Supporting Information

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SI Experimental Procedures

Antibodies and Chemicals. The antibodies used for this study are as follows: 389(P) p70, 240/244(P) S6, α-tubulin, 4EBP1, 51(P) eIF2α, 981(P) PERK, cleaved PARP, and PCNA were all purchased from Cell Signaling Technologies. Antibodies to GLS and GDH were purchased from Abcam. P62/SQSTM1 antibody was purchased from Sigma. GAPDH antibody was purchased from Ambion. LC2 antibody was from Novus Biological. The following chemicals were used in this study: rapamycin (Calbiochem), EGCG (Calbiochem), 17AAG (Sigma), DMSO (Sigma), DM-α-ketoglutaric acid (Sigma), sodium pyruvate (Sigma), BSO (Sigma), GSH-MEE (Sigma), glutamate (Sigma), vitamin C (Sigma), NAC (Sigma), monobromobimane (Biochemika), dichlorofluorescin diacetate (DCFDA) (Invitrogen-Molecular Probes), 968 (Specs), AUY922 (Selleckchem), BIIB021 (Selleckchem), and BPTES (provided by Takashi Tsukamoto, Johns Hopkins, Baltimore).

Cell-Viability Measurements. All cell-viability experiments were conducted with PI exclusion assay as described (1). For all phase images, the Nikon Eclipse TE300 camera was used, and images were taken at the indicated time points.

Determination of ROS. Before treating the cells with the small molecules, cells were rinsed with 1× PBS. Cells were incubated with 1× PBS containing 10 μM DCFDA (Invitrogen–Molecular Probes) for 5 min at 37 °C. PBS was removed, and small molecules dissolved in experimental medium were added if necessary. Once the experimental time was achieved, medium was collected in a 15-cm conical tube. Cells were then washed with saline solution and collected in the same tube. Trypsin (500 μL for a 10-cm^2 well) was added, cells were incubated on ice for 5 min, and trypsin was collected in the same 15-cm tube. The wells were washed with saline solution and collected in the 15-cm tube. Samples were centrifuged (180 × *g*, 4 °C) for 3 min. Cell pellets were resuspended in 300 μL of 1× PBS containing 0.3% BSA (filtered in 0.2 or 0.45 μm). Lastly, test samples were analyzed by FACS with a FITC filter.

Cell Lysis and Immunoblotting. Cells were washed once with cold PBS and solubilized on ice either in a regular lysis buffer [40 mM Hepes (pH 7.4), 1 mM EDTA, 120 mM NaCl, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM Na_3VO_4, and 0.3% CHAPS] or in a low-salt lysis buffer [40 mM Hepes (pH 7.4), 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM Na_3VO_4, and 0.3% CHAPS] supplemented with protease inhibitors (250 μM PMSF, 5 μg/mL Pepstatin A, 10 μg/mL Leupeptin, and 5 μg/mL Aprotinin). Cleared cell lysates were obtained by centrifugation at 16,000 × *g* for 10 min at 4 °C and analyzed by immunoblotting.

Intracellular Glutamate Level Measurements. To measure glutamate levels, we used the Amplex Red Glutamic Acid/Glutamate Oxidase Assay from Invitrogen–Molecular Probes (MP12221) according to the manufacturer's instructions.

TEM. Cells were fixed with 2% glutaraldehyde/2% formaldehyde in cacodylate buffer, followed by 1% osmium tetroxide. The samples were embedded in epoxy resin and viewed with a FEI Tecnai 12 transmission electron microscope operated at 80 kV.

siRNA Transfections. Twenty-five nM siRNAs were transfected in cells right after being seeded at a density of 30–50% confluency depending on experiments by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocols. Cells were harvested 36–60 h after transfection as described in the figure legends. All siRNAs used were obtained from Dharmacon.

Quantitative RT-PCR Analysis. Total cellular RNA was purified from cultured cells by using the RNeasy mini kit (Qiagen) following the manufacturer's protocol. For quantitative real-time PCR (qRT-PCR), RNA was reverse-transcribed by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The resulting cDNA was analyzed by qRT-PCR using the QuantiTect SYBR Green qPCR System (Qiagen). A QuantiTect Primer Assay was used to amplify the target gene (GCLC catalog no. QT00174300 and GSS catalog no. QT00150304) and normalization control gene (GAPDH catalog no. QT01658692) (Qiagen). All reactions were run on an ABI 7900HT Fast Real-Time PCR instrument with a 15-min hot start at 95 °C followed by 40 cycles of a three-step thermocycling program: denaturation, 15 s at 94 °C; annealing, 30 s at 55 °C; and extension, 30 s at 70 °C. Melting curve analysis was performed at the end of every run to ensure that a single PCR product of the expected melting temperature was produced in a given well. A total of three biological replicates × four technical replicates was performed for each treatment group. Data were analyzed by using the comparative Ct method (ΔΔCt method).

Metabolite Analysis of Spent Medium. Glutamine concentrations were measured in fresh and spent medium (after 72 h of culture in the presence or absence of drugs) by using a Yellow Springs Instruments 7100. Glutamine levels were normalized to cell numbers. The medium used for these experiments did not contain pyruvate and were supplemented with 10% dialyzed FBS.

Animal Studies. All animal work was performed in accordance with protocols approved by the Children's Hospital Boston Institutional Animal Care and Use Committee. Female intact CB17-SCID mice were used as described (2, 3). For xenograft tumor establishment, 2×10^6 cells were inoculated bilaterally into the posterior back region of mice. Five weeks after cell inoculation, mice bearing s.c. tumors were randomized into four groups: vehicle control ($n = 5$; 10% DMSO in corn oil, 100 μL/d, oral gavage in 0.2% methyl cellulose), 17-AAG ($n = 5$; 80mg/kg per day; oral gavage in 0.2% methyl cellulose), BPTES ($n = 5$; 40 mg/kg per day, oral gavage in 0.2% methyl cellulose), and 17-AAG plus BPTES ($n = 5$; 80 mg/kg per day, 40 mg/kg per day, oral gavage in 0.2% methyl cellulose). Drug treatment was initiated 5 wk after cell inoculation. Tumor area (width × length) was measured weekly by using a calipers. Tumor volume was calculated by the formula: $V = (\text{width})^2 \times \text{length}/2$.

Bioluminescent Reporter Imaging

Ten minutes before imaging, animals were injected with luciferin (Xenogen) (120 mg/kg, i.p.). Bioluminescent signals were recorded using the Xenogen IVIS System. Total photon flux of tumors was analyzed (2).

Immunohistochemical Staining

Histology sections were prepared from mouse tumors after 10% formalin fixation and cutting into 10-μm sections. Slides were deparaffinized, and antigen retrieval was performed by using Dako Target Retrieval Solution, pH 6. Immunohistochemical staining was performed by using antibodies against

PCNA (Cell Signaling Technologies) for cell proliferation, TACS 2 TdT-DAB in Situ Apoptosis Detection Kit (Trevigen) for cell death, and a Histostain-Plus Detection Kit (Life Technologies). After staining, images were captured by using an Olympus FluoView FSX100 microscope (Fig. 6 *E* and *F*) (sbars, 50 μM).

1. Choo AY, et al. (2010) Glucose addiction of TSC null cells is caused by failed mTORC1-dependent balancing of metabolic demand with supply. *Mol Cell* 38(4):487–499.
2. Yu JJ, et al. (2009) Estrogen promotes the survival and pulmonary metastasis of tuberin-null cells. *Proc Natl Acad Sci USA* 106(8):2635–2640.
3. Parkhitko A, et al. (2011) Tumorigenesis in tuberous sclerosis complex is autophagy and p62/sequestosome 1 (SQSTM1)-dependent. *Proc Natl Acad Sci USA* 108(30): 12455–12460.

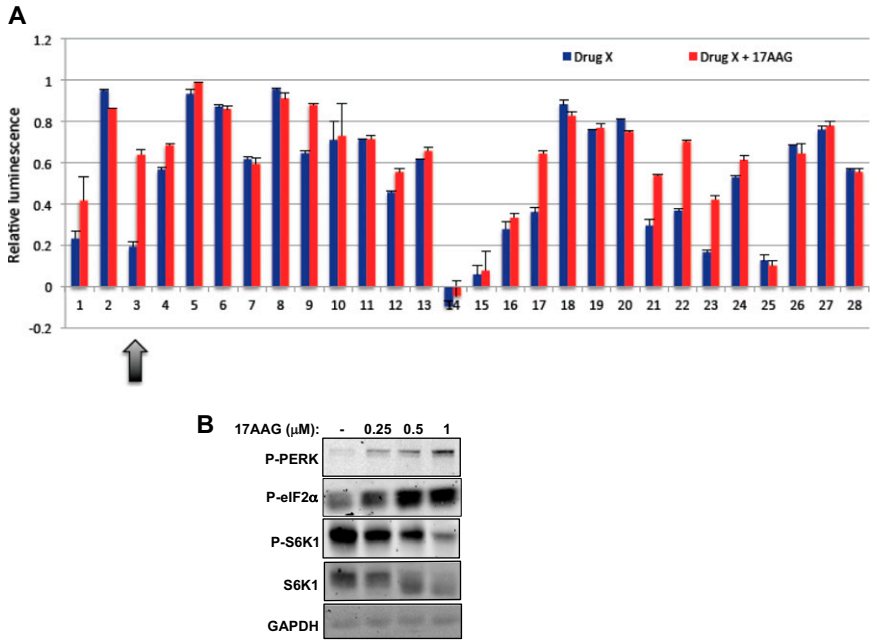

Fig. S1. A targeted small-molecule screen identifies GLS inhibition to sensitize *Tsc2*$^{-/-}$ MEFs to Hsp90 inhibition. (*A*) Cell viability of *Tsc2*$^{-/-}$ MEFs using the CellTiter-Glo. Relative luminescence was measured in *Tsc2*$^{-/-}$ MEFs after 72 h of treatment with small molecules and 17AAG (0.3 μM). The mean is shown; error bars represent SEM. (*B*) Immunoblot analysis of PERK, P-S6K1, S6K1, P-eIF2α, and GAPDH in *Tsc2*$^{-/-}$ MEFs treated with increasing concentrations of 17AAG as indicated for 72 h.

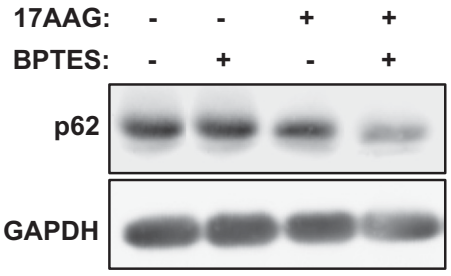

Fig. S2. The combined inhibition of GLS and Hsp90 results in decreased viability and morphological changes of *Tsc2*$^{-/-}$ MEFs. Immunoblot analysis of p62 in *Tsc2*$^{-/-}$ MEFs treated with DMSO, 17AAG (0.5 μM), and BPTES (10 μM) as indicated for 24 h.

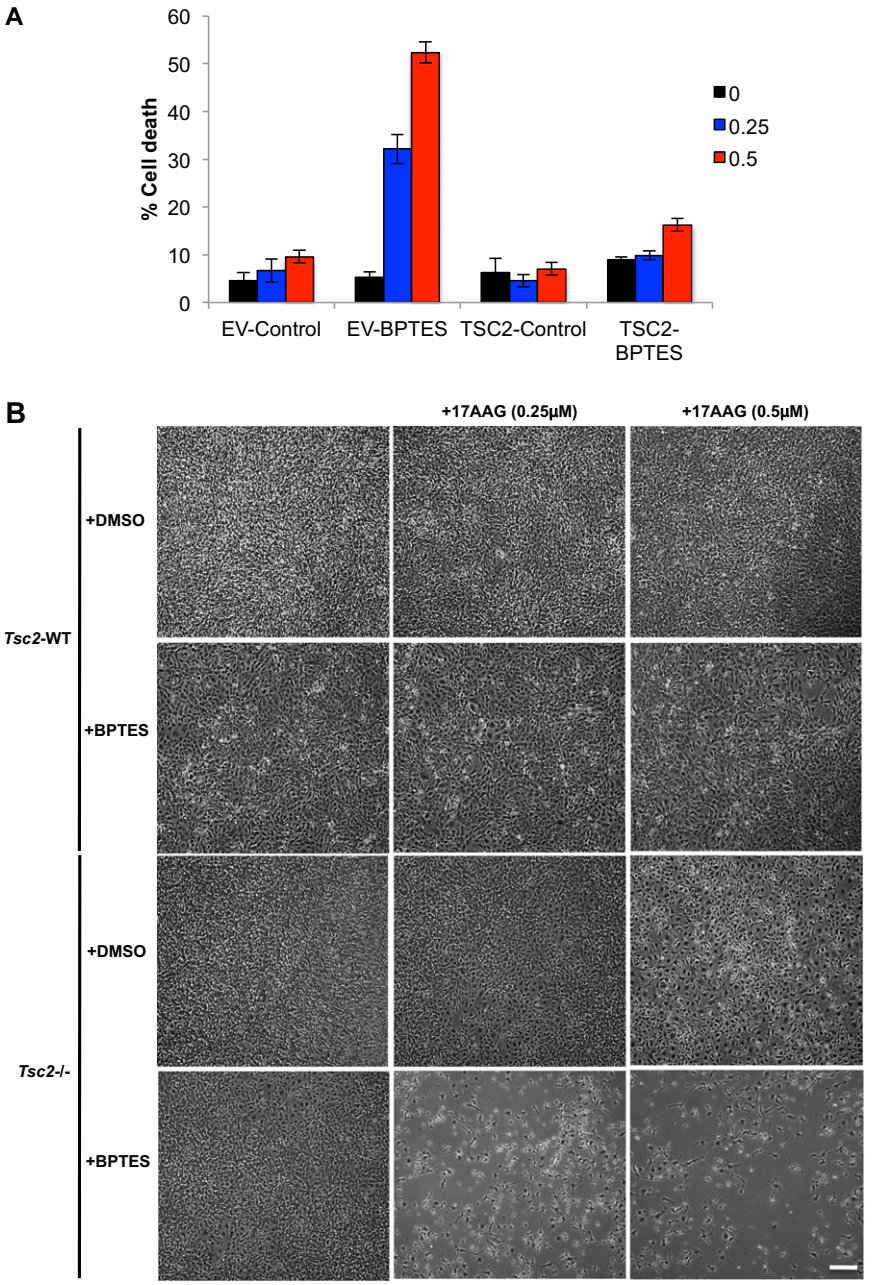

Fig. S3. (Continued)

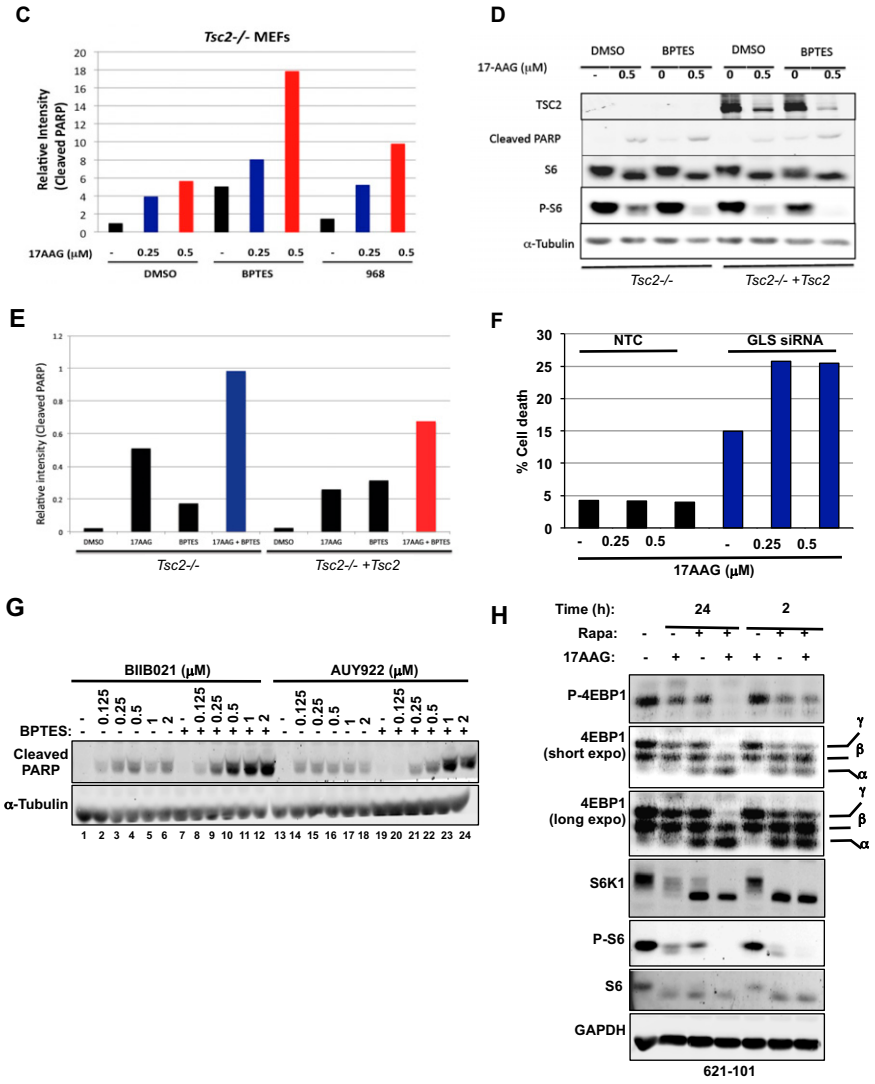

Fig. S3. Inhibition of glutamine anaplerosis and Hsp90 causes potent apoptosis in *Tsc2*$^{-/-}$ cells; Hsp90 inhibition reverses rapamycin insensitive mTORC1 phenotypes. (*A*) Cell death of ELT3 and TSC2-reexpressing ELT3 cells after 96 h of treatment with 17AAG (0.25 and 0.5 μM) and with or without BPTES (10 μM) was measured via PI exclusion assay. The mean is shown; error bars represent SEM ($n > 3$). (*B*) *Tsc2*$^{-/-}$ MEFs and WT MEFs were treated with DMSO, 17AAG (0.25 and 0.5 μM), and BPTES (10 μM) as indicated for 72 h. Phase microscopy was used to observe cell viability. (*C*) Relative intensity of cleaved PARP was measured in *Tsc2*$^{-/-}$ MEFs treated with the indicated compounds for 24 h. (*D*) Immunoblot analysis of TSC2, cleaved PARP, S6, P-S6, and α-tubulin in *Tsc2*$^{-/-}$ MEFs and *Tsc2*$^{-/-}$ +*Tsc2* MEFs treated with DMSO, 17AAG (0.5 μM), and BPTES (10 μM) for 24 h. (*E*) Relative intensity of cleaved PARP was measured in *Tsc2*$^{-/-}$ MEFs and *Tsc2*$^{-/-}$ +*Tsc2* MEFs treated with the indicated compounds for 24 h. (*F*) Cell death of *Tsc2*$^{-/-}$ MEFs after RNAi-mediated knockdown of GLS with or without 17AAG (0.25 and 0.5 μM) was measured via PI exclusion assay. (*G*) Immunoblot analysis of cleaved PARP and α-tubulin in *Tsc2*$^{-/-}$ MEFs treated with the indicated compounds (Hsp90 inhibitors: BIIB021, AUY922, and BPTES) for 24 h. (*H*) Immunoblot analysis of P-4EBP1, 4EBP1, S6K1, P-S6, S6, and GAPDH in 621-101 cells treated with rapamycin (20 ng/mL), 17AAG (1 μM), or the combination of both for the indicated time points.

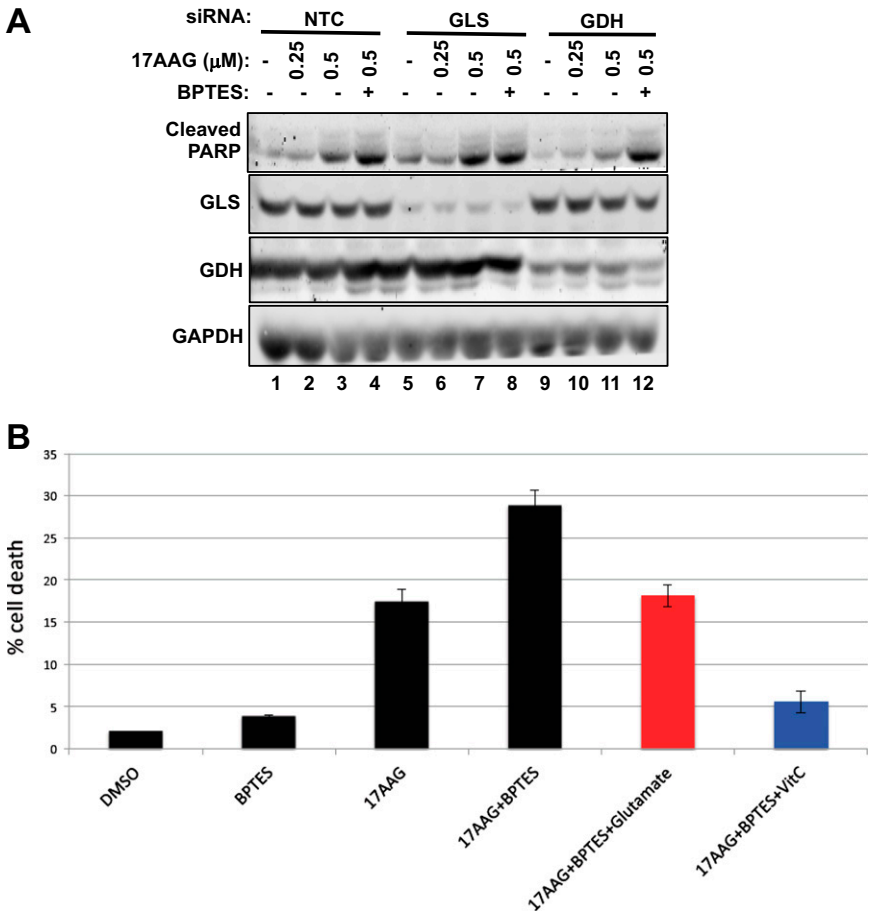

Fig. S4. GDH inhibition does not sensitize cells to Hsp90 inhibition. (*A*) Immunoblot analysis of cleaved PARP, GLS, GDH, and GAPDH in *Tsc2*$^{-/-}$ MEFs after RNAi-mediated knockdown of GLS or GDH with the indicated compounds. (*B*) Cell death of *Tsc2*$^{-/-}$ MEFs treated with DMSO, 17AAG (0.5 μM), BPTES (10 μM), glutamate (4 mM) and vitamin C (100 μM) for 48 h.

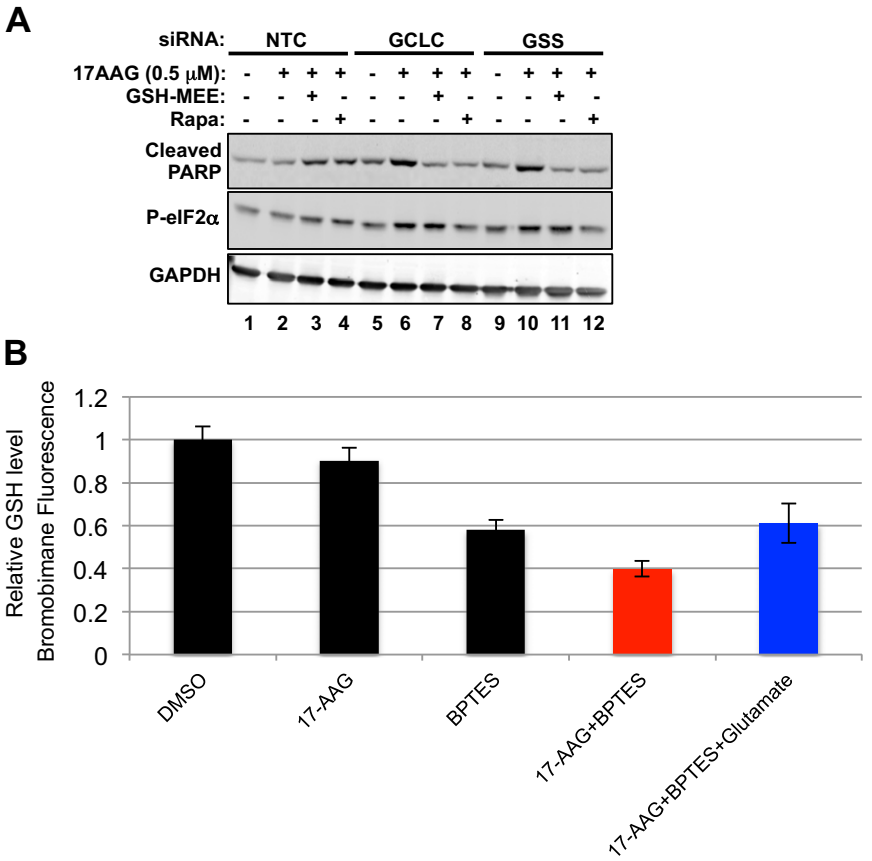

Fig. S5. Deregulated redox balance is responsible for the apoptosis induced by BPTES and 17AAG. (*A*) Immunoblot analysis of cleaved PARP, p-eIF2α and GAPDH in *Tsc2*$^{-/-}$ MEFs after RNAi-mediated knockdown of GCLC or GSS with the indicated compounds. (*B*) Intracellular GSH levels were measured in *Tsc2*$^{-/-}$ MEFs treated with DMSO, BPTES (10 μM), 17AAG (0.5 μM), BPTES plus 17AAG, or BPTES plus 17AAG plus glutamate for 24 h.

Table S1. Small-molecule library

Oxamate	Compound C
DON	Metformin
BPTES	AG1478
968	Gelfitinib
EGCG	LY294002
Mechlorethamine	Torin
Taxol	SB 203580
Etoposide	AZD6244
Cisplatin	PS2012
MCC	2-deoxyglucose
BEZ235	Orlistat
Rapamycin	Methotrexate
KU0063794	Gemcitabine
AICAR	Phenformin