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Rewiring Cellular Morphology Pathways With Synthetic Guanine Nucleotide Exchange Factors

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Supplementary Methods

Protein construction, expression, and purification

Synthetic GEFs. GEF domains (DH and/or DH-PH) were cloned by polymerase chain reaction from human or mouse first-strand cDNA. pET-19b (Novagen) derived plasmids encoding synthetic GEFs were constructed using conventional restriction-enzyme molecular biology (sequence details in Tables S1, S2, and S3). Proteins were expressed as hexahistidine fusions in *Escherichia coli* BL21(DE3)RIL, and purified by chromatography on Ni-NTA resin (Qiagen). Hexahistidine tags were removed by incubation with tobacco etch virus (TEV) protease at room temperature. Uncleaved protein, free hexahistidine tag, and protease were removed by subsequent incubation with Ni-NTA resin. Proteins were dialyzed into 20 mM Tris, 50 mM NaCl, 2 mM DTT, pH 7.5 (for *in vitro* nucleotide exchange assay) or Microinjection Buffer (PBS, 200 mM NaCl, 20 mM HEPES, 5 mM $MgCl_2$, pH 7.4), flash frozen in liquid nitrogen, and stored at -80°C.

GTPases. For nucleotide exchange assays, fragments of human Cdc42 (residues 1-179), human Rac1 (residues 1-177), and human RhoA (residues 1-190, C190S) lacking C-terminal prenylation sequences were expressed as hexahistidine fusions and purified as described above for synthetic GEFs. GTPases were further purified on a Source Q column (Amersham). Residual bound

nucleotide was removed by dialysis in 20 mM Tris, 50 mM NaCl, 5 mM EDTA, 2 mM DTT, pH 7.5. GTPases were loaded with GDP or methylanthraniloyl-GDP (mant-GDP, Molecular Probes) by incubation with excess nucleotide. Nucleotide exchange was quenched by addition of 50-fold molar excess of $MgCl_2$, and excess nucleotide was removed by dialysis into GEF Assay Buffer (20 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, 1% glycerol, 1 mM DTT, pH 7.5). GTPases loaded with GDP were flash frozen in liquid nitrogen, and stored at -80°C. GTPases loaded with mant-GDP were stored at 4°C, and used within 1 week of nucleotide loading.

For microinjection experiments, full-length human Cdc42 and Rac1 were expressed as Glutathione S-Transferase (GST) fusions in BL21(DE3)RIL (pGEX-4T1 vector, Amersham). GTPases were purified on glutathione-agarose resin (Sigma). GST tags were removed by incubation with thrombin (Calbiochem) at room temperature. Uncleaved fusion protein and free GST were removed by subsequent incubation with glutathione-agarose, and thrombin was removed by incubation with benzamidine-sepharose (Amersham). GTPases were further purified on a Source Q column, dialyzed into Microinjection Buffer, flash frozen in liquid nitrogen, and stored at -80°C.

PKA. The catalytic (C_α) subunit of PKA was cloned from mouse first-strand cDNA, and expressed as a hexahistidine fusion in BL21(DE3)pLysS. PKA was purified on Ni-NTA resin, the hexahistidine tag was removed by incubation with TEV protease, and PKA was further purified on a Source S (Amersham) column. PKA was dialyzed into 20 mM Tris, 50 mM NaCl, 1 mM EDTA, 2 mM DTT, pH 7.5, flash frozen, and stored at -80°C.

Verification of PKA-sensitive interaction module

The candidate (RRRESIV) and mutant (GVKESLV) ligands (Fig. S1) were expressed as GST-fusions in BL21(DE3)RIL, and immobilized on glutathione-agarose resin. GST-peptides were

tested for phosphorylation by incubation with PKA in the presence of 200 μM ATP at 30°C. Phosphorylated and unphosphorylated GST-peptides were incubated with 25 μM His6-syntrophin PDZ (expressed and purified as previously described[31]) for 15 minutes at 4°C. Glutathione-agarose beads were washed and resuspended in SDS-PAGE loading buffer. Samples were separated by SDS-PAGE and transferred to nitrocellulose for Western blotting. To assess phosphorylation, nitrocellulose membranes were visualized with Phospho-(Ser/Thr) PKA Substrate Antibody (Cell Signaling Technology). To assess binding to syntrophin PDZ domain, membranes were visualized with His-probe (Santa Cruz Biotechnology).

in vitro nucleotide exchange assays

Qualitative assays. Dissociation of mant-GDP from Cdc42 was measured using a SpectraMax Gemini XS (Molecular Devices) fluorescence multi-well plate reader (25°C, excitation: 360 nm, emission: 440 nm). Solutions were pre-equilibrated at 25°C for 10 minutes, and the reaction was initiated by mixing solutions of GEF/GDP and Cdc42(mant-GDP). Final concentrations were 1 μM Cdc42(mant-GDP), 200 nM GEF, 200 μM GDP in GEF Assay Buffer.

Quantitative assays. Relative activities of synthetic GEFs were quantified using a variation of the above assay in which an increase in fluorescence was observed following the incorporation of mant-GDP into GTPases[10] (see Fig. S2 for raw data). Solutions were pre-equilibrated at 25°C for 10 minutes, and the reaction was initiated by mixing solutions of GEF/mant-GDP and GDP-loaded GTPase. Final concentrations were 1 μM Cdc42(GDP), 25 nM GEF, 400 nM mant-GDP in GEF Assay Buffer for reactions involving synthetic GEFs based on Intersectin. For reactions involving GEFs based on Trio, final concentrations were 1 μM Rac1(GDP), 250 nM GEF, 400 nM mant-GDP.

Activity was quantified by determining the slope of the initial linear phase of the exchange reaction, and normalized to reactions involving no GEF and DH or DH-PH alone: relative activity = $(\text{slope}_{\text{experimental}} - \text{slope}_{\text{no GEF}})/(\text{slope}_{\text{DH/DH-PH alone}} - \text{slope}_{\text{no GEF}})$. Relative activity measured in this fashion, under these conditions, was linear with respect to GEF concentration. We prefer quantifying activity using initial slope (as opposed to fitting to exponential functions) because it does not require making any assumptions about reaction mechanism.

Activation by PKA. GEFs were pre-incubated with PKA at a 1:10 kinase:GEF molar ratio, in GEF Assay Buffer supplemented with 200 μM ATP, at 30°C for 30 minutes. (The relatively high kinase:GEF ratio was chosen to ensure complete or near-complete phosphorylation.) GEF/kinase mixture was then used in exchange assays as described above. For quantitative assays, activity was normalized to equivalently treated samples containing no GEF or DH/DH-PH alone.

Microinjection experiments

Cell culture. Rat embryo fibroblasts (REF52) were grown at 37°C and cultured in Dulbecco's modified Eagles's media (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) in a humidified incubator with 6.3% CO_2. Cells were cultured and plated at low passage number.

Microinjection. For microinjection experiments, cells were plated on ethanol washed non-gridded glass coverslips (Bellco) and grown as sub-confluent monolayers overnight and serum-starved in DMEM medium containing 0.5% FBS for 24 hours prior to injection. Microinjection was performed using an Eppendorf 5246 pressure system and an Eppendorf 5171 microinjector. Proteins were diluted in Microinjection Buffer and injected into the cytosol of cells. 3000 MW, anionic, lysine fixable, fluorescein-labeled dextran (Molecular Probes) was used as an injection marker at a concentration of 1 mg/mL (Fig. S6). Constitutively-active Intersectin DH-PH was

titrated to give maximal filopodial output at 2 mg/mL (in the microinjection needle). Subsequently, all other GEFs were injected at the molar equivalent of 2 mg/mL Intersectin DH-PH. We estimate the final cellular concentration of injected GEFs to be 0.1-1 μM, which is consistent with experimentally measured concentrations of Rho GEFs[32]. Additionally, the GEFs were co-injected with their associated GTPases (0.5 mg/mL) to facilitate scoring. Importantly, filopodia and lamellipodia are not induced in cells microinjected with Cdc42 or Rac1 alone.

Fixed cell experiments. Injected cells were incubated for 30 minutes at 37°C, treated with indicated concentration of forskolin (Alexis Biochemicals)[33], and incubated for an additional 30 minutes. Cells were washed twice with PBS and replaced in pre-incubated DMEM containing 0.5% FBS for 30 minutes at 37°C and 6.3% CO_2 to allow for recovery from any deleterious effects of the drug. Cells were then fixed in 3.7% formaldehyde/PBS for 1 hour at room temperature. After fixation, cells were washed three times for 5 minutes with PBS, permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 3 minutes at room temperature followed by three PBS washes for 5 minutes each. Non-specific binding was blocked using 1% bovine serum albumin (BSA, Sigma) in PBS for 5 minutes. Filamentous actin was stained by incubation with rhodamine-phalloidin (0.2 mg/mL in block solution, Molecular Probes) for 1 hour at 37°C in a humidified incubator. Coverslips were washed three times for 5 minutes in PBS, rinsed in distilled water, and mounted onto glass slides using 10 μL Immu-mount (Shandon) containing 0.04% paranitrodiphenylene (PADA, Sigma) as an anti-fade agent. Slides were visualized on Axiovert 200M or S100 microscopes (Zeiss); fluorescent micrographs were captured using the Axiovision software (Zeiss). Morphological phenotypes were scored in a blind fashion (without knowledge of the experimental condition). Cells displaying at least 5 protrusive spikes were scored positive for filopodia, and cells that displayed dense peripheral actin staining were scored positive for lamellipodia (no cells were observed with both filopodia and lamellipodia). The percentage of cells with each phenotype was calculated by dividing the number of cells with the scored phenotype by the total number of cells scored.

Time-lapse microscopy. REF52 cells were plated on glass bottom dishes (MatTek) in DMEM supplemented with 10% FBS, and serum-starved 24 hours prior to injection. Cells were microinjected with indicated protein constructs and placed in a humidified thermo chamber ventilated with a heated air-CO_2 mixture (6.3% CO_2, 37˚C) (Zeiss Tempcontrol 37-2 digital, Heating Insert, CTI-Controller). Live injected cells were identified by fluorescein-labeled dextran and images were captured every 20 seconds with a 63X DIC oil lens (Zeiss). For stimulation with forskolin, the chamber was opened, media was aspirated, and 2 mL pre-incubated DMEM with 0.5% FBS supplemented with 10 μM forskolin in DMSO was added. Cells were refocused and imaging resumed at 20 second intervals. Cells were observed using an Axiovert 200M microscope (Zeiss); micrographs were captured using the Axiovision software (Zeiss).

Table S1. Regulatory modules used for construction of synthetic GEFs

Domain	*Parent Protein*	*Residues*
PDZ	mouse α-syntrophin	77-171
GBD	rat N-WASP	196-274
GBD*	rat N-WASP	209-274
C	rat N-WASP	458-489

Table S2. Synthetic GEFs described in this study

GEF	*Composition*	*Substrate*	*Input*
GEF1	[PDZ]-GT-[Intersectin DHPH]-TGRRRESIV	Cdc42	Activated by PKA
GEF1*	[PDZ]-GT-[Intersectin DHPH]-TGVKESLV	Cdc42	Not activated by PKA
GEF2	[PDZ]-GT-[Trio N-term. DH]-TGRRRESIV	Rac1	Activated by PKA
GEF2*	[PDZ]-GT-[Trio N-term. DH]- TGVKESLV	Rac1	Not Activated by PKA
GEF3	[GBD]-GT-[Trio N-term. DH]-TG-[C]	Rac1	Activated by Cdc42(GTP)
GEF3*	[GBD*]-GT-[Trio N-term. DH]-TG-[C]	Rac1	Not activated by Cdc42(GTP)

Table S3. GEFs tested for regulation by PKA-sensitive interaction module
(Qualitative activities are relative to most-active GEF tested for each GTPase)

Parent Protein	*Frag-ment*	*Residue Numbers*	*Activity of DH(PH) alone vs.*			*Activity of PDZ-DH(PH)-RRRESIV*		
			Cdc42	*Rac1*	*RhoA*	*vs.*	*- PKA*	*+ PKA*
Intersectin 1L (Human)[10]	DH	1229-1445	+++	+	-	Cdc42	+	+++
	DHPH	1229-1580	++++	+	-	Cdc42	++	++++
Trio (Human)[12]	DH	1284-1477	-	++	+	Rac1	+	++
	DHPH	1284-1594	-	++++	+	Rac1	+++	++++
Tiam1 (Mouse)[34]	DH	1033-1259	-	+	-	not tested		
	DHPH	1033-1406	-	++++	+	Rac1	+	+
Prex1 (Human)[35]	DH	38-258	++++	++++	+	Cdc42	+	+
	DHPH	38-415	insoluble			not tested		
Tim (Human)[10]	DH	1166-1367	-	-	++++	RhoA	++	++
	DHPH	1166-1527	insoluble			not tested		

Table S4. Specificities of GEF1 and GEF2
(Qualitative activities scaled as in Table S3)

	- PKA			*+ PKA*		
Synthetic GEF	***Cdc42***	***Rac1***	***RhoA***	***Cdc42***	***Rac1***	***RhoA***
GEF1	++	+	-	++++	+	-
GEF2	-	+	+	-	++	+

Supplementary References

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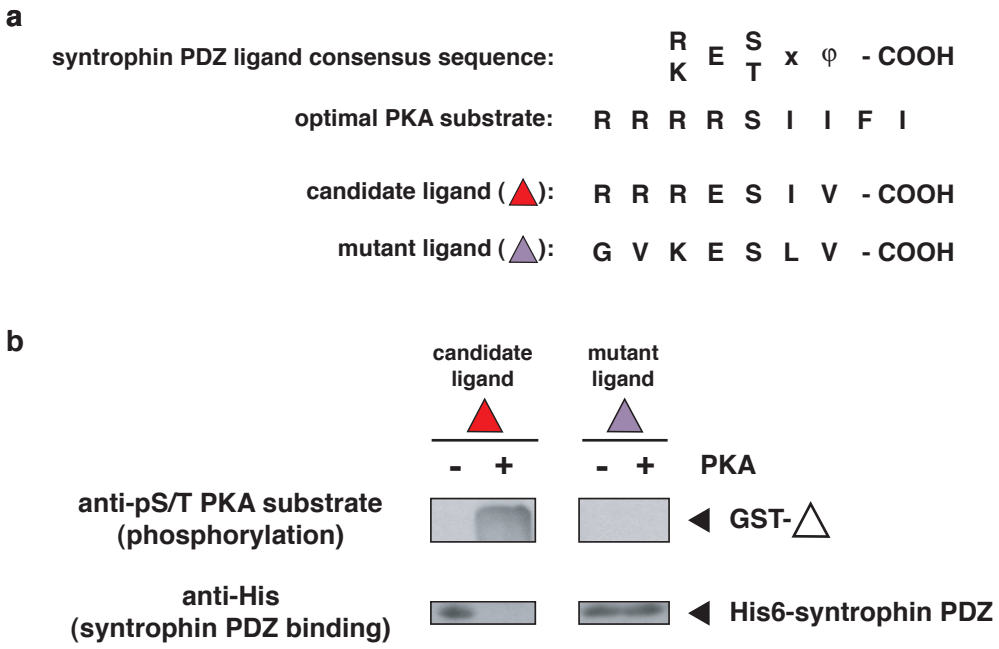

Figure S1 Design of PKA-sensitive interaction module. **a**, The syntrophin PDZ domain binds ligands that are close in sequence to optimal PKA substrates. A hybrid sequence (candidate ligand, red triangle) should be a PDZ ligand as well as a PKA substrate. The mutant ligand (purple triangle) acts only as a PDZ ligand. **b**, The candidate peptide was phosphorylated by PKA, and interacted with the syntrophin PDZ, but only in its non-phosphorylated form. The mutant ligand interacted with the PDZ domain, and was not phosphorylated by PKA. Phosphorylation was visualized using anti-pS/T PKA substrate Western blotting, and PDZ binding was assessed using GST pulldown experiments.

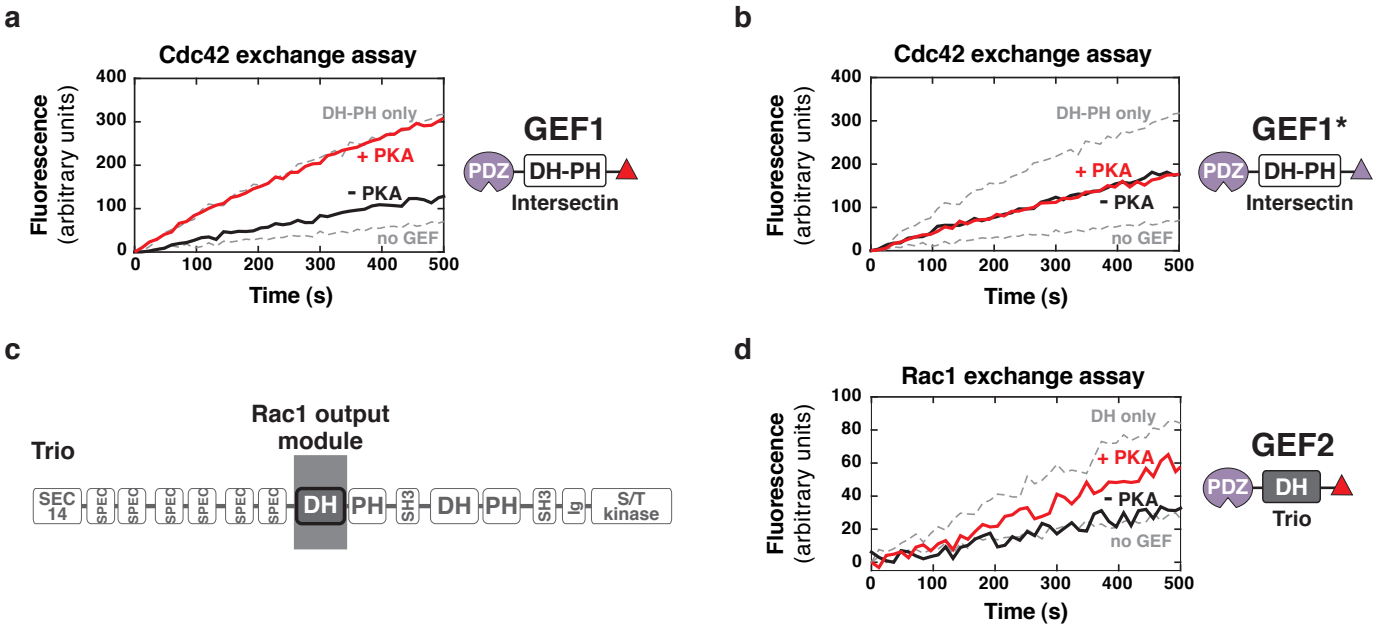

Figure S2 Sample raw fluorescence data used to quantify synthetic GEF activity. **a**, Loading of mant-GDP into Cdc42 catalyzed by **GEF1** (solid black line) or **GEF1** pre-treated with PKA (red line). Dotted lines represent spontaneous exchange (no GEF) or exchange catalyzed by Intersectin DH-PH. **b**, Cdc42 nucleotide exchange catalyzed by **GEF1***. **c**, Schematic of Trio domains. The N-terminal DH domain was used to construct **GEF2**. **d**, Loading of mant-GDP into Rac1 catalyzed by **GEF2** (solid black line) or **GEF2** pre-treated with PKA (red line). Dotted lines represent spontaneous exchange or exchange catalyzed by Trio DH. All curves are representative of at least three independent experiments.

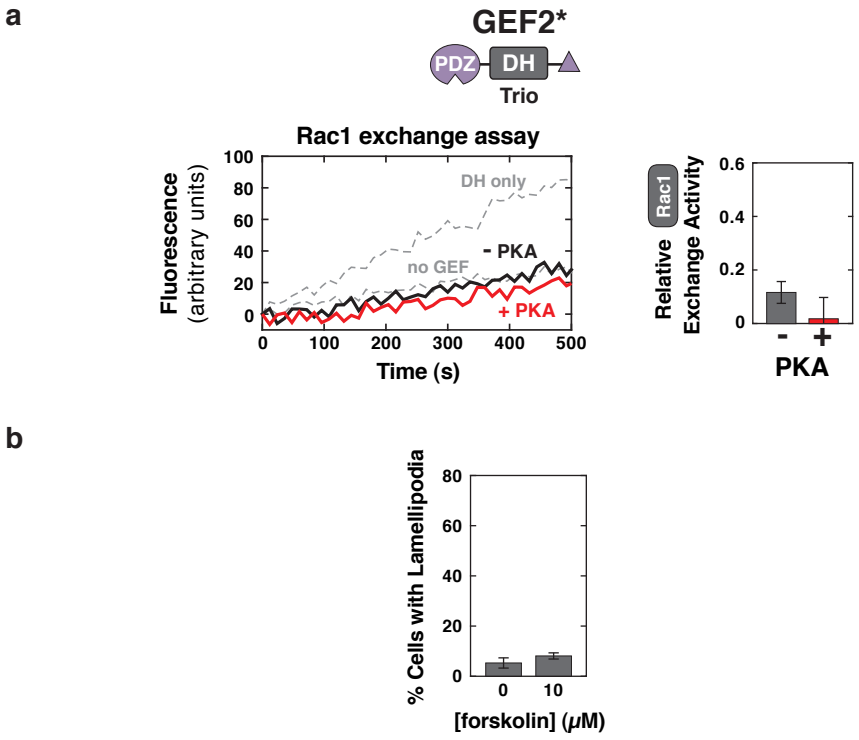

Figure S3 GEF2* is repressed, but not activated by PKA. **a**, Loading of mant-GDP into Rac1 catalyzed by **GEF2*** (solid black line) or **GEF2*** pre-treated with PKA (red line). Dotted lines represent spontaneous exchange or exchange catalyzed by Trio DH. All curves are representative of at least three independent experiments. Bar graph shows quantitated activities. Error bars represent SD of three experiments. **b**, Lamellipodia were not significantly stimulated by forskolin in cells injected with **GEF2***. Error bars represent SD of three experiments (at least 50 cells scored per experiment).

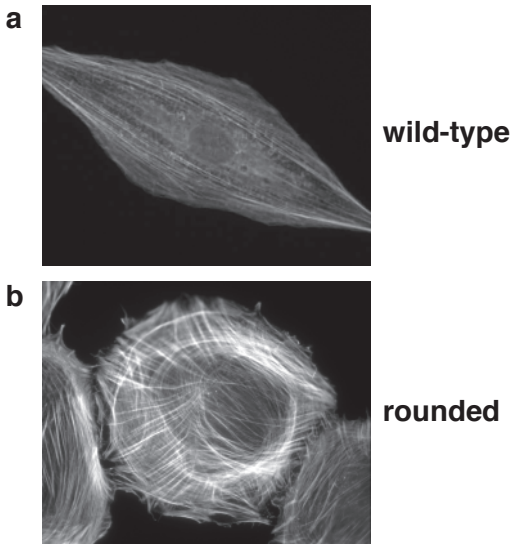

Figure S4 Rounded phenotype observed after microinjection of Intersectin DH-PH without co-injection of Cdc42. **a**, Wild-type morphological phenotype. REF52 cell was mock-injected with fluorescein-dextran only. **b**, Microinjection of Intersectin DH-PH induced filopodia or the rounded phenotype (but never both) in the majority of cells. The relative distribution of cells exhibiting the two phenotypes was inconsistent between experiments.

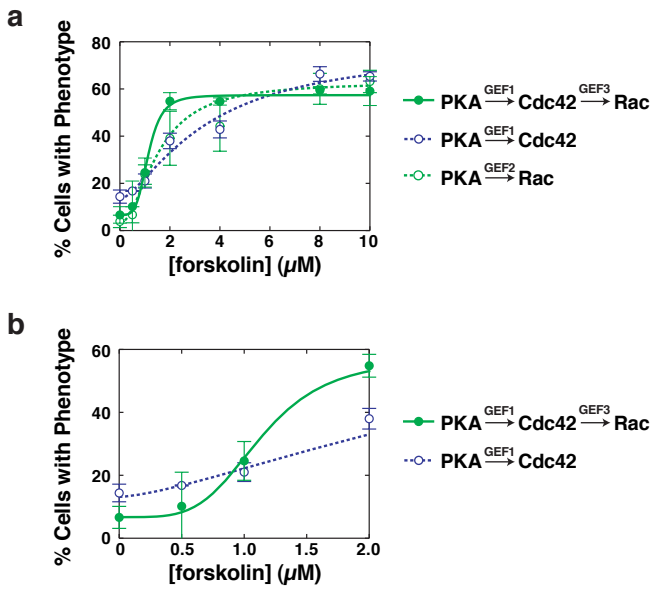

Figure S5 Comparison of **GEF1**-**GEF3** cascade to direct single-GEF circuits. **a**, Morphological responses of cells injected with **GEF1** and **GEF3** (lamellipodia, solid green line), **GEF1** (filopodia, dashed blue line), and **GEF2** (lamellipodia, dashed green line). Data points and error bars represent mean and SD of three experiments (at least 50 cells scored per experiment). **b**, Comparison of **GEF1**-**GEF3** cascade (solid green line) to direct single-GEF circuit mediated by **GEF1** (dashed blue line). The cascade had lower basal response (no forskolin stimulation), but was more sensitive to low concentrations (1-2 μM) of forskolin.

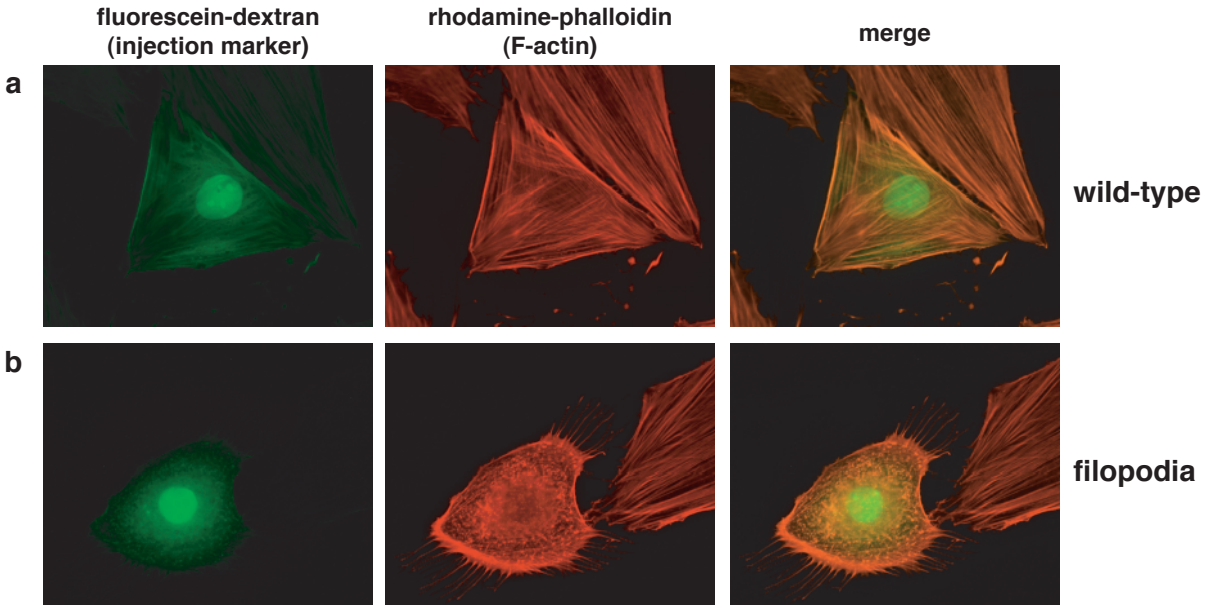

Figure S6 Methodology for Scoring Fixed Cells. Microinjected REF52 cells were identified by the presence of a fluorescein-labeled dextran marker (left panel). Filamentous actin was stained with rhodamine-conjugated phalloidin to visualize the morphology of the cytoskeleton (middle panel) and allow for scoring of injected cells. **a**, Microinjection of Cdc42 alone did not induce any morphological phenotype. **b**, Microinjection of constitutively-active Intersectin DH-PH and Cdc42 induced filopodia.