Oncogenic EGFR signaling activates an mTORC2-NF-κB pathway that promotes chemotherapy resistance

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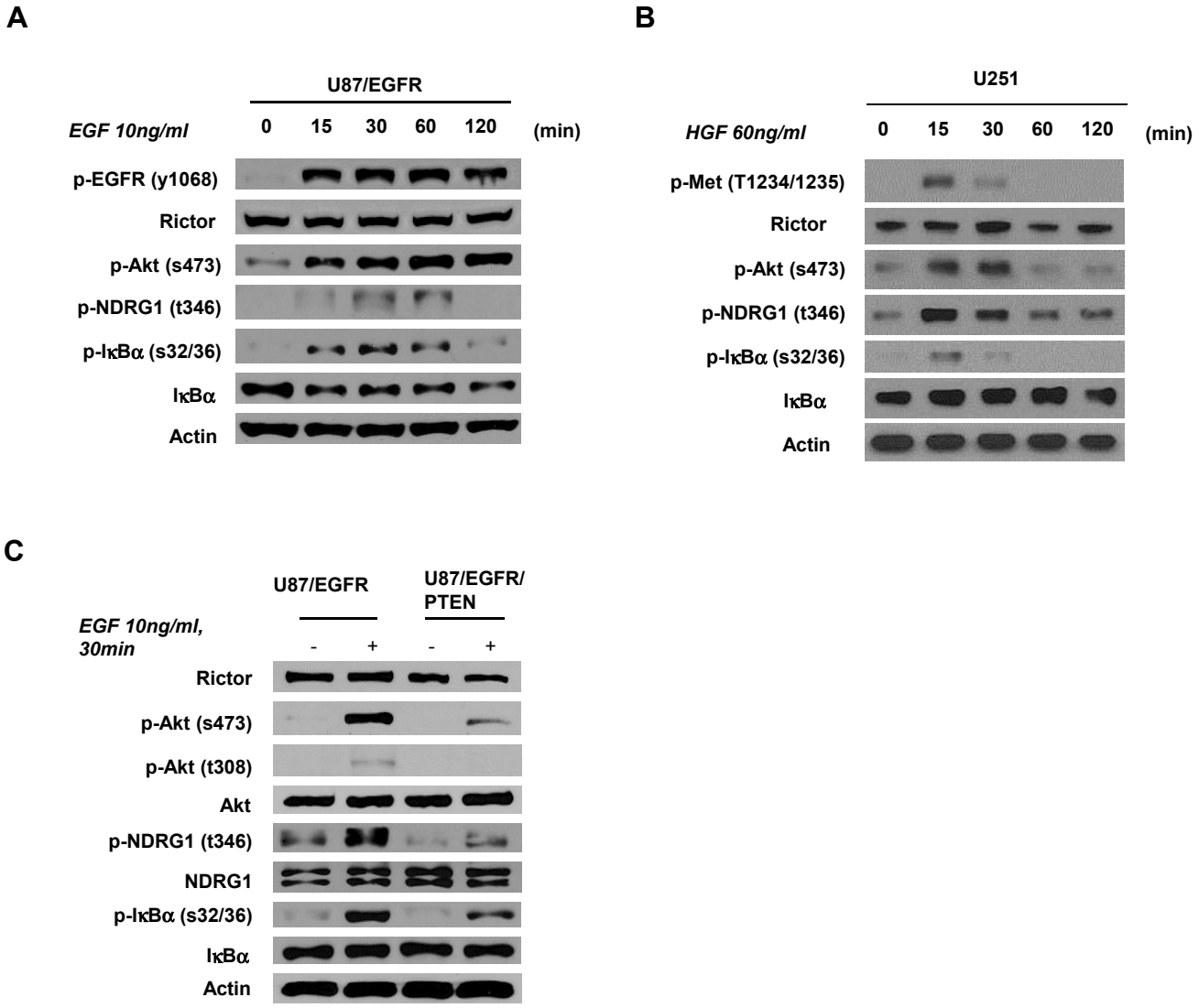
SUPPLEMENTAL INFORMATION

Supplemental Figures and Tables

Supplementary Figure 1.



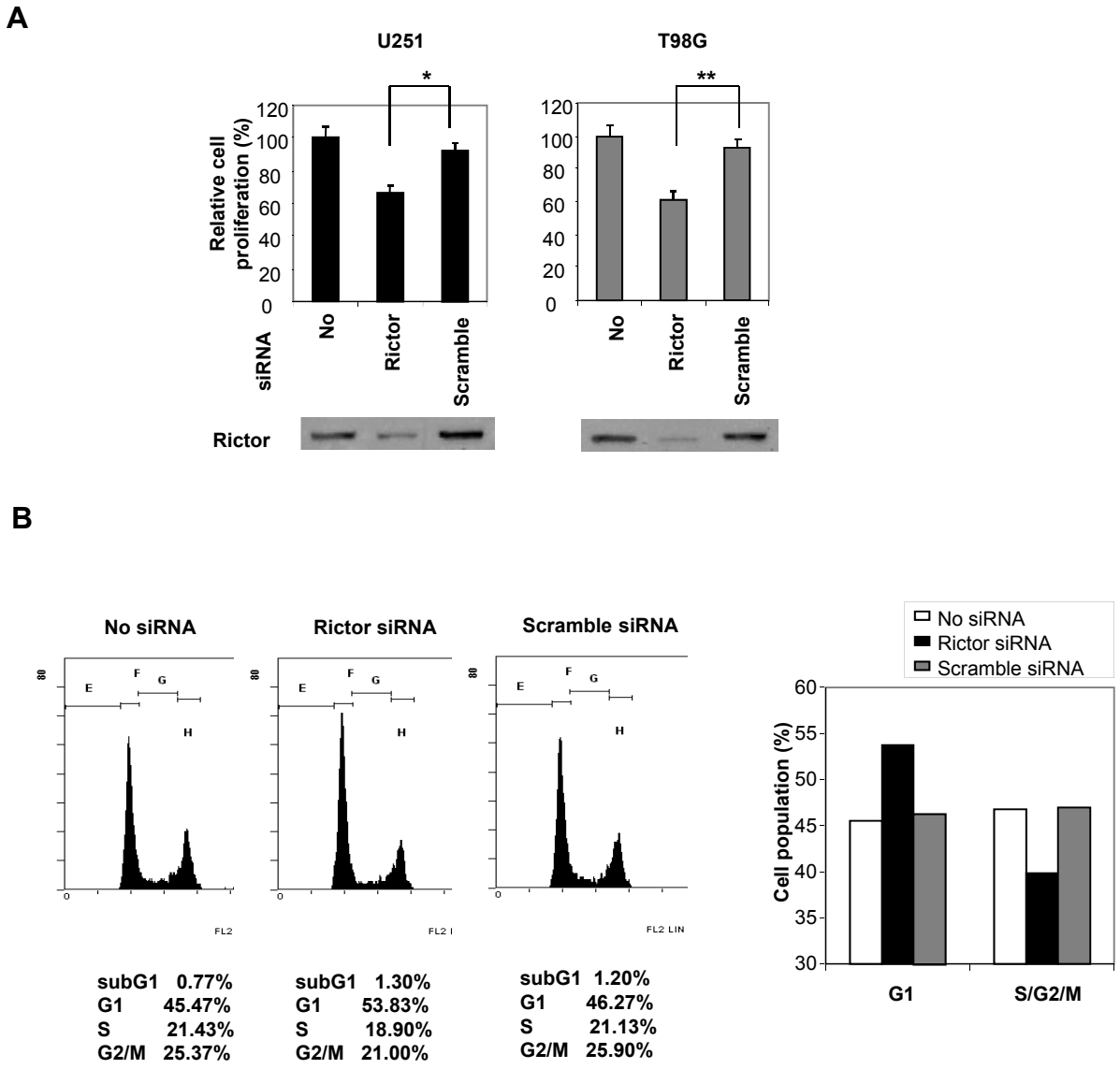
Supplementary Figure 1. EGFR signaling activates mTORC2 and NF-κB, which is partially suppressed by PTEN; HGF/c-MET signaling also activates mTORC2 and NF-κB; related to Figure 1

(A) Effect of EGF stimulation (10ng/ml) over time on mTORC2 and NF-κB in U87/wild-type EGFR cells.

(B) Effect of Met signaling using HGF (60ng/ml) stimulation on mTORC2 and NF-κB in U251 GBM cells.

(C) Effect of PTEN reconstitution on mTORC2 and NF-κB signaling in U87/wild-type EGFR cells with EGF-stimulation.

Supplementary Figure 2.

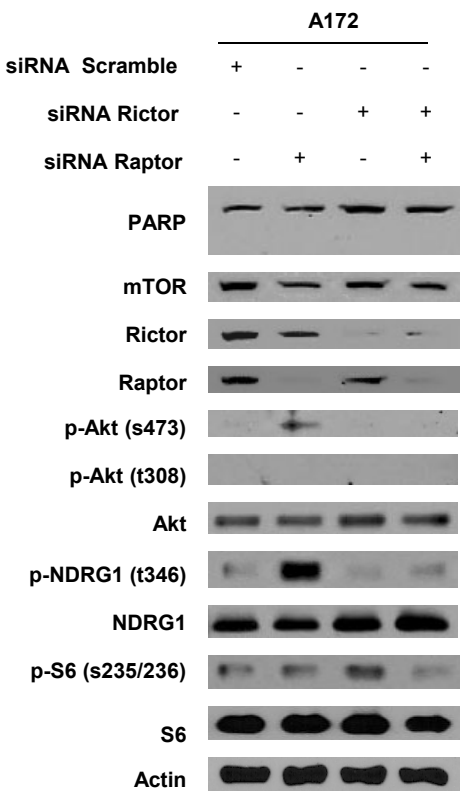


Supplementary Figure 2. mTORC2 promotes cell proliferation in multiple GBM cell lines, related to Figure 2

(A) Relative cell proliferation assay of U251 and T98G GBM cell lines with knockdown of Rictor. Relative cell growth was calculated with the cell proliferation assay. Data represent the mean +/- SEM of three independent experiments (Statistically significant with *$p<0.01$, **$p<0.001$).

(B) U87/EGFRvIII cells were transfected with Rictor siRNA or scrambled control siRNA constructs for 48 hours and then stained with propidium iodine (PI). The percentage of sub-G1, G1, S, G2/M population was calculated based on the results of cell cycle analysis by FACScan flow cytometry.

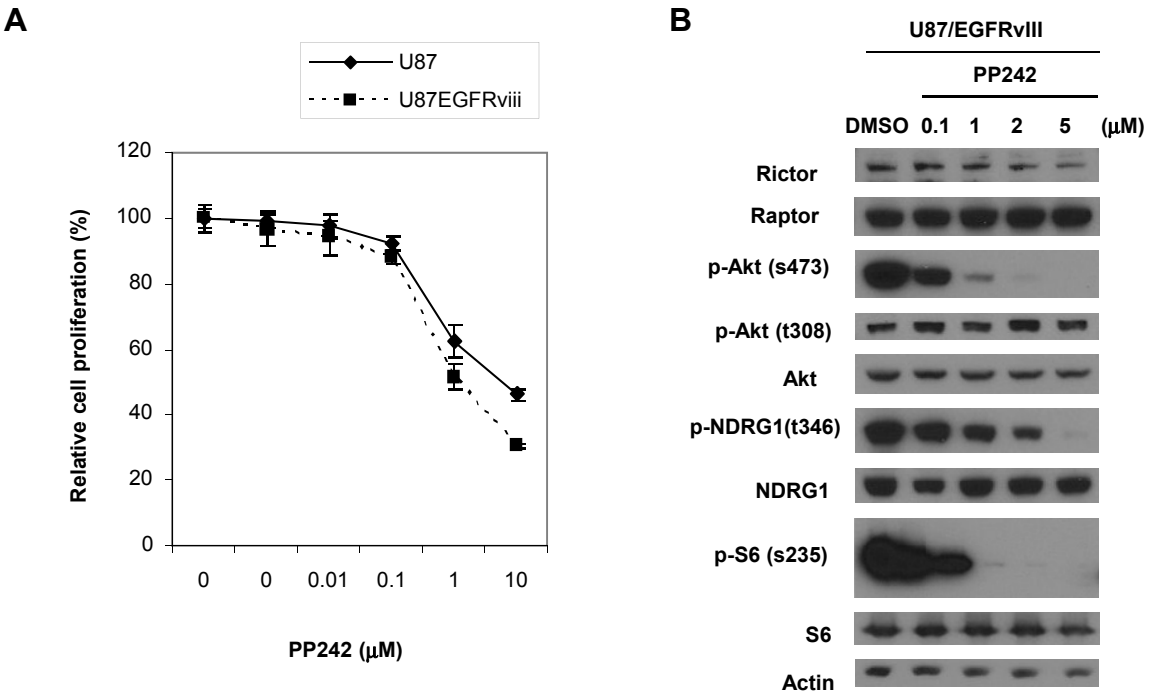
Supplementary Figure 3.



Supplementary Figure 3. Raptor knockdown increased mTORC2 signaling which is abrogated by Rictor knockdown in A172 GBM cells, related to Figure 2

Immunoblot analysis using indicated antibodies of A172 glioma cells with the siRNA against Raptor, Rictor or scrambled control.

Supplementary Figure 4.

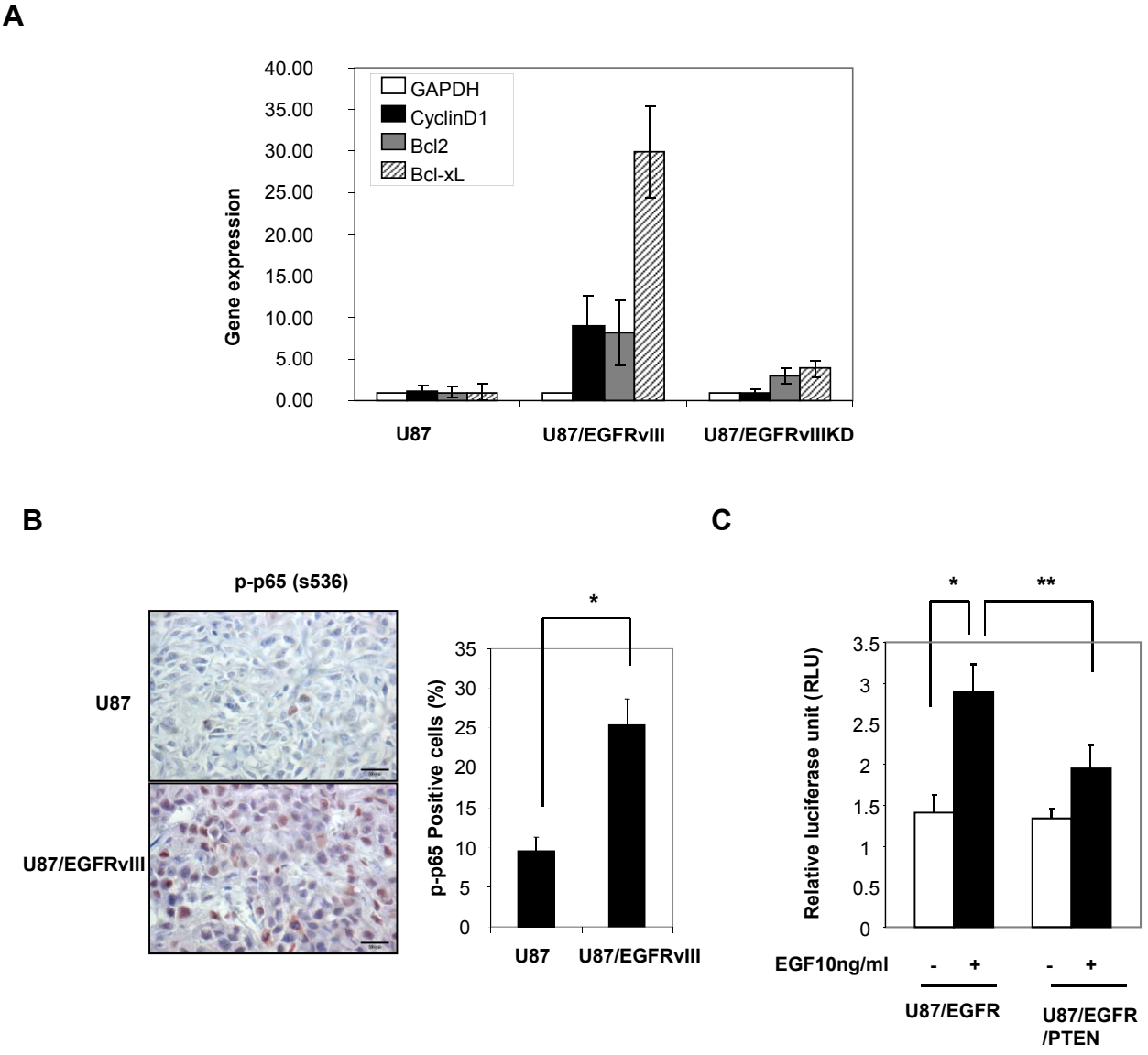


Supplementary Figure 4. mTOR kinase inhibitor, PP242 regulates cell proliferation in U87 and U87/EGFRvIII GBM cell lines, related to Figure 2

(A) Relative cell proliferation assay of U87 and U87/EGFRvIII cells treated with mTOR kinase inhibitor PP242.

(B) Immunoblot analysis using indicated antibodies of U87/EGFRvIII cells treated with PP242.

Supplementary Figure 5.



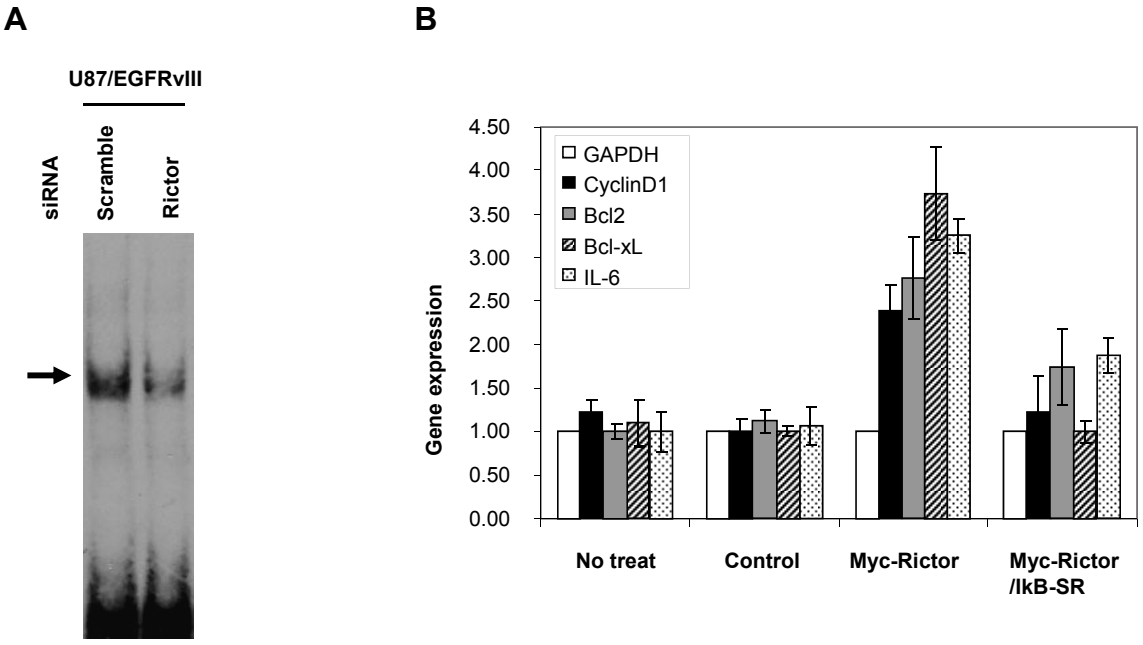
Supplementary Figure 5. EGFRvIII promotes NF-κB activity, related to Figure 3

(A) Assessment of changes in mRNA levels of NF-κB target gene expression using RT–PCR method. Data represent the mean +/- SEM of three independent experiments.

(B) Representative immunohistochemical images demonstrating p-p65(S536) to assess NF-κB signaling. Scale bar, 20 μm. Quantitative image analysis of p-p65(S536) expression was performed with NIH image. Data represent the mean +/- SEM of three independent experiments (Statistically significant with *$p<0.01$).

(C) Luciferase reporter assays targeting NF-κB signal transduction using EGF-stimulated U87/wild-type EGFR cells with PTEN reconstruction or not (measured as relative luciferase/luminescence units). Data represent the mean +/- SEM of three independent experiments (Statistically significant with *$p<0.01$, **$p<0.05$).

Supplementary Figure 6.

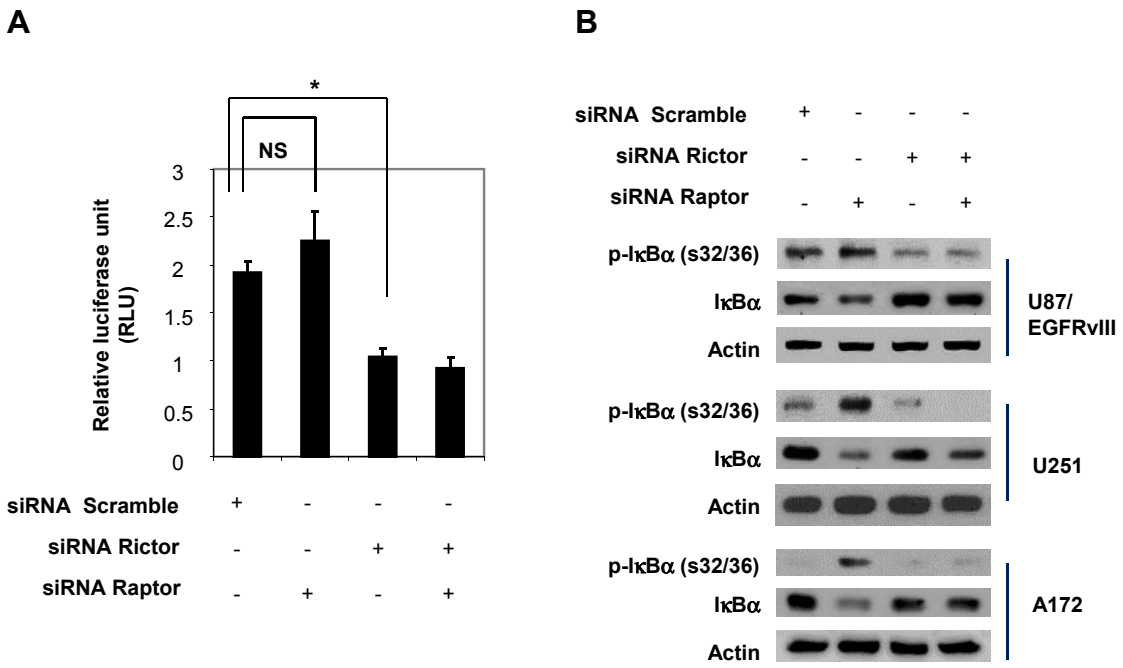


Supplementary Figure 6. EGFRvIII promotes NF-κB activity through mTORC2, related to Figure 3

(A) EMSAs were performed using nuclear extracts from U87/EGFRvIII cells with knockdown of Rictor and scramble. Arrow denotes the DNA/protein EMSA complex. Arrow denotes the DNA/protein EMSA complex.

(B) Assessment of changes in mRNA levels of NF-κB target gene expression in U87 cells transfected with myc-Rictor expressing vector and adenovirus encoding IκBα-SR using RT–PCR method. Data represent the mean +/- SEM of three independent experiments.

Supplementary Figure 7.

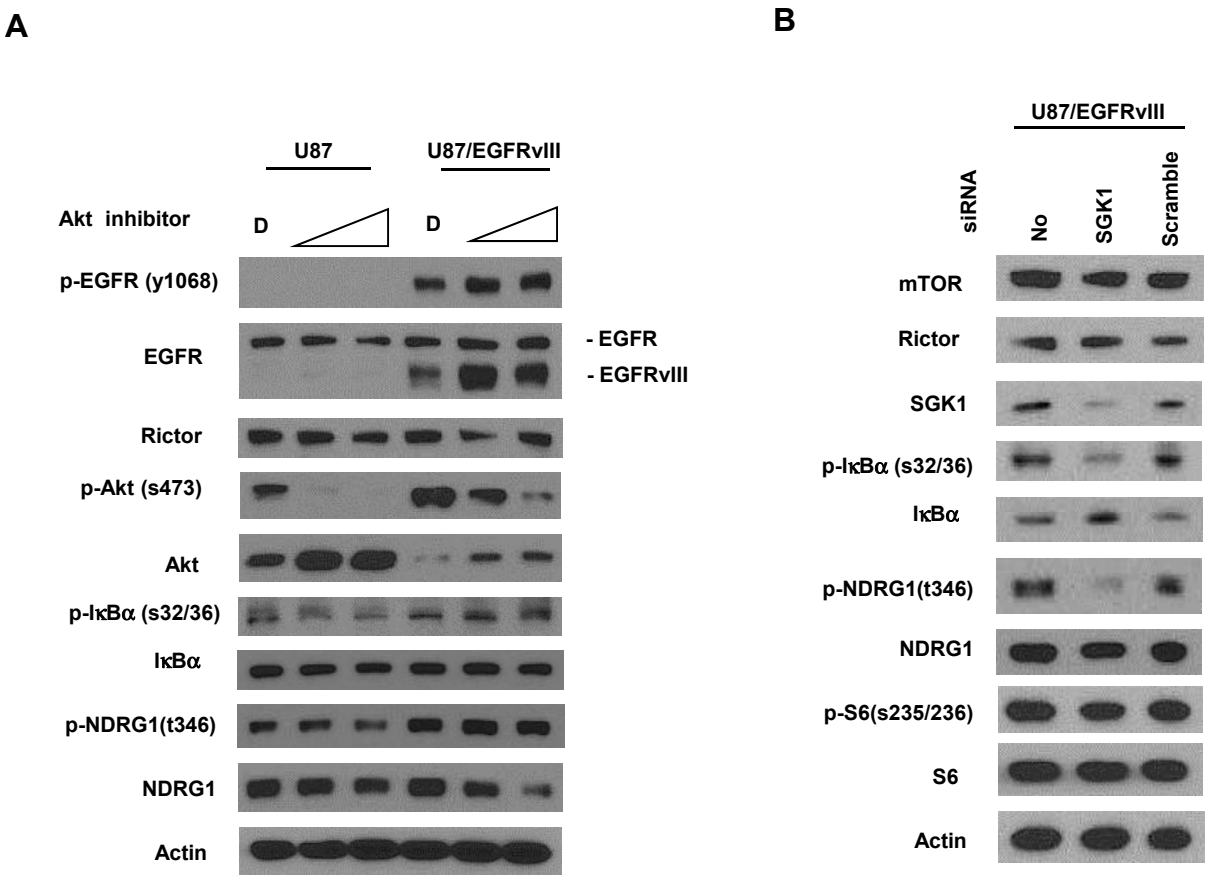


Supplementary Figure 7. mTORC2 but not mTORC1 activates NF-κB signaling in GBM cells, related to Figure 3

(A) U87/EGFRvIII cells were transfected with the NF-κB-dependent luciferase reporter and siRNA against Raptor, Rictor, and scramble, as indicated. Luciferase reporter assays were performed 48hr after transfection. Levels of luciferase are compared with the siRNA scrambled control. Data represent the mean +/- SEM of three independent experiments (Statistically significant with *$p<0.01$, NS; not significant).

(B) Immunoblot analysis of p-IκBα(S32/36) of U87/EGFRvIII, U251, and A172 GBM cells using same lysate as used in Figure 2E and Supplementary Figure S3.

Supplementary Figure 8.

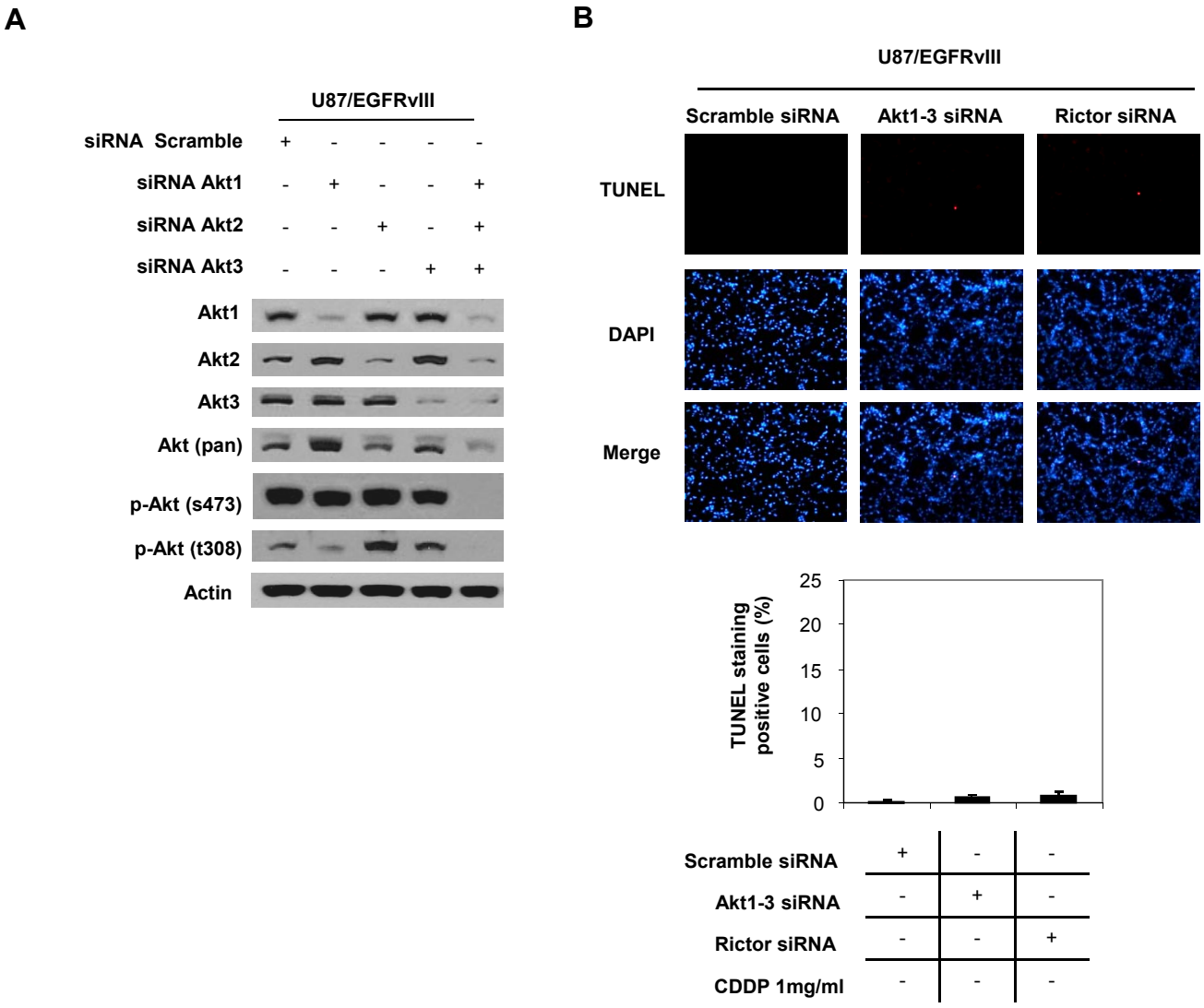


Supplementary Figure 8. mTORC2 promotes NF-κB activity through SGK1, independent of Akt, related to Figure 3

(A) Immunoblot analysis using indicated antibodies of U87 and U87/EGFRvIII cells treated with Akt inhibitor (1-2.5μM) (D; DMSO).

(B) Immunoblot analysis using indicated antibodies of U87/EGFRvIII cells with the siRNA against SGK1.

Supplementary Figure 9.

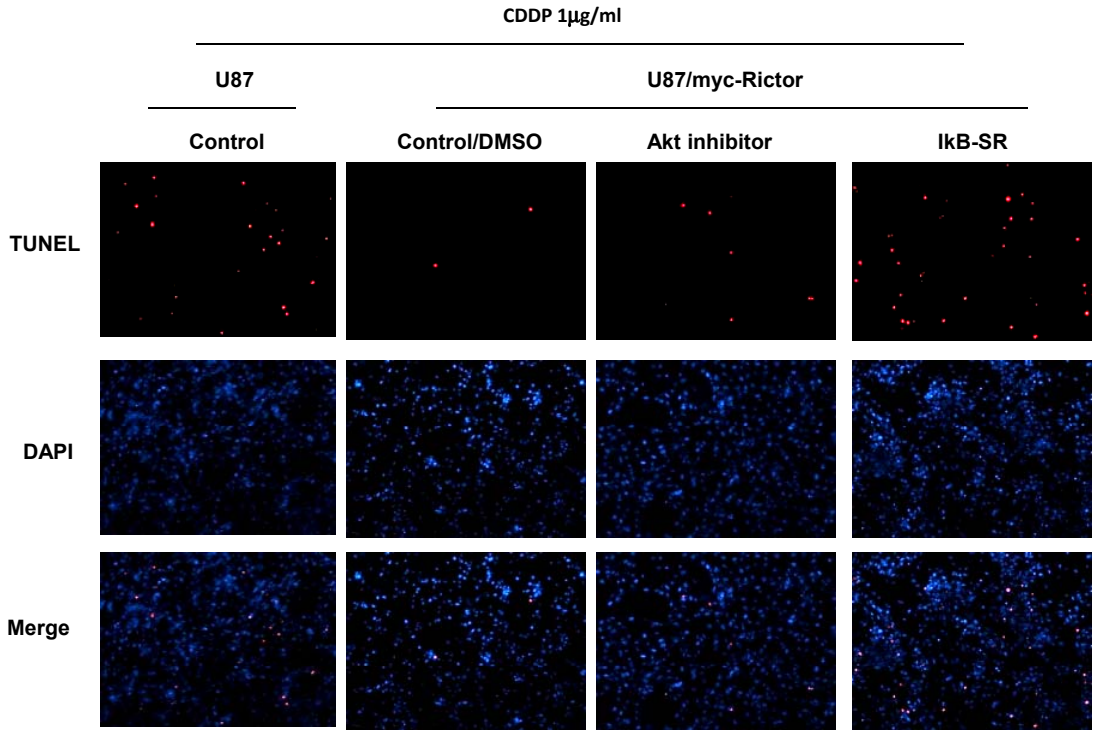


Supplementary Figure 9. Akt or Rictor knockdown doesn't induce cell death in U87/EGFRvIII cells, related to Figure 4

(A) Immunoblot analysis using indicated antibodies of U87/EGFRvIII cells with the siRNA against Akt1, Akt2 and Akt3, and all.

(B) TUNEL staining in U87/EGFRvIII cells transfected with siRNA against Akt1-3, Rictor and scrambled control. The percentage of apoptotic cells was calculated as the percentage of TUNEL-positive cells out of 400 cells for each group using NIH image. Data represent the mean +/- SEM of three independent experiments. Images are magnified x100.

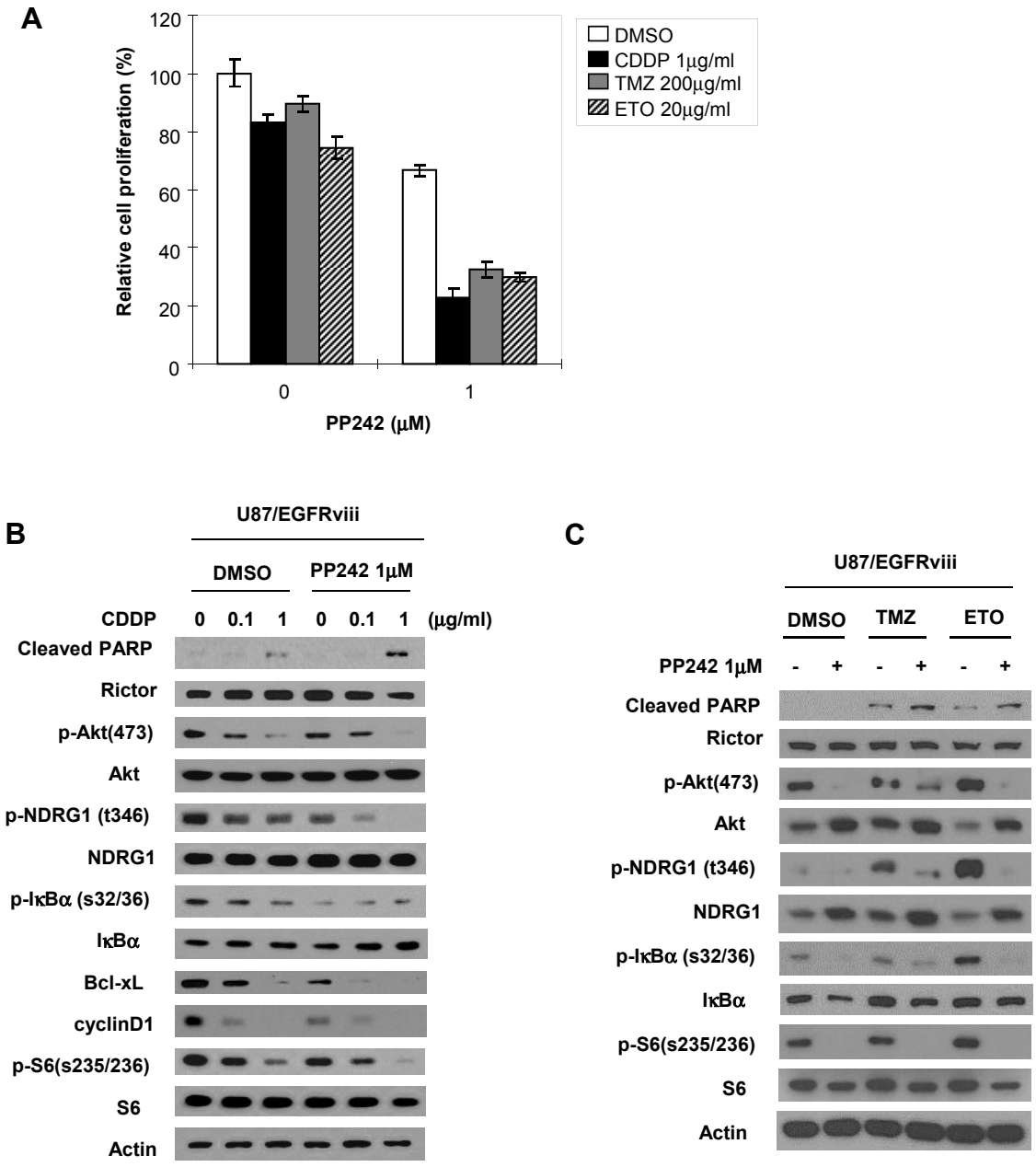
Supplementary Figure 10.



Supplementary Figure 10. mTORC2 mediates chemotherapy resistance through NF-κB, in a manner independent of Akt, related to Figure 4

TUNEL staining in myc-Rictor expressing U87 cells treated with Akt inhibitor (2.5μM) or transfected adenovirus encoding IκBα-SR under cisplatin (CDDP) treatment (1μg/ml). Images are magnified x100.

Supplementary Figure 11.



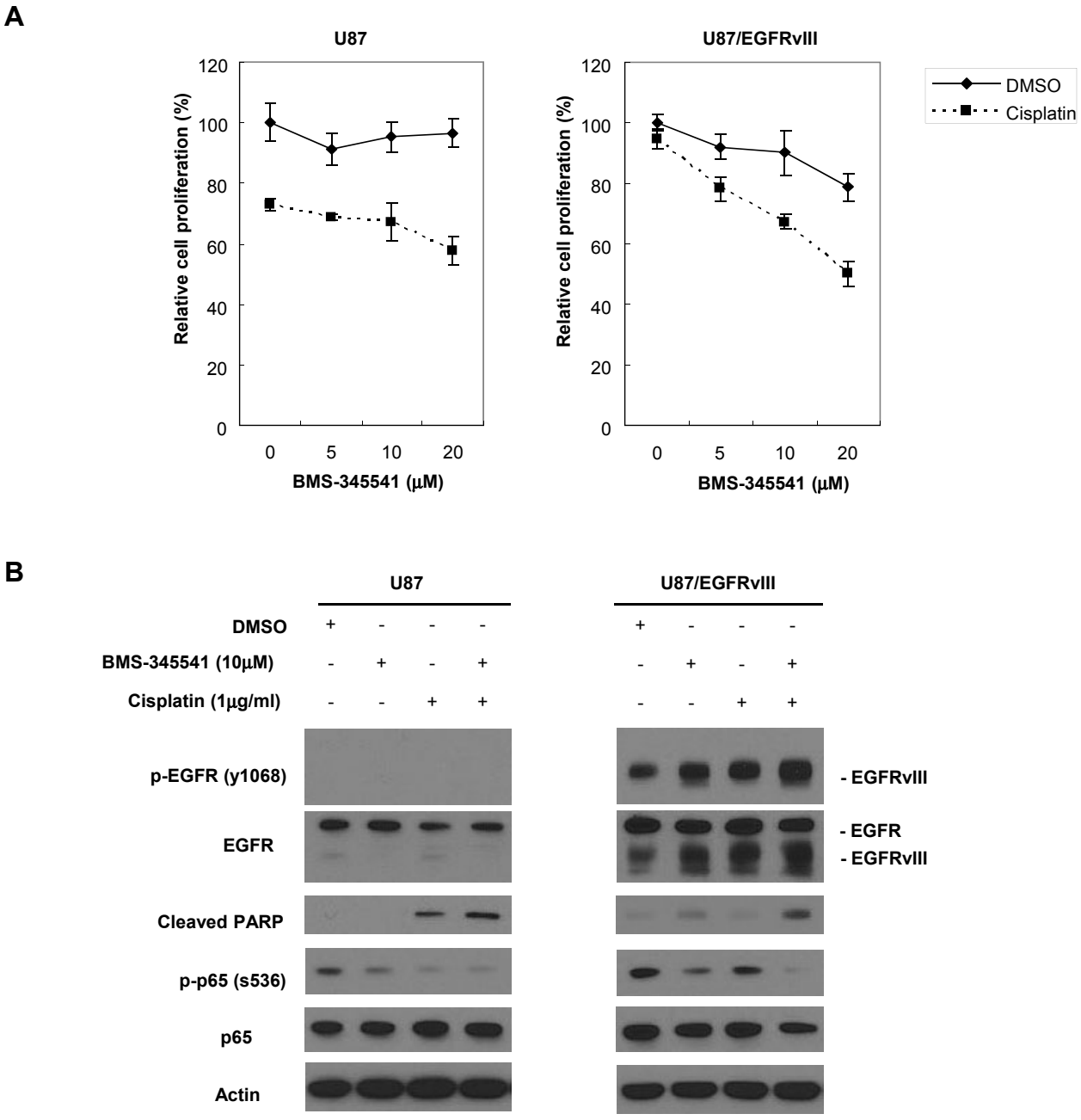
Supplementary Figure 11. Pharmacological inhibition of mTORC2 reverses chemotherapy resistance, related to Figure 4

(A) Relative cell proliferation assay of U87/EGFRvIII cells treated with Cispaltin (CDDP, 1μg/ml), Temozolomeide (TMZ, 200μg/ml) and Etoposide (ETO, 20μg/ml) combined with PP242 (1μM).

(B) Immunoblot analysis using indicated antibodies of U87/EGFRvIII cells treated with Cisplatin (0, 0.1 and 1μg/ml) and/or PP242 (1μM).

(C) Immunoblot analysis using indicated antibodies of U87/EGFRvIII cells treated with Temozolomide (TMZ, 200μg/ml) or Etoposide (ETO, 20μg/ml) combined with PP242 (1μM).

Supplementary Figure 12.

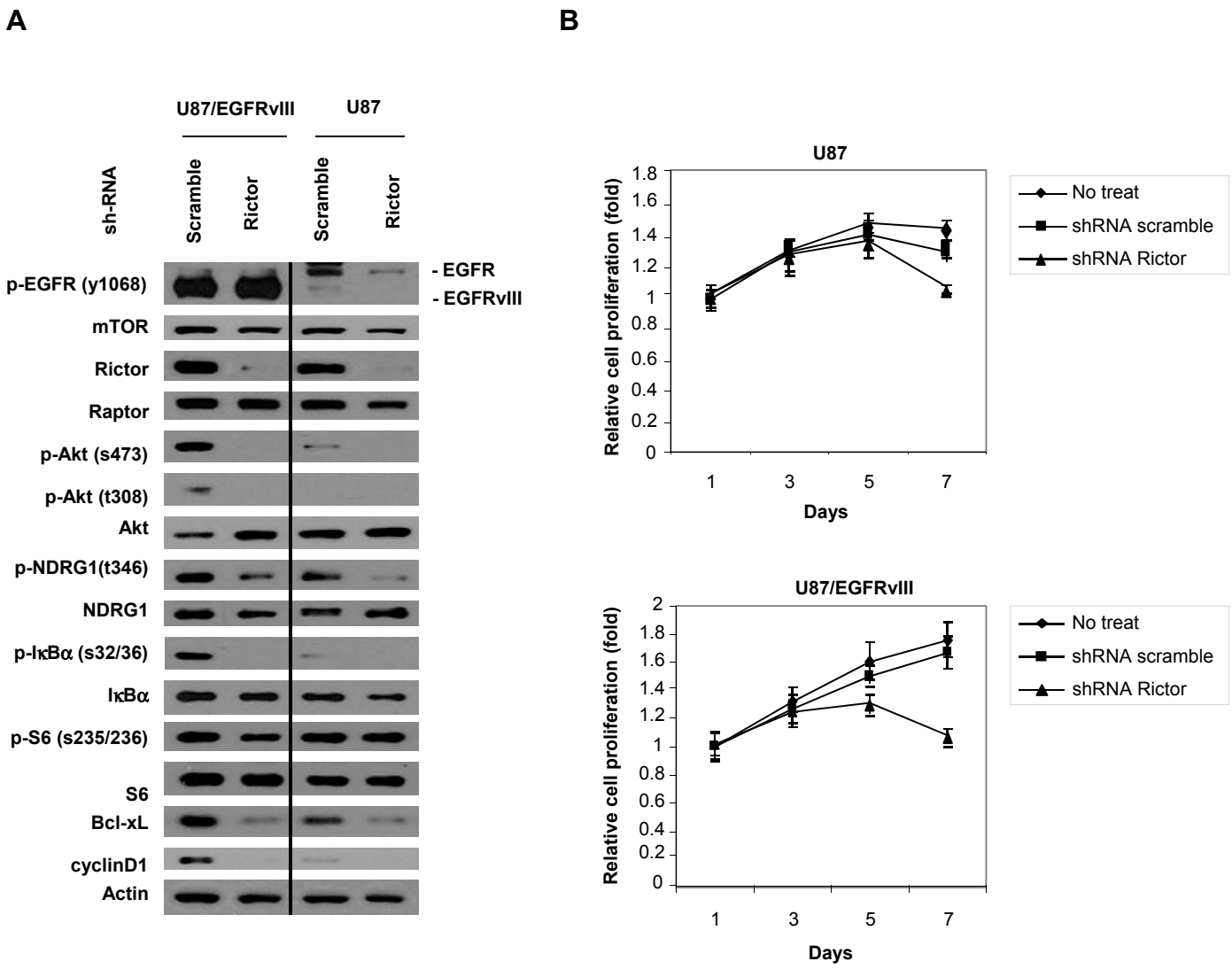


Supplementary Figure 12. IKK inhibitor BMS-345541 sensitizes U87/EGFRvIII cells to Cisplatin, related to Figure 4

(A) Relative cell proliferation assay of U87 and U87/EGFRvIII cells treated with BMS-345541 and/or Cisplatin.

(B) Immunoblot analysis using indicated antibodies of U87 and U87/EGFRvIII cells treated with BMS-345541 and/or Cisplatin.

Supplementary Figure 13.



Supplementary Figure 13. Stable knockdown of Rictor in GBM cells used for *in vivo* study, related to Figure 5

(A) Immunoblot analysis using indicated antibodies of U87 and U87/EGFRvIII cells 2 weeks after transfected with shRNA Rictor or control scramble.

(B) Relative cell proliferation assay of U87 and U87/EGFRvIII cells which is expressing stable knockdown of Rictor and control scramble under serum-free condition.

Supplementary Table1. Tissue microarray (TMA) analysis in GBM samples, related to Figure 6

A. Immunohistochemical staining of proteins in tissue microarrays of GBM samples and adjacent normal brain.

		Tumor	Normal
Rictor	+	150	39
	-	84	44
Total number		234	83
Positive rate (%)		**64.10**	46.99
P-value		***p*<0.01**	

		Tumor	Normal
p-NDRG1 (T346)	+	132	34
	-	94	43
Total number		226	77
Positive rate (%)		**58.41**	44.16
P-value		***p*<0.05**	

		Tumor	Normal
p-p65 (S536)	+	139	35
	-	95	48
Total number		234	83
Positive rate (%)		**59.40**	42.17
P-value		***p*<0.01**	

B. Co-expression of immunohistochemical markers in GBM samples on tissue microarrays.

		p-Akt(S473)		**p-NDRG1(T346)**		**p-EGFR(Y1068)**	
		+	-	+	-	+	-
Rictor	+	114	11	93	50	40	65
	-	48	15	39	38	19	23
Total number		162	26	132	88	59	88
P-value		***p*<0.01**		***p*<0.05**		***NS***	

*252 tumor cores and 91 normal cores from 140 patients on two tissue microarrays.

** Numbers may not add up to 252 or 91 because of missing cores.

P-value was determined by Chie square for independence test.

Supplementary Table2. Real-time PCR primer sequences for CyclinD1, Bcl2, Bcl-xL, IL-6 and GAPDH.

Gene		Sequence	Fragment size
CyclinD1	F	5’- GAGGAAGAGGAGGAGGAGGA -3’	236bp
	R	5’- GAGATGGAAGGGGGAAAGAG -3’	
Bcl-2	F	5’- GGATGCCTTTGTGGAACTGT -3’	236bp
	R	5’- AGCCTGCAGCTTTGTTTCAT -3’	
Bcl-xL	F	5’- TCTGGTCCCTTGCAGCTAGT -3’	196bp
	R	5’- CAGGGAGGCTAAGGGGTAAG -3’	
IL-6	F	5’- ATGCAATAACCACCCCTGAC -3’	167bp
	R	5’- GAGGTGCCCATGCTACATTT -3’	
GAPDH	F	5’- TTCGACAGTCAGCCGCATCTTCTT -3’	110bp
	R	5’- CAGGCGCCCAATACGACCAAATC -3’	