Supplemental Material for "Selective role for RGS12 as a Ras/Raf/MEK scaffold in nerve growth factor-mediated differentation" by Melinda D. Willard et al. (2007)

Supplemental Materials and Methods:

Cell Culture and Transfection

All cells were cultured at 37°C in 5% CO_2 humidified air. HEK 293T, COS-7, and N1E-115 cells were each maintained in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. HEK 293T and COS-7 cell cultures, seeded the day before at 800,000 and 160,000 cells per well, respectively of a 6-well dish, were transfected with a total of 1.5 μg plasmid DNA using Fugene 6 (Roche) at a ratio of 3:1 (Fugene:DNA). Empty pcDNA3.1 vector DNA was used to maintain a constant total amount of DNA per well. PC12 cells were maintained in DMEM supplemented with 10% horse serum/5% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Depending on the length of the assay, PC12 cells were seeded at 260,000 or 130,000 cells per well in a 12-well culture dish, and grown for 24 hr. Cells were transfected with a total of 300 ng plasmid DNA per well of a 12-well dish using LipofectAMINE 2000 (L2K) (Invitrogen) at a ratio of 7:1 (L2K:DNA). CHO-K1 cells were maintained in EMEM supplemented with non-essential amino acids, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. CHO-K1 cells were seeded at 1.6 x 10^6 cells per 100 mm dish, and grown for 24 hr prior to transfection with a total of 12 μg DNA using Fugene 6 at a ratio of 3:1 (Fugene:DNA).

Immunofluorescence Microscopy

PC12 or HEK 293T cells were plated on PDL-coated chamber slides or coverslips (BD Biocoat) and then transfected the next day and treated as described in the figure legends. Cells were subsequently fixed with 4% (w/v) paraformaldehyde, permeabilized with 100% methanol (-20°C) for 30 s, washed three times with PBS containing 1% (w/v) nonfat dry milk, 150 mM sodium acetate, pH 7, and then blocked in 1% nonfat dry milk/PBS for 15 min. Cells were incubated with primary antibodies for 1 hr at room temperature, or overnight at 4°C, then incubated with either Alexa Fluor 488 or 594 (Invitrogen) secondary antibodies for 1 hr at room temperature in the dark. Cells were then stained with the nuclear stain 7-aminoactinomycin D (7-AAD) (Gill et al., 1975) (Invitrogen) as per manufacturer's specifications. Cells were then washed four times with PBS, and Fluorsave anti-fade reagent (Chemicon, Temecula, CA) was

added to each coverslip before mounting. Confocal images were collected using a Fluoview 300 laser scanning confocal imager (Olympus, Tokyo, Japan) on an IX70 fluorescence microscope with a PlanApo 60x oil objective (Olympus). Fluorescent images, X-Y sections at 0.28 μm, were collected sequentially at 800 x 600 resolution with 2x optical zoom.

Subcellular Fractionation

Subcellular fractionation was performed as described (Crouch and Simson, 1997), with minor modifications. PC12 cells were grown to 90% confluence in a 100 mm dish, and rinsed two times with PBS. All subsequent fractionation steps were performed at 4°C. Cells were lysed in 2 ml of lysis buffer: 10 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 300 mM sucrose, 0.05% (v/v) Triton X-100, 17 μg/ml calpain inhibitor I, 7 μg/ml calpain inhibitor II, 2 mM Na_3VO_4, and protease inhibitors (Roche). Cells were then homogenized by 28 strokes with a 1 ml Wheaton Tenbroeck Tissue Grinder (Fisher). Lysate was then fractionated into nuclear, crude cytoskeletal, cytosol, and membrane fractions. Cells were centrifuged at 800 x *g* for 6 min at 4°C. Supernatant was removed and set aside on ice. Pellet was washed with 1 ml buffer and the 800 x *g* spin repeated twice. Supernatant was discarded, and resultant nuclear fraction pellet was resuspended in 150 μl lysis buffer. The post-nuclear supernatant was centrifuged at 16,300 x *g* for 25 min at 4°C. Supernatant was removed and set aside on ice as membrane and cytosolic material. Pellet was resuspended in 1 ml buffer and re-centrifuged. Supernatant was discarded, and resultant crude cytoskeletal fraction pellet was resuspended in 150 μl lysis buffer. Membrane and cytosolic material was then centrifuged for 18 hr at 48,000 rpm (~100,000 x *g*) at 4°C in a TLA-55 rotor (Beckman Coulter, Inc., Fullerton, CA). Supernatant (cytosolic fraction) was removed and placed in a fresh tube. The membrane fraction pellet was resuspended in 150 μl lysis buffer. All fractions were sonicated in an ice water bath for 5 min, and equalized with a BCA Protein Assay (Pierce).

Immunoprecipitation and Western Blotting

If fractionation was not required, cells were simply lysed 48 hr post-transfection in cold lysis buffer (20 mM Tris pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1% (v/v) Triton X-100, protease inhibitors; or for phospho-protein detection: 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM NaPPi, 200 μM Na_3VO_4, 1% (v/v) Triton-X-100, protease inhibitors). Cell lysates were sonicated in an ice water bath for 5 min and then centrifuged at

13,000 x *g* for 20 min at 4°C. A portion of the supernatant was removed and mixed 1:1 with 5x Laemmli sample buffer ("lysate" samples). Remaining supernatant was combined with appropriate antibody and rotated overnight at 4°C. Protein A/G agarose (Santa Cruz) was added 12 hr later. Each sample was then rotated at 4°C for 2 hr, washed four times with 1 ml lysis buffer, eluted with 2.5x Laemmli sample buffer, boiled for 5 min, subjected to SDS-PAGE and transferred to nitrocellulose. Western blotting was performed using aforementioned primary antibodies, secondary anti-mouse or -rabbit IgG antibody-HRP conjugates (GE Healthcare), and enhanced chemiluminescence (ECL™, ECL Plus™, or SuperSignal® West Femto from GE Healthcare and Pierce, respectively).

Surface Plasmon Resonance (SPR) Biosensor Measurements

SPR binding assays were performed at 25°C on a BIAcore 3000 (BIAcore Inc., Piscataway, NJ) at the UNC Department of Pharmacology Protein Core Facility. N-terminally biotinylated, synthetic peptides of the MEK2 C-terminus (wildtype: RTLRLKQPSTPTRTAV-COOH; "triple-alanine" tail mutant: RTLRLKQPSTPTRAAA-COOH) were separately bound to streptavidin-coated sensor surfaces (Sensor Chip SA, BIAcore) as per manufacturer's instructions to a density of 400 resonance units (RU). Binding analyses were performed using HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Surfactant P20) as the running buffer. Purified His_6-RGS12-PDZ/PTB or GST-RGS12-PDZ fusion proteins (Richman et al., 2005) were diluted in HBS-EP buffer and injected at a 20 μl/min flow rate over all test flow-cell surfaces simultaneously using the KINJECT command. Surface regeneration was performed, when necessary, using 20 μl injections of 1 M NaCl/50 mM NaOH at a flow rate of 20 μl/min. Binding curves were generated using BIAevaluation software version 3.0 (BIAcore Inc.) and plotted using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA).

Plasmids, Antibodies, Agonists, and other Materials

cDNA encoding human MEK1 and MEK2 were subcloned in frame with an N-terminal FLAG-tag into pCMV2 (Eastman Kodak). pcDNA3.1-based expression constructs encoding N-terminal myc-His_6 tagged full-length RGS12 and tandem RBD domain region (aa 961-1105 of GenBank AAC53176) were generated by heterostagger PCR cloning (Kimple et al., 2004). The open reading frame of FGFR1 (GenBank accession BC015035) was subcloned into a pcDNA3.1 derivative, encoding an N-terminal HA tag, using PCR amplification and ligation independent

cloning as described (Willard et al., 2007). Site-directed mutagenesis was performed using the QuikChange system (Stratagene, La Jolla, CA) to generate the histidine-995-to-leucine mutant of RGS12, the glycine-12-to-valine mutant of Rap2B, and the truncation and loss-of-function point mutations to the pcDNA3-FLAG-TrkA expression construct, including kinase-inactive K547A (Meakin et al., 1999), Y499A (loss-of-function mutation to Shc-binding site) (Meakin et al., 1997; Obermeier et al., 1993; Stephens et al., 1994), and Y794F/V797A (loss-of-function mutation to PLCγ-binding site) (Loeb et al., 1994; Meakin et al., 1997; Obermeier et al., 1993). Rat RGS12 was subcloned in-frame with either an N-terminal or a C-terminal YFP-epitope tag into pEYFP-C1 or pEYFP-N1 (Clontech), respectively. 7-aminoactinomycin D (7-AAD) and all Alexa Fluor conjugates were from Invitrogen (Carlsbad, CA). Receptor agonists were obtained as follows: Nerve Growth Factor 2.5S (mouse NGF) (Roche), human epidermal growth factor (Sigma), human basic fibroblast growth factor (Sigma), and human platelet derived growth factor BB homodimer (Upstate). The TrkA selective kinase inhibitor K-252a (*Nocardiopsis* sp. alkaloid) was obtained from EMD Biosciences, Inc. (San Diego, CA).

Table S1: Plasmid Sources

Plasmid(s)	Source
Wildtype and constitutively-activated (GV) mutants of triple hemagglutinin (3x HA) tagged human H-Ras, M-Ras, R-Ras, Rap1A, and Rap1B in pcDNA3.1	UMR cDNA Resource Center (www.cdna.org)
Human MEK1 and MEK2 in pUSEamp	Upstate Cell Signaling Solutions (Charlottesville, VA)
Human FGFR1 in pCMV-SPORT6 (NCBI GenBank accession BC015035)	Open Biosystems (Huntsville, AL)
Wildtype and activated mutants of HA-tagged H-Ras, K-Ras, and N-Ras in pCGN	Dr. Channing Der (UNC-Chapel Hill)
Activated mutants of H-Ras (G12V) and B-Raf (V600E) in pBABE; HA-ERK1 in pcDNA3	Dr. Channing Der (UNC-Chapel Hill)
3xHA-human A-Raf in modified pACTAG2	Dr. D. Anderson (Univ. of Saskatchewan)
HA-tagged human B-Raf and human c-Raf-1-FLAG in pcDNA3	Dr. K.-L. Guan (Univ. of Michigan, Ann Arbor)
Human A-Raf with an N-terminal FLAG-epitope tag in pCMV-FLAG-6b	Dr. Jeffrey Frost (Univ. of Texas Health Science Center, Houston)
N-terminal FLAG constructs of B-Raf deletion mutants in pCMV-FLAG-6b	Dr. Jeffrey Frost (Univ. of Texas Health Science Center, Houston)
Human B-Raf with a C-terminal FLAG-epitope tag in pLNCX	Dr. Deborah Morrison (NCI, Frederick, MD)
Rat TrkA with an N-terminal FLAG epitope tag in pcDNA3	Dr. Francis Lee (Weill Medical College of Cornell University)
Wildtype FLAG-tagged Rap2B in pCMV2	Dr. Lawrence Quilliam (IUPUI)
N-terminally HA-tagged InaD-PDZ1, Numb PTBo, full-length RGS12, RGS12-PDZ/PTB, and RGS12-PDZ expression vectors	(Sambi et al., 2006)
Rat ERK2 in the GFP fusion vector pEGFP-N1	(DeFea et al., 2000)

Table S2: Antibody Sources

Antibody (antibodies)	**Source**
Anti-HA-horseradish peroxidase (HRP) 3F10, anti-HA 12CA5, and anti-myc 9E10	Roche
Anti-myc-HRP 4A6 and anti-Rap-1	Upstate
Anti-RGS12 (A-14), -B-Raf (C-19), -EGFR (1005), -FGFR (C-17), -A-Raf (C-20), -MEK2 (A-1), -Lamin A/C (346), and -goat IgG HRP	Santa Cruz Biotechnology
Anti-tubulin-βIII, -β-actin, -pan-cadherin, -M2 FLAG HRP, and -M2 FLAG	Sigma (St. Louis, MO)
Anti-p44/42 ERK MAP kinase, anti-phospho-p44/42 (Thr202/Tyr204) ERK MAPK, and anti-phospho-TrkA (Tyr490)	Cell Signaling Technology (Danvers, MA)
Anti-rabbit IgG HRP and anti-mouse IgG HRP	GE Healthcare (Piscataway, NJ)
Anti-Ras OP-40	Calbiochem (San Diego, CA)
Anti-Living Colors A.v. peptide (GFP/CFP/YFP)	Clontech Inc. (Mountain View, CA)
Anti-H-Ras 146	Dr. Adrienne Cox (UNC-Chapel Hill)
Anti-EEA1	BD Biosciences (San Jose, CA)
Anti-lysosomal-associated membrane protein-1 (LAMP1) H4A3 mouse antibody	Developmental Studies Hybridoma Bank (Univ. of Iowa, Iowa City, IA)
Anti-RGS12-PDZ/PTB and anti-RGS12-RGS-box	(Martin-McCaffrey et al., 2005)

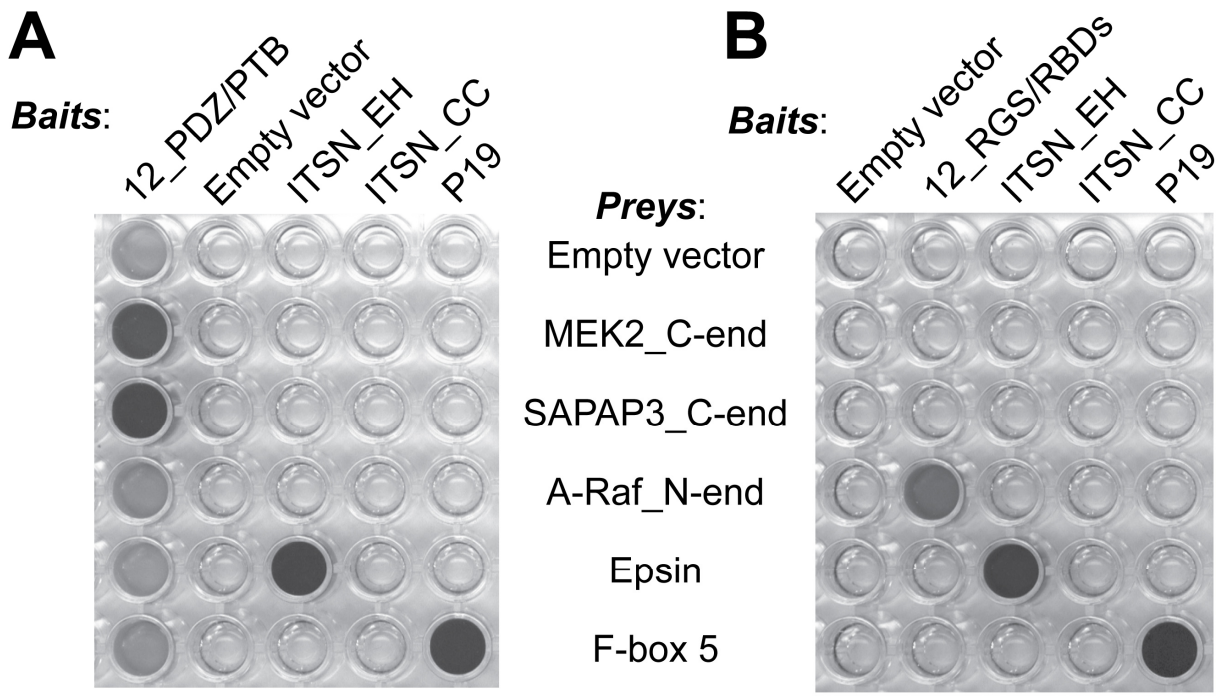

Figure S1. Validation of yeast two-hybrid interactions with RGS12-derived PDZ/PTB tandem (A) and RGS/RBD domain (B) fusion proteins. To confirm initial yeast two-hybrid positives, pairwise mating of yeast expressing bait or prey plasmids was performed in interaction-selection medium (histidine/adenine dropout plus 1 mM 3AT). Yeast were subsequently lysed and incubated for 7 hr (panel **A**) or 16 hr (panel **B**) at 37^{o}C with chlorophenyl red-β-D-galactopyranoside as chromogenic substrate for the β-galactosidase reporter of bait/prey interaction. Test baits included empty vector (pBUTE) and the following proteins cloned in-frame with the GAL4 DNA-binding domain: mouse RGS12 PDZ/PTB domain N-terminus (aa 1-369; panel **A**), rat RGS12 spanning the RGS domain and both RBDs (aa 702-1115; panel **B**), the EF-hand or coiled-coil domains of intersectin, and the human SKP1 homolog p19. Test prey included empty GAL4 activation domain vector (pGADC1), MEK2 C-terminus (aa 324-401), SAPAP3/Dlgap3 C-terminus (aa 814-977), an N-terminal portion of the MAP3K kinase A-Raf (aa 36-153), mouse Epsin, and human F-box 5 protein. The latter two prey serve as positive controls for interaction with the ITSN EF-hand domain and p19 baits, respectively (Lisztwan et al., 1998; Sengar et al., 1999).

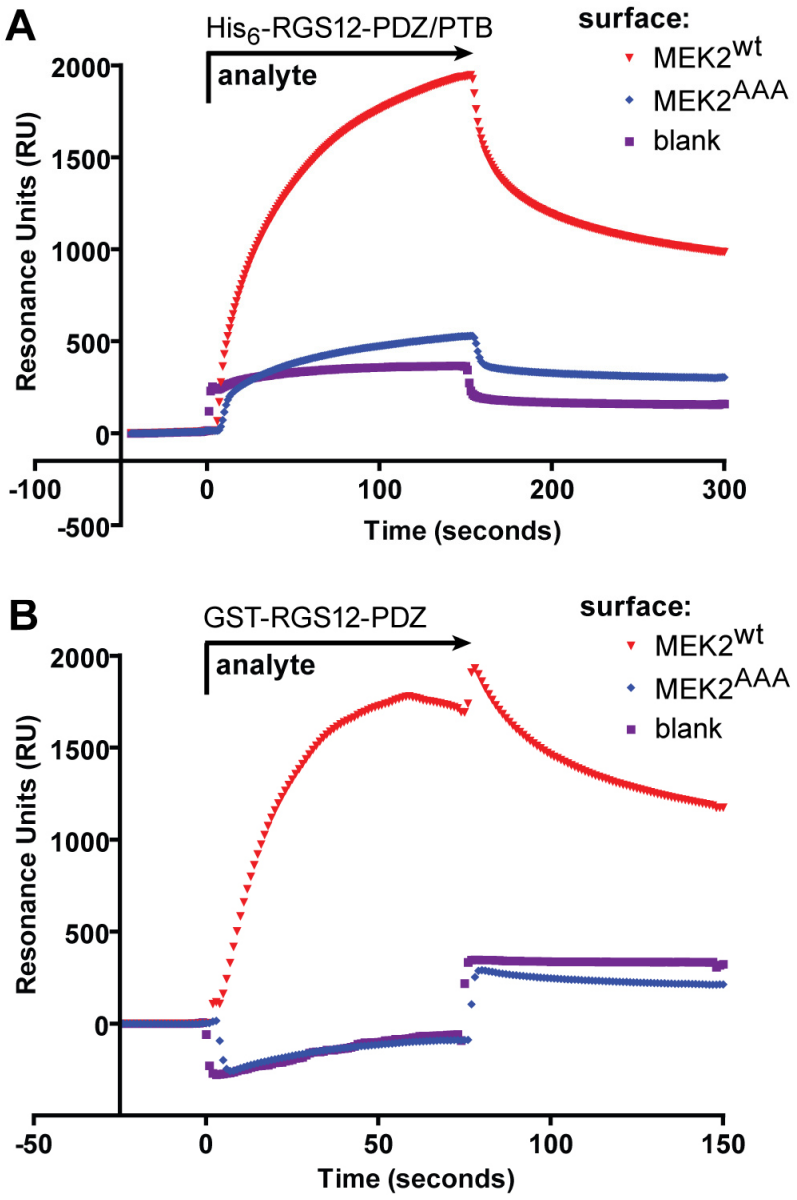

Figure S2. RGS12-derived PDZ/PTB tandem and PDZ domain fusion proteins specifically interact with the wildtype MEK2 C-terminal tail *in vitro*. Simultaneous SPR measurements of binding to a biotinylated peptide comprising the last 16 amino acids of MEK2 (wt), to the same peptide but ending with triple-alanine (AAA), and to a blank flow cell after injection (time 0 s, flow rate 20 µl/min) of 50 µl of 5 µM His_6-RGS12-PDZ/PTB fusion protein (panel **A**) or 50 µl of 5 µM GST-RGS12-PDZ fusion protein (panel **B**) at 25°C.

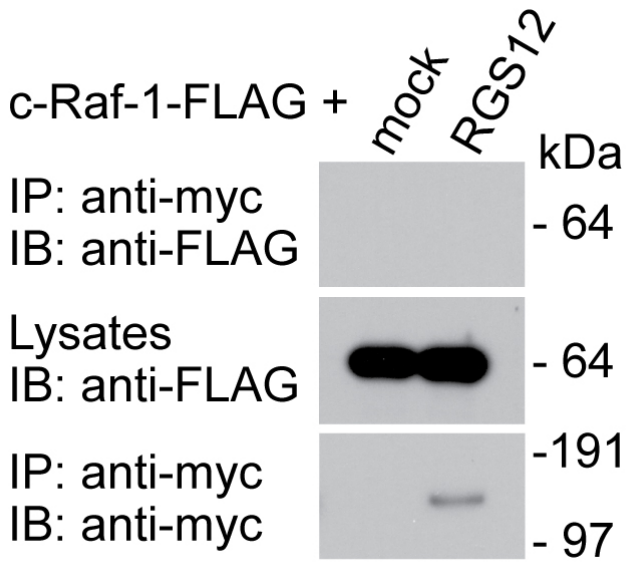

Figure S3. c-Raf-1 does not co-immunoprecipitate with RGS12. FLAG-epitope tagged c-Raf-1 and myc-epitope tagged, full-length RGS12 were transiently co-expressed in HEK 293T cells. Cell lysates obtained 48 hr after transfection were immunoprecipitated (IP) with anti-myc antibody; IP samples and cell lysates were subsequently immunoblotted (IB) as indicated above.

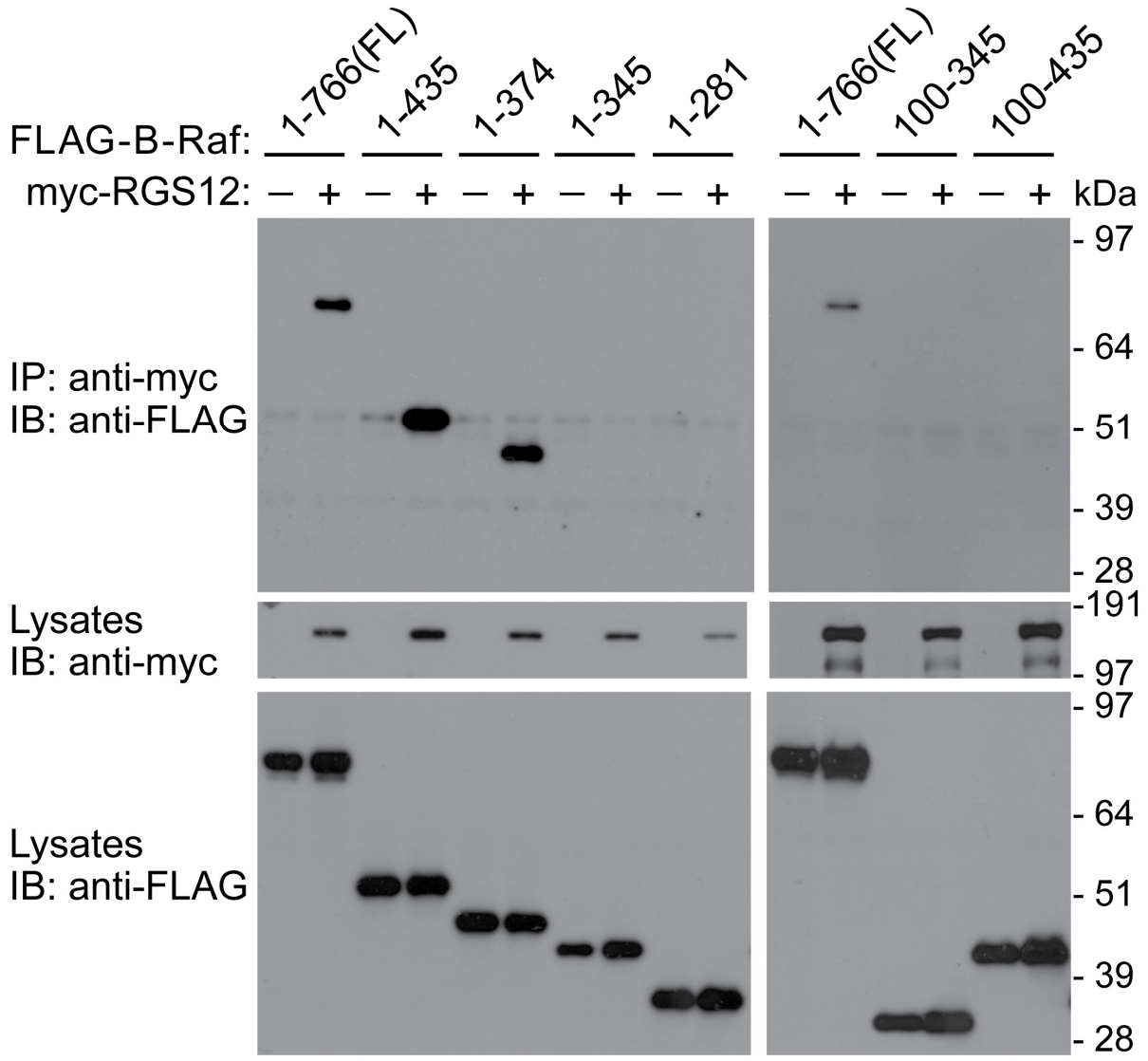

Figure S4. The N-terminal 374 amino-acids of B-Raf co-immunoprecipitate with RGS12. FLAG-epitope tagged B-Raf truncation mutants (Tran et al., 2005) and myc-epitope tagged, full-length RGS12 were transiently co-expressed in HEK 293T cells. Cell lysates obtained 48 hr after transfection were immunoprecipitated (IP) with anti-myc antibody; IP samples and cell lysates were subsequently immunoblotted (IB) as indicated above.

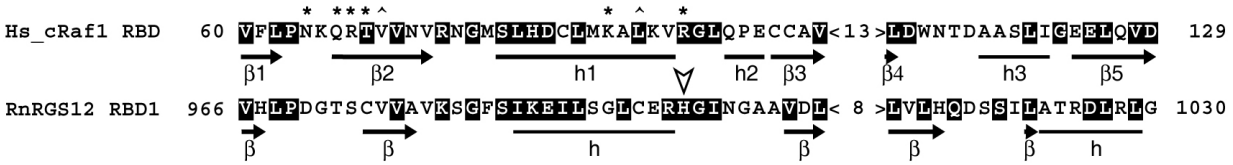

Figure S5. Predicted structural similarity between the Ras-binding domain (RBD) of human c-Raf-1 with the first, N-terminal RBD of rat RGS12. Comparison of known secondary structure of human c-Raf-1 (Protein Data Bank id 1GUA) with the predicted secondary structure of the first RBD within rat RGS12, the latter as derived from the 3D-PSSM protein fold recognition server (http://www.sbg.bio.ic.ac.uk/3dpssm/). Asterisks (*) and carats (^) denote c-Raf-1 residues involved in polar or hydrophobic interactions, respectively, with the effector-binding domain of bound Ras-family GTPase (PDB 1GUA). β, beta-strand secondary structure; h, alpha- or 3_{10}-helical structure. Open arrowhead denotes position of histidine-995 residue mutated to leucine within rat RGS12 to create a loss-of-function for Ras GTPase binding.

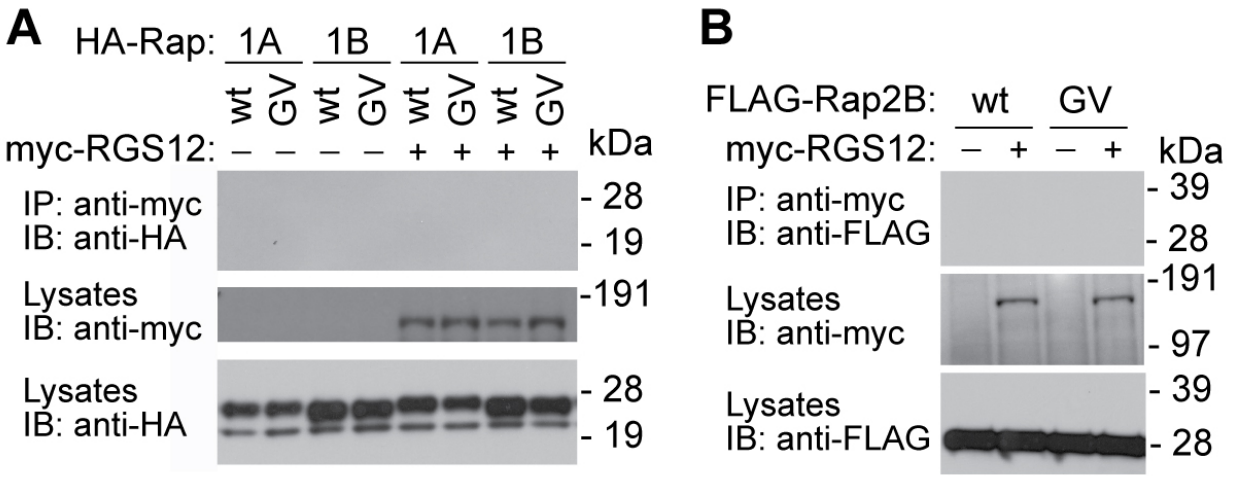

Figure S6. Rap-family GTPases do not co-immunoprecipitate with RGS12. Wildtype (wt) and constitutively-activated (GV), HA-epitope tagged Rap1A and Rap1B (panel **A**) or FLAG-epitope tagged Rap2B (panel **B**) were transiently co-expressed in HEK 293T cells along with myc-epitope tagged, full-length RGS12. Cell lysates obtained 48 hr after transfection were immunoprecipitated (IP) with anti-myc antibody; IP samples and cell lysates were subsequently immunoblotted (IB) as indicated above.

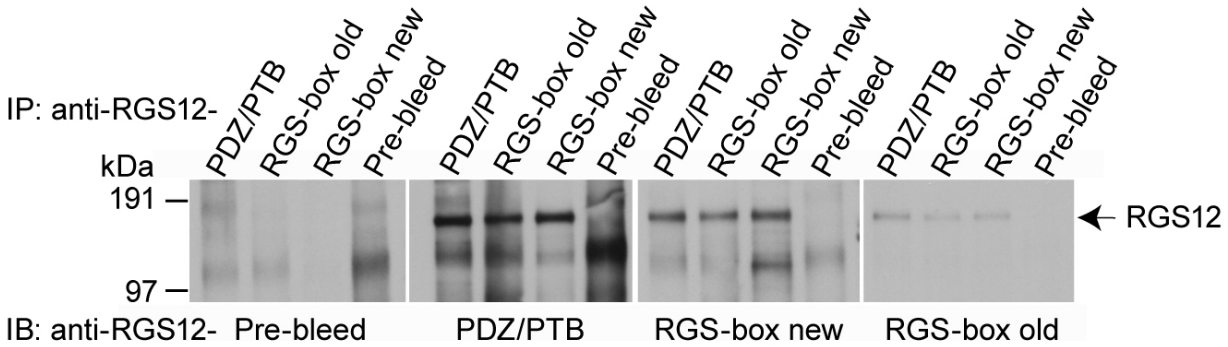

Figure S7. Validation of anti-RGS12 rabbit antisera and demonstration of endogenous RGS12 expression in PC12 rat pheochromocytoma cells. Rabbits were immunized with recombinant protein spanning the first 440 amino-acids of rat RGS12 (encompassing the N-terminal PDZ and PTB domains: "PDZ/PTB") or spanning residues 664-885 (encompassing the central RGS-box). Resultant antisera (or serum from pre-immunized rabbits ["pre-bleed"]) were used to immunoprecipitate (IP) proteins from PC12 cell lysate as indicated; immunoprecipitating proteins were resolved by SDS-PAGE, electroblotted onto nitrocellulose, and subsequently immunoblotted with indicated antisera, as per methods described in *Materials and Methods*. Note that "RGS-box old" describes rabbit antiserum previously described (Schiff et al., 2000), but subsequently exhausted and hence replaced via a new round of rabbit immunization using the same immunogen.

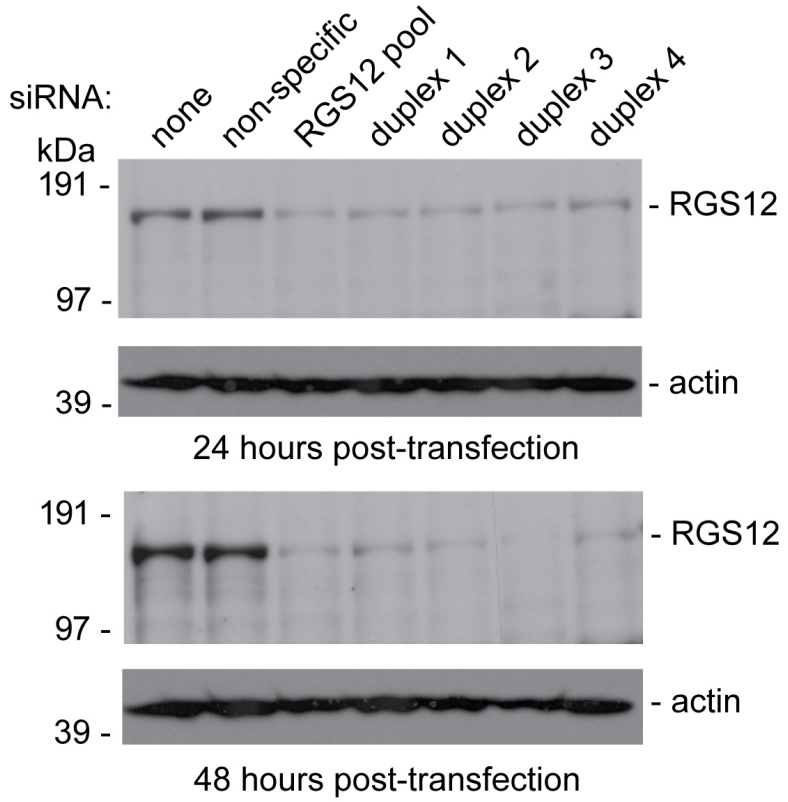

Figure S8. Four distinct siRNA oligonucleotide duplexes each result in knockdown of endogenous RGS12 expression. PC12 cells were transiently transfected with siRNAs as described in *Materials and Methods* and resultant cell lysates (obtained 24 or 48 hr later) were immunoblotted for RGS12 and actin as indicated. "RGS12 pool" refers to Dharmacon Research's SMARTpool designed against the rat *Rgs12* mRNA, comprised of four siRNA oligonucleotide duplexes targetting the following specific mRNA sequences: duplex 1 (5'-GGC CGA AAC UUG ACU CUA A-3'); duplex 2 (5'-GAA CAC UAG GCA AGU CUA A-3'); duplex 3 (5'- GCA ACA GGG UGC UUG UAG U-3'); duplex 4 (5'- CAU GAC AGU UUA CAG GCU A-3').

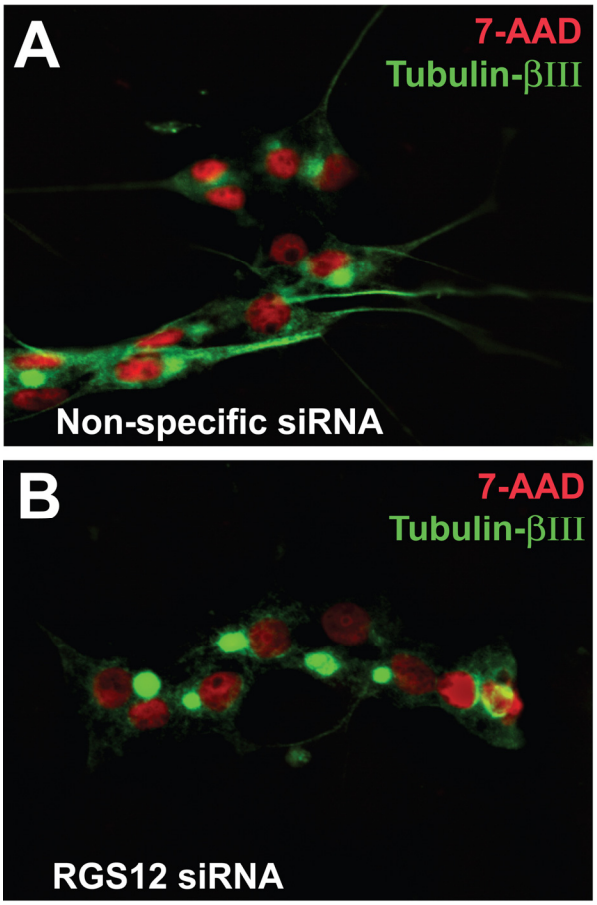

Figure S9. RGS12 is critical for NGF-mediated neurite outgrowth in PC12 cells.
PC12 cells were transfected with non-specific siRNA (panel **A**) or RGS12 siRNA (panel **B**) for 24 hr, and then treated with 100 ng/ml NGF for an additional 48 hr. Cells were fixed, permeabilized, and stained as described in *Supplemental Materials and Methods* with the differentiation marker anti-tubulin-βIII (*green*) and the nuclear stain 7-aminoactinomycin D (7-AAD; *red*) prior to analysis by confocal microscopy.

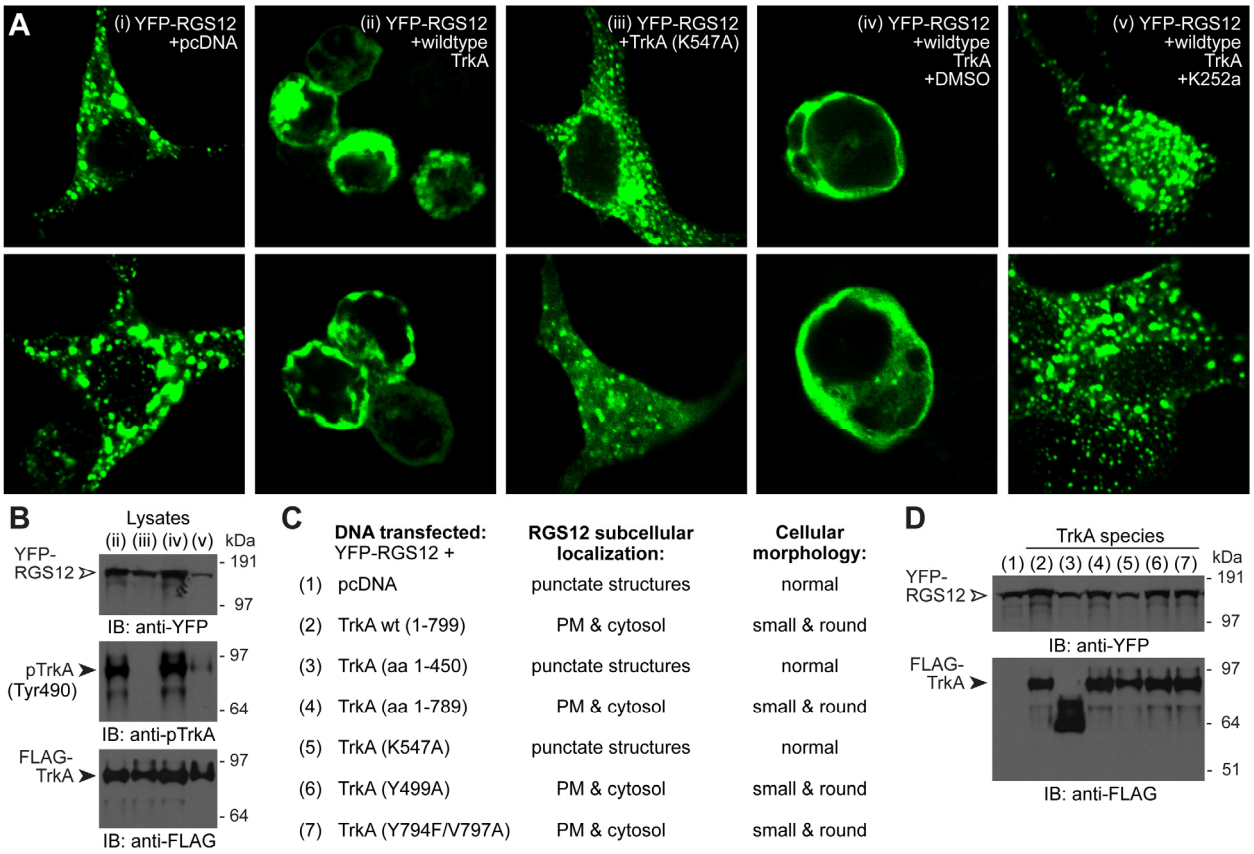

Figure S10. Relocalization of YFP-RGS12 from puncta upon TrkA co-expression is dependent on TrkA kinase activity. (**A**) HEK 293T cells were co-transfected with YFP-RGS12 and either (i) pcDNA3.1 empty vector, (ii) FLAG-TrkA wildtype, (iii) FLAG-TrkA kinase-inactive mutant (K547A), (iv) FLAG-TrkA wildtype (24 hr post-vehicle treatment [DMSO]), and (v) FLAG-TrkA wildtype (24 hr post-K-252a treatment [100 nM]) expression vectors and imaged by confocal microscopy for YFP-RGS12 localization. (**B**) HEK 293T cells were transfected as described in panel A. 48 hr post-transfection, cells were lysed and immunoblotted to verify expression of YFP-RGS12, FLAG-TrkA, and the phosphorylation state of TrkA (i.e., phosphotyrosine-490). (**C**) HEK 293T cells were co-transfected with YFP-RGS12 and either (1) pcDNA3.1 empty vector, (2) FLAG-TrkA wildtype (aa 1-799), (3) FLAG-TrkA (aa 1-450; kinase domain-deleted), (4) FLAG-TrkA (aa 1-789; C-terminal tail-deleted), (5) FLAG-TrkA kinase-inactive (K547A), (6) FLAG-TrkA (Y499A; loss-of-function mutation to Shc-binding site), and (7) FLAG-TrkA (Y794F/V797A; loss-of-function mutation to PLCγ-binding site) and imaged by confocal microscopy. (**D**) HEK 293T cells were transfected as described in panel C. 48 hr post-transfection, cells were lysed and immunoblotted to verify expression of YFP-RGS12 and FLAG-TrkA (wildtype and mutants thereof).

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