The Tuberous Sclerosis Protein TSC2 Is Not Required for the Regulation of the Mammalian Target of Rapamycin by Amino Acids and Certain Cellular Stresses*

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Amino acids positively regulate signaling through the mammalian target of rapamycin (mTOR). Recent work demonstrated the importance of the tuberous sclerosis protein TSC2 for regulation of mTOR by insulin. TSC2 contains a GTPase-activator domain that promotes hydrolysis of GTP bound to Rheb, which positively regulates mTOR signaling. Some studies have suggested that TSC2 also mediates the control of mTOR by amino acids. In cells lacking TSC2, amino acid withdrawal still results in dephosphorylation of S6K1, ribosomal protein S6, the eukaryotic initiation factor 4E-binding protein, and elongation factor-2 kinase. The effects of amino acid withdrawal are diminished by inhibiting protein synthesis or adding back amino acids. These studies demonstrate that amino acid signaling to mTOR occurs independently of TSC2 and involves additional unidentified inputs. Although TSC2 is not required for amino acid control of mTOR, amino acid withdrawal does decrease the proportion of Rheb in the active GTP-bound state. Here we also show that Rheb and mTOR form stable complexes, which are not, however, disrupted by amino acid withdrawal. Mutants of Rheb that cannot bind GTP or GDP can interact with mTOR complexes. We also show that the effects of hydrogen peroxide and sorbitol, cell stresses that impair mTOR signaling, are independent of TSC2. Finally, we show that the ability of energy depletion (which impairs mTOR signaling in TSC2−/− cells) to increase the phosphorylation of eukaryotic elongation factor 2 is also independent of TSC2. This likely involves the phosphorylation of the elongation factor-2 kinase by the AMP-activated protein kinase.

The regulation of mRNA translation in mammalian cells involves the coordinated control of a number of components of the translational apparatus. Several of them are regulated through signaling via the mammalian target of rapamycin (mTOR)1 (1, 2). These include the ribosomal protein S6 kinases (S6Ks) (3), the translational repressor protein, eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (2), and elongation factor eEF2 and its cognate kinase eEF2 kinase (4). The phosphorylation of these proteins is regulated in a rapamycin-sensitive (i.e. mTOR-dependent) manner and serves to modulate the activity of each of them.

It is well established that the phosphorylation of these proteins is regulated by insulin and amino acids. In many types of cells, withdrawal of amino acids results in dephosphorylation of the S6Ks, 4E-BP1 and eEF2 kinase, and prevents the phosphorylation of these proteins by insulin (reviewed in Refs. 1, 3, 5, and 6). Together, these effects operate such that amino acid withdrawal switches off key steps in the process of translation, which clearly makes physiological sense given that amino acids are the precursors for this process. The fact that they regulate multiple proteins that lie downstream of mTOR suggests that they, in some way, modulate the activity of mTOR. However, it is not known how amino acids exert these effects. mTOR forms a complex with other proteins (e.g. the scaffold protein raptor (7, 8)). Some evidence has been presented to suggest that amino acids decrease the binding of raptor to mTOR (7), although the mechanism is unclear. Other data indicate that, in vertebrate cells, mTOR signaling is actually regulated by intracellular rather than extracellular amino acids (9, 10). Thus, the pool of amino acids that regulates mTOR signaling probably receives contributions from extracellular amino acids and from amino acids generated by protein breakdown, including autophagy (10).

Recently, important advances have been made in understanding the control of mTOR signaling by stimuli such as insulin. This involves the proteins termed hamartin and tuberin (also called TSC1 and TSC2) that together act to suppress mTOR signaling (11). Recent data show that TSC2 functions as a GTPase-activator protein (GAP) for the small G-protein Rheb, which in its GTP-ligated state activates mTOR signaling, although the mechanism by which it does this is unclear (reviewed in Ref. 12). Phosphorylation of TSC2 by protein ki-

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1 The abbreviations used are: mTOR, mammalian target of rapamycin; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; AMPK, AMP-activated protein kinase; BCAA, branched-chain amino acids; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle medium; DOG, 2-deoxyglucose; D-PR5, Dulbecco's phosphate-buffered saline; eEF, eukaryotic elongation factor; eEF2, eukaryotic initiation factor; GAP, GTPase-activator protein; MEFs, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; PI 3-kinase, phosphatidylinositol 3-kinase; Rheb, Ras homologue enriched in brain; S6, ribosomal protein S6; S6K1, S6 kinase 1; TSC, tuberous sclerosis complex; HA, hemagglutinin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; AICAR, AICA-riboside (5-amino-4-imidazolecarboxamide riboside); HEK, human embryonic kidney; ACC, acetyl-CoA carboxylase.
nase B (also termed Akt) is thought to inactivate its GAP function, thus activating Rheb and mTOR. Because insulin activates protein kinase B via PI 3-kinase, this provides a mechanism by which insulin (and other agents that activate PI 3-kinase) can turn on mTOR signaling. There is currently a high level of interest in this area due, for example, to connections between mTOR signaling and a range of human diseases. These include tuberous sclerosis, which is caused by mutations in the genes for TSC1 or TSC2, and certain types of cancer. These include tuberous sclerosis, which is caused by mutations in the genes for TSC1 or TSC2, and certain types of cancer
(13). A limitation in both those earlier studies is that these cells did not completely lack TSC2 but harbored a mutated form of the protein or residual TSC2. Nevertheless, the idea has developed that TSC2 is involved in the amino acid-sensing mechanisms that control mTOR signaling. Consistent with this, Tee et al. (11) showed that overexpression of TSC2/1 impaired the ability of amino acids to activate S6K1. Conversely, ectopic expression of Rheb activated mTOR signaling in amino acid-deprived cells. Furthermore, Zhang et al. (19) reported that nutrient starvation still impaired mTOR signaling in cells lacking TSC2. However, in their study the effects of amino acids were only partial, leaving open the possibility that there exists a TSC2-independent mechanism by which amino acids control mTOR signaling, and no comparison with the corresponding TSC2+/− cells was conducted. Furthermore, although there are several targets for the mTOR pathway, each of which is phosphorylated at multiple sites, previous work has focused on the role of TSC2 in the amino acid control of S6K1. Regulation of targets of mTOR signaling may involve more than one output from mTOR; for example, 4E-BP1 contains two regulatory motifs that act in distinct ways to modulate the phosphorylation of different sites (20–22).

Other work has shown that ATP depletion leads to impairment of mTOR signaling, through the activation of the AMP-activated protein kinase (AMPK) (23). This is likely mediated by the direct phosphorylation of TSC2 by AMPK (24). Earlier work showed that depletion of cellular ATP, or other treatments that activate the AMPK, cause pronounced increases in the phosphorylation of eEF2 (25–27). Although more recent data suggest that this could be mediated through TSC2, AMPK also directly phosphorylates the kinase that acts on eEF2, eEF2 kinase, and this is associated with its activation (26). We therefore considered it important to exploit the availability of TSC2 null cells to test the involvement of TSC2 in the control of eEF2 and eEF2 kinase by ATP depletion and other situations that activate AMPK.

Finally, other stressful conditions lead to an impairment of mTOR signaling as assessed by effects on S6K1, ribosomal protein (RP) S6, and 4E-BP1 (see Refs. 28–32 and reviewed in Ref. 33). Many stressful conditions (e.g. hyperosmolarity) lead to activation of the p38 mitogen-activated protein kinase pathway which, via the downstream kinase mitogen-activated protein kinase-activated protein kinase-2, may modulate the function of TSC2 (34, 35). We therefore also wished to test whether the effects of other stresses on mTOR signaling required TSC2.

Here we have performed a detailed analysis of the effects of amino acid starvation and other stresses on multiple targets of mTOR signaling in TSC2−/− and TSC2+/− cells. In contrast to earlier studies, we find that amino acid deprivation does indeed impair mTOR signaling in TSC2−/− cells. This implies (i) that TSC2 is not essential for the effects of amino acids on mTOR signaling and thus (ii) that additional mechanisms exist, independent of TSC2, for the control of mTOR by amino acids. Furthermore, our data indicate that the effects of ATP depletion and AMPK activation on the phosphorylation of eEF2 are also independent of TSC2, as are the effects of selected cell stresses on mTOR signaling. These data imply that regulation of targets of the mTOR pathway involves mechanisms that operate independently of TSC2, so that additional regulatory inputs operate to control mTOR signaling in mammalian cells.

MATERIALS AND METHODS

Chemicals and Biochemicals—Cycloheximide (CHX) was obtained from Sigma. Rapamycin was from Calbiochem, and Immobilon P membranes were from Millipore (Bedford, UK). Unless otherwise indicated, all antisera were from New England Biolabs (Hitchin, Hertfordshire, UK). Antisera for eEF2 kinase and eEF2 (total and phospho-specific) were described previously (36). Cell culture media and MEM essential vitamins were obtained from Invitrogen; bovine serum albumin (fatty acid-free) was from Roche Applied Science. mGTP-Sepharose CL4B was from Amersham Biosciences. AICA-riboside (5-amino-4-imidazole-carboxamide riboside) (AICAR) was from Toronto Research Chemicals. Unless otherwise indicated, all other reagents were obtained from Sigma or Merck. Antibodies for AMPK (α1 and α2 isoforms) and for acetyl-CoA carboxylase phosphorylated at Ser-79 were kindly provided by Professor Graham Hardie (Dundee, Scotland). Antibodies for eEF2 kinase phosphorylated at Ser-77 were described earlier (36).

Cell Culture, Treatment, and Lysis—TSC2−/− mouse embryonic fibroblasts (MEFs) and the corresponding TSC2+/− cell line were generously provided by Dr. David Kwiatkowski (Harvard University, Boston (20)). These MEF cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Prior to treatment, 90% confluent MEF cells were starved of serum (see legends for details) for 16 h. To starve cells of external amino acids, plates were rinsed once in Dulbecco’s phosphate-buffered saline containing 10 mM d-glucose (D-PBS/glucose) and then incubated in the same solution plus MEM vitamins diluted 1:25 (v/v) (for the times indicated in the figures). Cells were then treated with insulin or rapamycin where indicated (at the concentrations and final concentrations and times indicated in the figures). For certain control experiments, a mixture of amino acids was added to the D-PBS/glucose for the duration of the amino acid starvation. Alternatively, amino acids were added back (for 30 min) to cells that had been kept in D-PBS/glucose for 90 min. The composition of the amino acid mixture is exactly as given in Ref. 10.

For treatment of cells with stress-inducing agents, cells were grown to near confluence prior to exposure to sodium arsenite, hydrogen peroxide, or sorbitol for 30 min at the indicated concentrations. For experiments to address the role of AMPK in regulating targets of the mTOR pathway, 90% confluent MEF cells were placed in low glucose medium (see text for details). Cells were then treated with AICAR or 2-deoxyglucose at the final concentrations and times indicated in the figures. Control cells received an appropriate dose of the relevant vehicle.

Cells were lysed by scraping into our standard extraction buffer (50 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na3VO4, 1 mM benzamidine hydrochloride, 0.1% (v/v) β-mercaptoethanol, 1% (v/v) Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of pepstatin, leupeptin, and antipain (pH 7.4)). Lysates were briefly centrifuged at 13,000 rpm in an Eppendorf 5415D microcentrifuge to remove debris. Protein concentrations in the resulting supernatant were determined using the Bradford method (37). To immunoprecipitate eEF2 kinase, supernatants were removed to fresh tubes and then rotated at 4 °C in the presence of protein G-Sepharose prebound to anti-eEF2 kinase antibody. Analysis of the association of 4E-BP1 with eIF4E was performed by affinity-purifying eIF4E from...
cell lysates followed by SDS-PAGE and Western blotting as described previously (38).

Assays for eEF2 Kinase and AMPK—eEF2 kinase and AMPK were assayed as described previously (26). AMPK was assayed after immunoprecipitation using the “AMARA” peptide.

Guanine Nucleotide Binding to Rheb in Vivo—HEK293 cells grown on 10-cm² plates were incubated in 5 ml of phosphate-free medium containing 0.5 mM of [32P]orthophosphate for 3 h. These cells were then harvested in buffer A (50 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, 1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 1% (v/v) Triton). FLAG-tagged Rheb was immunoprecipitated for 1 h with anti-FLAG antibodies coupled to protein G-Sepharose. Immunoprecipitates were washed twice each with buffer A and buffer B (50 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, 0.1% (v/v) Triton) in the presence of protease inhibitors. 32P-Labeled GTP and GDP were eluted from Rheb using 20 μl of elution buffer (0.5 mM GDP, 0.5 mM GTP, 5 mM dithiothreitol, 5 mM EDTA, 0.2% SDS) at 68 °C for 20 min and then resolved by TLC on polyethylenimine cellulose plates (Sigma) with KH₂PO₄/[32P]GTP and [32P]GDP levels were quantified by Phosphor-Imager analysis.

Vectors—N-terminal Ha-tagged Rheb/RB7K was generated by subcloning Rheb from FLAG-Rheb/pK7 into pKH3, FLAG-tagged TSC1, TSC2, and Rheb were generated as described previously (39). The vector encoding AU1-tagged mTOR was kindly provided by Dr. Robert Abraham (National Institutes of Health, San Diego).

Immunoprecipitation of Rheb-mTOR Complexes—HEK293 cells overexpressing AU1-mTOR and either FLAG-Rheb or HA-Rheb were harvested in 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 10 mM MgCl₂, 25 mM NaCl, 25 mM β-glycerophosphate, 0.2% CHAPS (Calbiochem). Rheb was immunoprecipitated for 2 h with either anti-FLAG (M2, Sigma) or anti-HA (Roche Applied Science) antibodies bound to protein G-Sepharose. Immunoprecipitates were washed three times in extraction buffer and then subjected to SDS-PAGE and Western blot analysis for detection of both mTOR and Rheb. mTOR was detected using mTABI antibodies, kindly provided by Professor Dick Denton (University of Bristol, UK).

Determination of BCAA Concentrations—Where indicated TSC2−/− cells were starved of serum for 16 h and also in some cases (indicated) of amino acids for the times shown, followed in some cases by treatment with the protein synthesis inhibitor cycloheximide as indicated in figure legends. For BCAA measurement experiments only, cells were lysed in 0.3% perchloric acid. Samples of cleared, neutralized cell lysates were incubated with BCAA dehydrogenase as described (40). Assays were then diluted with 500 μl of water and fluorescence intensity read at an excitation wavelength of 340 nm and an emission wavelength of 460 nm. Fluorescence blanks were prepared for each sample by substituting the glycerol vehicle for enzyme. The assays were calibrated using leucine standards (0–200 μmol/liter) in place of cell supernatants.

Gel Electrophoresis, Immunoblotting, and Other Procedures—These procedures were performed as described previously (41). For the analysis of the mobilities of different forms of 4E-BP1, resolving gels containing 15% (w/v) acrylamide and 0.35% (w/v) methylenebisacrylamide were used. To study the phosphorylation of the ribosomal protein, S6 gels containing 15% (w/v) acrylamide and 0.1% (w/v) methylene bisacrylamide were used. All other standard resolving gels contained 12.5% (w/v) acrylamide and 0.1% (w/v) methylenebisacrylamide. Equal amounts of cell protein were loaded into each lane, and equal loading of the samples was generally further confirmed by Western blot analysis with the appropriate antisera. Blots were developed using ECL.

Reproducibility and Quantitation—Where indicated, the data have been quantitated using the ImageJ software (available at reb.info.nih.gov/jj/). The ratios arrived at in this way are arbitrary and do not indicate, for example, true stoichiometries. In all cases, data are representative of at least three independent experiments, where similar data were obtained. Other information about the analysis of the data is given in the relevant figure legends.

RESULTS AND DISCUSSION

Effects of Amino Acid Starvation on Rheb—Although the above data indicate that TSC2 is not required for the effects of amino acid starvation on mTOR signaling, it was still conceivable that Rheb might be affected. We therefore first studied whether amino acid starvation affected the relative amounts of GTP and GDP associated with Rheb. Antisera for Rheb are available commercially. However, despite our repeated attempts to employ them, they proved unsuitable for the analysis of endogenous Rheb, e.g. in MEFs. In Western blots, the antibodies detected multiple bands, raising questions about their specificity, and when used to try to immunoprecipitate Rheb from [32P]orthophosphate-labeled cells, no bound GDP or GTP was detected, suggesting either that Rheb levels are too low to permit this kind of analysis or that the antibodies work poorly in immunoprecipitation. We therefore expressed Rheb in an epitope (FLAG)-tagged form. However, it is very hard to achieve efficient transfection of MEFs, and we therefore exploited an easily transfectable cell type, human embryonic kidney (HEK) 293 cells. Amino acid starvation inhibits mTOR signaling markedly in HEK293 cells, as judged here from the phosphorylation states of 4E-BP1 and S6 (11). When expressed at high levels, Rheb tends to be found almost entirely in its GTP-bound form (42). This could be a consequence of the abnormally high levels, and we therefore considered it important to study Rheb when it was expressed at levels similar to those of the endogenous protein (Fig. 1A, lowest panel, where endogenous and exogenous Rheb run as a poorly resolved doublet). As expected, the data show that expression of Rheb increases the phosphorylation of 4E-BP1 (Fig. 1A). Most importantly, amino acid withdrawal reverts 4E-BP1 phosphorylation to basal levels. Thus, low level expression of Rheb does not overcome the effects of amino acid withdrawal, in contrast to the situation when Rheb is highly overexpressed (39).

Amino acid withdrawal caused a reproducible decrease in the proportion of Rheb bound to GTP from about 40% to around 25% (Fig. 1B). By using the available reagents, we cannot yet address the issue of whether amino acid starvation regulates the level of GTP-bound Rheb in TSC2 null cells.

One possible explanation for the effects of amino acid withdrawal on the GDP/GTP loading of Rheb could be that amino acid withdrawal leads to activation of the TSC1/2 complex, bringing about the hydrolysis of Rheb-bound GTP. It was therefore important to assess whether the effects of amino acid withdrawal on mTOR signaling required TSC2.

Amino Acid Withdrawal Impairs mTOR Signaling in TSC2−/− Cells—Some earlier studies have attempted to address the same issue, but the experimental protocols and cell lines used were such that it was unclear whether the cells used completely lacked functional TSC2. To obviate these problems, we have employed MEFs that have been engineered to be devoid of TSC2 (19). We confirmed previously that the TSC2−/−...
of starvation (Fig. 2A). The shift toward the less phosphorylated more mobile forms was less complete in the TSC2<sup>−/−</sup> cells than in the TSC2<sup>1+/+</sup> MEFs (Fig. 2A). Possible reasons for this are discussed and studied below. To assess the intracellular activity of S6K1, we employed an antibody that detects phosphorylated S6; as expected from the behavior of S6K1, S6 phosphorylation was almost undetectable in TSC2<sup>−/−</sup> cells and was high in the TSC2<sup>1+/+</sup> cells in the presence of amino acids (Fig. 2A). In the TSC2<sup>−/−</sup> cells, removal of amino acids caused a marked decrease in S6 phosphorylation by 90 min, although, like S6K1 phosphorylation, it remained elevated above the levels seen in the TSC2<sup>1+/+</sup> cells. There was a reproducible tendency for phosphorylation of S6 and S6K1 to recover to some extent at later times, and this is also examined further below.

These data show that amino acid starvation does impair mTOR signaling in TSC2<sup>−/−</sup> cells. However, this could be an effect specific to S6K1, the only protein studied by other workers in this regard (11, 18, 19), rather than a general effect on mTOR signaling. We therefore also studied the phosphorylation of two other proteins that are regulated by mTOR signaling. They are 4E-BP1 (44) and eEF2 kinase (36). 4E-BP1 migrates as several distinct species on SDS-PAGE, which differ in their state of phosphorylation. The overall levels of 4E-BP1 are similar in the two cell lines (Fig. 2B). However, in the TSC2<sup>−/−</sup> cells the basal level of phosphorylation of 4E-BP1 was elevated relative to the TSC2<sup>1+/+</sup> cells as is evident from its reduced electrophoretic mobility and by the greatly increased signal seen for the phosphospecific antisera for the N-terminal threonine site (Thr-389) and for Ser-64 (Fig. 2B). However, the overall intensity of the signal for Thr-69 was similar in the two cell lines. In response to amino acid starvation for 90 min, phosphorylation of Thr-389 and Ser-64 was lost in the TSC2<sup>1+/+</sup> cells and decreased somewhat in the TSC2<sup>−/−</sup> cells, especially in the case of Ser-64 (Fig. 2B). Again, phosphorylation tended to recover at later times, as for S6K1. Amino acid starvation had no discernible effect on the overall level of phosphorylation at Thr-69. The mobility shifts seen in Fig. 2B reflect changes in the phosphorylation of Ser-65; this event gives rise to the γ-species. This result is surprising given that its phosphorylation, like that of Ser-64, is dependent upon the same regulatory motif on 4E-BP1, the TOS motif, and is sensitive to rapamycin (22). In contrast, phosphorylation of Thr-36/45 is mediated via the N-terminal RAP1 motif (22); because amino acid withdrawal did cause dephosphorylation of these sites, it appears that the input mediated by this motif is sensitive to the amino acid status of the cell.

We have shown previously that insulin promotes the phosphorylation of human eEF2 kinase at Ser-78 via the mTOR pathway and that phosphorylation of the corresponding site in mouse eEF2 kinase (Ser-77) is enhanced in TSC2<sup>−/−</sup> cells relative to TSC2<sup>1+/+</sup> controls (36). As shown in Fig. 2C, amino acid starvation almost completely abolished the phosphorylation of Ser-77 in the TSC2<sup>−/−</sup> cells. Little if any signal was seen for this site in the TSC2<sup>1+/+</sup> cells, although the overall levels of eEF2 kinase were actually higher. Phosphorylation of eEF2 itself was, as expected, lower in the TSC2<sup>−/−</sup> cells than in the TSC2<sup>1+/+</sup> ones (Fig. 2C). Amino acid starvation resulted in a modest increase in the phosphorylation of eEF2 in the TSC2<sup>−/−</sup> cells, but little if any change in the level of eEF2 phosphorylation was seen in the TSC2<sup>1+/+</sup> cells (Fig. 2D).

This set of data for multiple independent targets of the mTOR pathway clearly shows that amino acid starvation still causes inactivation of mTOR signaling in cells that lack TSC2. Most importantly, this indicates that the effects of amino acid withdrawal do not require this protein and that TSC2 does not mediate directly the amino acid input into mTOR. It therefore
seems likely that the decrease in the level of GTP bound to Rheb, as elicited by amino acid starvation (Fig. 1), is not because of activation of TSC2. If there is a guanine nucleotide exchange factor for Rheb, then this may, perhaps, be regulated by amino acids instead.

Effects of Transferring Cells to D-PBS/glucose Are Because of Intracellular Amino Acids—As the effects of amino acid starvation were already maximal by 90 min, and to verify that the effects were due to amino acid withdrawal and not some other effect, we performed the following additional experiments. At shorter times of amino acid withdrawal, the effects on the phosphorylation of S6K1, S6, and 4E-BP1 are only partial and therefore 90 min of amino acid withdrawal is required for the maximal effect (Fig. 3). To confirm that the effects are due to lack of amino acids, rather than anything else that might result from shifting the cells to D-PBS/glucose, we either kept the cells in D-PBS/glucose containing a mixture of amino acids or added back amino acids for 30 min after starving the cells of amino acids for 5 h. Inclusion of amino acids in the PBS-glucose prevented the dephosphorylation of S6K1 and 4E-BP1 (Fig. 3, A–D, penultimate lane in each case). Similarly, when amino acids were resupplied after 90 min of amino acid withdrawal, phosphorylation of S6K1 and 4E-BP1 recovered at least to the initial levels seen in cells kept in DMEM (Fig. 3, A–D, last lane in each case). These data suggest that the effects on mTOR signaling of shifting cells into D-PBS/glucose are due entirely to the addition or removal of amino acids.

As a sensitive functional readout for effects of changes in 4E-BP1 phosphorylation, we subjected cell lysates to chromatography on mGTP-Sepharose a resin that binds eIF4E and thus any associated proteins. In TSC2−/− cells, the substantial amount of 4E-BP1 bound to eIF4E under amino acid-fed conditions was not increased further by amino acid starvation (Fig. 3E). In contrast, in TSC2−/− cells, no detectable 4E-BP1 binding was seen in the presence of amino acids, but 4E-BP1 binding was seen following amino acid deprivation. This effect was prevented by keeping cells in medium containing amino acids or reversed by readdition of amino acids after a period of amino acid withdrawal (Fig. 3E, last two lanes). These data are entirely consistent with the changes in the phosphorylation of 4E-BP1 already noted.

The data suggest that amino acid starvation does impair mTOR in TSC2−/− cells. This being so, one would expect that the ability of insulin to activate mTOR signaling would be decreased under this condition. As shown in Fig. 3F, insulin stimulates the phosphorylation of S6K1 in TSC2+/+ cells, as indicated by the signal observed using the S6K1 anti-Thr(P)-389 antibody. Insulin greatly increases the signal seen in the TSC2−/− cells, at least to the level seen in the TSC2 null cells. In the TSC2−/− cells, as reported above, amino acid deprivation decreases Thr(P)-389 phosphorylation, and this is barely affected by the addition of insulin (Fig. 3F). This suggests that the ability of insulin to stimulate mTOR signaling is impaired by starving TSC2−/− cells for amino acids. The data obtained with the “total” S6K1 antibody show that the mobility of S6K1 is also unaffected by insulin in amino acid-starved TSC2−/− cells, although S6K1 remains partially phosphorylated under these conditions (cf. Fig. 2).

We have noted previously (10) that treatment of amino acid-starved Chinese hamster ovary cells with protein synthesis inhibitors restores mTOR signaling, which we interpreted as providing evidence that the mTOR pathway responds to intracellular amino acids. To ascertain whether the amino acid control of mTOR signaling functions in a similar way in MEFs in the cells used here, we treated some of the amino acid-deprived cells with CHX. As shown in Fig. 4, A–C, this resulted in substantial restoration of the high levels of phosphorylation of S6K1 and S6 but had no effect on the much lower levels of phosphorylation of these proteins in TSC2−/− cells. The phosphorylation of eEF2K at Ser(P)-77 was increased by treatment of cells with CHX (Fig. 4B). Addition of CHX also decreased the level of phosphorylation of eEF2, which otherwise increases in the TSC2−/− cells following amino acid withdrawal (Fig. 4C). In the case of 4E-BP1, CHX had little effect on the phosphorylation of Ser-64 or Thr-36/45, although it did cause a shift in its migration toward the slowest moving γ-species (Fig. 4D). The
suggestions that the partial nature of the effect of amino acid withdrawal on these proteins in TSC2−/− cells is not because of mTOR-independent signaling to them, and suggests that amino acid withdrawal only has a partial effect on mTOR signaling. One reason could be that cells might still contain residual levels of amino acids even after an extended period of incubation in amino acid-free medium. Several studies have shown that the most important amino acids for controlling mTOR signaling are BCAA such as leucine (5). We therefore considered it important to assess the levels of BCAA in MEFs subjected to differing treatments.

As shown in Fig. 5, starvation of cells for 90 min led to a marked decrease in intracellular amino acid levels, although significant levels of BCAA were still detectable. Levels rose again somewhat at later times. This may explain the partial recovery in mTOR signaling that was evident at later times in Fig. 2. Because mTOR signaling acts to repress autophagy (45), and mTOR signaling is impaired in response to amino acid withdrawal, the rise in amino acid levels at later time points could be due to stimulation of autophagy. We attempted to use the compounds 3-methyladenine and methylamine (which can block autophagy (46, 47)), but we obtained complex and confusing data, which may reflect the abilities of these compounds to interfere with other processes, such as phosphatidylinositol 3-kinase signaling (48, 49).

FIG. 5. Determination of intracellular BCAA concentrations in TSC2−/− cells. Where indicated TSC2−/− cells were starved of serum for 16 h and in some cases (indicated) also of amino acids for the times shown. Ins denotes that serum-starved cells were treated with insulin at 100 nM for 30 min. CHX denotes that amino acid-starved cells (90 min) were treated with CHX (10 μM) for 30 min. Cells were lysed in 0.3 M perchloric acid, and BCAA levels were measured as described under "Materials and Methods." Data are given using serum-starved cells as the control (= 100%) and are represented as mean (±S.D.) of the data from three independent experiments.

Treatment of the amino acid-starved cells with CHX caused a marked increase in BCAA levels to roughly those seen in amino acid-replete cells (Fig. 5). This likely explains the ability of CHX treatment to restore mTOR signaling under these conditions, supports the idea that mTOR signaling is regulated by intracellular amino acids in MEFs, and agrees with our earlier findings (10).

Rheb Can Be Co-immunoprecipitated with mTOR—Data shown above reveal that amino acid starvation decreases the proportion of Rheb in its GTP-bound form. Because G-proteins generally exert their function by binding to their downstream effectors, we asked whether Rheb could interact with mTOR, and if so, whether this interaction was influenced by the amino acid status of the cells. Because of the low levels of endogenous Rheb and mTOR in MEFs, and the impossibility of transfecting them to high efficiency, we again opted to study this in HEK293 cells. HEK293 cells were therefore transfected with vectors encod-
The present study. Starving the cells for amino acids had no effect on the amount of Rheb that co-immunoprecipitated with mTOR (Fig. 6A). A concern here was that for these experiments we needed to express Rheb at rather high levels where the proportion bound to GTP or GDP was not altered by amino acid starvation. This was a potential reason why amino acid starvation did not alter the amount of mTOR associated with overexpressed Rheb.

To address this issue, we made use of mutants of Rheb that cannot bind to guanine nucleotides (50, 51). As shown in Fig. 6B, these mutants (Rheb(D60V) and Rheb(D60K)) expressed as well as wild type Rheb in HEK 293 cells. They also bound to mTOR, as assessed by co-immunoprecipitation, and indeed bound better than the wild type Rheb protein (Fig. 6B). These data show that Rheb does not need to be bound to guanine nucleotide to interact with the mTOR complex. This finding suggests that nucleotide binding to Rheb does not favor its association with mTOR and raises the key question of whether, and how, this interaction is directly related to the ability of Rheb to promote mTOR signaling. Addressing this is beyond the scope of the present investigation.

It was nonetheless of interest to study whether Rheb/mTOR binding was influenced by expression of TSC1/2, negative regulators of Rheb and mTOR signaling. As shown in Fig. 6C, expression of TSC1/2 markedly decreased the binding of Rheb to mTOR. This effect is presumably not due to the ability of TSC2 to promote GTP hydrolysis by Rheb and could, for example, instead reflect direct competition between Rheb and TSC1/2 for binding to mTOR. Rapamycin did not affect Rheb/mTOR binding (Fig. 6C), showing that it is unlikely that the inhibitory effect of the FKBP12-rapamycin complex on mTOR function is because of displacement of Rheb from mTOR.

Effects of Other Stresses—We have reported previously that certain other stress conditions also result in the impairment of mTOR signaling, as judged from their effects on targets of the pathway such as S6K1 and 4E-BP1 (28). These stresses include oxidative stress (elicited by treating cells with H2O2), osmotic stress (sorbitol), and other chemical stresses (sodium arsenite). For comparison, their effects along with those of amino acid starvation are summarized in Table I.

We treated TSC2−/− MEFs with a range of concentrations of the above stress-inducing agents, and we then analyzed the cell lysates by Western blot to study the effects on the phosphorylation of 4E-BP1 and S6K1. In MEFs, arsenite actually tended to stimulate mTOR signaling as indicated by its effects on S6K1 and 4E-BP1 (data not shown; see Table I). Arsenite has been reported to have rather complex effects on mTOR signaling in other cells (28, 52). In contrast, treatment of cells with H2O2 or sorbitol increased the binding of 4E-BP1 to eIF4E, a sensitive readout for inhibition of mTOR signaling (Fig. 7). Sorbitol also caused the dephosphorylation of S6K1 (Fig. 7), and H2O2 induced increased phosphorylation of eEF2, both indicative of impaired mTOR signaling (Fig. 7). H2O2 did not reproducibly inhibit S6K phosphorylation; we do not know the reason for this. Sorbitol actually decreased the phosphorylation of eEF2, and this may reflect the input from other stress-activated protein kinases that actually lead to inhibition of eEF2 kinase and eEF2 phosphorylation and may be activated by sorbitol (53). The observation that certain stresses impair the high basal level of mTOR signaling in the TSC2−/− cells shows that TSC2 is not required for the inhibitory effects of these stress conditions. Furthermore, this implies that H2O2 and sorbitol do not exert their effects on mTOR signaling by activating TSC2 (24).

To examine whether the stresses affected the Rheb/mTOR

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The text describes the interaction between Rheb and mTOR in the context of amino acid and stress signaling. It discusses the role of TSC1/2 and rapamycin in regulating mTOR complex association with Rheb, and it explores other stress conditions that affect mTOR signaling. The study highlights the complexity of mTOR signaling and the interplay between different stressors and their effects on the mTOR complex.

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2 X. Wang and A. Tee, unpublished data.
interaction, it was necessary to express them as epitope-tagged proteins, and we therefore employed HEK293 cells to perform these studies. The use of these cells to study the effects of cell stresses on targets of the mTOR signaling pathway in TSC2−/− cells. TSC2−/− MEFs were grown to near confluence prior to exposure to hydrogen peroxide or sorbitol for 30 min at the concentrations indicated. The notation at the top is valid for all panels. A and C, lysates were analyzed by SDS-PAGE and Western blotting using the indicated antisera. B, eIF4E (and associated proteins) were purified from 300 μg of cell lysate protein (using mGTP-Sepharose) and then analyzed by SDS-PAGE/immunoblotting using antiserum for eIF4EB and eIF4E. * indicates a nonspecific band that is consistently detected by the anti-phospho-S6K antisera, and the position of S6K is shown by the arrow. Multiple arrows denote the differently phosphorylated forms of S6K1 resolved on this gel system. Where indicated, the data have been quantitated. Results are shown as ratio of phospho- to total protein for eEF2 (C) and the ratio of 4E-BP1 to eIF4E for the mGTP-pull down (B).

Effects of ATP-depleting Agents on eEF2 Phosphorylation Are Independent of TSC2—As outlined above, AMPK may also be able to regulate the phosphorylation of eEF2 through a TSC2-independent route, involving direct phosphorylation of eEF2 kinase and its activation (26). To examine the relative importance of these TSC2-dependent and -independent pathways for controlling eEF2, we again utilized the TSC2−/− cells.

To study the effects of ATP depletion on the phosphorylation of eEF2 (and other proteins), we first treated these MEF cells with DOG. This sugar can be taken up into cells and phosphorylated by hexokinase but cannot be further metabolized. Because the hexokinase reaction consumes ATP, but none can be regenerated by further metabolism of the product, this treatment decreases cellular ATP levels, increasing AMP and activating AMPK. DOG treatment led to activation of AMPK in both cell lines as assessed by studying the phosphorylation of Ser-79 in ACC, a well characterized substrate for AMPK (Fig. 8A). Under basal conditions, eEF2 phosphorylation was low in TSC2−/− cells and even lower (undetectable) in TSC2−/− cells, which is consistent with the facts that mTOR signaling is activated in the former (reviewed in Refs. 12 and 64) and that mTOR signaling negatively regulates eEF2 phosphorylation (4). In response to treatment with DOG, the level of eEF2 phosphorylation rose markedly in both cell types. In principle, this increase could either reflect activation of AMPK (which can phosphorylate eEF2 kinase (26)) or inhibition of mTOR protein synthesis negatively promotes eEF2 phosphorylation (24). In contrast, in TSC2−/− cells, treatment with DOG had no effect on the phosphorylation of S6. This observation is consistent with the idea that TSC2 provides a link (via AMPK (24)) between ATP depletion and inhibition of mTOR signaling and indicates that the increase in eEF2 phosphorylation induced by DOG in the absence of TSC2 is not because of impairment of mTOR signaling (24).

The phosphorylation of ribosomal protein S6 was therefore studied as a surrogate readout for mTOR signaling. Treatment of TSC2−/− cells with DOG caused a marked fall in the level of S6 phosphorylation (Fig. 8A), as expected from the negative influence of AMPK on mTOR signaling (24). In contrast, in TSC2−/− cells, treatment with DOG had no effect on the phosphorylation of S6. This observation is consistent with the idea that TSC2 provides a link (via AMPK (24)) between ATP depletion and inhibition of mTOR signaling and indicates that the increase in eEF2 phosphorylation induced by DOG in the absence of TSC2 is not because of impairment of mTOR signaling (24).

To study this more closely, we treated cells with a range of concentrations of DOG. To assess the efficacy of DOG in activity AMPK, we employed two complementary approaches. In the first, AMPK was immunoprecipitated from cell lysates and then assayed for activity against a peptide substrate. This revealed that in both TSC2−/− and TSC2−/− cells, AMPKmediated activation of AMPK (Fig. 8B). In the second approach, we employed the anti-ACC Ser(1)-79 antibody. This revealed that DOG increased ACC phosphorylation in both TSC2−/− and TSC2−/− cells (Fig. 8D). If anything, activation of AMPK (as indicated by both criteria) was slightly greater in the TSC2−/− cells than in the controls. To render the cells more sensitive to low DOG, we changed the medium to one containing lower concentrations of d-glucose (5.6 mM) than the medium usually used for MEFs (which contains 25 mM d-glucose). This results in a small increase in basal eEF2 phosphorylation (cf. Fig. 2A). About 9–10 h later, we treated the cells with a range of concentrations of DOG. Over such a range, the level of phosphorylation of eEF2 increased progressively in both the TSC2−/−

### Table I

| Condition | Cell type | Phosphorylation of 4E-BP1 or 4E-BP1/eIF4E binding | Phosphorylation of S6 | Phosphorylation of S6K1 | Phosphorylation of eEF2 | Rheb/mTOR binding |
|-----------|-----------|-----------------------------------------------|---------------------|-----------------------|----------------------|-------------------|
| Amino acids | MEF | ↑ | ND | ND | ↑ | ND |
| H2O2 | MEF | ↑ | ND | ND | ↑ | ND |
| Sorbitol | MEF | ↑ | ND | ND | ↑ | ND |
| Arsenite | MEF | ↑ | ND | ND | ↑ | ND |

Note: ↑ and ↓ indicate increases and decreases, respectively; — indicates no change; ND indicates not determined. MEF refers to the TSC2−/− MEFs.

**Fig. 7. Effects of cell stresses on targets of the mTOR signaling pathway in TSC2−/− cells.** TSC2−/− MEFs were grown to near confluence prior to exposure to hydrogen peroxide or sorbitol for 30 min at the concentrations indicated. The notation at the top is valid for all panels. A and C, lysates were analyzed by SDS-PAGE and Western blotting using the indicated antisera. B, eIF4E (and associated proteins) were purified from 300 μg of cell lysate protein (using mGTP-Sepharose) and then analyzed by SDS-PAGE/immunoblotting using antiserum for eIF4EB and eIF4E. * indicates a nonspecific band that is consistently detected by the anti-phospho-S6K antisera, and the position of S6K is shown by the arrow. Multiple arrows denote the differently phosphorylated forms of S6K1 resolved on this gel system. Where indicated, the data have been quantitated. Results are shown as ratio of phospho- to total protein for eEF2 (C) and the ratio of 4E-BP1 to eIF4E for the mGTP-pull down (B).

**Summary of effects of stress conditions on targets for mTOR signaling and Rheb/mTOR association**

| Condition | Cell type | Phosphorylation of 4E-BP1 or 4E-BP1/eIF4E binding | Phosphorylation of S6 | Phosphorylation of S6K1 | Phosphorylation of eEF2 | Rheb/mTOR binding |
|-----------|-----------|-----------------------------------------------|---------------------|-----------------------|----------------------|-------------------|
| Amino acids | MEF | ↑ | ND | ND | ↑ | ND |
| H2O2 | MEF | ↑ | ND | ND | ↑ | ND |
| Sorbitol | MEF | ↑ | ND | ND | ↑ | ND |
| Arsenite | MEF | ↑ | ND | ND | ↑ | ND |

Note: ↑ and ↓ indicate increases and decreases, respectively; — indicates no change; ND indicates not determined. MEF refers to the TSC2−/− MEFs.
and the TSC2−/− cells (Fig. 8C). To monitor mTOR signaling in these experiments, we examined the state of phosphorylation of eEF2 and other targets for mTOR signaling. A, TSC2+/+ and TSC2−/− MEFs (90% confluent) were incubated in DMEM (containing 5.6 mM d-glucose) plus fetal calf serum and treated with 10 mM DOG for 30 min. Lysates were subjected to SDS-PAGE and Western blotted for the following: ACC phosphorylated at Ser-79, eEF2 phosphorylated at Thr-56, eEF2 and S6 phosphorylated at Ser-235/236, eEF2 and other targets for mTOR signaling was less susceptible to low concentrations of DOG than in the TSC2+/+ cells. Nonetheless, as already noted, eEF2 phosphorylation increased to a similar extent in both. This is again consistent with the operation of the mechanism proposed by Dennis et al. (55) where, because of the high Km of mTOR for ATP, severe reductions in cellular ATP levels impair signaling downstream of mTOR.

To avoid this effect, we used an alternative approach to activate AMPK that avoids actual ATP depletion. AICAR is metabolized within cells to yield an activator of AMPK (56, 57). As shown in Fig. 8D, AICAR treatment led to increased phosphorylation of eEF2 in both the TSC2+/+ and the TSC2−/− cells. This indicates that the effect of AMPK is independent of TSC2; indeed, if anything, the effect AICAR was greater in the TSC2−/− cells than in the TSC2+/+ controls. This finding is consistent with earlier data for other types of cells (25–27) where AICAR has been shown to increase the phosphorylation of eEF2, and demonstrates that agents that specifically activate AMPK also elicit increased phosphorylation of eEF2 in the cell type used here (MEFs). In the TSC2−/− cells, AICAR caused a dephosphorylation of 4E-BP1 as evidenced by its shift toward more mobile, less phosphorylated, species (Fig. 8D). In contrast, no significant shift was seen in TSC2+/+ cells, which again is consistent with a requirement for TSC2 to link activation of AMPK to inhibition of mTOR signaling (24).

When cells were exposed to AICAR for differing times, 4E-BP1 underwent rapid dephosphorylation in the TSC2−/− cells but was not affected by AICAR in the TSC2+/+ cells, consistent with a requirement for TSC2 to link AMPK activation to inhibition of mTOR signaling (Fig. 8E). In contrast, phosphorylation of eEF2 was elevated by AICAR in both cell types and to similar extents, although it increased more slowly in the TSC2−/− cells. This again shows there are contributions from TSC2-dependent and -independent inputs into the control of eEF2 phosphorylation.

Finally, we wished to examine effects of ATP depletion or AMPK activation on eEF2 kinase itself. We have shown recently (36) that phosphorylation of human eEF2 kinase at Ser-78, which is adjacent to its CaM-binding site, plays a key
role in its regulation through mTOR signaling. Phosphorylation of this site increases in response to insulin, and this effect requires signaling through mTOR (36). Phosphorylation of Ser-78 impairs the binding of eEF2 kinase to CaM, thus decreasing its activity. The data above suggest that moderate activation of AMPK increases eEF2 phosphorylation in TSC2-/- cells, even in the absence of an effect on mTOR signaling. To study this further, we made use of a phosphospecific antibody for Ser-78 that we have developed (36) and that recognizes the corresponding site, Ser-77, in rodent eEF2 kinase. Ser-78 phosphorylation was detectable in TSC2-/- and TSC2+/+ cells under control conditions (Fig. 9A). The strength of the signal for phospho-Ser-77 was greater in the TSC2-/- cells than in the controls, even though the overall amount of eEF2 kinase is lower in them, indicating that, as expected, basal phosphorylation of Ser-77 is higher in the TSC2-/- cells (10). In response to treatment with low concentrations of DOG or with AICAR, Ser-77 phosphorylation decreased markedly in the TSC2-/- cells. In contrast, no change was observed in the TSC2+/+ cells. This confirms that these conditions do not affect mTOR signaling or phosphorylation of the key regulatory site in eEF2 kinase in the TSC2-/- cells. Thus, the increase in eEF2 phosphorylation observed in TSC2-/- cells in response to agents that activate AMPK is apparently not because of modulation of regulatory inputs from mTOR to eEF2 kinase. Instead, it seemed likely that it involves modulation of eEF2 kinase by AMPK-mediated phosphorylation. The major phosphorylation site involved here is Ser-398 (26). However, despite exhaustive attempts we were unable to observe a signal for this site using our phosphospecific antiserum (26), probably due to the low levels of eEF2 kinase present in the MEFs.

The mechanism by which agents that increase eEF2 phosphorylation, such as AICAR and DOG, exert this effect is likely through the activation of eEF2 kinase (see Ref. 26). We therefore tested the effects of DOG or AICAR on eEF2 kinase activity in TSC2-/- or TSC2+/+ cells. As shown in Fig. 9B, AICAR and DOG each activate eEF2 kinase to similar extents in the TSC2-/- or TSC2+/+ cells. Data from multiple experiments are collated and presented in Fig. 9C. They are consistent with the observed changes in eEF2 phosphorylation and indicate that regulation of mTOR signaling is not required for the major effect of AMPK activation on the control of eEF2.

Conclusions—The data presented here clearly show that TSC2 is not required for the effects of amino acid starvation on proteins that are regulated by mTOR. Two previous reports (11, 18) have indicated that TSC2 antagonizes the effects of amino acids on this pathway. One interpretation of these data is that TSC2 forms part of the amino acid sensing mechanism and relays information about this, presumably through Rheb, to the mTOR complex. In this model, amino acid sufficiency would act to inhibit TSC2 function and thus promote mTOR signaling. Amino acid withdrawal would thus be expected to activate TSC2, leading to inhibition of Rheb and mTOR. Because we find that amino acid withdrawal still impairs mTOR signaling in cells that are devoid of TSC2, this model cannot be correct. They appear to rule out the possibilities that TSC2 itself operates as an amino acid sensor or as the recipient of information from the sensing mechanism. Our data substantially extend those of Zhang et al. (19), who observed partial impairment of mTOR signaling (S6K1) in response to amino acid withdrawal in TSC2-/- cells. The effect of amino acid withdrawal was also only partial in the Eker rat-derived cell line studied by Gao et al. (18), an observation which they attributed to the fact that this line is not null for TSC2. A further possibility would be that the effects of amino acids are only partially dependent upon and/or mediated through TSC2. Our data provide an alternative explanation, i.e. that the partial nature of the defects in mTOR signaling reflects the production of amino acids from intracellular sources (autophagy), which provides sufficient amounts of these compounds to support some level of mTOR function. The effects of amino acid withdrawal in the TSC2-/- cells are reversed when cells are treated with CHX to prevent amino acids being consumed by protein synthesis (see also Ref. 10).

Tee et al. (11) also reported that the TSC1/2 complex inhibits amino acid-stimulated mTOR signaling. Their study (11) and that of Pan and co-workers (18) preceded the identification of Rheb as the target of the GAP activity of TSC2. It is thus likely that the inhibitory effects of TSC1/2 observed by Tee et al. (11) reflect the ability of high level expression of the TSC1/2 complex to drive hydrolysis of Rheb-bound GTP, thus “countering” the amino acid input into mTOR. The data can be interpreted as suggesting that amino acids promote formation of Rheb-GTP. They could do this by stimulating the (unknown) guanine nucleotide exchange factor for Rheb. Alternatively, they may provide an input to mTOR that is independent of
Rheb. These possible mechanisms are depicted schematically in Fig. 10.

An important prediction from this study is that mammalian cells possess a so far unidentified amino acid sensor, because TSC2 does not appear to have this function. A key goal must now be to identify the amino acid-sensing mechanism that controls mTOR signaling. Our data also show that the effects of hyperosmolarity and H₂O₂-induced oxidative stress on mTOR signaling do not require TSC2, again indicating that there are TSC2-independent mechanisms for regulating mTOR function.

The present data also show, for the first time, that Rheb forms complexes that also contain mTOR, although we cannot conclude whether there is actually a direct interaction between mTOR and Rheb. Although this could provide a mechanism by which Rheb regulates mTOR, our data also raise questions in that the Rheb/ mTOR signaling do not require TSC2, again indicating that there are TSC2-independent mechanisms for regulating mTOR function.