SUPPLEMENTAL MATERIAL

Plasmid cDNAs: All GIT1 constructs were described previously,[1] including GFP- GIT1(WT), Flag-GIT1(WT) and Xpress-GIT1(WT). Briefly, the full-length mGIT1 was cloned into either Flag-tagged, Xpress-tagged or GFP-tagged vector and resulted in Flag-GIT1(WT), Xpress-GIT1(WT) or GFP-GIT1(WT).[2] All GIT1 mutants including Flag-GIT1(Y293F), Flag-GIT1(Y392F), Xpress-GIT1(Y321F), GFP-GIT1(Y293A) and GFP-GIT1(Y392A) were created by using QuikChange site-directed mutagenesis kits. The sequence and reading frame of GIT1 mutants were confirmed by sequencing analysis.

Cell Culture: A7r5 rat smooth muscle cells (A7r5 SMC) were purchased from ATCC. A7r5 SMC and HEK 293 cells were grown in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen). SYF–/–, SYF+/+ fibroblast cell lines derived from src–/– yes–/– fyn–/–, src+/+ yes–/– fyn–/– mouse embryos respectively, were a generous gift from Jonathan A. Cooper (University of Washington) and cultured in DMEM supplemented with 10% fetal bovine serum and 500 μg/ml G418.

Transient transfection siRNA or cDNA and Infection with adenovirus: For siRNA transfection, A7r5 SMC were transiently transfected with 100 nM control siRNA or GIT1 siRNA with Lipofectamine 2000 reagent in OptiMEM medium at 90% confluence. Rat GIT1 siRNA (AAGCTGCCAAGAAGAAGCTAC) and control non-silencing siRNA (AATTCTCCGACACGTGTCACT) were described previously[3] and ordered from Ambion. After 6 hours, medium was replaced by a complete DMEM medium with 10% serum. For plasmid transfection, HEK293 cells were transfected with LipofectAMINE/plus, and A7r5 cells

were transfected with Lipofectamine 2000 in OptiMEM medium at 90% confluence described previously.[3] After 6 hours, the medium was replaced by a complete medium with 10% serum. For virus infection, cells were infected with 100MOI adenovirus, after 48 hours cells were treated and fixed for immunostaining or harvested for western blot analysis.

Cell lysate preparation: Cells were rinsed with ice-cold phosphate-buffered saline (PBS; 150mM NaCl, 20mM Na_2PO_4, pH 7.4) twice on ice and harvested in lysis buffer (150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 5mM NaF, 1mM Na_3VO_4 plus 1:1000 protein inhibitor cocktail) and centrifuged at 10,000rpm for 10 minutes. The protein concentration was determined by the Bradford assay.

Immunoprecipitation and immunoblotting: For immunoprecipitation, 1μg of the indicated antibody, including PLCγ (BD Bioscience), FlagM2 [4], and Xpress (Invitrogen), was added to 500μg cell lysates and incubated overnight at 4°C. On the second day, 30 μl protein A/G-agarose beads (Life Technologies, Inc) were added and the incubation continued for 1 hour at 4°C. Following two washes with cell lysis, the immunoprecipitates were subjected to western blot analysis. For immunoblots, cell lysates were separated by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes, and incubated with appropriate primary antibodies including GIT1 (Santa Cruz, 1:1000), Actin (Santa Cruz, 1:3000), PLCγ (BD Bioscience, 1:1000), FlagM2 (Sigma, 1:3000), Xpress (Invitrogen, 1:1000), pPLCγ (Y783) (Cell signaling, 1:1000), and 4G10 (upstate, 1:1000). After the membranes were washed for 3 times, the membranes were incubated with fluorescence-conjugated secondary antibodies (Molecular

Probe, 1:10000) for 1 hour, then immunoreactive proteins were visualized by an Odyssey infrared imaging system (LI-COR Biotechnology).

Statistical Analysis: All values are expressed as mean ± SD of three independent experiments performed in triplicate. Data analysis was done by Student's t test. A $p < 0.05$ was considered statistically significant.

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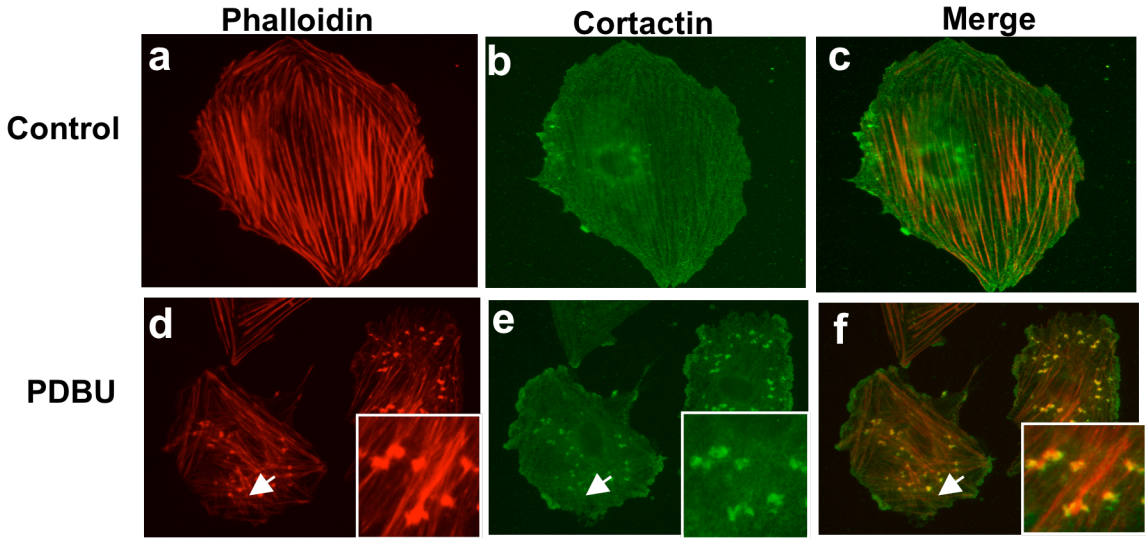
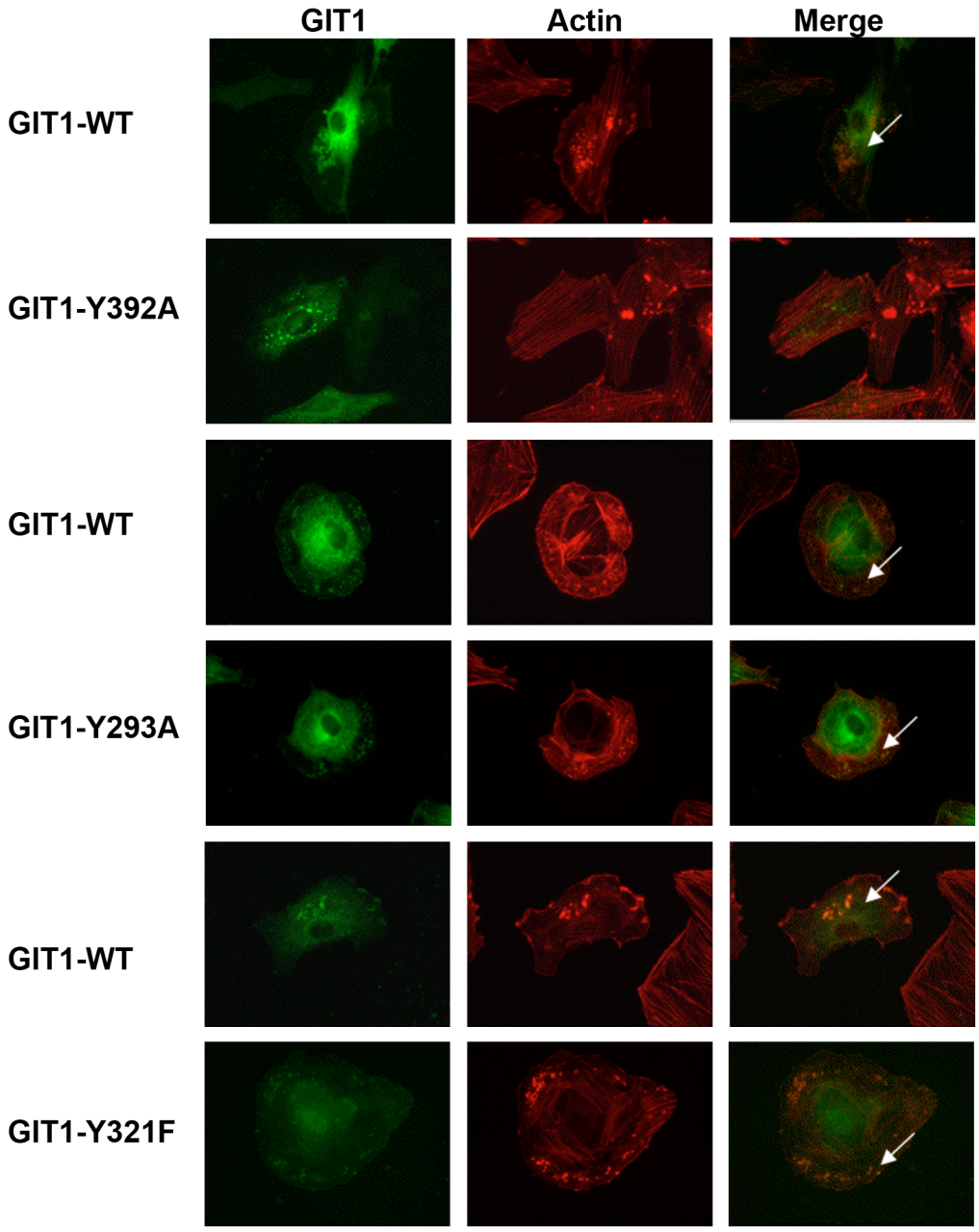
Supplemental Figure I



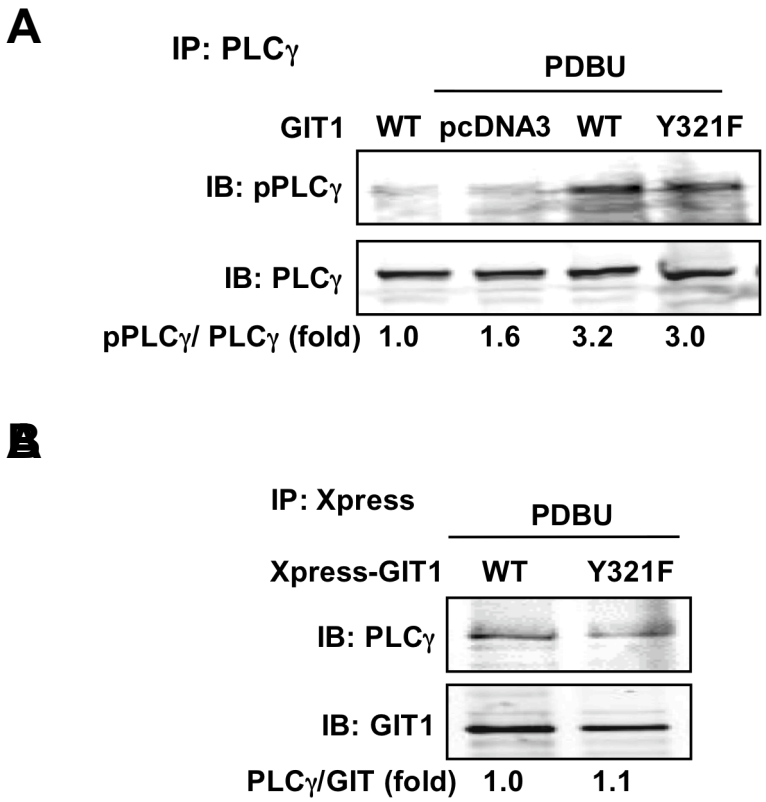
Supplemental Figure I. Representative immunofluorescent images showing F-actin (Phalloidin) and cortactin localization in podosomes. A7r5 cells were starved for 6 hours and then treated with or without 1μM PDBU for 60 min. Cells were fixed and double-stained for either cortactin or TRITC-Phalloidin. F-actin is in red. Cortactin is in green. Merged images are shown in panel c and f. Arrows indicate podosomes. Insets are enlarged images of podosome-enriched areas.

Supplemental Figure II



Supplemental Figure II. Ability of specific GIT1 mutants to form podosomes. A7r5 cells were transfected with GFP-GIT1 mutants- GIT1(WT), GIT1(Y293A) and GIT1(Y321F) , **GIT1(** Y392A) and stimulated with PDBU for 60 minutes. Cells were then stained with phalloidin to detect F-actin. Merged images were used to assess co-localization of GIT1 mutants with podosomes. Arrows indicate podosome positive structures.

Supplemental FigureIII



Supplemental Figure III: (A) HEK 293 were co-transfected with PLCγ and pcDNA3 vector or Xpress-GIT1 (WT) or Xpress-GIT1 (Y321F) for 24 hours and then starved for 6 hrs. Cells were treated with 1 μM PDBU for 10 min. Cell lysates were immunoprecipitated with PLCγ antibody and immunobloted with pPLCγ antibody. Blot was reprobed with PLCγ antibody (lower panel). (B) Immnoprecipitation was performed with Xpress antibody and blot was probed for PLCγ. Blot was reprobed for GIT1. Fold changes with respect to control are indicated.

Supplemental Figure IV

Cell type	A7r5 SMC	HEK293	Neuron
Location	Podosomes	Focal adhesions	Synapses
Potential targeting proteins	PIX, PAK	FAK, Paxillin	Grb4, CaMK, PIX
GIT1 phosphorylation site	Y392	Y321	Y392
Signaling pathways	Src ↓ pGIT1Y392 ↓ pPLCγ	Src ↓ pGIT1Y321 ↓ pMEK1 ↓ pERK1/2	Src ↓ pGIT1Y392 ↓ Grb4
Reference	1, 5-7	2, 8	9, 10

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