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NEDD4 Degrades TUSC2 to Promote Glioblastoma Progression

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Declaration of Interest Statement

Authors declare no conflict of interests.

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Abstract

Whether tumor suppressor candidate 2 (TUSC2) plays an important role in glioblastoma (GBM) progression is largely unknown. Whether TUSC2 undergoes polyubiquitination is unknown. Herein, we report that TUSC2 protein expression is reduced/lost in GBM compared to normal brain due to protein destabilization; TUSC2 mRNA is equally expressed in both tissues. NEDD4 E3 ubiquitin ligase polyubiquitinates TUSC2 at residue K71, and the TUSC2-K71R mutant is resistant to NEDD4-mediated proteasomal degradation. Analysis of GBM specimens showed NEDD4 protein is highly expressed in GBM and the level is inversely correlated with TUSC2 protein levels. Furthermore, TUSC2 restoration induces apoptosis and inhibits patient-derived glioma stem cells (PD-GSCs) *in vitro* and *in vivo*. Conversely, TUSC2-knockout promotes PD-GSCs *in vitro* and *in vivo*. RNA-Seq analysis and subsequent validations showed GBM cells with TUSC2- knockout expressed increased Bcl-xL and were more resistant to apoptosis induced by a Bcl-xL-specific BH3 mimetic. A TUSC2- knockout gene signature created from the RNA-seq data predicts poor patient survival. Together, these findings establish that NEDD4-mediated polyubiquitination is a novel mechanism for TUSC2 degradation in GBM and that TUSC2 loss promotes GBM progression in part through Bcl-xL upregulation.

Keywords

TUSC2; tumor suppressor; glioblastoma; NEDD4; glioma stem cells

1. INTRODUCTION

Glioblastoma (GBM), a grade IV astrocytoma, is the most common primary brain cancer in adults, the most intractable glioma, and is associated with a dismal prognosis of 8–15 months [1–3]. The majority of GBM (~90%) develop *de novo* without clinical or histological evidence of a less malignant precursor lesion. Genetically engineered mouse models and large-scale genome sequencing by The Cancer Genome Atlas (TCGA) have shown a number of oncogenes (EGFR, PDGFRA, PIK3CA, K-Ras, H-Ras, hTERT, and AKT) and tumor suppressors (p53, Rb, PTEN, $p16^{INK4A}$/$p14^{ARF}$, and NF1) to play potential roles in the development of GBM [4, 5].

TUSC2 (tumor suppressor candidate 2; also known as FUS1) is a known lung cancer suppressor [6]. Lerman & Minna identified several genes, including TUSC2, on deleted chromosome 3p21.3 as putative tumor suppressor genes in lung cancer [7]. 3p21.3 deletion was later found to be rare in lung cancer (1.1%; TCGA) and most other cancer types, except for mesothelioma (36%) [8] and renal clear cell carcinoma (12%; TCGA). No evidence of methylation was found in the TUSC2 gene promoter region in lung cancer samples [9], however it was reported to be partially methylated in head and neck squamous

cell carcinomas and normal salivary rinses, but unmethylated in normal mucosa [10]. TUSC2 somatic mutations have not been found in any cancer specimens according to TCGA, although infrequent mutations have been reported in lung cancer cell lines [9]. Despite infrequent TUSC2 deletion and lack of promoter methylation, TUSC2 mRNA is frequently reduced in lung cancer (~80%), which has been attributed to transcriptional and posttranscriptional mechanisms such as regulation by microRNAs (miRNAs) [7, 11–13]. However, regulation of TUSC2 expression through other mechanisms has not been reported.

The exact functions of TUSC2 remain unclear with published studies. TUSC2 has been shown to induce G1 cell cycle arrest, apoptosis [9, 14, 15], regulate calcium signaling [16], modulate tyrosine kinase and Ser/Thr kinase activity [15, 17–20], alter gene expression [8], and enhance tumor-suppressive miRNAs expression [21]. TUSC2 is primarily localized in the mitochondria and is detected in the cytoplasm [7, 22]. N-myristoylation of TUSC2 is required for its tumor suppressive role in lung cancer; however, whether TUSC2 protein undergoes ubiquitination has not been reported in any tumor or cell type [23].

A comprehensive study of TUSC2 as a tumor suppressor in GBM and normal brain has not been conducted. Whether TUSC2 protein stability is regulated by polyubiquitination or proteasomal degration has not been investigated. Only two studies have investigated TUSC2 expression in gliomas, reporting that TUSC2 protein is expressed at higher levels in normal brain or low-grade gliomas than in high-grade gliomas [13, 21]. However, these studies have only investigated regulation of TUSC2 mRNA expresion in GBM but not post-translational regulation of TUSC2 protein. In this study, we report that TUSC2 protein expression, but not mRNA expression, is frequently reduced in established GBM cell lines, primary GBM cells, patient-derived glioma stem cells (PD-GSCs), and patient samples compared to normal brain. We found that TUSC2 protein is destabilized in GBM but not in astrocytes. TUSC2 degradation is due to polyubiqutination at Lysine-71 (K71) mediated by the E3 ubiquitin ligase, neural precursor cell expressed developmentally downregulated protein 4 (NEDD4). We further showed that TUSC2 restoration in GBM cells and PD-GSCs induced apoptosis and reduced neurosphere formation *in vitro*, and inhibited PD-GSC intracranial growth *in vivo*. Conversely, TUSC2 knockout (TUSC2-KO) enhanced neurosphere formation, GBM growth and survival both *in vitro* and *in vivo*. Using RNA-Seq to gain new insights into the role of TUSC2 in GBM, we found that GBM cells with TUSC2-KO expressed higher levels of anti-apoptotic protein Bcl-xL, and that thse cells are more reistant to Bcl-xL inhibition via a Bcl-xL-selective BH3 mimetic, compared to control cells. Collectively, we provided novel evidence that TUSC2 protein is commonly lost in GBM via NEDD4-mediated polyubiquitination and degradation, and that TUSC2 plays a tumor suppressive role in GBM by inducing apoptosis and suppressing GSCs.

2. MATERIALS AND METHODS

2.1 Cell lines, PD-GSCs, and patient GBM specimens

NHA (normal human astrocytes), C8-S (mouse astrocytes), U251MG, LN18, LN229, T98G, and U87MG cells were obtained from ATCC (Manassas, VA, USA) and cultured according to their recommendations. Luciferase-expressing low-passage human GBM G48a cells were established by Dr. Waldemar Debinski [24]. Primary GBM cell lines, BTCOE4525,

BTCOE4536, BTCOE4710, BTCOE4795, and BTCOE4810 were developed and validated from patient tumors at the Wake Forest Brain Tumor Center of Excellence (Winston-Salem, NC, USA) [25]. Immortalized human astrocytes were a kind gift from Dr. Russell O. Pieper at University of California San Fransisco [26]. PD-GSCs were kind gifts from Drs. Erik Sulman and Krishna Bhat at University of Texas MD Anderson Cancer Center [27]. GSC-11 and GSC-23 belong to the proneural subtype whereas GSC-20 and GSC-28 are mesenchymal. PD-GSCs were passaged as neurospheres in serum-free DMEM/F12 growth medium supplemented with B27 (2%), FGF (10ng/mL), and EGF (100ng/mL) in order to preserve stem-like properties. Normal brain tissue (BNC17011) and glioma tissue microarrays (GL2083) were purchased from US Biomax (Rockville, MD, USA). Additional GBM patient samples were from the Wake Forest Brain Tumor Center of Excellence [25]. We have obtained written informed consent from the patients; the studies were approved by Wake Forest Insitutional Review Board and were conducted in accordance with recognized ethical guidelines.

2.2 Western blotting and Immunohistochemistry (IHC)

Immunoblotting and immunohistochemistry were performed as we previously described [26, 28]. Antibodies for immunoblotting include TUSC2 (Abcam (Cambridge, UK); ab70182), β-actin (Cell Signaling Technology/CST (Danvers, MA, USA); 8H10D10), NEDD4 (CST; C5F5), MDM2 (Santa Cruz Biotechnology/SCBT (Dallas, TX, USA); SMP14), DTL (SCBT; B-8), UBE3C (SCBT; S-14), PTEN (Millipore (Burlington, MA, USA); 04–035), Bcl-xL (CST; 54H6), STAT3 (CST; 12640), PARP (CST; 46D11), NEDD4 (ProteinTech; 67845) and TUSC2 (ThermoFisher; MA5–24739). Antibodies for IHC included TUSC2 (ProteinTech (Rosemont IL, USA); #11538–1-AP), NEDD4 (CST; #C5F5), and Ki67 (NeoMarkers (Portsmouth, NH, USA); #RB-9043-R7). Histologic scores (H-Scores) were computed from both % positivity (A%, A=1–100) and intensity (B=0–3) using the equation, H-Score=A × B.

2.3 Immunofluorescence staining and confocal microscopy

Immunofluorescence staining was performed as previously described [29]. Briefly, 10 μm-thick normal mouse brain slices or G48a cells attached to slides were immunostained with antibodies including TUSC2 (ProteinTech; #11538–1-AP; 1:100), Nestin (Invitrogen (Carlsbad, CA, USA); #MA1–110; 1:50), GFAP (Abcam; #ab10062; 1:1000), Olig2 (Invitrogen; #MA5–15810; 1:250), HA-tag (Abcam; #ab18181, 1:1000), Alexa Fluor 488-conjugated secondary antibody (Invitrogen; #A11029; 1:500), and Alexa Fluor 594-conjugated secondary antibody (Invitrogen; #A11037; 1:500), stained with DAPI (Vector Labs (Burlingame, CA, USA); #H-1500), and then subjected to confocal microscopy. Images were overlayed using ImageJ.

2.4 Animal studies

Two animal studies were carried out using 6–8 week female nude mice (Charles River; Wilmington, MA, USA). In the first study, GSC-28 stably expressing the dox-inducible TUSC2 lentiviral vector were injected at a concentration of 1–5 × 10^5 cells in 5 μL PBS into the right frontal lobe using a stereotaxic frame. Mice were anesthetized with a ketamine/xylazine mixture and a burr hole was drilled along the coronal suture through a scalp

incision according to an approved IACUC protocol. Mice were treated with doxycycline hyclate (Sigma-Aldrich; St. Louis MO, USA) administered at 2 mg/mL supplemented with 5% sucrose in the drinking water maintained in darkened bottles and refreshed twice weekly. For bioluminescent imaging, xenograft-bearing mice were injected intraperitoneally with d-luciferin at 100 mg/kg body weight and then imaged weekly using PerkinElmer IVIS100 imager (Waltham, MA, USA). In the second study, exponentially growing G48a cells stably expressing either control gRNA/Cas9 lentivirus (G48a-Control-gRNA) or TUSC2 gRNA/Cas9 lentivirus (G48a-TUSC2-KO) were injected at a concentration of 1–5 × 10^5 cells in 5 μL PBS into the right frontal lobe using a stereotaxic frame. Mice were monitored for tumor growth as described for the first study.

2.5 Statistical analyses

Data are presented as mean±SE. Student's t-test, one-way ANOVA, Chi-square analysis, Fisher's Exact test, and survival analyses were performed using GraphPad Prism and Sigma Plot version 11.0.

2.6 Data Availability Statement

Data from the Next Generation RNA-sequencing will be uploaded to Mendeley Data.

3. RESULTS

3.1 TUSC2 protein, but not mRNA expression, is frequently lost in GBM.

TUSC2 mRNA and protein expression in GBM compared to normal brain tissues has not been well investigated. Herein, we examined a panel of established GBM cell lines, low-passage primary GBM cell lines, GBM patient samples, and astrocytes (human and mouse) for TUSC2 mRNA and protein expression. TUSC2 mRNA levels in GBM and astrocytes were similar whereas TUSC2 protein was either lost or reduced in GBM compared to astrocytes (Fig. 1A). In agreement with our mRNA data, analysis of a publicly available dataset (Gene Expression Omnibus/GEO; GSE4290) indicated that TUSC2 mRNA expression did not differ between normal brain samples and GBM samples (Fig. 1B), or normal brain and across glioma grades (Fig. 1C) [30]. Using TCGA, we found limited methylation within the TUSC2 gene promoter in GBM patients (Fig. 1D). Next, we used immunohistochemistry (IHC) to examine GBM specimens (N=63) and normal brain tissues (N=80) for TUSC2 expression, and found TUSC2 protein to be frequently reduced or lost in GBM samples (Fig. 1E,F). To determine if TUSC2 is expressed in the predicted cells of origin for GBM [31, 32], we conducted immunofluorescence staining and confocal microscopy and found TUSC2 protein to be expressed in Nestin-positive neural stem cells, glial fibrillary acidic protein (GFAP)-positive astrocytes, and Olig2-positive oligodendrocytes in both human (Fig. 1G) and mouse brains (Supplementary Fig. 1). TUSC2 primarily localizes to the mitochondria but is also found within the cytoplasm and not found in the nucleas, which is consistant with our findings [16, 23]. Collectively, these results demonstrate that TUSC2 protein is expressed in normal brain tissues, including GBM cells of origin, but is frequently reduced or lost in GBM, with no change in TUSC2 mRNA expression in GBM compared to normal brain.

3.2 TUSC2 protein is destabilized in GBM via proteasome-mediated degradation.

To elucidate the mechanism of TUSC2 protein loss in GBM, we first asked whether TUSC2 protein loss is due to protein destabilization. To determine endogenous TUSC2 stability in human astrocytes versus GBM, we treated astrocytes and G48a with protein synthesis inhibitor cycloheximide (CHX) for 0–12 hrs followed by western blotting (WB) to determine protein half-life. Results showed that TUSC2 half-life was approximately 6 hours in GBM, but >12 hours in astrocytes, indicating that TUSC2 protein is preferentially destabilized in GBM compared to astrocytes (Fig. 2A; Supplemental Fig. 2). We further observed that TUSC2 degradation was abrogated by proteasome inhibitor MG132, indicating that TUSC2 protein degradation is mediated by the proteasome (Fig. 2A; Supplemental Fig. 2). Because polyubiquitinated proteins are targeted for proteasome-mediated degradation, we examined whether TUSC2 is polyubiquitinated in GBM. We overexpressed TUSC2 in TUSC2-low U251MG cells, immunoprecipitated TUSC2, and performed WB to determine levels of TUSC2 ubiquitination. WB analysis showed U251MG overexpressing TUSC2 contained increased levels of polyubiquitinated TUSC2 compared to control (Fig. 2B). We further observed that MG132 led to increased accumulation of polyubiquitinated TUSC2 in U251MG (Fig. 2C). These results indicate, for the first time, that TUSC2 protein stability is decreased in GBM compared to astrocytes and that TUSC2 is polyubiquitinated leading to subsequent proteasomal degradation.

3.3 NEDD4 is overexpressed in GBM and physically interacts with TUSC2.

E3 ubiquitin ligases are responsible for substrate specificity in the ubiquitin-proteasome system and previous literature suggests that dysregulation of E3 ligases is linked to cancer development [33]. Therefore, we reasoned that the E3 ligase(s) mediating TUSC2 polyubiquitination are likely more highly expressed in GBM than in normal brain. To identify these E3 ligases, we analyzed GEO dataset GSE4290 for expression of 62 known E3 ligases (Fig. 2D). We identified four E3 ligases, DTL, NEDD4, MDM2, and UBE3C, expressed at higher levels in GBM than normal brain (≥ 2-fold; $p<0.05$; Fig. 2E). We next examined whether these four E3 ligases physically interact with TUSC2 through immunoprecipitation (IP) followed by WB in TUSC2 transfected U251MG treated with 5 uM ZVAD and found that upon TUSC2 pull-down, only NEDD4 was bound to TUSC2 (Fig. 2F). Furthermore, when we immunoprecipitated NEDD4, we found TUSC2 to co-immunoprecipitate with NEDD4 (Fig. 2G). We further performed immunofluorescence staining and confocal microscopy on TUSC2 and NEDD4 transfected G48a cells treated with 10 uM MG132 and 5 uM ZVAD and found that TUSC2 and NEDD4 colocalize in the cytoplasm of GBM cells (Fig. 2H). These data indicate that NEDD4 is upregulated in GBM and physically interacts with TUSC2.

3.4 NEDD4 polyubiquitinates TUSC2 at K71 in GBM, leading to TUSC2 degradation.

We next examined whether NEDD4 mediates TUSC2 polyubiquitination. NEDD4 overexpression led to decreased TUSC2 protein in TUSC2-positive G48a and U87MG cells (Fig. 3A). Conversely, NEDD4 knockdown using two different NEDD4-targeting shRNAs resulted in increased TUSC2 protein in U87MG cells (Fig. 3B). To demonstrate there is no off-target effects of NEDD4 overexpression or knockdown, we found expression of STAT3,

not a NEDD4 substrate, to be unaffected by NEDD4. To further investigate whether NEDD4 directly ubiquitinates TUSC2, we performed a cell-free ubiquitination assay and observed that recombinant NEDD4 polyubiquitinated recombinant TUSC2 (Fig. 3C). Recombinant PTEN was used as the positive control since PTEN is a reported substrate for NEDD4 [34]. Next, we conducted IP-WB using recombinant TUSC2 and NEDD4 proteins in the cell-free ubiquitination assay; results showed that NEDD4 and TUSC2 directly interact (Fig. 3D). TUSC2 protein contains six lysine (K) residues which can be targeted for ubiquitination (Fig. 3E-Top). To identify NEDD4-targeted lysine(s) in TUSC2, we used mass spectrometry to analyze NEDD4-ubiquitinated recombinant TUSC2, which revealed that K71, K84, and K93 were ubiquitinated (Fig. 3E-Bottom; Supplemental Fig. 3A,B). Because mass spectrometry could not distinguish mono- versus poly-ubiquitinated peptides, we created three TUSC2 mutants (K71R, K84R, and K93R) to examine whether loss of each lysine residue abrogates polyubiquitination by NEDD4. Wild-type (WT) and mutant TUSC2 proteins were immunoprecipitated and subjected to the cell-free NEDD4 ubiquitination assay. We found that NEDD4-mediated polyubiquitination was drastically reduced on TUSC2-K71R, but not K84R or K93R, suggesting that NEDD4 targets K71 (Fig. 3F). Importantly, expression of the TUSC2-K71R was not reduced by NEDD4 overexpression (Fig. 3G); TUSC2-K71R is more stable than the TUSC2-WT in G48a-TUSC2-KO cells (Fig. 3H; Supplemental Fig. 3C,D).

GSCs are a subpopulation of GBM cells that are highly resistant to standard-of-care therapy and lead to tumor recurrence [35]. Because of the aggressivenss of GSCs, we examined what affect TUSC2 stability would have on the GSC population. We first examined GBM stemness by transfecting GSC-28, with either TUSC2-WT or the TUSC2 lysine mutants. We found that TUSC2-K71R significantly decreased GSC-28 neurosphere forming abilities as compared to WT and other TUSC2 mutants (Fig. 3I). Furthermore, we examined TUSC2 mediated-tumor suppression through apoptosis, using the TUNEL assay, and found that TUSC2-K71R significantly increased GSC-28 neurosphere apoptosis compared to WT (Fig. 3J). Together, these data indicate that NEDD4 polyubiquitinates TUSC2 at K71 residue, leading to subsequent proteasomal degradation and reduced tumor suppressive effects.

3.5 TUSC2 and NEDD4 proteins are inversely expressed in GBM and normal brain tissues.

To further examine the relationship between TUSC2 and NEDD4, we examined protein expression of TUSC2 and NEDD4 in GBM cells, PD-GSCs, and astrocytes. We found that TUSC2 is expressed at lower levels in PD-GSCs and GBM cells compared to astrocytes and NEDD4 expression is inversely correlated with TUSC2 levels (Fig. 4A). IHC analysis of 63 GBM specimens and 80 normal brain tissues revealed NEDD4 protein expression to be significantly increased in GBM patient samples compared to normal brain (Fig. 4B,C). Chi-square analysis of the IHC data indicated that NEDD4 and TUSC2 are significantly inversely expressed in patient tissue samples (Fig. 4D). Pair-wise analysis indicated that NEDD4 and TUSC2 are inversely correlated in individual GBM samples (Fig. 4E). Since TUSC2 mRNA expression does not differ between GBM and normal brain samples (Fig. 1A–B), while TUSC2 and NEDD4 protein expression are inversely correlated (Fig. 4A–E), we examined the prognostic value of NEDD4 mRNA as a surrogate marker for

TUSC2 protein expression. Analysis of three patient datasets showed that NEDD4 mRNA expression was not significantly correlated with GBM patient survival (TCGA, N=165; Fig. 4F); however, in two combined multicohort glioma datasets (TCGA, N=667; Rembrandt, N=397) [36], high NEDD4 mRNA expression is associated with poorer overall survival in patients with gliomas (Fig. 4G–H). Together, these results indicate that TUSC2 and NEDD4 protein expression is inversely correlated in GBM, and that NEDD4 is correlated with worse overall survival in glioma patients.

3.6 Restoring TUSC2 expression induces apoptosis and inhibits PD-GSCs *in vitro* and *in vivo*.

To further evaluate the effect of TUSC2 on GSCs, we overexpressed TUSC2 in four PD-GSC lines and found it to suppress their neurosphere-forming abilities (Fig. 5A–B). Overexpression of TUSC2 in U251MG GBM cells significantly reduced colony formation and neurosphere forming ability (Fig. 5C). To further investigate TUSC2-mediated tumor suppression, we generated a doxycycline (Dox)-inducible TUSC2 expression construct and establish the GSC-28-indTUSC2 line (Fig. 5D). We found that induction of TUSC2 expression inhibited neurosphere formation in two different GSC-28-indTUSC2 clones (Fig. 5E). TUSC2 induction in GSC-28-indTUSC2 neurospheres led to increased apoptosis as indicated by TUNEL (Fig. 5F). We next investigated the role of TUSC2 in GBM tumor-initiation *in vivo*. We injected GSC-28-indTUSC2 cells into the frontal lobe of nude mice and treated mice with either no Dox, Dox two days before orthotopic implantation with continued Dox treatment until the end of the study (Fig. 5G-Top; pre-induction), or Dox treatment following tumor establishment, to mimic TUSC2-based gene therapy in pre-clinical and clinical studies for patients with lung cancer [12, 17, 19, 37–41] (Fig. 5G-Bottom; post-induction). In the pre-induction group, TUSC2 re-expression significantly impaired xenograft development compared to the control (Fig. 5H, Supplemental Figure 4). In the post-induction group, tumor growth inhibition trended towards significance (p=0.052) (Fig. 5H). Tumor engraftment rate in the pre-induction group, but not the post-induction group, was significantly decreased in response to TUSC2 re-expression (Fig. 5I). We also found that TUSC2 re-expression at the time of inoculation prolonged host survival (Fig. 5J). Finally, TUNEL staining of resected brain samples revealed that TUSC2 restoration significantly induced tumor apoptosis in both pre- and post-induction groups (Fig. 5K). These novel findings reveal that TUSC2 acts as a tumor suppressor in GBM, in part, by inducing apoptosis and inhibiting GSCs.

3.7 Loss of TUSC2 expression promotes GBM aggressiveness *in vitro* and *in vivo*.

To determine the impact of TUSC2 protein loss on GBM, we knocked down TUSC2 expression with three different TUSC2-targeting siRNAs and two different TUSC2-targeting guide RNAs (gRNAs). Knockdown of TUSC2 via siRNA or gRNA was efficient, resulting in enhanced neurosphere fomation of G48a cells (Fig. 6A–B). We further developed a lentivirus carrying TUSC2-targeting gRNA and CRISPR/Cas9 to stably knockout TUSC2 expression in G48a cells (Fig. 6C). G48a-TUSC2-KO cells exhibited increased neurosphere formation compared to G48a-Control-gRNA (Fig. 6D). Next, we implanted the isogenic lines into the brains of 6 week-old nude mice and monitored tumor growth via bioluminescence imaging. TUSC2-KO significantly promoted intracranial GBM growth

and proliferation, as indicated by increased bioluminescent signal (Fig. 6E) and Ki-67 IHC staining (Fig. 6F–H). Next, we performed TUNEL staining and found a reduction in apoptosis in TUSC2-KO tumors compared to the control (Fig. 6I). Together, these data demonstrate that loss of TUSC2 expression promotes a stem-cell like phenotype *in vitro*, and increased tumor growth and proliferation and reduced apoptosis *in vivo*, therefore supporting the role of TUSC2 as a tumor suppressor in GBM.

3.8 TUSC2 suppresses GBM growth by modulating cellular apoptotic machinery.

The exact functionality for TUSC2 remains unclear. Only limited information is available for TUSC2 in GBM. To fill these knowledge gaps, we performed RNA-Seq on isogenic G48a lines with TUSC2-KO or Control-gRNA that were grown as neurospheres, to elucidate the effect of TUSC2 loss on the GBM transcriptome (Fig. 7A). Upon TUSC2 depletion, expression of 1240 genes were significantly altered ($p<0.05$), including 683 upregulated genes and 557 downregulated genes (excluding duplicate or uncharacterized genes) (Supplemental Table 1). Notably, the anti-apoptotic gene *BCL2L1* (Bcl-xL) was upregulated in TUSC2-KO cells. Confirmation through qPCR demonstrated that Bcl-xL was upregulated in G48a-TUSC2-KO cells (Fig. 7B) and downregulated in Dox-treated GSC28-indTUSC2 cells (Fig. 7C). Further assessment through WB demonstrated an increase in Bcl-xL protein expression in G48a-TUSC2-KO cells, and the inverse effect on GSC28-indTUSC2 cells (Fig. 7D). To further elucidate the connection between TUSC2 and Bcl-xL, we treated G48a-Control-gRNA and G48a-TUSC2-KO cells with a Bcl-xL specific BH3 mimetic, A-1331852, at 1 uM for 48 hours, and determined the extent of apoptosis using TUNEL assay and PARP cleavage-WB. Results showed that A-1331852 induced significant apoptosis in G48a-Control-gRNA cells but not G48a-TUSC2-KO cells (Fig. 7E–G), demonstrating that TUSC2-KO GBM cells are more resistant to Bcl-xL inhibition.

Next, we asked whether TUSC2 loss can be used as a prognostic indicator for GBM. Not only does TUSC2 mRNA expression not predict GBM survival (Fig. 7H), but we cannot use it to indicate TUSC2 protein expression in GBM due to decrease protein stability mediated by NEDD4. To indicate TUSC2 protein expression status, we used the RNA-Seq data to generate two gene signatures that include 683 significantly upregulated genes (TUSC2-KO-Up Gene Signature) or 557 significantly downregulated genes (TUSC2-KO-Down Gene Signature) in the G48a-TUSC2-KO cells compared to G48a-Control-gRNA cells ($p<0.05$). We found that the TUSC2-KO-Up Gene Signature was associated with worse overall survival in GBM (Fig. 7I). In contrast, the TUSC2-KO-Down Gene Signature was not associated with patient survival (Supplemental Fig. 5A). Similar results were found upon further refinement of the TUSC2-KO gene signatures to include only genes with Log2 fold-change at $p<0.01$ for use in Kaplan-Meier survival analysis (Supplemental Figure 5B–C). To further examine the link between TUSC2 and NEDD4, we conducted GSEA analysis of the TCGA GBM cohort and observed that patient tumors with high NEDD4 Gene Signature [42] were enriched for the TUSC2-KO-Up Gene Signature, further establishing the inverse relationship between TUSC2 and NEDD4 (Supplemental Figure 5D–E). In contrast, patient stratification based on NEDD4 mRNA levels did not show enrichment for either TUSC2-KO Gene Signatures (Supplemental Figure 5F–G). Similar to the TUSC2-KO-Up Gene

Signature, the NEDD4 Gene Signature was found to correlate with poor patient survival (Fig. 7J).

Because we observed enrichment of the TUSC2-KO-Up Gene Signature in patients with high NEDD4 Gene Signature, we then stratified patients based on the status of both gene signatures. We found patients with high NEDD4 and high TUSC2-KO-Up Gene Signatures had worse overall survival than those with low NEDD4 and low TUSC2-KO-Up Gene Signatures (Fig. 7K). Furthermore, we created a combined NEDD4+TUSC2-KO-Up Gene Signature and found it to predict poorer GBM patient survival (Fig. 7L). Together, these data indicate that TUSC2 may exert its tumor suppressive role in GBM, in part, by indirectly reducing expression of Bcl-xL to promote apoptosis, and that TUSC2-KO-Up Gene Signature alone, NEDD4 Gene Signature alone, and in combination can be used as novel prognostic indicators for patients with GBM.

4. DISCUSSION

We made the following important novel observations in this study: a) TUSC2 protein expression is frequently reduced or lost in GBM cells and patient samples, while mRNA levels are not affected; b) TUSC2 is preferentially degraded in GBM cells compared to normal astrocytes, and this effect is driven by NEDD4-mediated polyubiquitination of TUSC2; c) NEDD4 targets K71 residue of TUSC2; d) NEDD4 and TUSC2 are inversely expressed in GBM patient samples and normal brain; e) re-expression of TUSC2 induces apoptosis in GBM and PD-GSCs, and inhibits neurosphere formation *in vitro* and PD-GSC tumor growth *in vivo*; f) knockdown and knockout of TUSC2 expression in TUSC2-positive GBM cells increases their neurosphere-forming ability *in vitro* and promotes tumor growth *in vivo*; g) TUSC2 exerts its tumor suppressive role, in part, by indirectly reducing expression of Bcl-xL and thereby inducing apoptosis; and h) a TUSC2-KO-Up Gene Signature, alone and in combination with the NEDD4 Gene signature, is a novel prognostic indicator of poor GBM patient survival. Through these findings, this study serves as an important step towards defining the novel tumor suppressor role of TUSC2 in GBM and thereby, advances the biological understanding of molecular mechanisms of GBM development and progression.

Our molecular analyses revealed that TUSC2 protein expression, but not mRNA expression, is decreased or lost in the majority of GBM cases compared to normal brain tissues. Interestingly, a previous study reported that TUSC2 protein is expressed at lower levels in high-grade gliomas than low-grade gliomas [21]; however, this study did not examine normal brain for TUSC2 protein expression nor examine normal brain or gliomas for TUSC2 mRNA. In agreement with our observations, a recent study analyzed four normal brain and four gliomas for TUSC2 protein expression, and found normal brain to express more TUSC2 than gliomas [13]. The lack of difference in TUSC2 mRNA levels in normal brain and GBM samples was observed in our sample cohort and a GEO cohort; however, a recent study found TUSC2 mRNA levels to be higher in normal brain than gliomas [13]. Nevertheless, these observations strongly suggest that TUSC2 protein loss or reduction may play an important role in GBM development or gliomagenesis. Indeed, we found that TUSC2 is expressed in all three proposed cells of origin for GBM, namely, astrocytes,

neural stem cells, and oligodendrocytes. Since the vast majority of GBM cases present at grade IV without evidence of precancerous lesions, future work to further elucidate the direct role that TUSC2 loss plays in the malignant transformation of normal brain cells to GBM is warranted.

A previous study indicated that TUSC2 myristoylation is essential for its stability in lung cancer although the underlying mechanism was not elucidated [23]. Based on our discovery that NEDD4 mediates TUSC2 polyubiquitination leading to proteasomal degradation in GBM, it is possible that TUSC2 myristoylation prevents TUSC2 from being polyubiquitinated by NEDD4 and possibly other E3 ligases. This should be explored in a future study. Additionally, it is also possible that additional E3 ligases may target TUSC2 in GBM, and that TUSC2-targeting E3 ligases may be tumor-specific. Future efforts are warranted to explore other potential TUSC2-targeting E3 ligases.

Consistent with our findings, others have also shown that NEDD4 is overexpressed in GBM cells and patient tissues [43, 44]. Patients with NEDD4 overexpression and low TUSC2 could benefit from TUSC2 gene therapy. TUSC2 gene therapy has undergone a Phase I clinical trial with a cohort of 31 lung cancer patients demonstrating some success with 5 patients achieving stable disease [40]. In this clinical trial, the patients exhibited successful uptake of the TUSC2 plasmid with an increase in TUSC2 expression in the tumors, and determined a maximum tolerated dose of 0.06 mg/kg [40]. TUSC2 nanoparticles in combination with erlotinib, an EGFR inhibitor, is in a current Phase I/II trial for NSCLC (NCT01455389). The findings presented in this study suggest the potential utility of TUSC2 gene therapy as a treatment option for GBM. Forced TUSC2 expression from gene therapy may abrogate NEDD4 mediated TUSC2 loss in GBM, leading to increased apoptosis, decreased tumor growth, and a reduction in GBM stemness. The effectiveness of this treatment will depend largely on successful permeability across the blood-brain barrier and the blood-tumor barrier.

NEDD4-specific inhibitors have not been developed while proteasome inhibitors are promising therapy that helps restore tumor suppressor expression. In addition to TUSC2 and PTEN, other tumor suppressors, such as p53, Rb, and p27, also undergo post-translational polyubiquitination and degradation, indicating that proteasome inhibitors or E3 ligase specific inhibitors may be a promising treatment strategy [45]. Bortezamib is a selective inhibitor of the 26S proteasome that has shown efficacy against multiple myeloma and success against other solid tumors in mouse models [46, 47]. In fact, treatment of two GBM cell lines, U87 and U251, with bortezamib has shown decreased cell viability, decreased cell proliferation, and increased autophagy and apoptosis [46]. Bortezamib has also shown efficacy againtst multiple myeloma, myeloid leukemia, and renal cancer cells when used in combination with other treatments such as TRAIL/Apo2L [47]. Marizomib is a second generation 26S proteasome inhibitor that is blood-brain barrier permeable [48]. Treatment of GBM cells in 3D spheroids with marizomib in combination with IZI1551 (TRAIL-receptor agonist) has shown induction of apoptosis, demonstrating a potential use of proteasome inhibitors in combination with other therapies for GBM [48]. Furthermore, a study investigated NEDD4 and bortezamib in multiple myeloma and found that bortezamib treatment was most effective in multiple myeloma tumors with high NEDD4

[49]. suggesting that GBM tumors with high NEDD4 expressing may be more receptive to proteasome inhibition. These findings demonstrate the need for further development of NEDD4-targted therapy, as well as, future testing of proteasome inhibitors as a potential solution to negate tumor suppressor protein loss due to increased proteasomal degradation.

We observed that TUSC2 induces GBM apoptosis, which may occur through decreasing expression of Bcl-xL. This finding is consistent with previous studies reporting that TUSC2 induces mitochondrial apoptosis in lung cancer cells [12, 37, 38]. We also found that TUSC2- positive GBM cells are more sensitive to a Bcl-xL specific BH3 mimetic, A-1331852, as compared to GBM with TUSC2-KO, demonstrating a potential connection between TUSC2, Bcl-xL and apoptosis. However, the mechanisms underlying TUSC2-mediated apoptosis in GBM are likely complex. For example, previous studies have shown that re-expression of TUSC2 in lung cancer induces MDM2 downregulation and accumulation of p53 and APAF1, key factors in mitochondrial-associated apoptosis [12, 37]. In lung cancer, TUSC2 can directly interact with APAF1 and regulate its activity [15, 37]. Whether these events also occur in GBM will need to be examined in future studies, as well as the mechanism by which TUSC2 is linked to Bcl-xL downregualtion.

Our present study found that loss of TUSC2 expression resulted in global gene transcription alterations in GBM cells. Interestingly, TUSC2 re-expression has been reported to lead to alterations in the expression level of pro- and anti-tumorigenic genes in malignant mesothelioma cells [8]. Identifying TUSC2-affected genes that are shared by multiple tumor types is an important step in elucidating TUSC2 functionality that is conserved across cancer types. Furthermore, we showed that the genes upregulated in response to TUSC2-KO predict poor overall survival in patients with GBM (Fig. 7H). This is in agreement with a report showing that loss of TUSC2 protein expression is associated with poor survival in patients with lung cancer [50].

It is interesting to find that NEDD4 mRNA expression is associated with poorer overall survival in patients with gliomas in general but not GBM (Figs. 4F–H). The potential causes of the difference are likely complex. It could be attributed to NEDD4 protein levels in gliomas in general versus GBM, as well as, difference in NEDD4 E3 ligase activity in gliomas in general versus GBM. Additional explanation is the relatively small sample size of 165 for the TCGA GBM dataset (Fig. 4F) compared to larger sample sizes of 397 and 767 for gliomas (Figs. 4G and 4H). For GBM which is the most aggressive form of gliomas, additional factors in conjunction with NEDD4 may serve as a better prognostic indicator than NEDD4 mRNA alone. In support of this speculation, our data showed that in GBM, NEDD4 activity indicated by the gene signature score, is a better predictor of survival than NEDD4 mRNA levels (Fig. 7G versus Fig. 4F). As suggested by our data in Figs. 7H–L, concomitant status of NEDD4 and TUSC2 may be a better prognostic indicator for GBM disease progression and outcome than either one alone. In fact, combined NEDD4 activation signature and TUSC2-KO activation signature robustly predicts poorer survival of GBM patients (Fig. 7L; p=0.0004).

The mechanisms of TUSC2-mediated global gene transcription regulation has yet to be elucidated, and likely occur through regulating downstream transcription factors. For

example, TUSC2 has been reported to mediate accumulation of the transcription factor p53, leading to alterations in cell survival genes [12, 37]. TUSC2 also regulates calcium in the mitochondria, altering reactive oxygen species levels, which directly affects NFκB function, ultimately regulating genes involved in inflammation [16]. TUSC2 has also been connected to the EGFR pathway, which results in AKT activation and subsequent downstream activation of transcription factors, such as c-MYC [20]. Further investigation into the mechanisms of TUSC2 regulation of global gene transcription is an important future task.

Our results establish a novel mechanism of TUSC2 loss in GBM through NEDD4 mediated proteasomal degradation. We revealed that TUSC2 rescue increases survival and decreases tumor growth *in vivo*, demonstrating that TUSC2 gene therapy may be a beneficial treatment for GBM patients. TUSC2-KO RNA-Seq analysis showed that our TUSC2-KO derived gene signature is associated with worse overall patient survival, demonstrating TUSC2 loss as a potential prognostic marker in patients. Overall, we established TUSC2 as a novel tumor suppressor and prognostic indicator in GBM, and NEDD4 as a novel mediator of TUSC2 protein stability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Availability of data and materials

All data analyzed are included in this article and additional information is available upon request.

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Highlights

- TUSC2 protein is destabilized in GBM but not in its cell-of-origin astrocytes.
- NEDD4 E3 ligase polyubiquitinates TUSC2 protein at Lys71, leading to TUSC2 degradation in a proteasome-dependent manner.
- TUSC2 restoration induces apoptosis and inhibits glioma stem-like cells *in vitro*, and suppressed growth of intracranial glioma stem-like cells-derived xenografts *in vivo*.
- TUSC2-knockout inhibits apoptosis and promotes glioma stem-like cells *in vitro*, and enhanced growth of intracranial glioma stem-like cells-derived xenografts *in vivo*.
- TUSC2-knockout altered GBM transcriptome, including inducing expression of anti-apoptotic Bcl-xL; TUSC2-knockout Gene Signature predicts poor GBM patient survival.

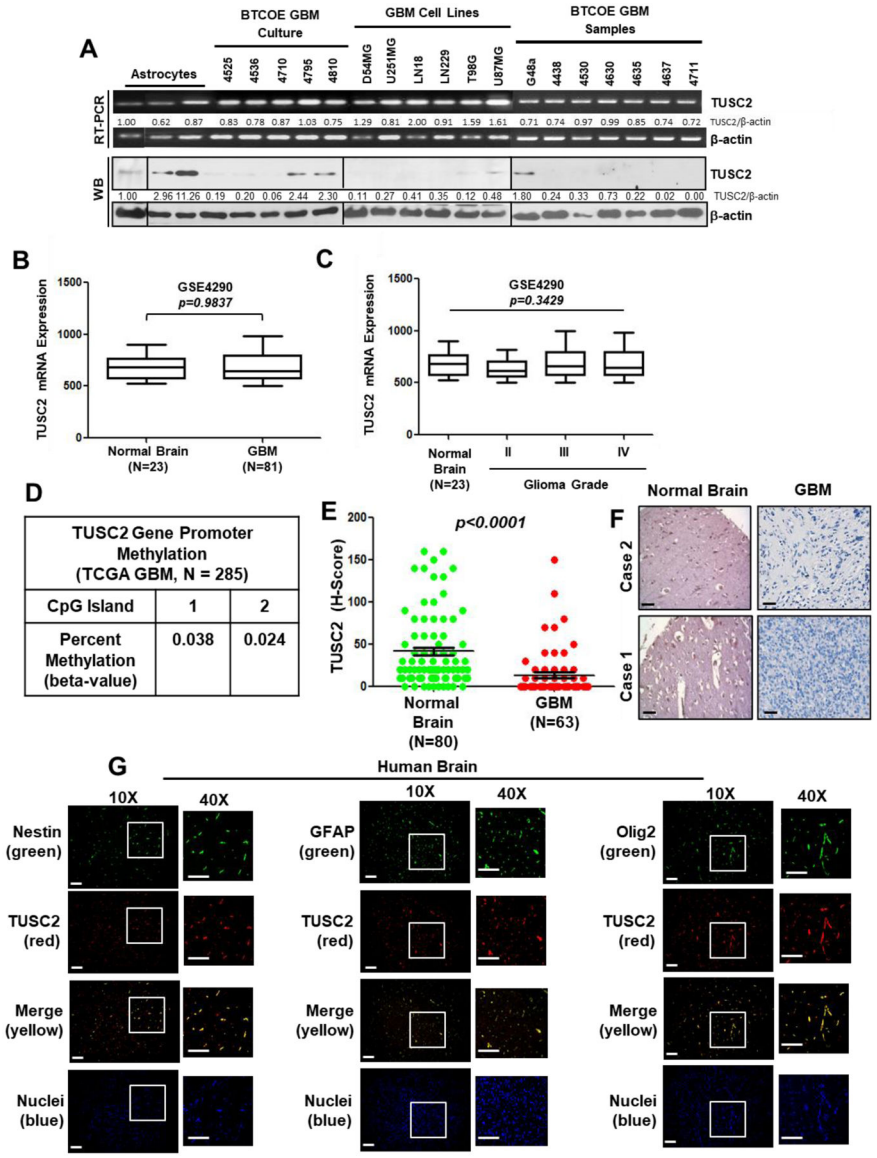

Figure 1. TUSC2 protein, but not mRNA expression, is frequently lost in GBM.

A)TUSC2 mRNA is equally expressed in astrocytes (NHA, C8-S and immortalized human astrocytes respectively) and GBM cells and patient samples (top panel; RT-PCR), but TUSC2 protein expression is significantly decreased in GBM cell lines and patient samples (lower panel; western blots/WB). Densitometric analysis was completed using ImageJ and values are placed below blot and were normalized to NHA. **B,C)** Analysis of GSE4290 dataset showed that TUSC2 mRNA was equally expressed between normal brain (N=23) and GBM (N=81), and across glioma grades (N=45, N=31, N=81 respective to grade). **D)** Human TUSC2 gene promoter is rarely methylated in GBM patient samples. TUSC2 gene promoter methylation status was retrieved from the TCGA GBM dataset. Percentage of patients with methylated TUSC2 gene promoter is shown (N=285). **E)** TUSC2 protein is highly expressed in normal brain samples (N=80), but not in GBM patient tumors (N=63), as shown by IHC. Immunostained sections were scored by a pathologist to derive H-scores.

F) Representative IHC images were taken at 10X magnification, scale bar indicates 50 μm. **G)** Normal healthy human brain tissue was subjected to immunofluorescence staining using antibodies specific to TUSC2, Nestin (neural stem cell marker), GFAP (astrocyte marker), and Olig2 (oligodendrocyte marker). Overlaid images with yellow co-staining indicate co-expression of TUSC2 and lineage-specific markers. Images were taken at 10X magnification, scale bar indicates 50 μm, increased magnificaiton images are at 40X. Data are presented as mean±SE. Student's t-test and ANOVA were used to calculate p-values.

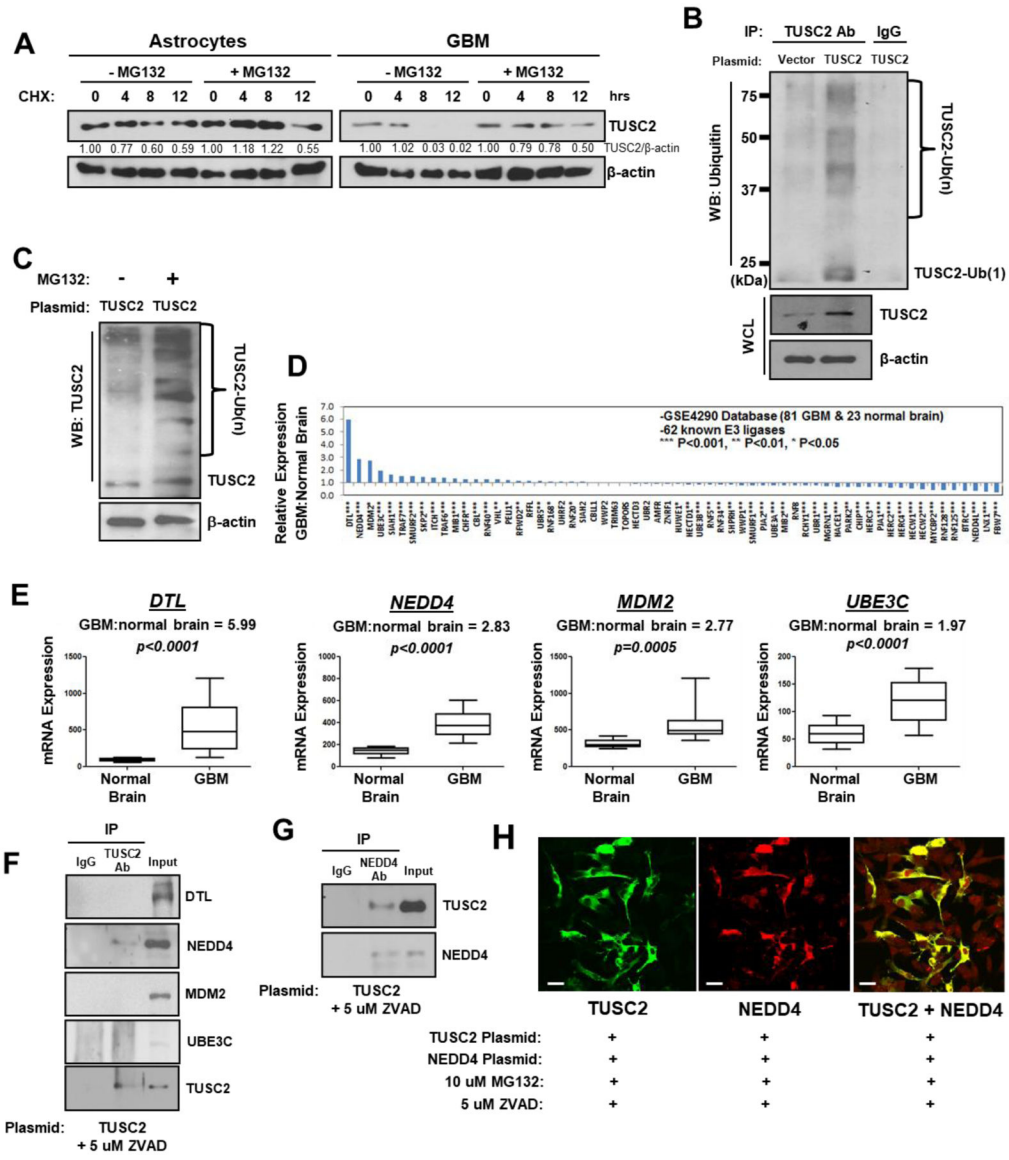

Figure 2. TUSC2 protein is destabilized in GBM via proteasome-mediated degradation; TUSC2 physically interacts with NEDD4 E3 ligase.

A) Astrocytes and G48a GBM cells were treated with 10 μg/mL cycloheximide (CHX) and either with or without proteasome inhibitor MG132 (10 μM). Densitometric analysis was completed using ImageJ and values are placed below blot. **B)** TUSC2 is polyubiquitinated in GBM cells. TUSC2 was overexpressed in TUSC2-low U251MG cells and subjected to immunoprecipitation (IP) with anti-TUSC2 antibody (Ab). IgG was used as an IP control. Resulting immunoprecipitates were analyzed by WB for ubiquitin (Ub). **C)** MG132 inhibits degradation of polyubiquitinated TUSC2. U251MG cells were treated with or without MG132 (10 μM). Resulting lysates were subjected to WB for TUSC2. **D,E)** Analysis of the GEO dataset (GSE4290) identified four E3 ubiquitin ligases overexpressed in GBM (N=81) compared to normal brain (N=23) samples ($p<0.05$; ≥2-fold). **F)** NEDD4 binds to TUSC2 in GBM cells. TUSC2 was overexpressed in U251MG cells treated with 5 μM ZVAD and subjected to IP with a TUSC2 Ab. IgG was used as control for IP. Resulting

immunoprecipitates were subjected to WB for DTL, NEDD4, MDM2, UBE3C, and TUSC2. **G)** TUSC2 is bound to NEDD4 in GBM cells. TUSC2 was overexpressed in U251MG cells treated with 5 μM ZVAD and were subjected to IP with a NEDD4 Ab and WB for TUSC2. **H)** TUSC2 and NEDD4 co-localize *in vitro*. G48a cells were transfected with TUSC2 and NEDD4 and treated with 10 μM MG132 and 5 μM ZVAD, and then subjected to immunofluorescence staining and confocal microscopy with images taken at 20X, scale bar indicates 100 μm. TUSC2 is indicated in green, NEDD4 in red. Data are presented as mean±SE. Student's t-test was used to calculate p-values.

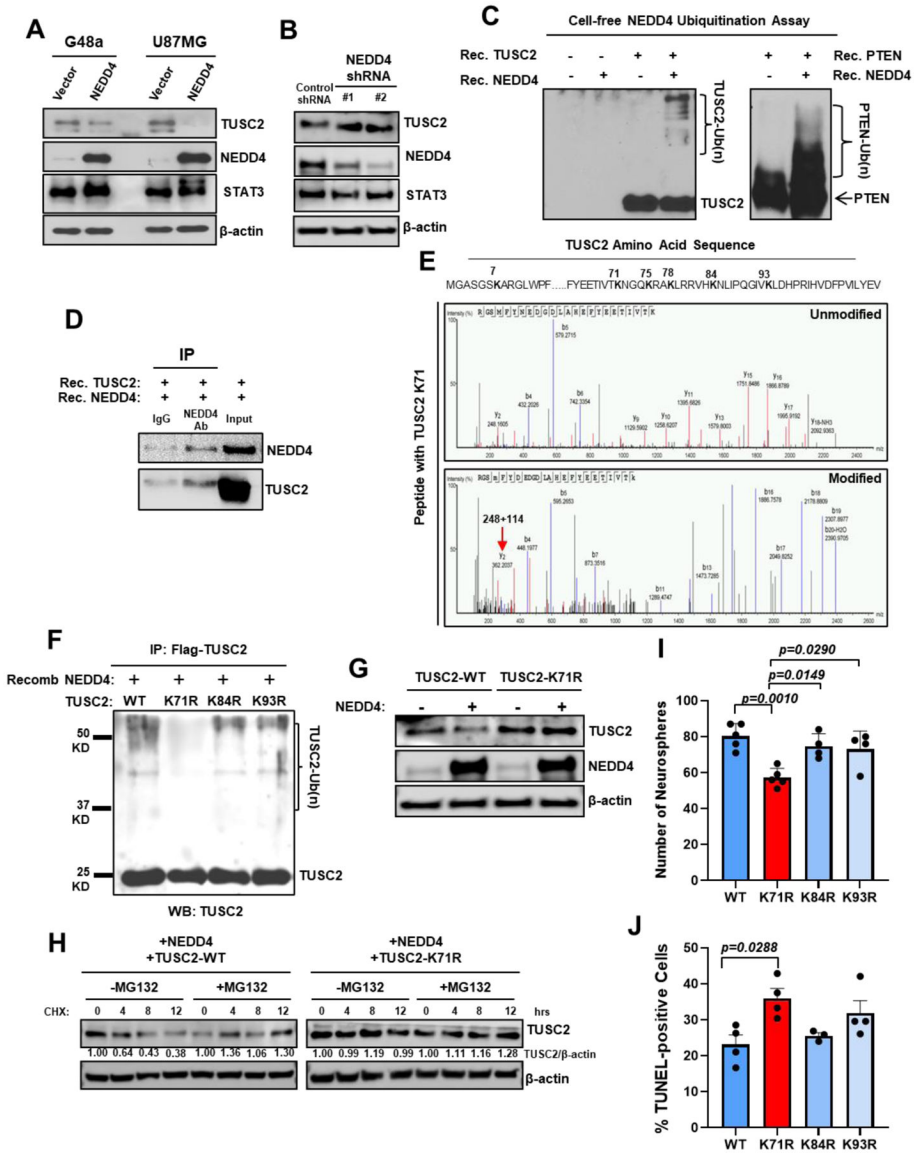

Figure 3. NEDD4 polyubiquitinates TUSC2 at K71 in GBM, leading to TUSC2 degradation.

A) NEDD4 overexpression decreased TUSC2 protein expression. NEDD4 was overexpressed in two different TUSC2-positive GBM cell lines and the resulting lysates were subjected to WB. **B)** NEDD4 knockdown by two different NEDD4 shRNAs increased TUSC2 expression in U87MG cells, as shown by WB. **C)** In a cell-free ubiquitination assay, recombinant NEDD4 ubiquitinated recombinant TUSC2. Reaction was subjected to WB with a TUSC2 Ab. PTEN was used as a positive control for NEDD4 ubiquitination activity. **D)** TUSC2 directly binds NEDD4. Following a cell-free ubiquitination assay, the product was subjected to IP with NEDD4 antibody and TUSC2 antibody for WB. **E)** TUSC2 has six lysine residues (top). Mass spectrometry showed that K71 on TUSC2 is ubiquitinated by NEDD4 (+114). Ubiquitinated and un-ubiquitinated TUSC2 products from the NEDD4 cell-free ubiquitination assay were isolated from the SDS-PAGE gel and subjected to mass spectrometry. **F)** TUSC2-K71R mutant lost the ability to be polyubiquitinated by NEDD4.

G48a-TUSC2-KO cells were transfected with wild-type Flag-TUSC2, K71R, K84R, or K93R Flag-TUSC2 mutant, and subjected to IP. Resulting immunoprecipitates were then subjected to the cell-free NEDD4 ubiquitination assay with recombinant NEDD4. **G)** TUSC2-K71R protein level was not affected by NEDD4 overexpression. U87MG cells were transfected with TUSC2 or TUSC2-K71R, and either NEDD4 or empty vector. Resulting lysates were subjected to WB. **H)** TUSC2-K71R mutant displayed increased protein stability compared to wild-type TUSC2. G48a-TUSC2-KO cells transfected with NEDD4, and either TUSC2 or TUSC2-K71R were treated with 10 μg/mL CHX, with or without proteasome inhibitor MG132 (10 μM). Densitometry was completed using ImageJ and is placed below blot. **I,J)** TUSC2 K71R decreases GBM stemness and increases apoptosis. GSC28 cells were transfected with either TUSC2-WT, K71R, K84R, and K93R mutants for 48 hours, and were reseeded for neurosphere assay (N=5 per group) or stained for TUNEL (N=4 per group). Data are presented as mean±SE. ANOVA was used to calculate p-values.

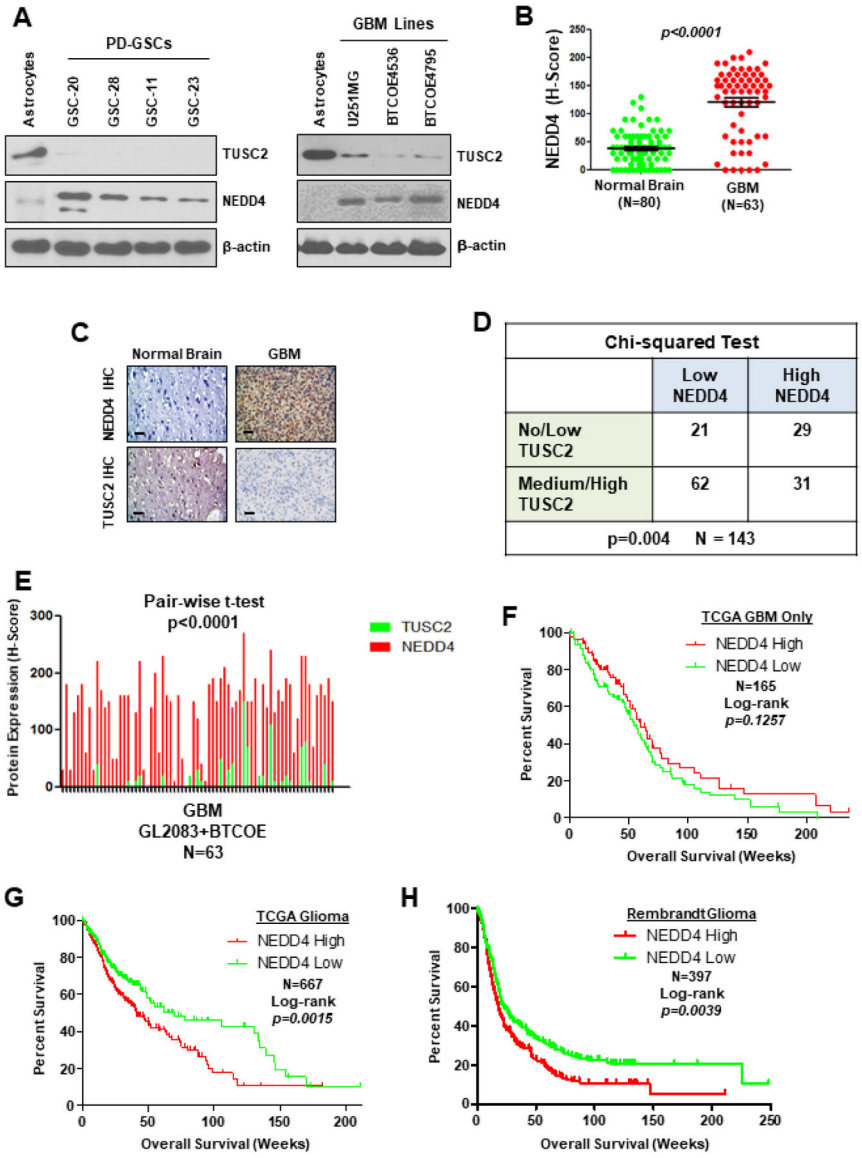

Figure 4. TUSC2 and NEDD4 proteins are inversely expressed in GBM, PD-GSCs, and normal brain samples.

A) TUSC2 and NEDD4 are inversely expressed between cultured GBM and PD-GSCs, and astrocytes, as shown by WB. **B)** NEDD4 expression was higher in GBM samples (N=63) compared to normal brain samples (N=80), as shown by IHC. Immunostained sections were scored by a pathologist to derive H-scores. Student's t-test was used to compute p-values. **C)** Representative IHC images of immunostained tissues. Images were taken at 10X magnification, scale bar indicates 50 μm. **D,E)** TUSC2 and NEDD4 are inversely expressed. In Panel D, two-by-two Chi-squared analysis of brain and GBM tissues was conducted. In Panel E, pair-wise t-test was used. **F-H)** High NEDD4 mRNA expression predicts poorer overall survival in glioma patients. Three datasets, TCGA GBM Only (N=165) (**F**), TCGA Combined Glioma (N=667) (**G**), and Rembrandt Combined Glioma (397) (**H**), were analyzed by Kaplan-Meier survival analysis based on NEDD4 expression. Mantel-Cox log-rank test was used to compute p-values. Data are presented as mean±SE.

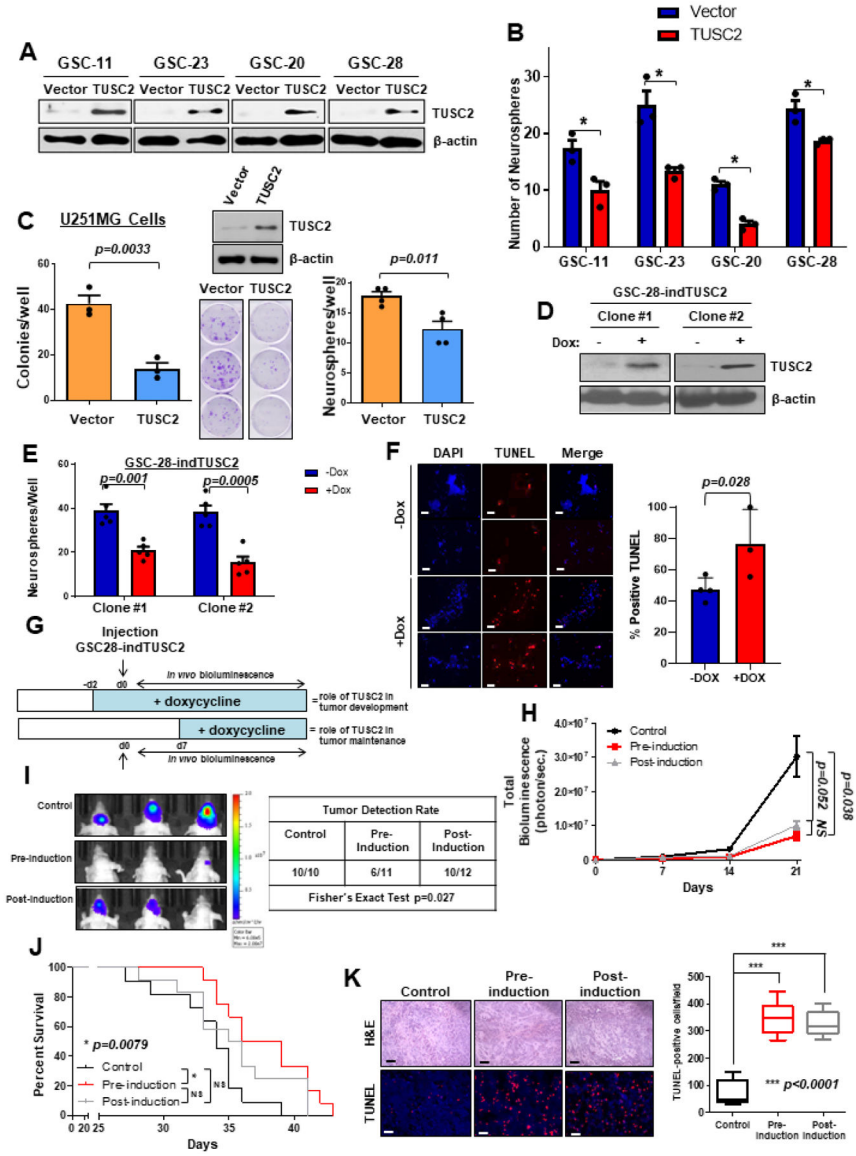

Figure 5. Restoring TUSC2 expression induces apoptosis and inhibits PD-GSCs *in vitro* and *in vivo*.

A,B) TUSC2 re-expression in PD-GSCs inhibited neurosphere formation. PD-GSCs were transfected with either TUSC2 or a control vector. TUSC2 expression was confirmed by WB (A). PD-GSCs were seeded and counted 7 days later (N=3 per group) (B). **C)** TUSC2 re-expression inhibited colony and neurosphere formation in cultured GBM cells. U251MG cells transfected with either TUSC2 or empty vector were seeded for colony formation assay (250 cells/well) (N=3 per group) or neurosphere formation assay (1000 cells/well) (N=4 per group). TUSC2 expression was confirmed by WB. **D)** Generation of PD-GSCs with doxycycline (dox)-inducible TUSC2 lentiviral vector (GSC28-indTUSC2). Two separate clones were generated and dox-induction (1 μg/mL) of TUSC2 expression was confirmed by WB. **E)** Dox-induced re-expression of TUSC2 inhibited neurosphere-forming capacity of GSC-28. GSC28-indTUSC2 cells were treated with dox (1 μg/mL) to induce TUSC2 expression, seeded for the assay, and counted after 7 days (N=4 per group). **F)** TUSC2

re-expression induced apoptosis. GSC28-indTUSC2 neurospheres were treated with dox (1 μg/mL) for 72 hours, attached to microscope slides using cytospin centrifugation, and subjected to TUNEL and DAPI staining (N=4 per group). Neurospheres were analyzed using a confocal microscope with images taken at 10X magnification, scale bar indicates 50 μm. **G)** Schema for GSC28-indTUSC2 orthotopic implantation animal experiment. Isogenic luciferase-expressing GSC28-indTUSC2 cells were injected into the right frontal lobe of female nude mice (N=10–12 per group) and tumor growth was assessed weekly via bioluminescent imaging. Pre-induction group mice started receiving water supplemented with Dox (2 mg/mL) or 5% sucrose 2 days prior to intracranial cell implantation. Post-induction group started receiving water supplemented with Dox (2 mg/mL) and 5% sucrose 7 days after intracranial implantation. **H)** TUSC2 re-expression prevented tumor development in the pre-induction group. Bioluminescent images were analyzed, and the mean total bioluminescent flux was plotted. **I)** TUSC2 re-expression reduced tumor development rates. Representative bioluminescent images of actively growing tumors at day 21. Differences in tumor detection rate were determined using 2-by-3 Fisher's exact test. **J)** TUSC2 pre-induction prolonged survival in mice bearing GSC28-indTUSC2 xenografts. Kaplan-Meier survival curves with Mantel-Cox log-rank test used to determine p-values. **K)** TUSC2-expressing xenografts displayed significantly increased apoptosis as shown by TUNEL staining with corresponding H&E. Images were taken at 10X magnification, scale bar indicates 50 μm. Representative xenografts (N=5 per group) from each group were analyzed for apoptosis. ANOVA was used to compute p-values.

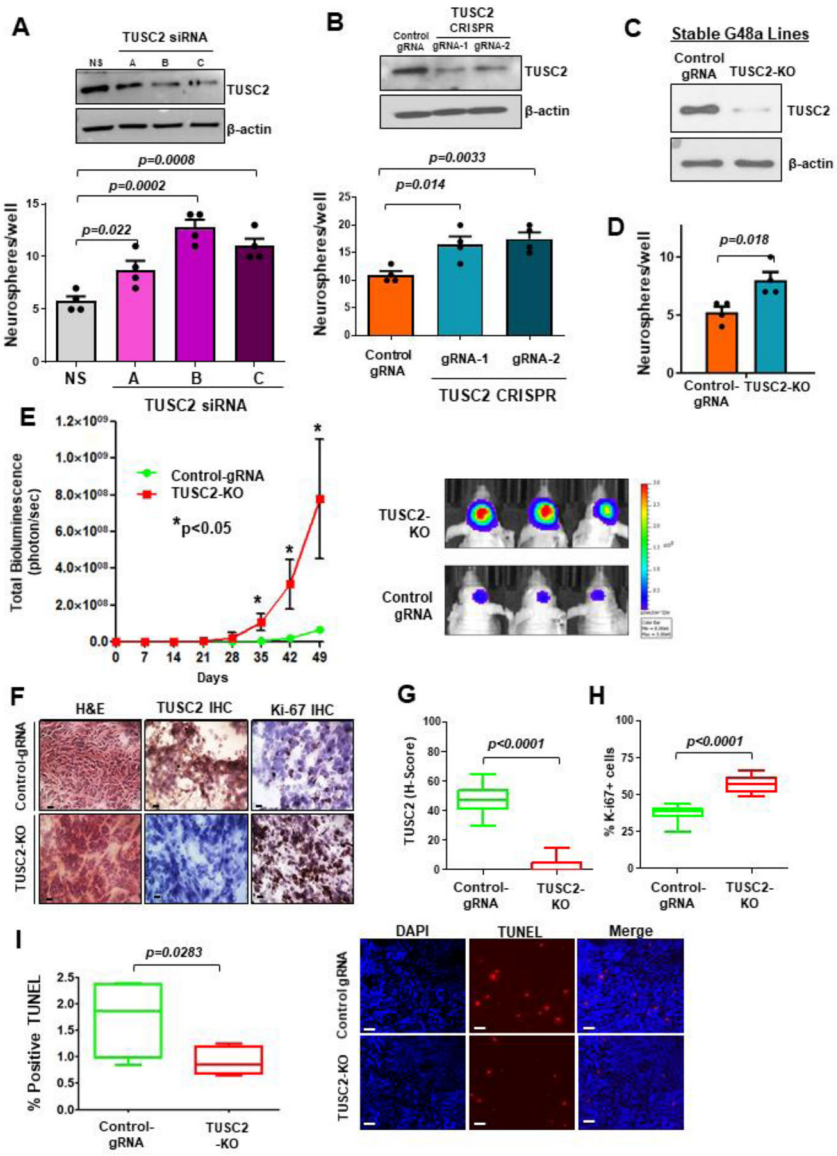

Figure 6. Loss of TUSC2 expression promotes GBM aggressiveness *in vitro* and *in vivo*.

A,B) TUSC2 knockdown promotes neurosphere formation in GBM cells. TUSC2-positive G48a cells transfected with three different TUSC2-targeting siRNAs (N=4 per group) (A) or two unique TUSC2 CRISPR/Cas9 guide RNA (gRNA) constructs (N=4 per group) (B) were subjected to a neurosphere assay. Results for WB (top panel) and neurosphere assay (bottom panels) are shown. **C)** Isogenic G48a GBM cells carrying lentiviral control CRISPR/Cas9 gRNA or TUSC2-targeting gRNA (TUSC2-KO) were subjected to WB. **D)** Stable G48a-TUSC2-KO cells displayed increased neurosphere-forming ability (N=4 per group). **E)** TUSC2-KO promoted GBM orthotopic xenografts growth. Isogenic luciferase-expressing G48a-TUSC2-KO and G48a-Control-gRNA cells were injected into the right frontal lobe of female nude mice (N=9 per group) and tumor growth was assessed weekly via bioluminescent imaging. Representative images of actively growing tumors at Day 56 are shown. **F-H)** TUSC2-KO xenografts were more proliferative than the

control xenografts. Representative xenografts (N=5 per group) were subjected to H&E staining and IHC for TUSC2 and Ki-67. Images taken at 20x magnification; scale bar indicates 100 μm. Immunostained sections were scored by a pathologist and H-scores were calculated. **I)** Xenografts were examined for apoptosis using TUNEL staining (N=3 per group). Xenografts with Control-gRNA, still containing TUSC2, contained higher positive TUNEL signal as compared to the TUSC2-KO xenograft samples, as seen in the graph and representative images on the right. Images were taken at 10X magnification, scale bar indicates 50 μm. Student's t-test and was used to compute p-values.

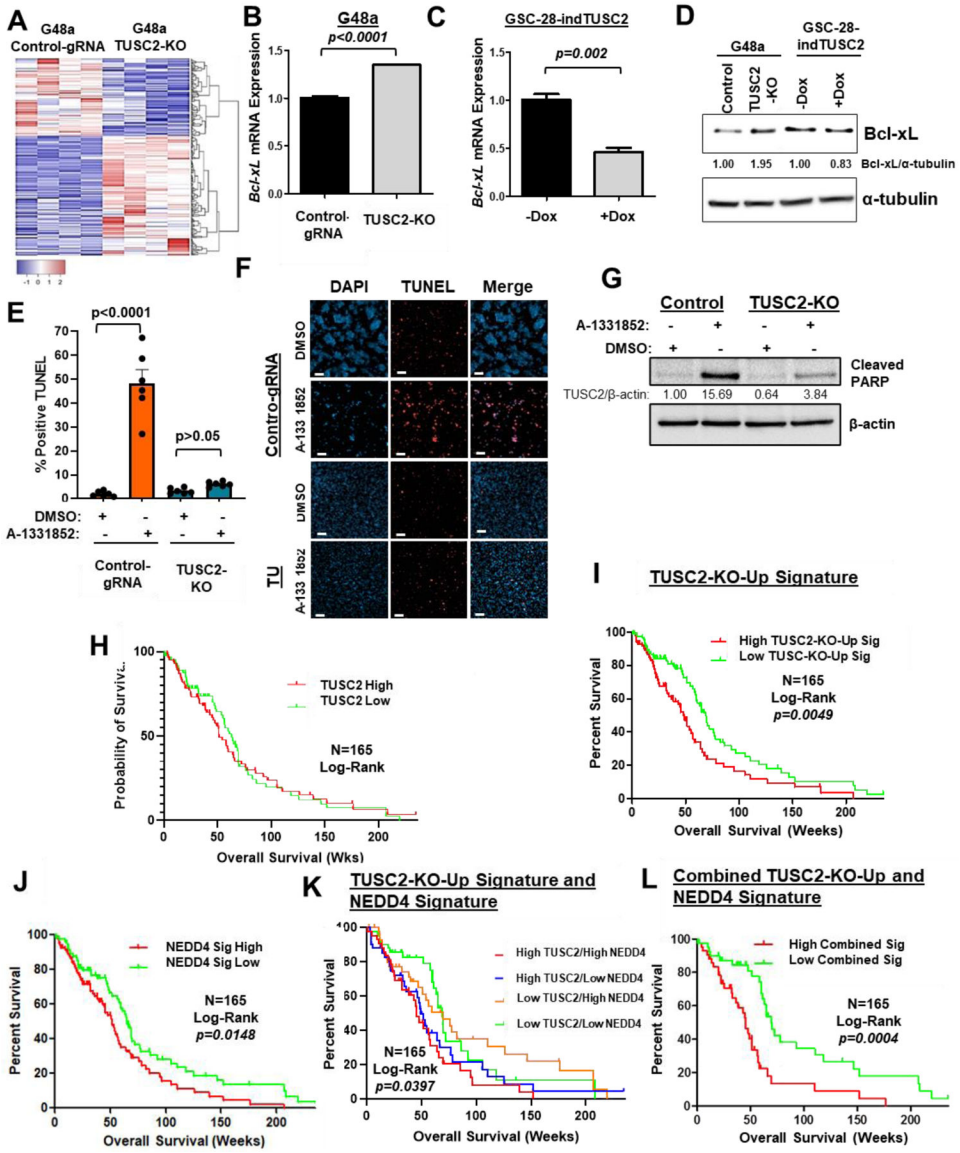

Figure 7. TUSC2 suppresses GBM growth by modulating cellular apoptotic machinery.

A) RNA-Seq analysis of G48a-TUSC2-KO and G48a-Control-gRNA cells. Heatmap shows per-sample expression values of differentially expressed genes (N=4 per group; $p<0.05$). **B-D)** Bcl-xL was validated as a TUSC2-modulated gene, as shown by RT-qPCR (B, C) (N=3 per group) and WB (D) in two cell lines. **E-G)** TUSC2-KO cells are resistant to A-1331852 treatment. G48a-Control-gRNA and G48a-TUSC2 KO cells were treated with vehicle or 1 uM of A-1331852 for 48 hours and were then subjected to TUNEL and WB (N=6 per group). Representative TUNEL images were taken at 10X magnification, scale bars indicate 75 μm (F). Corresponding WB probing for PARP (G). Densitometric analysis was completed using ImageJ and values are placed below blot. **H)** TUSC2 mRNA is not predictive of GBM patient survival (TCGA dataset) (N=165). **I)** The NEDD4 gene signature is predictive of overall worse survival in GBM patients (TCGA dataset) (N=165) with high NEDD4 signature patients having worse overall survival. **J)** The TUSC2-KO-Up

gene signature is a prognostic indicator for poor overall survival in GBM patients (TCGA dataset) (N=165), with high TUSC2-KO-Up gene signature patients having overall worse survival. **K)** Patients with high NEDD4 and high TUSC2-KO-Up Gene Signatures had the worst overall survival rate (N=165). **L)** The NEDD4 and TUSC2-KO-Up gene signatures were combined and patients with high expression of the combination signature had a worse overall survival (N=165). Kaplan-Meier survival curves were plotted with data from the TCGA GBM dataset. Student's t-test, and Mantel-Cox log-rank test was used to calculate p-value.