Supporting Information

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SI Materials and Methods

Plasmids and Reagents. Mouse RSK2-Y707A and -K100A mutants in pKH3 vector have been described previously (1). The wild-type (WT) RSK2 was obtained by site-directed mutagenesis (Agilent) from RSK2-K100A mutant. The WT-RSK2 was further subcloned into p3xFlag-CMV-10 (Sigma), pCMV-Myc (Clontech), N-terminal GST-fused bacterial expression vector of pGEX-4T1 (GE Healthcare), and a MBP-His_6 vector of pMal-His_6 constructed upon pMal-c2x vector (Addgene) in which an additional His_6 epitope tag was engineered directly after the maltose-binding protein (MBP) expression sequences. The pMal-His6 vector allows expression of both MBP and His_6 epitope tags at the N-terminal of proteins of interest. All resulting constructs were fully validated by DNA sequencing. Different RSK2 mutants as described in the text were subsequently generated by site-directed mutagenesis and DNA sequenced. The N-terminal kinase domain (NTKD, 1–427 aa) and the C-terminal kinase domain (CTKD, 428–740 aa) of RSK2 were amplified by PCR and subcloned to p3xFlag-CMV-10 vector and DNA sequenced. The HA-tagged human Rho family of small GTPases, including WT forms, constitutively active (CA) and dominant negative (DN) mutants of RhoA–C, Rac1–3, and Cdc42, were kindly provided by John H. Kehrl, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, and subsequently subcloned into pCMV-Myc (BD-Clontech), pEBG mammalian GST expression (John H. Kehrl), and bacterial pMal-His_6 expression vectors. The GFP-tagged WT-ARHGEF12 (also called leukemia-associated RhoGEF, LARG) construct was kindly provided by Philip B. Wedegaertner, Thomas Jefferson University, Philadelphia. WT-LARG was subcloned into pCMV-Myc and pGEX-4T1 vectors by PCR and DNA sequenced. LARG-S190A, -S1176A, -S1288A, and DN-LARG (ΔDH/PH, Δ787–1132 aa) mutants were subsequently generated by mutagenesis and verified by DNA sequencing. The following reagents were purchased: epidermal growth factor (EGF), tumor necrosis factor alpha (TNFα) (R&D Systems), phorbol 12-myristate 13-acetate (PMA) and 1× proteinase inhibitor mixture (CalBiochem), RSK2 inhibitor BI-D1870 (Santa Cruz Biotechnology), G418 (Geneticin), and puromycin (Life Technologies). Antibodies against RSK2 were purchased as follows: (rabbit monoclonal antibody, clone D21B2, 5528; Cell Signaling), RSK1 (clone D6D5, 8408; Cell Signaling), c-Myc (clone 9B11, 2276; Cell Signaling), phosphothreonine (9381; Cell Signaling), and phospho-AKT substrate-specific antibody (which recognizes peptides and proteins containing phospho-Ser/Thr preceded by Lys/Arg at positions −5 and −3) (9611, Cell Signaling), His (H-15, sc-803; Santa Cruz Biotechnology), RhoA (119, sc-179; Santa Cruz), RhoA/B/C (H-70, sc-28565; Santa Cruz Biotechnology), phosphoserine (clone 3C171, MA1-91609; Invitrogen), and phosphotyrosine (clone 4G10, 05-321; Millipore), ARHGEF2 (OWL-44338, One World Lab), ARHGEF12 (OWL-C18388, Anbo; One World Lab), and ARHGAP24 (OWL-44340, One World Lab), HA (clone 12CA5, ab16918; Abcam), actin (clone AC15, A1978; Sigma), and Flag (M2, F3165; Sigma). The horseradish peroxidase (HRP)-conjugated goat anti-mouse (115-035-003) or anti-rabbit IgG (111-035-003), Alexa 594-conjugated goat anti-mouse IgG (115-585-003) antibodies, and Alexa 594-conjugated streptavidin (016-590-084) were purchased from Jackson Immunoresearch Laboratories. The biotinylation of anti-HA and anti–c-Myc monoclonal antibodies were generated using a biotinylation kit of EZ-link Sulfo-NHS-SS-Biotin (Thermo). Protein-G Sepharose (Millipore), Ni-NTA His-Bind resin (Novagen), glutathione agarose resin (Sigma), and recombinant active RSK2 proteins (Sigma) were purchased.

Lentivirus-Mediated Silencing of Rho GTPases. The silencing of individual Rho isoforms was achieved by transient transduction of recombinant lentivirus containing Rho isoform-specific shRNAs. TRC lentiviral shRNA system was adapted and purchased (GE Healthcare-Dharmacon). The sense targeting sequences of isoform-specific shRNAs are: shRhoA [AAACACATCAGTATAACATCG (47708), AAGATCCTTCTTATTCCCAAC (47709), TTTGCTGAACACTCCATGTAC (47710), ATGAGCAAGCATGTCTTTCCA (47711), AAGCCAACTCTACCTGCTTTC (47712)]; shRhoB [AACTCGTCCTTACTGAACACG (47848), TTACTGAACACGATCAGCAGG (47849), AAGCACATGAGAATGACGTCG (47850), ATAGTTCTCGAAGACGGTGGG (47851), TAGTCGTAGGCTTGGATGCGC (47852)]; and shRhoC [AATGTCCGCAATATAGTTCTC (47863), TATAGTTCTCAAAGACAGTAG (47864), AAAGGCACTGATCCGGTTCGC (47865), AAGCACATGAGGATGACATCA (47866), TGCCTCAGGTCCTTC TTATTC (47867)]. The recombinant lentivirus (rLenti) containing expression cassettes that express shRNA and puromycin resistance gene were packaged in HEK293T cells following the instructions provided by the Broad Institute (https://portals.broadinstitute.org/gpp/public/resources/protocols) except the calcium phosphate was used to replace Lipofectamine for DNA transfection. Briefly, HEK293T cells (4×10^6 cells per 100-mm-diameter dish) were transfected with equal amounts of plasmids containing different Rho isoform-specific shRNA constructs together with helper plasmids of pCMV-Δ8.91 and pMD2.G-VSV-G following standard calcium transfection protocol, and then incubated in rLenti production medium. The supernatant containing released rLenti was harvested and HEK293T cells and cell debris were removed by centrifugation at $3{,}011 \times g$, 4 °C for 10 min. Aliquots of rLenti were then stored at −80 °C before use. To transduce the rLenti-shRNA into U87MG cells, cells were pretreated with polybrene (2 μg/mL; Sigma) at 37 °C for 1 h before being infected with 100 μL of rLenti-shRNA supernatant. To examine requirement of Rho isoform for RSK2-induced cell migration and invasion, cells were transfected with 2 μg of empty Flag vector (Flag-EV), Flag-RSK2-Y707A, or Flag-RSK2-T577E after appropriate rLenti-shRNA infection. Twenty-four hours later, U87MG cells were subjected to puromycin (2 μg/mL; Life Technologies) and G418 (400 μg/mL; Life Technologies) selection to enrich the cells expressing both shRNA and Flag-tagged proteins before being applied to the analysis.

Protein Expression, Analysis, and Immunoblotting. U87MG cells were lysed using a kinase lysis buffer [Hepes (pH 7.4), 20 mM; NaCl, 150 mM; KF, 50 mM; β-glycerolphosphate, 50 mM; EGTA (pH 8.0), 2 mM; Na_3VO_4, 1 mM; Triton X-100, 1%; glycerol, 10% and 1× protease inhibitor mixture (Calbiochem)] and the detergent-soluble fraction of lysates was then subjected to individual analysis. Proteins were fractionated using SDS/PAGE and transferred onto Protran nitrocellulose membrane (GE Healthcare). Immunoblotting was initiated by incubation with 1% casein (Sigma) in PBS plus 0.1% Tween-20 (PBST) at room temperature (RT) for 1 h, and then incubated with the manufacturer-suggested dilution of primary antibody for 1–4 h followed by three washes with PBST. The membranes were subsequently incubated with either HRP-conjugated secondary antibodies or HRP-conjugated streptavidin in 1% casein-PBST solution at RT for 1 h. The immunoblotting signal was acquired by exposure of the immunoblots to X-ray film (Denville) following an incubation with chemiluminescence at RT for 1 min (HyGlo, Denville).

Recombinant Protein Expression and Purification. *Escherichia coli* strain BL21-DE3 cells transformed with bacterial expression vectors were grown in 2× YT (trypton, 15 g; yeast extracts, 10 g; NaCl, 5 g, per liter) with vigorous shaking to OD_{600} of 0.5–0.6, and the expression of recombinant protein was initiated by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) (ThermoScientific) to a final concentration of 100 μM. The bacterial cultures were further incubated by vigorously shaking at either RT for 3 h or 18 °C overnight. The cells were harvested and resuspended in appropriate cell suspension buffer. For GST-fused proteins, the cells were resuspended with GST lysis buffer [GLB, Tris·HCl (pH 8.0), 50 mM; NaCl, 200 mM; DTT, 1 mM; PMSF, 1 mM], while MBP-His_6–fused proteins with Ni-NTA bind buffer [NBB, NaH_2PO_4 (pH 8.0), 50 mM; NaCl, 300 mM; imidazole, 10 mM; β-mercaptoethanol (2-ME), 5 mM]. The cells were then incubated with lysozyme at a final concentration of 1 mg/mL on ice for 1 h and then sonicated (1 min per time) on ice three times. The clear cell extracts were collected after centrifugation at 4 °C, 100,000 × *g*, 30 min, and incubated with appropriate resins at 4 °C for 3 h with end-to-end rotation. The resins were then recovered and extensively washed, and the bound proteins were eluted. The released proteins were further dialyzed against 20 volumes of appropriate buffer at 4 °C three times (1 h for the first two times and overnight for the third dialysis). Glycerol was added to the purified recombinant proteins to 10% (vol/vol), and the recombinant protein aliquots were stored at −80 °C before use. For GST-fused proteins, cell lysates were incubated with glutathione-conjugated agarose (Sigma). The glutathione resins were then washed with 10 volumes of GLB three times, GLB supplemented with 0.5 M NaCl twice, and GLB three times. The enriched GST-fused recombinant proteins were eluted by three volumes of glutathione free acid (50 mM in GLB) and further dialyzed and stored as described above. For MBP-His_6–tagged proteins, cell lysates were incubated with Ni-NTA His Bind resins and the resulting resins were extensively washed with 10 volumes of Ni-NTA wash buffer [NaH_2PO_4 (pH 8.0), 50 mM; NaCl, 300 mM; imidazole, 20 mM; 2-ME, 5 mM] five times and the bound His_6-tagged proteins were eluted by three volumes of Ni-NTA elution buffer [NaH_2PO_4 (pH 8.0), 50 mM; NaCl, 300 mM; imidazole, 400 mM; 2-ME, 5 mM]. The purified proteins were then dialyzed before storage as described above.

In Vitro Protein–Protein Interaction Analysis. Purified recombinant GST- or MBP-His_6–fused RSK2 proteins, MBP-His_6–fused Rho GTPases, and GST-fused LARG proteins were used for the in vitro interaction analysis. To determine the interaction between RSK2 and different Rho GTPases, different GST-tagged RSK2 proteins and MBP-His_6–fused Rho family of GTPases (0.8 μM, per protein) were mixed in the presence of 20 μL of either glutathione agarose or Ni-NTA His Bind resin in a final volume of 1 mL in appropriate buffers. After end-to-end rotation at 4 °C for 3 h, the resin was extensively washed as described above. The bound proteins were released by boiling in 1× Laemmli loading buffer for 5 min. The presence of GST-RSK2 proteins or MBP-His_6-Rho GTPases in the precipitates was determined by immunoblotting with either anti-GST or anti-His antibodies. To determine the direct interaction between RSK2 and nucleotide-bound Rho family of GTPases, MBP-His_6-Rho GTPases, including RhoA, RhoB, RhoC, and Rac1, were preloaded with either GTPγS or GDP (Sigma). In brief, MBP-His_6-Rho proteins were diluted to a final concentration of 80 pM in a 200-μL reaction system comprising 20 mM Tris·HCl (pH 7.5); 100 mM NaCl; 1 mM EDTA (pH 8.0), 1 μg/mL BSA; 1 mM DTT, together with either 100 μM of GTPγS plus 1 mM of GDP or 1 mM of GDP alone. The GTP/GDP loading to Rho GTPases was initiated by adding 0.4 μL of $MgCl_2$ (0.5 M) to a final concentration of 1 mM and further incubated in a 30 °C water bath with agitation for 20 min. A total of 50 μL of the GTPγS- or GDP-loaded Rho GTPases was subsequently incubated with 50 μL of recombinant GST-RSK2 (40 pM) together with 100 μL of glutathione-Sepharose in a binding buffer containing 20 mM Tris·HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT at 4 °C for 2 h with end-to-end rotation. Recombinant GST proteins were used as a negative control. The glutathione-Sepharose beads were recovered by brief centrifugation at 4 °C and extensively washed with GLB buffer four times. Bound proteins were released by boiling in 1× Laemmli sample buffer and subjected to fractionation using SDS/PAGE, while 1% of the GTPγS- or GDP-loaded Rho GTPases were loaded as input controls. The presence of MBP-His_6-Rho GTPases in the GST pulldown pellets was then determined by immunoblotting with anti-His antibody. Dual direction in vitro interaction analyses was used to determine the direct interaction between RSK2 and LARG. For GST pulldown, 500 nM of GST-LARG proteins as indicated in Fig. 6*D* were incubated with 500 nM of MBP-His_6-RSK2-WT in a final volume of 1 mL in GLP buffer. For Ni-NTA pulldown, 50 nM of MBP-His_6-RSK2 proteins as indicated in Fig. 6*E* were incubated with 50 nM of GST-LARG-WT in a final volume of 1 mL in NBB. The bound MBP-His_6-RSK2-WT to glutathione agarose resin (Sigma) was detected by anti-His immunoblotting (Fig. 6*D*), while the bound GST-LARG-WT to Ni-NTA resin detected by immunoblotting with anti-ARHGEF12 (LARG) antibody (Fig. 6*E*). The equal input levels of recombinant proteins were detected by Coomassie blue staining and shown.

Immunostaining and Cell Morphogenesis Analysis. The colocalization of RSK2 and Rho family of GTPases was analyzed using U373MG cells. To analyze the colocalization of RSK2 with different isoforms of Rho GTPases, U373MG cells seeded on two-chamber glass slides (BD-Falcon) at a density of $1 \times 10^3/cm^2$ were transfected with Flag-RSK2-Y707A in the presence of HA-EV, HA-RhoA-WT, HA-RhoB-WT, or HA-RhoC-WT overnight and further incubated in complete DMEM for another 24 h. The cells were washed with ice-cold PBS three times and then fixed with 4% paraformaldehyde (PFA) in PBS at RT for 15 min followed by three washes with ice-cold PBS. The cells were then permeabilized with 0.1% Triton X-100 in PBS at RT for 5 min and washed with PBS three times. The slides were incubated in a blocking PBS containing 5% goat serum at RT for 1 h before being immunostained with anti-Flag monoclonal antibody (M2, 1:1,000 dilution in PBS) at 4 °C overnight. The slides were washed with ice-cold PBS three times and incubated with Alexa Fluor 488 (green)-conjugated secondary goat anti-mouse IgG at RT for 1 h (to visualize Flag-RSK2). After three washes with PBS, the slides were further stained with biotinylated anti-HA monoclonal antibody at 4 °C overnight and visualized by Alexa 594 (red)-conjugated streptavidin at RT for 1 h (to visualize HA-tagged Rho isoforms). The slides were sealed with mounting media containing DAPI, and pictures were captured using a LeicaTCS SP5 confocal microscope equipped with image analysis software of LAS AF.

In Vitro Kinase Analysis. The potential ability of RSK2 to phosphorylate Rho isoforms was examined by in vitro kinase assays using purified MBP-His_6–tagged recombinant WT-RhoA or -RhoC as substrates. In brief, RSK2 proteins were incubated with 500 ng of MBP-His_6-Rho proteins in a final volume of 30 μL of kinase assay buffer [KAB, Tris·HCl (pH 7.5), 20 mM; $MgCl_2$, 10 mM; Na_3VO_4, 0.1 mM; DTT, 2 mM; 2-ME, 5 mM; ATP, 200 μM] at 30 °C for 30 min with agitation. The reaction was terminated by adding 4× Laemmli loading buffer (10 μL) and boiling for 5 min. The phosphorylation of Rho isoforms was then determined by immunoblotting with phosphoserine, phosphothreonine, and/or phospho-AKT substrate (which recognizes an AGC kinase consensus sequences of RXXS/T)-specific antibodies.

RSK2 proteins acquired from different sources were used. For bacterial purified RSK2 proteins, 2 μg of GST-fused RSK2-WT, active RSK2-Y707A, and DN-RSK2-K100A were used, while Flag-tagged RSK2 proteins were recovered from transfected U87MG cells as indicated. A total of 2 mg of total lysates prepared from transfected U87MG cells was subjected to immunoprecipitation with 2 μg of anti-Flag monoclonal antibodies and 20 μL of protein-G Sepharose at 4 °C with end-to-end rotation for 1 h. The resin was washed with KLB twice and KAB lacking ATP twice. One-tenth of the resulted immunoprecipitates were saved for immunoblotting with anti-Flag monoclonal antibody to ensure efficient recovery of transfected RSK2 proteins. The remaining immunoprecipitates were resuspended with 30 μL of KAB containing indicated substrate for in vitro kinase assay.

In Vitro ADP-Glo Kinase Assay. RSK2 kinase activity toward Rho GTPases was also determined using an in vitro ADP-Glo kinase assay (Promega) with purified recombinant Rho proteins as substrates, following manufacturer's instructions. In brief, 100 ng of recombinant active RSK2 (Sigma) was incubated with 3.32 μM of recombinant MBP-His_6 alone (as negative control), MBP-His_6-RhoA-WT, MBP-His_6-RhoC-WT, MBP-His_6-Rac1-WT, or 180 μM of S6K1 polypeptides (230–239 aa) as a positive control (SignalChem), in a kinase reaction buffer [Tris·HCl (pH 7.5), 40 mM; $MgCl_2$, 20 mM; BSA, 0.1 mg/mL; DTT, 2 mM; Na_3VO_4, 2 mM and ATP, 50 μM] to a final volume of 25 μL. Each reaction was performed in triplicate. The kinase reaction was performed at RT with agitation for 45 min and 25 μL of ADP-Glo reagent was then added to stop the kinase reaction and to deplete the unconsumed ATP by agitation at RT for 30 min. A total of 50 μL of kinase detection reagent was subsequently added to convert ADP to ATP and to introduce luciferase and luciferin to detect ATP. The reaction was further incubated at RT for 45 min and the luminescence intensity was detected by a luminometer (Synergy 2, equipped with Gen5 software; BioTek). RSK2 kinase activity was indicated by the luminescence unit and the fold induction was calculated by dividing the luminescence units in the experimental samples against that in the negative MBP-His_6 control.

1. Gawecka JE, et al. (2012) RSK2 protein suppresses integrin activation and fibronectin matrix assembly and promotes cell migration. *J Biol Chem* 287:43424–43437.

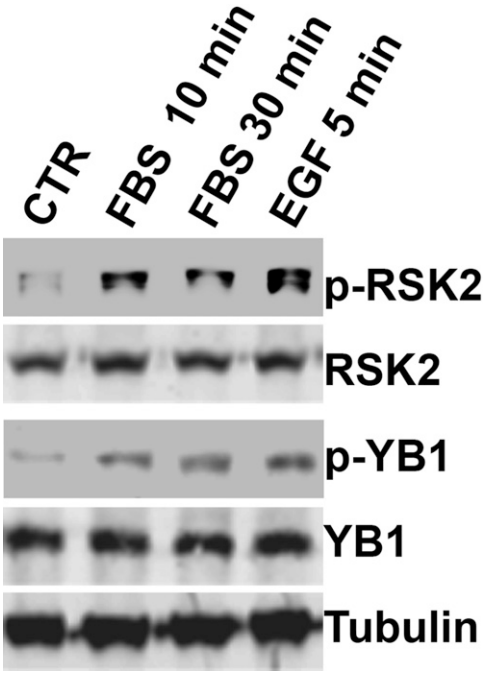

Fig. S1. FBS and EGF activate RSK2 in U87MG cells. Serum-starved U87MG cells were treated with FBS or EGF at the indicated concentrations and RSK2 activity was measured using RSK-phospho-S386 or phospho-YB1 (a RSK2 substrate) antibodies.

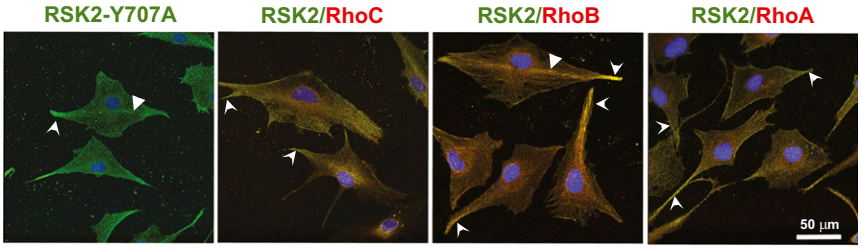

Fig. S2. Colocalization of RSK2 and Rho isoforms. U373MG cells seeded on glass slides were transfected with the indicated constructs. Flag-tagged RSK2-707A was stained with anti-Flag antibody and visualized by Alexa Fluor 488-conjugated secondary antibody, HA-tagged WT-RhoA, -B, and -C proteins were immunostained with biotinylated anti-HA antibody and visualized by Alexa 594-conjugated streptavidin, the nucleus is visualized by DAPI. Merged images are shown.

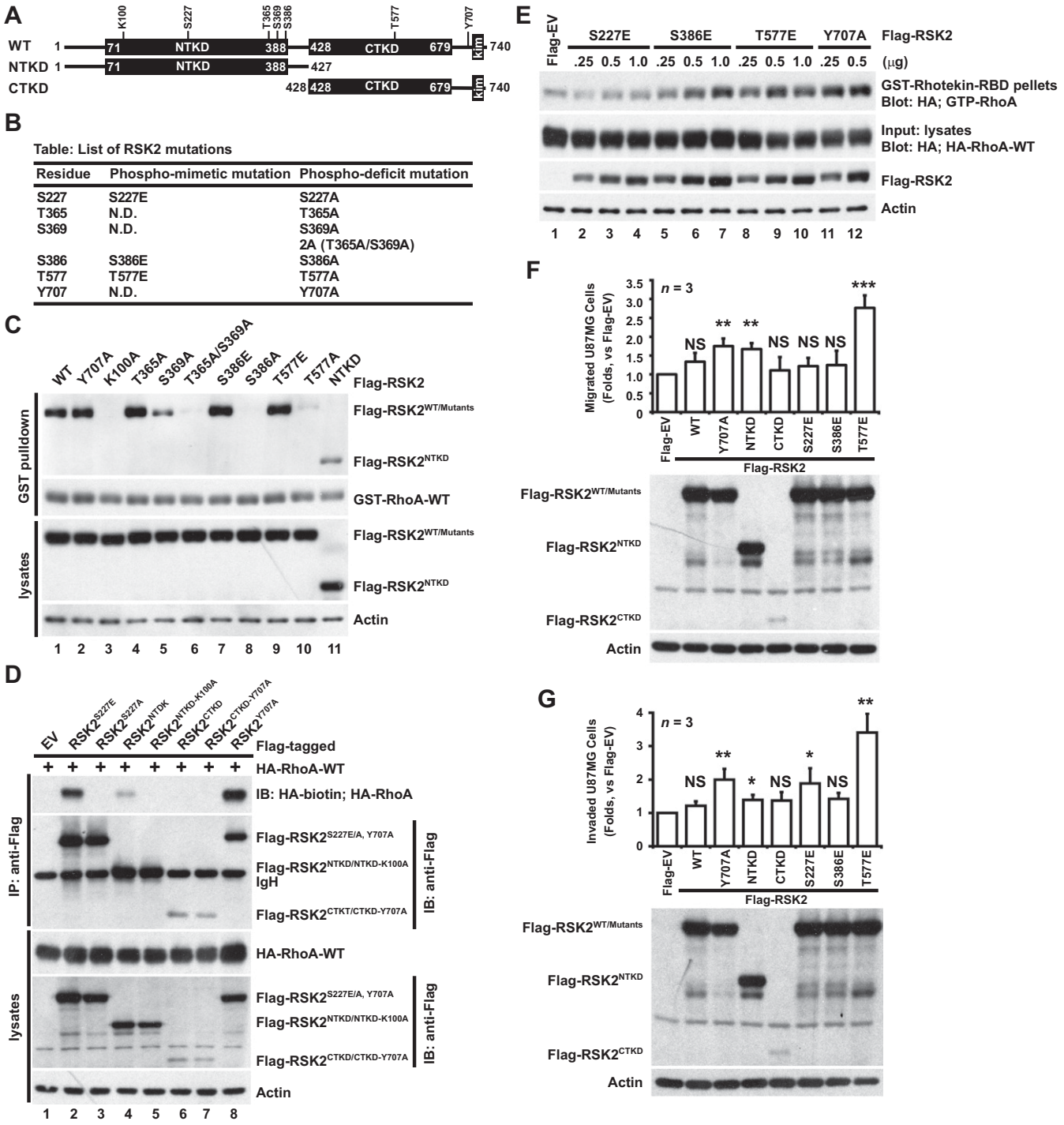

Fig. S3. RSK2 residue T577 is essential for RSK2-stimulated Rho signaling in cell migration and invasion. (*A*) RSK2 protein structure and two truncations. NTKD, N-terminal kinase domain; CTKD, C-terminal kinase domain. (*B*) RSK2 point mutations tested. N.D., not done. (*C* and *D*) Mapping the minimal RSK2 sequences required for association with RhoA. U87MG cells expressing HA- or GST-RhoA-WT were cotransfected with the indicated Flag-RSK2 constructs. A total of 2 mg of cell lysates was subjected to GST pulldown (*C*) or anti-Flag immunoprecipitation (*D*). Protein levels of RSK2 and RhoA proteins in precipitates or total cell lysates were detected by Western blot and are shown. Actin levels served as loading controls. Results are representative of three independent experiments. (*E*) RSK2 phosphorylation mimic mutants activate RhoA. U87MG cells expressing HA-RhoA-WT were transfected with the indicated phosphomimetic mutations of RSK2 and serum starved for 24 h before lysates were prepared for RhoA activation analysis. GTP-RhoA levels were determined by immunoblotting the GST-Rhotekin-RBD precipitates with anti-HA antibody. The levels of loaded HA-RhoA-WT and the overexpressed Flag-RSK2 mutants are shown. Actin levels were used as loading controls. Results are representative of three independent experiments. (*F* and *G*) Phosphomimetic mutation at T577 residue plays a predominant role in cell migration and invasion. U87MG cells were transiently transfected with the indicated Flag-RSK2 constructs, subjected to G418 selection, and then serum starved for 24 h. Flag-EV transfection was used as a negative control and Flag-RSK2-Y707A served as a positive control. Single cell suspension was prepared for cell migration (*F*) and invasion (*G*) analysis. Results are presented as mean ± SD of three independent experiments each performed in triplicate. Refer to Dataset S1*E* for all *P* values. The expression levels of Flag-RSK2 proteins from a representative experiment are shown.

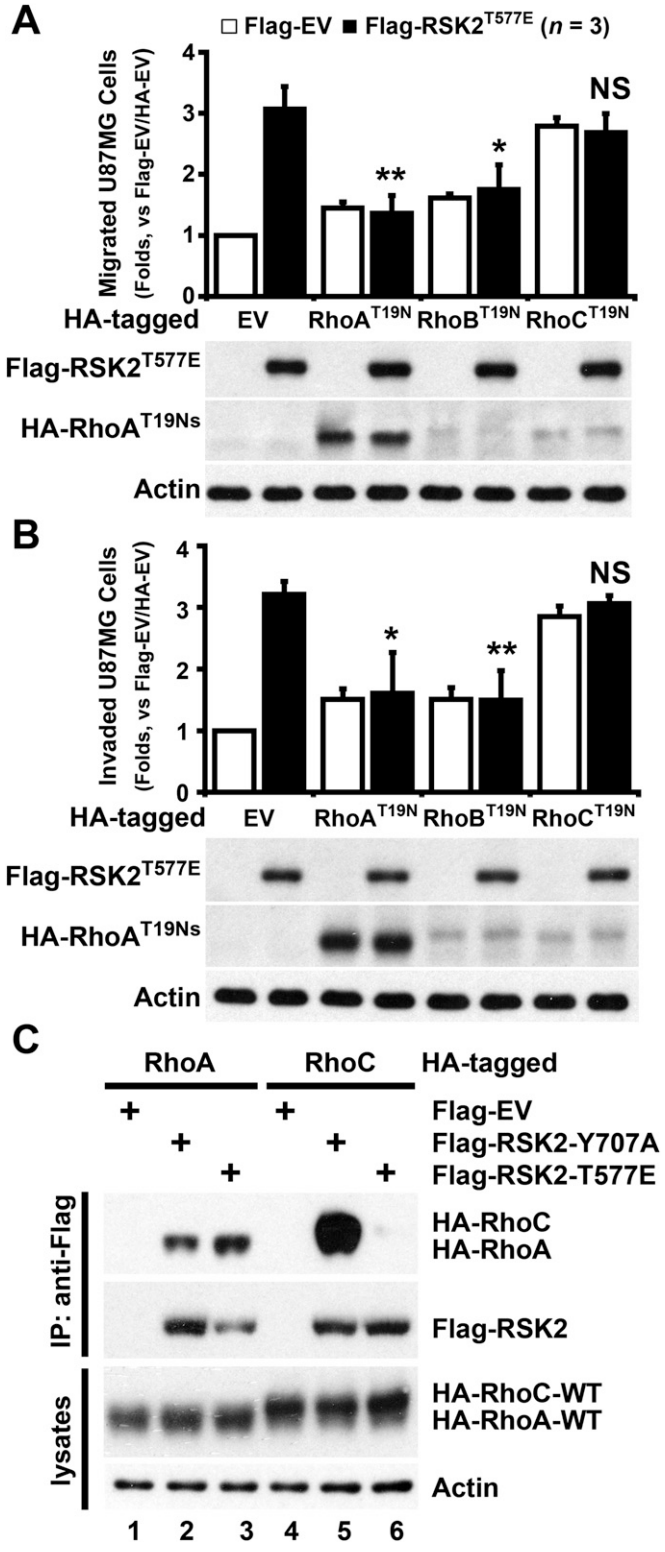

Fig. S4. RSK2-T577E mutant-induced Rho signaling contributes to U87MG cell migration and invasion. (*A* and *B*) RSK2-T577E mutant-induced cell migration and invasion depends on RhoA and RhoB, but not RhoC. The role of Rho isoform signaling on RSK2-T577E mutant-mediated U87MG cell migration (*A*) or invasion (*B*) was performed as described in Fig. 3 *A* and *B*. Results are shown as mean ± SD of three independent experiments each performed in triplicate. Refer to Dataset S1*F* for all *P* values. (*C*) RSK2-T577E does not bind RhoC. U87MG cells were cotransfected with Flag-EV, Flag-RSK2-Y707A, or Flag-RSK2-T577E and HA-RhoA-WT or HA-RhoC-WT. Transfected cells were serum starved for 24 h before lysate preparation. RSK2 proteins were precipitated with an anti-Flag antibody and coprecipitated HA-tagged RhoA or RhoC was detected by immunoblotting with biotinylated anti-HA antibody. Representative data from three independent experiments are shown.

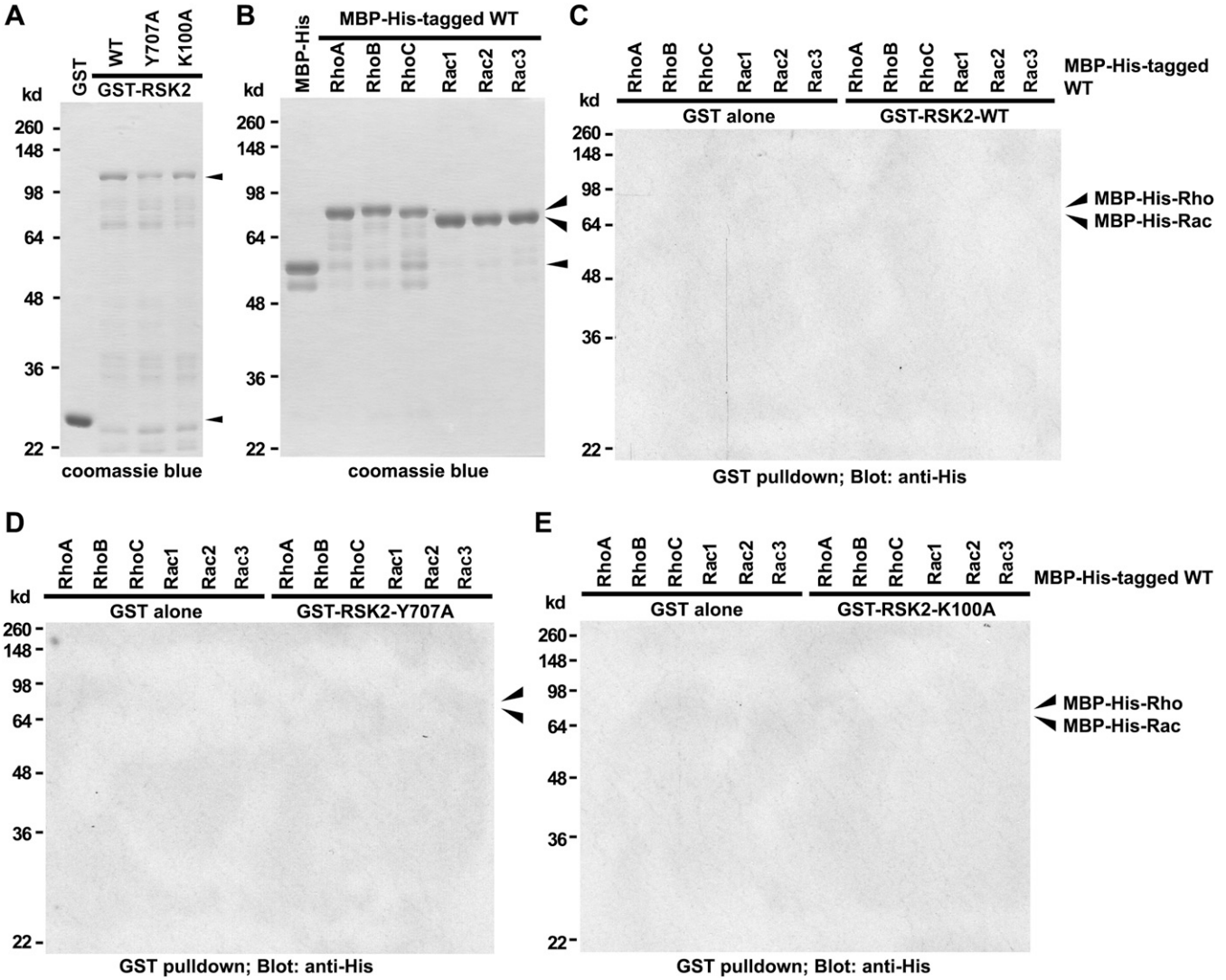

Fig. S5. RSK2 does not bind to naïve Rho GTPases in vitro. (*A* and *B*) Recombinant GST-fused RSK2 or MBP-His_6–fused Rho GTPase proteins were expressed and purified from an *E*. coli strain of BL21-DE3 and 1 μg of purified proteins was fractionated by SDS/PAGE and visualized by Coomassie blue staining. (*C*–*E*) RSK2 does not directly interact with naïve Rho family GTPases. Recombinant MBP-His_6–fused Rho GTPases (0.8 μM) were incubated with recombinant GST-RSK2-WT (*C*), GST-RSK2-Y707A (*D*), GST-RSK2-K100A (*E*), or GST alone as control (*C*–*E*) (0.8 μM) in a total volume of 1 mL for GST pulldown analysis. Precipitates were then resolved by SDS/PAGE and the presence of MBP-His_6–fused proteins was determined by immunoblotting with anti-His (H15) antibody. Representative results of four separate experiments are shown.

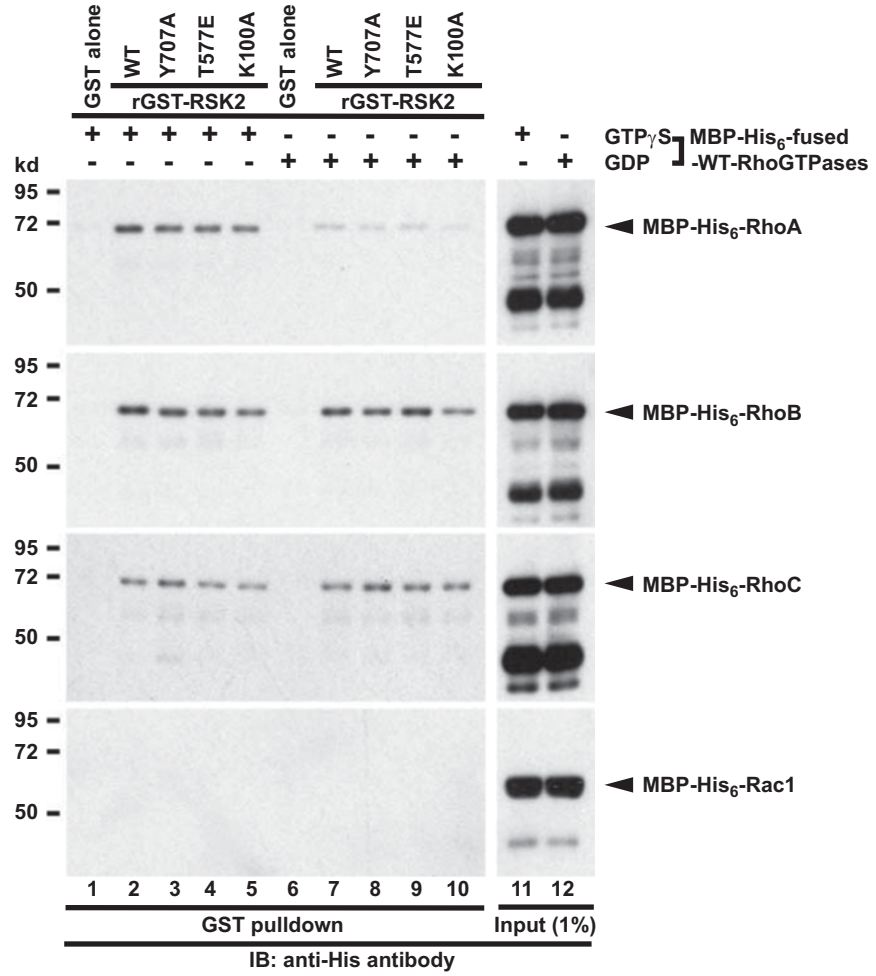

Fig. S6. RSK2 binds directly to Rho proteins in a nucleotide-dependent manner. The direct interaction between RSK2 and Rho GTPases was determined by incubation of nucleotide-preloaded MBP-His_6-Rho-WT and GST-RSK2 proteins. Bound MBP-His_6-RhoA (*Upper*), -RhoB (*Upper Middle*), -RhoC (*Lower Middle*), or Rac1 (*Lower*) were retrieved by GST pulldown and detected by anti-His immunoblotting. Results are representative of three independent experiments.

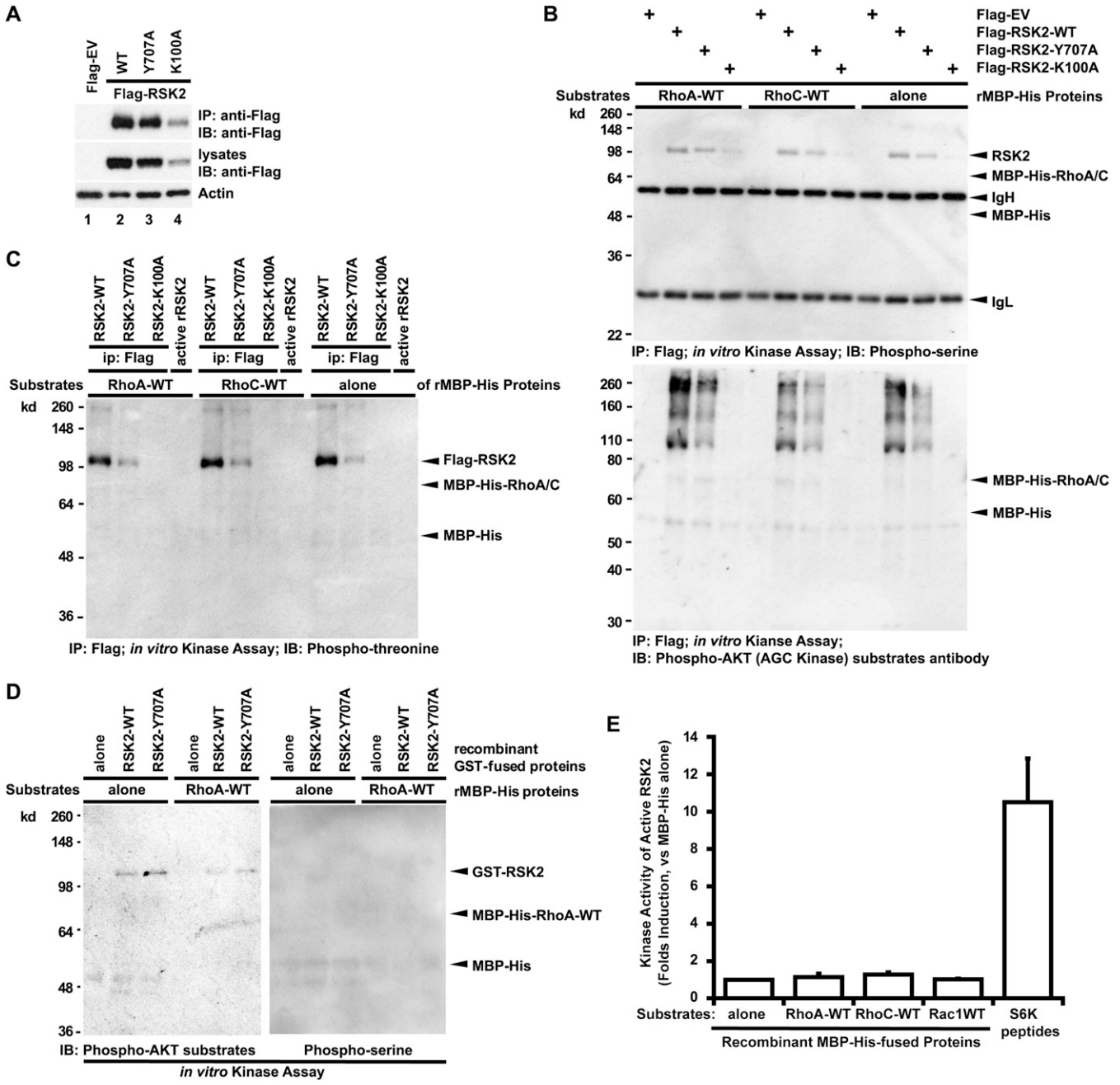

Fig. S7. Activated RSK2 does not induce phosphorylation of Rho GTPases. (*A*) U87MG cells were transfected with the indicated RSK2 constructs and the Flag-tagged RSK2 proteins were recovered by immunoprecipitation using anti-Flag monoclonal antibody. A total of 0.5% of the resulting precipitates was fractionated for immunoblotting to ensure the efficient recovery of the Flag-RSK2 proteins. (*B* and *C*) Immunoprecipitated RSK2 does not phosphorylate RhoA or -C. In vitro kinase assays were carried out by incubating recovered Flag-RSK2 proteins (from 2 mg total lysates) with 500 ng of recombinant MBP-His_6–fused RhoA-WT or RhoC-WT as substrates in kinase assay buffer. MBP-His_6 alone was used as a negative control. A commercial active recombinant RSK2 protein (active rRSK2) was used as control (*C*). The potential phosphorylation of Rho isoforms was determined by immunoblotting with phosphoserine, phospho-AKT substrates (*B*), or phosphothreonine (*C*) antibodies. Results shown are representative of three individual experiments. (*D*) Recombinant active RSK2 does not phosphorylate RhoA. A total of 2 μg of recombinant GST-fused RSK2 proteins or GST alone were incubated with 500 ng of recombinant MBP-His_6-RhoA-WT or MBP-His_6 alone as substrates for the in vitro kinase assay. Phosphorylation was determined by immunoblotting using either phospho-AKT substrates or phosphoserine antibodies. A representative of three separate experiments is shown. (*E*) Active RSK2 does not phosphorylate RhoA, RhoC, or Rac1 in the ADP-Glo kinase assay. Recombinant active RSK2 (100 ng per reaction) was incubated with 3.32 μM of MBP-His alone, MBP-His-RhoA-WT, MBP-His-RhoC-WT, MBP-His-Rac1-WT, or 180 μM of S6K1 polypeptides (as a positive control) in a 25-μL reaction system and the RSK2 kinase activity was determined using the ADP-Glo kinase assay kit.

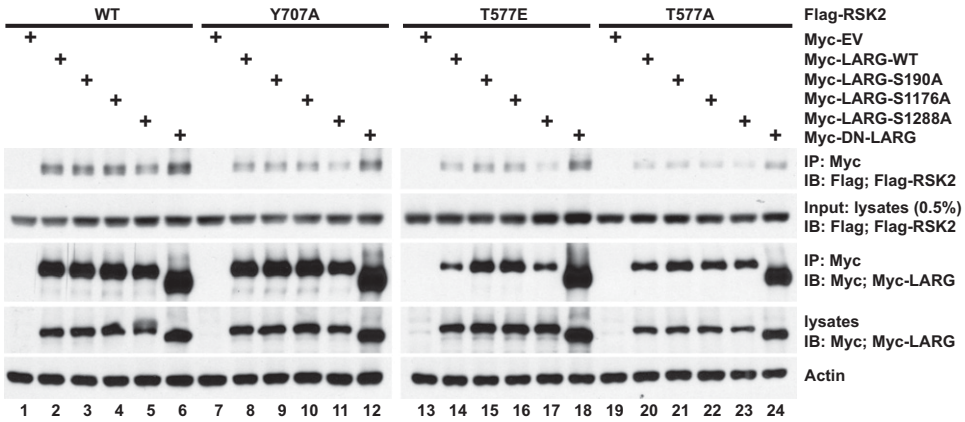

Fig. S8. LARG-S1288A mutation did not disrupt RSK2-LARG interaction. U87MG cells cotransfected with the indicated combination of Flag-RSK2 and Myc-LARG constructs were serum starved for 24 h. Myc-LARG was immunoprecipitated and coprecipitated Flag-RSK2 proteins were detected by anti-Flag immunoblotting. The Flag-RSK2–expressing cell lysates were loaded to determine input levels and the levels of Myc-LARG protein in the precipitates were also determined. The results are representative of three separate experiments.

Other Supporting Information Files

Dataset S1 (PDF)