Supplemental Information

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

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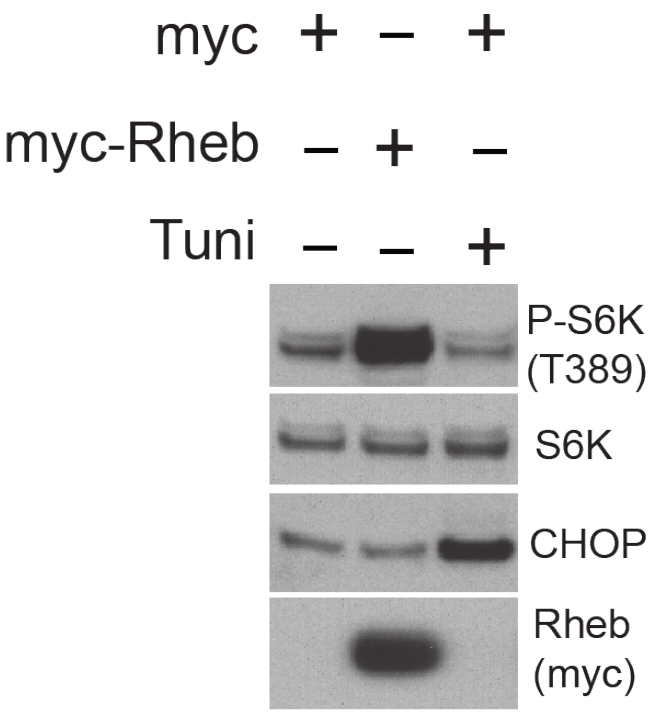

Figure S1

Figure S1, **related to Figure 1**. **Rheb does not induce CHOP expression.** Western blotting analysis of myc or myc-Rheb expressing cells were probed for the indicated protein. Tunicamycin (100 nM) is used as a control.

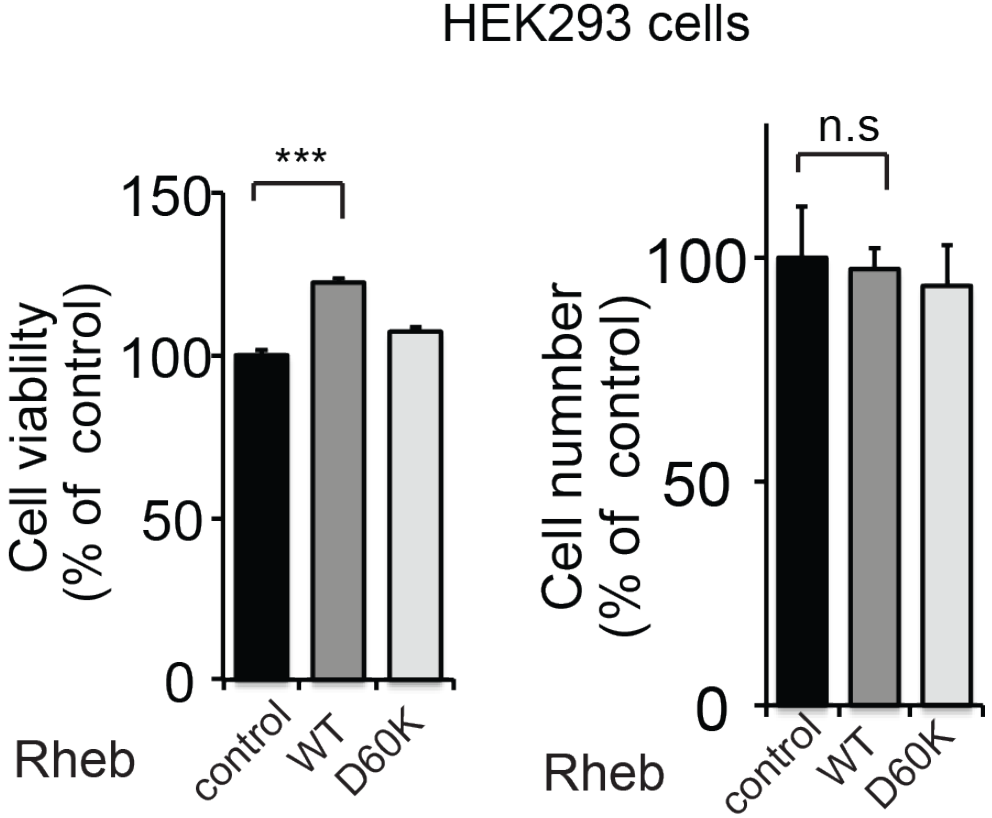
HEK293 cells
***
150
100
50
0
Cell viability
(% of control)
Rheb
control
WT
D60K
n.s
100
50
0
Cell numnber
(% of control)
Rheb
control
WT
D60K

Figure S2

Figure S2, related to Figure 1. Effect of Rheb on cell viability and proliferation. (A) MTS cell viability assay, and (B) the hemocytometer counting of number of cells in HEK293 cells expressing myc, myc-Rheb WT or Rheb D60K. (***p<0.001 vs. myc, Student *t* test). Data are means ± SEM from 3 experiments.

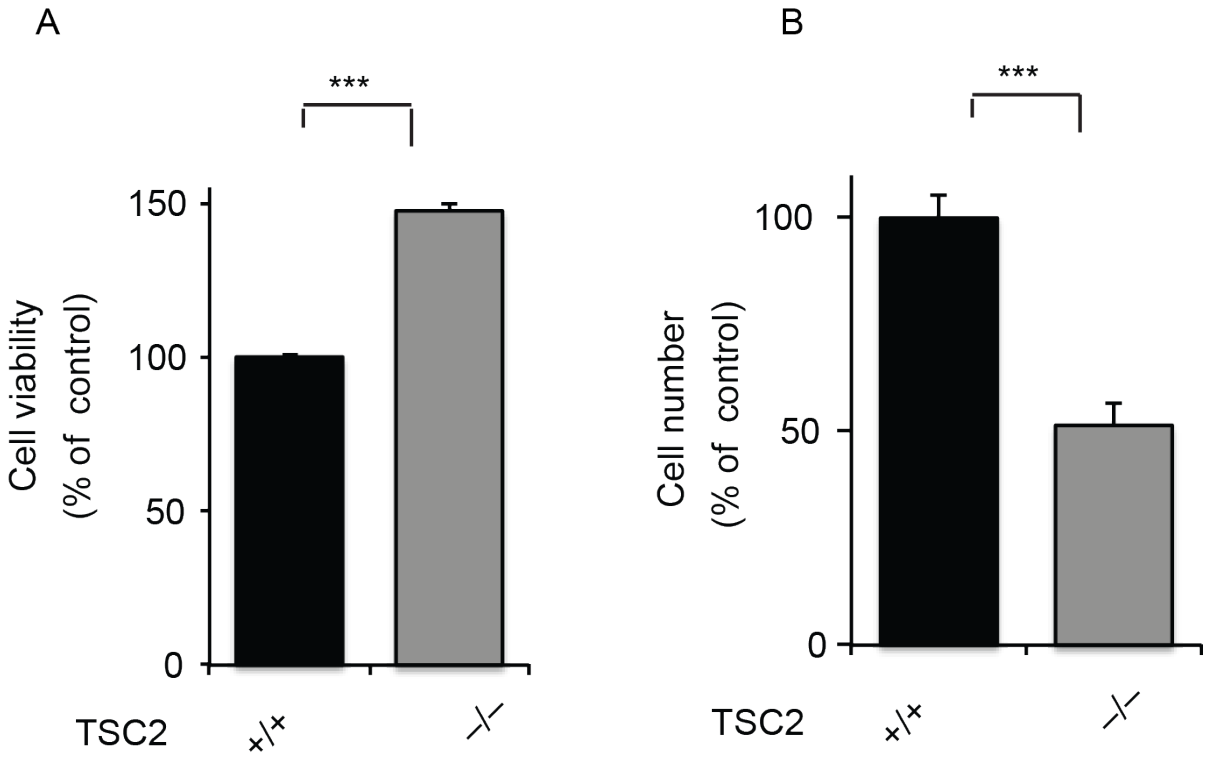
A
***
150
100
50
0
Cell viability
(% of control)
TSC2
+/+
-/-
B
***
100
50
0
Cell number
(% of control)
TSC2
+/+
-/-

Figure S3

Figure S3, related to Figure 1. *TSC2*$^{-/-}$ MEFs cell proliferate slower than *TSC2*$^{+/+}$ MEFs. Equal number of TSC2 WT (+/+) cells and KO (-/-) MEFs was seeded and after 48 hr, cell viability (MTS assay) or cell numbers (hemocytometer) were estimated (***p<0.001 vs. *TSC2*$^{+/+}$, Student *t* test). Data are means ± SEM from 3 experiments.

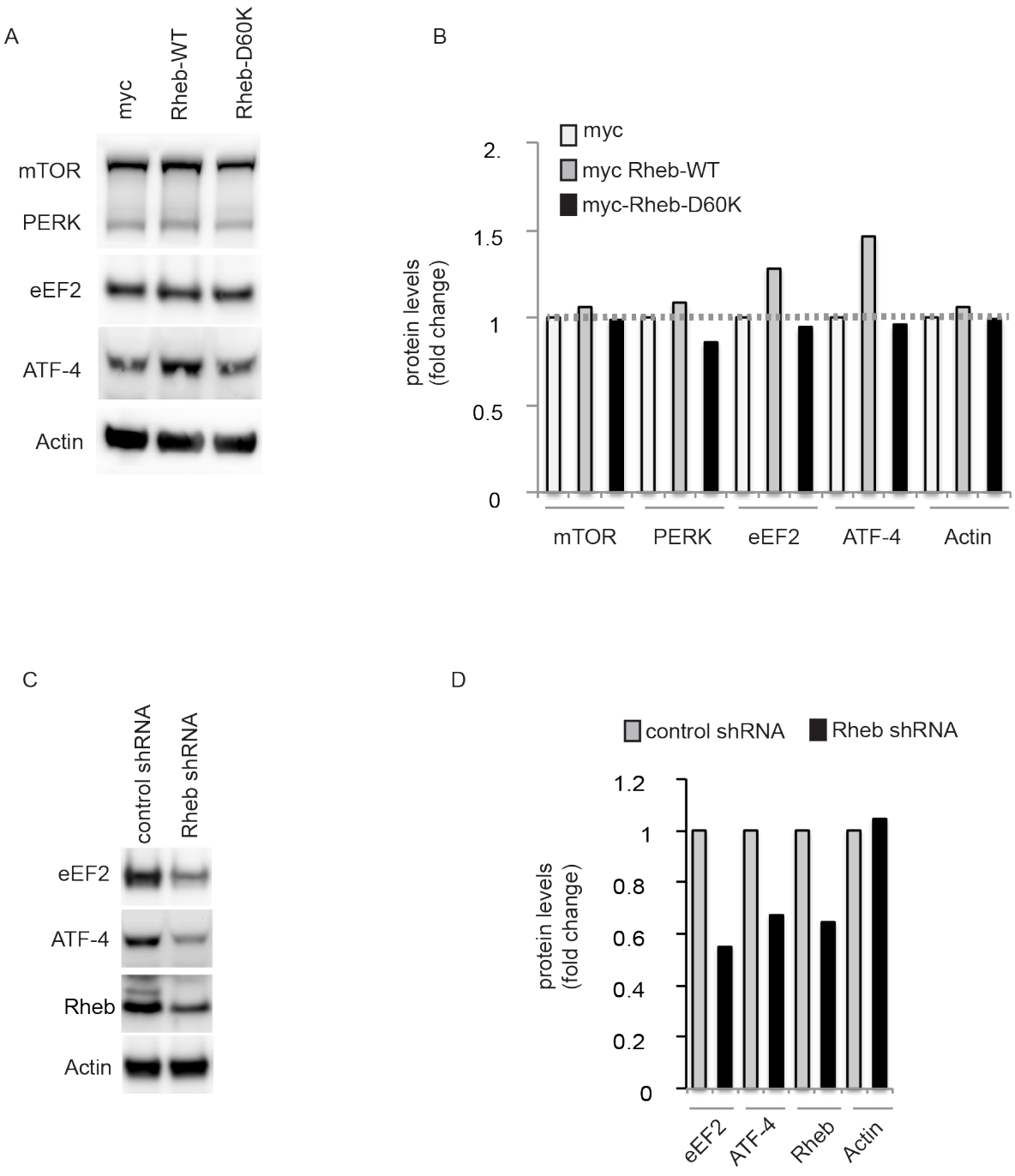
A
myc
Rheb-WT
Rheb-D60K
mTOR
PERK
eEF2
ATF-4
Actin
B
myc
myc Rheb-WT
myc-Rheb-D60K
protein levels
(fold change)
2.
1.5
1
0.5
0
mTOR
PERK
eEF2
ATF-4
Actin
C
control shRNA
Rheb shRNA
eEF2
ATF-4
Rheb
Actin
D
control shRNA
Rheb shRNA
protein levels
(fold change)
1.2
1
0.8
0.6
0.4
0.2
0
eEF2
ATF-4
Rheb
Actin

Figure S4

Figure S4, related to Figure 2. Rheb alters the protein levels of eEF4 and ATF-4. (A) Western blot analysis and (B) relative levels of indicated proteins in HEK293 cells expressing myc or myc Rheb WT or Rheb D60K. (C) Western blot analysis and (D) relative levels of indicated proteins in HEK293 cells expressing control shRNA or Rheb-shRNA.

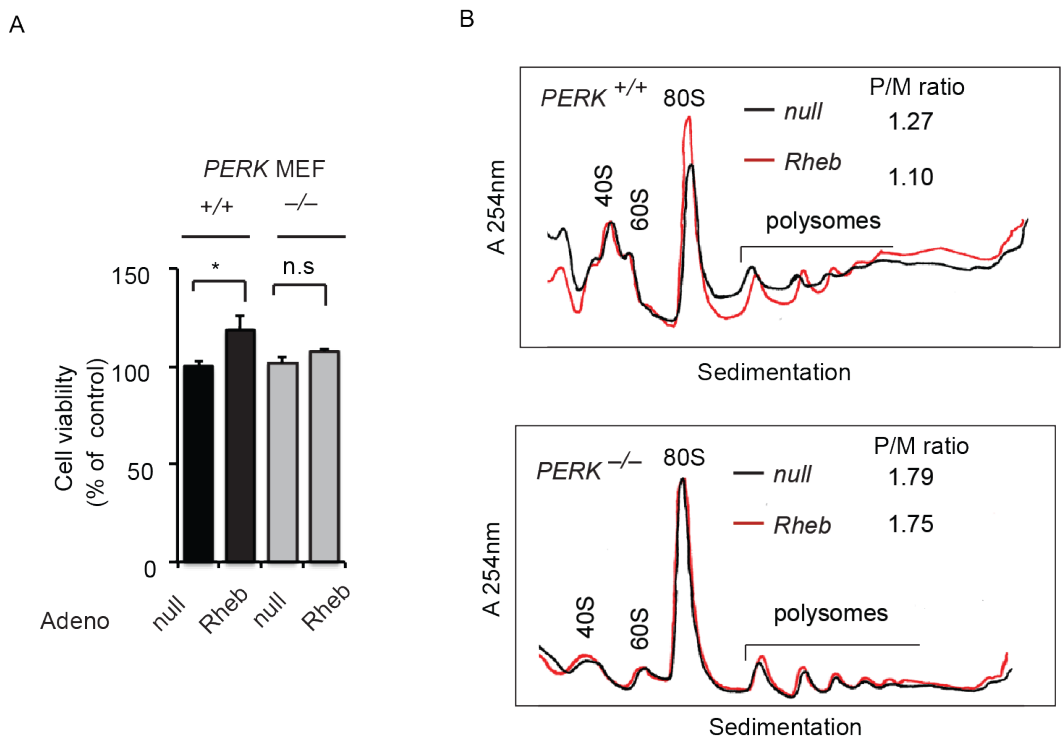
A
PERK MEF
+/+
–/–
*
n.s
150
100
50
0
Cell viability (% of control)
Adeno
null
Rheb
null
Rheb
B
PERK +/+
80S
null
Rheb
P/M ratio
1.27
1.10
40S
60S
polysomes
A 254nm
Sedimentation
PERK –/–
80S
null
Rheb
P/M ratio
1.79
1.75
40S
60S
polysomes
A 254nm
Sedimentation
C
PERK MEF
+/+
–/–
n.s
*
150
100
50
0
Cell number (% of control)
Adeno
null
Rheb
null
Rheb

Figure S5

Figure S5, related to Figure 3. Effect of Rheb on *PERK* MEF viability, polysome profiling and proliferation. (A) MTS assay of cell viability, (B) polysome profiling, and (C) hemocytometer counting of cell number in $PERK^{+/+}$ and $PERK^{-/-}$ MEFs infected with null or Rheb expressing adenovirus. (*p<0.05; **p<0.01 vs. null, Student *t* test). Data are means ± SEM from 3 experiments.

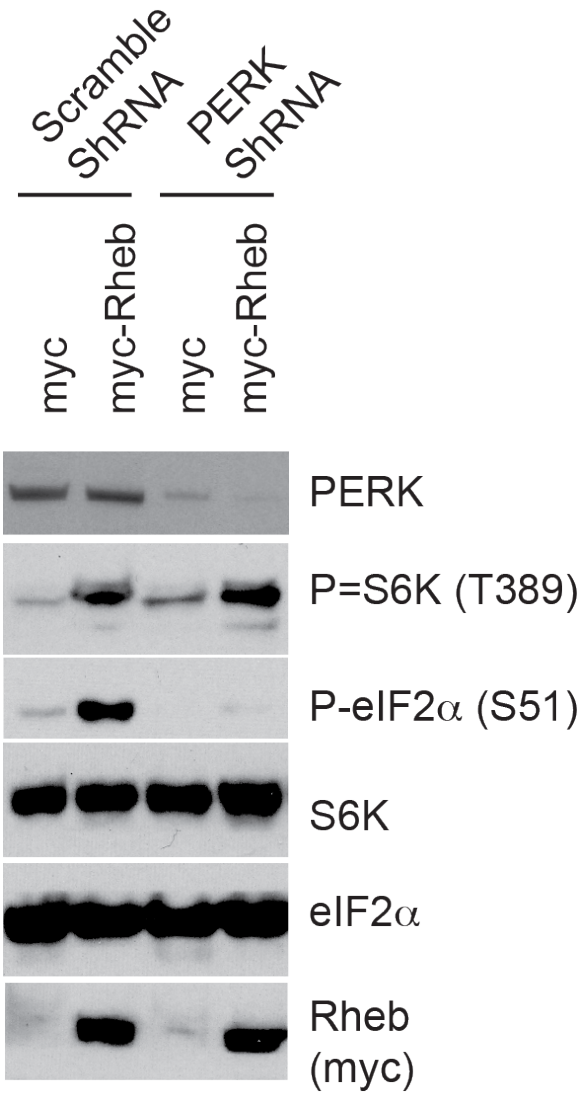
Scramble ShRNA
PERK ShRNA
myc
myc-Rheb
myc
myc-Rheb
PERK
P=S6K (T389)
P-eIF2α (S51)
S6K
eIF2α
Rheb (myc)

Figure S6

Figure S6, related to Figure 3. Rheb GTPase fails to stimulate P-eIF2α in PERK depleted HEK293 cells. Western blotting analysis of indicated protein from HEK293 cells expressing, myc or myc-Rheb cDNA together with control shRNA or PERK shRNA.

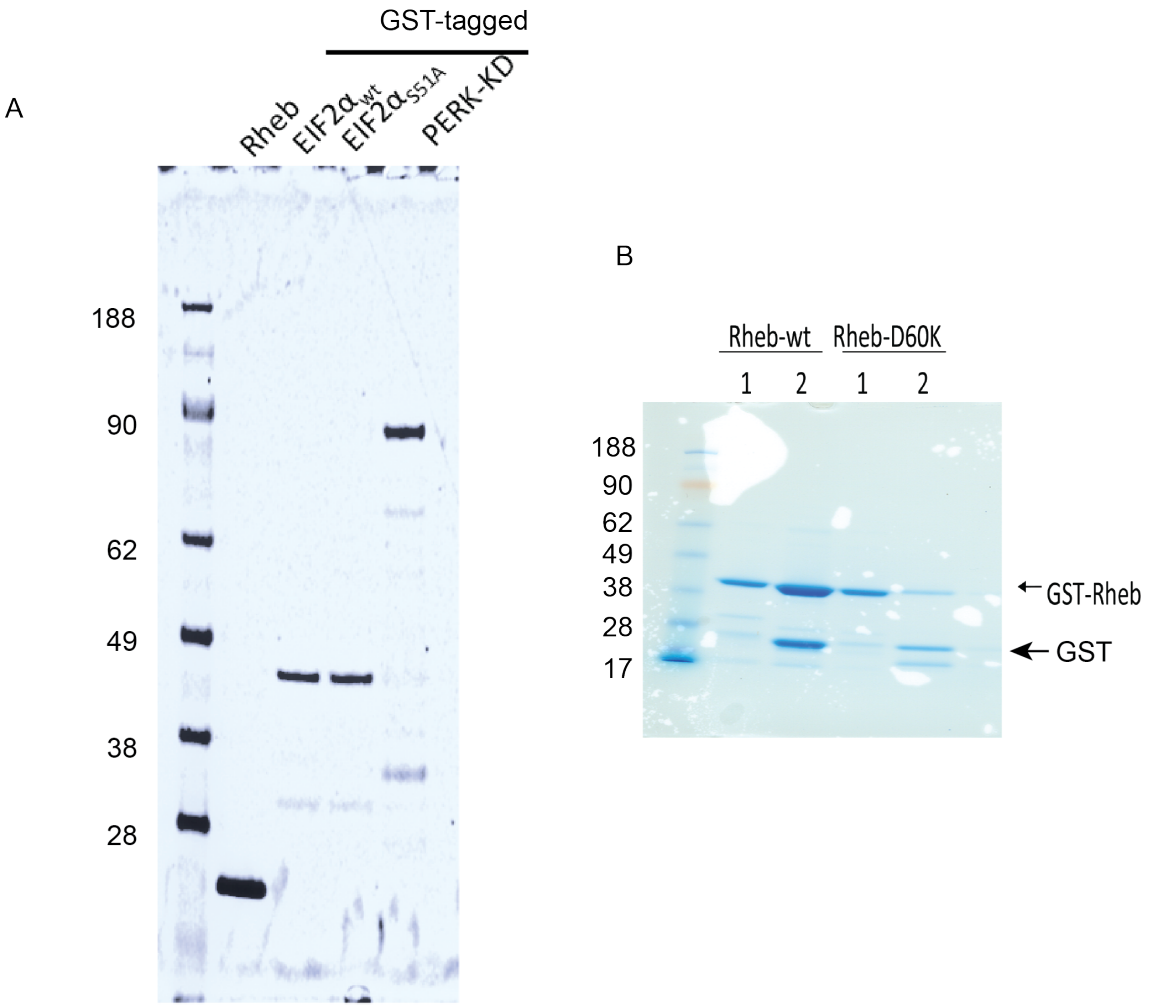
A
GST-tagged
Rheb
EIF2α wt
EIF2α S51A
PERK-KD
188
90
62
49
38
28
B
Rheb-wt
Rheb-D60K
1
2
1
2
188
90
62
49
38
28
17
GST-Rheb
GST

Figure S7

Figure S7, **refers to quality of purified protein. Commassie gel depicting the purity of recombinant proteins.** (A) GST-eIF2α, GST-PERK-KD (kinase domain) or Rheb WT. (B) GST-Rheb WT and GST-Rheb D60K, with partial cleavage of GST fusion proteins.

SUPPLMENTAL EXPERIMENTAL PROCEDURES

Reagents, Plasmids and Antibodies

Myc-tagged Rheb WT and D60K was a gift from Kun-Liang Guan. Rheb-WT and D60K were subcloned into pCMV-GST or pGEX-6P2 vectors as described before (Subramaniam et al., 2010). The scrambled shRNA lentiviral control vector was from Addgene, and Rheb shRNA424 was from Sigma (TRCN0000010424, Sigma). PERK shRNA was from Sigma (TRCN0000001401)(Bobrovnikova-Marjon et al., 2010). Antibodies for Rheb, GST and Myc were obtained from Santa Cruz (sc130398, sc138 and sc40 respectively). Antibodies against eIF2α (5324), P-eIF2α (3398) mTOR (2972), pS6K T389 (9205), pS6 S235/236 (4858), p4EBP S65 (9451), pAkt S473 (4060), and p44/42 Erk1/2 (4695), PERK (3192), GCN2 (3302), ATF-4 (11815), S6K (9202), S6 (2217), 4EBP1 (9644), CHOP (5554) and other translational regulators were from Cell Signaling Technology Inc. Glutathione-Sepharose beads were from Amersham Biosciences, and Protein G/Protein A agarose suspension was obtained from Calbiochem. Rapamycin, U0126, and wortmannin were from Selleckchem. GST-tagged PERK-WT with kinase domain (PERK-KD) or PERK-K618R defective in kinase activity (PERK- ΔKD) was from Addgene, GST-tagged eIF2α WT and eIF2α S51A were from Thomas Dever (National Institute of Health, Bethesda), and recombinant Rheb was purchased from PROSPEC, Israel. The protease inhibitor tablets were from Roche, and phosphatase inhibitor cocktails were from Sigma. Pre-made adenoviral-Rheb or adeno-control (null) particles were purchased from Applied Biologicals (British Columbia, Canada). AAV-GFP and AAV-Rheb were produced as described before (Shahani et al., 2013). Cell viability MTS assay kit was purchased from Promega.

Briefly, brain tissue was snap frozen then homogenized in RIPA buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with protease inhibitor cocktail (Roche). Protein lysates were loaded and separated by 4-12% Bis-Tris Gel (Invitrogen) transferred to PVDF membranes and probed with the indicated antibodies. Secondary antibodies were HRP-conjugated (Jackson Immuno Research Inc). Chemiluminescence was detected using WesternBright Quantum (Advansta). Where indicated tunicamycin (100nM) was added for the indicated time points in control shRNA or Rheb shRNA treated cells before proceeding to Western blotting.

Recombinant protein purification

Recombinant proteins are produced as indicated in the supplementary procedure. GST-PERK-KD or GST-PERK- ΔKD, GST-eIF2α-WT and GST-eIF2α-S51A proteins were expressed as described earlier (Tyagi et al., 2009). Briefly, proteins were expressed in *E. coli* BL21DE3 strain after induction with isopropyl β-D-1-thiogalactopyranoside (500 μM) for 20hr at 20°C. Cells were lysed by sonication in a buffer containing 50 mM Tris/HCl (pH 8.2), 1 mM dithiothreitol, 100 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM benzamidine, and 2 mM phenylmethylsulfonyl fluoride followed by centrifugation at 30,000 X *g*. The supernatant was interacted with GSH beads, and the matrix was washed with a similar buffer. This was followed by washes with a buffer containing 50 mM Tris/HCl (pH 8.2), 1 mM dithiothreitol, 100 mM NaCl, 2 mM EDTA, and 0.1% Triton-X. Proteins were eluted with a buffer containing 50 mM Tris/HCl (pH 8.2), 1 mM dithiothreitol, 50 mM NaCl, and 10 mM reduced glutathione. This was followed by dialysis with a buffer containing 50 mM Tris/HCl (pH 8.2), 1 mM dithiothreitol, and 50 mM NaCl. Where indicated the GST was cleaved from GST-tagged proteins, by prescisson protease cleavage, as describe before (Subramaniam et al., 2010). Proteins were stored

in aliquots at -70°C until further use. The quality of purified protein is shown in Figures S7A, S7B.

Co-immunoprecipitation, and *in vitro* Binding

Myc (control) and myc-PERK WT plasmids were transfected, using Lipofectamine 2000, into HEK293 cells in DMEM plus 10% fetal calf serum (Invitrogen). At 48hr post-transfection cells were lysed in lysis buffer (50 mM Tris/HCl (pH 8.2), 100 mM NaCl, and 1% NP-40 with protease inhibitor cocktail). Supernatants were incubated with Myc IgG and protein A/G agarose beads for 16hr at 4°C. Subsequently, beads were washed three times with lysis buffer and loaded on 4-12% Bis-Tris Gel (Invitrogen) and transferred to PVDF membranes. Western blotting was carried out using Rheb antibody. PERK protein was detected using Myc antibody. For *in vitro* binding assay, equimolar concentrations of recombinant purified GST or GST-tagged PERK, were incubated with Rheb in binding buffer containing 50 mM Tris/HCl (pH 8.2), 1 mM dithiothreitol, 100 mM NaCl, and 1% NP-40 for 16hr at 4°C with glutathione beads, and Rheb was detected through Western blotting. In tunicamycin experiments, the tunicamycin (100nM) or DMSO (0.5%) was added to the cells expressing GST or GST-Rheb for 6-9hrs, and the cells were lysed in lysis buffer and GST pulldown was carried out as indicated above.

Experiments with Mouse Embryonic Fibroblasts

TSC2 MEFs were grown in DMEM plus 10% serum. PERK MEFs and GCN2 MEFs were grown in full media (DMEM and 10% serum plus 1% non-essential amino acids, NEAA). For Adeno-Rheb infection, the PERK and GCN2 MEFs were grown in full media for 24 hr (in 12-well plate). The full media was changed to serum free DMEM + NEAA media and infected with Adeno-null or Adeno-Rheb (3 µl/well of adenovirus, 5×10^{10} optical particle unit, opu/µl) for 36-48 hr. The serum free media was replaced with full media for

2 hr, and the cells lysed to detect P-eIF2α and other proteins as indicated in the manuscript. For [^{35}S]-Met labeling, TSC2$^{-/-}$ MEFs were cultured in DMEM + serum for 48 hr and then replaced with methionine/cysteine-free DMEM + serum with 50-100 μCi/well of [^{35}S]-Met for 2 hr, followed by lysis with 2% SDS lysis buffer. Equal volumes of lysates were loaded onto the gel and transferred onto PVDF membrane to detect S35 labeling using a phosphoimager (name). Total protein was detected by staining the membrane with Ponceau S (Sigma). The S35 signal was quantified using Fiji-software, followed by graphical plot and statistical analysis using MS Excel.

Polysome profiling

Briefly, cells grown in 10 cm dishes (2-3 dishes of 70-80% confluence) were treated with cycloheximide (100μg/ml) for 15 minutes, washed once in 1x PBS + 100μg/ml cycloheximide and then lysed in homogenization buffer [15 mM Hepes-KOH [pH 7.4], 7.5 mM MgCl2, 100 mM KCl, 2 mM DTT, 1.0% Triton X-100, 100μg/ml cycloheximide, and 1X EDTA-free protease inhibitors (Roche) and RNAsin (20units/ml)]. Cell lysates were triturated 5 times with 26G needle, spun at 12000 rpm (5 min), loaded onto a linear density (10%-50% W/W) sucrose gradient (15 mM Hepes-KOH, 7.5 mM MgCl2, 100 mM KCl, 2 mM DTT, 100 μg/ml cycloheximide) and then subjected to ultracentrifugation (40,000 rpm/2hr, 4^{o}C) in a Beckman SW41 rotor. The gradient samples were run through UV reader (ISCO UA-6), and the polysome profiles were recorded. For polysome/monosome ration the area under the peaks was measured using Fiji-software.

SUPPLMENTAL REFERENCES

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