

# Supporting Information

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## SI Methods

**Antibodies, Equipment, and Reagents.** The anti-phospho-LKB1 [Thr366 (human)/Thr363 (rodent)] antibody was the kind gift of Dr. Dario Alessi (University of Dundee, Dundee, Scotland). Anti-S6K, anti-phospho-S6K (Thr389), anti-S6, anti-phospho-S6 (Ser235/236), anti-4E-BP1, anti-phospho-4E-BP1 (Thr37/46), anti-phospho-CHK2 (Thr68), anti-p53, anti-phospho-p53 (Ser15), anti-LKB1, anti-AMPK, anti-phospho-AMPK (Thr172), anti-ACC, anti-phospho-ACC (Ser79), and anti-LC3B were purchased from Cell Signaling Technologies. Other antibodies used were anti- $\gamma$ -tubulin and anti-Flag M2 (Sigma), anti-ATM (GeneTex), anti-TSC2 (Epitomics), anti-phospho-ATM (Ser1981) (R&D Systems), Anti-Myc, [mouse monoclonal (9E10)], and GAPDH (Santa Cruz Biotechnology). HRP-conjugated secondary antibodies were obtained from Santa Cruz. Hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 3%) was purchased from Sigma approximately once per month and diluted in sterile water immediately before each treatment. Etoposide (Sigma) was resuspended in DMSO to make a 20 mM stock solution. Compound C (Calbiochem) was resuspended in DMSO. Doxorubicin (Sigma) was resuspended in water. Bovine catalase (Sigma) was dissolved in 50 mM potassium phosphate buffer. N-acetyl cysteine (NAC) (Sigma) was dissolved in water immediately before use. Acridine orange was purchased from Sigma. Rapamycin (LC Laboratories) was resuspended in DMSO before dilution in the vehicle for in vivo studies as described below. Leptomycin B was purchased from LC Laboratories prediluted in ethanol. Cells were irradiated in closed plates with a Rad Source 2000 irradiator and then returned to a 37 °C incubator until harvesting.

**Cell Culture.** MCF7 cells [American Type Culture Collection (ATCC)] were grown in Improved Modified Eagle's Medium (IMEM) supplemented with 10% FBS. MCF7 cells stably expressing GFP-LC3 were maintained in RPMI 1640 supplemented with 10% FBS. ATM<sup>+/+</sup> human lymphoblasts (GM02184) and ATM<sup>-/-</sup> human lymphoblasts (GM01526) (both from Coriell Cell Repositories) were maintained in RPMI 1640 supplemented with 15% FBS. Tsc2<sup>+/+</sup>/p53<sup>-/-</sup> and Tsc2<sup>-/-</sup>/p53<sup>-/-</sup> MEFs (kind gifts from Dr. D. Kwiatkowski, Harvard Medical School, Boston, MA) were grown in Dulbecco's MEM supplemented with 10% FBS. HEK293 cells (kind gift from Dr. Y. Hu, University of Texas M. D. Anderson Cancer Center, Smithville, TX) were grown in Dulbecco's MEM supplemented with 10% FBS. HeLa S3 cells (ATCC) were grown in F12K medium (ATCC) supplemented with 10% FBS, and the derived clones expressing WT or mutant LKB1 were maintained in the same media containing 800  $\mu\text{g}/\text{mL}$  G418. Ishikawa cells (ATCC) were maintained in DMEM/F12 (1:1) supplemented with 10% FBS. p53<sup>+/+</sup> and p53<sup>-/-</sup> FVB MEFs (kind gifts from Dr. D. Johnson, University of Texas M. D. Anderson Cancer Center, Smithville, TX) were grown in DMEM supplemented with 10% FBS. Primary fibroblasts were derived from genotype-confirmed ATM<sup>+/+</sup>, ATM<sup>+/-</sup>, and ATM<sup>-/-</sup> embryos at about E13. These

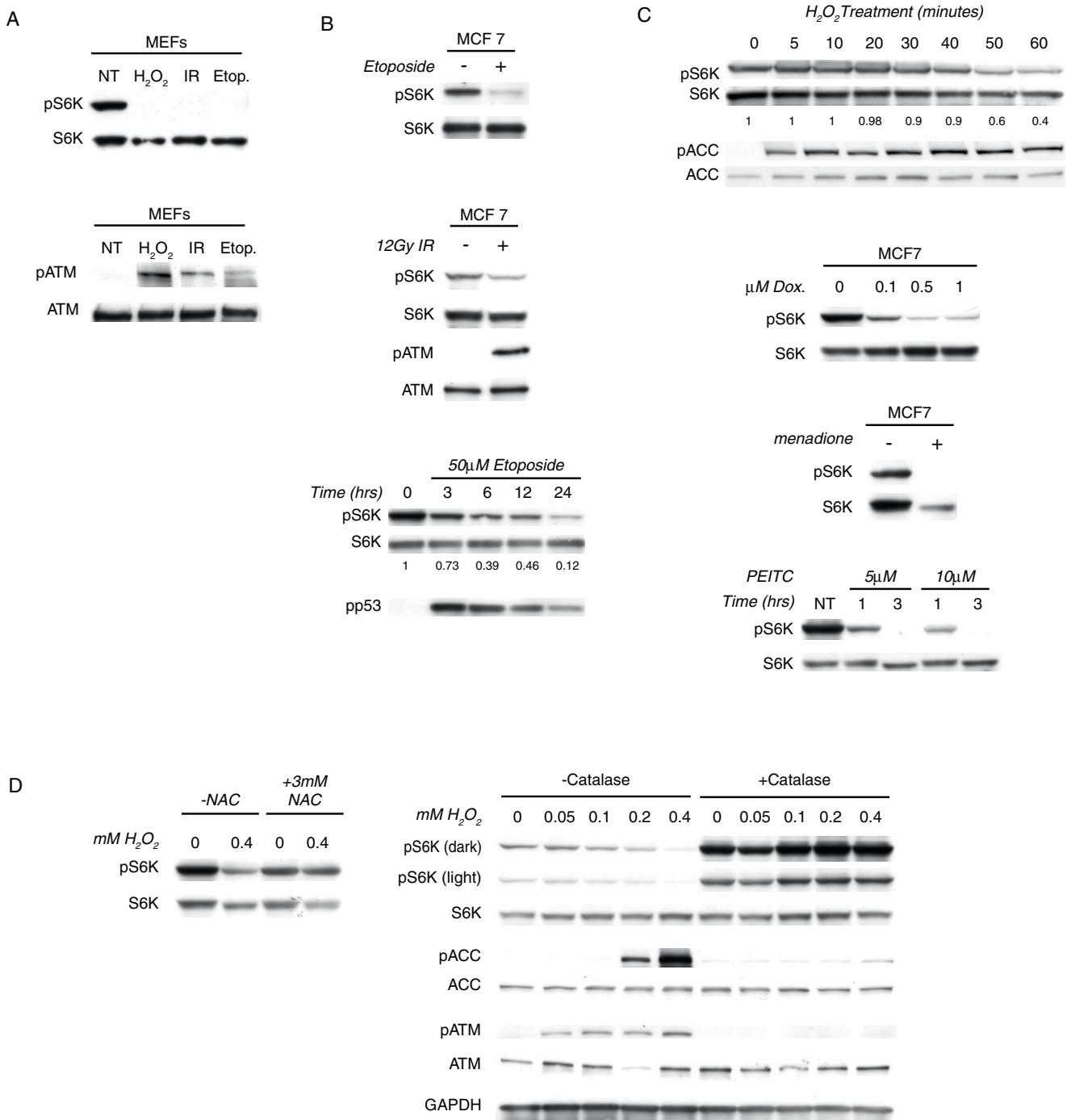
cells were maintained in DMEM supplemented with 10% FBS, except for the ATM<sup>-/-</sup> lines that were maintained in 15% FBS. SKOV3 cells, and derived cells stably expressing GFP-LC3 were maintained in RPMI 1640 supplemented with 10% FBS.

**Preparation of Cell Lysates.** Cells were lysed in lysis buffer [20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% Triton-X100, 2.5 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, and 1 mM  $\beta$ -glycerophosphate]. The following inhibitors were added immediately before buffer was added to cells: 1 mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, and protease inhibitor mixture (Roche).

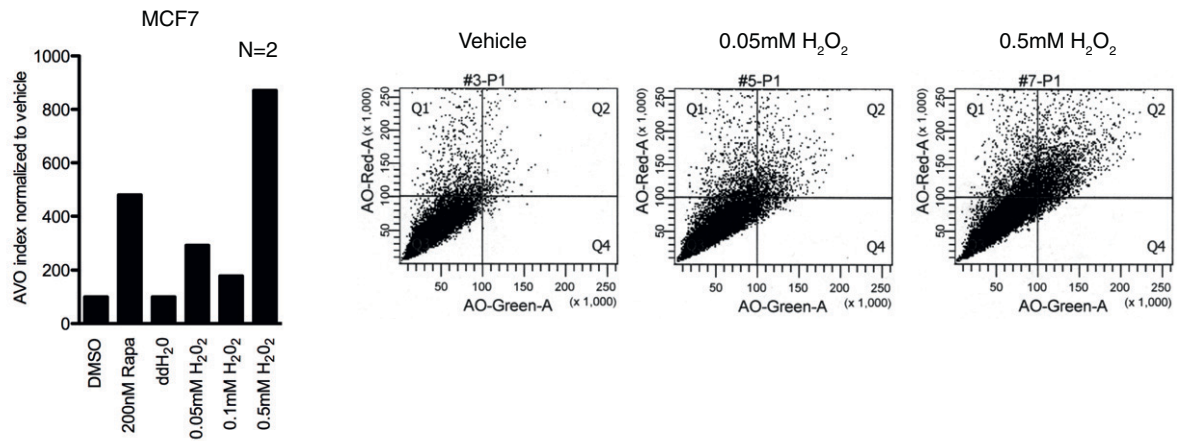
**Acidic Vesicle Detection with Acridine Orange.** MCF7 cells were plated on a 15-cm plate at 20 cells/mm<sup>2</sup> and allowed to equilibrate for 24 h. Cells were exposed to vehicle, rapamycin, or H<sub>2</sub>O<sub>2</sub> for 15 h, trypsinized, counted, centrifuged at 1,000 rpm for 5 min, and then resuspended to 2,200 cells/ $\mu\text{l}$ . Acridine Orange was added at 1  $\mu\text{g}/\mu\text{l}$  to visualize acidic vesicular organelles and propidium iodide 0.01  $\mu\text{g}/\mu\text{l}$  for cell integrity. Samples were processed by flow cytometry using the blue laser and 620/20 (AO-red) and 530/30 (AO-green) filters. Gates were normalized to vehicle treated samples to determine percent AVO positive cells for each sample.

**siRNA Experiments.** Chemically synthesized siRNA SMARTpools were obtained from Dharmacon (TSC2, M-003029-02; ATM, L-003201-00-05; LKB1, L-005035-00; p53, M-003329-01). The negative control siRNA used was siCONTROL [RISC-free 1 (D-001220-01)], which contained at least four mismatches to all known human, mouse, and rat genes. The oligos were resuspended in 1 $\times$  buffer to a concentration of 20  $\mu\text{M}$ . MCF-7 cells were plated in 35 mm plates approximately 36 h before transfection. The stock solutions of siRNA were diluted 1:100 (making a final siRNA concentration of 10 nM) in 1 $\times$  buffer, and DharmaFECT1 transfection reagent (Dharmacon) was diluted 1:50 in OptiMEM medium (Invitrogen). The diluted siRNA and transfection reagent were then incubated for 30 min at room temperature in a 1:2 ratio in a total volume of 600  $\mu\text{L}$ . The cells were washed with OptiMEM, and regular IMEM medium was added to a total volume of 4 mL per plate, after which the lipid micelles were added and dispersed across the monolayer. Knockdown efficiency was determined by Western analysis at 48 h after transfection; hydrogen peroxide or DNA damaging agents were added as indicated in the figure legends.

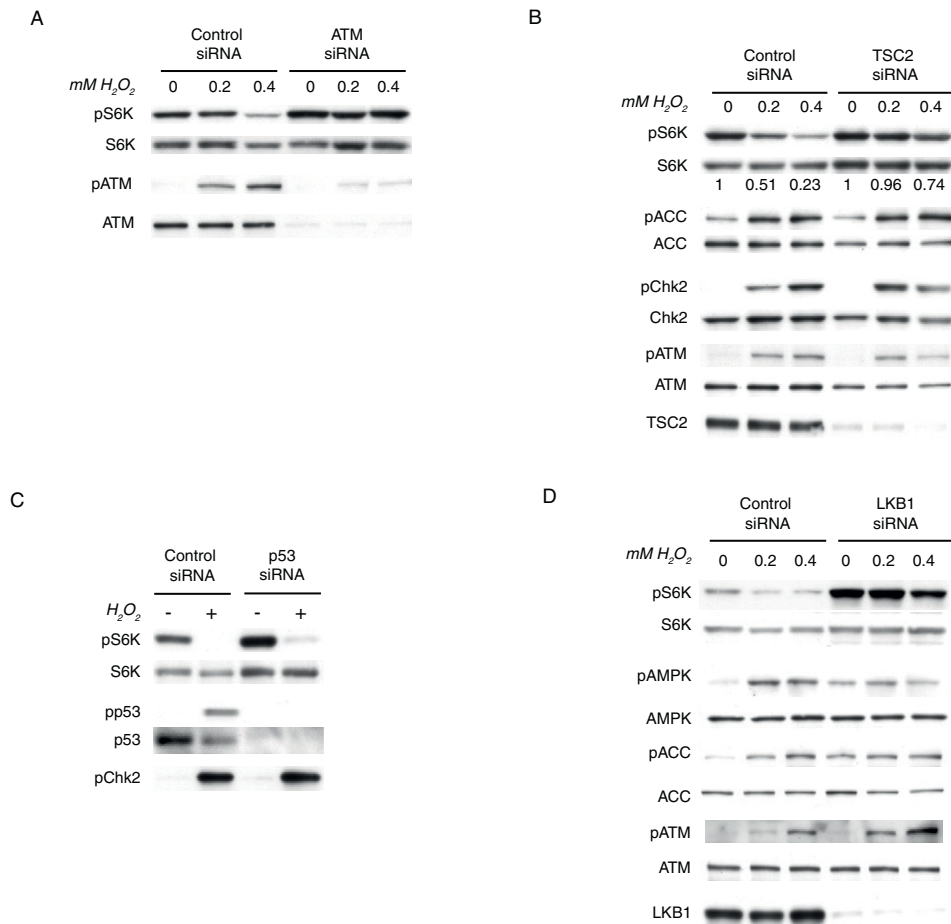
**Measurement of Intracellular ROS.** Cells were incubated with 10  $\mu\text{M}$  of DCFDA in the culture medium at 37 °C for 30 min and washed twice with cold PBS, and the resulting fluorescence measured with a Synergy HT Multidetection Microplate Reader (BioTeK Instruments, Inc.) at an excitation wavelength of 485/10 nm and an emission wavelength of 528/20 nm.



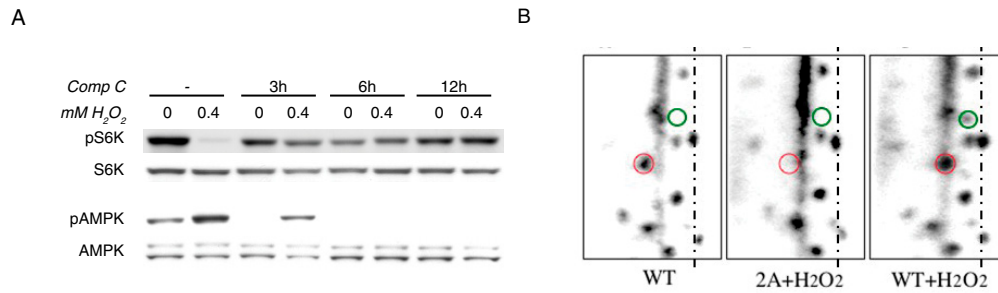
**Fig. S1.** Repression of mTORC1 in response to damage (A) *Tsc2<sup>+/+</sup>* MEFs were irradiated with 12 Gy IR or treated with 1 mM H<sub>2</sub>O<sub>2</sub> and harvested after 1 h, or treated with 50 μM etoposide for 24 h before harvesting. Lysates were examined by Western analysis for the indicated proteins. (B) MCF7 cells were treated with 50 μM etoposide or vehicle for up to 24 h or irradiated with 12 Gy and harvested after 6 h. (C) MCF7 cells were treated with 1 mM H<sub>2</sub>O<sub>2</sub> for the indicated times, and harvested for Western analysis. Doxorubicin treatments were 24 h and 100 μM menadione was for 1 h. MCF7 PEITC treatments are as indicated in the figure. For H<sub>2</sub>O<sub>2</sub> treatment, numbers indicate densitometric ratios for phosphorylated S6K/total S6K, with a baseline value of 1.0 for the zero time point. (D) Western analysis of MCF7 cells preincubated with 3 mM NAC or 2950U bovine catalase for 1 h before treatment with the indicated doses of H<sub>2</sub>O<sub>2</sub>.



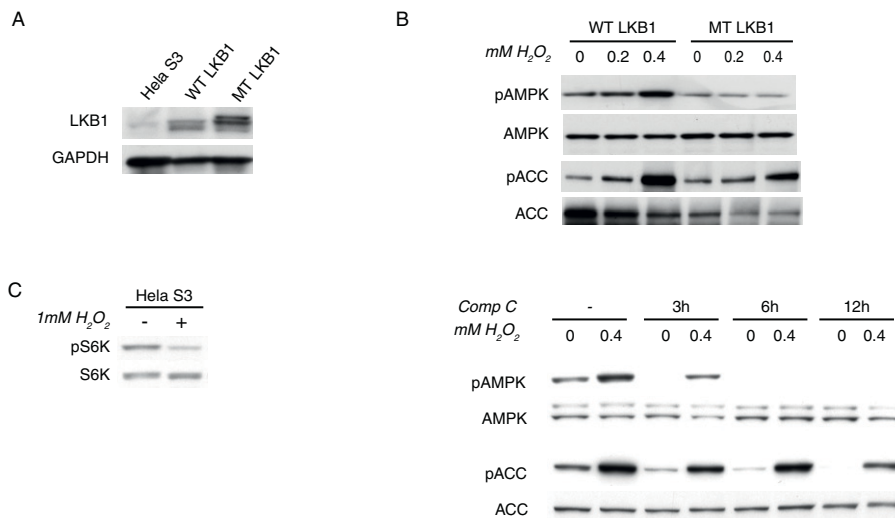
**Fig. S2.** Induction of autophagic vesicles by ROS. MCF7 cells were treated with the indicated doses of  $H_2O_2$  or rapamycin as a positive control and acidic vesicles stained with acridine orange. Flow cytometry was used to quantify red fluorescence. The number of cells in quadrants 1 and 2 as shown in the scatter plots are quantitated for a representative experiment in the graph ( $n = 2$  independent biological replicates performed).



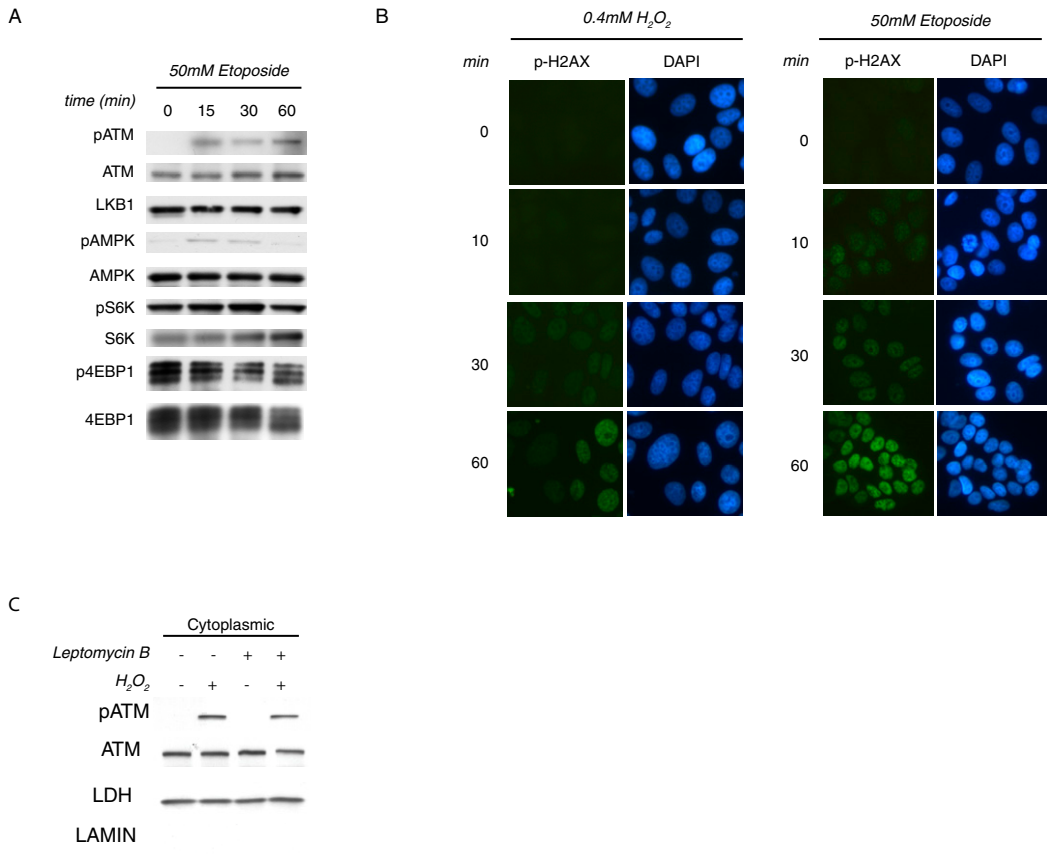
**Fig. S3.** siRNA knockdown of pathway proteins attenuates response to ROS. Western analysis of MCF7 cells transfected with the indicated siRNAs (A) ATM, (B) TSC2, (C) p53, and (D) LKB1 and treated with the indicated concentrations of  $H_2O_2$ . For B, numbers indicate densitometric ratios for phosphorylated S6K/total S6K with baseline set at one for vehicle.



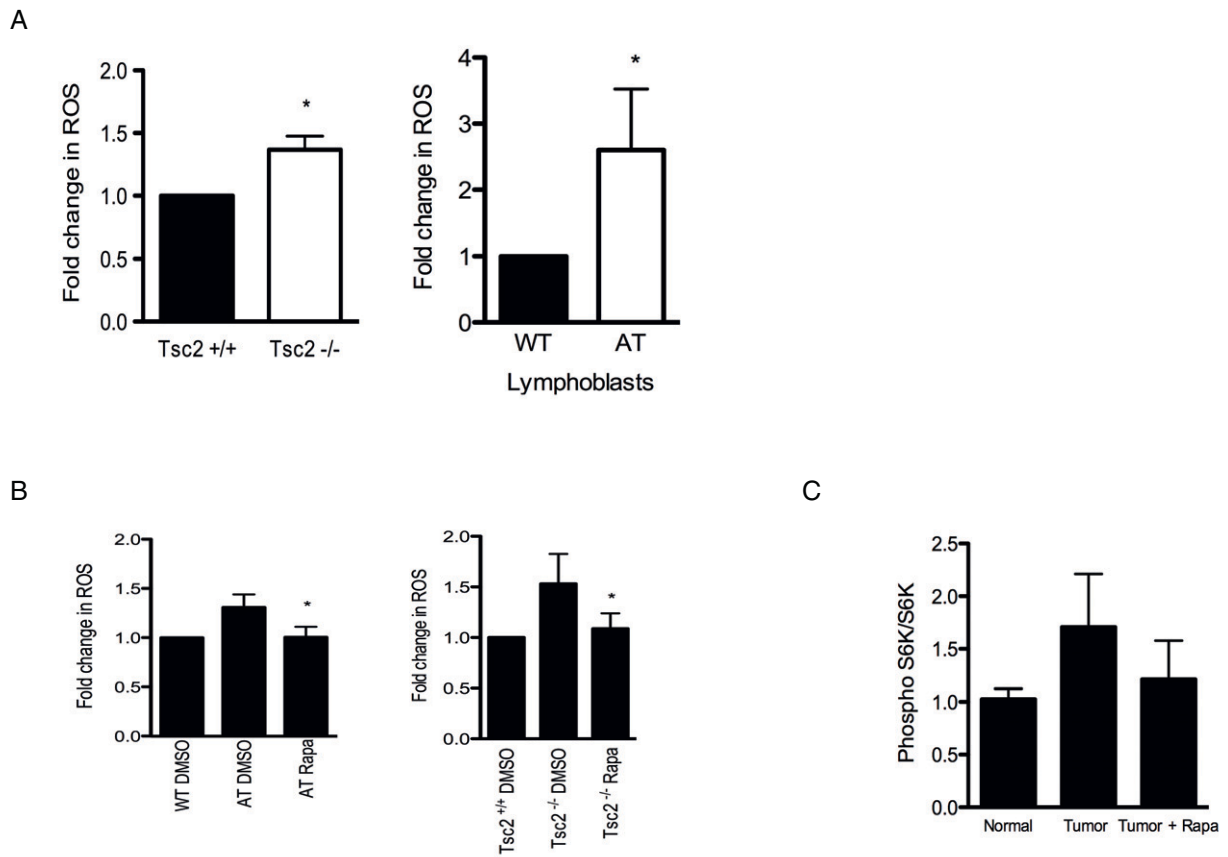
**Fig. 54.** AMPK phosphorylation of TSC2 mediates repression of mTORC1 (A) Western analysis of MCF7 cells treated with 20  $\mu$ M Compound C (AMPK inhibitor) for the indicated times before treatment with H<sub>2</sub>O<sub>2</sub> for 1 h. (B) 2D phosphopeptide mapping of TSC2. HEK293 cells transfected with wild-type (WT) TSC2 or a TSC2 mutant lacking the AMPK phosphorylation sites at T1271 and S1387 that cannot be phosphorylated by AMPK (2A) were labeled with <sup>32</sup>P-phosphate, digested and peptides separated by 2D gel electrophoresis.



**Fig. 55.** HeLa S3 clone validation and disconnect between AMPK and ACC phosphorylation. (A) Western analysis of LKB1 expression in HeLa S3 parental and transfected clones. (B) Western analysis of lysates used in Fig. 4B and Fig. 54A demonstrating ACC phosphorylation in the absence of AMPK phosphorylation at Thr172. (C) Western analysis of HeLa S3 cells treated as indicated.



**Fig. S6.** Induction of DNA damage does not acutely activate ATM signaling to AMPK (A) Western analysis of MCF7 cells treated with etoposide at times indicated. (B) Immunofluorescence staining of phospho-H2AX in H<sub>2</sub>O<sub>2</sub> and etoposide treated MCF7 cells. (C) Cytoplasmic fraction from MCF7 cells treated with H<sub>2</sub>O<sub>2</sub> in the presence or absence of 100 ng/mL leptomycin B.



**Fig. S7.** Impact of rapamycin on ROS and mTORC1 signaling (A) ROS levels in untreated Tsc2- and ATM-deficient cells. Error bars are standard deviation (mean  $\pm$  SD). (B) ROS levels in rapamycin-treated (200 nM, 24 h, DMSO vehicle) Tsc2- and ATM-deficient cells as indicated. Error bars are standard deviation (mean  $\pm$  SD). (C) Graph is quantitation of densitometric ratio of phospho-S6K/total S6K in thymi of starved mice treated as indicated. ( $n = 8$  normal,  $n = 4$  thymi with histologically confirmed tumors,  $n = 2$  tumors+rapa). Error bars represent SEM (mean  $\pm$  SEM).