Supplementary Materials

Supplementary Figures

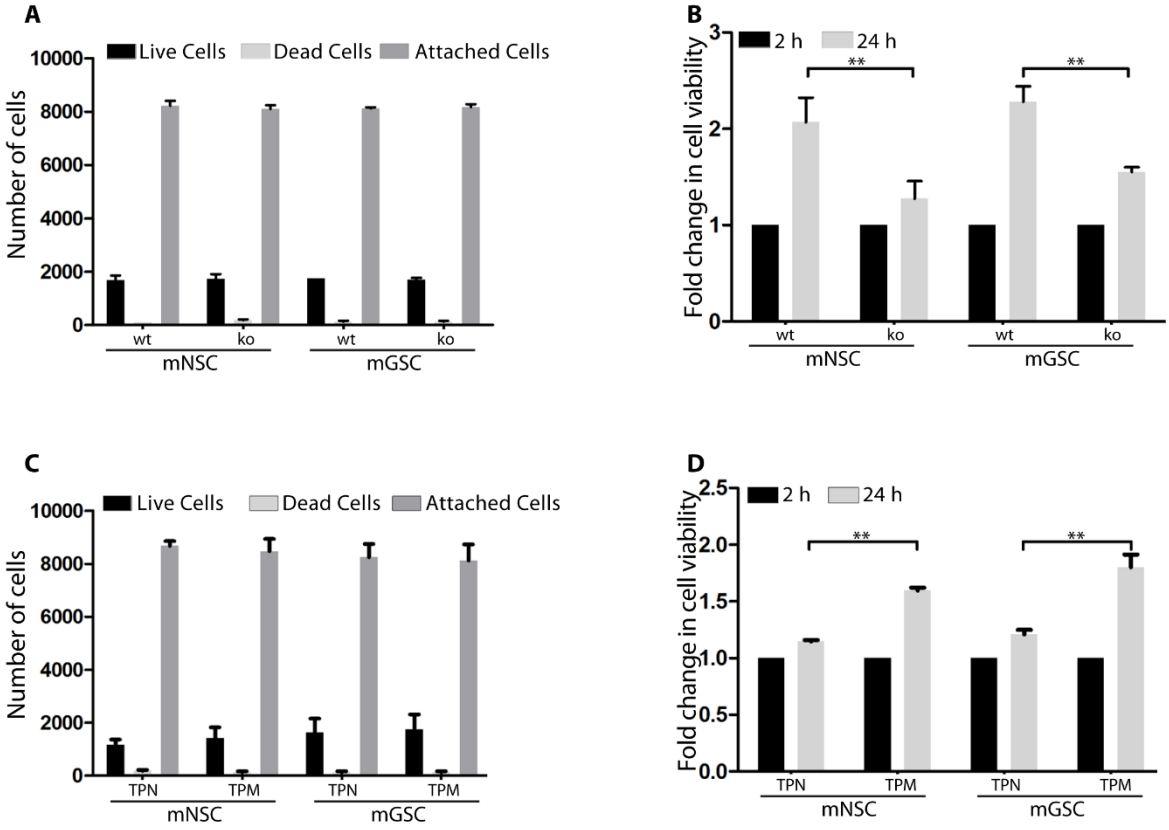

Figure S1. Analysis of cell viability, substrate attachment and proliferation in mNSCs and mGSCs as controls for migration assay. Related to Figure 1. (A) Quantification of total number of floating (live and dead) and attached cells using trypan blue exclusion assay 2 hrs post-seeding of Olig2 wild-type (wt) and Olig2 knockout (ko) mNSCs and mGSCs. (**B**) Quantification of viable cells after 2 hrs and 24 hrs post-seeding of Olig2-wt and Olig2-ko mNSCs and mGSCs for migration assay. Bar graphs represent fold increase in viable cells at 24 hrs compared to 2 hrs. (**C)** Quantification of total number of floating (live and dead) and attached cells 2 hrs post-seeding of TPM and TPN expressing mNSCs and mGSCs. (**D**) Quantification of viable cells after 2hrs and 24 hrs post-seeding of TPM and TPN-expressing mNSCs and mGSCs.

Bar graphs represent fold increase in viable cells at 24 hrs compared to 2 hrs. Data represent mean ± SD of two independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

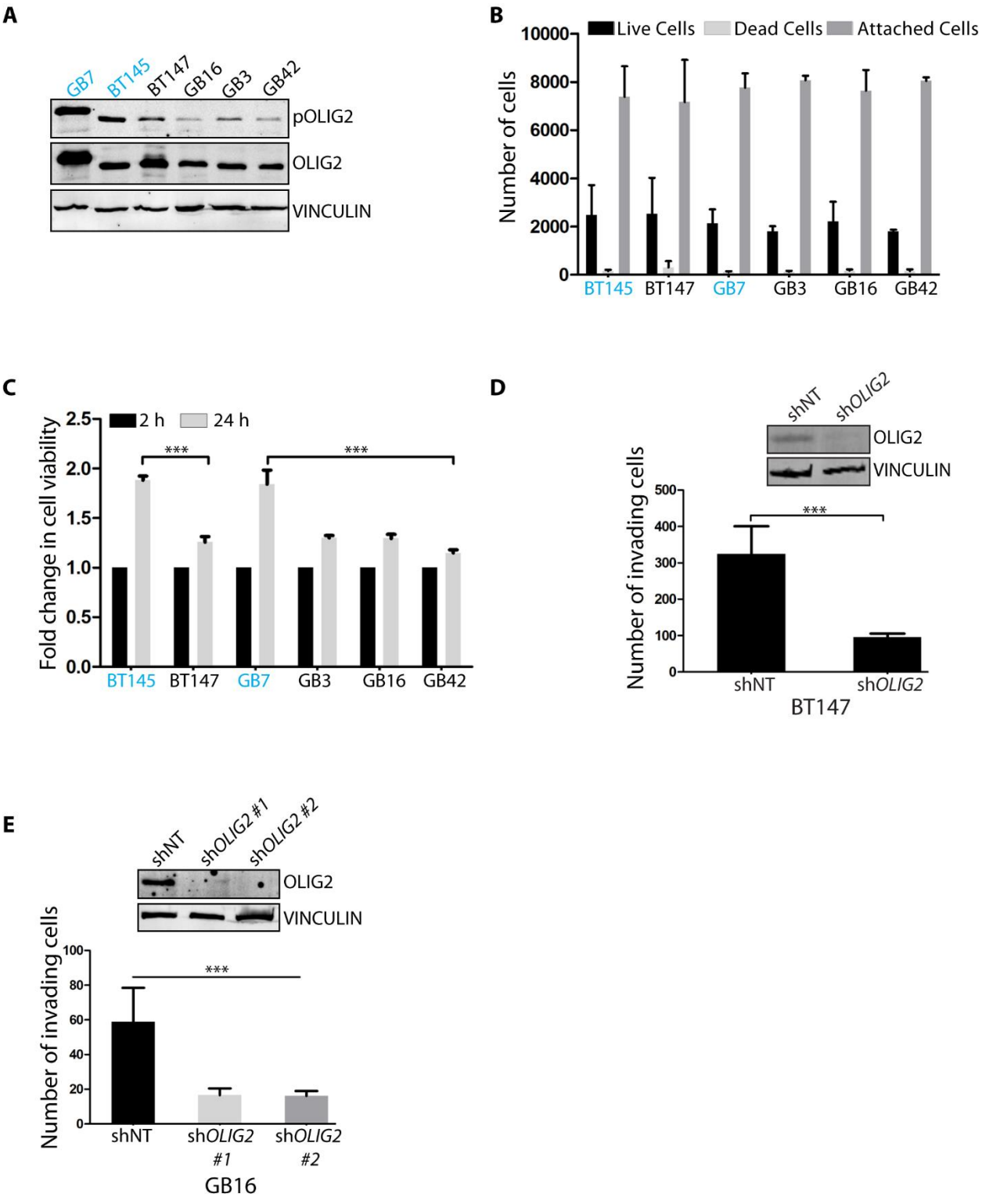
A
GB7
BT145
BT147
GB16
GB3
GB42
pOLIG2
OLIG2
VINCULIN
B
Live Cells
Dead Cells
Attached Cells
Number of cells
10000
8000
6000
4000
2000
0
BT145
BT147
GB7
GB3
GB16
GB42
C
2 h
24 h
Fold change in cell viability
2.5
2.0
1.5
1.0
0.5
0.0
***
***
BT145
BT147
GB7
GB3
GB16
GB42
D
shNT
shOLIG2
OLIG2
VINCULIN
Number of invading cells
500
400
300
200
100
0
***
shNT
shOLIG2
BT147
E
shNT
shOLIG2 #1
shOLIG2 #2
OLIG2
VINCULIN
Number of invading cells
100
80
60
40
20
0
***
shNT
shOLIG2 #1
shOLIG2 #2
GB16

Figure S2. **Control experiments for migration assay in hGSCs. Related to Figure 2. (A)** Western blot analysis for pOLIG2, OLIG2 and VINCULIN across six hGSC lines. (**B**) Quantification of total number of floating (live and dead) and attached cells using trypan blue exclusion assay 2 hrs post-seeding of hGSCs. (**C**) Quantification of viable cells after 2 hrs and 24 hrs post-seeding of pOLIG2^{low} (BT147, GB3, GB16 and GB42) and pOLIG2^{high} (BT145 and GB7) hGSCs. Bar graphs represent fold increase in viable cells at 24 hrs compared to 2 hrs. (**D**) Quantification of invading BT147 cells after transduction with control non-target hairpin (shNT) or sh*OLIG2*. Inset shows western blot for OLIG2 and VINCULIN. (**E**) Knockdown with second hairpin targeting different region in OLIG2 ORF also decreases invasion in GB16 hGSCs. Inset shows western blot for OLIG2 and VINCULIN.

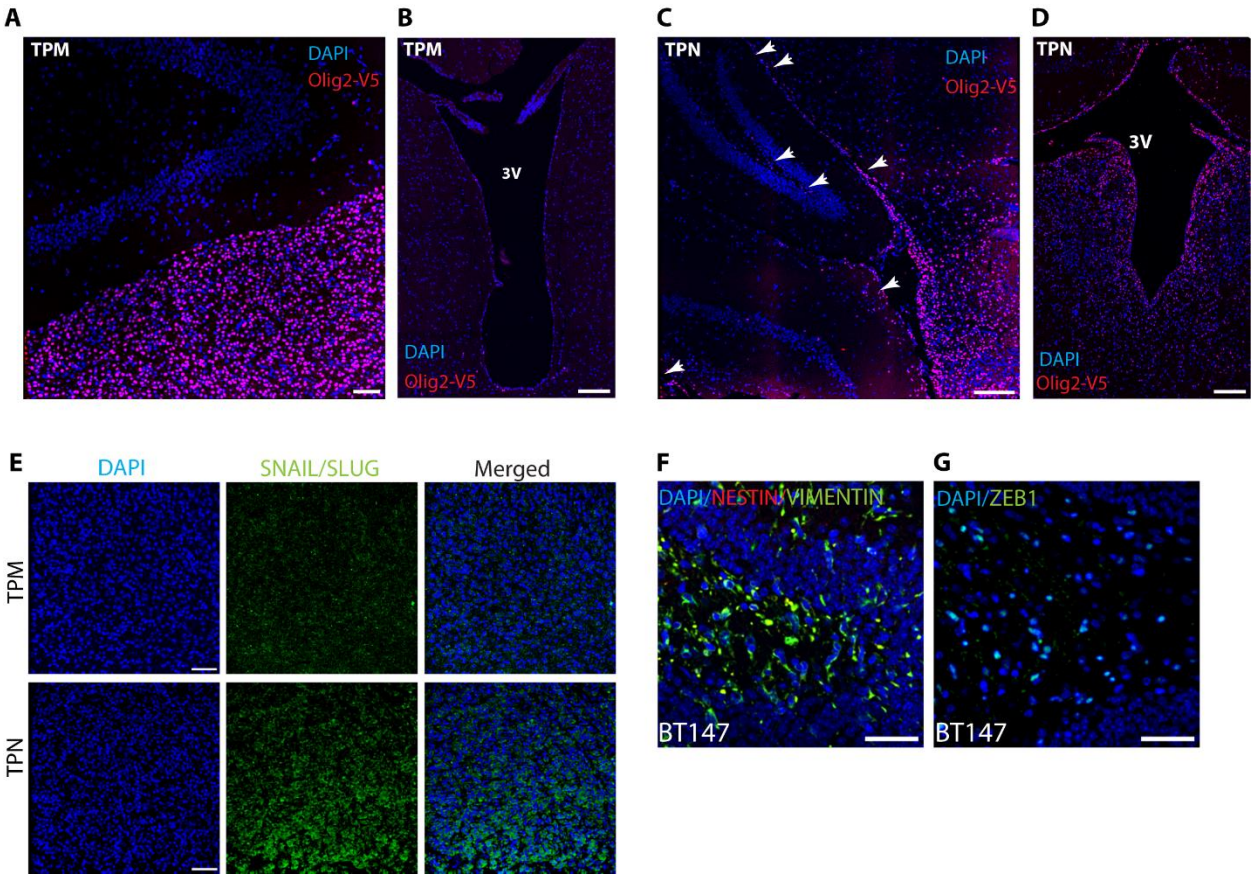

Figure S3. Cells expressing unphosphorylated Olig2 or pOLIG2^{low} are highly invasive in vivo. Related to Figure 3. (A-D). Immunostaining for V5-tag detect TPM- and TPN-expressing cells in tumors. (**A-B**) Very few V5-tagged TPM-expressing cells (red) invade away from the tumor core within the hippocampal (**A**) or the ventricular areas (**B**). (**C-D**) TPN-expressing cells are highly infiltrative and V5-tagged cells could be detected in the hippocampal (**C**) and ventricular regions (**D**) (arrowheads). Scale bars for **(A-B)** is 100 µm, **(C)** is 150 µm, **(D)** is 100 µm. (**E**) Immunostaining of TPM and TPN tissue with EMT marker Snail/Slug antibody (green= Snail/Slug; blue = DAPI). Scale bars = 50 µm. (**F**) Immunostaining of hNESTIN (red) and VIMENTIN (green) in the dentate gyrus regions of BT147 tumor tissue (blue = DAPI). **(G)** Immunostaining with anti-ZEB1 (green) in the dentate gyrus regions of BT147 (blue = DAPI). For (**F-G)**, Scale bars = 50 µm

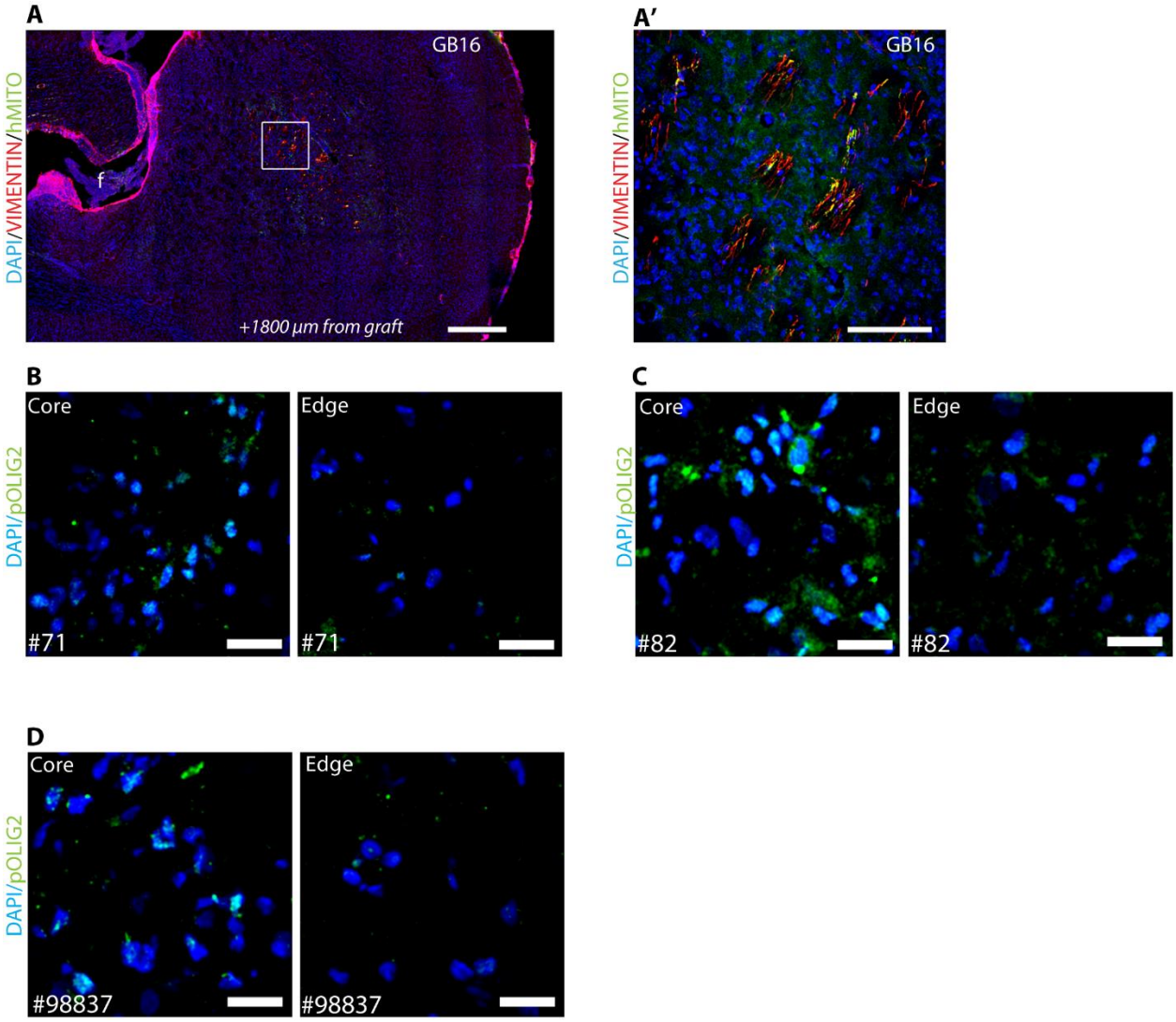

Figure S4. Human GSCs expressing pOLIG2 ^{low} are infiltrative while pOLIG2 expressing cells are predominantly found at the tumor core in GBM tissues. Related to Figure 4. (A) Representative images of GB16 xenograft. Maximum posterior area reached by GB16 cells, correspond to +800 microns from the injection site**. (A')** GB16 cells were detected by costaining of VIMENTIN and h-Mitochondria. f: Fimbria. Scale bars for **(A)** is 500 µm, **(B)** is 100 µm. **(B-D)** Immunostaining of pOLIG2 across the representative GBM tissues shows expression in the core but not at the infiltrative edge. For (**B-D)**, Scale bars = 25 µm.

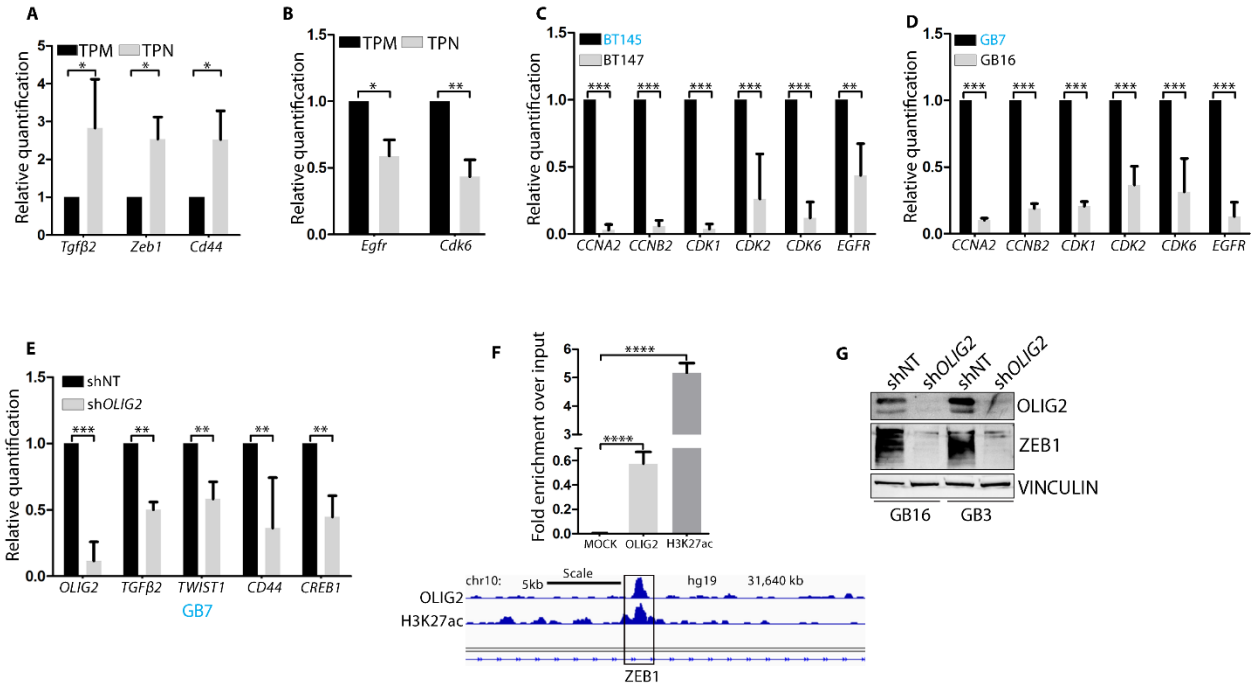

Figure S5. OLIG2 phosphorylation-dependent regulation of proliferation and invasion markers in GSCs. Related to Figure 5. (A) Quantitative RT-PCR analysis of invasion genes in TPN-expressing cells relative to TPM-expressing mGSCs. (**B**) Quantitative RT-PCR analysis of proliferation genes in TPN-expressing cells compared to TPM-expressing mGSCs (**C** and **D**) Quantitative RT-PCR analysis of proliferation and cell cycle related genes in pOLIG2^{low} (BT147 and GB16) cells compared to pOLIG2^{high} (BT145 and GB16) cells. (**E**) Quantitative RT-PCR analysis of invasion genes in GB7 cells transduced with sh*OLIG2*-expressing lentivirus as compared to cells transduced with non-target control (shNT). (**F**) Directed ChIP assay using OLIG2 and H3K27ac antibodies in GB16 hGSCs to validate association of OLIG2 with ZEB1 intronic enhancer region. ChIP-seq track obtained from Suva et al (Suva et al., 2014) (bottom panel). (**G**) Western blot analysis with anti-OLIG2 and anti-hZEB1 of lysates from GB16 and GB3 cells after transduction with shNT control or sh*OLIG2* lentivirus.

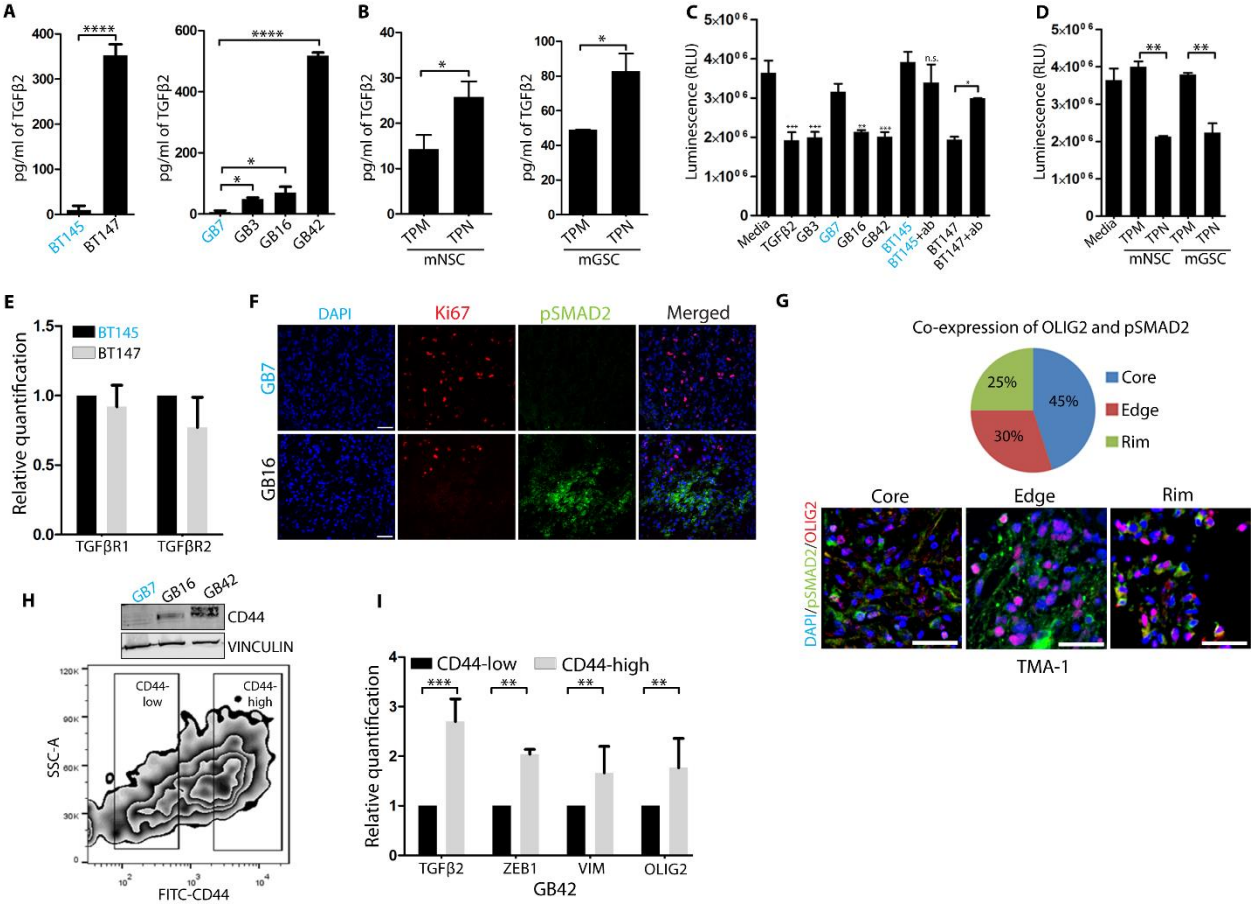

Figure S6. OLIG2 phosphorylation-dependent regulation of TGFβ2 signaling. Related to Figure 6. (**A**) ELISA assay to detect secreted Tgfβ2 levels in cell culture supernatants from mNSCs and mGSCs expressing TPM or TPN forms of Olig2. (**B**) ELISA assay to detect secreted TGFβ2 levels in cell culture supernatants from hGSCs as indicated. (**C-D**) Quantification of TGFβ-induced decrease in cell viability of CCL-64 cells. CCL-64 cells were incubated with neural stem cell media alone, cell culture supernatants from hGSCs, or BT145 and BT147 cell culture media pre-incubated with TGFβ2 antibody for 1 hr (**C**). CCL-64 cells were incubated with neural stem cell media alone, cell culture supernatants from mNSCs and mGSCs expressing TPM or TPN cells. Bar graphs in **A-D** represent mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001. **(E)** Quantitative RT-PCR analysis of TGFβRI and

TGFβRII genes in pOLIG2^{low} (BT147) cells compared to pOLIG2^{high} (BT145) cells. (**F**) Immunostaining of GB7 and GB16 patient tissues with pSMAD2 (green), Ki67 (red) and DAPI (blue). (**G**) Pie-chart depicting distribution of cells co-expressing OLIG2 and pSMAD2 at the tumor Core, Edge, and Rim regions in the glioma invasion TMA (upper panel). Representative images from Core, Edge, and Rim regions from TMA for immunostaining with pSMAD2 (green), OLIG2 (red) and DAPI (blue) (lower panel). (**H**) Western blot analysis for CD44 and VINCULIN levels in three different patient-derived GSCs (upper panel). Cell sorting was performed to collect CD44^{low} and CD44^{high} population in invasive GB42 hGSCs (lower panel). (**I**) QRT-PCR analysis for *TGFβ2*, *ZEB1*, *VIM* and *OLIG2* in CD44^{low}- and Cd44^{high}-sorted GB42 cells. Scale bars = 50 μm

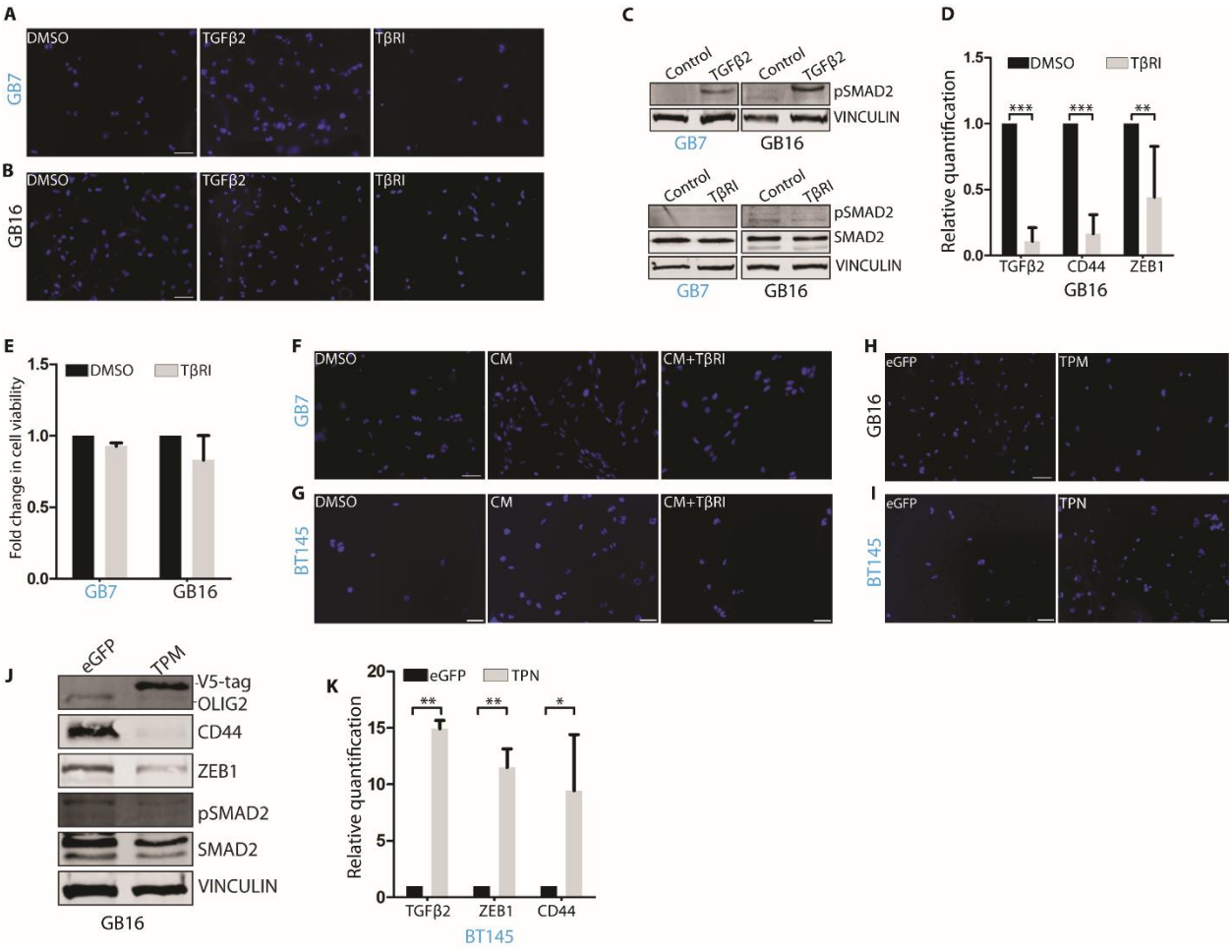

Figure S7. Inhibition of TGFβ signaling blocks OLIG2-mediated invasion. Related to Figure 7. (**A- B**) Representative images of invaded GB7 and GB16 in transwell assays performed in the presence or absence of 10 ng/mL TGFβ2 or pretreatment with 2 μM TβRI for 8 hours **(C)** Immunoblots depicting increase in pSMAD2 levels after treatment of GB7 and GB16 with TGFβ2 (top panel). Immunoblot for pSMAD2 and SMAD2 levels after treatment with TGFβ2 or TβRI treatment in GB7 and GB16 hGSCs (bottom panel). (**D**) qRT-PCR analysis of invasion genes after TβRI treatment in GB16 hGSCs. **(E)** Quantification of cell viability after 8 hrs of treatment with TβRI treatment compared to DMSO treated control in GB7 and GB16 cells. **(F)** Representative images of DAPI-stained nuclei from transwell assay with GB7 cells pretreated with GB16 conditional media (CM) for 48 hr in absence or presence of TβRI inhibitor.

(G) Representative images of DAPI-stained nuclei from transwell assay with BT145 cells pretreated with BT147 conditional media (CM) for 48 hr in absence or presence of TβRI. **(H)** Representative images of invading GB16 cells transduced with control eGFP or TPM-expressing retrovirus. **(I)** Representative images of invading BT145 cells transduced with control eGFP or TPN-expressing retrovirus. **(J)** Western blot analysis of GB16-eGFP and GB16-TPM cell lysates probed with indicated antibodies. **(K)** Quantitative RT-PCR analysis of invasion genes in BT145 transduced with TPN mutant as compared to control eGFP transduced cells. Bar graphs represent mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$. Scale bars = 50 μm

References:

SUVA, M. L., RHEINBAY, E., GILLESPIE, S. M., PATEL, A. P., WAKIMOTO, H., RABKIN, S. D., RIGGI, N., CHI, A. S., CAHILL, D. P., NAHED, B. V., et al. 2014. Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells. *Cell*, 157**,** 580-94.

Table S1. GBM subtype classification of human GSCs.

Patient #	Diagnosis	IDH1	PDGFRA	OLIG2	DLL3	PTEN	PCNA	TOP2A	EGFR	CD44	CHI3L1	Subclass
BT145	primary GBM	-	+	+++	-	-	+	+	+++	-	-	**CL**
BT147	primary GBM	-	+	++	+	-	-	-	+	+	+	**PN**
GB3	primary GBM	-	+	++	+	+	-	-	-	+	+	**PN**
GB7	primary GBM	-	-	+++	-	-	+	+	+++	-	-	**CL**
GB16	primary GBM	-	+	++	-	-	-	-	-	++	++	**PN**
GB42	primary GBM	-	-	+	-	-	-	-	-	++	++	**MES**

Supplemental methods:

Tissue processing for immunofluorescence:

Moribund animals were sacrificed with a lethal intraperitoneal injection of 2.5% Avertin (2,2,2-Tribromoethanol, Sigma-Aldrich; T48402; *tert*-Amyl alcohol, Sigma-Aldrich, A1685). Tissues were fixed through intracardial perfusion with Ringer's solution (Electron Microscopy Sciences; 11763-10) supplemented with 40 mM $NaNO_2$, 2 mM $NaCHO_3$, and 50 IE/mL heparin, followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were subsequently cryoprotected with incubation in a solution of 30% sucrose in PB for 48 hours before being frozen and cut into 40 µm coronal sections using a cryostat (Microm HM550, Thermo Scientific; ThermoFisher Scientific Inc.).

Immunofluorescence.

To block unspecific staining when mouse-raised primary antibodies were used, sections were initially incubated with goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.; 115-007-003) 1:10 in 0.1 M PB 0.4% Triton-X (i.e., PBTx). After a permeabilization and blocking incubation for 2 hours at room temperature (RT) in PBTx supplemented with 10% heat-inactivated goat serum (GS), the sections were incubated with the primary antibodies for 12 hours at 4°C in PBTx, 2% GS. Incubation with secondary antibodies was performed for 2 hours at 4°C in PBTx, 2% GS. Nuclear counterstaining was achieved with DAPI (0.5 µg/mL). The following primary antibodies and dilutions were used: mouse anti–Ki67 (1:100, Dako; Agilent Technologies), mouse anti-human Mitochondria (1:100, EMD Millipore), chicken anti-Vimentin (1:1,000, Millipore), rabbit anti-Olig2 (1:400, Millipore; AB9610), rabbit anti-pOlig2 (1:200, (Sun et al., 2011), mouse anti-Nestin (1:200, Neuromics), chicken anti-eGFP (1:500, Aves), rabbit anti- Zeb1 (1:500, Sigma), rabbit anti-Snail+Slug (1:100, Abcam), rabbit anti-pSmad2

(1:100, Cell Signaling Technology), , rabbit anti-Firefly Luciferase (1:250, Abcam), rabbit anti-RFP (1:250, Abcam) and mouse anti-V5-tag (1: 100, AbD Serotec). Secondary antibodies were used at 1:1,000 dilutions (goat anti-mouse Alexa Fluor 568, and goat anti-rabbit Alexa 488 [Invitrogen; ThermoFisher Scientific Inc.]). For nuclear counterstaining, DAPI (1:1,000 (5mg/ml) Sigma-Aldrich) was used. Coverslips were mounted using Aqua Mount (Thermo Scientific; ThermoFisher Scientific Inc.).

Protein Isolation and Western Blot Analysis

Cells were lysed in RIPA buffer and supplemented with protease and phosphatase inhibitors (ThermoFisher Sceintific). Whole-cell extracts were quantified using the Bradford Protein Assay Kit (Pierce) and were then separated on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk and probed with specific antibodies. Primary antibodies used were anti-OLIG2, anti-pOLIG2 (Sun et al., 2011), anti-pSMAD2 (Cell Signaling Technology), anti-SMAD2 (Cell Signaling Technology), anti-CD44, anti-V5 tag (AbD Serotec), anti-ZEB1 (Sigma-Aldrich), and anti-Vinculin (Abcam plc). Blots were then incubated with a fluorescence-conjugated secondary antibody and developed with Li-Cor Odyssey (Li-Cor Inc.).

ChIP Primers.

The following ChIP primers were used in this study: (Olig1+36kb), 5-TGACTACATCCCCAGGACAA-3 and 5-ATCTCACTTGGGCTTTTGGT-3; TGFβ2, 5-GTTGGCTGAATCCACCAGGA-3 and 5-TCTCATGTGGTCACGACAGC-3; ZEB1, 5-ACGGTACAGTTGCTCTGGAAA-3 and 5-TTGACGCAGACACTACGAAAGA-3, non-target 1, 5-GGCAGGGCTATTTCCATCCA-3 and 5-ACCTATCCGGTGGACTCACT-3; non-target 2, 5-CTCGTCACATTGGAGAGTGG-3 and 5-CAGGAGCTTGGTTTCCTCAG-3.

Virus Production and Packaging

Lentiviral packaging and production for sh*OLIG2* and shNT have been described previously (Mehta et al., 2011). The following sequences for sh*OLIG2* were used in this study: 5-CAAGAAGGACAAGAAGCAAAT-3 for sh*OLIG2#1* and ATAATAACTACAATGCACC for sh*OLIG2#2*. Retroviruses expressing triple phospho-mutants of Olig2 were packaged and produced, as previously described (Meijer et al., 2014; Sun et al., 2011).

Quantification of loss of Surface Attachment, and Cell Viability Assay.

Equal number of cells (10,000) were seeded on two laminin-coated Oris (BCG) 96-well plates for each cell line analyzed. The stoppers were removed after 2 hrs and the media analyzed for presence of dead/live cells using trypan blue exclusion assay. Viable attached cells were analyzed at 2 hr and 24 hr post incubation to assess differences in proliferation after 24 hrs using Celltiter Glo assay (Promega).

TGFβ Growth Inhibition Bioassay.

The mink lung epithelial cells (CCL-64) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% Pen-Strep at 37°C in 5% CO2. The bioassay was performed as previously described {Uhl, 2004 #408}. Briefly, CCL-64 cells were plated in a 96-well plate (5 x10^3 cells/well) and incubated at 37°C. Next day CCL-64 cells were rinsed with DMEM and supernatants from mNSCs, mGSCs, hGSCs (seeded 48 hrs prior to the bioassay), Neural Stem Cells (NSC) media and TGFβ2 or TGFβ2 antibody were added. The plate was incubated for 72 hrs at 37°C. Decrease in cell viability was assessed by CellTiter-Glo Luminescent Cell Viability assay (Promega).