Supplemental Information

Figure S1. Data supporting Figure 1

(A) Insulin dose response. Serum starved (16 hr) HeLa cells were stimulated with the indicated doses (nM) of insulin (15 min). Lysates were probed with the indicated antibodies.

(B) Insulin stimulation does not result in the acute dissociation of TSC complex components over a time course. Serum starved (16 hr) HeLa cells were stimulated with insulin (1 μM, 15 min) or left unstimulated prior to lysis and immunoprecipitation (IP) with a TSC1 antibody unique from that used in Figure 1C. Immunoblots of IPs were probed with the indicated antibodies.

(C) Insulin stimulation does not result in the acute dissociation of TSC complex components over a time course in MEFs. Treatments as in (B) except using MEFs.

(D) Phosphorylated TSC2 remains bound to TSC1 and TBC1D7 in MEFs. Treatments as in (B) except using MEFs and a phospho-T1462-TSC2 antibody for IPs.

(E) Standard curve of molecular weights for gel-filtration chromatography fractions. Purified protein standards, numbered here and listed in Extended Experimental Procedures, were individually run on the same chromatography system used to fractionate lysates in Figure 1E. The elution volume was transformed into a Kav value (elution volume-void volume/column volume-void volume) and graphed against the base-10 logarithm of the predicted molecular weight (kDa). A second-order polynomial curve was fitted to the data with the equation from this curve used to estimate the corresponding molecular weights of fractions in Figure 1E.

Figure S2. Data supporting Figure 2.

(A) A subpopulation of TSC2 colocalizes with the Golgi but is not affected by insulin stimulation. Serum starved (16 hr) HeLa cells were stimulated with insulin (1 μM, 15 min) prior to immunofluorescent labeling of TSC2 (red) and the cis-Golgi marker GM130 (green).

(B) TSC2 does not localize to mitochondria. HeLa cells were treated as in (A) prior to immunofluorescent labeling of TSC2 (red) and the mitochondrial marker CytC (green).

(C) Validation of a PMP70 antibody for immunofluorescent labeling in siRNA-control cells (siControl) or cells with siRNA-mediated knockdown of PMP70 (siPMP70).

(D) TSC2 does not localize to peroxisomes. HeLa cells were either grown in 10% FBS (16 hr) or serum starved (16 hr) and stimulated with insulin (1 μM, 15 min) prior to immunofluorescent labeling of TSC2 (red) and the peroxisomal marker PMP70 (green).

(E) Immunoblot of lysates showing signaling under conditions used in Figure 2A. Concurrently with the processing of cells in Figure 2A, serum starved (16 hr) HeLa cells were stimulated with insulin (1 μM) for the indicated times and collected as lysates. Immunoblots of lysates were probed with the indicated antibodies.

(F) Dose-dependent response to insulin of the TSC2-LAMP2 colocalization. Serum starved (16 hr) HeLa cells were stimulated with 0, 50 nM, 100 nM and 1 μM of insulin for 15 min prior to immunofluorescent labeling of

TSC2 and LAMP2. Colocalization was quantified and both the mean percent colocalization (blue) and the respective SEMs (red) at the given doses were graphed with dual y-axes.

(G) Insulin stimulation results in the acute dissociation of TSC2 from the lysosome in MEFs. Serum starved (16 hr) MEFs were stimulated with insulin (1 μM, 15 min) prior to immunofluorescent labeling of TSC2 (red) and LAMP1 (green). Percent colocalization was graphed as a mean±SEM. *$p< 1X10^{-8}$.

(H) Confirmation that the nocodazole treatment causes microtubule depolymerization. Serum starved (16 hr) HeLa cells were treated with vehicle (DMSO) or 5 μM nocodazole for 1hr prior to labeling of actin with rhodamine-Phalloidin (red) and microtubules with an anti-α-Tubulin antibody (green).

(I) TSC2-LAMP2 colocalization is unaffected by lysosome dispersion caused by microtubule depolymerization with nocodazole. HeLa cells were treated as in (H) prior to immunofluorescent labeling of TSC2 (red) and LAMP2 (green). Colocalization was quantified as in (G).

Figure S3. Data supporting Figure 3.

(A) Lyso-TSC2 assembles into the TSC complex with TSC1 and TBC1D7. HeLa cells were transfected with empty vector (vec), flag-tagged wild type TSC2 (WT-TSC2), or flag-tagged Lyso-TSC2 and grown in full serum (48 hr). Lysates and Flag immunoprecipitates from lysates of transfected cells were probed with the indicated antibodies.

(B) Lyso-TSC2 is constitutively localized to the lysosome under full serum growth conditions in HEK293E cells. HEK293E cells were transfected with flag-tagged WT-TSC2 or Lyso-TSC2 (48 hr) and grown under full serum growth condition prior to immunofluorescent labeling of Flag-tag (WT-TSC2 or Lyso-TSC2; red) and LAMP2 (green).

(C) Lyso-TSC2 inhibits the phosphorylation of endogenous S6K1 under full-serum growth conditions. Immunoblots of lysates from HEK293E cells transfected as in (A) were probed with the indicated antibodies.

(D) Lyso-TSC2 inhibits the phosphorylation of co-expressed S6K1 under full-serum growth conditions. HEK293E cells were co-transfected with HA-S6K1 and either empty vector (vec), flag-tagged WT-TSC2, or flag-tagged Lyso-TSC2 (48 hr) and grown as in (A). Immunoblots of lysates and anti-HA-S6K1 immunoprecipitates were probed with the indicated antibodies.

Figure S4. Data supporting Figure 4.

(A) Representative immunofluorescence images quantified in Figure 4B. Serum starved (16 hr) HeLa cells were stimulated with insulin (1 μM, 15 min) prior to immunofluorescent labeling of mTOR or TSC2 (red) and LAMP2 (green).

Figure S5. Data supporting Figure 5.

(A) Representative immunofluorescence images quantified in Figure 5B. HeLa cells starved of serum (16 hr) and amino acids (50 min) were re-stimulated with amino acids (10 min) prior to immunofluorescent labeling of Rheb1 (red) and LAMP1 (green).

(B) Representative immunofluorescence images quantified in Figure 5C. Serum starved (16 hr) HeLa cells were stimulated with insulin (1 μM, 15 min) prior to immunofluorescent labeling of Rheb1 (red) and LAMP1 (green).

(C) Rheb localizes to the lysosome in MEFs, and this association is not affected by insulin stimulation. MEFs were treated and immunofluorescently labeled as in (B). Percent colocalization is graphed as a mean±SEM.

(D) Higher-resolution confocal images of acute insulin-stimulated dissociation of TSC2 from Rheb. HeLa cells were treated as in (B) and immunofluorescently labeled for TSC2 (red) and Rheb1 (green). Percent colocalization is graphed as in (C). *$p< 1X10^{-9}$.

Figure S6. Data supporting Figure 6.

(A) Representative immunofluorescence images quantified in Figure 6C. HeLa cells with siRNA-mediated knockdown of Rheb1 and 2 (siRHEB1/2) or control siRNA-treated cells (siC) were serum starved (16 hr) and stimulated with insulin (100 nM, 15 min). Cells were immunofluorescently labeled for mTOR (red) and LAMP2 (green).

(B) Representative immunofluorescence images quantified in Figure 6F. HeLa cells were serum starved (16 hr) with or without FTI-277 (FTI; 10μM) and then stimulated with insulin (1 μM, 15 min). Cells were immunofluorescently labeled for TSC2 (red) and Rheb1 (green).

(C) Representative immunofluorescence images quantified in Figure 6G. HeLa cells were treated as in (B) prior to immunofluorescent labeling of mTOR (red) and LAMP2 (green).

(D) Representative immunofluorescence images quantified in Figure 6H. HeLa cells were treated as in (B) prior to immunofluorescent labeling of mTOR (red) and Rheb1 (green).

(E) TSC2-dependent binding of endogenous TSC complexes to Rheb. Lysates of serum starved HeLa cells stably expressing a control shRNA against luciferase (shLUC) or one targeting TSC2 (shTSC2) were subjected to affinity purifications with GST or GST-Rheb preloaded with GDP or non-hydrolyzable GTPγS. Immunoblots of lysates and GST-pulldowns were probed with the indicated antibodies and GST-fused bait proteins were detected with Ponceau S stain.

Figure S7. Data supporting Figure 7.

(A) Effects of inhibiting PI3K and Akt on insulin-stimulated signaling in MEFs. Serum starved (16 hr) MEFs were pretreated (30 min) with wortmannin (100 nM), MK2206 (2 μM) or vehicle (DMSO) and then stimulated with insulin (1 μM, 15 min). Immunoblots of lysates were probed with the indicated antibodies.

(B) Inhibition of PI3K or Akt blocks insulin-stimulated dissociation of TSC2 from the lysosome in MEFs. MEFs were treated as in (A) prior to immunofluorescent labeling of endogenous TSC2 (red) and LAMP1 (green). Percent colocalization is graphed as a mean ± SEM. *p< $1X10^{-8}$ for comparison with insulin stimulation in vehicle-treated cells.

(C) Inhibition of Akt blocks the insulin-stimulated decrease in affinity of TSC complex components for Rheb. HeLa cells were serum starved for 16 hr, treated with 2 μM MK2206 or vehicle (DMSO) for 30 min, and then stimulated with 1 μM insulin for 15 min. Proteins were pulled down from lysates with GDPβS-loaded GST-Rheb. Immunoblots of pulldowns and lysates were probed with indicated antibodies or Ponceau S stain to detect GST-Rheb.

(D) PTEN null MEFs exhibit constitutive Akt, TSC2 and S6K phosphorylation, which are reversed by the Akt inhibitor MK2206. Serum starved (16 hr) MEFs were treated (30 min) with the Akt inhibitor MK2206 (2 μM) or vehicle (DMSO). Immunoblots of lysates were probed with the indicated antibodies.

(E) Inhibition of Akt rapidly increases TSC2 localization to the lysosome in PTEN null MEFs. PTEN null MEFs were treated as in (D) prior to immunofluorescent labeling of endogenous TSC2 (red) and LAMP1 (green). Percent colocalization was quantified as in (B). *p< $1X10^{-11}$ for comparison with untreated control.

EXTENDED EXPERIMENTAL PROCEDURES

Cell Culture

All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Corning/Cellgro, 10-017-CV), without pyruvate, with 10% fetal bovine serum (FBS; Sigma). *Tsc2*$^{-/-}$ (*p53*$^{-/-}$) MEFs and their littermate-derived wild-type counterparts were kindly provided by D. J. Kwiatkowski (Harvard Medical School)(Zhang et al., 2003).. *Pten*$^{-/-}$ MEFs were kindly provided by P.P. Pandolfi (Harvard Medical School)(Chen et al., 2005). For amino acid starvation and stimulation experiments, subconfluent cells were incubated in DMEM with or without 10% dialyzed serum (dFBS; Life Technologies/Gibco, 26400-036) for 16 hr, then amino acid starved by replacing the media with DMEM lacking all amino acids (Life Technologies/Gibco, ME120086L2; custom ordered) with or without dFBS for 50 min, and finally the media was replaced with standard DMEM (stimulated) or fresh amino-acid free DMEM (unstimulated) with or without dFBS for 10 min. For insulin stimulation, subconfluent cells were serum starved in DMEM for 16 hr and without a media change were stimulated with 1 μM insulin (Sigma, I9278) for 15 min, unless otherwise noted. In Figure 4C, serum starvation was followed by 50 min amino acid starvation prior to insulin stimulation. Reagents used in specific cell culture experiments include Nocodazole (Sigma, M1404), FTI-277 (Sigma, F9803), AICAR (Toronto Research Chemicals Inc., A611700), MK2206 (ChemExpress), and wortmannin (EMD Millipore, 681675).

Immunofluorescence microscopy

HeLa and HEK-293E cells cultured at ~70% confluency were seeded into 6 well culture dishes containing glass coverslips (Zeiss, 474030-9000-000) at 0.25 million cells per well. MEFs and PC3 cells were seeded at 0.15 million cells per well. Following attachment (5 hr for HeLa, MEFs, PC3 and 24 hr for HEK-293E), cells were washed twice with warm serum-free medium and incubated in serum-free medium or medium containing 10% dialyzed FBS for no longer than 16 hr prior to the treatments indicated. Cells were then washed 3 times with PBS and fixed with 4% paraformaldehyde in PBS (ThermoScientific, 28908) for 15 min at room temperature. Cells were then washed 3 times with PBS and permeabilized with PBS containing 0.2% Triton X-100 for 10 min at room temperature. Following 1-hr blocking in Odyssey blocking buffer (Licor, 927-40000) diluted 1:1 with PBS at room temperature, coverslips were incubated for 12 hr at 4ºC in primary antibodies diluted in blocking buffer containing 0.2% Triton X-100. Following three 5 min washes in PBS, coverslips were incubated for 1 hr at room temperature in the dark in secondary antibodies diluted in blocking buffer containing 0.2% Triton X-100. Following three 5 min washes with PBS, coverslips were mounted in Fluoromount G (SouthernBiotech, 0100-01). For fluorescently tagged primary antibodies, the coverslips were incubated for 1 hr at room temperature in the dark with antibodies diluted in blocking buffer containing 0.2% Triton X-100 and following three 5 min washes with PBS were mounted in Flouromount G.

The following primary antibodies were used for immunofluorescence (validation refers to demonstration that immunofluorescent labeling in cultured cells is lost with knockout or RNAi-mediated knockdown of the antigen): mouse anti-LAMP2 (H4B4 from Santa Cruz, sc-18822; 1:100; recognizes only human LAMP2), rat anti-LAMP1 (DSHB, 1D4B; 1:100; recognizes only mouse LAMP1; validated in (Albertti et al., 2010)), rabbit anti-LAMP1 (CST, 9091; 1:200), rabbit anti-TSC2 (CST, 4308; 1:1000; validated in (Dibble et al., 2012)), rabbit anti-mTOR (CST, 2983; 1:200; validated in (Sancak et al., 2008)), mouse anti-Rheb (Abnova, H00006009-M01; 1:1000; validated in this study), mouse anti-TBC1D7 (Sigma, SAB1400543; 1:500; validated in this study), mouse anti-Cytochrome C (Santa Cruz, sc-13561; 1:100), mouse anti-GM130 (BD Biosciences, 610822; 1:300), mouse anti-PMP70 (Sigma, SAB4200181; 1:1000; validated in this study), Alexa Fluor 555 Phalloidin (CST, 8953; 1:20), Alexa Fluor 488 α-Tubulin (CST, 8058; 1:100), anti-rabbit Flag (CST, 2368; 1:800). The following secondary antibodies were used for immunofluorescence: anti-rabbit, anti-mouse and anti-rat Alexa Fluor 488 (Life Technologies/Molecular Probes, A21206, A11001, A11006; 1:1000), anti-mouse Alexa Fluor 594 (Life Technologies/Molecular Probes, A11032; 1:1000), and anti-rabbit Cy3 (Jackson ImmunoResearch, 111-165-144; 1:1000). All antibody stocks that were not in glycerol were divided into single-use aliquots and frozen. Once thawed for use these aliquots were used immediately and were not reused or refrozen.

For most experiments, pseudo-confocal images were acquired with a Zeiss Axiotome fluorescence microscope with Axiovision software and the Apotome feature engaged. Objectives included the Plan-Apochromat 63X/1.4 oil immersion objective for HeLa and 293E cells and Plan-Apochromat 40X/1.4 oil immersion objective for MEFs used with Immersol 518F (Carl Zeiss, 12-624-66A) immersion oil. These images are 1388X1024 pixels with a pixel size of 150 nm. For quantitative analyses, the raw images were deconvoluted using the Axiovision software and the central plane of a z-stack containing 6-8 contiguous focal planes (~0.3 μm each) was used. Threshold intensities were set to exclude the background signal from siRNA knockdown samples. Thresholded Pearson's correlation coefficients, and colocalization percentages (determined by Mander's colocalization coefficient - the number of pixels from the red channel that overlap with pixels from the green channel divided by the total number of pixels detected in the red channel above the threshold value (x 100) were calculated using the Axiovision software. For each condition, images of 5 representative fields were captured and a total of 25 to 50 cells were analyzed.. Identical exposure times and magnifications were used for all comparative analyses. In all figures, representative cells are shown at the same exposure and magnification, and in merged color images, colocalization is indicated by yellow and orange regions.

All confocal images were acquired with a Plan-Apochromat 63X/1.4 oil immersion objective using the LSM 510META confocal laser microscope (Zeiss). The thickness of each optical slice was set to 0.38 μm for both the red (1Airy unit, pinhole size 108 μm) and green channel (pinhole size 98 μm), with pixel depth of 12-bit, scan speed of 6, and line averaging of 2. The images were 2048X2048 pixels with a pixel size of 100 nm. Colocalization analysis was performed using Volocity software (Perkin Elmer), and threshold intensities were

set as described above. A thresholded Pearson's correlation coefficient and colocalization percentage (Mander's colocalization coefficient) were calculated for each individual cell on a maximum intensity projection image of the z-stacks (6-8 contiguous focal planes). Identical settings were used across five different representative fields, and 40 to 50 cells were analyzed per condition.

DNA Constructs and RNAi

All cDNA constructs are human sequences. The TSC2-S939A/S981A/S1130A/S1132A/T1462A (TSC2-5A) mutant was described previously (Zhang et al., 2009). Stable isogenic lines of *Tsc2*$^{-/-}$ (*p53*$^{-/-}$) MEFs were generated by infection with retroviruses encoding empty vector, wild-type TSC2, or TSC2-5A, followed by selection with 100 to 200 µg/ml hygromycin B (Life Technologies/Invitrogen, 10687-010). Lower concentrations of hygromycin B (100 µg/ml) were used to select for cells expressing lower levels of TSC2 in order to visualize changes in localization by immunofluorescence. To generate Lyso-TSC2 and its wildtype control (WT-TSC2), the *TSC2* cDNA with an N-terminal Flag tag was cloned into the SalI and AscI sites of a modified pLKO.1 lentiviral vector (pLJM1) with or without a sequence encoding the lysosomal targeting motif of p18/LAMTOR1, which is 39 amino acids at the N-terminus**:** MGCCYSSENEDSDQDREERKLLLDPSSPPTKALNGAEPN (generous gift from D. M. Sabatini). After sequence verification, the plasmids were introduced into cells via transient transfections or following production of lentivirus. For co-transfection experiments, subconfluent *Tsc2*$^{-/-}$ (*p53*$^{-/-}$) MEFs or HEK293E cells in 10-cm dishes were transfected with pRK7-HA-S6K1 (1 µg), described previously (Romanelli et al., 1999). and either the WT-TSC2 or Lyso-TSC2 constructs (12 µg) with Lipofectamine2000, (Life Technologies/Invitrogen, 11668019) according to the manufacturer's instructions. 48 hr post-transfection, cells were split 1:2 into 10-cm dishes and starved for 16 hr in serum-free media, followed by stimulation with 100 nM insulin for 15 min.

siRNA-mediated knockdowns were done using 20-nM ON-TARGETplus siRNA pools (ThermoScientific/Dharmacon) transfected into cells with 3-µL RNAiMAX Lipofectamine (Life Technologies/Invitrogen, 13778-150) per ml of medium, according to the manufacturers' instructions. 48-hr post-transfection, the cells were split and plated for subsequent experiments. Cells were processed for the given experiments at 72-hr post-transfection. HeLa cells with stable shRNA-mediated knockdowns of TSC2 or TBC1D7 or their control shGFP-expressing counterparts were maintained in DMEM/10% FBS with 2µg/mL puromycin and were described previously (Dibble et al., 2012). Puromycin was excluded during experimental treatments.

Cell lysis, fractionation, immunoprecipitation and antibodies

For cell lysates, cells were rinsed once with cold PBS and lysed in cold NP-40 lysis buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM EDTA, 1% NP-40 (Igepal CA-630), 5% glycerol, 10 mM sodium pyrophosphate, 10 mM glycerol 2-phosphate, 50 mM NaF, 0.5 mM sodium orthovanadate, and 1:100 protease inhibitors (Sigma,

P8340)), except for size-exclusion chromatography and GST affinity purification experiments in which cells were lysed in CHAPS lysis buffer (see below). Lysates were centrifuged at 16,000g for 10 min at 4ºC to remove insoluble material and protein concentrations were normalized using Bradford reagent. Normalized lysates of equal volume were incubated with the immunoprecipitation antibody with rocking at 4ºC for 2-4 hr plus an additional 2 hr following addition of 20 µl of a 1:1 slurry of buffer and pre-washed protein A/G-agarose beads (Thermo Scientific/Pierce, 20421). Beads with immunocomplexes were centrifuged briefly at 3000g, washed three times in lysis buffer, and heated in laemmli SDS-sample buffer at 95ºC for 5 min.

For fractionation of lysates into heavy membrane and light membrane/cytosolic fractions, HeLa cells from two near-confluent 15-cm dishes per treatment were washed with cold PBS, scraped into cold PBS, pelleted by centrifugation at 800 g for 2 min at 4ºC, and re-suspended in 300 µl cold hypotonic lysis buffer (10 mM HEPES, pH 7.2, 10 mM KCl, 1.5 mM $MgCl_2$, 20 mM NaF, 100 µM sodium orthovanadate, 250 mM sucrose with freshly added protease inhibitors). Cells were mechanically lysed by drawing 4 times through a 23G needle and then centrifuged at 500 g for 10 min at 4ºC, yielding a post-nuclear supernatant (PNS). The PNS was centrifuged at 20,000 g for 2 hr to separate the soluble supernatant (light membrane/cytosolic fraction) from the insoluble pellet (heavy membrane fraction). The pellet was resuspended in RIPA buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% Na deoxycholate, 5% glycerol, 10 mM sodium pyrophosphate, 10 mM glycerol 2-phosphate, 50 mM NaF, 0.5 mM sodium orthovanadate, and 1:100 protease inhibitors). Protein concentrations of heavy membrane fractions were normalized separately from light membrane/cytosolic fractions prior to addition of laemmli SDS sample buffer, SDS-PAGE, and immunoblotting.

Antibodies used for immunoprecipitation include TSC2 mouse mAb (Life Technologies (LT), 37-0500), TSC2 rabbit mAb (Cell Signaling Technology (CST), 4308), TSC1 mouse mAb (LT, 37-0400), TSC1 rabbit mAb (CST, 6935), TBC1D7 mouse pAb (Sigma, SAB1400543; note this antibody is used only for immunoprecipitations and not for immunoblotting), phospho-T1462-TSC2 rabbit mAb (CST, 3617), phospho-S939-TSC2 rabbit pAb (CST, 3615), Flag antibody-agarose bead conjugate (Sigma, A2220), and HA antibody-agarose bead conjugate (Sigma, A2095). All antibodies used for immunoblotting are from CST except Actin (Sigma, A5316), LAMP2 (Santa Cruz, sc-18822), rat anti-LAMP1 (Developmental Studies Hybridoma Bank, 1D4B), LDH (Abcam, ab2101) and Rheb (Abnova, H00006009-M01). The TBC1D7 antibody developed by CST (Dibble et al., 2012) was used for immunoblotting. The residues recognized by the phospho-specific antibodies used for immunoblots are *p*-Akt (S473), *p*-TSC2 (T1462), *p*-S6K1 (T389), *p*-4E-BP1 (S65), *p*-S6 (S240/S244), and *p*-PRAS40 (T246).

For immunopurification of the lysosome from cross-linked lysates with an anti-TSC2 antibody (Figure 7F), MEFs from two near-confluent 15-cm dishes per sample were washed once with cold PBS, gently scraped into 10 ml cold fractionation buffer (140 mM KCl, 250 mM sucrose, 2 mM EGTA, 10 mM $MgCl_2$, 25 mM HEPES, pH

7.4, 5 mM glucose), pelleted by centrifugation at 400 g for 3 min at 4ºC, and re-suspended in 600 µl of hypotonic lysis buffer (140 mM KCl, 250 mM sucrose, 2 mM EGTA, 10 mM $MgCl_2$, 25 mM HEPES, pH 7.4, 5 mM glucose, 1 mM orthovanadate, 1 µM microcystin , 2x protease inhibitors, and 1 mM GDPβS) containing 2.5 mg/ml of the cross-linking agent DSP (Thermo Scientific/Pierce, 22585). Cells were mechanically lysed by drawing through a 23G needle 8 times, incubated at 4ºC for 30 min, and quenched for DSP using 0.2 M Tris-HCl (pH 7.4). Lysates were centrifuged at 700 g for 10 min at 4ºC, yielding a post-nuclear supernatant (PNS). Normalized PNS samples of equal volume were pre-cleared for 1 hr with protein A/G-agarose beads (Thermo Scientific/Pierce, 20421) and incubated with TSC2 antibody (CST, 4308) with rocking for 12 hr at 4ºC and then for an additional 3 hr following addition of 20 µl of a 1:1 slurry of buffer and pre-washed protein A/G-agarose beads. Bead-immunocomplexes were washed five times in fractionation buffer before heating in 2x NuPAGE LDS Sample Buffer containing 5% β-mercaptoethanol at 70ºC for 10 min.

GAP Assays

In vitro Rheb-GAP assays were performed as described previously (Dibble et al., 2012; Tee et al., 2003). Briefly, HeLa cells were lysed in 1% NP40 lysis buffer and endogenous TSC complexes were immunoprecipitated with a TSC1 antibody (CST, 6935), compared to an IgG control (CST, 3900), from combined lysates of two 15 cm dishes per reaction. Immunopurified complexes on protein-A/G agarose beads were incubated with ~250 ng of soluble recombinant GST or GST-Rheb preloaded with GTP[α-^{32}P] at 30ºC for 1 hr. Rheb-bound GTP and GDP were separated by thin-layer chromatography (TLC) and quantified using a phosphorimager in three independent experiments.

Size-exclusion chromatography

Cells from a near-confluent 10-cm dish were washed with cold PBS and lysed on ice in 500 µL CHAPS lysis buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM EDTA, 0.3% w/v CHAPS, 10 mM pyrophosphate, 10 mM glycerol-2-phosphate, 50 mM NaF) and freshly added protease inhibitors at 1:100 (Sigma, P8340). Lysates were rocked at 4ºC for 15 min and centrifuged at 16,000 g for 15 min to remove insoluble material. Supernatants were normalized for protein concentration between samples, and then passed through a 0.22 µm filter. Samples were processed and analyzed using a Fast Protein Liquid Chromatography (FPLC) system consisting of an AKTA PURIFIER 10 core system and a Frac-900 fraction collector controlled with UNICORN software connected to a 24-ml Superose 6 10/300 GL column (GE Healthcare, 45-002-901), which has a separation range from 5 kDa to 5 MDa. Before each run the system was equilibrated with two to three column volumes of lysis buffer. For each sample, 250 µl (~1% of column volume) was loaded into a 1 ml sample loop by the partial filling method described in the FPLC product manual, and injected into the column with 1.2 ml of lysis buffer. Samples were eluted from the column and collected as 0.5 ml fractions using CHAPS lysis buffer with protease inhibitors at a constant flow rate of 0.25 ml/min. For SDS-PAGE, following addition of laemmli SDS sample buffer to the fractions and heating for 5 min at 95ºC, all fractions from the same treatment were

loaded on a single 26-lane 4-12% Bis-Tris gel (Life Technologies, WG1403BX10). To generate a standard curve, elution volumes were determined for purified proteins and protein complexes of known molecular weights which were run in CHAPS lysis buffer unless otherwise noted. All standard samples contained at least 100 mM NaCl to minimize weak interactions with the column media and were passed through a 0.22μM filter immediately prior to use. Peak position of these standard proteins was determined using UNICORN software based on absorbance at 280 nm. In some cases the peak elution volume was confirmed by determining the protein concentrations of collected fractions with Bradford reagent. Elution volumes were transformed into Kav values (elution volume-void volume/column volume-void volume) and graphed against the base-10 logarithms of the predicted molecular weights (kDa). A second-order polynomial curve was fitted to the data with the equation from this curve used to estimate the corresponding molecular weights of fractions in Figure 1E. Standards as numbered in Figure S1E included: Void volume, Dextran Blue (elutes in the void volume due to its very large hydrodynamic radius; Sigma, MWGF1000); 1. 26S Proteasome, double capped (2.5 MDa; Enzo Life Sciences BML-PW9310-0050) run in 10 mM HEPES pH7.4, 100 mM NaCl, 25 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 10% glycerol; 2. 70S E. coli Ribosome (2.3 MDa; New England Biolabs, P0763S) run in 20 mM HEPES, pH 7.4, 10 mM $Mg(OAc)_2$, 100 mM NaCl, 0.3% CHAPS; 3. IgM (970 kDa; Jackson Immunoresearch, 009-00-012); 4. alpha2-macroglobulin (725 kDa; Sigma, M6159); 5. thyroglobulin (669 kDa; Sigma, MWGF1000); 6. urease (545 kDa; Sigma, U7752), 7. beta-galactosidase (465 kDa; Sigma, 48275); 8. apoferritin (443 kDa; Sigma, MWGF1000); 9. catalase (245 kDa; Sigma, C40); 10. β-amylase (200 kDa; Sigma, MWGF1000); 11. alcohol dehydrogenase (150 kDa; Sigma, MWGF1000); 12. BSA (66 kDa; Sigma, MWGF1000); 13. ovalbumin (44 kDa; Sigma, A2512); 14. carbonic anhydrase (29 kDa; Sigma, MWGF1000).

GST-Rheb affinity purifications

Cells from a near-confluent 15-cm dish per treatment were lysed in CHAPS lysis buffer with protease inhibitors but without NaF (lysis buffer with NaF yielded similar results) at 4ºC, centrifuged at 16,000 g for 15 min to remove insoluble material, and normalized for protein concentration between samples. GST, GST-Rab5A, and GST-Rheb expressed in E. coli were purified as described for the GAP assay. GST-proteins were captured from bacterial lysates with glutathione-agarose beads, washed 3X in Rheb wash buffer (50 mM HEPES, pH7.4, 500 mM NaCl, 2 mM EDTA, 0.2% Triton X-100 with 1 mM DTT and 1:100 protease inhibitors added fresh), washed 2X in loading buffer (50 mM HEPES, pH 7.4, 1 mM EDTA, 5 mg/mL BSA, and 1:200 protease inhibitors), and loaded with no nucleotide or 500 μM GTPγS, GDPβS, or GDP in loading buffer for 15 min at 30C with gentle agitation. Following loading, samples were placed on ice and nucleotide binding was locked in by adding 20 mM $MgCl_2$ for at least 5 min before use. Loaded GST-Rheb or GST controls on glutathione-beads (20 μL 1:1 bead/buffer slurry) were added to 1 mL of lysate to which either 5 mM EDTA had been added for the nucleotide-free treatment or 10 mM $MgCl_2$ and 500 μM of the appropriate nucleotide. Samples were rocked for 6 hr at 4ºC. Beads were washed briefly 4 times with CHAPS lysis buffer plus 10 mM $MgCl_2$ and then heated in ~40 μL 1X SDS sample buffer at 95ºC for 5 min.

SUPPLEMENTAL REFERENCES

Albertti, L.A., Macedo, A.M., Chiari, E., Andrews, N.W., and Andrade, L.O. (2010). Role of host lysosomal associated membrane protein (LAMP) in Trypanosoma cruzi invasion and intracellular development. Microbes Infect *12*, 784-789.

Chen, Z., Trotman, L.C., Shaffer, D., Lin, H.K., Dotan, Z.A., Niki, M., Koutcher, J.A., Scher, H.I., Ludwig, T., Gerald, W.*, et al.* (2005). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature *436*, 725-730.

Dibble, C.C., Elis, W., Menon, S., Qin, W., Klekota, J., Asara, J.M., Finan, P.M., Kwiatkowski, D.J., Murphy, L.O., and Manning, B.D. (2012). TBC1D7 is a third subunit of the TSC1-TSC2 complex upstream of mTORC1. Mol Cell *47*, 535-546.

Romanelli, A., Martin, K.A., Toker, A., and Blenis, J. (1999). p70 S6 kinase is regulated by protein kinase Czeta and participates in a phosphoinositide 3-kinase-regulated signalling complex. Mol Cell Biol *19*, 2921-2928.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science (New York, NY *320*, 1496-1501.

Tee, A.R., Manning, B.D., Roux, P.P., Cantley, L.C., and Blenis, J. (2003). Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol *13*, 1259-1268.

Zhang, H., Cicchetti, G., Onda, H., Koon, H.B., Asrican, K., Bajraszewski, N., Vazquez, F., Carpenter, C.L., and Kwiatkowski, D.J. (2003). Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-Akt signaling through downregulation of PDGFR. J Clin Invest *112*, 1223-1233.

Zhang, H.H., Huang, J., Duvel, K., Boback, B., Wu, S., Squillace, R.M., Wu, C.L., and Manning, B.D. (2009). Insulin stimulates adipogenesis through the Akt-TSC2-mTORC1 pathway. PLoS One *4*, e6189.