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Supplemental Information

FOSL1 promotes proneural-to-mesenchymal transition of glioblastoma stem cells via UBC9/CYLD/NF-κB axis

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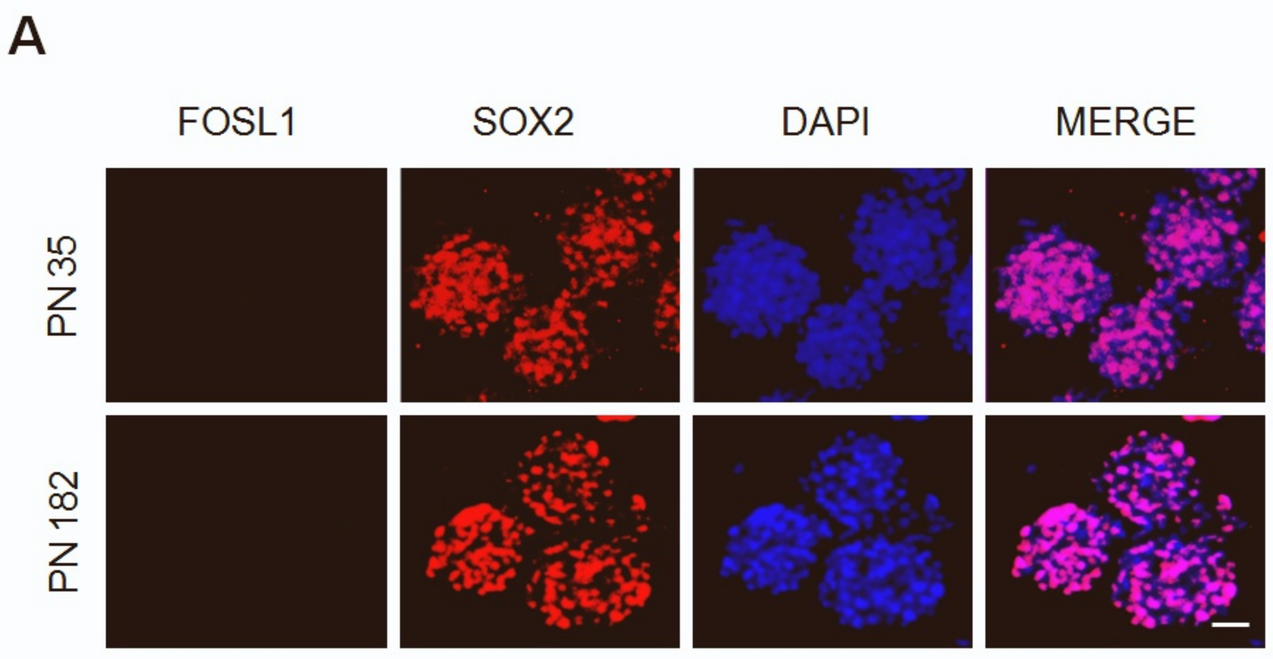
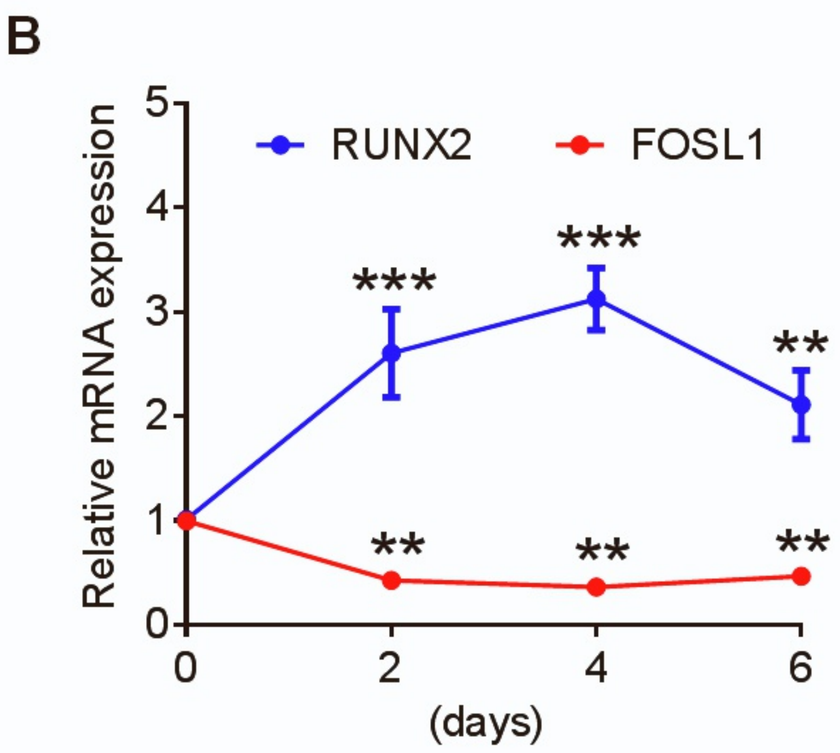
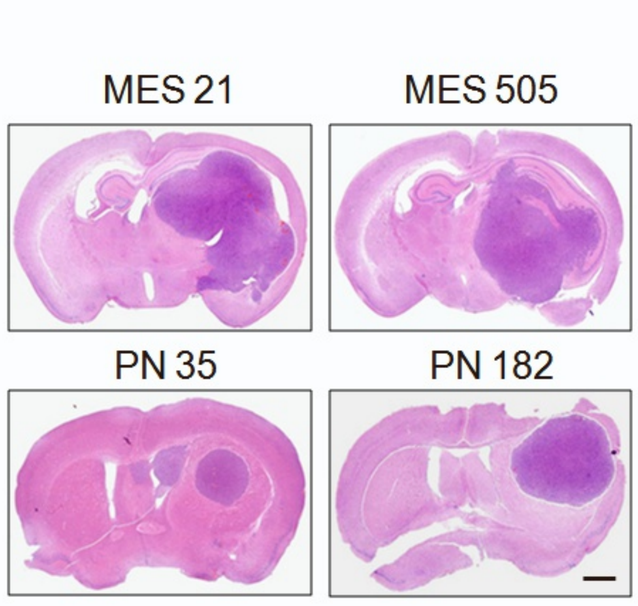
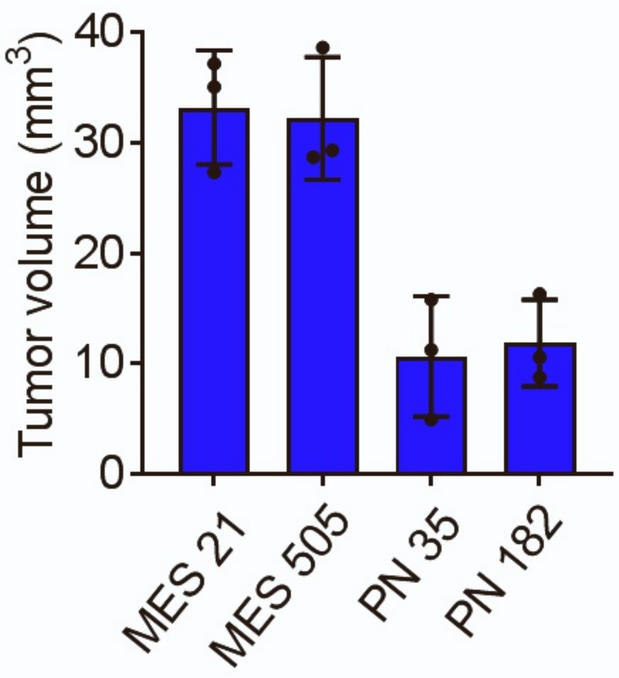
Supplemental Figure 1









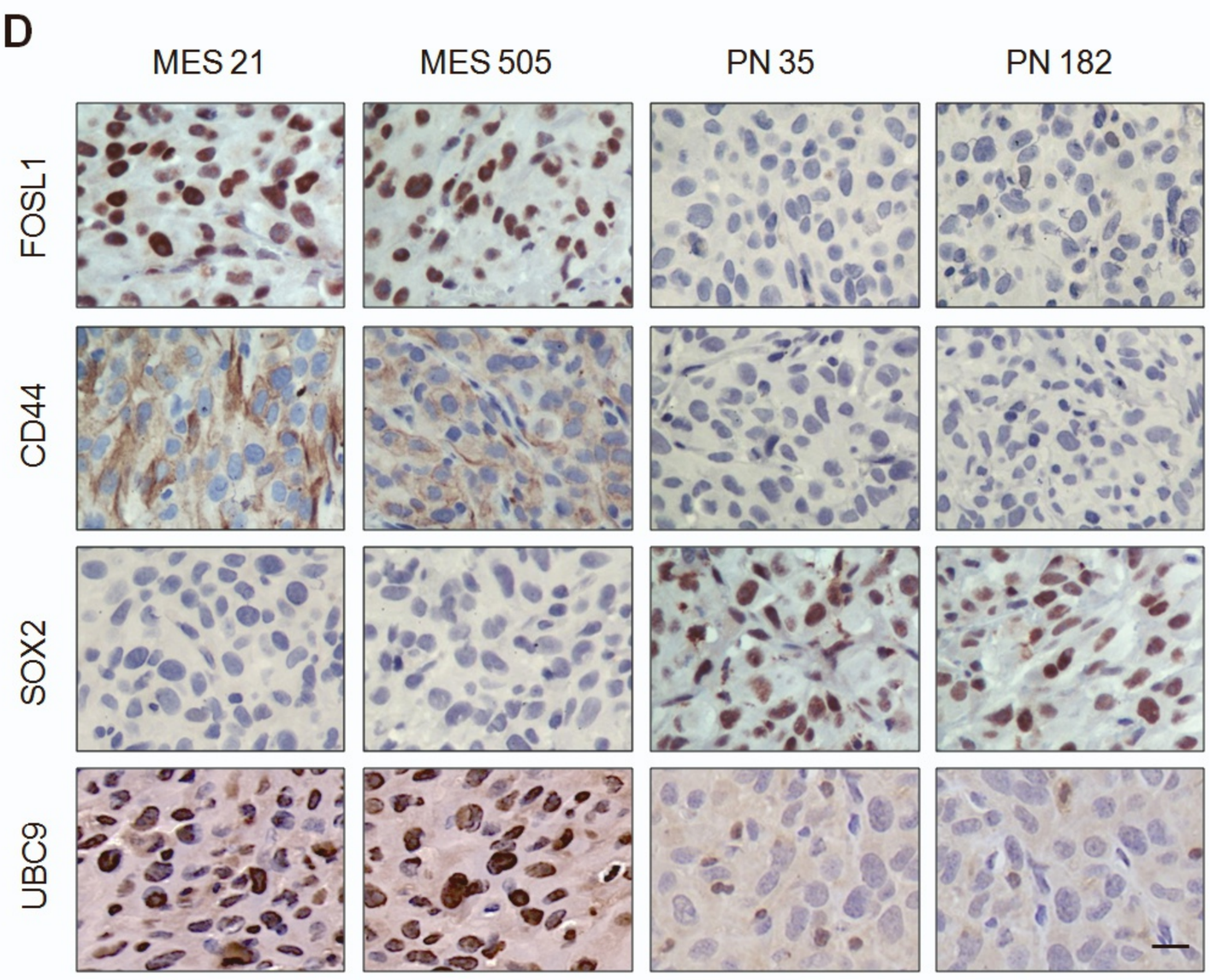
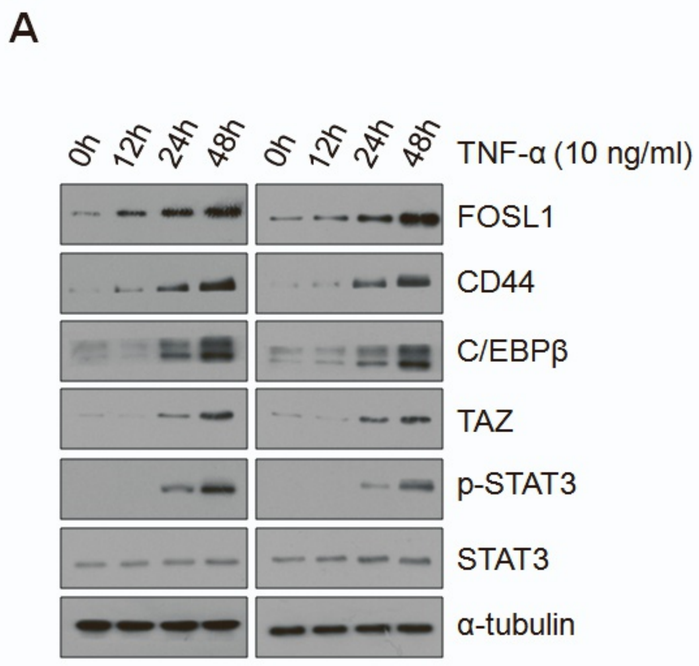
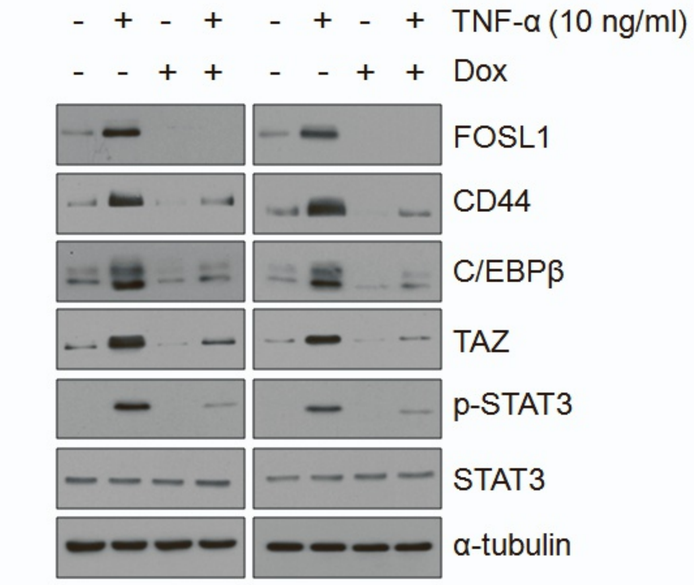


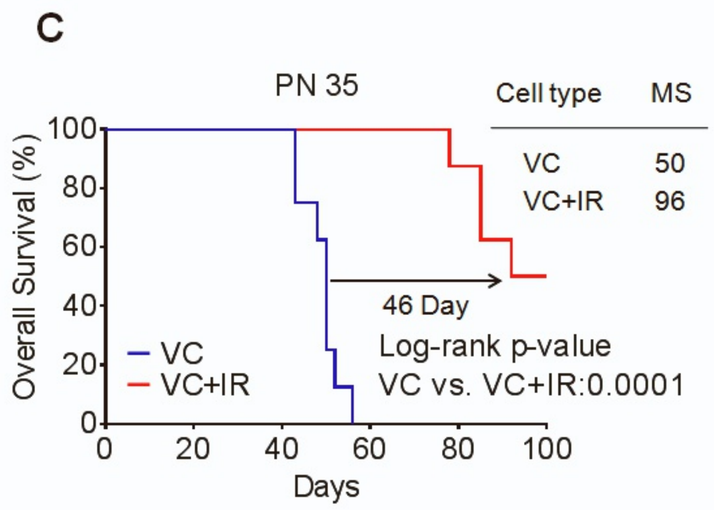
Figure S1. FOSL1 maintains the MES phenotype in GSCs

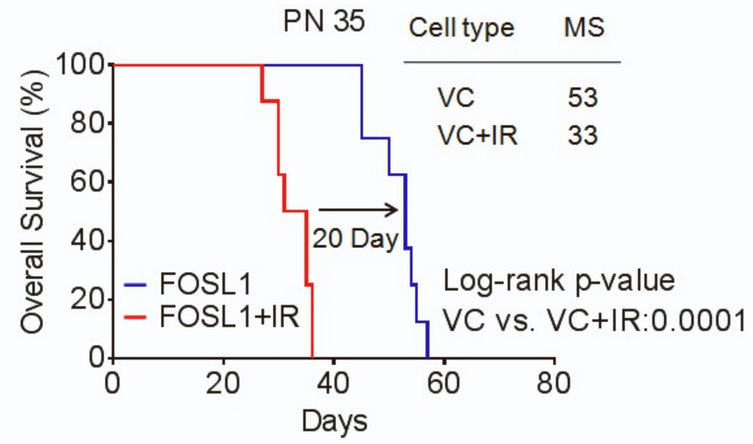
(**A**) Representative IF images of FOSL1 and SOX2 expression in PN 35 and PN 182 GSCs. FOSL1 was labeled in green and SOX2 in red. Nuclei were counterstained with DAPI (blue). Scale bar, 25 μm. (**B**) qRT-PCR analysis of mRNA expression of FOSL1 and an osteogenic differentiation marker RUNX2 in MES 21 GSC during osteogenic differentiation induction. (**C**) Representative images of intrancranial xenograft tumors derived from MES 21, MES 505, PN35 and PN 182 GSCs. The bar graph shows the mean tumor volume. (**D**) Representative IHC staining images of FOSL1, CD44, SOX2 and UBC9 in the orthotopic xenograft mouse model using MES 21, MES 505, PN35 and PN 182 GSCs. Scale bar, 20 μm. Data are represented as means ± SD of 3 independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, 2-tailed Student's t test.

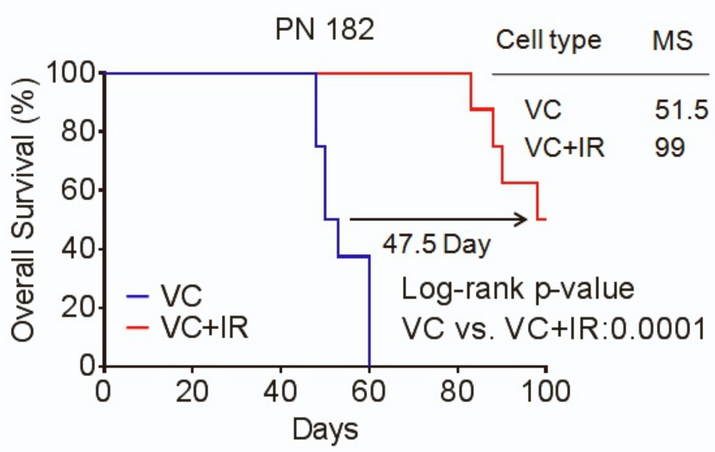
Supplemental Figure 2

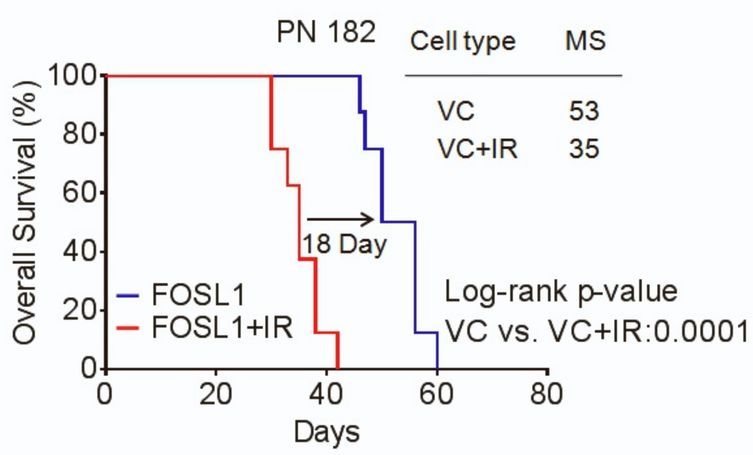


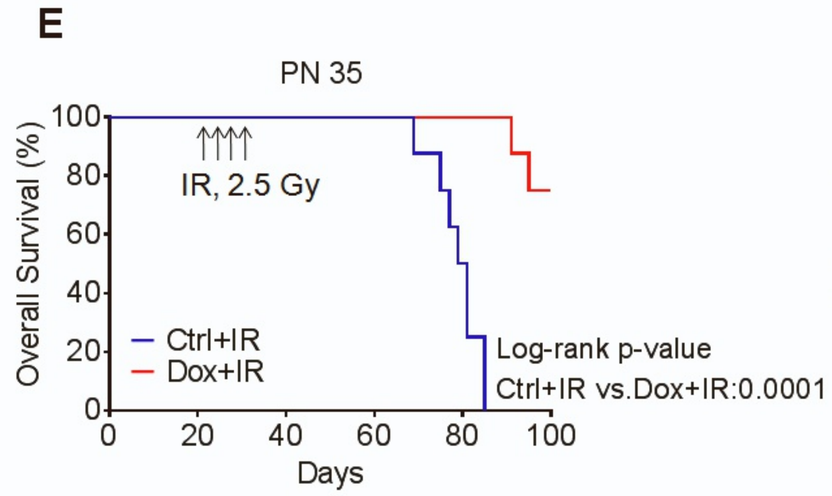


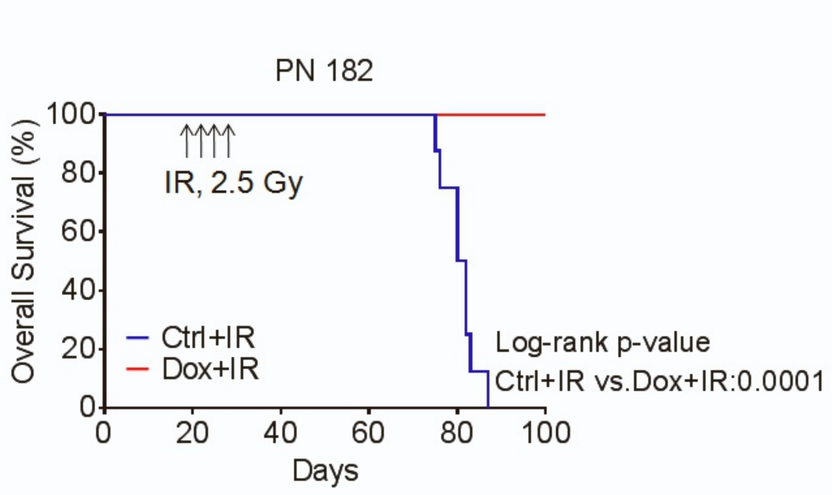


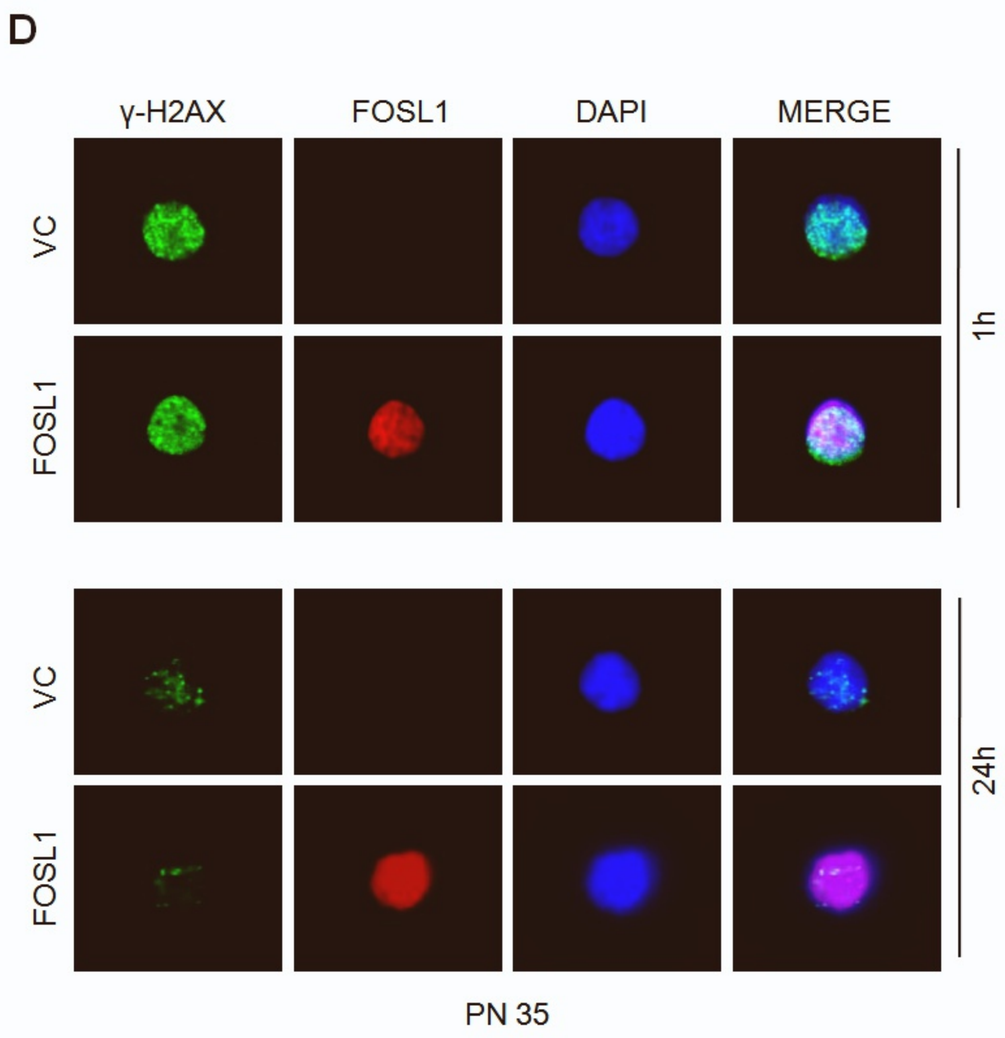












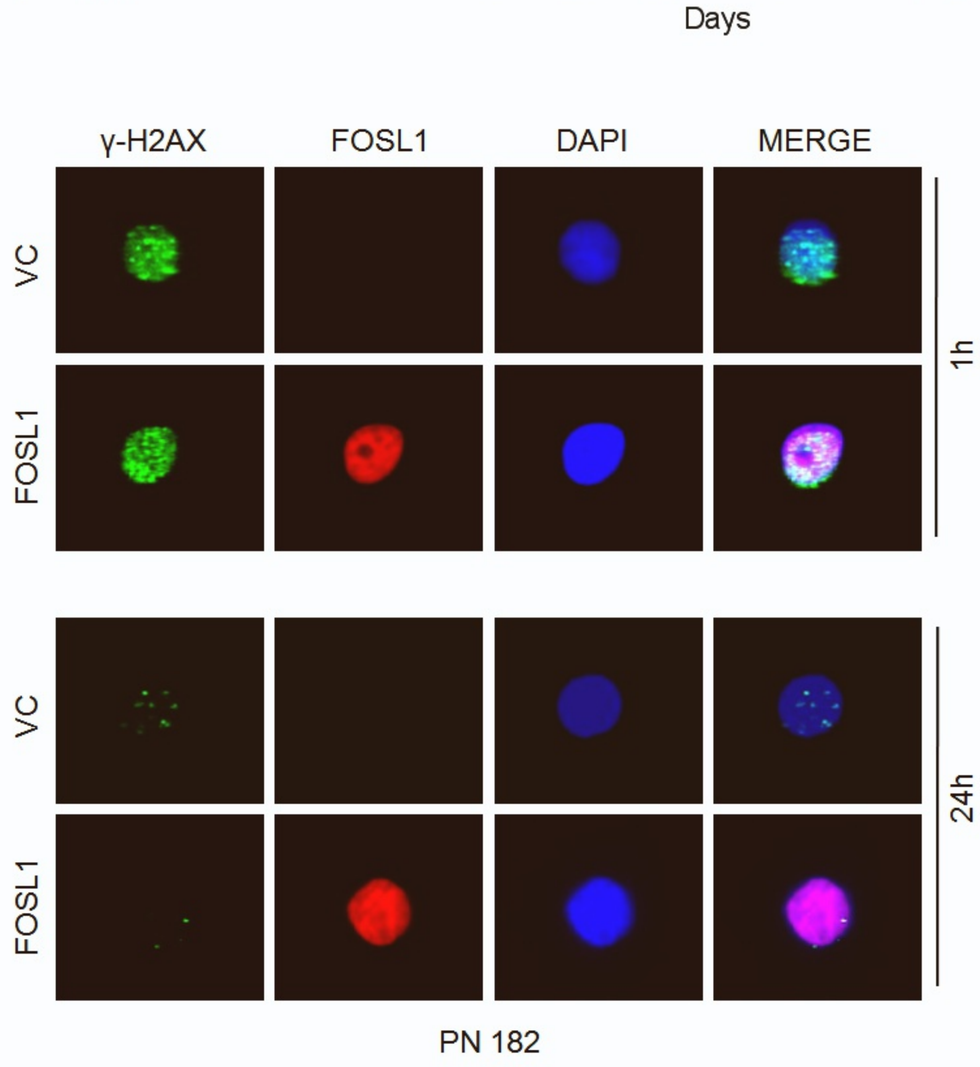


Figure S2. FOSL1 facilitates IR-induced PMT and radioresistance of PN GSCs

(**A**) IB analysis of FOSL1, CD44, C/EBPβ, TAZ, p-STAT3 and STAT3 in PN 35 and PN 182 GSCs at the indicated time points after treatment with 10 ng/ml TNF-α. α-tubulin as internal control. (**B**) IB analysis of indicated antibodies in PN 35 and PN 182 GSCs transduced with Dox-inducible lentiviral vectors expressing FOSL1 shRNA or control shRNA in the presence or absence of 10 ng/ml TNF-α for 48 h. α-tubulin as internal control. (**C**) Kaplan-Meier survival curves of mice intracranially implanted PN 35 and PN 182 GSCs with vector control or FOSL1 overexpression following IR treatment. IR was performed on week 3 after implantation for 4 consecutive days at 2.5 Gy/day. (**D**) Representative IF images of γH2AX foci in FOSL1 or vector control-transduced PN 35 and PN 182 GSCs for 1 h and 24 h post-IR. (**E**) Kaplan-Meier survival curves of mice intracranially implanted PN 35 and PN 182 GSCs with Dox-inducible lentiviral vectors expressing FOSL1 shRNA or control shRNA in response to IR. The arrows indicate the time of IR.

Supplemental Figure 3

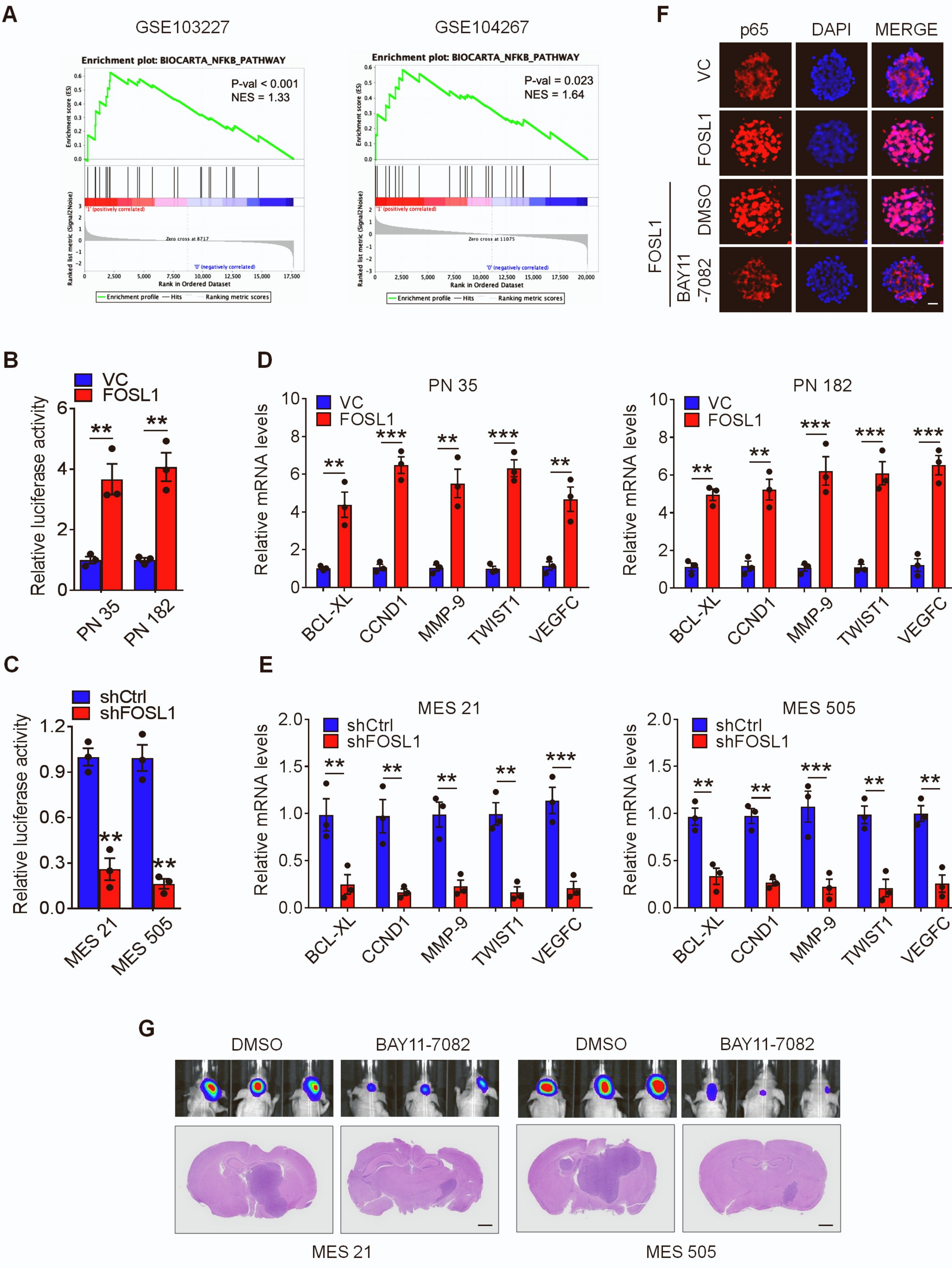


Figure S3. FOSL1 induces activation of NF-κB signaling

(**A**) GSEA analysis showing correlation between expression of FOSL1 and the enrichment of NF-κB targets in primary human GBM specimens (GSE103227 and GSE104267). (**B**) Relative luciferase reporter activity of NF-κB in PN 35 and PN 182 GSCs transduced with vector control or FOSL1. (**C**) Relative luciferase reporter activity of NF-κB in MES 21 and MES 182 GSCs transduced with Ctrl shRNA or FOSL1 shRNA. (**D**) qRT-PCR analysis of NF-κB target genes (*BCL-XL*, *CCND1*, *MMP-9*, *TWIST1* and *VEGFC*) in PN 35 (A) and PN 182 (B) GSCs transduced with vector control or FOSL1. (**E**) qRT-PCR analysis of NF-κB target genes (*BCL-XL*, *CCND1*, *MMP-9*, *TWIST1* and *VEGFC*) in MES 21 (C) and MES 182 (D) GSCs transduced with shCtrl or shFOSL1. (**F**) IF staining for p65 in PN 182 GSCs expressing exogenous FOSL1 or vector control, with or without a specific NF-κB inhibitor BAY 11-7082 treatment. p65 was labeled in red. Nuclei were counterstained with DAPI (blue). Scale bar, 20 μm. (**G**) Representative BLI images of mice bearing xenografts derived from MES 21 and 505 GSCs, with or without BAY 11-7082 treatment (upper). Representative H&E stained brain sections in indicated MES GSCs-derived xenografts treated with DMSO or BAY 11-7082. Scale bar, 1mm (lower). Data are represented as means ± SD of 3 independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, 2-tailed Student's t test.

Supplemental Figure 4

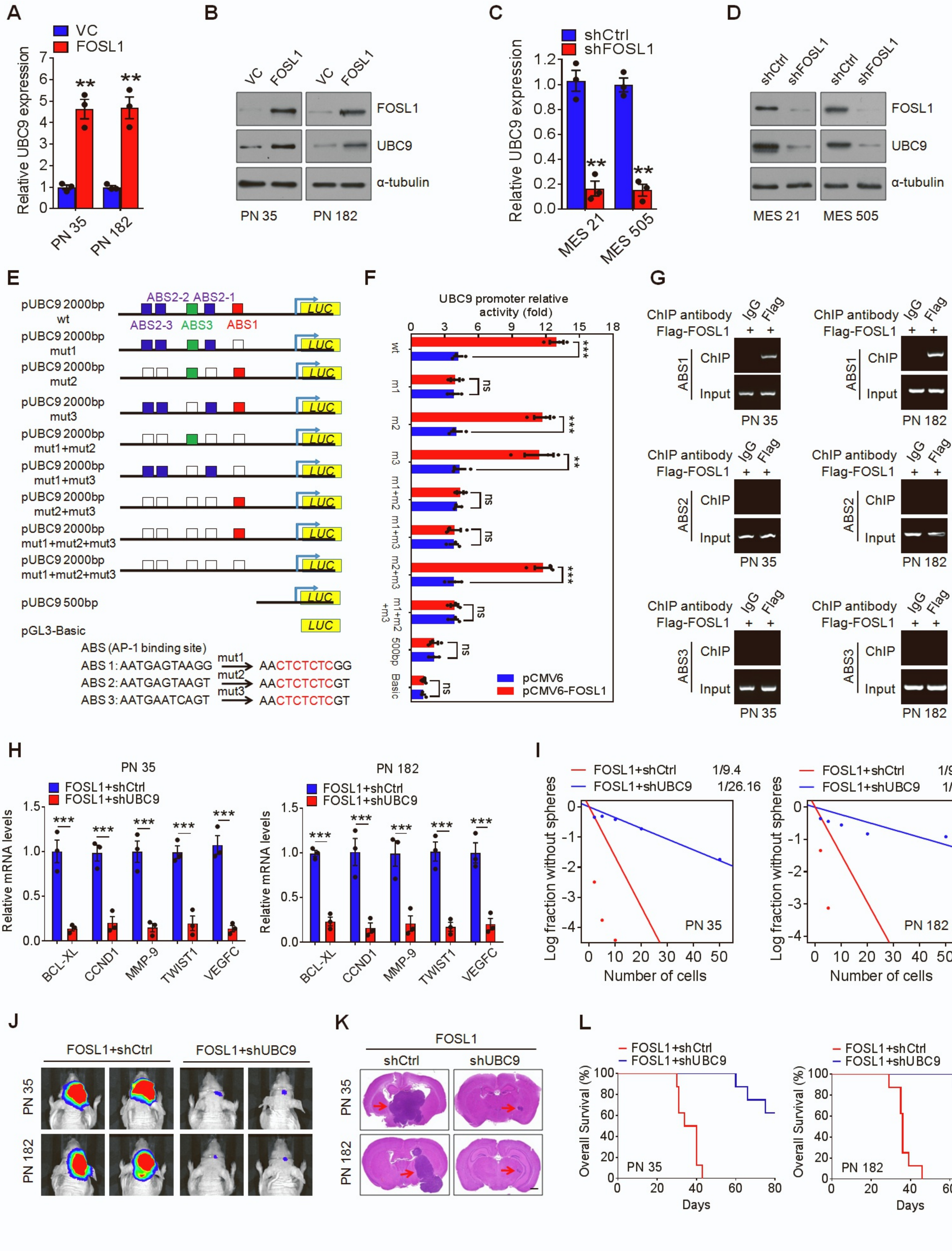


Figure S4. FOSL1 promotes CYLD SUMOylation to impair deubiquitination of NF-κB signaling intermediaries

(**A** and **B**) qRT-PCR (A) and IB (B) analysis of UBC9 mRNA and protein levels in PN 35 and PN 182 GSCs transduced with vector control or FOSL1. **p < 0.01. (**C** and **D**) qRT-PCR (C) and IB (D) analysis of UBC9 mRNA and protein levels in MES 21 and MES 505 GSCs transduced with control shRNA or FOSL1 shRNA. (**E**) Schematic representation of the human UBC9 regulatory region showing the positions of the five putative AP1-binding sites and the corresponding mutant binding sites. (**F**) WT or mutated UBC9 promoter constructs were cotransfected with pCMV-FOSL1 into HEK293T cells, and the dual luciferase activity was measured. (**G**) ChIP assays on AP1-binding site 1, 2 or 3 of UBC9 promoter were performed in Flag-FOSL1-transduced PN 35 and PN 182 GSCs. (**H**) qRT-PCR analysis of NF-κB target genes (*BCL-XL*, *CCND1*, *MMP-9*, *TWIST1* and *VEGFC*) in PN 35 and PN 182 GSCs expressing exogenous FOSL1 or vector control, with or without UBC9 depletion. (**I**) Limiting dilution neurosphere-forming assay in PN 35 and PN 182 GSCs with indicated modifications. (**J** and **K**) Representative images of BLI (J) and H&E-stained brains (K) of mice intracranially implanted with luciferase-labeled PN 35 and PN 182 GSCs with indicated modifications. Colored scale bars represent photons/s/cm2/steradian. Red arrows indicate tumors. Scale bar, 1 mm. (**L**) Kaplan-Meier survival curves of mice bearing PN 35 and PN 182 GSCs orthotopic xenografts with indicated modifications. Data are represented as means ± SD of 3 independent experiments. ***P* < 0.01, ****P* < 0.001, 2-tailed Student's t test.

Supplemental Figure 5

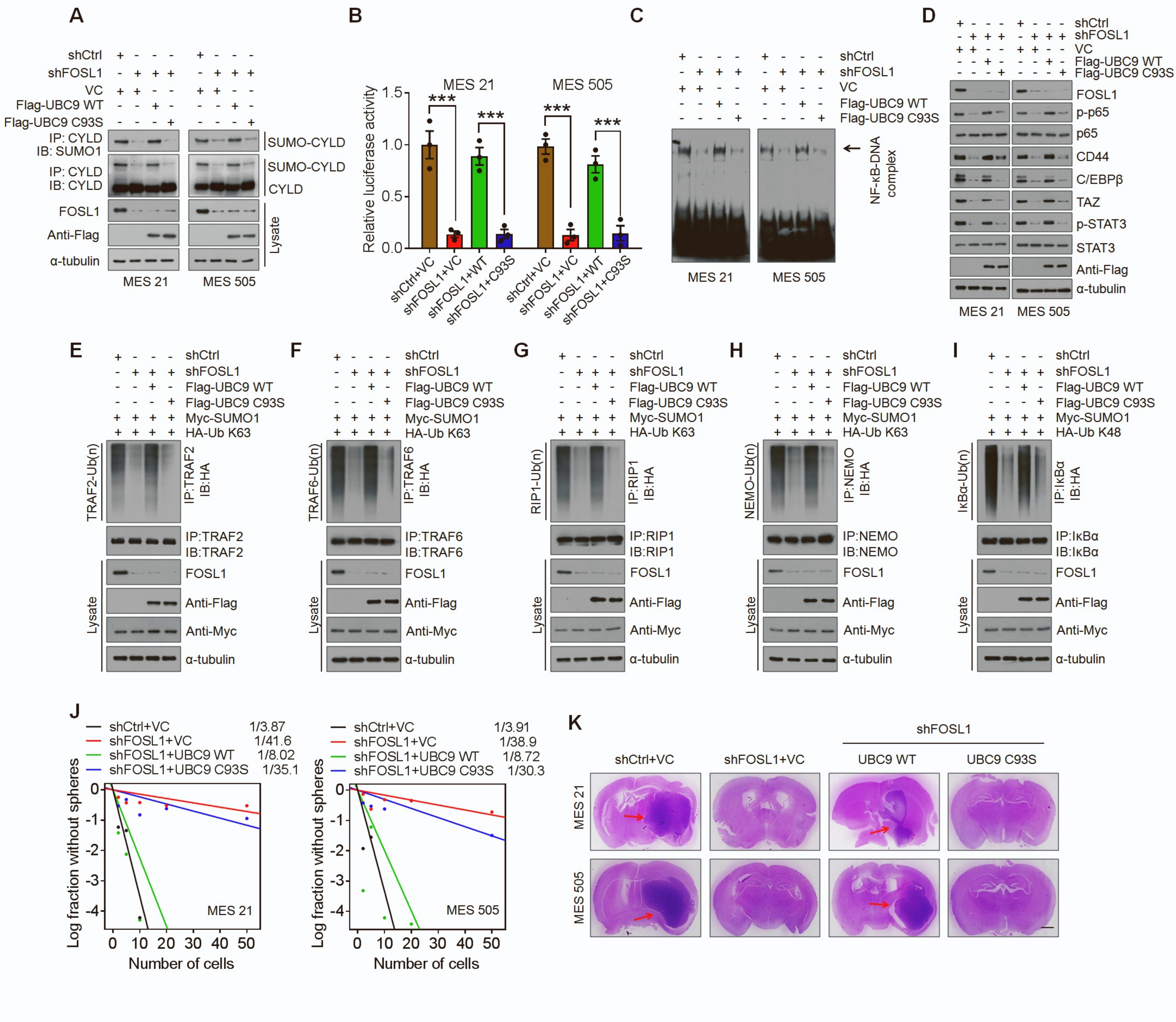


Figure S5. FOSL1 facilitates CYLD SUMOylation, NF-κB activation and PMT via transcriptionally activating UBC9

(**A**) MES 21 and MES 505 GSCs were transduced with shCtrl or shFOSL1, reconstituted with WT or C93S UBC9. CYLD was immunoprecipitated and then incubated with anti-SUMO1 antibody or anti-CYLD antibody, respectively. (**B**) Luciferase-reported NF-κB activity in MES 21 and MES 505 GSCs with indicated modifications. (**C**) EMSA analysis of NF-κB DNA binding activity in MES 21 and MES 505 GSCs with indicated modifications. (**D**) IB analysis of FOSL1, p-p65 (Ser536), p65, CD44, C/EBPβ, TAZ, p-STAT3 (Tyr705) and STAT3 in MES 21 and MES 505 GSCs with indicated modifications. (**E-H**) K63-linked polyubiquitin chains of TRAF2 (E), TRAF6 (F), RIP1 (G) and NEMO (H) were detected in MES 21 GSCs with indicated modifications. (**I**) K48-linked polyubiquitin chains of IκBα was analyzed in MES 21 GSCs with indicated modifications. (**J**) Limiting dilution neurosphere-forming assay in MES 21 and MES 505 GSCs with indicated modifications. (**K**) Representative H&E stained brain sections of mice that received indicated MES 21 and MES 505 GSCs. Red arrows indicate tumors. Scale bar, 1 mm. Data are represented as means ± SD of 3 independent experiments. ***$P < 0.001$, 2-tailed Student's t test.

Supplemental Figure 6

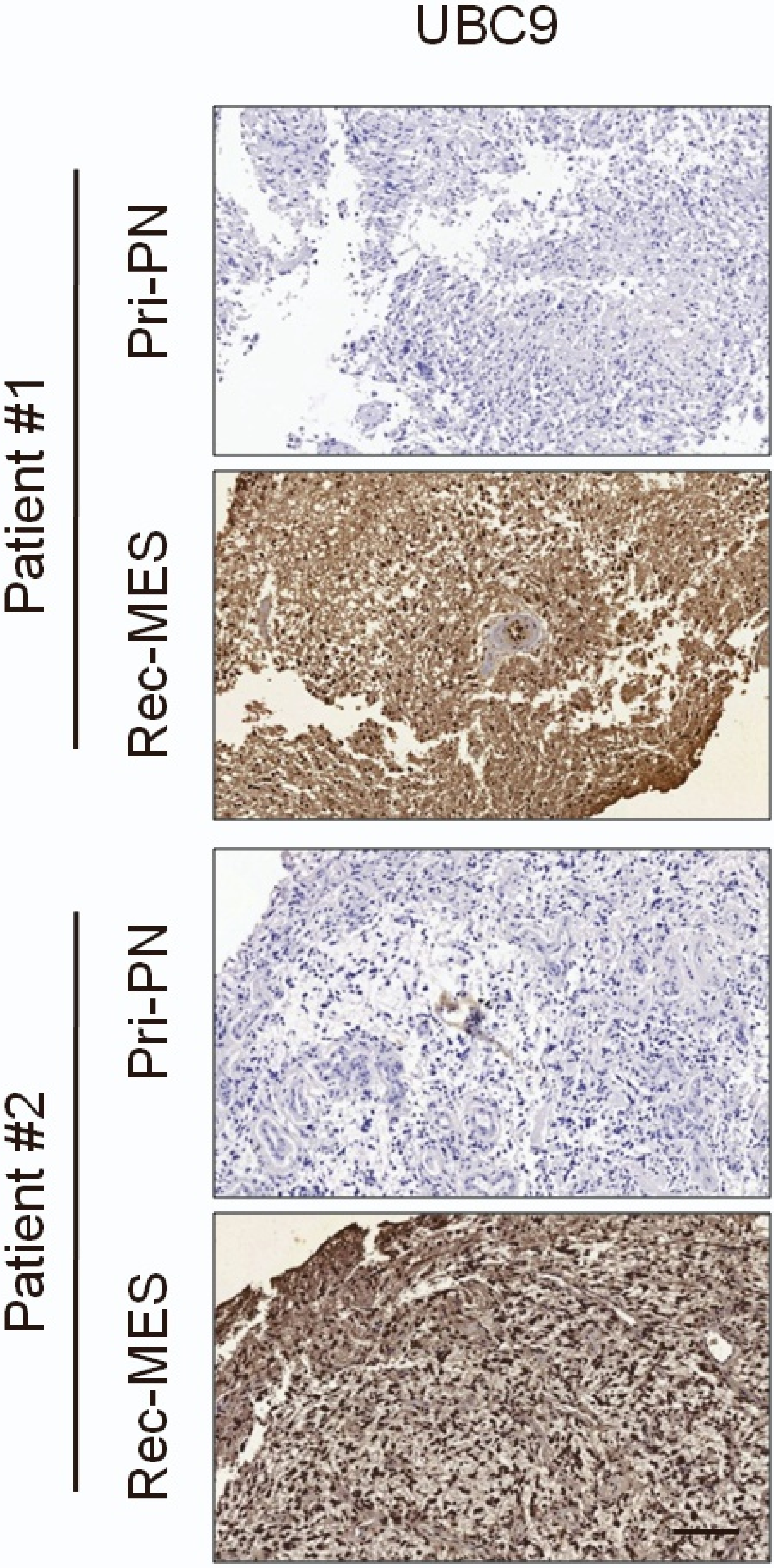


Figure S6. Clinical relevance of FOSL1/NF-κB-driven PMT in human GBM

Representative UBC9 expression levels are shown in consecutive sections of 2 matched pairs of primary PN tumors and corresponding relapsed MES tumors. Scale bars, 50 μm.

Patient-derived GSC Inventory

Cell Line Name	GSC subtype	Pathological Diagnosis	Clinical Status
35	Proneural	Glioblastoma, WHO IV	Primary
182	Proneural	Glioblastoma, WHO IV	Primary
21	Mesenchymal	Glioblastoma, WHO IV	Primary
505	Mesenchymal	Glioblastoma, WHO IV	Primary

Table S1, Related to Figure 1. Patient-derived GSC Inventory

STR Analysis of GSC Used for This Study

	Cell Line Name			
	35	182	21	505
Amelogenin	X	X	X	X,Y
CSF1PO	11, 12	9, 12	12	10, 12
D13S317	10, 11	8, 11	8, 13	10, 13
D16S539	9, 11	11, 12	11, 13	9
D5S818	12, 13	12, 13	12	11, 13
D7S820	8, 9	8, 11	9, 10	9, 11
THO1	9.3	9	6, 9.3	6, 9.3
TPOX	8, 11	9	9, 11	8, 11
vWA	15, 16	16, 17	14, 19	15, 17

Table S2, Related to Figure 1. STR Analysis of GSC Used for This Study

Characterization of the sequences of shRNAs, cDNA and ChIP-qPCR primers, and DNA probes

Sequences for shRNAs	
TRC shRNA Control	N/A
FOSL1 shRNA #1	ACTCATGGTGTTGATGCTTGG
FOSL1 shRNA #2	AATGAGGCTGTACCATCCACT
SMARTvector Lentiviral Controls	N/A
FOSL1 shRNA(Targer 3'-UTR)	AATATGGTCAGTCTCATTA
UBC9 shRNA #1	TTGGCAGTAAATCGTGTAGGC
UBC9 shRNA #2	AAATGGGTGGTCTTTCCTCCA
Sequences for cDNA primers	
UBC9-F	ACAGTGTGCCTGTCCATCTTAGAG
UBC9-R	TGTTTTGGCAGTAAATCGTGTAGG
BCL-XL-F	TCCTTGTCTACGCTTTCCACG
BCL-XL-R	GGTCGCATTGTGGCCTTT
CCND1-F	CCTCTGTGCCACAGATG
CCND1-R	GGGTCACACTTGATCACTC
MMP9-F	ACGACGTCTTCCAGTACCGA
MMP9-R	TTGGTCCACCTGGTTCAACT
TWIST1-F	TCCGCGTCCCACTAGCA
TWIST1-R	TTCTCTGGAAACAATGACATCTAGGT
VEGFC-F	GTGTCCAGTGTAGATGAACTC
VEGFC-R	ATCTGTAGACGGACACACATG
GAPDH-F	GAAGGTGAAGGTCGGAGTC
GAPDH-R	GAAGATGGTGATGGGATTTC
Sequences for ChIP-qPCR primers	
UBC9-ChIP-F	GGCGGGAATGAATGAATG
UBC9-ChIP-R	TTCCCTCCCCTACTCCGT
Sequences for NF-κB Biotin end-labeled DNA probes	
NF-κB DNA probes-F	AGTTGAGGGGACTTTCCCAGGC
NF-κB DNA probes-R	GCCTGGGAAAGTCCCCTCAAC

Table S3, Related to the Methods. Characterization of the sequences of shRNAs, cDNA and ChIP-qPCR primers, and DNA probes.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER	
Antibodies			
Anti-FOSL1 (For WB/IP)	Cell Signaling Technology	Cat#5281S	RRID:AB_10557418
Anti-FOSL1 (For IF/IHC)	Santa Cruz Biotechnology	Cat#sc-28310	RRID:AB_627632
Anti-CD44	Cell Signaling Technology	Cat#3570S	RRID:AB_10693293
Anti-CD44 (For FACS)	Abcam	Cat#ab51037	RRID:AB_ 868936
Anti-CD133	Abcam	Cat#ab19898	RRID:AB_470302
Anti-SOX2	Abcam	Cat#ab79351	RRID:AB_10710406
Anti-OLIG2	Abcam	Cat#ab81093	RRID:AB_1640746
Anti-IKKα	Abcam	Cat#ab32041	RRID:AB_733070
Anti-IKKβ	Abcam	Cat#ab124957	RRID:AB_10975710
Anti-phospho-IKKα/β (Ser180/181)	Abcam	Cat#ab55341	RRID:AB_883038
Anti-SUMO1	Abcam	Cat#ab32058	RRID:AB_778173
Anti-SUMO2/3	Abcam	Cat#ab81371	RRID:AB_1658424
Anti-SUMO4	Abcam	Cat#ab126606	RRID:AB_11128131
Anti-Mouse IgG	Cell Signaling Technology	Cat#5415S	RRID:AB_10829607
Anti-Rabbit IgG	Cell Signaling Technology	Cat#3900S	RRID:AB_1550038
Anti-TRAF2	Abcam	Cat#ab126758	RRID:AB_11145260
Anti-TRAF6	Cell Signaling Technology	Cat#8028S	RRID:AB_10858223
Anti-RIP	Abcam	Cat#ab72139	RRID:AB_2178115
Anti-NEMO	Abcam	Cat#ab178872	
Anti-IκBα	Abcam	Cat#ab32518	RRID:AB_733068
Anti-TAZ	Abcam	Cat#ab84927	RRID:AB_1925489
Anti-C/EBPβ	Abcam	Cat#ab32358	RRID:AB_726796
Anti-STAT3	Cell Signaling Technology	Cat#9139S	RRID: AB_331757
Anti-phospho-STAT3 (Tyr 705)	Cell Signaling Technology	Cat#9145S	RRID: AB_2491009
Anti-p65	Abcam	Cat#ab16502	RRID:AB_443394
Anti-phospho-p65 (Ser536)	Abcam	Cat#ab86299	RRID:AB_1925243
Anti-phospho-histone H2AX (Ser139)	Abcam	Cat#ab26350	RRID:AB_470861
Anti-α-tubulin	Abcam	Cat#ab7291	RRID:AB_2241126
Anti-UBC9	Abcam	Cat#ab75854	RRID:AB_1310787
Anti-Ubiquitin	Cell Signaling Technology	Cat#3933S	RRID:AB_2180538
Anti-CYLD	Cell Signaling Technology	Cat#4495S	RRID:AB_10557111
Anti-Myc-Tag	Cell Signaling Technology	Cat#2276S	RRID:AB_331783
Anti-HA-Tag	Cell Signaling Technology	Cat#3724S	RRID:AB_1549585
Anti-Flag-Tag	Sigma-Aldrich	Cat#F3165	RRID:AB_259529
Anti-Flag-Tag (For ChIP)	Cell Signaling Technology	Cat#14793S	
Goat Anti-Rabbit IgG H&L (HRP)	Abcam	Cat#ab6721	RRID:AB_955447
Goat Anti-Mouse IgG H&L (HRP)	Abcam	Cat#ab6789	RRID: AB_955439
Alexa Fluor ® 594 goat anti-rabbit IgG H+L	Thermo Fisher Scientific	Cat#A11012	RRID: AB_141359
Alexa Fluor ® 488 goat anti-rabbit IgG H+L	Thermo Fisher Scientific	Cat#A11070	RRID: AB_142134
Alexa Fluor ® 594 goat anti-mouse IgG H+L	Thermo Fisher Scientific	Cat#A11005	RRID: AB_141372
Alexa Fluor ® 488 goat anti-mouse IgG H+L	Thermo Fisher Scientific	Cat#A11017	RRID: AB_143160

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Recombinant DNA		
TRC shRNA Control	Dharmacon	Cat#RHS4080
FOSL1 shRNA #1	Dharmacon	Cat#TRCN0000019540
FOSL1 shRNA #2	Dharmacon	Cat#TRCN0000019542
SMARTvector Lentiviral Controls	Dharmacon	Cat#VSC11649
FOSL1 shRNA (Target 3'UTR)	Dharmacon	Cat#V3SH11240-225891219
UBC9 shRNA #1	Dharmacon	Cat#TRCN0000007205
UBC9 shRNA #2	Dharmacon	Cat#TRCN0000007206
CYLD shRNA #1	Dharmacon	Cat#TRCN0000039629
CYLD shRNA #2	Dharmacon	Cat#TRCN0000039630
pLenti6.2/V5-DEST Vector	Invitrogen	Cat#V36820
pLenti6.2-FOSL1	This study	N/A
pLenti6.2-UBC9 WT	This study	N/A
pLenti6.2-UBC9 C93S	This study	N/A
pCDH-CMV-MCS-EF1-puro	System Biosciences	Cat#CD510B-1
pCDH-Flag-FOSL1	This study	N/A
pCDH-HA-FOSL1	This study	N/A
pCDH-HA-UBC9	This study	N/A
pCDH-HA-CYLD	This study	N/A
pCDH-Flag-CYLD WT	This study	N/A
pCDH-Flag-CYLD K40R	This study	N/A
pCDH-Flag-UBC9 WT	This study	N/A
pCDH-Flag-UBC9 C93S	This study	N/A
pRK5-HA-Ubiquitin-K63	Addgene	Cat#17606
pRK5-HA-Ubiquitin-K48	Addgene	Cat#17605
pcDNA3.1	Invitrogen	Cat#V79020
pcDNA3.1-Myc-SUMO1	This study	N/A
pcDNA3.1-Myc-SUMO2	This study	N/A
pcDNA3.1-Myc-SUMO3	This study	N/A
pcDNA3.1-Myc-SUMO4	This study	N/A
pLenti-CMV-Puro-LUC	Addgene	Cat#17477
pGL4.32[luc2P/NF-κB-RE/Hygro]	Promega	Cat#E8491
pGL4.75[hRluc/CMV]	Promega	Cat#E6931
Experimental Models: Organisms/Strains		
Athymic nude mice	Animal Core Facility of Nanjing Medical University	N/A
Experimental Models: Cell Lines		
HEK293T	ATCC	Cat# CRL-3216; RRID: CVCL_0063
Normal human astrocytes	ScienCell	Catalog #1800
PN 35	This study	N/A
PN 182	This study	N/A
MES 21	This study	N/A
MES 505	This study	N/A

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Biological Samples		
GBM patient specimen	Department of Neurosurgery, the First Affiliated Hospital of Nanjing Medical University	Nanjing, China
GBM Tissue Microarray	Department of Neurosurgery, the Second and fourth Affiliated Hospitals of Harbin Medical University	Harbin, China
Chemicals, Peptides, and Recombinant Proteins		
DMEM/HIGH GLUSOSE	HyClone	Cat#SH30243.01
LM-DME/F12	HyClone	Cat#SH30023.01
Astrocyte Medium	ScienCell Research Laboratories	Catalog #1801
Fetal Bovine Serum	Gibco/Thermo Fisher Scientific	Cat#10100147
Penicillin-Streptomycin	Gibco/Thermo Fisher Scientific	Cat#15140122
B27 Supplement (50X)	Gibco/Life technologies	Cat#1889394
N-2 Supplement (100X)	Gibco/Life technologies	Cat#1890761
EGF human	Sigma-Aldrich	Cat#074M4114V
FGF-basic human, recombinant	Sigma-Aldrich	Cat#0413AFC08
LIF human	Sigma-Aldrich	Cat#086M4128V
Lipofectamine 3000	Invitrogen/Thermo Fisher Scientific	Cat#L3000-015
X-tremeGENE HP DNA Transfection Reagent	Roche	Cat#6366236001
Opti-MEM I Reduced Serum Medium	Gibco/Thermo Fisher Scientific	Cat#31985070
Pierce Protein A/G Agarose	Thermo Fisher Scientific	Cat#20421
Protease and Phosphatase Inhibitor Cocktail	Sigma-Aldrich	Cat#PPC1010
TNF-α	Sigma-Aldrich	Cat#SLBS9022
BAY11-7082	Selleck	Cat#S2913
PVDF Western Blotting Membranes	Roche Diagnostics	Cat#28745500
SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Fisher Scientific	Cat#34096
N-Ethylmaleimide	Sigma-Aldrich	Cat#E3876
VECTASHIELD Antifade Mounting Medium with DAPI	Vector Laboratories	Cat#H-1200
Critical Commercial Assays		
ViraPower™ II Lentiviral Gateway™ Expression System	Invitrogen	Cat#K36720
Papain Dissociation System	Worthington Biochemical	Cat#LK003176
Misfolded Protein Ubiquitination Kit	Ubiquitin-Proteasome Biotechnologies	Cat#J5110
RNeasy Mini Kit	Qiagen	Cat#74106
iScript cDNA Synthesis Kit	Bio-Rad	Cat#1708891
iTaq™ Universal SYBR® Green Supermix	Bio-Rad	Cat#172-5124
Ion 550™ Chip Kit	Thermo Fisher Scientific	Cat#A34538
CellTiter-Glo® Luminescent Cell Viability Assay	Promega	Cat#G7571
QuikChange Site-Directed Mutagenesis Kit	Agilent Technologies	Cat#200519
LightShift Chemiluminescent EMSA Kit	Thermo Fisher Scientific	Cat#20148

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Click-iT™ EdU Alexa Fluor™ 488 Imaging Kit	Invitrogen	Cat#C10337
MycoAlert™ Mycoplasma Detection Kit	Lonza	Cat#LT07-418
DAB Substrate Kit	Abcam	Cat# ab64238
Dual Luciferase Reporter Assay Kit	Promega	Cat#E1910
Sequence-Based Reagents		
All primers and oligonucleotides are listed in Table S3	This study	N/A
Software and Algorithms		
Software TriTek Comet Score Freeware v1.5	http://www.autocomet.com	TriTek Corporation
Extreme Limiting Dilution Analysis	http://bioinf.wehi.edu.au/software/elda/	ELDA
TCGA RNA Sequence Analysis	https://tcga-data.nci.nih.gov/docs/publications/gbm_exp/	N/A
AP-1 Binding Site Prediction	http://jaspar.genereg.net/	JASPAR
GraphPad Prism	https://www.graphpad.com/scientificsoftware/prism/	GraphPad Software
FlowJo	https://www.flowjo.com/solutions/flowjo	FLOWJO, LLC
ImageJ	https://imagej.nih.gov/ij/download.html	ImageJ
Pannoramic Viewer	http://www.3dhistech.com/pannoramic_viewer	3DHISTECH Ltd.