Materials and Methods

Materials

Reagents were obtained from the following sources: antibodies to phospho-S2481 mTOR, phospho-T389 S6K1, phospho-S235/S236 S6, phospho-T37/T46 4E-BP1, phospho-S65 4E-BP1, phosho-T308 Akt, phospho-S473 Akt, phospho-T246 PRAS40, phospho-T346 NDRG1, phospho-S302 IRS1, phospho-S636/S639 IRS1, phospho-T202/T204 Erk1/2, phospho-tyrosine, phospho-Y1135/1136 IGF1-Rb/phospho-Y1150/Y1151 InRb, phospho-Y1131 IGF-1Rb/phospho-Y1146 InRb, phospho-Y980 IGF-1Rb, phospho-S150 Grb10, phospho-S428 Grb10, phospho-S476 Grb10, Akt, S6K1, 4E-BP1, TSC2, FLAG, rictor, IRS1, IRS2, Nedd4, Erk1/2, IGF-1R, InR, p85 PI3K, and human Grb10 from Cell Signaling Technology; an antibody to mouse Grb10 and HRP-labeled anti-mouse and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; an antibody to IRS1 from Upstate/Millipore; FLAG M2 affinity gel, ATP, staurosporine, cycloheximide, FKBP12, L-glutathione, amino acids, insulin, IGF-1, EGF, and PDGFbb from Sigma-Aldrich; CIP from New England Biolabs, MG-132 from Calbiochem; [γ-^{32}P]ATP and [^{35}S]cysteine and methionine from Perkin-Elmer; FuGENE 6, PhosSTOP, and Complete Protease Cocktail from Roche; rapamycin from LC Laboratories, PF-4708671 from Tocris Bioscienes, DMEM from SAFC Biosciences; Inactivated Fetal Calf Serum (IFS), MagicMedia E. coli expression medium and SimplyBlue Coomassie G from Invitrogen, amino acid-free RPMI from US Biological, Superose 6 10/300 GL and HiLoad 16/60 Superdex 200 from GE Healthcare; BCA assay reagent, protein G-sepharose and immobilized glutathione beads from Pierce; Ni-NTA agarose from Qiagen; Whatman grade P81 ion exchange chromtagraphy paper from Fisher Scientific, QuikChange XLII mutagenesis kit and BL21(DE3) Competent Cells from Stratagene. Torin1 was provided by Nathanael Gray (Harvard Medical School) (*1*).

Cell lines and tissue culture

All cells (HEK-293E, HEK-293T, HeLa, MEFs) were cultured in DMEM with 10% IFS and antibiotics. HEK-293Es were generously provided by John Blenis (Harvard Medical School), TSC2$^{+/+}$ p53$^{-/-}$ and TSC2$^{-/-}$ p53$^{-/-}$ MEFs by David Kwiatkowski (Harvard Medical School), S6K1$^{+/+}$ S6K2$^{+/+}$ and S6K1$^{-/-}$ S6K2$^{-/-}$ MEFs by Mario Pende (INSERM U845, Medical School, Paris Descartes University). rictor$^{+/+}$ p53$^{-/-}$ and rictor$^{-/-}$ p53$^{-/-}$ MEFs have been described previously (*2*). Stable expression cell lines were created using constructs described below according to established procedures (*3*).

Quantitative mass spectrometry

Cell lysis and protein digestion: Actively proliferating HEK-293E cells were serum starved for 4 hrs, treated with 100 nM rapamycin, 250 nM Torin1, or DMSO for 1 hr, stimulated with 150 nM insulin for 20 minutes, and then lysed. Actively proliferating MEFs in fresh media were treated with 100 nM Torin1 or DMSO for 1 hr and then lysed. Cells were lysed with 8 M urea, 20 mM HEPES pH 8.0, 1 mM sodium orthovanadate, 2.5 mM pyrophosphate, and 1 mM glycerophosphate. Protein concentration was determined using the BCA assay. Proteins were reduced with 10 mM dithiothreitol for 30 min at 56ºC, then alkylated with 55 mM iodoacetamide for 1 hr at room temperature in the dark. Cell lysates were diluted to a final urea concentration of 1.6 M with 50 mM ammonium bicarbonate, and digested with trypsin (substrate:enzyme = 50) at 37ºC overnight with end-over-end rotation. The resulting peptide solutions were acidified with 10% TFA, and desalted on a Waters C18 solid phase extraction plate. Eluted peptides were divided into ~100ug aliquots, lyophilized to complete dryness, and stored at -80ºC until needed.

iTRAQ labeling: For both samples, duplicates were performed such that peptides were independently labeled and analyzed by LC-MS/MS twice. Desalted peptides were labeled with iTRAQ (*4*) reagents according to the manufacturer's instructions (400µg or 800ug in total for 4plex or 8plex, respectively). Briefly, 100 µg aliquots of dried peptides were reconstituted with 30 µL 0.5 M triethylammonium bicarbonate. One tube of iTRAQ reagent (114, 115, 116, or 117 for 4plex; 113, 114, 115, 116, 117, 118, 119, or 121 for 8plex) was reconstituted with 70 µL ethanol (4plex) or 50 µl of isopropanol (8plex), and added to each peptide solution. The reaction was allowed to proceed for 1 hr (4plex) or 2 hr (8plex) at room temperature. Derivatized peptides were combined, dried by vacuum centrifugation, and desalted on a Waters C18 solid phase extraction plate. iTRAQ labeled peptides were lyophilized to complete dryness and stored at -80ºC until needed. The MEF samples were labeled with iTRAQ 4plex reagent ($TSC2^{+/+}$ Vehicle: 114; $TSC2^{+/+}$ Torin1: 115; $TSC2^{-/-}$ Vehicle: 116; $TSC2^{-/-}$ Torin1: 117). The HEK-293E samples were labeled with iTRAQ 8plex (Starved: 113; Insulin: 114; Torin: 117 or 115 for the first and second replicates, respectively; Rapamycin: 118) with the remaining channels used for other analyses not discussed here.

Phosphopeptide enrichment: Magnetic Ni-NTA agarose beads (100 µL of a 5% bead suspension/400 µg tryptic peptides) were treated with 400 µL of 100 mM EDTA, pH 8.0 to remove Ni(II). NTA-agarose beads were then charged with 200 µL of 100 mM aqueous FeCl3 solution (*5*). Beads were washed 4x with 400 µL 80% acetonitrile/0.1% TFA to remove excess

metal ions. iTRAQ labeled peptides were reconstituted with 80% MeCN/0.1% TFA at a concentration of 1-2 µg/µL, were then mixed with the beads. The mixture was incubated for 30 min at room temperature with end-over-end rotation. After removing the supernatant, beads were washed 3x with 400 µL 80% acetonitrile/0.1% TFA, and 1x with 400 µl of 0.01% acetic acid. Phosphopeptides were eluted with 50 µL of 20 mM ammonium formate buffer pH 10.

LC-MS/MS analysis: Enriched phosphopeptides were separated into 40 fractions by high-pH reversed phase and strong anion exchange chromatography, respectively, followed by low-pH reversed phase LC-MS/MS on a QSTAR Elite (AB Sciex, Foster City, CA) hybrid quadrupole time-of-flight mass spectrometer. The spectrometer was operated in data-dependent mode with dynamic exclusion. A precursor was selected for MS/MS when its signal intensity was at least 50 counts, and its charge state is 2+, 3+ or 4+. Up to 5 most abundant precursors in each MS scan could be selected for MS/MS, and then excluded for 20 sec. The MS/MS scan was acquired for 0.5 sec with a multiplier value of 4.

Data processing: MS/MS spectra from each acquisition were extracted and converted into .mgf files, and then searched against NCBI mouse and human RefSeq databases (downloaded June 2008) using Mascot. Precursor and product ion tolerances were set at 200 ppm and 0.2 Da respectively. Search parameters included tyrpsin specificity with up to 2 missed cleavages, fixed carbamidomethylation on cysteine, fixed iTRAQ modification on N-terminus and lysine (8plex for HEK-293E, 4plex for MEF), variable deamidation on asparagine and glutamine, variable oxidation on methionine and variable phosphorylation on serine, threonine and tyrosine. The search results were further collated using our Multiplierz software framework (*6, 7*) for peptides above a mascot score cutoff of 25, corresponding to a FDR of ~1%. iTRAQ reporter ion intensities were extracted and corrected for isotope impurities. Results from multiple fractions were combined for each sample. iTRAQ reporter ion signals were summed for each unique phosphopeptide. A small aliquot from the supernatant of phosphopeptide enrichment was also analyzed for each sample. The intensity values of iTRAQ reporter ion were summed for each channel across all identified peptides and used to correct for minor variation in source protein amount in each labeled sample.

Hit identification: The following $\log_2$(ratios) were calculated based on the corrected ion intensities: (Starved/Insulin), (Rapamycin/Insulin), (Torin1/Insulin) for HEK-293E samples and ($TSC2^{+/+}$ Torin1/ $TSC2^{+/+}$ vehicle), ($TSC2^{-/-}$ vehicle/ $TSC2^{+/+}$ vehicle), and ($TSC2^{-/-}$ Torin1/ $TSC2^{-}$

$^{/-}$ vehicle) for the MEF samples. The binary logarithms were then median centered and median absolute deviation (MAD) scaled separately for each technical replicate. The mTOR-regulated phosphopeptides ("hits") were those with a robust z-score (MADs away from the median) of at least -2.5 in $\log_2$(Torin1/Insulin) for HEK-293E or $\log_2$(TSC2$^{-/-}$ Torin1/ TSC2$^{-/-}$ vehicle) for MEF. Those phosphopeptides detected in both replicates had to score below the threshold both times in order to be counted among the regulated phosphopeptides. The phosphopeptides which qualified as "rapamycin-sensitive" or "TSC2-upregulated" were those whose $\log_2$(Rapamycin/Insulin) or (TSC2$^{-/-}$ vehicle/ TSC2$^{+/+}$ vehicle) met the -2.5 MAD or +2.5 MAD cutoffs, respectively. Enrichment of control peptides was determined using Fisher's Exact Test and a list of well-accepted mTOR pathway phosphorylation sites which were detected in the datasets (Table S1). Correlation of features was measured using Spearman's rank correlation coefficient and significance was tested using a ρ test. p-values ~ 0 are lower than the smallest number able to be represented computationally.

Heat map visualization: The HEK-293E mTOR-regulated phosphopeptides were sorted based on their $\log_2$(Rapamycin/Insulin) robust z-score and negative outliers were set to -10 in the heatmap for improved visualization. Conditions were clustered using complete-linkage hierarchical clustering with the Euclidian distance metric. Statistical analyses and data processing were performed in R 2.11.1 and Bioperl 1.6 (*8*).

Pathway analysis: Enrichment was determined at the protein level by collapsing the mTOR-regulated phosphopeptides at the -2 MAD cutoff from both datasets into gene symbols. Analysis was performed using DAVID (*9, 10*), and SP_PIR_KEYWORDS, the GO FAT categories, Interpro domains, and Biocarta and KEGG pathways. Terms were defined as enriched if they contained at least 2 hits from the screen and had p-values < 0.01 and a FDR < 10%.

Positional scanning peptide library screening

PSPL screening: mTORC1 purification from HEK-293T cells stably expressing FLAG-raptor and Rheb purification from transiently transfected HEK-293T cells were performed as described previously (*11, 12*). PSPL screening was done according to the published protocol (*13*) with the final reaction conditions as follows: 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 4 mM $MnCl_2$, 1 mM DTT, 50 ng Rheb, 150 ng mTORC1, 50 μM biotinylated peptides, 50 μM ATP, 2 μCi [γ-^{32}P]ATP. Each reaction consists of purified mTORC1, Rheb, radiolabeled ATP, and a mixture of peptides

containing one fixed residue relative to the central phospho-acceptor and other residues randomized. After incubating for 6 hrs at 30ºC, aliquots of the reactions were spotted onto streptavidin membrane and analyzed by PhosphorImaging.

Data analysis: A position specific scoring matrix (PSSM) was generated based on the normalized average intensities of the spots in the PSPL plot. A PSSM entry S_{ij} for residue *i* in position *j* was calculated using the formula

$$S_{ij} = \left(I_{ij} - \bar{I}_j\right)\frac{s_{Ij}}{\bar{I}_j}$$

where I_{ij} is the normalized average intensity of the spot for residue *i* in position *j*, $\bar{I}_j$ is the mean of the average normalized intensities in position *j*, and s_{Ij} is the standard deviation of the average normalized intensities in position *j*. The exponential of the PSSM ($e^{S_{ij}}$) was used as in input for the Scansite algorithm to predict likely mTOR phospho-acceptor sites (*14*). Confidence thresholds for predictions were set based on the 0.2nd, 1.5th, and 5th percentile of the empiric score distributions of all serine and threonine sites in NCBI human and mouse RefSeq databases (downloaded June 2008). This setting corresponds to high, medium, and low stringency, respectively, in the standard Scansite configuration. For the classification of the mTOR-regulated phosphopeptides, those phosphopeptides containing sites whose Scansite scores were below the 5th percentile were considered as possessing a putative mTOR motif. AGC kinase motifs were determined by querying the sites for an (R/K)X(R/K)XX(S*/T*) sequence.

cDNA manipulations and mutagenesis

The cDNA for human GRB10 (NCBI NM_001001550.2 isoform c) in the pOTB7 vector was obtained from OpenBiosystems. The GRB10 cDNA was amplified by PCR, and the product was subcloned into the XhoI and NotI sites of the pMSCV retroviral vector for stable expression or the Xba1 and Xho1 sites of pET303/CT-His vectors for bacterial expression. The HA-GST-S6K1-pRK5 and GST-4E-BP1-pGEX-4T constructs were described previously (*12, 15*).

The Grb10-pMSCV and HA-GST-S6K1-pRK5 were mutagenized with the QuikChange XLII mutagenesis kit with oligonucleotides obtained from Integrated DNA Technologies. The Grb10 mutants used in our experiments are (amino acid numbering according to NCBI NM_005311.4 isoform a although all clones used in this study are the human isoform c): 5A = S150A T155A S158A S474A S476A Grb10, 9A = S104A S150A T155A S158A S426A S428A

S431A S474A S476A, 2D = S474D S476, and 5D = S150D T155D S158D S474D S476D. T229 of S6K1 in the HA-GST-pRK5 vector was mutated to an alanine to attenuate its catalytic activity.

Cell treatments, lysis, immunoprecipitations, and phosphatase treatment

For growth factor stimulation, almost confluent cells were rinsed once and incubated in serum-free DMEM for times as indicated in figure legends, and then stimulated for 15 or 20 minutes. Inhibitors and doses of growth factors were added as indicated. Insulin and IGF-1 were most commonly used at 100 or 150 nM and 100 ng/ml, respectively. Amino acid starvation was done as described previously (*16*).

Cells rinsed once with ice-cold PBS and lysed in ice-cold lysis buffer (50 mM HEPES [pH 7.4], 40 mM NaCl, 2 mM EDTA, 1 mM orthovanadate, 50 mM NaF, 10 mM pyrophosphate, 10 mM glycerophosphate, and 1% Triton X-100 or 0.3% CHAPS (for immunoprecipitations), and one tablet of EDTA-free protease inhibitors per 25ml. The soluble fractions of cell lysates were isolated by centrifugation at 13,000 rpm for 10 minutes by centrifugation in a microfuge. For immunoprecipitations, one PhosSTOP tablet was added per 25 ml of CHAPS lysis buffer, and primary antibodies were added and the lysates incubated with rotation overnight at 4°C. 50% slurry of protein G-sepharose was then added and the incubation continued for an additional 1 hour. Immunoprecipitates were washed three times with lysis buffer containing 150 mM NaCl. Immunoprecipitated proteins were denatured by the addition of sample buffer, boiled for 5 minutes, resolved by SDS-PAGE, and analyzed by immunoblotting as previously described (*17*).

For FLAG purification, FLAG M2 affinity resins were washed 2 times in lysis buffer, added to pre-cleared lysates, and incubated with rotation for 2 hours at 4°C. FLAG-Grb10 purified from a 10 cm plate of insulin stimulated $TSC2^{+/+}$ MEFs stably expressing FLAG-Grb10 was phosphatase treated while still bound to FLAG resin. The resin-bound FLAG-Grb10 washed once in 1X NEBuffer 3, divided among the reaction tubes, and incubated for 60 minutes at 37°C in 20 µl of buffer alone, buffer with 20 units of CIP, or 20 units of CIP previously inactivated by boiling for 10 minutes. FLAG-Grb10 from serum starved or Torin1-treated cells was incubated for 60 minutes at 37°C in buffer alone. The reactions were stopped with the addition of sample buffer, boiled, and analyzed by immunoblotting.

mTORC1 kinase assays

HA-GST-S6K1 (T229A) was purified from transiently transfected HEK-293T cells treated with 250 nM Torin1 for one hour and lysed in Triton lysis buffer. The cleared lysates were incubated with glutathione resin for 2 hours at 4°C, eluted as described previously (*12*), concentrated, quantified, and stored in 50% glycerol at -20°C. BL21(DE3) cells carrying GST-4E-BP1-pGEX-4T were grown in MagicMedia for 24 hours, and lysed by sonication in ice-cold Triton lysis. GST-4E-BP1 purification proceeded as detailed for HA-GST-S6K1, but was further purified by gel filtration using a HiLoad 16/60 Superdex 200 column and stored at -80°C. BL21 (DE3) cells carrying pET303-Grb10 were grown in MagicMedia for 24 hours, and lysed by sonication in ice-cold His-tag lysis buffer (25 mM Hepes [pH 7.4], 500 mM NaCl, 5 mM Imidazole, 1% Triton + protease inhibitor tablets). The cleared lysate was incubated with Ni-NTA agarose, incubated for 20 minutes at 4°C, washed, eluted in 150 mM imidazole-containing buffer, and further purified by gel filtration using a HiLoad 16/60 Superdex 200 column and stored at -80°C.

mTORC1 was purified from HEK-293T cells stably-expressing FLAG-raptor as described (*11*). Kinase assays were preincubated for 10 minutes at 4°C before addition of ATP, and then for 30 minutes at 30°C in a final volume of 20 µl consisting of: kinase buffer (25 mM HEPES, pH 7.4, 50 mM KCl, 10 mM MgCl2, 1 µM staurosporine), active mTORC1, 500 nM substrate, 50 µM ATP, 2 µCi [γ-^{32}P]ATP, and when indicated 250 nM Torin1 or 250 nM rapamycin/FKBP12. Samples were stopped by the addition of 10 µl of sample buffer, boiled for 5 minutes, and analyzed by SDS-PAGE followed by autoradiography.

Grb10 phosphorylation site mapping

mTORC1 kinase assays were performed as detailed above, except with a one hour reaction time and in the presence of 500 µM cold ATP. For the *in vitro* kinase assay samples, urea was added to a final concentration of 1.6 M. Proteins were reduced with 10 mM DTT at 56ºC for 30 min, alkylated with 55 mM iodoacetamide for 1 hr at room temperature in the dark, then digested with trypsin at 37ºC overnight. The solution was then acidified with 10% TFA. Peptides were extracted using c18 ZipTip, were lyophilized to dryness, and were stored at -80ºC until needed. A third of each sample was analyzed by LC/MS/MS on an orbitrap velos mass spectrometer in data-dependent mode. Identified phosphopeptides were manually validated and combined into a single list. A targeted MS method was then created to perform MS/MS on those selected phosphopeptides, as well as the top 5 most abundant precursors in each cycle. The *in vitro* kinase samples were then re-analyzed with the targeted MS method.

Peak intensity values of extract ion chromatogram were obtained for both the phosphorylated and unphosphorylated forms. Their ratios were used to compare the phosphorylation level in different samples. FLAG-Grb10 immunoprecipitates from HEK-293E cells stably expressing Grb10 were separated on SDS-PAGE and stained with Coomassie. The bands corresponding to Grb10 were excised and digested in situ. Peptides were extracted and analyzed using the targeted MS method. Phosphorylation levels on identified phosphopeptides were estimated as described above. p-values were determined by a one-tailed Mann-Whitney t-test.

Antibody detection of Grb10

The human Grb10 antibody (CST) detects one isoform of Grb10 while the mouse Grb10 antibody detects multiple isoforms (Santa Cruz). Phosphospecific antibodies against S150, S428, and S476 of Grb10 were provided as bleeds from Cell Signaling Technology. All could be used to detect the phosphorylation of immunoprecipitated human Grb10, and the antibodies against S150 and S476 could also be used to probe mouse lysates.

Lentiviral shRNAs

TRC lentiviral shRNAs (*18*) targeting Grb10 and Nedd4 were obtained from the RNAi consortium (Broad Institute of MIT and Harvard) (*18*). The TRC identifications for each shRNA are as follows:

Human GRB10 shRNA #1: TRCN0000063686; NM_001001549.1-1459s1c1
Mouse Grb10 shRNA #1: TRCN0000109915; NM_010345.2-2392s1c1
Mouse Grb10 shRNA #2: TRCN0000109917; NM_010345.2-1841s1c1
Mouse Nedd4 shRNA #1: TRCN000009235; XM_486230.1-1319s1c1
Mouse Nedd4 shRNA #2: TRCN000009236; XM_486230.1-2082s1c1

The shGFP control and the shRNA targeting mouse raptor were previously described and validated (*1, 19*). The mouse Grb10 shRNA #1 was also additionally cloned into a pLKO.1 derivative, pLKO_TRC016, obtained from the RNAi consortium (Broad Institute of MIT and Harvard) with a blasticidin resistance gene. Virus production was performed as previously described (*19*). Virus-containing supernatants were collected 48 hours after transfection, filtered to eliminated cells, and target cells were infected in the presence of 8 μg/ml polybrene. 24 hours later, cells were selected with puromycin or blasticidin and analyzed starting at the 3rd day after infection. Grb10 knockdown cells were passaged with persistent antibiotic selection.

For replacement of endogenous murine Grb10 with human Grb10 isoform c, $TSC2^{-/-}$ $p53^{-/-}$ MEFs were infected with retroviruses (pMSCV) expressing empty vector, FLAG-Grb10, FLAG-Grb10 5A, or FLAG-Grb10 9A mutants, and selected for 4 days in puromycin. The resulting stable cell lines were subsequently infected with lentiviruses expressing either the control hairpin or mouse Grb10 shRNA #1 in pLKO_TRC016 that recognizes the 3' untranslated region of the mouse Grb10 mRNA, but not the human cDNA. Cells were then kept in puromycin and additionally selected in blasticidin, and analyzed starting at the 7^{th} day after lentiviral infection. Grb10 replacement cells were passaged with persistent dual antibiotic selection.

Pulse chase

Cells were labeled with 1 mCi [^{35}S]methionine/cysteine (1175 Ci/mmol) in 15 ml methionine- and cysteine-free DMEM at 37°C for 2 hours and chased with DMEM and 10% IFS supplemented with nonradiolabeled methionine (2.5 mM) and cysteine (0.5 mM) at 37 °C for the indicated times. mTOR inhibitors were added to the chase as indicated. Cells were lysed in 1% SDS in PBS. Immunoprecipitations were performed in Nonidet P-40 lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and protease inhibitors) with 0.1% SDS and 30 µl of anti-FLAG M2-agarose for 3 hr at 4°C. Immunoprecipitates were boiled in sample buffer, subjected to 10% SDS-PAGE, and visualized by autoradiography or quantified by PhosphorImaging. p-values were determined by a two-way ANOVA.

Grb10 mRNA expression analysis

For quantification of Grb10 mRNA expression, total RNA was isolated from cells grown in the indicated conditions and reverse-transcription was performed. The resulting cDNA was diluted in DNase-free water (1:100) before quantification by real-time PCR. Data are expressed as the ratio between the expression of Grb10 and the housekeeping gene Rplp0. p-values were determined by a two-tailed Mann-Whitney t-test.

The following primers were used for quantitative real-time PCR:
Grb10 (M. musculus):
Forward: ACAGGATCATCAAGCAACAA
Reverse: TCTTTGTGAAGTCCAATAAC
Rplp0 (M. musculus):
Forward: TAAAGACTGGAGACAAGGTG
Reverse: GTGTACTCAGTCTCCACAGA

FIGURE S1

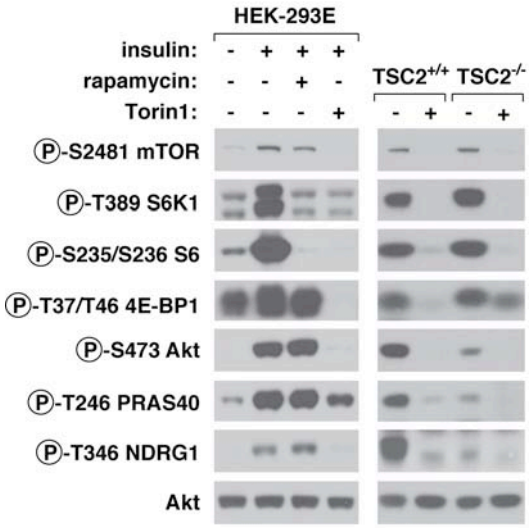


Fig. S1. Known mTOR pathway phosphorylations in HEK-293E cells serum starved for 4 hrs, treated with 100 nM rapamycin, 250 nM Torin1 or vehicle control for 1 hr, and then stimulated where indicated with 150 nM insulin for 20 min or $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs treated with 100 nM Torin1 or vehicle control for 1 hr behaved as expected. Cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the specified proteins.

FIGURE S2

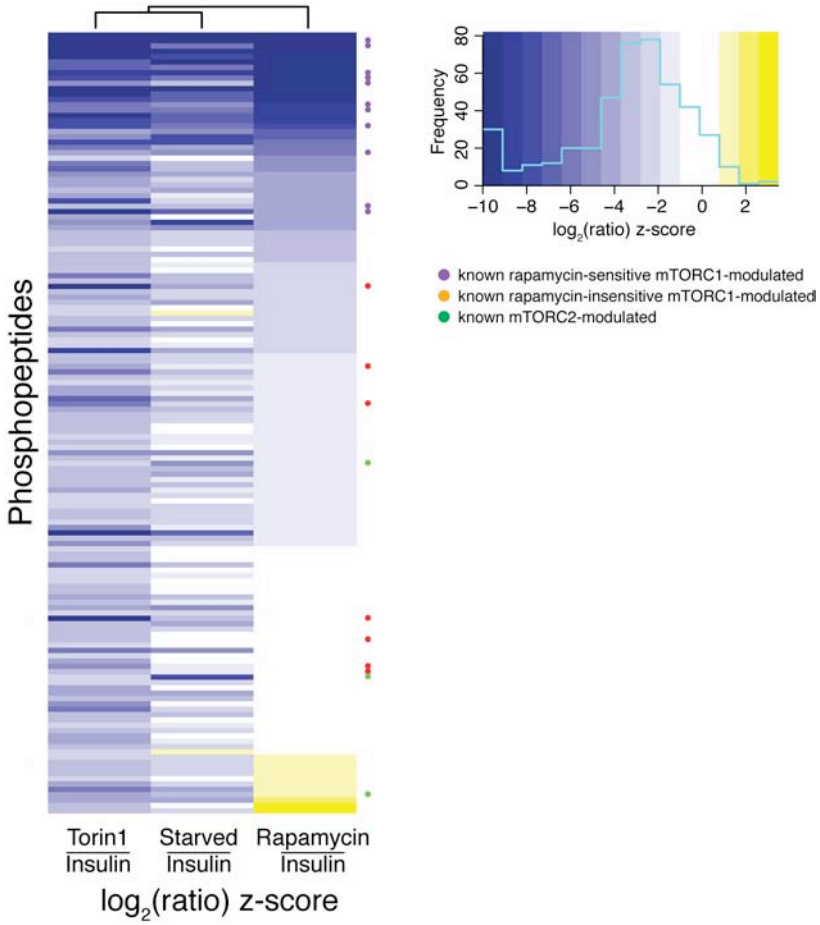


Fig. S2. Heat map visualization of the robust z-scores for the HEK-293E mTOR-regulated phosphopeptides reveals the similarity between serum starvation and Torin1 treatment, the ability to differentiate rapamycin-sensitive from -insensitive sites, and the greater extent of inhibition of rapamycin-sensitive phosphorylation sites with Torin1. Conditions were hierarchically clustered while phosphopeptides were sorted based on rapamycin-sensitivity. Yellow indicates positive z-scores, and blue indicates negative z-scores. Those z-scores less than -10 were binned together to aid in visualization. Known mTOR-modulated sites are indicated.

FIGURE S3

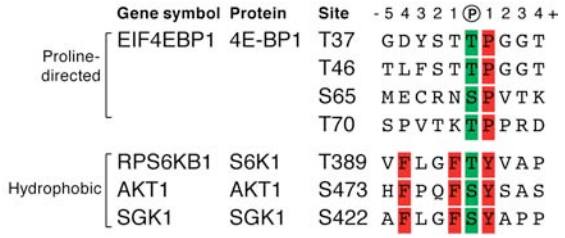


Fig. S3. mTOR phosphorylates two distinct motifs: proline-directed sites present in substrates like 4E-BP1 and the hydrophobic motif present in AGC family kinases. The numbering of the positions is relative to the central phospho-acceptor serine or threonine.

FIGURE S4

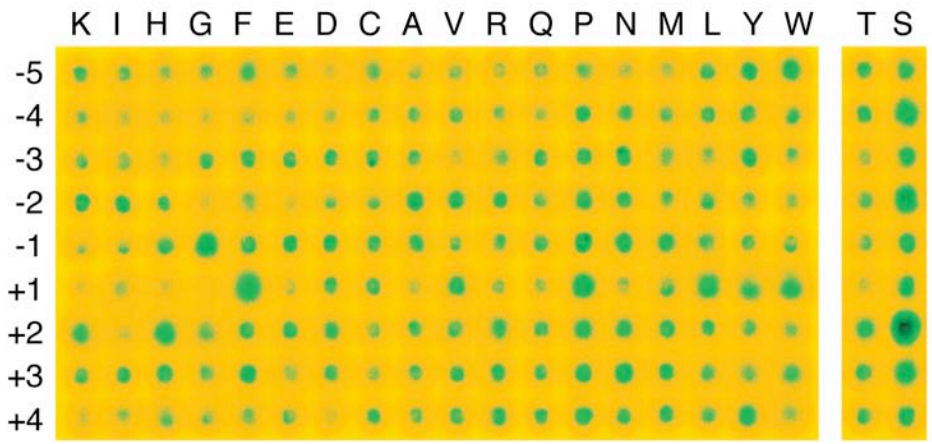


Fig. S4. In vitro phosphorylation of a PSPL with mTORC1 purified from HEK-293T cells stably expressing FLAG-raptor in the presence of Rheb and radiolabeled ATP. Each reaction consists of a mixture of biotinylated peptides containing one fixed residue relative to the central phospho-acceptor and other residues randomized. Aliquots of each reaction were spotted onto a streptavidin membrane and developed by PhosphorImaging. The scan was pseudo-colored to aid in visualization. The numbering of the positions is relative to the central phospho-acceptor serine or threonine.

FIGURE S5

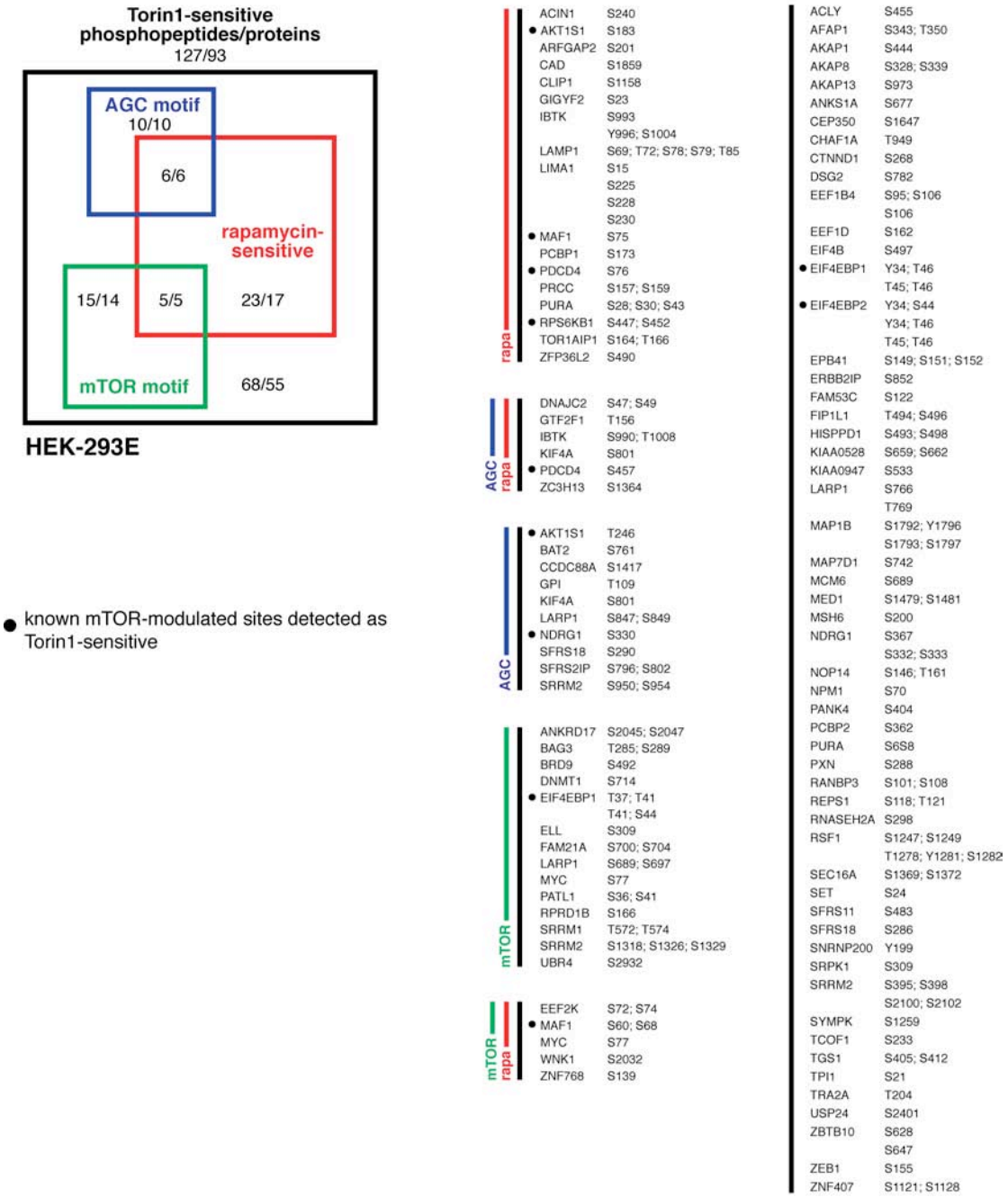
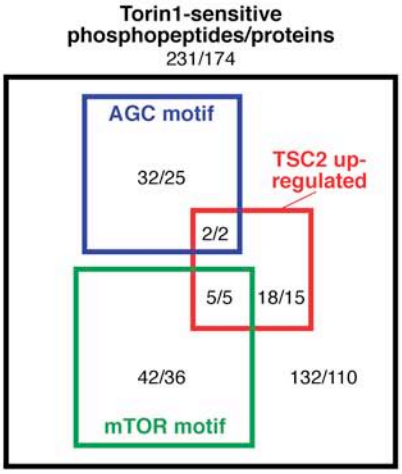


Fig. S5. Classification of the mTOR-regulated phosphopeptides in HEK-293E cells organized by rapamycin sensitivity (-2.5 MAD (log_2 (Rapamycin/Insulin), consistency with the mTOR motif (5th percentile by Scansite) or presence of an AGC motif ((R/K)X(R/K)XX(S*/T*)). The numbers before the slash represent the number of unique phosphopeptides while the numbers after the slash represent the number of unique proteins represented by those phosphopeptides within each category. The phosphorylation sites present in the phosphopeptides and the known mTOR-modulated sites detected as Torin1-sensitive are indicated to the right.

FIGURE S6



MEF

● known mTOR-modulated sites detected as Torin1-sensitive

TSC2 up

●	EIF4EBP1	T44; T45
	EPB4.1L3	T584
	GORASP1	T363; S365; S367
	ITSN1	T890
	MAP4K4	S691
	MCF2L	T583
	OSBP	S188; S191
	PHLDB2	T358
		S359
		T546
		S551
	RPS6KC1	S697; S708
	SLK	S643; S647
	SMCR8	S413
	SMTNL2	S339
	SNX18	S20
	TMEM135	S221
	TNKS1BP1	S1437

AGC TSC2 up

●	NDRG1	S330
	REEP1	S152

mTOR

	2810004N23.	T53; S67
	AFAP1	S665; S669
		S666; S669
	AHNAK	S5525; S5536
		S5553; T5571
	AKAP11	S987
		T1092
	ATXN2L	T31; S32
	BC059842	S3116
	CDC42BPB	S1692
	CHFR	S231
	DDX18	T101
	DLG5	S1209
	DPYSL3	S522
	EHMT2	S287
●	EIF4EBP2	T37; T41
	EIF4G1	T212
	FLNA	S1338
	FOXK1	S431
		S427; S431
	FOXK2	S547
	IRS2	S616
	JUN	T242; S246
	LARP1	S1010; S1018
	MCL1	T144
	MTAP1B	S1247; S1260
	NES	S919
	NFRKB	S351
	NUMA1	S1973; T1982
		S1974; T1982
	NUP188	T1515
	PATL1	T194
	PHLDB1	S1007; S1009
	POLR2A	S1882
	PTPN13	S1073; S1077
	RC3H1	S535
	ST5	S30; S32; S42
	TAF3	S229
	TBC1D10B	S382; S386; S389
	TOPORS	S1016
	WIPI2	S395

mTOR TSC2 up

	ARHGAP29	S1040
	CUX1	S793
	GRB10	S103; S104
	PHLDB2	S17
	SMCR8	S416

AGC

	AHNAK	S5555; S5557
●	AKT1S1	T247
	ATXN1	S751
	BAT2	S759; S761; S764
	BRWD1	S1782; S1784; S1789
	CARHSP1	S53
	EHMT2	S285
	EIF4B	S174; S176
	FAM178A	S318
	HDGFRP2	S450
		S450; S454
	LMNA	S292
	100045983	S983
	LYSMD2	S33
●	MDM2	S183
	MYCBP2	S3577
●	NDRG1	T328; S330
		S330; S332
		S330; S332; S333
	PDCD11	S1468
	PLEC1	S4245
	POM121	S367
●	RPS6	S236; S240
	SETD1A	S1176
	SFRS18	S290
	SRPK2	S488; S490
		S488; S491
	TOPORS	T859; S861; S863
	UTP18	T200; S201; S202; S206

	4922501C03.	S451
	ACIN1	S387; S389
	ADARB1	T686
	AFAP1	S684
	AKAP12	S1645
●	AKT1S1	S184
		S204; S213
	ALAD	S215
	ALS2	S486
	AMIGO3	S257
	ANKRD11	S1829
	ANXA2	S127
	ATN1	S77
	ATP2B2	S1185
	ATXN2L	S304
	AW146020	S118
	BRE	S222
	CCDC12	S165
	CCNL1	S341; S344
		S344; S347
	CEBPB	S183
	CHD8	S2213
	CNPY2	S115
	CSNK1G3	S367
	CTPS	S575
	CXCR7	S350
	DGCR8	S377
	DLK1	S357
	DTL	S654
	EDC3	S131
●	EIF4EBP1	Y33; T45
		S34; T49
		S34; T35; T49
●	EIF4EBP2	Y34; T46
		T45; T46
	EIF5B	S184; S187
	EP400	T885; S886
	EPS8	S810; S814
	F11R	S288
	FAM117B	T12
	FIP1L1	S447
	FNIP1	S306
	FOSL1	S267
	FOXK1	S222; S225; S243
		S225; S229; T231
		S243
	GM5780	S534; S536
	GRB10	S99
	GTF2F1	S385
	HGS	S310
	HIRIP3	S207; S208

	HNRNPF	S63
	HNRNPUL2	S166
	IBTK	S994
	IL1RAP	S566
	KIRREL	S671; S674; S676
	KTN1	S90
	LARP1	S900; S902
		S1010; T1013
		S1108
	LARP4	S377
		S377; S379; S382
	LIG1	S922; S923
	LMO7	S1156
	LOC637796	S146; S151
	LRP12	T849
	MBIP	S22
	MCM2	T25; S26; S27
	MCM6	S699
	MDC1	S168; S176
	MRE11A	S686
	MYADM	S22
	NES	S963
		T1130
		S1860; S1861
	NRADD	S226
	NUMA1	S1973
	NUP133	S40
	OLFR244	Y184; T186; T189; S190
	OXR1	T115
	PDCD11	S1470
	PEX5L	S441
	PLEKHG3	S1123; S1129
	PPAN	T233
	PPIG	S252; S254; S255; S257
	PTPN13	S1506
	PUM2	S82
	SCAP	S850
	SFRS2IP	S498; S502
	SLC22A14	T346; S358
	SLC4A7	S89
	SLK	S348
	SNTB2	S208; S211
	SRRM2	S971; S972
		S1243; S1247
		S1244; S1247
		T1478; S1481
		S1956
		S2548; S2550; S2552
	SSRP1	S667
	SSX2IP	S313
	STEAP3	S20
	STX7	S129
	SUPT6H	T1532
	TBC1D5	S561
	TCF12	S582
	TCF20	S1397
	TCFAP4	S121
	TCFE3	S413
	TMPO	S384
	TNS3	S686
	TOMM20	S135
	TPD52L2	S96
	TRIM28	S596
	TRIM47	S504
	TRIM68	S388
	TRIP10	S296; S299
	UBE4B	S88
	UBXN7	S367
	UHRF2	S668
	ULK1	S638
	WDR33	S1204
	WDR44	T351
	WIBG	S138
	ZEB1	S681
		T680
	ZFP36L1	S334
	ZFP509	S282; S288

Fig. S6. Classification of the mTOR-regulated phosphopeptides in MEFs organized by upregulation in the absence of TSC2 (+2.5 MAD $\log_2$(TSC2$^{-/-}$ vehicle/TSC2$^{+/+}$ vehicle), consistency with the mTOR motif (5th percentile by Scansite) or presence of an AGC motif ((R/K)X(R/K)XX(S*/T*)). The numbers before the slash represent the number of unique phosphopeptides while the numbers after the slash represent the number of unique proteins represented by those phosphopeptides within each category. The phosphorylation sites present in the phosphopeptides and the known mTOR-modulated sites detected as Torin1-sensitive are indicated to the right.

FIGURE S7

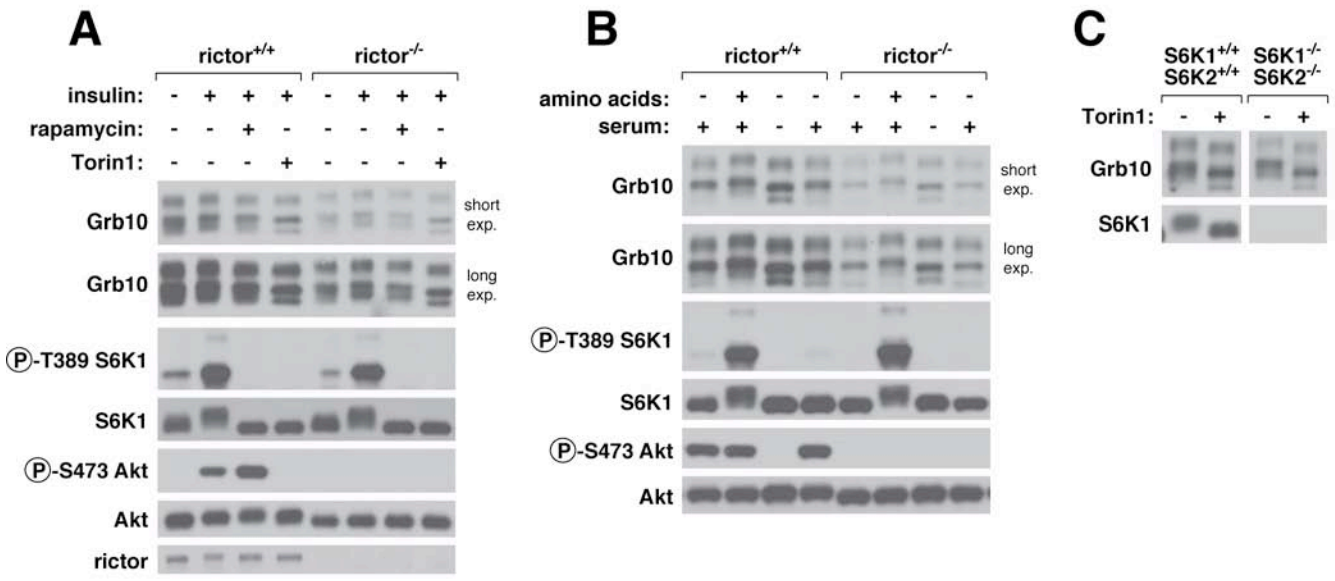


Fig. S7. mTORC2 and S6K are not required for Grb10 phosphorylation. (A) rictor$^{+/+}$ and rictor$^{-/-}$ MEFs were serum starved for 4 hours, treated with either 100 nM rapamycin, 250 nM Torin1, or vehicle control for 1 hr, and then stimulated where indicated with 150 nM insulin for 15 min. Cell lysates were analyzed by immunoblotting. (B) rictor$^{+/+}$ and rictor$^{-/-}$ MEFs were deprived of amino acids or both amino acids and serum for 50 min, and then stimulated with either amino acids or serum for 10 min where indicated and analyzed by immunoblotting. (C) S6K1$^{-/-}$ S6K2$^{-/-}$ or control cells were treated with 250 nM Torin1 or vehicle control for 1 hr and analyzed by immunoblotting.

FIGURE S8

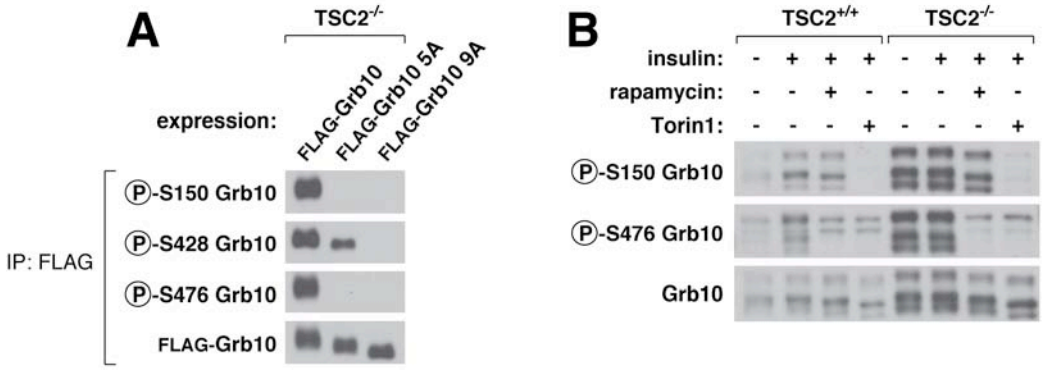


Fig. S8. Validation of phospho-specific antibodies against S150, S428, and S476 Grb10. (A) FLAG-immunoprecipitates from TSC2$^{-/-}$ MEFs stably expressing FLAG-Grb10, 5A (S150A T155A S158A S474A S476A), or 9A (5A + S104A S426A S428A S431A) were analyzed by immunoblotting. (B) TSC2$^{+/+}$ and TSC2$^{-/-}$ MEFs were deprived of serum for 4 hrs, treated with 100 nM rapamycin, 250 nM Torin1, or vehicle control for 1 hr, and then stimulated where indicated with 150 nM insulin for 15 min. Cell lysates were analyzed by immunoblotting.

FIGURE S9

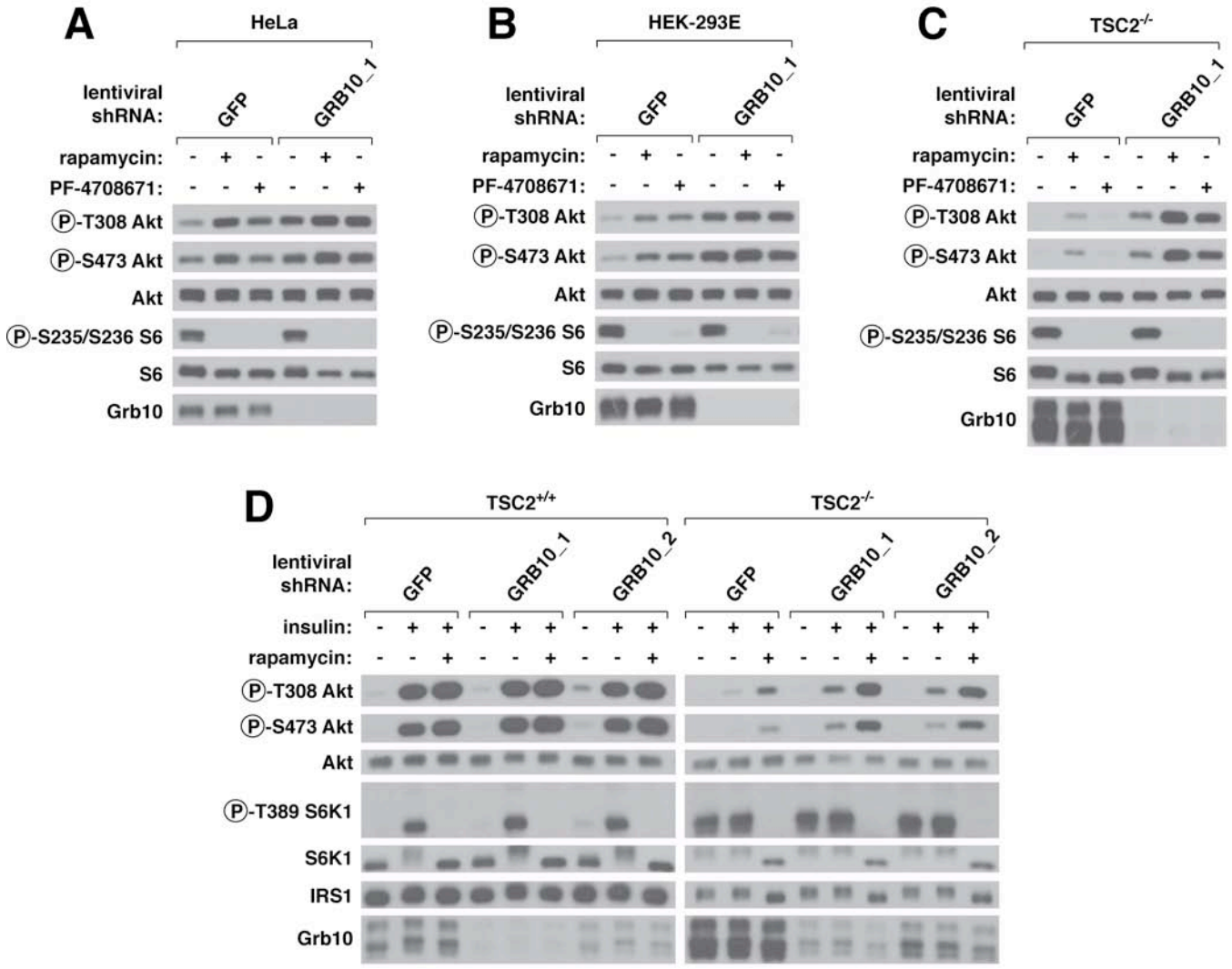


Fig. S9. The effects of Grb10 suppression and S6K inhibition are additive. (A) HeLa and (B) HEK-293E cells expressing shRNAs against GFP or human Grb10 were treated for 100 nM rapamycin, 10 μM PF-4708671 (an S6K inhibitor), or vehicle control for 1 hr, and lysates were analyzed by immunoblotting. (C) $TSC2^{-/-}$ MEFs expressing shRNAs against GFP or mouse Grb10 were starved for 1 hr in the presence of either 100 nM rapamycin, 10 μM PF-4708671, or vehicle control, and then all samples were stimulated with 100 nM insulin for 15 min and analyzed by immunoblotting. (D) $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs expressing shRNAs against GFP or mouse Grb10 were starved for 4 hrs, treated with rapamycin or vehicle control for 1 hr, and then stimulated with 100 nM insulin where indicated for 15 min and analyzed by immunoblotting.

FIGURE S10

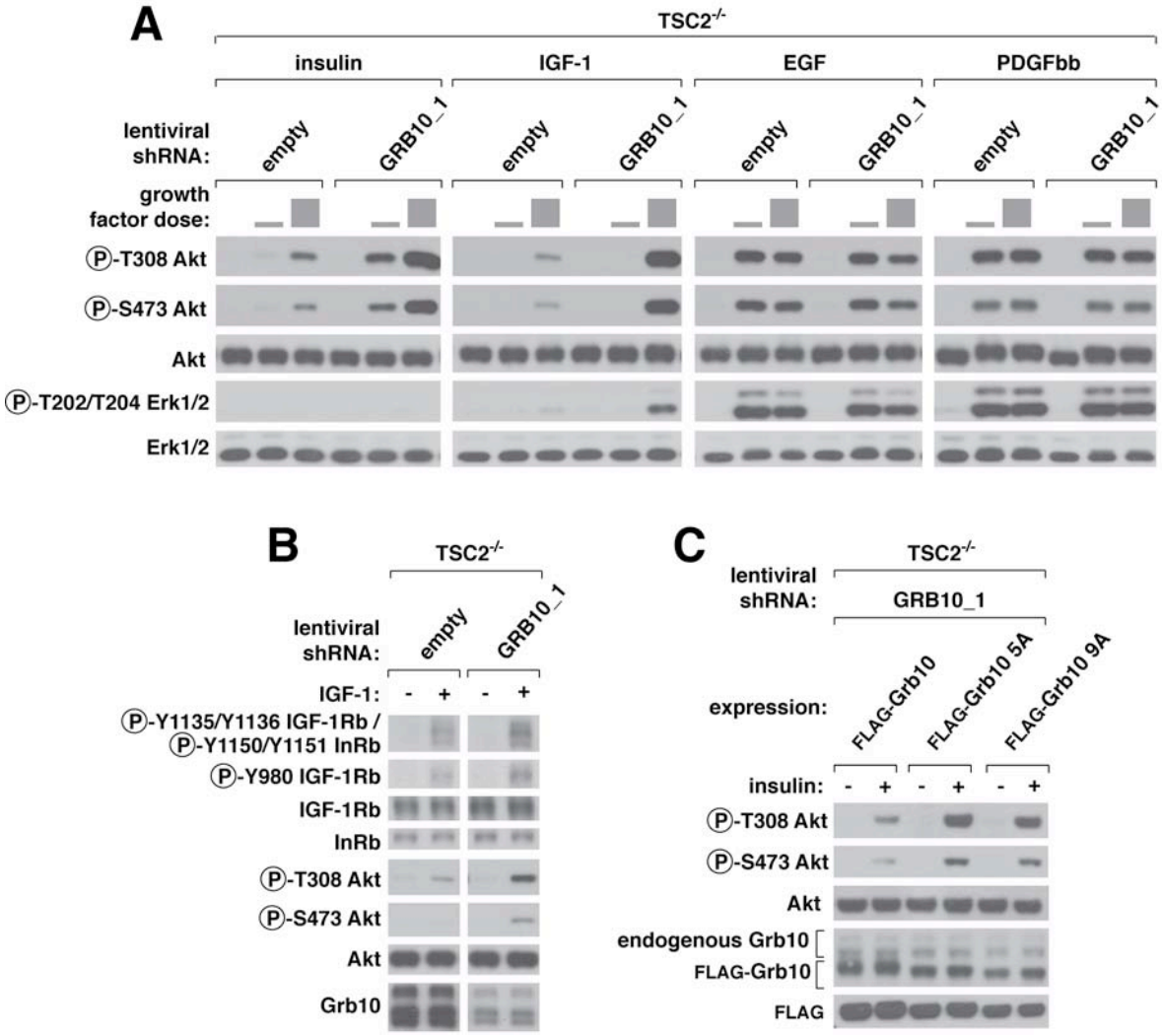


Fig. S10. Suppression of Grb10 restores the insulin and IGF-1 sensitivity of TSC2$^{-/-}$ cells and phosphorylation is important for Grb10 function. (A) TSC2$^{-/-}$ MEFs expressing either an empty vector or shRNA against Grb10 were starved for 4 hrs, stimulated where indicated with 10 or 100 nM insulin, 10 or 100 ng/ml IGF-1, 10 or 100 ng/ml EGF, or 10 or 100 ng/ml PDGFbb for 15 minutes, and analyzed by immunoblotting. (B) TSC2$^{-/-}$ MEFs expressing shRNAs against GFP or mouse Grb10 were serum starved for 4 hrs and then stimulated where indicated with 100 ng/ml IGF-1 for 15 min and analyzed by immunoblotting. (C) TSC2$^{-/-}$ MEFs coexpressing an shRNA targeted against the mouse Grb10 3'UTR and a FLAG-Grb10 (human), 5A (S150A T155A S158A S474A S476A) or 9A (5A + S104A S426A S428A S431A) cDNA expression construct were starved for 4 hrs and then stimulated with 100 nM insulin for 15 min where indicated and analyzed by immunoblotting.

FIGURE S11

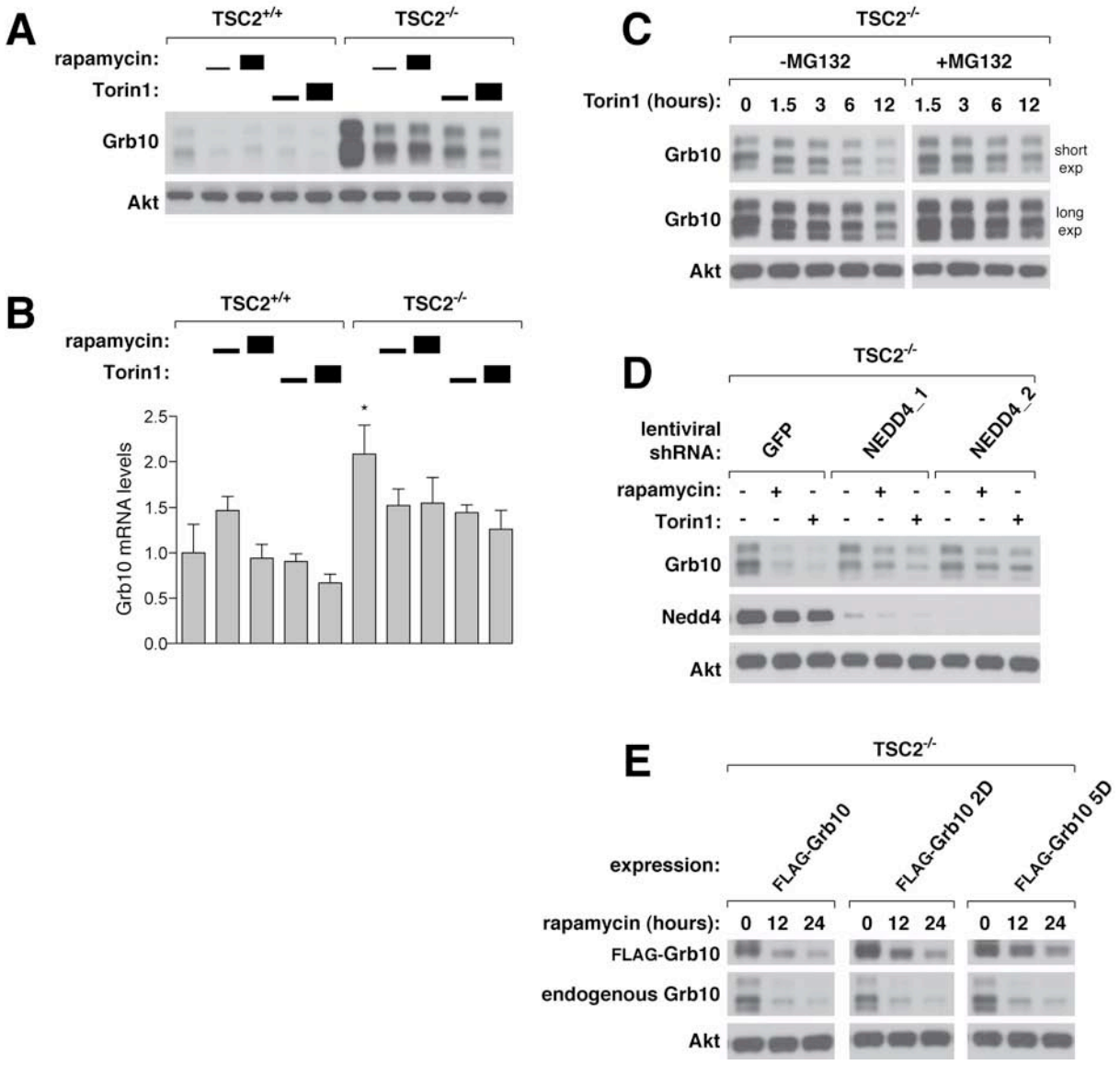


Fig. S11. mTORC1 phosphorylation regulates Grb10 stability. (A) Abundance of Grb10 in $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs treated with 10 or 100 nM rapamycin, Torin1, or vehicle control for 24 hrs. Cells were lysed and analyzed by immunoblotting. (B) RNA was isolated from $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs treated as in (A). Grb10 mRNA was measured by qRT-PCR and normalized to the level of Rplp0 mRNA. Data are means ± s.e.m. (n=7); *Mann-Whitney t-test p-value < 0.05 for differences between vehicle treated $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs. Other comparisons are not significant. (C) Abundance of Grb10 in $TSC2^{-/-}$ MEFs treated with 100nM Torin1 or vehicle control for the indicated number of hrs in the absence or presence of 10 μM MG132. Cells were lysed and analyzed by immunoblotting. (D) Abundance of Grb10 in $TSC2^{-/-}$ MEFs expressing shRNAs against GFP or mouse Nedd4 and treated with 100 nM rapamycin, Torin1, or vehicle control for 24 hrs. Cells were lysed and analyzed by immunoblotting. (E) Abundance of

endogenous Grb10 or exogenous FLAG-Grb10 in $TSC2^{-/-}$ stably expressing FLAG-Grb10, 2D (S474D S476 D), or 5D (S150D T155D S158D S474D S476D) mutants treated with 20 nM rapamycin or vehicle control for the indicated lengths of time. Cells were lysed and analyzed by immunoblotting.

FIGURE S12

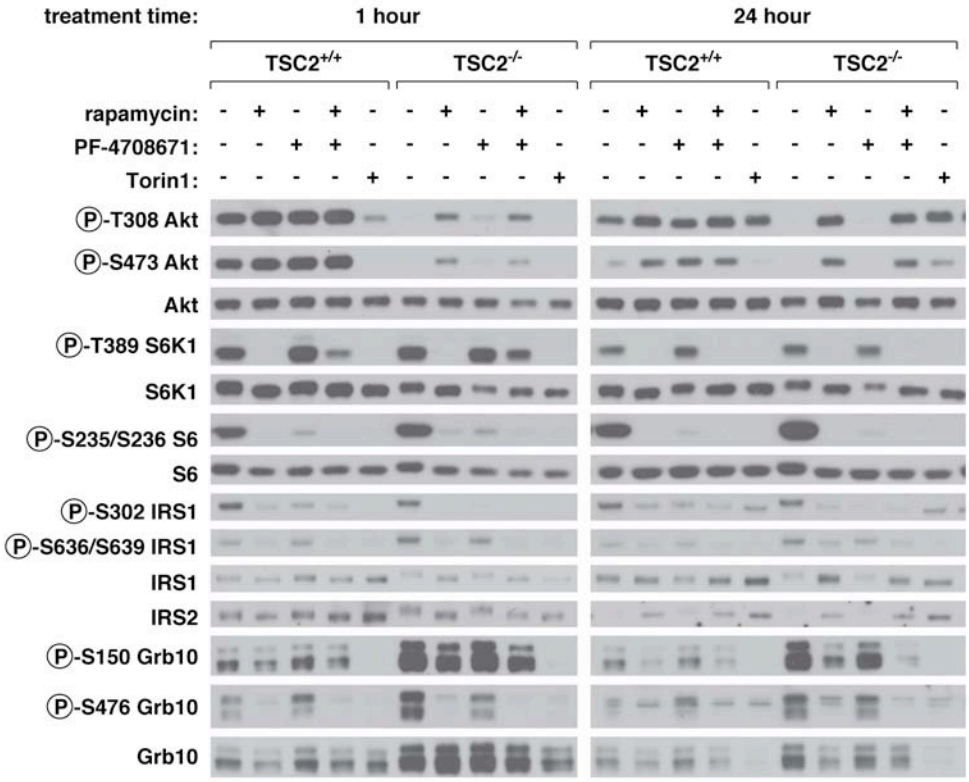


Fig. S12. mTORC1 acutely regulates the phosphorylation and chronically regulates the stability of Grb10 and IRS proteins. $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs expressing shRNA constructs against GFP or Grb10 were starved for one or 24 hrs in the presence of 100 nM rapamycin, 10 μM PF-4708671, 100 nM Torin1, or vehicle control as indicated, and then all samples were stimulated with 100 nM insulin for 15 min. Cells lysates were analyzed by immunoblotting.

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