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SUMO-Specific peptidase 3 mediates the SUMO3 modification of BECN1 to repress cell autophagy in gliomas

Running title: SENP3 affects glioma cell autophagy via BECN1

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Abstract

Objective: SUMO Specific Peptidase 3 (SENP3) is involved in the occurrence and development of various cancers. However, its effects on gliomas have been barely reported. Herein, this research was designed to probe the potential mechanisms of SENP3 mediating beclin-1(BECN1) SUMO3 modification in autophagy in gliomas.

Methods: SENP3 expression in gliomas was analyzed through bioinformatic information. Clinical samples of glioma tissues were collected and frozen. SENP3 expression was evaluated with western blot. In glioma cells, autophagy- and apoptosis-related proteins, viability, and apoptosis were assessed with western blot and immunofluorescence, the cell counting kit-8, and flow cytometry, respectively. The SUMO modification of BECN1 and interactions between BECN1 and PIK3C3 were identified with Ni-NTA pull-down and co-immunoprecipitation assays, respectively. The tumor formation assay was carried out in nude mice for in vivo validation.

Results: Bioinformatics analysis predicted the overexpression of SENP3 in gliomas, which was confirmed in clinical samples and glioma cells. SENP3 silencing promoted autophagy and apoptosis and inhibited viability in glioma cells, which was counteracted by further autophagy inhibition. Mechanistically, SENP3 facilitated BECN1 deSUMOylation to mediate the SUMO3 modification of BECN1, thus impeding the formation of BECN1-PIK3C3 complexes. The loss of the SUMO part in BECN1 lowered the protein expression of LC3 and the value of LC3B II/LC3B I in glioma cells. Additionally, SENP3 silencing boosted autophagy and repressed tumor growth in mice, which was neutralized by further autophagy repression.

Conclusion: SENP3 fosters the deSUMOylation of BECN1 to block the formation of BECN1-PIK3C3 complexes, thus restraining glioma cell autophagy.

Key words: SUMO-Specific Peptidase 3; BECN1-PIK3C3; SUMO; Gliomas; Autophagy
**Introduction**

Gliomas are the most frequently occurring primary tumor of the central nervous system, representing the majority of malignant brain tumors (Chen et al., 2017; Gusyatiner et al., 2018). The brain microenvironment, age-associated changes in the cell of origin, repressed immunity surveillance, and mutation accumulation may increase the risk of glioma occurrence (Cahill et al., 2018). Gliomas are very refractory and current therapies exert limited effects on improving patient prognosis (Xu et al., 2020). Hence, research for innovative therapeutic directions is warranted.

Autophagy is a cellular process that sequesters proteins and damaged or aged organelles within double-membrane autophagosomes, resulting in component degradation (Onorati et al., 2018). Autophagy exerts vital functions in various biological processes, and its dysregulation was reported to be related to different human diseases, including neurodegenerative diseases, cardiovascular diseases, and cancers (Cheng, 2019; Chung et al., 2020). Notably, the regulation of autophagy was known to be relevant to the progression of gliomas (Ulasov et al., 2018). However, the specific mechanism of autophagy in gliomas warrants further investigation.

SUMOylation, a reversible modification at the post-translational level, is an important mechanism implicated in the pathophysiological processes of diseases (Chang et al., 2020). The promotion of SUMOylation was reported to facilitate glioblastoma (GBM) growth (Zhang et al., 2020). SUMOylation can be erased by Small Ubiquitin-like Modifier (SUMO)-specific proteases (SENPs), whose aberrant regulation is involved in cancer development (Tokarz et al., 2021). Among SENPs, SUMO-Specific Peptidase 3 (SENP3) is a deSUMOylating enzyme that manipulates numerous pathways and contributes to disease progression (Wang et al., 2022). Furthermore, SENP3 has been shown to influence protein deSUMOylation, promoting the growth and development of bladder and gastric cancers (Ren et al., 2014; Li et al., 2022). Of note, a prior study demonstrated the upregulation of SENP3 in GBM (Li and Meng, 2021). In addition, SENP3 was found to have an impact on autophagy in hepatic cells (Liu et al., 2020), implying a potential involvement of SENP3 in glioma cell autophagy.
BECN1 (beclin-1) is an indispensable protein in the modulation of autophagy, which is abnormally expressed in several kinds of tumors, and influences tumor development (Li et al., 2021; Sun et al., 2015). As reported, the BECN1 and Phosphatidylinositol 3-Kinase Catalytic Subunit Type 3 (PIK3C3) complex plays a part in initiating autophagy (Xu et al., 2023). It was also documented that BECN1-independent autophagy is related to the modulation of GBM cell growth (Ding et al., 2021). Moreover, prior research revealed that SENP3 was involved in the deSUMOylation of BECN1, thus affecting the formation of BECN1-PIK3C3 in cells (Liu et al., 2020). Based on the above findings, a hypothesis was proposed that SENP3 might mediate BECN1-PIK3C3 formation via SUMOylation to control glioma development. Hence, our research was conducted to probe the specific mechanism of SENP3 in glioma cell autophagy by modulating BECN1-PIK3C3 formation.

Materials and Methods

Bioinformatics

Unified and standardized pan-cancer datasets The Cancer Genome Atlas, TARGET, and Genotype-Tissue Expression (PANCAN, N=19131, G=60499) were downloaded from The University of California Santa Cruz (UCSC) database (https://xenabrowser.net/). The expression data of ENSG00000161956 (SENP3) in each sample, including Solid Tissue Normal, Primary Solid Tumor, Primary Tumor, Normal Tissue, Primary Blood-Derived Cancer-Bone Marrow, and Primary Blood-Derived Cancer - Peripheral Blood samples, were extracted from these datasets. Each expression value was treated with log2 (x+0.001) replacement. After excluding cancer types with less than three samples, expression data for 34 cancer types were acquired. The expression in differences between normal and tumor samples from each cancer type was calculated with the use of R software (version 3.6.4). Unpaired Wilcoxon Rank Sum and Signed Rank Tests were employed to analyze significant differences.
Collection of clinical samples

Forty glioma tissue samples resected during neurosurgeries and ten nontumorous brain tissue samples (control) resected during surgical decompression for traumatic brain injury were collected in Liuyang Jili Hospital between 2020 and 2022. Inclusion criteria were as follows: patients with major pathological lesions in the midbrain, pontine, medulla, and oblongata diagnosed with glioma through histopathological analysis and who did not receive chemotherapy, radiotherapy, or other treatments. These tissue samples were frozen in liquid nitrogen for later use. This research was approved by the Ethics Committee of Liuyang Jili Hospital (ethics number: 202207015; approval date: July 15, 2022), followed the Declaration of Helsinki, and written informed consent was obtained from all patients included.

Construction of lentiviruses

Lentivirus silencing vectors [short hairpin RNA (sh)] pSIH1-H1-cop green fluorescent protein (GFP) (SI501A-1, System Biosciences, Palo Alto, CA, USA) were purchased for the construction of lentivirus-based SENP3 silencing vectors. Lentiviruses were identified in cell supernatants obtained 48 hours after using lentivirus packaging kits (A35684CN, Invitrogen, Carlsbad, CA, USA) to package SENP3 silencing or negative control (NC) lentivirus particles into HEK-293T cells. The titer was $1 \times 10^8$ TU/mL.

Culture and grouping of cells

The HA1800 (MZ-0843) human normal astroglia cell line and the TJ905 (MZ-1484), U138 (MZ-2452), and LN-229 (MZ-1620) glioma cells were obtained from MINGZHOUBIO (Zhejiang, China) and cultured in RPMI 1640 media containing 10% fetal bovine serum, in an incubator (37°C, 5% CO₂). These cells were then digested by 0.25% trypsin for cell passage. They were seeded in 6-well plates ($3 \times 10^5$ cells/well) and then harvested when cell confluence reached 70%-80%.

Cells were assigned into the following groups: sh-NC (transfected with sh-NC lentivirus vectors), sh-SENP3 (transfected with sh-SENP3 lentivirus vectors), sh-SENP3 + chloroquine (CQ,
autophagy inhibitor, HY-17589A, MedChemExpress LLC, Shanghai, China) (transfected with sh-SENP3 lentivirus vectors and with 10 µM CQ treated), and sh-SENP3 + dimethyl sulfoxide (DMSO) (transfected with sh-SENP3 lentivirus vectors and with an equal volume of DMSO treated) (Liu et al., 2020). The transfection sequence of the sh-SENP3 lentiviral vector was CATTGGTCCCTCATCTCTGTT.

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

Total RNA was extracted from cell or tissue samples with the use of Trizol (15596026, Invitrogen), followed by transcription into cDNAs with an RT kit (RR047A, Takara, Otsu, Shiga, Japan). Primers were synthesized by Shanghai Sangon (Shanghai, China) and the specific primer sequences of SENP3 were forward: 5'-ATCCACCTGGAGGTGCATTGGT -3' and reverse: 5'-TCTTTACCGCCTCTGCCTGTAG-3'. The sample was treated with the 2 × SYBR Green PCR Master mix kits (SR1110, Solarbio, Beijing, China) and underwent qRT-PCR reactions in a real-time fluorescence quantitation PCR instrument (ABI7500, ABI, Foster City, CA, USA). The 20 µL reaction system was made up of 9 µL SYBR Mix, 0.5 µL forward primers, 0.5 µL reverse primers, 2 µL cDNAs, and 8 µL RNase Freed H2O. Three duplicate wells were set up for each sample and the Ct value of each well was recorded, with β-actin as the internal reference. The 2^ΔΔCt method was employed for the calculation of the relative expression of products. Each experiment was repeated in triplicate.

**Western blot**

In strict accordance with the manufacturer’s instructions, 4°C precooled-Radio-Immunoprecipitation assay (RIPA) cell lysis buffers (R0010, Solarbio) containing phenylmethylsulfonyl fluoride were utilized to extract the total proteins of cells or tissues. The protein concentration of each sample was measured with bicinchoninic acid kits (20201ES76, Yeasen Company, Shanghai, China). Proteins were transferred to a polyvinylidene fluoride
membrane (Millipore, Billerica, MA, USA) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and sealed for 1 h in 5% bovine serum albumin at room temperature. Subsequently, primary antibodies anti-rabbit SENP3 [#5591, 1:1000, Cell Signaling Technologies (CST), Beverly, MA, USA], microtubule-associated protein 1 light chain 3 (LC3, #43566S, 1:1000, CST), B-cell lymphoma-2 (Bcl-2, #3498, 1:1000, CST), Bcl-2-Associated X (Bax. #2772, 1:1000, CST), GFP (#2555, 1:1000, CST), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ab8245, 1:5000, Abcam, Cambridge, UK) were added dropwise to the membranes and incubated overnight at 4°C. Following washing with Tris-buffered saline with Tween 20 (TBST), horseradish peroxidase-labeled goat-anti-rabbit secondary antibody Immunoglobulin G (IgG, ab6721, 1:5000, Abcam) liquid was supplemented for 1h culture at room temperature. Next, membranes were washed and placed in electrogenerated chemiluminescence reaction liquid (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) for the developing reaction to proceed at room temperature. After developing and fixing (Bio-Rad ChemiDoc™), the results were observed.

**Immunofluorescence**

After three PBS washes, the slide containing TJ905 cells on the plate was fixed in 4% paraformaldehyde for 12 min. After 5-min antigen retrieval in sodium citrate buffers (C1010, Solarbio), normal goat serum (SL038, Solarbio) was added dropwise to the slide, followed by 30-min blocking at room temperature. After that, primary antibody LC3 (autophagosome marker) anti-rabbit antibody (1:1000, ab48394, Abcam) was added for overnight culture at 4°C. Next, cells were incubated with secondary antibody goat-anti-rabbit IgG (Alexa Flug 488, 1:1000, ab150077, Abcam) for 1h avoiding light at room temperature. After 5-min treatment with nuclear marker 4',6-Diamidino-2-Phenylindole (C0065, Solarbio) away from the light, micrographs were captured using a fluorescence microscope (Olympus BX51, Olympus Corp, Tokyo, Japan), and Image J software was applied for quantitative analysis of fluorescence intensity.
Development of plasmids and treatment of gene mutations

GFP-BECN1-amplified BECN1 fragments were inserted in the HindIII and KpnI sites of p3xFLAG-CMV-10 plasmids (E7658, Sigma-Aldrich, St. Louis, MO, USA) to develop Flag-BECN1 plasmids, obtained from Transheep (Shanghai, China). Based on the Flag-BECN1 construct, QuikChange mutagenesis kits (210, 518, Agilent Technologies, Santa Clara, CA, USA) were used to generate the Flag-BECN1 Lys to Arg mutant construct, K380R, through site-directed mutagenesis, namely the SUMO partially-deleted BECN1 mutation.

Ni-nitrilotriacetic acid (Ni-NTA) pull-down assay

GFP-SENP3, Flag-BECN1, and RH-SUMO3 (obtained from Transheep, Shanghai, China) were transected in TJ905 cells, and after 48h cells were scrapped from the surface in lysis buffers as per the protocols. Afterward, cell lysates were supplemented with Ni²⁺-NTA agarose resin, shaken, and incubated overnight at 4°C. After rinsing the resin thoroughly with four different kinds of washing liquids (binding buffer, wash buffer, elution buffer, and stripping buffer) at room temperature, the samples were eluted using an elution buffer, and SDS-PAGE and western blot detection were performed. Anti-RH (34650) antibody was purchased from Qiagen (Düsseldorf, Hilden, Germany), and the anti-flag antibody from CST (#14793).

Co-immunoprecipitation (Co-IP)

RIPA cell lysis buffers (R0010, Solarbio) were utilized to lyse cells on ice. One mg of protein was attained from each sample and adjusted to the same volume with IP lysis buffers. Flag monoclonal antibodies (#14793, 1:50, CST) were added for the IP reaction and incubated overnight at 4°C on a silent mixer. The following day, 20 µL of Protein A + G beads were added and incubated for 2h, after which impurities were eluted with IP lysis buffers. After 5-min centrifugation at 2500 rpm and 4°C, samples underwent elution five times. After another centrifugation, supernatants were carefully discarded and 20 µL of loading buffer (0.02 mol/L) was
added to each well. Samples were subsequently subjected to SDS-PAGE and western blot. The antibody PIK3C3 (1:200, ab227861, Abcam) was used, and the experiment was repeated in triplicate.

**Cell counting kit (CCK)-8**

The CCK-8 assay was used to determine glioma cell viability using a kit (CK04, Dojindo, Kumamoto, Japan). Glioma cells at the logarithmic phase were seeded in 96-well plates at 1\cdot10^4 cells/well for 24h pre-culture. Subsequently, cells were transfected with different plasmids for 48h. After completing transfection, 10 µL CCK-8 reagents were added at 0h, 24h, 48h, and 72h and incubated for 3h at 37°C. The optical density (OD) values of each well at 450 nm were determined on a microplate reader and the OD value levels were directly proportional to the number of proliferative cells in the medium. The growth curve was drawn, and the experiment was repeated in triplicate.

**Flow cytometry**

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining was applied to detect cell apoptosis. The treated cells were cultured in a 37°C and 5% CO₂ incubator, after which they were subjected to two PBS washes, centrifugation, and re-suspending in 200 µL of binding buffers. Following that, 10 µL Annexin V-FITC (ab14085, Abcam; final concentration: 20 µg/mL) and 5 µL PI (final concentration: 50 µg/mL) were added to the cells, gently mixed, and left for 15 min at room temperature while avoiding light. Following the addition of 300 µL of binding buffers, a flow cytometer was used to determine cell apoptotic conditions (FITC: 488 nm excitation wavelength and 525 nm emission wavelength; PI: 535 nm excitation wavelength and 615 nm emission wavelength). Cell apoptotic rate = late cell apoptotic rate (Q1-LR) + early cell apoptotic rate (Q1-UR) (early apoptotic cells have a preapoptotic stage, which is characterized by morphological and biochemical changes; late apoptotic cells belong to the late apoptotic stage, and
the process of cell death and decomposition is more obvious).

**Tumor formation assay in nude mice**

Twenty-four specific pathogen-free grade male BALB/c nude mice (6 weeks, 15-18 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and randomly arranged into three groups (sh-NC + DMSO, sh-SENP3 + DMSO, and sh-SENP3 + CQ groups: 8 nude mice in each group). Specifically, TJ905 cells stably transfected with sh-NC and sh-SENP3 were made into cell suspensions (2×10⁶ cells/mL) and subcutaneously injected into nude mice for the development of the subcutaneous xenograft model, followed by five successive weeks of CQ injection via the tail vein at a dosage of 10 mg/kg/d. The mice in the control group were injected with the same volume of DMSO. Tumor growth was examined one week following injection, and data were collected every three days. On the 25th day after injection, the nude mice were euthanized via cervical dislocation, and tumor tissues were removed and weighed on a scale; next, proteins were extracted for western blot analysis. The measurement for each group was repeated in triplicate. All our animal experiments were approved by the Animal Care and Use Committee and met the principles of experimental animal management and use of Liuyang Jili Hospital.

**Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay**

The TUNEL Apoptosis Detection Kit-DAB [abs50022, AIBIXIN, Shanghai, China] was used to detect cell apoptosis. Specifically, tumor tissue sections were incubated with the TdT/nucleotide complex for 1 h at room temperature, washed with PBS, and then treated with nuclear labeling with horseradish peroxidase and diaminobenzidine. Subsequently, sections were counter-stained with hematoxylin, followed by observation and photography of apoptotic cells (nuclei were stained brown in positive cells) under the optical microscope. Five visual fields were randomly selected to monitor cell apoptosis for each section and the apoptotic rate (%) was the percentage of positive
cells. The apoptotic rate = the number of positive cells/the number of total cells × 100%.

**Statistical analysis**

GraphPad Prism 8.0 was utilized for statistical analysis, and data measurement was displayed as mean ± standard deviation. The independent sample *t*-test was adopted to analyze data with normal distribution in two groups and the one-way analysis of variance test to compare data among multiple groups. Tukey's test was employed for post-hoc verification. *p* < 0.05 was considered statistically significant.

**Results**

**High SENP3 expression was found in glioma tissues and cells**

To explore the expression and effect of SENP3 in gliomas, we downloaded pan-cancer datasets with unified standardization from the UCSC database and extracted the expression data of ENSG00000161956 (SENP3) in each sample for differential analysis. In comparison with normal tissues, SENP3 was highly expressed in the tissues of GBM and low-grade glioma (LGG) (Figure 1A).

Next, clinical samples of glioma tissues were harvested for testing SENP3 expression. Figure 1B-C shows that SENP3 expression was substantially upregulated in glioma tissues. Afterward, glioma cells were cultured, and qRT-PCR and western blot results showed that SENP3 expression was clearly higher in glioma cell lines TJ905, U138, and LN-229 versus that in the normal astroglia cell line HA1800 (Figure 1D-E). The highest SENP3 expression was observed in TJ905 cells, hence these were selected for the following experiments. Conclusively, the deSUMOylating enzyme SENP3 shows high expression in glioma tissues and cells.
SENP3 Silencing restrained the progression of gliomas by promoting cell autophagy

To ascertain the impact of SENP3 on glioma cell autophagy, we first silenced SENP3 in TJ905 cells. Western blot results show that after SENP3 silencing, SENP3 protein expression was greatly lowered in TJ905 cells (Fig. 2A). Immunofluorescence data uncovered that SENP3 silencing noticeably enhanced the protein levels of LC3 (an autophagy marker) in TJ905 cells (Figure 2B). Western blot analyses of autophagy-associated proteins LC3B II/LC3B I showed the enhanced ratio of LC3B II to LC3B I in TJ905 cells after the silencing of SENP3, while the autophagy inhibitor CQ reversed the promoting effect of SENP3 silencing on cell autophagy (Fig. 2C). These data indicated that SENP3 silencing facilitated autophagy in glioma cells.

Flow cytometry and CCK-8 (Figure 2D-E) demonstrated that suppressing SENP3 increased apoptosis and decreased the viability of TJ905 cells, whilst CQ treatment nullified these effects (Figure 2D-E). Western blot results unveiled that after SENP3 was silenced, Bax protein expression increased and Bcl-2 protein expression declined in TJ905 cells, which was abrogated by CQ treatment (Fig. 2F). Collectively, SENP3 silencing accelerated apoptosis and autophagy in glioma cells.

SENP3 mediated the SUMO3 modification of BECN1 via the promotion of BECN1 deSUMOylation

To confirm the existence of SUMOylation in BECN1, labeled BECN1 was transfected into TJ905 cells, and SUMO3 (5 mM, 10 mM, and 15 mM) was added. Ni-NTA pull-down results disclosed that the SUMOylation levels of BECN1 were augmented in cells in a SUMO3 dose-dependent manner (Fig. 3A), which implied that the BECN1 protein can be modified by SUMOylation. Next, after the transfection of labeled SENP3 into TJ905 cells, the SUMOylation levels of BECN1 clearly declined in a SENP3 dose-dependent manner (Fig. 3B). In summary, SENP3 can accelerate the deSUMOylation of BECN1 to mediate the SUMO3 modification of BECN1.
SENP3 impeded the formation of BECN1-PIK3C3 complexes to depress glioma cell autophagy

Subsequently, the downstream mechanism of BECN1 in gliomas was identified. Initially, the SUMOylation site of BECN1 was mutated to acquire the SUMO partially-deleted BECN1 mutant, K380R. Co-IP results showed a notably weakened interaction between K380R and PIK3C3 (Figure 4A), indicating the repressive effects of BECN1 deSUMOylation on the formation of BECN1-PIK3C3 complexes. Immunofluorescence and western blot demonstrated that K380R evidently lowered the protein expression of LC3 and the ratio of LC3B II to LC3B I in TJ905 cells (Figure 4B-C), which illustrated that BECN1 deSUMOylation can suppress autophagy in glioma cells.

Following the transfection of labeled SENP3 into TJ905 cells, Co-IP data showed that SENP3 reduced the interaction between BECN1 and PIK3C3 (Fig. 4D). In conclusion, SENP3 promotes BECN1 deSUMOylation, thus curtailing the formation of BECN1-PIK3C3 complexes and the autophagy of glioma cells.

SENP3 silencing promoted autophagy and suppressed tumor growth in vivo

To further probe the oncogenic capability of SENP3 through autophagy in vivo, the tumor formation assay was conducted in nude mice. Results reflected reductions in both the volume and weight of the xenograft in the sh-SENP3 + DMSO group relative to the sh-NC + DMSO group. However, the sh-SENP3 + CQ group had enhanced tumor volume and weight versus the sh-SENP3 + DMSO group (Figure 5A-C). Western blot results presented that, compared with the sh-NC + DMSO group, SENP3 protein expression was reduced and the ratio of LC3B II to LC3B I was elevated in tumor tissues of the sh-SENP3 + DMSO group. Conversely, the sh-SENP3 + CQ group displayed a markedly decreased ratio of LC3B II to LC3B I in tumor tissues in comparison with the sh-SENP3 + DMSO group (Fig. 5D). TUNEL data demonstrated an elevation in apoptosis in the sh-SENP3 + DMSO group versus the sh-NC + DMSO group, and a decline in apoptosis in the sh-SENP3 + CQ group versus the sh-SENP3 + DMSO group (Fig. 5E). These findings indicate that
the silencing of SENP3 fosters autophagy and impedes tumor growth in mice

Discussion

Gliomas are diverse and classified based on their histologic type and malignancy grade; the majority of them show extensive infiltration into the central nervous system parenchyma (Perry et al., 2016). Given that gliomas are incurable, further research is necessary to enhance the prognosis and reduce cognitive dysfunction in patients (Gusyatiner et al., 2018). To enrich the theoretical molecular basis of related research, this study discussed the mechanism of the SENP3-BECN1-PIK3C3 axis in autophagy during gliomas and revealed that SENP3 curtailed the formation of BECN1-PIK3C3 complexes by inducing BECN1 deSUMOylation, thereby suppressing autophagy in gliomas in vivo and in vitro.

Autophagy is regarded a crucial mechanism in gliomas (Chu et al., 2019; Liu et al., 2019; Meyer et al., 2021). During the modulation of autophagy, BECN1 plays an important part and its inhibition was recorded to suppress autophagy in malignant glioma cells (Zou et al., 2016). It was reported that BECN1 upregulation participated in the reduction of cell growth in glioma cells (Chen et al., 2016). Furthermore, the depressing effects of BECN1 deSUMOylation on autophagy were revealed in an earlier work (Liu et al., 2020). As a result, we hypothesized that BECN1 deSUMOylation might be involved in glioma cell autophagy. Intriguingly, this speculation was confirmed by our results, that the loss of the SUMO part of BECN1, namely the deSUMOylation of BECN1, lowered the protein expression of autophagy marker LC3 (Schaaf et al., 2016)) and the value of LC3B II/LC3B I in glioma cells. As reported, the LC3B II/LC3B I ratio is reduced during the repression of autophagy (Oku et al., 2019). Obviously, it can be concluded that BECN1 deSUMOylation impedes glioma cell autophagy. A previous study elaborated that BECN1 forms BECN1 complexes to manipulate autophagy by interacting with other accessory proteins such as PIK3C3 (Sun et al., 2015). Similarly, we previously observed that the BECN1 and PIK3C3 complex was weakened along with BECN1 deSUMOylation-induced inhibition of autophagy. PIK3C3 is
vital for sustaining autophagy (Su et al., 2018). The interactions of BECN1 with PIK3C3 and other factors form BECN1-related complexes, manipulating the process of autophagy (Sun et al., 2015). Consistent with our evidence, Zhang et al. also found that the repressed formation of BECN1-PIK3C3 complexes might contribute to the suppression of autophagy (Zhang et al., 2015).

SENP3 belongs to the SUMO system and was recognized to facilitate cell proliferation, metastasis, and tumor development in several cancers, including GBM (Cheng et al., 2017; Li, Meng, 2021). Concordantly, our online database prediction exhibited the obvious upregulation of SENP3 in gliomas, which was also confirmed in glioma clinical samples and cells. However, research into the particular role of SENP3 in glioma growth is scarce. Interestingly, our data elucidated that the silencing of SENP3 increased LC3 protein levels, the LC3B II/LC3B I ratio, and apoptosis while decreasing proliferation in glioma cells, which was annulled by further treatment with the autophagy inhibitor. Yang et al. previously demonstrated that suppressing SENP3 promoted death and suppressed growth in osteosarcoma cells (Yang et al., 2020). Moreover, SENP3 silencing reduced the weight and volume of tumors in our in vivo nude mouse model, along with enhanced trends in autophagy and apoptosis in xenograft tissues. Meanwhile, the interaction of BECN1 with SENP3 was unveiled by earlier research, showing that SENP3 boosted the deSUMOylation of BECN1 and weakened the formation of BECN1-PIK3C3 complexes to affect autophagy in mouse livers (Liu et al., 2020). We also explored their relationship in our experiments and noted that SENP3 promoted BECN1 deSUMOylation and modulated its SUMO3 modification to reduce the formation of BECN1-PIK3C3 complexes, thus reducing glioma cell autophagy.

To summarize, our findings suggest that SENP3 may inhibit autophagy and accelerate tumor progression in gliomas by suppressing BECN1-PIK3C3 complex formation via the promotion of BECN1 deSUMOylation. Based on our findings, SENP3 and BECN1 are proposed as potential therapeutic targets for gliomas. While we only investigated SENP3, other factors that may have an impact on SUMO3 still need to be investigated, such as SENP1, SENP2, SENP5, SENP6, and SENP7.
Acknowledgment

N/A

Declaration of interest

The authors report no relationships that could be construed as a conflict of interest.

Author contribution

HPJ and KWB conceived the ideas. HPJ, KWB, and CHB designed the experiments. HPJ, KWB, and TJ performed the experiments. HPJ, KWB, and CHB analyzed the data. HPJ provided critical materials. HPJ, KWB, CHB, and TJ wrote the manuscript. KWB supervised the study. All the authors have read and approved the final version for publication.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The current research was approved by the Ethics Committee of Liuyang Jili Hospital (ethics number: 202207015; approval date: July 15, 2022) and abided by the Declaration of Helsinki, and written informed consent was obtained from all patients included.
Legends

**Figure 1** SENP3 expression is high in glioma tissues and cells. A: Analysis of differentially expressed SENP3 in each sample of pan-cancer datasets from the UCSC database; B: qRT-PCR detection of SENP3 mRNA expression in tumor tissues from glioma patients, glioma = 40, normal = 10; C: Western blot tests of SENP3 protein expression in tumor tissues from glioma patients, glioma = 40, normal = 10; D: qRT-PCR results of SENP3 mRNA expression levels in glioma cells; E: Western blot results of protein expression levels of SENP3 in glioma cells. All the above values were measurement data and presented as mean ± standard deviation. The independent sample t-test was adopted for comparisons between two groups. *p<0.05, compared with the normal group; #p<0.01, compared with the HA1800 group. The experiment was conducted in triplicate. SENP3, SUMO-Specific Peptidase 3; UCSC database, The University of California Santa Cruz database; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; mRNA, messenger RNA.

**Figure 2** SENP3 silencing facilitates glioma cell autophagy. A: Western blot detections of SENP3 protein expression in glioma cells after the silencing of SENP3; B: Immunofluorescence observations of autophagy marker LC3 in glioma cells; C: Western blot measurement of expression of autophagy-related proteins (LC3B II/LC3B I) in glioma cells; D: CCK-8 assessment of proliferative ability in glioma cells; E: Flow cytometry observations of apoptotic conditions in glioma cells; F: Western blot testing of apoptotic protein (Bax and Bcl-2) expression. All the above values were measurement data and presented as mean ± standard deviation. The independent sample t-test was adopted for comparisons between two groups. *p<0.05, compared with the sh-NC group; #p<0.05, compared with the sh-NC or sh-SENP3 + DMSO groups. The experiment was repeated in triplicate. SENP3, SUMO-Specific Peptidase 3; LC3, microtubule-associated protein 1 light chain 3; CCK-8, cell counting kit-8; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-Associated X.
Figure 3 SENP3 expedites BECN1 deSUMOylation and regulates the SUMO3 modification of BECN1. A: After the transfection of labeled BECN1 and addition of SUMO3 in TJ905 cells, the Ni-NTA pull-down assay was carried out to detect the SUMOylation levels of BECN1 in cells; B: After the transfection of labeled SENP3 in TJ905 cells, the Ni-NTA pull-down assay was applied to assess SUMOylation modification levels of BECN1 in cells. SENP3, SUMO-Specific Peptidase 3; BECN1, beclin 1; Ni-NTA, Ni-nitrilotriacetic acid.

Figure 4 SENP3 restrains glioma cell autophagy by suppressing BECN1-PIK3C3 complex formation. A: Co-IP assay to evaluate the interactions between BECN1 and PIK3C3; B: Immunofluorescence to determine the contents of autophagy marker LC3; C: Western blot to measure the ratio of autophagy-associated protein LC3B II to LC3B I; D: Co-IP assay to check the interaction between BECN1 and PIK3C3. All the above values were measurement data and presented as mean ± standard deviation. The independent sample t-test was adopted for comparisons between two groups. *p<0.05, compared with the WT-BECN1 group. The experiment was repeated in triplicate. SENP3, SUMO-Specific Peptidase 3; BECN1, beclin 1; PIK3C3, Phosphatidylinositol 3-Kinase Catalytic Subunit Type 3; LC3, microtubule-associated protein 1 light chain 3; Co-IP, co-immunoprecipitation.

Figure 5 SENP3 silencing blocks the growth of gliomas and promotes autophagy in mice. A: Tumor formation in nude mice; B: Tumor volumes from each group of nude mice; C: Tumor weights from each group of nude mice; D: Western blot to determine SENP3 protein expression and the value of LC3B II/LC3B I in tumor tissues; E: TUNEL detections of apoptotic conditions in tumor tissues. All the aforementioned results were obtained by measurement and are presented as mean ± standard deviation. One-way Analysis of Variance was adopted for comparisons among multiple groups and Tukey's post hoc test was used for post hoc comparisons. N = 8, *p<0.05, compared with the sh-NC + DMSO group; #p<0.05, compared with the sh-SENP3 + DMSO group. SENP3, SUMO-Specific Peptidase 3; LC3, microtubule-associated protein 1 light chain 3; TUNEL,
Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling.

References

cell death. Autophagy 17, 3424-3443.
**A**

RH-SUMO3 -

Flag-BECN1 + + + +

---

Ni-NTA

Input

IB:RH

IB:Flag SUMO

IB:Flag BECN1

IB:GAPDH

**B**

GFP-SENP3 - -

RH-SUMO3 - + + +

---

Ni-NTA

Input

IB:RH

IB:Flag

IB:GFP

IB:Flag

IB:GAPDH

IB:GAPDH
**HISTOLOGY AND HISTOPATHOLOGY**

**A**

![Image of samples](image)

**B**

**Tumor volume (mm³)**

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<th>Time (days)</th>
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<td>7, 10, 13, 16, 19, 22, 25</td>
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<tr>
<td>sh-SENP3+DMSO</td>
<td>7, 10, 13, 16, 19, 22, 25</td>
</tr>
<tr>
<td>sh-SENP3+CQ</td>
<td>7, 10, 13, 16, 19, 22, 25</td>
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**C**

**Tumor weight (g)**

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<tr>
<td>sh-SENP3+CQ</td>
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**D**

**Western Blot Analysis**

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<tr>
<td>LC3B II</td>
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<tr>
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<tr>
<td>GAPDH</td>
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**E**

**Apoptosis Rate (%)**

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<tr>
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<tr>
<td>sh-SENP3+CQ</td>
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* * *