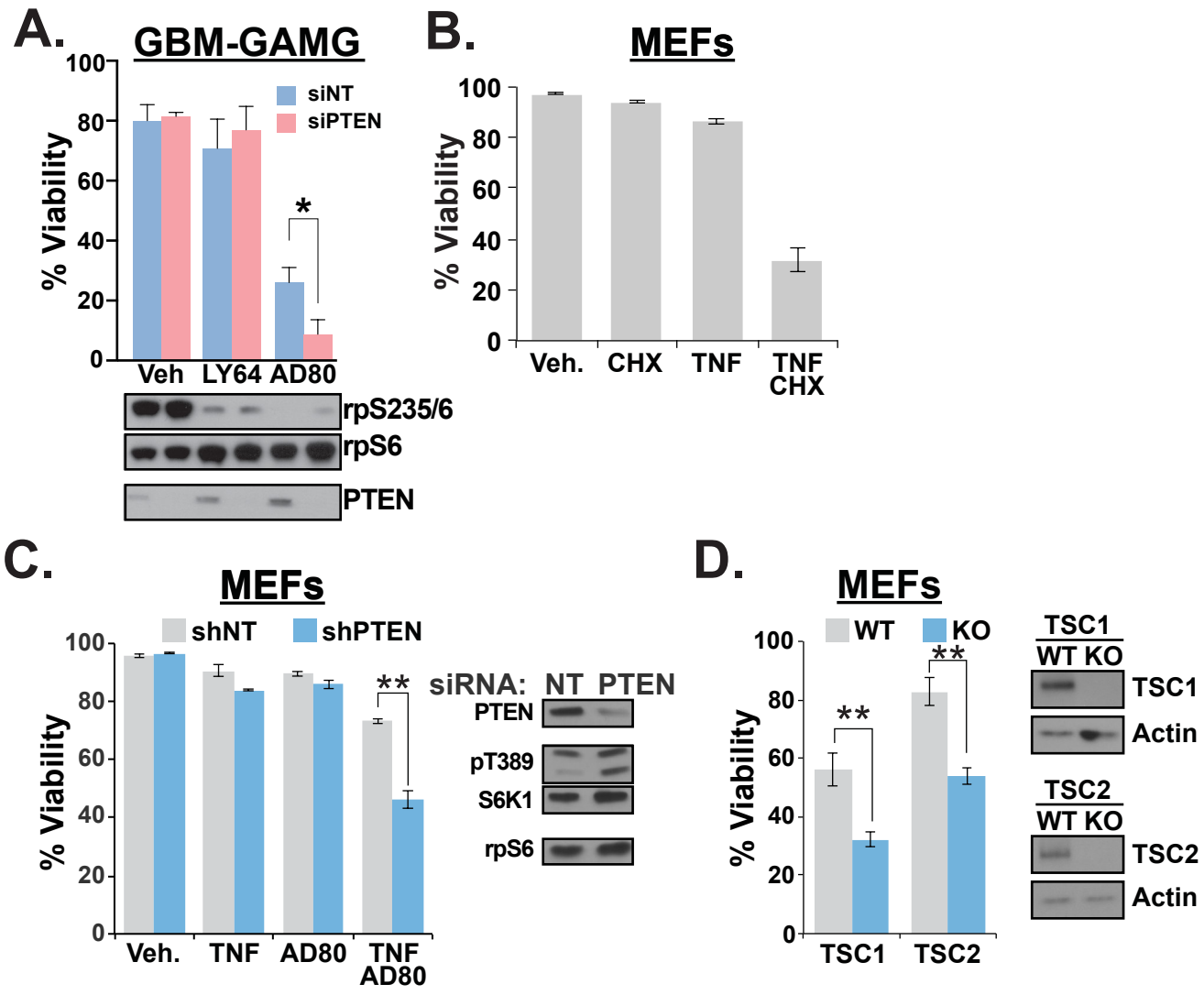

Figure S1, related to Figure 1: Cytotoxic efficacy of S6K1 inhibitors. **(a)** GAMG glioblastoma cells were transfected with siNT or siPTEN, then cultured in vehicle control, 5 μM LY-2779964, or 10 μM AD80. Mean ±SD viability was determined by CytoTox Glo assay in triplicate after 72 hours of culture (top); immunoblot analysis was performed in parallel wells after 3 hours of culture (bottom). *, $p<0.05$ by two-tailed t-test; n=3. **(b)** Vector control MEF cells were treated with Vehicle control, 10 μg/ml cycloheximide (CHX), 30 ng/ml TNFα, or CHX + TNFα for 22 hours. Mean viability measured from triplicate wells were by Cytotox Glo +/- standard deviation. n=2. **(c)** Stably transduced shNT or shPTEN MEFs were cultured with vehicle control, 30ng/ml TNFα, 10 μM AD80, or TNFα + AD80 as shown for 8 hours. Viability was measured as in (a), n=3. **(d)** TSC1 or TSC2 knockout MEFs, or their respective WT counterparts, were treated with TNFα + AD80 as in (b). Viability was measured as in (a), n=3.

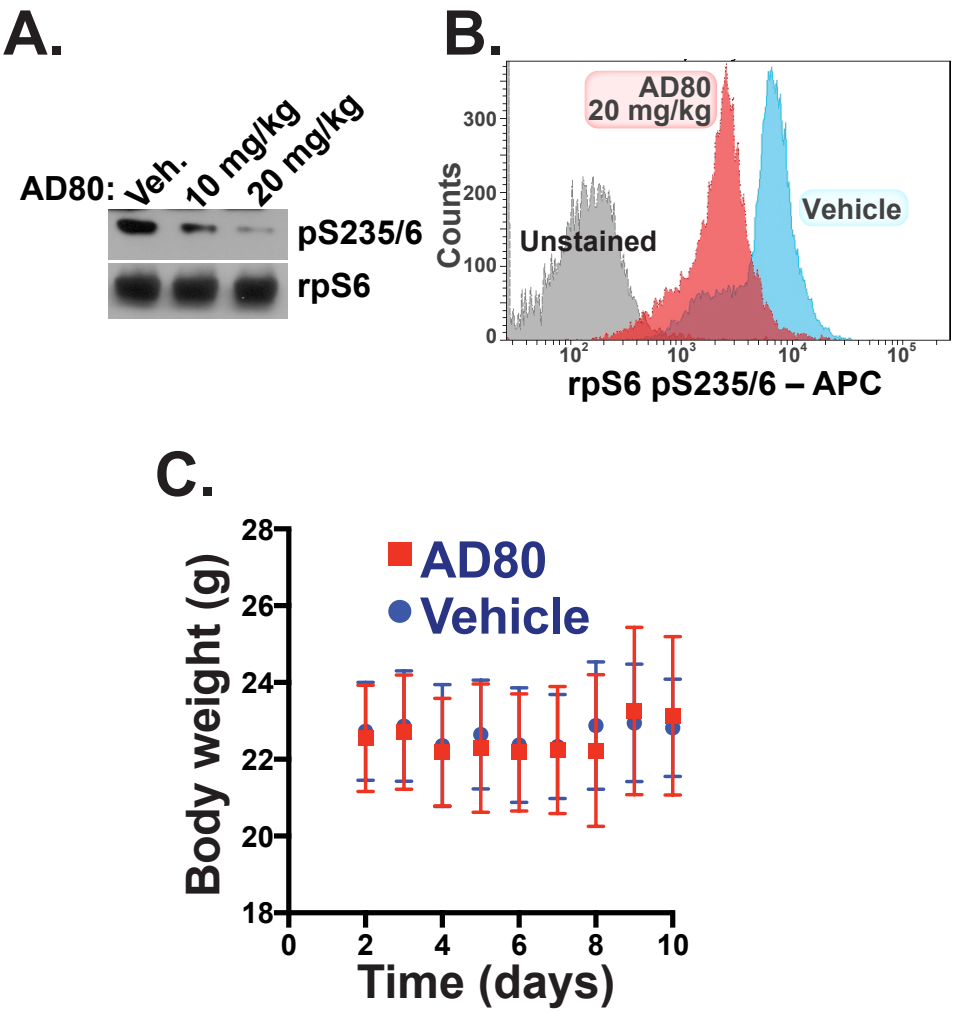

Figure S2, related to Figure 1f: AD80 *in vivo* therapy. AD80 was injected i.p. at 20 mg/kg. **(a)** Six hours after injection, spleen lysates were prepared for rpS6 immunoblots. **(b)** A single cell suspension of bone marrow cells was tested for rpS6 phosphorylation by intracellular staining and flow cytometry. n=3. **(c)** Mean +/- SD of body weight during AD80 therapy. There was no statistical difference in body weight during among the groups during the course of treatment (two-tailed t test).

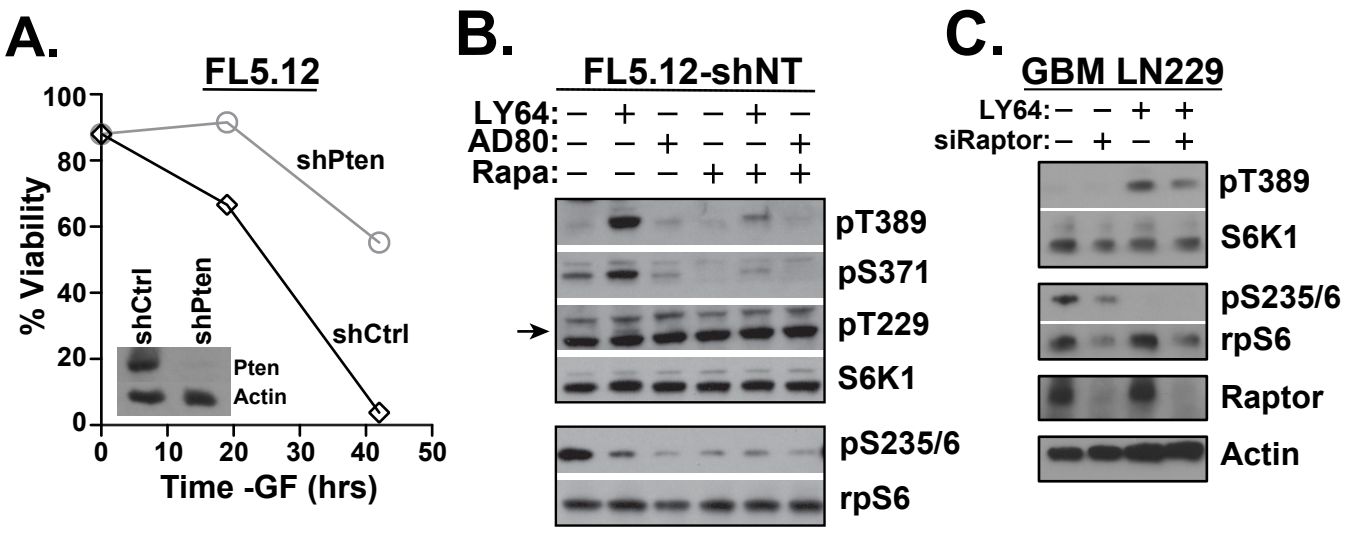

Figure S3, related to Figure 2. S6K1 pathway dynamics. **(a)** IL-3 dependent FL5.12 cells stably transduced with shNT or shPTEN were cultured in the absence of IL-3 (GF). shPTEN conferred a survival advantage. **(b)** Comparison of 1 μM LY-2779964 and 4 μM AD80 effects on regulatory phosphorylation of S6K1 in FL5.12 shNT cells. The intensity of pT389 increase is not mirrored in pS371 or pT229. n=2. **(c)** LN229 cells were transfected with non-targeting (-) or raptor-targeting siRNA prior to treatment with 5 μM Ly-2779964. siRaptor reduced S6K1 T389 phosphorylation. n=3.

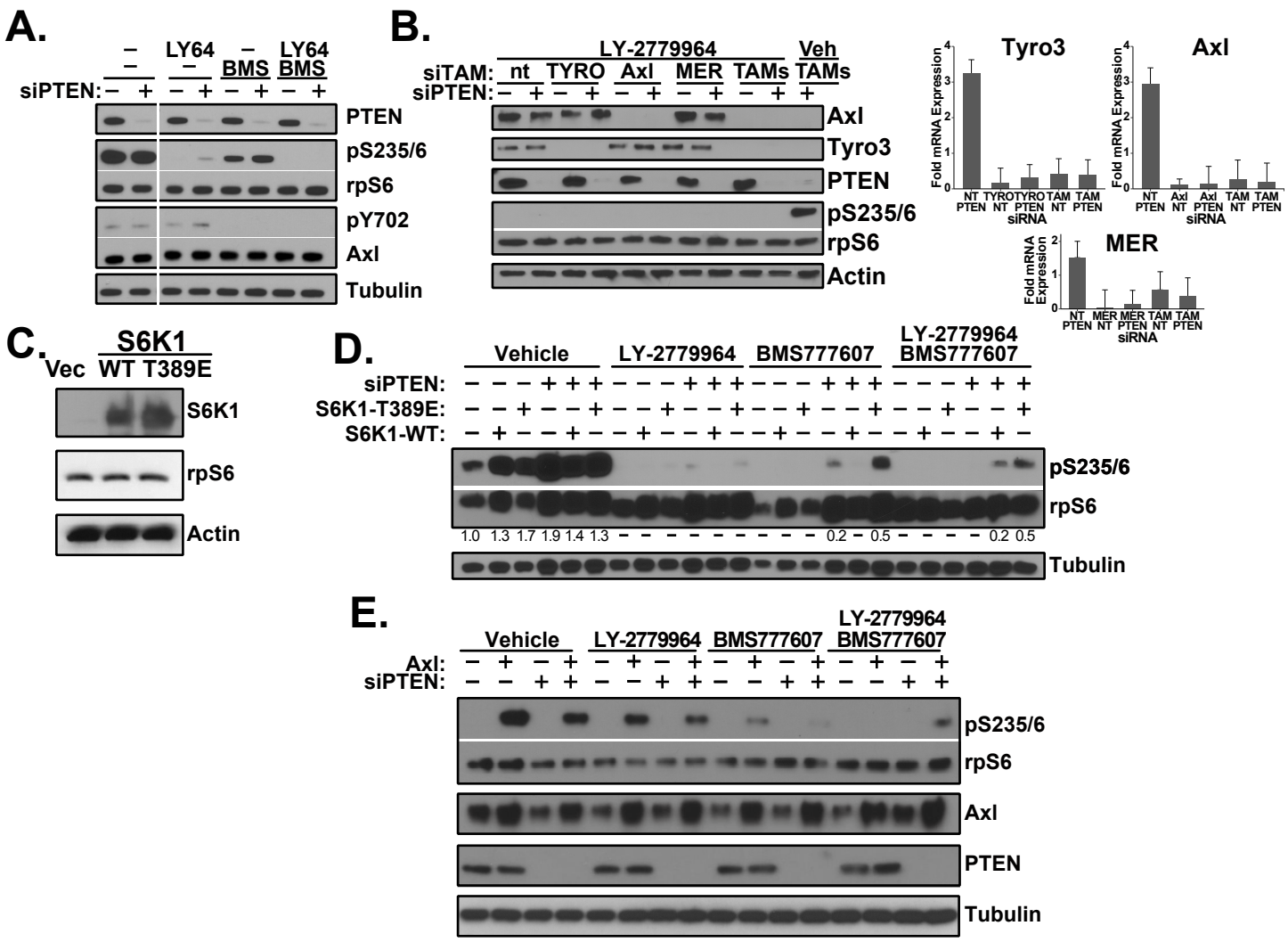

Figure S4, related to Figure 4. S6K1/TAM kinase combination targeting. **(a)** rpS6 phosphorylation in siNT or siPTEN LN229 cells treated with vehicle control, 5 μM LY-2779964, and/or 10 μM BMS-777607 for 3 hours. **(b)** TAM kinase knockdown LN229 cells were cultured in conditions as indicated for 3 hours. *Left*, Immunoblots confirm protein knockdown and LY64 efficacy. MER was not detecable. *Right*, Fold changes in TAM mRNA abundance, referenced to siNon-Targeting control. PTEN-knockdown induced TYRO3 and Axl mRNA, but this is not reflected in total protein levels. **(c)** Expression of S6K1 WT and T389E in LN229 cells. **(d)** LN229 cells from (c) were treated for 3 hours as indicated prior to analysis. The ratio of pS235/6:rpS6 signal, standardized to Vector/vehicle control is shown. n=2. **(e)** LN229 cells transduced with vector control or Axl expression constructs were treated for 3 hours as indicated. Axl expression sustained S6 phosphorylation in PTEN-deficient cells.

Supplemental Tables

Table S1, related to Figure 4a. KiNativ analysis of AD80 inhibition in PTEN-deficient LN229 cells. Cells were treated with 10 uM AD80 for 3 hours prior to shipment of flash frozen packed cell pellet to ActivX for KiNativ screening.

Table S2, related to Figure 4a. Ingenuity Regulator Analysis results. Kinases identified as inhibited by AD80 in the KiNativ and Dar et al. datasets (Figure 4a) were submitted for Ingenuity Regulator Analysis.

Supplemental Experimental Procedures

Cell culture. IL-3-dependent FL5.12 cells and the FL5.12-derived cells were cultured in serum-containing RPMI supplemented with murine recombinant IL-3 as previously described (Khatri et al., 2010). Pten-deficient FL5.12 cells with stable incorporation of shPten and shControl were previously described (Tandon et al., 2011). For growth factor withdrawal experiments, cells were washed 2-3 times in RPMI, then cultured in complete serum-containing medium but lacking IL-3. 293T and MEF cells were cultured in DMEM supplemented with 10% FBS, penicillin and streptomycin. Serum starvation was performed for 24-48 hours.

PTEN-deficient leukemia. Four Mx1-Cre^{+} PTEN$^{fl/fl}$ mice (donors) were injected three times q.o.d. with i.p. PIPC at a dose of 12.5 μg per gram of body weight. Upon evidence of leukemia in donors, bone marrow was harvested. 2E6 BM cells were transplanted into CD45.1+ Boy/J mice that had been exposed to 700 rads γ-irradiation in a GC-40 instrument. Four days post-transplant, mice were randomly selected for groups treated q.d. for 10 days i.p. with vehicle control or 20 mg per kg of body weight AD80. Housing of mice was intermingled among groups. Transplanted mice were monitored for signs of leukemia as evidenced by isolation behavior, splenomegaly, lymphadenopathy or reduced grooming, which was documented by spleen weight and skewed hematopoiesis detected by surface marker analysis in a flow cytometer. The experiment was conducted with approval and under guidelines from the University of Cincinnati IACUC office. Log-Rank analysis was performed in Graphpad Prism software.

Inhibitors. All inhibitors were dissolved in DMSO. LY-2779964 was provided by Eli Lilly and Company, rapamycin from LC Laboratories, WYE354 from SelleckChem, AD57 (#13975) from Cayman Chemical. PF-4708671 was purchased from Sigma. AD80 and DG2 were synthesized as described and generously provided by Dr. Kevan Shokat, UCSF (Dar et al., 2012). AD80 was also obtained from Cayman Chemical. BMS-777607, GSK690693, and trametinib were from LC Labs. Concentrations of drugs selected for each cell line were determined from dose curve analysis. For *in vivo* administration, AD80 was dissolved in normal saline containing 5% PEG 400, 5% Tween 80, and 12.5% DMSO, and administered i.p. to each mouse at a dosage of 20mg/kg.

Immunoblot. Immunoblots were performed with the following antibodies: S6K1 (#9202), S6K1 pT389 (#9234 or #9205), S6K1 pT421/S424 (#9204), S6K1 pS371 (#9208) rpS6 (#2317), rpS6 pS235/236 (#4856 or #4858), PTEN (#9188), ULK1 pS757 (#6888), Axl (#8661), Axl pY702 (#5724), 4EBP1 (#9452) and 4EBP1 pS65 (#9451), Erk (#9102), pErk (9101) from Cell Signaling Technology, S6K1 pT229 (44-918G) from Invitrogen. Contrast, brightness, crop and levels adjustments were performed in Illustrator and Photoshop software (Adobe), or Designer and Photo (Affinity). Antibody arrays were PathScan RTK signaling antibody arrays from Cell Siganling (#7982).

Viability measurement. To assess viability of FL5.12 cells, samples from cell culture suspensions were removed from culture. Propidium iodide (Invitrogen, #P3566) was added to a final concentration of 2 μg/ml. Triplicate technical replicates of each sample were performed on a FACS cytometer to determine PI exclusion (viability). Adherent cell viability was determined in triplicate wells of the same plate using CytoTox Glo (Promega #G9291) to measure loss of plasma membrane integrity by addition of a luminescent substrate that is cleaved upon release of cell-intrinsic protease activity. Luminescent counts released in response to apoptotic stimuli were indexed to total counts released after addition of detergent to calculate % viability. Two-tailed T-test analysis was performed using Microsoft Excel or R.

siRNA Knockdown. FL5.12 cells were nucleofected as described previously (Tandon et al., 2011). Briefly, 5E6 cells were nucleofected with 5 μg siS6K1 (Acell) or matched si-Non-Targeting using Nucleofector II (Amaxa Biosystems) and the G-016 program. Cells were rested 16-24 hours post nucleofection prior to further manipulation. MEF and GBM cells were transfected with 25 or 50 nM siPTEN (siGENOME: M-003023-02-0005 Dharmacon) or non-targeting control (D-001210-01-05 Dharmacon) using Lipofectamine 3000 (Life Technologies) and Opti-MEM I Reduced Serum Media according to manufacture protocol. Cells were left on transfection media and reagents for 72-hours before being re-plated with DMEM + 10% FBS and rested for 24-hours before further addition of inhibitors.

S6K1 and Axl expression. p70 WT S6K1-FLAG and T398E S6K1-FLAG constructs were expressed in the MIT retroviral vector. MEF or LN229 cells were transduced with the retrovirus prepared in 293T cells using 8 μg/ml of polybrene 2-3 times. MEF cells were harvested 48 hours after the first infection. Analysis of the Thy1.1 marker

expressed from MIT in LN229 cells indicated >90% transduction, and cells were subsequently maintained as stably transduced populations. Reconstitution of WT S6K1 and T389E S6K1 was confirmed by western blot analysis. Lentiviral particles containing pLOC (Dharmacon) driving expression of Axl or TurboRFP (control) were delivered to LN229 cells in serial transductions. Transduced populations were selected in Blasticidin (1μg/ml) for 10 days, then maintained as stably transduced populations.

Polysome profiles. 1E7 PTEN-deficient cells were cultured in complete media lacking only IL–3, treated with the indicated drugs or vehicle for 3 hour, washed twice with ice cold hypotonic buffer (5 mM Tris-HCl [pH 7.5], 1.5 mM KCl, and 2.5 mM $MgCl_2$), collected, and lysed in 400 μl of extraction buffer (5 mM Tris-HCl [pH 7.5], 1.5 mM KCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100, 0.5% sodium deoxycholate, 120 U/ml RNAse inhibitor, and 3 mM DTT). Lysates were loaded onto 0.5 M-1.5 M sucrose density gradients (20 mM Tris-HCl [pH 8.0], 80 mM NaCl, and 5 mM $MgCl_2$) and centrifuged at 36,000 rpm for 2 hour at 4ºC. Gradients were fractionated and optical density at 254 nm was continuously recorded.

^{3}H-Leucine incorporation. 1E5 MEF S6K1$^{-/-}$ cells re-expressing MIT vector, WT S6K1, or S6K1 E389 were cultured in each well of a 6-well plate, treated with the indicated drugs or vehicle for 2 hour, and then were labeled with 10 μCi of [4,5]-^{3}H-leucine for 2 hour. After labeling, cells were washed with ice cold PBS once and lysed with 1 ml of 10% trichloroacetic acid (TCA). The resultant precipitated proteins were washed with 5% TCA twice, and were solubilized in 500 μl of 0.1 M NaOH. The radioactivity was determined by liquid scintillation using a Beckman LS6500 multi-purpose scintillation counter.

KiNativ analysis. LN229 cells transfected with siPTEN were cultured in medium containing 0.1% serum and 10 μM AD80 (or vehicle control) for 3 hours. Packed cell pellets were flash frozen in liquid nitrogen then submitted for KiNativ analysis (ActivX). Statistically significant differences from KiNativ analysis comparing vehicle versus AD80 treatment were matched for individual kinases and plotted against published *in vitro* recombinant kinase inhibitor activity data from Dar et al, Nature 2012. Results indicate "direct" targets of AD80 that are inhibited by ≥50% in both assay, context-dependent or "indirect" targets that are inhibited ≥50% in KiNativ data only, and cellular "adaptive response" kinases which exhibited ≤-15% inhibition in KiNativ and ≤20% inhibition in the recombinant assay. A list of kinases inhibited by AD80 was analyzed using Ingenuity software.

Statistical analysis. Plots showing % Viability, ^{3}H-leucine incorporation, RT-qPCR RNA levels are the means of triplicate analyses plotted +/- the standard deviation. Statistical differences among groups were calculated using two-tailed t tests in Microsoft Excel or R Package using Holm's correction for multiple comparisons. The Log-Rank test was used to calculate the significance of the difference in the Kaplan-Meier curve using GraphPad Prism software.