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Doctor of Philosophy

ZBTB7A-deficiency promotes tumorigenesis of
glioblastoma by transcriptionally activating EPB41L5

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ZBTB7A-deficiency promotes tumorigenesis of
glioblastoma by transcriptionally activating EPB41L5

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ZBTB7A-deficiency promotes tumorigenesis of glioblastoma by transcriptionally activating EPB41L5

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Abstract

Glioblastoma multiforme (GBM), the most aggressive and malignant glioma, has a poor prognosis. Although patients with GBM are treated with surgery, chemotherapy, and radiation therapy, GBM is highly resistant to treatment, difficult to treat, and expensive. In this study, we analyzed the Gene Expression Profiling Interactive Analysis dataset, the Cancer Genome Atlas dataset, and the Gene Expression Omnibus array data. ZBTB7A (also called FBI1/POKEMON/LRF) was found to be highly expressed in low-grade GBM, but significantly reduced in patients with GBM. ZBTB7A is a transcription factor that plays an important role in many developmental stages, including cell proliferation. The activation of epithelial-mesenchymal transition (EMT) in cancer is a key process in cancer progression and metastasis. Erythrocyte membrane protein band 4.1 Like 5 (EPB41L5) is an essential protein for EMT progression and metastasis in various types of cancer. We found that ZBTB7A-depleted U87 cells exhibited GBM progression and metastasis. Based on RNA sequencing data, ZBTB7A directly binds to the promoter of the EPB41L5 gene, reducing its expression and inhibiting GBM progression. We demonstrated that ZBTB7A dramatically inhibited GBM tumor growth through transcriptional repression of EPB41L5. Thus, both ZBTB7A and EPB41L5 may be potential biomarkers and novel therapeutic targets for GBM treatment. Taken together, we discovered the role of a novel tumor suppressor, ZBTB7A, that directly inhibits GBM progression, and EPB41L5 is a therapeutic target protein for patients with GBM and an essential protein for the development of GBM therapeutics.

Keywords: ZBTB7A, EPB41L5, Glioblastoma, Tumorigenesis, EMT

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Introduction

The most common and fatal primary tumor is glioblastoma multiforme (GBM), the highest grade (IV) human brain tumor¹⁻⁴. Despite surgical interventions such as chemotherapy and radiation therapy, GBM is now resistant to all treatments and is a fatal type of brain tumor with a 14–16 months survival rate⁵⁻⁹. The development of resistance to standard treatment regimens is a major cause of low survival¹⁰⁻¹², and because GBM is highly invasive and aggressive, the patient's prognosis is poor, despite various drug treatment options^{13,14}. According to cohort molecular profiling studies, genetic abnormalities frequently occur in specific gene sets such as PTEN, TP53, and IDH1 in patients with GBM¹⁵. Thus, survival or guide treatment decisions of patients with GBM are difficult to predict¹⁶, suggesting that there are other factors contributing to aberrant gene expression because of the molecular heterogeneity of tumor tissue. Therefore, the development of novel therapeutic strategies to increase the survival rate of GBM requires the analysis of gene, protein, and molecular mechanisms through gene profiling, and it is critical to identify key GBM diagnostic markers.

ZBTB7A, also known as FBI1, POKEMON, or LRF, is a member of the POK family and a pleiotropic transcription factor containing zinc fingers and protein 7A¹⁷⁻¹⁹. Transcription factors of the POK family act as transcription activators or repressors by specifically binding to DNA elements located on the DNA-binding domain of target genes²⁰, and can also repress transcription by recruiting corepressor complexes²¹. In addition, overexpression of ZBTB7A is associated with tumorigenesis and metastasis in various human cancers²². For example, ZBTB7A inhibits transcription by directly binding to GLUT3, PFKF, and PKM, all of which are important factors in glycolysis, as well as the promoter of the androgen receptor gene in prostate cancer^{20,23-26}. In addition, ZBTB7A acts as a tumor suppressor; the loss of ZBTB7A in a PTEN-deficient environment promotes tumor growth in mouse prostate cancer²⁴. However, the mechanisms of gene regulation by ZBTB7A in GBM cells remain unknown.

EPB41L5 (Erythrocyte membrane protein band 4.1 Like 5) is a mesenchymal-specific protein induced during EMT (Epithelial-mesenchymal transition) that promotes the disruption of cell-cell adhesion kinetics²⁷. Overexpression of EPB41L5 in epithelial cells causes ZO-1 disorganization as it destabilizes E-cadherin and affects the tightness of cell junctions^{28,29}.

EPB41L5 is highly expressed in gastric, kidney, and breast cancers and promotes invasion, metastasis, and EMT³⁰⁻³³. It is also considered a key factor in the metastasis of various types of cancer by promoting EMT, proliferation, migration, and invasion in esophageal squamous cell carcinoma²⁷. However, the role and function of EPB41L5 in GBM remain unclear.

In this study, we confirmed that the expression of ZBTB7A was very low in GBM through the analysis of The Cancer Genome Atlas (TCGA) dataset, and the decreased expression of ZBTB7A was correlated with lower survival rates of GBM. Interestingly, ZBTB7A is associated with cell migration and upregulation of ZBTB7A significantly reduced migration, invasion, and proliferation in GBM. It was found to ZBTB7A negatively regulates GBM tumor progression by directly binding to the promoter of *EPB41L5*, an important gene involved in cell mobility, and repressing its transcription. Our study revealed a novel tumor suppressor role for ZBTB7A, which directly inhibits GBM tumorigenesis. GBM was brought to power by ZBTB7A, helping achieve high-performing visual effects. Our findings revealed a novel tumor suppressor role for ZBTB7A, which directly inhibits the tumorigenesis of GBM. In addition, we discovered that EPB41L5 is a key marker of GBM tumorigenesis, which is regulated by the transcriptional repression of ZBTB7A. Therefore, we suggest that the co-regulation of ZBTB7A and EPB41L5 is an essential therapeutic strategy for treating GBM.

Materials and methods

1. Cells, plasmids, and reagents

Human A172, LN18, LN229, U118, U343, U373, and U87 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Biowest, L0103) supplemented with 10% fetal bovine serum (FBS; Biowest, S1480) and 1% antibiotics (Hyclone, SV30010). All cells were cultured at 37 °C in a 5% CO₂ incubator.

Effectene transfection reagent was purchased from QIAGEN (301427). The Myc-tagged ZBTB7A constructs were subcloned into the pSG5 vector. The pCDH-CMV-MCS-EF1-Puro overexpression vector was purchased from System Biosciences (CD510B-1).

The shRNAs were obtained from Mission-shRNA (Sigma-Aldrich). Stably overexpressed LN229, U343, and knockdown U87 cells were cultured under puromycin selection (1.5 µg/mL). The shRNA clone IDs are listed in Table 1.

2. In vitro migration, invasion, colony-forming, and proliferation assay

After stably overexpressed LN229, U343, and knockdown U87 cells reached approximately 70% confluence, cells were wounded with a 200 µL pipette tip, washed with DPBS, and incubated at 37°C. Wound healing assay was observed for 12 h and 24 h at the scraping line. Wound healing was observed for 12 h and 24 h, and the area around the scratched line was photographed.

Invasion assay was performed using an 8.0 µm pore polycarbonate membrane insert (Corning, 353097). Transwell inserts coated with Matrigel (Corning, 354234) were used, and 2×10^4 cells/well were plated in the upper chamber. The lower chamber was filled with 600 µL of serum-free medium. After incubation for 48 h, cells infiltrated with 100% methanol were fixed. After staining with 1% crystal violet (Sigma-Aldrich, V5265), the infiltrated cells were observed under a microscope.

For the colony-forming assay, 1×10^3 cells were plated in 6-well plates. After culturing the cells at 37°C for 2 weeks, colonies formed on each of the three plates were measured. Cell proliferation was assessed according to the manufacturer's protocol (Invitrogen, C35011) using the CyQuant Direct Cell Proliferation Assay Kit. All experiments were performed in

triplicate and cell proliferation was determined using a VICTOR™ X3 Multilabel Plate Reader (PerkinElmer).

3. Western blot analysis

Cells were harvested by washing and scraping with cold phosphate-buffered saline (PBS) for western blotting. The cells were lysed with lysis buffer for 30 min on ice. Lysates were centrifuged at 13,000 rpm for 20 min at 4 °C, and the protein concentration of the supernatant was measured using Pierce 660 nm Protein Assay Reagent (Thermo, 22660).

Total cell lysate proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose membranes.

The transferred membranes were blocked by incubation for 1.5 h in 5% w/v non-fat Difco skim milk (BD Biosciences, 232100) blocking buffer.

The primary antibodies were incubated together for 3 h or overnight at 4 °C. After washing, the secondary antibody was incubated for 1.5 h.

Results were visualized by the developer after western blot analysis. The antibodies used were anti-ZBTB7A (Abcam, ab70208), anti-EPB41L5 (Thermo, PA5-58009), anti-β-actin (Sigma-Aldrich, A5441), anti-Myc (MBL, M192-3), anti-N-cadherin (sc-59987), anti-β-catenin (sc-7963), anti-Vimentin (sc-32322) (Santa Cruz Biotechnology), and anti-Snail (Cell Signaling, 3879S). All secondary antibodies were purchased from Thermo (31430 and 31460).

4. RNA extraction and quantitative polymerase chain reaction

Total RNA was isolated using an AccuPrep Universal RNA Extraction Kit (BIONEER, K-3141) according to the manufacturer's instructions. Total RNA was converted to cDNA by cDNA synthesis using the PrimeScript RT Reagent Kit (Takara, 2680A).

For real-time quantitative polymerase chain reaction (qPCR), primer sequences used for the *ZBTB7A*, *EPB41L5*, *APOE*, *MDK*, *MMP9*, *IL27RA*, *SEMA3A*, *FGF7*, *IL1B*, *PAK3*, *ADAM18*, *SERPINF1*, *TGFB2*, *HYAL1*, *THBS1*, *IL33*, *ITGA2*, *ACVRL1*, *CXCL8*, *S100A4*, *TMEM100*, *DACT3*, *HGF*, *GLIPR2*, *SNAI1*, *EFNA1*, *POLR1B*, *CDH2* (N-cadherin), *CTNNB1* (β-catenin), *VIM* (Vimentin), and *GAPDH* genes are listed in Table 1.

qPCR was performed using a real-time PCR kit (ELPIS Bio, EBT-1801), and all samples were normalized using $\Delta\Delta C_t$ method. All expression values are expressed as fold change, and the reaction was repeated three times.

5. Luciferase reporter gene assay

The human *EPB41L5* gene promoter was prepared and purified by PCR amplification of human genomic DNA from HEK293T cells for a specific region (-900 to 100 nucleotides) of the *EPB41L5* gene. It was cloned into the pGL4.21 (*luc2P/Puro*) vector (Promega, E6761) using *KpnI* and *XhoI* restriction enzymes.

Luciferase analysis of the *EPB41L5* reporter gene and β -galactosidase gene was transiently transfected with 100 ng and 400 ng per well, respectively. After incubation for 24 h, the luciferase assay system (Promega, E1501) was used according to the manufacturer's instructions. The colorimetric substrate ortho-nitrophenyl-galactoside was used to measure β -galactosidase activity at 405 nm absorbance. Normalization was expressed as luciferase activity/ β -galactosidase activity (fold change).

6. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed on U343 cells, stably overexpressed U343 cells and ZBTB7A-knockdown U87 cells using the Pierce Magnetic ChIP Kit (Thermo, 26157). Nuclear lysates were precipitated with anti-ZBTB7A, anti-Myc, normal rabbit (sc-2027), and mouse (sc-2025) IgG (Santa Cruz Biotechnology) overnight at 4 °C with A/G magnetic beads. DNA obtained following the manufacturer's instructions was analyzed using qPCR. The ChIP primers (-833/-649, -314/-177, -202/-58, and -130/-56) used for qPCR are shown in Table 1.

7. Animal studies

At the University of Ulsan College of Medicine, all mouse experiments were performed under an approved protocol (2020-12-353). This study was conducted in accordance with the guidelines of the International Animal Care and Use Committee guidelines.

Briefly, stably overexpressed U343 (5×10^5) cells were collected and injected subcutaneously into the flank of female BALB/c-nu mice. After 5 weeks, all mice were sacrificed.

Tumor volume was measured every 2–3 days and calculated using the formula $V = (L \times W^2)/2$ (L; longer, W; width). Subsequently, the tumor was excised, weighed, and photographed.

The cells used for the orthotopic xenograft mouse model were stable knockdown U87 (1×10^6) cells, which were injected into the right hemisphere. After 4 weeks, all mice were sacrificed. For all animal experiments, mice were randomly allocated to each experimental group.

8. Immunohistochemistry analysis

The immunohistochemistry (IHC) detection kit was purchased from Abcam (ab64264). Experiments were performed according to the manufacturer's instructions. Anti-ZBTB7A, anti-EPB41L5, and Ki67 (Abcam, ab16667) were diluted 1:300.

Images were analyzed using ImageJ software (Java 1.8.0_112, NIH) and an IHC profiler (special plug-in).

9. Analysis of dataset for human samples and cell lines

Various tumor/normal differential expression data were obtained from the Gene Expression Profiling Interactive Analysis (GEPIA) database (<http://gepia.cancer-pku.cn/index.html>). Data on human TCGA-GBM (n = 538), TCGA-GBMLGG (n = 667), overall survival, and correlation were obtained from the GlioVis database (<http://gliovis.bioinfo.cnio.es/>).

Affymetrix Human Genome U133A Array (GPL96) CEL files were obtained from the Freije dataset from Gene Expression Omnibus (GEO) with accession number GSE4412. The Affymetrix ID used was 213299_at.

Human glioma cancer cell line (n = 59) expression data were obtained from the Cancer Cell Line Encyclopedia (CCLE) database (<https://sites.broadinstitute.org/ccle/>).

The target gene prediction data bound by ZBTB7A were obtained from the ChIP-Atlas database (<https://chip-atlas.org/>).

The predicted ZBTB7A binding motif sequence and the promoter sequence of *EPB41L5* were analyzed using JASPAR (<https://jaspar.genereg.net/>) and the Eukaryotic Promoter Database (EPD; <https://epd.epfl.ch//index.php>).

10. mRNA-sequencing analysis

Total RNA was isolated using Trizol reagent (Invitrogen, 15596018), and RNA quality and quantification were performed using an Agilent 2100 bioanalyzer (Agilent Technologies) and an ND-2000 Spectrophotometer (Thermo).

Library preparation was performed using the NEBNext Ultra II Directional RNA-Seq Kit (NEW ENGLAND BioLabs, E7760L). Using a Poly(A) RNA Selection Kit, mRNA was extracted (LEXOGEN, 157.96) and used according to the manufacturer's instructions for cDNA synthesis and shearing. Indexing was performed using Illumina indices 1–12 and was enriched by PCR.

The mean fragment sizes were evaluated, and libraries were checked using a TapeStation HS D1000 Screen Tape (Agilent Technologies). Quantification was performed using the StepOne Real-Time PCR System (Life Technologies), and high-throughput sequencing was performed using NovaSeq 6000 (Illumina).

FastQC was used to control the quality of the raw sequencing data, and adapters and low-quality reads (<Q20) were removed using FASTX_Trimmer and BBDMap. TopHat was used to map the trimmed reads to the reference genome, and fragments per kilobase per million read (FPKM) values were used to estimate gene expression levels. FPKM values were normalized using EdgeR in R (R Development Core Team).

Data mining and graphical visualization were performed using Microsoft Excel (Office 365), GSEA (GSEA_4.1.0, Broad Institute), and ExDEGA (Ebiogen).

11. Statistical analysis

Statistical significance was determined using Student's *t*-test or one-way ANOVA. Prism (Prism 8.0.1, GraphPad) and Excel (Microsoft) were used. All values are reported as the mean \pm SD. *p*-value < 0.05 were considered significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

12. Availability of data and materials

The raw mRNA-seq data reported in this document has been deposited with GEO under accession number GSE196320, available at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196320>.

Table 1. List of primer for shRNA, qPCR, ChIP

shRNA		
shZBTB7A#1	Sigma-Aldrich	TRCN0000137332
shZBTB7A#2	Sigma-Aldrich	TRCN0000136851
shZBTB7A#3	Sigma-Aldrich	TRCN0000137998
shZBTB7A#4	Sigma-Aldrich	TRCN0000138671
shZBTB7A#5	Sigma-Aldrich	TRCN0000138519
shZBTB7A#6	Sigma-Aldrich	TRCN0000138716

ChIP	
-833/-649	F : AATCGCAAACCCTCACTGCC R : ACGAGATACGACAGACAACCA
-314/-177	F : GAATCTAGCAGCCAGCTCCA R : TCCGCGGCTGGTTAAAGG
-202/-58	F : CAGCGCCCTTTAAACCAG R : GAGCGCTCCGCTTTAAAC
-130/-56	F : CCGGTTTCTCTCCAGTCGC R : TAGACGCCGAGAAATGGCG

qPCR	
ZBTB7A	F : CATCTGCGAGAAGGTCATCCA R : AGGTCGTAGTTGTGGGCAAAG
EPB41L5	F : GAAAGAAGGCCAGCAACG R : AGATCTCATCCCCAAGCCT
APOE	F : GTTGCTGGTCACATTCTGG R : GCAGGTAATCCAAAAGCGAC
MDK	F : CGCGGTCGCCAAAAGAAAG R : TACTTGCACTCGGCTCCAAAC
MMP9	F : TGTACCGCTATGTTACACTCG R : GGCAGGGACAGTTGCTTCT
IL27RA	F : GACCCTTGGGCGACTTGAA R : TCTGGAGGTGTAACCTGGAGG
SEMA3A	F : GTGCCAAGGCTGAAATTATCCT R : CCCACTTGCACTTCTCTTCT
FGF7	F : TCCTGCCAATTTGCTCTACA R : CAGGGCTGGAACAGTTCACAT
IL1B	F : ATGATGGCTTATTACAGTGCCAA R : GTCGGAGATTCTAGCTGGA
PAK3	F : CCAGGCTTCGCTCTATCTTCC R : TCAAACCCCATGAATCGTATG
ADAM18	F : GTCACAGTCCACGGAAGATT R : CAGCATATCCTTGTAATGGCA
SERPINF1	F : TTCAAAGTCCCGTGAACAAG R : GAGAGCCCGTGAATGATGG
TGFB2	F : CAGCACACTCGATATGGACCA R : CCTCGGGCTCAGGATAGTCT
HYAL1	F : CGATATGGCCCAAGGCTTTAG R : ACCACATCGAAGACACTGACAT
THBS1	F : AGACTCCGCATCGCAAAGG R : TCACCACGTTGTTGCAAGGG
IL33	F : GTGACGGTGTGATGGTAAGAT R : AGCTCCACAGAGTTCCTTG
ITGA2	F : CCTACAATGTTGGTCTCCCGA R : AGTAACCAGTTGCCTTTGGATT
ACVRL1	F : CGAGGGATGAACAGTCTGG R : GTCATGCTGAGGCGATGAAG
CXCL8	F : TTTTGCCAAGGAGTGCTAAAGA R : AACCTCTGCACCCAGTTTTC
S100A4	F : TTCTGGTTTGATCCTGACTGCT R : TGTCCCTGTTGCTGTCCAAG
TMEM100	F : TGCTGTGGTTGCTTTCATCG R : CTCTCCCGTCTCTGGCTTTC
DACT3	F : CCCAGCGTCTGCTGCTTTA R : CGATTCGCTCTCCCGTAAC
HGF	F : ACCCTGGTGTTCACAAGCA R : GCAAGAATTTGTGCCGGTGT
GLIPR2	F : ATGGGCAAGTCAGCTTCCAAA R : GCCTCAGAATACTGTTGAGCCT
SNAI1	F : ACCCTGGTGTTCACAAGCA R : GCAAGAATTTGTGCCGGTGT
EFNA1	F : TCAGGCCATGACAATCCAC R : GTGACCGATGCTATGTAGAACC
POLR1B	F : TCCGAATGTTGATTATGCCTCG R : TGACAGCGGAATGTTCTTCCC
CDH2	F : CCATCACTCGGCTTAATGGT R : GATGATGATGCAGAGCAGGA
CTNNB1	F : AGCTTCCAGACACGCTATCAT R : CGGTACAACGAGCTGTTTCTAC
VIM	F : CCCTCACCTGTGAAGTGAT R : TCCAGCAGCTTCCGTAGGT

Results

ZBTB7A expression was decreased in GBM cells and patients

We investigated the RNA expression of *ZBTB7A* in GBM patients using various databases. Profiling of various tumor samples and normal brain (NB) tissues from the TCGA and the Genotype-Tissue Expression (GTEx) projects in GEPIA confirmed that *ZBTB7A* expression was lower in GBM and lower-grade glioma tissues than in NB tissues (Fig. 1A–C). Analysis of TCGA-GBM and TCGA-GBMLGG datasets using the GlioVis database revealed that *ZBTB7A* expression was significantly decreased in GBM, and it was confirmed that the expression of *ZBTB7A* was decreased in high-grade tumors. (Fig. 1D, E). In addition, GEO profiling revealed that the expression of *ZBTB7A* in the Freije dataset (GSE4412) was decreased in GBM, as was observed in the TCGA-GBMLGG dataset (Fig. 1F). Furthermore, the prognosis of patients with GBM was poor in the GBM patients with *ZBTB7A* downregulation (Fig. 1G) according to Kaplan–Meier survival analysis based on the TCGA-GBMLGG dataset.

To determine whether the expression of *ZBTB7A* was decreased in GBM tissues compared to NB tissues based on the results of the analysis of various databases, we performed IHC using tissue microarrays (TMAs). The expression of *ZBTB7A* was significantly decreased in GBM tissues (Fig. 2A). The expression of *ZBTB7A* was confirmed using the CCLE database before the in vitro experiment, and *ZBTB7A* expression was found to be relatively low in LN229, LN18, A172, and U343 cells and relatively high in U118 and U87 cells (Fig. 2B). In addition, when the CCLE database was used to analyze *ZBTB7A* protein expression in the GBM cell line, nearly identical results were obtained (Fig. 2C).

Based on these results, the expression of *ZBTB7A* is decreased in both GBM cell lines and GBM patients, and decreased expression of *ZBTB7A* at the mRNA and protein levels indicates a poorer prognosis.

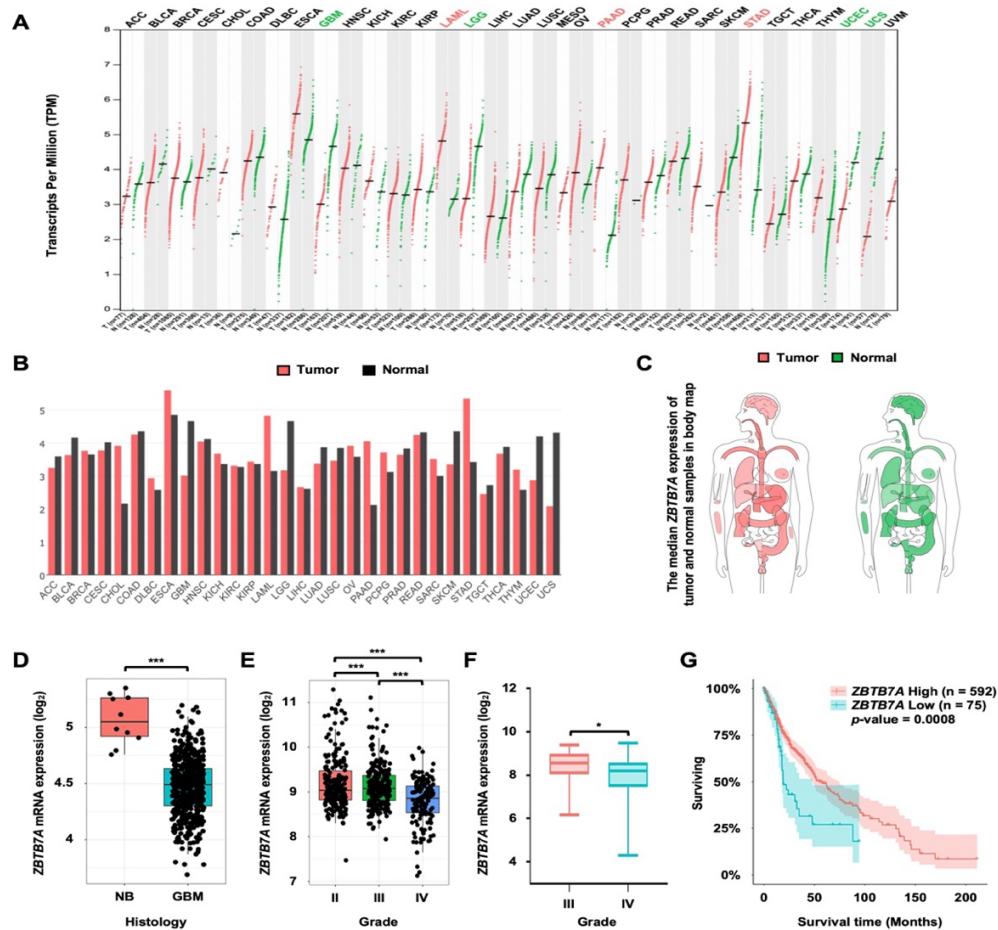


Fig. 1 ZBTB7A expression was decreased in GBM patients

A, B *ZBTB7A* expression in cancers. *ZBTB7A* expression profiles across tumor samples compared to NB samples for 33 TCGA tumor types using GEPIA. The mean $\log_2(\text{TPM}+1)$ was used as the signature score. Each point on the dot plot represents the sample, and the height of the bars in the bar plot represents the median expression of a specific tumor type or NB. **C** Median *ZBTB7A* expression in tumor and NB samples in the interactive bodymap (GEPIA). **D** mRNA expression of human *ZBTB7A* in NB and GBM samples from TCGA-GBM (GlioVis). NB, $n = 10$; GBM, $n = 528$. NB – GBM, $p = 3.1\text{E-}13$. **E** mRNA expression levels of *ZBTB7A* in gliomas grades II, III, and IV in TCGA-GBMLGG (GlioVis). Grade II, $n = 226$; III, $n = 224$; IV, $n = 150$. II – III, $p = 6.4\text{E-}01$; II – IV, $p = 7.8\text{E-}12$; III – IV, $p = 4.4\text{E-}09$. **F** mRNA *ZBTB7A* expression analysis using Freije dataset (GEO; GSE4412). Grade III, $n = 26$; IV, $n = 59$. **G** Kaplan–Meier curves representing the survival of patients with low versus high expression of *ZBTB7A* in TCGA-GBMLGG (GlioVis). $*p < 0.05$, $***p < 0.001$.

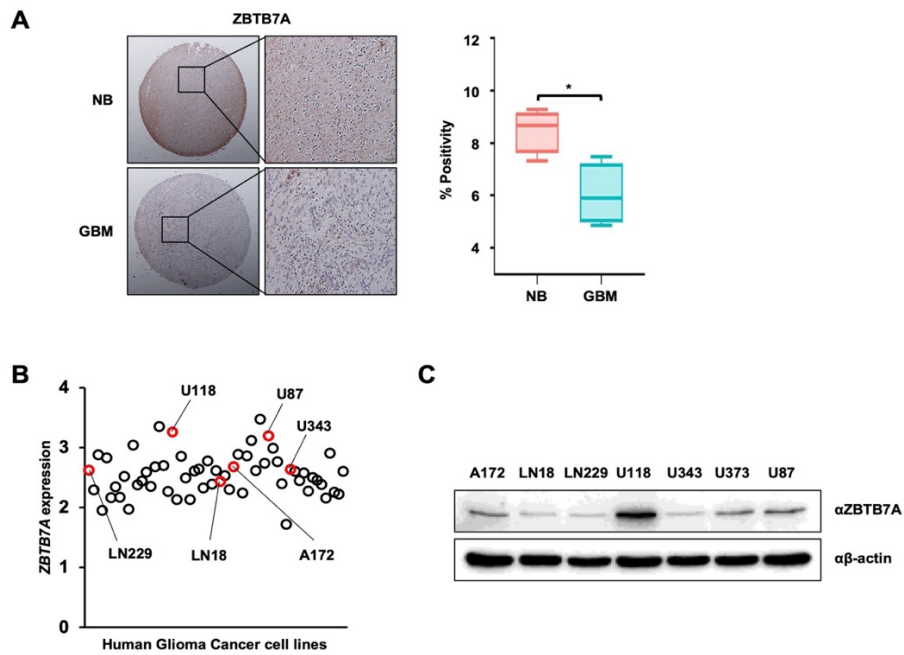


Fig. 2 ZBTB7A expression was decreased in GBM cells

A IHC analysis of TMAs in human NB and GBM samples using anti-ZBTB7A antibody. NB, $n = 4$; GBM, $n = 4$. **B** Analysis of the *ZBTB7A* expression profiles in glioma cell lines using CCLE. **C** ZBTB7A protein expression levels in GBM cells. Scale bars: 50 μm . $*p < 0.05$.

ZBTB7A-knockdown promotes GBM tumorigenesis in vitro

We examined the tumorigenicity of U87 cells, a cell line with relatively high expression of ZBTB7A, after transfection with ZBTB7A shRNA or control shRNA lentiviral plasmid (Fig. 3A; Control shRNA and shZBTB7A#1–6) to determine the probable role of ZBTB7A in GBM development. In addition, we overexpressed ZBTB7A in LN229 and U343 cells, which have relatively low ZBTB7A expression. To investigate the tumorigenicity of ZBTB7A-depleted U87 cells, we assessed the protein and mRNA expression levels of ZBTB7A (Fig. 3B, C).

To gain insight into the mechanism of action of ZBTB7A, we performed mRNA sequencing analysis. Of the 1124 genes, 597 were upregulated and 527 were downregulated (DEG analysis) (Fig. 3D). We also performed gene ontology analysis of 1124 upregulated and downregulated genes. These results showed that multicellular organisms were enriched for genes involved in processes, cell motility, and the movement of cellular components (Fig. 3E). GSEA revealed that all genes involved in the three processes were more enriched in the GBM group than in the control group when ZBTB7A was knocked down (Fig. 3F), suggesting that ZBTB7A is generally involved in biological processes related to cell motility.

When the expression of ZBTB7A was knockdown, cell proliferation was significantly increased in ZBTB7A-knockdown U87 cells (Fig. 4A), and cell migration and invasion also increased in ZBTB7A-knockdown U87 cells compared with control U87 cells (Fig. 4B, C). Additionally, we performed colony-forming assay. As a result, colony formation significantly increased in ZBTB7A-knockdown U87 cells (Fig. 4D). Therefore, our results suggest that ZBTB7A acts as a tumor suppressor in GBM.

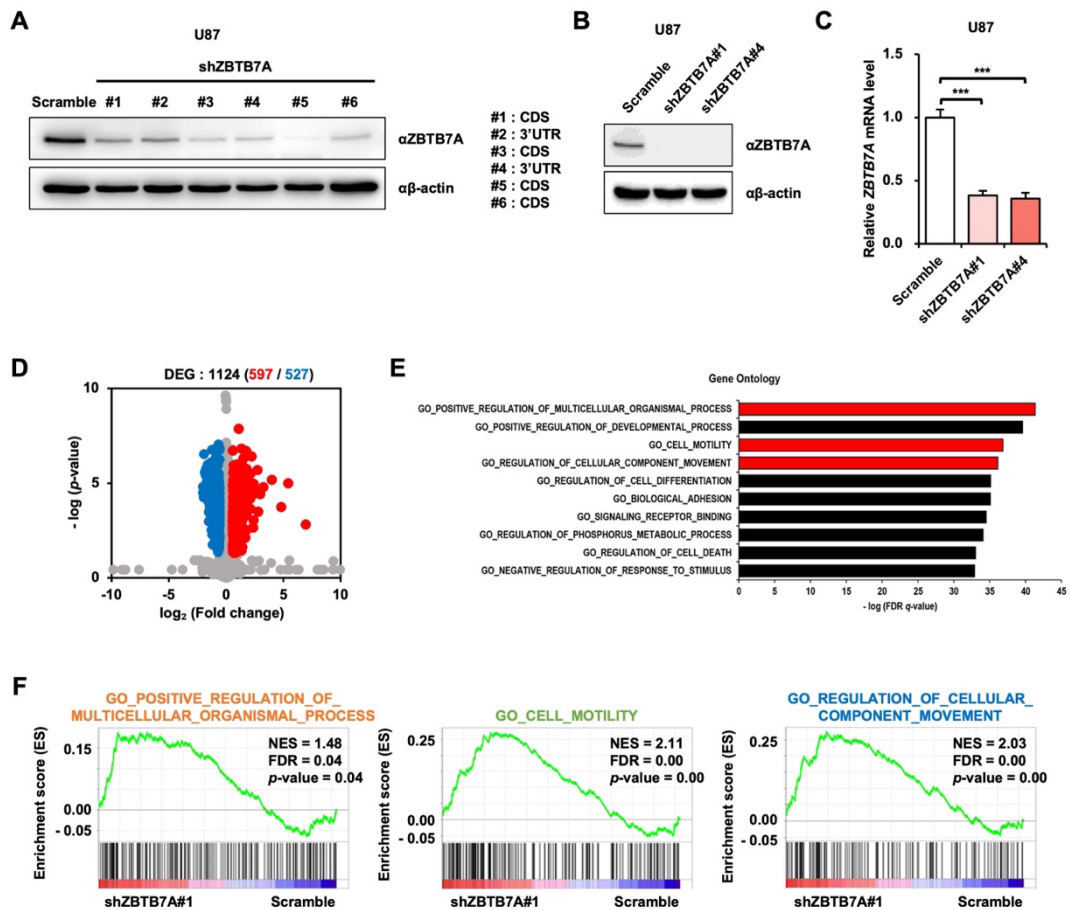


Fig. 3 ZBTB7A-knockdown is associated with cell motility and the movement of cellular components

A Expression of ZBTB7A-knockdown U87 cells. **B** Western blot analysis of ZBTB7A in shZBTB7A U87 cells. **C** Western blot and qPCR analysis of ZBTB7A silencing in U87 cells. **D** Differentially expressed gene analysis of mRNA-seq of shZBTB7A U87 cells (red dots; upregulated genes, blue dots; downregulated genes, FC \pm 1.5 and p -value $<$ 0.05). **E** Gene Ontology enrichment analysis for up- and downregulated genes scramble versus shZBTB7A. **F** GSEA reveals differences in the expression of genes involved in cell migration. NES (normalized enrichment score), FDR (fold discovery rate), *** $p <$ 0.001.

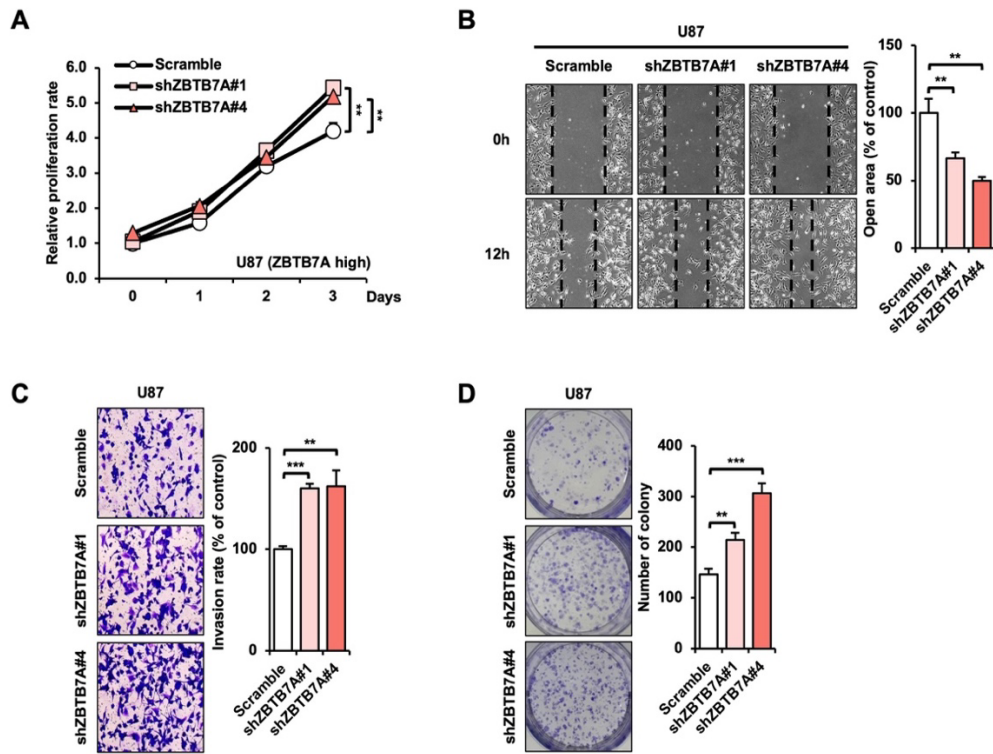


Fig. 4 ZBTB7A-knockdown promotes cell proliferation, migration, invasion, and colony formation in U87 cells

A Cell proliferation assay was performed after shZBTB7A U87 cells for 3 d. **B** Migration assay of shZBTB7A U87 cells after 12 h. **C** Transwell invasion assay of shZBTB7A U87 cells after 48 h. **D** Effect of ZBTB7A-knockdown on colony formation in shZBTB7A U87 cells.

** $p < 0.01$, *** $p < 0.001$.

ZBTB7A-overexpressing suppresses GBM tumorigenesis in vitro

To confirm the previous results, we expressed ZBTB7A in LN229 and U343 cells, which have relatively low expression levels of ZBTB7A. The protein and mRNA expression levels of ZBTB7A were assessed in ZBTB7A-expressing LN229 and U343 cells (Fig. 5A, B; cells with overexpression: pCDH ZBTB7A and control cells: pCDH CTR).

To analyze the biological function of ZBTB7A in GBM cells, we performed various cell-based assays, such as cell proliferation, migration, invasion, and colony-forming assay in ZBTB7A-overexpressing LN229 and U343 cells. As shown in Fig. 5C, cell proliferation was decreased in ZBTB7A-overexpressing GBM cells compared to that in control cells. The migration, invasion, and colony formation ability of GBM cells were significantly decreased in ZBTB7A-overexpressing GBM cells compared to control cells (Fig. 5D–F), indicating that ZBTB7A inhibits tumorigenesis in GBM cells.

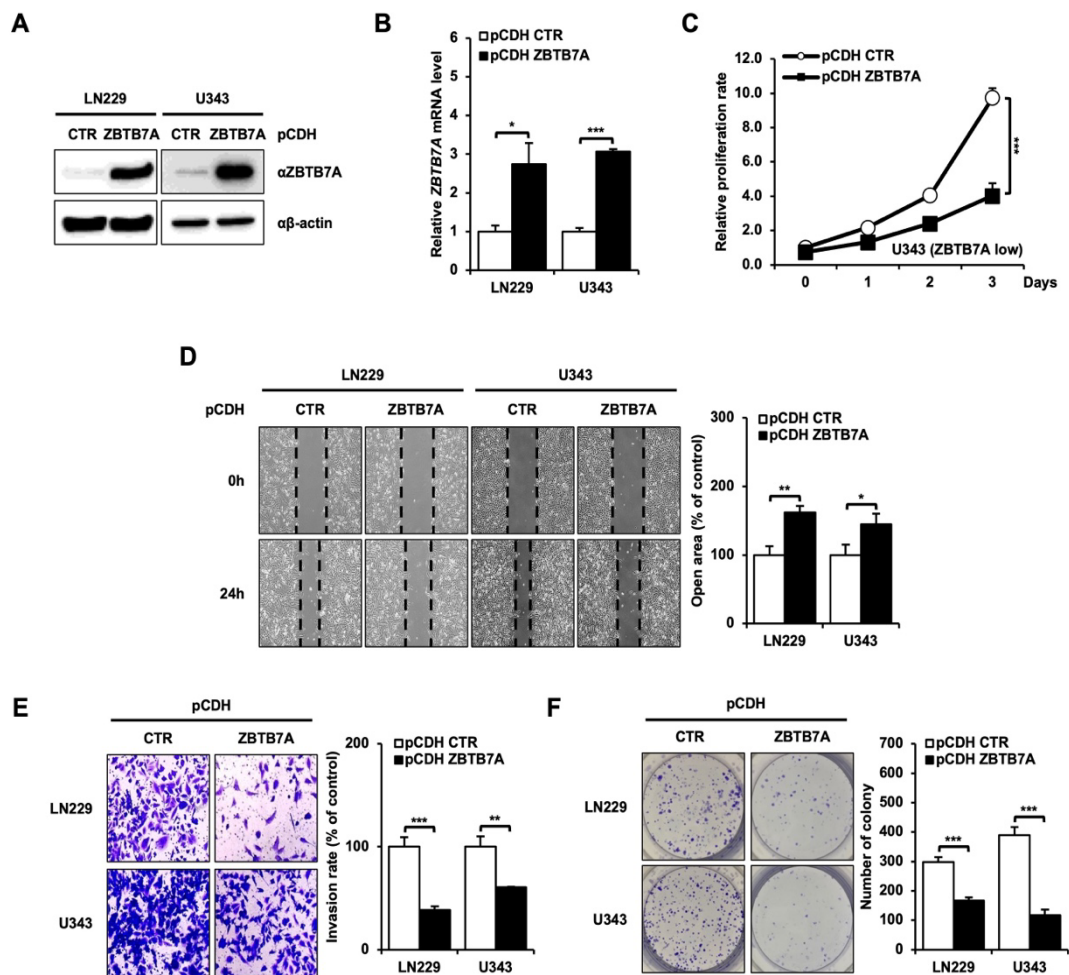


Fig. 5 ZBTB7A-overexpressing inhibits cell proliferation, migration, invasion, and colony formation in LN229 and U343 cells

A Western blot analysis of ZBTB7A expression in pCDH ZBTB7A LN229 and U343 cells. **B** mRNA level of *ZBTB7A* in pCDH ZBTB7A LN229 and U343 cells. **C** Cell proliferation assay was performed after pCDH ZBTB7A U343 cells for 3 d. **D** Migration assay of pCDH ZBTB7A LN229 and U343 cells after 24 h. **E** Transwell invasion assay of pCDH ZBTB7A LN229 and U343 cells after 48 h. **F** Effect of ZBTB7A overexpression on colony formation in pCDH ZBTB7A LN229 and U343 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

EPB41L5 is the target gene of ZBTB7A in GBM cells and patients

Next, we analyzed the genes related to these three processes in ZBTB7A-knockdown GBM cells. The upregulated genes overlapping with ZBTB7A were knocked down in three processes, and 18 genes were upregulated in all processes (Fig. 6A). The expression of 18 overlapping genes was confirmed using a heatmap (Fig. 6B). Therefore, 18 genes were upregulated when ZBTB7A expression was low, suggesting that there is an inverse correlation between the expression of ZBTB7A and the 18 target genes. To confirm the mRNA sequencing data, the mRNA expression levels of 18 target genes were analyzed in ZBTB7A-knockdown and overexpression cells using qPCR (Fig. 6C, D).

Also, we analyzed the target genes of ZBTB7A in ZBTB7A-knockdown cancer cells using the ChIP-Atlas database and predicted that the target gene binds to suppress tumorigenesis as a transcription factor. We found that EPB41L5, a protein essential for cell migration and involved in EMT (Fig. 6E).

In addition, correlation analysis was performed on the TCGA-GBM dataset to confirm that the 18 target genes obtained from our mRNA-seq data had an inverse correlation with ZBTB7A, even in patient samples. We confirmed the inverse correlation of MDK, IL33, and EPB41L5 with ZBTB7A for three of the 18 target genes (Fig. 7A, B). Therefore, we examined whether the mRNA levels of *MDK*, *IL33*, and *EPB41L5* increased in ZBTB7A shRNA-expressing GBM cells. As shown in Fig. 7C, the mRNA expression of *MDK*, *IL33*, and *EPB41L5* was inversely correlated with that of ZBTB7A. In addition, the expression of *EPB41L5* mRNA was significantly decreased in ZBTB7A-overexpressing GBM cells but not in *MDK* and *IL33* cells (Fig. 7D). Thus, EPB41L5 expression is directly affected by ZBTB7A expression.

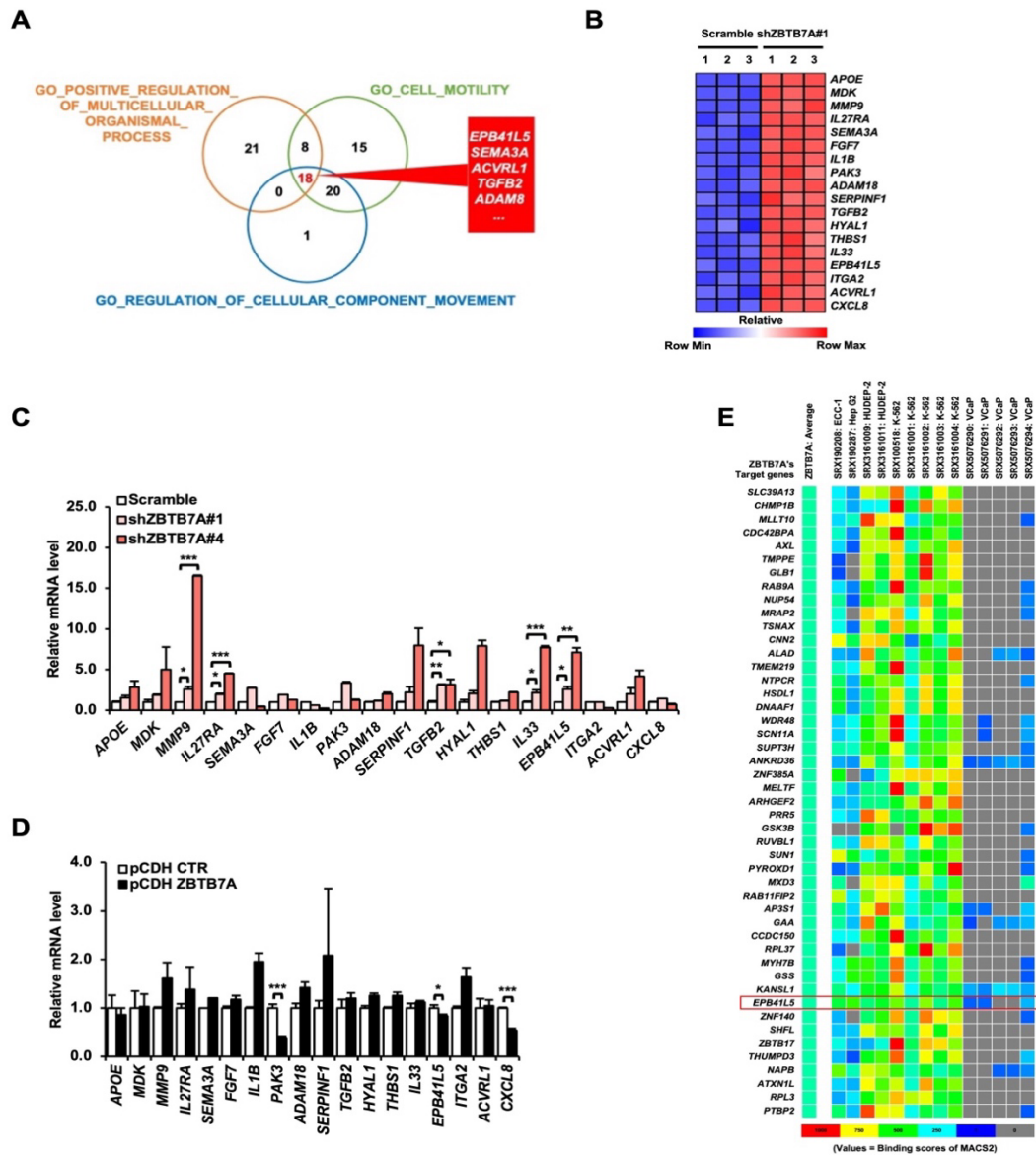


Fig. 6 *EPB41L5* is the target gene of ZBTB7A in GBM cells

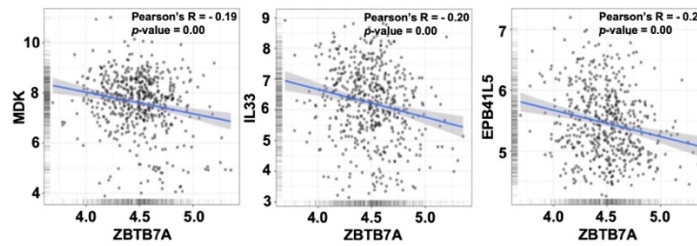
A The Venn diagram shows an overlap between GSEA results. **B** Heatmap of expression and enrichment of 18 overlapping genes in GSEA results. **C**, **D** mRNA expression levels of 18 overlapping genes in shZBTB7A U87 cells and pCDH ZBTB7A U343 cells. **E** Target gene and MACS2 binding score of potential ZBTB7A at ± 1 kb distance using CHIP-Atlas. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

A

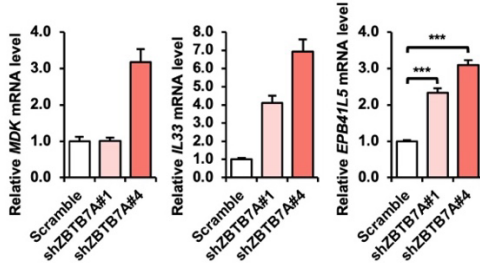
Gene Symbol	Fold change estimate	statistic	p.value	parameter	conf.low	conf.high	method	alternative	
APOE	42.780	-0.04	-0.91	0.37	536.00	-0.12	0.05	Pearson's product-moment correlation	two.sided
MDK	5.319	-0.19	-4.58	0.00	536.00	-0.27	-0.11	Pearson's product-moment correlation	two.sided
MMP9	4.310	-0.05	-1.16	0.25	536.00	-0.13	0.03	Pearson's product-moment correlation	two.sided
IL27RA	3.537	0.11	2.67	0.01	536.00	0.03	0.2	Pearson's product-moment correlation	two.sided
SEMA3A	3.023	0.02	0.53	0.60	536.00	-0.06	0.11	Pearson's product-moment correlation	two.sided
FGF7	2.701	0.02	0.53	0.60	536.00	-0.06	0.11	Pearson's product-moment correlation	two.sided
IL1B	2.612	0.06	1.43	0.15	536.00	-0.02	0.15	Pearson's product-moment correlation	two.sided
PAK3	2.609	0.10	2.38	0.02	536.00	0.02	0.19	Pearson's product-moment correlation	two.sided
ADAM8	2.597	0.17	3.88	0.00	536.00	0.08	0.25	Pearson's product-moment correlation	two.sided
SERPINF1	2.593	0.01	0.28	0.78	536.00	-0.07	0.1	Pearson's product-moment correlation	two.sided
TGF β 2	2.480	0.03	0.80	0.43	536.00	-0.05	0.12	Pearson's product-moment correlation	two.sided
HYAL1	2.243	0.12	2.89	0.00	536.00	0.04	0.21	Pearson's product-moment correlation	two.sided
THBS1	2.114	0.06	1.40	0.16	536.00	-0.02	0.14	Pearson's product-moment correlation	two.sided
IL33	2.111	-0.20	-4.63	0.00	536.00	-0.28	-0.11	Pearson's product-moment correlation	two.sided
EPB41L5	2.082	-0.20	-4.67	0.00	536.00	-0.28	-0.11	Pearson's product-moment correlation	two.sided
ITGA2	2.077	0.14	3.39	0.00	536.00	0.06	0.23	Pearson's product-moment correlation	two.sided
ACVRL1	2.027	0.09	2.11	0.04	536.00	0.01	0.17	Pearson's product-moment correlation	two.sided
CXCL8									

Gene not available for this dataset

B



C



D

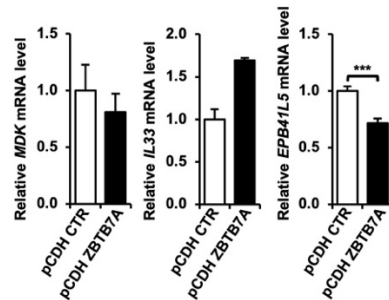


Fig. 7 EPB41L5 inversely correlates with ZBTB7A in patients

A Correlation of 18 overlapping genes with ZBTB7A in the TCGA-GBM dataset (Gliovis).

B Negative correlation of the gene expression levels of MDK, IL33, and EPB41L5 with ZBTB7A (Gliovis). **C, D** mRNA expression levels of MDK, IL33, and EPB41L5 in shZBTB7A U87 cells and pCDH ZBTB7A U343 cells. *** $p < 0.001$.

Additionally, we confirmed that ZBTB7A and EPB41L5 had an inverse correlation at the protein level based on analysis of ZBTB7A-knockdown U87 cells and ZBTB7A-overexpressing LN229 and U343 cells, respectively (Fig. 8A–D). Furthermore, the protein expression levels of ZBTB7A and EPB41L5 were inversely correlated in GBM cell lines (Fig. 8E). The expression of EPB41L5 in tissues of patients with GBM (based on GBM TMAs) was observed using IHC staining, and the expression of EPB41L5 increased in the high-grade GBM tissues (Fig. 8F, G).

In a previous study, we reported that the expression of EPB41L5 induced by TGF- β 1 promotes the migration and invasion of gastric cancer cells by TGF- β signaling³⁴. Thus, we further confirmed whether the knockdown of ZBTB7A plays an important role in the EMT process due to increased cellular motility. When ZBTB7A was knocked down in GBM cells, the EMT process was induced, and the related core genes were enriched (Fig. 9A, B), indicating that ZBTB7A-knockdown activates GBM growth-related genes for the development and progression of GBM. Also, ZBTB7A-knockdown significantly increased mRNA levels of related core genes including EPB41L5 (Fig. 9C). Overall, ZBTB7A controls GBM tumorigenesis by regulating EPB41L5 expression.

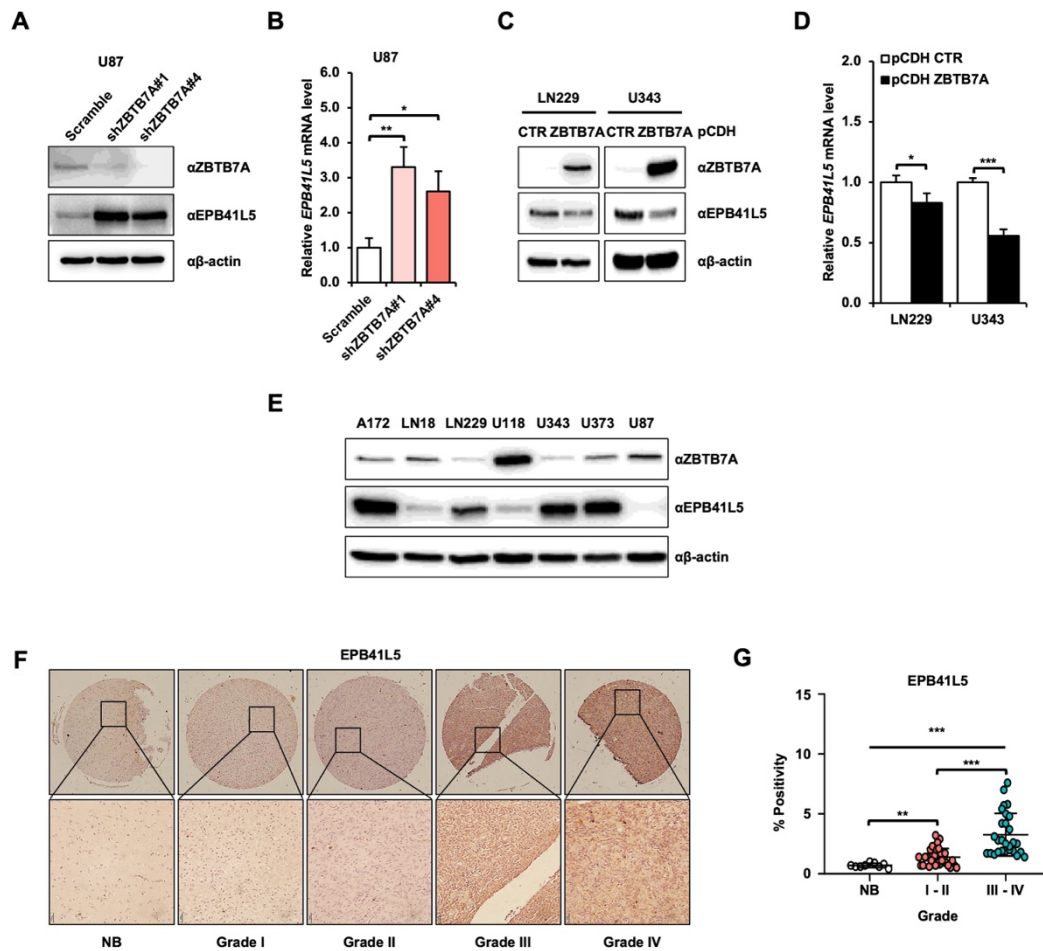


Fig. 8 ZBTB7A negatively regulates EPB41L5 in GBM cells and patients

A Western blot analysis of ZBTB7A and EPB41L5 in shZBTB7A U87 cells. **B** *EPB41L5* mRNA level in shZBTB7A U87 cells. **C** Western blot analysis of ZBTB7A and EPB41L5 in pCDH ZBTB7A LN229 and U343 cells. **D** *EPB41L5* mRNA level in pCDH ZBTB7A LN229 and U343 cells. **E** Western blot analysis of ZBTB7A and EPB41L5 in GBM cell lines. **F** EPB41L5 IHC staining in increasing grades of glioma using TMA blots. **G** IHC analysis of EPB41L5 expression in TMA blots. NB, $n = 9$; Grade I – II, $n = 30$; III – IV, $n = 28$. Scale bars: 50 μm . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

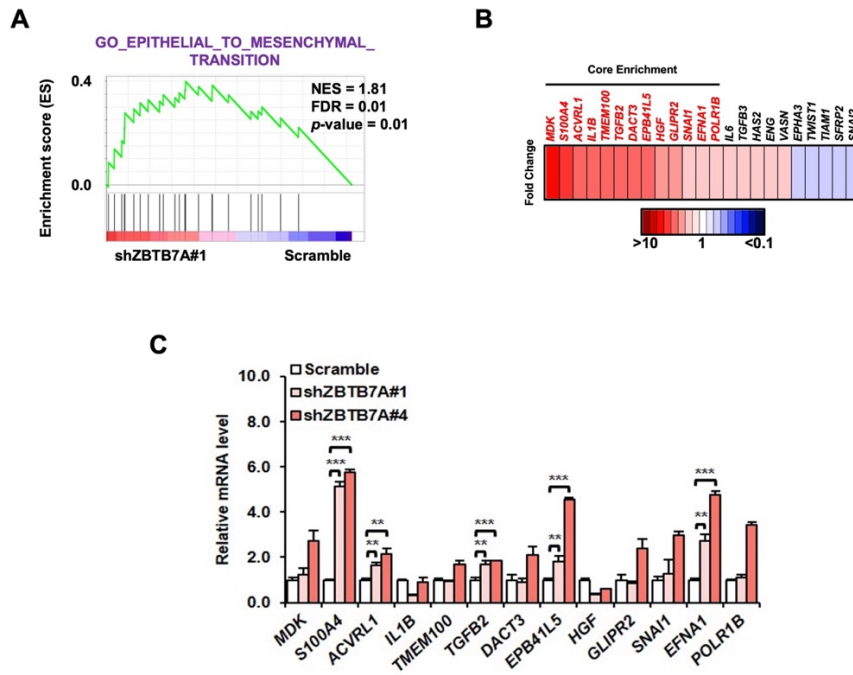


Fig. 9 ZBTB7A-knockdown induces EMT process

A GSEA reveals differences in the expression of genes involved in EMT. **B** EMT-related core enrichment gene fold change value. **C** qPCR analysis of EMT-related core enrichment genes.

** $p < 0.01$, *** $p < 0.001$.

ZBTB7A binds to the *EPB41L5* promoter to repress the transcriptional activity

To investigate whether ZBTB7A and EPB41L5 are inversely correlated, we performed a promoter assay to confirm that ZBTB7A binds to the promoter of EPB41L5 as a transcription factor and negatively regulates its transcriptional activity. The binding motif sequence of ZBTB7A was confirmed using the JASPAR database, and the region where ZBTB7A binds was predicted in the 1 kb region (-900 to 100) of the *EPB41L5* promoter through the EPD database (Fig. 10A, B). We also obtained overlapping results from the JASPAR and EPD databases for four regions that were significant for ZBTB7A binding in the EPB41L5 promoter (Fig. 10C). In fact, the 1 kb region of the *EPB41L5* promoter was cloned into the pGL4.21 vector to perform the luciferase assay, and the ZBTB7A ORF was cloned into the Myc-tagged pSG5 vector for cotransfection into U343 cells with relatively low ZBTB7A expression. ZBTB7A was transfected in a dose-dependent manner, and we confirmed a gradual decrease in the transcriptional activity of EPB41L5 (Fig. 10D).

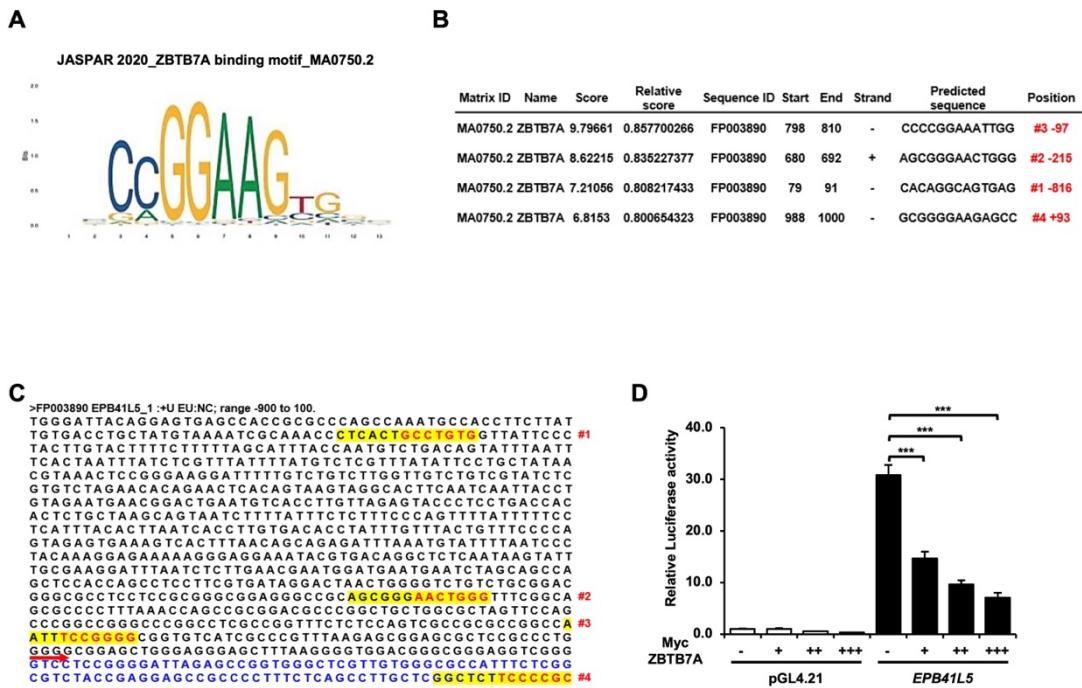


Fig. 10 ZBTB7A inhibits transcriptional activity of EPB41L5

A, B Schematic of the 1 kb *EPB41L5* gene promoter and the ZBTB7A binding element within the ZBTB7A binding motif of JASPAR. **C** *EPB41L5* promoter sequence from -900 to 100 region (EPD). **D** ZBTB7A suppresses *EPB41L5* promoter activity by luciferase assay. *** $p < 0.001$.

To analyze transcriptional activity, the 1 kb full-length *EPB41L5* (-900 to 100: #1, -500 to 100: #2, -150 to 100: #3, and -40 to 100: #4) promoter regions were truncated (Fig. 11A). We tested whether ZBTB7A inhibits transcription by binding to regions of the *EPB41L5* promoter using a ChIP assay.

ZBTB7A was significantly recruited to the #3 region of the *EPB41L5* promoter in ZBTB7A-expressing U343 cells compared to control cells (Fig. 11B). Furthermore, we measured transcriptional activity through the transfection of *EPB41L5* promoter-deleted mutants (*EPB41L5* promoter truncated constructs) in ZBTB7A-overexpressing U343 cells or control U343 cells. The transcriptional activity of *EPB41L5* promoter wild-type/deletion mutants was significantly reduced in ZBTB7A-overexpressing U343 cells compared to the control group, and when the -150 to 100 promoter regions (#3) were truncated, the transcriptional activity was drastically reduced in the ZBTB7A-overexpressing U343 cells and control cells (Fig. 11C). Based on these results, we confirmed that the promoter region, including the *EPB41L5* #3 (-150 to 100) position, was a significant ZBTB7A binding region.

To further confirm whether the *EPB41L5* #3 (-150 to 100) position is the ZBTB7A binding region, the *EPB41L5* promoter construct (#3-WT) or the specific binding sequence deleted form of the *EPB41L5* promoter construct (#3-MT) was transfected into ZBTB7A-overexpressing U343 cells or control cells, and promoter activity was assessed using a luciferase assay. The transcriptional activity of the ZBTB7A binding sequence deleted in the *EPB41L5* promoter (#3-MT) was not significantly different between ZBTB7A-expressing U343 cells and control cells compared with the wild-type *EPB41L5* promoter (#3-WT) (Fig. 11D).

In addition, we performed a luciferase assay to determine whether transcriptional activation of *EPB41L5* was increased by ZBTB7A-knockdown. The transcriptional activation of *EPB41L5* was greatly increased in the -150 to 100 region (#3) of the *EPB41L5* promoter but not in the #3 mutant promoter after ZBTB7A-knockdown (Fig. 11E).

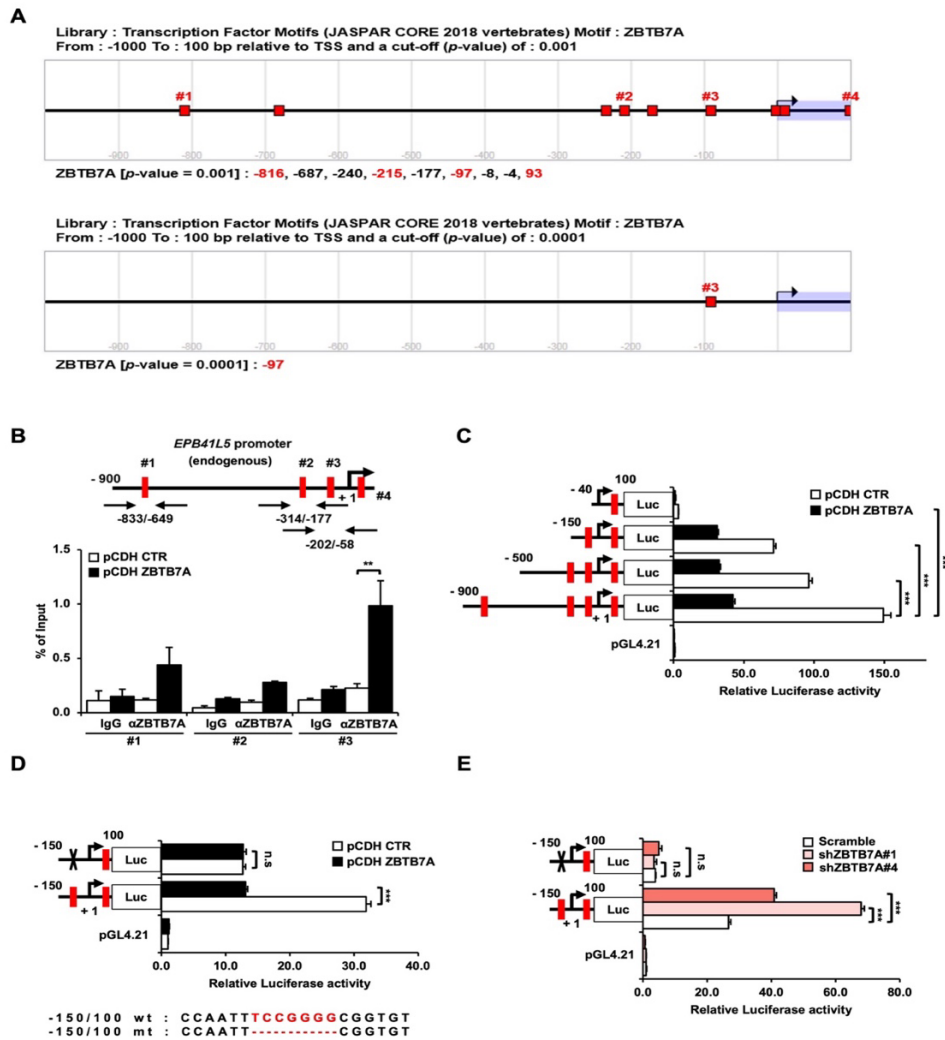


Fig. 11 ZBTB7A binds specifically to the *EPB4IL5* promoter

A Predicted binding site to which the ZBTB7A motif can bind in the *EPB4IL5* promoter region (-900 to 100) (EPD). **B** The upper panel shows the region amplified using ChIP primers in the *EPB4IL5* promoter. The lower panel shows the amount of DNA precipitated using anti-ZBTB7A or IgG. **C** Repression by ZBTB7A in the *EPB4IL5* promoter required a binding motif of ZBTB7A, as shown by a luciferase assay. The left panel shows the relative cleavage of the *EPB4IL5* promoter. **D** Of the four potential ZBTB7A binding sites of the *EPB4IL5* promoter, the most important (the ZBTB7A#3 position) was deleted. Transcriptional activity of the depleted promoter was determined via luciferase assay. **E** The transcriptional activity of the *EPB4IL5* promoter with deletion of the #3 position in shZBTB7A U87 cells was investigated by luciferase assay. n.s; not significant, ** $p < 0.01$, *** $p < 0.001$.

Next, we investigated whether R399L binds to the *EPB41L5* promoter region through site-directed mutagenesis of R399L in the zinc finger domain of ZBTB7A. ChIP assays were performed in U343 cells transfected with the empty control vector, wild-type (wt) Myc-ZBTB7A, and mutant-type (mt) Myc-ZBTB7A. mtZBTB7AR399L failed to bind to position #3 of the *EPB41L5* promoter (Fig. 12A). As expected, mtZBTB7AR399L did not inhibit the transcriptional repression of EPB41L5 compared to wtZBTB7A, and the wt/mt Myc-ZBTB7A protein was normally expressed in all cells (Fig. 12B).

We also confirmed that mtZBTB7AR399L failed to repress the transcriptional activity of EPB41L5. We examined the transcriptional activity in the GBM cell lines LN229, U343, U118, and U87 transfected with the empty vector, wtZBTB7A, and mtZBTB7A (Fig. 12C). Thus, we suggest that ZBTB7A negatively regulates the transcriptional activity of EPB41L5 by binding to the ZBTB7A #3 position of the *EPB41L5* promoter.

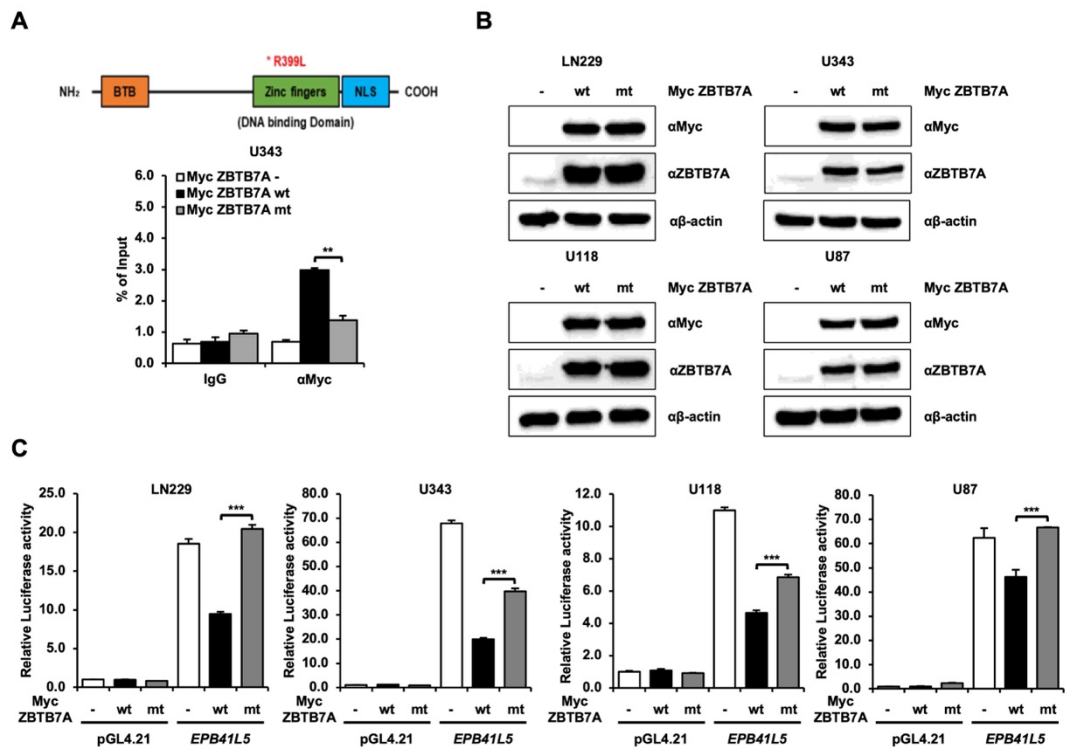


Fig. 12 Mutation of ZBTB7A rescues the transcriptional activity of EPB41L5

A The domain structure of the ZBTB7A protein and the design of the ZBTB7A zinc finger mutant R399L. Then, Myc ZBTB7A wt and mt were transfected in U343 cells to show the amount of DNA precipitated by Myc antibody or IgG, and the -202/-58 region was amplified with a ChIP primer. **B** Protein levels of Myc ZBTB7A wt and mt in LN229, U343, U118 and U87 cells. **C** To determine the ability of ZBTB7A R399L to repress the activity of the *EPB41L5* promoter, we performed luciferase assays in LN229, U343, U118, and U87 cells. ** $p < 0.01$, *** $p < 0.001$.

ZBTB7A-knockdown increases the transcriptional activation of EPB41L5

To confirm the same results when ZBTB7A-knockdown, the mRNA expression of EPB41L5 and ZBTB7A was confirmed in shZBTB7A-expressing U87 cells, and the transcriptional activity of EPB41L5 was measured in shZBTB7A-expressing U87 cells that target the 3'UTR *EPB41L5* promoter, mtZBTB7A expression greatly induced the transcriptional activity of EPB41L5. The activities of wtZBTB7A and the control were similar (Fig. 13A–C). Additionally, a ChIP assay was performed in shZBTB7A-expressing U87 cells cotransfected with the *EPB41L5* promoter and wtZBTB7A or mtZBTB7A. In the previous experiments, mtZBTB7A failed to bind the *EPB41L5* promoter, and wtZBTB7A did not bind the #3 mutation region of the *EPB41L5* promoter (Fig. 13D).

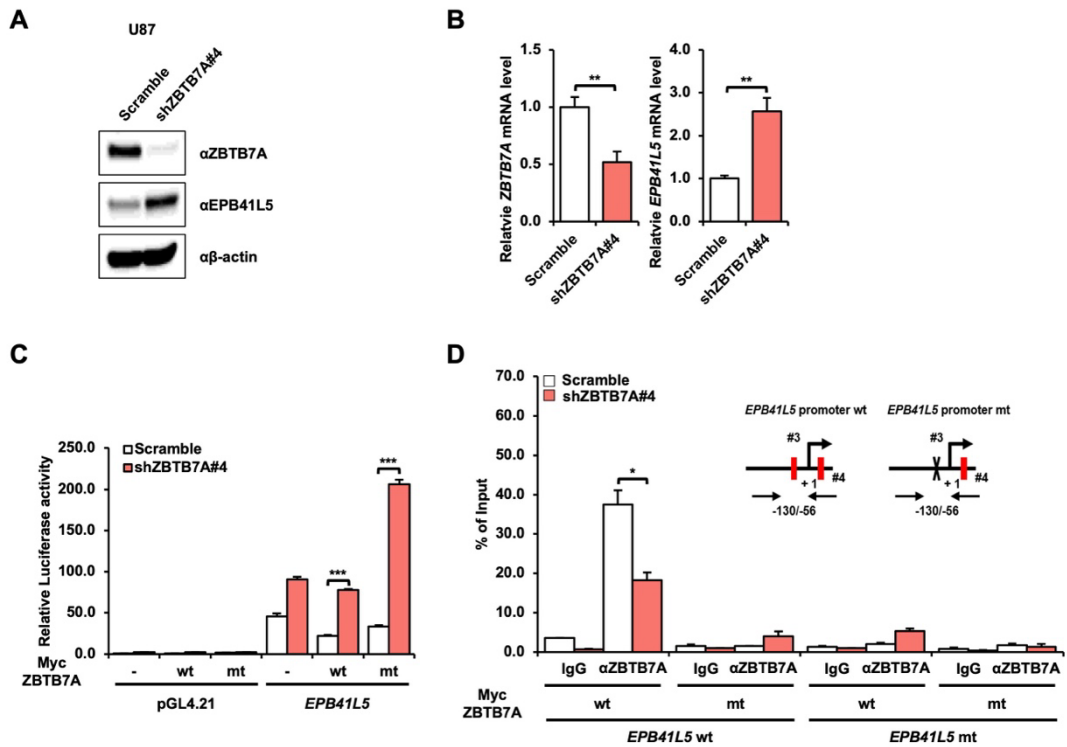


Fig. 13 ZBTB7A negatively regulates the transcriptional activity of EPB41L5

A Western blot analysis of shZBTB7A#4 U87 cells. **B** mRNA levels of *ZBTB7A* and *EPB41L5* in shZBTB7A#4 U87 cells. **C** To measure the ability of ZBTB7A R399L to repress promoter activity when ZBTB7A protein expression was low, we performed a luciferase assay in shZBTB7A#4 U87 cells. **D** The -130/-56 region was amplified with ChIP primers, showing the amount of DNA precipitation by ZBTB7A antibody or IgG by cotransfection of Myc ZBTB7A and *EPB41L5* promoters in shZBTB7A#4 U87 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

ZBTB7A-overexpressing suppresses GBM tumorigenesis in vivo

To confirm that ZBTB7A had the same inhibitory effect on GBM cell migration, invasion, and colonization, control cells (pCDH CTR) and ZBTB7A-expressing U343 cells (pCDH ZBTB7A) were injected subcutaneously into nude mice, and the tumor weight was monitored every 2 days for 6 weeks. As expected, tumor growth on ZBTB7A-expressing U343 cells was further reduced compared to that in control cells (Fig. 14A, B).

In addition, we confirmed whether the tumor volume and weights were suppressed in ZBTB7A-expressing U343 cells compared to the control cells (Fig. 14C, D). The survival rate was also higher in ZBTB7A-expressing U343 cells than in the control group (Fig. 14E) and whether the protein expression of ZBTB7A was normal in the ZBTB7A-expressing tumor using Western blotting (Fig. 14F).

We conducted the expression of EPB41L5 was significantly decreased in ZBTB7A-overexpressing tumor tissues (Fig. 14G, H). Based on these results, we suggest that ZBTB7A acts as a transcriptional repressor that inhibits tumorigenesis by targeting EPB41L5 in GBM.

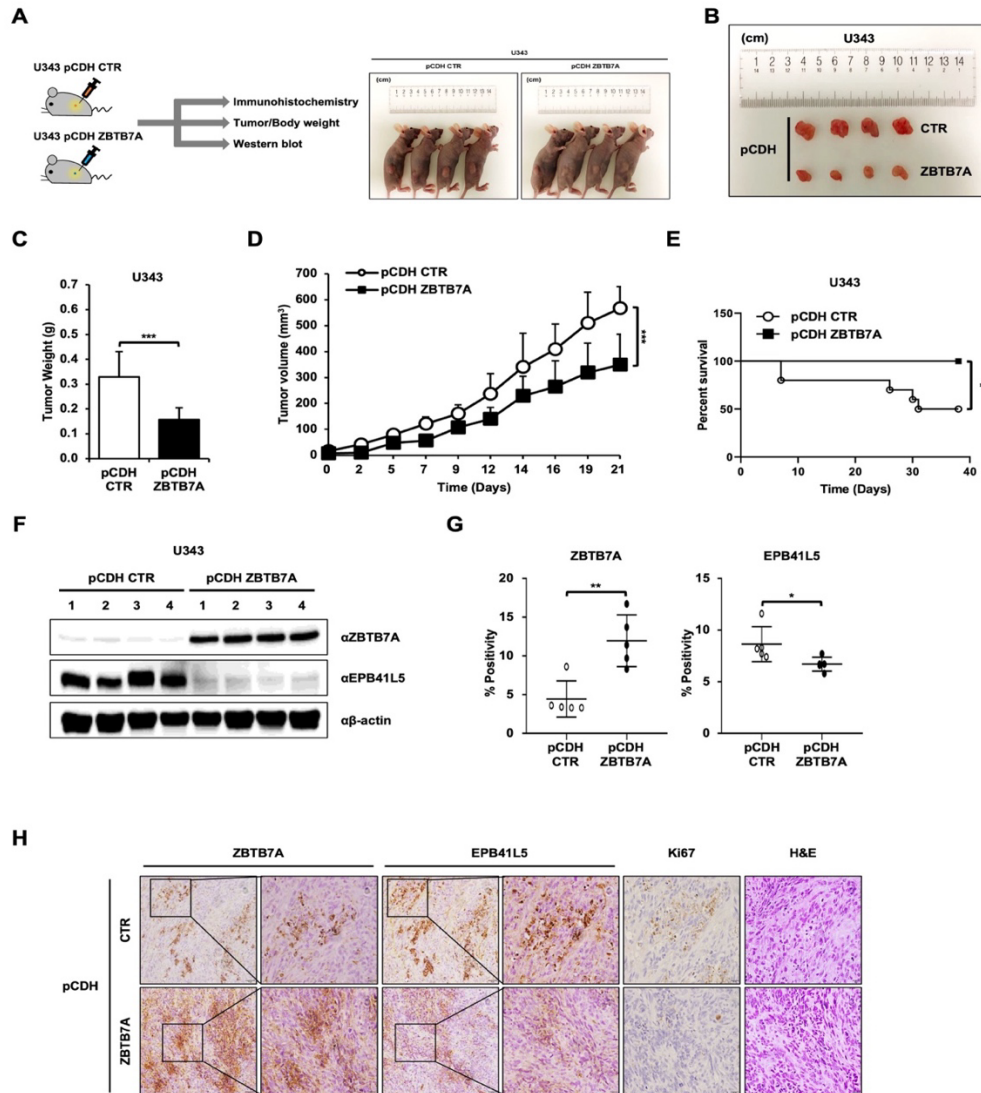


Fig. 14 ZBTB7A-overexpressing inhibits tumorigenesis

A Schematic diagram and photograph of a subcutaneous tumor experiment using nude mice. **B** Tumor size confirmed 5 weeks after subcutaneous injection of pCDH ZBTB7A U343 cells ($n = 4$ mice/group). **C** The weight of each tumor was measured. ($n = 8$ mice/group). **D** Tumor volume was calculated according to the formula at 2–3 d intervals for 5 wk ($n = 5$ mice/group). **E** The survival rate of a mouse model injected subcutaneously with pCDH ZBTB7A U343 cells. **F** Western blot analysis of ZBTB7A and EPB41L5 in pCDH ZBTB7A U343 subcutaneous tumors. **G** IHC analysis of pCDH ZBTB7A U343 subcutaneous tumors using IHC profiler ($n = 5$ mice/group). **H** IHC staining using anti-ZBTB7A, EPB41L5, Ki67 antibodies and H&E staining. Scale bars: 50 μm , 20 μm . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

ZBTB7A-knockdown increases GBM tumorigenesis through the transcriptional activation of EPB41L5

We conducted the following experiment to determine whether ZBTB7A-knockdown promotes EMT and tumorigenesis in GBM. The protein and mRNA expression of EMT markers (N-cadherin, β -catenin, and Vimentin) was confirmed in shZBTB7A U87 cells, and both protein and mRNA levels were increased (Fig. 15A, B). When the correlation between ZBTB7A and EMT markers was analyzed using patient samples from the TCGA-GBM and TCGA-GBMLGG datasets, ZBTB7A and EMT markers showed a significant negative correlation (Fig. 15C).

In addition, we monitored the development and progression of tumors derived from shZBTB7A-expressing GBM cells in an orthotopic xenograft mouse model. Control U87 and shZBTB7A U87 cells were injected into the right corpus striatum of the mouse brain. After observation for 4 weeks, the GBM tumor volume was significantly increased in a mouse injected with shZBTB7A-expressing U87 cells compared to that in the control mice, and the protein expression levels of ZBTB7A and EPB41L5 in GBM tumor tissues were inversely correlated (Fig. 15D, E). As a result of confirming the expression of EMT markers together with EPB41L5 through IHC analysis, it was also significantly increased (Fig. 15F). Taken together, our results show that the knockdown of ZBTB7A induces transcriptional activation of EPB41L5 and promotes GBM tumorigenesis via the EMT process.

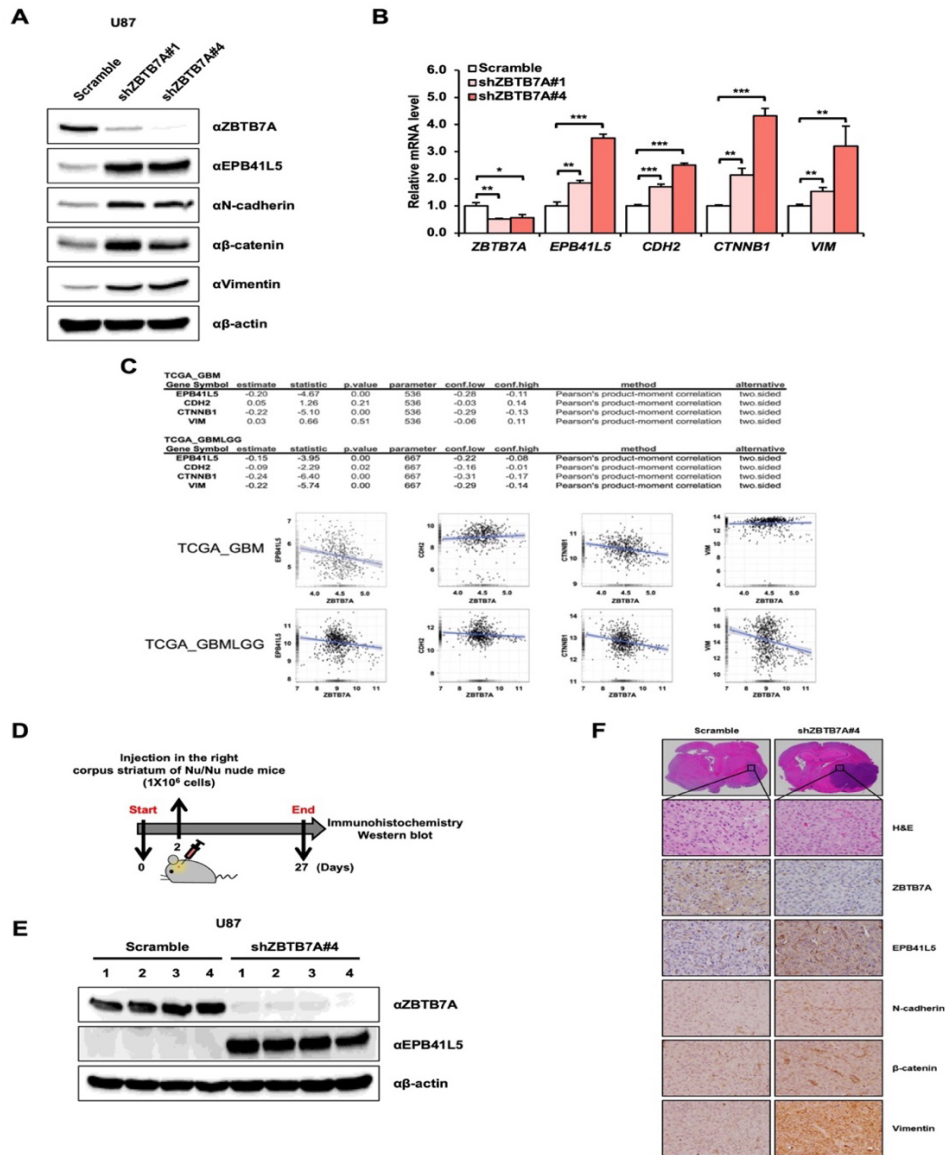


Fig. 15 ZBTB7A-knockdown promotes EMT and tumorigenesis

A, B Expression of EMT markers by Western blot and qPCR analyses in shZBTB7A U87 cells. **C** Correlation analysis between ZBTB7A and EMT markers using TCGA-GBM (GlioVis). **D, E** Schematic of the experimental procedure of orthotopic GBM nude mouse models injected with shZBTB7A#4 U87 cells and Western blot analysis of ZBTB7A and EPB41L5 of tumor sample ($n = 4$ mice/group). **F** H&E staining and IHC staining of anti-ZBTB7A, EPB41L5, and EMT marker (N-cadherin, β -catenin, and Vimentin) in orthotopic GBM nude mouse models injected with shZBTB7A#4 U87 cells. Scale bars: 20 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

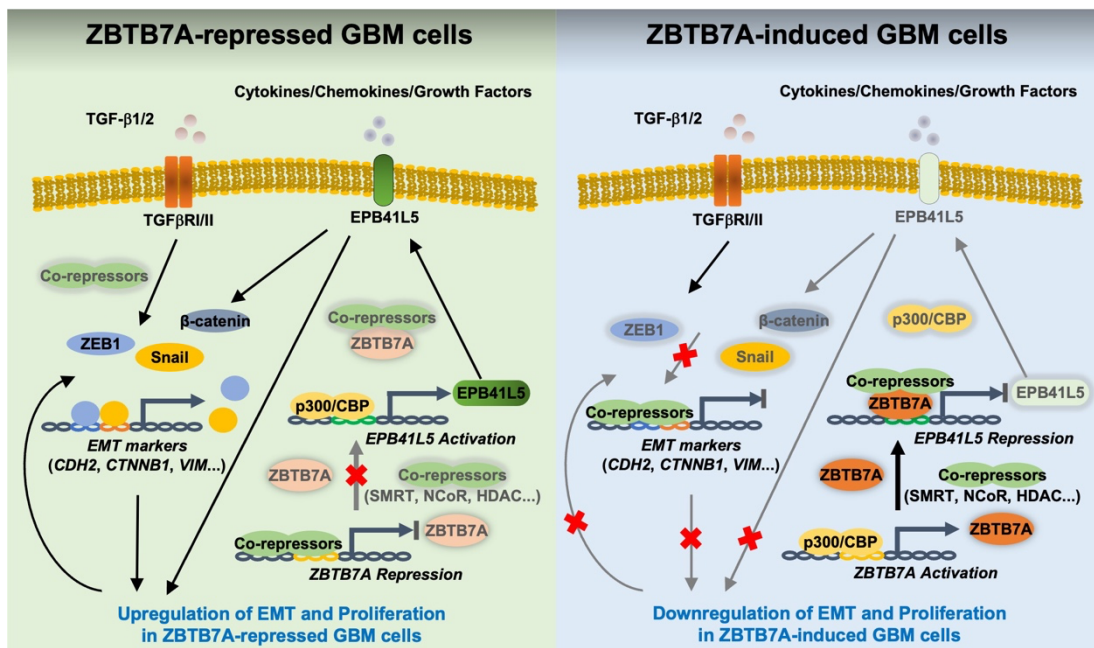


Fig. 16 Graphical abstract

Discussion

GBM is a highly mobile, invasive, and lethal primary tumor that is resistant to all therapies and has a poor prognosis³⁵. Although the frequent occurrence of genetic abnormalities has been reported in various cohort profiling studies, survival is difficult to predict. This result implies that there are other factors influencing abnormal gene expression. ZBTB7A, a transcriptional activator or repressor known to play an important role in tumorigenesis and metastasis in various human cancers, is downregulated in GBM²⁵. ZBTB7A is required for the regulation of tumor growth in cancers, including GBM. However, it is not clear how ZBTB7A functions as a transcriptional activator or repressor of tumor growth in GBM cells.

In our study, ZBTB7A provided insight into the mechanism of a tumor suppressor in GBM, and its deficiency significantly increased the tumor growth of GBM. In particular, among the various targets regulated by ZBTB7A, the *EPB41L5* gene, which is involved in cell migration and the EMT process, was discovered through ChIP-Atlas and mRNA-seq analyses. In a previous study, we reported that EPB41L5 was induced by TGF- β signaling in gastric cancer and increased cell mobility and invasiveness and the expression of various EMT markers³⁴.

Therefore, because the expression of ZBTB7A is low in GBM, we confirmed that GBM growth is regulated through overexpression of ZBTB7A. ZBTB7A inhibited the migration, invasion, and proliferation of GBM cells. However, the growth of GBM tumors was promoted by ZBTB7A-knockdown, and GBM tumor volume was significantly increased in the in vivo mouse GBM model. In addition, we subcutaneously injected ZBTB7A-overexpressing U343 cells into nude mice. ZBTB7A overexpression did not promote tumor growth in vivo in GBM models, but it suppressed the expression of EPB41L5, a target of ZBTB7A. Furthermore, shZBTB7A-expressing U87 cells enhanced GBM tumorigenesis in an in vivo orthotopic xenograft mouse model of GBM.

To clarify our findings, mRNA sequencing analysis was performed to confirm the mechanism by which ZBTB7A, a transcriptional repressor, inhibits tumorigenesis by targeting EPB41L5 in GBM. When ZBTB7A was depleted in GBM cells, tumor migration and EMT-related gene expression were significantly increased. In this process, we verified once again

that EPB41L5 is an important target and confirmed that the survival rate of patients with GBM decreased with increasing EPB41L5 expression.

To demonstrate the transcriptional regulation of EPB41L5 by ZBTB7A as a transcriptional repressor, we predicted that the promoter of EPB41L5 had a ZBTB7A motif and found that ZBTB7A negatively regulated the transcription level of EPB41L5 by binding directly to -97 bp (*EPB41L5* #3 position) close to the EPB41L5 transcription start site. In addition, we clearly showed that the ZBTB7A-EPB41L5 axis could play a central role in promoting tumor growth in GBM by cooperatively promoting the EMT process. However, while we have demonstrated that the ZBTB7A-EPB41L5 axis significantly promotes GBM tumor growth, we do not yet understand how this axis is activated depending on the cellular microenvironment. Therefore, we considered the correlation between tumorigenesis and the tumor microenvironment essential for the growth of GBM cells through epigenetic regulation of the target gene with ZBTB7A.

Chromatin modification by the epigenome is important in determining the transcriptional activation of EPB41L5 by ZBTB7A, and ZBTB7A regulates the expression of target genes by recruiting epigenetic modifiers of corepressors, such as BCoR, NCoRs, HDACs, SIN3A, and MBD3³⁶⁻³⁸. Based on the report that ZBTB7A recruits various corepressors, we suggest that ZBTB7A is a central corepressor complex and has the potential to regulate transcription by controlling chromatin remodeling in the EPB41L5 promoter. In addition, GBM tumorigenesis is determined by the secretomes of the tumor microenvironment through epigenetic regulation of corepressors and coactivators by ZBTB7A. As a result, ZBTB7A-mediated EPB41L5 inhibition should be considered as a therapeutic strategy for GBM.

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Abstract (in Korean)

가장 공격적이고 악성이며 신경아교종인 교모세포종 (GBM)은 예후가 좋지 않다. GBM 환자는 수술, 화학 요법 및 방사선 요법을 통해 치료하지만 GBM 은 치료에 대한 저항성이 높아 치료가 어렵고 비용이 많이 든다. 본 연구에서 Gene Expression Profiling Interactive Analysis (GEPIA), Cancer Genome Atlas 및 Gene Expression Omnibus (GEO) array 데이터세트들을 분석했다. ZBTB7A (FBI1/POKEMON/LRF 라고도 함)는 낮은 등급의 신경아교종에서 높게 발현되지만 GBM 환자에서는 상당히 하향 조절되어 있는 것으로 밝혀졌다. ZBTB7A 는 세포 증식을 포함한 많은 발달 단계에서 중요한 역할을 하는 전사 인자이다. EMT (Epithelial-Mesenchymal Transition)의 활성화는 암 진행 및 전이의 핵심 과정이다. Erythrocyte membrane protein band 4.1 like 5 (EPB41L5)는 다양한 종류의 암에서 EMT 진행 및 전이에 필수적인 단백질이다. 우리는 U87 세포에서 ZBTB7A 결핍이 GBM 진행 및 전이를 유도한다는 것을 발견했다. RNA-seq 데이터를 기반으로 ZBTB7A 는 EPB41L5 유전자의 프로모터에 직접 결합하여 발현을 감소시키고 GBM 진행을 억제한다. 우리는 ZBTB7A 가 EPB41L5 의 전사 억제를 통해 GBM 종양 성장을 극적으로 억제한다는 것을 입증했다. 따라서, ZBTB7A 와 EPB41L5 는 GBM 치료를 위한 잠재적인 바이오마커이자 새로운 치료 표적이 될 수 있다. 전반적으로 우리는 GBM 진행을 직접적으로 억제하는 새로운 종양 억제인자인 ZBTB7A 의 역할을 발견했고 EPB41L5 를 GBM 환자를 위한 치료 표적 단백질로 확인했다.

중심단어: ZBTB7A, EPB41L5, Glioblastoma, Tumorigenesis, EMT