Supporting Information

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SI Methods

Cell Culture and Reagents. $Tsc2^{+/+}p53^{-/-}$ and $Tsc2^{-/-}p53^{-/-}$ MEFs (1), as well as HEK293 cells, were cultured in DMEM supplemented with 10% FBS, 100 μg/mL of penicillin and 100 μg/mL of streptomycin. The 621-101 cells (2) and ELT3 cells (3) were cultured in IIA complete medium. Rapamycin was obtained from LC Laboratories or Biomol. Chloroquine diphosphate salt and propidium iodide were obtained from Sigma-Aldrich.

Antibodies, shRNA, and Immunoblot Analysis. Phospho-S6 (Ser235/236) and β-actin antibodies were obtained from Cell Signaling Technology, tuberin antibody from Abcam, LC3 antibody from Novus Biologicals, GFP and p62/SQSTM1 antibodies from Santa Cruz Biotechnology, and p62/SQSTM1 and α-tubulin antibodies from Sigma-Aldrich. shRNA constructs against TSC2 and p62/SQSTM1 were obtained from Sigma-Aldrich. For immunoblot analyses, cells were washed with PBS and harvested in Nonidet P-40 buffer. Whole-cell lysates were resolved by electrophoresis, and proteins were transferred onto PVDF membrane (Immobilon P; Millipore), blocked in Tris-buffered saline Tween-20 buffer (Cell Signaling Technology), and probed with the indicated antibodies in this buffer.

Transmission Electron Microscopy. Cells were fixed with 2% glutaraldehyde/2% formaldehyde in cacodylate buffer, followed by 1% osmium tetroxide. The samples were embedded in epoxy resin. The samples were viewed with a FEI Tecnai 12 transmission electron microscope operated at 80 kV, with 9300× magnification.

Cell Viability Assays. Cell viability was measured with the propidium iodide exclusion assay (4). Here 1 μg/mL of propidium iodide was added to culture medium for 5 min before collection. The medium was collected, and the plates were washed briefly with saline. The remaining cells on the plate were detached with trypsin and added to the media collection tube. The tube was centrifuged and the pellet analyzed for propidium iodide exclusion with a FACS Aria Cell Sorter (BD Biosciences).

Oxygen Consumption Assay. Intact cellular respiration was measured using the Seahorse Bioscience XF24 analyzer. Before the respiration assay, cells were rinsed and cultured in DMEM running medium [8.3 g/L of DMEM (Sigma-Aldrich), 200 mM GlutaMax-1 (Invitrogen), 100 mM sodium pyruvate (Sigma-Aldrich), 25 mM D-glucose (Sigma-Aldrich), 63.3 mM NaCl (Sigma-Aldrich), and phenol red (Sigma-Aldrich), pH 7.4], according to the manufacturer's protocol. Oxygen consumption was measured under basal conditions or in the presence of the mitochondrial inhibitors oligomycin (0.5 μM). Levels of oxygen consumption were normalized to cell number.

ATPLite Luminescence Assay. ELT3 cells were grown in DMEM with 10% FBS or in nutrient-restricted DMEM (no FBS, no L-glutamine, glucose 1 g/L) in 96 opaque white well plates. At 24 h after cell seeding, cells were treated with vehicle, CQ (10 μg/mL), rapamycin (20 nM), or CQ plus rapamycin for 24 h. ATP levels were measured with the Perkin-Elmer ATPLite system according to the manufacturer's instructions.

Immunohistochemistry. Sections were deparaffinized, incubated overnight with primary antibodies at 4 °C in a humidified chamber and then rinsed and incubated with biotinylated secondary antibodies for 30 min at room temperature. Slides were counterstained with Gill's hematoxylin.

Induction, Treatment, and Measurement of Subcutaneous Tumors and Scoring of Renal Lesions. $Tsc2^{-/-}p53^{-/-}$ MEFs or ELT3 cells were inoculated bilaterally into the posterior back region of 6-wk-old immunodeficient CB17 SCID or CD-1 nude mice (Taconic). Tumor length, width, and depth were measured with a Vernier caliper by an investigator blinded to the experimental conditions. When tumors reached 100 mm^2, mice were randomly assigned to i.p. CQ at a dose of 50 mg/kg, rapamycin (Biomol) at a dose of 3 or 6 mg/kg, or sterile PBS daily treatment. The animal studies were approved by the Children's Hospital Boston Animal Care and Use Committee. $Tsc2^{+/-}$ mice were studied in either the CB57BL/6 or the A/J genetic background. The severity of renal lesions in the $Tsc2^{+/-}$ mice was scored using a quantitative index that was developed and validated in multiple previous studies (5, 6), incorporating standardized gross inspection and microscopic histological parameters.

Statistics. The two-tailed Student *t* test was used for in vitro and in vivo studies. The Mantel–Cox log-rank test was used for the analysis of control or p62 shRNA.

1. Onda H, et al. (2002) Tsc2 null murine neuroepithelial cells are a model for human tuber giant cells, and show activation of an mTOR pathway. *Mol Cell Neurosci* 21:561–574.
2. Yu J, Astrinidis A, Howard S, Henske EP (2004) Estradiol and tamoxifen stimulate LAM-associated angiomyolipoma cell growth and activate both genomic and nongenomic signaling pathways. *Am J Physiol Lung Cell Mol Physiol* 286:L694–L700.
3. Howe SR, et al. (1995) Rodent model of reproductive tract leiomyomata: Establishment and characterization of tumor-derived cell lines. *Am J Pathol* 146:1568–1579.
4. 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* 38:487–499.
5. Lee L, et al. (2005) Efficacy of a rapamycin analog (CCI-779) and IFN-γ in tuberous sclerosis mouse models. *Genes Chromosomes Cancer* 42:213–227.
6. Pollizzi K, Malinowska-Kolodziej I, Stumm M, Lane H, Kwiatkowski D (2009) Equivalent benefit of mTORC1 blockade and combined PI3K–mTOR blockade in a mouse model of tuberous sclerosis. *Mol Cancer* 8(38):1–9.

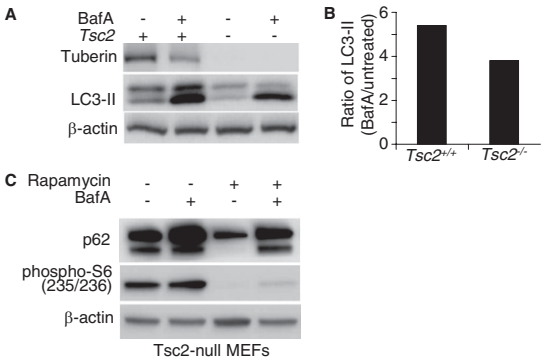

Fig. S1. Autophagic flux is intact in *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs. (*A*) Immunoblot analysis of LC3-II in *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs treated with 10 nM bafilomycin A or vehicle for 6 h. (*B*) Densitometry analysis of LC3-II performed in *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs treated with 10 nM bafilomycin A or vehicle for 6 h and normalized to β-actin. The ratio of LC3-II (treated/untreated) in *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs is shown. (*C*) Immunoblot analysis of p62 and phospho-S6 (235/236) in Tsc2-null ELT3 cells treated with 20 nM rapamycin, 10 nM bafilomycin A, or both for 24 h.

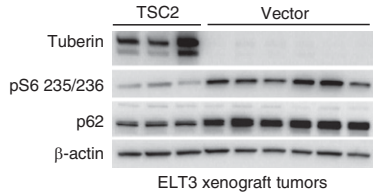

Fig. S2. Reexpression of TSC2 suppresses accumulation of p62 in ELT3 xenograft tumors. Immunoblot analysis of p62/SQSTM1 in xenograft tumors from TSC2-null ELT3 cells (V3; *n* = 3) compared with ELT3 cells reexpressing TSC2 (T3; *n* = 6).

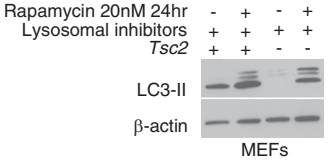

Fig. S3. mTORC1 inhibition increases LC3-II levels in *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs in the presence of lysosomal inhibitors E64d and pepstatin. Immunoblot analysis of LC3-II in *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs treated with 20 nM rapamycin or vehicle for 24 h and with the lysosomal inhibitors E64d and pepstatin during the last hour of rapamycin treatment to prevent the degradation of LC3-II.

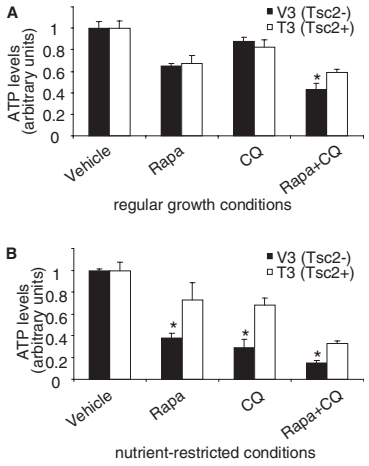

Fig. S4. TSC2-null ELT3 cells are more sensitive to dual inhibition of autophagy and mTORC1 compared with TSC2-reexpressing cells. ATP levels were measured using the PerkinElmer ATPlite assay system after treatment of ELT3 cells expressing either empty vector (V3) or TSC2 (T3) cultured in either DMEM + 10% FBS (*A*) or nutrient-restricted DMEM (no FBS, no L-glutamine, 1 g/L of glucose) (*B*) with CQ (2.5 μg/mL), rapamycin (20 nM), or both for 24 h. **P* < 0.05.

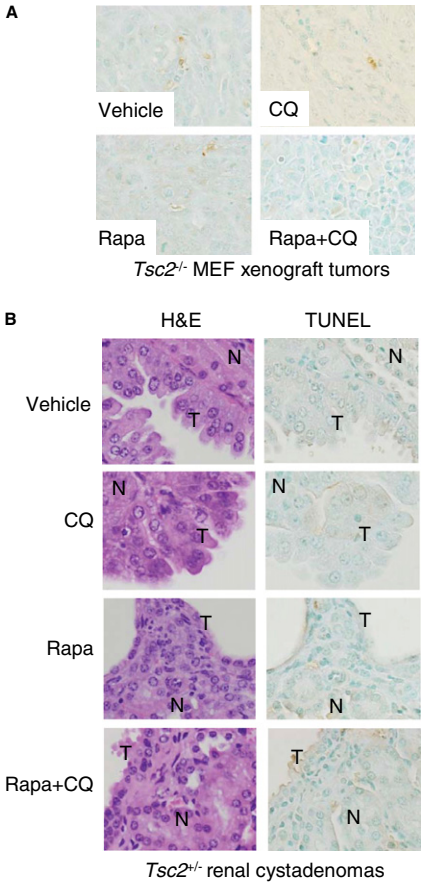

Fig. S5. Effect of dual treatment with rapamycin and CQ on cell survival in TSC2-null tumors. TUNEL staining of $Tsc2^{-/-}$ MEF xenograft tumors treated with vehicle, CQ (50 mg/kg/d), rapamycin (6 mg/kg/d), or rapamycin (6 mg/kg/d) plus CQ (50 mg/kg/d) for 2 wk (*A*) and $Tsc2^{+/-}$ mice in A/J background treated with vehicle, rapamycin (6 mg/kg, 3 times/wk), CQ (50 mg/kg/d), or both for 1 wk (*B*). Representative H&E- and TUNEL-stained lesions are shown. N, normal kidney. T, cystadenomas. (Original magnification, 20×.)

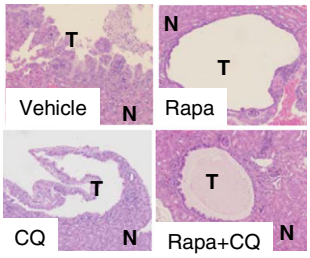

Fig. S6. Dual inhibition of autophagy and mTORC1 is more effective in suppressing $Tsc2^{+/-}$ renal cystadenomas development than inhibition of either one alone. Representative H&E-stained lesions from mice receiving each treatment. Lesions are quantitated in Fig. 2*D*. N, normal kidney. T, cystadenomas. (Original magnification, 20×.)

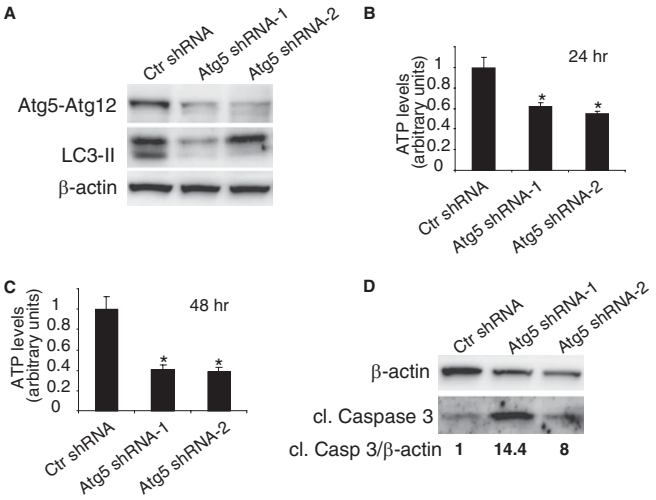

Fig. S7. ATG5 down-regulation suppresses ATP production and cell survival in Tsc2-null cells. (*A*) Immunoblot analysis of ATG5 and LC3-II in *Tsc2*$^{-/-}$ MEFs infected with control or two different *Atg5* shRNAs. (*B* and *C*) ATP levels measured using the ATPlite assay and normalized to cell number in *Tsc2*$^{-/-}$ MEFs with control or *Atg5* shRNAs cultured in DMEM + 10% FBS after 24 h (*B*) or 48 h (*C*). (*D*) Immunoblot analysis of cleaved caspase-3 in *Tsc2*$^{-/-}$ MEFs with control or *Atg5* shRNAs cultured in nutrient-restricted DMEM (no FBS, no L-glutamine, 1 g/L of glucose) for 16 h.