochemistry; OS, overall survival; TCGA, The Cancer Genome Atlas; CPTAC, Clinical Proteomic Tumor Analysis Consortium; CGGA, Chinese Glioma Genome Atlas; GBM, glioblastoma multiforme.

ACC1 knockdown on SDH, ROS levels and migration/invasion (Fig. 4E-Q). These findings suggest that knockdown of ACC1 promotes histone acetylation by increasing acetyl-CoA levels, which subsequently upregulates the expression of DNMT1. Consequently, hypermethylation of SDH promoter inhibits SDH expression, leading to increased ROS production and ultimately facilitating cell migration and invasion.

ACC1 elevates DNMT1 expression through P300 interaction. To assess the involvement of P300 in the upregulation of DNMT1 induced by ACC1 knockdown, siRNA was utilized to interfere with P300 expression (Fig. 5A, B, M and N). Compared with in cells with ACC1 knockdown alone (shACC1 + siNC), the silencing of P300 in ACC1 knockdown cells (shACC1 + siP300) significantly reduced the ACC1 knockdown-induced increase in DNMT1 expression (Fig. 5C, M and N). Additionally, compared with shACC1 + siNC, interference with P300 (shACC1 + siP300) restored SDH expression (Fig. 5D, M and N) and reduced SDHB promoter methylation (Fig. 5E and F). Compared with shACC1

+ siNC, P300 knockdown (shACC1 + siP300) also attenuated the increased ROS and enhanced migration/invasion observed upon ACC1 knockdown (Fig. 5G-N). Overall, the results indicate that ACC1 knockdown enhances the availability of substrates for P300 by elevating acetyl-CoA levels, resulting in increased DNMT1 expression, SDH hypermethylation and elevated ROS levels, which ultimately promotes cell migration and invasion.

Low ACC1 expression is associated with poor prognosis in patients with glioma. To further assess the association between ACC1 levels and the prognosis of patients with glioma, immunohistochemical staining of ACC1 was performed using a glioma tissue microarray (Fig. 6A). Analysis of clinical data indicated a significant reduction in overall survival (OS) among patients with grades III-IV glioma, compared with those with grades I-II (Fig. 6B). Furthermore, evaluation based on immunohistochemistry scores revealed significantly lower ACC1 levels in tumor tissues from patients with grades III-IV glioma compared with those with grades I-II (Fig. 6C).

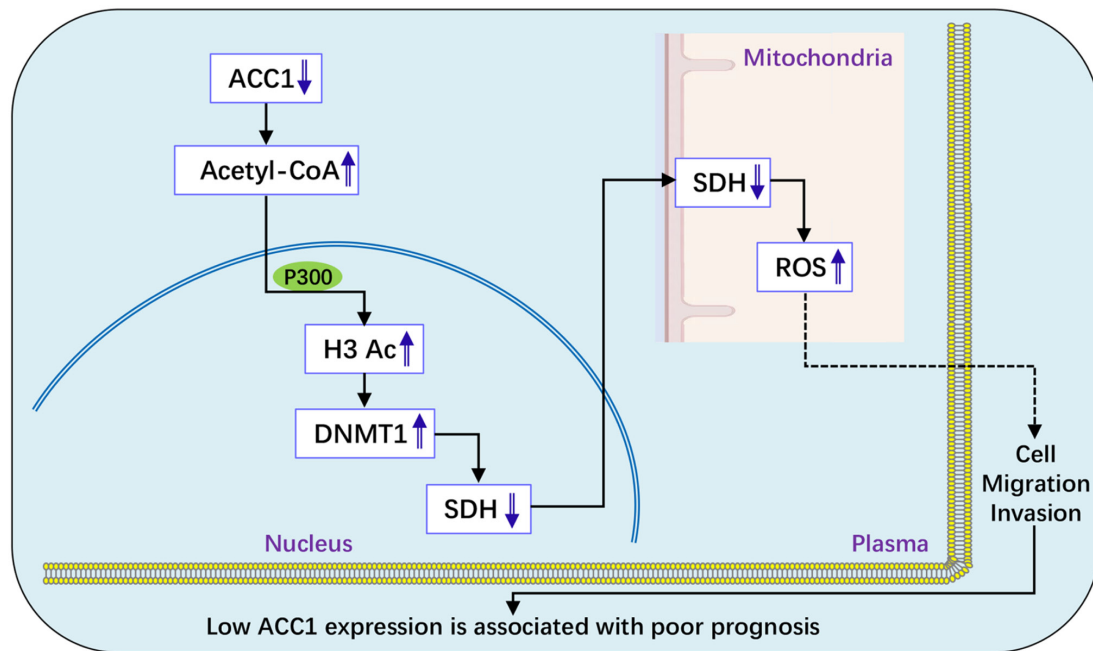


Figure 7. ACC1 knockdown triggers a cascade of events in U251 cells, increasing acetyl-CoA levels and enhancing P300 substrate availability. This prompts an increase in DNMT1 expression, leading to hypermethylation of the SDH promoter, reduced SDH expression and increased ROS levels. Ultimately, these molecular alterations promote the migration and invasion of U251 cells. Down-regulation of ACC1 is associated with poor prognosis and a high-grade of glioma. ACC1, acetyl-CoA carboxylase 1; DNMT, DNA methyltransferase; SDH, succinate dehydrogenase; ROS, reactive oxygen species; Ac, acetylation.

Notably, patients exhibiting low ACC1 expression experienced significantly worse OS outcomes compared with those with high ACC1 expression (Fig. 6D), with a positive correlation observed between survival time and ACC1 expression levels (Spearman $r=0.2341$; $P=0.0091$; Fig. 6E). Moreover, data from TCGA (Fig. 6F) and CPTAC databases (Fig. 6G) corroborated the downregulation of ACC1 in glioma tissues compared with in normal tissues (28). Finally, analysis of three datasets from the CGGA (31) database (mRNA_array_301, mRNAseq_325 and mRNAseq_693) revealed that patients with glioma with low ACC1 expression exhibited worse OS (Fig. 6H-J) and were at a higher risk of recurrence, compared with those with high ACC1 expression (Fig. 6K). In summary, the aforementioned findings highlight the association between low ACC1 expression and adverse outcomes in patients with glioma.

Discussion

The results of the present study demonstrate the multifaceted role of ACC1 in glioma progression, impacting proliferation, migration, invasion and prognosis. The study first assessed ACC1 expression across four glioma cell lines, revealing a differential pattern: U87 cells demonstrated lower ACC1 levels, whilst U251, LN229 and T98G cells exhibited higher expression. This pattern closely mirrored the ACC1 mRNA levels observed in U87, U251, T98G and LN229 cells, according to the Cancer Cell Line Encyclopedia database (32). However, abnormal SDH activity was observed exclusively in U251 cells. To further explore the mechanism underlying this specificity, the research focused on the molecular characteristics of U251 cells.

Understanding glioma cell origin, classification and heterogeneity of glioma cells is essential for both research and therapy. Although U87, U251, T98G and LN229 are all

World Health Organization grade IV glioma cell lines derived from astrocytic transformation, they originate from patients with diverse ethnic backgrounds, ages, sexes and tumor locations, resulting in distinct genetic backgrounds and molecular characteristics (33,34). According to TCGA classification, U251 and LN229 belong to the classical subtype, whilst U87 and T98G represent the mesenchymal subtype, with notable differences in gene expression profiles and signaling pathway activity (35). Morphologically, U251 and T98G exhibit fibroblast-like characteristics, whereas U87 and LN229 display more epithelial-like features, further illustrating the phenotypic diversity among glioma cells (33). In this context, the finding that ACC1 knockdown reduced SDH expression specifically in U251 cells highlights this intrinsic heterogeneity and underscores the importance of molecular subtyping and precise diagnostic strategies in glioma.

SDH activity is associated with ROS levels in several cancers. SDH mutations increase ROS and promote tumor development in paraganglioma, pheochromocytoma (11) and gastrointestinal stromal tumors (12). Redox imbalance is also implicated in glioma progression (17). Elevated ROS levels have been reported to drive glioma stem cell proliferation (36) and enhance the proliferation and migration/invasion in glioma cell lines (37,38). Our previous findings demonstrated that ACC1 knockdown in U251 cells increases ROS and promotes migration/invasion via ERK1/2 phosphorylation (Wang *et al*, unpublished data). The results of the current study further support the importance of ROS in glioma pathogenesis.

Epigenetic mechanisms, including DNA methylation and histone modifications, serve crucial roles in cancer progression, including in glioma (39). Elevated histone acetylation has been reported to promote invasion and metastasis across several tumors, including glioma (40). The dynamic balance

of histone acetylation and deacetylation modulates DNMT1 activity, a key regulator of epigenetic processes, as supported by multiple studies (41-45). Specifically, the histone acetyltransferase P300 directly interacts with the DNMT1 promoter, enhancing chromatin acetylation and DNMT1 gene transcription (46). Furthermore, P300 functions as a critical transcriptional regulator of DNMT1, as highlighted by its role in cancer progression, as demonstrated by Li *et al* (46) in breast cancer. In U251 glioma cells, the present study demonstrated that suppressing ACC1 increased the levels of acetyl-CoA, consequently promoting protein acetylation catalyzed by histone acetyltransferases, including that of P300. This is likely to induce increased histone acetylation, potentially reshaping gene expression profiles and fueling glioma advancement. Additionally, this may enhance the binding affinity of P300 to the DNMT1 promoter, resulting in increased DNMT1 acetylation, elevated DNMT1 expression and subsequent epigenetic modifications. Moreover, recent studies have highlighted the role of DNMT1-mediated hypermethylation of the SDHB promoter in reducing SDHB expression, which, in turn, is associated with elevated levels of ROS and contributes to adrenal cortical dysfunction (47). In line with this, the findings of the present study in U251 glioma cells suggest that increased DNMT1 expression induces hypermethylation of the SDH promoter. This epigenetic modification elevates ROS levels, fostering U251 glioma cell migration and invasion.

Furthermore, the *in vitro* results in the present study demonstrate that ACC1 knockdown promoted glioma cell migration and invasion, suggesting a role for ACC1 in glioma progression. This finding aligned with that of the clinical tissue microarray analysis, which revealed that lower ACC1 expression was associated with a worse prognosis in patients with glioma. This was further supported by survival data from the CGGA database. Together, these results indicate that ACC1 may serve as a potential prognostic marker in glioma. Moreover, analysis of the CPTAC glioma dataset revealed consistent downregulation of all SDH subunits in high-grade gliomas, suggesting that SDH suppression is a common feature of aggressive glioma phenotypes. This is consistent with previous studies linking reduced SDH expression with poor prognosis in patients with high-grade gliomas (20). However, as ACC1 knockdown was associated with SDH reduction specifically in U251 cells in the present study, SDH levels and their association with ACC1 expression in patient tissues were not evaluated, leaving the clinical relevance and subtype-specific significance of this relationship uncertain. These findings highlight the need for further clinical validation to determine whether SDH dysregulation contributes to ACC1-mediated glioma progression across distinct glioma subtypes.

Notably, despite adherence to standardized protocols, the present study was unable to establish a stable orthotopic xenograft model using U251 cells, and this limitation precluded *in vivo* validation of the *in vitro* findings. Therefore, future studies should employ patient-derived xenograft models to evaluate the physiological relevance of the results of the present study.

In conclusion, the results of the present study demonstrated that low ACC1 expression is associated with poor prognosis

in patients with glioma and that ACC1 knockdown enhances glioma cell proliferation, migration and invasion. Specifically, in U251 cells, ACC1 knockdown promotes migration and invasion through the acetyl-CoA/P300/DNMT1/SDH/ROS pathway (Fig. 7). These findings highlight ACC1 as a potential therapeutic target and underscore the need for personalized treatment strategies for patients with glioma.

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Availability of data and materials

The data generated from this study are available upon request from the corresponding author.

Authors' contributions

XXW, YW, YZL and BSZ conceived and designed the study. XXW and YW performed the investigation and data analysis. XXW, WLZ, WQY and JPT conducted the experiments and bioinformatics analysis. YZL and BSZ supervised the research. XXW and YZL drafted the manuscript. XXW and YZL confirm the authenticity of all the raw data. All authors edited, read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval for the glioma tissue microarray was obtained by Shanghai Outdo Biotech Co., Ltd. (approval no. SHYJS-C P-1801018).

Patient consent for publication

Not applicable.

Competing interests

The authors that they have declare no competing interests.

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