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RGS16 inhibits signalling through the Gα13–Rho axis

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Gα13 stimulates the guanine nucleotide exchange factors (GEFs) for Rho, such as p115Rho-GEF[1]. Activated Rho induces numerous cellular responses, including actin polymerization, serum response element (SRE)-dependent gene transcription and transformation[2]. p115Rho-GEF contains a Regulator of G protein Signalling domain (RGS box) that confers GTPase activating protein (GAP) activity towards Gα12 and Gα13 (ref. 3). In contrast, classical RGS proteins (such as RGS16 and RGS4) exhibit RGS domain-dependent GAP activity on Gαi and Gαq, but not Gα12 or Gα13 (ref 4). Here, we show that RGS16 inhibits Gα13-mediated, RhoA-dependent reversal of stellation and SRE activation. The RGS16 amino terminus binds Gα13 directly, resulting in translocation of Gα13 to detergent-resistant membranes (DRMs) and reduced p115Rho-GEF binding. RGS4 does not bind Gα13 or attenuate Gα13-dependent responses, and neither RGS16 nor RGS4 affects Gα12-mediated signalling. These results elucidate a new mechanism whereby a classical RGS protein regulates Gα13-mediated signal transduction independently of the RGS box.

Rho is involved in tumour cell growth, metastatic invasion and migration, and overexpression of activated Gα13, Rho exchange factors and Rho results in cellular transformation[1,2,5]. RGS16 mRNA is upregulated in response to genotoxic stress in MCF-7 breast carcinoma cells with aberrant expression of the tumour suppressor p53 (ref 6). Because of this potential physiological link in regulating cell growth, we investigated whether RGS16 has a function in Gα13-evoked cellular responses. First, we tested the ability of RGS16 to regulate morphological changes provoked by active Gα13 in 1321N1 astrocytoma cells. These cells undergo Rho- and Rho-GEF-dependent reversal of stellation and rounding when micro-injected with activated Gα12(Q229L; Gα12QL) or Gα13(Q226L; Gα13QL)[7]. We used RGS4 or the RGS domain of p115Rho-GEF for comparative studies, as p115(RGS) selectively blocks Gα12- or Gα13-mediated responses, including rounding[7,8]. Both Gα12QL and Gα13QL induced reversal of stellation and rounding in approximately 60–70% of injected cells, compared with only approximately 10% rounding in cells injected with vector, RGS16, RGS4 or p115(RGS) (Fig. 1a, b). Rounding in cells co-injected with either RGS16 or p115(RGS) and Gα13QL was reduced by approximately 30–40% (Fig. 1b). No inhibition was observed with RGS4, and neither RGS4 nor RGS16 inhibited Gα12QL-stimulated cell rounding. These results demonstrate that RGS16 selectively inhibits the morphological changes evoked by activated Gα13 to a similar degree as that of p115(RGS), an inhibitor of Gα13 pathways.

To determine whether RGS16 regulates other responses mediated by Gα13, we measured transcription of an SRE-dependent reporter gene (SRE.L-luciferase) induced by Gα13QL in human embryonic kidney (HEK)-293T cells. Transfection of Gα13QL resulted in an approximately18-fold increase in SRE.L reporter activity compared with vector-transfected cells (Fig. 1c). Although transfection of RGS4, RGS16, or p115(RGS) elicited no significant changes in basal SRE.L activity, overexpression of either RGS16 or p115(RGS) inhibited Gα13QL-evoked SRE.L activity by approximately 50%, whereas RGS4 had no effect. Semi-quantitative immunoblotting with recombinant standards estimated protein concentrations of approximately 50 nM for RGS16 and approximately100 nM for Gα13QL. Thus, a 1:2 molar ratio of RGS16:Gα13QL was sufficient to inhibit Gα13QL-induced SRE.L activation. To determine whether RGS16 functions upstream of Rho to inhibit Gα13QL-evoked SRE.L activation, we measured SRE.L reporter activity elicited by active RhoA. None of the three RGS constructs inhibited RhoA-mediated SRE.L activation, indicating that RGS16 and p115(RGS) function upstream of RhoA to inhibit Gα13-dependent activation of this pathway (see Supplementary Information, Fig. S1a). Consistent with this hypothesis, transfection of p115(RGS) reduced Gα12QL-evoked SRE.L activation by approximately 50%, whereas overexpression of neither RGS16 nor RGS4 significantly inhibited the response (Fig. 1d; also see Supplementary Information, Fig. S1b). In addition, RGS16 GAP activity was not required for the inhibition of Gα13-coupled SRE.L activation, as an RGS16 mutant lacking Gαi GAP activity (EN90/91A)[9] inhibited the response in a manner similar to wild-type RGS16 (data not shown).

To determine whether RGS16 inhibits the signalling cascades initiated by receptor stimulation of endogenous Gα13, we measured SRE.L and RhoA activation induced by lysophosphatidic acid (LPA) in MCF-7 cells, which express endogenous Gα13, RGS16 and LPA receptors[6,10,11]. Although transfection of RGS4 did not inhibit LPA-induced SRE.L activity, RGS16 overexpression blocked the response by approximately

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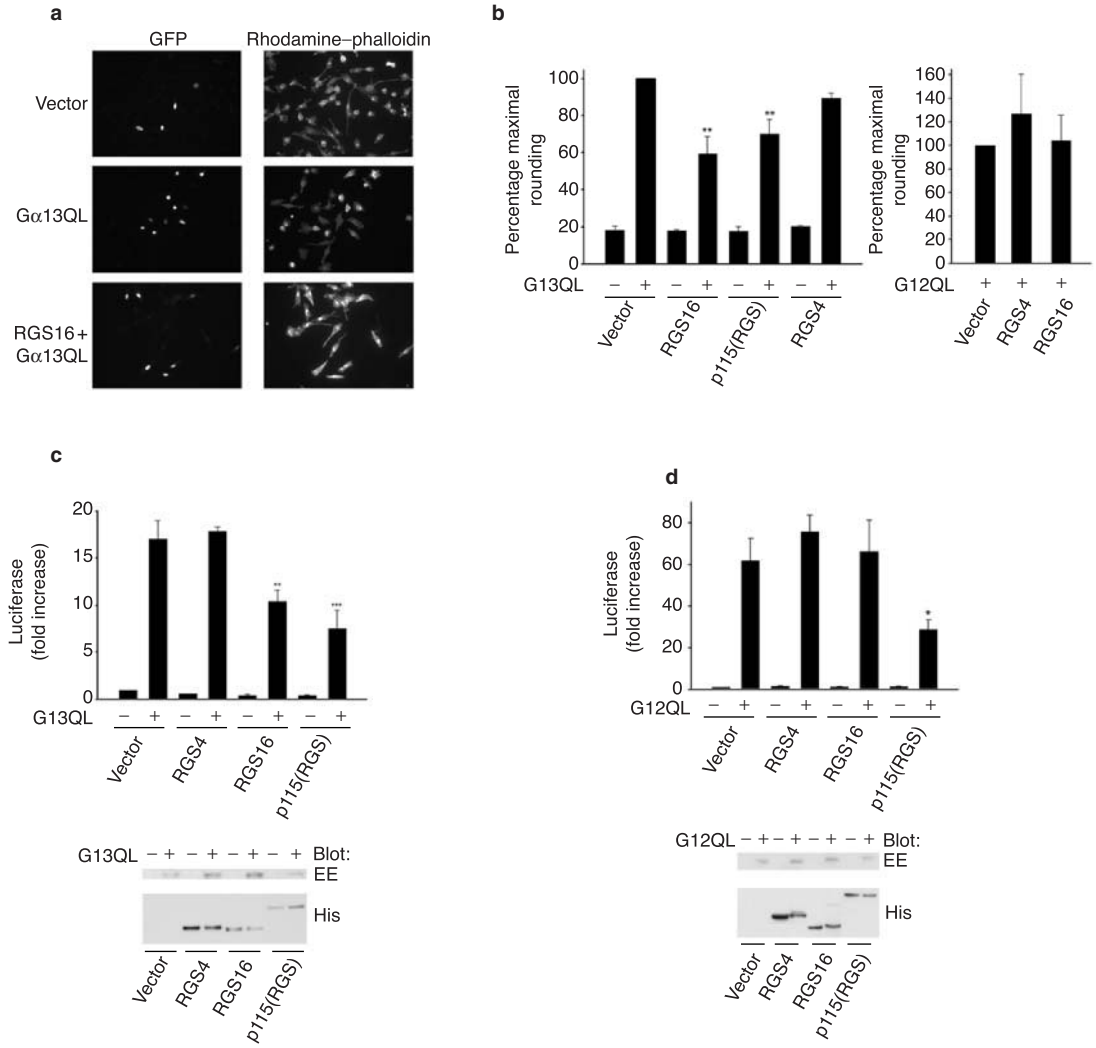

Figure 1 Regulation of Gα13-mediated rounding and SRE activation by RGS16. GFP and either RGS16, RGS4, or p115(RGS) plasmids were microinjected into 1321N1 cells with or without Gα12QL or Gα13QL. (**a**) Injected cells were identified by GFP (left), whereas staining with rhodamine-phalloidin, a marker of actin polymerization, assessed cell rounding (right) in cells expressing vector, Gα13QL, or Gα13QL + RGS16. (**b**) Bar graph shows the percentage of maximal rounding induced by Gα13 or Gα12 (set at 100%) for each condition (mean ± S.E.M. of 3 experiments, $^{**}p < 0.01$). (**c**) Empty vector, RGS16, RGS4, or p115(RGS) plasmids were transfected into HEK 293T cells with SRE.L-luciferase and β-gal reporters with or without Gα13QL. Values represent fold-increase in activity compared with cells transfected with vector alone (mean ± S.E.M. of three experiments; $^{**}p = 0.01$, $^{***}p = 0.001$).(**d**) Experiment similar to **c**, except that cells were co-transfected with Gα12QL. Bar graph represents fold increase in normalized luciferase values compared to vector-transfected cells (mean ± S.E.M. of three independent experiments; $^{*}p < 0.05$).

50%, and transfection of p115(RGS) almost completely eliminated LPA-induced reporter activity (Fig. 2a). As LPA receptors may couple to Gα12 in addition to Gα13 (ref. 10), this finding could partially reflect p115(RGS) binding to both Gα12 and Gα13, whereas RGS16 would be expected to inhibit only the Gα13-dependent component.

Some RGS proteins attenuate signals that induce their expression. Platelet-activating factor (PAF) stimulation of B-lymphocytes upregulates RGS1 levels, and RGS1, in turn, inhibits PAF-evoked MAP kinase activation in these cells[12]. We determined whether LPA stimulation of MCF-7 cells was associated with changes in endogenous RGS16 expression, which might implicate RGS16 in the regulation of LPA signalling. Whereas Gα13 and p115Rho-GEF levels remained relatively constant or declined, RGS16 levels increased after LPA treatment (Fig. 2b). Thus, increased expression of RGS16 resulting from LPA exposure could induce a feedback desensitization loop by attenuating LPA-evoked, Gα13-mediated signalling.

As overexpression of RGS16 inhibited LPA-evoked SRE.L activation, we reduced expression of endogenous RGS16 by RNAi and evaluated SRE.L reporter activity in LPA-treated cells. Transfection of RGS16 siRNA, but not empty vector or RGS4 siRNA, decreased the amount of endogenous RGS16 with no significant effect on the expression of Gα13, Gα12, or RhoA (Fig. 2c). RGS16 knockdown enhanced LPA-induced SRE.L activation, whereas RGS4 siRNA had no effect. Stimulation of Gαq by LPA receptors may also mediate Rho-induced SRE.L activation[13]. Therefore, we used RNAi to evaluate the importance of Gαq in LPA-evoked SRE.L reporter stimulation. Although Gαq-specific siRNA reduced the level of Gαq by more than 80%, Gαq knockdown was associated with increased LPA-stimulated SRE.L activity. Thus, Gαq is not required for LPA-induced SRE.L activation in MCF-7 cells. The increase in LPA-stimulated reporter activity in

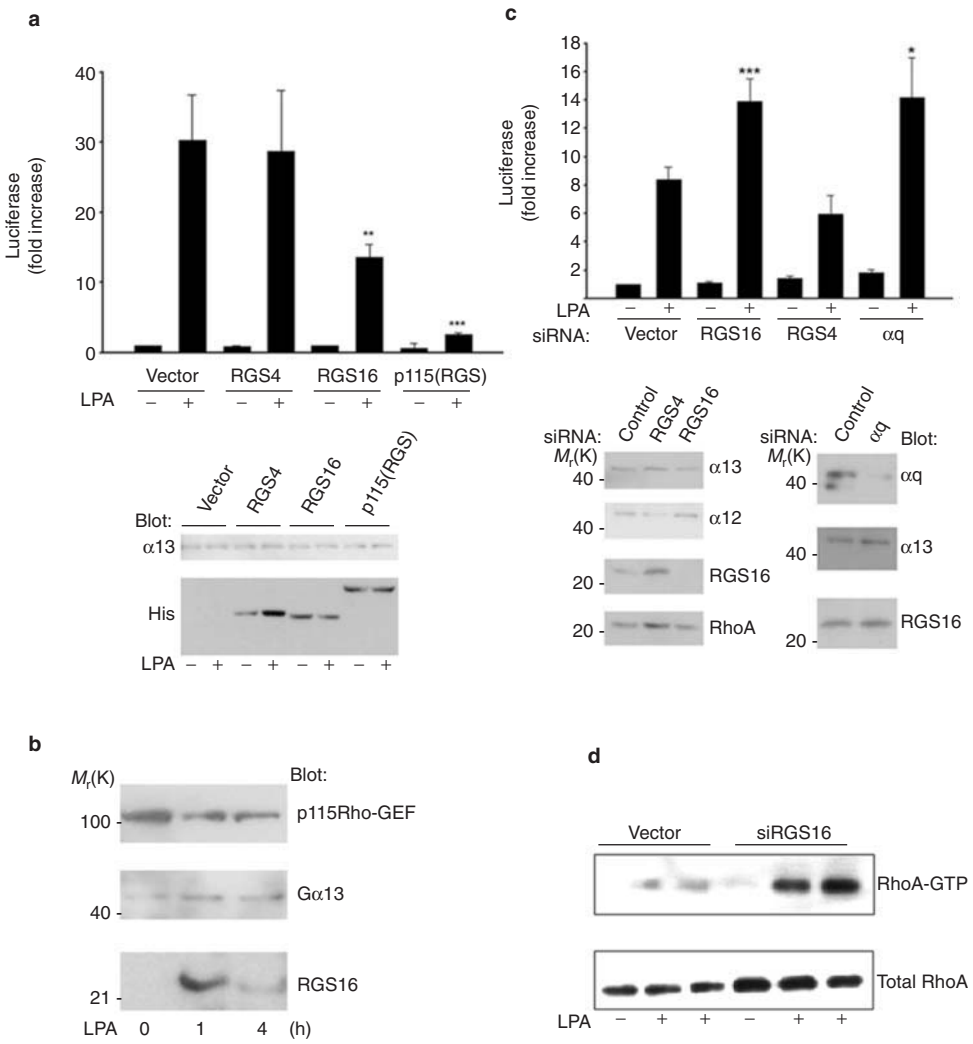

Figure 2 Regulation of LPA-evoked, Gα13-mediated signalling in MCF-7 cells. (**a**) Cells were transfected with RGS4, RGS16, or p115(RGS), together with reporters and stimulated with LPA (10 μM). Fold increase in SRE.L activity compared with vector-transfected cells (mean ± S.E.M. of 3–5 experiments; $^{**}p = 0.01$; $^{***}p = 0.001$) is shown. (**b**) Cells were treated with LPA (10 μM) for the indicated times, and lysates (80 μg protein) were immunoblotted with indicated antibodies. (**c**) Cells transfected with vector or siRNAs for RGS16, RGS4, or Gαq together with the SRE.L reporter were stimulated with LPA (1 μM). Normalized luciferase activity is shown in the bar graph (fold-increase compared with vector-transfected cells) representing five independent experiments ($^{**}p = 0.01$; $^{*}p < 0.05$). Lysates (80 μg protein) from untreated cells transfected with the indicated siRNA plasmids were immunoblotted as indicated. (**d**) MCF-7 cells were stimulated with LPA before extraction of GTP-bound RhoA, immunoblotting, and densitometry. RhoA–GTP values were normalized to total RhoA for each sample ($p = 0.01$). Blot shown is representative of two experiments.

cells with reduced Gαq could represent enhanced coupling of receptors to Gα12 or Gα13.

To determine if decreased levels of RGS16 affected the SRE.L activation induced by a Gα13-independent stimulus, we treated cells with nocodazole, which disrupts microtubules and stimulates a Rho-GEF (GEF-H1) to promote SRE.L activation[14]. Treatment of MCF-7 cells transfected with either empty vector or plasmids encoding siRNAs specific for RGS4 or RGS16 with nocodazole induced an approximately 3.5-fold activation of SRE.L (see Supplementary information, Fig. S1c). This result confirms that the effect of RGS16 knockdown on LPA-evoked SRE.L reporter activity occurs upstream of Rho activation.

To evaluate the effect of RGS16 knockdown on Gα13-stimulated Rho-GEF activity directly, we used the Rho-binding domain of Rhotekin to extract active (GTP-bound) RhoA from LPA-treated MCF-7 cells. In control cells, LPA produced a 3.5-fold increase in RhoA activation, compared with an approximately 10.3-fold response in cells treated with siRNA to RGS16 (Fig 2d). This result is consistent with the hypothesis that RGS16, at physiological levels of expression, serves as an endogenous inhibitor of Gα13-mediated RhoA activation in MCF-7 cells.

Because RGS16 seems to function upstream of Rho to inhibit Gα13- but not Gα12-mediated SRE.L activation and rounding, we hypothesized that RGS16 could bind Gα13 directly. We examined the interactions between RGS16, RGS4, or p115Rho-GEF and Gα proteins in cellular membranes. Both RGS4 and RGS16 co-precipitated Gαi and Gαq from membrane lysates pre-treated with aluminum magnesium fluoride (AMF), whereas p115Rho-GEF did not bind either Gαi or Gαq (Fig. 3a). None of the three RGS proteins coprecipitated Gαs. RGS16, but not RGS4, also co-purified Gα13, independent of the presence of AMF. Neither RGS16 nor RGS4 co-precipitated Gα12, whereas

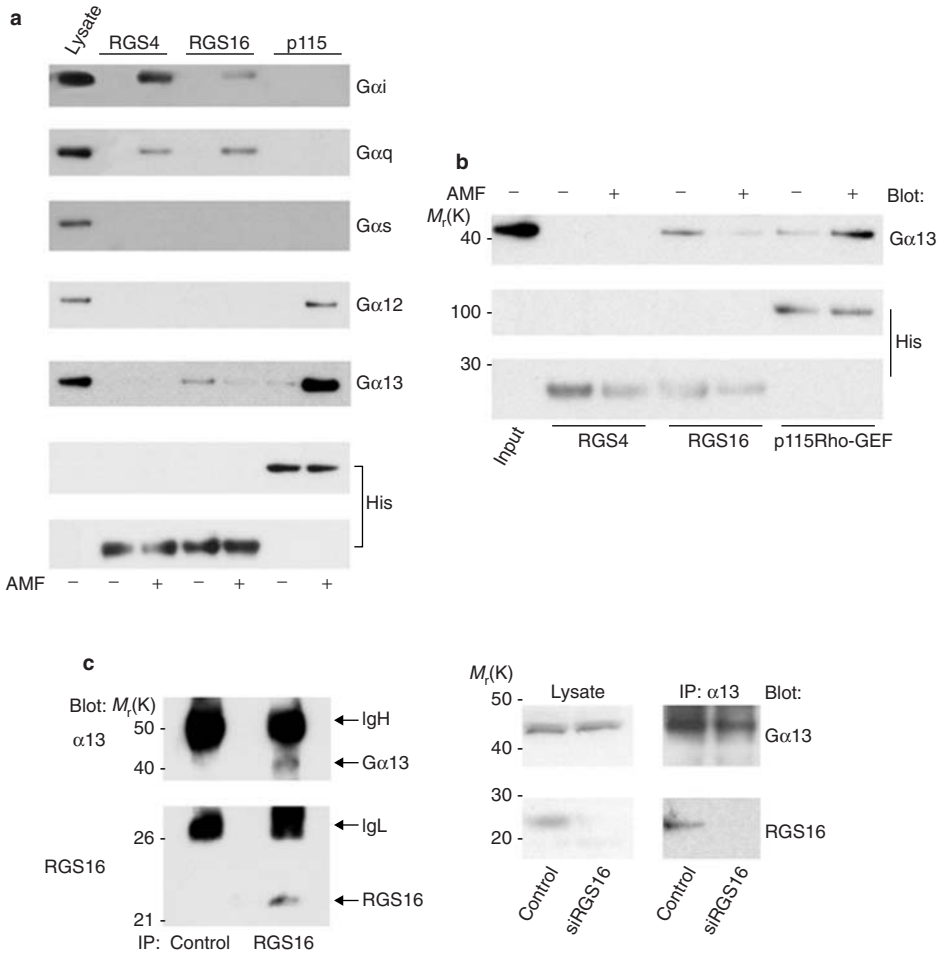

Figure 3 RGS16, but not RGS4, selectively binds Gα13. (**a**) His-tagged RGS4, RGS16, and p115Rho-GEF were immobilized on Ni^{2+} agarose and incubated with NG108 cell lysates (Gαs, Gα12 and Gα13) or bovine brain membrane extracts (Gαi, Gαq) in the presence or absence of AMF. Co-purified proteins were detected with antibodies against the indicated Gα subunits and RGS proteins with anti-His. (**b**) Ni^{2+}-bound His_6RGS4, RGS16, or p115Rho-GEF was incubated with recombinant Gα13. Co-purified proteins were detected by immunoblotting. (**c**) Polyclonal antibodies raised against RGS16, Gα13, or control rabbit IgG were used to immunoprecipitate endogenous proteins from lysates of MCF-7 cells transfected with vector or siRGS16 plasmids as indicated. RGS16 and Gα13 were detected by immunoblotting with the same antibodies used for immunoprecipitation.

p115Rho-GEF coprecipitated Gα12 only in the presence of AMF. These results suggest that RGS16 binds Gα13 in addition to its known G protein partners.

To establish whether there is a direct association between RGS16 and Gα13, we evaluated co-precipitation of recombinant proteins. Although purified Gα13 failed to bind RGS4, it bound RGS16 in the presence or absence of AMF. p115Rho-GEF bound Gα13–AMF more efficiently than Gα13–GDP (Fig. 3b). AMF-dependent binding of RGS16 to Gα13 might be observed if RGS16 were a Gα13 GAP (ref. 15). RGS16 did not exhibit GAP activity on purified Gα13 in single turnover assays (data not shown).

One outcome of the RGS16–Gα13 interaction could be interference with RGS16 GAP activity toward Gαi. To test this hypothesis, we measured GTP hydrolysis by Gαi in the presence or absence of RGS16 and Gα13. RGS16 enhanced the GTPase activity of Gαi, whereas a molar excess of Gα13 over Gαi (approximately 300-fold) did not alter RGS16 GAP activity (see Supplementary Information, Fig. S2). The affinity of RGS16 for Gαi (for which the K_d is < 100 nM; ref. 16) may be higher than its affinity for Gα13, one possible explanation for this result. Alternatively, Gα13 could bind RGS16 at a site distinct from the RGS domain (the region required and sufficient for RGS GAP activity on Gαi[17]) enabling it to interact with Gαi and Gα13 simultaneously.

To confirm that the interaction between RGS16 and Gα13 occurs in a native system, we immunoprecipitated endogenous RGS16 from MCF-7 cells using an antibody raised against the RGS16 holoprotein[18] without addition of exogenous nucleotide or AMF. We detected endogenous Gα13 from cell lysates that co-precipitated with RGS16 (Fig. 3c, left). To exclude the possibility that RGS proteins cross-reactive with this antibody could be present in the immunoprecipitates, we purified RGS16 with antiserum raised against a unique RGS16 carboxy-terminal peptide. This antibody also efficiently immunoprecipitated RGS16 from MCF-7 cells, and we detected co-precipitated Gαi only in the presence of AMF and Gα13 in the presence or absence of AMF (see Supplementary Information, Fig. S3). Furthermore, RGS16 was also detectable in anti-Gα13 immunoprecipitates from control cells, but not siRGS16 cells (Fig. 3c, right panel). Thus, Gα13 and RGS16 are binding partners in a physiological setting.

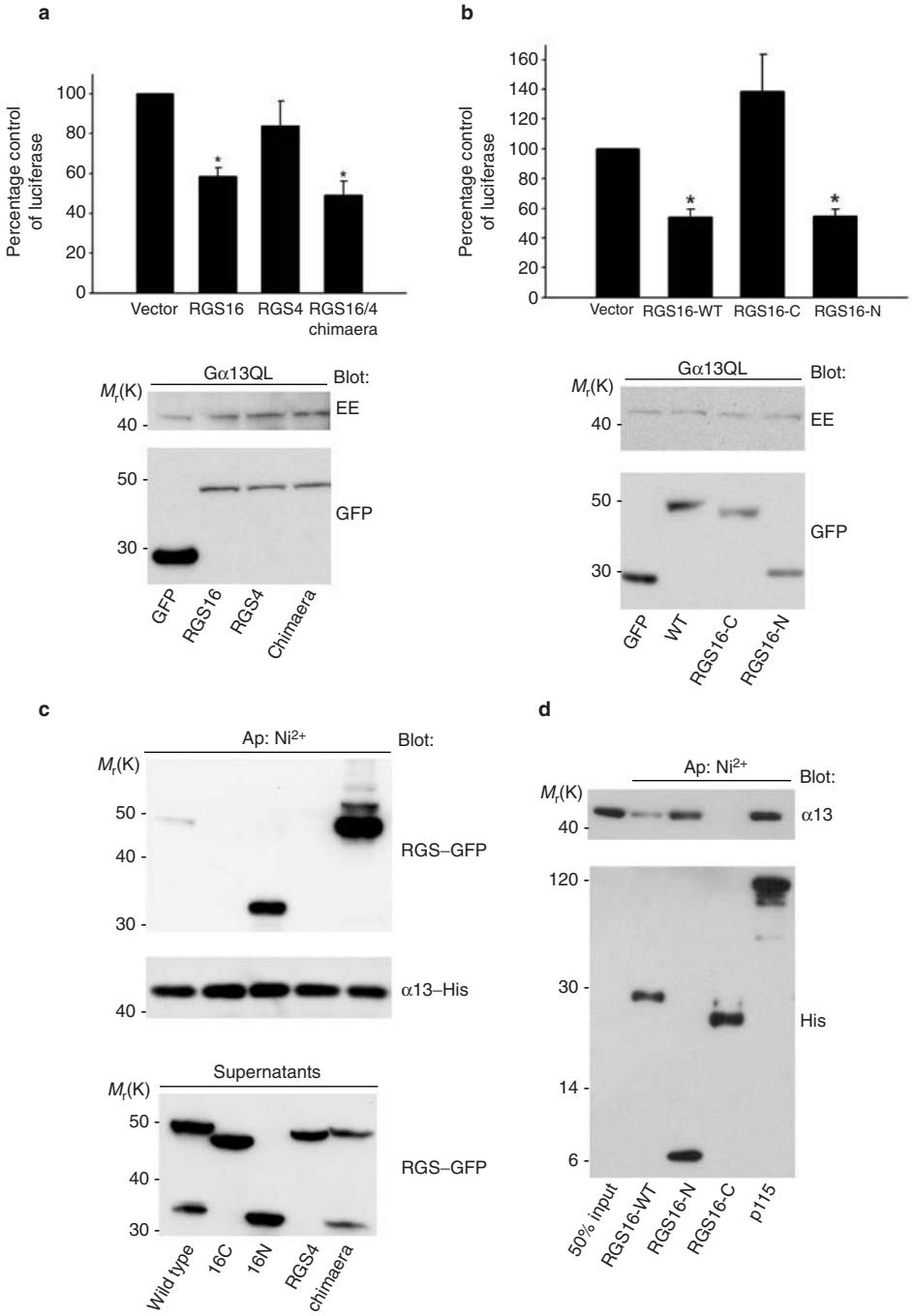

Figure 4 RGS–Gα13 interaction and inhibition of Gα13QL signalling requires the RGS16 amino terminus. (**a**) SRE.L activity was determined in HEK 293T cells expressing GFP or various RGS–GFP constructs, together with Gα13QL and reporters. Bar graph represents β-Gal-normalized luciferase values expressed as the percent of vector (GFP)-transfected cells (mean ± S.E.M.) from seven experiments (*$p < 0.05$). (**b**) Percentage of Gα13QL-stimulated SRE activity in vector-transfected cells in cells expressing RGS16 WT (wild type), RGS16-N (residues 1–31) or RGS16-C (residues 32–202) deletion mutants (*$p < 0.01$). (**c**) Cells were co-transfected with Gα13-V5–His and either GFP, RGS16 WT, RGS16-N (1–31), RGS16-C (32–202), or a RGS16(1–87)–RGS4(88–205) chimera fused to GFP. Gα13 was extracted using Ni^{2+} agarose, and co-purified proteins (top) and post-purification supernatants (bottom) were analysed with anti-GFP. (**d**) His-tagged RGS16-WT, RGS16-N (1–31), RGS16-C (32–202), or p115Rho-GEF was mixed with recombinant Gα13. RGS proteins were affinity purified, and Gα13 was detected by immunoblotting.

To identify the region(s) necessary for RGS16 inhibition of Gα13-dependent SRE.L activation, we first constructed a chimera encoding amino acids 1–87 in RGS16 fused to the last 117 amino acids of RGS4. The RGS16/4 chimera inhibited Gα13QL-induced SRE.L activation similar to wild-type RGS16 (Fig. 4a), indicating that the RGS16 amino terminus (amino acids 1–87) is likely responsible for the observed effect of RGS16. To further delineate which region in RGS16 is involved in the regulation of Gα13-dependent SRE.L activation, we

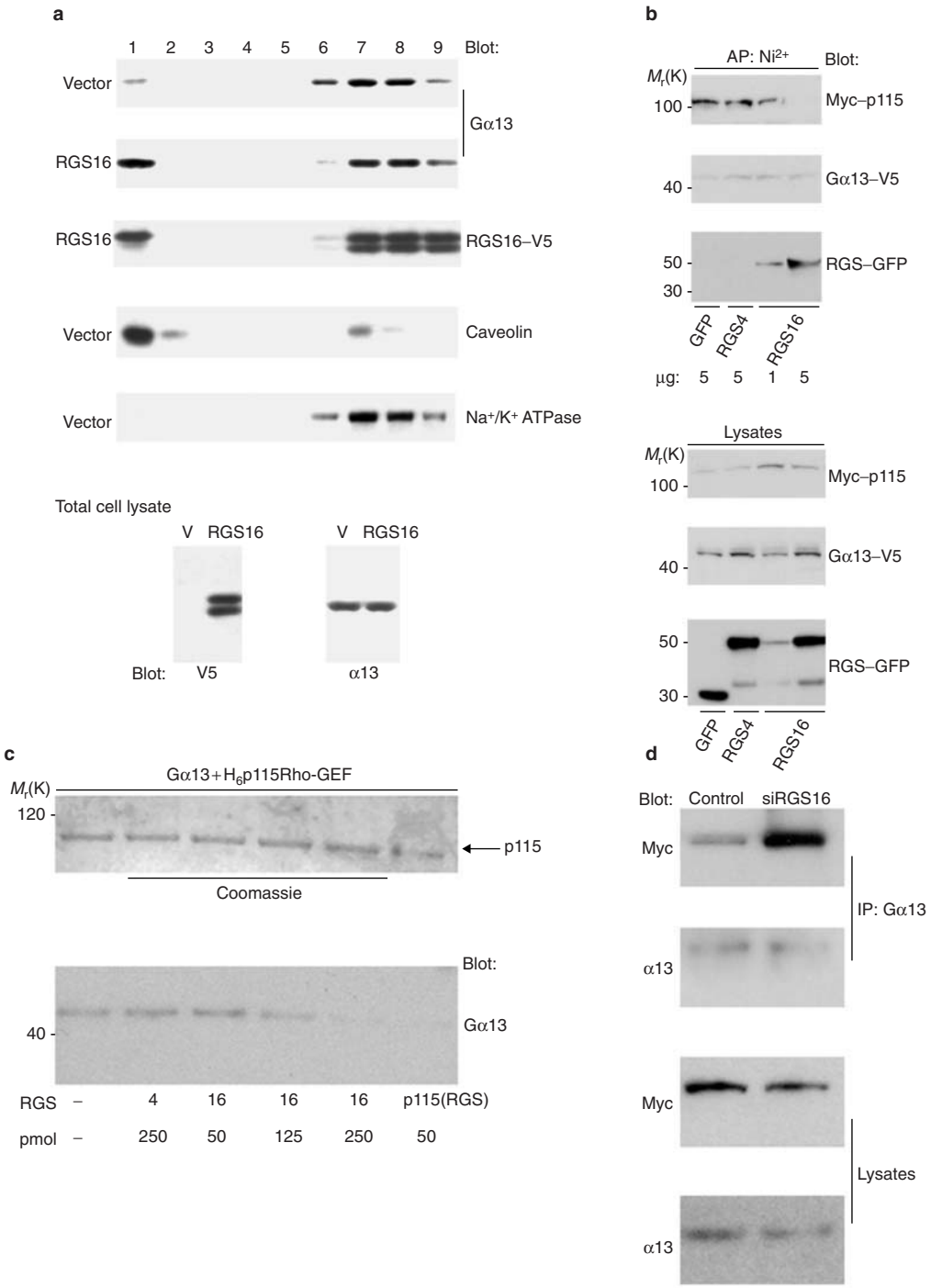

Figure 5 RGS16 is associated with Gα13 translocation to lipid rafts and reduced Gα13 binding to p115Rho-GEF. (**a**) HEK 293T cells were transfected with vector alone or RGS16-V5–His. Total cell lysates (bottom) or cells treated with cold 0.5% Triton X-100 and separated by Optiprep gradient centrifugation (top) were immunoblotted as indicated. (**b**) HEK 293T cells transfected with Gα13–V5–His, myc–p115Rho-GEF, GFP, RGS4, or RGS16 (1 or 5 μg). Lysates were affinity purified and immunoblotted with specific antibodies as shown. (**c**) His_6p115Rho-GEF was purified with nickel agarose in the presence of Gα13 and RGS4, p115(RGS), or increasing amounts of RGS16. Gα13 bound to p115Rho-GEF-coupled beads was detected by immunoblotting. Coomassie blue staining of the membrane verified equal amounts of p115Rho-GEF in each sample. (**d**) Control or siRGS16 cells were transfected with myc–p115Rho-GEF. Endogenous Gα13 was immunoprecipitated with anti-Gα13, and co-purified proteins were identified by immunoblotting as indicated.

assayed amino-terminal and carboxy-terminal RGS16 deletion mutants fused to green fluorescent proteins (GFP). When compared with SRE.L activity in vector-transfected cells, cells expressing RGS16-N (amino acids 1–31) exhibited reduced Gα13QL-evoked SRE.L activation, whereas cells transfected with a mutant lacking the amino-terminus (RGS16-C, amino acids 32–202) displayed SRE.L activity

similar to control (Fig. 4b). Thus, the RGS16 amino-terminus (amino acids 1–31) is necessary and sufficient for inhibition of Gα13-mediated SRE.L activation.

To determine if the RGS16 amino terminus is required for Gα13 interaction, we evaluated binding of Gα13 to GFP fusions of full length RGS16, RGS16 truncation mutants, RGS4, or the RGS16/4 chimera. Full-length RGS16–GFP co-purified with Gα13, whereas neither RGS16-C nor RGS4 co-precipitated (Fig. 4c). Surprisingly, both RGS16-N and the RGS16/4 chimera bound Gα13 more efficiently than wild-type RGS16, suggesting a stronger interaction with the RGS16 amino terminus than with full-length RGS16. To confirm that the RGS16 amino terminus binds directly to Gα13, we evaluated Gα13 co-precipitation of purified, His-tagged RGS proteins. Recombinant Gα13 co-eluted with either wild-type RGS16 or RGS16-N, but not with RGS16-C (Fig. 4d). These data indicate that the first 31 amino acids of RGS16 are both necessary and sufficient for Gα13 binding.

As RGS16 binding to Gα13 did not require a specific nucleotide, it seems unlikely that RGS16 modulates the Gα13 GTPase cycle or receptor–heterotrimer coupling. Alternatively, RGS16 could inhibit Gα13 function by affecting Gα13 modification or localization. Gα13 undergoes palmitoylation on amino-terminal cysteine residues, which is critical for plasma membrane localization and receptor interactions[19]. We tested whether RGS16 expression affects Gα13 palmitoylation by metabolically labelling cells with ^{3}H palmitate. Transfection of RGS16 did not inhibit tritium incorporation into Gα13, indicating that Gα13 palmitoylation was not affected by RGS16 expression (see Supplementary Information, Fig. S4). Although RGS16 did not interfere with Gα13 palmitoylation, we speculated that its interaction with Gα13 could change Gα13 localization at the membrane. RGS16 localizes to DRMs, whereas Gα13, unlike other Gα subunits, does not[20, 21]. We immunoblotted DRM fractions from HEK 293T cells in the presence and absence of RGS16 transfection. We observed an approximately threefold increase in the levels of Gα13 in the DRM fraction of RGS16-transfected cells (Fig. 5a). Caveolin and Na^{+}/K^{+} ATPase were used as markers for proteins included in (caveolin) and excluded from (Na^{+}/K^{+} ATPase) DRMs. RGS16 transfection did not change the DRM localization of p115Rho-GEF (data not shown) or the total expression levels of Gα13 (Fig. 5a, bottom). Thus, RGS16 alters the membrane localization of Gα13, which further supports a physiological interaction.

Because a considerable amount of Gα13 remained in the non-DRM fractions after RGS16 transfection, it was unclear whether the shift of Gα13 to DRMs could fully explain the functional effects of RGS16 on Gα13 signalling. As RGS16 is not a Gα13 GAP, we hypothesized that it might instead inhibit Gα13 from binding to a downstream effector. To test this possibility, we measured co-precipitation of Gα13–His and p115Rho-GEF in the presence of GFP, RGS4, or RGS16. Gα13 extracted equivalent quantities of p115Rho-GEF from cells expressing either GFP or RGS4 (Fig. 5b). RGS16 co-purified with Gα13, and increasing amounts of RGS16 correlated with reduced levels of Gα13-bound p115Rho-GEF. To confirm that decreased Gα13–p115Rho-GEF association was a direct result of RGS16 binding, we purified recombinant Gα13 and RGS proteins with His-tagged p115Rho-GEF. The addition of RGS4 in molar excess did not disrupt Gα13 binding to p115Rho-GEF, whereas an equimolar amount of p115(RGS) completely blocked binding (Fig. 5c). Increasing quantities of RGS16 reduced the amount of Gα13 bound to p115Rho-GEF in a concentration dependent-manner. A quantity of RGS16 approximately fivefold greater than that of p115(RGS) was required to inhibit Gα13 binding to full-length p115Rho-GEF, suggesting that the relative affinity of Gα13 for RGS16 is at least fivefold less than its affinity for the RGS domain of p115Rho-GEF.

Although RGS16 appeared to block the Gα13-p115Rho-GEF interaction and inhibited Gα13-stimulated Rho activation in cells (see Fig. 2d), it unexpectedly failed to inhibit Gα13 stimulation of recombinant p115 Rho-GEF activity *in vitro* (data not shown). Because membrane, receptor, and phospholipid interactions profoundly affect the activity of RGS proteins[22], this result suggests that an additional co-factor, modification, or conformation of either protein could be required for the regulation of Gα13-stimulated Rho-GEF activity by RGS16 that we observed in MCF-7 cells.

To confirm that RGS16 can function as an inhibitor of the Gα13-p115Rho-GEF interaction in cells, we measured p115Rho-GEF–Gα13 binding in cells with reduced RGS16 levels. Although we immunoprecipitated similar quantities of Gα13 from control and siRGS16 cells, the amount of co-associated p115Rho-GEF was significantly increased in cells with decreased RGS16 expression (Fig. 5d). This result suggests that disruption of the Gα13–p115Rho-GEF complex is a potential mechanism whereby RGS16 can attenuate Gα13-dependent signalling.

This study describes a unique function for RGS16, a classical (R4) RGS protein lacking additional well-defined domains, in the regulation of Gα13-mediated signalling and highlights a new region outside the RGS box directly involved in G protein binding, but not GAP activity. Although the affinity of Gα13 for RGS16 is apparently less than its affinity for p115Rho-GEF, RGS16 binding to Gα13 is associated with Gα13 redistribution within the membrane and reduced effector interaction. As the RGS16 membrane binding domain and Gα13 interaction sites overlap[23], it will also be of interest to determine how Gα13 binding affects RGS16 regulation of Gαi and Gαq and the DRM localization of RGS16. Our study suggests that RGS16 could oversee distinct signalling pathways evoked by a single receptor coupled to several Gα subclasses. □

METHODS

Cells, proteins, and plasmids. HEK 293T and MCF-7 cells were purchased from ATCC (Manassas VA). NG108 cells were the gift of Warner Klee (NIDDK/NIH, Bethseda, MD), and bovine brain extracts were purchased from Calbiochem (San Diego, CA). We transfected cells in either six-well plates or 100 mm tissue culture dishes using Superfect reagent (Qiagen, Valencia, CA) and harvested cells 24 h after transfection. We prepared His_6RGS16 and RGS4 from *Escherichia coli* as described previously[24, 25]. Baculoviruses directing expression of Gα13, Gβ1, and His_6Gγ2 were used to generate recombinant Gα13 essentially as described elsewhere[26]. pCMV5–myc–p115Rho-GEF was the kind gift of Matthew Hart (Onyx Pharmaceuticals, Richmond, CA), and the plasmid encoding GST–Rhotekin RBD was obtained from Martin Schwartz. EE–Gα12QL, EE–Gα13QL, and HA–Rho(G14V) were obtained from the Guthrie Research Institute (Sayre, PA) and RhoA(Q63L) from J. Silvio Gutkind (NIDCR/NIH). Gα13 and the RGS domain (encoding residues 1–252) of p115 were generated by polymerase chain reaction (PCR) and subcloned into TOPO–pcDNA3.1–V5/His. Plasmids pcDNA3.1/V5–His–RGS16 and pcDNA3/myc–His–RGS4 have been described previously[18, 25]. The coding region of full-length p115Rho-GEF was generated by PCR and cloned into TOPO–pBlueBac-V5/His. We generated recombinant baculovirus and purified p115–His_6 on nickel agarose according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). We generated RGS16, RGS16(1–31, 'RGS16-N') RGS16 (32–202, 'RGS16-C'), and RGS4 PCR fragments and subcloned the products into pEGFP–N2 (Clontech). We constructed the RGS16/4 chimera by subcloning a PCR fragment encoding RGS16 residues 1–87 and flanking SspI sites into an endogenous SspI site in RGS4–pEGFP.

His-tagged RGS16 (32–202, 'RGS16-C') was generated by cloning the appropriate PCR fragment into pET28a (Novagen, Madison, WI) and purified as described for the wild-type protein. We generated RGS16 (1–31)–His_6 ("RGS16-N") by subcloning a PCR fragment into TOPO–pET152D (Invitrogen) in frame with thioredoxin. The RGS domain of p115 (amino acids 1–252) was generated by PCR and cloned into pGEX–4T (Amersham Biosciences, Piscataway, NJ). Protein expression was induced in *E. coli* BL21(DE3) using IPTG, and recombinant proteins were affinity purified using Ni^{2+}/NTA (**nitriloacetic acid**) agarose or glutathione sepharose according to the manufacturer's instructions. Thioredoxin was cleaved from the RGS16 fragment using Enterokinase max (Invitrogen), leaving RGS16 (1–31) with a carboxy-terminal His tag. EK-max was removed from the preparation using EK-Away resin (Invitrogen).

Affinity purifications and immunoblotting. RGS-G protein binding experiments with recombinant proteins and pull-downs were performed essentially as described[27]. For co-immunoprecipitation of RGS16 from MCF-7 cells, we lysed cells in detergent buffer (20 mM Tris at pH 7.5, 150 mM sodium chloride, 1% Triton X-100, 0.25% deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM sodium fluoride plus protease inhibitors) and incubated clarified lysates with either normal rabbit IgG, anti-RGS16, or anti-Gα13 antisera. Immune complexes were recovered with 30 μl Protein A-agarose (Invitrogen), and immunoprecipitates were washed five times with lysis buffer. Antibodies against the myc, GFP, and His tags, Gαi, Gαq, Gαs, Gα12, Gα13, p115Rho-GEF, and RhoA were purchased from Santa Cruz (Santa Cruz, CA), anti-V5 from Invitrogen, and anti Glu–Glu (EE) from Covance. The polyclonal antibody against RGS16 (CT265) and the carboxy-terminal anti-Gα13 antibody (HD) have been described previously[20, 21]. Antiserum against a carboxy-terminal RGS16 peptide (CGSPAEPSHT) was generated in rabbits and affinity purified using resin coupled to the immunizing peptide (Quality Controlled Biochemicals, Hopkinton, MA).

Single turnover GTPase assays. Nonmyristoylated Gαi1 was expressed in *E. coli* host strain BL21(DE3) as an amino-terminal S–Tag fusion with subsequent cleavage of the affinity tag during purification using S-protein Agarose (Novagen). Single turnover GTP hydrolysis by Gαi1 (1 μM) was assayed as previously described[28], except that reactions were carried out in the presence of 5 mM free magnesuim. The final concentration of GTP-bound Gαi1 was estimated to be approximately 0.3 nM.

Metabolic labelling, immunoprecipitation and detergent resistant membrane (DRM) preparation. We performed these procedures as previously described[20, 21]. We isolated DRM fractions using solubilization in cold 0.5% Triton X-100 and centrifugation on a 5/30/35 OptiPrep gradient[21].

Reversal of stellation (rounding). We measured cell rounding as described previously[7].

SRE assays. We evaluated activation of SRE wild type (Stratagene, La Jolla, CA) or SRE.L-Luc (containing a mutation in the ternary complex factor binding site, obtained from Kozo Kaibuchi, NAIST, Ikoma, Nara, Japan) reporter plasmids exactly as described elsewhere[27]. All luciferase values were normalized to β-gal values.

Rhotekin binding assay. Subconfluent MCF-7 cells were serum starved overnight and stimulated in 100 mm dishes with LPA (10 μM) for 3 min. at 37°C. We extracted RhoA-GTP as previously described[29] and quantitated RhoA-GTP levels by immunoblotting and densitometry. RhoA–GTP values were normalized to total RhoA values for each sample before statistical analysis.

Suppression of gene expression by short, interfering RNAs (siRNAs). The plasmid pSUPER (from Reuven Agami), which contains the human H1 RNA polymerase promoter, was used as the backbone for insertion of short sequence-specific oligomers predicted to form stem–loop–stem structures as described[30]. The sequences for siRNA were: human *rgs16*—GCGATACTGGGAGTACTGG and CGCTTCCTGAAGTCGCCTG; human *rgs4*—CCGCCGCTTCCTCAAGTCT; human *Gαq*—GGGACGCCCGCCGGGAGCT. 2 μg of the siRNA plasmid or pSUPER without an insert were transfected into MCF-7 cells transiently or stably together with pCDNA3 as a selection marker using Superfect (Qiagen). Clones were selected using Geneticin (Invitrogen, Carlsbad, CA).

Statistical analysis. Data were analysed using In Stat software (Graph Pad Software Inc, San Diego, CA). Where indicated, statistical significance was determined using repeated measures ANOVA with Tukey-Kramer or Dunnett post-hoc tests or student's *t* tests. A two tailed p value < 0.05 was considered statistically significant. UVP LabWorks software (Upland, CA) was used to quantitate band intensity of immunoblots.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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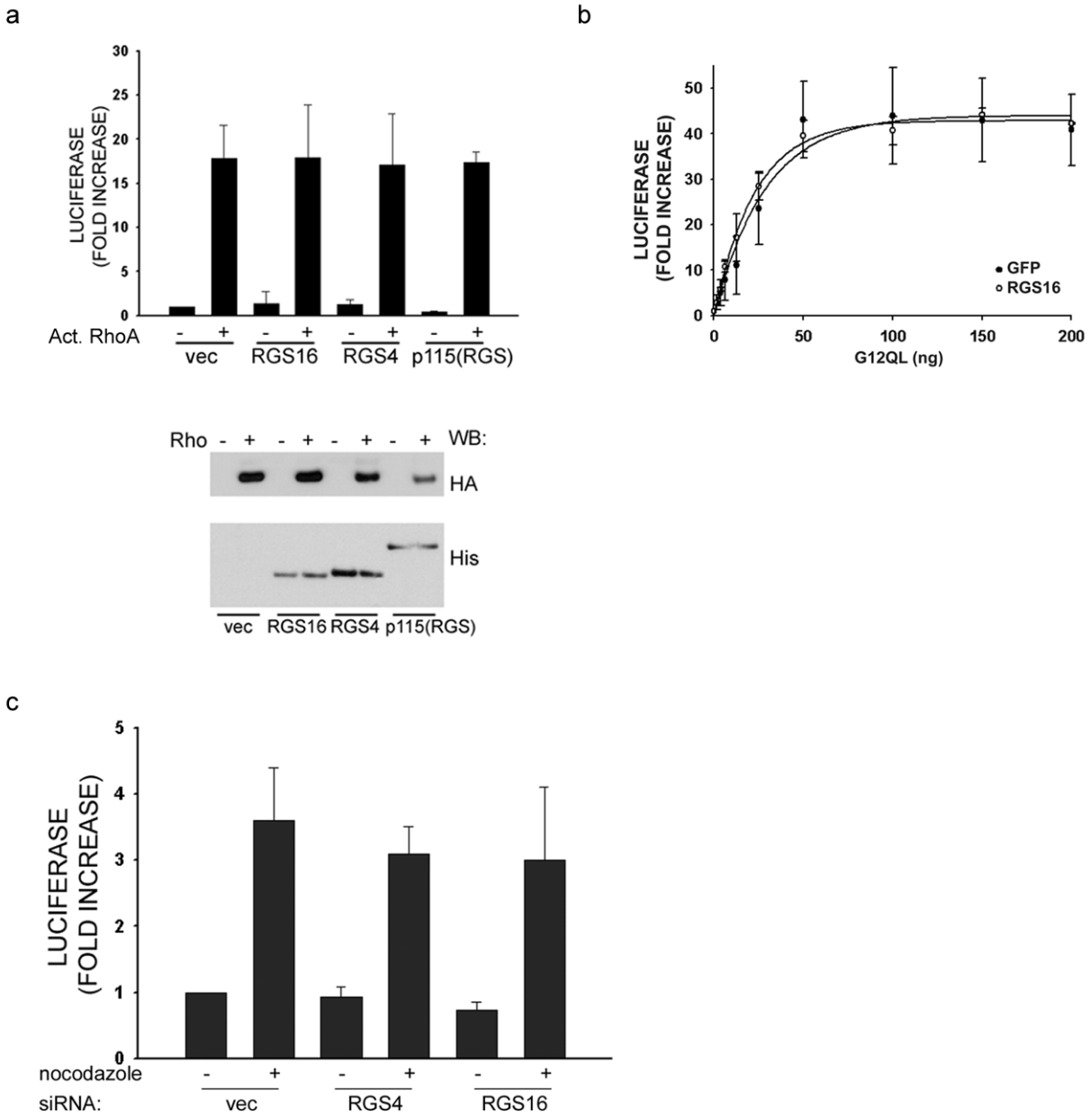

Figure S1 **RGS16 acts upstream of Rho to inhibit Gα13QL-induced SRE.L activation. a**, SRE.L activity induced by constitutively active Rho. Rho(Q63L) or HA-Rho(G14V) and the SRE.L reporter were transiently transfected with empty vector or the indicated RGS proteins in HEK 293T cells. Luciferase activity was measured as in previous experiments. Bar graph represents fold- increase (mean +/- S.E.M. of 5 experiments) for each condition compared to vector-transfected cells. **b,** Experiment similar to Fig. 1d except that a range of concentrations of Gα12QL was used to determine the effect of RGS16 at sub-maximal doses. **c,** RGS16 does not inhibit SRE.L activation induced by a Gα13-independent stimulus. MCF-7 cells were transfected with empty vector or plasmids containing siRNAs specific for *rgs4* or *rgs16* and luciferase activity determined after incubation with nocodazole (10 μM).

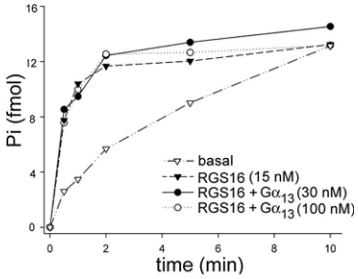

Figure S2 **Gα13 does not interfere with RGS16 GAP activity on Gαi.** GTP hydrolysis by Gαi was measured during a single catalytic turnover in the presence of RGS16 with or without a molar excess of Gα13 (30 or 100 nM).

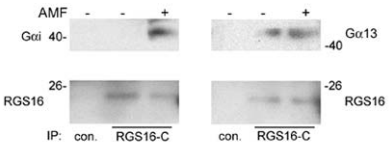

Figure S3 **Co-immunoprecipitation of Gαi and Gα13 with an RGS16-specific antibody.** Pre-immune (con) or anti-peptide antisera raised against the RGS16 carboxy-terminus (RGS16-C) was utilized to immunoprecipitate endogenous RGS16 from MCF-7 cells in the presence or absence of AMF. Co-purified Gαi or Gα13 was detected with specific antibodies as indicated.

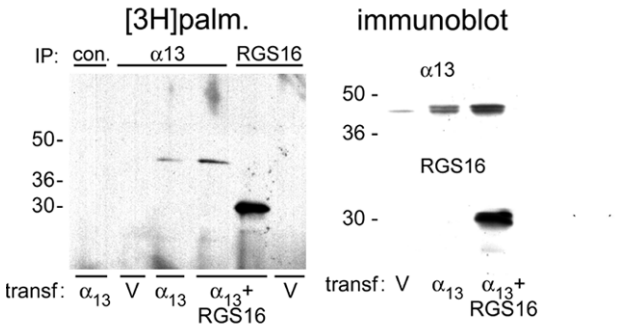

Figure S4 **RGS16 does not block Gα13 palmitoylation.** [^{3}H] palmitate incorporation into Gα13 after RGS16 co-transfection. HEK 293T cells were transfected with vector alone (V) or a plasmid directing expression of Gα13 in the presence or absence of RGS16. Cells were metabolically labeled with [^{3}H]palmitate followed by homogenization and cellular fractionation into particulate and soluble fractions. Particulate fractions were immunoprecipitated with control IgG or antibodies against Gα13 or RGS16. Immunoprecipitates were resolved by SDS-PAGE and prepared for fluorography. Protein expression was verified by immunoblotting an aliquot of the particulate fraction (30 μg protein) with the indicated antibodies.