**Cell Reports**

**Rheb Inhibits Protein Synthesis by Activating the PERK-eIF2α Signaling Cascade**

**Graphical Abstract**

Known role for Rheb GTPase

- Rheb
- mTOR kinase
- 4EBP1 P
- S6K P
- Protein synthesis

New role for Rheb GTPase

- Rheb
- PERK kinase
- eIF2α P

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**In Brief**

Tyagi et al. now show that the ubiquitously expressed GTPase Rheb binds and activates the ER-associated kinase PERK and suppresses protein synthesis, thus elucidating the cellular mechanisms by which Rheb GTPase balances protein translation.

**Highlights**

- Rheb suppresses protein synthesis in vitro and in vivo
- Rheb induces phosphorylation of eIF2α independent of mTORC1
- Rheb binds directly to PERK and increases its activity in vitro
Rheb Inhibits Protein Synthesis by Activating the PERK-eIF2α Signaling Cascade

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SUMMARY

Rheb, a ubiquitous small GTPase, is well known to bind and activate mTOR, which augments protein synthesis. Inhibition of protein synthesis is also physiologically regulated. Thus, with cell stress, the unfolded protein response system leads to phosphorylation of the initiation factor eIF2α and arrest of protein synthesis. We now demonstrate a major role for Rheb in inhibiting protein synthesis by enhancing the phosphorylation of eIF2α by protein kinase-like ER kinase (PERK). Interplay between the stimulatory and inhibitory roles of Rheb may enable cells to modulate protein synthesis in response to varying environmental stresses.

INTRODUCTION

Protein synthesis is orchestrated by an array of proteins, which activate or inhibit translation based on their phosphorylation status. The mTOR system enhances protein synthesis in response to nutrients and growth factors. Cellular stress, on the other hand, triggers processes that inhibit translation in order to conserve cellular resources. Phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) is a major mediator of this inhibitory system and involves four discrete kinases: protein kinase-like ER kinase (PERK), general control nonderepressible 2 (GCN2), protein kinase RNA-activated (also known as protein kinase R; PKR), and the heme-regulated inhibitor (HRI) (Donnelly et al., 2013).

There exists an inverse relationship between mTOR signaling and phospho-eIF2α. Under pathological conditions, such as apoptosis, hypoxia, serum, and nutrient deprivation, mTOR activity is downregulated whereas phospho-eIF2α is upregulated, leading to diminished global protein synthesis (Deng et al., 2008; Hara et al., 1998; Kim et al., 2002; Koumenis et al., 2002; Liu et al., 2006; Schneider et al., 2008; Tee and Proud, 2001). In some instances, mTOR and eIF2α may act in parallel. For example, deletion of TSC2, an inhibitor of mTOR signaling, augments signaling both by mTOR and by phospho-eIF2α (Ozcan et al., 2008). Pharmacologic and genetic studies suggest that mTOR signaling does influence phospho-eIF2α. For instance, the mTOR inhibitor rapamycin is known to upregulate phospho-eIF2α in some cells (Anand and Gruppuso, 2006; Kato et al., 2012; Kubota et al., 2003; Matsuo et al., 2005). Similarly, heat inactivation of mTOR potentiates phospho-eIF2α in yeast cells, whereas the genetic deletion of PTEN, a negative regulator of mTOR, reduces phospho-eIF2α in cancerous cells (Mounir et al., 2009; Valbuena et al., 2012).

The GTPase Rheb is well established as an inducer of mTOR thereby augmenting protein synthesis. Here, we demonstrate that Rheb plays a major role in inhibiting protein synthesis by enhancing PERK-mediated phospho-eIF2α levels. This action may underlie, in part, the reciprocal relationship of mTOR and eIF2α signaling.

RESULTS

GTPase Rheb Inhibits Protein Synthesis

The canonical mTOR pathway involves TSC1/2 binding to and inhibiting Rheb, preventing activation of mTOR signaling by Rheb (Inoki et al., 2003). Rheb, acting via mTOR, is generally regarded as a physiologic stimulant of protein synthesis (Hall et al., 2007; Wang et al., 2008). By contrast, in human embryonic kidney 293 (HEK293) cells, we observe that overexpression of Rheb is associated with diminished protein synthesis, as measured by incorporation of [35S]-Met (Figure 1A). Tunicamycin, an ER stressor, elicits cell stress and inhibited protein synthesis (Figure 1A). We confirm that overexpression of Rheb augments phospho-S6 kinase, an index of mTOR signaling (Figure S1). The marked stimulation by tunicamycin of CHOP levels (Figure S1), indicating that diminished incorporation of [35S]-Met...
by Rheb (Figure 1A) may not be due to cellular stress. Inhibition of protein synthesis by Rheb is dependent upon its guanine-nucleotide binding, as it is absent with Rheb-D60K (Figure 1B), which cannot bind guanosine triphosphate (GTP) or guanosine diphosphate (Aspura and Tamaoki, 2004). Polysome profiles revealed the Rheb wild-type (WT)-overexpressing cells showed a reduced polysome/monosome ratio (Figure 1C). We next assessed whether Rheb can directly modulate protein synthesis in vitro (Figure 1D). Rheb WT, but not Rheb D60K, markedly decreases luciferase mRNA translation, an effect also elicited by active PERK kinase, a known inhibitor of protein synthesis (Harding et al., 1999). Rheb overexpression increased the viability of the HEK293 cells in an MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)]-2H-tetrazolium]; inner salt) assay (which measures mitochondrial activity), but it did not significantly alter the cell number (counted using a hemocytometer) (Figure S2). In TSC2 depleted fibroblasts, which did not significantly alter the cell number (counted using a hemocytometer) (Figure S2), consistent with previous report compared to TSC2 intact cells (Auerbach et al., 2011). TSC2 depleted fibroblasts also exhibited reduced polysome/monosome ratio (Figure 1F) and diminished cell numbers, compared to TSC2 intact cells (Figure S3). Thus, GTPase Rheb can act as a negative regulator of protein synthesis.

Rheb Enhances the Phosphorylation of eIF2α, which Inhibits Protein Synthesis

By what mechanism might Rheb inhibit protein synthesis? We examined regulation of eIF2α, whose phosphorylation is associated with inhibition of protein synthesis (Harding et al., 1999). Overexpressing Rheb (2- to 4-fold more than endogenous Rheb) in the absence or presence of serum markedly stimulates phosphorylation of eIF2α at serine-51 with an increase comparable to the stimulation of mTOR signaling monitored as phospho-S6 kinase/phospho-4EBP1 (Figure 2A). This action of Rheb is dependent upon its guanine-nucleotide binding, as levels of phospho-eIF2α, like those of phospho-S6 kinase and phospho-4EBP1, are markedly reduced in the presence of Rheb-D60K (Figure 2B).

Rheb influences phospho-eIF2α levels in intact animals. Thus, injections of adeno-associated viral particles expressing Rheb directly into the hippocampus elicit a substantial increase in phospho-eIF2α and phospho-S6 kinase (Figure 2C). Further evidence that Rheb is required for phosphorylation of eIF2α comes from experiments depleting Rheb by small hairpin RNA (shRNA) leading to a profound reduction in phospho-eIF2α as well as phospho-S6 kinase/phospho-4EBP1 (Figure 2D). Rheb also altered the protein expressions of eIF2α, the target of mTORC1 signaling (Browne and Proud, 2002; Hay and Sonenberg, 2004), and ATF-4, the target of phospho-eIF2α signaling (Lu et al., 2004). While Rheb WT overexpression increased the eIF2α/ATF-4 protein levels, Rheb shRNA treatment attenuated those levels (Figures S4A–S4D). Note that although ATF-4 levels go up, CHOP, one of the ATF-4 target genes, is not increased (Figure S1). Although mechanisms are unclear, CHOP can also be induced through ATF-4-independent pathways. It has been shown that ATF-4−/− mouse embryonic fibroblasts (MEFs) elicit CHOP expression induced by thapsigargin (Ma and Hendershot, 2004) and that ATF-4 is necessary, but not sufficient, for the induction of CHOP expression under certain conditions (Harding et al., 2000). Thus, Rheb might selectively induce ATF-4, but not its downstream target, CHOP, under the experimental conditions used in this study.

We then tested whether Rheb is necessary for ER-stress-mediated phospho-eIF2α and protein synthesis. As expected, the ER stressor tunicamycin increased phospho-eIF2α levels and decreased the phosphorylation of an mTORC1 target, 4EBP1, in a time-dependent manner, consistent with previous reports (Deldicque et al., 2011; Li et al., 2008), and that increase was markedly reduced in Rheb shRNA-treated cells (Figure 2E). While Rheb depletion (~50%) did not markedly affect the [35S] Met-incorporation in control conditions, it prevented the loss of [35S] Met-incorporation induced by tunicamycin (Figure 2F). Thus, Rheb physiologically regulates ER-stress-induced phospho-eIF2α levels and protein synthesis.

Rheb Induces Phosphorylation of eIF2α Independent of mTORC1, PI3K, and MAPK Signaling

Experiments with rapamycin rule out mTOR as mediating Rheb’s effects on phospho-eIF2α. Rapamycin virtually abolishes stimulation by Rheb of phospho-S6 kinase and phospho-4EBP1 but has negligible effects on phospho-eIF2α (Figure 3A). This finding buttresses the conclusion that phosphorylation of eIF2α does not involve mTORC1, consistent with previous reports (Bunpo et al., 2009; O’Connor et al., 2008). In contrast to phosphorylation of eIF2α, which is not mediated by mTORC1, phosphorylation of other elongation factors does involve mTORC1. Thus, rapamycin prevents phosphorylation of eIF4A and eIF4B, targets of S6K (Shahbazian et al., 2006), but not eIF2α (Figure 3B).

Kinase inhibitors provide additional evidence of specificity (Figure 3C). Thus, wortmannin, which inhibits PI3 kinase, abolishes phosphorylation of Akt and greatly reduces phospho-S6 kinase but does not alter phosphorylation of eIF2α or Erk.
PD98095, which inhibits the mitogen-activated protein kinase (MAPK) pathway, greatly reduces phospho-Erk but does not influence phospho-eIF2α, phospho-Akt, or phospho-S6 kinase. By contrast, rapamycin, as expected, abolishes phospho-S6 kinase but fails to influence phospho-eIF2α, phospho-Erk or phospho-Akt (Figure 3C). Thus Rheb induces phosphorylation of eIF2α independent of mTORC1, PI3K and MAPK signaling.

**Rheb Elicits Phosphorylation of eIF2α via PERK**

Four protein kinases are known to phosphorylate eIF2α: GCN2, PERK, PKR, and HRI (Wek et al., 2006). Of these, GCN2 and PERK account for the great bulk of eIF2α phosphorylation and so have been the focus of our attention. Adenoviral-mediated Rheb expression enhances phosphorylation of eIF2α or reduces [35S] Met labeling comparably in GCN2 WT and GCN2-deleted MEFs (Figures 3D and 3E). By contrast, the Rheb-mediated increase in the phospho-eIF2α levels or suppression of [35S] Met-labeling is markedly reduced in PERK deleted MEFs (Figures 3F and 3G). Rheb overexpression also increased the viability and reduced the polysome/monosome ratio in PERK-intact MEFs compared to PERK-deleted MEFs (Figures SSA and SSB). In these overexpression studies, we found exogenously expressed Rheb levels are 2- to 4-fold higher than endogenous Rheb levels. Interestingly, Rheb overexpression had no significant effect on cell viability but decreased the proliferation of PERK-deleted MEFs, indicating that loss of PERK might interfere with Rheb’s effect on cell proliferation (Figure S5C). We also confirmed importance of PERK in Rheb-mediated phospho-eIF2α in HEK293 cells transiently transfected with PERK shRNA (Figure S6). Thus, Rheb modulates phospho-eIF2α and affects protein synthesis predominantly via PERK.

We assessed the physical association of Rheb and PERK and how Rheb modulates PERK activity in vitro. An intimate association of PERK and Rheb is evident in the robust binding of overexpressed PERK with endogenous Rheb, or overexpressed Rheb with endogenous PERK, in HEK293 cells (Figures 4A and 4B). Binding between these two proteins is direct. Rheb WT, compared to Rheb D60K, binds strongly to PERK, as demonstrated with purified proteins in vitro (Figure 4C).

Further evidence for the importance of Rheb in the phosphorylation of eIF2α comes with PERK kinase experiments showing a concentration-response relationship for Rheb’s stimulation of eIF2α phosphorylation in vitro (Figure 4D). This activity is GTP dependent, as Rheb D60K does not elicit PERK-kinase activity in vitro (Figure 4E). This phosphorylation is selective for eIF2α-S51, as it is abolished with S51A mutations (Figure 4F). Since ER stress upregulated the phosphorylation of eIF2α but downregulated mTORC1 signaling (Figure 2E), consistent with previous reports (Deldicque et al., 2011; Li et al., 2009), we wondered whether this inverse signaling might occur due to an enhanced Rheb interaction with PERK during ER stress. Although tunicamycin treatment abolished the Rheb-mTOR interaction, Rheb-PERK interactions remained unaffected (Figure 4G). Thus, depending upon the cellular status, such as ER stress, the Rheb-mTOR interaction would be dismantled, but Rheb-PERK interactions might be stabilized. Though this interaction may not be increased further by ER stress, we cannot rule out the possibility that this Rheb-PERK complex is more active under stress conditions. Thus, under ER stress conditions, Rheb might selectively associate with PERK to facilitate reduction of protein synthesis through eIF2α signaling.

**DISCUSSION**

A canonical cascade for protein synthesis involves interactions of Rheb with mTOR to activate the mTORC1 protein synthesis pathway (Wang and Proud, 2006). In the present study, we demonstrate an alternative pathway in which Rheb inhibits protein synthesis by stimulating the phosphorylation of eIF2α. In terms of the overall protein synthetic activity of cells, inhibition of protein synthesis by Rheb may be its principal role, as overexpressing Rheb decreases cellular protein synthesis.

The mechanism whereby Rheb inhibits protein synthesis appears to involve enhancement of phosphorylation of eIF2α predominantly by the PERK protein kinase. Thus, deletion of PERK, but not another protein kinase, GCN2, markedly abolishes Rheb-mediated enhancement of phospho-eIF2α. Rheb overexpression appears to decrease extracellular regulated kinase (Erk) phosphorylation, a signaling linked to protein synthesis (Kelleher et al., 2004). This is consistent with previous findings in which deletion of mTOR or Rheb elicits phosphorylation of Erk (Carracedo et al., 2008; Kelleher et al., 2004; Li et al., 2011). Whether Rheb-mediated reduction of Erk signaling is a feedback loop to regulate protein synthesis remains unclear. Nevertheless, rapamycin, which increases Erk signaling (Carracedo et al., 2008), actually inhibits rather than promotes protein synthesis in certain cells (Huo et al., 2011). Thus, the significance of cross-talk between Rheb and Erk signaling and what role they play in protein synthesis remains unclear.

Inhibition of protein synthesis in response to stressful stimuli is a major mechanism to protect cells from stress-elicted cell death. Phosphorylation of eIF2α by PERK is regarded as a principal means whereby cell stress downregulates protein synthesis. In the absence of cell stress, eIF2α forms a ternary complex...
Figure 3. Rheb Induces Phospho-eIF2α Independent of mTORC1 and Promotes Phospho-eIF2α Predominantly through PERK

(A) Western blotting of indicated proteins of HEK293 expressing cDNAs of myc (control) or myc-Rheb WT were grown in DMEM with serum (+) or without serum (−) or pretreated with the inhibitors rapamycin (250 nM) or DMSO (0.5%, control).

(B) Western blotting of indicated proteins in HEK293 cells expressing myc or myc-Rheb and pretreated with rapamycin or DMSO as in (A).

(C) Western blotting of indicated proteins in HEK293 cells expressing myc or myc-Rheb and pretreated with inhibitors of MAPK (PD98059, 50 μM) or PI3K (wortmannin, 100 nM), rapamycin, or DMSO.

(legend continued on next page)
with tRNA and the 43S ribosome, which initiates translation (Kimball, 1999). With cell stress, phosphorylation of eIF2α prevents the formation of the ternary complex, abrogating new protein formation.

Though Rheb is well known to activate mTOR, the physiologic rationale for this action has not been altogether clear. Our findings suggest that Rheb may function as a molecular switch between stimulation and inhibition of protein synthesis (see Graphical Abstract). Thus, by binding to mTOR kinase, Rheb stimulates protein synthesis. On the other hand, when it binds to PERK to enhance phosphorylation of eIF2α, protein synthesis is inhibited. Under what cellular contexts these opposite functions of Rheb are involved remains less clear. When cells are actively growing, Rheb-mTOR circuitry might promote protein synthesis, and when cells are under stress, for example during uncontrolled growth, the Rheb-PERK circuitry might reduce protein synthesis. This later notion is supported by our binding experiments in which we found that under ER-stress conditions (where mTORC1 activation is reduced and P-eIF2α signaling is increased), Rheb loses its interaction with mTOR but remains bound to PERK (Figure 4G). As cell growth and ER stress are intimately connected (Tsang et al., 2010), such dynamic binding of Rheb may regulate normal physiology to reciprocally alter mTOR and phospho-eIF2α signaling, as observed in various cell types (Anand and Gruppuso, 2006; Deng et al., 2002; Hara et al., 1998; Kato et al., 2012; Kim et al., 2002; Koumenis et al., 2002; Kubota et al., 2003; Liu et al., 2006; Matsuo et al., 2005; Schneider et al., 2008; Teo and Proud, 2001).

Our model raises the possibility that a hitherto-unidentified posttranslational modification of Rheb—perhaps phosphorylation—mediates the reciprocity of Rheb’s capacities to stimulate or inhibit protein synthesis. Bioinformatic analysis (GPS phosphorylation prediction software) indicates that Rheb possesses several consensus sequences for phosphorylation, for example, threonine 38 for protein kinase B, serine 130 for ribosomal S6 kinase, serine 179 for CAM kinase, and tyrosine 67 for tyrosine kinase. Phosphorylation of Rheb at serine 130 by p38-regulated kinase inhibits Rheb-mediated mTORC1 activation (Zheng et al., 2011). Whether phosphorylation or other posttranslational modifications, such as acetylation, differentiate Rheb’s interactions with mTOR versus PERK remains to be seen.

The Rheb/PERK/eIF2α system may influence major organismic activities. Recently, we found Rheb GTPase can regulate BACE1, a principle enzyme implicated in Alzheimer’s disease (AD) pathology (Shahani et al., 2014). PERK and phospho-eIF2α levels are linked to BACE1 regulation and AD-related behavioral deficits (Dev and Ohno, 2010; Ma et al., 2013; O’Connor et al., 2008). We also found huntingtin serves as a new effector of Rheb GTPase to activate mTORC1 to modulate Huntington disease (HD)-related symptoms (Pryor et al., 2014). Though the role of phospho-eIF2α signaling in HD symptoms is not clear, a recent study implicated phospho-eIF2α in HD cellular toxicity (Leitman et al., 2014). Moreover, Rheb through phospho-eIF2α may regulate ER stress—a feature linked to all major age-related diseases, such as diabetes, Parkinson’s disease, AD, and HD (Chang et al., 2002; Harding et al., 1999; Hoozemans et al., 2007; Kang et al., 2011; Kouroku et al., 2007). Thus, Rheb-eIF2α circuitry may have multiple cellular functions with implications in physiological as well as pathological conditions.

**EXPERIMENTAL PROCEDURES**

**Reagents, Antibodies, and Proteins**

Most of the reagents were purchased from Sigma, unless mentioned otherwise. Details regarding cDNA, antibodies, viral particles, and other reagents used can be found in the Supplemental Experimental Procedures.

**Cell Culture, Transfections, and Amino Acid Treatments**

HEK293 cells were grown in DMEM (Gibco 11965-092) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 5 mM glutamine. In brief, cells were seeded in 3.5 or 6 cm plates. After 18-24 hr, the cells were transfected with cDNA constructs using polyfect (Qiagen) as per the manufacturer’s instructions. For the serum starvation protocol, after 48 hr, the growth media was replaced with DMEM with or without 10% FBS. Cells were kept in these media for 1-2 hr and lysed, followed by western blotting.

**Immunoblotting, Commmunoprecipitation, and In Vitro Binding**

Glutathione-affinity and western blotting and in vitro binding protocols were carried out essentially as described before (Shahani et al., 2014; Subramaniam et al., 2009, 2010). More details can be found in the Supplemental Experimental Procedures.

**Cell Viability and Proliferation Assay**

HEK293 cells (transfected with indicated vectors) or MEFs (infected with null or Rheb adenoviruses) were grown in 24-well plates, and after 48 hr, the viability was assessed using an MTS assay, as per the manufacturer’s protocol (Promega). For the proliferation assay, an equal number (1 x 10⁶) of HEK293 cells or MEFs were seeded in 24-well plates transfected or infected with Rheb, and after 48 hr, cell numbers were counted using a hemocytometer.

**Recombinant Protein Purification**

Recombinant proteins were produced essentially as indicated before (Tyagi et al., 2009). More information can be found in the Supplemental Experimental Procedures.

**PERK In Vitro Kinase Assay**

In vitro kinase assays with recombinant bacterially expressed glutathione S-transferase (GST)-PERK-KD or GST-PERK-ΔKD were performed at 30°C for 30 min in 30 μl of kinase buffer (20 mM HEPES [pH 7.5], 50 mM KCl, 1 mM DTT, 2 mM MgCl₂, 0.1 mM ATP) containing 1 μg of purified GST-eIF2α-WT or GST-eIF2α-S51A proteins as substrate. Phosphorylation of eIF2α was detected using western blotting with phospho-eIF2α antibody. Where indicated, purified Rheb WT or Rheb D60K protein (50 or 100 ng) was added to the in vitro kinase reaction.

**In Vitro Translation Assay**

In vitro translation assays were performed as described earlier (Harding et al., 1999). Briefly, to measure translational inhibition by Rheb and PERK, purified...
Figure 4. Rheb GTPase Directly Binds and Activates PERK In Vitro

(A) Western blotting/glutathione-binding affinity analysis of indicated proteins in HEK293 cells expressing GST or GST-Rheb.
(B) Western blotting analysis of immunoprecipitated (myc-IgG) proteins in HEK293 cells expressing myc or myc-PERK (and corresponding input).
(C) Western blotting of glutathione-GST affinity-purified recombinant proteins (and corresponding input) containing equimolar concentrations of untagged Rheb WT or Rheb D60K, and GST-PERK-KD (kinase domain).
(D) Western blotting indicated proteins of in vitro PERK kinase activity containing PERK-KD with eIF2α in the absence or presence of untagged Rheb (50 and 100 ng).
(E) Western blotting indicated proteins of in vitro PERK kinase activity containing PERK-KD with eIF2α in the presence of GST-Rheb WT or GST-Rheb D60K (100 ng).
(F) Western blotting indicated proteins of in vitro PERK kinase activity with GST-eIF2α WT and GST-eIF2α S51A.
(G) Western blotting/glutathione-binding affinity analysis of indicated proteins in HEK293 cells transfected with GST or GST-Rheb in the presence of DMSO (−, 0.5%) or tunicamycin (+, 100 nM).

**p < 0.01; *p < 0.05; n.s, not significant (Student’s t test). Data are means ± SEM from three experiments.
50 ng GST-PERK-KD or GST-PERK-ΔKD and 500 ng of GST-Rheb WT or GST-Rheb-D60K were preincubated for 10 min at room temperature with 12 μl of an in vitro translation mixture (Promega) before the addition of 0.5 μg in vitro-transcribed capped luciferase mRNA. The [35S] Met-labeled luciferase protein was resolved by 4%–12% Bis-Tris gel (Invitrogen).

Intrahippocampal AAV Injection
Mouse protocols were carried out under the guidelines approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute. Adeno-associated virus (AAV)-Rheb was stereotaxically injected into the hippocampus of adult (postnatal day 90) male C57BL/6 mice, and tissue/protein extraction and western blotting was carried out as described before (Shahani et al., 2014).

L-[³⁵S]-Met Labeling of HEK293 Cells
Myc (control), myc-Rheb WT, or myc-Rheb D60K vectors (1 or 2 μg each) were transfected, using polyfect, into HEK293 cells in DMEM plus 10% serum and grown in six-well plates (Invitrogen). After 2 days, the media was replaced with labeling media (methionine/cysteine-free DMEM media, Invitrogen) and 100 μCi/well of [³⁵S]-Met for 30 min, and cells were directly lysed either in 2× Laemmli buffer with β-mercaptoethanol or in Tris buffer with 100 μg of cycloheximide before loading onto the gels. The gels were blotted onto a polyvinylidene fluoride membrane, then subjected to autoradiography (Bio-Rad), protein staining (ponceau), or the detection of phosphorylation of eIF2α and other indicated proteins by western blotting. For tunicamycin (Tuni) experiments, Tuni (100 nM) was added 3 hr before changing the medium to labeling media with [³⁵S]-Met.

Experiments with Mouse Embryonic Fibroblasts
TSC2−/− and TSC2±/− MEFs were from David J. Kwiatkowski (Brigham and Women’s Hospital, Boston). PERK−/− and PERK±/− MEFs were from David Ron (University of Cambridge, Cambridge), and GCN2−/− and GCN2±/− MEFs were from ATCC. More details can be found in the Supplemental Experimental Procedures.

Polysome Profiling
Polysome profiling was essentially carried out as described before (Theoreen et al., 2012). For more details, see the Supplemental Experimental Procedures.

Statistical Analysis
Data were expressed as means ± SEM as indicated. All experiments were performed at least in triplicate and repeated twice. Statistical analysis was performed with a Student’s t test (Microsoft Excel).

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cellrep.2015.01.014.

AUTHOR CONTRIBUTIONS
S. Subramaniam initiated the project and conducted initial cell culture/biochemical work. R.T. carried out binding, in vitro translation, and in vitro kinase and TSC2[³⁵S]-Met work. N.S. carried out PERK/GCN2 MEF work, cell viability and proliferation work, and polysome profiling, L.G. assisted with MEF-related work. M.F. carried out polysome profiling, M.F. and K.K. shared the polysome-related reagents and provided helpful discussion. W.P. performed hippocampal injections with the help of S. Swamkar, P.F.W., and P.Y.C. provided reagents and helpful discussion. S. Subramaniam and S.H.S. wrote the manuscript with input from co-authors.

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