Received: 23 January 2018 | Revised: 8 May 2018 | Accepted: 16 May 2018

DOI: 10.1111/cas.13646

ORIGINAL ARTICLE

WILEY Cancer Science

Ubiquitin-specific protease 22 acts as an oncoprotein to maintain glioma malignancy through deubiquitinating B cell-specific Moloney murine leukemia virus integration site 1 for stabilization

Guan-Zhong Qiu[1,2] | Xiao-Yuan Mao[3,4,5] | Yue Ma[1] | Xing-Chun Gao[6] | Zhen Wang[1] | Ming-Zhu Jin[7] | Wei Sun[8] | Yong-Xiang Zou[8] | Jing Lin[9] | Hua-Lin Fu[1,10] | Wei-Lin Jin[1,10]

[1]Department of Instrument Science and Engineering, Key Lab. for Thin Film and Microfabrication Technology of Ministry of Education, School of Electronic Information and Electronic Engineering, Institute of Nano Biomedicine and Engineering, Shanghai Jiao Tong University, Shanghai, China

[2]Department of Neurosurgery, General Hospital of Jinan Military Command, Jinan, China

[3]Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha, China

[4]Institute of Clinical Pharmacology, Central South University, Changsha, China

[5]Human Key Laboratory of Pharmacogenetics, Changsha, China

[6]Shanxi Key Laboratory of Ischemic Cardiovascular Disease, Institute of Basic Medical Medicine, Xi'an Medical University, Xi'an, China

[7]Shanghai Jiao Tong University School of Medicine, Shanghai, China

[8]Department of Neurosurgery, Shanghai Changzheng Hospital, Second Military Medical University, Shanghai, China

[9]Department of Neurosurgery, The General Hospital of Western Air Force, Chengdu, China

[10]National Centers for Translational Medicine, Shanghai Jiao Tong University, Shanghai, China

Correspondence
Wei-Lin Jin, Department of Instrument Science and Engineering, Institute of Nano Biomedicine and Engineering, Shanghai Jiao Tong University, Shanghai, China
Emails: weilinjin@yahoo.com; weilinjin@sjtu.edu.cn

Funding information
Special Presidential Foundation of General Hospital of Jinan Military Command (Grant/Award Number: '2016BS04'), Shanghai Jiao Tong University Med-X Fund (Grant/Award Number: 'YG2015MS20'), National Natural Science Foundation of China (Grant/Award Numbers: '81402063' ,'81602193', '81671293', '81702944').

Ubiquitin-specific protease 22 (USP22) is a member of the "death-from-cancer" signature, which plays a key role in cancer progression. Previous evidence has shown that USP22 is overexpressed and correlates with poor prognosis in glioma. The effect and mechanism of USP22 in glioma malignancy, especially cancer stemness, remain elusive. Herein, we find USP22 is more enriched in stem-like tumorspheres than differentiated glioma cells. USP22 knockdown inhibits cancer stemness in glioma cell lines. With a cell-penetrating TAT-tag protein, B cell-specific Moloney murine leukemia virus integration site 1 (BMI1), a robust glioma stem-cell marker, is found to mediate the effect of USP22 on glioma stemness. By immunofluorescence, USP22 and BMI1 are found to share similar intranuclear expression in glioma cells. By analysis with immunohistochemistry and bioinformatics, USP22 is found to positively correlate with BMI1 at the post-translational level only rather than at the transcriptional level. By immunoprecipitation and in vivo deubiquitination assay, USP22 is found to interact with and deubiquitinate BMI1 for protein stabilization. Microarray analysis shows that USP22 and BMI1 mutually regulate a series of genes

Guan-Zhong Qiu, Xiao-Yuan Mao, and Yue Ma contributed equally to this work.

involved in glioma stemness such as *POSTN*, *HEY2*, *PDGFRA* and *ATF3*. In vivo study with nude mice confirms the role of USP22 in promoting glioma tumorigenesis by regulating BMI1. All these findings indicate USP22 as a novel deubiquitinase of BMI1 in glioma. We propose a working model of the USP22-BMI1 axis, which promotes glioma stemness and tumorigenesis through oncogenic activation. Thus, targeting USP22 might be an effective strategy to treat glioma especially in those with elevated BMI1 expression.

KEYWORDS
BMI1, cancer stemness, deubiquitination, glioma, USP22

1 | INTRODUCTION

Glioma is one of the most common intracranial primary tumors.[1] For years, despite conventional therapeutic strategies including surgery, radiation, and chemotherapy, median survival was hardly improved.[2] As a small side population in the tumor bulk, glioma stem-like cells (GSC) have been widely regarded as the main cause of glioblastoma multiforme refractoriness.[3,4] Hence, specific targeted therapies especially aiming at eliminating GSC will render great significance to therapeutic advancement and prognostic improvement.

The polycomb gene B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) is a core member of the polycomb repressive complex 1 involved in cell development by epigenetic modification.[5] Multiple studies have shown that BMI1 is frequently overexpressed and responsible for tumorigenesis in many types of cancers, such as colorectal cancer and lung adenocarcinoma.[6,7] BMI1 is a robust stemness-related factor in glioma. It can not only promote proliferation, migration, and invasion of differentiated glioma cells,[8] but can regulate self-renewal, apoptosis, and immunogenicity of GSC.[9] However, the mechanism for BMI1 overexpression in glioma remains not fully understood.

Ubiquitin-specific protease 22 (USP22) is a member of deubiquitinases (DUB) in mammals, which contains an N-terminal zinc-finger domain for substrate interaction and a C-terminal ubiquitin-specific peptidase domain for protein deubiquitination.[10] As a component of the human Spt-Ada-Gcn5-acetyltransferase complex, USP22 is involved in epigenetic modification for gene transcription.[11] Moreover, USP22 can also catalyze protein stabilization through cleaving the Ub moieties from target substrates to prevent them from proteolytic degradation.[12-14] USP22 is a member of the 11-gene death-from-cancer signature; thus, it is deemed as a putative cancer stem-cell marker, which has been extensively studied in a broad range of cancers.[15-18] However, the effect and mechanism of USP22 in glioma malignancy, especially cancer stemness, remains largely unknown.

Here, we show that USP22 is more enriched in stem-like tumorspheres than in differentiated glioma cells. USP22 knockdown inhibits glioma stemness partially by downregulating the protein level but not the transcriptional level of BMI1. Clinical analysis with glioma tissues also shows that USP22 correlates with the protein level rather than the transcriptional level of BMI1. USP22 interacts with and deubiquitinates BMI1 for post-translational stabilization. USP22 and BMI1 jointly regulate a series of genes involved in glioma stemness such as *POSTN*, *HEY2*, *PDGFRA* and *ATF3* in both glioma cell lines and clinical tissues. USP22 inhibition attenuates glioma tumorigenesis in the xenograft model through downregulating BMI1 expression. These findings not only indicate USP22 as a novel deubiquitinase of BMI1, but the presence of a USP22-BMI1 axis to mediate glioma stemness and tumorigenesis by oncogenic activation. Thus, targeting USP22 may be a promising strategy to treat glioma with BMI1 overexpression.

2 | MATERIALS AND METHODS

2.1 | Clinical samples

Thirty glioma samples were obtained from surgeries carried out between 2013 and 2015 in the Department of Neurosurgery at Xiangya Hospital. Five normal brain tissues were acquired from patients with traumatic brain edema that underwent partial brain resection. All procedures related to acquiring the samples from the patients were consented by the patients and were approved by the ethics committee of Xiangya Hospital. General information of clinicopathological features of 30 glioma patients is listed in Table S1.

2.2 | Cell culture

Established glioma cell lines, including LN229, U251, U87MG, and A172, as well as the HA astroglial cell line, were purchased from the Chinese Academy of Sciences Cell Bank. Authenticity of the cancer cell lines was tested by short tandem repeat profiling. All cell lines were cultured in DMEM medium (Gibco, Waltham, MA, USA) supplemented with 10% (v/v) FBS (Gibco), 100 U/mL penicillin (Gibco), and 100 U/mL streptomycin (Gibco) in a 5% CO_2 atmosphere. GSC were cultured in a serum-free medium (SFM) composed of DMEM/F12 (Gibco), 20 ng/mL basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA), 20 ng/mL epidermal growth factor (Sigma, St Louis, MO, USA), and 20 μg/mL B27 supplement (Life Technologies, Carlsbad, CA, USA).

2.3 | Viral packaging and lentivirus transfection

The oligonucleotides shown in Table S2 were annealed and cloned into vector LV3 (pGLVH1/GFP + Puro) (Shanghai GenePharma, Shanghai, China) to generate specific shRNA-expressing plasmids. The lentivirus transfection procedure has been described in a previous study.[8]

2.4 | Construction and transduction of pTAT-HA recombinant protein in vitro

The construction and transduction procedure of the cell-penetrating peptide TAT has been described in a previous study.[19] In a typical procedure, the DNA sequence encoding BMI1 and USP22 was PCR-amplified from a full-length human BMI1 (BC011652; Proteintech Group, Rosemont, IL, USA) and USP22 (BC126898; Proteintech Group) expression construct. Afterwards, the DNA sequence was inserted into the pTAT-HA vector (gift from Dr Steven Dowdy, The University of California, USA). After protein size and purity were analyzed by immunoblotting, the recombinant protein was added externally to the neurosphere culture medium at a concentration of 0.2 μM with 2-hour incubation. The transducible effects of the TAT fusion proteins were analyzed by western blot.

2.5 | Tumorsphere formation assay

In a typical procedure, 2×10^3 dissociated cells of each group were synchronously cultured in a 60-mm floating Petri dish containing SFM; afterwards, tumorsphere formation was observed. For 10-14 days, tumorsphere diameters in 30 randomly selected microscopic fields were calculated and collected for statistical analysis.

2.6 | Limiting dilution assay

Attached and sphere cells were dissociated and plated on 96-well plates with 0.2 mL of SFM. Final cell dilutions ranged from 120 cells/well to 1 cell/well with 0.2 mL SFM. Cultures were fed with 0.025 mL of SFM every 2 days until the 7th day. Then, the percentage of wells without spheres for each cell-plating density was calculated and plotted against the number of cells per well. Regression lines were plotted, and x-intercept values were calculated, which represented the number of cells required to form at least 1 tumorsphere in each well.

2.7 | Tumor migration and invasion assays

The procedure of tumor migration and invasion assays is indicated in Appendix S1.

2.8 | Quantitative RT-PCR analysis

The procedure of quantitative RT-PCR analysis has been described in the previous study.[8] HPRT1 was selected as the endogenous control in the assay. The PCR primer sequences are listed in Table S2.

2.9 | Western blot

The procedure of western blot analysis has been described in the previous study.[8] The antibodies used were USP22 (ab4812), POSTN (ab172615), and ATF3 (ab200655) purchased from Abcam (Cambridge, UK); GFP (M20004), Flag (M20008) and HA (M20003) from Abmart (Shanghai, China); PDGFRA (sc-21789), α-Tubulin (sc-53646), β-Actin (sc-47778), and GAPDH (sc-47724) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); BMI1 (66161-1-Ig) and HEY2 (10597-1-AP) were from Proteintech Group.

2.10 | Immunoprecipitation

For exogenous immunoprecipitation, after 48 hours of expression plasmid transfection into HEK293FT cells, cells were collected and lysed in a chilled nuclear lysis buffer (50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 1% Nonidet P40) with 2% (v/v) complete proteinase inhibitor (CPI) and $NaVO_3$. Cells were sonicated, and the supernatants were collected by centrifugation at 14 000 *g* for 15 minutes at 4°C. Flag-conjugated protein A + G agarose beads (Sigma) were added to the samples; afterwards, the samples were incubated for 7-9 hours at 4°C with gentle rotation. For endogenous immunoprecipitation, whole-cell lysate of abundant U251 cells (about 1×10^7) in the nuclear lysis buffer was divided equally into 2 microcentrifuge tubes and incubated with anti-USP22 antibody (ab4812; Abcam) and anti-IgG antibody (ab109489; Abcam) at 4°C respectively. After 6 hours, non-tagged protein A + G beads (Santa Cruz Biotechnology) were added to each microcentrifuge tube and the mixed samples were incubated at 4°C overnight. After that, the beads were precipitated by centrifugation at 8000 *g* for 30 seconds at 4°C. Then, the beads were washed 4 times with 1 mL ice-cold lysis buffer, and the immunoprecipitated proteins were released from the beads by boiling the sample buffer for 2 minutes. The proteins were detected by western blot assay as previously mentioned.

2.11 | Protein stability assay

Protein stability was analyzed by seeding the equivalent cell number and, 24 hours later, the sample was treated with 10 mg/mL cycloheximide (CHX) (Sigma) for the indicated time course. To analyze BMI1 proteasomal degradation contributions, HEK293FT cells were transfected with GFP-BMI1[20] and selected with puromycin for 5 days, including a culture in DMEM/FBS media for 72 hours and 10 μM MG132 (Sigma) treatment for 6 hours.

2.12 | Immunohistochemistry staining

Tumors were fixed with 10% formalin, followed by paraffin embedding and sectioning. The protocol has been described in a previous study.[19] Percentages of positive tumor cells scored 0 (0% to 25%), 1 (26% to 50%), 2 (51% to 75%), and 3 (76% to 100%). The scores were graded as follows: 0 (negative), 1 (weak), 2 (moderate), or 3 (strong) positively according to the staining intensities.

The final scores were the arithmetic product of the percentage and staining intensity, that is, (−) = 0, (+) = 1 to 3, (++) = 4 to 6, (+++) = 7 to 9.

2.13 | Immunofluorescence and H&E staining

Procedure of immunofluorescence has been described in a previous study.[8] Primary antibodies used were USP22 (ab4812; Abcam) and BMI1 (66161-1-Ig; Proteintech). Slides were photographed using a confocal microscope (Olympus, Tokyo, Japan). For H&E staining, fixed tumors were embedded in paraffin, cut into 4-μm sections, and stained with H&E (Sigma). Slides were photographed using an optical microscope (Leica, Wetzlar, Germany).

2.14 | In vivo ubiquitination assay

HEK293FT cells were transfected with GFP-BMI1 (gift from Dr Michael Hendzel, University of Alberta, Canada), pCDNA3.1+-HA-Ub WT (gift from Dr Bin Wang, University of Texas MD Anderson Cancer Center, USA), and Flag-USP22 WT/C185S plasmids (gift from Dr Sharon Dent, University of Texas MD Anderson Cancer Center, USA) as indicated. The cells were treated for 6 hours with 10 μM MG132 (Sigma) at 42 hours post-transfection; afterwards, the cells were lysed. The samples were immunoprecipitated using anti-HA agarose (BioTool, Kirchberg, Switzerland).

2.15 | Animal studies

U87MG cells stably expressing USP22 and BMI1 shRNA or empty vector controls were implanted in the flanks of 4-week-old female SCID mice (2.0×10^6/200 μL per mouse, 5 mice per cell line). After 4 weeks, the mice were killed, and tumors were collected. Tumor volume (V) was determined by measuring the longest diameter (a) and the shortest diameter (b) according to the formula V (mm^3) = $(b)^2 \times a/2$. All mouse experiments were carried out in accordance with institutional guidelines and regulations of the government. All mouse experiments were also approved by the Ethical Board of Shanghai Jiao Tong University.

2.16 | Microarray analyses

RNA extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was labeled and hybridized onto the Agilent Human Gene Expression Analysis platform (8*60K, Design ID: 039494) by Oebiotech Co., Ltd (Shanghai, China). Statistical analyses and data normalization were conducted using Genespring GX software (Agilent Technologies, Santa Clara, CA, USA). Genes with 2-fold change in expression were considered differentially regulated by USP22 and BMI1. Genes were mapped onto KEGG pathways using DAVID version 6.7 (https://david-d.ncifcrf.gov/). The microarray data reported in this article have been deposited in the Gene Expression Omnibus (accession number GSE46059).

2.17 | Statistical analysis

All experiments were carried out in triplicate and repeated at least 3 times unless indicated otherwise. Data are expressed as mean ± SD. Statistical significance between the 2 groups was calculated by unpaired Student's t test using GraphPad PRISM software. In experiments involving more than 1 group for comparison, ANOVA was used with a suitable post-hoc test. Statistical significance in the multifactor correlation analysis was calculated by chi-squared test with SPSS software. Gene expression correlation was analyzed by Pearson correlation coefficient. Statistical significance is indicated by asterisks (*). P-value <0.05 was considered statistically significant, that is, $*P < .05$, $**P < .01$, and $***P < .001$.

3 | RESULTS

3.1 | USP22 knockdown attenuates cancer stemness in glioma cells

Considering USP22 is a putative cancer stem cell marker, we explored its effect on glioma stemness. We first compared USP22 expression between stem-like tumorspheres and relatively differentiated adherent cells by western blot. We found that USP22 was more enriched in both the U251 and U87MG cell-derived stem-like tumorspheres (Figure 1A). To study the function of USP22 on glioma malignancy, a pair of USP22 shRNAs were constructed and validated by western blot. The more effective shRNA-2 was chosen for subsequent experiments (Figure 1B). Then, we conducted a limiting dilution assay to evaluate the effect of USP22 on the self-renewal capacity of GSC. The number of cells required to generate at least 1 tumorsphere per well was significantly increased after USP22 depletion in both the U251 (119.5 vs 51.3, $P = .038$) and U87MG (146.6 vs 50.9, $P = .01$) cells (Figure 1C). To avoid potential off-targets of shRNA-2, we conducted a rescue assay on neurosphere formation with a pTAT-HA-USP22 recombinant protein in U251 cells. First, the result of western blot showed that the recombinant USP22 protein could rescue the effect of USP22 silencing in U251 cells (Figure 1D). Then, we found that knockdown of USP22 attenuated the neurosphere formation by decreasing the diameter in U251 cells, which could be reversed by the recombinant USP22 protein. (Figure 1E,F). By qRT-PCR analysis, attenuation of glioma stemness could be partially explained by transcriptional inhibition of stemness-related factors *Oct4* and *Nanog* as well as by elevation of differentiation markers *GFAP* and *Tuj1* (Figure S1). These findings indicate the important role of USP22 in maintaining glioma stemness and malignancy.

3.2 | USP22 sustains glioma stemness partially through maintaining BMI1 expression

Considering BMI1 is a potential target of USP22 in other cancers,[21,22] we attempted to examine whether BMI1 may mediate the effect of USP22 on glioma stemness. First, we examined the effect of USP22 on BMI1 expression at both post-translational and transcriptional level in U251 and U87MG cells. USP22 knockdown

remarkably decreased BMI1 protein level (Figure 2A,B), but it barely affected BMI1 mRNA level in glioma cell lines (Figure 2C,D). Then, we purified a transducible TAT-BMI1 protein to conduct a functional rescue assay in U251 cells (Figure 2E). First, we ensured the biological activity of TAT-BMI1 protein by detecting its effect on rescuing tumorsphere formation in BMI1-knockdown U251 cells (Figure 2F). Afterwards, the TAT-BMI1 protein was introduced into the USP22-knockdown U251 cells (Figure 2G). Compared with the control group, average tumorsphere diameter in the USP22-knockdown group was significantly decreased, but it was rescued by the TAT-BMI1 protein (Figure 2G). These results showed that BMI1 mediates the effect of USP22 on glioma stemness.

3.3 | USP22 deubiquitinates BMI1 for protein stabilization

Although BMI1 expression can be maintained by USP22, the exact mechanism is elusive. To examine whether USP22 can interact with BMI1, we coexpressed exogenous GFP-BMI1 with either the wild-type or catalytic inactive (C185S) mutant USP22 in HEK293FT cells.

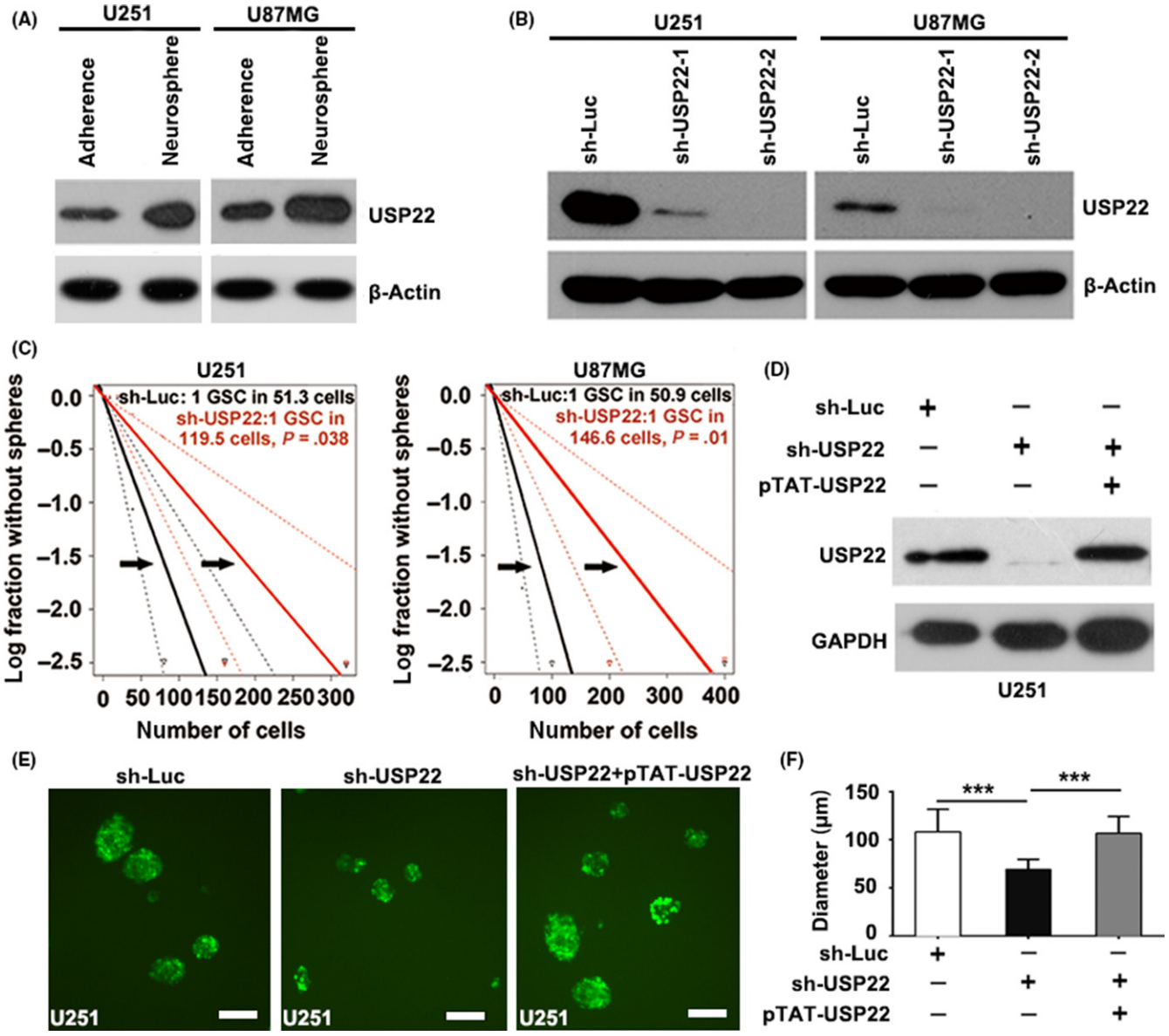

FIGURE 1 Ubiquitin-specific protease 22 (USP22) knockdown attenuates glioma stemness. A, USP22 overexpression in glioma stem-like cells (GSC) compared with differentiated glioma cells in glioma cells. B, USP22 shRNA efficiency validation by western blot in glioma cells. C, Effect of USP22 on self-renewal of GSC was evaluated by limited dilution assay. Number of cells required to generate at least 1 tumorsphere per well was significantly increased after USP22 depletion in both U251 (119.5 vs 51.3, $P = .038$) and U87MG (146.6 vs 50.9, $P = .01$) cells. D, Detection of USP22 expression in negative control, USP22 knockdown and pTAT-USP22 treatment groups in U251 cells by western blot. E, Representative morphology of tumorspheres from the negative control, USP22 knockdown and pTAT-USP22 treatment groups in U251 cells. F, Tumorsphere diameter was decreased in USP22 knockdown GSC derived from U251 cells, which was rescued by the recombinant pTAT-USP22 protein. Student's t test. $^{***}P < 0.001$. Bar, 100 μm

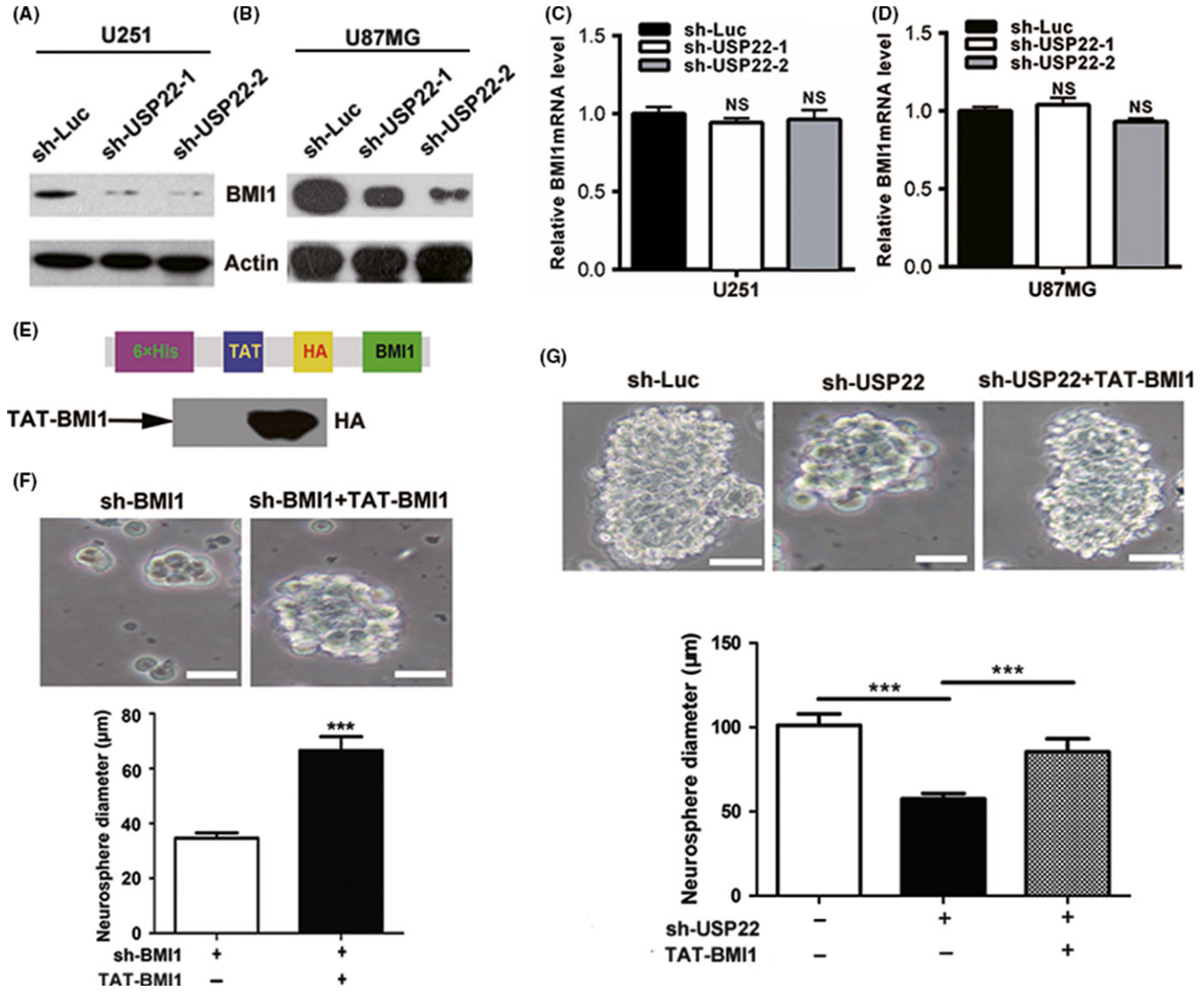

FIGURE 2 Ubiquitin-specific protease 22 (USP22) sustains glioma stemness partially through maintaining B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) expression. A, B, USP22 knockdown downregulated BMI1 protein level in both cell lines. C, D, USP22 knockdown hardly affected BMI1 mRNA level in both cell lines. E, Construction and transduction of pTAT-HA-BMI1 protein in U251 cells were validated by western blot. F, Representative images of the TAT-HA-BMI1 protein rescue effect on the tumorsphere volume in BMI1 knockdown U251 glioma stem-like cells (GSC). Scale bar, 50 μm. G, TAT-HA-BMI1 protein rescue effect on the diameter of USP22 knockdown U251 tumorspheres. Scale bar, 50 μm. Student's *t* test. ***$P < .001$

After GFP-BMI1 immunoprecipitation, both the associated WT-USP22 and CI-USP22 were detected. However, BMI1 binding to USP22 was obviously decreased by C185S mutation (Figure 3A), which suggests an interaction between USP22 and BMI1 with the affinity, in part, related to the deubiquitination activity. To further investigate whether the effect of USP22 on BMI1 expression is mediated through deubiquitination, we coexpressed GFP-BMI1 and HA-Ub with either WT-USP22 or CI-USP22 in HEK293FT cells. After GFP-BMI1 immunoprecipitation, we observed that BMI1 was heavily ubiquitinated (lane 1, Figure 3B). However, WT-USP22 coexpression (not CI-USP22) almost completely abolished BMI1 ubiquitination (lane 2 vs lane 3, Figure 3B). To test whether USP22 can stabilize BMI1 protein by antagonizing the Ub proteasome pathway, we coexpressed GFP-BMI1 with Flag-USP22 or vector control in HEK293FT cells and examined BMI1 degradation. We found that compared with the WT-USP22 group, GFP-BMI1 protein level in the CI-USP22 group was significantly decreased (lane 2 vs lane 3, Figure 3C); however, it can be reversed by the proteasome inhibitor MG132 (lane 4, Figure 3C). With the use of translational inhibitor CHX to block the synthesis of new proteins, GFP-BMI1 degraded rapidly in HEK293FT cells transfected with a control vector (Figure 3D, upper image). Moreover, WT-USP22 prolonged GFP-BMI1 half-life compared with that in cells transfected with control vector or CI-USP22 (Figure 3D, lower image). To examine the interaction between endogenous USP22 and BMI1 in glioma cells, we used either USP22 antibody or control IgG-conjugated agarose beads to immunoprecipitate endogenous BMI1 in U251 cells. We found that compared with no BMI1 in the control IgG group (Figure 3E, lane 1),

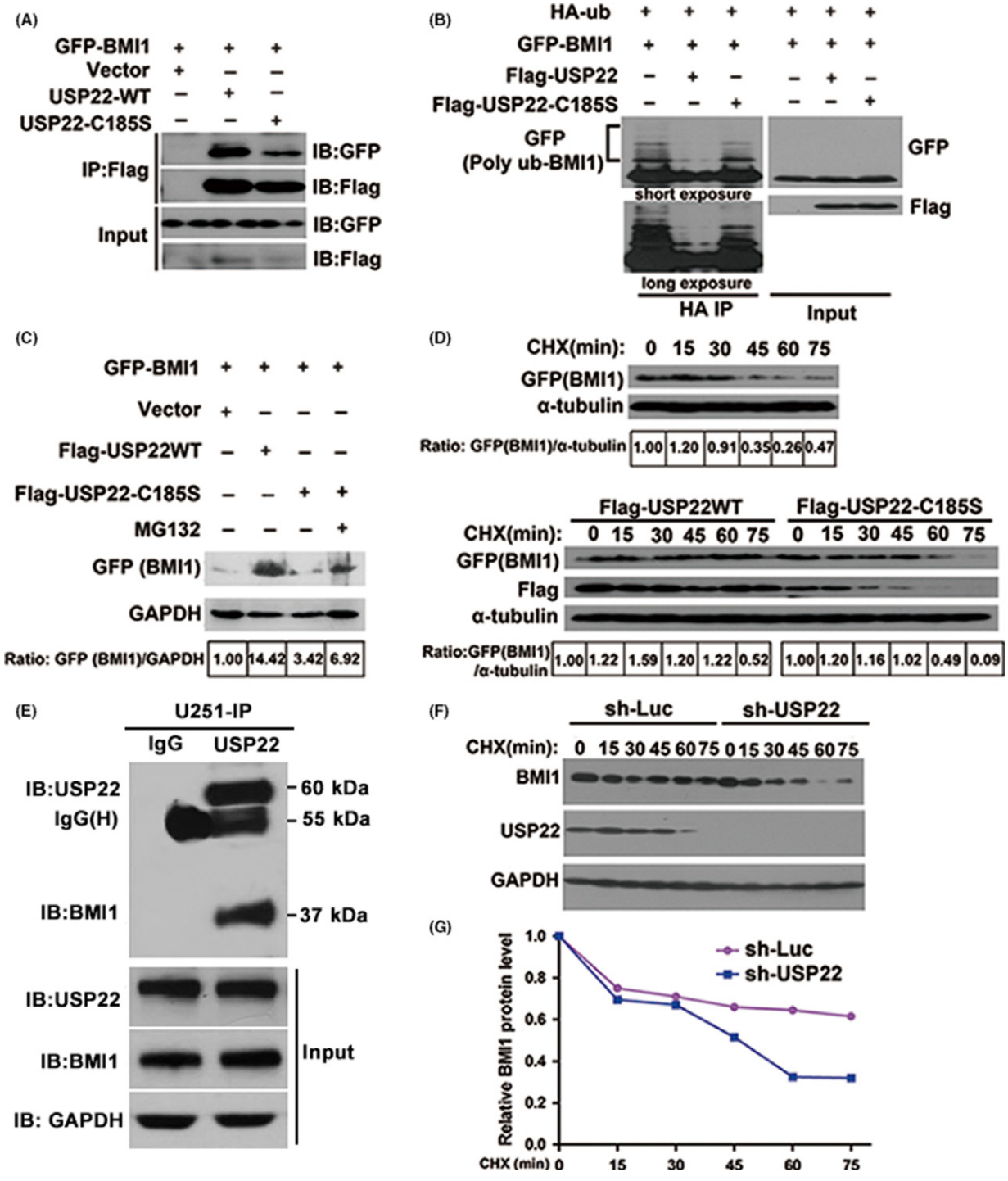

FIGURE 3 Ubiquitin-specific protease 22 (USP22) deubiquitinates B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) for protein stabilization. A, GFP-BMI1 was coexpressed with Flag-USP22WT or Flag-USP22C185S in HEK293FT cells. Interaction between USP22 and BMI1 was analyzed by immunoprecipitation using Flag antibody. B, GFP-BMI1 and HA-Ub were coexpressed with Flag-USP22WT or Flag-USP22C185S in HEK293FT cells. BMI1 was immunoprecipitated and detected by GFP antibody. BMI1 polyubiquitination was detected by HA antibody. C, GFP-BMI1 was coexpressed with Flag-USP22WT or Flag-USP22C185S in HEK293FT cells. Effect of USP22 on BMI1 stability was analyzed by western blot. GFP-BMI1 protein level in the CI-USP22 group was significantly decreased; however, this phenomenon can be reversed by the proteasome inhibitor MG132. D, GFP-BMI1 was coexpressed with Flag-USP22WT or Flag-USP22C185S in HEK293FT cells. After treating cells with cycloheximide (CHX) for the indicated time intervals, BMI1 and USP22 expressions were analyzed by western blot using GFP and Flag antibodies, respectively. E, Sample was collected from U251 cells for endogenous immunoprecipitation with anti-USP22 antibody. IP sample was analyzed by western blot with antibodies against USP22 and BMI1. F, G, U251 cells were transfected with USP22 or scramble shRNA. After cells were treated with CHX, expressions of endogenous BMI1 and USP22 were analyzed by western blot. BMI1 expression intensity for each time point was quantified by densitometry. Afterwards, the results were plotted

endogenous BMI1 was detected in the USP22 antibody group (Figure 3E, lane 2). To test whether the endogenous BMI1 is also deubiquitinated by USP22 in glioma cells, we knocked down endogenous USP22 in U251 cells pretreated with CHX and found that endogenous BMI1 also became unstable and degraded rapidly (Figure 3F,G). These results indicate that USP22 interacts with BMI1 and protects BMI1 from degradation through a deubiquitination mechanism in glioma cells.

3.4 | USP22 correlates with BMI1 at the protein level only

As USP22 is required for BMI1 protein stability without affecting its transcription in glioma cells, we also explored the correlation of USP22 and BMI1 at both transcriptional and post-translational levels in clinical glioma samples. Through immunofluorescence staining, we found that both USP22 and BMI1 were not only localized in the nucleus of U251 and U87MG cells, but also shared similar intranuclear expression (Figure 4A). By immunohistochemistry (IHC) analysis, we found a significant correlation of USP22 and BMI1 at protein level in 30 clinical samples (Figure 4B,C). On the contrary, there was no correlation between USP22 and BMI1 at transcriptional level by analysis of gene transcriptional profiling of 153 glioma patients from the Human Protein Atlas (http://www.proteinatlas.org/) (Figure 4D).

3.5 | USP22 and BMI1 mutually regulate various genes involved in glioma malignancy and stemness

Considering the expressional correlation of USP22 and BMI1, we then explored the correlation of their downstream gene expression profiles in glioma cells. Using differential expression microarray analysis, approximately 506 genes with at least a 2.0-fold change were mutually regulated by USP22 and BMI1 in the U251 cells. Among these genes, 320 genes were downregulated, and 186 genes were upregulated (Figure 5A,B). Pathway analysis of 506 commonly

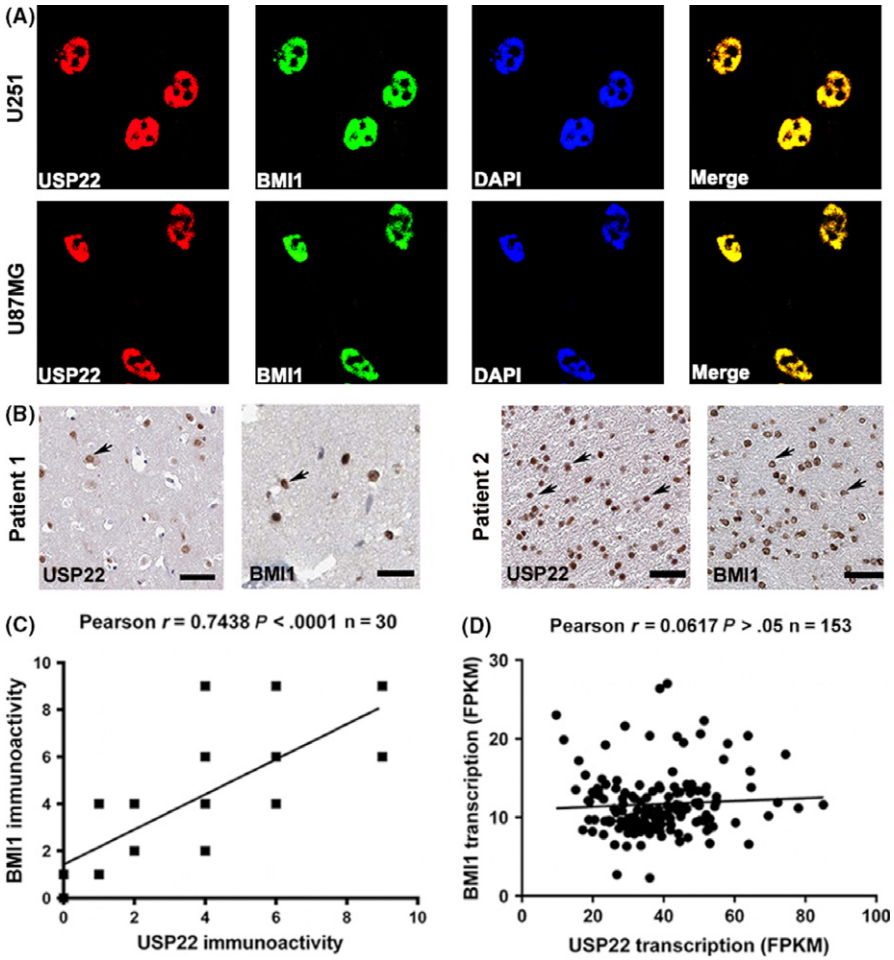

FIGURE 4 Ubiquitin-specific protease 22 (USP22) correlates only with B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) at the protein level. A, USP22 and BMI1 are not only both localized in the nucleus of U251 and U87MG cells but have similar intranuclear expression. Nuclei are shown by DAPI staining. B, Representative immunohistochemistry images of USP22 and BMI1 expression in different glioma patients. Arrows point to the nuclear IHC staining of USP22 and BMI1. Bar, 100 μm. C, USP22 correlates with BMI1 at protein level in 30 glioma samples. Pearson $r = .7438$, $P < .0001$. D, There is no correlation between the transcriptional level of USP22 and BMI1 in 153 TCGA (The Cancer Genome Atlas) glioma samples from the Human Protein Atlas. $P > .05$

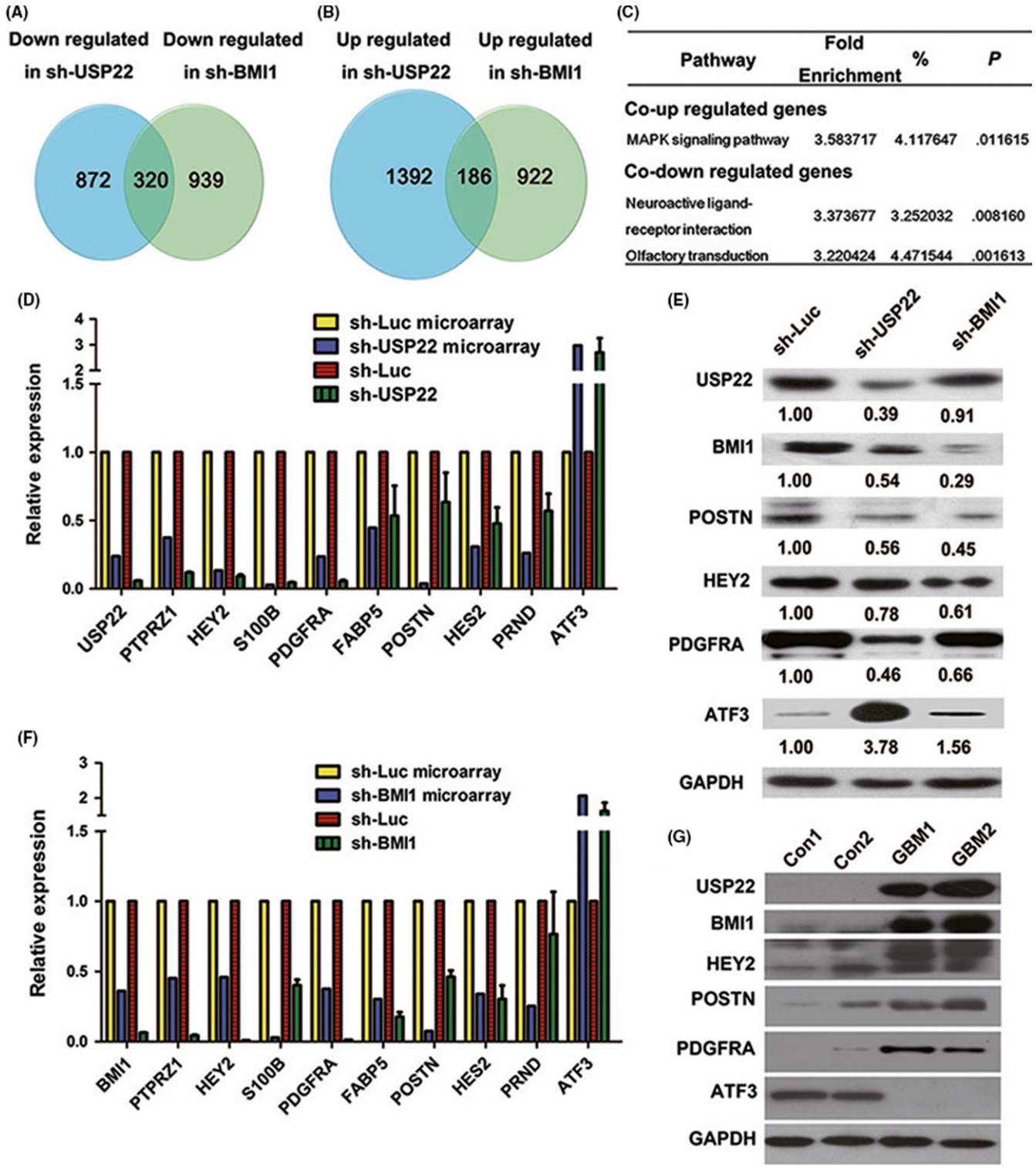

FIGURE 5 Ubiquitin-specific protease 22 (USP22) and B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) mutually regulate various genes involved in glioma malignancy and stemness. A, B, Venn diagram showing the intersection of genes between the downregulated genes or the upregulated genes with at least a 2.0-fold decrease in USP22 knockdown or BMI1 knockdown U251 cells, respectively. C, Representative KEGG pathway enrichment analysis for 506 genes identified from (A) and (B). D, F, Validation of 9 mutually regulated gene targets from (C) in the USP22 knockdown and BMI1 knockdown U251 cells by qRT-PCR, respectively. E, Western blot validation of 4 mutually regulated gene targets from (D) and (F). G, Expression pattern of USP22, BMI1 and the 4 mutually regulated gene targets was detected in clinically normal and glioma tissues by western blot. Con, control; GBM, glioblastoma multiforme

regulated genes showed significant enrichment in several core signaling pathways, such as MAPK for co-upregulated genes and neuroactive ligand-receptor interaction and olfactory transduction for co-downregulated genes (Figure 5C). We first confirmed the fidelity of the microarray results by analyzing the mRNA expression of 9 mutually regulated genes with qRT-PCR, which were tightly associated with glioma stemness or malignancy (Figure 5D,F; Table S3). Subsequently, we investigated the protein expression of 4 out of the 9 genes in U251 cells by western blot and found that the protein level alteration was consistent with mRNA changes in the microarray data (Figure 5E). Finally, we further detected the expression of 4 selected targets in the clinically normal or tumor samples and found that the alteration of these candidate downstream genes was also consistent with the result in the microarray validation (Figure 5G). In summary, USP22 and BMI1 mutually regulate a broad range of genes involved in glioma stemness and malignancy.

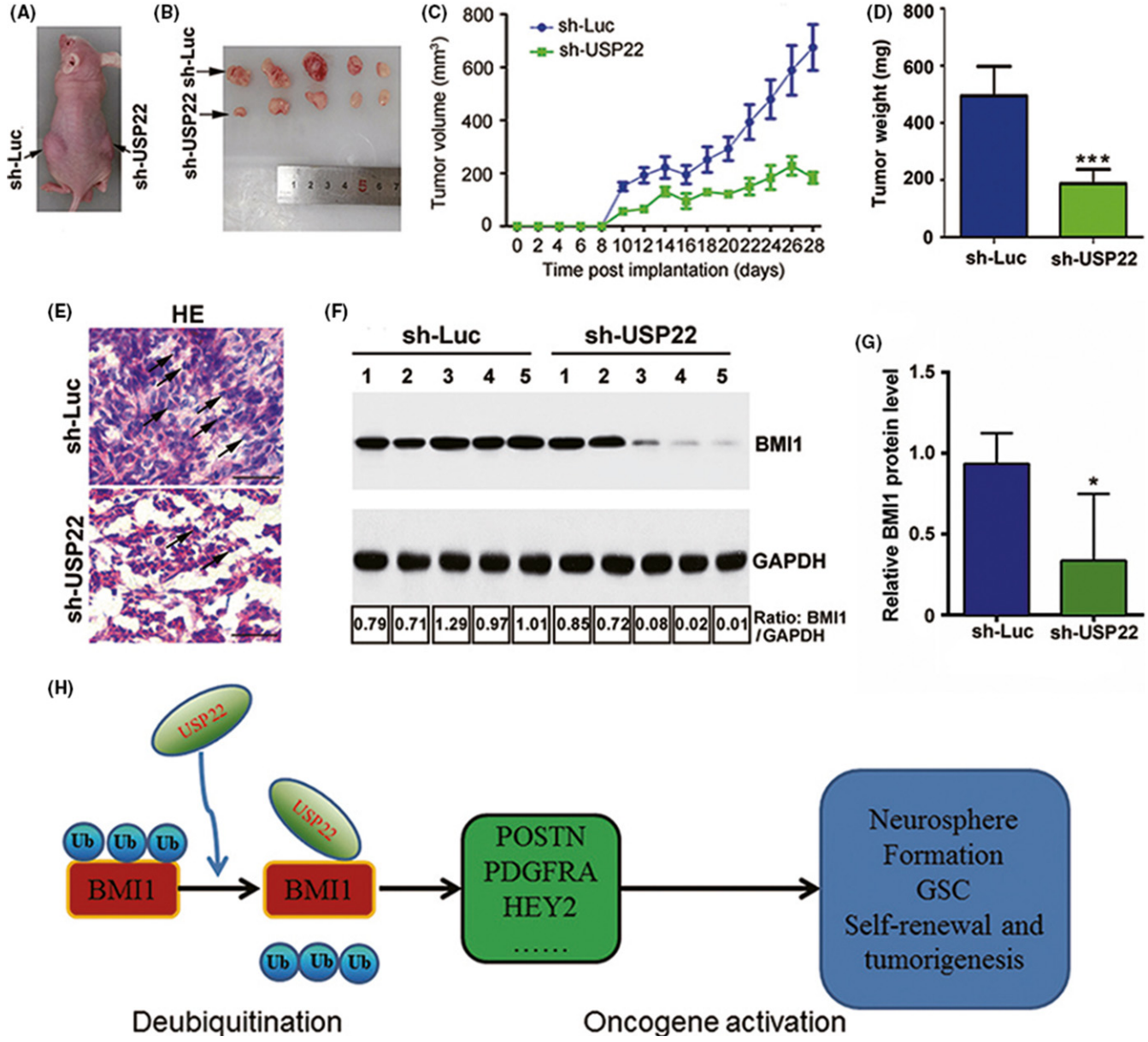

FIGURE 6 Ubiquitin-specific protease 22 (USP22) knockdown inhibits glioma tumorigenesis through reducing B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) expression in vivo. A, Nude mice were s.c. injected with USP22 control and knockdown U87MG cells to generate xenograft tumors. B, Photographs represent standard tumor removal in animals. C, Mice treated with USP22 knockdown U87MG cells statistically decreased tumor volume starting from the 8th day. At the 28th day post-implantation, tumor volume in the USP22 knockdown group was 183.50 ± 21.12 mm^3 compared with 674.73 ± 86.62 mm^3 in the control group (***$P < .001$). D, Statistical comparison of tumor weight between the 2 groups. E, Representative H&E staining of xenograft tumor cells in the 2 groups. Arrows point to the nucleus of implanted glioma cells. Bar, 25 μm. F, Tumor tissues in the 2 groups were subjected to western blot to detect BMI1 expression. GAPDH was the sample loading control. G, Statistical comparison of BMI1 expression between the 2 groups (*$P < .05$). H, Proposed working model of USP22-BMI1 axis to illustrate that USP22 deubiquitinates and stabilizes BMI1 to promote glioma stemness and progression by oncogenic activation. GSC, glioma stem-like cells

3.6 | USP22 knockdown inhibits glioma tumorigenesis through reducing BMI1 expression in vivo

After we confirmed the role of USP22 in gliomagenesis in vitro, we tried to further verify its oncogenic effect in vivo. Through subcutaneous xenograft assay in a nude mouse model with U87MG cells, we found that USP22 knockdown significantly inhibited tumor growth (Figure 6A,B). Both tumor volume and weight of USP22-knockdown mice were significantly decreased compared with those of control mice (Figure 6C,D). Furthermore, xenograft tissue H&E staining showed decreased tumor cell numbers in the USP22-knockdown mice compared with the control mice (Figure 6E, black arrows). To confirm USP22 regulation on BMI1 in vivo, we compared BMI1 protein grayscale in the transplanted tumors between the 2 groups. BMI1 protein level in the USP22-knockdown group was significantly lower than that in the control group (Figure 6F,G, $*P < .05$). Thus, a working model of the USP22-BMI1 axis was proposed whereby USP22 stabilizes BMI1 by deubiquitination to promote glioma stemness and tumorigenesis through oncogenic modulation of various downstream genes such as *POSTN*, *HEY2*, *PDGFRA* and *ATF3* (Figure 6H).

4 | DISCUSSION

Disorder of protein control resulting from DUB deregulation usually promotes malignant transformation in various cancers including glioma.[23-25] In the present study, we again confirm the overexpression of USP22 and its potential value in glioma prognosis (Figure S2), which is consistent with the findings of Liang et al.[26] However, the effect of USP22 on glioma malignancy, especially stemness, as well as the underlying mechanism are still elusive. Herein, we uncover the role of USP22 in maintaining glioma stemness. Higher USP22 expression in stem-like tumorspheres indicates USP22 as a cancer stem-cell marker in glioma. As cancer stemness is critical for malignant behaviors, it may well explain the roles of USP22 in promoting glioma migration and invasion (Figure S3) as well as proliferation and anti-apoptosis.[27]

Previous literature has mentioned expressional correlation of USP22 and BMI1 in gastric and liver cancers.[28,29] In our study, a similar correlation exists in glioma. It is interesting that the correlation of USP22 and BMI1 is at the protein level only and USP22 knockdown hardly affects BMI1 transcription. As BMI1 is a short half-life protein mainly subject to Ub-dependent proteasomal degradation,[30] this suggests that USP22 is probably required for BMI1 stability only. Despite our previous work showing BMI1 stabilization by USP22 in gastric cancer,[31] whether this regulation is dependent on proteasome function and the deubiquitinating enzyme activity of USP22 has not been elucidated. In the present study, by comparing the effect between wild-type and catalytically inactive USP22 in an in vivo deubiquitination assay, we first show that USP22 is a novel deubiquitinase of BMI1, which relies on its enzymatic activity to protect BMI1 from proteasomal degradation.

These findings prompt us to make a further exploration of the common targets of USP22 and BMI1. By microarray analysis, we observed a large overlap in their downstream gene profiles. The 4 selected genes *POSTN*, *HEY2*, *PDGFRA* and *ATF3* are all reported regulators of glioma stemness.[32-37] Hitherto, a series of tumor-suppressor genes have been identified as direct targets of BMI1 in glioma, which is transcriptionally repressed by BMI1 such as *CDKN1A*, *CDKN2A*, *ALX3*, *CBX7*, *GFI1*, *IL5RA* and *PTPRD1*.[37-39] Hence, we also compared the profile of these genes between BMI1-knockdown and USP22-knockdown cells in the microarray. The results showed that knockdown of USP22 could also upregulate most of these genes to a different extent, which was consistent with the profile of BMI1 inhibition (Figure S4). These findings indicate the presence of a USP22-BMI1 axis to promote glioma malignancy through oncogenic activation by regulating a complicated network of downstream targets. Thus, our study may bring about better understanding of the function and molecular mechanism of USP22 in glioma malignancy. In the future, evidence from patient-derived glioma cells and an orthotopic implantation xenograft model will provide further compelling support for our study.

To conclude, USP22 is a novel deubiquitinase of BMI1 with pivotal roles in glioma stemness and tumorigenesis. We uncover a USP22-BMI1 axis, which promotes glioma malignancy through oncogenic activation of a series of gene targets. Considering BMI1 regulation by USP22 in other malignancies,[28,29,31] we speculate a common existence of the USP22-BMI1 axis in cancers. Hence, targeting the USP22-BMI1 axis may not only be a promising alternative approach to treat glioma, but may also be used to treat other types of cancer.

ACKNOWLEDGMENTS

We thank Prof. Guo-Han Hu (Department of Neurosurgery in Shanghai Changzheng Hospital) for experimental support. This research was supported by the National Natural Science Foundation of China (81702944, 81671293, 81402063, and 81602193), Special Presidential Foundation of General Hospital of Jinan Military Command (2016BS04) and Shanghai Jiao Tong University Med-X Fund (YG2015MS20).

CONFLICT OF INTEREST

Authors declare no conflicts of interest for this article.

ORCID

Jing Lin http://orcid.org/0000-0001-6387-6366
Wei-Lin Jin http://orcid.org/0000-0001-8011-2405

REFERENCES

1. Louis DN, Perry A, Reifenberger G, et al. The 2016 World Health Organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol*. 2016;131:803-820.
2. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 2005;352:987-996.

3. Bao S, Wu Q, McLendon RE, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature*. 2006;444:756-760.
4. Lathia JD, Mack SC, Mulkearns-Hubert EE, Valentim CL, Rich JN. Cancer stem cells in glioblastoma. *Genes Dev*. 2015;29:1203-1207.
5. Abdouh M, Hanna R, El Hajjar J, Flamier A, Bernier G. The Polycomb Repressive Complex 1 Protein BMI1 is required for constitutive heterochromatin formation and silencing in mammalian somatic cells. *J Biol Chem*. 2016;291:182-197.
6. Kreso A, van Galen P, Pedley NM, et al. Self-renewal as a therapeutic target in human colorectal cancer. *Nat Med*. 2014;20:29-36.
7. Yong KJ, Basseres DS, Welner RS, et al. Targeted BMI1 inhibition impairs tumor growth in lung adenocarcinomas with low CEBPα expression. *Sci Transl Med*. 2016;8:350ra104.
8. Tu Y, Gao X, Li G, et al. MicroRNA-218 inhibits glioma invasion, migration, proliferation, and cancer stem-like cell self-renewal by targeting the polycomb group gene BMI1. *Cancer Res*. 2013;73:6046-6055.
9. Abdouh M, Facchino S, Chatoo W, Balasingam V, Ferreira J, Bernier G. BMI1 sustains human glioblastoma multiforme stem cell renewal. *J Neurosci*. 2009;29:8884-8896.
10. Lee KK, Florens L, Swanson SK, Washburn MP, Workman JL. The deubiquitylation activity of Ubp8 is dependent upon Sgf11 and its association with the SAGA complex. *Mol Cell Biol*. 2005;25:1173-1182.
11. Lang G, Bonnet J, Umlauf D, et al. The tightly controlled deubiquitination activity of the human SAGA complex differentially modifies distinct gene regulatory elements. *Mol Cell Biol*. 2011;31:3734-3744.
12. Lin Z, Yang H, Kong Q, et al. USP22 antagonizes p53 transcriptional activation by deubiquitinating Sirt1 to suppress cell apoptosis and is required for mouse embryonic development. *Mol Cell*. 2012;46:484-494.
13. Atanassov BS, Evrard YA, Multani AS, et al. Gcn5 and SAGA regulate shelterin protein turnover and telomere maintenance. *Mol Cell*. 2009;35:352-364.
14. Atanassov BS, Dent SY. USP22 regulates cell proliferation by deubiquitinating the transcriptional regulator FBP1. *EMBO Rep*. 2011;12:924-930.
15. Glinsky GV, Berezovska O, Glinskii AB. Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer. *J Clin Invest*. 2005;115:1503-1521.
16. Zhang XY, Pfeiffer HK, Thorne AW, McMahon SB. USP22, an hSAGA subunit and potential cancer stem cell marker, reverses the polycomb-catalyzed ubiquitylation of histone H2A. *Cell Cycle*. 2008;7:1522-1524.
17. Zhang XY, Varthi M, Sykes SM, et al. The putative cancer stem cell marker USP22 is a subunit of the human SAGA complex required for activated transcription and cell-cycle progression. *Mol Cell*. 2008;29:102-111.
18. Melo-Cardenas J, Zhang YS, Zhang DD, Fang D. Ubiquitin-specific peptidase 22 functions and its involvement in disease. *Oncotarget*. 2016;7:44848-44856.
19. Ding MH, Wang Z, Jiang L, et al. The transducible TAT-RIZ1-PR protein exerts histone methyltransferase activity and tumor-suppressive functions in human malignant meningiomas. *Biomaterials*. 2015;56:165-178.
20. Ismail IH, Gagné JP, Caron MC, et al. CBX4-mediated SUMO modification regulates BMI1 recruitment at sites of DNA damage. *Nucleic Acids Res*. 2012;40:5497-5510.
21. Liu YL, Jiang SX, Yang YM, Xu H, Liu JL, Wang XS. USP22 acts as an oncogene by the activation of BMI-1-mediated INK4a/ARF pathway and Akt pathway. *Cell Biochem Biophys*. 2012;62:229-235.
22. Hu J, Liu YL, Piao SL, Yang DD, Yang YM, Cai L. Expression patterns of USP22 and potential targets BMI-1, PTEN, p-AKT in non-small-cell lung cancer. *Lung Cancer*. 2012;77:593-599.
23. Amm I, Sommer T, Wolf DH. Protein quality control and elimination of protein waste: the role of the ubiquitin-proteasome system. *Biochim Biophys Acta*. 2014;1843:182-196.
24. Qiu GZ, Sun W, Jin MZ, Lin J, Lu PG, Jin WL. The bad seed gardener: deubiquitinases in the cancer stem-cell signaling network and therapeutic resistance. *Pharmacol Ther*. 2017;172:127-138.
25. Jin WL, Mao XY, Qiu GZ. Targeting deubiquitinating enzymes in glioblastoma multiforme: expectations and challenges. *Med Res Rev*. 2017;37:627-661.
26. Liang J, Zhang X, Xie S, et al. Ubiquitin-specific protease 22: a novel molecular biomarker in glioma prognosis and therapeutics. *Med Oncol*. 2014;31:899-904.
27. Li ZH, Yu Y, Du C, Fu H, Wang J, Tian Y. RNA interference-mediated USP22 gene silencing promotes human brain glioma apoptosis and induces cell cycle arrest. *Oncol Lett*. 2013;5:1290-1294.
28. Yang DD, Cui BB, Sun LY, et al. The co-expression of USP22 and BMI-1 may promote cancer progression and predict therapy failure in gastric carcinoma. *Cell Biochem Biophys*. 2011;61:703-710.
29. Zhai R, Tang F, Gong J, et al. The relationship between the expression of USP22, BMI1, and EZH2 in hepatocellular carcinoma and their impacts on prognosis. *Onco Targets Ther*. 2016;9:6987-6998.
30. Sahasrabuddhe AA, Dimri M, Bommi PV, Dimri GP. βTrCP regulates BMI1 protein turnover via ubiquitination and degradation. *Cell Cycle*. 2011;10:1322-1330.
31. Ma Y, Fu HL, Wang Z, et al. USP22 maintains gastric cancer stem cell stemness and promotes gastric cancer progression by stabilizing BMI1 protein. *Oncotarget*. 2017;8:33329-33342.
32. Park SY, Piao Y, Jeong KJ, Dong J, de Groot JF. Periostin (POSTN) regulates tumor resistance to antiangiogenic therapy in glioma models. *Mol Cancer Ther*. 2016;15:2187-2197.
33. Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes Dev*. 2004;18:901-911.
34. Jin X, Kim SH, Jeon HM, et al. Interferon regulatory factor 7 regulates glioma stem cells via interleukin-6 and Notch signaling. *Brain*. 2012;135:1055-1069.
35. Dougherty JD, Fomchenko EI, Akuffo AA, et al. Candidate pathways for promoting differentiation or quiescence of oligodendrocyte progenitor-like cells in glioma. *Cancer Res*. 2012;72:4856-4868.
36. Jackson EL, Garcia-Verdugo JM, Gil-Perotin S, et al. PDGFR alpha-positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signaling. *Neuron*. 2006;51:187-199.
37. Gargiulo G, Cesaroni M, Serresi M, et al. In vivo RNAi screen for BMI1 targets identifies TGF-β/BMP-ER stress pathways as key regulators of neural- and malignant glioma-stem cell homeostasis. *Cancer Cell*. 2013;23:660-676.
38. Venugopal C, Li N, Wang X, et al. Bmi1 marks intermediate precursors during differentiation of human brain tumor initiating cells. *Stem Cell Res*. 2012;8:141-153.
39. Sibin MK, Chetan G, Lavanya C, Jeru MM, Bhat DI. Correlation of P16 and Bmi1 gene expression in human high grade glioma. *Mol Cytogenet*. 2014;7(Suppl 1):9.

SUPPORTING INFORMATION

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

How to cite this article: Qiu G-Z, Mao X-Y, Ma Y, et al. Ubiquitin-specific protease 22 acts as an oncoprotein to maintain glioma malignancy through deubiquitinating B cell-specific Moloney murine leukemia virus integration site 1 for stabilization. *Cancer Sci*. 2018;109:2199–2210. https://doi.org/10.1111/cas.13646