

GDNF regulates lipid metabolism and glioma growth through RET/ERK/HIF-1/SREBP-1

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Abstract. Cancer cells rewire their metabolism to meet the demands of growth and survival and this metabolic reprogramming has been recognized as an emerging hallmark of cancer. However, the respective mechanisms remain elusive and the contribution of aberrant lipid metabolism to the malignant phenotypes of glioma are unclear. The present study demonstrated that glial-derived neurotrophic factor (GDNF) is highly expressed in glioma and associated with poor clinical outcomes. In addition, there was a significant correlation between GDNF/rearranged during transfection (RET)/ERK signaling and sterol regulatory element-binding protein-1 (SREBP-1) expression in glioma cells. Pharmacological or genetic inhibition of GDNF-induced RET/ERK activity downregulated SREBP-1 expression and SREBP-1-mediated transcription of lipogenic genes. Additionally, GDNF regulated SREBP-1 activity by promoting hypoxia-inducible factor-1 α (HIF-1 α) mediated glucose absorption and hexosamine biosynthetic pathway mediated SREBP cleavage-activating protein N-glycosylation. In addition, the inhibition of SREBP-1 reduced the *in vitro* GDNF-induced glioma cell proliferation. The results elucidated the complex relationship between GDNF/RET/ERK signaling and dysregulated glycolipid-metabolism, which shows great potential to uncover novel metabolic vulnerabilities and improve the efficacy of targeted therapies.

Introduction

Malignant glioma is the most common and severe primary malignant intracranial tumor in adults with higher morbidity and recurrence. The current standard of care for newly diagnosed glioma is maximal safe resection followed by radiotherapy along with concomitant and adjuvant temozolomide (1). Relatively poor prognosis, fast recurrence and multi-drug resistance are some of the main challenges in combating brain tumors (2,3). Therefore, there is an urgent need to determine novel molecular targets in gliomas to develop more potent and effective therapies for patients. Although large-scale genome sequencing efforts have defined oncogenes and tumor suppressor genes mutation in glioma, most of these mutations have not been subjected to targeted therapy (4). Thus, a deeper insight into the biology properties and vulnerabilities of these tumor can potentially yield therapeutic impact.

Metabolic reprogramming is one of the hallmarks of cancer because tumors can alter metabolic pathways to meet the biosynthetic, bioenergetic and redox requirements of malignancy. In addition, elevated lipid metabolism is a common pathophysiological characteristic of metabolic diseases and cancer (5-7). Alterations in the metabolism of fatty acids has received renewed interest in cancer research because, in addition to their main function as structural components of the membrane matrix, they are important secondary messengers and can serve as fuel sources for energy production (7). In these processes, sterol regulatory element-binding proteins (SREBPs) have a critical regulatory function. SREBPs are a family of transcription factors that control the expression of genes important for the uptake and synthesis of cholesterol, fatty acids and phospholipids (8,9). The activity of these genes is regulated by SREBP cleavage-activating protein (SCAP), which is a polytopic membrane protein that forms complexes with membrane-bound SREBPs in the endoplasmic reticulum (ER) (10).

Several researchers investigating metabolism reprogramming in tumor tissue have attempted to elucidate the interactions between oncogenic signaling and cell metabolic processes. In this context, glial-derived neurotrophic factor (GDNF) is highly expressed in a number of human cancers without mutation (11,12). The ligand-binding component of GDNF is a glycosyl-phosphatidylinositol-anchored GDNF family receptor α 1 (GFR α 1), which is a well-characterized oncogene that only associates with its transmembrane

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co-receptor rearranged during transfection (RET) following ligand binding (13,14). Elevated GDNF expression enhances RET activation, which is crucial for the development and progression of gliomas (12,15-18). The ERK pathway is one the most important signaling cascades among all MAPK signal transduction pathways, which is required for RET induced proliferation (19) and lipid metabolism (20). Previous studies have indicated that elevated GDNF/RET signaling is associated with additional glucose absorption or lipid metabolism in tumorigenesis (21,22). However, the molecular mechanisms underlying the correlation between GDNF/RET/ERK signaling and dysregulated glycolipid-metabolism in glioma have remained largely unknown. In the present study, the activation of GDNF/RET/ERK signaling promoted the hexosamine biosynthetic pathway (HBP) and cascade-induced lipid metabolism. In addition, HBP was crucial for the correlation between oncogenic signaling and fuel availability to SREBP-1-dependent lipid metabolism.

Materials and methods

Reagents and samples. Sodium pyruvate (cat. no. P5280), lactate (cat. no. 1614308), D-glucose (cat. no. NIST917C), GlcNAc (cat. no. A3286), RPI-1 (cat. no. R8907), azaserine (cat. no. A4142), tunicamycin (cat. no. T7765) and OSMI-1 (cat. no. SML1621) were purchased from MilliporeSigma. Glioma and normal brain tissue samples were collected from the Department of Neurosurgery, First Affiliated Hospital of Zhengzhou University between August 2019 and September 2021. Tumors were classified histopathologically according to the 2016 World Health Organization classification (23). The study was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University (approval no. 2020-KY-155).

Cell culture. U251 and U87 human glioma cell lines were purchased from the American Type Culture Collection, the cell lines were authenticated by short tandem repeat (STR) analysis (HKGene, Inc.). All cell lines were normally cultured in complete Dulbecco's modified eagle medium (DMEM; Thermo Fisher Scientific, Inc.) supplemented with 10 mM glucose, 10% fetal bovine serum (HyClone; Cytiva), 100 U/ml penicillin-streptomycin (HyClone; Cytiva) and 2 mM glutamine in a humidified atmosphere of 5% CO₂ at 37°C. Cells in the mid-log phase of growth were used for the experiments.

Transfection of siRNA. U251 and U87 human glioma cells were transfected at 80% confluence with SCAP siRNA (cat. no. sc-36462; Santa Cruz Biotechnology, Inc.), hypoxia-inducible factor 1 (HIF-1) siRNA (cat. no. sc-35561; Santa Cruz Biotechnology, Inc.), SREBP-1 siRNA (cat. no. sc-36557; Santa Cruz Biotechnology, Inc.) and control siRNA (cat. no. sc-37007; Santa Cruz Biotechnology, Inc.) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, prior to treatment, Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.)/siRNA complexes were prepared in Opti-MEM medium, at the ratio of 1 µl Lipofectamine 2000 per 20 pmole siRNA. Cells then were then treated at 37°C for 48 h before being replaced with complete medium for the

desired duration. The transfection efficiency of the cells was verified by western blotting.

Cell proliferation assays. Cell proliferation assays was performed as previously described (24). Briefly, U251 (1x10⁴/cells/200 µl), U87 (1x10⁴/cells/200 µl) glioma cells were seeded onto 96-well microplate and cultured at 37°C for 24 h and then treated with target compounds at given concentrations at 37°C for indicated periods. The cytotoxicity to glioma cells was determined with an MTT assay. Viability was expressed as a ratio to the absorbance value at 490 nm of the control cells, and the OD value was measured using a microplate reader (BioTek Instruments, Inc.).

Synergy analysis. A synergy analysis was performed as previously described (24). Briefly, the Chou-Talalay method and CalcuSyn software (version 1.0) (25) were used to determine the dose effect of combination therapy. For this synergy analysis, RPI-1 was combined with Fatostatin at a constant ratio for glioma cells at a dosage determined by the IC₅₀ of each drug. Interaction was quantified based on a combination index (CI) to assess synergism (CI <1), additive effect (CI=1), and antagonism (CI >1).

Western blotting. The collected U251, U87 glioma cells and tissue lysates were prepared using RIPA buffer (Beyotime Institute of Biotechnology) and total protein concentration was quantified using a bicinchoninic acid (BCA) assay kit. For the SCAP glycosylation analysis, the protein samples were treated with PNGase F according to the manufacturer's instructions (MilliporeSigma). Equal protein amounts (30-50 µg) were electrophoresed on 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis gels and the separated proteins were transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk at room temperature for 2 h, the membranes were probed with primary antibodies against acetyl CoA carboxylase (ACC; 1:1,000; cat. no. 3662, Cell Signaling Technology, Inc.), HIF-1 (1:1,000; cat. no. 36169, Cell Signaling Technology, Inc.), SCAP (1:1,000; cat. no. 13102, Cell Signaling Technology, Inc.), SREBP-1 (1:1,000; cat. no. sc-365513, Santa Cruz Biotechnology, Inc.), fatty acid synthase (FASN) (1:1,000; cat. no. 3180, Cell Signaling Technology, Inc.), RET (1:1,000; cat. no. 14556, Cell Signaling Technology, Inc.), phosphorylated (p)-RET (1:1,000; cat. no. SAB4504530, MilliporeSigma), ERK (1:1,000; cat. no. 5013, Cell Signaling Technology, Inc.), p-ERK (1:1,000; cat. no. 4370, Cell Signaling Technology, Inc.), stearoylCoA desaturase-1 (SCD1) (1:1,000; cat. no. 2794, Cell Signaling Technology, Inc.), β-tubulin (1:1,000; cat. no. 2128, Cell Signaling Technology, Inc.) and lamin B (1:1,000; cat. no. 13435, Cell Signaling Technology, Inc.), at 4°C for 12 h. Subsequently, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:5,000; cat. no. ZB-2301; OriGene Technologies, Inc.) or HRP-conjugated goat anti-mouse IgG (1:5,000; cat. no. ZB-2305, ZSGB-BIO; OriGene Technologies, Inc.) secondary antibodies at room temperature for 2 h. Immunoreactivity was visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Reverse transcription-quantitative (RT-q) PCR. RT-qPCR assays was performed as previously described (24). Briefly,

U251 (1×10^6 /cells/5 ml) and U87 (1×10^6 /cells/5 ml) glioma cells were plated in 60 mm dishes and allowed to grow to 60-70% confluence and then treated with target compounds at given concentrations at 37°C for 24 h. Total RNA from the U251 and U87 glioma cells was extracted using a TRIzol® reagent (Invitrogen, Thermo Fisher Scientific, Inc.). Following which, the RNA was reverse-transcribed to cDNA using Trans-Script First-Strand cDNA Synthesis SuperMix (TransGen Biotech Co. AT301); both procedures were performed according to the manufacturer's instructions. RT-qPCR reactions were performed using a SYBR green PCR master mix (TransGen Biotech, China) on a MxPro-Mx3005P real-time PCR system (Agilent Technologies, USA) and β -tubulin was used as the control. The qPCR conditions were as follows: Initial denaturation at 95°C for 30 sec; followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The relative expression of target genes was calculated using the $2^{-\Delta\Delta C_q}$ method (26). The following primers were used: β -tubulin, F: 5'-GTGGTACGGAAGGAGGCAGAGA-3', R: 5'-AACGGAGGCAGGTGGTGACA-3'; GDNF, F: 5'-TCACTGACTTGGGTCTGGG-3', R: 5'-TCA AAGGCGATGGGTCTGC-3'; SREBP-1, F: 5'-CCATGGATTGCACTTTCGAA-3', R: 5'-CCAGCATAGGGTGGGTCAA-3'; SCD1, F: 5'-CACTTGGGAGCCCTGTATGG-3', R: 5'-TGAGCTCCTGCTGTTATGCC-3'; FASN, F: 5'-TGAGCAGACGAGAGCACCTT-3', R: 5'-CGATGTTGTAGATGGCGGCTGAG-3'; ACC, F: 5'-TTCCTCCACCTTGTCTCAGCGGA-3', R: 5'-GTCAGAGAAGCAGCCCATCACT-3'.

Immunofluorescence and immunohistochemistry. Immunofluorescence analysis was performed as previously described (24). Briefly, the treated cells were immunostained with an antibody to SREBP-1 (1:100; cat. no. sc-365513, Santa Cruz Biotechnology, Inc.) at 4°C overnight and subsequently incubated with fluorochrome-conjugated secondary antibody (1:100; cat. no. ZF-0311; OriGene Technologies, Inc.) for 0.5 h at room temperature in darkness. The nuclei were counterstained with DAPI at room temperature for 20 min. The SREBP-1 expression was monitored by confocal microscopy. Quantitative evaluation of SREBP-1 nuclear intensity was performed with ImageJ (v 1.8, National Institutes of Health).

Glucose uptake assay. U251 (1×10^5 /cells/500 μ l) and U87 (1×10^5 /cells/500 μ l) glioma cells were plated in 48-well microplate and cultured at 37°C for 24 h and then treated with target compounds at given concentrations at 37°C for 24, 48 or 72 h. Subsequently, 50 μ M 2-NBDG (cat. no. 72987; MilliporeSigma) was added to the cells at 37°C for 1 h and the U251 and U87 glioma cells were washed in Hank's balanced salt solution buffer for three times. The fluorescent intensity was then measured using laser confocal microscopy at excitation and emission wavelengths of 467 and 542 nm, respectively.

Comparative expression and survival analysis. The preprocessed level 3 RNA-seq data and corresponding clinical information of cancer patients were collected from The Cancer Genome Atlas (TCGA) database (<http://cancergenome.nih.gov/>) and the normal samples RNA data were acquired from the Genotype-Tissue Expression (GTEx) databases (<https://www.gtexportal.org/>).

Statistical analysis. The experiments were independently performed in triplicate and the results were presented as mean values \pm standard deviation. Unpaired student's t-test was used to analyze the differences between two groups and one-way analysis of variance (ANOVA) followed by Tukey's test was used for the comparison among multiple groups. Patients were divided into high and low groups according to the 50% cut off point of GDNF and SREBP-1 expression and Kaplan-Meier survival analysis was used to analyzed significance between groups. All statistical analyses and experimental graphs were performed by GraphPad Prism version 8.0 software (GraphPad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

GDNF/RET signaling is upregulated in glioma and promotes lipid metabolism. The relative expression level of GDNF mRNA in normal brain and in low- and high-grade glioma tissues was determined by RT-qPCR. The results indicated that GDNF mRNA expression was upregulated in glioma compared to normal tissue (Fig. 1A). In addition, GDNF mRNA levels increased with pathological grade of glioma tissue (Fig. 1B). The GDNF mRNA levels between normal brain tissue and glioma tissue were then compared using RNA sequencing (RNA-seq) data from the GTEx database and The Cancer Genome Atlas (TCGA) database (<http://cancergenome.nih.gov/>). The results also showed that GDNF expression were upregulated in glioma compared to normal human tissue (Fig. 1C) and high GDNF gene expression was associated with poor prognosis in glioma (Fig. 1D).

In the presence of GDNF, SREBP-1 was activated (nSREBP-1) in a dose- and time-dependent manner. In addition, there was an increase in the protein levels of FASN, SCD1 and ACC, which are downstream targets genes of SREBP-1 (Fig. 1E and F). The immunofluorescence analysis showed that the nuclear fluorescence intensity of the SREBP-1 signal was significantly higher in U251 glioma cells treated with GDNF than in control cells (Fig. 1G). The RT-qPCR results showed that GDNF stimulation enhanced the SREBP-1 expression and activated the expression of SREBP-1 regulated genes involved in lipid metabolism (Fig. 1H). However, SREBP-1 expression in gliomas and its relationship with tumor malignancy remains to be elucidated.

The mRNA expression of SREBP-1 was more enriched in glioma than in normal human brain tissues, according to the RNA-seq data from the GTEx database and The Cancer Genome Atlas (TCGA) database (Fig. 1I). Therefore, it was decided to explore the prognostic value of SREBP-1 in gliomas based on the TCGA datasets. The results showed that glioma patients with higher SREBP-1 expression presented worse overall survival than those with lower SREBP-1 expression (Fig. 1J). Furthermore, the results of the present study showed that GDNF activates SREBP-1 through the RET/ERK signaling pathway (Fig. 1K). Therefore, GDNF pharmacologically blocked the activity of RET/ERK signaling with RPI-1 (GDNF/RET inhibitor), which significantly reduced the SREBP-1 activity (nSREBP-1; Fig. 1K). The MTT assay demonstrated that GDNF significantly promoted glioma cell proliferation in a dose- and time-dependent manner and that

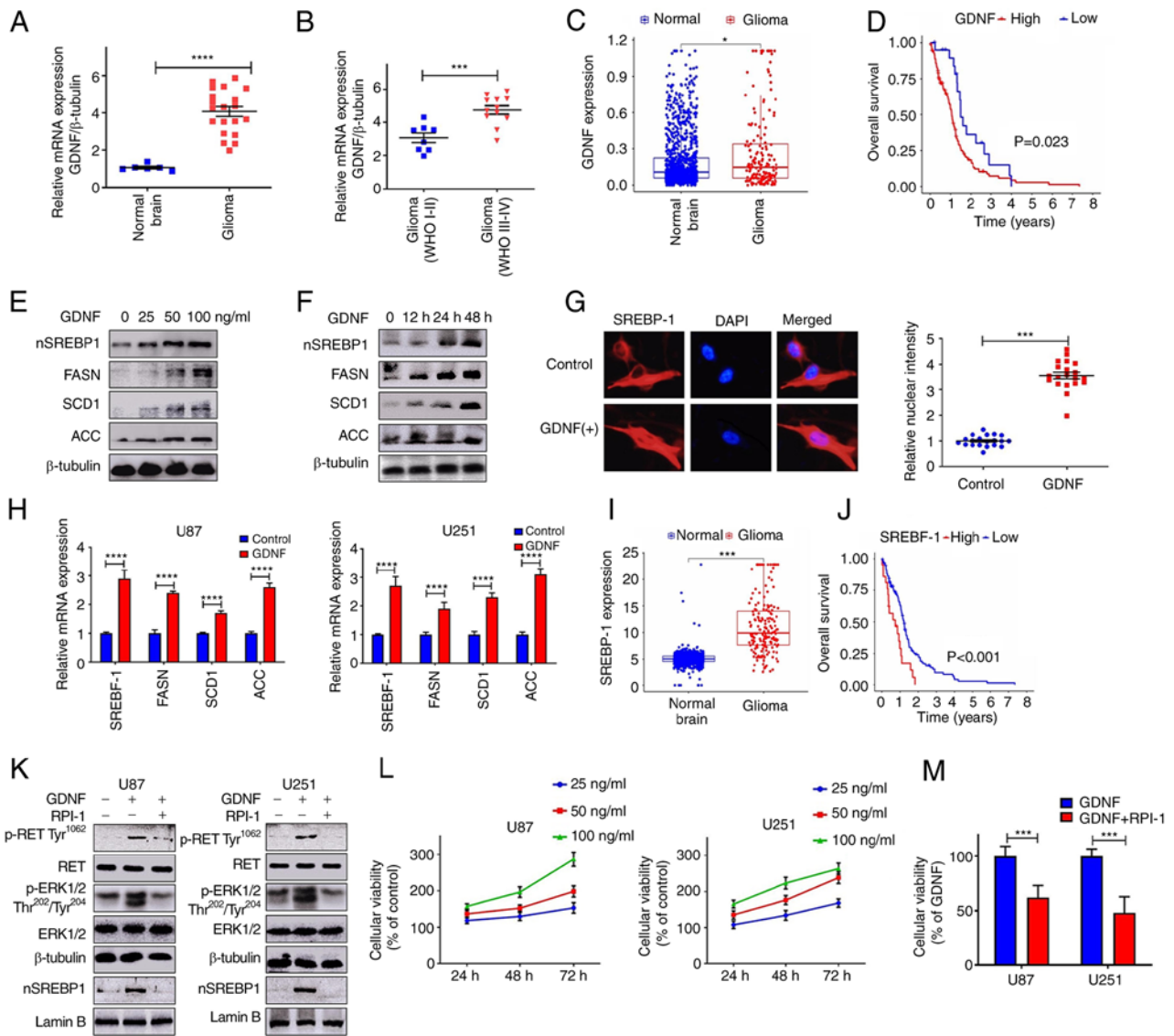


Figure 1. GDNF/RET signaling is upregulated in glioma and promotes lipid metabolism. mRNA expression of GDNF in normal brain tissues and glioma (A) RT-qPCR and (C) TCGA data analysis. (B) Association between GDNF expression and degree of malignancy (RT-qPCR). (D) Survival curves for patients with low and high GDNF mRNA expression levels in glioma (TCGA database). Western blot analysis of total cell lysates (FASN, SCD1, ACC) or nuclear extracts (nSREBP-1) from U251 and U87 glioma cells cultured in DMEM complete medium with (E) different dose of GDNF for 48 h or (F) with 50 ng/ml GDNF at indicated times. (G) Immunofluorescence staining of SREBP-1 (red) and DAPI (blue) from U251 glioma cells were cultured in DMEM complete medium and treated with 50 ng/ml GDNF for 48 h. Quantitative evaluation of SREBP-1 nuclear intensity with ImageJ (n=20). Images were captured at x200 magnification. (H) RT-qPCR analysis of mRNA levels in U251 and U87 glioma cells cultured in DMEM complete medium with or without GDNF (50 ng/ml) for 24 h. (I) SREBP-1 mRNA expression in normal brain tissues and glioma (TCGA data analysis). (J) Survival curves for patients with low and high SREBP-1 mRNA expression levels in glioma (TCGA database). (K) Western blot analysis of p-RET, p-ERK and nSREBP-1 levels in U251 and U87 glioma cells that were cultured in DMEM complete medium with or without 50 ng/ml GDNF in the presence or absence of 20 μ M RPI-1 (GDNF/RET inhibitor) for 48 h. (L) U251 and U87 glioma cells cultured in DMEM complete medium with different dose of GDNF for 48 h or with 50 ng/ml GDNF at indicated times. Relative viability of glioma cells detected by MTT assay. (M) Relative viability of glioma cell cultured in DMEM complete medium with 50 ng/ml GDNF in the presence or absence of 20 μ M RPI-1 for 48 h. Significance was determined by unpaired Student's t test (* P <0.01, *** P <0.001, **** P <0.0001). GDNF, glial-derived neurotrophic factor; RET, rearranged during transfection; RT-qPCR, reverse transcription-quantitative PCR; TCGA, The Cancer Genome Atlas; FASN, fatty acid synthase; SCD1, stearoylCoA desaturase-1; ACC, acetyl CoA carboxylase; SREBP-1, sterol regulatory element-binding protein-1; p-, phosphorylated.

the inhibition of RET/ERK signaling could significantly reverse this biological effect (Fig. 1L and M).

GDNF was overexpressed in glioma and was associated with poor clinical outcome and highly expressed GDNF promoted the expression of SREBP-1, which is a transcription factor with a central role in lipid metabolism. Accordingly, patients with high expression of SREBP-1 presented poor prognosis. Although the present study revealed that SREBP-1 was activated by the GDNF/RET/ERK signaling pathway,

the mechanisms underlying the oncogenic signaling to the SREBP-1 function remains to be elucidated.

GDNF/RET activates SREBP-1 via glucose-mediated hexosamine biosynthetic pathway. Tumorigenesis is associated with increased glucose consumption and lipogenesis. Studies have suggested that elevated GDNF/RET signaling is associated with enhanced glucose uptake or lipogenesis in tumorigenesis (21,22). The present study demonstrated that GDNF promoted lipid

metabolism by upregulating SREBP-1. GDNF also promoted glucose absorption (Fig. 2A and B) in a dose- and time-dependent manner and it pharmacologically blocked the activity of RET/ERK signaling with RPI-1, thereby significantly preventing glioma cells to absorb glucose (Fig. 2C). To investigate whether glucose was involved in SREBP-1 activation, U87 and U251 glioma cells were plated with GDNF with and without glucose and SREBP processing was analyzed by RT-qPCR, western blot and immunofluorescence microscopy. As shown in Fig. 2D and E, GDNF stimulation presented no effect on the activation of SREBP-1 in a glucose-free medium, despite the strong activation of the RET/ERK signaling pathway. By contrast, GDNF stimulation promoted SREBP-1 activity in the presence of glucose and it pharmacologically blocked the activity of RET/ERK signaling with RPI-1, which completely abolished the GDNF-mediated activation of SREBP-1 expression. The fluorescence imaging indicated that GDNF stimulation was unable to promote the nuclear translocation of SREBP-1 in the absence of glucose and the addition of glucose restored the GDNF-mediated SREBP-1 nuclear translocation (Fig. 2F). Although GDNF did not elevate SREBP-1 activity without glucose, RT-qPCR analysis showed that it still promoted SREBP-1 mRNA expression and was inhibited by the RET inhibitor RPI-1. However, there was no change in the downstream target gene expression of SREBP-1 (Fig. 2G). Combined with the results described above, this suggested that GDNF/RET/ERK promoted SREBP-1 mRNA, protein expression and glucose absorption and that glucose is important for the activation of SREBP-1.

To investigate the glucose function in SREBP-1 activation, glucose and its intermediate metabolites, namely N-acetylglucosamine (GlcNAc; HBP), lactate or pyruvate (glycolysis pathway), were added in a glucose-free medium to U251 and U87 glioma cells, respectively. The results showed that GlcNAc was as effective as glucose in enhancing SREBP-1 activity, whereas lactate and pyruvate presented no effect (Fig. 2H). GFPT is the rate-limiting enzyme of HBP and glioma cells were treated with azaserine (GFPT inhibitor). As expected, azaserine inhibited the glucose-mediated SREBP-1 activity, but did not inhibit the SREBP-1 activity mediated by GlcNAc (Fig. 2I). The addition of GlcNAc, which has been widely used to increase HBP production, restored SREBP-1 protein activity in U87 and U251 glioma cells, which was previously reduced by the azaserine treatment (Fig. 2J). However, HBP inhibition presented no effect on the expression of SREBP-1 mRNA (Fig. 2K). In addition, in both glioma cell lines tested, the toxicity of azaserine was at least partially reversed by GlcNAc supplementation (Fig. 2L). These results demonstrated that GDNF/RET can promote glucose absorption and subsequently activate SREBP-1 by accelerating HBP synthesis.

HBP promotes SCAP N-glycosylation and consequent activation of SREBP-1. Proteases cleaving SREBPs are activated by SCAP (10). In the present study, the knockdown of SCAP using siRNA reduced the GDNF- and glucose-mediated activation of SREBP-1 (Fig. 3A). Nohturfft *et al* (27) and Cheng *et al* (28) show that the N-glycosylation status of SCAP affects its protein function. However the NetNGlyc server prediction of glycosylation sites using artificial neural networks (<http://www.cbs.dtu.dk/services/NetNGlyc/>) showed that SCAP presented both N- and O-glycosylation

sites (Fig. 3B). UDP-N-acetyl glucosamine (UDP-GlcNAc), which is the end product of HBP, is the substrate for O- and N-glycosylations (29). Therefore, whether GDNF regulated SCAP levels by regulating its N- or O-glycosylation was investigated. Tunicamycin (inhibitor of N-glycosylation) and OSMI-1 (inhibitor of O-glycosylation) were added to U251 and U87 glioma cells, respectively, in a GDNF/glucose medium. As shown in Fig. 3C, tunicamycin inhibited the protein activity of SREBP-1, whereas OSMI-1 did not. Immunofluorescence analysis showed that in U251 glioma cells treated with tunicamycin, the nuclear fluorescence intensity of the SREBP-1 signal was significantly lower than in control cells and there was no clear change in the OSMI-1 group (Fig. 3D). To further confirm the function of SCAP N-glycosylation for the regulation of SREBP-1 activity, U251 glioma cells were cultured with GDNF with and without glucose. The N-glycosylation was investigated using PNGase F and the differences between glycosylated and deglycosylated proteins were evaluated by western blot tests. As shown in Fig. 3E, the treatment combining GDNF and glucose induced more total SCAP proteins and its glycosylated forms, which was associated with elevated SREBP-1 activation. As expected, GDNF- and glucose-mediated SCAP N-glycosylation and SREBP-1 activity were simultaneously inhibited by the RET inhibitor (RPI-1), GFPT inhibitor (azaserine) and N-glycosylation inhibitor (tunicamycin) (Fig. 3F-H). These results demonstrated that GDNF elevated the SREBP-1 activity through mechanisms involving the upregulation of SCAP N-glycosylation.

GDNF/RET signaling promotes glucose absorption by upregulating HIF-1. Although the results indicated that GDNF/RET/ERK signaling promotes glucose absorption and SREBP-1 activation, the mechanisms through which GDNF/RET promotes glucose absorption are still unknown. The western blot results showed that the hypoxia-inducible factor 1 (HIF-1) protein levels increased significantly when U251 and U87 glioma cells were treated with GDNF in glucose medium and the GDNF-induced changes in HIF-1 expression were associated with SREBP-1 activation (Fig. 4A). HIF-1 is crucial for the reprogramming of cancer metabolism as it activates the transcription of genes that encode glucose transporters and glycolytic enzymes (30). In the present study, the knockdown of HIF-1 using siRNA reduced the GDNF-mediated glucose absorption (Fig. 4B), which was associated with the terminated SREBP-1 activation (Fig. 4C). Although the present study showed again that GDNF induced SREBP-1 activation depended on upregulated RET/ERK/HIF-1 signaling pathway, but knockdown of HIF-1 using siRNA had no effect on GDNF induced RET/ERK expression (Fig. 4C). Immunofluorescence analysis also showed that the nuclear fluorescence intensity of the SREBP-1 signal was significantly lower in U251 glioma cells treated with siHIF-1 than in the cells of the GDNF group (Fig. 4D). RT-qPCR analysis showed that knockdown of HIF-1 using siRNA reduced the SREBP-1 downstream target gene expression, but presented no apparent effect on SREBP-1 mRNA expression (Fig. 4E). In addition, GlcNAc supplementation restored the SREBP-1 protein activity (Fig. 4F) and cell toxicity (Fig. 4G) in U251 and U87 glioma cells, which were previously reduced by the siHIF-1 treatment. Therefore, it was hypothesized that the GDNF/RET/ERK signaling pathway

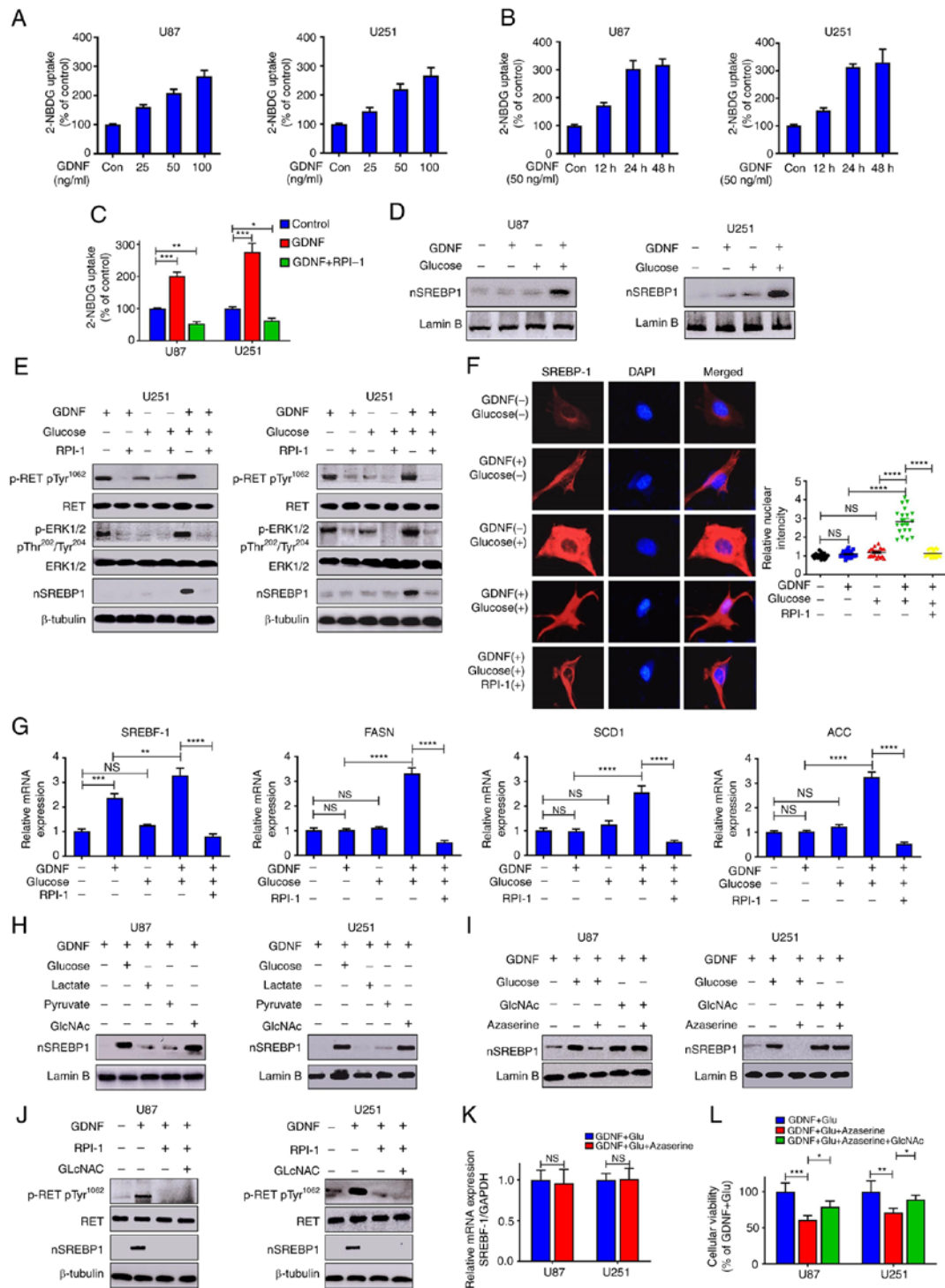


Figure 2. GDNF/RET signaling pathway promotes glucose absorption and subsequently activates SREBP-1 through HBP. U251 and U87 glioma cells cultured in DMEM complete medium with (A) different dose of GDNF for 48 h or with (B) 50 ng/ml GDNF at indicated times and (C) in the presence or absence of 20 μ M RPI-1 (GDNF/RET inhibitor). Glucose uptake ability of glioma cells evaluated by fluorescent glucose 2-NBDG. (D and E) Western blot analysis of total cell lysates (RET/p-RET, ERK/p-RET) or nuclear extracts (nSREBP-1) from U251 and U87 glioma cells cultured in DMEM glucose-free medium, treated with 50 ng/ml GDNF, 10 mM glucose or in combination with 20 μ M RPI-1 for 48 h. (F) Immunofluorescence staining of SREBP-1 (red) and DAPI (blue) from U251 glioma cells cultured in DMEM glucose-free medium and treated with 50 ng/ml GDNF or 25 mM glucose or in combination with 20 μ M RPI-1 for 48 h. Quantitative evaluation of SREBP-1 nuclear intensity using ImageJ (n=20). Images were captured at x200 magnification. (G) RT-qPCR analysis of mRNA levels in U251 and U87 glioma cells cultured in DMEM glucose-free medium and treated with 50 ng/ml GDNF, 10 mM glucose, or 20 μ M RPI-1 for 24 h. (H and I) Western blot analysis of nuclear extracts (nSREBP-1) from U251 and U87 glioma cells cultured in DMEM glucose-free medium with or without 10 mM glucose, 10 mM lactate, 10 mM pyruvate, or 20 mM HBP or in combination with 20 μ M azaserine (HBP inhibitor) for 48 h. (J) Western blot analysis of total cell lysates (RET/p-RET) or nuclear extracts (nSREBP-1) from U251 and U87 glioma cells cultured in DMEM glucose-free medium, treated with or without GDNF or in combination with 20 μ M RPI-1 or 20 mM HBP for 48 h. (K) RT-qPCR analysis of mRNA levels in U251 and U87 glioma cells cultured in DMEM complete medium and treated with 50 ng/ml GDNF and in the presence or absence of 20 μ M azaserine for 24 h. (L) U251 and U87 glioma cells cultured in DMEM complete medium treated with 50 ng/ml GDNF and in combination with 20 μ M azaserine or 20 mM HBP for 48 h. Relative viability of glioma cells detected by MTT assay. (* P <0.05; ** P <0.01; *** P <0.001; **** P <0.0001; NS: not significant). GDNF, glial-derived neurotrophic factor; RET, rearranged during transfection; SREBP-1, sterol regulatory element-binding protein-1; HBP, N-acetylglucosamine; p-, phosphorylated; RT-qPCR, reverse transcription-quantitative PCR.

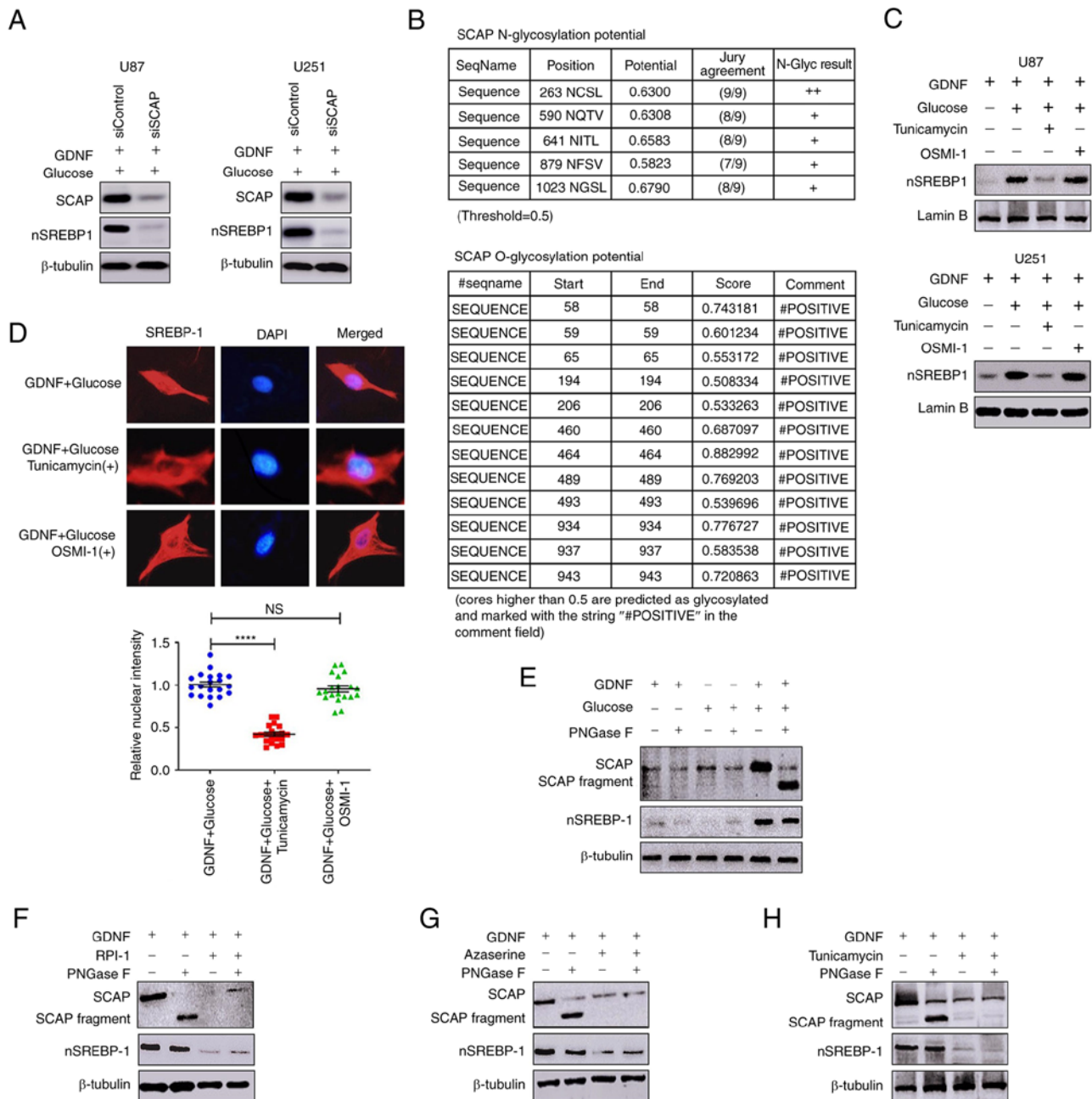


Figure 3. HBP regulates SREBP-1 activity by promoting SCAP N-glycosylation. (A) Western blot analysis of SCAP and nuclear SREBP-1 levels in U251 and U87 glioma cells after SCAP gene silenced by siRNA, cultured in DMEM complete medium treated with 50 ng/ml GDNF for 48 h. (B) NetNGlyc server showed that SCAP presents both N- and O-glycosylation. (C) Western blot analysis of nuclear extracts from U251 and U87 glioma cells cultured in DMEM glucose-free medium, treated with or without 50 ng/ml GDNF or in combination with tunicamycin (N-glycosylation inhibitor) (2 μ g/ml) or 20 μ M OSMI-1 (O-glycosylation inhibitor) for 48 h. (D) Immunofluorescence staining of SREBP-1 (red) and DAPI (blue) from U251 glioma cells cultured in DMEM complete medium, treated with 50 ng/ml GDNF or in combination with 2 μ g/ml tunicamycin or 20 μ M OSMI-1 for 48 h. Quantitative evaluation of SREBP-1 nuclear intensity using ImageJ (n=20). Images were captured at x200 magnification. (E) Western blot analysis of total cell lysates (SCAP N-glycosylation levels) or nuclear extracts (nSREBP-1) from U251 glioma cells cultured in DMEM glucose-free medium, treated with 50 ng/ml GDNF or/and 10 mM glucose for 48 h. Western blot analysis of total cell lysates (SCAP N-glycosylation levels) or nuclear extracts (nSREBP-1) from U251 glioma cells cultured in DMEM complete medium, treated with 50 ng/ml GDNF or in combination with (F) 20 mM RPI-1 (GDNF/RET inhibitor), (G) 20 μ M azaserine (HBP inhibitor) and/or (H) 2 μ g/ml tunicamycin (N-glycosylation inhibitor) for 48 h. Significance was determined by unpaired Student's t test (****P<0.0001; NS: not significant). HBP, N-acetylglucosamine; SREBP-1, sterol regulatory element-binding protein-1; SCAP, SREBP cleavage-activating protein; GDNF, glial-derived neurotrophic factor.

regulated the expression of SREBP-1 and HIF-1, whereas the SREBP-1 activity is regulated by HIF-mediated glucose absorption (Fig. 5A).

SREBP-1 suppression inhibits GDNF-induced glioma cell growth. SREBP-1 functions as a transcription factor that

activates specific genes involved in cholesterol and fatty acid metabolism. SREBP-1 and its downstream target gene can be effectively regulated by GDNF and the knockdown of SREBP-1 can reduce GDNF-mediated SREBP-1 and its downstream target gene expression (Fig. 5B) and it can completely reverse the GDNF-induced cell proliferation (Fig. 5C).

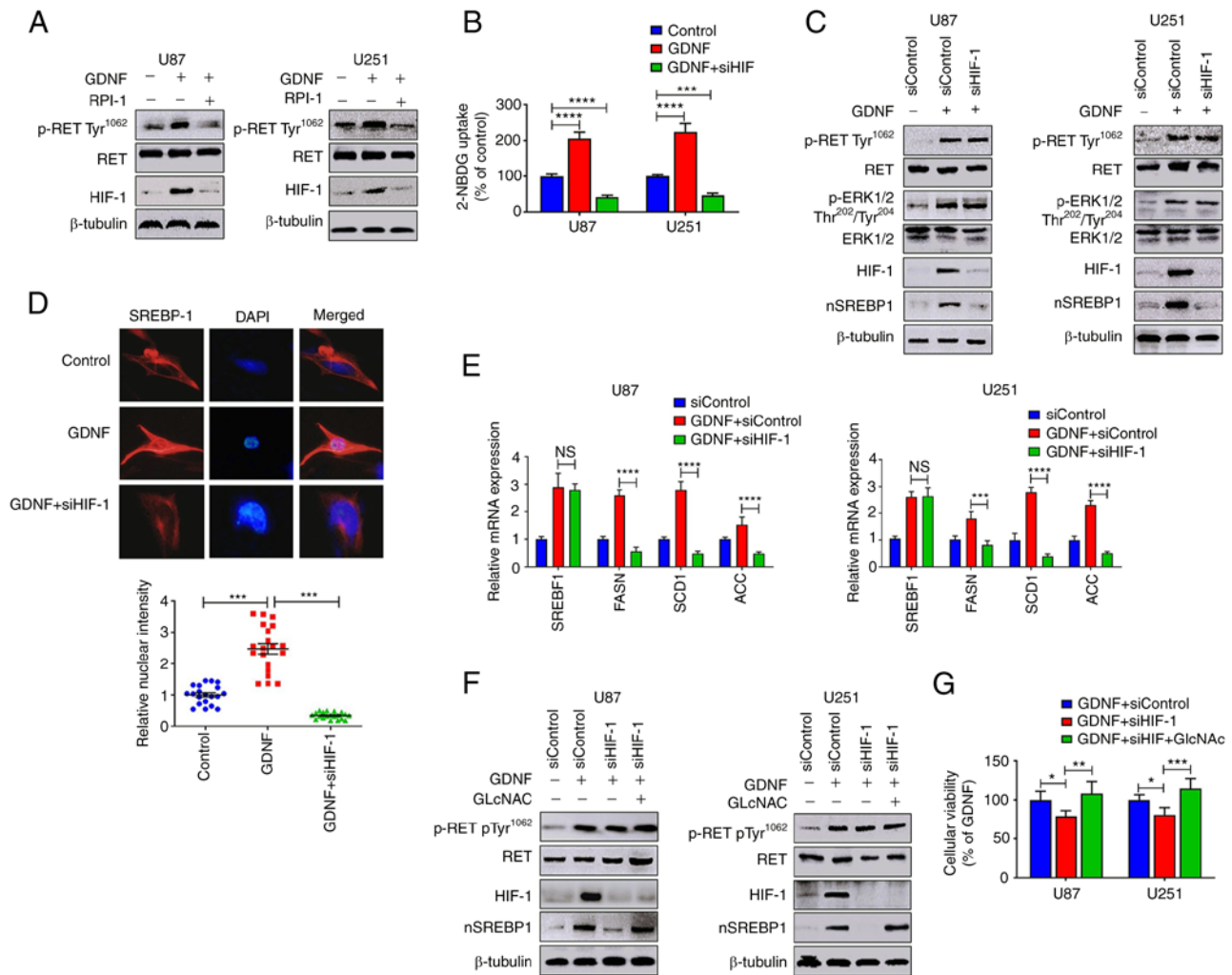


Figure 4. GDNF/RET Signaling promotes glucose absorption by upregulating HIF-1. (A) Western blot analysis of p-RET, HIF-1 levels in U251 and U87 glioma cells that were cultured in DMEM complete medium with or without 50 ng/ml GDNF in the presence or absence of 20 μ M RPI-1 (GDNF/RET inhibitor) for 48 h. U251 and U87 glioma cells were transfected with siHIF-1 for 48 h and cultured in DMEM complete medium with or without 50 ng/ml GDNF; (B) Glucose uptake ability of glioma cells was evaluated by fluorescent glucose 2-NBDG; (C) Protein expression determined by western blotting; (D) Immunofluorescence staining of SREBP-1 (red) and DAPI (blue) and quantitative evaluation of SREBP-1 nuclear intensity with ImageJ (n=20). Images were captured at x200 magnification. (E) Reverse transcription-quantitative PCR analysis of mRNA levels in U87 and U251 glioma cells following knock down of HIF-1 and cultured in DMEM complete medium with or without 50 ng/ml GDNF for 24 h. U251 and U87 glioma cells transfected with siHIF-1 for 48 h and cultured in DMEM complete medium with 50 ng/ml GDNF or 20 mM HBP. (F) Protein expression was determined by western blotting. (G) Relative viability of glioma cells detected by MTT assay. Significance was determined by unpaired Student's t test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS: not significant). GDNF, glial-derived neurotrophic factor; RET, rearranged during transfection; HIF-1, hypoxia-inducible factor 1; p-, phosphorylated; RET, rearranged during transfection; HBP, N-acetylglucosamine.

Fatostatin, a chemical inhibitor of the SREBP pathway (31), shows high antitumor activity for a number of cancers (32,33), but its effects on glioma cells are largely unknown. The present study showed that fatostatin reversed the GDNF induced SREBP-1 activity (Fig. 5D). The nuclear fluorescence intensity of the SREBP-1 signal was significantly lower in U251 glioma cells treated with GDNF and fatostatin than in GDNF-treated cells (Fig. 5E). In addition, in both glioma cell lines, GDNF-induced cell activity was completely reversed by the supplementation with fatostatin, which inhibited the growth of glioma cells in a dose- and time-dependent manner (Fig. 5F).

It is often ineffective to treat cancer only using traditional methods involving the inhibition of a single oncogene pathway or enzyme (7). Therefore, the combination of drugs and chemotherapeutic agents is becoming a popular therapeutic option. Accordingly, because GDNF/RET regulates SREBP-1

activity, the present study investigated if the GDNF/RET inhibitor could enhance the cytotoxicity of the SREBP-1 inhibitor. A proliferation assay was performed in which glioma cells were treated with RPI-1 and fatostatin at a constant ratio according to their respective IC₅₀. The combination of RPI-1 and fatostatin provided a antiproliferative effect stronger than that of single agents and showed synergistic effect when they were used in combination [combination index (CI)<1.0; Fig. 5G]. These results suggest that the inhibition of SREBP-1 combined with GDNF/RET signaling pathway might be a new therapeutic method for glioma.

Discussion

Despite the increase in life expectancy for patients with GBM under optimal treatment, current therapy options are

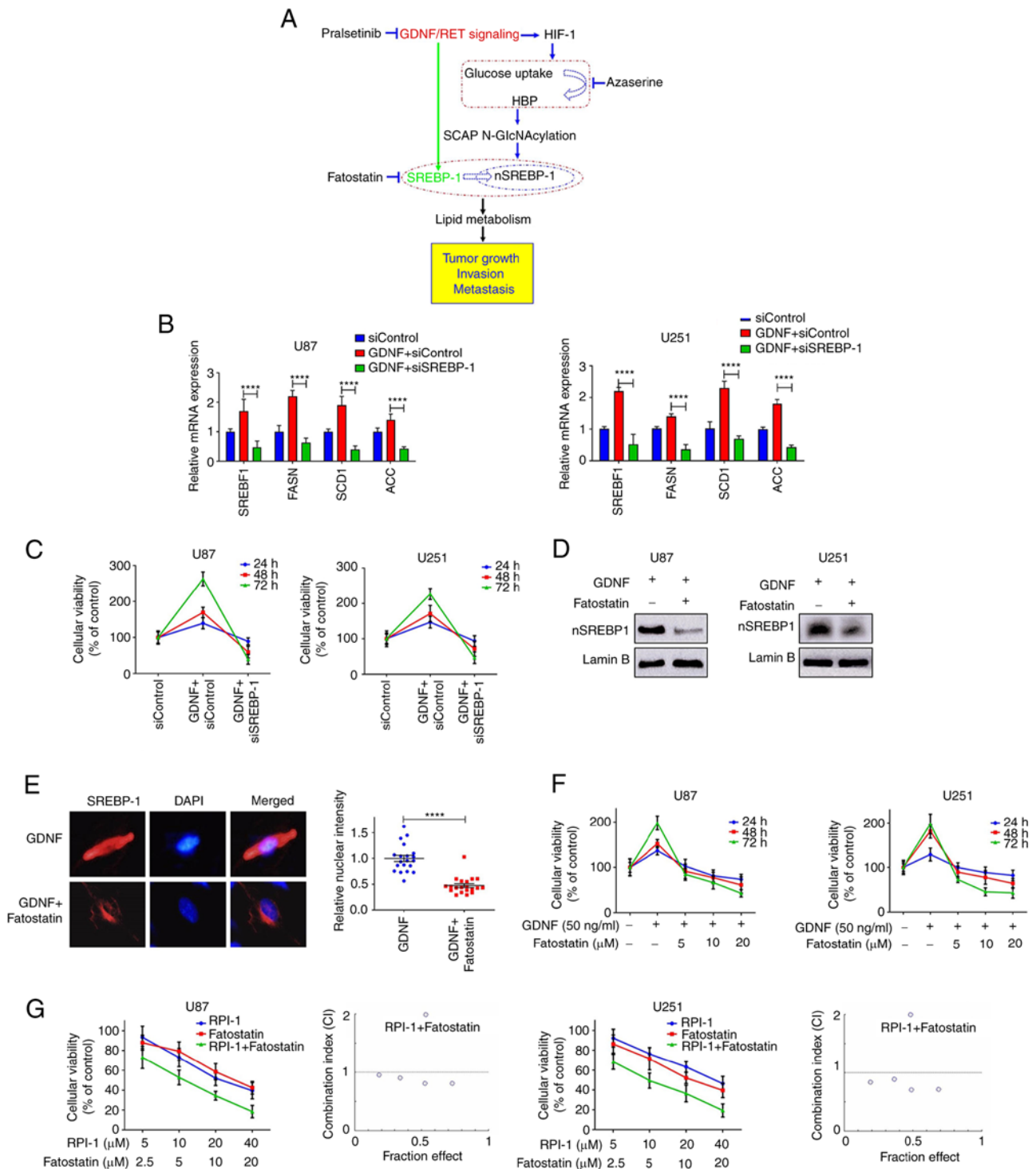


Figure 5. SREBP-1 suppression inhibits GDNF-induced glioma cell growth. (A) Proposed mechanism of SREBP-1 regulation by GDNF. U251 and U87 glioma cells transfected with siSREBP-1 for 48 h and cultured in DMEM complete medium with or without 50 ng/ml GDNF. (B) mRNA levels were analyzed by reverse transcription-quantitative PCR and (C) Relative viability of glioma cells were detected by MTT assay. U251 and U87 glioma cells cultured in DMEM complete medium with 50 ng/ml GDNF in the presence or absence of 20 μ M fatostatin (SREBP-1 inhibitor) for 48 h; protein expression was determined by (D) western blotting and (E) immunofluorescence staining for SREBP-1 (red) and DAPI (blue) and quantitative evaluation of SREBP-1 nuclear intensity provided using ImageJ (n=20). Images were captured at x200 magnification. (F) U251 and U87 glioma cells cultured in DMEM complete medium with 50 ng/ml GDNF and treated with different dose of fatostatin for 48 h or with 20 μ M fatostatin at indicated times; relative viability of glioma cells were detected by MTT assay. (G) U251 and U87 glioma cells cultured in DMEM complete medium with 50 ng/ml GDNF and treated with different dose of RPI-1 and fatostatin; Relative viability of glioma cells were detected by MTT assay; the Fa-CI plots show the combination index (CI) value for each fractional effect; curves were generated using CalcuSyn 1.0 software (CI <1, synergism; CI=1, additive effect; CI >1, antagonism). (****P<0.001; NS: not significant). SREBP-1, sterol regulatory element-binding protein-1; GDNF, glial-derived neurotrophic factor; CI, combination index.

considered palliative and GBM is essentially an incurable disease. Therefore, new treatments for GBM have been widely

investigated. It is unlikely that inhibiting single oncogene pathways or enzymes is sufficient to harness the full potential

of a targeted therapy because of the heterogeneity of cancer cells. A common feature of cancer cells is their ability to rewire their metabolism to sustain the production of adenosine triphosphate and macromolecules needed for cell growth, division and survival (5). Particularly, the importance of altered lipid metabolism in cancer patients has received renewed interest because, in addition to their main role as structural components of the membrane matrix, they are important secondary messengers and can serve as fuel sources for energy production (7,34). Therefore, research focusing on the complex correlation between oncogenic signaling and dysregulated lipid-metabolism has a great potential to uncover novel metabolic vulnerabilities and improve the efficacy of targeted therapies.

GDNF is a family of neurotrophins with similarities to the transforming growth factor β regulatory proteins (11,35) and it has been identified as a potent neurotrophic factor for a variety of neuronal cell populations (36). GDNF is biosynthesized in glial cells and might be relevant to the development of gliomas (37). The present study showed that GDNF highly expressed in glioma is associated with poor clinical outcome and promoted glioma cell proliferation through RET/ERK signaling pathway. Cruceru *et al* (38) show that high expression of ERK can also promote the differentiation and metastasis of glioma. A previous study shows that ERK promotes lipid metabolism (20) and SREBPs are key transcriptional regulators of lipid metabolism and cellular growth (39,40). The results of the present study showed that there was a clear correlation between GDNF/RET/ERK signaling and SREBP-1 expression in glioma cells and revealed that patients with high SREBP-1 expression also have a poor prognosis. Therefore, it is important to clarify the relationship and mechanism between oncogenic signaling (GDNF/RET/ERK) and glioma cell lipid metabolism.

The inactive precursors of SREBPs reside in ER membranes bound with SCAP, the present study showed that GDNF/RET signaling pathway contributed SREBP-1 transfer to the cell nucleus and the activated SREBP-1 promoted FASN, SCD1 and ACC expression. However, the regulation mechanism of the SREBP-1 activity in glioma remains to be elucidated. The GDNF upregulation and RET ligand-receptor interaction might participate in the glucose-induced cancer progression (21). Cheng *et al* (28) suggest that glycosylation stabilizes SCAP and reduces its association with Insig-1, thereby allowing the movement of SCAP/SREBP to the Golgi bodies and the consequent proteolytic activation of SREBP. Although NetNGlyc server predicted that SCAP presented both N- and O-glycosylation sites, the results of the present study showed that only SCAP N-glycosylation plays a critical role in SREBP-1 activity. UDP-GlcNAc, the end product of glucose metabolism via HBP, is the substrate for O- and N-glycosylations. In order to study how GDNF regulates SCAP N-glycosylation, further research was conducted. The study showed that GDNF promoted glucose absorption through RET/ERK signaling pathway and that GDNF had no effect on the activation of SREBP in glucose-free medium, suggesting that glucose served a crucial role in the GDNF-mediated SREBP-1 activation. GDNF/RET/ERK signaling was highly expressed in glioma cells and promoted the expression of HIF-1, which has been shown to play a crucial role in the

reprogramming of cancer metabolism by activating transcription of genes encoding glycolytic enzymes and glucose transporters (30,41). Although the results of the present study do not confirm this, it determined that HIF-1 serves an important role in the glucose-mediated SREBP-1 activation and knockdown of HIF-1 using siRNA reduced the GDNF- and glucose-mediated SREBP-1 activation. Highly expressed HIF-1 accelerated HBP and promoted N-glycosylation of SCAP and consequent activation of SREBP-1. GDNF-mediated SREBP-1 activity was simultaneously inhibited by the RET inhibitor (RPI-1), GFPT inhibitor (azaserine) and N-glycosylation inhibitor (tunicamycin). Although the present study helped clarify the relationship between GDNF/RET/ERK signaling and dysregulated glycolipid-metabolism, the regulatory pathways responsible for the activation of these processes remain unclear because the established carcinogenesis mechanisms cannot fully explain multiple metabolic rearrangements in glioma cells, such as how GDNF/RET/ERK promotes SREBP-1 mRNA expression and whether GDNF mediated HIF-1 expression is associated with glioma cell microenvironment such as hypoxia.

Due to the high number of genetic alterations observed in glioma, a number of which occur concurrently, combining anticancer drugs can lead to a synergistic toxic effect against tumor cells and reduce damage to normal cells. GDNF/RET and SREBP-1 are both crucial for cancer growth (8,11,34). The results of the present study showed that the combination of GDNF/RET inhibitor RPI-1 treatment and SREBP-1 inhibitor fatostatin induced a synergistic anti-tumoral response in glioma cells. Current therapy options for glioma patients are considered palliative and the results of the present study provided a background to improve the efficacy of targeted therapies for these patients. The development of such therapies are important especially considering the heterogeneity and mutability of cancer cells and the current inhibition of a single oncogene pathway or enzyme by traditional treatments.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZYY and YKX conceived the project and planned the experiments. ZYY, MW and HJL performed experiments. ZYY, WZL and YKX analyzed results. ZYY and WZL wrote the paper and edited the manuscript. ZYY, HJL and YKX confirmed the authenticity of all data. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University (approval no. 2020-KY-155).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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