Supporting Information

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SI Materials and Methods

Cell Lines

For neurosphere cultures, small glioblastoma (GBM) samples were dissociated with a firepolished glass pipette and resuspended at 50,000 cells per mL in neurosphere medium, containing Dulbecco's modified Eagle medium (DMEM)/F12 medium (Invitrogen) supplemented with B27 [final concentration 2% (vol/vol), Invitrogen], basic fibroblast growth factor (bFGF) (20 ng/mL, Peprotech), epidermal growth factor (EGF) (50 ng/mL, Peprotech), penicillin/streptomycin [1% (vol/vol), Invitrogen], and heparin (5 mg/mL, Sigma).

Antibodies and Reagents. We used antibodies directed against the following: phosopho-Akt Ser473, Akt, phospho-S6 Ser235/236, S6, phospho-Erk, Erk, CyclinD1, cleaved polyADP ribose polymerase (PARP) (Cell Signaling); β-actin, cyclin-dependent kinase inhibitor 1 (p21) (Sigma); phospho-EGF receptor (EGFR) Tyr1086 (Invitrogen); EGFR (Millipore); promyelocytic leukemia (PML) (for Western blotting, Abcam; for immunohistochemisry, Santa Cruz). Reagents used are rapamycin, As_2O_3, polybrene (Sigma), erlotinib (ChemieTex), and pp242 (Chemdea). Stock solutions of inhibitor for rapamycin were made by dissolving in ethanol, erlotinib, and pp242 were made by dissolving in DMSO (Sigma) and stored at −20 °C. Inhibitors were added to each well at final concentrations of 10 nM, 10 μM, and 2 μM respectively. An equal concentration of ethanol or DMSO served as control. As_2O_3 was diluted by PBS and 10 M NaOH, then pH was adjusted at 8.0 by 12 M HCl.

Plasmid, Retroviral Infection, and siRNA transfection. To generate retrovirus, either the Phoenix 293T Amphotropic retroviral packaging line was transfected with pLNCX-hemagglutinin (HA) tag-expression or pLNCX constructs by using FuGENE 6 Transfection Reagent (Roche). At 48 h after transfection, viral supernatants were collected from packaging cell lines, filtered through a 0.45 μM filter (Millipore), and added to glioblastoma cells with polybrene at a final concentration of 8 μg/mL After 4-h incubation, fresh medium was replenished. The infected cells were incubated for 1–2 d and then selected in G418 containing medium (0.4 mg/mL; Omega Scientific) in limiting dilution. Multiple clonal cell lines were obtained by G418 selection and screened by Western blot.

Cell Proliferation and Death Assays. For cell proliferation, cells were seeded in 96-well plates and incubated in each day course. For analyzing the effect of drugs, cells were seeded in 96-well plates and were treated after 48 h with different drugs indicated in each experiment in medium containing 1% FBS. Relative proliferation to control cells with vehicle treatment was checked with a WST-1 Cell Proliferation Assay Kit (Millipore). Cells were incubated 2 h after the addition of tetrazolium salt WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-^{2}H-tetrazolium, monosodium salt] (Millipore) at 5% CO_2 and 37 °C, and the absorbance of the treated and untreated cells was measured with a microplate reader (Bio-Rad) at 420–480 nm. Cell death was assessed by Trypan blue exclusion (Invitrogen).

Cell Cycle Analyses. Cells were fixed in 70% ethanol diluted in PBS, and the samples were stored at −20 °C. The fixed cells were resuspended in PBS containing 20 μg/mL propidium iodide (Sigma) and 10 μg/mL RNase A (Sigma) and incubated for 10 min at 37 °C. Flow cytometric analysis was performed by using FACSCalibur flow cytometer (Becton Dickinson).

TUNEL Staining. Cells were placed in 8-well chamber slides at 2×10^4 cells/well in 500 μL of growth medium and then incubated for 48 h in each condition of treatment. After that, cells were fixed in 4% paraformaldehyde (in PBS, pH 7.4) for 1 h at room temperature, washed in PBS, and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate on ice for 2 min. Slides were then washed twice in PBS before incubation with TUNEL Reaction Mixture (Roche) at 37 °C for 1 h in the dark. Slides were washed three times in PBS and coverslipped with ProLong Gold antifade reagent with DAPI (Invitrogen). TUNEL-positive cells were visualized with a fluorescene microscope (Olympus BX-61). Ten separate, randomly chosen fields on each chamber were imaged, and the numbers of TUNEL-positive cells and whole nuclei were counted.

Western Blotting. Cultured cells were lysed and homogenized with RIPA buffer (Boston Bioproducts), phosphatase inhibitor, and protease inhibitor mixture (Thermo Scientific). Equal amounts of protein extracts were separated by electrophoresis on 4–12% NuPAGE Bis-Tris Mini Gel (Invitrogen) and then transferred to a nitrocellulose membrane (GE Healthcare). The membrane was blocked for 1 h in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk and then probed with various primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase. The immunoreactivity was revealed with SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Immunohistochemical Assays, Tissue Microarrays, and Image Analysis-Based Scoring. Paraffin-embedded tissue blocks were sectioned by using the University of California, Los Angeles Pathology Histology and Tissue Core Facility. Immunohistochemical staining was performed as described (1, 2). Slides were counterstained with hematoxylin to visualize nuclei. Paraffin-embedded tissue sections underwent immunohistochemical analysis in which the results were scored independently by two pathologists who were unaware of the findings of the molecular analyses. Quantitative image analysis to confirm the pathologists' scoring was also performed with Soft Imaging System software (Olympus). We have demonstrated the utility of this quantitative method for measuring drug-specific effects in paraffin-embedded tissue samples from GBM patients enrolled in clinical trials with targeted agents (1, 2).

Tissue microarrays (TMAs) were used to analyze PML, Ki-67, and phospho-S6 immunohistochemical staining in 140 GBM patient samples. TMAs enable tumor tissue samples from hundreds of patients to be analyzed on the same histological slide. We constructed two GBM TMAs by using a 0.6-mm needle to extract 252 representative tumor tissue cores and 91 adjacent normal brain tissue cores from the paraffin-embedded tissue blocks of 140 primary GBM patients. These cores were placed in a grid pattern into two recipient paraffin blocks, from which tissue sections were cut for immunohistochemical analysis of PML, Ki-67, and phospho-S6. These TMAs have been used for other studies (3, 4). Among 140 cases, 87 GBM patient tissue cores were available for analysis based on sufficient high quality tissue. Staining intensity was scored independently by two pathologists who were unaware of the findings of the molecular analyses.

Tissue sections were cut from blocks of formalin-fixed paraffin tumor tissue from glioblastoma patients treated with rapamycin or erlotinib. Tumor specimens were obtained according to a protocol approved by the Institutional Review Board of University of California, Los Angeles. Details regarding the results from these trials have been published (1, 2). Each set of paired pre- and posttreatment tumor tissues for rapamycin and erlotinib trial were examined, respectively. Five-micrometer tissue sections were stained with antibodies directed against PML; digital scores for PML were based on absolute staining intensity of tumor cells as quantified after false-color conversion. Sections were photographed by using a DP25 II camera mounted on an Olympus BX43 microscope at 40× magnification. Three images were captured per slide from representative regions of the tumor. Borders between individual cells were approximated by using a separator function of the Soft Imaging Software (with the parameter of Smooth and Fine/Coarse, 2 and 10, respectively). Quantitative analysis was done by using HSI color algorithm based on hue, saturation and intensity. Saturations of the separated cell in the images were quantified in the red-brown hue range to exclude the negative staining area with hematoxylin nuclear staining. For PML staining scoring, separated cells were quantified with red brown hue range (cells with positive nuclear staining) and total hue range (all cells) after cell border separation and proportion of positive cells was calculated based on these numbers (1, 2). To compare the staining intensity of all slides, mean saturation of total cells on each image was quantified and calculated. One thousand five hundred to two thousand cells per case (on average) were measured for each slide.

Immunofluorescence Analysis. Cells were fixed with 4% paraformaldehyde in PBS for 10 min and washed twice in PBS. Next, cells were permeabilized with 0.1% Triton X-100 in PBS for 3 min, washed four times with PBS and blocked with 10% FCS in PBS for 2 h at room temperature. Cells were incubated with primary antibodies in PBS containing 3% BSA at 4 °C for overnight. Primary antibodies were revealed by a 4-h incubation with appropriate fluorescence-conjugated secondary antibodies (Invitrogen) in a 200-fold dilution in PBS containing 3% BSA. For double staining, cells were incubated with the two antibodies under the same conditions. Slides were washed three times in PBS and coverslipped with ProLong Gold antifade reagent with DAPI (Invitrogen). The images under fluorescence microscope (Olympus) at magnification 40× or confocal microscope at 63× (Carl Zeiss) were captured.

Statistical Analysis. Results are shown as mean ± SEs of the mean (SEM). χ^2 for independence test was used to assess correlations between various molecular markers on TMAs. For nonparametric clinical trial data, Wilcoxon rank test was used. Other comparisons in cell proliferation assays, cell death assays, and TUNEL staining were performed with Student's t test as, by analysis of variance, appropriate. $P < 0.05$ was considered as statistically significant.

1. Cloughesy TF, et al. (2008) Antitumor activity of rapamycin in a Phase I trial for patients with recurrent PTEN-deficient glioblastoma. *PLoS Med* 5(1):e8.
2. Mellinghoff IK, et al. (2005) Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N Engl J Med* 353(19):2012–2024.
3. Guo D, et al. (2009) EGFR signaling through an Akt-SREBP-1-dependent, rapamycin-resistant pathway sensitizes glioblastomas to antilipogenic therapy. *Sci Signal* 2(101):ra82.
4. Lu KV, et al. (2009) Fyn and SRC are effectors of oncogenic epidermal growth factor receptor signaling in glioblastoma patients. *Cancer Res* 69(17):6889–6898.

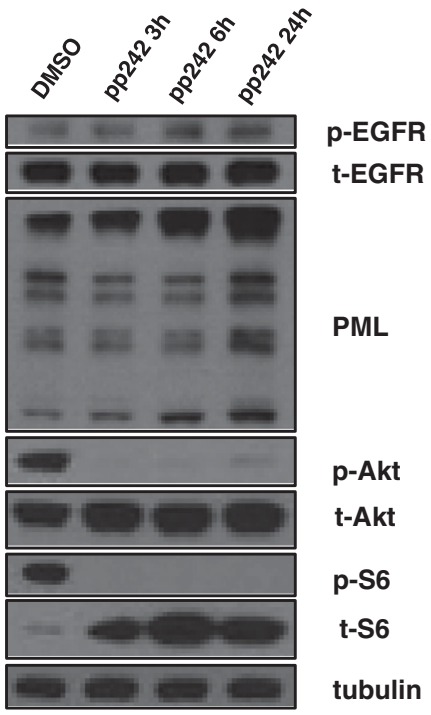

Fig. S1. mTOR inihibitor pp242 induces an increased PML expression in U251 GBM cell lines. Effect of the mTOR kinase inhibitor pp242 on PML expression in U251 cells is shown. Cells were cultured in serum-free condition. Cells were cultured in serum-free conditions.

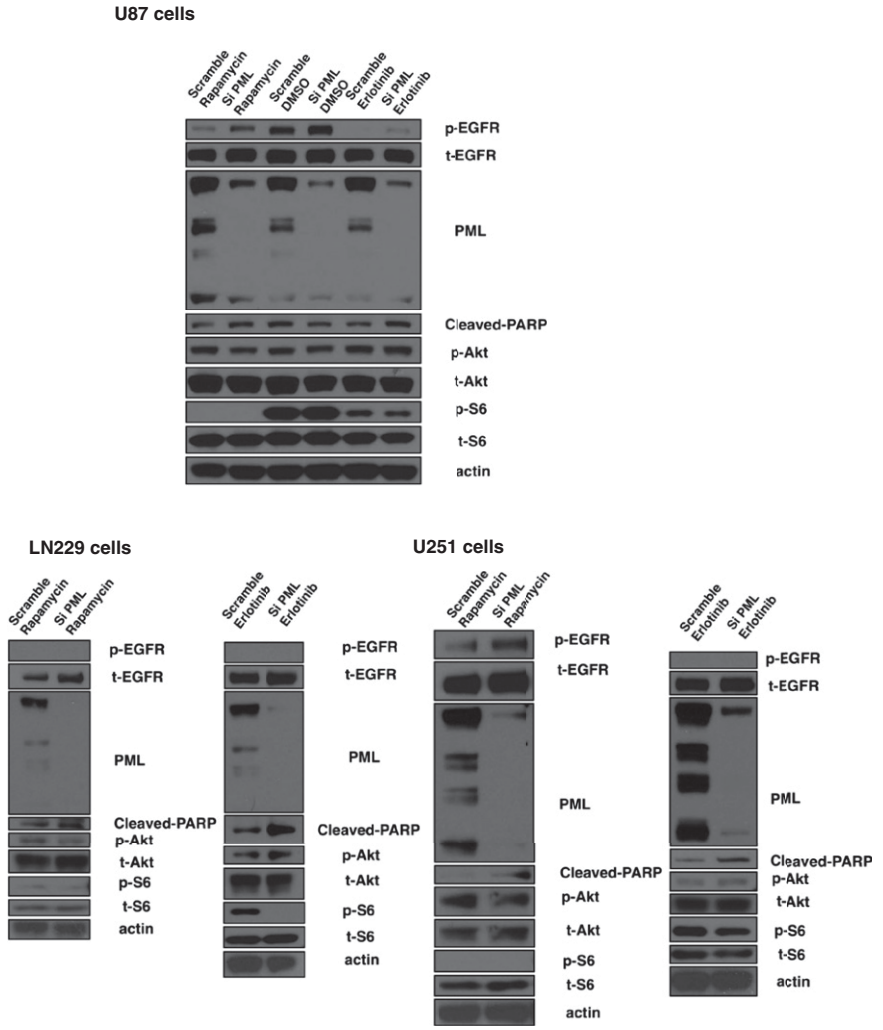

Fig. S2. PML knockdown sensitizes GBM cell lines to drug treatment. Western blotting analysis of cleaved-PARP, PI3K/Akt/mTOR signaling pathway and cell cycle-related proteins performed on lysates from U87, LN229, and U251 cells treated with a combination of PML knockdown and rapamycin or erlotinib.

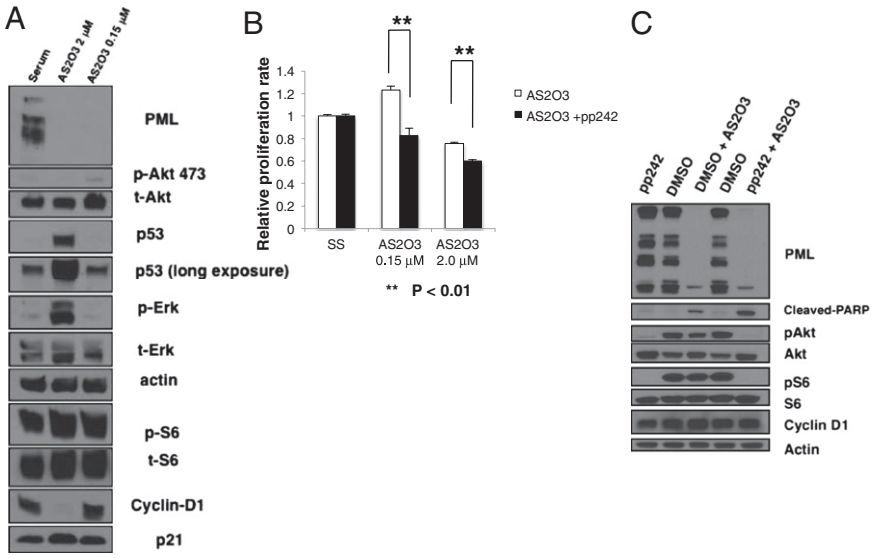

Fig. S3. pp242 and As_2O_3 cause synergistic GBM cell death in vitro. (*A*) Western blot analysis of PI3K/Akt/mTOR signaling pathway and cell cycle-related proteins performed on lysates from U87 cells treated with/without As_2O_3. (*B*) Effect of combined treatment with mTOR kinase inihibitor pp242 and As_2O_3 on growth of U87 cells analyzed by WST assay. *P* value was determined by Student's *t* test. (*C*) Western blotting analysis of cleaved-PARP, PI3K/Akt/mTOR signaling pathway, and cell cycle-related proteins performed on lysates from U87 cells treated with a combination of pp242 (2 μM) and As_2O_3 (2 μM).

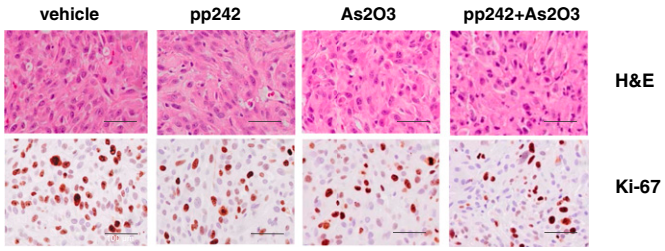

Fig. S4. Ki-67 immunostaining of pp242, As_2O_3, or combination-treated U87 GBM xenografts. Immunohistochemical images showing H&E and Ki-67 immunostained images. (Scale bars: 100 μm.)