**Drosophila Ric-8 interacts with the Gα_{12/13} subunit, Concertina, during activation of the Folded gastrulation pathway**

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INTRODUCTION

G protein–coupled receptors (GPCRs) are a highly conserved family of transmembrane receptors that evolved to detect a wide range of signals, including neurotransmitters, hormones, odorants, and light. These receptors have a characteristic topology that spans the membrane via seven α-helices and are oriented with their N-termini toward the extracellular space, their C-termini inside the cell, and three interhelical loops on each side. Ligand binding allows the cytoplasmic domains of the GPCR to activate heterotrimeric G-proteins, downstream signaling molecules that consist of a GTP-binding α subunit that exists in 1:1:1 stoichiometry with a β and a γ subunit.

Abbreviations used: Cta, Concertina; Fog, Folded gastrulation; GDI, guanine-nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; GPCR, G protein–coupled receptor; Mito, mitochondrially tagged; S2R+, S2-receptor +.

ABSTRACT  Heterotrimeric G proteins, composed of α, β, and γ subunits, are activated by exchange of GDP for GTP on the Gα subunit. Canonically, Gα is stimulated by the guanine-nucleotide exchange factor (GEF) activity of ligand-bound G protein–coupled receptors. However, Gα subunits may also be activated in a noncanonical manner by members of the Ric-8 family, cytoplasmic proteins that also act as GEFs for Gα subunits. We used a signaling pathway active during Drosophila gastrulation as a model system to study Ric-8/Gα interactions. A component of this pathway, the Drosophila Gα_{12/13} subunit, Concertina (Cta), is necessary to trigger actomyosin contractility during gastrulation events. Ric-8 mutants exhibit similar gastrulation defects to Cta mutants. Here we use a novel tissue culture system to study a signaling pathway that controls cytoskeletal rearrangements necessary for cellular morphogenesis. We show that Ric-8 regulates this pathway through physical interaction with Cta and preferentially interacts with inactive Cta and directs its localization within the cell. We also use this system to conduct a structure–function analysis of Ric-8 and identify key residues required for both Cta interaction and cellular contractility.

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family of proteins (e.g., GPR1/2 in Caenorhabditis elegans and Pins in Drosophila melanogaster). Ric-8 binding inactive Gz facilitates GDP release and promotes the formation of a transient nucleotide-free state, which allows Gz-GTP exchange by cytosolic excess of GTP (Tall et al., 2003). In addition, Ric-8 binds to and drives dissociation of Gz-GDP complexed with a GDI, subsequently freeing Gz to engage other effectors (Hampoolz et al., 2005; Tall and Gilman, 2005; Thomas et al., 2008). Recently Ric-8 was identified as a chaperone involved in the biosynthesis of mammalian Gz subunits and their subsequent localization to the plasma membrane (Gabay et al., 2011). Thus several lines of evidence indicate that Ric-8 regulates multiple aspects of Gz function.

A growing body of data has implicated the Ric-8 family of proteins as important accessory molecules involved in heterotrimeric G protein signaling in a variety of developmental processes (for review see Hinrichs et al., 2012). Ric-8 is a highly conserved cytosolic protein that was originally identified in a screen for proteins required for Gz_1 signaling in the C. elegans nervous system (Miller et al., 2000). Since then, Ric-8 has been implicated as a regulator of signaling in events as diverse as fungal pathogenesis and development (Li et al., 2010; Wright et al., 2011) to modulation of mammalian vision, taste, olfaction, and bone formation (Tönissoo et al., 2003, 2010; Von Dannecker et al., 2005, 2006; Dhingra et al., 2008; Kerr et al., 2008; Fenech et al., 2009; Yoshikawa and Touhara, 2009; Grandy et al., 2011; Maldonado-Agurto et al., 2011). Ric-8 plays a well-defined role in spindle orientation during mitosis of asymmetrically dividing cells. During early divisions of the C. elegans embryo Ric-8 acts through Gz family members to establish the position of the mitotic spindle through modulation of pulling forces along the anterior–posterior axis (Miller and Rand, 2000; Afshar et al., 2004, 2005; Couwenbergs et al., 2004). Similarly, in Drosophila, Ric-8 functions through Gz to align the mitotic spindle in both neuroblast and sensory organ precursor cells (David et al., 2005; Hampoolz et al., 2005; Wang et al., 2005). Recent findings also show that Ric-8 is important for spindle alignment in asymmetric cell division in mammalian tissue culture (Woodard et al., 2010). In addition to spindle positioning, Ric-8 regulates cytoskeletal rearrangements during dorsal ruffle formation via Gz13 in mammalian tissue culture (Wang et al., 2011). These data demonstrate that Ric-8, through its interaction with Gz subunits, functions to regulate diverse processes during G protein signaling events, including cytoskeletal behavior.

Drosophila gastrulation is a powerful model system with which to study heterotrimeric G protein signaling within a developmental context. During this process, the Drosophila blastoderm undergoes a series of highly orchestrated cell movements to drive subsets of cells into the interior of the embryo to establish the germ layers. One of the hallmarks of gastrulation is the invagination of a subset of epithelial cells along the ventral midline to form a structure called the ventral furrow (Leptin, 1995). Furrow formation is driven by concerted cellular shape changes in which apical constriction of the actin network by myosin II has the net effect of driving the internalization of the mesodermal precursor cells (Dawes-Hoang et al., 2005; Martin et al., 2009). Genetic analysis of this pathway has identified several components believed to act sequentially to trigger apical constriction. First, the midline epithelial cells destined to invaginate secrete an extracellular protein, Folded gastrulation (Fog), from their apical domains. Fog acts as an autocrine signal and binds to an unidentified transmembrane receptor that then signals through a heterotrimeric G protein complex containing the Drosophila Gz_12/13 subunit, Concertina (Cta; Costa et al., 1994; Morize et al., 1998). Mutations in the GB13F and Gy1 subunits exhibit gastrulation defects and, presumably, comprise the βγ subunits of the heterotrimer along with Cta (Wang et al., 2005). Cta activates a guanine nucleotide exchange factor, Rhof2, relocating Rhof2 from the plus-end tips of growing microtubules to the cortex, where it is docked by its interaction with a transmembrane protein, T48 (Rogers et al., 2004; Nikolaidou and Barrett, 2004; Kölsch et al., 2007). Rhof2 then activates the small G protein Rho1, which activates myosin II at the apical domain via Rho kinase (Rok), thus producing contraction (Barrett et al., 1997; Nikolaidou and Barrett, 2004; Dawes-Hoang et al., 2005). Mutations in any Fog pathway component interfere with the timing or execution of normal gastrulation. This pathway also has been implicated in epithelial remodeling during later stages of development (Nikolaidou and Barrett, 2004). Drosophila gastrulation events are highly analogous to epithelial remodeling in other multicellular organisms, most notably neural tube formation in the developing vertebrate embryo, and downstream signaling components are conserved between invertebrates and vertebrates (Lecuit and Lenne, 2007). Thus we are using the Drosophila Fog signaling pathway as a model system to investigate general mechanisms of signaling during tissue remodeling.

Given the central importance of Cta to Drosophila gastrulation, it is a useful model to study potential interactions between Ric-8 and Gz_12/13-class subunits. Previous studies showed that Ric-8 mutants exhibit gastrulation defects that resemble Cta loss of function (Hampoolz et al., 2005; Wang et al., 2005). Further, Ric-8 was recently shown to regulate the cortical stability of actin in cells of the ventral furrow undergoing apical constriction (Kanasaki et al., 2013). However, the mechanistic details through which Ric-8 functions in this process remain to be determined. Here we examine the role of Ric-8 in the Fog pathway using a novel cell-based assay for Fog-induced cellular contractility. Using RNA interference (RNAi), we show that Ric-8 is necessary for Fog signaling and that it functions within the pathway at the level of Cta. We also find that Ric-8 directly interacts with Cta and exhibits higher affinity for inactive Cta mutants (GTP-free). We present biochemical data that show that Ric-8 preferentially binds and specifically acts to localize inactive Cta downstream of Fog/GPCR signaling. Finally, we conduct a mutagenesis screen of conserved charged amino acid residues and identify specific residues within Ric-8 required for Cta function and/or establishment of a binding interface between the two molecules. On the basis of our results, we propose a model in which Ric-8 acts downstream of Fog pathway activation to localize/scaffold inactive Cta, potentiating Fog signaling to drive persistent cellular constriction.

RESULTS
Reconstitution of Fog-stimulated cellular contractility in a cultured cell model
To study the effect of Fog signaling on cell morphology, we developed a cell culture system to allow us to replicate in vivo signaling events. We began by engineering a stable S2 cell line that expresses full-length Fog tagged at its C-terminus with the Myc epitope under an inducible metallothionein promoter (S2:Fog-Myc). Costa et al. (1994) originally hypothesized that Fog is a secreted protein based on hydropathy analysis of the protein’s primary sequence, which revealed the presence of an N-terminal 12-amino acid hydrophobic region predicted to function as a signal sequence. Later analysis of Fog localization in cells of the embryonic ventral furrow and posterior midgut showed that the protein localized to membrane-bound organelles targeted for the apical surface of the blastoderm epithelium (Dawes-Hoang et al., 2005). To test whether Fog is secreted from S2:Fog-Myc cells, we induced its expression with copper sulfate for 48 h, collected the conditioned medium, and concentrated it.
Thus, as found in tissues in the Drosophila blastoderm preceding cellular shape change, ectopic Fog-Myc is expressed in S2 cells as a secreted protein.

We next screened an assortment of immortalized Drosophila cell lines for their ability to respond to Fog-conditioned medium. Previously we showed that activation of the Rho1 pathway in S2 cells caused the cells to adopt a contracted morphology (Rogers et al., 2004). We therefore used this read-out to test the ability of S2 cells, S2-receptor + (S2R+) cells, and several immortalized lines derived from imaginal discs to respond to Fog. S2R+ cells are a subline derived from S2 cells that express receptors not found in S2 cells (Yanagawa, 1998). Neither S2 cells nor the other epithelial lines we tested changed their shape in response to Fog perfusion (unpublished data). However, S2R+ cells exhibited a robust morphological response upon perfusion with Fog. S2R+ cells adopt a flattened, discoid morphology when plated on concanavalin A (ConA)–treated coverslips. Within 10 min of Fog treatment the cells adopted a “puckered” shape and pushed their nuclei and organelles up and away from the coverslip. At the same time, radial, phase-dark furrows appeared at the cell periphery and moved centripetally to the center of the cell (Figure 1B). One of the downstream effects of Rho pathway signaling is activation of nonmuscle myosin II by phosphorylation of the motor’s regulatory light chain (RLC). Therefore we treated S2R+ cells with concentrated Fog or control cell medium and examined the RLC phosphorylation state using phospho-specific antibodies. Immunofluorescence with phosphorylated-RLC antibodies revealed an overall increase in phosphorylation, along with a dramatic incorporation of myosin II into actomyosin purse-string structures (Figure 1C). To verify that Fog was acting via the canonical pathway involved in gastrulation, we used RNAi to deplete Cta, RhoGEF2, or Rho from S2R+ cells before Fog treatment. RNAi targeting Cta, Rho-GEF2, or Rho prevented cellular constriction after Fog treatment (Figure 1, B and D), as did pretreatment of S2R+ cells with the Rho kinase small-molecule inhibitor Y-27632 (unpublished data). Previous work revealed that embryos mutant for the $\beta$ subunit, $\beta_{13F}$, and the $\gamma$ subunit, $\gamma_{1}$, exhibited gastrulation phenotypes similar to Cta mutants (Wang et al., 2005). We introduced RNAi targeted to these subunits and predicted that they comprise the heterodimer that associates with Cta. We found that these treatments blocked Fog-mediated contractility (Figure 1D). Thus we conclude that treatment of S2R+ cells with Fog activates the identical signaling pathway used in cellular contraction during Drosophila gastrulation.
Ric-8 is necessary for Fog pathway activation in Drosophila S2R+ cells

Next we tested the hypothesis that Ric-8 acts in the Fog pathway. We designed double-stranded RNAs (dsRNAs) to target the coding region or the 5’ and 3’ untranslated regions (UTRs) of the Ric-8 mRNA and found that each effectively depleted Ric-8 from S2R+ cells (Supplemental Figure S1). When tested in the contractility assay, Ric-8-depleted cells were unable to contract after treatment with Fog (Figure 1B and D). The effect of our RNAi was specific, as we were able to rescue the ability of S2R+ cells to respond to Fog by expressing Ric-8-green fluorescent protein (GFP) in cells depleted of endogenous Ric-8 (Figure 2, A and B). Ectopic overexpression of Ric-8–GFP was not sufficient, however, to induce contractility in the absence of Fog (unpublished data). From these data, we conclude that Ric-8 is a necessary component of the Fog signaling cascade.

To identify where Ric-8 is functioning within the Fog signaling pathway, we performed a series of epistasis experiments using RNAi. Overexpression of Myc-Cta-Q303L (CtaQL), a mutation predicted to lock Cta in its GTP-bound conformation (Xu et al., 1993), in S2R+ cells is sufficient to trigger contractility in the absence of Fog (see later discussion of Figure 4A). However, expression of Myc-CtaQL in S2R+ cells depleted of endogenous Ric-8 does not drive cellular constriction (Figure 2, C and D). To verify that the inability of Myc-CtaQL to trigger constriction in Ric-8–depleted cells was not due to the absence of overall Cta protein, we created dual-expression constructs with two distinct metallothionein promoters within the same vector: 1) preceding full-length Myc-Cta and 2) preceding the coding sequence for mCherry. We then transfected these constructs into S2 cells treated with either control or Ric-8 dsRNA and compared levels of wild-type Myc-Cta, constitutively active Myc-CtaQL, and constitutively inactive Myc-Cta-G302A (CtaGA). Mutation of glycine 302 to alanine is predicted to trap Cta in either its GDP-bound conformation or in a nucleotide-free state, based on homology with similar mutations in other Gα12/13 family members (Gohla et al., 1999). Using these constructs, we show that levels of ectopic Myc-Cta are not affected by Ric-8 depletion (Figure 2E). In the converse experiment, S2R+ cells depleted of endogenous Cta and overexpressing Ric-8–GFP do not contract in response to Fog. However, overexpression of RhoGEF2, which is directly downstream of Cta, in either Cta- or Ric-8–depleted cells is sufficient for cellular constriction (Figure 2F). Therefore Ric-8 functions upstream of RhoGEF2, implying a role for Ric-8 at the level of the putative GPCR, Cta, or the βγ subunits. It has been well documented in Drosophila and other systems that Ric-8 does not interact with Ga when it is complexed with its βγ subunits (Tall et al., 2003; Hampoelz et al., 2005; Wang et al., 2005). There is no evidence that Ric-8 interacts with a receptor in receptor-dependent activation of Ric-8, although this possibility has not been directly tested.
we used immunoprecipitation to test the hypothesis that Ric-8 and Cta directly interact. A disadvantage of this strategy is that antibodies against Cta have not been published and our attempts to develop them were unsuccessful. However, we found that Myc-Cta is functional and able to restore Fog sensitivity to S2R+ cells depleted of endogenous Cta by RNAi (Supplemental Figure S2, A and B); thus we used this construct as a proxy for endogenous protein. We transfected Myc-Cta into S2 cells and immunoprecipitated with an anti-Myc monoclonal antibody, and found that endogenous Ric-8 coprecipitated (Figure 3A). As expected from our rescue experiments, Ric-8–GFP also coimmunoprecipitated with Myc-Cta (Figure 3B). Thus, Ric-8 and Cta are able to interact in Drosophila tissue culture cells.

Given that Ric-8 functions as a GEF for Goα subunits in other systems, we wanted to test the hypothesis that Ric-8 exhibits a preferred interaction with GTP-free Cta. To do this, we used an inactive version of Cta, Cta\(\text{GA}\), Cta\(\text{GA}\) fails to rescue the contractility effects of S2R+ cells depleted of endogenous Cta (Supplemental Figure S2B). To determine whether nucleotide association affected Ric-8 interaction, we cotransfected Ric-8–GFP together with wild-type Myc-Cta, Myc-Cta\(\text{QL}\), or Myc-Cta\(\text{GA}\) into S2 cells. We prepared lysates from transfected cultures, immunoprecipitated GFP, and compared the amount of Myc-Cta in each sample by quantitative immunoblot. We found that Ric-8 binding to Cta is dependent on the nucleotide state of Cta, as pull-downs performed with constitutively inactive Myc-Cta\(\text{GA}\) and constitutively active Myc-Cta\(\text{QL}\) showed greater and lesser binding affinity to Ric-8, respectively, than wild-type Myc-Cta (Figure 3, B and C). These data indicate that Ric-8 discriminates between Cta nucleotide states and preferentially binds to inactive Cta.

**Ric-8 acts to selectively localize nucleotide-free Cta within the cell**

Ric-8 is necessary to localize Cta and Goα to the cortex in Drosophila embryonic epithelium (David et al., 2005; Hampoelz et al., 2005; Wang et al., 2005; Kanesaki et al., 2013). In S2 cells, Myc-Cta normally localizes to the plasma membrane and exhibits enrichment in membrane ruffles. On Ric-8 depletion, we saw a reduction of Cta at the plasma membrane and an absence in ruffles (Supplemental Figure S3). We therefore wanted to test the hypothesis that Ric-8 functions to localize Cta and determine whether its nucleotide state plays a role in this interaction. Our strategy was to coexpress Myc-Cta along with a version of Ric-8–GFP that was mistargeted to mitochondria by tagging it with residues 310–338 of Listeria ActA (mitochondrially tagged [Mito]-Ric-8–GFP). We expressed Mito-Ric-8–GFP in S2 cells and showed that it localized to the mitochondria (Supplemental Figure S4). When wild-type Myc-Cta or Myc-Cta\(\text{QL}\) was coexpressed with Mito-Ric-8–GFP, neither Cta construct exhibited discrete localization (Figure 4, A and B). However, coexpression of Myc-Cta\(\text{GA}\) and Mito-Ric-8–GFP resulted in robust accumulation of Cta to the mitochondria (Figure 4, A and B). These data indicate that Ric-8 acts to selectively localize inactive Cta within the cell.

Our results suggest Ric-8 could act to localize Cta to the cell cortex to mediate Fog signaling. To test this model, we transfected S2R+ cells depleted of endogenous Ric-8 with either Ric-8–GFP or Mito-Ric-8–GFP and scored for the ability of each construct to rescue contractility. Ric-8–GFP restored the normal constriction of Ric-8 depleted cells; however, cells expressing Mito-Ric-8–GFP exhibited a significantly diminished response to Fog (Figure 4C). These results suggest that either Ric-8 needs to be cytoplasmic to function correctly or the Mito-Ric-8 is sequestering endogenous Cta to the mitochondria.

**Ric-8 directly binds Cta and exhibits higher affinity for the inactive form of Cta**

Given the likelihood that Ric-8 interacts with the Goα in our system, we used immunoprecipitation to test the hypothesis that Ric-8 and Cta interact. A disadvantage of this strategy is that antibodies against Cta have not been published and our attempts to develop them were unsuccessful. However, we found that Myc-Cta is functional and able to restore Fog sensitivity to S2R+ cells depleted of endogenous Cta by RNAi (Supplemental Figure S2, A and B); thus we used this construct as a proxy for endogenous protein. We transfected Myc-Cta into S2 cells and immunoprecipitated with an anti-Myc monoclonal antibody, and found that endogenous Ric-8 coprecipitated (Figure 3A). As expected from our rescue experiments, Ric-8–GFP also coimmunoprecipitated with Myc-Cta (Figure 3B). Thus, Ric-8 and Cta are able to interact in Drosophila tissue culture cells.

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FIGURE 4: Ectopic localization of Ric-8 drives mislocalization of constitutively inactive Cta and attenuates the efficacy of Fog signaling. (A) Targeting Ric-8 to the mitochondria causes CtaGA but not Myc-Cta or Myc-CtaQL to localize to the mitochondria. S2 cells were transfected with Ric-8-GFP, or Mito-Ric-8-GFP, and Myc-Cta, Myc-CtaGA, or Myc-CtaQL and stained for anti-Myc and anti-GFP. Insets, enlarged views of boxed images in Mito-Ric-8-GFP and Cta variants. Scale bar, 20 μm. (B) Colocalization of Mito-Ric-8-GFP/Cta is greater with inactive Cta than with wild-type or constitutively active Cta. Colocalization index was determined using the Manders coefficient (the overlap of Concertina with Ric-8-Mito–GFP; ±SEM; error bars, p < 0.05). (C) Mislocalized Ric-8 fails to compensate to drive Fog-induced cellular constriction. S2R+ cells depleted of endogenous Ric-8 transfected with GFP, Ric-8-GFP, and Mito-Ric-8-GFP were treated with Fog and scored for their ability to constrict (±SEM; error bars, p < 0.05). (D) Evolutionarily conserved residues contribute to localization of CtaGA. Mito-tagged versions of Ric-8-GFP mutants that exhibit decreased Myc-CtaGA binding (Figure 5, B and C) were coexpressed with Myc-CtaGA. Whereas mutants 9, 10, and 13 colocalized with Myc-CtaGA, mutant 1 did not. Insets, enlarged images of boxed areas. Scale bar, 20 μm.
Ric-8 binds to Cta through an interface of conserved residues

Although previous work provided insight into the structure of Ric-8, a rigorous investigation of specific residues important for interactions with Gα has not been performed. Ric-8 is predicted to be composed of 10 Armadillo repeats (Figuerroa et al., 2009). Armadillo repeats adhere to a canonical fold and global elongated structure (Coates, 2003). The Olate group recently used molecular modeling to construct an in silico model of the Ric-8 structure (Figuerroa et al., 2009). On the basis of sequence conservation of Ric-8 across species (Supplemental Figure S5), we made 14 cluster mutations in Ric-8–GFP, targeting conserved electrostatic residues likely to be surface exposed and that were exposed in the Ric-8 model (Table 1 and Figure 5A). These mutations consisted of charge reversals, with the intent not only to diminish, but also to repel an interaction with Cta. We coexpressed the Ric-8–GFP mutants with Myc-Cta, Myc-CtaQL, or Myc-CtaGA in S2 cells and assessed their ability to interact. Several of our Ric-8–GFP mutant constructs exhibited altered affinities for Cta and are described later. The mutants span the length of the protein and are ordered in succession from N-terminus to C-terminus. Although Ric-8–GFP robustly bound Myc-CtaGA, it exhibited lower-affinity interactions with Myc-Cta and Myc-CtaQL (Figure 2, B and C). Owing to this weaker binding, Ric-8–GFP pull-down experiments performed with either Myc-Cta or Myc-CtaQL displayed a high degree of variance, making it difficult to determine interaction strengths between these molecules. Therefore we focused our analyses on Ric-8–GFP and Myc-CtaGA.

We identified four Ric-8–GFP mutants with clustered point mutations (1, 9, 10, and 13) that had significantly reduced binding to Myc-CtaGA (Figure 5, B and C, and Table 1). To further parse out the individual residues responsible for this interaction, we made single point mutants for each cluster of more than one mutated amino acid (Supplemental Figure S6 and Table 1). Mutant 10 is a singular mutation, so it was not tested again. We identified specific residues within mutants 1, 9, and 13 that attenuated the ability of Cta to bind Ric-8 (Figure 6, A and B, and Table 1). Although not statistically significant, both Myc-Cta and Myc-CtaQL variants exhibited decreased binding to mutant 1 and moderate to high binding to mutants 9, 10, and 13 (Supplemental Figure S7, A–D). The fact that mutants 9, 10, and 13 inhibited binding to Myc-CtaGA but did not strongly affect Myc-Cta or Myc-CtaQL binding indicates that the C-terminal region of Ric-8 may be important for high-affinity binding seen specifically in the Ric-8/GTP–free Cta interaction, whereas the N-terminal residues found in mutant 1 could be important for global Ric-8 association and function.

We next tested the hypothesis that the residues mediating Ric-8/Cta interactions are required for Fog signaling. We depleted endogenous Ric-8 from S2R+ cells and transfected the cells with the clustered and individual point mutant variants of Ric-8-GFP. We then...

| Ric-8 mutation | Binding assay (with Cta<sub>GA</sub>) | Cellular constriction assay | Ric-8 mutation | Binding assay (with Cta<sub>GA</sub>) | Cellular constriction assay |
|----------------|--------------------------------------|---------------------------|----------------|--------------------------------------|---------------------------|
| Mutant 1       | ++++                                 | +++                       | Mutant 1       | R401E                               | N/A                       |
| R71E           | ++                                   | ++                        | Mutant 2       | D402K                               | N/A                       |
| R75E           | ++                                   | +++                       | Mutant 3       | E408K                               | N/A                       |
| D76E           | -                                     | -                         | Mutant 4       | E414E                               | +++                       |
| Mutant 2       | ++                                   | -                         | Mutant 5       | T485A                               | +++                       |
| E134K          | N/A                                  | N/A                       | Mutant 6       | N415A                               | +                         |
| K137E          | N/A                                  | N/A                       | Mutant 7       | E486K                               | +                         |
| D180K          | N/A                                  | N/A                       | Mutant 8       | E487K                               | +                         |
| K182E          | N/A                                  | N/A                       | Mutant 9       | E488K                               | +++                       |
| Mutant 3       | -                                     | -                         | Mutant 10      | E491K                               | +++                       |
| Mutant 4       | -                                     | -                         | Mutant 11      | E492K                               | +                         |
| E231K          | N/A                                  | N/A                       | Mutant 12      | E493K                               | ++                        |
| K234E          | N/A                                  | N/A                       | Mutant 13      | E494K                               | +++                       |
| Mutant 5       | -                                     | -                         | Mutant 14      | E495K                               | +++                       |
| N285A          | N/A                                  | N/A                       | Mutant 15      | E496K                               | +++                       |
| N289A          | N/A                                  | N/A                       | Mutant 16      | E497K                               | +++                       |
| Mutant 6       | -                                     | +++                       | Mutant 17      | E498K                               | +++                       |
| R383E          | N/A                                  | N/A                       | Mutant 18      | E499K                               | +++                       |
| R386E          | N/A                                  | N/A                       | Mutant 19      | E500K                               | +++                       |
| R389E          | N/A                                  | N/A                       | Mutant 20      | E501K                               | +++                       |
| Mutant 7       | -                                     | +++                       | Mutant 21      | E502K                               | +++                       |

Summary of data collected from Ric-8/Cta<sub>GA</sub> binding and contractile assays. The individual point mutants comprising each cluster are denoted below the clustered point mutants. Individual point mutants tested experimentally have binding and contractility scores; the remainder were untested (N/A). The effect of each mutant in the pathway is measured in strength from (–) no effect in assay, (+) weak effect, (++), moderate effect (+++), to very strong effect (++++). For the binding assay a mutant with a very strong effect (++++) showed little to no binding in pull-down experiments, while mutants with no effect (–) were capable of robustly binding CtaGA. In the contractility assay mutants with no effect (–) rescued contractility to wild-type levels, while mutants with a strong effect (++++) dramatically affected the ability of cells to constrict in response to Fog. Mutants that had strong effects both in binding and contractility assays are highlighted in yellow.

**TABLE 1:** Cluster mutations used in Ric-8 binding and Fog-induced cellular contractility assays.
pattern in the residues that prevented pathway activation to the individual residues deficient in binding Cta in the pull-down assay (Figure 6, A and B, and Table 1). This suggests that residues R71, R75, R414, D484, T485, and E487 within Ric-8 act to establish a binding interface with Cta and are important for successful G protein signaling downstream of Fog.

To determine whether Ric-8 mutants with low Cta-binding affinity affected Cta localization, we made Mito-tagged versions of cluster mutants 1, 9, 10, and 13. We transfected S2 cells with the Mito-Ric-8–GFP cluster mutants and Myc-CtaGA and screened for colocalization of the two proteins. Myc-CtaGA did not colocalize with Mito-Ric-8–GFP mutant 1, whereas, surprisingly, colocalization of Myc-CtaGA was seen with Mito-Ric-8–GFP mutants 9, 10, and 13 (Figure 4D). Hypothetically, mutants 9, 10, and 13 could be affecting the binding kinetics of Myc-CtaGA, which may account for the colocalization of the two in our mistargeting assays, as well as the absence of interaction within the pull-down assay.

Mutants 1, 9, and 13 had low binding affinity to CtaGA, as well as decreased contractility in Fog-treated S2R+ cells; however, mutant 10 rescues contractility to near-wild-type levels (Figure 5, B and C, and Table 1). It is possible that mutant 10, while impeding the binding interface between Ric-8 and Cta, is sufficient in its interaction to function in pathway activation. Intriguingly, mutants 6–8 are capable of binding Cta, as shown in pull-down assays (Figure 5, B and C, and Table 1) but have diminished ability in activating the pathway (Figure 5C and Table 1), suggesting that these residues may have an important functional role outside of binding.

The majority of the residues that affected both binding and Fog-induced contraction rescue map to a conserved face of the Arm repeats and show a potential clamp-like binding of Ric-8 to Cta (Supplemental Figure S6). Our findings suggest that residues within the inner face of the N-terminus (R71 and R75) of Ric-8 may act to facilitate global interaction with Cta, whereas residues found in the C-terminus (R414, D484, T485, and E487) modulate binding based on nucleotide specificity. Whereas previous work found residues important in Gα subunits for facilitating interaction with Ric-8 (Thomas et al., 2011), here we identify some of the first residues found to be important for Ric-8 binding to a Gα.

**DISCUSSION**

We established a novel assay for testing potential Fog pathway components and found that in Drosophila tissue culture Ric-8 is
FIGURE 6: Individual residues derived from Ric-8 cluster mutants comprise key interaction sites for Cta binding and function. (A) Individual Ric-8 point mutants from cluster mutants (1, 9, and 13) negatively regulate binding to Myc-CtaGA. Cells were transfected with GFP, Ric-8–GFP, or individual Ric-8–GFP point mutants and CtaGA. IPs were performed with GFP-binding protein and probed with anti-GFP and anti-Myc. (B) Quantification of the IP experiments in A (black bars). The pull-down/input ratios were determined using quantitative densitometry and normalized to Ric-8–GFP (±SEM; error bars, p < 0.05). S2R+ cells were depleted of endogenous Ric-8, transfected with Ric-8–GFP or individual Ric-8–GFP point mutants, and scored for the percentage of transfected cells constricting within the population (±SEM; hatched bars). Dashed lines indicate where two separate gels have been combined. (C) Proposed model for Ric-8 function within the Fog signaling pathway. Ric-8 initially acts to chaperone the folding of Cta and is released before Cta association with Gαi13F and γ1. The heterotrimer is targeted to the plasma membrane, where it interacts with a GPCR for Fog. Fog binding activates Cta through exchange of GDP for GTP. Cta-GTP activates RhoGEF2, and RhoGEF2’s GAP activity catalyzes hydrolysis of GTP to GDP. Ric-8 may then either bind Cta-GDP or stabilize a nucleotide-free version of Cta. Ric-8 then localizes the inactive Cta for reactivation and reinsertion into the Fog signaling pathway. Ric-8 initially acts to chaperone the folding of Cta and is released before Cta association with Gαi13F and γ1. The heterotrimer is targeted to the plasma membrane, where it interacts with a GPCR for Fog. Fog binding activates Cta through exchange of GDP for GTP. Cta-GTP activates RhoGEF2, and RhoGEF2’s GAP activity catalyzes hydrolysis of GTP to GDP. Ric-8 may then either bind Cta-GDP or stabilize a nucleotide-free version of Cta. Ric-8 then localizes the inactive Cta for reactivation and reinsertion into the Fog signaling pathway, either by returning it to the heterotrimer to be reactivated by the GPCR (A) or through disassociation of GDP, which facilitates GTP binding, and subsequent pathway reinsertion directly upstream of RhoGEF2 (B).
levels of Cta are not dramatically affected in the absence of Ric-8 (Figure 2E); in addition, we see some rescue in cells depleted of endogenous Ric-8, overexpressing constitutively active Cta (Figure 2C), indicating that at least a small amount of Cta is localized correctly and functional. Therefore, whereas plasma membrane levels of Cta are affected by Ric-8 (Kanesaki et al., 2013; present work), overall levels of protein are not (Figure 2E). One possibility, given that constitutively active Cta was still able to rescue, is that Ric-8 could be important for Gα cycling at the site of receptor activation, which is believed important for spatial regulation of Gα signaling (Ross, 2008).

Although signaling nodes involving GPCRs, Gα subunits, and Ric-8 have been extensively studied, little is known about the structure of Ric-8 and how it interacts with Gα. We used a predicted model (Figueroa et al., 2009) of Ric-8 as a conceptual basis to visualize mutants and identify key conserved residues important for Cta binding, nucleotide specificity, and execution of productive G protein pathway activation. Based on these data, our structure-function assay of Ric-8 identified four cluster mutations (1, 9, 10, and 13; Supplemental Figure S6) that inhibits CtaGA binding, of which three (1, 9, and 13) also failed to rescue Fog-induced constriction to wild-type levels. Of these four mutants, we found that only mutant 1 (in the N-terminus of Ric-8) had an inhibitory effect on binding to wild-type, constitutively active, and constitutively inactive Cta, whereas mutants 9, 10, and 13 (in the C-terminus of Ric-8) were deficient only in binding inactive Cta. The Itoh lab found that a truncated version consisting of the N-terminal half (residues 1–301) of Ric-8 was sufficient to bind Gαq (Nishimura et al., 2006). In accordance with these data, we suggest that residues in mutant 1 are important for non–nucleotide-specific Cta interaction, whereas residues in mutants 9, 10, and 13 confer nucleotide-specific recognition of Cta. This study presents the first evidence of specific residues within Ric-8 facilitating interaction with Gαq.

Several mutants had effects in only the binding or contractile assays. Mutant 10 inhibited binding, whereas mutants 6–8 prevented Fog-induced constriction. Mutant 10 was able to modestly rescue cellular constriction but exhibited decreased binding to Cta, implying that this mutant is still functional but perhaps folded in a manner unproductive for robust binding to Cta; this may be due to its proximity to its ectopic location at the mitochondria, whereas mutants 9, 10, and 13 confer nucleotide-specific recognition of Cta. This study presents the first evidence of specific residues within Ric-8 facilitating interaction with Gαq.

On the basis of our characterization of Ric-8 and data from the literature, we propose the following model (Figure 6C). Ric-8 acts to initially chaperone the folding of Cta, allowing Cta, Gβ13F, and Gγ1 to form a complex that is then transported to the plasma membrane (Gabay et al., 2011; Kanesaki et al., 2013). On Fog/GPCR interaction, GTP-bound Cta is released from the Gβγ heterodimer and interacts with RhoGEF2 (via its RGS domain), causing hydrolysis of GTP to GDP. Specific, evolutionarily conserved residues regulate the binding of GDP-bound Cta to Ric-8, or, alternatively, Ric-8 stabilizes a nucleotide-free version of Cta. This allows Cta to bypass destruction and be reinserted into the Fog pathway to activate downstream targets.

### MATERIALS AND METHODS

**Tissue culture, transfection, and RNAi**

S2 and S2R+ cell lines were obtained from the Drosophila Genome Resource Center (Bloomington, IL), and propagated as previously described (Rogers and Rogers, 2008). S2 cells were maintained in SF900 SFM (Invitrogen, Carlsbad, CA) and S2R+ cells in Shields and Sang medium (Invitrogen) supplemented with 5% heat-inactivated fetal bovine serum (Invitrogen). S2 and S2R+ cells were transfected with 2 μg/μl DNA using the Amaza Nucleofector system with Kit V using program G-30 (Lonza, Basel, Switzerland), or with FuGENE HD (Promega, Madison, WI; except the Mito-tag constructs, for which 1 μg/μl DNA was used). For individual RNAi treatments, cells at 75–90% confluency in 6- or 12-well plates were treated every other day for at least 10 d with 15 μg/ml dsRNA. dsRNAs were produced using Promega Ribomax T7 kit according to instructions. Primers used for dsRNA synthesis are as follows, with all preceded by the T7 sequence (5′-TAATACGACTCACTATAGG-3′). Control, forward (fwd), 5′-TAATATTGAACTTATATTATGTTGTTGTCACCGCCGATATAGGTCAC-3′, and Control, reverse (rev), 5′-ATTCGATATACGCTATCGATGTCCACCTATTATTAC-3′, to amplify a region from the pBluescript plasmid; Cta, fwd, 5′-TGCTGGGTCGCTGCTCTCGCCTAGG-3′; ric-8, rev, 5′-CAGCGCAGCCGCTGAGGATGGG-3′; and ric-8, rev, 5′-CTCGGCTGCTGCTGCTGAGGATGGG-3′.

In the early dividing C. elegans embryo (Afshar et al., 2005), D. melanogaster neuroblasts and epithelium (David et al., 2005; Hampoelz et al., 2005; Wang et al., 2005; Kanesaki et al., 2013), and several mammalian tissue culture cell lines (Woodard et al., 2010; Gabay et al., 2011; Wang et al., 2011) Ric-8 localizes Gαq subunits to the plasma membrane. Our data suggest that there is an additional level regulating Gαq localization that is dependent on the nucleotide-bound state of Gαq. We identified a cluster of residues that may facilitate this interaction with Cta. Clustered Ric-8 mutants, deficient in binding CtaGA in immunoprecipitation assays, when tagged with a sequence directing them to the mitochondria had varying effects in their ability to ectopically localize CtaGA. Mito-Ric-8 mutant 1 did not recruit CtaGA to its ectopic location at the mitochondria, whereas Mito-Ric-8 mutants 9, 10, and 13 triggered mitochondrial mislocalization of CtaGA (Figure 4D). Of interest, mutants 9, 10, and 13 exhibited decreased binding to constitutively inactive Cta, CtaGA, but not to wild-type or constitutively active Cta, CtaGA (Figure 5, B and C, and Supplemental Figure S7). This implies that these residues may confer temporally regulated nucleotide specific recognition sites for Cta.

**Contractility assay**

S2R+ cells were resuspended and plated on concanavalin A (MP Biomedicals, Solon, OH)–coated coverslips, allowed to spread for 1–3 h, and then treated for 10 min with concentrated Fog-conditioned medium or medium harvested from S2 cells. To produce Fog-conditioned medium, we created a stable S2 cell line carrying a Fog-Myc expression construct driven by an inducible metallothionein promoter. Fog-stable S2 cells were grown to 75–90% confluency in T150 flasks before SF-900 media was exchanged for Schneider’s media (Invitrogen) and induced with 1 mM CuSO4 for 48 h. Cells were then pelleted, and the supernatant was concentrated using protein concentrators (Millipore, Billerica, MA) to ~2.5–5% of the original volume. For control media, the same process was applied to non-Fog-expressing S2 cells. Control (S2) and Fog-concentrated media were diluted 1:1 with Schneider’s media before application. For each experiment, we
scoring the number of cells within a population that contracted in response to Fog treatment, repeating each condition at least three times, and counted ≥500 cells. Error bars were calculated using SE.

Molecular biology
The Fog-Myc expression construct was generated using PCR to amplify the coding sequence of the gene and introduce a 5′ EcoRI site, a C-terminal Myc tag, and a 3′ NotI site to allow cloning into pMT-5V/His (Invitrogen). Construction of N-terminally Myc-tagged Cta and C-terminally GFP-tagged RhoGEF2 constructs was described previously (Rogers et al., 2004). The dual-expression constructs were created by subcloning Myc-Cta constructs into pMT-5V-histidine (Hist) containing a second transcriptional unit for membrane-mCherry marker containing the sgr promoter and 3′ UTR (Martin et al., 2010). To generate the expression construct for constitutively active Rho kinase, we used PCR to amplify the catalytic domain (amino acids 1–506) from a cDNA (expressed sequence tag clone LD15203) and introduced a 5′ EcoRI site and a 3′ NotI site and incorporated the Myc epitope tag at the 5′ end of the coding sequence. This insert was then subcloned into pMT-A for inducible expression. Full-length Ric-8a cDNA was subcloned using the Gateway TopoD pEentr system (Invitrogen) into a final Zeocin-selectable pIZ backbone that has a metallothionein promoter, Gateway (Invitrogen) LR recombination sites in the multiple cloning site, and a C-terminal enhanced GFP tag. All mutagenesis was preformed on this construct using KOD Xtra Hot Start Polymerase (Novagen, Gibbstown, NJ). Mitochondrial localization of Ric-8 was achieved by N-terminally attaching Listeria monocytogenes ActA residues 310–338 (Pistor et al., 1994).

Immunoprecipitation and immunoblotting
We bacterially expressed a His- and Fc-tagged GFP-binding protein (Fc-GFP-BP). The Fc-GFP-BP was first purified on a Ni column, and the eluted Fc-GFP-BP fractions were incubated with protein A beads. GFP-binding protein was covalently linked to the beads using 20 mM dimethylpimelimidate (Sigma-Aldrich, St. Louis, MO). Before use in immunoprecipitation (IP) experiments, beads were washed with IP lysis buffer (50 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100, 2.5 mM phenylmethysulfonyl fluoride, and Complete EDTA-Free Protease Inhibitor Cocktail [Roche, Indianapolis, IN]).

S2 cells used for IPs were transfected as described and induced 24 h later with 1 mM CuSO4. The next day cells were resuspended, pelleted, and washed before lysing with IP lysis buffer. Samples were removed for input controls, and the rest of the sample was incubated with GFP-binding-protein beads. Samples were resuspended in SDS–PAGE sample buffer and boiled for 10 min. SDS–PAGE sample buffer was also added to input samples and boiled for 10 min. Samples were run on SDS–PAGE gels and transferred to nitrocellulose membranes for Western blotting using anti-Myc9e10 (Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA) and anti-GFPJL8 (Clontech, Mountain View, CA). For immunoblot quantitation the pull-down input ratios were determined using densitometry with ImageJ software (National Institutes of Health, Bethesda, MD) on scanned film images. All immunoblot quantitation was performed at least three times on three distinct blots. Error bars represent SEM. Asterisks in Figures 3, B and C, SC, and 6B and Supplemental Figure S7B indicate p < 0.05 determined using Student’s t test.

Microscopy
Cells were plated onto ConA-coated coverslips and prepared for imaging as previously described (Rogers and Rogers, 2008), differing only in the addition of 0.08% formic acid to the fixation solution of cells stained with the anti-ATP synthase α subunit antibody. Antibodies used for immunofluorescence were as follows: anti-phospho-myosin light chain 2 (Ser-19; Cell Signaling Technology, Danvers, MA), anti-Ric-8 (gift from William Chia, National University of Singapore), anti-Fog (gift from Eric Wieschaus, Princeton University), anti-dsRed (Clontech), anti-GFPJL8 (Clontech), anti-Myc9e10 (DSHB), anti–ATP synthase α subunit (MitoSciences, Eugene, OR), and anti-DM1α and Alexa Fluor 564–phalloidin (Invitrogen). All cells were imaged using a CoolSnap HQ charge-coupled device camera (Roper Scientific, Tucson, AZ) mounted on an Eclipse Ti-E and driven by Nikon Elements software (Nikon, Melville, NY), except cells in Figure 4, which were imaged using a total internal reflection fluorescence system (Nikon) mounted on an inverted IX81 microscope using an Andor Clara Interline camera (Andor Technology, Belfast, UK) and driven by Nikon Elements software, and cells in Supplemental Figure S3, which were imaged using an inverted IX81 (Olympus, Tokyo, Japan) with a CoolSnap ES camera (Photometrics, Tucson, AZ) driven by MetaMorph software (Molecular Devices, Sunnyvale, California). Colocalization index was determined using the Manders coefficient powered by the ImageJ plug-in JACoP (for more information see Bolte and Cordelières, 2006). Photoshop CS3 (Adobe, San Jose, CA) was used to adjust input levels so that the main range of signals spanned the entire output grayscale and to adjust brightness and contrast.

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3470 | K. A. Peters and S. L. Rogers
Molecular Biology of the Cell
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