SUPPLEMENTARY METHODS

siRNA Sequences and Cloning

For GIT1 and GIT2 vector based shRNA, complimentary 80-87bp oligonucleotides containing BamH1 and HindIII overhangs were annealed and cloned into pReSI-H1-Puro or pReSI-H1-GFP. pReSI vectors were designed by Hirokazu Kurata and kindly provided by DNAX Research Institute (Palo Alto, CA). shRNA target sequences used in these studies: Crk (ggagatcaagagtttgattca); GIT1 (gacctcagcaagcaactacac); GIT2 shRNA-1 (acagactagccttctatctct); GIT2 shRNA-2 (gaaacaggccacaaccaatgt); GIT2 shRNA-3 (ggatcaggtcagaaaccatat); GIT2 shRNA-4 (gatacacgtagctgttacaga); and GIT2 shRNA-5 (ggacttcccttcgtttactga). *Silencer*^{TM} custom siRNA duplexes against βPIX (Ambion; ID#s 214584, 119398, and 119397) correspond to siRNA-1 (cgacaggaaugacaaucac), siRNA-2 (ccagugagaaguuaaguuctt), and siRNA-3 (gcaaaugcucgtacagucutt), respectively.

Generation of rabbit anti-GIT2 antibodies

Fragments derived from the longest form of GIT2 (amino acids 475-638; accession #NM_057169) and the homologous region of GIT1 (amino acids 480-649) were cloned in pGEX-4T-1 (Amersham). Recombinant proteins were produced according to the manufacturers protocol. Purified GST-GIT2 was used to immunize rabbits (Pocono Rabbit Farm and Laboratory Inc.). To eliminate antibodies that cross-reacted with GIT1, 15 ml of GIT2 immune serum was incubated overnight at 4^0C with 10 mg of GST-GIT1 coupled to glutathione sepharose. GIT1 cross-reactive antibodies were removed by centrifugation.

For verification of the results obtained of our rabbit-GIT2 antibodies, we also detected endogenous GIT2 by an IP-western protocol using commercially available antibodies. For these experiments, cells were lysed in 0.5 ml SDS Lysis Buffer (50 mM Tris at pH 8.1, 0.5% SDS, 100 mM NaCl, 5 mM EDTA) and boiled for 5 min. An equal volume of Triton Dilution buffer (2.5% Triton X-100, 100 mM Tris [pH 8.6], 100 mM NaCl, and 5mM EDTA) was added to each lysate for a final volume of 1 ml. Lysates were cooled on ice and vortexed at 4^0C for 20 min to facilitate shearing of DNA. Clarified lysates were incubated overnight at 4^0C with 1 μg of goat anti-GIT2 (Santa Cruz Biotechnology sc-5416). Immunoprecipitates were transferred to PVDF and blotted with monoclonal PKL antibody (BD Biosciences). No substantial differences in specificity of the two protocols were detected in experiments were the expression of GIT molecules was depleted by knockdown.

Reversible formaldehyde cross-linking

HeLa cells cultured in 15 cm-diameter dishes (2-4 plates) were fixed by the addition of formaldehyde to the culture medium at a final concentration of 1%. Cross-linking was allowed to proceed for 10 min at room temperature and stopped by the addition of glycine at a final concentration of 0.125 M. Fixed cells were washed twice with TBS (20 mM Tris at pH 7.4, 150 mM NaCl) and scrape harvested in 6 ml SDS Lysis Buffer (50 mM Tris at pH 8.1, 0.5% SDS, 100 mM NaCl, 5 mM EDTA, and protease inhibitors). Cells were pelleted by centrifugation at 250 g, and suspended in 4 mL of IP Buffer (100 mM Tris at pH 8.6, 0.3% SDS, 1.7% Triton X-100, and 5 mM EDTA). Cells were disrupted by sonication with a 1/4-in.-diameter tapered probe for 20 sec in a Branson 250 sonicator at a power setting of 4 and 100% duty cycle. For each immunoprecipitation, 1 mL (equivalent to approximately one 15 cm dish) of lysate was precleared by addition of 25 μL of CL4B beads, followed by centrifugation at 14,000 rpm in tabletop microcentrifuge. Samples were immunoprecipitated

overnight at 4°C with 1μg rabbit anti-GIT1 serum (sc-13961) or 5μl anti-GIT2 serum. Immune complexes were recovered by the addition of 10 μL protein G-Sepharose beads, followed by an incubation for 2 h at 4°C. Immunoprecipitates were washed three times with IP Buffer and eluted in 80 μl 2x Laemmli Buffer. Crosslinks were reversed by heating overnight at 65^0C.

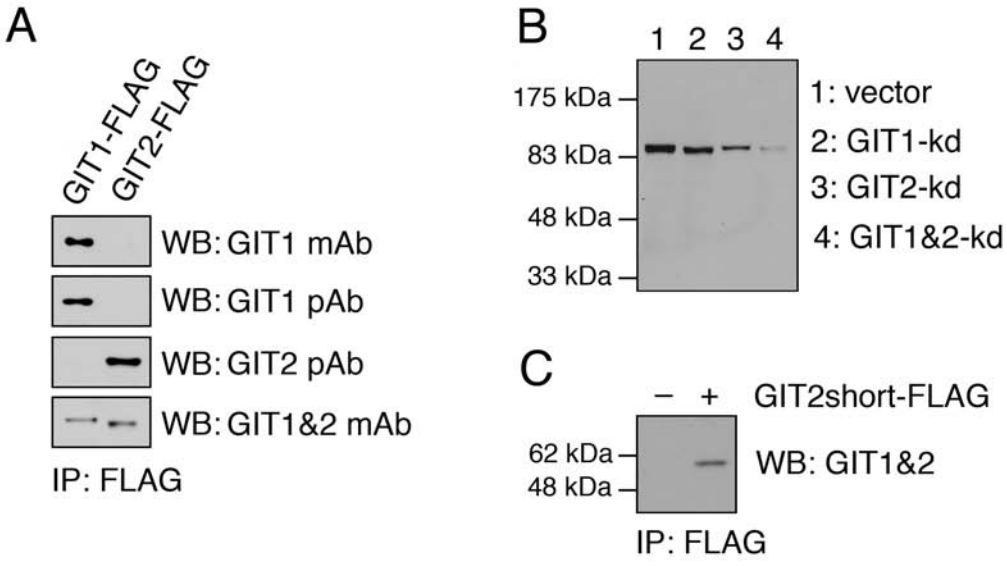

Figure S1. Specificity of antibodies used in this study. **(A)** HeLa cells were transfected with FLAG-tagged GIT1 and GIT2. 48h post-transfection cells were lysed in 0.5% SDS lysis buffer and boiled for 5 min to disrupt GIT1-GIT2 heterodimers. An equal volume of 2.5% Triton dilution buffer was added and the subsequent precleared lysates immunoprecipitated with 1 μg of monoclonal anti-FLAG antibody (SIGMA). Immunoprecipitates were blotted with mouse anti-GIT1 (BD Biosciences), rabbit anti-GIT1 (Santa Cruz Biotechnology), rabbit anti-GIT2 (generated in our lab), and mouse anti-PKL (p95PKL; BD Biosciences) antibodies. Note that mouse anti-PKL, which was generated against the chicken ortholog of GIT2, recognizes both GIT1 and GIT2 (bottom panel; GIT1&2). **(B)** Uncropped and overexposed version of GIT1&2 blot depicted in Fig. 1B. Control (lane 1), GIT1 (lane 2), GIT2 (lane 3) and GIT1&2 (lane 4) knockdown cell lysates were blotted with mouse anti-PKL. Note that HeLa cells express predominantly GIT1 (upper band) and the longest form of GIT2 (lower band). **(C)** HeLa cells were transfected with FLAG-tagged GIT2-short and cell lysates were immunoprecipitated with monoclonal anti-FLAG antibody. Immunoprecipitates were blotted with mouse anti-PKL antibody, demonstrating that this antibody detects GIT2-short. The ability of mouse anti-PKL to detect GIT2-short suggests that this antibody recognizes an epitope shared by all predicted GIT2 forms (Premont et al., 2000). Taken together with the blot shown in panel **B** this supports our finding that HeLa cells express predominantly GIT1 and GIT2-long.

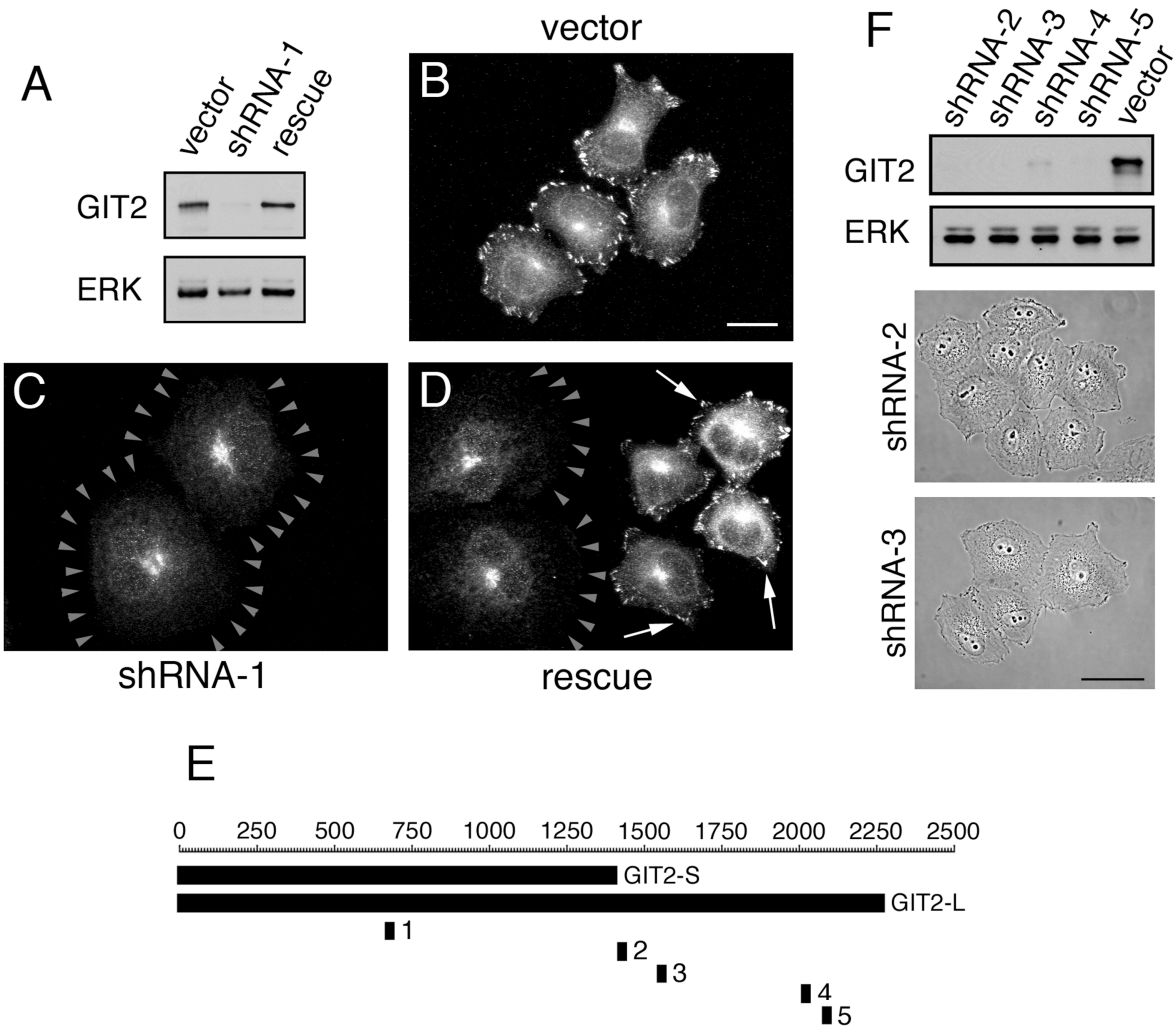

Figure S2. Enhanced cell spreading and FA loss results from the reduction of GIT2-long. (**A-D**) HeLa cells were transfected with vector alone, GIT2 shRNA-1, or GIT2 shRNA-1 in combination with a GIT2-long cDNA in which the binding site for shRNA-1 has been eliminated by silent mutagenesis ("rescue") and blotted for GIT2. Staining with GIT2 antibodies shows that introduction of exogenous GIT2-long (arrows) restores normal cell size and FA structure in GIT2 shRNA-1 treated cells (B-D). Arrowheads delineate membrane border of GIT2 knockdown cells. (**E**) Target regions of GIT2-shRNAs used in this study. Note that GIT2 shRNA-1 used throughout this study targets sequence common to all GIT2 forms (Premont et al., 2000). (**B**) HeLa cells were transfected with control or GIT2 shRNA2-5 and blotted for GIT2 or ERK (left panel). Phase contrast images of HeLa cells transfected with shRNA-2 or shRNA-3 (right panels).

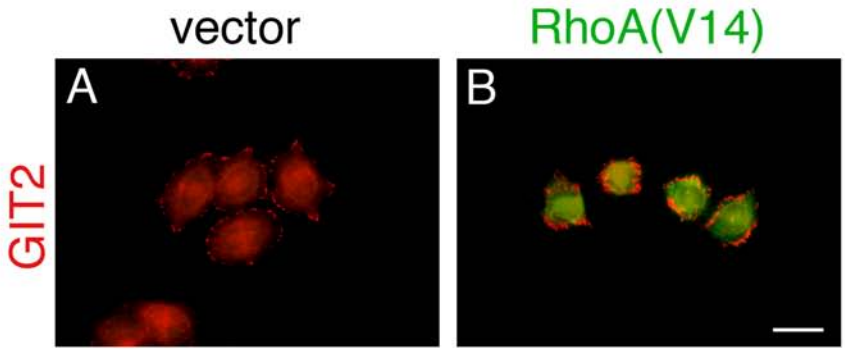

Figure S3. GIT2 localizes to mature focal adhesions. HeLa control cells (**A**) or cells transfected with myc-tagged RhoA(V14) (**B**) were stained 24h post-transfection with antibodies specific for GIT2 (red) and myc (green). RhoA(V14) expression results in the generation of robust focal adhesions to which GIT2 localizes.