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Supplementary Materials for

SH3 Domain–Based Phototrapping in Living Cells Reveals Rho Family GAP Signaling Complexes

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The PDF file includes:

Supplementary Materials

SUPPLEMENTARY MATERIALS AND METHODS

Label-Free Quantitation of Free Style 293 lysate

Label-free quantitation and integration of qualitative peptide identifications was performed using Rosetta Elucidator (v 3.3, Rosetta Inpharmatics, Seattle, WA). Triplicate LC-MS/MS analysis of a Free Style 293 lysate were imported and subjected to chromatographic retention time alignment using the PeakTeller® algorithm. Quantitation of all detectable signals in the precursor MS spectra was performed within Elucidator by calculating peak height of the corresponding peptide level extracted ion chromatograms. Protein quantities were calculated using the average MS response from the two or three highest abundant peptides based on a modified strategy initially described by Silva et. al. (*1*). Absolute quantities were determined by normalizing MS response factors to that of yeast alcohol dehydrogenase spiked into the lysate at 50 fmol/ug.

Immunostaining of HBE cells

Culturing and transfection of Human Bronchial Epithelial (HBE) cells were as described for HEK293 cells. Immunostaining for HBE cells was also performed the same way as for HEK293 cells except for the use of 1% paraformaldehyde for fixation. The primary antibodies used for staining were polyclonal mouse anti-ARHGAP12 (Abcam, 1:100) and rabbit anti-ZO-2 (C-term) (Invitrogen, 1:100).

REFERENCE

1. J. C. Silva, M. V. Gorenstein, G. Z. Li, J. P. Vissers, S. J. Geromanos, Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. *Mol Cell Proteomics* **5**, 144-156 (2006).

Fig. S1.Expression levels of Rho family GAP SH3 domains and their interactors. (**A**) Schematic of vector used to express pBpa containing bait SH3 domains. (**B**) Relative levels of each SH3 domain bait after transfection. n=2. (**C**) Graph depicting the quantification of the most abundant proteins in the lysate of Free Style 293 cells to three orders of magnitude. **D**) Chart showing the number of PI cluster proteins identified in lysates. Only 10.8% of PI cluster proteins and one SH3 domain ligand were identified as abundant proteins in (C) using label-free LC/MS/MS quantification of Free Style 293 lysates.

Figure S1

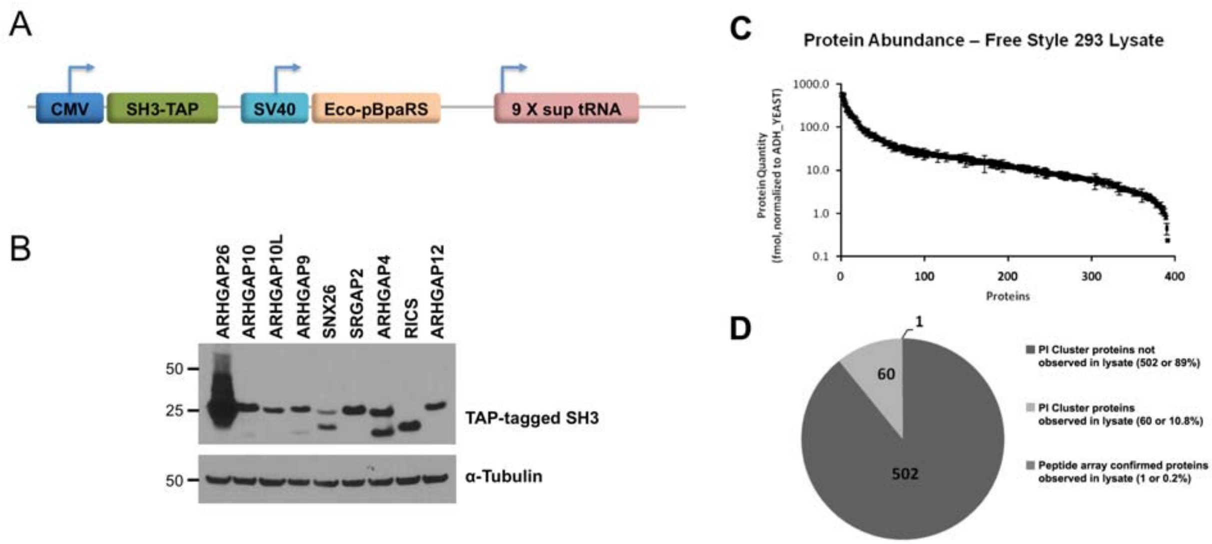


Fig. S2. Identification of SH3 domain binding proteins by peptide array-based in vitr*o* binding assay and coimmunoprecipitation. **A-I**) Proteins in Rho GAP SH3 domain-specific protein interaction (PI) clusters shown in Fig. 3A were searched for PXXP motifs. Peptides (18mer) containing the PXXP motifs were synthesized on cellulose membranes, incubated with purified GST-tagged wild-type Rho GAP SH3 domain, and immunoblotted with anti-GST antibody. The peptide array immunoblots were densitometrically quantified. The binding strength was normalized to the strongest interaction. **J**) HEK293 cells co-expressing palladin-GFP with vector or full-length wild-type SRGAP2-Flag were subjected to immunoprecipitation by Flag antibody. **K**) HEK293 cells co-expressing ARHGAP4 -Flag with GFP only or GFP-tagged full-length DIAPH1, were subjected to immunoprecipitation by GFP antibody. Co-precipitation was analyzed by Western blot analyses using indicated antibodies. Representative blots are shown from n=3.

Figure S2. ARHGAP26/SH3 - *in Vitro* Binding Assay

A

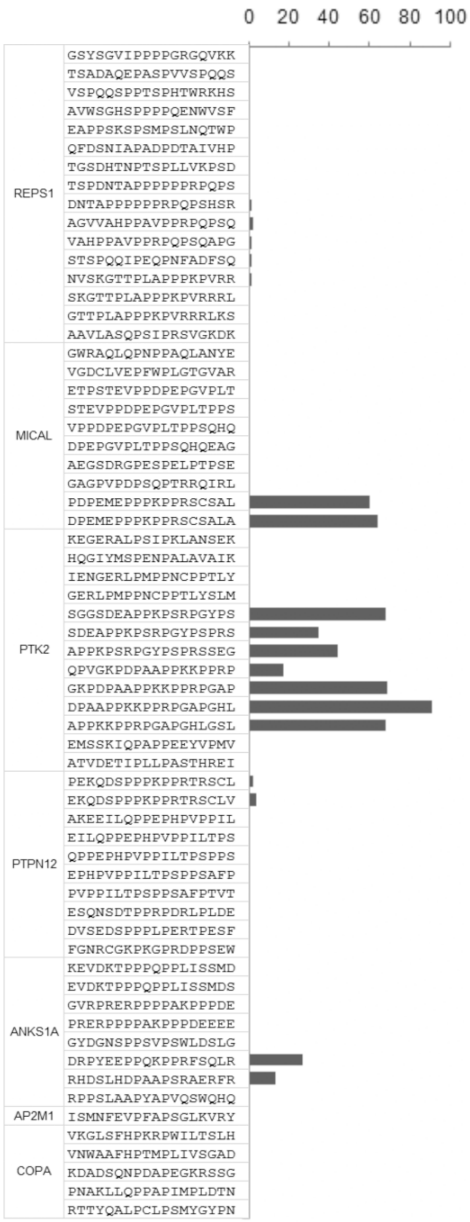


Figure S2. ARHGAP10/SH3 - *in Vitro* Binding Assay

B

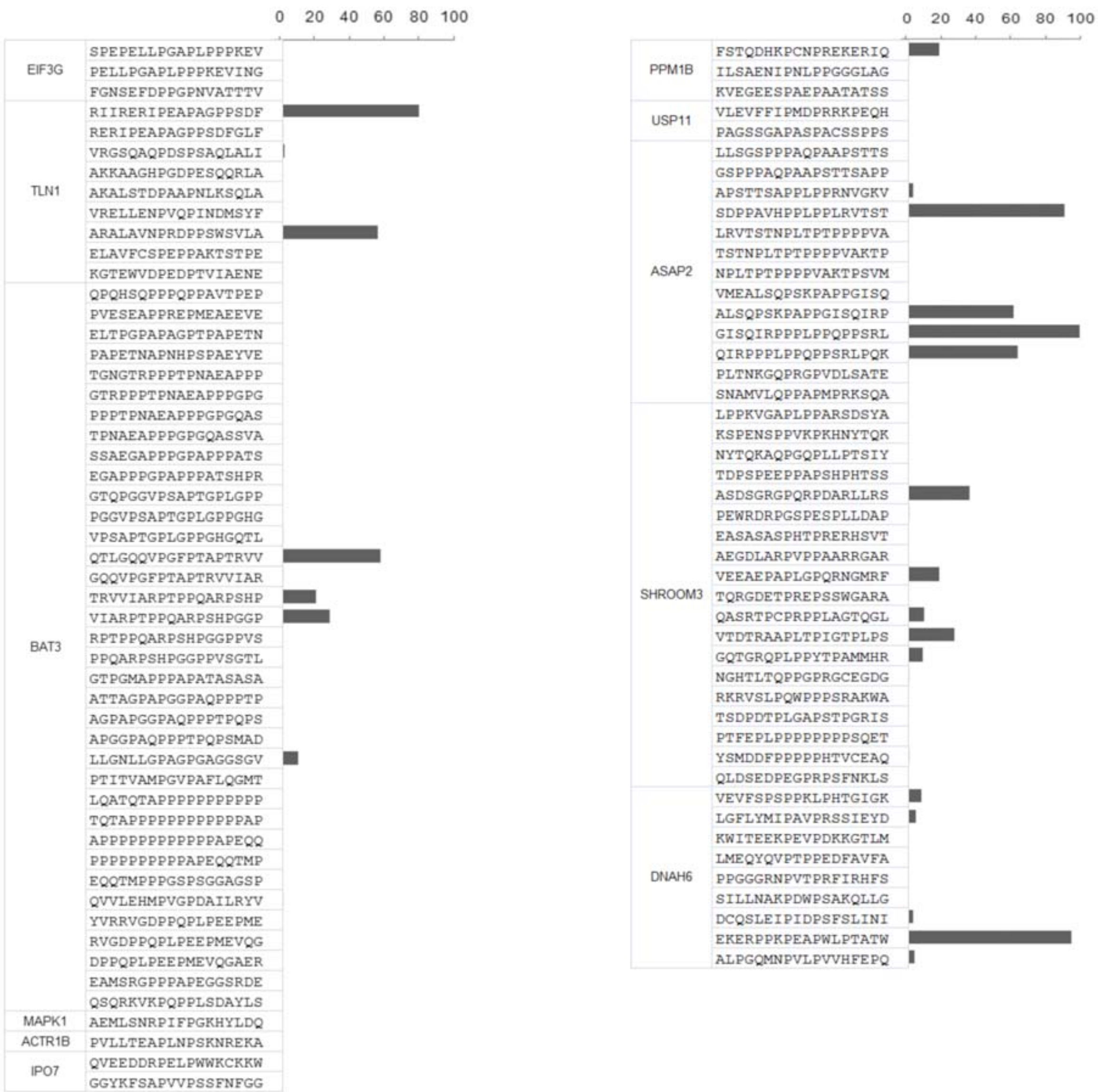


Figure S2. ARHGAP10L/SH3 - *in Vitro* Binding Assay

C

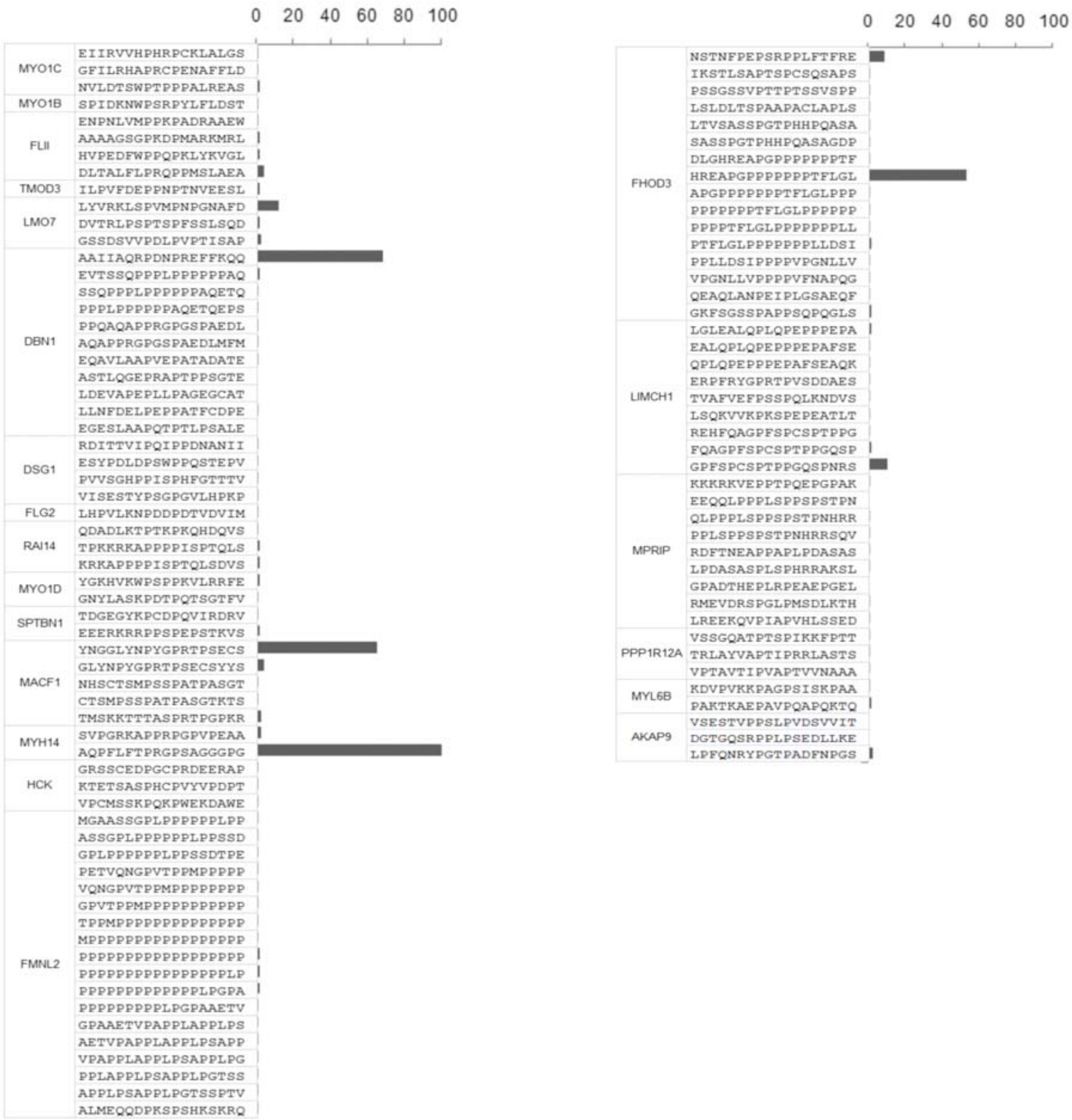


Figure S2. ARHGAP9/SH3 - *in Vitro* Binding Assay

D

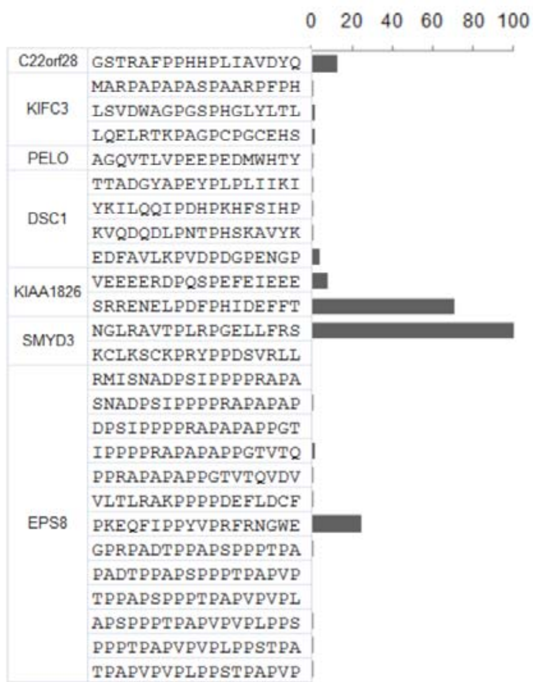


Figure S2. SNX26/SH3 - *in Vitro* Binding Assay

E

Figure S2. SRGAP2/SH3 - *in Vitro* Binding Assay (1/2)

F1

Figure S2. SRGAP2/SH3 - *in Vitro* Binding Assay (2/2)

F2

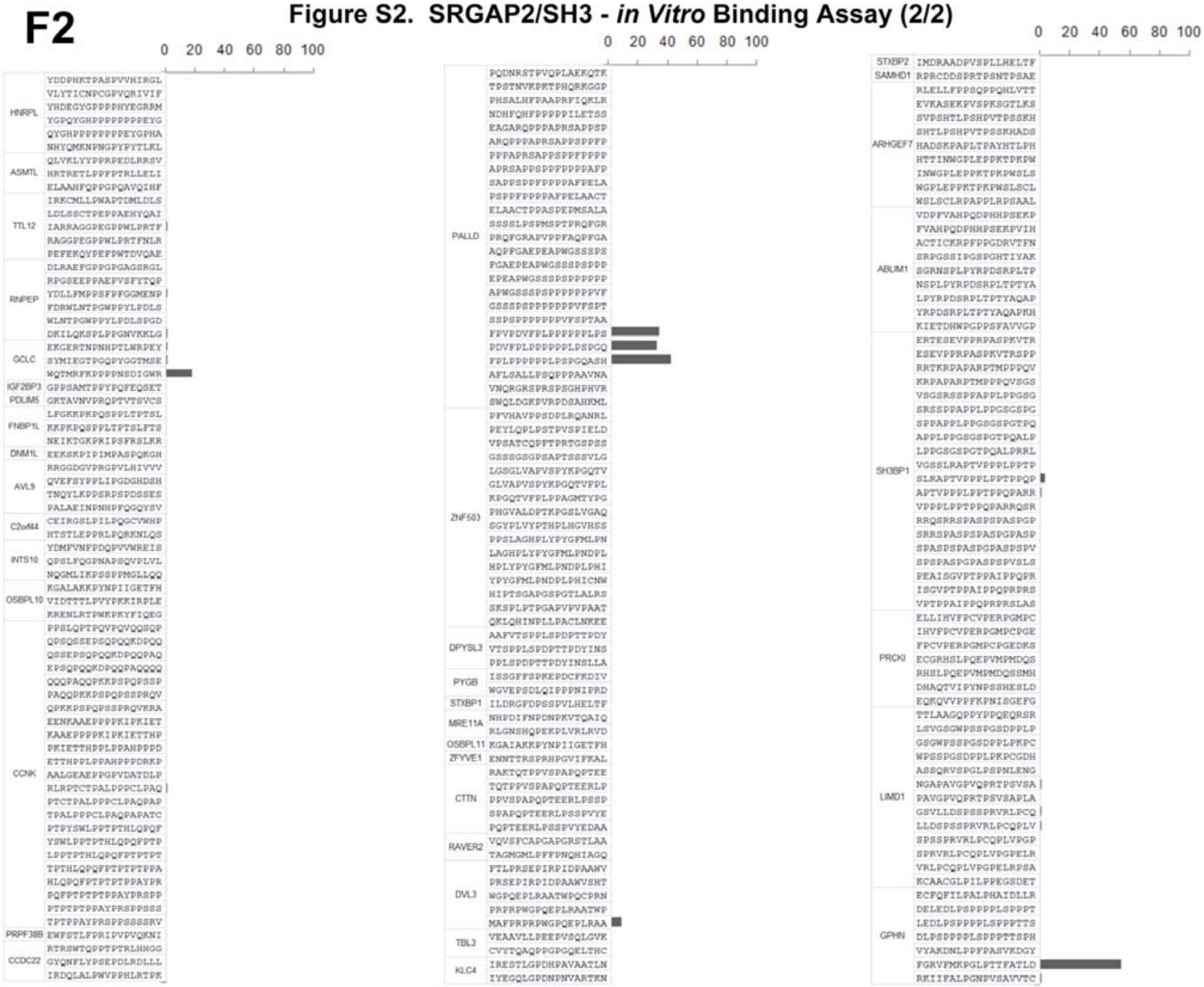


Figure S2. ARHGAP4/SH3 – *in Vitro* Binding Assay (1/2)

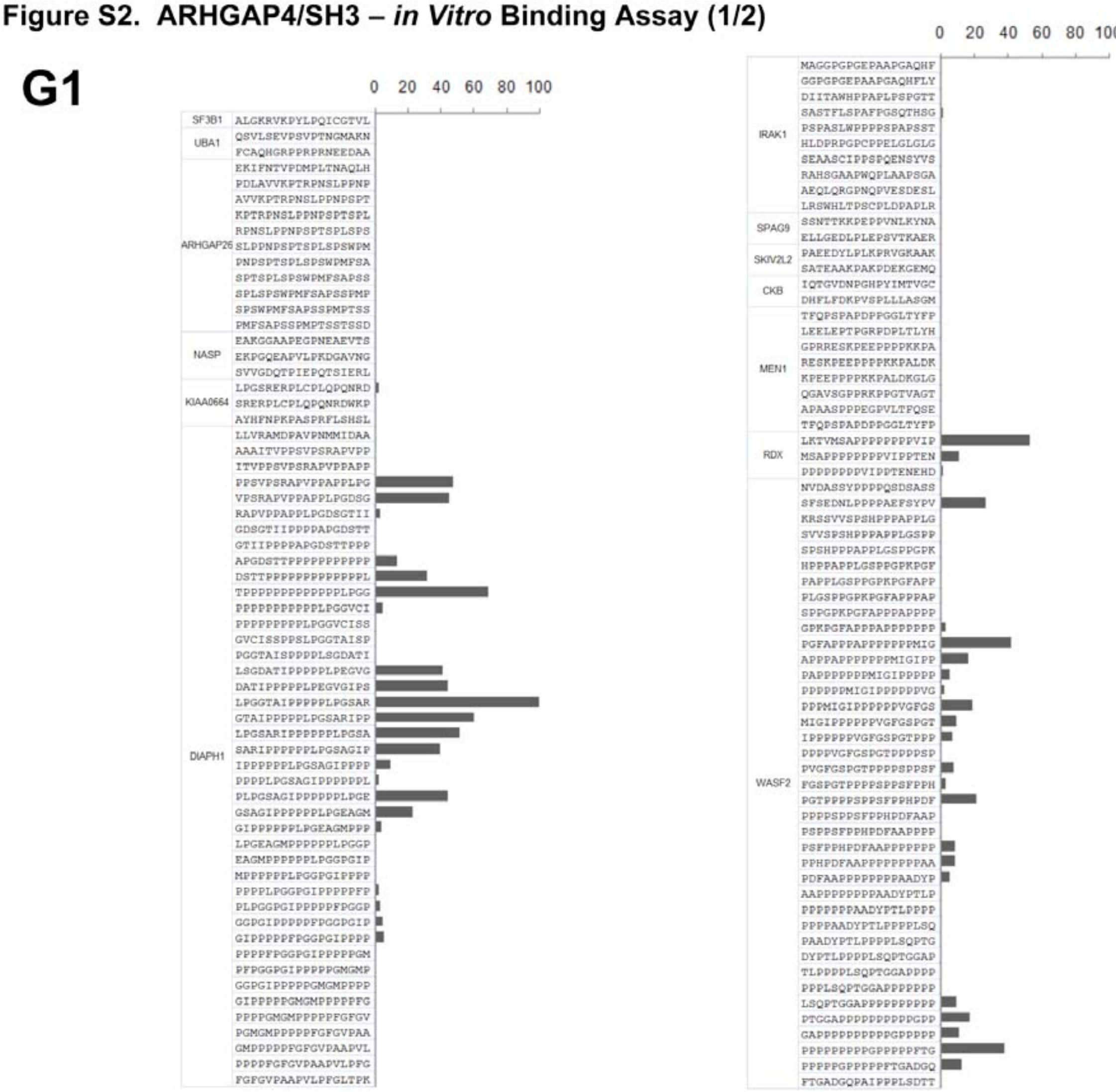


Figure S2. ARHGAP4/SH3 - *in Vitro* Binding Assay (2/2)

G2

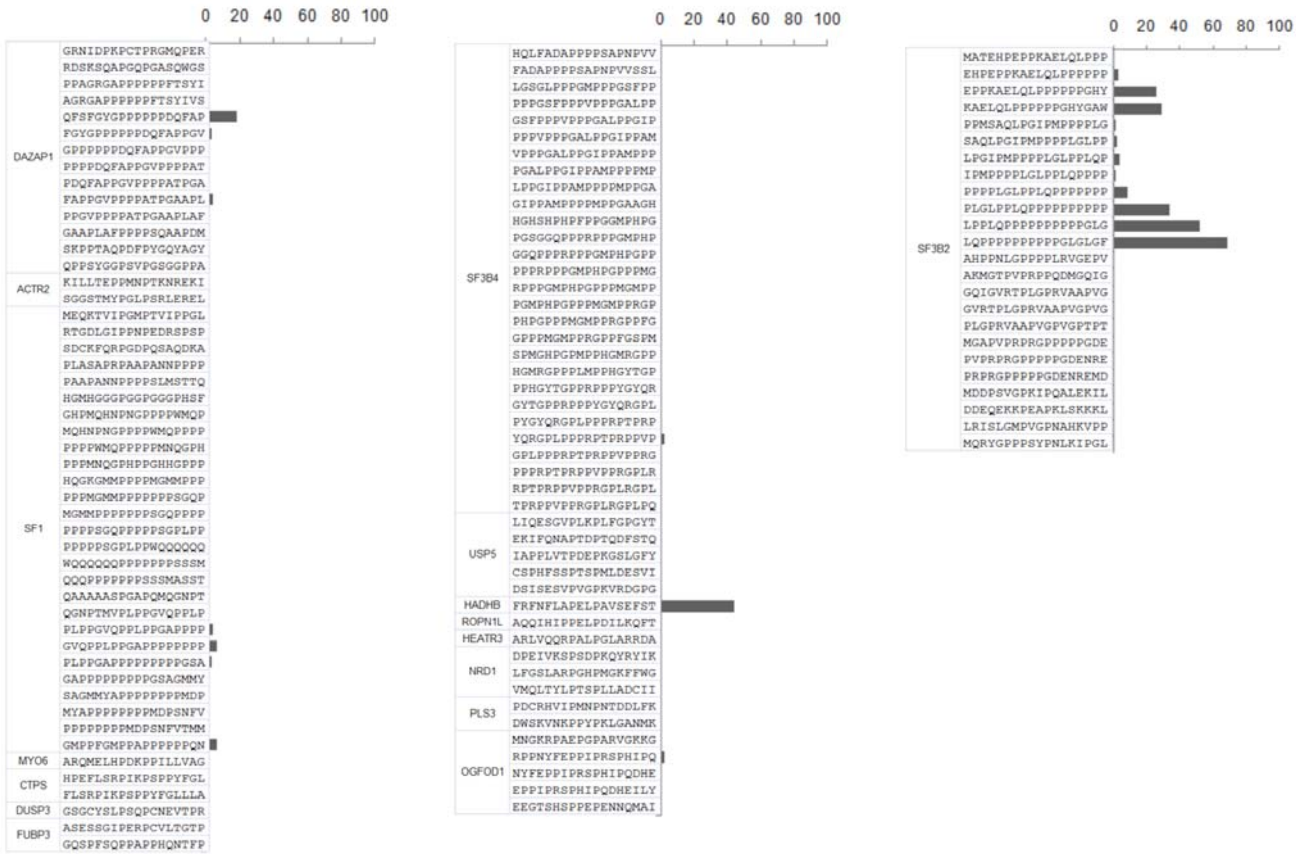


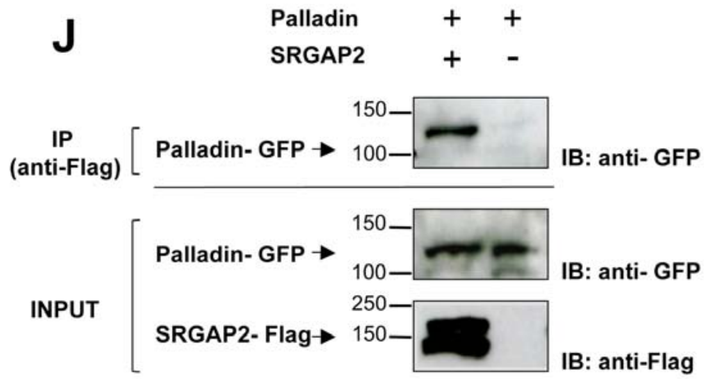
Figure S2. RICS/SH3 - *in Vitro* Binding Assay

H

Figure S2. ARHGAP12/SH3 - *in Vitro* Binding Assay

I

Figure S2. Co-immunoprecipitation assay



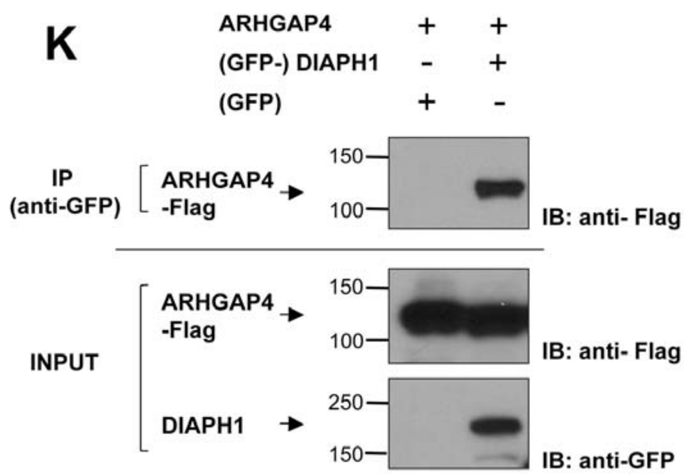


Fig. S3. Rho family GAP PI cluster interactomes and colocalization data. **A-I**) Protein-protein interaction networks of Rho GAPs were constructed from SH3 domain PI clusters based on peptide array and published interactome data. Circle nodes represent proteins in the PI clusters specific to individual RhoGAP SH3 domains. Hexagon nodes represent sub-specific proteins that were association with two RhoGAP SH3 domains. These two types of nodes are colored to reflect the mean normalized spectral counts (see spectral scale). Diamond nodes in cyan represent Rho GTPases added to visualize their potential links to the networks. Brown edges represent novel SH3-ligand interactions, and orange edges represent known interactions reproduced by this study. Thickness of these edges reflects the relative binding affinity determined by the in vitro binding assays. Blue and cyan edges that have a constant thickness indicate previously known interactions. Cyan edges exhibit known interactions with Rho GTPases. **J**) Co-localization of Endogenous ARHGAP12 and Tight Junction Protein TJP2 at cell-cell junctions. Human bronchial epithelial cells were fixed and stained with antibodies against indicated proteins. Scale bars = 10μm.

Figure S3. Rho GAP Interactome – ARHGAP26

A

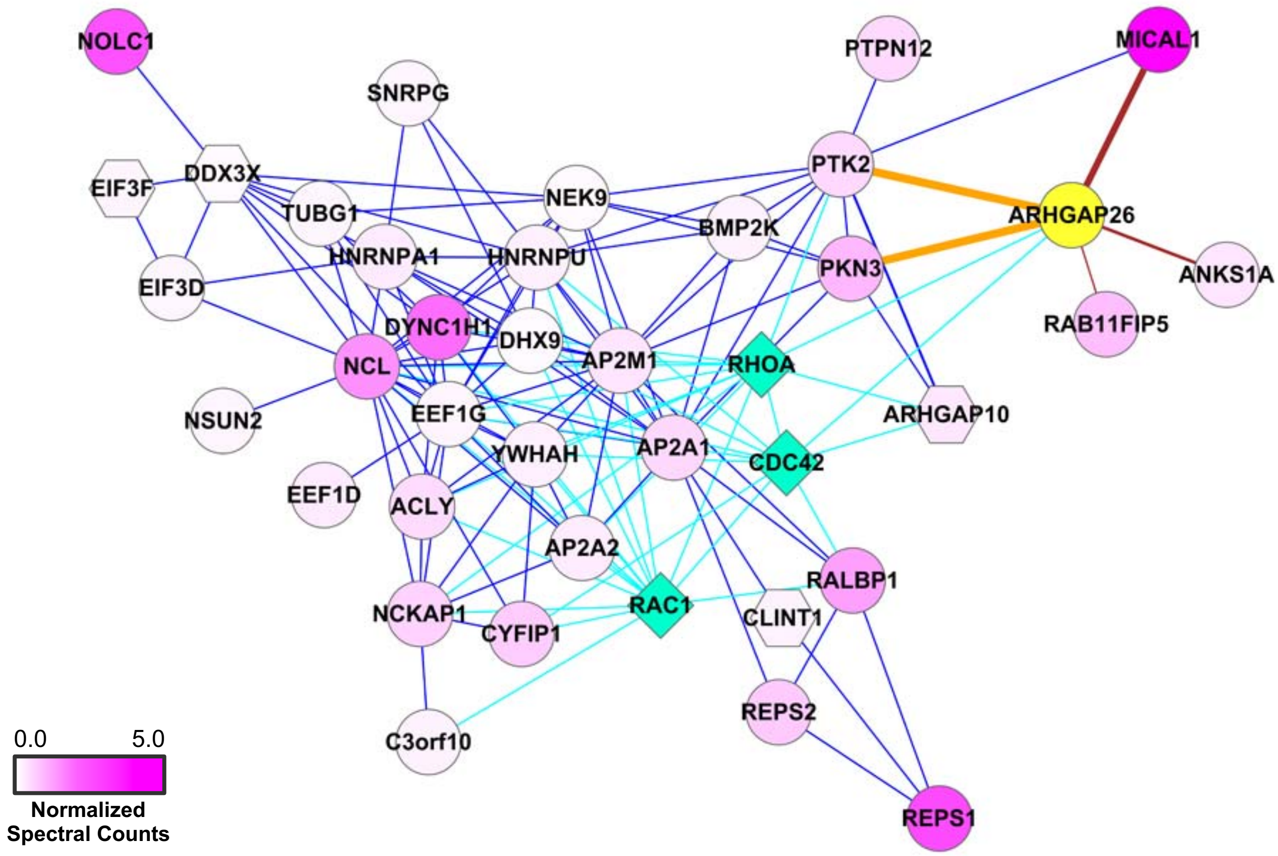


Figure S3. Rho GAP Interactome – ARHGAP10

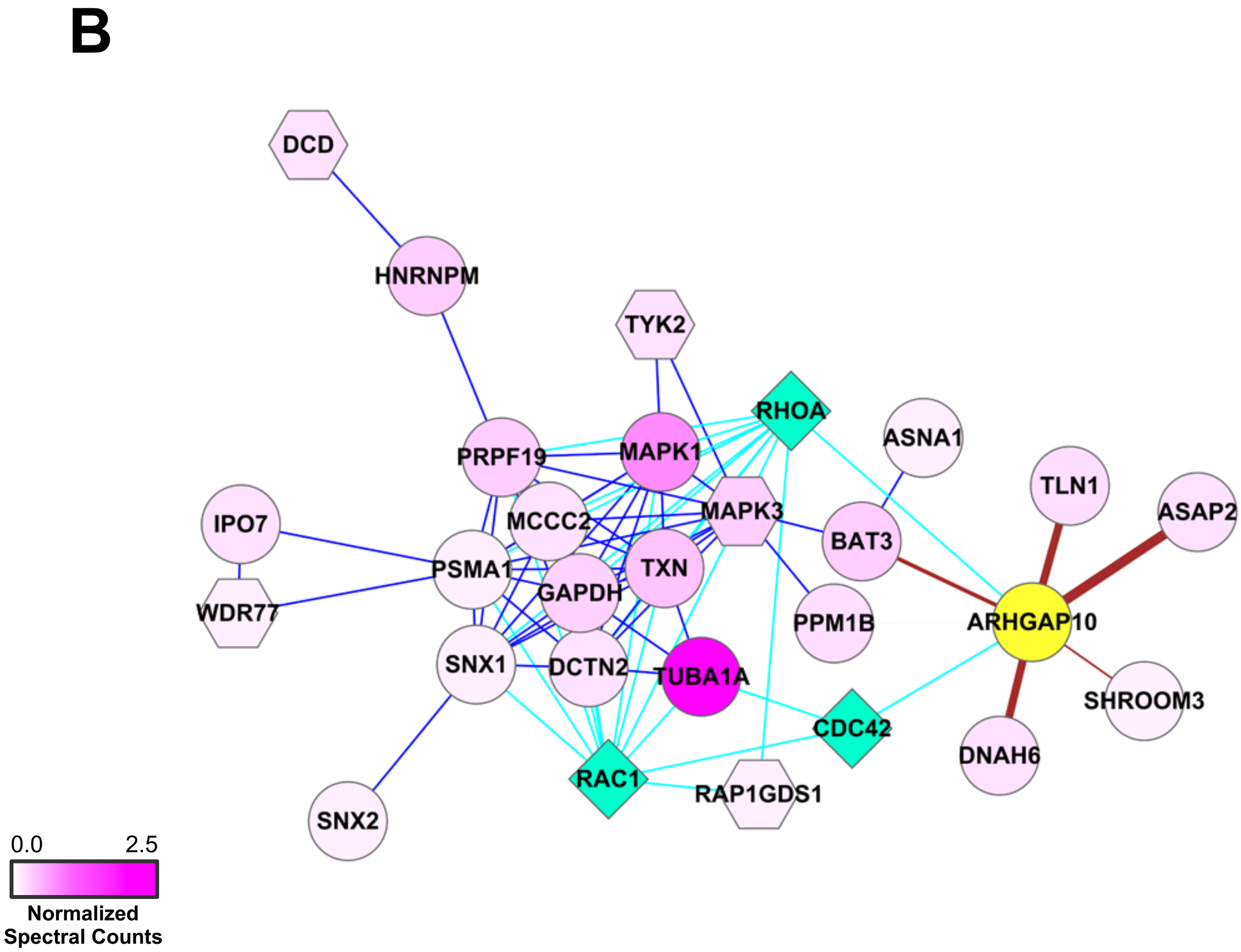


Figure S3. Rho GAP Interactome – ARHGAP10L

C

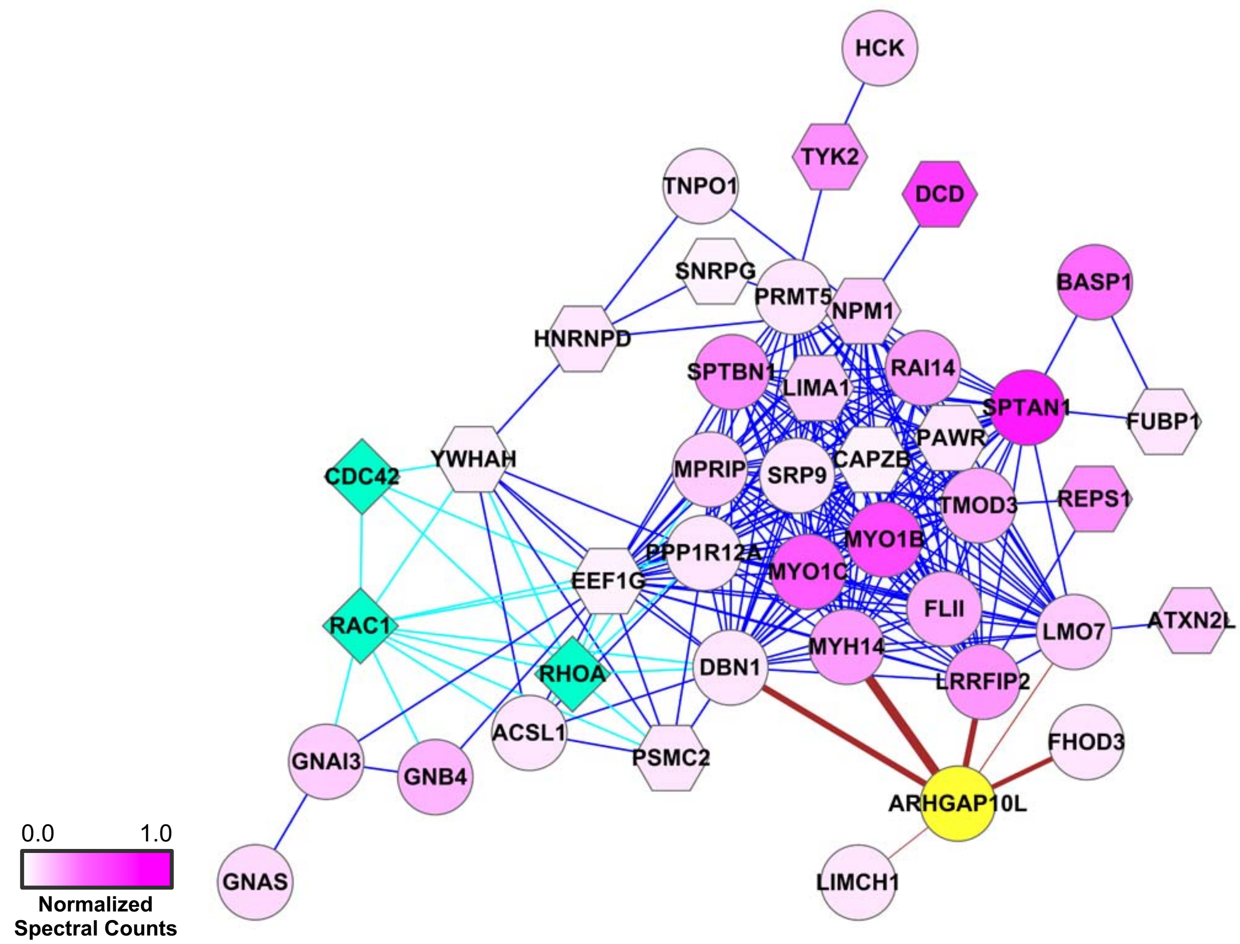


Figure S3. Rho GAP Interactome – ARHGAP9

D

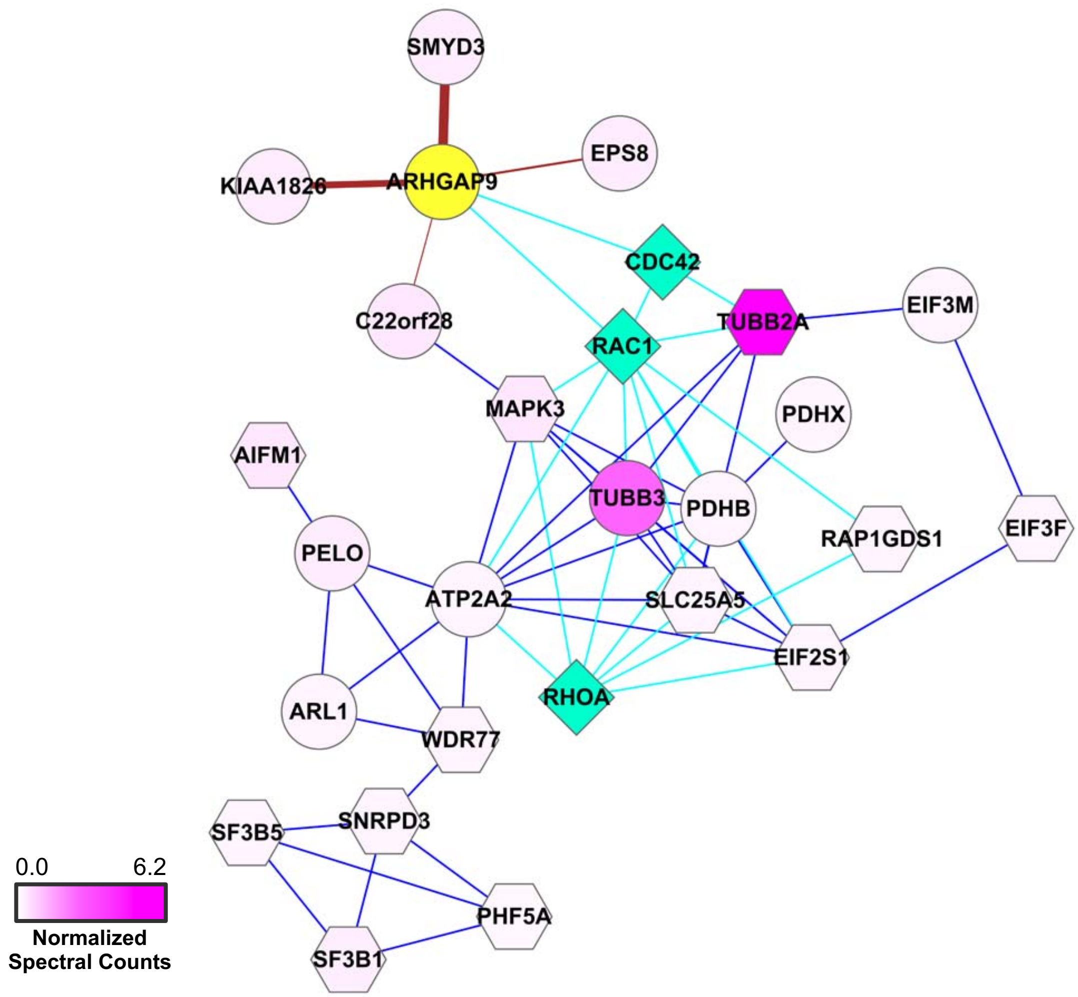


Figure S3. Rho GAP Interactome – SNX26

E

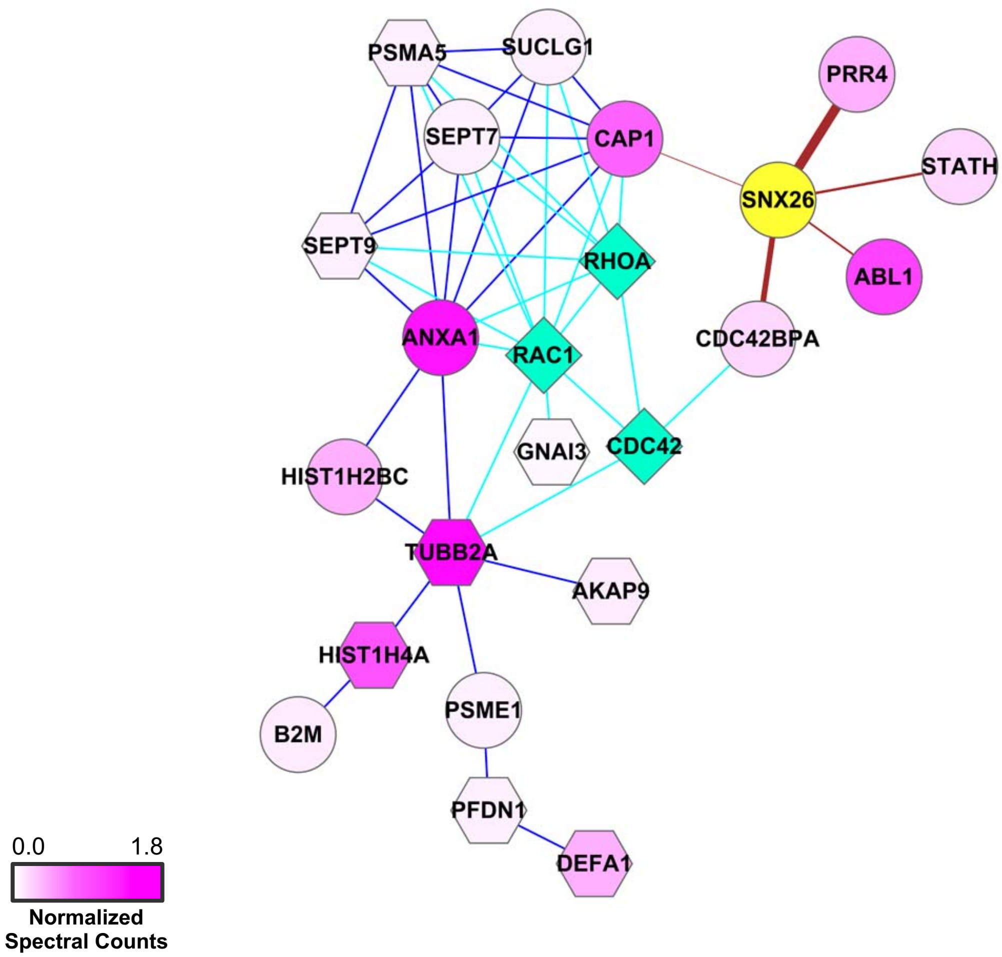


Figure S3. Rho GAP Interactome – SRGAP2

F

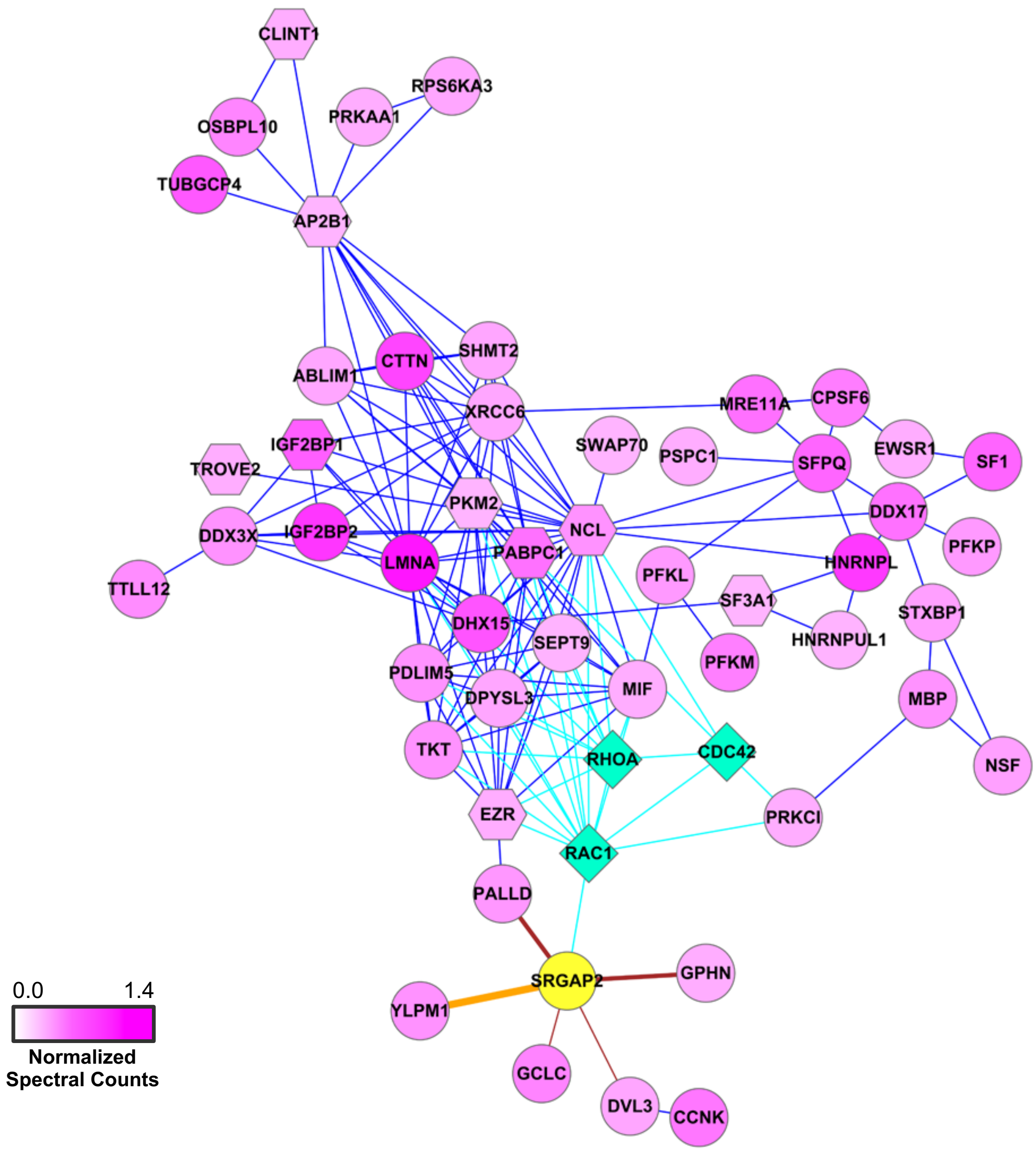


Figure S3. Rho GAP Interactome - ARHGAP4

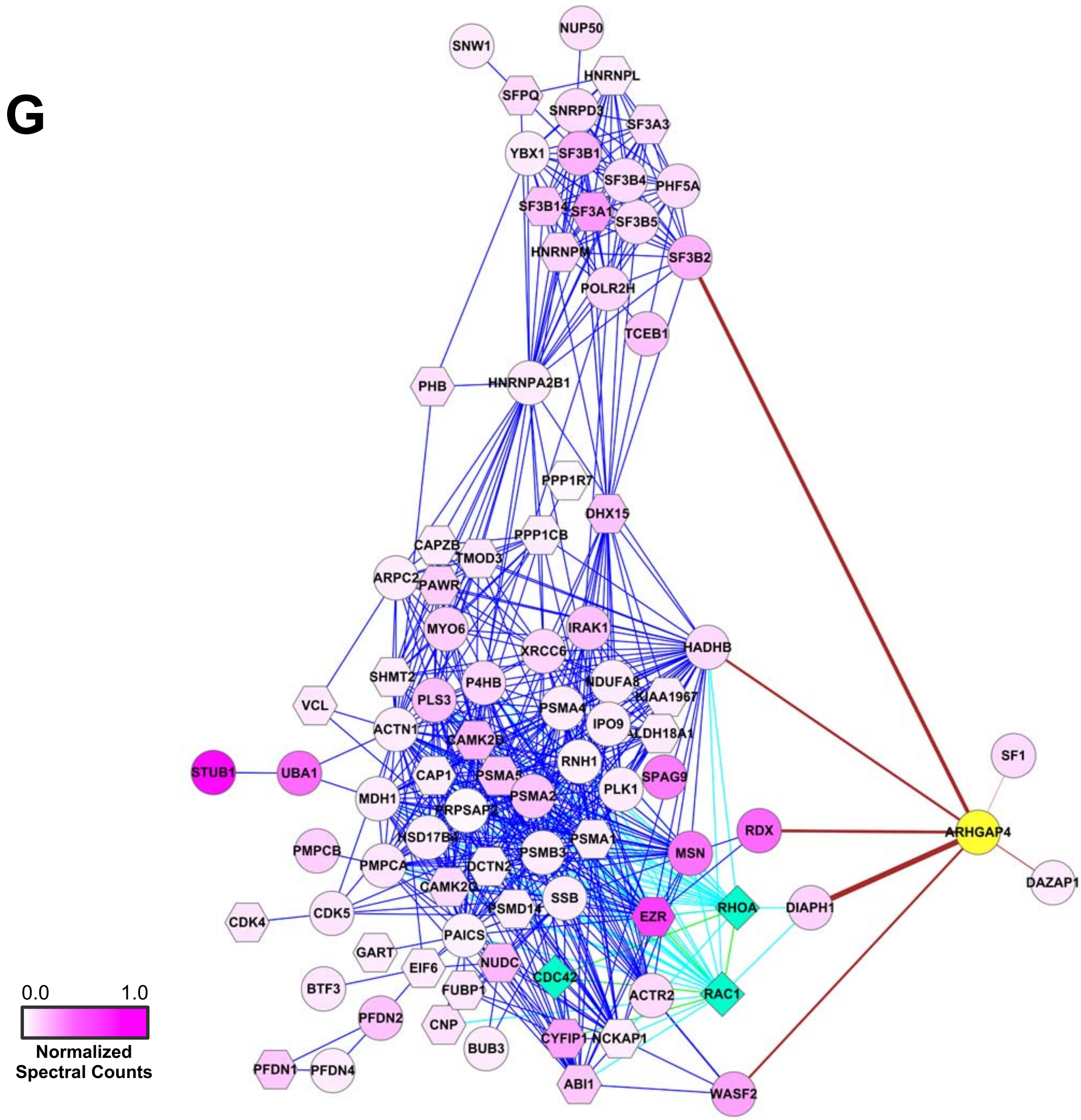


Figure S3. Rho GAP Interactome – RICS

H

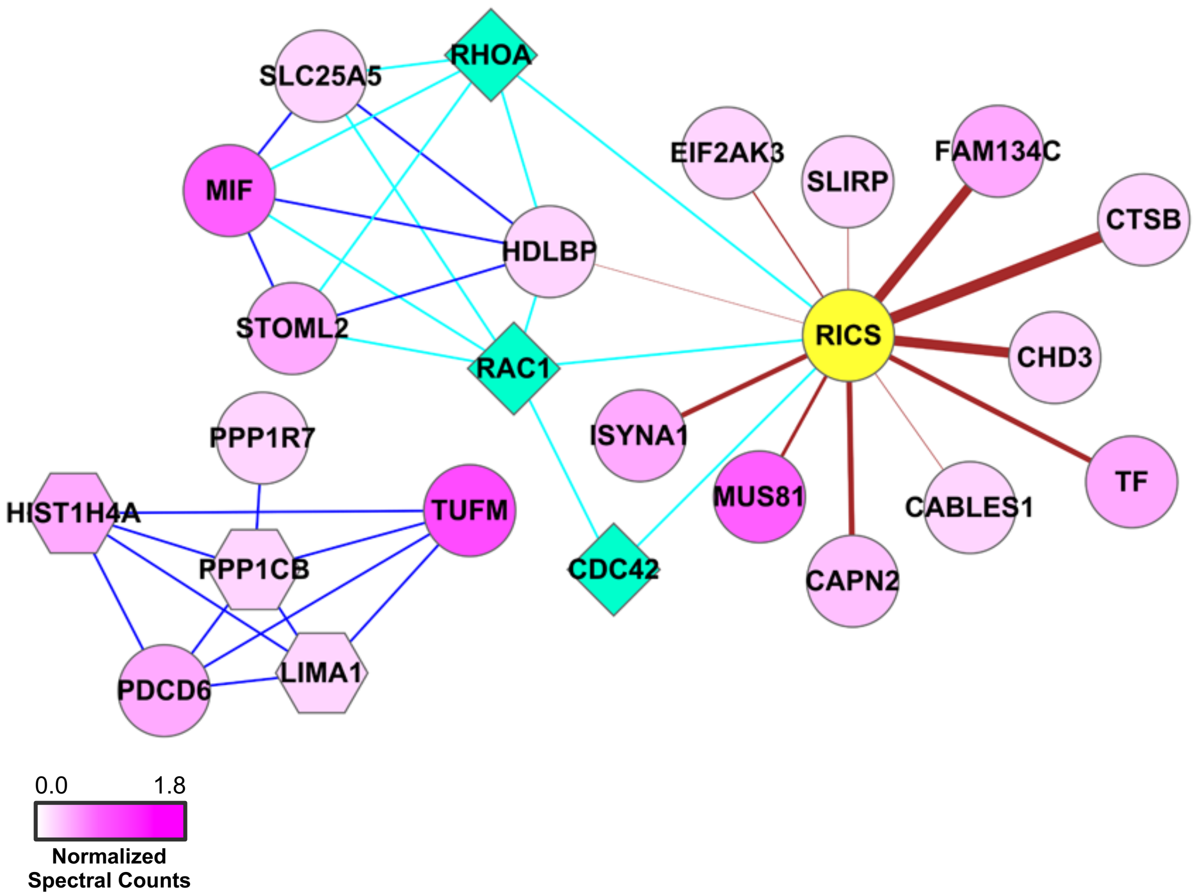


Figure S3. Rho GAP Interactome – ARHGAP12

I

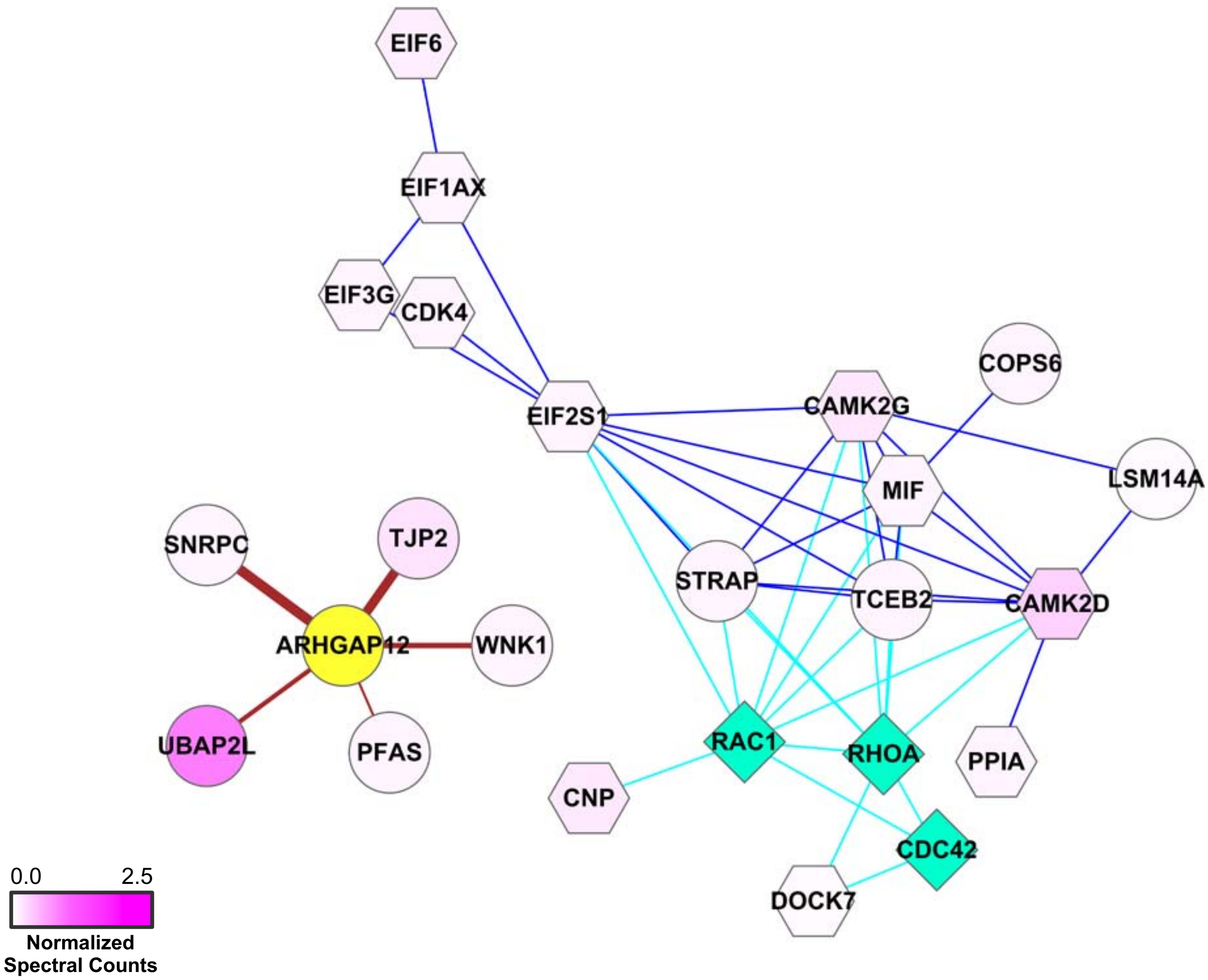


Figure S3. Co-localization Data

J

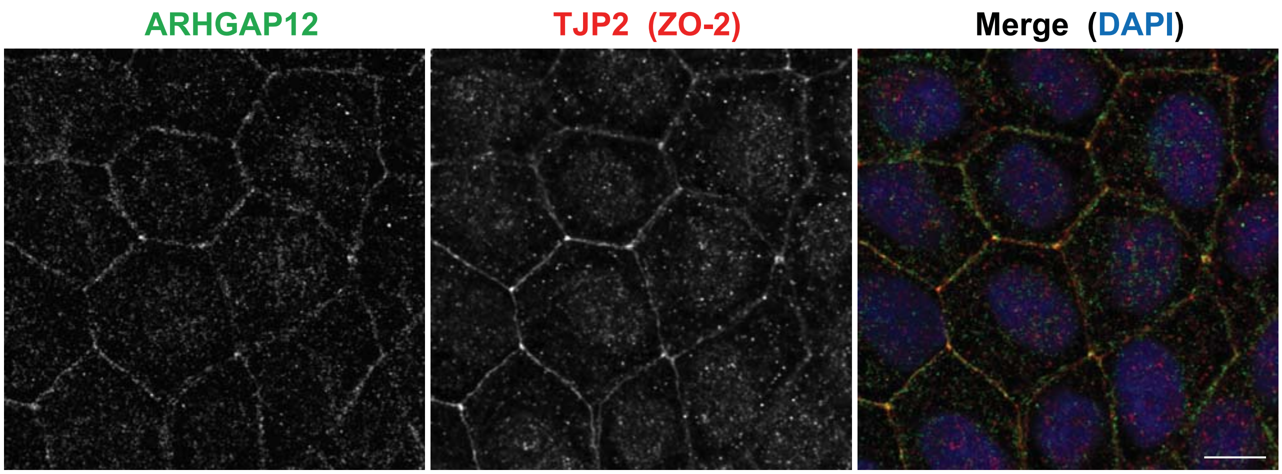
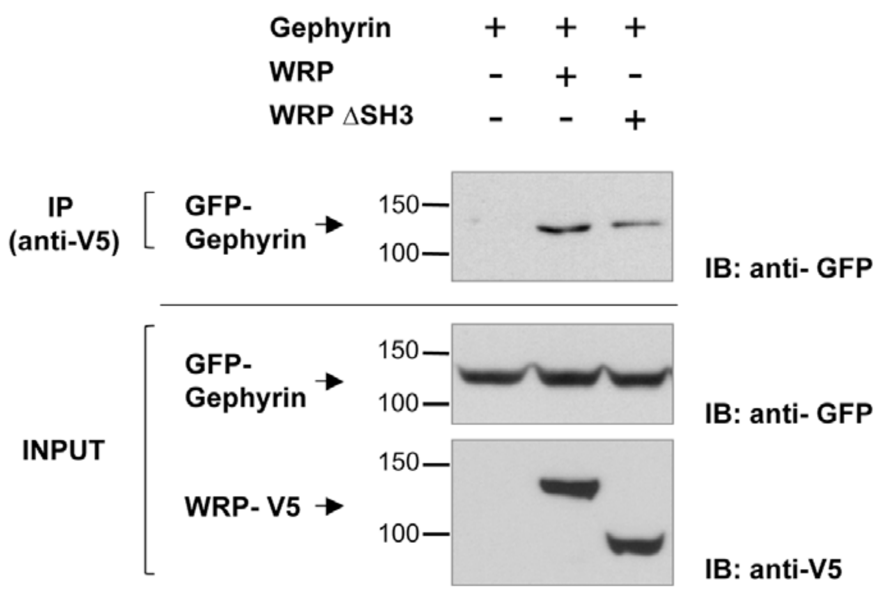


Fig. S4. Gephyrin's Interaction with WRP and colocalization with VIAAT. **A**) HEK293 cells co-expressing GFP-gephyrin with vector, V5-tagged full-length WRP or WRP lacking its SH3 domain, were subjected to immunoprecipitation by V5 antibody. Co-precipitation was analyzed by Western blot analysis using indicated antibodies. Representative blot from n=3 is shown. **B**) Co-localization of endogenous gephyrin and inhibitory synapse presynaptic marker VIAAT in hippocampal neurons. Dissociated hippocampal neurons were fixed at DIV12 and stained with gephyrin antibody mAb7a and VIAAT antibody. Scale bars = 10μm.

A



B

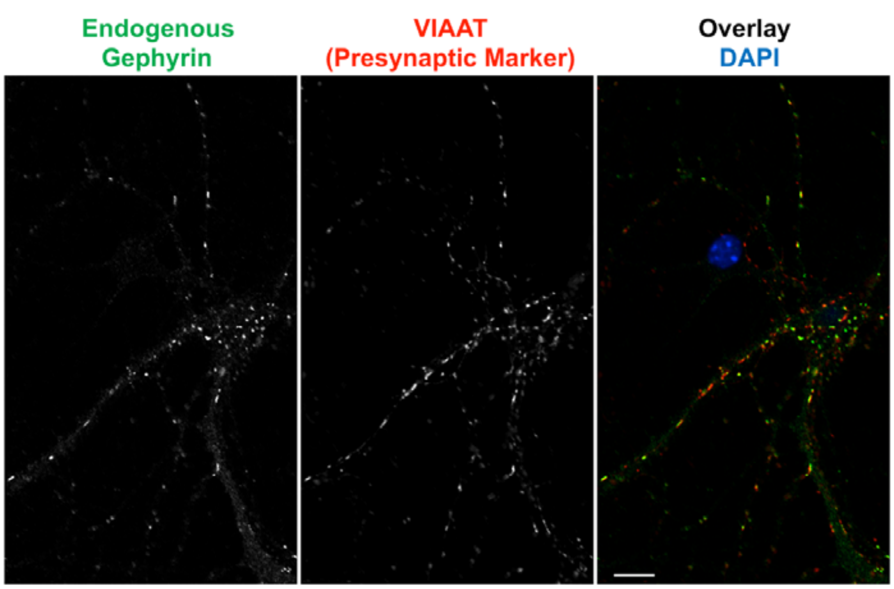


Fig. S5. Endogenous gephyrin is highly colocalized with inhibitory synapse presynaptic marker VIAAT in vivo. **A**) Stratum radiatum of CA1 region in the hippocampal formation from P40 $WRP^{+/+}$ and $WRP^{-/-}$ mice was stained with mAb7a and VIAAT antibodies. Scale bars = 10μm. **B**) Quantification of the gephyrin and VIAAT puncta density. n=3 mice. From each mouse, 4 brain slices were processed for immunostaining and 2 images/slice were obtained. The data are represented as mean ± SEM.