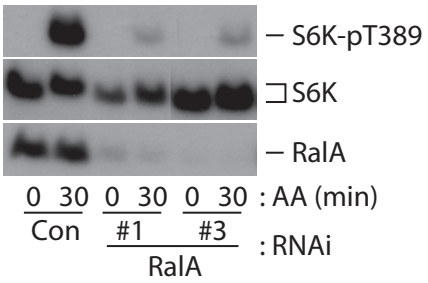
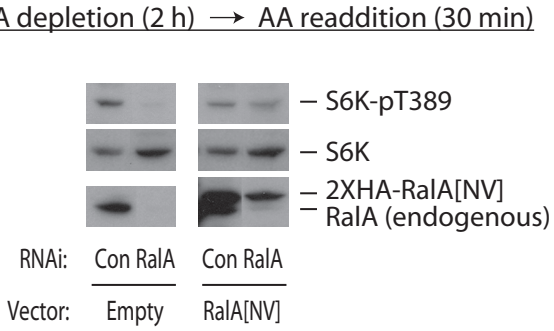


Supplemental Figure 1. Two independent siRNAs against RalA inhibit RalA expression and amino acid-induced S6K phosphorylation.

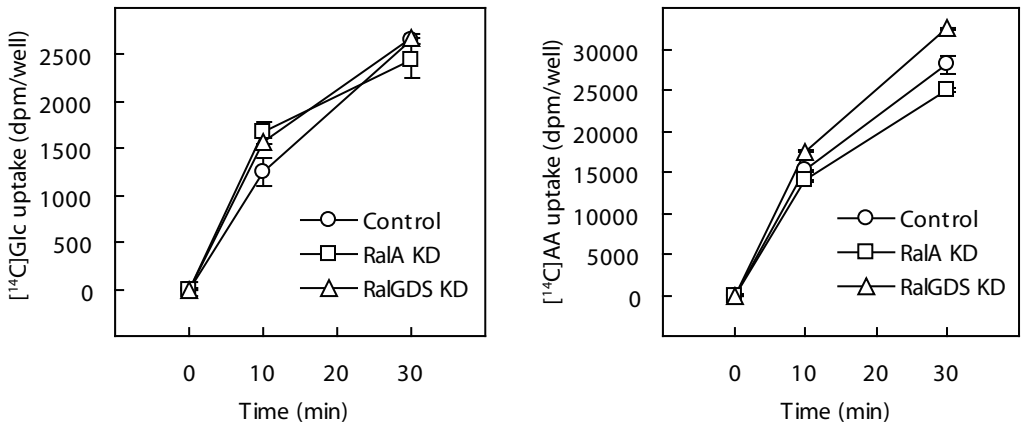
HeLa cells were transfected with siRNA as indicated. After 2-day culture, cells were starved for amino acids for 2 h. Cells were then treated with amino acids for indicated times, followed by immunoblot analysis. Unprocessed original gel images are represented in Supplemental Figure 5.



Supplemental Figure 2. RNAi-resistant form of RalA overcomes the inhibitory effect of RalA knockdown on amino acid-induced S6K phosphorylation.

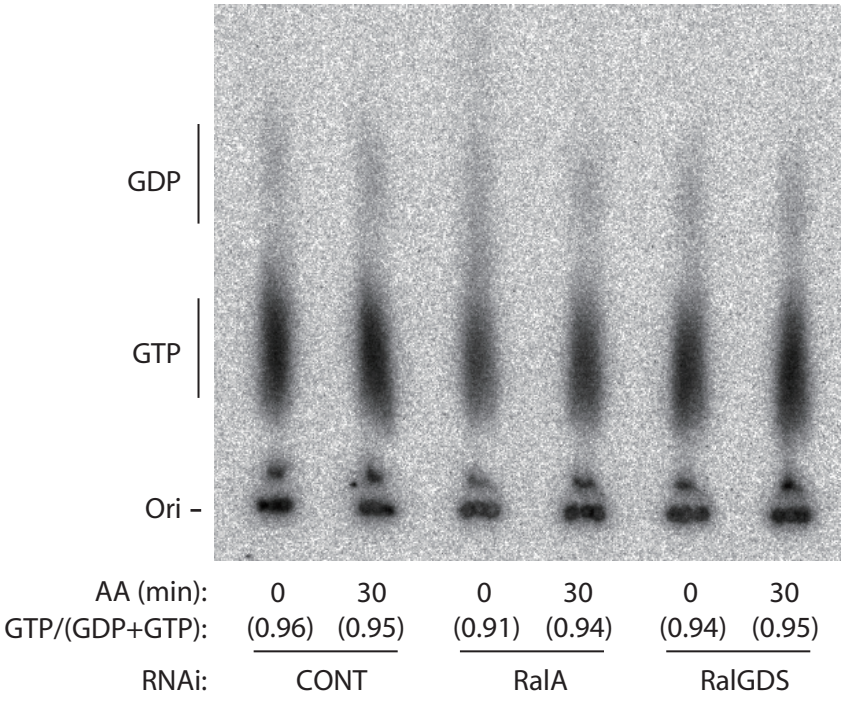
RNAi-resistant RalA mutant (for RALA RNAi #3) was generated by introducing silent mutations at codon 150 (asparagine, aat/aac) and 151 (valine, gtt/gtg). RalA cDNA bearing the silent mutations with tandem HA-tag at the N-terminus was cloned into BamHI/XhoI site of pcDNA3 (Invitrogen) to generate 2XHA-RalA[N150V151]/pcDNA3.

HeLa cells were transfected with control siRNA or RalA siRNA #3. After 1-day culture, cells were further transfected with either 2XHA-RalA[N150V151]/pcDNA3 or empty vector, and cultured for 1 day. Cells were then starved for amino acids for 2 h, then treated with amino acids for 30 min, followed by immunoblot analysis. Unprocessed original gel images are represented in Supplemental Figure 5.

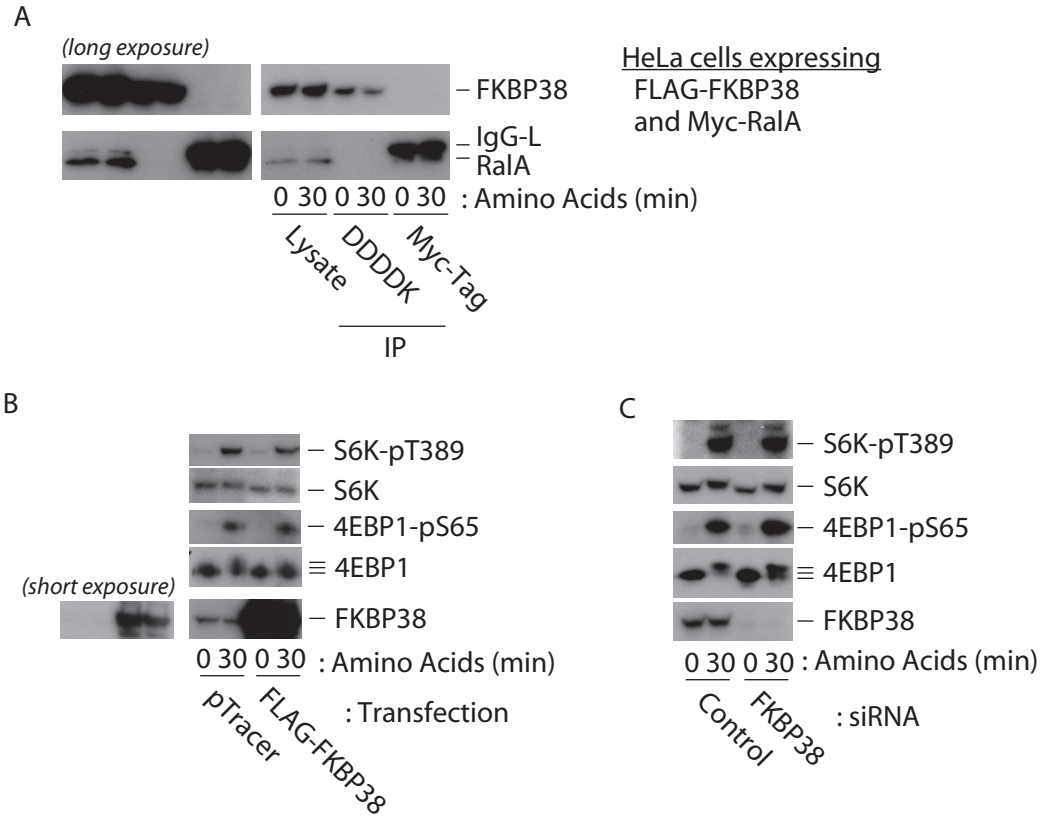


Supplemental Figure 3. Knockdown of RalA and RalGDS does not inhibit nutrient uptake.

HeLa cells grown on 24-well plate were transfected with control siRNA (open circles), siRNA against RalA (open squares), or siRNA against RalGDS (open triangles). After 2-day culture, cells were starved for glucose (Left panel) or amino acids (Right panel) for 2 h. For glucose uptake assay (Left panel), cells were treated with 1 mM [^{14}C]glucose (2 μCi/ml, GE Healthcare). For amino acid uptake assay (Right panal), cells were treated with amino acids (by addition of standard RPMI1640 medium) with [^{14}C]amino acid mix (2 μCi/ml, ARC). After the incubation for indicated times, cells were washed with ice-cold PBS and lysed in 0.25 ml of 0.03% SDS solution. Radioactivity in the lysate was determined by liquid scintillation counter and represented as average±difference from duplicated determinations in a typical study.



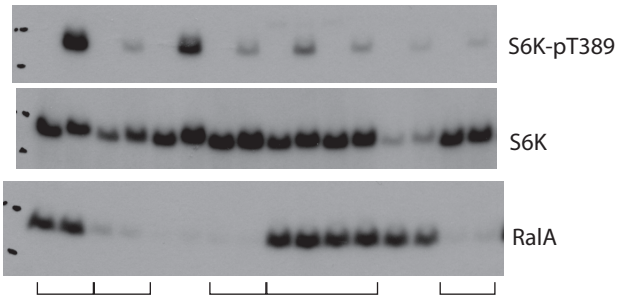
Supplemental Figure 4. Knockdown of RalA and RalGDS does not affect activation status of Rheb.
HeLa cells were transfected with control siRNA, siRNA against RalA, or siRNA against RalGDS. After 1-day culture, cells were further transfected with myc-Rheb/pCAGGS plasmid DNA and further cultured for 1 day. Cells were then starved for amino acids and radiolabeled in amino acid-free RPMI1640 medium supplemented by [^{32}P]orthophosphate (Perkin Elmer, 0.15 mCi/ml) for 2 h. After the incubation in standard RPMI1640 medium (for amino acids stimulation) for indicated times, cells were washed with PBS and lysed in ice-cold Lysis buffer (50 mM Tris-HCl [pH 7.5], 120 mM NaCl, 1% NP-40, 10 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, 25 mM NaF, 25 mM β-glycerophosphate, 15 mM Na-pyrophosphate, 0.05 μM microcystin LR, 1 mM Na-orthovanadate, 0.02% β-mercaptoethanol, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM PMSF, 1 mM benzamidine-HCl). All procedures after this step were performed at 4C. Lysates were mixed with Protein G Sepharose beads (GE Healthcare) conjugated with anti-myc 9B11 antibody (Cell Signaling Technology) and incubated for 2 h. Beads were collected by centrifugation and washed with Lysis buffer extensively. Then bound nucleotides were extracted from the beads in Nucloetide elution buffer (10 mM Na-EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM GTP, 0.5 mM GDP) by the incubation at 68C for 20 min. Eluted nucleotides were separated on PEI-cellulose TLC plate with 0.75 M KH_2PO_4 (pH 3.4). Radioactive spots were analyzed by BAS2000 imaging analyzer and calculated ratios were represented. Similar results were obtained from repeated experiments.



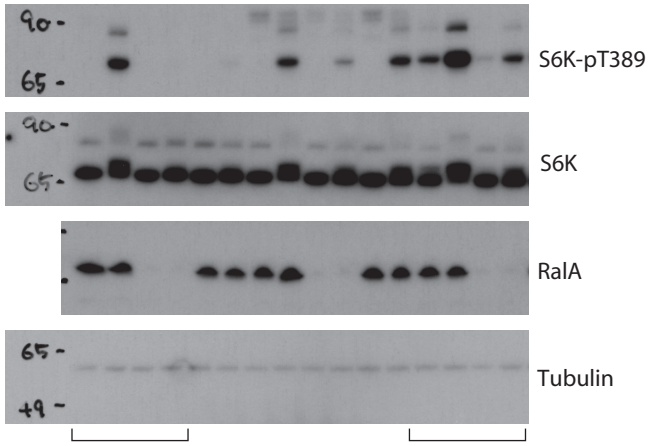
Supplemental Figure 5. RalA does not interact with FKBP38.

(A) HeLa cells were transfected with FLAG-FKBP38/pTracer and Myc-RalA/pCMV5. After 1 day in culture, cells were deprived of amino acids for 1 h. Cells were then exposed to amino acids for the indicated times, and lysed in a buffer consisting of 50 mM Na-Hepes (pH 7.4), 1% NP-40, 25 mM NaF, 15 mM Na-pyrophosphate, 25 mM β-glycerophosphate, 120 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na-orthovanadate, 0.02% β-mercaptoethanol, 0.1 μM microcystin LR, and protease inhibitor cocktail. After removal of insoluble materials, FLAG-FKBP38 and Myc-RalA were immunoprecipitated using anti-DDDDK (MBL) and anti-Myc 9B11 (Cell Signalling) antibodies, respectively, with Protein G Sepharose beads (GE Healthcare). Immunoprecipitants were washed with the same buffer, followed by immunoblot analysis with anti-FKBP38 (R&D systems) and anti-RalA (R&D systems). FLAG-FKBP38/pTracer plasmid DNA was generated by cloning of full-length human FKBP38 cDNA into pTracer-CMV vector (Invitrogen) with the addition of FLAG-tag sequence at the 5'-end. (B) HeLa cells were transfected with empty pTracer or FLAG-FKBP38/pTracer. After 1 day in culture, cells were deprived of amino acids for 1 h. Cells were then exposed to amino acids for the indicated times followed by immunoblot analysis. (C) HeLa cells were transfected with control siRNA or siRNA against FKBP38 (Ambion AM51331). After 1 day in culture, cells were deprived of amino acids for 1 h. Cells were then exposed to amino acids for the indicated times followed by immunoblot analysis.

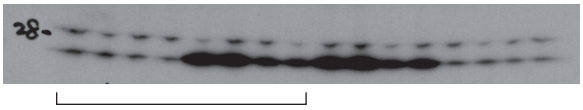
(Figure 2B and Supplemental Figure 1)



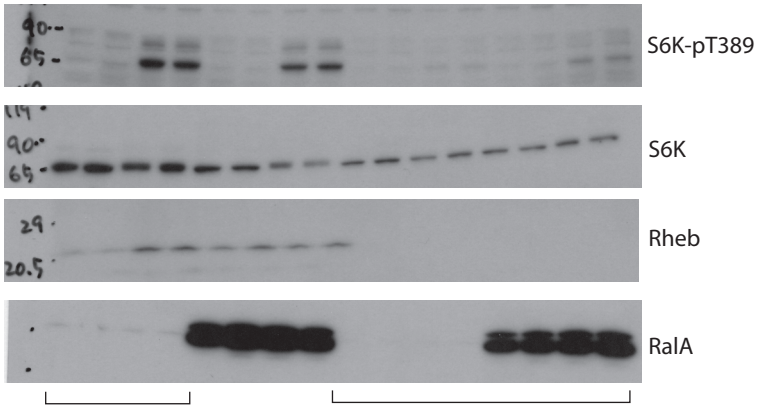
(Figure 4A)



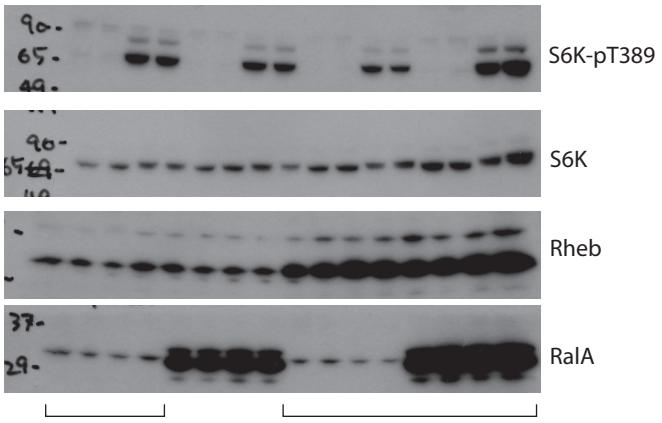
*Same samples (as used above) were loaded with different order.



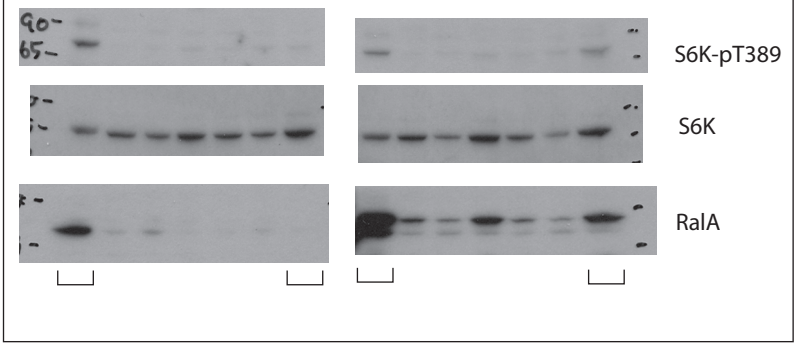
(Figure 4C)



(Figure 4D)



(Supplemental Figure 2)



Supplemental Figure 6. Unprocessed original images for Figures 2 and 4, and Supprelemtal Figures 1 and 2.

Images used (extracted) in each figure were indicated with brackets.