Supplementary Information

Supplementary Methods

Coimmunoprecipitations of Gα13 with myc-tagged LARG constructs

Cos1 cells were cotransfected with Gα13WT (0.5 μg) and the indicated myc-tagged LARG constructs by using the Lipofectamine PLUS reagent (Invitrogen). Transfected cells were incubated at 37°C in 10% CO_2 for 24 h. Cells were lysed in the 1.25 × lysis buffer A (50 mM Tris-HCl, pH 7.5/ 150 mM NaCl/ 1% Nonidet P-40/ 1 mM EDTA/ 1 mM DTT/ 10 mM β-glycerophosphate/ 10 mM Na_3VO_4/ 10 mM $MgCl_2$/ 100 μM ATP/ protease inhibitors) with 6.25 μM GDP and centrifuged at 100,000 × g for 30 min. The supernatants were incubated at 4°C in the presence or absence of AMF (30 μM $AlCl_3$/ 10 mM $MgCl_2$/ 10 mM NaF) for 10 min, and then, with anti-myc antibody 9E10 (Covance) for 60 min. The samples were added to protein G-agarose PLUS beads (Santa Cruz Biotechnology) and further incubated at 4°C for 60 min and washed three times with 1 × lysis buffer A with or without AMF. The immunoprecipitates were subjected to SDS/PAGE and analyzed by immunoblotting with anti-myc antibody 9E10 and anti-Gα13 antibody (Santa Cruz Biotechnology).

SRE-Luciferase Assay and Subcellular Localization of LARG

HeLa cells (6×10^4 cells per well) were plated onto 24-well plates 1 day before transfection. Cells were cotransfected with SRE.L-luciferase reporter plasmid (0.1 μg), pCMV-βgal (0.1 μg), and the indicated cDNAs. The cells were cultured in the presence of 10% FCS for 5 h and then serum-starved for 24 h. Luciferase activities in cell extracts were measured according to the manufacturer's instruction (Promega). Total amounts of transfected DNA were kept constant among wells by supplementing

the empty vector DNA. β-Galactosidase activities of cell lysates were used to normalize for the transfection efficiency.

The expressions of LARG constructs and Gα13 in total cell lysate, crude cytosol, and membranes fractions of Hela cells were detected by immunoblotting using anti-myc antibody 9E10 and anti Gα13 antibody. Particulate debris was removed by centrifugation at 700 × g for 5 min. Cytosol and Membranes were then separated by centrifugation of samples at 100,000 × g for 30 min. Samples were boiled in SDS and visualized by immunoblot analysis using anti-myc antibody 9E10 and anti-Gα13 antibody.

Measurement of Rho activation in cells

Endogenous RhoA in the activated GTP-bound state was measured by their association with GST-Rhotekin RBD as described by Ren and Schwartz (1). GST-Rhotekin RBD was expressed and purified by a procedure described previously. HeLa cells were transfected with the LARG constructs and/or Gα13QL by Lipofectamine 2000 reagent (Invitrogen) in the presence of 10% FCS according to the manufacturer's protocol. The cells were serum-starved for 24 hours after 5 h of transfection, and then lysed with 450 ml of 1 x lysis buffer A on ice for 5 min. Lysates were clarified by centrifugation at 20,000 × g for 5 min. Each lysate was incubated for 15 min at 4°C with 30 μg of GST-Rhotekin RBD immobilized to glutathione-Sepharose 4B beads (GE healthcare). The beads were washed three times with the wash buffer (50 mM Tris-Hcl/ 150 mM NaCl/ 0.5% Nonidet P-40/ 1mM EDTA/ 1mM DTT/ 10mM β-glycerophosphate/ 10mM Na_3Vo_4/ 10 mM $MgCl_2$/ 100 μM ATP/ protease inhibitors). Bound proteins were eluted by boiling in SDS sample buffer and subjected to Western blot analyses using a monoclonal antibody against

RhoA (Santa Cruz Biotechnology). To verify the expressions of myc-LARG mutants and Gα13, anti-myc antibody and anti-Gα13were used for immunoblotting, respectively.

In vitro RhoGEF Assay

(1) GDP dissociation from RhoA: The guanine nucleotide exchange activity of a RhoGEF protein can be assayed by monitoring the dissociation of GDP from RhoA as described previously (2). This assay was performed using Gα13WT. Gα subunits were preincubated with AMF (30 μM $AlCl_3$/ 5 mM $MgCl_2$/ 10 mM NaF) for 10 min on ice, and further mixed with LARG proteins. The RhoA loaded with [^{3}H]GDP (100 nM, 2000 cpm/pmol) was incubated with the GEF reaction mixtures at 20°C in a final volume of 20 ml of GEF dissociation assay buffer (50 mM Tris-HCl, pH 7.5/ 50mM NaCl/ 1 mM EDTA/ 1 mM DTT, 10 mM $MgCl_2$/ 5 μM GTPγS/ 0.1% $C_{12}E_{10}$/ ALF). The reactions were stopped by the addition of 2 ml washing buffer (20 mM Tris-Hcl, pH 7.5/ 40 mM $MgSO_4$/ 100 mM NaCl), followed by filtration through BA-85 filters (Schleicher & Schuell). The amount of [^{3}H]GDP that remains on the filters was measured by a liquid scintillation counter, and that of GDP dissociation from gg-RhoA was then determined.

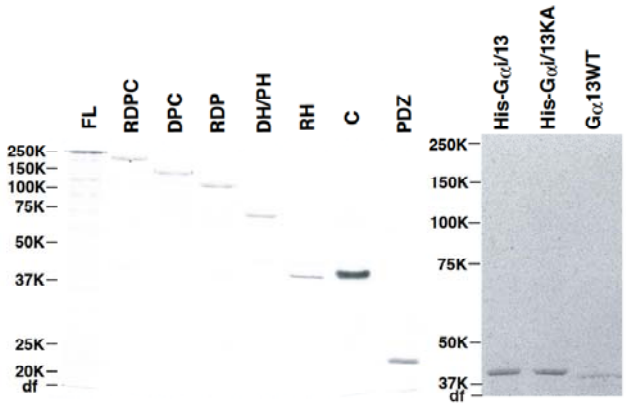
(2) GTPγS binding assay—The GDP-GTP exchange activity of a RhoGEF can also be assayed by monitoring GTPγS binding to Rho GTPase. This assay was performed using His-Gαi/13 or His-Gαi/13K204A. G protein α subunits were activated by incubating with AMF for 10 min on ice, and mixed with LARG proteins for 30 min on ice. 200 nM RhoA was incubated with the GEF reaction mixtures at 20°C in a final volume of 20 μl of GEF binding assay buffer (50 mM Tris-HCl, pH 7.5/ 50mM NaCl/ 0.5 mM EDTA/ 1 mM DTT, 5 mM $MgCl_2$ / 0.05% $C_{12}E_{10}$/ ALF/ 5 μM GTPγS/

[^{35}S]GTPγS ~2000 cpm/pmol). The reactions were stopped by the addition of 2 ml washing buffer, followed by filtration through BA-85 filters. The amount of [^{35}S]GTPγS that remained on the filters was measured by a liquid scintillation counter, and that of GTP binding to gg-RhoA was then determined.

Cell rounding assay

The day before transfection, MDCKII cells were grown on glass coverslips in DMEM containing 10% FCS. Cells were transiently transfected with 1.5 μg of the indicated myc-tagged RH-RhoGEFs or vector in the presence or absence of 0.05 μg of Gα13QL, or 2.0 μg of flag-tagged V14RhoA. The cells were kept in the presence of 10% FCS for 5 h and serum-starved for 24 h. Cells were then fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 15 min. The fixed cells were incubated with mouse anti-myc antibody 9E10 and rabbit anti-Gα13 (Santa Cruz Biotechnology) antibody for 1 h at room temperature and then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG, Texas Red-conjugated donkey anti-rabbit IgG, and Alexa Fluor 350-conjugated phalloidin (Molecular Probes Inc) simultaneously, and visualized by fluorescence microscopy. The cells (100-200 per slip) were scored for rounding based on morphology visualized by actin staining.

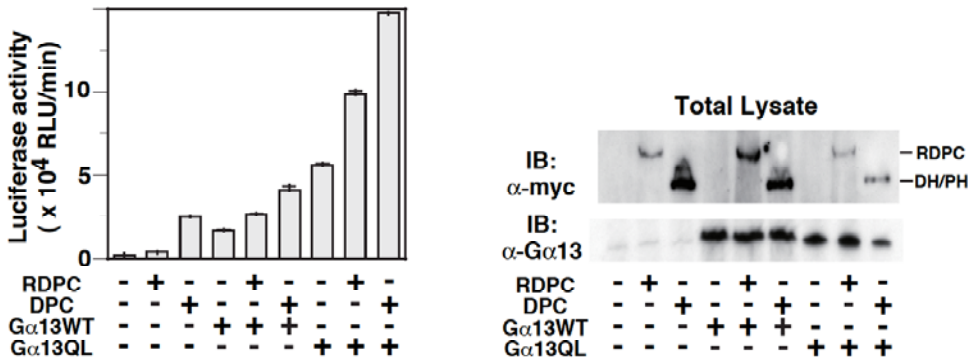
Supplementary Fig. 1



Supplementary Fig. 1 SDS-PAGE of purified recombinant LARG and Gα13 proteins used for *in vitro* analysis. The proteins (2 μg each) were separated by SDS-PAGE and stained with Coomassie brilliant blue.

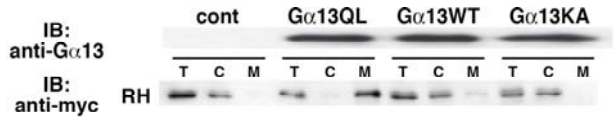
Supplementary Fig. 2

A **B**



Supplementary Fig. 2 Potentiation of SRF activation by LARG with Gα13QL but not Gα13WT. A, HeLa cells were cotransfected with 0.1 μg of SRE.L-luciferase reporter plasmid and the indicated constructs: Gα13WT (0.01 μg), Gα13QL (0.01 μg), myc-LARG-RDPC (0.1 μg), myc-LARG-DPC (0.1 μg), myc-LARG-DH/PH (0.01 μg), myc-LARG-C (0.1 μg). SRF activities of cell lysates were measured 24 h after transfection as described in Supplementary Methods. B, The expression of the LARG proteins and Gα13WT or Gα13QL in the same procedure as A.

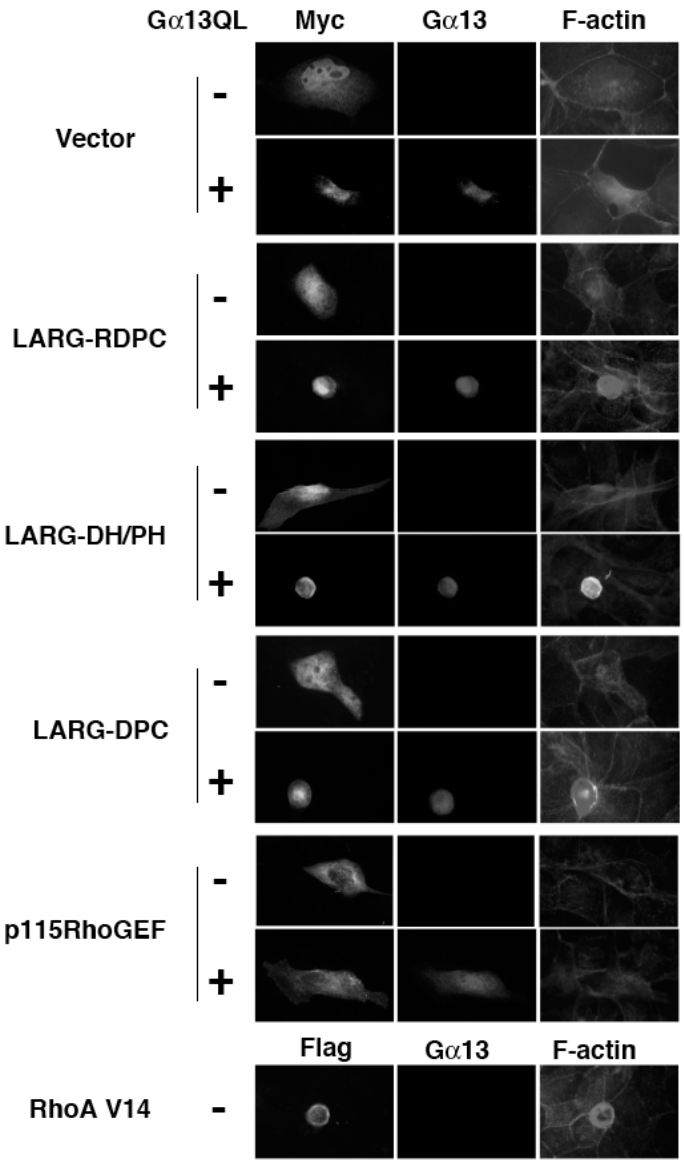
Supplementary Fig. 3



Supplementary Fig. 3

Recruitment of the LARG RH domain to the plasma membrane by constitutively active Gα13. HeLa cells were cotransfected in the same procedure as in the Fig. 2C with the indicated plasmids: Gα13QL (0.01 μg), Gα13WT (0.01 μg), Gα13KA (0.01 μg), myc-LARG-RH (0.1 μg). The expressions of LARG constructs in total cell lysate (T), crude cytosolic (C), and membrane (M) fractions and Gα13 in total lysates was detected by immunoblotting using anti-myc antibody or anti-Gα13 antibody.

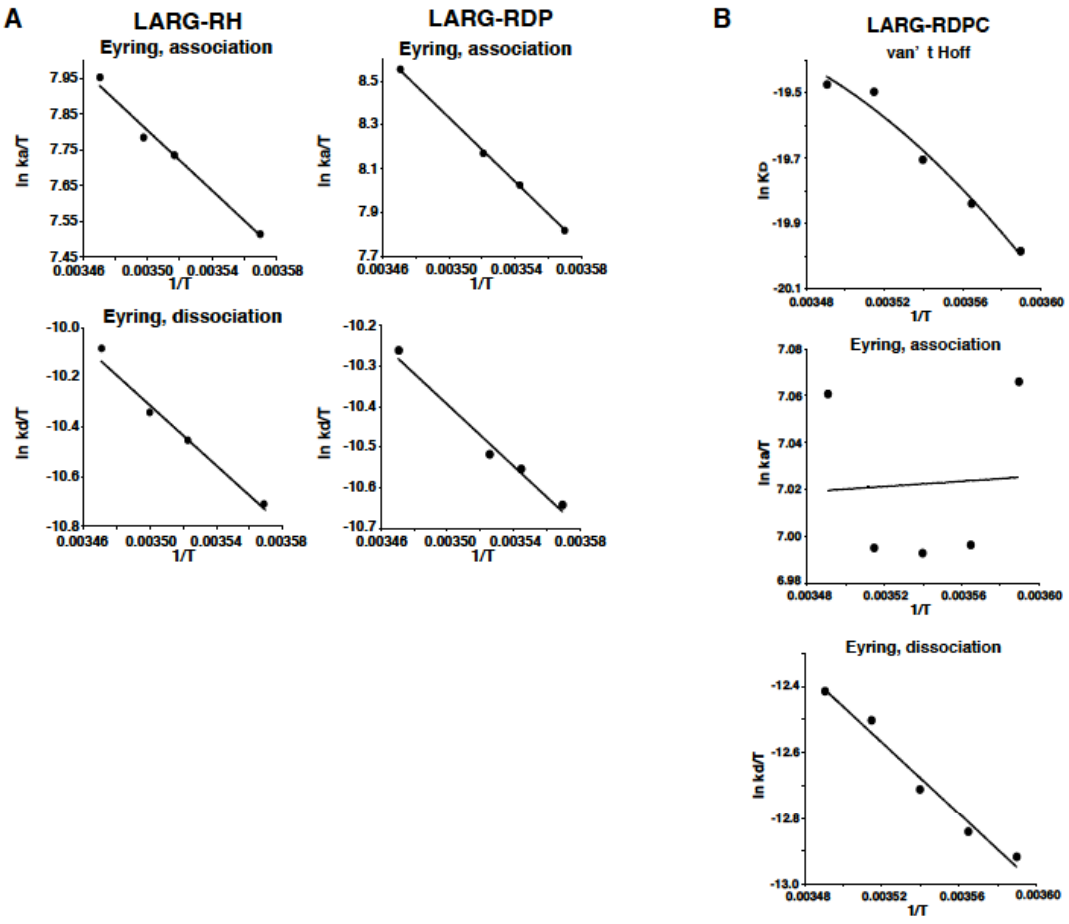
Supplementary Fig. 4



Supplementary Fig. 4 Cell rounding induced by LARG constructs with the constitutively active Gα13 in MDCKII cells. A representative image from four independent experiments. MDCKII cells were transiently transfected with the indicated myc-tagged LARG constructs in the presence or absence of Gα13QL, or flag-tagged V14RhoA alone. The cells were serum-starved for 24 hours after 5 h of

transfection, then fixed, and triply stained with anti-myc antibody, anti-Gα13 antibody, and phalloidin for filamentous actin. Transfected cells were visualized by fluorescence microscopy, identified, and scored for rounding indicating the involvement of RhoA activation as described under Supplementary Methods.

Supplementary Fig. 5



Supplementary Fig. 5 Therymodynamic Analysis of Gα13-LARG interaction. **A. B.** Thermodynamic analysis of the Gα13/LARG-RDPC complex formation and dissociation. van't Hoff plots and Eyring plots of the experimental data are shown. The procedure was the same as **Fig. 4**.

Supplementary Reference

1. Ren, X. D., and Schwartz, M. A. (2000) *Methods Enzymol* **325**, 264-272
2. Suzuki, N., Nakamura, S., Mano, H., and Kozasa, T. (2003) *Proc Natl Acad Sci U S A* **100**, 733-738

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