Supporting Information

Choo *et al*. 10.1073/pnas.0809136105

SI Materials and Methods

Cell Lines and Reagents. HEK293, HeLa, DU145, MCF7, U2OS, $p53^{-/-}$ MEFs, and $p53^{-/-}$ $TSC2^{-/-}$ MEFs were all cultured in DMEM plus 10% FBS media. The MEFs were a generous gift from Brendan Manning and David Kwiatkowski. PC3 cells were cultured in F12K Ham's media with 10% FBS. Transfection was carried out with FuGENE 6 (Roche), Lipofectamine 2000 (Invitrogen), or calcium phosphate. Antibodies to 4E-BP1, BP1 Thr-37/46, BP1 Thr-70, S6(P), eIF4E, eIF4E Ser-209, S6K1, S6K1 Thr-389, Creb Ser-133, Tsc2 Thr-1462, Rictor, and Akt Ser-473 were purchased from Cell Signaling Technology. Antibodies to eIF4G, actin, HA, and 4E-BP1 Ser-65 were purchased from Santa Cruz Biotechnology. Antibodies to Raptor and mTOR were from Diane Fingar. UO126, actinomycin D, cycloheximide, and rapamycin were purchased from Sigma. All CDK inhibitors (kenpaullone, roscovitine, and purvalanol A), PI-103, wortmannin, UO126, and staurosporine were from Calbiochem.

Translational Assays. Detailed descriptions of the translational assays were described elsewhere (1, 2). m7GTP Sepharose beads were purchased from Amersham and were used to precipitate cap-binding proteins (1, 2). For the Cap versus IRES assays, a 10-cm plate was transfected with 10 μg of the dual luciferase vector and incubated for 12 h. After, the cells were equally split into 8 different 6-cm plates. After 3 h, the cells were treated with either ethanol or rapamycin. Seventy-two hours after treatment, the cells were lysed, normalized to protein content, and measured for *Renilla*/firefly activity.

Kinase Assays. S6K1 kinase assays were done as previously described (3). S6K2 kinase assays were done with exogenously expressed S6K2 with an HA tag because no commercial antibody that immunoprecipitates S6K2 has been reported. For the in vitro mTORC1 kinase assays, HEK293 cells were lysed in detergent-free buffer containing 40 mM Hepes, 120 mM NaCl, 1 mM EDTA, 10 mM β-glycerolphosphate, 5 mM EGTA, 1 mM $NaVO_4$, and appropriate protease inhibitors. The cells were lysed by sonication twice for 8 s using the W-385 sonicator from Heat Systems Ultrasonics. The mTORC1 complex was immunoprecipitated with an anti-raptor antibody targeting the internal region or Raptor (885–901). After washing, the immunoprecipitated proteins were incubated with 125 ng/RxN of recombinant FKBP12 (catalog no. 325902; Calbiochem) and indicated concentrations of rapamycin for 45 min. The kinase assays were carried out in a buffer containing both $MnCl_2$ and $MgCl_2$ with GST-4EBP1 as the substrate for 25 min.

RNA Interference. siRNAs against human mTOR and Raptor were purchased from Qiagen. The sequences for the human Raptor siRNAs include 5′-CTCCGCACTCTTTATCCATTT-3′ and 5′-AGGGCCCTGCTACTCGCTTTT-3′. The siRNA against mTOR was a validated pool purchased from Qiagen and Darmacon. To achieve efficient mTOR knockdowns, the Qiagen and Darmacon siRNAs were mixed at a ratio of 1:1. siRNA against Rictor was purchased from Darmacon (OTP pools).

In Vitro Translational Assay. The pcDNA3-rLuc-PolioIRES-fLuc vector was cut with XhoI to linearize the plasmid. The mMESSAGE mMACHINE T7 kit was used for capped-RNA transcription, and the Poly(A) Tailing Kit was used for Poly(A) addition (Ambion). The capped-PolyA mRNA was purified, quantified, and used for in vitro translation reaction with [^{35}S]methionine addition (Promega rabbit reticulocyte lysate nuclease-treated reaction kit). Recombinant GST-4E-BP1 is described elsewhere (4).

1. Holz MK, Ballif BA, Gygi SP, Blenis J (2005) mTOR and S6K1 mediate assembly of the translation preinitation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* 123:569–580.
2. Roux PP, *et al*. (2007) RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. *J Biol Chem* 282:14056–14064.
3. Roux PP, *et al*. (2004) Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. *Proc Natl Acad Sci USA* 102:667–667.
4. Schalm SS, Fingar DC, Sabatini DM, Blenis J (2003) TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. *Curr Biol* 13:797–806.

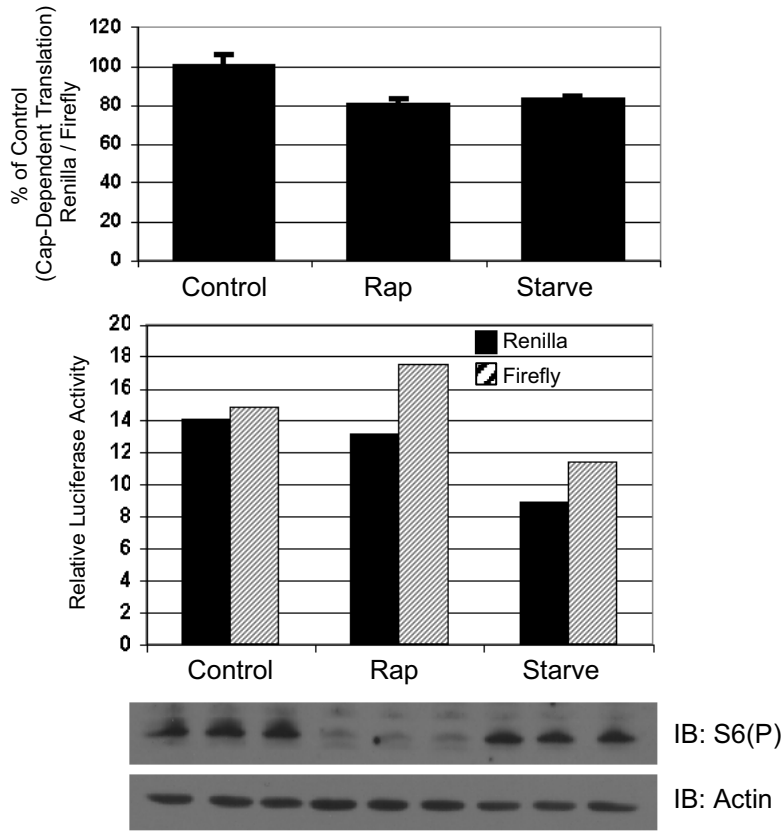

Fig. S1. In vivo cap-dependent translational assays were conducted with a dual renilla-firefly luciferase assay with the Polio virus IRES driving firefly expression. One microgram of DNA was transfected into HEK293 cells in 6-well plates and 24 h later were either starved or treated with rapamycin for 24 h more. On the bottom, the raw data for control, rapamycin, and starved samples are shown.

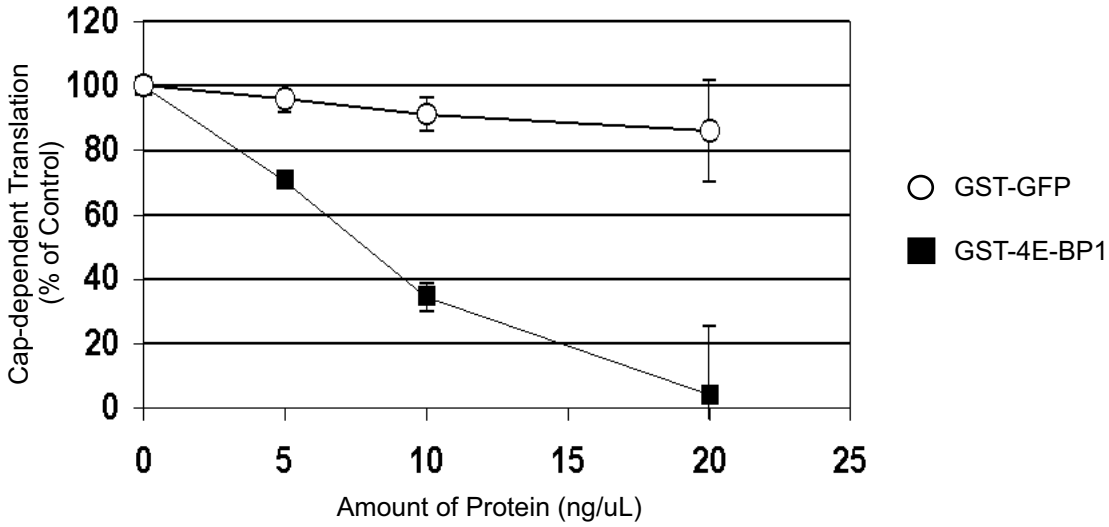

Fig. S2. 4E-BP1 is a potent repressor of cap-dependent translation in vitro. An in vitro translational assay was carried out with capped-polyA rLuc-PolioIRES-fLuc mRNA. Recombinant GST-GFP or GST-4EBP1 was added to the reaction at various concentrations. The reaction was carried out for 90 min at 30 °C.

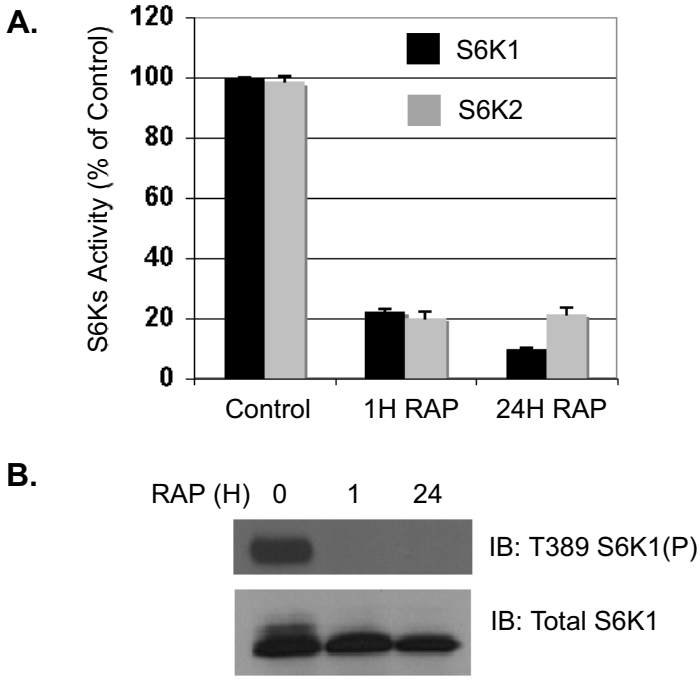

Fig. S3. Rapamycin inhibits the activation and phosphorylation of S6K1 at both 1 and 24 h of treatment. (*A*) Kinase activities of S6K1 and S6K2 were measured at 1 h and 24 h post rapamycin treatment. (*B*) Lysates from rapamycin-treated cells were blotted for T389 phosphorylation site on S6K1.

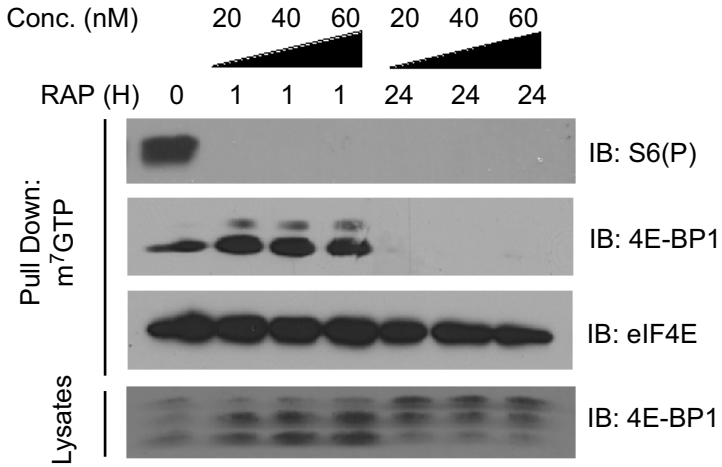

Fig. S4. Increasing concentrations of rapamycin from 20–60 nM were added to HEK 293 cells, and 4E-BP1 phosphorylation was measured by the cap-binding assay and 4E-BP1 gel shift.

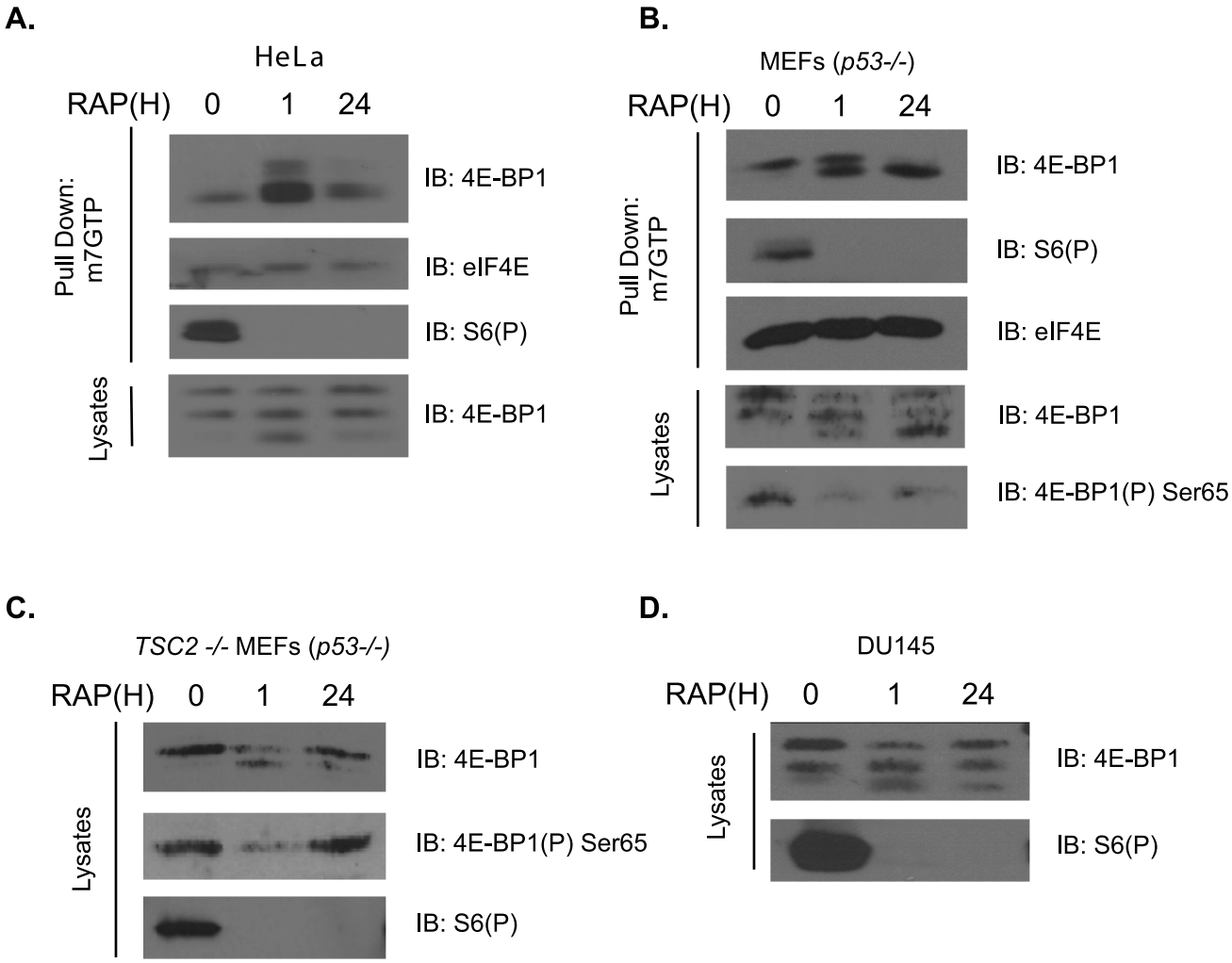

Fig. S5. Rapamycin-induced 4E-BP1 phosphorylation is observed in different cell lines. HeLa cells (*A*), $p53^{-/-}$ MEFs (*B*), $TSC2^{-/-}$ $p53^{-/-}$ MEFs (*C*), and DU145 cells (*D*) were treated with rapamycin (20 nM) for 24 h and analyzed for the ability of 4E-BP1 to bind m7GTP Sepharose, and/or 4E-BP1 phosphorylation was measured.

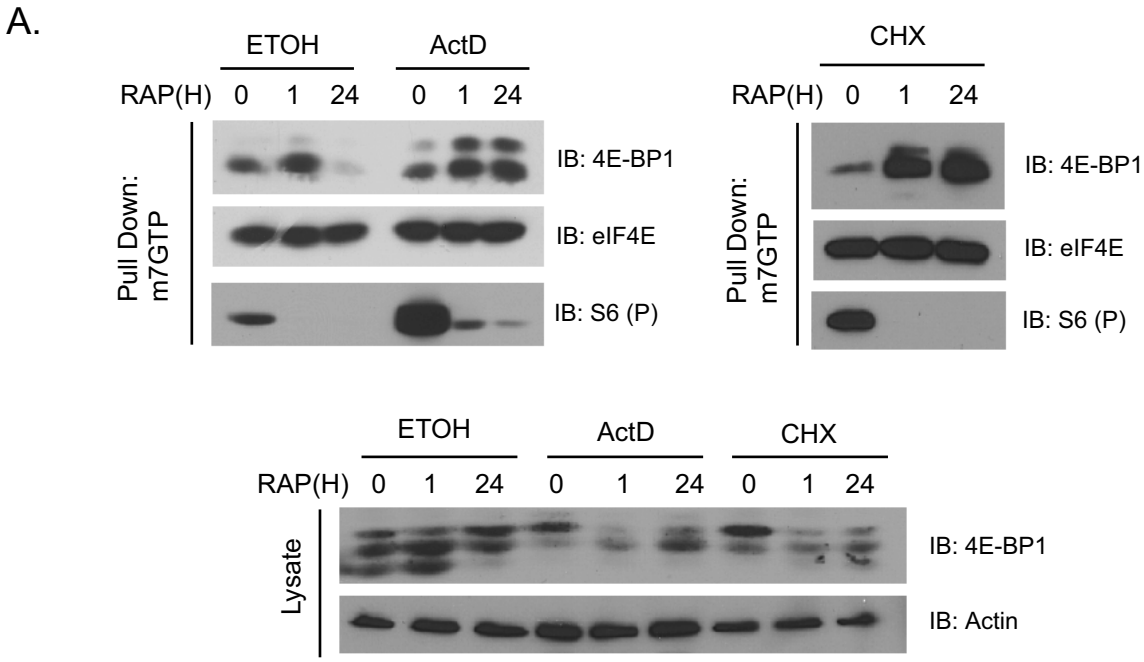

Fig. S6. Rapamycin controls 4E-BP1 phosphorylation through a process that requires de novo protein synthesis. HEK293 cells were treated with DMSO for 24 h or rapamycin (20 nM) for 1 or 24 h. In addition, these cells were also treated with actinomycin D (5 μg/ml), cycloheximide (10 μg/ml), or ethanol for 24 h to block de novo transcription or translation. Actinomycin D, cycloheximide, or vehicle control was added to 0-, 1-, and 24-h rapamycin-treated groups at the same time as the 24-h rapamycin treatment. These samples were then analyzed for 4E-BP1 and S6 phosphorylation.

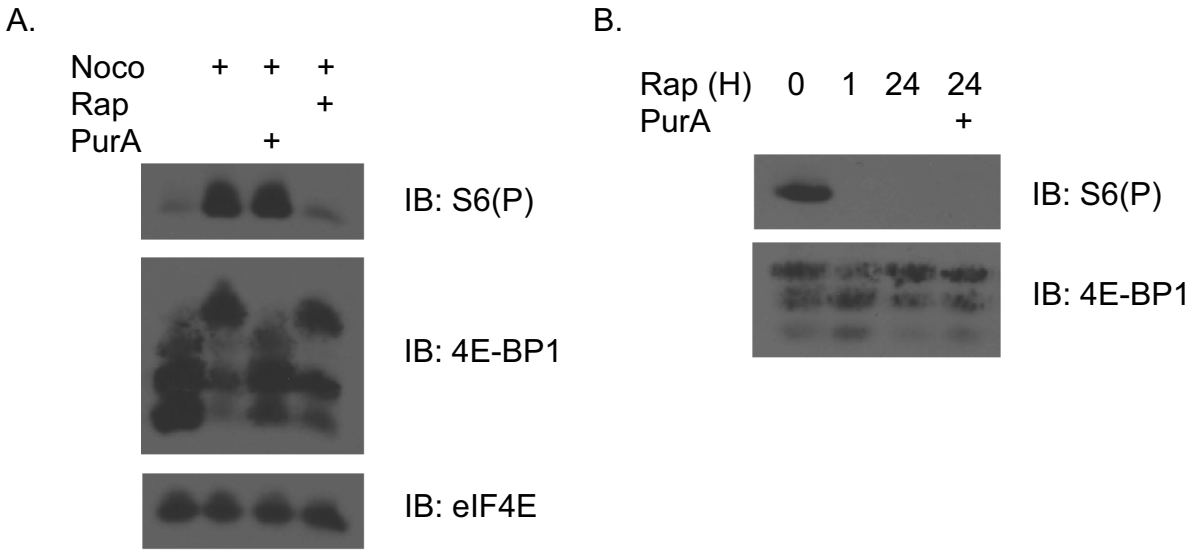

Fig. S7. Cell cycle arrest and CDKs are not involved in rapamycin-mediated 4E-BP1 phosphorylation. (*A*) Nocodazole (100 ng/ml) was added to HEK293 cells for 24 h followed by either rapamycin (20 nM) or purvalanol A (10 μM) for 3 h and analyzed for 4E-BP1 phosphorylation. (*B*) HEK293 cells were treated with rapamycin for 24 h and then treated with purvalanol A for 3 h before lysis.

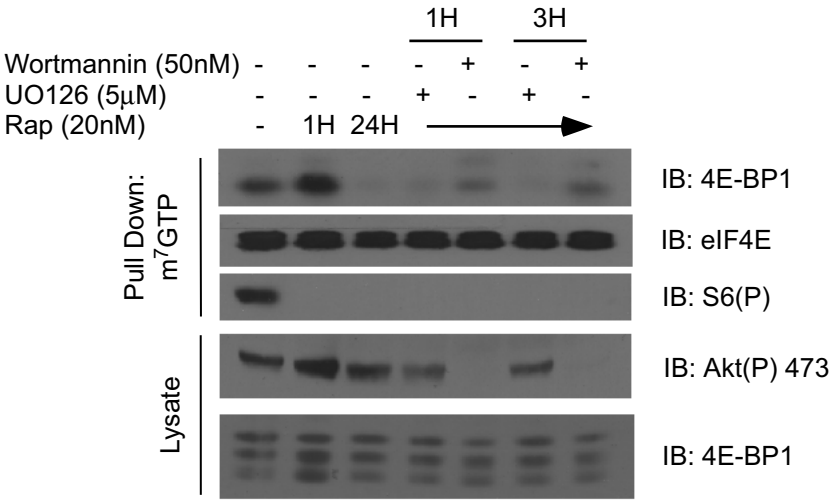

Fig. S8. UO126 (5 μM), a MEK inhibitor, or Wortmannin (50nM) was added to samples that have been treated with rapamycin for 24 h. The inhibitors were added for either 1 or 3 h, and 4E-BP1 phosphorylation was analyzed.

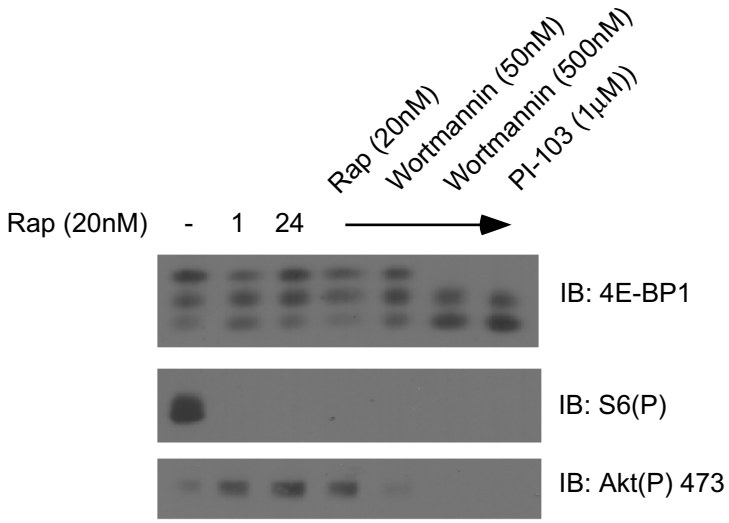

Fig. S9. HEK 293 were treated with rapamycin for 24 h and either control (DMSO), Rap (20nM), Wortmannin (50 and 500 nM), or PI-103 (1μM) were added for 1 h prior to lysis and analyzed for 4E-BP1 phosphorylation.

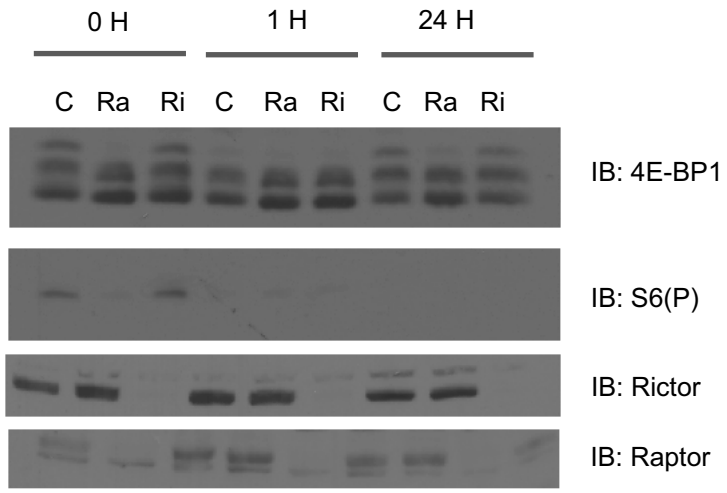

Fig. S10. mTORC1 but not mTORC2 is required for rapamycin-induced 4E-BP1 phosphorylation. siRNAs targeting either Raptor or Rictor was transfected into HEK293 cells. Twenty-four hours after transfection, rapamycin was added and incubated for 24 h. One-hour samples were treated with rapamycin for 1 h before lysis, and controls were treated with ethanol. The lysates were blotted for Raptor, Rictor, and S6 and 4E-BP1 phosphorylation. Con, control siRNA; Ra, siRaptor; Ri, siRictor.

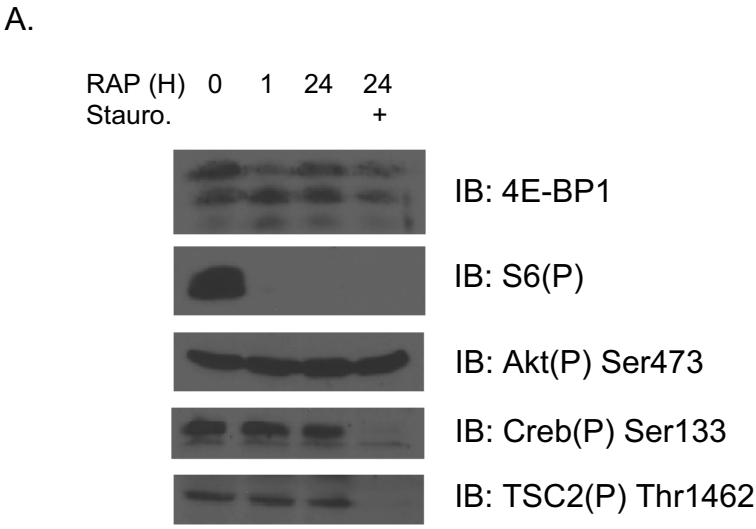

Fig. S11. Rapamycin-mediated 4E-BP1 phosphorylation is insensitive to the global kinase inhibitor staurosporine in vivo. HEK293 cells were treated with rapamycin for 24 h and then treated with staurosporine (500 nM) for 1 h before lysis. 4E-BP1, S6 Ser-235/36(P), Creb Ser-133(P), TSC2 Thr-1462(P), and Akt 473(P) were blotted.

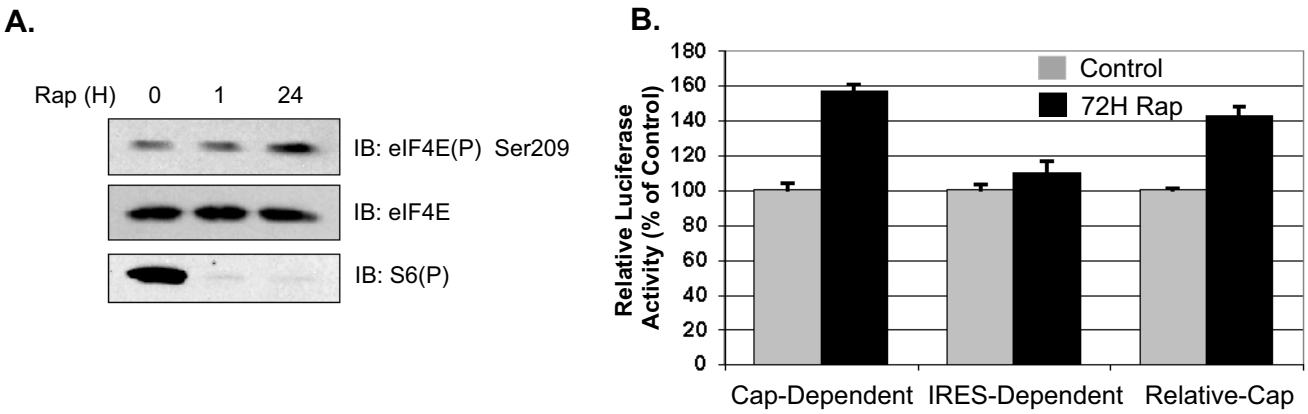

Fig. S12. Long-term rapamycin treatment increases cap-dependent translation. (*A*) The lysates from rapamycin-treated cells were blotted for eIF4E ser209 phosphorylation. (*B*) Cap versus IRES dependent translation was measured as described in *Materials and Methods*. Rapamycin was treated for 72 h.

B.

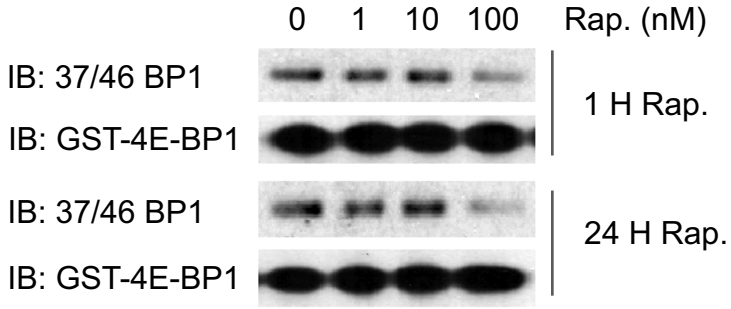

Fig. S13. Both short-term and long-term rapamycin-treated mTORC1 complexes are sensitive in vitro to rFKBP12-rapamycin treatment. HEK293 cells were treated with rapamycin for either 1 h or 24 h, and the mTORC1 complex was immunoprecipitated. A total of 125 ng of GST-FKBP12 was added to each reaction with the indicated amounts of rapamycin (details are described in *Materials and Methods*). The activity of mTORC1 toward 4E-BP1 was measured by 37/46 phosphorylation on 4E-BP1.

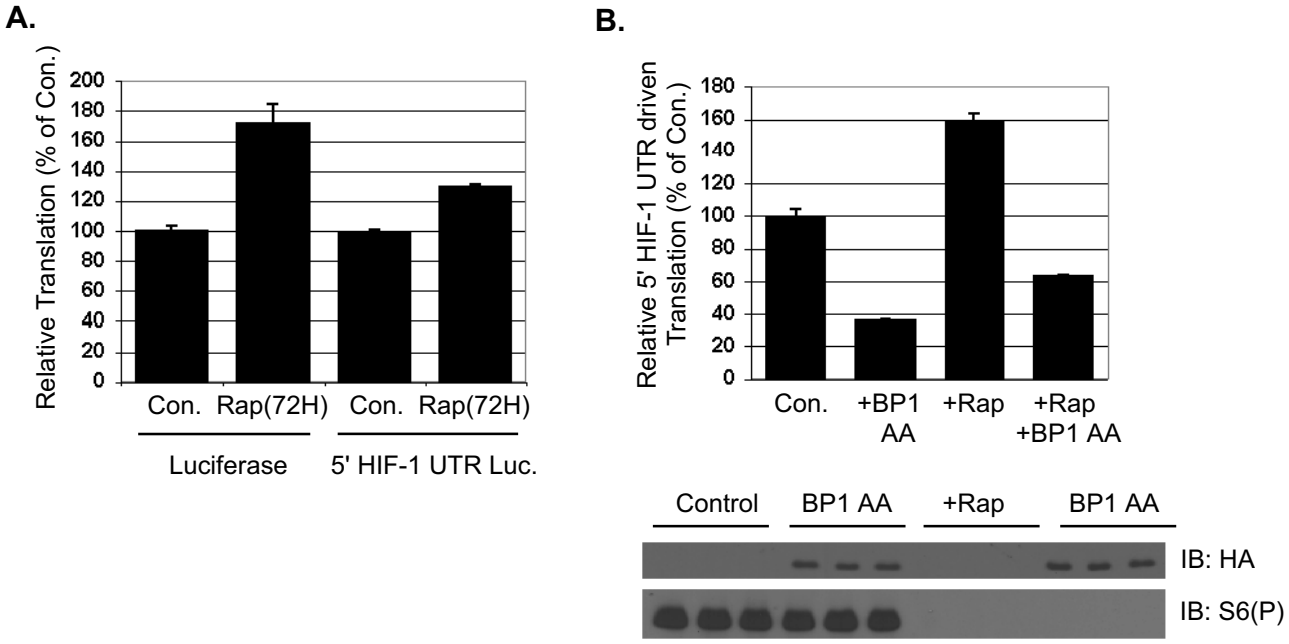

Fig. S14. 4E-BP1 hyperphosphorylation induced by rapamycin determines a cell's sensitivity to rapamycin in attenuating cap-dependent translation. (*A*) HEK293 cells were transfected with a reporter vector that is regulated with or without the structured 5′ UTR of HIF-1α. Three hours after transfection, rapamycin or ethanol was added for 72 h and analyzed. (*B*) The experiment was conducted as in Fig. 4*F*, except the HIF-1α 5′ UTR-driven translational vector was used. HA 4E-BP1 AA levels and phosphor-S6 levels are also shown.