List of Supplementary Materials:

Supplemental Experimental Procedures

Supplemental Figures

Supplemental Table

Supplemental Experimental Procedures

Cell proliferation and death assays

Cells were placed in 96-well plates at $2x10^3$ cells/well in 100 μl of growth medium and then incubated for 48 hours in each treatment condition. Cell proliferation was examined with Cell Count Reagent SF (Nacalai Tesque) according to the manufacturer's instructions. The absorbance of the treated and untreated cells was measured with a microplate reader (Thermo Scientific) at 450 nm. Cell death was assessed by trypan blue exclusion (Nacalai Tesque).

Western blotting

Cultured cells or snap-frozen tissue samples were lysed and homogenized with a Lysis buffer AM1 and phosphatase inhibitor and protease inhibitor cocktail (Active Motif). Equal amounts of protein extracts were separated by electrophoresis on 4-12% NuPAGE Bis-Tris Mini Gels (Invitrogen) and then transferred to a nitrocellulose membrane (GE Healthcare) with the XCell II Blot Module (Invitrogen). The membrane was blocked for 1 hour in Tris-buffered saline containing 0.1% Tween20 and 5% nonfat milk and then probed with various primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase

(HRP). The immunoreactivity was revealed with Super Signal West Pico Chemiluminescent Substrate or the West Femto Trial Kit (Thermo Scientific).

TUNEL staining

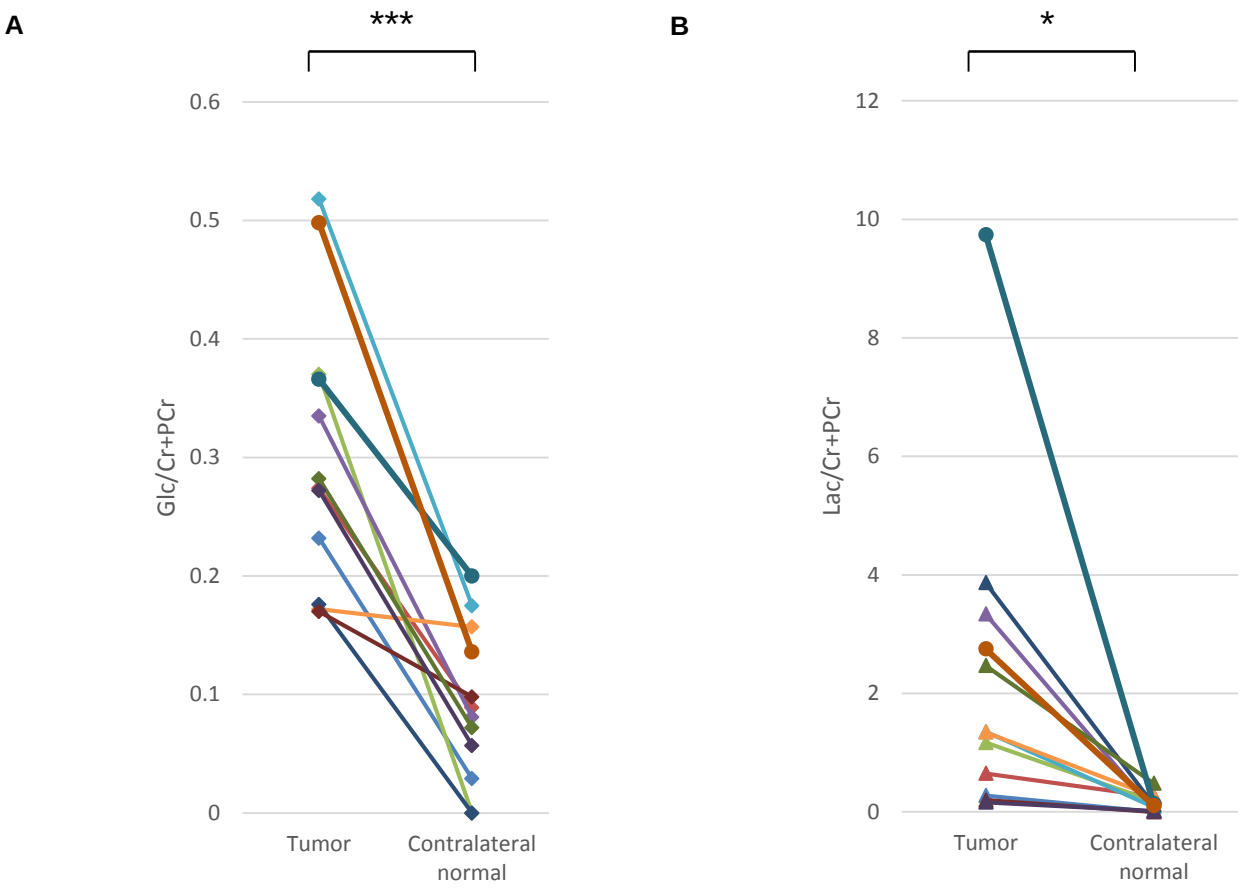
Cells were placed in 6-well chamber slides at $1x10^5$ cells/well in 5 ml of growth medium and then incubated for 48 hours in each treatment condition. Apoptotic cells were evaluated with the In Situ Cell Death Detection Kit, Fluorescein and following the manufacturer's protocol (Roche). Nuclei were stained blue by 4′, 6-diamidino-2-phenylindole (DAPI) (Invitrogen). TUNEL-positive cells were visualized with a fluorescein microscope (Keyence BZ-9000, Japan). The percentage of apoptosis was calculated as the percentage of TUNEL-positive cells out of 400 cells from each group using the NIH images.

ATP levels

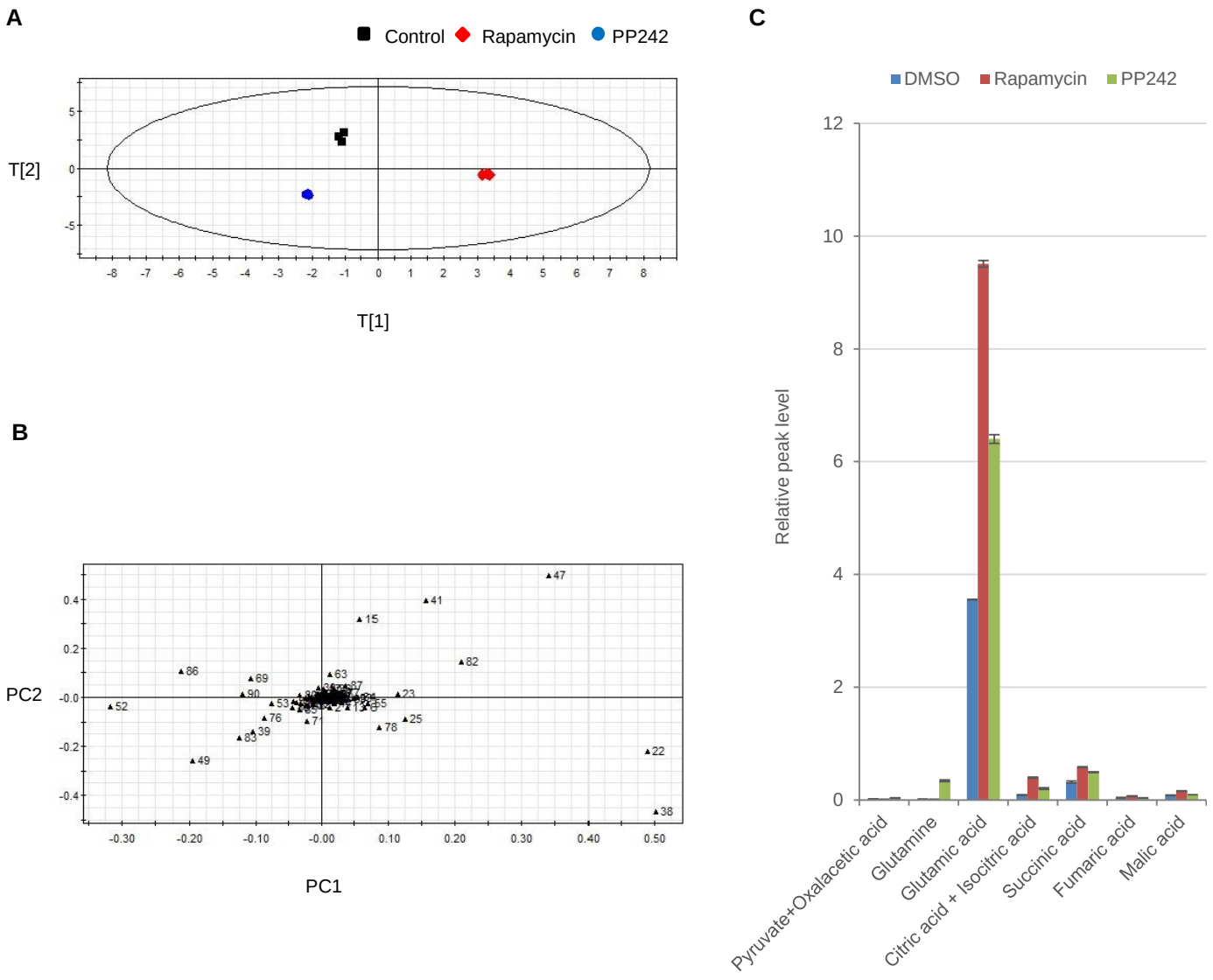
Intracellular ATP levels were measured following the manufacturer's instructions (ATP bioluminescence assay kit HD II, Roche).

Rota-Rod

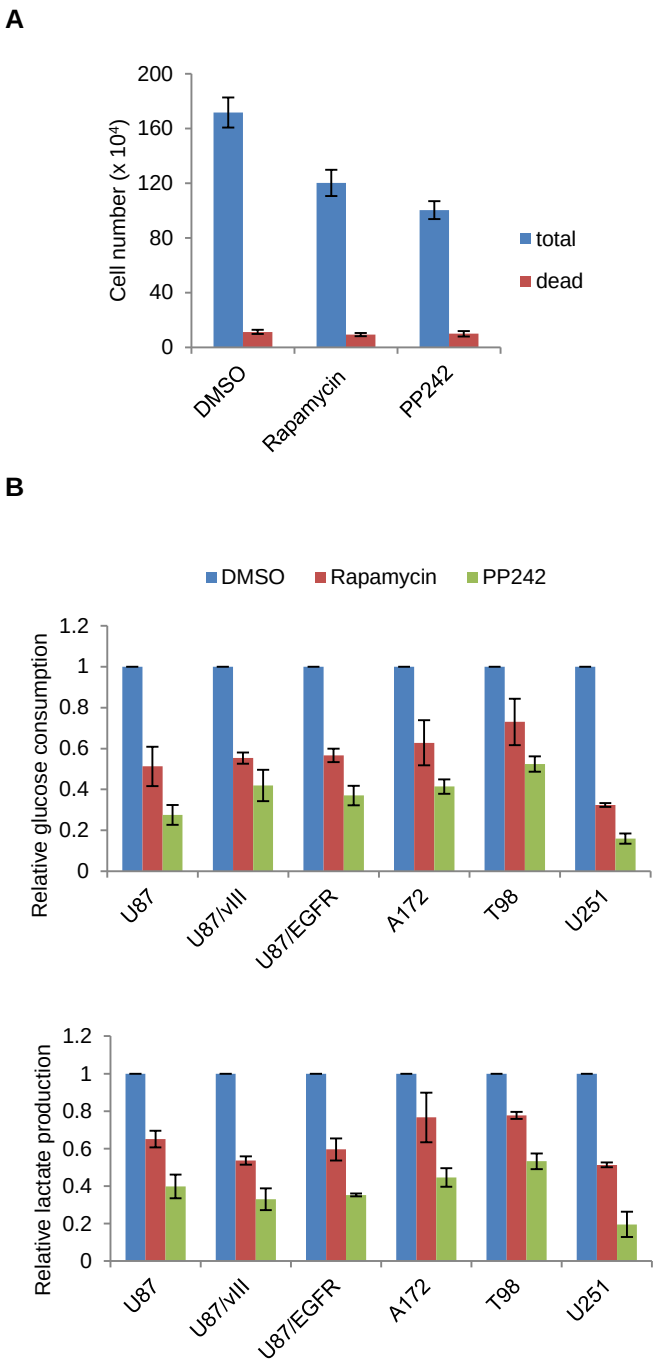
Motor coordination was measured by using a rota-rod treadmill for mice (Muramachi, Japan). Four groups of immunodeficient BALB/cAJcl-nu/nu mice were used and treated for 15 days with intraperitoneal injections of PP242 (5 mg/kg), Compound968 (5 mg/kg), or a combination of PP242 (5 mg/kg) and Compound968 (5 mg/kg). All mice were tested at the last day of treatment course. After practice trial 3 times, mice were placed on the rota-rod apparatus at a constant speed of 15 r.p.m., and the time to fall from the rod was measured with a cutoff time established at 5 min.



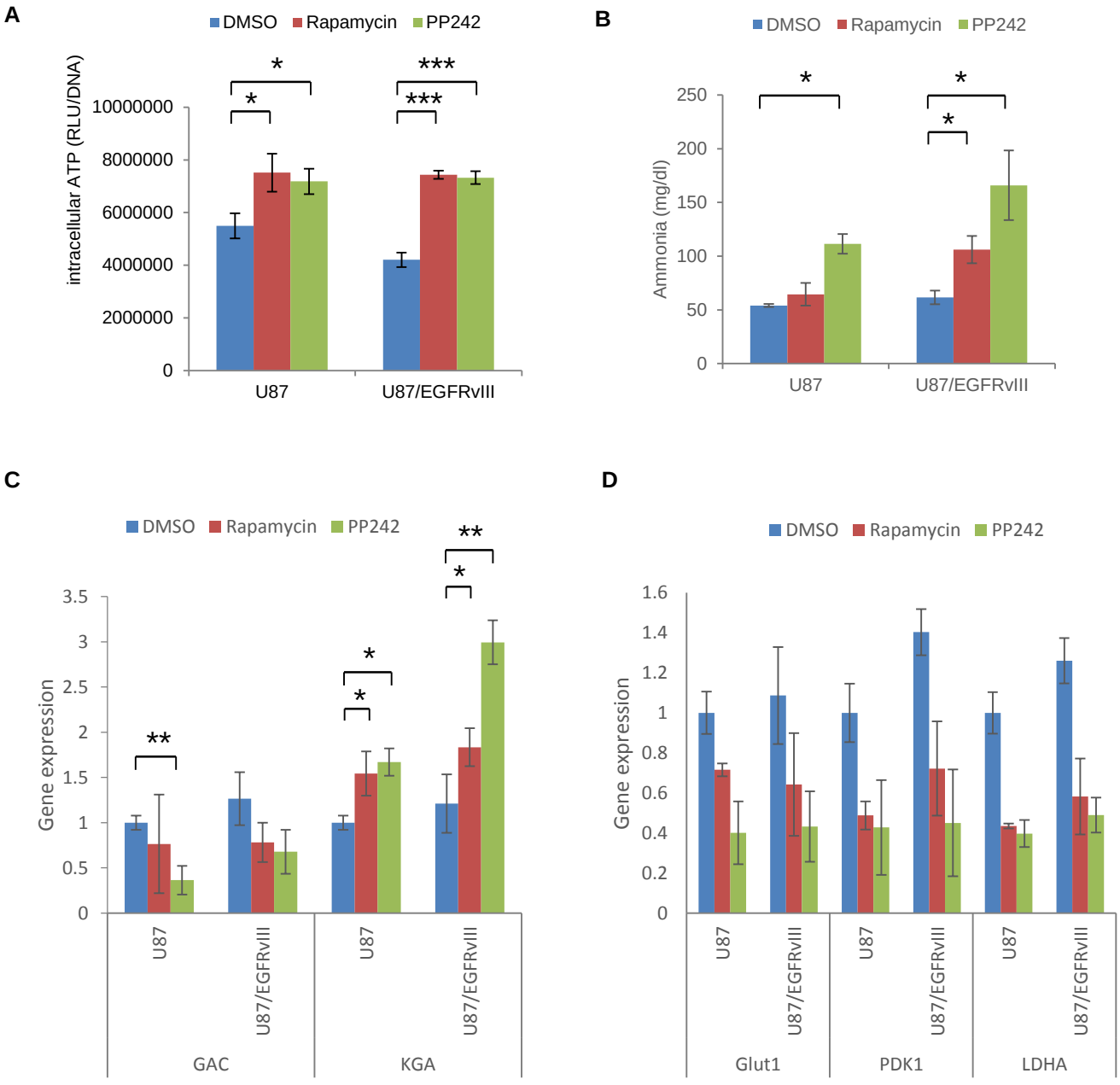
Supplemental Figure 1.
Glucose and lactate levels increase in the tumor of GBM patients, related to Figure 1.
(**A**) Glucose level on MRS studies for tumors and contralateral normal brain regions in 12 GBM patients. The relative level of glucose (Glc) was calculated with respect to creatine and phosphocreatine (Cr and PCr) for regions of interest (ROIs) of tumor and contralateral normal brain. ****p<0.001*, according to 2-tailed Student's *t* test. (**B**) Lactate level on MRS studies for tumors and contralateral normal brain regions in 12 GBM patients. The relative level of lactate (Lac) was calculated with respect to creatine and phosphocreatine (Cr and PCr) for regions of interest (ROIs) of tumor and contralateral normal brain. **p<0.05,* according to 2-tailed Student's *t* test.



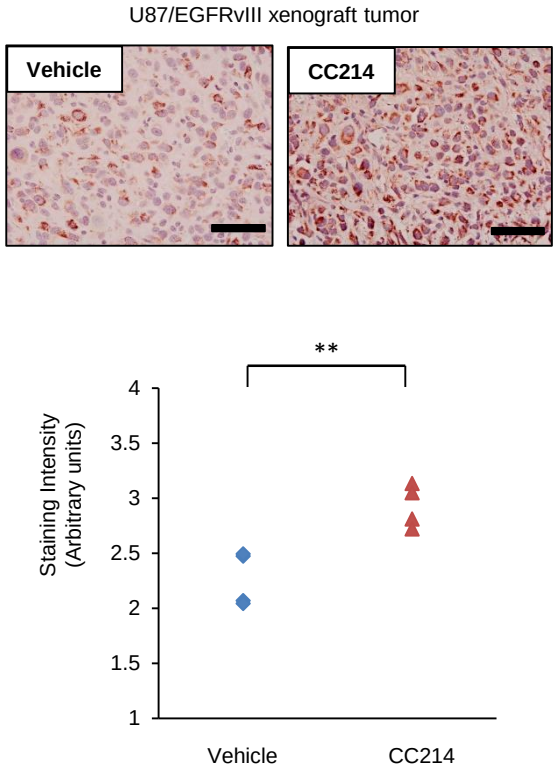
Supplemental Figure 2.
Principle component analysis (PCA) of metabolites identified as differentially expressed among U87/EGFRvIII GBM cells treated rapamycin, PP242, and control DMSO, related to Figure 2.
(**A**) PCA score plots (upper) discriminating among U87/EGFRvIII GBM cells treated with 1 nM rapamycin, 1 mM PP242, and control DMSO for 48 hours. The black squares, red diamonds, and blue circles indicate control group (n = 3), rapamycin treatment group (n = 3), and PP242 treatment group (n = 3), respectively. The principal components PC1 (t[1], a horizontal axis) and PC2 (t[2], a vertical axis) described 55.1 and 41.8% of the variation, respectively. (**B**) PCA loading plots (lower) were calculated on the basis of score plots. The number of metabolites indicated in Supplemental Table 1 (ex, 22; aspartic acid, 25; citric or isocitric acid, 38; glutamic acid, 78; succinic acid). **(C)** Metabolites targeting glutaminlysis and the TCA cycle, pyruvate and oxaloacetic acid, glutamine, glutamic acid, citric and isocitric acid, succinic acid, fumaric acid and malic acid, were extracted and profiled. Data represent the mean ± SEM of three independent samples, respectively.



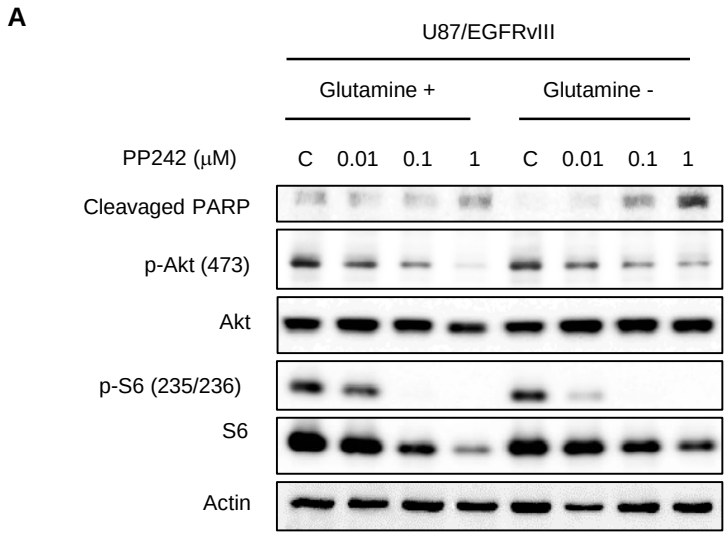
Supplemental Figure 3.
Cell proliferation and glucose metabolism are regulated by mTOR-targeted treatments, related to Figure 3.
(**A**) U87/EGFRvIII cells were treated with 1 nM rapamycin, 1 μM PP242, and control DMSO for 48 hours. Cell death was measured by trypan blue exclusion. Data represent the mean ± SEM of three independent experiments. (**B**) Relative glucose consumption and lactate production in U87 isogenic cells and other GBM cell lines (A172, T98 and U251 cells) treated with control DMSO versus 1 nM rapamycin or 1 μM PP242. . Data represent the mean ± SEM of three independent experiments.

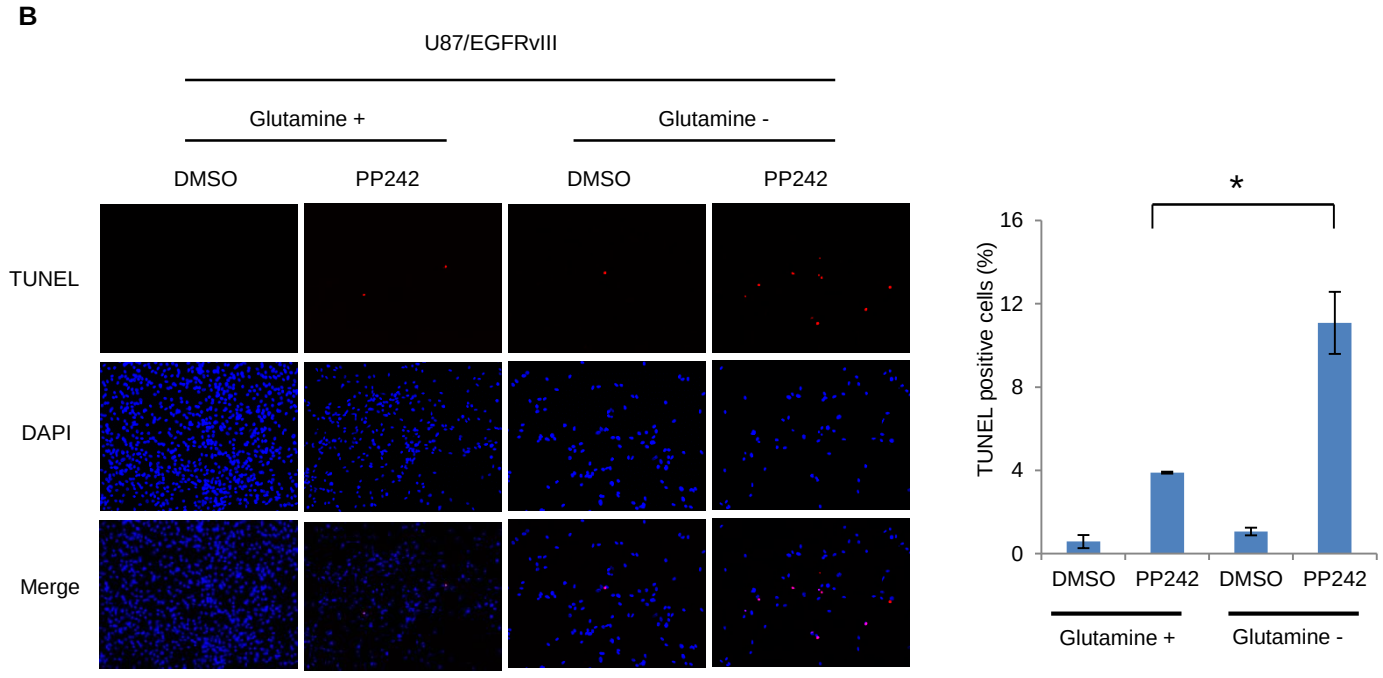


Supplemental Figure 4.
Alteration in intracellular ATP, ammonia, and metabolic gene induced by mTOR inhibition treatments, related to Figure 3.
(**A**) ATP levels of U87 and U87/EGFRvIII cells treated with 1 nM rapamycin, 1 μM PP242, or control DMSO for 48 hours. ATP levels were determined by the luciferine-luciferase-based assay on aliquots containing an equal number of live cells. Data represent the mean ± SEM of three independent experiments. **P<0.05*, ****P<0.001,* according to 2-tailed Student's *t* test. (**B**) Intracellular levels of ammonia in U87 and U87/EGFRvIII GBM cells treated with 1 nM rapamycin, 1 μM PP242, or control DMSO for 48 hours. Data represent the mean ± SEM of three independent experiments. **P<0.05,* according to 2-tailed Student's *t* test. (**C**) mRNA levels of GAC and KGA, two splicing isoforms of GLS, in U87 and 87/EGFRvIII cells. Cells were treated with 1 nM rapamycin, 1 μM PP242, or control DMSO for 48 hours. Data represent the mean ± SEM of three independent experiments.**P<0.05*, ***P<0.01,* according to 2-tailed Student's *t* test. KGA and GAC; glutaminase splice variants. (**D**) mRNA levels of glycolysis enzymes including Glut1, PDK1, and LDHA in U87 and 87/EGFRvIII cells. Cells were treated with 1 nM rapamycin, 1 μM PP242, or control DMSO for 48 hours. Data represent the mean ± SEM of three independent experiments. Glut1; Glucose Transporter 1, PDK1; Pyruvate Dehydrogenase Kinase 1, LDHA; Lactate Dehydrogenase A.

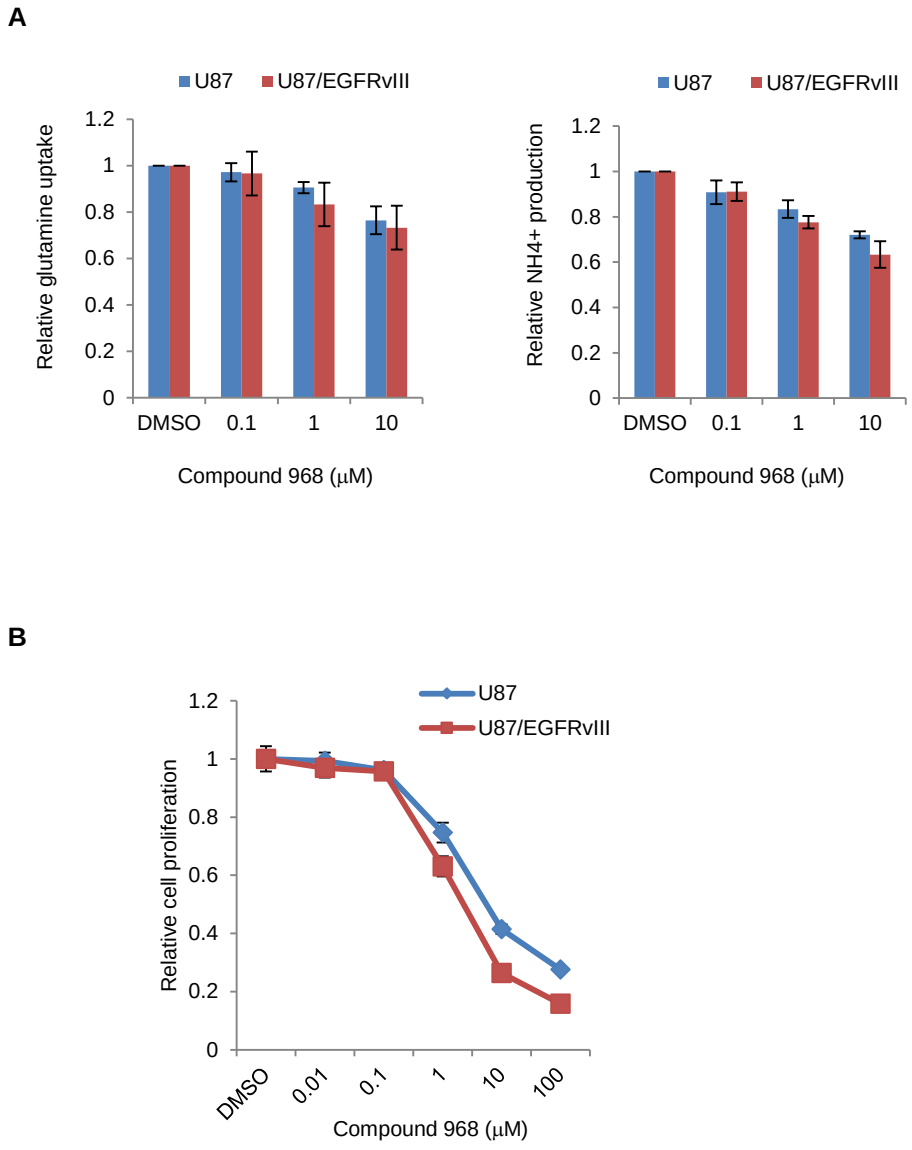


Supplemental Figure 5.
Compensatory elevation of GLS protein *n vivo* after mTOR inhibitor treatment, related to Figure 3.
Immunohistochemical images of GLS obtained from EGFRvIII-expressing U87 xenografts that received treatment with CC214 (n=2) or control DMSO (n=2). Tissues were counterstained with hematoxylin. Scale bar, 50 μm. Relative staining intensity was measured from four independent images for each group. ***p<0.01,* according to 2-tailed Student's *t* test.

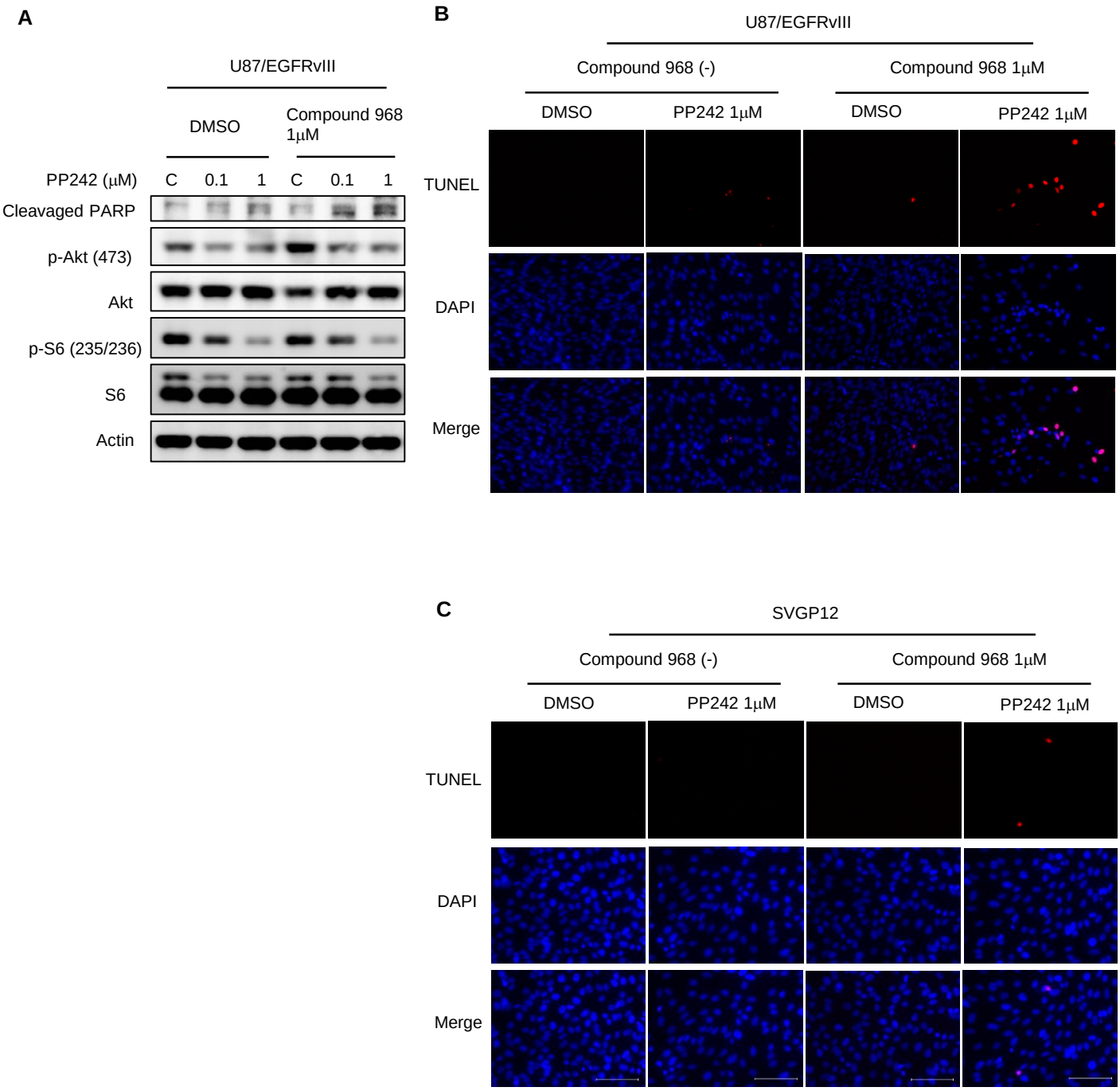




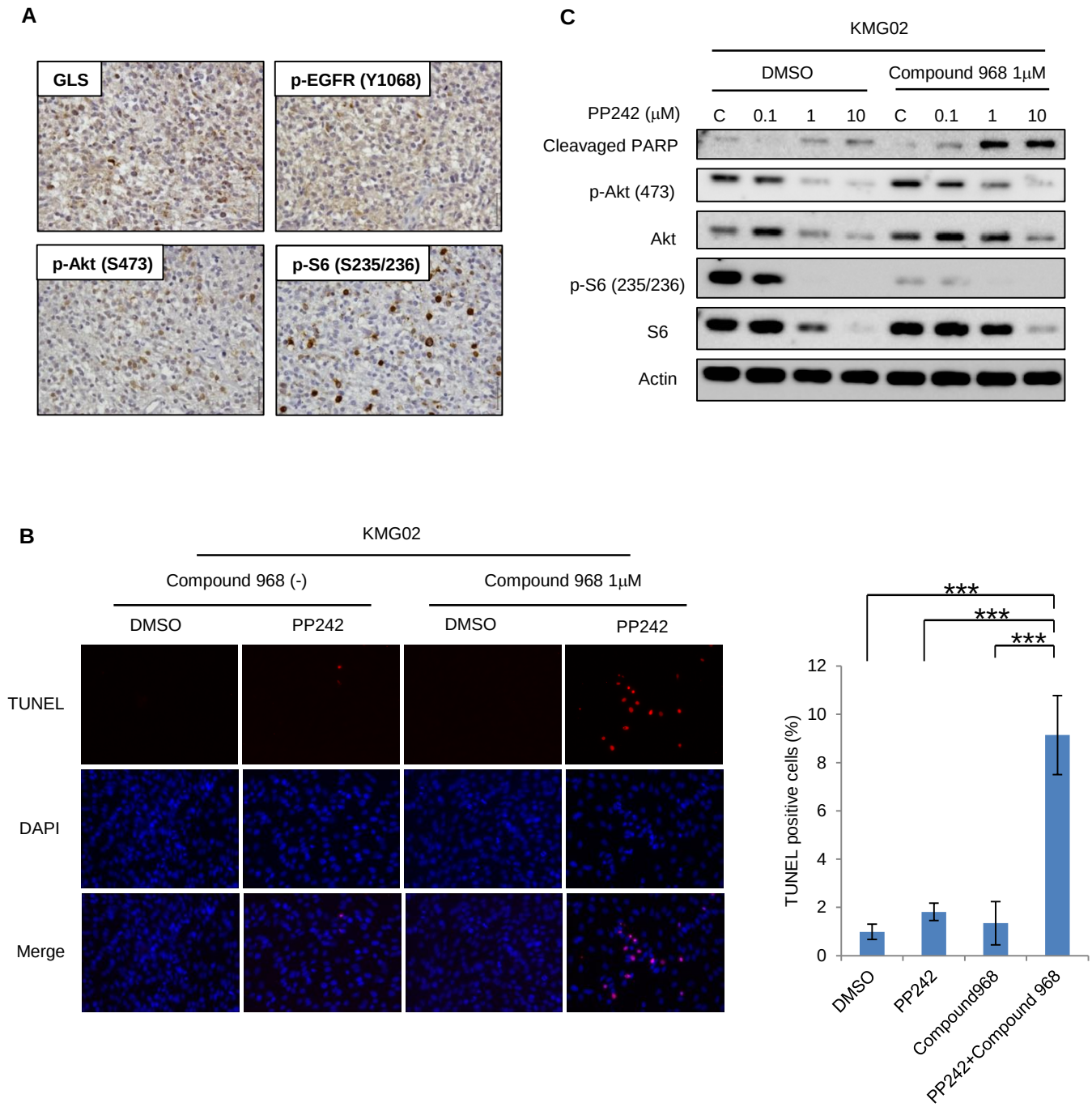
Supplemental Figure 6.
Glutamine is required for GBM cells to survive impairment of mTOR signaling, related to Figure 4.
(**A**) Biochemical analysis of U87/EGFRvIII cells treated with indicated concentrations of PP242 or control DMSO with glutamine depletion or not in 1% FBS medium for 3 days. (**B**) Representative images of U87/EGFRvIII cells with TUNEL staining. Cells were treated with 1 μM PP242 or control DMSO with/without glutamine in 1% serum culture medium for 3 days. Quantification of TUNEL-positive cells was performed with the NIH image analysis Image J. Data represent the mean ± SEM of five independent images for each group. **p<0.05,* according to 2-tailed Student's *t* test. Images are magnified x100. TUNEL; TdT-mediated dUTP nickend labeling.



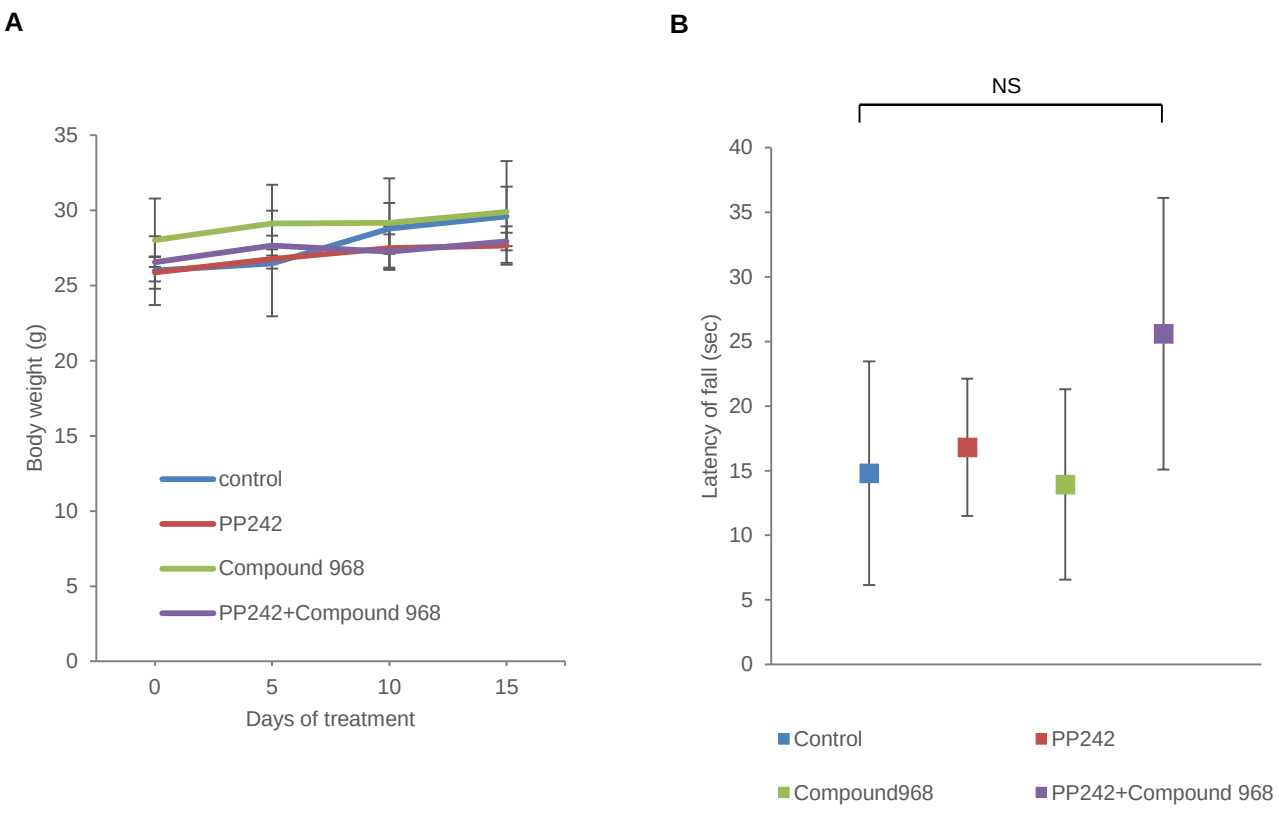
Supplemental Figure 7.
Pharmacological inhibition of GLS suppresses GBM cell proliferation, related to Figure 4.
(**A**) Relative glutamine uptake and $NH4^+$ production in U87 and U87/EGFRvIII cells treated with the indicated concentrations of Compound 968 (GLS inhibitor) for 2 days. Data represent the mean ± SEM of three independent. (**B**) U87 and U87/EGFRvIII cells were treated with the indicated concentrations of Compound 968 for 2 days. Relative cell growth was calculated with the cell proliferation assay. Data represent the mean ± SEM of three independent experiments.



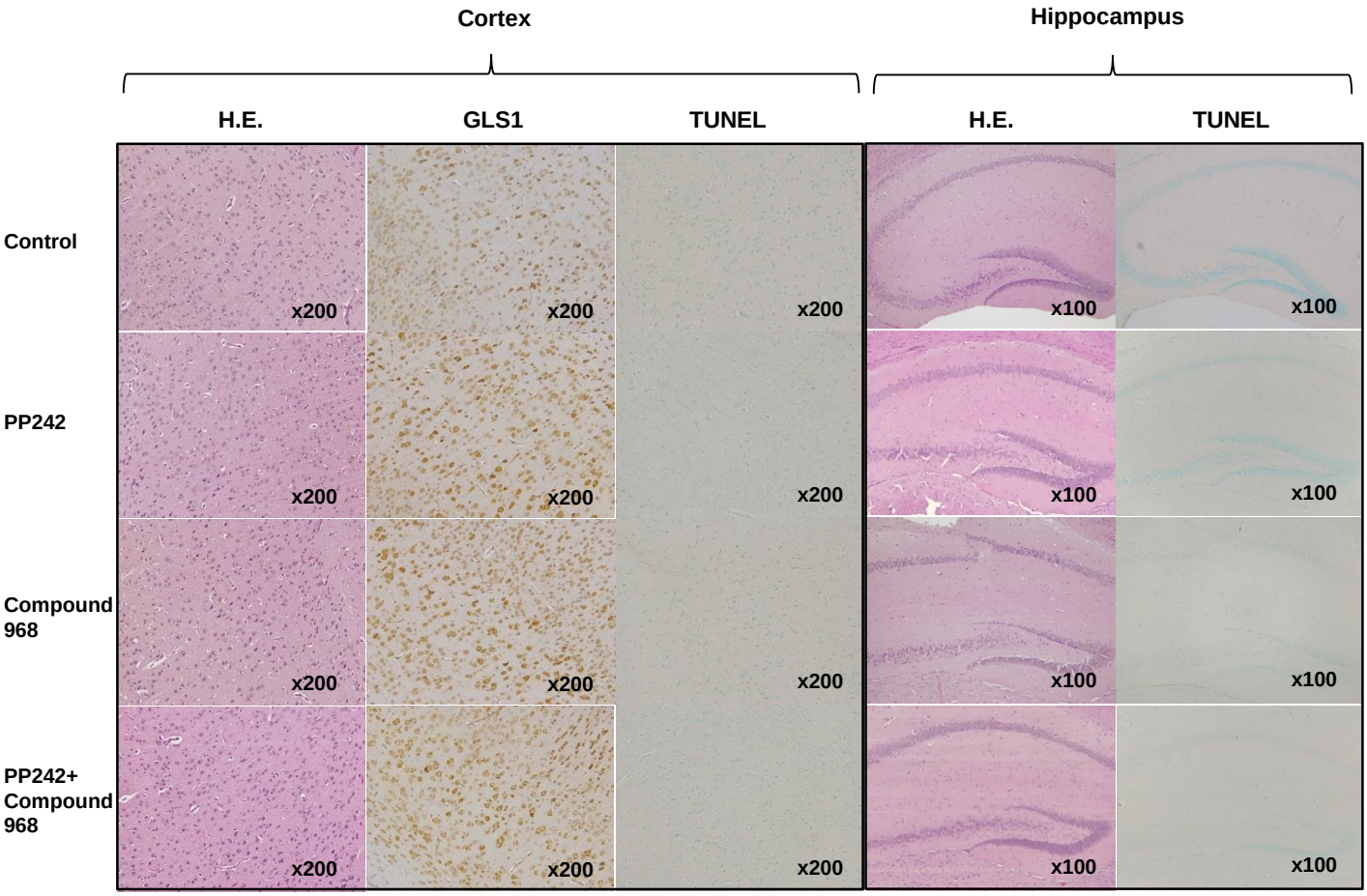
Supplemental Figure 8.
GLS inhibition sensitizes GBM cells to mTOR-targeted treatment, but did not normal astrocyte cells, related to Figure 4.
(**A**) Westernblot analysis using indicated antibodies of U87/EGFRvIII cells treated with indicated concentrations of PP242 and/or 1 μM Compound 968 (GLS inhibitor) for 2 days. C; Control DMSO. (**B** and **C**) Representative images of U87/EGFRvIII (B) and SVGP12 (C) cells with TUNEL staining. Cells were treated with 1 μM PP242 and/or 1 μM Compound 968 for 2 days. Quantification of TUNEL-positive cells was indicated in Figure 3E. Images are magnified x200. TUNEL; TdT-mediated dUTP nickend labeling.



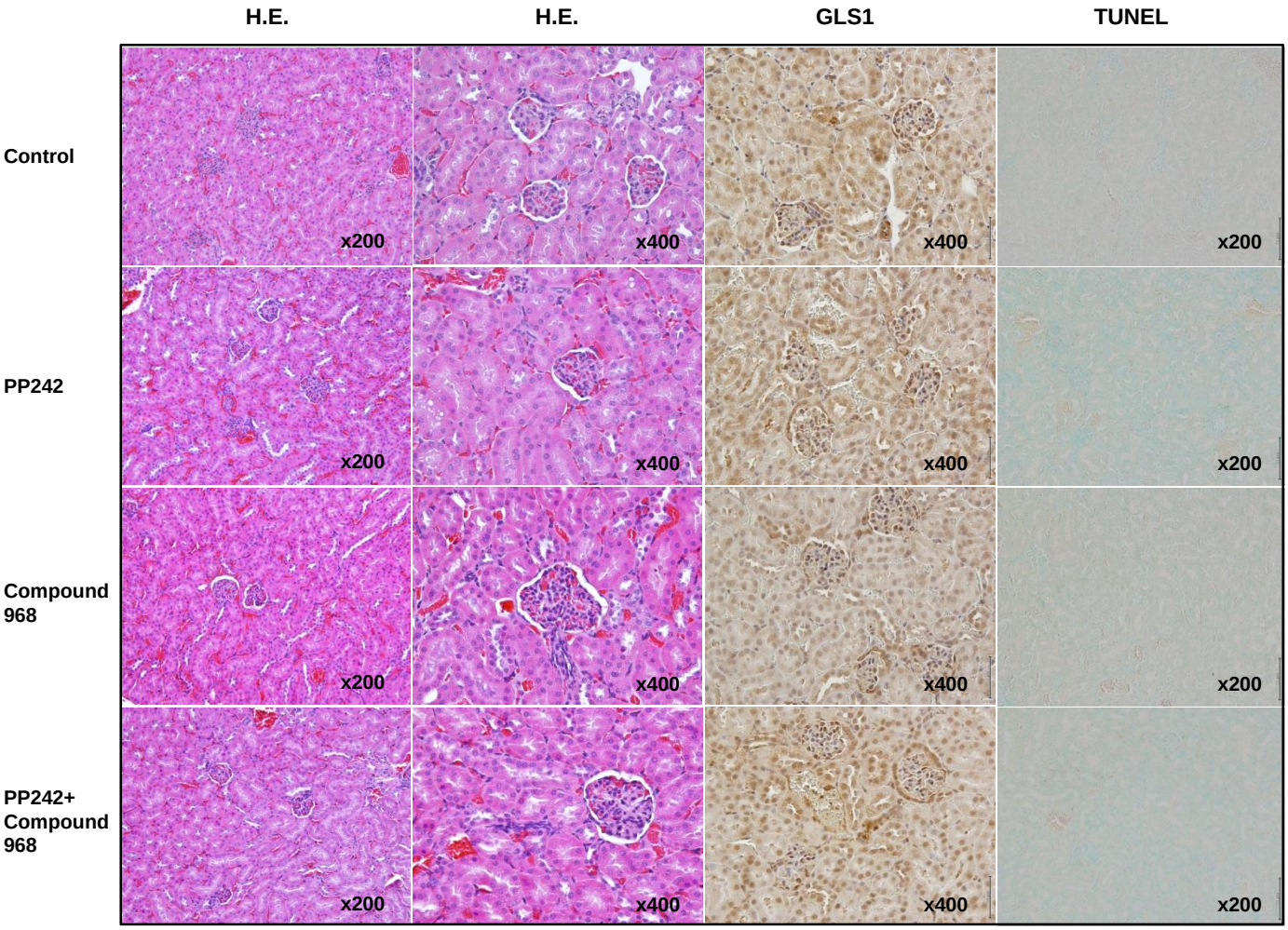
Supplemental Figure 9.
GLS inhibition reverses mTOR-targeted therapy resistance in patient-derived GBM cells, related to Figure 4.
(**A**) Immunohistochemical staining analysis of GLS, p-EGFR(Y1068), p-Akt(S473) and p-S6(S235/236) in patient-derived GBM cells (KMG02). Images are magnified x200. (**B**) Representative images of KMG02 cells with TUNEL staining. Cells were treated with 1 μM PP242 and/or 1 μM GLSi (Compound 968) for 2 days. Quantification of TUNEL-positive cells was performed with the NIH image analysis Image J. Data represent the mean ± SEM of five independent images for each group. Tukey-Kramer honest significance testing was performed for multiple comparison testing. ****p<0.001*. Images are magnified x200. TUNEL; TdT-mediated dUTP nickend labeling. (**C**) Immunoblot analysis using indicated antibodies of KMG02 cells treated with indicated concentrations of PP242 and/or 1 μM Compound 968 for 2 days. C; Control DMSO.



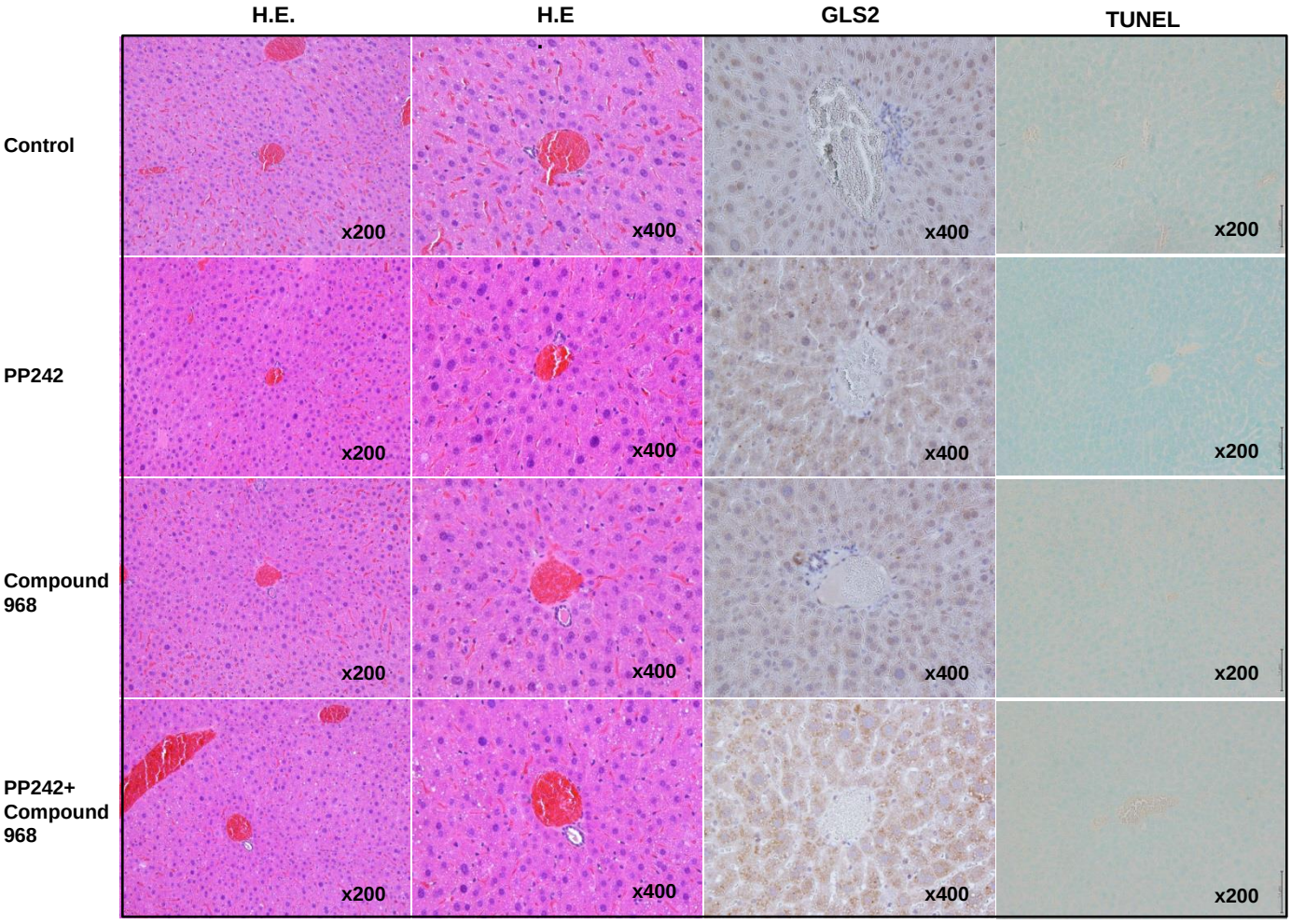
Supplemental Figure 10.
Effects of PP242 and/or Compound 968 on body weight and motor function in mice, related to Figure 6.
(**A**) Changes in body weight of non tumor-bearing mice treated with intraperitoneal injections of PP242 (5 mg/kg) and/or Compound 968 (5 mg/kg). The control group received an equal volume of DMSO. Treatment started 15 days after implantation. Data represent the mean ± SEM of four mice for each group. (**B**) Motor coordination in mice treated with daily intraperitoneal injection of PP242 and/or Compound968. Data represent the mean ± SEM of four mice for each group. Tukey-Kramer honest significance testing was performed for multiple comparison testing. NS; not significant.



Supplemental Figure 11.
Effects of PP242 and/or Compound 968 on brain in mice, related to Figure 6.
Histological findings of mouse brain (cortex and hippocampus) treated with pp242, Compound 968 alone, and combination. No significant difference of morphology was observed among each treatment in H.E. stain. No significant difference of GLS1 (kidney type glutaminase) expression was observed among each treatment. Dead cells were not observed in each treatment groups at all in TUNEL assay. Original magnification; x200 : H.E. cortex, GLS1 cortex, and TUNEL cortex, x100: H.E. hippocampus and TUNEL hippocampus. H.E.; Hematoxylin and eosin, TUNEL; TdT-mediated dUTP nickend labeling.



Supplemental Figure 12.
Effects of PP242 and/or Compound 968 on kidney in mice, related to Figure 6.
Histological findings of mouse kidney treated with pp242, Compound 968 alone, and combination. No significant difference of morphology was observed among each treatment in H.E. stain. GLS1 (kidney type glutaminase) protein expression levels and patterns were almost similar in immunohistochemical analysis. Dead cells were not observed in each treatment groups at all in TUNEL assay. Original magnification; x200 in H.E. left and TUNEL, x400; H.E. right and GLS1 stain. H.E.; Hematoxylin and eosin, TUNEL; TdT-mediated dUTP nickend labeling.



Supplemental Figure 13.
Effects of PP242 and/or Compound 968 on liver in mice, related to Figure 6.
Histological findings of mouse liver treated with pp242, Compound 968 alone, and combination. No significant difference of morphology was observed among each treatment in H.E. stain. In immunohistochemical analysis, GLS2 (liver type glutaminase) protein expression levels and patterns were almost similar in each treatment. There was not dead cell in each treatment group in TUNEL assay. Original magnification; x200 in H.E. left and TUNEL, x400; H.E. right and GLS2 stain. H.E.; Hematoxylin and eosin, TUNEL; TdT-mediated dUTP nickend labeling.

Supplemental Table1. Metabolites identified in GC-MS analysis, related to Figure 2.

No.	Metabolite Name				
1	1,6-Anhydroglucose	**31**	Fructose	**61**	N-Acetyl-L-Aspartic acid
2	2,3-Bisphospho-glycerate	**32**	Fructose-6-Phosphate	**62**	n-Caprylic acid
3	2-Aminobutyric acid	**33**	Fumaric acid	**63**	Nicotinamide
4	2-Aminoethanol	**34**	Galactitol	**64**	N-Methylethanolamine
5	2-Aminoisobutyrate	**35**	Galactosamine	**65**	Nonanoic acid
6	2-Aminopimelic acid	**36**	Galacturonic acid	**66**	Ornithine
7	2-Dehydro-D-gluconate	**37**	Glucose	**67**	Oxalate
8	2'-Deoxyribose-5'-Phosphate	**38**	Glutamic acid	**68**	Pantothenate
9	2-Thiouracil	**39**	Glutamine	**69**	Phenylalanine
10	3-Hydroxyisovaleric acid	**40**	Glycerol-2-Phosphate	**70**	Proline
11	Acetoacetic acid	**41**	Glycine	**71**	Putrescine
12	Aconitate	**42**	Glycyl-Glycine	**72**	Pyroglutamic acid
13	Adenine	**43**	Heptadecanoate	**73**	Pyruvate+Oxalacetic acid
14	Adenylosuccinic acid	**44**	Histidine	**74**	Ribitol

15	Alanine	**45**	Homocysteine	**75**	Sarcosine
16	Allantoin	**46**	Hypoxanthine	**76**	Serine
17	Allothreonine	**47**	Inositol	**77**	Spermidine
18	Anthranilic acid	**48**	Kynurenine	**78**	Succinic acid
19	Arabinose-5-phosphate	**49**	Lactic acid	**79**	Sucrose
20	Ascorbic acid	**50**	Lactitol	**80**	Tagatose
21	Asparagine	**51**	Lauric acid	**81**	Tartarate
22	Aspartic acid	**52**	Leucine	**82**	Taurine
23	b-Alanine	**53**	Lysine	**83**	Threonine
24	Cadaverine	**54**	Lyxose	**84**	trans-4-Hydroxy-L-proline
25	Citric acid + Isocitric acid	**55**	Malic acid	**85**	Tryptophan
26	Citrulline	**56**	Malonic acid	**86**	Tyrosine
27	Creatinine	**57**	Maltotriose	**87**	Uracil
28	Cysteic acid	**58**	meso-erythritol	**88**	Urea
29	Cysteine Sulfonic acid	**59**	Methionine	**89**	Uridine
30	Cysteine+Cystine	**60**	N-a-Acetyl-L-Lysine	**90**	Valine

				91	Xylitol