

PELP1 promotes glioblastoma progression by enhancing Wnt/ β -catenin signaling

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Abstract

Background: Glioblastoma (GBM) is a deadly neoplasm of the central nervous system. The molecular mechanisms and players that contribute to GBM development is incompletely understood.

Methods: The expression of PELP1 in different grades of glioma and normal brain tissues was analyzed using immunohistochemistry on a tumor tissue array. PELP1 expression in established and primary GBM cell lines was analyzed by Western blotting. The effect of PELP1 knockdown was studied using cell proliferation, colony formation, migration, and invasion assays. Mechanistic studies were conducted using RNA-seq, RT-qPCR, immunoprecipitation, reporter gene assays, and signaling analysis. Mouse orthotopic models were used for pre-clinical evaluation of PELP1 knock down.

Results: Nuclear receptor coregulator PELP1 is highly expressed in gliomas compared to normal brain tissues, with the highest expression in GBM. PELP1 expression was elevated in established and patient-derived GBM cell lines compared to normal astrocytes. Knockdown of PELP1 resulted in a significant decrease in cell viability, survival, migration, and invasion. Global RNA-sequencing studies demonstrated that PELP1 knockdown significantly reduced the expression of genes involved in the Wnt/ β -catenin pathway. Mechanistic studies demonstrated that PELP1 interacts with and functions as a coactivator of β -catenin. Knockdown of PELP1 resulted in a significant increase in survival of mice implanted with U87 and GBM PDX models.

Conclusions: PELP1 expression is upregulated in GBM and PELP1 signaling via β -catenin axis contributes to GBM progression. Thus, PELP1 could be a potential target for the development of therapeutic intervention in GBM.

Key Points

- PELP1 facilitates proliferation, migration, and invasion of glioblastoma cells
- PELP1 functions as a coactivator of β -catenin and contributes to glioblastoma progression

Glioblastoma (GBM) is the most common brain neoplasm that exhibits dismal prognosis.^{1,2} Despite advancements in current multimodal treatment strategies, GBM patients have a median

survival that is ~20 months, and the 5-year survival rate after diagnosis is ~13%.³ GBMs are highly infiltrative, mutable in nature, and often exhibiting extensive cellular heterogeneity,⁴

Importance of the Study

In this study, we identified that nuclear receptor coregulator PELP1 is highly expressed in gliomas compared to normal brain tissues. Global RNA-Seq studies identified that PELP1 regulates several genes involved in proliferation, migration, and invasion of GBM cells. Mechanistic studies demonstrated that PELP1 interacts with and functions as a coactivator of β -catenin. Knockdown of PELP1 resulted in a

significant increase in survival of mice implanted either with orthotopic GBM xenografts or GBM PDX models. The current study provides the first evidence that PELP1 function as a coactivator of β -catenin and that the PELP1/ β -catenin axis is essential for GBM progression. This study significantly advances our understanding of how PELP1 exerts its oncogenic functions in GBM and suggests PELP1 may be a novel target for therapeutic intervention for GBM.

angiogenesis, a hypoxic and immunosuppressive microenvironment and resistance to standard treatments.^{5,6} GBM development is suspected to involve alterations in multiple tumor suppressors as well as oncogenes.^{7,8} Understanding the molecular pathways that contribute to GBM progression are urgently needed and could elucidate novel therapeutic targets for GBM.

Proline-, glutamic acid-, and leucine-rich protein 1 (PELP1) is a scaffolding protein that functions as a coregulator for several nuclear receptors (NRs) including estrogen receptor (ER), androgen receptor (AR), and glucocorticoid receptor (GR).^{9–13} PELP1 signaling is implicated in the progression of several cancers.^{14–21} Recent studies demonstrated that PELP1 is highly expressed in the brain.^{22–25} However, little is known about the status and role of PELP1 in GBM pathogenesis.

PELP1 plays an essential role in several pathways²⁶ including NR signaling, proliferation, cell cycle progression, ribosomal biogenesis, and DNA damage response.^{27,28} Notably, PELP1 has a histone binding domain,²⁹ recognizes histone modifications and interacts with several chromatin-modifying complexes including KDM1A,²⁹ HDAC,³⁰ PRMT,³¹ and CARM1.³² PELP1 interacts with MTP53, regulates its recruitment, and alters epigenetic marks at target gene promoters.³³ These emerging findings implicate PELP1's oncogenic signaling in cancer progression by modulating multiple pathways. Yet, it remains unclear whether PELP1 regulated pathways play a role in GBM progression.

In the present study, we investigated the role and significance of PELP1 in GBM pathogenesis. Using glioma tissue microarray, we found that PELP1 is highly expressed in high grade gliomas. Silencing of PELP1 resulted in an inhibition of GBM cell viability, survival and invasion. RNA sequencing of GBM cells and mechanistic studies revealed that PELP1 acts as a coactivator of β -catenin. Our results demonstrate that the knockdown of PELP1 suppressed the Wnt signaling pathway in GBM cells, and improved survival of the mice bearing GBM.

Collection (ATCC, Manassas, VA) and maintained as per ATCC guidelines. All the cells were passaged in the laboratory for fewer than 15 passages after receipt or resuscitation. Normal human astrocytes were purchased from ScienCell Research Laboratories (Carlsbad, CA) and maintained in astrocyte medium. Neurobasal medium and B27 serum-free supplement were obtained from Invitrogen (Carlsbad, CA). All model cells utilized were free of Mycoplasma contamination and was confirmed by using Mycoplasma PCR Detection Kit (Sigma Chemical Co, St. Louis, MO). Short tandem repeat (STR) polymorphism analysis of the cells was used to confirm their identity. Patient-derived primary GBM cells were isolated from surgically discarded specimens obtained from patients undergoing surgery using an UT Health San Antonio Institutional Review Board–approved protocol and their characterization was earlier described.^{34–37} All patients were provided a copy of informed consent for the use of the discarded tissues in research. All the studies were conducted in accordance with the declaration of Helsinki and the standards defined by UTHSA Institutional Review Board. Primary GBM lines GBM-082209, GBM-101310, GBM-111010, GBM-080409, GBM-090909, and GBM-092208 were cultured in neurobasal medium supplemented with B27 serum-free supplement, EGF (20 ng/mL), bFGF (20 ng/mL), LIF (10 ng/mL), and heparin (5 μ g/mL) as described.^{34,37} PELP1 On-Targetplus-SMART pool siRNAs (cat#L004463-00-0050) which consists a pool of four siRNA duplexes which are designed to target distinct sites within the *PELP1* gene were obtained from Thermo Scientific (Waltham, MA). The PELP1 antibodies were obtained from Bethyl, and β -catenin, phospho- β -catenin (Thr41/Ser45), MMP-2, and MMP-3 antibodies were purchased from Cell Signaling Technology (Beverly, MA). PELP1 specific shRNA lentivirus plasmids (PELP1-shRNA1:cat#TRCN0000159193; PELP1-shRNA2:cat#TRCN0000159673), β -actin and all secondary antibodies were purchased from Sigma Chemical Co (St. Louis, MO). Ki67 antibody was purchased from Abcam (Cambridge, MA). Glioblastoma cells stably expressing PELP1-shRNA were generated using human specific Lentiviral PELP1-shRNA particles. Stable clones were selected with puromycin selection (1 μ g/mL) and pooled clones were used for all the studies. Lentiviral particles expressing nontargeted short hairpin RNA (shRNA) were used to generate control cells.

Materials and Methods

Cell Lines and Reagents

Human GBM cell lines U87, U251, T98G, LN229, U138, and M059J were obtained from the American Type Culture

Cell Lysis and Western Blotting

Whole cell lysates were prepared from GBM cells using RIPA buffer containing protease and phosphatase inhibitors (Sigma Chemical Co, St. Louis, MO). Total proteins (50 μ g) were mixed with SDS sample buffer and run on SDS-PAGE gels. The resolved proteins were transferred onto nitrocellulose membranes and the blots were blocked with 5% non-fat dry milk powder for 1 h at room temperature. Primary antibody incubation was carried out at 4°C for overnight followed by incubation with secondary antibodies for 1 h at room temperature. Blots were developed using the ECL kit (Thermo Scientific, Waltham, MA).

Cell Proliferation and Clonogenic Assays

Cell proliferation rates were measured by using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). GBM cells transduced with either control shRNA or PELP1 shRNA1 or PELP1 shRNA2 were seeded in 96 well plates (2×10^3 cells/well). After various time intervals, the total ATP content as an estimate of total number of viable cells was measured on automatic Fluoroskan Luminometer according to the manufacturer's instructions. For the clonogenic assays, U251 and T98G cells stably expressing either control shRNA or PELP1 shRNA1 or PELP1 shRNA2 (500 cells/well) were seeded in 6-well plates and allowed to grow for an additional 8 days. The cells were then fixed in ice cold methanol and stained with 0.5% crystal violet solution to visualize the colonies. Colonies that contained ≥ 50 cells were counted.

Cell Migration and Invasion Assays

The cell migration rates of control and PELP1 silenced GBM cells were determined using a colorimetric QCM chemotaxis cell migration assay (EMD Millipore, Billerica, MA) as per the manufacturer's instructions. The invasive potential of control and PELP1 knockdown cells were determined using Corning® BioCoat™ Growth Factor Reduced Matrigel Invasion Chamber assay according to the manufacturer's instructions.

RNA Sequencing and RT-qPCR Analysis

U87 cells were transfected with either control siRNA or PELP1 siRNA using oligofectamine. After 72 h of transfection, total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). RNA sequencing and analysis was performed as described previously.³⁷ Selected genes were validated by quantitative real-time-PCR (RT-qPCR) using gene-specific primer sequences obtained from Harvard Primer Bank (<http://pga.mgh.harvard.edu/primerbank/>). Reverse transcription (RT) reactions were performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). RT-qPCR was performed using SYBR Green (Thermo Fisher Scientific). Results were normalized to the β -actin or GAPDH transcript levels and the difference in fold expression was calculated by using the delta-delta-CT method.

Immunoprecipitation and GST Pulldown Assays

U87 cells were treated with LiCl (20 mM) for 6 h and cell lysates were prepared using NP40/TritonX100-lysis buffer (50 mM Tris-HCl at pH 7.5, 0.2% Triton X-100, 0.3% Nonidet P-40, 150 mM NaCl, 25 mM NaF, 0.1 mM sodium orthovanadate) containing protease and phosphatase inhibitors. Cell lysates were immunoprecipitated with IgG or PELP1 or β -catenin antibodies, followed by Western blotting with PELP1 or β -catenin antibodies. For GST pulldown assays, U87 cell lysates were incubated with GST or PELP1-GST full length or various PELP1-GST deletion fragments, bound proteins were isolated by GST-pull-down assay, and the interaction of β -catenin with PELP1 was analyzed by Western blotting. PELP1-GST fragments were generated as described previously.²²

Reporter Gene Assays

Reporter gene assays were performed as described.³⁴ Briefly, U87, U251, T98G, 293T, and primary GBM cells were seeded in 24 well plates and incubated overnight, the cells were then transfected with pTOPFLASH, or pFOPFLASH plasmids along with PELP1 or FHL2 or control expression vectors using Fugene transfection reagent. pSV- β -Galactosidase or pRL control vector was used as an internal control. For some assays, after 48 h transfection, cells were stimulated with 20 mM LiCl for 12 h and luciferase activity was measured using the luciferase assay system (Promega, Madison, WI). Luciferase activity was expressed as percent of relative light units versus untreated transfected cells.

Tissue Microarrays and Immunohistochemistry

The tissue microarrays (TMA, Cat#GL2083a) were obtained from US Biomax, Inc (Rockville, MD). Each TMA comprised 0.6-mm cores taken from paraffin-embedded specimens that represent a total of 192 glioma tissues (132 cases of brain astrocytoma, 31 brain GBM, 7 brain oligo-astrocytoma, 9 brain oligodendroglioma, 11 brain ependymoma, 1 each of brain ganglioglioma and gliosarcoma) plus 8 each of adjacent normal brain tissue and normal brain tissue. Immunohistochemical analysis was performed as described previously.³⁶ We have excluded oligoastrocytomas from this study due to their mixed origin and ganglioglioma and gliosarcoma due to low sample number. The tumor specimens that were damaged during slide processing were also excluded in the analysis. PELP1 immunoreactivity on TMA was scored using Allred scoring system.³⁸ Briefly, the PELP1 staining intensity was scored on a scale between zero and three and the proportion of positive stained cells was rated as one between 0 and 1%, two between 1% and 10%, three between 10% and 33%, four between 33% and 66%, and five between 66% and 100%. Xenograft tumor sections were incubated overnight with Ki67, PELP1, MMP-2, MMP-3, and β -catenin primary antibodies for overnight. After secondary antibody incubation for 45 min, immunoreactivity was visualized by using the DAB substrate and counterstained

with haematoxylin (Vector Lab, Inc., Burlingame, CA). The proliferative index was calculated as percentage of Ki-67-positive cells and the staining intensity of PELP1, β -catenin, MMP-2, and MMP-3 on xenograft tumor sections was quantified using ImageJ analysis software. Briefly, the image was subjected to color deconvolution and the mean DAB intensity was measured using H DAB vector plug in and the resulting D-HSCORE values were plotted in histogram as described previously.³⁹

Orthotopic Tumor Assays

All animal experiments were performed after obtaining UT Health San Antonio Institutional Animal Care and Use Committee approval. Male athymic nude mice of 8–10 weeks old were purchased from Charles River Laboratories. U87 cells expressing control shRNA or PELP1 shRNA1 were labeled with the GFP-Luciferase reporter and 1×10^6 cells were injected orthotopically into the right cerebrum of a mouse using an established protocol.³⁷ For primary GBM cell implantation, NCI SCID/NCr mice were used. Primary GBM cells GBM-101310 (1×10^5) were injected orthotopically into the right cerebrum of SCID mouse. Tumor progression was monitored weekly using the Xenogen *in vivo* imaging system. Mice were euthanized and brains collected upon development of symptomatic neurological deficits or becoming moribund. Mouse survival was determined using the Kaplan–Meier survival curves and log-rank test using GraphPad Prism 6 software.

Statistical Analysis

Statistical differences between the groups were analyzed with unpaired Student *t*-test and one-way ANOVA using GraphPad Prism 6 software. All the data represented in the graphs are shown as means \pm SE. A value of $P < .05$ was considered as statistically significant.

Results

PELP1 Is Highly Expressed in Glioblastoma

PELP1 expression is upregulated in many cancers.⁴⁰ To examine the status of PELP1 in GBM, we performed immunohistochemistry (IHC) for PELP1 using human glioma tissue micro arrays that contained different grades of glioma and normal brain tissues. Tissues were scored for intensity of staining and IHC score as described previously.⁴¹ The representative staining for each tumor type is shown in [Figure 1A](#). Increased PELP1 expression was observed in all glioma subtypes compared to normal brain samples with the highest expression observed in GBM ([Figure 1B](#)). PELP1 immunoreactivity was predominantly observed in nucleus of normal brain, grade II astrocytoma, grade III astrocytoma, GBM, oligodendroglioma, ependymoma, anaplastic ependymoma, which showed positivity rates of 93.7% (15/16), 95.4% (104/109), 100% (21/21), 96.7% (30/31), 100% (9/9), 100% (4/4), 100% (7/7), respectively. The cytoplasmic positivity for PELP1 was observed in 56.2% (9/16) of

normal brain, 26.6% (29/109) of grade II astrocytoma, 61.9% (13/21) of grade III astrocytoma, 32.2% (10/31) of GBM, 11.1% (1/9) of oligodendroglioma, and 14.2% (1/7) of anaplastic ependymoma. Next, we examined the expression of PELP1 in normal astrocytes, established GBM cell lines and patient-derived GBM cells. As shown in [Figure 1C](#) and [D](#), all GBM cells lines showed higher PELP1 expression compared to normal astrocytes (NA). Collectively these results suggest that tumors and GBM cells express high levels of PELP1 as compared to normal brain tissue and astrocytes.

PELP1 Knockdown Reduces the Cell Viability and Survival of GBM Cells

To examine the functional role of PELP1 in GBM cells, PELP1 expression was silenced or knocked down using validated PELP1 specific siRNA and shRNA, respectively. U87, U251, T98G, and primary GBM cells were transduced with either control shRNA or PELP1 shRNA1 or PELP1 shRNA2 lentiviral particles. Cell viability assays revealed that PELP1 knock-down significantly reduced the cell viability of U87, U251, T98G, and GBM10 cells when compared to control shRNA expressing cells ([Figure 2A–D](#)). The knock-down of PELP1 in GBM cells was validated using Western blot analysis ([Figure 2E](#)). Furthermore, the effect of PELP1 knock-down on the survival of GBM cells was examined using clonogenic assays. As shown in [Figure 2F](#), both U251 and T98G cells that stably express PELP1shRNA1 or PELP1 shRNA2 showed a reduced number of colonies compared to control shRNA cells. These results suggest that knock-down of PELP1 reduced GBM cell viability and survival.

PELP1 Knockdown Reduces Migration and Invasion of GBM Cells

We next examined the effect of PELP1 knockdown on the migration and invasion of GBM cells. Migration assays were carried out using trans-well migration assays. As shown in [Figure 2G](#), PELP1 silencing significantly reduces the migration of U87 and T98G GBM cells when compared to control siRNA cells. Furthermore, the effect of PELP1 knockdown on invasion of GBM cells was examined using matrigel invasion chamber assay. As shown in [Figure 2H](#), knockdown of PELP1 significantly reduced the invasion of U87, U251, and primary GBM10 cells when compared to control shRNA cells. These results suggest that PELP1 knockdown reduced the migration and invasion of GBM cells.

Transcriptomic Analysis of PELP1 Modulated Genes

To understand the mechanisms by which PELP1 mediates GBM progression, we performed RNA-seq analysis using U87 cells that were transfected with either control siRNA or PELP1 specific siRNA. The silencing of PELP1 resulted in significant changes in the expression of 792 genes (1.5-fold change over control with adjusted P -value $< .05$) of which 521 genes were downregulated and 271 genes were upregulated. The complete list is available in the GEO database under accession number GSE131502. Among the groups, the

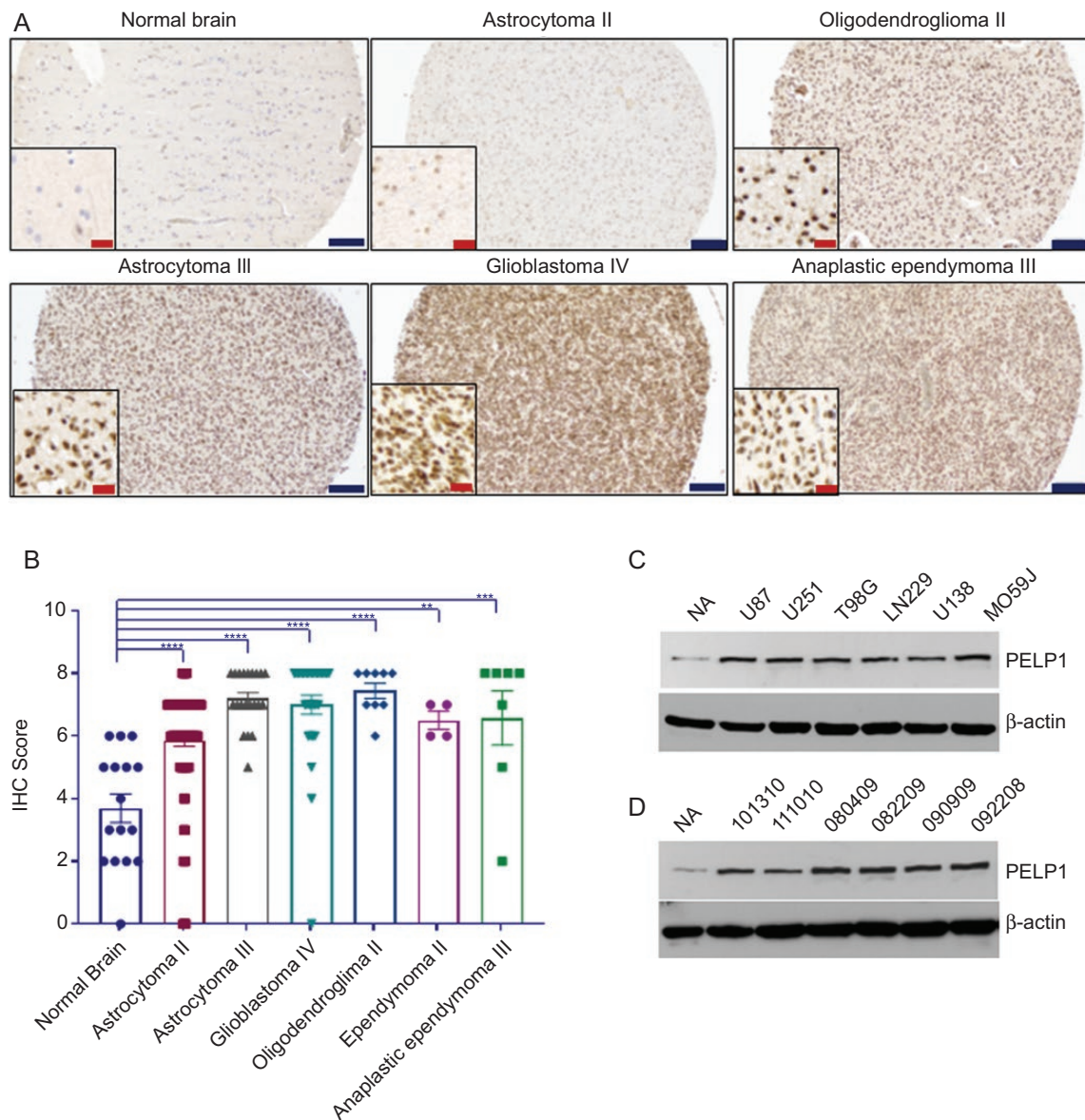


Figure 1. PELP1 is highly expressed in gliomas. (A) Glioma tissue array containing normal brain ($n = 16$), as well as grade II astrocytoma ($n = 109$), grade III astrocytoma ($n = 21$), grade IV glioblastoma ($n = 31$), grade II oligodendrogloma ($n = 9$), grade II ependymoma ($n = 4$) (representative data not shown), anaplastic ependymoma ($n = 7$) samples were subjected to immunohistochemical staining with PELP1 antibody as described in the methods. Scale bars represent $100 \mu\text{m}$ (blue color) and $20 \mu\text{m}$ (red color). (B) Quantitation of IHC was done as described in methods section. Data are represented as mean \pm SE. ** $P < .01$; *** $P < .001$; **** $P < .0001$. (C and D) Expression of PELP1 protein in established and patient-derived GBM cell lines was analyzed by Western blotting; NA, normal astrocytes.

differentially expressed genes were shown in the heat map (Figure 3A). The biological significance of differentially expressed genes was examined using ingenuity pathway analysis (IPA) (Figure 3B) and gene set enrichment analysis (GSEA) (Figure 3C). The IPA of differentially expressed genes between U87-control siRNA vs U87-PELP1 siRNA cells revealed the downregulation of genes related to inhibition of matrix metalloproteases, glioma invasion signaling, Wnt/ β -catenin signaling, and other pathways related to invasion and GBM signaling (Figure 3B). More importantly, GSEA

revealed that Wnt/ β -catenin signaling and epithelial mesenchymal transition gene sets showed negative correlation in PELP1 siRNA group compared to control (Figure 3C).

PELP1 Modulates Wnt/ β -Catenin Signaling in GBM Cells

Next, we tested whether PELP1 modulates the expression of Wnt/ β -catenin target genes using RT-qPCR assays in two

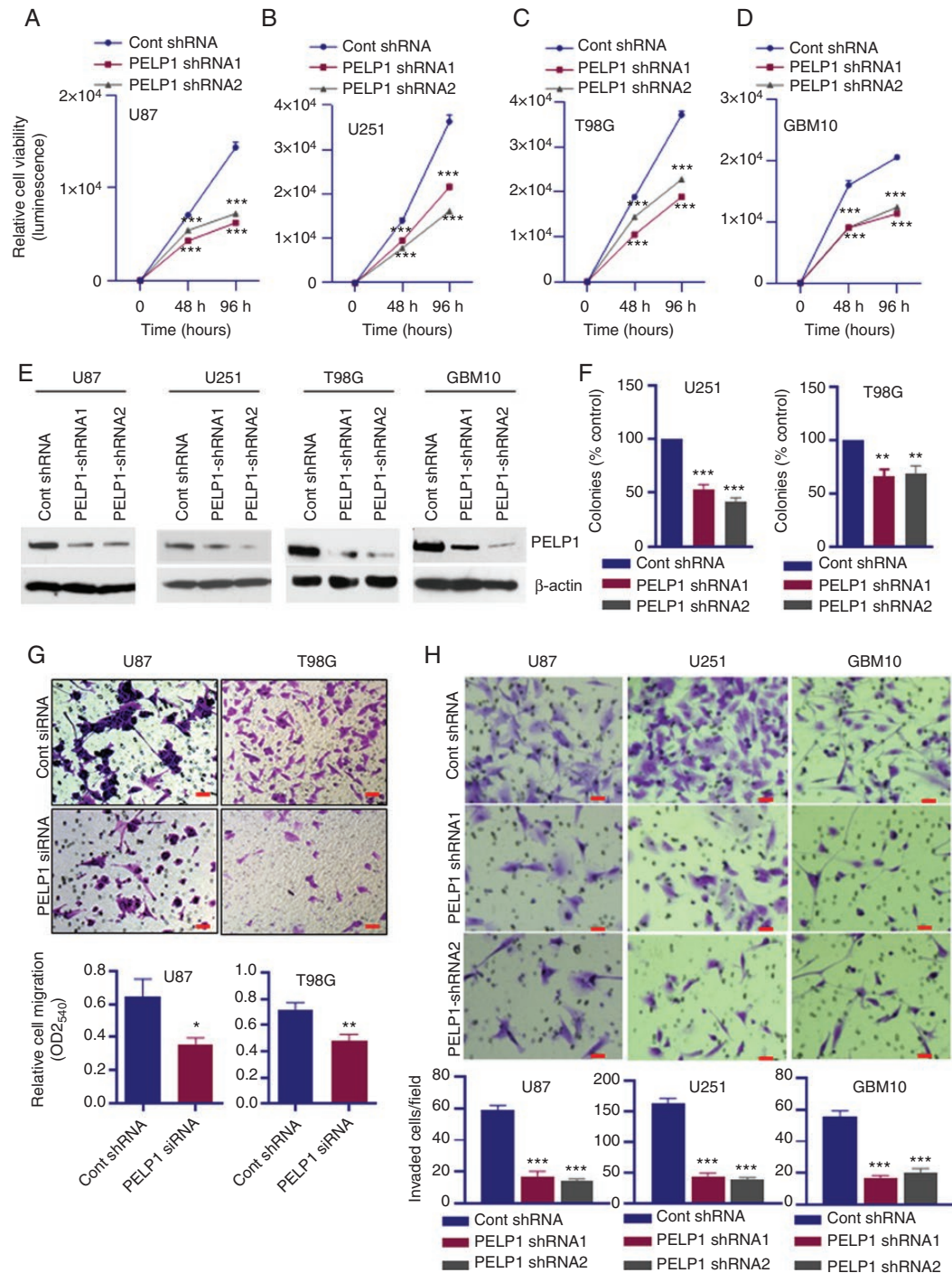


Figure 2. PELP1 knockdown reduced the cell viability and survival of GBM cells. U87 (A), U251 (B) T98G (C) and GBM10 (D) cells were transfected with either control shRNA or PELP1 shRNA1 or PELP1 shRNA2. The cell proliferation rates were measured using CellTiter-Glo assay. (E) Expression of PELP1 in shRNA model cells was confirmed by Western blotting. (F) Clonogenic survival of U251 and T98G cells stably expressing control shRNA or PELP1 shRNA1 or PELP1 shRNA2 was examined using colony formation assay. (G) Cell migration potential of U87 and T98G cells transiently expressing control siRNA or PELP1 siRNA was analyzed using QCM chemotaxis cell migration assay. Representative images of migrated cells in various groups are shown (upper panel) and migrated cells in terms of colorimetric intensity (OD) was shown (lower panel). Scale bars represent 100 μ m (red color). (H) Cell invasion potential of U87, U251, and GBM10 cells stably expressing control shRNA or PELP1 shRNA1 or PELP1 shRNA2 was analyzed using BioCoat invasion chamber assay. Representative images of invaded cells in various groups are shown (upper panel) and invaded cells were quantitated in five random fields (lower panel). Scale bars represent 100 μ m (red color). Data are represented as mean \pm SE. * $P < .05$; ** $P < .01$; *** $P < .001$.

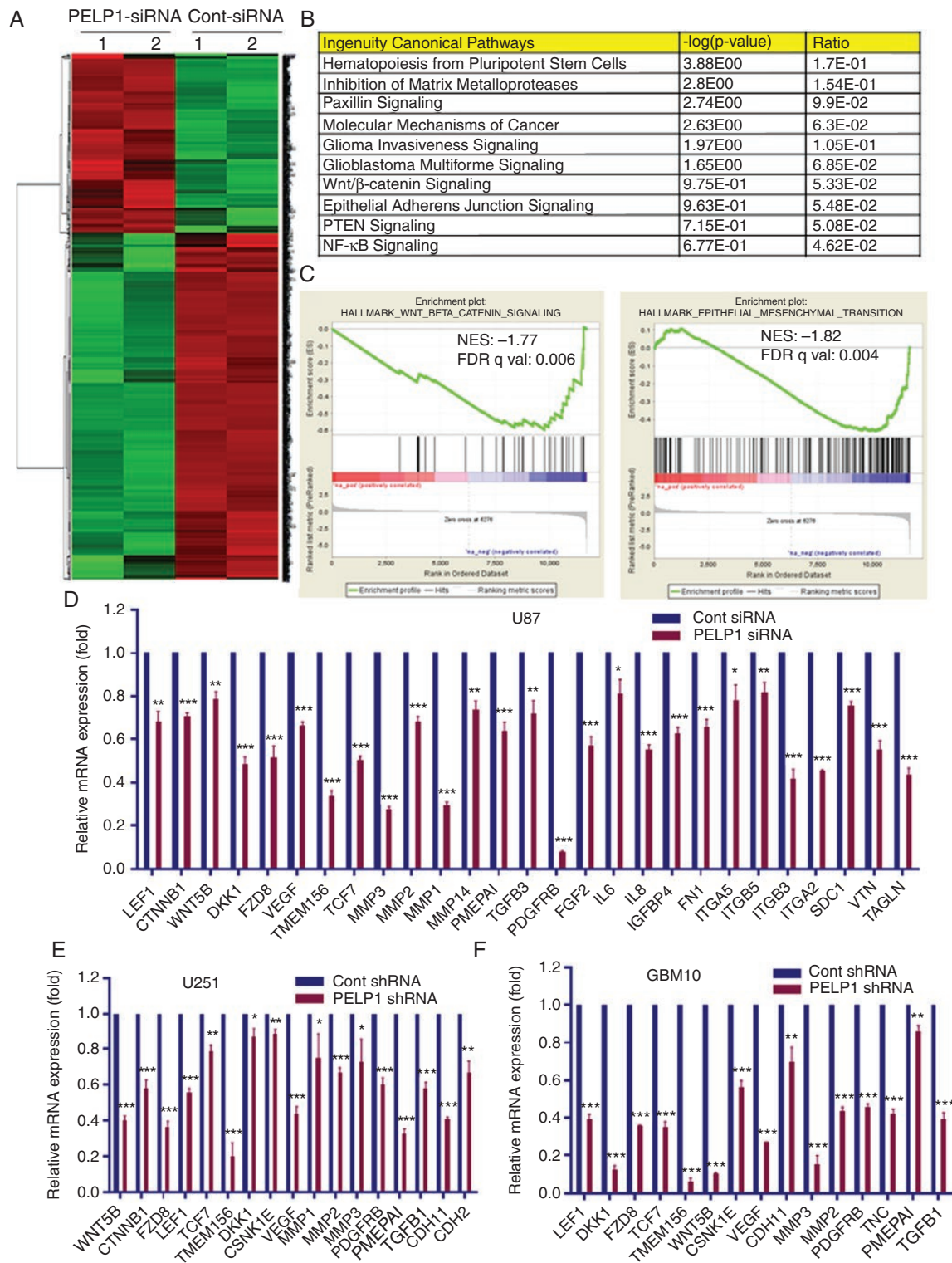


Figure 3. Analysis of global transcriptional changes modulated by PELP1 in GBM cells. U87 cells were transfected with control siRNA or PELP1 siRNA. After 72 h RNA was isolated and subjected to RNA sequencing. (A) Heat map of differentially expressed genes between control and PELP1 siRNA is depicted. (B) Differentially expressed genes were subjected to pathway analysis using IPA software, and the selected top canonical pathways are shown. (C) Gene set enrichment analysis (GSEA) testing correlation of PELP1-regulated genes with signatures of Wnt/ β -catenin signaling and epithelial mesenchymal transition gene sets. (D) Selective genes representing each pathway were validated in U87 control siRNA or PELP1 siRNA transfected cells, and U251 (E) and GBM10 (F) cells stably expressing control shRNA or PELP1 shRNA1. Data are represented as mean \pm SE. * $P < .05$; ** $P < .01$; *** $P < .001$.

established GBM cells (U87, U251) and a patient derived GBM cells (GBM10). As shown in [Figure 3D–F](#), the expression of several Wnt/ β -catenin signaling molecules such as *LEF1*, *CTNNB1*, *WNT5B*, *DKK1*, *FZD8*, and *TCF7* were significantly downregulated in PELP1 knockdown cells. Furthermore, the expression of genes involved in cellular invasion and EMT process such as matrix metalloproteases (*MMP-1*, *MMP-2*, *MMP-3*, *MMP-14*) and integrin molecules (*ITGA2*, *ITGB5*, *ITGA5*) were significantly downregulated in PELP1 knockdown cells compared to control cells. Collectively, these results suggest that PELP1 has the potential to modulate Wnt/ β -catenin signaling in GBM cells.

PELP1 Interacts with and Functions as Coactivator of β -Catenin

To test whether PELP1 interacts with β -catenin, we performed co-IP assays. Results showed that PELP1 interacts with β -catenin ([Figure 4A](#), left panel). Reciprocal co-IP also showed that β -catenin interacts with PELP1 ([Figure 4A](#), right panel). Further GST pull down assays also confirmed the interaction of PELP1 with β -catenin ([Figure 4B](#)). GST pulldown assays using various domains of PELP1 as GST fusions revealed that β -catenin interacts with the 400–600 aa region of PELP1 ([Figure 4C](#)). To test whether PELP1 acts as a coactivator for β -catenin, we used the TOPFLASH (TCF/LEF-Firefly luciferase) reporter assay. Co-transfection of PELP1 along with the reporter into U251 and GBM10 cells resulted in significant activation of the reporter ([Figure 4D](#) and [E](#)). Furthermore, knockdown of PELP1 significantly reduced reporter activity compared to control shRNA cells ([Figure 4F](#) and [G](#)). In addition, LiCl mediated activation of TOPFLASH reporter activity was significantly attenuated in PELP1 knockdown cells ([Figure 4H](#)).

PELP1 is Needed for FHL2 Coactivation Function of β -catenin.

Since PELP1 couples four and a half LIM domains protein 2 (FHL2) to nuclear receptors such as AR⁴² and because, FHL2 acts as coactivator of β -catenin,⁴³ we further examined whether the PELP1 and FHL2 interaction was vital for β -catenin mediated transcription using the TOPFLASH reporter. As expected, FHL2 significantly increased the reporter activity in U87 and T98G cells, however, FHL2 mediated increase in TOPFLASH activity was significantly attenuated in PELP1 knockdown cells ([Figure 5A](#)). We also confirmed PELP1/FHL2 regulation of TOPFLASH reporter using HEK293T cells. PELP1 and FHL2 co-transfection significantly increased the TOPFLASH reporter activity compared to PELP1 or FHL2 alone both in unstimulated and stimulated (LiCl treatment) conditions ([Figure 5B](#)). To corroborate with reporter gene activity, we subsequently examined expression of β -catenin target gene *Axin2* in PELP1 knockdown GBM cells and 293T cells following stimulation with LiCl. As shown in [Figure 5C](#), stimulation with LiCl significantly increased the expression of *Axin2* in GBM cells and HEK293T cells; and this increase was significantly compromised in PELP1 knockdown cells. To further study whether PELP1 knockdown affect the stability of β -catenin, we examined the status of phospho- β -catenin in control and PELP1

knockdown cells. Western blot analysis revealed that PELP1 knockdown substantially increased the phosphorylation of β -catenin compared to controls ([Figure 5D](#)) suggesting that PELP1 knockdown leads to destabilization of β -catenin.

PELP1 Knockdown Reduces GBM Progression *In Vivo* and Enhances the Mice Survival

To study the effect of PELP1 knockdown on *in vivo* tumor growth, we used, both U87 and patient-derived GBM10 cells as orthotopic xenografts. Luciferase-labeled U87 or GBM10 cells stably expressing control shRNA or PELP1 shRNA1 were injected into mouse brain and tumor progression was measured using the Xenogen *in vivo* imaging system. As shown in [Figure 6A](#) and [B](#), PELP1 knockdown significantly reduced tumor growth in U87 tumor bearing mice when compared to control mice. Furthermore, survival analysis demonstrated that PELP1 knockdown significantly improved the survival of U87 tumor bearing mice when compared to control mice ([Figure 6C](#)). More importantly, PELP1 knockdown significantly increased the survival of the mice implanted with primary GBM10 cells ([Figure 6D](#)). We further examined the expression levels of the proliferation marker Ki67 in control and PELP1 shRNA1 tumors using immunohistochemistry. As shown in [Figure 6E](#), Ki67 positive cells were significantly lower in PELP1 shRNA1 U87 tumors compared to control shRNA U87 tumors. IHC analysis of tumor sections revealed that PELP1 shRNA expressing U87 tumors had decreased expression of β -catenin, MMP-2, and MMP-3 than control shRNA expressing tumors ([Figure 6E](#)). These results suggest that PELP1 knockdown reduced *in vivo* GBM progression via downregulation of the β -catenin pathway.

Discussion

Proline-, glutamic acid-, and leucine-rich protein 1 (PELP1),¹⁴ originally cloned in our lab, plays a critical role in signal transduction by multiple nuclear receptors and transcription factors.^{27,40} PELP1 is expressed in many tissues, with the highest expression noted in the brain.^{22,23,44} PELP1 signaling plays an important role in the progression of many cancers, however, little is known about the role of PELP1 in GBM. In this study, we provided evidence that PELP1 is overexpressed in GBM and plays an important role in GBM cell proliferation, survival and invasion. Using mechanistic studies, we demonstrated that PELP1 directly interacts with β -catenin and modulates its transactivation functions. RNAseq studies identified β -catenin signaling as the top pathway modulated by PELP1 in GBM. Quantitative RT-PCR studies using multiple GBM models confirmed that PELP1 knockdown significantly reduced the expression of β -catenin target genes involved in survival and invasion. Furthermore, PELP1 knockdown significantly increased the survival of mice in orthotopic GBM models. Collectively, these results suggest that PELP1 plays a crucial role in GBM progression.

Oncogenic PELP1 signaling is implicated in the progression of several cancers including breast,¹⁴

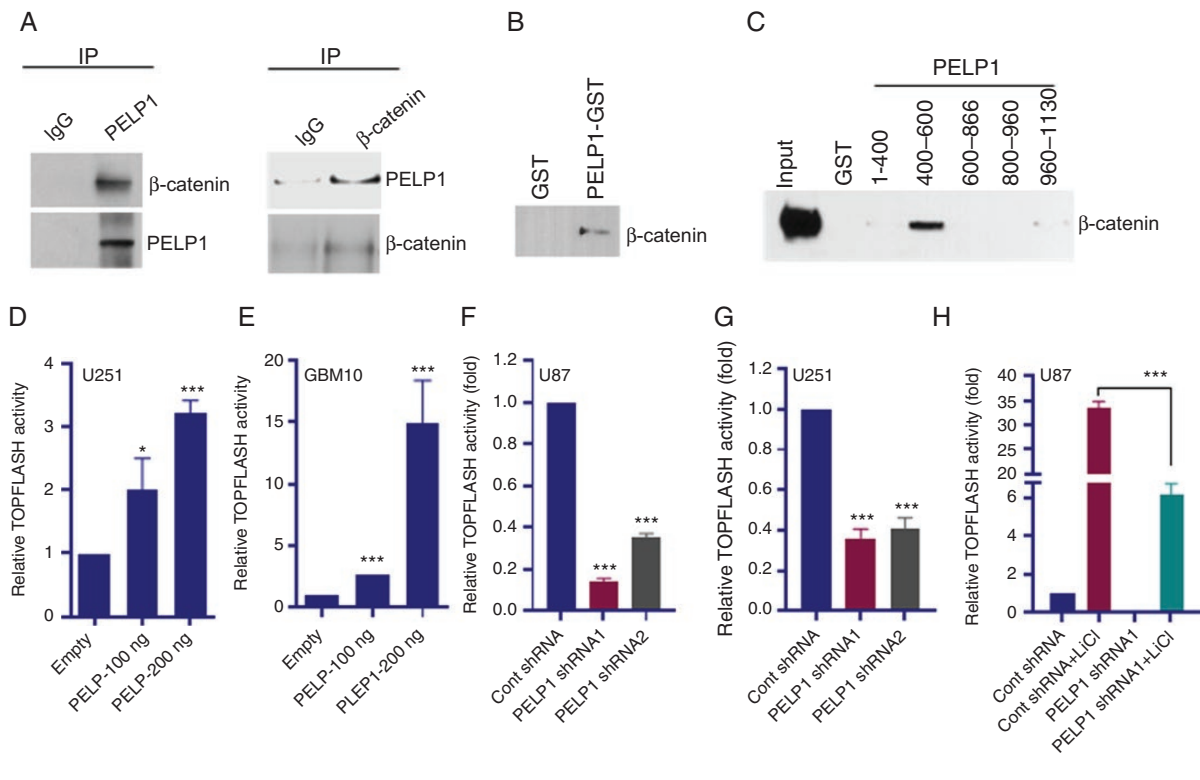


Figure 4. PELP1 interacts with and functions as a coactivator of β -catenin. (A) U87 cells were stimulated with LiCl (20 mM) for 6 h and lysates were subjected to immunoprecipitation with β -catenin antibody (right panel) or PELP1 antibody (left panel) and the interaction with PELP1 or β -catenin was confirmed using Western blotting respectively. (B) U87 cell lysates were used in pull-down assays using GST or PELP1-GST, and interaction of β -catenin with PELP1 was examined by Western blotting. (C) U87 cell lysates were incubated with GST or various PELP1-GST deletion fragments and immunoprecipitates were subjected to GST pull down and the interaction of β -catenin with PELP1-GST fragments was examined by Western blotting. U251 (D) and GBM10 (E) cells were transfected with TOPFLASH and β -galactosidase vectors along with either empty vector or PELP1 expression vector and after 48 h, reporter activity was measured as described in the Methods. (F and G) U87 and U251 cells stably expressing control shRNA or PELP1 shRNA1 or PELP1 shRNA2 were transfected with TOPFLASH and pRL vectors and after 48 h, luciferase activity was measured using dual luciferase assay system. (H) U87 control shRNA or PELP1 shRNA1 cells were transfected with TOPFLASH and β -galactosidase vectors and after 48 h, cells were stimulated with LiCl (20 mM) for 12 h and the reporter activity was measured. Data are represented as mean \pm SE. * $P < .05$; *** $P < .001$.

endometrial,¹⁵ ovarian,¹⁶ salivary,¹⁷ prostate,¹⁸ lung,¹⁹ pancreas,²⁰ and colon.²¹ In transgenic mouse models, PELP1 overexpression in the mammary glands contributes to mammary gland carcinoma, further supporting its oncogenic potential *in vivo*.⁴⁵ PELP1 expression is an independent prognostic predictor of shorter breast cancer-specific survival and disease-free interval.⁴⁶ PELP1 expression has also been identified as a prognostic factor for predicting poor survivorship in patients diagnosed with TNBC.^{47,48} Our results using glioma TMA of 208 patients revealed that PELP1 is highly expressed in glial tumors compared to normal brain tissues. Furthermore, an increase in PELP1 expression was strongly correlated with the degree of malignancy, and accordingly, the highest expression was noted in GBM (grade IV glioma). TCGA analysis of PELP1 transcript did not show an association of PELP1 mRNA with prognosis, and there is no upregulation of PELP1 mRNA in GBM compared to control and low grade tumors. In our study using IHC, we observed that PELP1

expression is greater in GBM, as compared to control brain and low grade astrocytomas. Several studies showed that PELP1 is post-translationally modified by phosphorylation, sumoylation, acetylation, and glutamylation at multiple sites, and these post-translational modifications controls PELP1 localization, modulates its interactions with adaptor proteins, and alters its stability, which contributes to its biological activity.^{20,49-51} Importantly, our recent study²² showed that PELP1 is phosphorylated by GSK3 β leading to its degradation. Since, PELP1 is modified post-translationally, and its biological activity as a coregulator is, in part, dictated by its protein levels, the TCGA mRNA expression data may not truly represent PELP1 status with patient survival, and IHC studies are more likely to provide biologically relevant information on PELP1 signaling that occur in tumors. Furthermore, PELP1 is highly expressed in neurons of specific brain regions, however, normal astrocytes exhibit little to no PELP1 expression. A recent study showed that PELP1 expression is associated with a worse

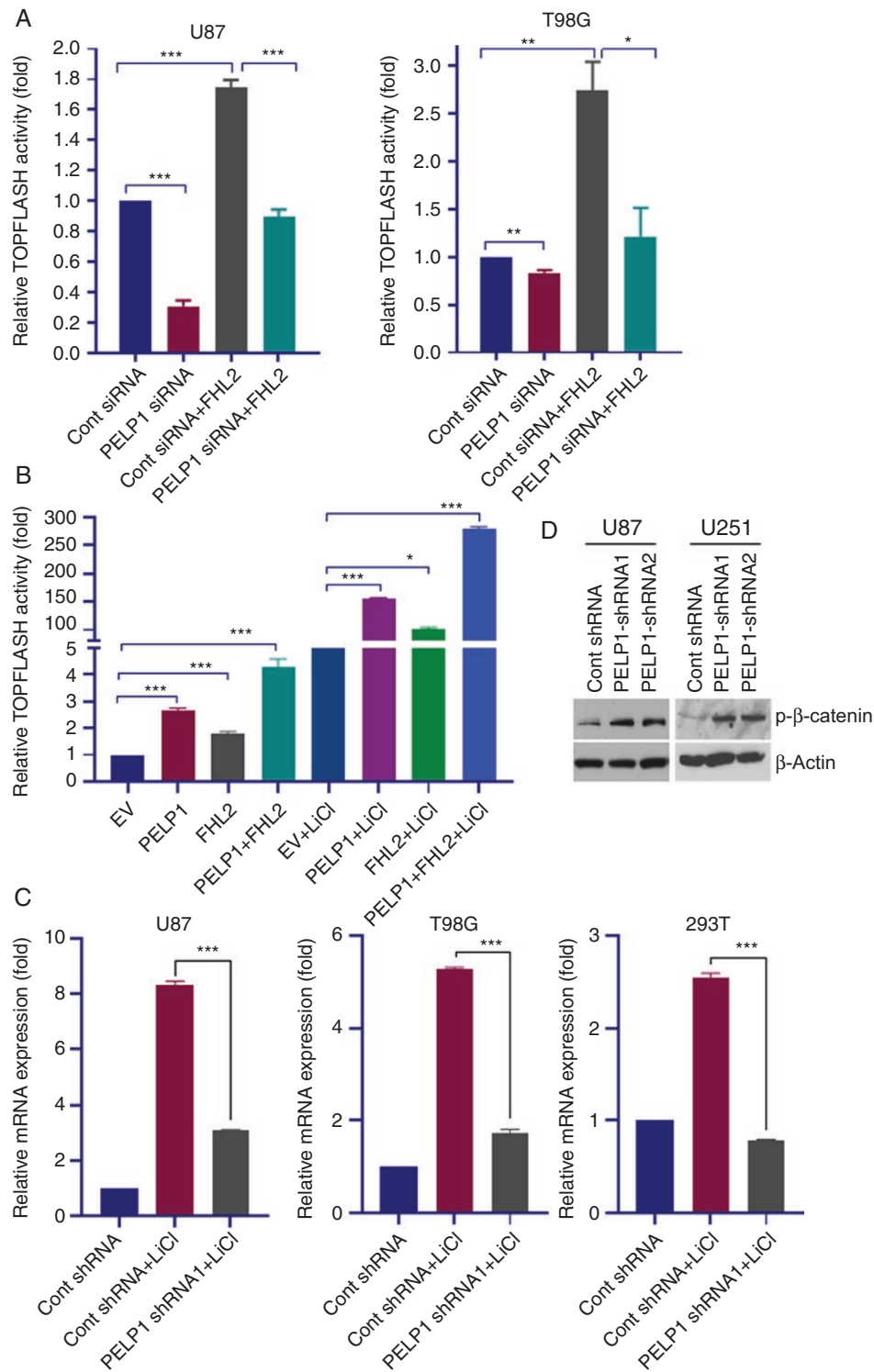


Figure 5. PELP1 is needed for FHL2 coactivation function of β -catenin. (A) U87 and T98G cells were transfected with control siRNA or PELP1 siRNA and after 24 h, cells were transfected again with TOPFLASH and β -galactosidase vectors along with either empty vector or FHL2 expression vector. After 48 h, TOPFLASH reporter activity was measured. (B) HEK293T cells were transfected with TOPFLASH and β -galactosidase vectors along with either empty vector or PELP1 or FHL2 expression vectors and after 48 h cells were stimulated with LiCl (20 mM) for 12 h and reporter activity was measured as described in the Methods. (C) U87, T98G, and 293T cells stably expressing control shRNA or PELP1 shRNA1 were stimulated with LiCl (20 mM) for 12 h and the expression of β -catenin target gene *Axin2* was examined using RT-qPCR. Data are represented as mean \pm SE. * $P < .05$; ** $P < .01$; *** $P < .001$. (D) U87 and U251 cells stably expressing control shRNA or PELP1 shRNA1 or PELP1 shRNA2 were subjected to western blotting with phospho- β -catenin antibody.

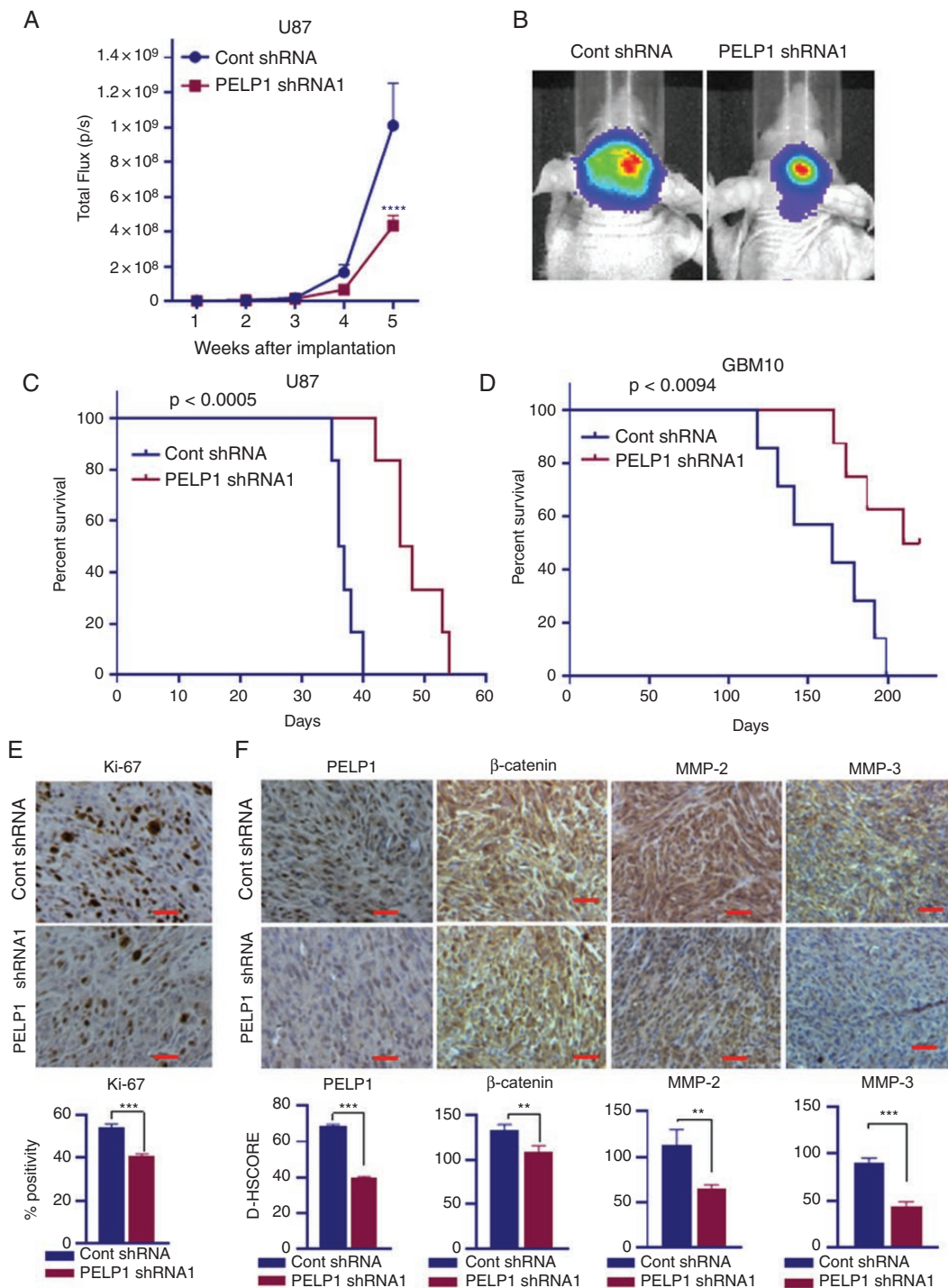


Figure 6. PELP1 knockdown reduced the GBM progression and increased survival of mice in orthotopic GBM models. (A) Athymic nude mice were implanted orthotopically into the right cerebrum with luciferase labeled U87-control shRNA or U87-PELP1 shRNA1 cells and the tumor growth in terms of luminescence was examined using Xenogen *in vivo* imaging system. (B) Representative tumor bearing mice are shown. (C) The survival of the U87 bearing mice was recorded and plotted using the Kaplan–Meier curve. (D) SCID/NCr mice were implanted with patient-derived primary GBM10 cells stably expressing control shRNA or PELP1 shRNA1 and survival of the mice was recorded and plotted using the Kaplan–Meier curve. (E) Mouse brains collected from U87 control shRNA and PELP1 shRNA1 groups, were fixed in formalin and processed for immunohistochemical staining for Ki67 and the proliferation index was calculated (lower panel). (F) PELP1, β -catenin, MMP-2 and MMP-3 immunohistochemistry was performed on tumor sections from U87 control shRNA and PELP1 shRNA1 groups. Representative images are shown (upper panel) and staining intensity was measured using ImageJ software and shown in histogram (lower panel). Scale bars represent 100 μ m (red color).

prognosis of astrocytoma in a small cohort of patients, and PELP1 expression is greater in GBM, as compared to low-grade gliomas,⁵² which is in agreement with our study.

PELP1 expression is associated with various genes involved in epithelial mesenchymal transition (EMT) and modulates the expression of metastasis-influencing microRNAs to promote cancer metastasis.³⁰ The β -catenin pathway plays an essential role in migration and invasion, and the EMT process in several malignancies.^{53–57} In this study, we found that knock down of PELP1 in GBM cells reduced their invasion and migration. Our RNA-seq results identified that PELP1 knockdown modulated several pathways related to the invasion and migration and GSEA results showed that PELP1 knockdown regulated genes were negatively correlated with the *EMT* gene set. Collectively, these findings support that PELP1 modulates GBM migration and invasion via modulation of the β -catenin pathway.

The Wnt signaling pathway plays a crucial role in normal embryonic development and abnormal pathological processes in vertebrates.⁵⁸ Activation of the Wnt pathway also plays a critical role in many cancers. Several studies including ours demonstrated that activation of Wnt/ β -catenin signaling contributes to the initiation and progression of gliomas and the components of this pathway were overexpressed in gliomas.^{59–62} β -Catenin is the critical mediator of canonical Wnt signaling. Recent studies demonstrated that β -catenin plays a crucial role in EMT processes of various malignancies,^{53–57} and promotes glioma aggressiveness through EMT.⁶³ Our study discovered, for the first time, that PELP1 functions as a novel coactivator of β -catenin. These results further implicate PELP1 as a mediator of the EMT process in GBM by functioning as a coactivator of the β -catenin pathway. Glioma stem cells (GSCs) plays a central role in tumor initiation, progression and therapy resistance of GBM.^{64,65} Importantly, Wnt/ β -catenin signaling pathway is critical in regulating GSCs.^{66–68} Our RNA-sequencing data identified that stem cell markers nestin, sox2, and vimentin were downregulated in PELP1 silenced cells suggesting that targeting PELP1 may be effective in reducing stemness of GBM via inhibition of Wnt/ β -catenin pathway. Recent studies also implicated Wnt/ β -catenin signaling as a regulator of resistance to standard chemotherapy of GBM.^{69–72} Since PELP1 functions as a coactivator of β -catenin signaling, inhibition of PELP1 may have a sensitizing effect with standard chemotherapy in GBM. Future studies are needed to confirm whether PELP1 utilizes the β -catenin pathway to modulate EMT, stemness, and chemoresistance in GBM.

PELP1 is scaffold protein that contains several protein–protein interaction motifs. PELP1 interacts with nuclear receptors using the nuclear receptor interaction motif LXXLL; Src kinase, using SH2 motifs; and PI3K, using PXXP motifs.⁴⁰ Notably, PELP1 has a histone binding domain, recognizes histone modifications and interacts with several chromatin-modifying complexes.^{29–32} In this study, we showed that PELP1 interacts with β -catenin and functions as a coactivator of its transcriptional functions. We previously showed that PELP1 interacts with FHL2 and synergistically activates the AR transcriptional functions.⁴² Interestingly, FHL2 also acts as a coactivator of β -catenin in

kidney and colon cancer cells.⁴³ In support of these studies, we showed that FHL2 activates β -catenin transactivation functions in GBM cells. Further PELP1 and FHL2 synergistically enhances β -catenin reporter activity suggesting that PELP1 modulation of β -catenin may involve multiple interactions in the transcription complex. Additional studies are needed to better understand the role of PELP1 and FHL2 in the modulation of β -catenin target genes.

In summary, the results of our study demonstrates that PELP1 protein expression is upregulated in GBM tissues, established GBM cells and primary GBM cells. Down regulation of PELP1 significantly reduced GBM growth and down regulated β -catenin signaling *in vitro* and *in vivo*. The present study provides the first evidence, to the best of our knowledge, of a coactivator function of PELP1 for β -catenin and that the PELP1/ β -catenin axis is essential for GBM progression. This study significantly advances our understanding of how PELP1 exerts its oncogenic functions in GBM and suggests that PELP1 may be a novel target for therapeutic intervention for GBM.

Keywords

β -catenin signaling | coregulator | glioblastoma | glioma | PELP1

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest statement: The authors declare no potential conflicts of interest.

Authorship statement

G.R.S., R.K.V., D.B., S.V., and A.J.B. designed the experiments and interpreted the results. G.R.S. and A.R.G. are involved in the IHC and scoring of TMAs. G.R.S., U.P.P., S.V., P.P.V., B.N., S.R.K., A.R.G., S.Z., conducted the experiments; G.R.S., R.K.V. and A.J.B., designed and conducted xenograft studies; R.K.V. and G.R.S., wrote the manuscript.

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