Identification of ubiquitin-specific protease 32 as an oncogene in glioblastoma and the underlying mechanisms

Sifang Chen1, Xi Chen1, Zhangyu Li1, Jianyao Mao1, Weichao Jiang1, Zhi Zhu2, Yukui Li1, Zhengye Jiang1, Wenpeng Zhao1, Guowei Tan1 & Zhanxiang Wang1,3,4

Glioblastoma (GBM) patients present poor prognosis. Deubiquitination by deubiquitinating enzymes (DUBs) is a critical process in cancer progression. Ubiquitin-specific proteases (USPs) constitute the largest sub-family of DUBs. Evaluate the role of USP32 in GBM progression and provide a potential target for GBM treatment. Clinical significance of USP32 was investigated using Gene Expression Omnibus databases. Effects of USP32 on cell growth and metastasis were studied in vitro and in vivo. Differentially expressive genes between USP32-knockdown U-87 MG cells and negative control cells were detected using RNA sequencing and used for Gene Ontology and Kyoto Encyclopedia of Genes and Genomic pathway enrichment analyses. Finally, RT-qPCR was used to validate the divergent expression of genes involved in the enriched pathways. USP32 was upregulated in GBM patients, being correlated to poor prognosis. USP32 downregulation inhibited cell growth and metastasis in vitro. Furthermore, USP32 knockdown inhibited tumorigenesis in vivo. In addition, USP32 was identified as a crucial regulator in different pathways including cell cycle, cellular senescence, DNA replication, base excision repair, and mismatch repair pathways. USP32 acts as an oncogene in GBM through regulating several biological processes/pathways. It could be a potential target for GBM treatment.
constitute the largest one. DUBs not only function to reverse ubiquitination but are also involved in protein trafficking, chromatin remodeling, cell cycle regulation, base excision repair, mismatch repair, and signaling pathway modulations, which are tightly associated with the development of cancer.

DUBs have emerged as attractive targets for targeted therapy in cancer after the clinical success of proteasomal inhibitors, such as bortezomib, ixazomib, and carfilzomib. NSC 144303 (G5) and NSC 632839 (F6), two DUBs inhibitors, are under pre-clinical trial. Several DUBs were reported to regulate multiple cellular processes such as apoptosis, proliferation, and stemness in GBM.

Methods

Cell lines and cell culture. 293T, SVG p12, U-87 MG, U-118 MG, U-251 MG, T98G, and A172 cells were purchased from Xiamen Immocell Biotechnology Co., Ltd (Xiamen, Fujian, China). All cells were maintained using Dulbecco’s modified Eagle’s medium (DMEM, IMMOCELL) containing 15% fetal bovine serum (FBS, Gibco) at 37 °C in an incubator with an atmosphere containing 5% CO₂ and 21% O₂.

High-content screening. Small interfering RNAs (siRNAs) targeting USPs were obtained from GenePharma (Shanghai, China). Table 1 describes these siRNAs. U-87 MG and U-251 MG cells were seeded into 96-well plates at a density of 1 × 10⁴ per well. Lipofectamine RNAiMAX (Life Technology, Carlsbad, CA, USA) was used for siRNA transfection at 37 °C. After a 36 h of transfection, the cells were incubated with 10 μL/well CCK-8 solution (Y easen, Shanghai, China). The cell viability was evaluated 2 h later by detecting the optical density at 450 nm (OD₄₅₀) using a microplate reader (Molecular Devices, San Francisco, CA, USA).

Data mining from Gene Expression Omnibus (GEO) databases. Dataset GSE59612 (normal 17, GBM 39) was downloaded from the GEO website (https://www.ncbi.nlm.nih.gov/geo/) to evaluate the differential expression of USP32 between normal tissues and tumor tissues. The mRNA expression data and survival information of GBM patients in datasets GSE74187 (GBM 60) and GSE83300 (GBM 50) were also downloaded. The association between USP32 expression level and prognosis was analyzed. GBM patients were divided into two groups (high and low) using the optimal cut-off value of USP32 expression level, which was determined using the surv_cutpoint function of the R package survminer via RStudio software (version 2021.09.0 + 351, https://www.rstudio.com/).

RT-qPCR. Total RNA from U-87 MG was obtained using an RNA Extraction Kit (Vazyme, Nanjing, Jiangsu, China) and reverse transcribed into cDNA using HiScript II Reverse Transcriptase (Vazyme). One hundred nanogram of cDNA was used for qPCR per well. qPCR was performed using a Bio-Rad CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA) with an AceQ qPCR SYBR Green Master Mix Kit (Vazyme). The thermocycling condition was 96 °C for 5 min, followed by 96 °C for 15 s, 60 °C for 25 s, and 72 °C for 20 s, for 45 cycles. The 2−ΔΔCt method was used to calculate the relative mRNA levels, which was calibrated to 18S RNA. The primers for RT-qPCR are shown in Table 2.

Western blotting. U-251 MG and U-87 MG cells were lysed using RIPA buffer (Vazyme). The protein concentrations of samples were measured using a bicinchoninic acid (BCA) protein quantification kit (Abcam, Shanghai, China). Samples (12 μg/lane) were loaded into a 12% SDS-PAGE gel for electrophoresis and then transferred onto a PVDF membrane (Roche, Basel, Switzerland). The membrane was incubated with primary antibody Anti-USP32 (1:1000, CAT#ab251903, Abcam) or Anti-GAPDH (1:2000, CAT#ab9485, Abcam) at 4 °C overnight, followed by incubation with secondary antibody HRP-conjugated Goat Anti-Rabbit IgG H&L (1:2000, CAT#ab6721, Abcam) at 28 °C for 30 min. The signals were visualized using the ECL detection system (Thermo Fisher Scientific, Waltham, MA, USA) and quantified by densitometry using Image J v1.48u.

USP32 knockdown by transfection. U-251 MG and U-87 MG cells were seeded into 6-well plates at a density of 5 × 10⁶ per well and transfected with 200 pmol/well siRNA NC, siUSP32-2261, or siUSP32-386 using Lipofectamine RNAiMAX. The cells were harvested and used for further experiments after incubation for 6–48 h at 37 °C.

CCK-8 assay for cell viability. U-251 MG and U-87 MG cells were tripinized after a 24 h of transfection and then seeded into 96-well plates at a density of 5 × 10⁴ per well. At different time points (0, 24, 48, 72 h), 10 μL/well CCK-8 solution was added into cells and the cell viability was evaluated by detecting OD₄₅₀.
| siRNAs          | Sense (5′–3′)               | Antisense (5′–3′)               |
|----------------|----------------------------|--------------------------------|
| siUSP1-1780    | GGGUUAAGUCUGCAACUAUU       | UUAGUGUCCAGACUUUAAACCUU        |
| siUSP1-1501    | GCAUAGGAAGGACAGAUAU       | AUAUCUGGCAACUCAUGAUCGCUU       |
| siUSP2-1339    | GGCGCUUGUGGCACUAU         | AAUAAUGGCAACAAAGGCUGG         |
| siUSP2-1921    | CGCCUGACCCGCACUAUU         | UUAAUGGACACUGGCAGAGCUU        |
| siUSP3-1109    | GGCGACAGAUCUCGAAAAGU       | ACUUCUACGACUGCCCUU            |
| siUSP3-1569    | GCCGGUUGACCUUCAAAGU       | UCUAAUGAGGUGAACAGCUU          |
| siUSP4-377     | GCGUGGAAUAACUCUAUAU       | UUAAGUAGUUAACUGCAACCU         |
| siUSP4-860     | GCAAAGUGUGAUGCAACAUU       | AUAUGCGUAAUCCAUGUUGUACU       |
| siUSP5-822     | GGACUGCGACUGUAACUAUU       | AUAGUGUACACUGCCGCUU           |
| siUSP5-1265    | GCCAGAAGCAAGGGAGAAGU       | AAGCGUUCUUCUGUGCGGCUU          |
| siUSP6-2274    | GGAAGGCAACUAUCUAUGAU       | UUCAUAAAGUAAUCUGUUGUACU       |
| siUSP6-2494    | GCCACAGGAACUAACUAUU        | UUAAGUGUACACUGCCGCUU           |
| siUSP7-2625    | GUGGUUGACCUUUAACUAUU       | AAUAGUAAAGACUAGCCGCUU          |
| siUSP7-603     | GCAUGUGCAAGGUAUAACUAU      | UUCAUAACUCGACUGCCCU            |
| siUSP8-873     | CCAAGAAGAAAGGGCAACCUU      | AAGCGUUCUUCUGUGCGGCUU          |
| siUSP8-3569    | GCAAGAAGCAACGUGGCUUUAUU   | UAAACCGACGUGUUGUUGCUU         |
| siUSP9-7849    | GCCGAUAGGGAACACUAUUU       | UUAAGAAGCUCACUGCGCUU           |
| siUSP9-2333    | CCCGCACUGAACAACAUU         | UAAAGAGUUCUGACGUG Gum          |
| siUSP9-2805    | CUGGUGACACCAUCUGAAUU       | AUCAUCAGUGAGGUGCGCUU           |
| siUSP9-8135    | CAGAGUGGCUUCGUUCAAUU       | UGACGCAAGAGACAGCUU            |
| siUSP10-630    | GCUUUUGGAGGGAAGAUCUAU      | UUAAGAAGCUCACUGCGCUU           |
| siUSP10-1175   | GCAACACCGGAAAGCAUAU        | UUAAGGUGUACACUGCGCUU           |
| siUSP11-1415   | GCAAGAUAUCUGUGACCUUUAU     | AAUAGGCAAGAGACAGCUU           |
| siUSP11-2088   | CUCUCUGGACAUUGCCCUU        | UAGCGUUCUUCUGUGCGGCUU          |
| siUSP12-314    | CUCUGCGGAUAUAGGAAAUU       | UUCUUCACUCGACUGCCCU            |
| siUSP12-648    | CUCUAACUAUAACUAUAGGCUU     | AGCAUAGUAUAUAGGAGG           |
| siUSP13-1685   | CGUGGCAGAAGAUCAAUUU        | UUAAGGUGUACACUGCGCUU           |
| siUSP13-2004   | GCCGUGAAGCAAACUAUAU        | AUCAUCAGUGAGGUGCGCUU           |
| siUSP14-655    | GCCUCAGGGCGAGUAAUU         | AAUAGGCAAGAGACAGCUU           |
| siUSP14-1632   | GCAUAGGCUUACGUUCAUU        | UUAAGGUGUACACUGCGCUU           |
| siUSP15-249    | GGAACAACCAAUAGUGUAADU      | UUAAGGUGUACACUGCGCUU           |
| siUSP15-1150   | GCAAGAUGGAAAGGCAGAUAU      | UUAAGGUGUACACUGCGCUU           |
| siUSP16-331    | GGAAGAUAUACULUACGAAUU      | UUAAGGUGUACACUGCGCUU           |
| siUSP16-469    | GCAUGGCCUAGACCAUAUU        | AAUAGGCAAGAGACAGCUU           |
| siUSP17-1431   | CCAUCAUCCUGAAGCAAAUU      | UUGCGUUCUUCACGACUGG           |
| siUSP17-1538   | GGAAGAUCAAAGGGAGGAAAU      | UUCUUCACUCGACUGCCCU            |
| siUSP18-527    | GCCGGCACUAACCCCUUAUU       | UCAAGGAGGUAAGGCACGUU          |
| siUSP18-1418   | CGCGCAUAUCUCCUGGUUAUU      | UUAACCGAAGAUCAGGCU            |
| siUSP19-2439   | GCCAUCAGCACAGCCCAUAU       | UAGCGGCUUUCGUAGGAGG           |
| siUSP19-2518   | GGGCGCAAGAGAGGGAAGAUAU     | AUCUUCACUCUGUGGCGGCUU          |
| siUSP20-249    | CCAUAGAGAGGUGACACUAU       | UUAAGGUGUACACUGCGCUU           |
| siUSP20-1041   | GCCCAGAAGAUGAGGAGGUU       | AAUAGGCAAGAGACAGCUU           |
| siUSP21-641    | CCAACUCAGCCCGUACCAUU       | UUAAGGACGGGCUAGUGG            |
| siUSP21-1353   | GCCAAGAAGAAGCCGUGAUAU     | AAUAGGCAAGAGACAGCUU           |
| siUSP22-1367   | GCCACAGGAAGUCCAAAAU        | UUAAGGUGAUCUGGUGCGGCUU        |
| siUSP22-695    | GAGAAGAAGAUCACCUGGAGAUA    | UUAAGGUGAUCUGGUGCGGCUU        |
| siUSP24-2719   | GCCAUCAUCAACUCAUAUU        | UUAAGGUGAUCUGGUGCGGCUU        |
| siUSP25-897    | GCCCAAGAAGGCUUCAAAGAUAU    | AAUAGGGAACAGCGGCUAUAAGGCUU    |
| siUSP25-1128   | GCCCGGUAUAACUAUGGAGUU      | UUAAGGGAACAGCGGCUAUAAGGCUU    |
| siUSP26-1600   | CCUAUAUGGUCACCUCAAU        | UUAAGGGAACAGCGGCUAUAAGGCUU    |
| siUSP26-2426   | GGGUUCGAUAAGAUAACUAUU      | UUAAGGGAACAGCGGCUAUAAGGCUU    |
| siUSP27-1339   | GCCGGCAGAAGUCCAGCUAUAU     | UUAAGGGAACAGCGGCUAUAAGGCUU    |
| siUSP27-855    | CUCUCACUGUGGCUACCUAUAU     | UUAAGGGAACAGCGGCUAUAAGGCUU    |
| siUSP28-1836   | GGGCCCAUAUCUAAUACUAU       | UUAAGGGAACAGCGGCUAUAAGGCUU    |
| siUSP28-841    | GCAUCCAGCGUAGCUUAAU        | AAUAGGGAACAGCGGCUAUAAGGCUU    |

Continued
| siRNAs        | Sense (5′–3′)               | Antisense (5′–3′)                  |
|--------------|-----------------------------|-----------------------------------|
| siUSP29-983 | CCCAUCACUGUUUAGAGGUUU       | AUUCCACUAACUGAGGGU                |
| siUSP29-1922| GGUGAGAAGAAGACGCAAU         | UUGUCUGUAUUCUCACCCU               |
| siUSP30-176 | CGACACGUAUAAGCUAUAU         | UAGAUCUAUGAUCGACG                 |
| siUSP30-521 | GGGCUCUGUGAGAUGCUUU         | AAGACAUCCCAACAGGACG               |
| siUSP31-954 | GGCUCUCUAGUCAGUCAU          | UACAGUGACUAUGGAGG                  |
| siUSP31-3922| GUCUCGAUUACCCAGCUUU         | AAGACUAUGGAGAUGGAGG                |
| siUSP32-2261| GGCAUAUAAGAAGGAGAU         | AUUCCUCUCUCUAAAGGAGG               |
| siUSP32-386 | GCCCCGUGAGCUCCAUAAU         | AUUAUGAGAAGCAACGAGU               |
| siUSP33-829 | CCCGUAUAACACACUAUU         | UUAACCAGAAGAUGGAG                   |
| siUSP33-597 | GGAAGAUGAGAUGCUAU          | AUUAUGAGAAGCAACGAGU               |
| siUSP34-1228| GGGACAGAUGAAGCACCACAUU    | AUUAUGAGAAGCAACGAGU               |
| siUSP34-3023| CCCUAUCAGGCUCCAUUAU      | UUAACCAGAAGAUGGAG                  |
| siUSP35-3188| CCCGACAGGACUGCCAUUU        | AUUAGCAGUCCUGAGG                   |
| siUSP35-1916| GCCGGAGAAUACGGAGCUUU       | AUUAGCAGUCCUGAGG                   |
| siUSP36-741 | CCAACUCCGCUCCACAUU        | UUAACCAGAAGAUGGAG                   |
| siUSP36-474 | GCAAAUUGUGUGCUCAAU       | UUAACCAGAAGAUGGAG                  |
| siUSP37-557 | CCAAGGACUUAUACGCUAUA      | UUAACCAGAAGAUGGAG                  |
| siUSP37-2235| GCCACUAGGCAAUU            | UUAACCAGAAGAUGGAG                  |
| siUSP38-301 | GUAUUUGGGAGAUU          | UUAACCAGAAGAUGGAG                  |
| siUSP38-1047| GGUUCUGAAAGGCGACCUUU     | UUAACCAGAAGAUGGAG                  |
| siUSP39-958 | GGAACCCUCGCAAUUUCUAUU     | UUAACCAGAAGAUGGAG                  |
| siUSP39-1375| GCAACUGAAGAAGCACCACAUU  | AUUAACCAGAAGAUGGAG                  |
| siUSP40-721 | CGACGAGAGCCGCUAAAUU       | UUAACCAGAAGAUGGAG                  |
| siUSP40-1212| GCCUCACUCUGAUAUUU        | UUAACCAGAAGAUGGAG                  |
| siUSP42-459 | GCCGCAGAUAUUGCGCAAU       | AUUAACCAGAAGAUGGAG                  |
| siUSP42-750 | GCAGAAGCAUGCUAGAAUU      | UUAACCAGAAGAUGGAG                  |
| siUSP43-813 | GCCACUUAACAGCAACAUU      | UUAACCAGAAGAUGGAG                  |
| siUSP43-2196| GGGCUCUAUACCCUGCUAUA     | UUAACCAGAAGAUGGAG                  |
| siUSP44-653 | GGGGUGAGAAGGAGGUAUU      | UUAACCAGAAGAUGGAG                  |
| siUSP44-1553| CGGCUCAGGAUUAUUGCUU      | UUAACCAGAAGAUGGAG                  |
| siUSP45-1377| GGGAGCAGAAGCACCACAUU    | AUUAACCAGAAGAUGGAG                  |
| siUSP45-753 | GCAUCGAGAUGACUACCUUU    | AUUAACCAGAAGAUGGAG                  |
| siUSP46-204 | GUCUCAGACGAGCUCUAUU     | UUAACCAGAAGAUGGAG                  |
| siUSP46-426 | CCAACCCAGGACAGCUAAUU    | UUAACCAGAAGAUGGAG                  |
| siUSP47-2463| GCCUGUUGGCCCUUGUAUAUU  | UUAACCAGAAGAUGGAG                  |
| siUSP47-3757| CGAGAAGCAGAGGUUGAAU    | UUAACCAGAAGAUGGAG                  |
| siUSP48-676 | GCCACUCAGDCUGCUUUAU     | UUAACCAGAAGAUGGAG                  |
| siUSP48-871 | GGCGUACUAUACGGCUAUA     | UUAACCAGAAGAUGGAG                  |
| siUSP49-1925| GGGUCUGAUGUGCGCUAUA    | UUAACCAGAAGAUGGAG                  |
| siUSP49-1825| GGAUCAGAAGAAGCACCACAUU | AUUAACCAGAAGAUGGAG                  |
| siUSP50-561 | GGCACAGAAGAAGCGCUU       | UUAACCAGAAGAUGGAG                  |
| siUSP50-692 | CCACUCAGGACACUCAUAU     | UUAACCAGAAGAUGGAG                  |
| siUSP51-895 | CCAUUAUGCUAGARGACCUUU   | UUAACCAGAAGAUGGAG                  |
| siUSP51-1225| CCAUUAUCUCUCUCAGCAAUU   | UUAACCAGAAGAUGGAG                  |
| siUSP52-896 | GCCGACAGAUAUCACAUAAU    | UUAACCAGAAGAUGGAG                  |
| siUSP52-1247| GGGCGCUCAUCUCACUAUU     | UUAACCAGAAGAUGGAG                  |
| siUSP53-3296| GAGCCAACACUCACUGAUAAU   | UUAACCAGAAGAUGGAG                  |
| siUSP53-1605| GGGGCGCUCAUCUCACUAAD    | UUAACCAGAAGAUGGAG                  |
| siUSP54-3654| GGGCCUCUUGAGGCAACUUA    | UUAACCAGAAGAUGGAG                  |
| siUSP54-2546| GAGGCGCUAUGAGCAGAUAU    | UUAACCAGAAGAUGGAG                  |
| siRNA NC     | CUCGACGGCUCGAGCUUU      | CUCGACGGCUCGAGCUUU               |

Table 1. Sequences of siRNAs targeting USPs.
Colony formation assay for cell growth. U-251 MG and U-87 MG cells were trypsinized and then seeded into 6-well plates at a density of 1 × 10^3 per well 6 h after transfection. The cells were continuously cultured at 37 °C for 7–10 days. The colonies were fixed with 4% methanal (Sangon Biotech, Shanghai, China) in phosphate buffer saline (PBS) at 28 °C for 15 min. After washing with PBS three times, the colonies were photographed using HUAWEI Mate 40 (Huawei, Shenzhen, China) and the number of colonies was counted using Image J v1.48u (NIH, Bethesda, MD, USA).

Microscopic analysis of EdU incorporation for cell proliferation. U-251 MG and U-87 MG cells were trypsinized after a 24 h of transfection and then seeded into 96-well plates at a density of 2 × 10^4 per well. Twelve hours later, the cells were labeled with 10 μM EdU solution (Beyotime, Shanghai, China) at 37 °C for 3 h. After fixation with 4% methanal in PBS at 28 °C for 20 min and subsequent permeation with 0.5% Triton X-100 (Sangon Biotech) in PBS at 28 °C for 10 min, the cells were incubated with 50 μL of 1 × Click Additive Solution (Beyotime) at 28 °C for 30 min. The nucleus was stained with 5 μg/mL DAPI (Y easen). Finally, the fluorescent dots were observed and photographed using a fluorescence microscope (MOTIC, Hongkong, China) and then photographed. Image J v1.48u was used to count the number of cells.

Flow cytometry-based cell cycle. U-251 MG and U-87 MG cells were collected after a 24 h of transfection and then fixed using 70% ethanol in PBS at − 20 °C for 6 h. The cells were then treated with 0.5% Triton X-100 (Sangon Biotech) at 28 °C for 25 min. Finally, the cells were stained with 20 μg/mL propidium iodide (PI, Vazyme) in the dark at 28 °C for 25 min and then placed into to the flow cytometer NovoCyte 1300 (ACEA, San Diego, CA, USA) for fluorescent detection within the PE-channel (Ex: 488 nm/Em: 578 nm).

Transwell assay for cell migration and invasion. U-251 MG and U-87 MG cells were trypsinized after a 24 h of transfection and then suspended in FBS-free DMEM at a density of 3 × 10^5/mL. In the transwell migration assay, 100 μL of suspended cells were placed into the upper chambers of transwell plates (NEST, Wuxi, Jiangsu, China). The lower chamber was supplied with 10 μL 10% FBS DMEM. In the Matrigel invasion assay, the membrane of the upper chamber was pre-coated with sixfold diluted Matrigel (Corning, Corning, NY, USA) before seeding cells. The migrated and invasive cells were fixed with 4% methanol in PBS and then stained with 1% crystal violet (Sangon Biotech) in PBS at 28 °C for 20 min, respectively. The stained cells were observed and photographed using a light microscope (MOTIC). The number of migrated/invasive cells were counted using Image J v1.48u in three random fields.

Construction of stable U-87 MG cell lines with USP32 knockdown (shUSP32). The lentiviral vector PLKO.1-TRC-Puro (Antihela, Xiamen, Fujian, China) was used to construct plasmid overexpressing short hairpin RNA targeting USP32 and shctrl plasmid. The primers for shUSP32 plasmid construction were designed based on the sequence of siUSP32-386 (Table 2). For lentiviral packaging, 293T cells (4 × 10^7/well) were seeded into 6-well plates and transfected with 3 μg shUSP32 or shctrl plasmid, 2 μg psPAX2 (Antihela),
and 1 μg pMD2.G (Antihela) using Lipofectamine RNAiMAX at 37 °C. After incubation for 48 h, the lentivirus was harvested and used to infect U-87 MG cells at a multiplicity of infection of 30 with the addition of 10 μg/mL polybrene (Thermo Fisher Scientific). Forty-eight hours after infection, U-87 MG cells were treated with 1.0 μg/mL puromycin (Yeasen) for 3 days, constructing shUSP32 and shctrl U-87 MG cells.

**Animal experiments.** Six-week-old female BALB/c nude mice were obtained from Vitalriver (Beijing, China). The subcutaneous injection of shctrl or shUSP32 cells (4 × 10⁶) was performed into the right flank of six mice. The long diameter (a) and short diameter (b) of tumors were measured every 4 days. The tumor volume (V) was calculated using the formula V = ab²/2²⁶. The mice were euthanized using isoflurane (RWD life science, Shenzhen, China) at day 48. Tumors were dissected off and photographed using HUAWEI Mate 40. The tumor was also weighed.

**RNA sequencing (RNA-Seq) and data analysis.** Total RNA from shctrl or shUSP32 U-87 MG cells was used as input material for the RNA sample preparations. miRNA was purified from total RNA using poly-T oligo-attached magnetic beads. The RNA-Seq library was built by Novogene (Beijing, China). After cluster generation using TruSeq PE Cluster Kit v3-cBot-HS (Illumia, San Diego, CA, USA) on a cBot Cluster Generation System (Illumia), the library preparations were sequenced on a Novaseq platform (Illumina). RNA-Seq data analysis was performed according to the protocol of Novogene. In brief, reads were aligned to the human transcriptome and genome hg19 using T Hisat2 v2.0.5. Transcripts and genes were quantified using featureCounts v1.5.0-p3. Differential expression analysis was performed using DESeq2 R package v1.20.0. Genes with an adjusted p value < 0.05 were considered as differentially expressed. GO and KEGG pathway enrichment analyses²⁷-²⁹ of differentially expressed genes were performed using the clusterProfiler R package.

---

**Figure 1.** High content screening based on cell viability to screen USPs may function in glioblastoma. Data are represented as mean ± standard deviation (SD) of three biological replicates.
Statistical analysis. All statistical analyses were performed using GraphPad Prism v8.2.1 (GraphPad Software, San Diego, CA, USA). The data are presented as mean ± standard deviation (SD) unless otherwise shown. ANOVA was performed with a post-hoc Tukey test for multiple comparisons among three groups. Unpaired Student’s t test was performed to compare differences between two groups. Survival curves were calculated using the Kaplan–Meier method, and the significance was determined by the log-rank test. Statistical significance was accepted at $p < 0.05$.

Ethics approval. Animal experiments were conducted in accordance with the national guidelines for the humane treatment of animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at Xiamen University. The study is reported in accordance with ARRIVE guidelines.

Results

High content screening. High content screening was performed to identify the USPs regulating GBM cell survival. As shown in Fig. 1, knockdown of USP1, USP8, or USP32 in U-87 MG cells inhibited cell viability by at least half. Knockdown of USP32, USP9X, or USP1 suppressed the viability of U-251 MG. Therefore, USP32 was chosen for further study.

USP32 expression level linked to poor prognosis. First, we investigated the clinical significance of USP32 using GEO dataset. As can be seen in Fig. 2A, the USP32 expression level was higher in GBM tissues compared to normal tissues. Moreover, higher USP32 expression level indicated poorer prognosis (Fig. 2B). Next, we evaluated the USP32 expression level in normal brain cells and GBM cells. The results showed that the USP32 mRNA and protein levels in GBM cells (U-118 MG, U-87 MG, A172, T98G, and U-251 MG) were higher than those in the normal brain cell SVG p12 (Fig. 2C,D). Furthermore, U-87 MG and U-251 MG have the highest USP32 expression levels. Based on these findings, we chose to knock down USP32 in U-87 MG and U-251 MG cells to study the function of USP32 in GBM.
Knockdown of USP32 inhibits cell growth. USP32 in U-87 MG and U-251 MG cells was knocked down using siRNAs. As shown in Fig. 3A,B, the mRNA and protein levels of USP32 were significantly reduced after transfection with siUSP32-2261 and siUSP32-386. Moreover, siUSP32-386 had higher knockdown efficiency.
than siUSP32-2261. Similarly, the CCK-8 assay indicated that USP32 knockdown significantly suppressed the viability of U-87 MG and U-251 MG cells (Fig. 3C). Cellular proliferation was analyzed using colony formation and EdU assays in U-87 MG and U-251 MG cells; Fig. 3D,E show that USP32 knockdown significantly reduced the number of colonies. In U-87 MG cells, the number of colonies decreased from 125 ± 11 to 80 ± 14 (p = 0.0058) for siUSP32-2261 and 46 ± 5 (p = 0.0003) for siUSP32-386. In U-251 MG cells, the number of colonies decreased from 48 ± 8 to 24 ± 3 (p = 0.0051) for siUSP32-2261 and 8 ± 3 (p = 0.0003) for siUSP32-386. The percentage of EdU⁺ cells was also significantly reduced by silencing USP32 (Fig. 3F). In U-87 MG cells, the percentage of EdU⁺ cells decreased from 25.5 ± 2.5% to 16.9 ± 2.0% (p = 0.0045) for siUSP32-2261 and 8.6 ± 1.1% (p = 0.0001) for siUSP32-386. In U-251 MG cells, the percentage of EdU⁺ cells decreased from 39.3 ± 1.4% to 28.3 ± 2.3% (p = 0.0016) for siUSP32-2261 and 24.2 ± 2.3% (p = 0.0003) for siUSP32-386 (Fig. 3G). Next, we investigated the effect of USP32 on cell cycle progression. The results show that USP32 knockdown promotes the arrest of cells in the G0/G1 phase (Fig. 3H,I). These findings suggested that silencing USP32 may inhibit cell growth due to cell-cycle arrest.

Knockdown of USP32 inhibits cell metastasis. Transwell migration and Matrigel invasion assays were performed to study the effect of USP32 on cell metastasis. Figure 4A shows that USP32 downregulation inhibited cell migration. In U-87 MG cells, the number of migrated cells was 330 ± 26, 186 ± 17, and 123 ± 13 for siRNA NC, siUSP32-2261 (p = 0.0003, vs siRNA NC), and siUSP32-386 (p < 0.0001, vs siRNA NC), respectively. In U-251 MG cells, the number of migrated cells was 431 ± 20, 305 ± 21, and 246 ± 13 for siRNA NC, siUSP32-2261 (p = 0.0004, vs siRNA NC), and siUSP32-386 (p < 0.0001, vs siRNA NC), respectively (Fig. 4B). This downregulation also inhibited cell invasion (Fig. 4C). In U-87 MG cells, the number of invasive cells decreased from 136 ± 22 to 53 ± 10 (p = 0.0010) for siUSP32-2261 and 24 ± 3 (p = 0.0002) for siUSP32-386. In U-251 MG cells, the number of invasive cells decreased from 136 ± 22 to 53 ± 10 (p = 0.0010) for siUSP32-2261 and 24 ± 3 (p = 0.0002) for siUSP32-386. In U-251 MG cells, the number of invasive cells were reduced from 156 ± 19 to 77 ± 10 (p = 0.0008) for siUSP32-2261 and 34 ± 6 (p < 0.0001) for siUSP32-386 (Fig. 4D). Taken together, these data indicate that USP32 knockdown inhibited cell metastasis.
Knockdown of USP32 inhibits tumor growth in vivo.

Next, stably USP32-knockdown U-87 MG cell lines were constructed to validate the function of USP32 in vivo. Figure 5A–C show that the stable cell lines were successfully constructed. The constructed cells were then subcutaneously injected into nude mice. As can be seen in Fig. 5D, the tumor in group shUSP32 grew more slowly compared to group shctrl. The dissected tumors are shown in Fig. 5E, being the weight lighter when group shUSP32 was compared to shctrl (Fig. 5F). Moreover, the mRNA and protein levels of USP32 were indeed lower in group shUSP32 than those in group shctrl (Fig. 5G–I). These data demonstrate that USP32 knockdown inhibits tumor growth in vivo.

Analysis of differentially expressive genes (DEGs).

Transcriptional sequencing was performed to find the differentially expressive genes between shctrl and shUSP32 U87-MG cells. The volcano plot indicates that 2017 genes were significantly upregulated and 2333 genes were significantly downregulated after USP32 knockdown (Fig. 6A). The heat map was used to show the distinguishable mRNA expression patterns between shUSP32 and shctrl samples (Fig. 6B).

GO and KEGG pathway enrichment analyses. All upregulated and downregulated genes were used for GO and KEGG enrichment analyses. In the GO enrichment analysis, 10 molecular functions such as cadherin binding and catalytic activity acting on DNA, 11 cellular components such as condensed chromosome and cell cycle checkpoint, and 9 biological processes such as mitotic cell cycle phase initiation and DNA replication were significantly modulated (Fig. 6C). KEGG pathway enrichment analysis discovers 20 pathways significantly linked to USP32 expression, including cell cycle and DNA replication pathways (Fig. 6D). The enriched DEGs in GO and KEGG pathway enrichment analyses are listed in Supplementary Table S1 and Supplementary Table S2, respectively. The expression profile of several DEGs associated with cell cycle, DNA replication, base excision repair, and mismatch repair are shown in Fig. 7A. RT-qPCR analysis confirmed that USP32 knockdown reduced the expression of these genes (Fig. 7B).
GBM accounts for 16% of primary brain tumors with an incidence rate of thirty-two per million population. GBM often causes poor prognosis and the median-survival time of patients is less than 2 years. It is urgent to find novel targets for brain-penetrating targeted therapies. Recently, more and more USPs were reported to play important roles in GBM progression. USPs with antitumor activity such as USP11 and USP28 promote cell viability after being silenced, which is consistent with the results in Fig. 1. In this study, USP32 knockdown inhibited cell growth and metastasis in vitro, and suppressed tumor growth in vivo, which suggests that USP32 acts as an oncogene in GBM and may serve as a potential target for GBM treatment.

EdU+ cells indicate the cells in DNA replication. DNA replication, occurring in the S phase of interphase during cell cycle, is an important step for cell proliferation and division. Results showed that the percentages of EdU+ cells and G0/G1-phase cells were reduced and increased after USP32 knockdown, respectively. Moreover, GO and KEGG pathway analyses revealed that this enzyme is involved in DNA replication and cell cycle processes or pathways. This suggests that USP32 may promote cells passing through the G0/G1 phase and initiate the DNA replication, promoting the proliferation of cancer cells, which is consistent with the study of Hu et al.

Base excision repair is an essential genome-maintenance pathway by which cells repair damaged DNA bases that arise at a high level during DNA replication. Failure to remove the damaged DNA bases causes increasing...
levels of mutation and chromosomal instability, finally resulting in carcinogenesis. DNA mismatch repair is a rescue system that conserves the DNA sequences by removing the erroneously mismatched, inserted, and deleted bases during DNA duplication and recombination. Defects in DNA mismatch repair are also associated with carcinogenesis. Moreover, DUBs are often involved in base excision repair and mismatch repair processes. Therefore, USP32 effect on the expression of molecules functioning in these processes was validated using RT-qPCR, although the adjusted \( p \)-values for base excision repair and mismatch repair pathways in KEGG pathway analysis were greater than 0.05. The results indicate that USP32 regulates the expressions of MCM3, MCM4, MCM6, MCM7, FEN1, NEIL3, and POLE, suggesting that USP32 plays an important role in base excision repair and mismatch repair.

There are few reports about elements of the ubiquitin–proteasome system involved in GBM metastasis. USP18 was reported to promote epithelial-mesenchymal transition in GBM cells by deubiquitinating and stabilizing Twist. UBE2T, a ubiquitin-conjugating enzyme, stabilizes GRP78 to promote the metastasis of GBM cells. Our study demonstrates that USP32 facilitates the migration and invasion of GBM cells, which supports that the ubiquitin–proteasome system plays an important role in the GBM metastasis.

Further experiments are needed to confirm the mechanism of action by which USP32 upregulates the expression of several genes (Fig. 7B). Further, the effect of USP32 modulation on cell function in normal glial cells and a broader panel of GBM cells will be evaluated. In addition, a collection of clinical samples to analyze the expression of this enzyme in GBM and normal tissue will also be included.

In conclusion, our study demonstrates that USP32 acts as an oncogene in GBM through regulating cell cycle, DNA replication, base excision repair, and mismatch repair. USP32 could be a potential target for GBM treatment.

Data availability
The data in this study will be made available from the corresponding author on reasonable request.

Figure 7. USP32 is involved in cell cycle, DNA replication, base excision repair, and mismatch repair processes/pathways. (A) The expression profile of several DEGs. (B) RT-qPCR analysis confirming the regulatory effect of USP32 on cell cycle, DNA replication, base excision repair, and mismatch repair processes/pathways. Data are represented as mean ± SD of three technical replicates.
37. Kit Leng Lui, S. et al. USP26 regulates TGF-β signaling by deubiquitinating and stabilizing SMAD7. *EMBO Rep.* 21, e49618. https://doi.org/10.15252/embr.201949618 (2020).

38. Nabais Sá, M. J. et al. Biallelic GINS2 variant p.(Arg114Leu) causes Meier–Gorlin syndrome with craniosynostosis. *J. Med. Genet.* https://doi.org/10.1136/jmedgenet-2020-107572 (2021).

39. Arczewska, K. D., Michalickova, K., Donaldson, I. M. & Nilsen, H. The contribution of DNA base damage to human cancer is modulated by the base excision repair interaction network. *Crit. Rev. Oncog.* 14, 217–273. https://doi.org/10.1615/critrevoncog.v14.i4.20 (2008).

40. Li, G. M. Mechanisms and functions of DNA mismatch repair. *Cell Res.* 18, 85–98. https://doi.org/10.1038/cr.2007.115 (2008).

41. Cai, X. et al. USP18 deubiquitinates and stabilizes Twist1 to promote epithelial-mesenchymal transition in glioblastoma cells. *Am. J. Cancer Res.* 10, 1156–1169 (2020).

42. Huang, P. et al. UBE2T promotes glioblastoma invasion and migration via stabilizing GRP78 and regulating EMT. *Aging (Albany NY)* 12, 10275–10289. https://doi.org/10.18632/aging.103239 (2020).

**Acknowledgements**

The study is supported by National Natural Science Foundation of China (No. 82072777).

**Author contributions**

W.Z.X. conceived and designed the study. C.S.F. collected the data. W.Z.X. and C.S.F. verified and analyzed the data. C.X., L.Z., M.J.Y., J.W., Z.Z., L.Y.K., J.Z.Y., Z.W.P., and T.G.W. performed the experiments and drafted the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-09497-y.

**Correspondence** and requests for materials should be addressed to Z.W.

**Reprints and permissions information** is available at www.nature.com/reprints.

**Publisher’s note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022