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Original Article

USP18 deubiquitinates and stabilizes Twist1 to promote epithelial-mesenchymal transition in glioblastoma cells

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Abstract: Aberrant activation of epithelial-mesenchymal transition (EMT) pathway drives the invasion and migration of multiple cancers including glioblastoma (GBM). Clinical interventions focused on inhibiting EMT are of increasing interest in the treatment of GBM. In the present study, we discovered that glioma tissues and cells, especially GBMs show significantly up-modulated ubiquitin-specific protease 18 (USP18) expression. Functionally, decreased USP18 expression attenuated GBM cell invasion and migration through repressing EMT. Moreover, a critical EMT-inducing transcription factor Twist1 that activates EMT, was identified as a downstream target of USP18. Mechanistically, USP18 interacts with Twist1, removes its ubiquitination off, and subsequently stabilizes it. Short hairpin RNA-mediated downregulation of USP18 accelerates Twist1 degradation, resulting in the inhibition of GBM cell invasion and migration in vitro and in a nude mouse model. Importantly, reconstituted expression of Twist1 almost completely rescues the inhibitory effect of USP18 depletion on GBM cell invasion, migration and tumor formation. Clinically, the expression levels of USP18 and Twist1 are positively relevant in GBM specimens, and high expression of USP18 correlates with patient's poor outcome. Finally, our findings unveil the crucial role of USP18 on GBM malignancy. Targeting USP18-Twist1 regulatory axis may open a novel avenue for GBM treatment.

Keywords: Glioblastoma, USP18, Twist1, invasion, migration, deubiquitination

Introduction

GBM, a grade IV astrocytoma, is a common, most destructive primary central nervous system tumor in adults with extensive invasive potential [1, 2]. Multiple treatments have improved in recent years, including surgery, radiotherapy, chemotherapy and molecular targeted therapy, the prognosis of GBM patients is still poor. Average overall survival after diagnosis is nearly 15 months [3-5]. The essence of their highly invasive nature became one of the biggest challenges in treating GBM [6]. Thus, novel molecular approaches directing GBM invasion inhibition may provide new hope for promoting the outcomes of GBM patients.

The process epithelial cells transdifferentiate into motile mesenchymal cells was known as EMT, which is indispensable in stem cell behavior, embryonic development and cancer invasion [7]. This switch is usually mediated by several crucial EMT-inducing transcription factors, which includes Twist1, Snail, ZEB1, ZEB2 and SLUG [7]. Growing evidence revealed that EMT-inducing transcription factors (EMT-TFs) were upregulated in various tumors and thus prompted tumor invasion and progression [8].

Protein ubiquitination, as a kind of reversible modifications, plays a key role in a myriad of cellular functions [9]. The deubiquitinases (DUBs), which are a broad group of proteases, could remove ubiquitin conjugated with substrates directly and impede ubiquitin-dependent signal transduction [10]. Thus far, more than 100 human DUBs have been recognized. These DUBs are mainly composed of six subfamilies: ovarian tumor proteases (OTUs), ubiquitin-specific proteases (USPs), ubiquitin

C-terminal hydrolases (UCHs), JAB1/MPN/Mov-34 metalloenzymes (JAMMs), Machado-Joseph disease proteases (MJDs), and motif interacting with ubiquitin-containing novel DUB family (MINDYs) [9, 11-13]. The DUBs have gained increasing attention emerging as pivotal modulators of many cellular processes and cancer initiation and progression [14, 15]. As a staple member of the USP subfamily, USP18 (also known as UBP43) has been reported to exert important effects in a diverse range of biological functions in various cell types, including cell development [16, 17], antibacterial response [18], autoimmune diseases initiation [19-21] and tumor development [22, 23]. There is increasing evidence that USP18 expression is dysregulated in various types of malignancies, and this protein exhibits a unique ability to modulate tumorigenesis. For example, USP18 deubiquitinates and stabilizes Kirsten rat sarcoma viral oncogene homolog (KRAS) to promote lung cancers aggression [24]. USP18 knockdown also promotes cervical cancer cells apoptosis through the activation of miR-7 [25]. Nevertheless, the role of USP18 in GBM is unknown.

In the present study, results suggested high USP18 expression can be detected in GBM tissues and cell lines. Functional and animal experiments consistently indicated that USP18 expression knockdown in GBM cell lines drastically impaired its capacity for growth, invasion and migration. Mechanistically, USP18 deubiquitinates and stabilizes Twist1, an important EMT-inducing transcription factor, thereby promoting GBM invasion and migration. Collectively, our work suggested that USP18 is an undoubted DUB of Twist1 and involves a novel important role in GBM progression.

Materials and methods

Cell culture and reagents

Human GBM cells U87MG, SW1783, HS683, LN229, U118, T98G and U251 were purchased from the American Type Culture Collection, and human embryonic kidney 293T (HEK293T) cells were obtained from Bena Culture Collection Technology (China). All these cells were cultured at 37°C in 5% CO_2 in Dulbecco-modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), glutamine, and nonessential amino acids. Normal human astrocytes (NHAs) were purchased from Lonza and maintained in astrocyte growth media supplemented with 5% FBS, rhEGF, insulin, GA-1000, glutamine and ascorbic acid. CHX and puromycin were acquired from Sigma Aldrich. MG132 were purchased from Millipore. Lipofectamine 3000 Transfection Reagent was purchased from Invitrogen.

Human tissue samples

138 GBM samples were obtained from the Department of Neurosurgery, the First Affiliated Hospital of Nanjing Medical University. Normal brain tissue (NBT) samples were taken from 10 patients who underwent decompressive craniectomy for severe traumatic brain injury. The histological features of all of the samples were confirmed by pathologists according to the WHO criteria. This study was reviewed and approved by the Ethical Committee of Nanjing Medical University and Anhui Medical University. Written informed consent was obtained from these patients.

RNA extraction and qRT-PCR analysis

Total cellular RNA from tissues and cells were extracted using TRIzol and first strand cDNA preparation was performed by the PrimeScript RT Master Mix (TaKaRa). RT-PCR was performed according to manufacturer's instructions using SYBR Green (Applied Biosystems). Relative mRNA expression levels of Twist1 and USP18 normalized to GAPDH were calculated by the standard ΔΔCT method. The sequences of PCR primers were as follows: Twist1 forward: 5'-GTCCGCGTCCCACTAGC-3'; Twist1 reverse: 5'-TCCATTTTCTCCTTCTCTGGAA-3'; USP18 forward: 5'-ATCCGGAATGCTGTGGATGG-3'; USP18 reverse: 5'-AGATATGCAGTTTCCTGCCAGT-3'; GAPDH forward: 5'-TGCACCACCAACTGCTTAGC-3'; GAPDH reverse: 5'-GGCATGGACTGTGGTCATGAG-3'.

Plasmid construction

USP18 WT, USP18 mutant C64S, and Twist1 were cloned into the CMV-MCS-EGFP-SV40-Neomycin vectors as indicated. U87MG and U251 cells stably expressing USP18-specific shRNA (shUSP18) or scrambled shRNA control (shCtrl) were constructed using a lentiviral shRNA technique. U87MG and U251 cells were

transfected with serial dilutions of lentiviral supernatant and selection using 5 mg/mL puromycin was started 48 hours after infection. In a parallel experiment, the same procedure was performed in HS683 and SW1783 cells. The human USP18 shRNA target sequences were listed as follows: USP18shRNA#1: CCGGCCAAACCAAGCTGACGAAGAACTCGAGTTCTTCGTCAGCTTGGTTTGGTTTTTG; USP18shRNA#2: CCGGCCATCATGCAAGTACCTGTTTCTCGAGAAACAGGTACTTGCATGATGGTTTTT; The target sequence of shRNA against Twist1 was listed as follows: CCGGGCATTCTGATAGAAGTCTGAACTCGAGTTCAGACTTCTATCAGAATGCTTTTT.

Western blotting and antibodies

Western blotting was performed as our study described previously [26]. The following primary antibodies were used in our study: Twist1 (ab50581, Abcam); USP18 (ab115618, Abcam); N-cadherin (ab76057, Abcam); E-cadherin (ab40772, Abcam); Vimentin (ab92547, Abcam); Snail (ab229701, Abcam); Fibronectin (ab2413, Abcam); ZEB1 (ab203829, Abcam); ZEB2 (NBP1-82991, Novus); β-actin (AP0060, Bioworld Technology); FLAG-tag (F3165, Sigma-Aldrich); HA-tag (ab9110, Abcam); Myc-tag (2276, Cell Signaling Technology).

Immunoprecipitation and in vivo deubiquitination assay

Cells were lysed in buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 μg/ml aprotinin, 1 μg/ml pepstatin, 1% Nonidet P-40, 1 mM EDTA and 0.25% deoxycholate). Total cell lysates (1000 μl) were incubated overnight with 1 μg of desired antibody conjugated to agarose beads (Roche Molecular Biochemicals) at 4°C. The beads were then washed and boiled with SDS loading buffer, and the immunoprecipitated protein complexes were analyzed using Western blotting. HA-ubiquitinated Flag-Twist1 or endogenous Twist1 was immunoprecipited using the indicated antibodies in denaturing conditions. Then the ubiquitination level of Twist1 was detected using antibody against HA.

In vitro deubiquitination assay

HEK293T cells cotransfected with HA-Ub and Flag-Twist1 were treated with 20 μM MG132 for 6 h followed by lysation. Flag-tagged ubiquitinated Twist1 was purified from these cells by immunoprecipitation using Flag-tag antibody. The immunoprecipitates were then eluted using Flag-tag peptide. Likewise, HEK293T cells transfected with either Myc-tagged USP18 WT or mutant C64S were treated with 20 μM MG132 for 6 h followed by lysation. Myc-tagged USP18 WT or mutant C64S was purified from these cells by immunoprecipitation using Myc-tag antibody. The immunoprecipitates were then eluted using Myc-tag peptide. The Flag-tagged ubiquitinated Twist1 was incubated with Myc-tagged USP18 WT or mutant C64S in deubiquitination buffer at 30°C for 12 h, and the reaction products were resolved in SDS-PAGE buffer followed by Western blotting analysis.

Immunohistochemistry (IHC)

The IHC assays were performed on human GBM samples or NBTs to test USP18 and Twist1 protein levels, and the methods used here have been described previously [26].

Wound healing assay

2 GBM cells transfected the indicated plasmids were seeded in 6-well plates containing coverslips, and a 20 μL plastic pipette was used to create an artificial wound. 24 h later, the injury area was photographed using microscope (Leica, Wetzlar, Germany). A migration index was used to evaluate the wound-healing effect of GBM cell lines.

Invasion assay

The 24-well BD Matrigel invasion chambers (BD Biosciences) were used to assess invasion of 2 GBM cell lines according to the manufacturer's protocol. U87MG or U251 cells resuspended in DMEM without serum were added to upper wells of the chamber. The lower chamber wells were filled with DMEM supplemented with 20% FBS. After incubation for 24 h, invading cells were taken and fixed with 100% methanol, and stained with 0.1% crystal violet. Then images of the bottom wells were captured in three independent 10 × magnification fields.

Xenograft model

Animals were randomly divided into four different groups (six mice per group). U87MG cells (5×10^6 cells) stably infected with luciferase-

encoding lentivirus were intracranially injected into 4-weeks-old to 5-weeks-old male nude mice. Tumor growth was monitored using in vivo bioluminescence imaging system. The mice were sacrificed when they were moribund, then brains were harvested, fixed with 4% paraformaldehyde, and embedded with paraffin for further study. All animal studies were approved by the Animal Welfare Ethical Review Committee of Nanjing Medical University and Anhui Medical University and completed in compliance with national guidelines for the care and use of laboratory animals.

Statistical analysis

All experimental data was expressed in the format of mean ± standard deviation. Student's t test was used for pairwise comparison, and one-way analysis of variance was used for multiple group comparison. Survival of patients and nude mice was analyzed using Kaplan-Meier analysis by Graphpad Prism 7 software. Value of $P < 0.05$ was considered to be statistically significant.

Results

Increased USP18 expression in GBM samples and cell lines

To test USP18 expression level in GBM, we performed a gene expression analysis using Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/) to compare USP18 expression profiles in a group of 163 GBM tissues and 207 normal brain tissues (NBTs). We found that USP18 expression level was significantly upregulated in these GBM specimens, and USP18 expression level was low in NBTs ($P < 0.05$, **Figure 1A**). We next measured the expression of USP18 in GBM tissues and NBTs obtained from 6 paired participants, and we found USP18 expression was considerably increased in GBM tissues in comparison to NBTs (**Figure 1B**). To figure out whether USP18 expression level was consistent in different grades of gliomas. We tested USP18 expression using Western blotting and immunohistochemistry (IHC) in 4 different grades of glioma tissues and NBTs, and the results displayed a significant upregulation of USP18 expression in GBMs (**Figure 1C**, **1D**). Moreover, Western blotting was used to further analyze USP18 expression level in normal human astrocytes (NHAs), 2 glioma cell lines (SW1783, HS683) and 5 GBM cell lines (U87MG, U251, T98G, LN229 and U118). We found that all GBM cell lines showed higher USP18 expression level than glioma cell lines. Importantly, USP18 expression level in NHAs was nearly undetectable (**Figure 1E**). Together, these results indicated that the upregulated USP18 level was of great significance and it may play a pivotal role in GBM malignancy.

USP18 promotes EMT through Twist1

A recent study has shown that higher USP18 level in bladder cancer was associated with extensive muscle invasion and poor survival of patients, and this study suggested USP18 mediated an invasive phenotype of bladder cancer [27]. Accordingly, because GBM tissues and cells consistently exhibited USP18 upregulation, we next sought to investigate whether USP18 can influence the invasion and migration capacity of GBMs. First, we evaluated knockdown efficiency of two short hairpin RNAs (shRNAs) targeting USP18 (shUSP18#1, shUSP18#2) in U87MG and U251 cells. Western blotting assay showed that U87MG and U251 cells transduced with shUSP18#1 and shUSP18#2 exhibited significantly lower USP18 expression than those cells transfected with scrambled control shRNA (shCtrl) (**Figure 2A**). It was known that EMT was thought to be at the root of invasion and migration of cancer cell [28]. Several important EMT-related molecules (N-Cadherin, E-Cadherin, Vimentin, Fibronectin) were involved in the EMT process. To explore whether USP18 could affect the invasion and migration ability of GBM through EMT-associated molecule markers. We detected the expression levels of these molecules after USP18 knockdown. As shown in **Figure 2A**, USP18 depletion remarkably decreased expression of the mesenchymal markers N-cadherin, Vimentin, Fibronectin and increased the epithelial marker E-cadherin expression in U87MG and U251 cells. We next evaluated the effect of USP18 on cellular migration using wound healing assays in U87MG and U251 cells. The results revealed that the migration ability of U87MG and U251 cells transfected with USP18 shRNA were markedly inhibited in comparison to those cells transfected with control shRNA (**Figure 2B**, **2C**). Moreover, the effect of USP18 on cellular invasion was also tested in these 2 GBM cell lines. Compared with the negative control groups, U87MG and U251 cells with

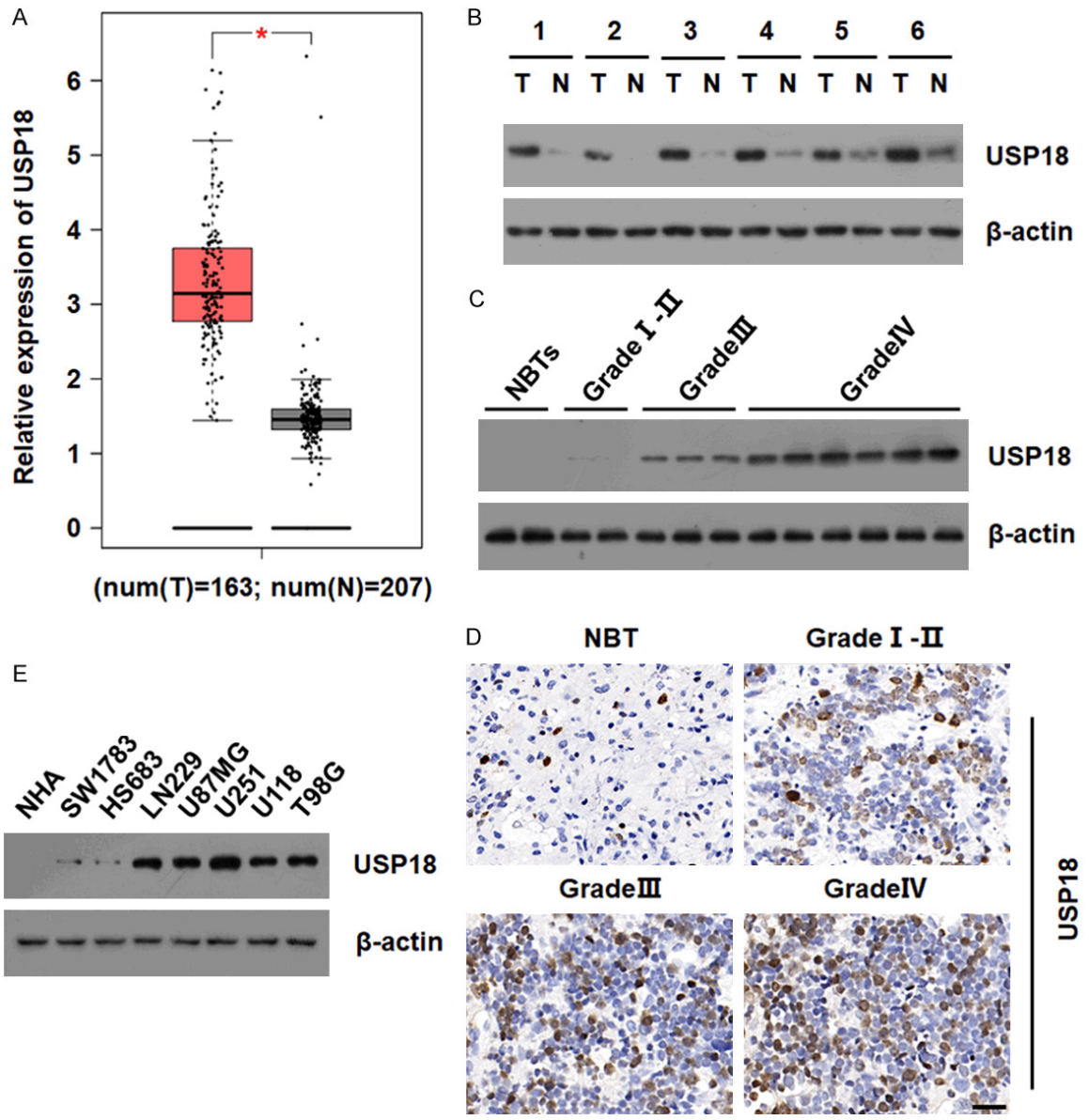
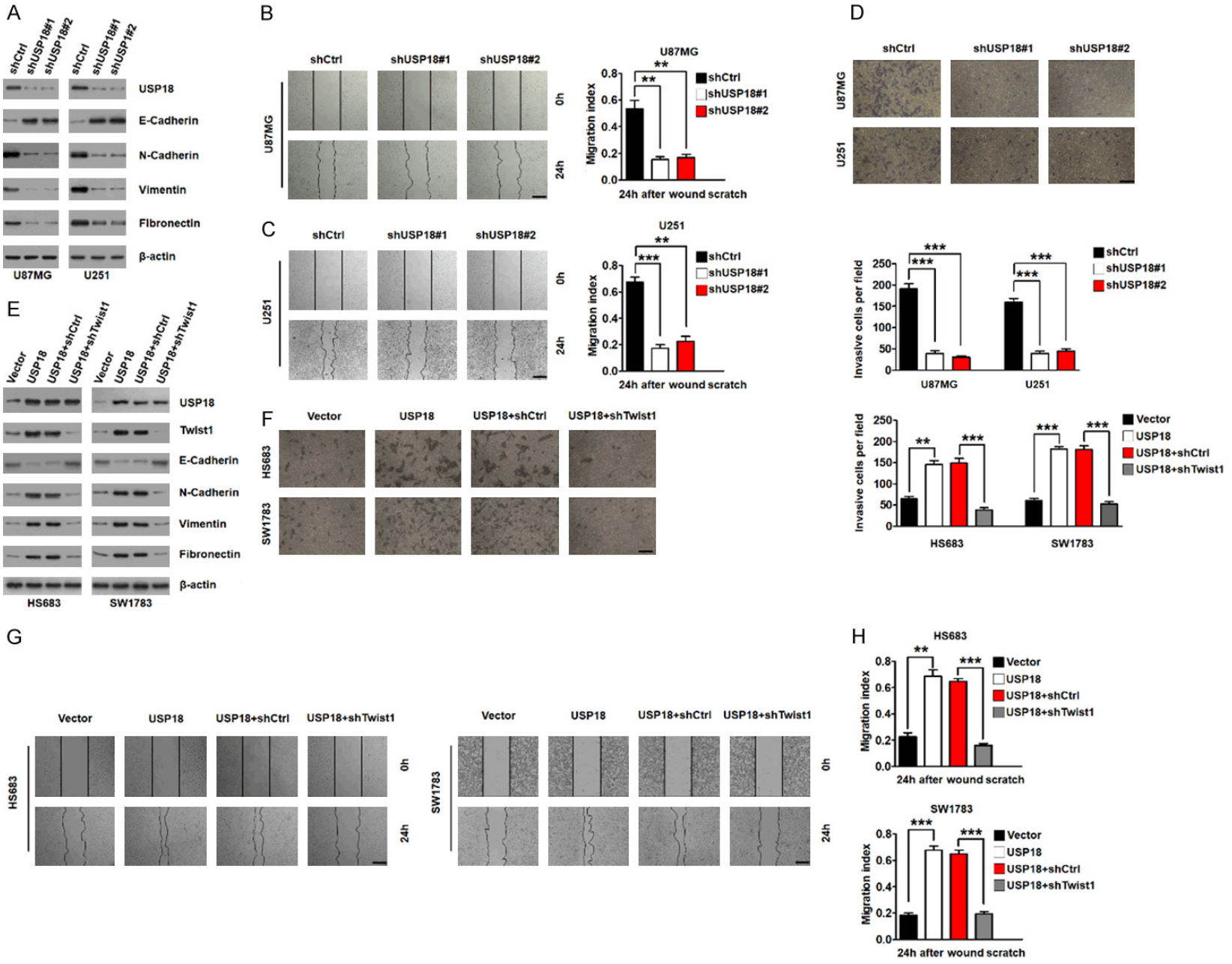


Figure 1. Elevated USP18 expression in GBM samples and cell lines. A. USP18 expression was analyzed using Gene Expression Profiling Interactive Analysis (*P < 0.05). B. Protein levels of USP18 were analyzed using Western blotting in 6 GBM samples and matched normal tissues (N = normal matched tissues, T = tumor). C. Protein levels of USP18 analysis using Western blotting in normal brain tissues (NBTs) and glioma specimens (grade I-IV). D. Protein levels of USP18 were analyzed by immunohistochemistry staining in NBTs and glioma tissues (grade I-IV). E. USP18 protein levels were determined using Western blotting analysis in normal human astrocyte (NHA), 7 glioma cells (SW1783, HS683, LN229, U87MG, U251, U118 and T98G). β-actin served as the loading control.

USP18 depletion showed a significant reduction in cell invasion ability (**Figure 2D**). Together, these data suggested that inhibition of USP18 suppresses GBM cells invasion and migration by blocking EMT.

To further determine whether USP18 promotes EMT through Twist1, we first overexpressed USP18 in HS683 and SW1783 cells. Western blotting analysis showed a significant increase of Twist1 and mesenchymal markers expression levels after USP18 overexpression, and this effect could be completely reversed by Twist1 silencing (**Figure 2E**). Consistent with these properties, ectopic expression of USP18 increased the invasion of glioma cells and that depletion of Twist1 reversed this effect (**Figure 2F**). Likewise, as shown in **Figure 2G** and **2H**,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shCtrl
shUSP18#1
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USP18
E-Cadherin
N-Cadherin
Vimentin
Fibronectin
β-actin
U87MG
U251
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U87MG
0h
24h
Migration index
24h after wound scratch
C
U251
D
Invasive cells per field
E
Vector
USP18
USP18+shCtrl
USP18+shTwist1
Twist1
HS683
SW1783
F
G
H

Figure 2. USP18 promotes EMT through Twist1. (A) Plasmids shUSP18#1, shUSP18#2 or shCtrl were stably transduced into U87MG and U251 cells. The effect of USP18 depletion on several EMT-associated proteins was analyzed using Western blotting. β-actin was used as the loading control. (B) Representative images of wound healing assay using U87MG cells transduced with shUSP18#1, shUSP18#2 or shCtrl. Quantification of wound healing assay is shown. **P < 0.01, n = 3 experiments. (C) Representative images of wound healing assay using U251 cells transduced with shUSP18#1, shUSP18#2 or shCtrl. Quantification of wound healing assay is shown. **P < 0.01, ***P < 0.001, n = 3 experiments. (D) Representative images of transwell invasion assay using U87MG and U251 cells transduced with shUSP18#1, shUSP18#2 or shCtrl. Quantification of transwell invasion assay is shown. ***P < 0.001, n = 3 experiments. (E) Indicated Plasmids were stably transduced into HS683 and SW1783 cells, the protein levels of Twist1 and several EMT-associated proteins were analyzed by Western blotting using indicated antibodies. β-actin served as the loading control. (F) Representative images of transwell invasion assay using HS683 and SW1783 cells transduced with the indicated plasmids. Quantification of transwell invasion assay is shown. **P < 0.01, ***P < 0.001, n = 3 experiments. (G, H) Representative images of wound healing assay using HS683 and SW1783 cells transduced with the indicated plasmids (G). Quantification of wound healing assay is shown (H). **P < 0.01, ***P < 0.001, n = 3 experiments.

knockdown of Twist1 remarkedly restored upregulated migration ability of HS683 and SW1783 cells caused by overexpression of USP18. These data supported the contention that USP18 promotes EMT via Twist1.

USP18 maintains Twist1 stability

Accumulating evidence has shown that the elevated expression of individual EMT-TFs, Twist1, Snail, ZEB1, ZEB2 or SLUG, has been found to activate the EMT process in invasive tumors [29-31]. To identify whether USP18 affect the expression level of EMT-TFs in GBM, we tested EMT-TFs protein expression in USP18-depleted U87MG and U251 cells. Intriguingly, Twist1 level, but not other EMT-TFs, was significantly decreased in USP18-depleted GBM cells in comparison to the negative control groups (**Figure 3A**). However, Twist1 mRNA expression levels in the USP18 silencing group showed no differences with the negative control group, suggesting that USP18 may modulate Twist1 protein degradation (**Figure 3B**). To investigate whether USP18's ability to upregulate Twist1 protein levels depends on its DUB activity, we transduced wild-type (WT) USP18 or mutant USP18-C64S, a catalytically inactive mutant [32], into U87MG and U251 cells. Then, we observed that WT USP18, but not USP18-C64S mutant increased the Twist1 protein levels (**Figure 3C**), suggesting that USP18 regulates Twist1 depends on its DUB activity. We next tested whether USP18 upregulated Twist1 expression levels through the ubiquitin–proteasome system. As shown in **Figure 3D**, U87MG and U251 cells with USP18 depletion showed a drastic reduction in Twist1 expression levels, and the proteasome inhibitor MG132 reversed this effect. Moreover, knockdown of USP18 promoted the degradation of Twist1 in U87MG cells following the protein synthesis inhibitor cycloheximide treatment (**Figure 3E**, **3F**). Similarly, the degradation of Twist1 in U251 cells was also accelerated after USP18 depletion under cycloheximide treatment (**Figure 3G**, **3H**). These results, taken together, suggested that USP18 maintains Twist1 stability in GBM cells.

USP18 interacts with and deubiquitinates Twist1

Next, to investigate whether USP18 could interact with Twist1. Myc-tagged WT USP18 or the mutant USP18-C64S combined with Flag-tagged Twist1 were coexpressed in HEK293T cells. Co-immunoprecipitation assays confirmed that Flag-tagged Twist1 could be detected in Myc-tagged WT USP18 or the mutant USP18-C64S immunoprecipitates (**Figure 4A**). Furthermore, we conducted co-immunoprecipitation experiments to demonstrate the interaction between endogenous USP18 and Twist1 in 2 GBM cell lines. The results indicated that endogenous Twist1 was present in USP18 immunoprecipitates (**Figure 4B** top), and the presence of endogenous USP18 was also detected in Twist1 immunoprecipitates (**Figure 4B** bottom). Together, these findings demonstrated that USP18 can interact with Twist1 in GBM cells.

We sought to determine whether Twist1 was regulated by USP18 through deubiquitination. First, we coexpressed HA-ubiquitin and Flag-tagged Twist1 combined with either Myc-tagged WT USP18 or the mutant USP18-C64S in HEK293T cells. We then treated Twist1 immunoprecipitates from cells with the proteasome inhibitor MG132, and found that Twist1 ubiquitination was heavily increased. Moreover, coexpression of Myc-tagged WT USP18, but not the

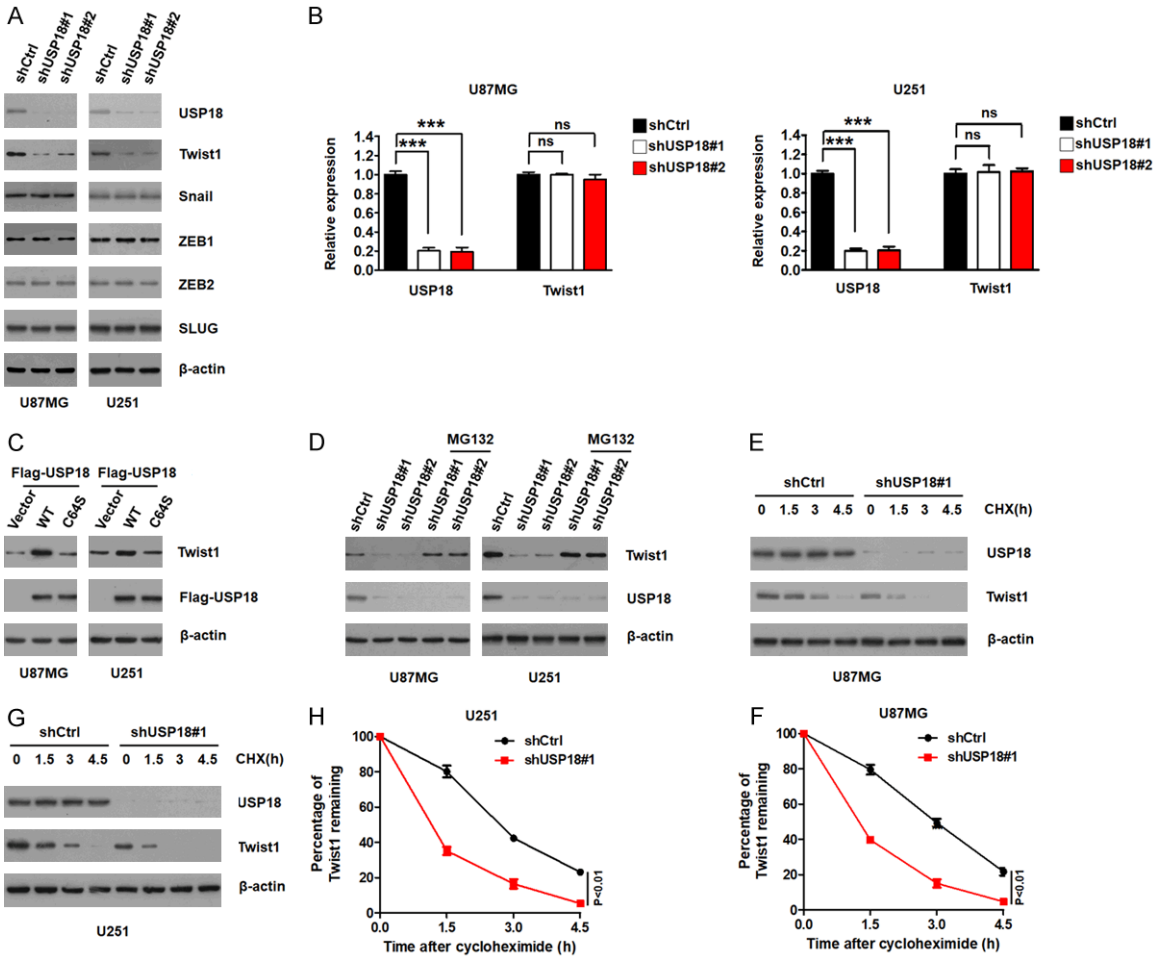

Figure 3. USP18 maintains Twist1 stability. (A) Plasmids shUSP18#1, shUSP18#2 or shCtrl were stably transduced into U87MG and U251 cells. Expression of USP18, Twist1, Snail, ZEB1, ZEB2 and SLUG were analyzed using Western blotting with the indicated antibodies. β-actin served as the loading control. (B) USP18 and Twist1 mRNA levels were analyzed by qRT-PCR in U87MG and U251 cells stably transduced with USP18 shRNAs or control shRNA. ***P < 0.001, ns: no significance, n = 3 experiments. (C) U87MG and U251 cells were transfected with plasmids expressing Flag-tagged wild-type USP18 or the mutant USP18-C64S and Twist1 protein level was detected with the indicated antibody. β-actin was used as the loading control. (D) U87MG and U251 cells transfected with two independent shRNAs against USP18 were treated with or without the proteasome inhibitor MG132 (20 μM, 8 hours), and then USP18 and Twist1 protein levels were analyzed. β-actin served as the loading control. (E-H) U87MG (E, F) and U251 (G, H) cells transfected with USP18 shRNA or control shRNA were treated with 50 μg/ml cycloheximide for different time intervals. Twist1 protein level was detected by Western blotting. Quantification of Twist1 expression relative to β-actin is shown. Data are mean ± SD. n = 3 independent experiments. **P < 0.01, using 2-tailed Student's t test.

mutant USP18-C64S almost completely abolished Twist1 ubiquitination (**Figure 4C**). Conversely, depletion of USP18 dramatically increased Twist1 ubiquitination (**Figure 4D**). To further investigate whether USP18 deubiquitinated Twist1 directly, we performed an in vitro deubiquitination assay. Briefly, we purified Flag-tagged ubiquitinated Twist1 and Myc-tagged WT USP18 or the mutant USP18-C64S from HEK293T cells, and then incubated ubiquitinated Twist1 with either WT USP18 or mutant C64S respectively. As shown in **Figure 4E**, WT USP18, but not the mutant C64S, specifically reduced Twist1 ubiquitination, suggesting USP18 was able to directly deubiquitinate Twist1. These data collectively indicated that USP18 is a specific deubiquitinase targeting Twist1.

Twist1 rescues inhibition of GBM cells migration, invasion and tumor formation mediated by USP18 depletion

To further investigate whether Twist1 plays an important role in USP18-mediated GBM cells invasion and migration. Twist1 or the negative vector control was transduced into USP18-

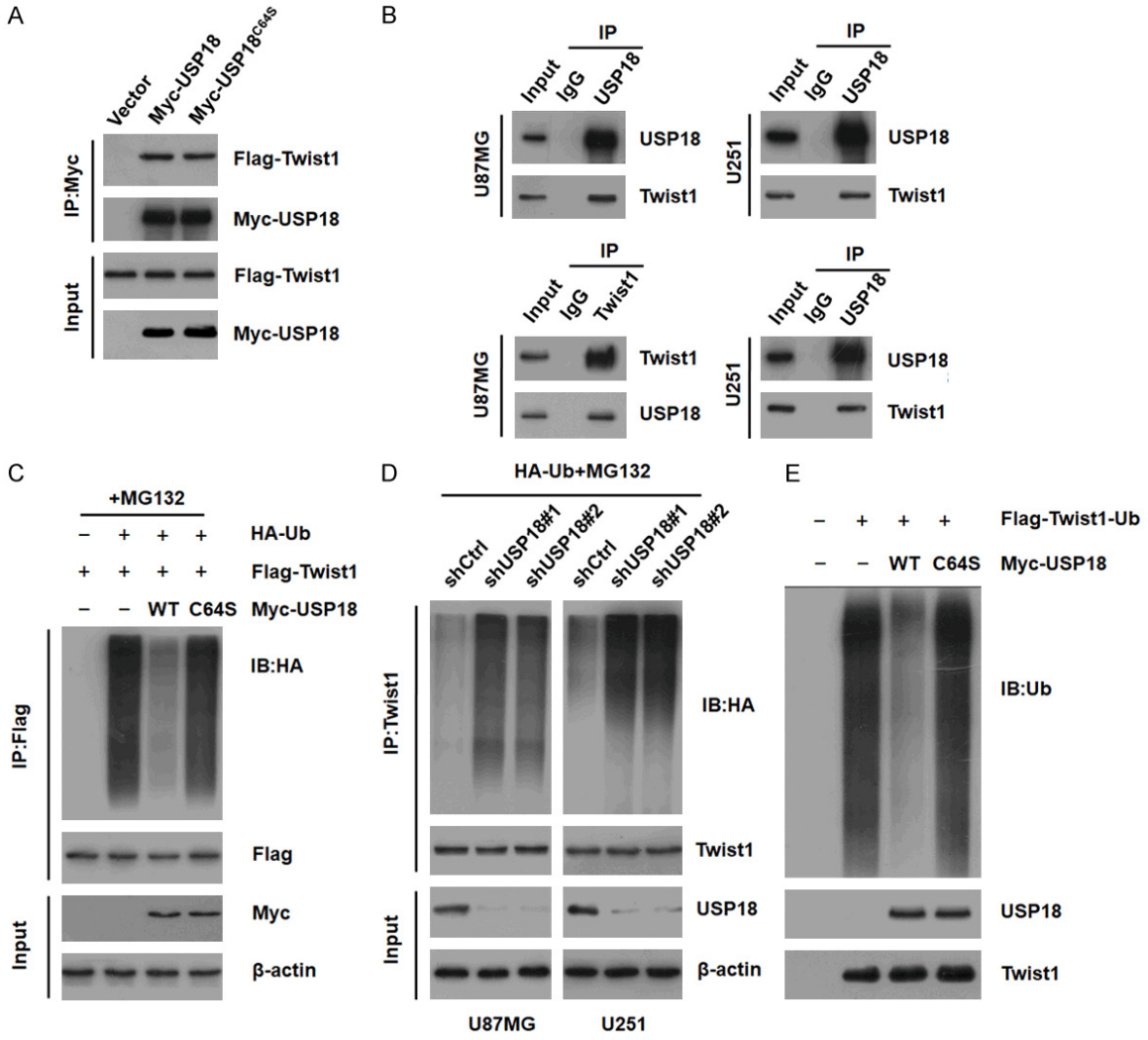

Figure 4. USP18 interacts with and deubiquitinates Twist1. A. Only Flag-Twist1 or in combination with Myc-tagged USP18 WT or mutant C64S were transduced into HEK293T cells, then cell lysates were immunoprecipitated with anti-Myc antibody, the immunoprecipitates were immunoblotted with the indicated antibodies. B. Cell lysates from U87MG and U251 cells were immunoprecipitated with antibodies against USP18 and Twist1 respectively, the immunoprecipitates were analyzed using Western blotting with the indicated antibodies. IgG served as negative control. C. Plasmids Flag-Twist1, HA-ubiquitin (HA-Ub), and either Myc-tagged USP18 WT or mutant C64S were cotransduced into HEK293T cells. Cells were treated with 20 μM MG132 for 6 h. Then cells were lysed for immunoprecipitation using anti-Flag antibody and analyzed by Western blotting. D. Plasmids HA-Ub and either USP18 shRNA or control shRNA were cotransduced into 2 GBM cell lines. These cells were treated with 20 μM MG132 for 6 h. Then cells were lysed for immunoprecipitation using Twist1 antibody and analyzed by Western blotting. E. USP18 deubiquitinates Twist1 in vitro. Flag-tagged ubiquitinated Twist1, Myc-tagged WT USP18 and the mutant USP18-C64S were purified from HEK293T cells. Purified ubiquitinated Twist1 was incubated with purified WT USP18 or the mutant C64S respectively, followed by Western blotting analysis using the indicated antibodies.

depleted U87MG and U251 cells. Western blotting assays confirmed that Twist1 protein level was elevated significantly in these cells after Twist1 transfection (**Figure 5A**). As shown in **Figure 5B** and **5C**, the migration ability of U87MG and U251 cells decreased after USP18 depletion, and this suppressive effect could be largely restored by Twist1 overexpression. Likewise, invasion assays suggested that downregulation of USP18 repressed GBM cells invasion and reconstituted expression of Twist1 almost completely reversed this effect (**Figure 5D**). We next conducted the in vivo assays and found that knockdown of USP18 drastically inhibited tumor formation and prolonged the survival of mice. Importantly, reintroduction of

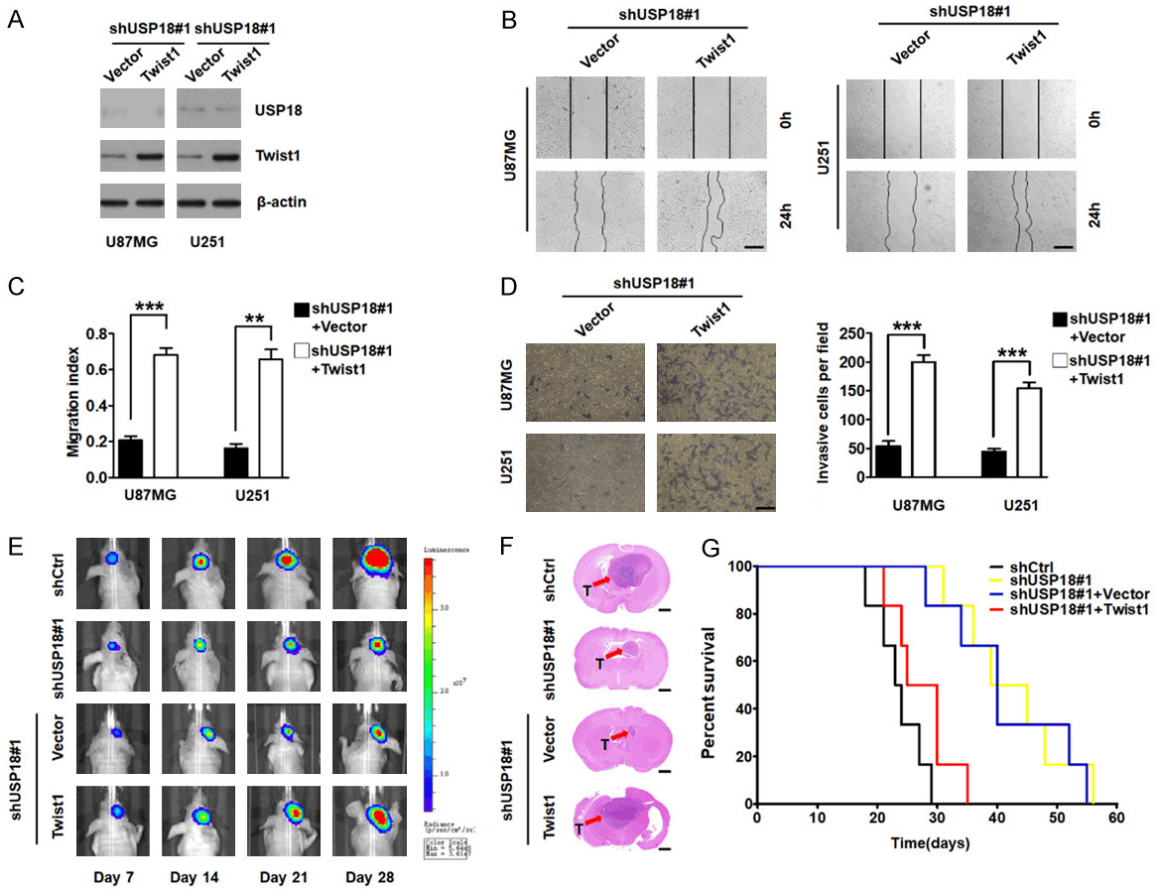

Figure 5. Twist1 rescues inhibition of GBM cells invasion and migration mediated by USP18 depletion. A. Plasmids USP18 shRNA and either Twist1 or control vector were cotransduced into U87MG and U251 cells. Then cell lysates were subjected to Western blotting with antibodies against USP18 and Twist1. B. Representative images of wound healing assay using U87MG and U251 cells transduced with the indicated plasmids. C. Quantification of wound healing assay is shown. **P < 0.01, ***P < 0.001, n = 3 experiments. D. Representative images of transwell invasion assay using U87MG and U251 cells transduced with the indicated plasmids. Quantification of transwell invasion assay is shown. ***P < 0.001, n = 3 experiments. E. Representative pseudocolour bioluminescence images of intracranial GBM xenografts bearing the indicated modified U87MG cells on the days as indicated. F. Representative H&E staining for tumor cytostructure. G. Survival curve of intracranial xenografts bearing the indicated modified U87MG cells.

Twist1 restored the effect of USP18 silencing on GBM tumorigenesis and survival time of mice (**Figure 5E-G**). These data suggested that USP18-Twist1 axis is critical for mediating the progression of GBM.

USP18 correlates with Twist1 protein levels and predicts poor outcome of GBMs

We next sought to clarify the potential clinical association between USP18 and Twist1, the expression of USP18 and Twist1 from 138 cases of human GBM tissues were detected using IHC. We found that GBM with high level of USP18 expressed high level of Twist1, conversely, GBM with low level of USP18 expressed low level of Twist1 (**Figure 6A**). Moreover, Immunostaining quantification and statistical analyses revealed that USP18 expression level correlates with Twist1 expression level directly (**Figure 6B**). We next attempted to identify whether USP18 expression was correlated with survival of GBM patients. Kaplan-Meier survival analysis indicated that GBM patients with elevated USP18 expression displayed poorer overall and disease-free survival compared with patients expressed relatively low levels of USP18 (**Figure 6C**, **6D**). In conclusion, our results strongly suggested that a positive correlation exists between USP18 and Twist1 expression levels, and higher expression of USP18 could predict shorter survival of GBM patients.

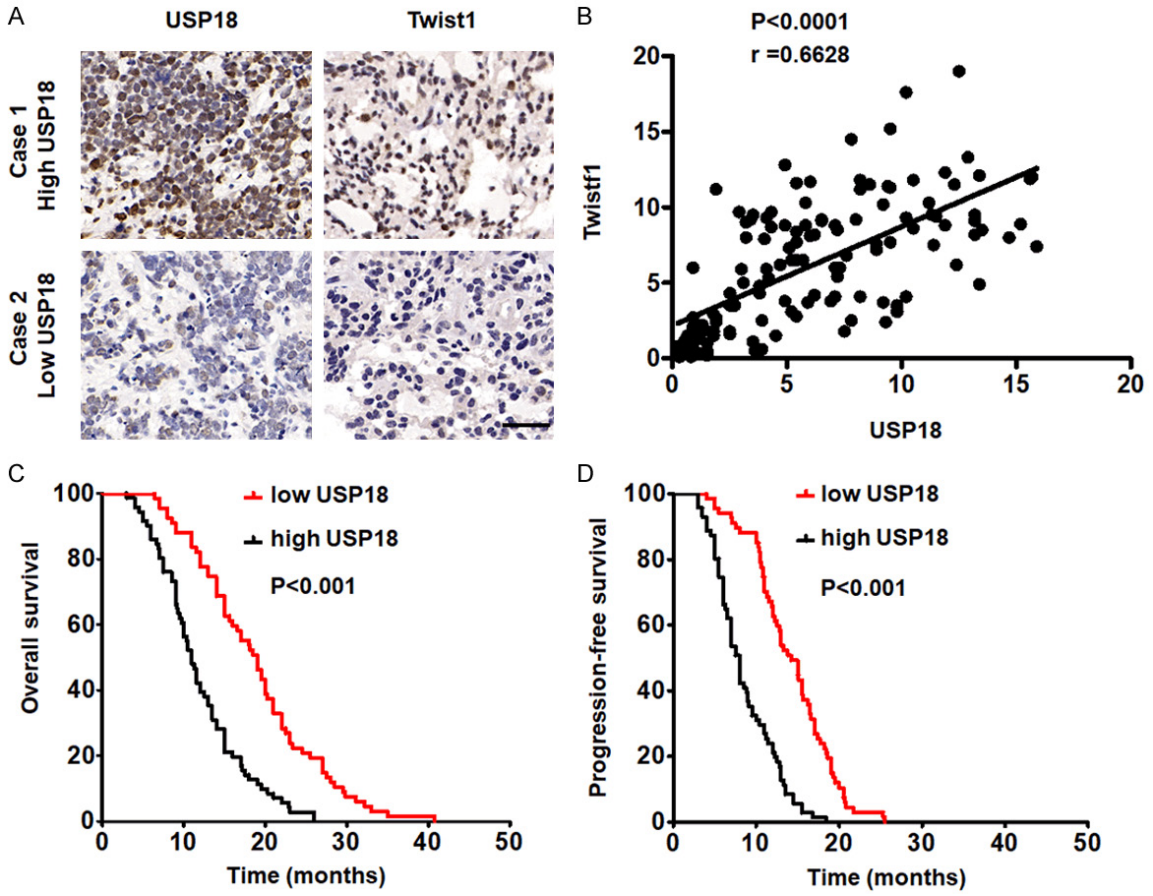

Figure 6. USP18 correlates with Twist1 protein levels and predicts poor survival of GBMs. A. Immunohistochemical staining detected the expression of USP18 and Twist1 in 138 human GBM specimens. Two representative specimens are shown. B. Correlation of USP18 and Twist1 expressions was analyzed using Spearman correlation test. C, D. The overall survival (left panel) and progression-free survival (right panel) of GBM patients with high (n = 67) or low (n = 71) USP18 expression were analyzed using a Kaplan-Meier model.

Discussion

As an important member of USP family, USP18 can influence the expression of various genes, which play crucial roles in the occurrence and progression of many human disorders. Recently, USP18 has been found to play a regulatory role in the pathogenesis of nonalcoholic fatty liver disease in human and transgenic mice hepatocytes [33]. Randall G et al. demonstrated that USP18 could modulate the antiviral activity of interferon against hepatitis C virus infection [34]. Noticeably, USP18 was also reported to be upregulated or downregulated in some malignancies, including lung cancer [24], breast cancer [35], melanoma [22], and muscle invasive bladder cancer [27]. However, the role of USP18 in GBM is waiting to discover. Our present study indicated that GBM cell lines and specimens frequently showed high expression of USP18. Based on analysis from 138 human GBM samples, we found that high USP18 level was positively associated with dismal outcome of GBM patients. Moreover, knockdown of USP18 suppressed GBM cell invasion in vitro and tumor formation in vivo, suggesting its therapeutic potential for GBMs. In spite of effective inhibitor against USP18 is not available currently, our results strongly suggest that further efforts targeting USP18-based therapeutics are fully warranted.

EMT usually refers to a cellular reprogramming process in which epithelial cells change their shape, increase motility and exhibit a mesenchymal phenotype [36]. And this process is thought to be responsible for a myriad of pro-

tumorigenic functions such as increased invasion, migration and repression of apoptosis and senescence [37, 38]. Twist1 is a key EMT-inducing transcription factor, which govern the transcription of EMT-related genes through upregulating the mesenchymal-associated proteins, such as N-Cadherin, Vimentin, and Fibronectin, and downregulating the epithelial-associated proteins, such as E-Cadherin [39]. Moreover, a variety of cancers exhibited Twist1 overexpression. For example, a study performed by Beck B et al. revealed that high-level Twist1 was detected in skin tumor [40]. Bladder urothelial carcinoma also overexpressed Twist1 proteins. More recently, emerging evidence has shown that elevated expression of Twist1 was observed in GBM [41]. However, the molecular mechanism underlying high level of Twist1 in GBM remains unclear. In this study, we first determined that USP18 exerts its biological function via deubiquitinating and stabilizing Twist1 in GBM. We demonstrated that USP18 depletion inhibits the expression of Twist1, whereas reconstituted expression of USP18 enhances Twist1 expression at post-transcriptional level. Moreover, we provided evidence that knockdown of USP18 suppresses GBM cells invasion, migration and tumor formation, reintroduction of Twist1 almost completely reverses the effect of USP18 depletion. Meanwhile, it was observed that Twist1 protein level are positively correlated with USP18 protein level in clinical GBM specimens. Thus, we concluded that Twist1 is indeed a downstream target of USP18 in GBM.

The invasive properties of GBMs into normal peripheral brain tissue has become a major hindrance that renders current treatments for the disease invalid. Substantial efforts have been made to elucidate the mechanisms underlying the invasive potential of GBM. Recently, studies revealed that elevated expression of Twist1 is linked to enhanced invasion of GBM [42]. In our study, we identified USP18 serves as a crucial mediator of GBM invasion and migration through directly interacting with and protecting Twist1 from degradation. Intriguingly, stabilization of Twist1 depended on the catalytic activity of USP18 because WT USP18 could stabilize Twist1, the mutant USP18-C64S had not this effect. Therefore, our findings suggest that a kind of therapeutics targeting USP18 is promising for GBM treatment.

In summary, this study identifies USP18 as a bona fide DUB of Twist1, resulting in enhanced invasion, migration and tumor formation of GBM cells. Targeting USP18-Twist1 signaling axis may thus provide a new sight into therapeutic intervention in GBM.

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Disclosure of conflict of interest

None.

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