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UBE2T promotes glioblastoma malignancy through ubiquitination-mediated degradation of RPL6

Xuxiu Tao[1,2] | Xia Wu[2,3] | Peijun Zhou[1,2] | Xuehui Yu[1,2] | Chen Zhao[1,2] | Xingzhi Peng[1,2] | Kun Zhang[1] | Liangfang Shen[1] | Jinwu Peng[4,5] | Lifang Yang[1,2]

[1]Department of Oncology, Key Laboratory of Carcinogenesis and Cancer Invasion of Ministry of Education, National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, China

[2]Cancer Research Institute, School of Basic Medicine Science, Central South University, Changsha, China

[3]Department of Pathology, The Second Xiangya Hospital, Central South University, Changsha, China

[4]Department of Pathology, Xiangya Hospital, Central South University, Changsha, China

[5]Department of Pathology, Xiangya Changde Hospital, Changde, China

Correspondence
Lifang Yang, Central South University, Xiangya Road 110, Changsha 410078, China.
Email: yanglifang@csu.edu.cn

Jinwu Peng, Central South University, Xiangya Road 110, Changsha, 410078, China.
Email: jinwupeng2005@aliyun.com

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Abstract

Glioblastoma (GBM) is the most frequent and aggressive malignant glioma. Due to patients' poor prognosis, it is of great clinical significance to determine new targets that may improve GBM treatment. In the present study, we showed that ubiquitin (Ub)-conjugating enzyme E2T (UBE2T) was significantly overexpressed in GBM and could promote proliferation, invasion, and inhibit apoptosis of GBM cells. Mechanistically, UBE2T functioned as the Ub enzyme of ribosomal protein L6 (RPL6) and induced the ubiquitination and degradation of RPL6 in an E3 ligase-independent manner through direct modification by K48-linked polyubiquitination, thus contributing to the malignant progression of GBM cells. Furthermore, inhibiting the expression of RPL6 by UBE2T could not only reduce the expression of wild-type p53, but also enhance the gain-of-function of mutant p53. Moreover, knockdown of UBE2T in LN229 cells obviously suppressed tumor growth in LN229 xenograft mouse models. Collectively, our study demonstrated that UBE2T promotes GBM malignancy through ubiquitination-mediated degradation of RPL6 regardless of the p53 mutation status. It will provide new candidates for molecular biomarkers and therapeutic targets for clinical application in GBM.

KEYWORDS
GBM, RPL6, UBE2T, ubiquitination

1 | INTRODUCTION

Glioma is a primary tumor derived from gliocyte, accounting for approximately 40% to 50% of intracranial brain tumors. Glioma is classified into I–IV grade based on different histopathological characteristics, among which grade IV glioma is the most malignant glioblastoma (GBM), representing the most common form of glioma in the adult population.[1] Because of the malignant proliferation, radiotherapy, and chemotherapy resistance and high recurrence rate of GBM, patients' median survival time is only 12–15 months.[2,3] Therefore, it is urgent to explore the molecular mechanism causing GBM to be more malignant than low-grade glioma (LGG).

Xuxiu Tao and Xia Wu Equal contributors.

Ubiquitination modification is to the process where ubiquitin (Ub) covalently combines with protein under the effect of Ub activating enzyme (E1), Ub conjugating enzyme (E2), and Ub ligase (E3). Ubiquitination plays an important role in enzyme activity, protein stability, and interaction between proteins.[4] Disorders in the regulation of ubiquitination could lead to abnormal gene expression, which affects the tumorigenesis and development of tumors.[5–7] E3 ligase TRIM22 bound IκBα, a negative regulator of NF-κB, accelerated its degradation by inducing K48-linked polyubiquitination and promoted tumor growth and treatment resistance in GBM.[8] The E3 ligase TRIM45 acts as a tumor suppressor in glioma. It maintained the stability of p53 protein through K63-linked polyubiquitination to inhibit the proliferation and promote the apoptosis of glioma cells.[9] Therefore, it is meaningful to study the mechanism of the high malignancy of GBM from the perspective of ubiquitination regulation.

Ub-conjugating enzyme E2T (UBE2T) is an E2 enzyme that possesses both E2 and E3 ligase activities. UBE2T acts as an oncogene in various types of tumors. Overexpression of UBE2T can enhance the ubiquitination and degradation of the tumor suppressor BRCA1 and promote the progression of breast cancer.[10] In gastric cancer cells, UBE2T directly degraded RACK1 in an E3 ligase-independent manner and activated the Wnt/β-catenin signaling pathway to promote cell proliferation and migration.[11] In non-small-cell lung cancer, UBE2T promoted epithelial-mesenchymal transition (EMT) via ubiquitination-mediated FOXO1 degradation and activation of the Wnt/β-catenin signaling pathway.[12] A recent report revealed that UBE2T can promote the ubiquitination degradation of Mule in liver cancer, thereby stabilizing the β-catenin and further participating in the tumorigenesis and self-renewal of liver cancer stem cells (CSCs).[13] Despite this fascinating research, the functions of UBE2T remain largely unknown in GBM progression.

In the present study, a transcriptome sequencing analysis was performed between the GBM and LGG tissues and cells, and the data revealed that UBE2T was highly expressed in GBM. Ribosomal protein L6 (RPL6) was identified as an interacting protein of UBE2T by mass spectrometry analysis. In addition, we proved that UBE2T promoted the ubiquitination-mediated degradation of RPL6, further inhibiting the expression of wild-type p53 (wt-p53) or enhancing the expression of mutant p53 (mut-p53) and thereby promoting the malignancy of GBM cells.

2 | MATERIALS AND METHODS

2.1 | Plasmids, small interfering RNA, and transfection

The pcDNA3.1-3xFlag-RPL6 plasmid was purchased from Youbio (F105654). The Ub point mutation plasmids K48R (HA-Ub-K48R) and K63R (HA-Ub-K48R) were constructed using a pRK5-HA-Ub-WT plasmid (#1760, Addgene) and point mutation kit (C215, Vazyme). Using prokaryotic plasmid pDEST17-His-UBE2T (#15808, Addgene) as a template, the His-UBE2T sequence was then cloned into the pLVX-EF1α-IRES-puro (VT2015, Youbio) vector using EcoRI/BamH1 restriction sites to construct the pLVX-His-UBE2T recombinant plasmid for eukaryotic expression.

For the transient transfection of plasmid and small interfering RNA, Lipofectamine^{3000} reagent (L3000015, Invitrogen) was used according to the instructions. The sequence of the small interfering RNA is shown in Table S1.

2.2 | Cell culture and Reagents

HEK 293T (ATCC CRL-11268), glioma cell lines HS683 (ATCC HTB-138, grade I), SHG44 (grade I), and GBM cell lines U251, U87 (ATCC HTB-14), LN229 (CRL-2611), U373 (ATCC HTB-17), and T98G (CRL-1690) were cultured in DMEM (Gibco BRL) medium supplemented with 10% FBS (HyClone).

Two UBE2T-shRNAs were, respectively, cloned into pLVX-shRNA1 (632177, Clontech) vector using EcoRI/BamH1 restriction sites to construct shUBE2T-1 and shUBE2T-2 plasmids. The sequence of shRNA is shown in Table S2. Recombinant lentivirus was obtained by co-transfecting 293T cells with shUBE2T and packaging plasmids (Gag-pol, pRSV-Rev, and pCMV-VSV-G, Addgene, 14887, 12253, 8454, respectively) with Lipofectamine^{3000} reagent (L3000015, Invitrogen) according to the instructions. The virus supernatant was collected after transfection of 48 h, and then GBM cells were infected. Stable cell lines (LN229/U87-shUBE2T-1, LN229/U87-shUBE2T-2, and LN229/U87-shVector) were obtained by screening with puromycin hydrochloride (60210ES25, Yeasen).

Cycloheximide (CHX) was purchased from Merck Millipore (66–81-9), and the proteasome inhibitor MG132 was purchased from MCE (HY-13259).

2.3 | Transcriptome sequencing

Using grade I glioma HS683 cells as control, GBM cell lines U251 and LN229 were RNA sequenced by Sangon Biotech to analyze the differentially expressed genes.

2.4 | Quantitative RT-PCR

Trizol reagent was purchased from Thermo Fisher Scientific (15596026) to extract total RNA following the instructions. Using the RevertAid First Strand cDNA Synthesis Kit (K1621, Thermo Fisher Scientific), the RNA was reverse-transcribed into cDNA, and quantitative PCR was performed using the ABI 7500 system and SYBR Green (4309155, Life Technologies). PCR primers are listed in Table S3.

2.5 | Antibodies, western blot, and immunoprecipitation

The cells were lysed in immunoprecipitation (IP) buffer (87787, Thermo Fisher Scientific) containing an inhibitor cocktail

(4693116001, Roche). The protein concentration was measured using a BCA Kit (23225, Pierce Chemical). The protein was separated by SDS-PAGE and then transferred to polyvinylidene fluoride membrane (Merck Millipore). The membrane was incubated with first antibody at 4°C overnight and then incubated with the second antibody for 1 h. Then the bands were observed with an enhanced chemiluminescence detection kit (36208-A, Yeasen) and analyzed using the ChemiDoc XRS System (Bio-Rad).

In the IP assay, the total cell protein extracts were incubated with Protein-G magnetic beads (10004d, Invitrogen) and pre-treated with antibody at 4°C overnight. The beads were washed with cell lysis buffer, and the immunoprecipitated samples were analyzed via immunoblotting.

The antibodies used were as follows: anti UBE2T (10105-2-AP), anti RPL6 (15387-1-AP), anti p53 (21891-1-AP), and anti RPL11 (16277–1-AP) purchased from Proteintech. Anti HDM2 (Sc-965) was purchased from Santa or Bioworld (BS1223). Anti RPL30 (A13690), anti Actin (AC026), and anti Flag (F1804) were purchased from Sigma. Anti Ub (ab7254) was purchased from Abcam.

2.6 | CCK8 assay

According to the instructions, the cell viability was measured through the CCK8 Kit (C0005, Targetmol). The cells of the control group and the treatment group were seeded in a 96-well plate for an appropriate time. The viability was measured as OD450 values after 2 h incubation with determination solution using a Microplate Reader (BioTek ELx800).

2.7 | Colony formation assay

The same number of cells of the control group and treatment group were seeded in a six-well plate. After incubation for 2 weeks, the cell colonies (>50 cells) were fixed with 4% paraformaldehyde and stained with 0.0125% crystal violet (V5265, Sigma), and the number of colonies was calculated.

2.8 | Wound healing assay

Cells grew to 80% confluence in a 24-well plate and were wounded by making a single scratch in the monolayer with a sterile 200-μL pipette tip. The migration of cells was monitored at time 0 h and 24 h with serum-free medium. The reduced distances were analyzed using Image J (National Institutes of Health, NIH).

2.9 | Transwell invasion assay

Transwell assays were conducted with 24-well (353047, Falcon) and transwell inserts (353097, Falcon) pre-coated with matrigel (356234, Corning). Then, 1×10^5 cells with serum-free medium were placed in the upper chamber and medium containing 10% FBS in the lower chamber, with incubation for an appropriate time. The invasive cells attached to the bottom surface of the filter were stained with crystal violet (V5265, Sigma) and were imaged under a conventional microscope (DMI3000B, Leica) and counted manually.

2.10 | Flow Cytometry

Cells were collected and processed according to the instructions for the Annexin-V FITC/PI Apoptosis Detection Kit (KGA107, KeyGEN BioTECH). A flow cytometer was used to analyze the cells (Flow Sight, Millipore).

2.11 | Mass spectrometry analysis

LN229 cells were lysed in IP buffer (87787, Thermo Fisher Scientific) containing an inhibitor cocktail (4693116001, Roche). The protein concentration was determined by BCA reagent (23225, Pierce). Subsequently, the cell lysates were incubated overnight with Protein-G magnetic beads (10004d, Invitrogen) and anti-UBE2T antibody (10105-2-AP, Proteintech) at 4°C. The magnetic beads were then sent to Bioprofile Company for mass spectrometry analysis (EASY-nLC1200, Thermo Fisher Scientific).

2.12 | Immunofluorescence

Cells were fixed and permeabilized with cold methyl alcohol (−20°C) for 10 min and then blocked in 5% donkey serum for 1 h and incubated with the primary antibody in PBS containing 1% BSA at 4°C overnight. The cells were incubated with fluorochrome-conjugated secondary antibody for 45 min. DAPI staining solution (E607303, BBI Life Sciences) was used to stain the cell nucleus. Cell samples were visualized on a confocal microscope with appropriate emission filters (LSM 510 META, Carl Zeiss).

2.13 | Animal experiment

All animals were raised and operated in accordance with the guidelines established by the Medical Research Animal Ethics Committee of Central South University. LN229-shVector and LN229-shUBE2T cells (8×10^6) were subcutaneously injected into the 4-week-old female BALB/c nude mice, with five in each group. Tumor formation was examined every 3 days. The tumor volume was calculated as volume (mm^3) = $d^2\times D/2$, where d and D were the shortest and the longest diameters, respectively. At the indicated time points, animals were killed, and tissues were collected and fixed with 10% buffered formalin for immunohistochemistry (IHC) analysis.

2.14 | Immunohistochemistry

Tissue sections of animal tumor and 40 clinical GBM pathological sections from the Department of Pathology of Xiangya Hospital (2019–2021) were collected. IHC was performed using a universal two-step detection kit (PV-9000, ZSGB-Bio) and a DAB kit (ZLI-9017, ZSGB-Bio). Then the sections were lightly counterstained with Mayer hematoxylin (ZLI-9610, ZSGB-Bio). The expression of each protein was semi-quantitatively evaluated using the method previously described.[14]

2.15 | Bioinformatics analysis

The CGGA database (http://www.cgga.org.cn/) and TCGA databases (https://portal.gdc.cancer.gov/) contain mRNA microarray data and clinical information on glioma and GBM patients. GraphPad software was used to analyze the survival curve.

2.16 | Statistical analysis

Data analysis was conducted using SPSS software. Statistical differences were determined using Student's *t*-test. All values are expressed as mean values ± SE of three individual experiments. A value of $p < 0.05$ was considered statistically. The relationship between UBE2T and RPL6 was analyzed using Pearson's method.

3 | RESULTS

3.1 | UBE2T is overexpressed in glioblastoma

Our previous work showed that there were 105 differentially expressed genes between the high and LGG tissue samples ($n = 5$) by transcriptome sequencing and now 26,801 differentially expressed genes between the GBM cells (U251 and LN229) and LGG cells (HS683) through cell transcriptome sequencing. Further, there were

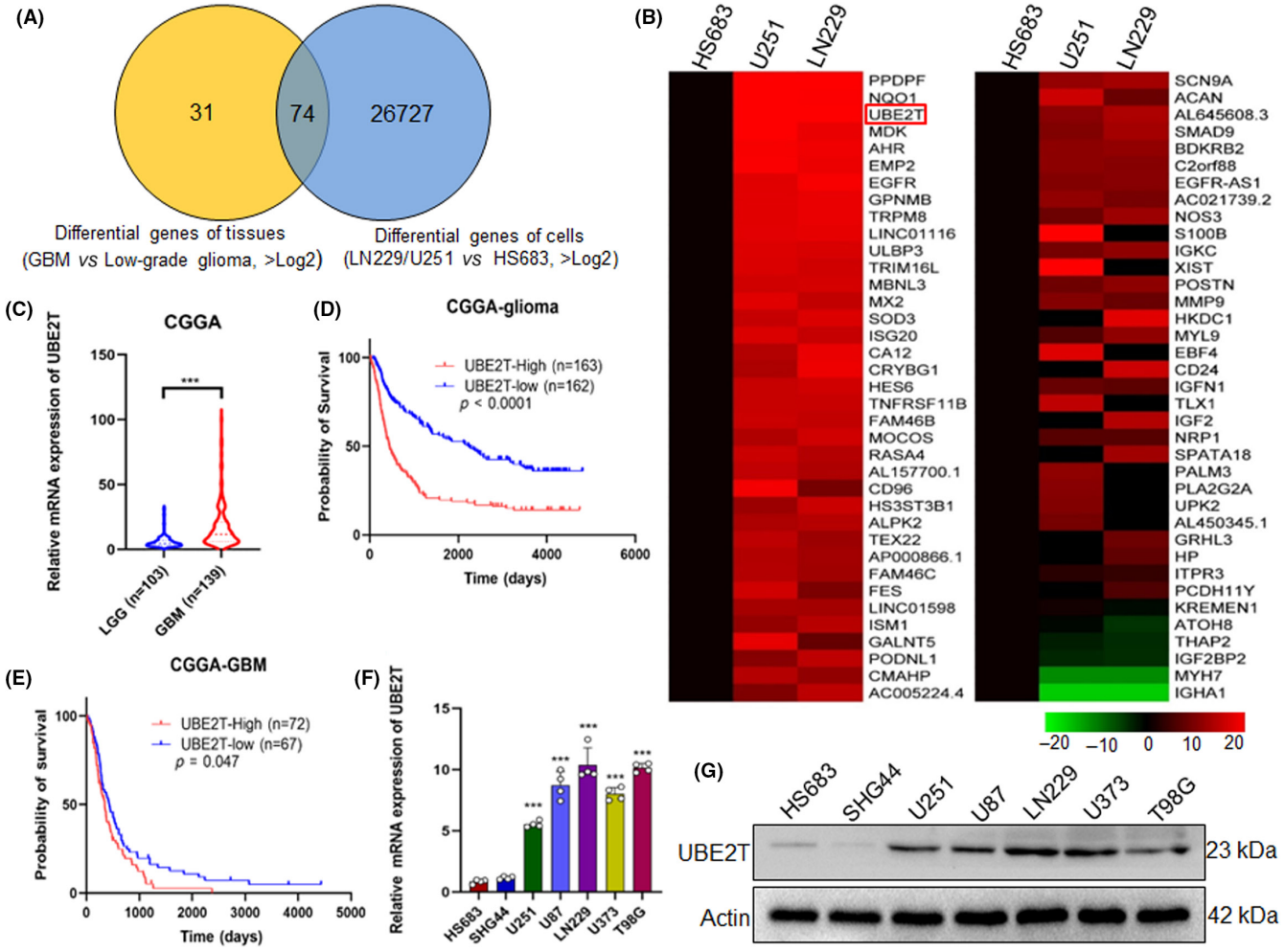


FIGURE 1 UBE2T is overexpressed in glioblastoma (GBM). (A) The differential gene expression was analyzed by transcriptome sequencing in tissue samples and cells. (B) The heatmap of 74 differentially expressed genes. (C) The mRNA expression of UBE2T was analyzed using the CGGA database and low-grade glioma (LGG) and GBM tissues. The overall survival of glioma (D) and GBM (E) was analyzed by CGGA database in differentially UBE2T expression. (F) The mRNA expression of UBE2T was analyzed by quantitative RT-PCR in glioma cells. (G) The protein expression of UBE2T was analyzed by western blot in glioma cells. ***$p < 0.001$.

74 differentially expressed genes in the two sets of data (Figure 1A), in which there were 69 genes upregulated and 5 genes downregulated in GBM cells. Among them, it can be found that UBE2T is the epigenetic regulatory molecule with the most obviously differential expression (Figure 1B). CGGA and TCGA database datas showed that UBE2T expression was higher in high-grade glioma than LGG ($p<0.001$) (Figure 1C, Figure S1A), and the high expression of UBE2T in glioma was correlated with a shorter survival time ($p<0.0001$) (Figure 1D, Figure S1B). In GBM, data from the CGGA database showed that the high expression of UBE2T was correlated with a poor prognosis as well (Figure 1E). However, the differential expression of UBE2T in GBM from TCGA was not correlated with prognosis (Figure S1C), which is not consistent with the data from CGGA. The reason might be related to the different clinical sample composition of different databases. Further, in the LGG cell lines HS683 and SHG44 and GBM cell lines U251, U87, LN229, U373, and T98G, quantitative RT-PCR and western blot have confirmed that UBE2T was highly expressed in GBM cells (Figure 1F, G). These results indicate that the overexpression of UBE2T in GBM may be related to the malignancy of GBM.

3.2 | UBE2T promotes cell proliferation, invasion, and anti-apoptosis of glioblastoma cells

To explore the function of UBE2T in GBM cells, stable cell lines (LN229-shUBE2T-1 and LN229-shUBE2T-2) and U251 cells transfected with the pLVX-His-UBE2T plasmid were used (Figure S2). CCK8 and colony formation assay were performed, and the data showed that the proliferation of LN229-shUBE2T cells was significantly decreased compared to LN229-sh-Vector cells. After overexpression of UBE2T in U251 cells, it obviously promoted cell proliferation (Figure 2A, B). The wound healing and transwell assay results demonstrated that the migration and invasion ability of the LN229-shUBE2T group were significantly reduced compared with the control group. After overexpression of UBE2T

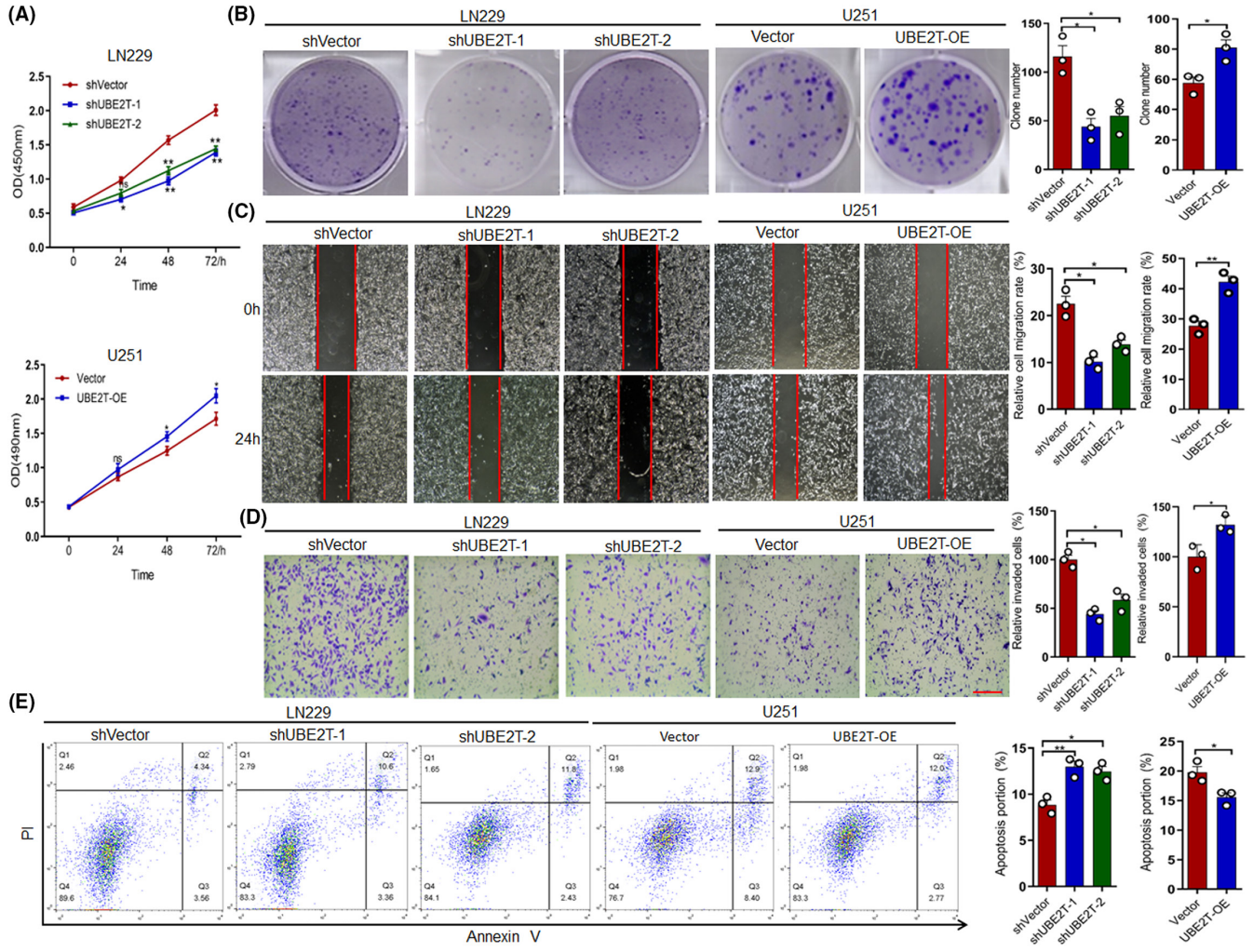


FIGURE 2 UBE2T promotes cell proliferation, invasion, and anti-apoptosis of glioblastoma (GBM) cells. The stable cell lines (LN229-shVector and LN229-shUBE2T cells) and U251 cells transfected with pLVX-Vector or pLVX-His-UBE2T plasmids were used. (A) Cell proliferation was analyzed by CCK8 and (B) colony formation assay. (C) Cell migration was analyzed by scratch assay. (D) Cell invasion was analyzed by transwell assay. Scale bar: 100 μm. (E) Cell apoptosis was analyzed by flow cytometry assay. ns: $p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$.

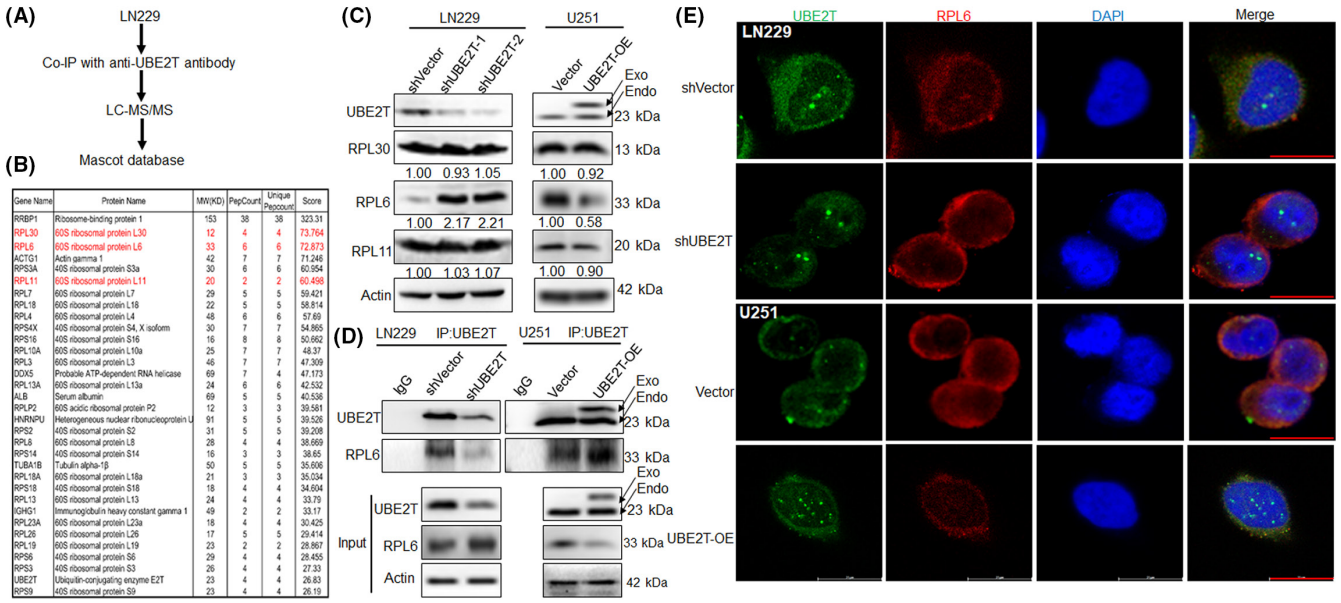
in U251 cells, the migration and invasion ability of cells was increased (Figure 2C, D). Flow cytometry experiment data revealed that the apoptosis of LN229-shUBE2T cells was significantly enhanced, while overexpression of UBE2T in U251 cells could reduce cell apoptosis (Figure 2E). The above results indicated that UBE2T enhances the proliferation, invasion, and anti-apoptosis of GBM cells.

3.3 | UBE2T interacts with RPL6 in glioblastoma cells

To identify the molecules interacting with UBE2T in GBM cells, mass spectrometry analysis was performed, the data showed that many members of the RPLs interacted with UBE2T (Figure 3A, B). With the score > 60 as the standard, interactions between RPL30, RPL6, and RPL11 and UBE2T were discovered. Western blot results demonstrated that only RPL6 expression increased in LN229-shUBE2T cells, and the expression of RPL6 was inhibited after overexpression of UBE2T in U251 cells. The expression of RPL30 and RPL11 did not change (Figure 3C). Further, the IP results showed that the interaction of UBE2T with RPL6 weakened with the decrease of UBE2T and strengthened with the overexpression of UBE2T (Figure 3D). Moreover, immunofluorescence experiment results revealed that the fluorescence intensity of UBE2T and RPL6 were negatively correlated, and both were located in the cytoplasm and exhibited co-localization (Figure 3E). Forty clinical specimens of GBM were further used for IHC analysis, and the results also showed that UBE2T was negatively correlated with RPL6 (Figure S3). These results proved that RPL6 could interact with UBE2T, which inhibited the RPL6 expression.

3.4 | UBE2T reduces RPL6 protein stability through ubiquitination-mediated degradation

To clarify how UBE2T could inhibit RPL6 expression, the quantitative RT-PCR results showed that mRNA expression of RPL6 did not change in LN229-shUBE2T cells or U251 cells overexpression of UBE2T compared with control groups (Figure S4). These results indicated that the regulation of RPL6 by UBE2T may be a post-translational modification. After LN229-shUBE2T and U87-shUBE2T cells were treated with the protein synthesis inhibitor CHX, the results revealed that it had prolonged the half-life of RPL6 protein compared with the control group, while after overexpression of UBE2T in U251, the RPL6 protein degradation rate was accelerated (Figure 4A). These results indicated that UBE2T affects the protein stability of RPL6. Further, when cells were treated with the proteasome inhibitor MG132, the data showed that the RPL6 expression in the sh-Vector group was lower than that in shUBE2T, and after treatment with MG132, the expression of RPL6 in the shVector group was increased. Similar results were shown for U251 and U251 overexpression of UBE2T cells (Figure 4B). These results indicated that UBE2T reduced the stability of RPL6 through the proteasome pathway. In vitro ubiquitination experiments showed that knockdown of UBE2T expression could inhibit the RPL6 ubiquitination in



Gene Name	Protein Name	MW(KD)	PepCount	Unique Pepcount	Score
RRBP1	Ribosome-binding protein 1	153	38	38	323.31
RPL30	60S ribosomal protein L30	12	4	4	73.764
RPL6	60S ribosomal protein L6	33	6	6	72.873
ACTG1	Actin gamma 1	42	7	7	71.246
RPS3A	40S ribosomal protein S3a	30	6	6	60.954
RPL11	60S ribosomal protein L11	20	2	2	60.498
RPL7	60S ribosomal protein L7	29	5	5	59.421
RPL18	60S ribosomal protein L18	22	5	5	58.814
RPL4	60S ribosomal protein L4	48	6	6	57.69
RPS4X	40S ribosomal protein S4, X isoform	30	7	7	54.865
RPS16	40S ribosomal protein S16	16	8	8	50.662
RPL10A	60S ribosomal protein L10a	25	7	7	48.37
RPL3	60S ribosomal protein L3	46	7	7	47.309
DDX5	Probable ATP-dependent RNA helicase	69	7	4	47.173
RPL13A	60S ribosomal protein L13a	24	6	6	42.532
ALB	Serum albumin	69	5	5	40.536
RPLP2	60S acidic ribosomal protein P2	12	3	3	39.581
HNRNPU	Heterogeneous nuclear ribonucleoprotein U	91	5	5	39.526
RPS2	40S ribosomal protein S2	31	5	5	39.208
RPL8	60S ribosomal protein L8	28	4	4	38.669
RPS14	40S ribosomal protein S14	16	3	3	38.65
TUBA1B	Tubulin alpha-1β	50	5	5	35.606
RPL18A	60S ribosomal protein L18a	21	3	3	35.034
RPS18	40S ribosomal protein S18	18	4	4	34.694
RPL13	60S ribosomal protein L13	24	4	4	33.79
IGHG1	Immunoglobulin heavy constant gamma 1	49	2	2	33.17
RPL23A	60S ribosomal protein L23a	18	4	4	30.425
RPL26	60S ribosomal protein L26	17	5	5	29.414
RPL19	60S ribosomal protein L19	23	2	2	28.867
RPS6	40S ribosomal protein S6	29	4	4	28.455
RPS3	40S ribosomal protein S3	26	4	4	27.33
UBE2T	Ubiquitin-conjugating enzyme E2T	23	4	4	26.83
RPS9	40S ribosomal protein S9	23	4	4	26.19

FIGURE 3 UBE2T interacts with RPL6 in glioblastoma (GBM) cells. (A) Schematic diagram of mass spectrometry. (B) The proteins interacting with UBE2T in LN229 cells by mass spectrometry. (C) The protein expression of UBE2T, RPL30, RPL6, and RPL11 were analyzed by western blot. Quantification of the protein band was performed using ImageJ software. (D) The interaction between UBE2T and RPL6 were analyzed by immunoprecipitation. (E) The interaction and colocation between UBE2T and RPL6 were analyzed by immunofluorescence. Scale bar: 20 μm.

LN229 and U87 cells (Figure 4C). Further, 293T cells were transfected with His-UBE2T, Flag-RPL6, and Ub plasmids, respectively. The data demonstrated that the overexpression of UBE2T increased the ubiquitination level of RPL6 (Figure 4D). Studies have shown that there are many types of ubiquitination involved in the requisition of target proteins, of which K48 polyubiquitination is mainly involved in degradation, while K63 polyubiquitination is predominately associated with stability and signal transduction.[15,16] The experiments further showed that UBE2T promotes the K48-linked but not K63-linked polyubiquitination of RPL6 (Figure 4E). These results indicated that UBE2T could enhance the degradation of RPL6 through direct modification by K48-linked polyubiquitination.

3.5 | UBE2T promotes the proliferation, invasion, and anti-apoptosis of glioblastoma cells through RPL6

To determine whether RPL6 was involved in the tumorigenic role of UBE2T, western blot was performed to detect the protein levels of RPL6 in glioma cells, and the results showed that RPL6 was minimally expressed in GBM compared with LGG cells (Figure S5). LN229-shUBE2T cells were transfected with RPL6 siRNA, or U251 cells were co-transfected by pLVX-His-UBE2T and pcDNA3.1-RPL6 plasmids (Figure S6). CCK8 and transwell assay results showed that knockdown of UBE2T significantly inhibited cell proliferation and

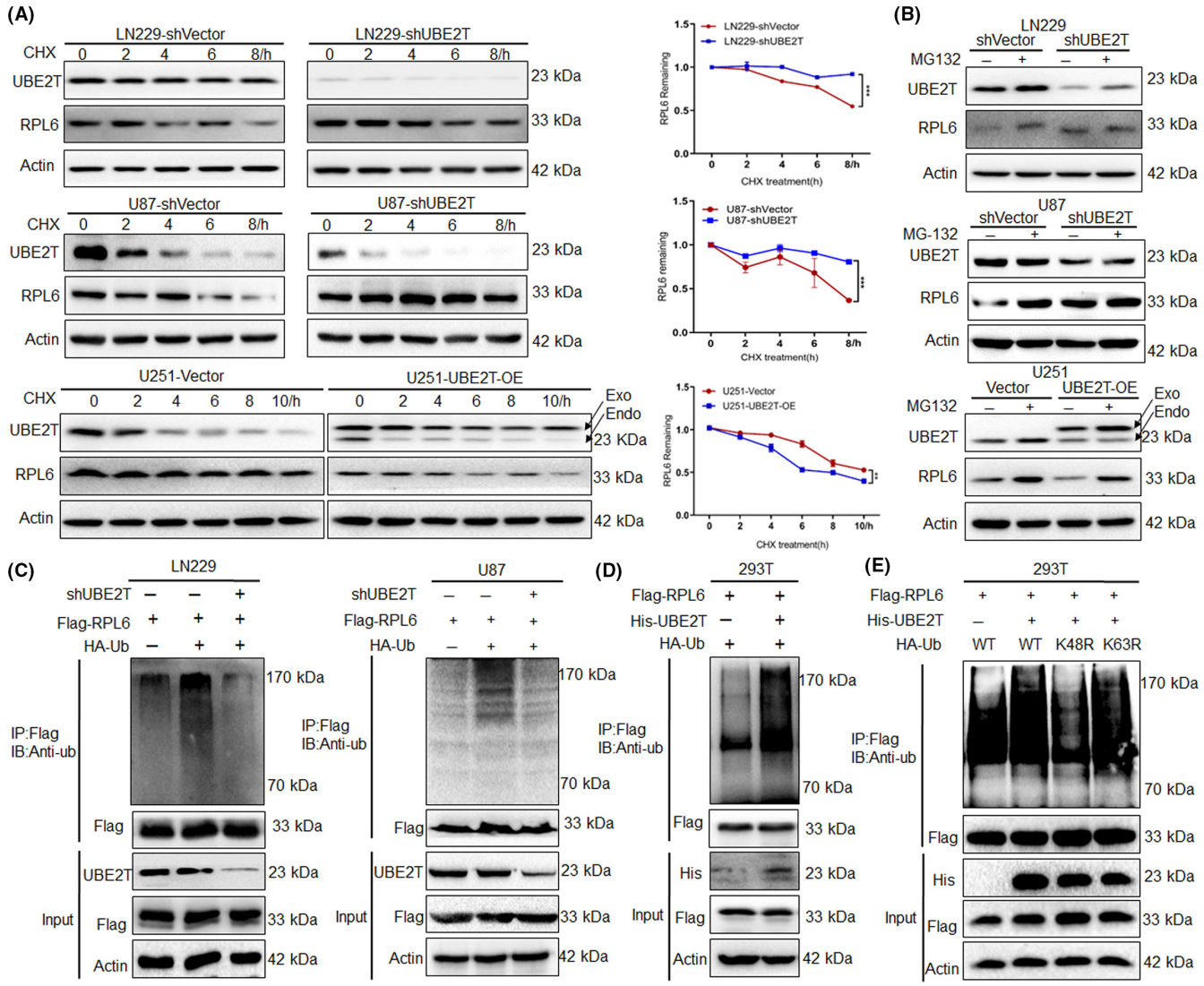


FIGURE 4 UBE2T reduces RPL6 protein stability through ubiquitination-mediated degradation. (A) The cells were treated with cycloheximide (CHX, 50 μg/mL) for the indicated duration, and the protein expression of UBE2T and RPL6 were analyzed by western blot. (B) The cells were treated with MG132 for 8 h, and the protein expression of UBE2T and RPL6 were analyzed by western blot. (C) LN229-shUBE2T and U87-shUBE2T cells were transfected with Flag-RPL6 and HA-Ub plasmids, and cell lysates were immunoprecipitated with an anti-Flag followed by immunoblotting against Ub antibody. (D) 293 T cells were transfected with Flag-RPL6, LVX-His-UBE2T, and HA-Ub plasmids; cell lysates were immunoprecipitated with an anti-Flag followed by immunoblotting against Ub antibody. (E) 293 T cells were transfected with Flag-RPL6, pLVX-His-UBE2T, and HA-Ub or K48R or K63R plasmids, respectively. Cell lysates were immunoprecipitated with an anti-Flag followed by immunoblotting against Ub antibody.

invasion, while further knockdown RPL6 by siRNA could rescue the inhibition caused by the knockdown of UBE2T. Overexpression of UBE2T in U251 cells could promote cell proliferation and invasion, and further overexpression of RPL6 would restrain this promotion (Figure 5A, B). Flow cytometry experiment data showed that knocking down RPL6 by siRNA in LN229-shUBE2T cells reversed the cell apoptosis caused by the knockdown of UBE2T. Overexpression of UBE2T inhibited U251 apoptosis, and after further overexpression of RPL6, it could significantly rescue the inhibition of apoptosis (Figure 5C). These results indicated that UBE2T can promote GBM malignancy through reduction of RPL6.

3.6 | RPL6 promotes wt-p53 expression and inhibits mut-p53 expression

Research has revealed that RPL6 can bind with E3 ligase HDM2 and inhibit HDM2 activity and then attenuate HDM2-mediated wt-p53 degradation.[17] LN229 are wt-p53 cells,[18–20] the expressions of RPL6 and p53 were both upregulated in LN229-shUBE2T cells. After overexpression of UBE2T in LN229 cells, the expressions of RPL6 and p53 were inhibited, indicating that UBE2T inhibited wt-p53 expression through RPL6. Interestingly, U251 cells are mut-p53 cells (p53 R273H).[21,22] Although knocking down of UBE2T in U251 cells could promote RPL6 expression, the mut-p53 expression was reduced. After overexpression of UBE2T in U251 cells, the expression of RPL6 was inhibited, while the expression of p53 protein also increased (Figure 6A). These results indicated that UBE2T inhibited the expression of RPL6 and further decreased the expression of wt-p53; however, it increased the mut-p53 expression. Further data showed that the overexpression of RPL6 could inhibit HDM2 expression and increase wt-p53 expression in LN229 cells. While in U251 and U373, another mut-p53 cells (p53 R273H),[23,24] knockdown of RPL6 could promote the expression of HDM2 and mut-p53 (Figure 6B). Moreover, the immunofluorescence data showed that there was co-localization between RPL6 and HDM2 in LN229 cells, and the fluorescence intensity of both is negatively correlated (Figure 6C). These results demonstrated that RPL6 plays a tumor suppressor role regardless of the wt-p53 or the mut-p53 cells of GBM.

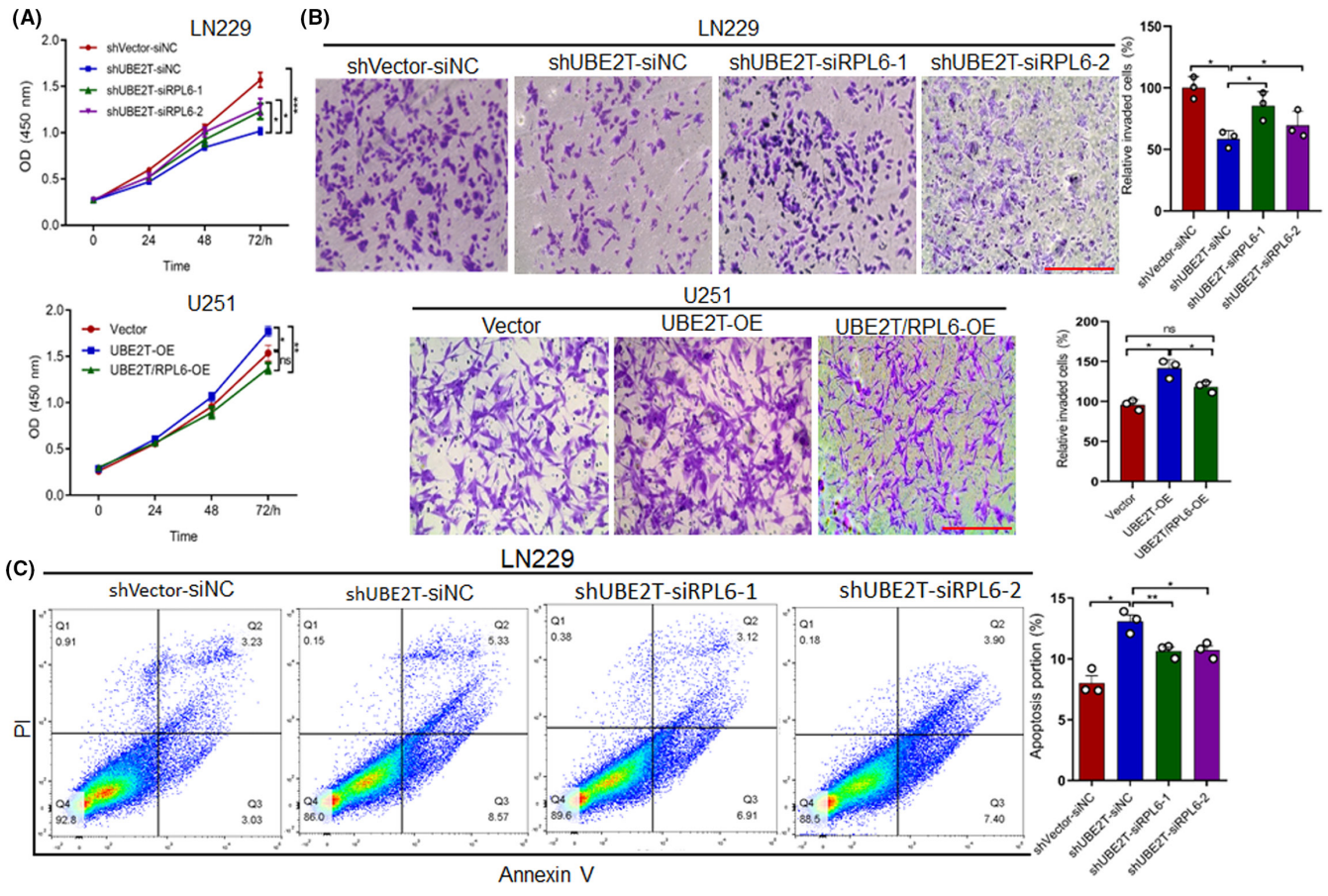


FIGURE 5 UBE2T promotes the proliferation, invasion, and anti-apoptosis of glioblastoma (GBM) cells through RPL6. LN229-shUBE2T cells transfected with RPL6 siRNA or U251 cells co-transfected with pLVX-His-UBE2T and pcDNA3.1-RPL6 plasmid were used. (A) Cell proliferation was analyzed by CCK8 assay. (B) Cell invasion was analyzed by transwell assay. Scale bar: 100 μm. (C) The apoptosis was analyzed by flow cytometry assay. ns: $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.7 | UBE2T knockdown suppressed tumor growth in vivo

LN229-shVector or LN229-shUBE2T cells were subcutaneous injected into the BALB/c nude mice to establish the xenograft tumor models. The results showed that the growth and volume of tumors in the shUBE2T group were significantly decreased compared to the shVector group (Figure 7A, B). IHC analysis found that the expression of UBE2T was decreased, while the expression of RPL6 and p53 were both increased in the shUBE2T group (Figure 7C). These results indicated that UBE2T may promote the growth of GBM by inhibiting the expression of RPL6 in vivo.

4 | DISCUSSION

Recent research reports that UBE2T is highly expressed in GBM, which promotes the invasion and migration of GBM cells by enhancing the stability of GRP78 and enhancing EMT.[25] In this study, taking LGG cells as the control, UBE2T with high expression was also analyzed in GBM. The results revealed that UBE2T promoted the proliferation, invasion, and anti-apoptotic ability of GBM cells through ubiquitination-mediated degradation of RPL6.

It has been found that E2 conjugation enzymes usually bind to E3 ligases to perform the function of ubiquitination substrate protein, but some E2 enzymes can directly bind to the substrate and transfer Ub to the lysine residue of the substrate protein as mixtures of E2/E3.[26] For example, E2 conjugation enzyme BRUCE directly ubiquitylates its substrate Smac/DIABLO in an E3 ligase-independent manner.[27] UBE2C enhances ubiquitination and degradation of p53, thereby promoting the proliferation and migration of endometrial cancer cells.[28] UBE2O functioned as a Ub enzyme of AMPKα2, promoting its ubiquitination and degradation, consequently activating the mTORC1 signal pathway and contributing to oncogenesis and metastasis of breast cancer.[29] UBE2O directly targets Mxi1 through conducting the ubiquitination and degradation of Mxi1 at the K46 residue, thus promoting tumorigenesis and radiation resistance of lung cancer.[30] UBE2T directly induces the ubiquitination and degradation of the tumor suppressor RACK1, thereby activating the Wnt/β-catenin signaling pathway to promote the progression of gastric cancer.[11] In this research, our result further confirmed that UBE2T could promote the ubiquitination-mediated degradation of RPL6 in an E3 ligase-independent manner in GBM cells and participate in the GBM malignancy.

The ribosomal proteins (RPs) not only participated in protein biosynthesis but also regulated DNA replication and repair, RNA

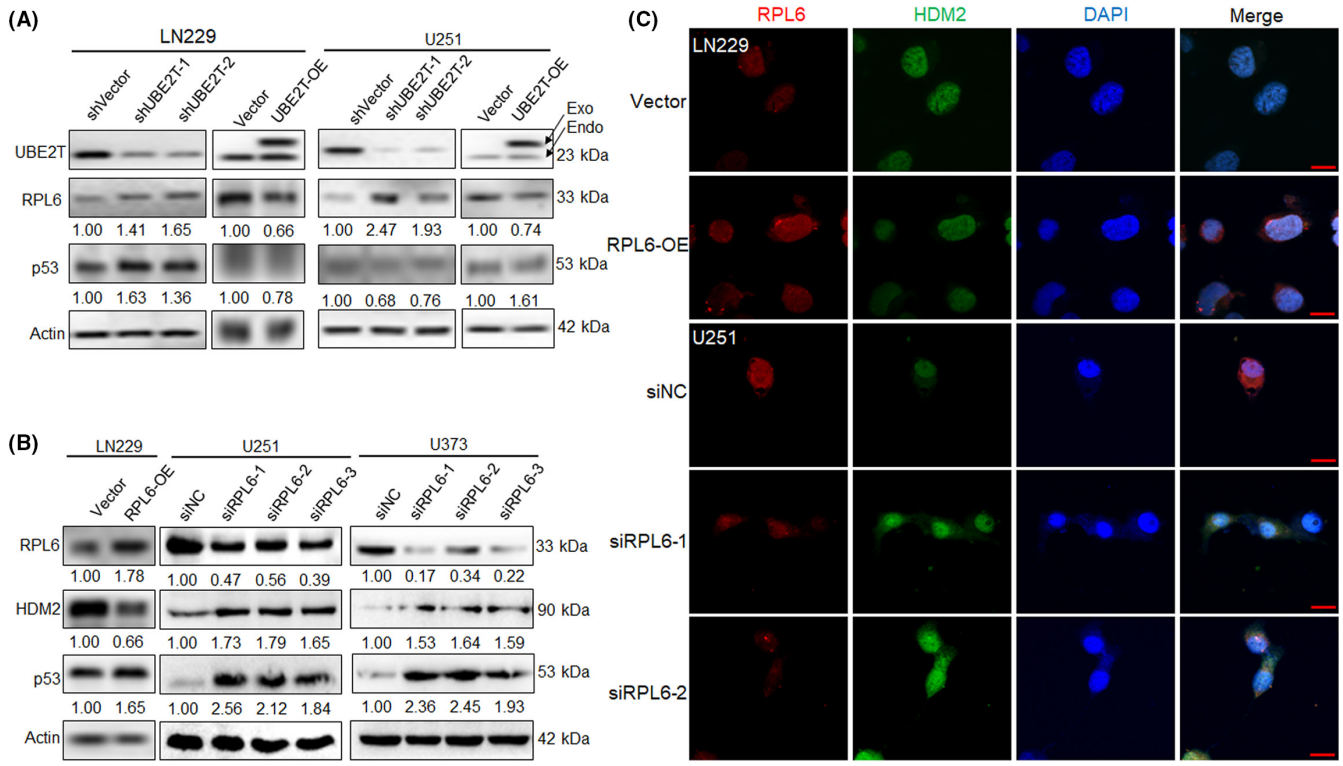


FIGURE 6 RPL6 promotes wt-p53 expression and inhibits mut-p53 expression. (A) LN229-shUBE2T stable cells or LN229 cells transiently transfected with pLVX-His-UBE2T plasmid and U251 cells transiently transfected with shUBE2T or pLVX-His-UBE2T plasmid, respectively. The protein expression of UBE2T, RPL6, and p53 were analyzed by western blot. (B) LN229 cells transfected with pcDNA3.1-RPL6 plasmid, and U251 and U373 cells transfected with siRPL6, respectively. The protein expression of RPL6, HDM2, and p53 were analyzed by western blot. (C) The interaction and colocation between RPL6 and HDM2 were analyzed by immunofluorescence. Scale bar: 20 μm. NC, negative control.

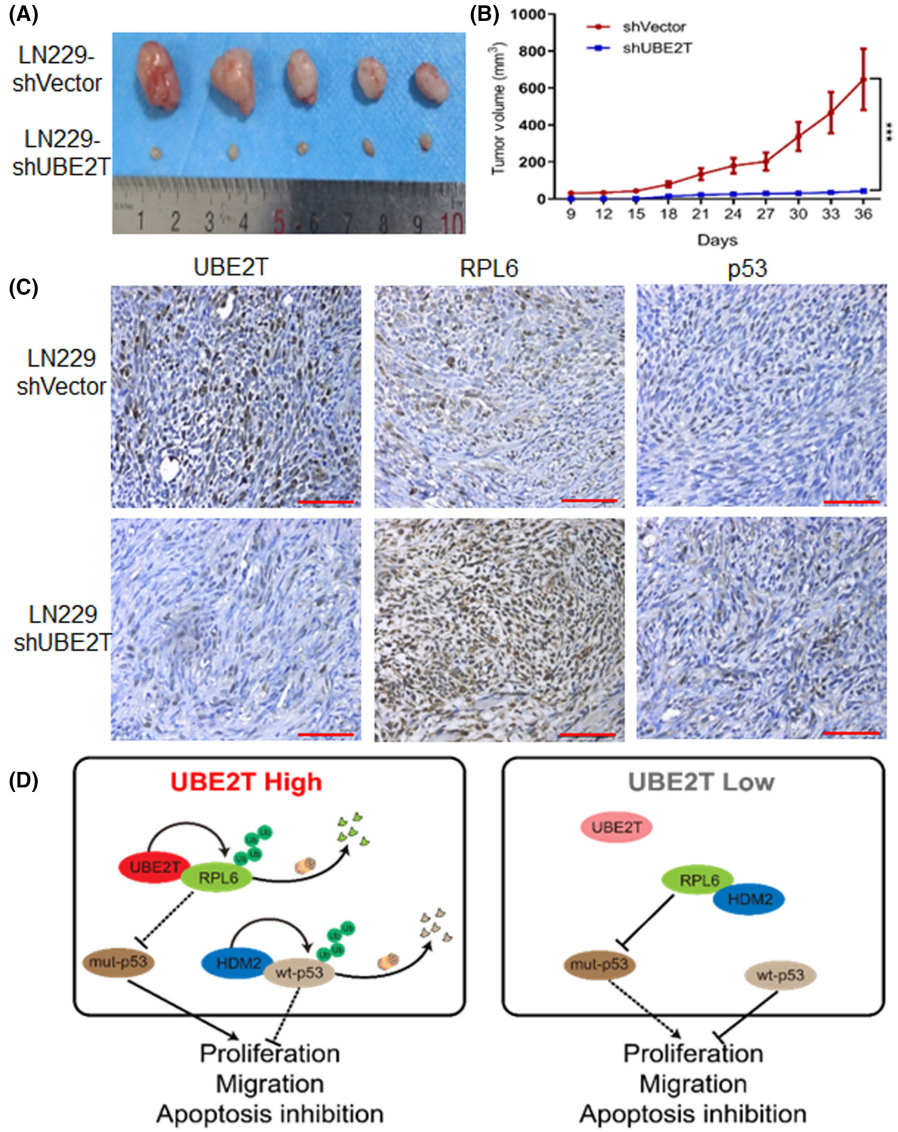


FIGURE 7 UBE2T knockdown suppressed tumor growth in vivo. $8x10^6$ LN22-shVector or LN229-shUBE2T cells were subcutaneously injected into nude mice to construct a xenograft tumor model. The tumor size of nude mice was detected every 3 days. The nude mice were killed and the tumor tissue was stripped at the appropriate time. (A) At the experimental end point, tumor xenografts were dissected and photographed. (B) Tumor volume was measured. (C) Representative immunohistochemistry staining of UBE2T, RPL6, and p53 protein levels in xenograft tumor tissues. Scale bar: 100 μm. (D) The schematic diagram of UBE2T's role in promoting the malignant progression of glioblastoma (GBM) through ubiquitination-mediated degradation of RPL6 and further regulating p53.

splicing and modification, and transcription thus participating in the cell proliferation, metastasis, and apoptosis.[31] Some studies have found that the overexpression of RPL6 promoted cell proliferation and drug resistance by inhibiting cell apoptosis, and leading to poor prognosis in gastric cancer.[32–34] By interacting with oncogene NOP2/Sun RNA methyltransferase 2, RPL6 promotes the tumor progression of gallbladder cancer.[35] However, other research revealed that the RPL6 expression was significantly downregulated in early gastric cancer tissues compared to nontumorous tissues.[36] In lung cancer, RPL6 acted as a tumor suppressor, it binds to HDM2 and inhibits its E3 Ubligase activity, thus preventing the ubiquitination-mediated degradation of p53 and further leading to cell cycle arrest and inhibiting the growth of tumor cells.[17] The above evidence shows that the role of RPL6 depends on specific cell status and the cell type in tumors. RPL6 has a dual function in regulating tumors. In this study, RPL6 is found to inhibit cell proliferation and promote apoptosis, and UBE2T plays a tumor-promoting function through ubiquitination-mediated degradation of RPL6 in GBM cells.

p53 plays a pivotal role in determining death or survival of cells, and is often deregulated in tumors. Indeed, there are p53 mutations in approximately 50% of human tumors, most of which are caused by point and missense mutations.[37] In GBM patients, the mutation rate of p53 is approximately 30%.[38,39] The mutations of p53 not only lead to loss of wild-type suppressor function but also gaining of the oncogenic function and phenotype, which contributes to the invasiveness of tumors.[40–42] For example, when using paclitaxel to treat malignant glioma cells, it was found that LN229 cells with wt-p53 showed stronger paclitaxel sensitivity than T98G cells with mut-p53.[18] In this work, the results demonstrated that UBE2T inhibited RPL6 in LN229 cells with wt-p53 and upregulated the expression of HDM2, thereby inhibiting wt-p53 and promoting the malignancy of LN229 cells. However, UBE2T inhibits RPL6 in U251 and U373 cells with mut-p53. Although it also promotes the expression of HDM2, it cannot inhibit the expression of p53. Instead, it promotes the expression of mut-p53, which in turn promotes the malignancy of U251 cells. These results revealed that the adverse regulation of p53 by UBE2T in wt- and mut- p53 cells through regulation of RPL6 both promoted

the GBM malignancy. It should be noted that there may be other regulatory mechanisms for UBE2T to promote the expression of mut-p53 through RPL6, which needs to be deeply studied in future work.

In summary, the present study showed that overexpression of UBE2T promoted the proliferation, invasion, and anti-apoptosis of GBM cells. It is further revealed that UBE2T inhibited the expression of RPL6 through ubiquitination modification, thereby promoting the GBM malignancy regardless of whether p53 was of wild or mutation status (Figure 7D).

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CONFLICT OF INTEREST

No potential conflicts of interest were disclosed.

ANIMAL STUDIES

All animal experiments were approved by the Medical Research Animal Ethics Committee of Central South University.

ORCID

Liangfang Shen https://orcid.org/0000-0002-6429-7531
Lifang Yang https://orcid.org/0000-0002-3012-8350

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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