The role of canonical and non-canonical regulators of heterotrimeric G protein signaling during *Drosophila melanogaster* morphogenesis

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Abstract

KIMBERLY ANN PETERS: The role of canonical and non-canonical regulators of heterotrimeric G protein signaling during Drosophila melanogaster morphogenesis (Under the direction of Dr. Stephen Rogers)

Morphogenesis of multicellular organisms requires precise regulation of cell movements and cell shape changes. The first morphogenetic movement to occur in Drosophila melanogaster embryogenesis is ventral furrow formation during gastrulation; wherein a subset of presumptive mesodermal cells undergoes cytoskeletal rearrangements to invaginate into the embryo. This process is regulated by a secreted ligand, Folded gastrulation (Fog), that binds a G-protein-coupled receptor (GPCR), which activates Concertina (Cta), a Ga12/13 protein, and triggers a signaling cascade resulting in contraction of the apical actomyosin network to drive cell shape changes leading to internalization of the ventral furrow.

We used an RNAi screen in Drosophila tissue culture cells targeting all known and putative GPCRs to identify a receptor required for Fog signaling which we named Mist (Mesoderm Invagination Signal Transducer). We determined that Mist is an essential component of the Fog signaling pathway, and is sufficient to mediate Fog susceptibility in otherwise Fog unresponsive cells. I further identified specific domains within the receptor involved in signal transduction. Mist loss-of-function in the Drosophila melanogaster embryo revealed a role for Mist in gastrulation, and exhibited defects similar to Fog mutants. Ultimately our examination of Mist function within cellular morphogenesis has defined a role
for Mist from its transcriptional regulation, to its function within developmental processes.

I also investigated the role for Ric-8, a conserved cytoplasmic protein, in Cta function. In *Drosophila* tissue culture I used RNAi to show that Ric-8 is necessary for Cta-induced cellular constriction triggered by ectopic Fog application. Biochemical analyses and molecular mis-targeting demonstrated that Ric-8 directly binds to- and localizes Cta, with a much higher affinity for constitutively inactive Cta. Further, I found that Ric-8 modulation directly impacts productive Fog signaling. Finally, by mutagenizing amino acids conserved across species I identified specific residues within Ric-8 required for Cta function and/or establishing a binding interface between the two molecules. These two projects converge to further our understanding of the regulation of Gα signaling during gastrulation events by two distinct mechanisms, canonical GPCR activation via Mist signaling and non-canonical modulation of Cta, by the highly conserved cytosolic protein Ric-8.
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List of Abbreviations and Symbols

Abl: Abelson kinase
Arr: Arrestin
*C. elegans*: Caenorhabditis elegans
ConA: Concanavalin A
Cta: Concertina
Ctp: Cut-up
*Drosophila*: Drosophila melanogaster
*E. coli*: Escherichia coli
Fog: Folded gastrulation
Gα: G alpha
Gβ: G beta
Gγ: G gamma
GAP: GTPase activating protein
GDP: Guanosine diphosphate
GEF: Guanine nucleotide exchange factor
GDI: Guanine nucleotide dissociation inhibitor
GPCR: G protein coupled receptor
GPR: G protein regulator
GPRK/GRK: G protein-coupled receptor kinase
GTP: Guanosine triphosphate
IP: Immunoprecipitation
Mist: Mesoderm invagination signal transducer
Mud: Mushroom body defects
MWCO: Molecular weight cut-off
Numa: Nuclear mitotic apparatus
OE: overexpression/overexpressing
P-RLC: Phosphorylated regulatory light chain
PMG: Posterior midgut
Pins: Partner of Inscuteable
PDZ: Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (Zo-1)
RLC: Regulatory light chain
RGS: Regulator of G protein signaling
Rok: Rho kinase
S2R+: S2 Receptors +
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP: Sensory organ precursor cell
VF: Ventral furrow
Wg: Wingless
Chapter I

Introduction

The rearrangement of tissues during development is a fundamentally important process for embryonic viability. One such mechanism of cellular repositioning occurs via apical constriction: wherein a defined set of cells constricts their apical membranes, driving internalization of the cells from an external sheet to an internal tube. Many organisms utilize apical constriction to move and rearrange cells during morphogenetic movements.

*Caenorhabditis elegans* (*C. elegans*) utilizes apical constriction to internalize endoderm; *Drosophila melanogaster* uses apical constriction to internalize mesoderm and endoderm as well as to form various structures, such as the trachea and salivary glands\(^1\); in mammals apical constriction is used during neurulation to drive the invagination of the neural tube\(^2\).

These examples of morphological events involving apical constriction in varying organisms highlight the ubiquitous nature of this mechanical shape change to drive cellular rearrangement. Interestingly, many of the previously described cellular movements employ similar signaling components.

**The Fog signaling pathway regulates early gastrulation movements in the *Drosophila melanogaster* embryo**

During the developmental process of gastrulation cells receive instructive inputs to determine their cell fate, and then rearrange to the appropriate positions in the embryo to
establish distinctive germ layers. The first morphogenetic movements that occur in *Drosophila* begin immediately after cellularization with the formation of the ventral furrow. A subset of presumptive mesodermal cells on the ventral side of the embryo constricts their apices to invaginate, as a tube, into the interior of the embryo\(^3\) (Figure 1.1).

These cells express the transcription factors Twist and Snail, which confer mesodermal identity\(^4\). Twist and Snail are essential for mesoderm invagination, as they regulate the transcription of gene products that provide signaling and structural components important for coordinated constriction\(^5,6\). Twist drives transcription of the secreted protein Fog in the ventral furrow\(^7\). Fog is an apically secreted protein\(^7,8\), that binds to a putative G-protein coupled receptor (GPCR), to activate the Ga12/13 family member, Concertina (Cta)\(^9,10\). Cta activates the guanine nucleotide exchange factor RhoGEF2\(^11,12\), which will stimulate the release of GDP from the small GTPase Rho, allowing Rho to bind GTP and be activated. The Rho effector, Rho Kinase (Rok), subsequently phosphorylates and activates non-muscle myosin II, while inhibiting myosin II phosphatase activity, allowing myosin II to bind F-actin and drive apical constriction in the ventral furrow\(^8\) (Figure 1.2).

The invagination of the ventral furrow is tightly regulated both spatially and temporally. In *Drosophila* embryos mutant for Fog or Cta, coordinated apical constriction is disrupted leading to a disorganization of cell shape changes and a delay in ventral furrow formation\(^7,10\). In embryos mutant for RhoGEF2 or Rok, unlike Fog or Cta mutants, the cells that make up the ventral furrow lose their basal myosin, but never accumulate apical myosin, and therefore are unable to complete ventral furrow formation, leaving the mesodermal precursors on the outside of the embryo\(^8\). The effect of Fog or Cta mutations are much weaker than the mutant phenotypes of RhoGEF2 or Rok in the ventral furrow, indicating
there are additional inputs in the Fog signaling pathway that are important for coordinated constriction. One of these additional inputs is the non-receptor tyrosine kinase, Abelson kinase (Abl), which has been shown to be important for organizing the actin cytoskeleton at the apical domains of ventral furrow cells; Abl mutants exhibit uncoordinated cell constriction and delayed ventral furrow formation\textsuperscript{13}. A transmembrane protein, T48, was also found to be involved in successful apical constriction during ventral furrow formation. This protein contains an -ITTEL sequence that binds the PDZ domain of RhoGEF2 to anchor RhoGEF2 at the membrane upon pathway activation. In \textit{Cta} or \textit{T48} mutants RhoGEF2 is still localized weakly at the apical membrane of cells in the ventral furrow. However, when \textit{Cta} and \textit{T48} gene expression is depleted in tandem, RhoGEF2 expression is completely abolished in ventral furrow cells\textsuperscript{5}. Despite the significant breadth of information describing this pathway, questions still remain. The most obvious of which, being the identity of the receptor that transduces the Fog signal. As the \textit{Gα}_{12/13}, \textit{Cta}, has been found to act genetically downstream of Fog\textsuperscript{9} it is likely that the unidentified receptor is a member of the G-protein coupled receptor (GPCR) family of proteins.

\textbf{Canonical activation of Ga proteins is driven by G protein coupled receptors}

GPCRs are a diverse class of receptors, comprising one of the largest groups of encoded genes in the human genome and a frequent drug target studied in pharmacological sciences\textsuperscript{14}. GPCRs relay external signals received by the cell to produce tightly regulated intracellular activation of signaling cascades. GPCRs are composed of 7-α helical domains spanning the membrane, with an extracellular N-terminus and an intracellular C-terminus. Agonist binding to the extracellular domain of GPCRs cause a conformational change in the
cytoplasmic domains of the receptor. This conformational change allows the GPCR to activate its associated Gα subunit by facilitating its release of GDP for GTP\textsuperscript{15}. GPCR signaling is compounded by the fact that GPCRs are capable of binding several ligands and G proteins, and G proteins in turn are able to interact with numerous receptors and intracellular effectors, allowing for highly complicated signaling networks\textsuperscript{16}.

Gα proteins form a heterotrimeric complex with Gβ and Gγ subunits. The stability of these proteins is dependent on this complex, as the formation of the Gαβγ heterotrimer is necessary for exit from the ER. Post-translational isoprenylation of the Gγ subunit and fatty acylation of the Gα subunits targets the complex to the plasma membrane, where Gαβγ can interact with GPCRs and downstream effectors\textsuperscript{17}. Activation of the Gα subunits by a GPCR triggers exchange of GDP for GTP causing disruption of the heterotrimer, allowing both Gα and Gβγ to activate intracellular signaling cascades. Once Gα hydrolyzes GTP to GDP, the inactive heterotrimer reforms\textsuperscript{18}. Gα proteins have slow intrinsic GTPase activity, however RGS (regulator of G protein signaling) domain containing family members, including the Fog pathway component RhoGEF2, act as GAPs (GTPase accelerating protein) for Gα subunits to potentiate hydrolysis of GTP to GDP\textsuperscript{19}.

Gα subunits fall into 4 major classes: Gα\textsubscript{i}, Gα\textsubscript{q}, Gα\textsubscript{s}, Gα\textsubscript{12} based on their sequence homology. The different classes of Gα family members also modulate different sets of effectors. For example Phospholipase-C is activated by Gα\textsubscript{q}, whereas adenyl cyclase is activated by Gα\textsubscript{s} and inhibited by Gα\textsubscript{i}\textsuperscript{16}. The Gα\textsubscript{12} family, which includes the molecules Gα\textsubscript{12} and Gα\textsubscript{13}, has been found to regulate pathways involved in cellular morphogenesis and migration through their downstream effector, the small GTPase Rho. The Gα\textsubscript{12} signaling pathways have been directly linked to cancer formation and other diseases such as leukemia.
and hypertension through their role in regulating cellular morphogenesis and movements\textsuperscript{20}.

**Ric-8 regulates $G\alpha$ protein signaling during development**

Ric-8 was originally identified in a screen for molecules resistant to inhibitors of cholinesterase (Ric) in *C. elegans*, establishing a role for Ric-8 in positively regulating the release of the neurotransmitter acetylcholine\textsuperscript{21}; Ric-8 has since been found to modulate secretion of neurotransmitters in several different model organisms\textsuperscript{22}. Shortly after Ric-8 was identified the same group published a paper showing that Ric-8 positions the centrosome during early asymmetric divisions of the *C. elegans* embryo\textsuperscript{23}. Asymmetric cell division plays an essential role in normal as well as abnormal development. Asymmetric cell division allows a dividing cell to partition its cell fate determinants, allowing daughter cells to inherit molecules, which will establish their identity, and subsequently generate cellular diversity. However, abnormal asymmetric cell division can produce aberrant numbers of cells and cell types, which can subsequently contribute to different cellular proliferative disease states, such as tumorigenesis\textsuperscript{24}. Further research of the role of Ric-8 in asymmetric cell division, in *C. elegans*\textsuperscript{25-28}, *Drosophila*\textsuperscript{29-31}, and mammalian tissue culture\textsuperscript{32} has demonstrated that Ric-8 is an essential component for spindle positioning during asymmetric cell division.

Tall et al., was the first to show that Ric-8 directly interacts with $G\alpha$ subunits using a yeast two-hybrid assay\textsuperscript{33}. Subsequent analysis has shown that the initial biosynthesis of $G\alpha$ subunits relies on Ric-8 as a chaperone\textsuperscript{34,35}, as does localization to the appropriate site within the cell. Ric-8 is essential for targeting $G\alpha$ subunits in *C.elegans* and *Drosophila* to the plasma membrane\textsuperscript{27,29-31}, as well as in mammalian tissue culture\textsuperscript{35}. Finally Ric-8 has been shown to protect and stabilize $G\alpha$ subunits from proteasomal degradation\textsuperscript{36,37}. Ultimately
Ric-8 regulates the behavior of Ga from its inception, to its (near) destruction.

Ric-8 preferentially interacts with GDP-bound Ga, and acts as a GEF (guanine nucleotide exchange factor) to disassociate GDP, forming a stabilized nucleotide-free state, until GTP binding. In mammals there are two genes encoding Ric-8 proteins, Ric-8A and Ric-8B. Ric-8A, has GEF activity for, and interacts with, Ga_i, Ga_q, and Ga_13, while Ric-8B interacts with Ga_s and Ga_q. In invertebrates there is only one functional version of Ric-8, indicating a divergence in evolutionary specificity of Ric-8 for Ga subunits between vertebrates and invertebrates. It will be interesting to see, in future studies, whether invertebrate Ric-8 is capable of interacting with any or all vertebrate Ga family members.

While Ric-8 is capable of binding monomeric Ga-GDP subunits in vitro, Ga-GDP does not exist in a monomeric form within the cell. Ga-GDP subunits are found either complexed with guanine nucleotide dissociation inhibitors (GDIs), that lock the Ga in an inactive, state by preventing its GDP release or within a heterotrimeric complex containing their βγ partners. Ric-8 cannot bind to Ga when it is part of the Gaβγ heterotrimeric complex. However, it has been shown that Ric-8 can bind Ga subunits complexed with GDIs; Ric-8 binding these complexes causes the dissociation of the GDI and release of the Ga. Examples of this process have been described during the asymmetric cell divisions of the one-cell C. elegans embryo, the neuroblast in Drosophila, and in mammalian tissue culture models.

Cell divisions in the C. elegans early embryo produce cells of different sizes due to the increased pulling forces on, and subsequent shift of the spindle to the posterior side of the embryo. Correct spindle positioning requires the Ga_i family members, GOA-1/GPA-16; the GDIs, GPR1/2; the GAP, RGS-7, and the non-canonical GEF, Ric-8. These components
work together in a cyclical process in which binding of Ric-8 to Ga\textsubscript{i} disrupts the GDP-Ga\textsubscript{i}-GPR1/2 complex. Ric-8 is then able to facilitate exchange of GDP for GTP on Ga\textsubscript{i}. To return Ga\textsubscript{i} to its GDP-bound state RGS-7 activates hydrolysis of GTP allowing GPR1/2 to bind again. This cycling is essential for correct spindle positioning\textsuperscript{27,28,39}. The GD\textsubscript{Is}, Loco and Pins (Partner of Inscuteable), \textit{Drosophila} orthologs of GPR1/2, Ric-8 and Ga\textsubscript{i} act analogously during asymmetric division of the \textit{Drosophila} neuroblast; although, in this system Loco possesses an RGS domain, allowing it to act as the GAP for GTP-Ga\textsubscript{i} within this pathway\textsuperscript{41}. In mammalian asymmetrically dividing cells, it has been proposed that a similar cyclical process involving GD\textsubscript{Is}, GEFs, and GAPs regulates spindle pulling forces through association with the microtubule spindle organizer, Numa (nuclear mitotic apparatus). Numa binds both microtubules and the GDI, LGN, but cannot bind both simultaneously. When Ric-8 binds LGN-Ga\textsubscript{i}-Numa, it causes dissociation of the complex, allowing Numa to interact with microtubules, and affect spindle positioning\textsuperscript{32,42}. Insight into how these pulling forces are translated into movement was found in flies, where it has been shown that the Numa homolog, Mud (mushroom body defects) forms a complex with Ctp (cut-up) the light chain of the minus-end directed motor, dynein\textsuperscript{43}.

The crystallographic structure of Ric-8 has not been solved; however, a model has been constructed using data from secondary sequence analysis and circular dichorism experiments with \textit{Xenopus laevis} Ric-8. Based on this data Ric-8 is composed of 10 repeated right-twisted alpha helical domains\textsuperscript{44}. The predicted model of Ric-8 is similar in structure to molecules known to act as scaffolding proteins, such as beta-catenin and alpha-importin\textsuperscript{45}; although, thus far, the only proteins found to directly bind Ric-8 are Ga subunits. It is unclear how Ric-8 and Ga subunits physically interact. However, a truncated version
composed the N-terminal half of mammalian Ric-8 was found to be sufficient for binding $G_{\alpha_q}^{46}$. It has also been shown that the 12 C-terminal residues of $G_{\alpha_{il}}$ are crucial for interacting with Ric-8A$^{34}$. While there is some evidence of residues important for $G_{\alpha}$ association with Ric-8, no specific amino acids have been identified within Ric-8 that facilitate interaction with a $G_{\alpha}$ subunit.

**Dissertation Goals**

To investigate the Fog signaling pathway we wanted to develop a *Drosophila* tissue culture assay in which we could apply ectopic Fog to cells to drive cellular constriction. As one of the first graduate students in Dr. Stephen Rogers’s lab I helped develop many tools and techniques to examine varying aspects of cellular morphogenesis. We first made a cell line that secreted ectopic Fog into the media. By concentrating that media and applying it to different *Drosophila* tissue culture cell lines, we were able to identify one cell type that responded to Fog by altering its cytoskeleton, S2R+ cells. The development of, and detailed protocol for performing this assay is presented in Chapter II of this dissertation.

A key missing component of the Fog signaling pathway is the receptor that transmits the Fog signal to activate the pathway. One aim of this dissertation is to show that we have identified a GPCR that drives contractility downstream of Fog. While deficiency screens have been performed for zygotic genes controlling gastrulation in *Drosophila*, and identified other members within the pathway such as T48$^5$, no GPCR has been revealed. Using *Drosophila* tissue culture put us at a unique advantage as we were able to quickly screen through a dsRNA library comprising all 138 known and putative GPCRs in the *Drosophila* genome$^{47,48}$. Doing this we found one GPCR that inhibited Fog-induced cellular constriction,
Mist. Upon identifying this receptor we further showed that Mist was capable of conferring Fog responsiveness to previously unresponsive cells, and identified specific domains important for its function. In the animal, we found that Mist drives cellular contraction in tissues known to be regulated by the Fog pathway through overexpression and targeted RNAi depletion of Mist. An in-depth analysis of the GPCR, Mist is presented in Chapter III of this dissertation.

In the initial characterization of embryos depleted of maternal and zygotic Ric-8 it was noted that phenotypes of Ric-8, strongly resembled Fog and Cta embryos. Therefore, we wanted to investigate how Ric-8 fit into the Fog signaling pathway. While clues abounded in the wealth of data linking Ric-8 to Gα function, little was known about Ric-8’s function within this pathway or how it interacts with Gα subunits in any system. Using our tissue culture assay, I showed that Ric-8 is an essential component and acts at the level of Cta in the Fog signaling pathway. To determine how Ric-8 influences the behavior of Cta I performed pulldown assays showing that Ric-8 preferentially binds and localizes GDP-bound Cta. The ability of Ric-8 to localize Cta plays a role in sustained pathway activation, as mis-targeting of Ric-8 negatively modulates the ability of S2R+ cells to respond to Fog. To gain further insight into the functional relationship of Ric-8 and Cta I made a series of evolutionarily conserved point mutants, and assessed their ability to rescue contractility in cells depleted of endogenous Ric-8, or to bind Cta in immunoprecipitation pulldown experiments. From these experiments I identified specific residues important for establishing a functional binding interface between Cta and Ric-8. Chapter IV contains a detailed description of these experiments demonstrating an essential role for Ric-8 in the Fog signaling pathway.

Finally, this dissertation as a whole is presented to describe to you how a novel
experimental system we devised resulted in two projects, identification of the GPCR, Mist, and characterization of the cytoplasmic protein Ric-8, that have advanced our understanding of a pathway essential for cellular shape changes that drive whole-sale tissue rearrangement.
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Figure 1.1 Ventral furrow formation in the developing Drosophila embryo. Yellow cells are presumptive mesoderm, which will undergo apical constriction. Red cells represent presumptive mesoderm that are internalized, but do not apically constrict. Arrows indicate direction of cell movements. Modified from Sawyer, et al.¹
Figure 1.2 Activation of the Fog signaling pathway drives cytoskeletal changes within the cell.
Chapter II

Establishing the Drosophila tissue culture cell line, S2R+, as a model for studying Fog pathway signaling

Preface

This work is currently in preparation for submission for publication. All experiments were designed by my advisor, Dr. Stephen Rogers, and myself. This manuscript was written by me, and edited by Dr. Stephen Rogers. I performed all of the experiments, except Figure 2.4C, which was performed by Dr. Stephen Rogers.

Introduction

Drosophila tissue culture has been established as a powerful system to address molecular and cell biological questions. Additionally, gain- or loss- of function analyses in Drosophila cell based assays are quick, easy and efficient due to high transfection efficiency, and the ability of cells to take up large dsRNA molecules directly from media. A previously studied cell line, Dm-D17-C3, was established as a model for investigating basic principals of cellular migration and the regulation of the cytoskeleton \(^{1,2}\). We wanted to develop a similar system to study a cell signaling pathway that drives cell shape changes during Drosophila development.

The Fog (Folded gastrulation) signaling pathway is used reiteratively during development of the Drosophila embryo to facilitate cell shape changes in movements of the ventral furrow and posterior midgut to establish germ layers \(^{3}\); to shape the epithelial imaginal
wing disc⁴; as well as to form the salivary glands⁵,⁶. The Fog pathway has been most well-studied in gastrulation during ventral furrow formation. From this system a signaling cascade has been described starting with the secreted protein Fog binding to the 7-transmembrane G protein coupled receptor (GPCR), Mist, to activate a \(\text{G}_\alpha_{12/13}\) protein (Concertina, Cta)⁷,⁸. The small GTPase Rho is activated by the Cta target RhoGEF2. Rho in turn activates Rho kinase resulting in the phosphorylation and activation of non-muscle myosin II⁴,⁶,⁸-¹⁰; myosin-II binds F-actin resulting in cellular constriction.

We wanted to recapitulate the Fog signaling pathway, from ligand binding to pathway activation (cellular constriction), in a cultured system. To accomplish this we needed to find a cell line that responded to ectopic application of Fog. S2 cells respond to overexpression of downstream Fog pathway components (i.e. RhoGEF2, Rho, Rho Kinase) by altering their cytoskeletal morphology, changing from a flattened pancake shaped cell to a bonnet shaped cell¹¹. The cell acquires a bonnet shape due to the activation of myosin which elicits constriction in an actin dependent manner to form a tight band of activated myosin at the base of a dome, where the organelles have been displaced due to constriction (Figure 2.1).

However, application of concentrated Fog has no effect on the cytoskeletal organization, or the cell morphology, of S2 cells, as these cells lack the receptor that binds Fog (Figure 2.1). We therefore began testing various immortalized cell lines, obtained from the *Drosophila* Genome Resource Center, for Fog responsiveness, and identified one cell line, S2R+ (S2 Receptors +), able to respond to Fog application. It has been shown that this cell line is responsive to the Wingless (Wg) ligand, unlike S2 cells, because these cells express the Wg receptor, Frizzled¹². Upon application of Fog, S2R+ cells undergo dramatic morphological changes. There is a re-localization of F-actin and active myosin, as visualized using an
antibody to the phosphorylated regulatory light chain of non-muscle myosin II (pRLC) (Figure 2.2). Using S2R+ cells we have established a means to investigate the Fog signaling pathway in a tissue culture based system.

Experimental data from the animal is invaluable to understanding morphological movements during embryogenesis. The transmission of inductive cues within a tissue from one cell or group of cells to another, how cells migrate and move past each another to reach their final location, and other questions involving tissue dynamics during cellular reorganization and movements to shape the embryo are well-suited to analysis in the *Drosophila* embryo. However, the system we have developed provides a streamlined approach to study the general mechanism of this signaling pathway through fast, easy, and efficient methods. The aim of this article is to describe the methodology we developed to investigate Fog-induced cellular constriction using S2R+ cells.

**Experimental Design**

**Maintenance, transfection and RNAi of S2R+ cells.**

S2R+ cells are a subclone\(^1\) of the S2 cell line, an immortalized population of cells originally derived from late stage embryos\(^1\). However, unlike S2 cells, S2R+ cells do not grow well in either SF900 or Schneider’s Media, and as found by Yanagawa, et al. the cells must be cultured in Shield and Sang M3 insect medium supplemented with 10% heat-inactivated FBS\(^1\). For routine passage, S2 cells can be diluted to \(~25\text{-}50\%\) confluency and continue to propagate; however, at this density, S2R+ cells will change their morphology from a mostly homogenous lawn of rounded cells to a sparse population of long, spindly cells.
that will shortly undergo cell death. We have therefore found it is best to maintain S2R+ cells at a confluency, near 50-75%, for continued propagation.

RNAi in S2R+ cells is performed identically to dsRNA treatment in S2 cells. As a positive control, we routinely use dsRNA targeted to the small GTPase Rho. Due to its role in cytokinesis, when cells are depleted of Rho they become very large and contain multiple nuclei compared to control dsRNA treated cells\(^1,2\). Rho is also a member of the Fog signaling pathway, and upon its depletion cells are no longer able to respond to Fog and undergo cellular contraction\(^4\) (Figure 2.3). This makes Rho an ideal positive control for determining the optimal length of dsRNA treatment, as well as a useful tool to verify that dsRNA treatment has progressed long enough to prevent Fog induced cellular constriction.

To achieve high transfection efficiency in S2 cells, we utilize the Amaxa electroporation system. However, S2R+ cells do not survive electroporation under these conditions, most likely due to the inability to tolerate the high current required for introduction of plasmid DNA. Instead we have found great success using the FuGENE HD system for transfection of S2R+ cells. After performing a dilution series, based on suggested parameters outlined in the product literature, we found that the optimal transfection conditions consist of using a 2:8 ratio (2ug of plasmid DNA/8uL of transfection reagent). The procedure for transfection of S2R+ cells is outlined below.

**Construction of the Fog expression vector and production of ectopic Fog**

We created a construct with tagged, full-length Fog under an inducible promoter using PCR to amplify the coding sequence of the gene and introducing a 5’ EcoRI site, a C-terminal Myc tag, and a 3’ NotI site to allow cloning into pMT-V5/His. The pMT promoter
is inducible by addition of copper sulfate. We transfected this construct, along with a plasmid encoding the antibiotic resistance gene for Hygromycin, into S2 cells and selected for cells containing the construct by treating the cells with increased doses of the drug Hygromycin B for ~4 weeks to make a stable cell line, S2:Fog-Myc.

One can transfet two constructs simultaneously, a plasmid encoding your gene of interest and a plasmid containing a eukaryotic antibiotic resistance gene, as an efficient way to generate a stable cell line. However, it means that while most cells will contain both plasmids, there is a subpopulation that only contains the plasmid with the antibiotic resistance gene. To overcome this problem one can use the OpIE2 promoter pIZ family of vector backbones. These plasmids contain a multiple cloning site, and a eukaryotic antibiotic resistance gene all within the same backbone. Either method is suitable to make a stable cell line with high levels of construct expression. A protocol outlining the procedure for creation of stable cell lines is presented below.

Before induction, collection and concentration of Fog containing media, cells were scaled up from 25cm² (~5mLs) to 150cm² (~20mLs) flasks. We wanted to maximize the amount of Fog produced and harvested per experiment. Therefore, we tested several variables to ascertain the appropriate medium and induction time for optimal expression and collection.

To verify that Fog is being expressed and secreted into the media, we separated media concentrated from S2:Fog-Myc cells and untransfected S2 cells on SDS-PAGE gels, blotted, and probed nitrocellulose with antibodies to recognize Fog. In S2:Fog-Myc expressing cells monoclonal anti-Myc and affinity purified anti-Fog antibodies recognized a band at ~150kDa, which is absent from media collected and concentrated from untransfected S2 cells (Figure
2.4A and Figure 4.1A). S2:Fog-Myc cells are maintained in SF900 media, however we found if the media is exchanged on the day of induction with Schneider’s media that we are able to collect more Fog protein per mL (Figure 2.4A). We tested various experimental conditions before finding an optimum length of induction, which we determined as ~48 hours (Figure 2.4B). Before each experiment, we tested the efficacy of the concentrated Fog. The process of concentrating Fog-containing media can yield potent Fog ligand, and we routinely determine the amount used for experiments by testing a dilution series of Fog/Schneider’s media. Generally the Fog we use for experiments is diluted 1:3 with Schneider’s media.

S2R+ cells start to respond within minutes of Fog application. We have found that treatment of S2R+ cells for 10 minutes is sufficient for robust S2R+ cell response (Figure 2.4C).

**Recapitulation of Fog Signaling events in S2R+ cells**

As previously described, activation of the Fog pathway during *Drosophila* gastrulation drives the activation of the acto-myosin cytoskeleton resulting in cellular contraction. We can activate the Fog signaling pathway in S2R+ cells simply through addition of concentrated ectopic Fog (Figure 2.1 and 2.2). To verify that addition of the Fog ligand was specifically affecting the Fog signaling pathway, we depleted cells of the essential pathway components Cta, RhoGEF2, and Rho, and using phase-contrast microscopy assessed their ability to undergo cellular constriction in response to Fog. Depletion of any of these components abrogates Fog responsiveness (Figures 1.3 and 4.1B). Therefore we have developed a system wherein we can directly activate the Fog pathway in S2R+ cells, using ectopic application of concentrated Fog-containing media. The aim of this paper is to explain, in detail, the procedural methods to make a stably expressing cell line that will secrete Fog into the
medium, and how to harvest and apply concentrated Fog to cells to create a robust system to investigate dynamics of the Fog signaling pathway.

Materials and Methods

Reagents

- S2R+ cells are available from the DGRC (http://dgrc.cgbi.indiana.edu/)
- SF900 (Gibco, cat no. 10902), Schneider’s (Gibco, cat no. 11720), and Shield and Sang M3 (Sigma-Aldrich, cat no. S3652) insect medium; All medias are supplemented with 1% antibiotic/antimycoctic and Shield and Sang M3 media is additionally supplemented with 10% heat-inactivated (see-below) FBS. All preparation of media is to be performed in a sterile laminar flow hood.
- Antibiotic/antimycoctic (Gibco, cat. no. 15240)
- Non-heat inactivated FBS (Gibco, cat. no. 26140); we heat-inactivate FBS at 55C, as we have found that commercially heat-inactivated FBS can inhibit sustained cell growth2.
- Hygromycin B solution (CellGro, cat. no. 30-240-CR) or Zeocin antibiotic (Invitrogen, cat. no. R250)

- Concanavalin A (MP Biomedicals, cat no. 150710)

Equipment

- Sterile laminar flow hood
- Treated tissue culture flasks (25cm²; Falcon, cat. no. 353014, 75cm²; Falcon, cat. no. 353135 and 150cm²; Falcon, cat. no. 353046)
- 6-well tissue culture plates (Falcon, cat no. 353064)

- Hemocytometer (Hausser Scientific, cat. no. 02-671-54)

- Amaxa Kit V (Lonza, cat. no. VCA-1003)

- Transfection vectors for creating stable cells lines such as the pMT promoter vector family (Invitrogen, cat. no. V412020) or the OpIE2 promoter containing pIZ vector (Invitrogen, cat. no. V8000)

- Polystyrene Petri dishes (35 mm × 10 mm; Falcon, cat no. 351008)

- Glass cover slips, (no. 1.5, 22 mm²; Corning, cat. no. 2940-225)

- Glass bottom plates (Maktek, cat. no. P35G-1.5-10)

- Microscope slides (25x75x1mm; Fisher Scientific, cat no. 12-544-2)

- FuGENE HD transfection reagent (Promega, cat. no. E2311)

- Sterile water (Fisher Scientific, cat. no. BP5611)

- Protein concentrators with a 30,000 molecular weight cutoff (MWCO) (Millipore, cat. no. UFC903008)

- Swinging bucket centrifuge

- Bright-field microscope capable of phase-contrast or DIC microscopy

**Equipment Set-up**

Preparation of coverslips and glass bottom plates for phase-contrast and fluorescence microscopy:

Prepare coverslips as previously described¹². To prepare glass-bottom plates for microscopy, add enough Concanavalin A (ConA) (0.5mg/mL) to cover the glass portion of the coverslip.
Immediately aspirate off and allow to air-dry. Glass bottom plates treated this way are viable for at least one month.

**Procedures**

**Note: Handing of live cells should always be performed in a sterile laminar hood.**

**Creating and storing a stable S2 cell line**

1. Remove 10uL from a dense flask of resuspended S2 cells. (Resuspend S2 cells by gently aspirating the media up into a pipette and running the media with some force over the attached cells). Using a bright-field microscope with either DIC or phase-contrast filters use a hemocytometer to determine the number of cells within the flask.

2. Transfect ~10^6 S2 cells with 1ug/uL of plasmid DNA as described in the instructions for Amaxa Kit V using the Amaxa nucleofector system program G-030.

3. Using a micropipette remove the transfected cells from the cuvette by adding 0.5mL of SF900 media. Add this mixture to 1mL of SF900 in a 6 well plate. Transfect 3 wells for each construct.

4. Allow the cells to recover from the transfection for 24 hours.

5. Start treating the cells with the appropriate antibiotic at low doses. For the two most commonly used antibiotics in our laboratory, Hygromycin B and Zeocin, start at 200ug/mL and 50ug/mL, respectively. Using a low dose of the drug at first is critical as many cells will undergo cell death, and if too many within the population die the paucity of cells will cause the remaining, vector-containing cells to die as well.

6. After the first treatment allow cells to recover for one week, and then resuspend all 3
wells and transfer cells to a 25cm² flask. The following day treat the cells with a low dosage of drug.

7. Over a period of a month gradually increase the amount of antibiotic, treating cells every 5-7 days, and passing cells into a new flask when necessary. A final amount of ~5X the initial drug dose should be used for the last treatment. The final doses of drug will select for cells containing high levels of transfected DNA.

8. To determine the level of protein expression for a stable cell line, plate cells out onto a ConA coated coverslip or glass bottom plate and fix and stain using an appropriate antibody. A protocol for preparing Drosophila tissue culture cells for microscopy has been previously described¹. Alternatively one can run cell lysate samples out on an SDS-PAGE gel to visualize and quantify protein expression levels.

9. Once you have established the desired stable cell line, it is imperative to freeze cells down as stocks for future use. Protocols for freezing cells down and thawing them out are previously described¹.

**Transient transfection of S2R+ cells**

1. Into 1mL of Shield and Sang M3 insect medium plate out S2R+ cells into one well of a 6-well plate to ~60-70% confluence. Allow several hours to overnight for attachment.

2. Prepare the transfection complex using FuGENE HD, per product literature guidelines, using a 2:8 ratio of DNA to transfection reagent, and sterile water as the medium.

3. After addition of the FuGENE HD transfection reagent wait at least 20 minutes before adding the mixture to cells. The media does not need to be changed before or after addition of transfection complexes.
4. If your gene of interest is under an inducible promoter wait 24 hours before induction, and 
a following 24 hours before experimental usage.

**Harvesting secreted Fog from S2 media**

1. Scale up growth of S2:Fog-Myc cells from one 25cm$^2$ (~5mLs), to one 75cm$^2$ flask 
   (~10mLs), ultimately to (2) confluent 150cm$^2$ flasks (~20mLs).
2. Remove SF900 media from flask and discard.
3. Slowly add 10mLs of Schneider’s media to flask and gently rock back and forth several 
times and discard. It is critical to not disrupt attachment of cells to tissue culture plastic, or 
allow too much time to pass in between addition and removal of media.
4. Add 20mLs of fresh Schneider’s media to flask.
5. Immediately after adding Schneider’s media add 100uL of 100mM CuSO4, and wait 48 
   hours.
6. Remove media and transfer to a conical tube. Discard cells and flask.
7. Spin media at 4000 rpm at 4°C for 15 minutes to clear media of any cellular detritus, and 
   transfer to a fresh conical tube, placing media on ice or at 4°C.
8. In batches, add 10mLs of pre-cleared media to protein concentrators and spin at 4000 rpm 
at 4°C for 30-45 minutes (or until you have decreased the amount of media to 20 percent 
of the original volume).
9. Remove concentrated media from the protein concentrator reservoir and transfer to a fresh 
tube, kept on ice or at 4°C. Concentrated Fog can be stored at 4°C for several months; 
however, longer periods of storage may result in bacterial contamination.
Testing the efficacy of concentrated Fog

1. Plate out S2R+ cells into 1mL of Shield and Sang M3 insect medium in a glass-bottom plate pre-coated with ConA to a density of ~50% confluence. Allow cells to attach for at least 1 hour. Cells are sufficiently attached when they become phase-dark and have wide, flattened lamellipodia.

2. Remove a small amount of Fog media from 4°C stock, and allow to warm up to room temperature.

3. Dilute concentrated Fog protein 1:1 with fresh Schneider’s media.

4. Discard media from glass bottom plate and carefully add Fog media only to the glass circle containing the S2R+ cells. Usage of 150 uL is sufficient to cover the area.

5. Wait 10 minutes and using phase-contrast or DIC microscopy determine the amount of cells undergoing morphological change. Cell shape changes are clearly evident under low magnifications, such as 10X and 20X.

6. If a 1:1 ratio of Fog to Schneider’s is sufficient for robust cellular constriction, continue a dilution series to determine the minimal amount of Fog necessary for robust constriction. Usually this is between a 1:3 and 1:5 ratio. If there is no or little contraction with diluted Fog, apply undiluted Fog.

Anticipated Results

Following these steps will allow the user to establish a system in which to test the morphological cell shape changes downstream of Fog application. S2R+ cells provide a tractable system for loss- and gain- of function studies due to the amenability of this tissue culture line to RNAi and transient transfection. S2R+ cells give a clear read-out of Fog
pathway activation, which can be seen using basic phase-contrast microscopy, saving time and reagents. Alternatively, commercially available antibodies are available to Fog pathway components allowing for visualization of cellular function using fluorescence microscopy. Finally, the ability to perfuse Fog into a chamber allows for live-imaging with high-resolution confocal microscopy.
References


Figure 2.1 Overexpression of downstream components of the Fog signaling pathway drive cellular constriction in S2 cells. Cells were transfected with RhoGEF2-GFP or Rho Kinase-Myc, and stained for pRLC (and for Rho kinase, anti-Myc). Scale bar: 20 µm.
Figure 2.2 S2R+ cells treated with ectopic Fog ligand respond by reorganizing their cytoskeleton. S2R+ cells were treated with control or Fog containing media and stained for pRLC (active myosin), Phalloidin (F-Actin) and DAPI (DNA). Scale bar: 20 µm.
Figure 2.3 S2R+ cells treated with dsRNA targeting Rho do not respond to ectopic Fog application, unlike control dsRNA treated cells. Rho dsRNA treated cells are multi-nucleated and much larger than control dsRNA treated cells. S2R+ cells were treated for 7 days with dsRNA targeting control or Rho dsRNA, and treated with either control of Fog containing media. Scale bar: 50 µm.
Figure 2.4 Optimization of ectopic Fog collection and application. (A) S2:Fog-Myc cells grown in Schneider’s media produce more Fog than cells grown in SF900 media as visualized by anti-Myc antibodies. (B) Media concentrated from S2 and S2:Fog-Myc cells and induced for either 24 or 48 hours. Longer induction times yield more Fog ligand. Blots probed with anti-Myc to recognize Fog. (C) S2R+ cells respond robustly to Fog application. S2R+ cells treated with concentrated Fog media, before Fog application (t=0), and after 10 minutes (t=10). Phase contrast microscopy.
Chapter III

Regulation of morphogenesis by intersecting expression patterns of *Drosophila* Fog and its receptor, Mist

Preface

This chapter represents a manuscript we have submitted and are currently making revisions for resubmission.

I originally performed a first pass of this screen making dsRNA, and depleting cells of all 44 known peptide-binding GPCRs\(^1\), assuming since Fog is a large peptide that it was probable that one of these GPCRs would bind Fog and activate the signaling pathway. This screen presented no candidates so we compiled a list of all known and putative GPCRs and we ordered plates of pre-made dsRNA from Harvard. Another graduate student in the lab, Alyssa Manning, performed the large dsRNA screen with all 138 known and candidate GPCRs\(^{1,2}\). All experiments for this manuscript were designed by Alyssa Manning, my advisor, Dr. Stephen Rogers, Dr. Mark Peifer, and/or myself. I performed all experiments in Figure 3.1, save Figure 3.1E; Figure 3.3B, and all Figures in Figure 3.4; and Figure S3.2B. All other experiments were performed by Alyssa Manning. This manuscript was written and edited by Alyssa Manning, Dr. Stephen Rogers, Dr. Mark Peifer and myself.

Abstract

Epithelial sheet remodeling is a morphogenetic process that shapes organs and tissues
and establishes the three embryonic germ layers during gastrulation. We have used an innovative approach to identify a key molecule, connecting transcriptional patterning to the cellular machinery involved in epithelial morphogenesis. Using a novel cell-based assay and RNAi screening, we have identified a *Drosophila* G-protein coupled receptor, Mist, which triggers apical constriction to drive epithelial folding at several stages in development, including gastrulation and wing disc morphogenesis. We show that Mist acts as a receptor for the secreted morphogen Folded gastrulation, and that its zygotic expression is regulated by the transcription factor, Snail. Overlapping expression of the ligand receptor pair provides temporal and spatial regulation of tissue morphogenesis.

**Introduction**

During embryogenesis sheets of epithelial cells are shaped to build organs, define tissue compartments, and establish the embryonic body plan\(^3,4\). The forces that drive these tissue-level rearrangements are produced by the actin cytoskeleton and its associated motor proteins and transmitted from cell-to-cell within epithelia by adherens junctions. Regulation of epithelial contractility and adhesion is governed by a complex interplay between maternally supplied proteins and patterned zygotic gene expression; understanding how these two sources of information interact to direct embryogenesis is a key question in the field of developmental biology\(^5\).

Studies in *Drosophila* have identified evolutionarily conserved molecules involved in apical constriction, a morphogenetic cell shape change that drives epithelial folding, and revealed key insights about the biophysical principles at work\(^6\). Genetic analyses identified a core-signaling pathway that triggers epithelial folding through apical constriction during
gastrulation, invagination of salivary glands, and folding of the wing imaginal disc epithelium, among others. This pathway is triggered by Folded gastrulation (Fog), a secreted protein thought to act as a ligand for an unidentified receptor on the epithelial cells that produce Fog. The downstream signaling cascade includes a heterotrimeric G-protein complex containing the Gα12/13 homologue Concertina (Cta). Cta is thought to activate RhoGEF2, which is recruited to the apical membrane by the transmembrane protein T48. RhoGEF2 in turn activates the small GTPase Rho1 to recruit and stimulate cytoskeletal contractile machinery, thereby inducing apical constriction. The Fog pathway has been best characterized during Drosophila gastrulation, when a transcriptional cascade triggers localized Fog expression. This initiates formation of both the ventral furrow (VF) to internalize the mesoderm and the posterior midgut (PMG) to internalize the endoderm. Thus, Drosophila gastrulation provides a classical and powerful model system to study a morphogenetic pathway from the level of gene expression to cytoskeletal regulation.

Materials and Methods

Cell Culture and RNAi
S2 and S2R+ cell lines were obtained from the Drosophila Genome Resource Center (Bloomington, IL), and cultivated as described previously. S2 cells were maintained in SF900 SFM (Invitrogen, Carsbad, CA) and S2R+ cells in Sang’s and Shield’s medium (Invitrogen) supplemented with 5% heat-inactivated FBS (Invitrogen). Double stranded RNAs were produced using Promega (Madison, WI) Ribomax T7 kit according to instructions, or ordered from the Drosophila RNAi Screening Center (Boston, MA). Primers
used for dsRNA synthesis are as follows and are all preceded by the T7 sequence (5’-
TAATACGACTCATATAGG-3’). Control-fwd: 5’-
TAAATTGTAAGCGTTAATATTTTG-3’ and Control-rev: 5’-
AATT CGATATCAAGCTTATCGAT-3’ to amplify a region from the pBluescript plasmid;
Cta-fwd: 5’- TGACCAAATTAACTCAAGAAGAAT-3’, Cta-rev: 5’-
TTCCAGGAACCTTATCAATCTCTTTG-3’; RhoGEF2-fwd: 5’-
ATGGATCAACCATCAATCAAAAAACGG-3’, RhoGEF2-rev: 5’-
TGTCCTGCATCCCTATGACCACAAGGC-3’; Rho-fwd: 5’-
GTAAAAACTTGCCCTTCTGATTGTCT-3’, Rho-rev: 5’-
ATCTGGTCTTCTCCTCTTTTGA-3’; Mist1-fwd: 5’-AATTGCAAATTGAGGCCAAG-3’; Mist1-rev: 5’-AGAGCATTGTGCGCTGACT-3’; Mist2-fwd: 5’-
CTCCATTGCGGATGTTG-3’; Mist2-rev: 5’-GGAACGTCCACCAGATGTT-3’. For
individual RNAi treatments, cells at 50-90% confluency in 6- or 12-well plates were treated
every other day for 7 days with 10\(\mu\)g/ml of dsRNA. Cells were resuspended and plated on
Concanavalin A (MP Biomedicals) coated coverslips, allowed to spread for 1 hour, then
treated for 10min with concentrated Fog-conditioned medium or medium harvested from
untransfected S2 cells (see below). For RNAi screening, 96-well plates containing dsRNAs
were heated to 95°C for 3min, and then the temperature was lowered 1°C per 30sec to room
temperature. 0.2-0.4 \(\mu\)g of a single dsRNA was added to each well of a 96-well plate; then
2.5x10^4 cells were plated in each well and incubated at 25°C for 6 days. Cells were
resuspended and 2.5x10^4 cells were plated in each well of a ConA-coated 96-well glass
bottom plate (Greiner, Frickenhausen, Germany) for 1 hour prior to Fog treatment. S2 cells
were transfected using the Amaxa nucleofector system with Kit V using program G-30
(Lonza, Basel, Switzerland). For quantifying numbers of cells contracted, each condition was repeated at least in three times and $\geq 100$ cells were counted per experiment. Statistical significance was determined with Student’s t-test.

**Production of recombinant Fog protein**

We engineered a stable Fog-secreting cell line by amplifying the Fog open reading frame and ligating it into the inducible pMT-V5/His A plasmid (Invitrogen). Stable Fog-producing cells were obtained by co-transfecting S2 cells pMT-Fog-Myc with pCoHygro hygromycin selection plasmid (Invitrogen) followed by antibiotic selection as directed by the manufacturer. Fog producing cells were plated at 70-90% confluency in 150cm$^2$ flasks for 24 hours, washed two times with Schneider’s SFM (Invitrogen), and induced for 48 hours in Schneider’s with 100µM CuSO$_4$. Medium was collected and clarified of cells by centrifugation at 4000 x g for 10 minutes. Cleared medium was concentrated 40x in Amicon 30 k centrifugal concentration devices (Millipore, Billerica, MA). Concentrated Fog containing medium or similar control medium was diluted 1:1 with fresh Schneider’s for use on cells.

**Immunofluorescence microscopy**

**Cells**

To visualize Mist, cells were plated on coverslips treated with ConA, fixed with 4% formaldehyde (EM Sciences, Gibbstown, NJ) in HL3 buffer (70 mM NaCl; 5 mM KCl; 1.5 mM CaCl$_2$-2H$_2$O; 20 mM MgCl$_2$-6H$_2$O; 10 mM NaHCO$_3$; 5 mM trehalose; 115 mM sucrose;
5 mM HEPES; pH to 7.2), and permeabilized with PBST (PBS+0.1% Triton X-100). Cells were blocked with 5% normal goat serum (Sigma-Aldrich, St. Louis, MO) in PBST and stained with anti-Mist antibody diluted into the same solution at 1:500. Following washing, cells were incubated with secondary antibodies (RhodamineX-conjugated goat anti-rabbit diluted 1:1000, Jackson Immunoresearch, West Grove, PA). After washing, the cells were mounted in fluorescent mounting medium (Dakocytomation, Glostrup, Denmark). We acquired images of the cells using a CoolSnap HQ CCD camera (Roper Scientific, Ottobrunn, Germany) on a Nikon Eclipse Ti inverted microscope driven by Nikon Elements software (Tokyo, Japan).

* ***Drosophila*** tissue

Embryos were collected on apple juice plates supplemented with yeast paste at 25°C, fixed in 4% formaldehyde in PBS/heptane, methanol divitilenized, and stained as above. DNA was stained with Hoescht 33342 diluted 1:10,000. Wing imaginal discs were collected by picking wandering 3rd instar larvae and dissecting them in PBS, leaving discs attached to the larval cuticles during staining. They were fixed in 4% formaldehyde in PBS with 0.1% Tween-20 and stained as above using mouse anti-actin antibody and Cy2-donkey anti-mouse secondary at 1:1000. Imaginal discs were mounted by dissecting wing discs from the larval cuticles in 70% glycerol in PBS. Images of embryos and imaginal discs were obtained using a Leica DMI 6000 microscope driven by LAS AF software (Leica Microsystems, Buffalo Grove, IL). Cross-sectioned embryos were prepared as previously described in Dawes-Hoang, et al. and imaged using a Zeiss LSM 710 and LSM software (Zeiss, Thornwood, NY), or a Vt-Hawk Swept-field confocal and Vox Cell-Scan software (Visitech, Sunderland, UK).
Immunoblotting

S2 or S2R+ extracts were produced by resuspending cell pellets in PBS + 0.1% Triton X-100. A small amount was reserved to measure protein concentration. SDS-PAGE sample buffer was then added and boiled for 5 minutes. Comparisons were made by normalizing protein loads to immunoblots performed with antibodies to α-tubulin.

Antibodies

The following antibodies were used in this study: mouse anti-α tubulin monoclonal DM1α (Sigma), used at 1:500 dilution; mouse anti-Neurotactin (DSHB), used at 1:50; rabbit anti-Twist (gift from Maria Leptin), used at 1:1000; mouse anti-GFP JL8 (Clontech), used at 1:500; sheep anti-Digoxygenin-alkaline phosphatase (Roche, Mannheim, Germany) used at 1:2000; sheep anti-Digoxygenin-POD (Roche) used at 1:50. In addition, streptavidin-alkaline phosphatase (Jackson Immunoresearch) was used at 1:1000. Antibodies to Mist were raised in rabbit against recombinant GST fusions with the COOH-terminal 100 residues of Mist by Pocono Rabbit Farm and Laboratories (Canadensis, PA) and used at 1:500 dilution (cells) or 1:5000 (sectioned embryos).

In Situ Hybridization

Probe preparation and in situ hybridization for embryos and imaginal discs was performed essentially as described in Kearney et al.\textsuperscript{16}. dsRNA mist probes were made with Digoxygenin-UTP to the entire predicted coding sequence. Fog probes were made with Biotin-RNA labeling kit (Roche) to the sequence amplified with the same T7-Fog primers.
used to make dsRNA for embryo injection (below). Alkaline phosphatase developing was performed in premixed BCIP/NBT (MP Biomedicals), while fluorescence developing was performed with a Cy5 TSA kit (Perkin-Elmer, Waltham, MA). Alkaline phosphatase developed tissues were mounted in 70% glycerol in PBS and imaged using a Zeiss Axio phot microscope, Sony 3XDD CCD video camera, and Zeiss Axiovision software.

**Embryo Injection**

Embryos were prepared as previously described in Carthew et al.\textsuperscript{17}, unless noted below. Primers used for dsRNA synthesis are as follows and are all preceded with the T7 sequence (5’-TAATACGACTCACTATAGG-3’). Control-fwd: 5’-TAAATTGTAAGCGTTAATATTTTG-3’ and Control-rev: 5’-AATTCGATATCAAGCTTATCGAT-3’; Fog-Fwd: 5’-ATATTTTTGAGAAGAAATTCCCCAC-3’, Fog-Rev: 5’-CTGTGGTATACTCGTCTTCCTC; Mist1 and Mist2: same as used in cell culture. Embryos were injected with a final concentration of 1 µg/µl for all dsRNAs. Embryos were removed from tape using a steady stream of heptane, fixed with 37% para-formaldehyde, and hand-peeled to remove the vitelline membrane. Images were obtained using a Zeiss LSM 710 and LSM software.

**Fly Stocks**

The following fly lines were used in this study: UAS-\textit{mist} RNAi, UAS-\textit{cta} RNAi (Vienna Drosophila Resource Center), \textit{moesin-GFP} (Edwards, et al. 1997), \textit{yellow white, fog}\textsuperscript{S4}/FM7 \textit{twist-GFP}, A9(wing disc specific)-GAL4, \textit{twist}\textsuperscript{1}/CyO, \textit{snail}\textsuperscript{18}/CyO, (from Bloomington
Drosophila Stock Center, Bloomington, Indiana), UAS-fog (gift from Eric Wieschaus, Princeton University). UAS-mist flies were made by Gateway cloning (Invitrogen) the coding region of mist into the pPW vector (Terence Murphy, Carnegie Institution), which was sent to Best Gene (Chino Hills, CA) for injection and recovery of transformants. Wings were collected by using forceps to remove single wings from CO₂-immobilized adults. Wings were placed on white paper and imaged with Nikon SMZ1000 dissecting scope and Nikon CoolPix camera.

Results

Mist acts as a receptor for Folded gastrulation

Although traditional genetic analyses of epithelial folding have identified many of the components involved, the Fog receptor has remained elusive. We used a functional genomic approach to identify receptors for Fog by reconstituting the signaling pathway in a cell-based assay and then used RNAi screening to systematically test candidate receptors. Our previous work showed that activating Rho1 in cultured Drosophila S2 cells induces a characteristic contracted morphology and we looked for a similar response in cells upon Fog application. We engineered a stable S2 cell line that expressed and secreted Fog, and used conditioned medium from these cells to screen several immortalized Drosophila tissue culture cell lines for a response to Fog. S2R+ cells exhibited a robust contractile response to Fog, but S2 cells and several other epithelial-derived cell lines failed to respond (Figure 3.1A and data not shown). RNAi depletion of components known to be involved in the epithelial folding pathway, including Cta, RhoGEF2, or Rho, prevented Fog-induced S2R+ cell contraction,
indicating that we had recapitulated this morphogenetic cascade in cultured cells (Figure 3.1B).

Since Cta acts downstream of Fog, we hypothesized that Fog signals through a G-protein coupled receptor (GPCR). To identify the receptor in our cultured cell model, we performed a targeted RNAi screen in S2R+ cells, depleting the 138 known and predicted GPCRs in the *Drosophila* genome (Table 3.1) and tested whether the cells contracted in response to Fog. A comparison of triplicate screens revealed that a single dsRNA corresponding to the uncharacterized gene CG4521 (previously called methuselah-like 1, or mthl1) consistently blocked Fog-induced contraction to the same extent as positive controls. This gene, designated here as *Mesoderm-invagination signal transducer* (Mist), encodes a predicted GPCR of the secretin receptor family. Mist is predicted to have a large 298 residue NH2-terminal extracellular domain, which is characteristic of this family, seven membrane-spanning helices, and a 93 residue cytoplasmic COOH-terminal domain (Figure 3.1C).

Antibodies against Mist recognized a single protein band on immunoblots of S2R+ cells, which was depleted upon treatment with *mist* dsRNA (S3.1A). Thus, Mist is necessary for the Fog response.

We next tested whether Mist was sufficient to confer Fog responsiveness. S2 cells do not express Mist, however, ectopic expression of full-length Mist endowed this cell line with the ability to contract in response to Fog (Figure 3.1D, E, and S3.1B). To determine if Mist’s extracellular domain is necessary for Fog signaling, we expressed in S2 cells a deletion construct retaining the signal sequence but lacking the predicted NH2-terminal ectodomain (*MistΔN*, Figure 3.1C). *MistΔN* failed to confer Fog responsiveness upon S2 cells, indicating that the extracellular domain of Mist is required for Fog signaling (Figure 3.1D).
In contrast, MistΔC, lacking the cytoplasmic domain could confer pathway activation, indicating the COOH-terminus is not essential for activating downstream effectors (Figure 3.1C, D). Together these data demonstrate that Mist is necessary for Fog signaling in cultured Drosophila cells, and that the receptor’s large, extracellular domain is required for signaling, perhaps acting as a ligand-binding surface.

*Mist regulates Fog dependent epithelial folding in the imaginal wing disc epithelium*

Genetic studies revealed that loss of the Fog pathway’s downstream effector, RhoGEF2, leads to aberrant folding patterns in the wing imaginal disc epithelium, resulting in malformed adult wings20. Reducing Fog or Cta protein levels enhances this phenotype. To test whether Mist is involved in epithelial folding during Drosophila wing development we first assessed *mist* RNA expression in wild-type wing discs. Strikingly, *mist* RNA was expressed in discrete stripes precisely correlating with the folds in the wing disc tissue (Figure 3.2A). *fog* RNA showed a similar pattern of specific expression in the folds, with additional expression in the wing pouch (Figure 3.2B). The overlapping of expression patterns in the folds of the tissue suggests that Mist works with Fog in establishing the folds in the developing wing disc epithelium.

To functionally test the role of Mist we manipulated its levels in wing discs by expressing transgenic *mist* dsRNA using a wing disc specific driver. *mist* RNAi discs displayed abnormal folding patterns, although these defects were no longer apparent in adult wings; phenotypically, these were very similar to wing discs expressing *cta* dsRNA (Figure 3.2C, D). To test the effects of overexpression, we drove ectopic Mist or Fog across the entire imaginal disc. Overexpression of either gene disrupted the stereotypical epithelial
folding pattern, with Fog overexpression exhibiting a stronger effect on imaginal disc misfolding (Figure 3.2C). Fog overexpression resulted in wrinkled adult wings, whereas Mist overexpression did not (Figure 3.2D, E). The Fog overexpression phenotype allowed us to explore the epistatic relationship between Mist and Fog. If Mist is the Fog receptor, it should be essential for wing disc misfolding caused by Fog overexpression. Co-expression of ectopic Fog and mist dsRNA completely rescued the misfolding phenotypes induced by Fog overexpression in both imaginal and adult wing tissues, indicating that Mist functions downstream of Fog (Figure 3.2C-E). The difference in phenotypes between mist RNAi alone and Fog overexpression with mist RNAi may be due to incomplete knockdown or synthetic effects. Together these data confirm a role for Mist downstream of Fog during wing disc morphogenesis in vivo, and indicate that proper expression levels and patterning of both components are important for wing disc folding.

**Mist is transcriptionally regulated by Snail in the ventral furrow**

As the Fog pathway is used repeatedly in epithelial folding throughout development, we hypothesized that Mist also promotes epithelial folding during gastrulation. We first examined the embryonic localization of mist RNA. Low levels are present in the blastoderm, suggesting a maternal contribution corroborated by ModEncode data\(^{21}\) (Figure 3.3A). In cellularizing embryos, mist transcription is strongly upregulated along the ventral side and posterior end in a stripe of cells corresponding to the VF and PMG primordia, and is absent in all other cells. During VF invagination Mist protein is localized to punctae at the apical contractile surfaces of VF cells (Figure 3.3B, S3.2). mist RNA expression remains strong in the mesoderm and midgut after invagination (Figure S3.3A). Thus mist expression is
specifically upregulated in contractile cells of the VF and PMG primordia shortly after zygotic transcription begins, allowing spatial and temporal regulation of morphogenesis.

We next examined the relationship between Mist and the genetic pathway specifying mesodermal precursor cells. The embryonic dorso-ventral axis is established by maternally supplied Dorsal protein acting through the zygotic transcription factors Twist and Snail, both of which are independently required for VF invagination\textsuperscript{22,23}. fog is a known transcriptional target of Twist in the early embryo, but the Snail targets involved in VF invagination remain unclear\textsuperscript{7,24-26}. To test if ventral mist expression is downstream of Twist or Snail, we performed \textit{in situ} hybridization to examine patterns of mist expression in wild-type, twist, and snail mutant embryos. Wild-type embryos exhibited robust expression of mist in the VF and PMG from cellularization through the beginning of germ band extension (embryonic stages 5 through 8, Figure 3.3A and insets in 3.3C). When we crossed snail heterozygous parents, 25\% of embryos, presumably snail homozygous mutants, lacked mist expression in the VF but retained expression in the PMG (Figure 3.3C, D). In contrast, almost all embryos from twist heterozygous parents exhibited wild-type mist expression with only a few lacking VF expression (Figure 3.3D, S3.3B). As Twist enhances Snail expression in the mesoderm\textsuperscript{27}, the low frequency of mist misexpression observed in the twist mutants likely reflects this. Thus Snail is required for mist expression specifically in the VF.

\textbf{Mist regulates ventral furrow formation in the developing \textit{Drosophila} embryo}

To determine whether Mist is involved in epithelial morphogenesis during VF formation we injected dsRNA into preblastoderm stage embryos. Control dsRNA injected embryos rarely exhibited morphogenetic defects, while \textgreater 50\% of mist dsRNA injected
embryos displayed disorganization of the ventral midline and/or failure of mesoderm invagination (Figure 3.4A, D-G). These defects closely resembled defects seen in fog dsRNA injected or fog hemizygous mutant embryos, though fog deficient embryos exhibited more severe defects than mist dsRNA injected embryos (Figure 3.4B, C, G). This discrepancy may be caused by incomplete knockdown of mist due to maternal contribution. The similarities in phenotypes between mist and fog depleted embryos is further evidence that the two act in conjunction during epithelial folding many times during development.

Discussion

The Fog pathway is a premier example of how transcriptional programming is translated into cell behavior, but a key component was missing from our knowledge. Our data strongly support that the Drosophila GPCR Mist is a receptor for the secreted factor Fog. We revealed the identity of a long-sought part of a morphogenetic pathway leading from the transcription factors Twist and Snail to the cellular machinery involved in triggering epithelial folding (Figure 3.4H). Mist also represents the first downstream transcriptional target activated by Snail to induce gastrulation movements. These data help explain how the branches of the Twist and Snail regulatory pathway are ultimately integrated, by driving independently patterned, yet overlapping expression of the ligand receptor pair (Figure 3.4I). We favor a model in which apical constriction is regulated during multiple points throughout Drosophila development by patterned expression of both Fog and Mist. In the ventral furrow, Twist activates production of Fog and T48 and reinforces Snail expression in the ventral presumptive mesoderm cells. Snail, in turn, promotes Mist expression. Fog is secreted and activates Mist via autocrine signaling, leading to activation of Cta, recruitment of RhoGEF2 to the apical membrane via T48, and localized contractility through the Rho pathway. The
patterned expression of receptor-ligand pairs is likely to reflect a general principle of embryonic morphogenesis in *Drosophila*, as well as in other organisms.

**Acknowledgments**

We thank S. Crews, B. Goldstein, and K. Slep for feedback on the manuscript. We thank M. Leptin and E. Wieschaus for reagents. This work was supported by grants from the NIH (RO1-GM081645 to SLR and RO1-GM47857 to MP) and the Arnold and Mabel Beckman Foundation (Beckman Young Investigator Award to SLR). KAP was supported by funding from the Lineberger Comprehensive Cancer Center.
References


Figure 3.1 Mist acts as a Fog receptor. (A) S2R+ and S2 cells treated with control- or Fog-conditioned media. (B) Percentage of S2R+ cells contracted in response to 10-minute treatment with control- or Fog-conditioned media after RNAi knockdown of known Fog pathway components Cta, RhoGEF2, and Rho. (C) Mist predicted structure. Top: 37aa signal sequence (pink), 298aa extracellular domain (light green), 7 predicted transmembrane domains (yellow, numbered with Roman numerals), and a 93aa intracellular domain (dark...
blue). Extracellular loops are dark green and intracellular loops light blue. Bottom: Mist truncations used in D. (D) Percentage of cells contracted in response to 10-minute control or Fog treatment after *mist* knockdown (in S2R+ cells) or overexpression on Mist constructs (in S2 cells). n.s.: not significant. (E) S2 cells transfected with untagged Mist treated with control or Fog media and stained for Mist (red). Scale bar A, E: 20µm.
Figure 3.2 Proper Mist and Fog expression is required for wing imaginal disc morphogenesis. (A-B). Left: *In situ* hybridization for *mist* (A) or *fog* (B) RNA in wildtype wing imaginal discs. Right: Higher magnification of boxed areas in left-hand panels. Arrowheads indicate furrows with RNA expression. *: RNA in wing pouch. (C). Actin staining of wing imaginal discs. The driver line alone exhibits the stereotypical wild-type folding pattern of the disc. *cta* RNAi or *mist* RNAi results in misfolding of the tissue. Overexpressing Mist or Fog leads to minor or major disruptions in tissue folding, respectively. The Fog OE disc has a gap between the two asterisks. Reducing *mist* levels in discs with overexpressed Fog greatly reduces the level of disc misfolding. White arrows: proper folds; yellow arrows: misfolding. (D). Percentages of wing imaginal discs and adult wings with morphogenetic defects. n= number of imaginal discs or adult wings scored for...
each condition. (E). Individual wings from Driver only, Fog OE, and mist RNAi Fog OE adults. Scale bar A-C: 100µm.
Figure 3.3 *mist* expression is upregulated in the ventral furrow downstream of Snail. (A). Fluorescent *in situ* hybridization for *mist* RNA (red) in wild-type embryos
counterstained for DNA (white). There is a low ubiquitous maternal contribution of mist RNA. At cellularization and into VF formation, mist RNA is restricted to a ventral stripe of cells. Bottom left is mist channel alone from bottom right image. Anterior is to the left. lat: lateral view; vent: ventral view. (B). Mist immunofluorescence in VF. During apical constriction Mist protein is localized apically and is enriched in the presumptive mesodermal cells. Top: Grazing apical section, insets show enlarged image of boxed area; bottom: cross section. Membranes are marked with Neurotactin (magenta) and Mist is in green. Arrows point to areas of Mist enrichment. (C). In situ hybridization to mist RNA in snail mutant embryos. Corresponding stages of wild-type embryos are shown in insets. (D). Percentages of stage 5-8 embryos with PMG only or PMG and VF localization of mist RNA. n=number of embryos scored for each condition. Scale bar A, C: 100μm; B: 25μm.
Figure 3.4 Mist depletion causes defects during VF invagination. (A). Moesin-GFP expressing embryo injected with control dsRNA has a normal, straight ventral midline (flanked by dotted lines). (B). *fog* hemizygous mutant exhibiting improperly internalized mesoderm. (Arrowheads indicate mesoderm on embryo surface). (C). *fog* dsRNA injected embryo displays a wide swath of mesoderm on the exterior surface (outlined with dotted line). (D). *mist* dsRNA injected embryo with minor morphological defects in the ventral midline.
(arrows). (E) *mist*-injected embryo with improper invagination of individual mesodermal cells, or (F) with a large area of mesoderm present on the exterior of the embryo. Cell membranes (green) are outlined with either Moesin-GFP (A, C-F), or Neurotactin (B). Mesoderm (magenta) is stained for Twist (A-F). (G) Quantification of morphological defects in dsRNA injected embryos and *fog* hemizygous mutants. n=number of embryos scored for each condition. (H) Model for Mist regulation and function within Fog signaling pathway. Colored boxes denote classification of Fog pathway components. Blue: Transcription factor, Yellow: Secreted protein, Red: Transmembrane protein, Green: Cytoplasmic protein. I. Schematic of *mist* and *fog* RNA expression in cellularizing embryos. Areas of overlapping expression are where the VF and PMG invaginate. Scale bar A: 100µm.
Figure S3.1 Mist is expressed in S2R+, but not S2 cells. (A). Western blot of S2R+ cell lysates for Mist, after control or mist dsRNA treatment. (B). Mist is expressed in S2R+ cultured cells, but absent from S2 cultured cells. α-tubulin used as loading control.
Figure S3.2 Mist is temporally and spatially localized during VF formation. Series of cross-sectioned embryos over time show that Mist is specifically enriched at the apical ends of cells that are actively undergoing apical constriction during VF formation. Brackets designate ventral furrow. Arrows and arrowheads indicate areas of Mist enrichment. Scale bar: 20 µm.
Figure S3.3 mist is expressed in embryos depleted of twist. (A). *mist in situ* hybridization in wild-type embryos shows mesodermal *mist* expression after VF and PMG invagination. (B). *mist in situ* hybridization in embryos from *twist* heterozygous parents shows both VF and PMG expression. vent: ventral view; lat: lateral view. Scale bars: 100µm.
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**Table 3.1 List of genes targeted with dsRNAs in cell culture screen.** Each was targeted by at least one dsRNA. Mist is highlighted in red.
Chapter IV

*Drosophila* Ric8 interacts with the Gα12/13 subunit, Concertina, during activation of the Folded gastrulation pathway

Preface

This work has been submitted for review for publication. All of the experiments were designed by my advisor Dr. Stephen Rogers, and myself. All experiments were performed by me. The manuscript was edited by Dr. Stephen Rogers and myself.

Abstract

Heterotrimeric G proteins are composed of α, β, and γ subunits and are activated by exchange of GDP for GTP on the Gα subunit, a reaction that leads to the separation of Gα and Gβγ to allow targeting of downstream effectors. Canonically, Gα is stimulated by the guanine-nucleotide exchange factor (GEF) activity of ligand-bound G protein coupled receptors (GPCRs). However, Gα subunits may also be activated in a non-canonical manner by members of the Ric-8 family, cytoplasmic proteins that also act as GEFs for Gα subunits. We have 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* Gα12/13 subunit, Concertina (Cta), is necessary to trigger acto-myosin contractility during gastrulation events. *Ric-8* mutants exhibit similar defects to *Cta* mutants in this process. Here we describe 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 a physical interaction with Cta, and that Ric-8 preferentially interacts with inactive Cta and directs its localization within the cell. We also used this system to conduct a structure-function analysis of Ric-8 and identified key residues required for interaction with Cta and cellular contractility. Our data demonstrate that evolutionarily conserved residues facilitate Ric-8/Cta interaction downstream of receptor activation to localize inactive Cta.

**Introduction**

G protein coupled receptors (GPCRs) are a highly conserved family of transmembrane receptors that have 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 7 α-helices and are oriented with their N-termini towards the extracellular space, their C-termini inside the cell, and three inter-helical 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 a 1:1:1 stoichiometry with a β and γ subunit. These three proteins form a tightly-bound inactive heterotrimer when Gα is in its GDP-bound state. Activation of the GPCR induces a conformational change that triggers its guanine nucleotide exchange factor (GEF) activity for Gα causing Gα to exchange bound-GDP for GTP. Active Gα-GTP dissociates from the Gβγ heterodimer and both species are able to regulate downstream effector molecules, such as ion channels and enzymes that produce second messengers. Gα subunits have an intrinsic GTPase activity that hydrolyzes GTP to GDP causing the complex to reform into its inactive state. This cycle of activation and inactivation may be modulated by accessory factors, such as RGS (regulator of G protein signaling) proteins that accelerate the rate of GTP hydrolysis.
by Ga subunits (for review see1-3). Thus, although the core regulatory component in heterotrimeric G protein signaling is the nucleotide-bound state of the Ga subunit, the activities of these molecules are affected by accessory factors that may reflect various signaling inputs into the pathways.

At the biochemical level Ric-8 has been found to act as a non-canonical GEF for multiple families of Ga subunits4,5 by associating with Ga-GDP, often complexed with a GDI (guanine-nucleotide dissociation inhibitor), such as the Go-Loco repeat containing family of proteins (for example GPR1/2 in C. elegans and Pins in Drosophila melanogaster). Ric-8 binding inactive Ga facilitates GDP release and promotes the formation of a transient nucleotide-free state, which allows Ga-GTP exchange by cytosolic excess of GTP4. Additionally, Ric-8 has been shown to bind to and drive dissociation of Ga-GDP complexed with a GDI, subsequently freeing Ga to engage other effectors6-8. Recently, Ric-8 was identified as a chaperone involved in the biosynthesis of mammalian Ga subunits and their subsequent localization to the plasma membrane9. Thus, it is evident that Ric-8 regulates multiple aspects of Ga function.

A growing body of evidence 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 see10). Ric-8 is a highly conserved cytosolic protein that was originally identified in a screen for proteins required for Gaq signaling in the C. elegans nervous system11. Since then, Ric-8 has been implicated as a regulator of signaling in events as diverse as fungal pathogenesis and development12,13 to modulation of mammalian vision, taste, olfaction and bone formation14-23. 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 Ga family members to establish the position of the mitotic spindle through modulation of pulling forces along the anterior-posterior axis.\(^{24-27}\). Similarly, in *Drosophila*, Ric-8 functions through Ga to align the mitotic spindle in both neuroblast and sensory organ precursor cells.\(^{7,28,29}\) Recent findings also show that Ric-8 is important for spindle alignment in asymmetric cell division in mammalian tissue culture.\(^{30}\)

In addition to spindle positioning, Ric-8 regulates cytoskeletal rearrangements during dorsal ruffle formation via Ga\(_{13}\) in mammalian tissue culture.\(^{31}\) These data demonstrate that Ric-8, through its interaction with Ga subunits, functions to regulate diverse processes during G protein signaling events, including cytoskeletal behavior.

*Drosophila* gastrulation has proven to be a powerful model system 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.\(^{32}\) 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.\(^{33,34}\)

Genetic analysis of this pathway has identified several components that are thought 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* Ga\(_{12/13}\) subunit, Concertina (Cta).\(^{35,36}\) Mutations in the Gβ13F and Gγ1
subunits exhibit gastrulation defects and, presumably, comprise the βγ subunits of the heterotrimer along with Cta\textsuperscript{28}. Cta activates a guanine nucleotide exchange factor, RhoGEF2, relocating RhoGEF2 from the plus end tips of growing microtubules to the cortex, where it is docked by its interaction with a transmembrane protein, T48\textsuperscript{37-39}. RhoGEF2 then activates the small G protein Rho1 which activates myosin II at the apical domain via Rho kinase (Rok), thus producing contraction\textsuperscript{33,38,40}. Mutations in any Fog pathway component interfere with the timing or execution of normal gastrulation. This pathway has been implicated in epithelial remodeling during later stages of development, as well\textsuperscript{38}. \textit{Drosophila} gastrulation events are highly analogous to epithelial remodeling in other multi-cellular organisms, most notably neural tube formation in the developing vertebrate embryo, and downstream signaling components are conserved between invertebrates and vertebrates\textsuperscript{41}. Thus, we are using the \textit{Drosophila} Fog signaling pathway as a model system to investigate general mechanisms of signaling during tissue remodeling.

Given the central importance of Cta to \textit{Drosophila} gastrulation, it is a useful model to study potential interactions between Ric-8 and Ga12/13-class subunits. Two previous studies showed that Ric-8 mutants exhibited gastrulation defects that resembled Cta loss-of-function\textsuperscript{7,28}, however, the mechanistic details through which Ric-8 functions in this process remain to be determined. Here, we examined the role of Ric-8 signaling in the Fog pathway using a novel cell-based assay for Fog-induced cellular contractility. We used RNAi to show that Ric-8 is necessary for Fog signaling and that it functions within the pathway at the level of Cta. Ric-8 directly interacted with Cta and exhibited higher affinity for inactive Cta mutants (GTP-free). We present biochemical data that shows Ric-8 preferentially binds and specifically acts, to localize inactive Cta downstream of Fog/GPCR signaling. Finally, by
mutating electrostatic amino acids conserved across species we identified specific residues within Ric-8 required for Cta function and/or establishing a binding interface between the two molecules. Based on our results we propose a model wherein Ric-8 acts downstream of Fog pathway activation to localize/scaffold inactive Cta, potentiating Fog signaling to drive persistent cellular constriction.

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\textsuperscript{42}. S2 cells were maintained in SF900 SFM (Invitrogen, Carsbad, CA) and S2R+ cells in Sang’s and Shield’s medium (Invitrogen) supplemented with 5% heat-inactivated FBS (Invitrogen). S2 and S2R+ cells were transfected with 2µg/µL of DNA using the Amaxa nucleofector system with Kit V using program G-30 (Lonza, Basel, Switzerland), or with Fugene HD (Promega) (except the Mito-tag constructs where 1µg/µL concentration of 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 days with 15µg/ml of dsRNA. dsRNAs were produced using Promega (Madison, WI) Ribomax T7 kit according to instructions. Primers used for dsRNA synthesis are as follows and are all preceeded by the T7 sequence (5’-TAATACGACTCACTATAGG-3’). Control-fwd: 5’-

TAAATTGTAAGCGTTAATTTTTG-3’ and Control-rev: 5’-

AATTTCGATATCAAGCTTATCGAT-3’ to amplify a region from the pBluescript
plasmid; Cta-fwd: 5’- TGACCAAATTAACTCAAGAAGAAT-3’, Cta-rev: 5’-
TTCCAGGAACTTATCAATCTCTTTTG-3’; Cta 5’UTR-fwd: 5’-
ATATACAGGCAAAAAATTATTATCACC CGCTTGTGTTCGC-3’, Cta 5’UTR-Rev: 5’-
CGCTGGCAAGCCACGCCTTGATGCTCGCASTTCTTCTATA-3’; RhoGEF2-fwd: 5’-
ATGGATCACCCATCAAATCAAAAAACGG-3’, RhoGEF2-rev: 5’-
TGTCCCCGATCCCTATGACCCTAAAGGC-3’; Rho-fwd: 5’-
GTA AAAACTTGCTTTCTGATGTCT-3’, Rho-rev: 3’-
ATCTGGTCTTCTTCTCTTTTTGA-3’ Ric-8-fwd: 5’-
GCAGGCGCCAGTGCCTGCGGC-3’, Ric-8-rev: 5’-CCGGAGATGT TTGTGTCAGCA-3’;
Ric-8 5’UTR-fwd: 5’-GCAAAGGCTGC GTCAC-3’, Ric-8 5’UTR-rev: 5’-
GTCGCCAACC GGTGCG-3’; Ric-8 3’UTR-fwd: 5’ GTATTGCGGGATCTG-3’, Ric-8
3’UTR-rev: 5’-GGCGTGTTATTTAA-3’.

**Contractility Assay**

S2R+ cells were resuspended and plated on Concanavalin A (MP Biomedicals) coated coverslips, allowed to spread for 1-3 hours, then treated for 10 minutes 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 1mM CuSO4 for 48 hours. Cells were then pelleted, and the supernatant was concentrated using protein concentrators (Millipore, Billerica, MA,) to approximately 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 was diluted 1:1 with Schneider’s media before application. For each experiment, we scored 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 standard error.

**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-V5/His (Invitrogen). Construction of N-terminally Myc-tagged Cta, and C-terminally GFP-tagged RhoGEF2 constructs was described previously. The dual expression constructs were created by sub-cloning Myc-Cta constructs into pMT-V5-His containing a second transcriptional unit for membrane-mCherry marker containing the sqh promoter and 3’ untranslated region. 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 (EST clone LD15203) and introduced a 5’ EcoRI site, 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 pEntr 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 eGFP tag. All mutagenesis was performed on this construct using KOD Xtreme Hot Start Polymerase (Novagen, Gibbstown, NJ). Mitochondrial localization of Ric-8 was achieved by N-terminally attaching *Listeria*
*monocytogenes* ActA residues 310-338. 

### 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 incubated with Protein A beads. GFP-binding protein was covalently linked to the beads using 20mM dimethylpimelimidate (DMP) (Sigma-Aldrich, St. Louis, MO). Before use in IP experiments beads were washed with IP lysis buffer (50 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 1mM Dithiothreitol, 0.5% Triton X-100, 2.5 mM Phenylmethylsulfonyl fluoride, and Complete EDTA-Free Protease Inhibitor Cocktail [Roche, Indianapolis, IN]).

S2 cells used for IPs were transfected (see above) and induced 24 hours later with 1mM CuS04. The following 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 minutes. SDS-PAGE sample buffer was also added to input samples, and boiled for 10 minutes. Samples were run on SDS-PAGE gels, and transferred to nitrocellulose membranes for western blotting using anti-Myc9e10 (DSHB, Iowa City, IA), and anti-GFPJL8 (Clontech). For immunoblot quantitation the pulldown:input ratios were determined using densitometry using ImageJ software (National Institutes of Health, Bethesda, MD) on scanned film images. All immunoblot quantitation was performed at least 3 times on 3 distinct blots. Error bars represent SEM.

### Microscopy
Cells were plated onto ConA coated coverslips and prepared for imaging as previously described\textsuperscript{42}. Antibodies used for immunofluorescence were, anti-phospho-myosin light chain 2 (Ser19) (Cell Signaling Technology, Inc., Danvers, MA), anti-Ric-8 (gift from William Chia), anti-Fog (gift from Eric Wieschaus), anti-dsRed (Clontech), anti-GFPJL8 (Clontech), anti-Myc9e10 (DSHB), anti-DM1α and Alexa Fluor 564 Phalloidin (Invitrogen). All cells were imaged using a CoolSnap HQ CCD camera (Roper Scientific) mounted on an Eclipse Ti-E and driven by Nikon Elements software (Nikon, Melville, NY) except cells in Figure 4.4 which were imaged using a TIRF system (Nikon) mounted on an inverted Ti-E microscope using an Andor-Clara Interline camera (Andor Technology, Belfast, UK) and driven by Nikon Elements software.

Results

Reconstitution of Fog-stimulated cellular contractility in a cultured cell model

In order to study the effect of Fog signaling on cell morphology, we developed a cell culture system to allow us to replicate \textit{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. 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\textsuperscript{35}. 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 epithelia\textsuperscript{33}. To
test whether Fog is secreted from S2:Fog-Myc cells we induced its expression with copper sulfate for 48 hours, collected the conditioned medium, and concentrated it ~20-fold. An affinity-purified antibody against the N-terminus of Fog recognized a single protein with a molecular weight of ~150 kD on immunoblots of conditioned medium from induced S2:Fog-Myc cells and the same sized band was also recognized by a monoclonal anti-Myc antibody. Neither antibody recognized the protein in conditioned medium collected from untransfected S2 cells (Figure 4.1A). 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\(^\text{37}\). We, therefore, used this read-out to test the ability of S2 cells, S2R+ cells, and several immortalized lines derived from imaginal discs, to respond to Fog. S2R+ cells are a sub-line derived from S2 cells that express receptors not found in S2 cells\(^\text{45}\). Neither S2 cells nor the other epithelial lines we tested changed their shape in response to Fog perfusion (data not shown). However, S2R+ cells exhibited a robust morphological response upon perfusion with Fog. S2R+ cells adopt a flattened, discoid morphology when plated on concanavalin A-treated coverslips. Within 10 minutes 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 4.1B). One of the downstream effects of Rho pathway signaling is activation of non-muscle 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 P-RLC (phosphorylated-regulatory light chain) antibodies revealed an overall increase in phosphorylation, along with a dramatic incorporation of myosin II into acto-myosin purse string structures (Figure 4.1C). 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 prior to Fog treatment. RNAi targeting Cta, RhoGEF2, or Rho prevented cellular constriction following Fog treatment (Figure 4.1B, D) as did pretreatment of S2R+ cells with the Rho-kinase small molecule inhibitor Y-27632 (data not shown). Previous work revealed that embryos mutant for the beta subunit, β13F, and the gamma subunit, γ1, exhibited gastrulation phenotypes similar to Cta mutants28. We introduced RNAi targeted to these subunits, predicting they comprise the heterodimer that associates with Cta, and found these treatments blocked Fog mediated contractility (Figure 4.1D). Thus, we conclude that treatment of S2R+ cells with Fog activates the identical signaling pathway utilized 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 dsRNAs to target the coding region or the 5' and 3' untranslated regions of the Ric-8 mRNA and found that each effectively depleted Ric-8 from S2R+ cells (Figure S4.1). When tested in the contractility assay, Ric-8-depleted cells were unable to contract following treatment with Fog (Figure 4.1B, 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-GFP in cells depleted of endogenous Ric-8 (Figure 4.2 A, B). Ectopic overexpression of Ric-8-GFP was not
sufficient to induce contractility in the absence of Fog, however (data not shown). 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\textsuperscript{46}, in S2R+ cells is sufficient to trigger contractility in the absence of Fog (Figure 4.4A). However, expression of Myc-CtaQL in S2R+ cells depleted of endogenous Ric-8 does not drive cellular constriction (Figure 4.2C, D). To verify 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 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 Ga12/13 family members (\textsuperscript{47} and personal communication with Ted Meigs). Using these constructs we show that levels of ectopic Myc-Cta are not affected by Ric-8 depletion (Figure 4.2E). In the converse experiment, S2R+ cells depleted of endogenous Cta and over-expressing Ric-8-GFP do not constrict upon Fog treatment. However, over-expression of RhoGEF2, which is directly downstream of Cta, in either Cta- or Ric-8-depleted cells is sufficient for cellular constriction (Figure 4.2 C, D). Therefore, Ric-8 functions upstream of RhoGEF2 implicating a role for Ric-8 at the level of either the putative GPCR, Cta, or the \( \beta \gamma \) subunits. It has been well documented, in \textit{Drosophila} and other systems, that Ric-8 does not
interact with Ga when it is complexed with its βγ subunits\textsuperscript{4,7,28}. To date there is no evidence that Ric-8 interacts with a receptor in receptor dependent activation of Ric-8, however this possibility has not been directly tested.

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

It is probable that in our system Ric-8 interacts with the Ga. Using immunoprecipitation we tested 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 own 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 (Figure S4.2 A, B), thus, we used this construct as a proxy for endogenous protein. We transfected Myc-Cta into S2 cells, immunoprecipitated with an anti-Myc monoclonal antibody, and found that endogenous Ric-8 co-precipitated (Figure 4.3A). As expected from our rescue experiments, Ric-8-GFP also co-immunoprecipitated with Myc-Cta (Figure 4.3B). Thus, Ric-8 and Cta are able to interact in *Drosophila* tissue culture cells.

Given that Ric-8 functions as a GEF for Ga 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\(_{GA}\). Overexpression of Myc-Cta\(_{GA}\) in S2R\(^+\) cells depleted of endogenous Cta inhibited Fog-mediated contractility (Figure S4.2B). To determine whether nucleotide association affected Ric-8 interaction we co-transfected Ric-8-GFP together with wild-type Myc-Cta, Myc-Cta\(_{QL}\), or Myc-Cta\(_{GA}\) 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 pulldowns performed with constitutively inactive Myc-Cta\textsubscript{GA} and constitutively active Myc-Cta\textsubscript{QL} showed greater and lesser binding affinity to Ric-8 respectively, as compared to wild-type Myc-Cta (Figure 4.3B, 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 plays a role in localizing Ga\textsubscript{i} to the cortex in *Drosophila* neuroblasts and sensory organ precursor cells\textsuperscript{7,28,29}; therefore, we wanted to test the hypothesis that Ric-8 also functions to localize Cta and determine whether its nucleotide state plays a role in this interaction. Our strategy was to co-express Myc-Cta along with a version of Ric-8-GFP that was mis-targeted to mitochondria by tagging it with residues 310-338 of *Listeria* ActA (Mito-Ric-8-GFP). When wild-type Myc-Cta or Myc-Cta\textsubscript{QL} was co-expressed with Mito-Ric-8-GFP, neither Cta construct exhibited discrete localization (Figure 4.4A). However, co-expression of Myc-Cta\textsubscript{GA} and Mito-Ric-8-GFP resulted in robust accumulation of Cta to the mitochondria (Figure 4.4A). These data indicate that Ric-8 acts to selectively localize inactive Cta within the cell.

Our results suggest a mechanism in which Ric-8 localizes 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 4.4B). Together, these findings clearly demonstrate that Ric-8 binds to, and can localize, Cta based on its nucleotide state. They further suggest that
Ric-8 is required for Cta cycling through its nucleotide state downstream of Fog pathway activation.

**Ric-8 binds to Cta through an interface of conserved residues**

While previous work has 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. Armadillo repeats adhere to a canonical fold and global elongated structure. The Olate group recently used molecular modeling to construct an *in silico* model of the Ric-8 structure. Based on sequence conservation of Ric-8 across species (Figure S4.3), we made fourteen 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 4.1, Figure 4.5A). These mutations consisted of charge reversals, with the intent to not only diminish, but repel an interaction with Cta. We co-expressed the Ric-8-GFP mutants with the three Cta variants (Myc-Cta, Myc-CtaQL, 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 below. The mutants span the length of the protein and are ordered in succession from N-terminus to C-terminus. While Ric-8-GFP robustly bound Myc-CtaGA, it exhibited lower affinity interactions with Myc-Cta and Myc-CtaQL (Figure 4.2B, C) and the Ric-8-GFP pulldown data displayed a high degree of variance with those two mutants making it difficult to determine the binding activity of Ric-8-GFP with Myc-Cta and Myc-CtaQL. Therefore, we focused our analyses on pulldowns performed with Myc-CtaGA.

We identified four Ric-8-GFP mutants (1, 9, 10, and 13) that had significantly reduced
binding to Myc-CtaGA by testing the ability of the cluster mutants to interact with Cta (Figure 4.5B, C and Table 4.1). 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 (Figure S4.4 and Table 4.1). 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 4.6A, B and Table 4.1). The remaining mutations had moderate to no effect on binding when co-expressed with Myc-CtaGA (Table 4.1). Although not statistically significant, both Myc-Cta and Myc-CtaQL variants exhibited decreased binding to mutant 1, and moderate-high binding to mutants 9, 10 and 13. These latter three mutants exhibited decreased interaction with Myc-CtaGA, however, they displayed increased affinity for Myc-Cta and Myc-CtaQL (Figure 4.5B, C and S5A-D). The fact that these mutations severely inhibited binding to Myc-CtaGA but did not strongly affect Myc-Cta and Myc-CtaQL binding suggests a model in which the C-terminal region of Ric-8 may be important for the high affinity binding seen specifically in the Ric-8/GTP-free Cta interaction, while the N-terminal residues are 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 treated the cells with Fog and assessed the ability of the transfected cells to rescue constriction. Of the fourteen clustered point mutants tested in the binding assay, six mutants: 1, 6, 7, 8, 9, and 13, failed to rescue Fog-induced constriction (Figure 4.5C and Table 4.1). Testing the individual point mutants within the cellular constriction assay revealed a similar pattern in the residues that prevented pathway activation to the individual residues deficient
in binding Cta in the pulldown assay (Figure 4.6A, B and Table 4.1). This suggests that residues R71, R75, R414, D484, T485, and E487 within Ric-8 are important for establishing a binding interface, as well as for successful G protein signaling.

Finally to determine if Cta localization was affected by binding mutants with low binding affinity 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 co-localization of the two proteins. Myc-CtaGA did not co-localize with Mito-Ric-8-GFP mutant 1, while, surprisingly, co-localization of Myc-CtaGA was seen with Mito-Ric-8-GFP mutants 9, 10 and 13 (Figure 4.4C). Hypothetically, mutants 9, 10, and 13 could be affecting the binding kinetics of Myc-CtaGA, which may account for the co-localization of the two in our mis-targeting 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 dramatically decreased contractility in Fog-treated S2R+ cells; however, mutant 10 rescues contractility to wild-type levels (Figure 4.5B, C and Table 4.1). It is probable 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 4.5B, C and Table 4.1) but have diminished ability in activating the pathway (Figure 4.5C and Table 4.1), suggesting that these residues may have an important functional role outside of binding. The majority of the residues that affected both binding and functional pathway rescue map to a conserved face of the Arm repeats, and show a potential clamp-like binding of Ric-8 to Cta (Figure S4.4). Our findings suggest that residues within the inner face of the N-terminus (R71 and R75) of Ric-8 facilitate global interaction with Cta, while residues found in the C-terminus (R414, D484, T485, and E487)
modulate binding based on nucleotide specificity. While previous work has found residues important in Gα subunits for facilitating interaction with Ric-8, here we identify some of the first residues found to be important for Ric-8 binding to a Gα.

Discussion

In this study we conducted an in-depth analysis of the interaction between Ric-8 and Cta downstream of the Fog-activated morphogenetic pathway. We established a novel assay for testing potential Fog pathway components and found that in Drosophila tissue culture Ric-8 is required for pathway activation and that Ric-8 not only binds the Gα12/13, Cta, but also preferentially binds the inactive, GTP-free version of Cta. We defined a role for Ric-8 as an escort/scaffold for inactive Cta by using artificially induced localization of Ric-8 to the mitochondria. Upon Ric-8 translocation we found that inactive Cta co-localized with ectopically localized Ric-8, while the cellular localization of wild-type and constitutively active Cta were unaffected. Additionally, when Ric-8 was mis-targeted to the mitochondria, cells were impaired in their ability to constrict in response to Fog application. We identified evolutionarily conserved residues within Ric-8 important for 1) establishing a binding interface between Ric-8 and Cta, 2) recognition of nucleotide specific variants of Cta, and 3) successful G protein signaling downstream of Fog pathway activation. These data establish a role for Ric-8 in Cta localization and attenuation of pathway signaling through Cta.

Our novel cell-based assay is ideal for examining Fog-induced activation of the Rho pathway, due to the ease in which we are able to deplete cells of specific proteins using RNAi, the rapidity of screening multiple genes simultaneously, and the ability to visualize pathway activation using a simple microscope-based examination. This assay opens
numerous possibilities for the identification of other pathway components, including the unidentified GPCR involved in transduction of the Fog signal, as well as investigation of general cellular functions such as mechanochemical force production and regulation of the acto-myosin cytoskeleton. Additionally, although not highlighted in this study, we are able to view Fog-induced cell morphological changes in real-time. This allows for further investigation of pathway components that specifically affect the kinetics with which cells are able to respond to Fog, and/or the longevity and persistence of pathway activation.

The fundamental importance of Ric-8 for productive $G\alpha$ signaling has been well documented. Ric-8 plays a key role in modulating the behavior of $G\alpha$ subunits in receptor-independent and dependent signaling events during asymmetric cell division, neurotransmitter release and maturation, both vertebrate and invertebrate gastrulation, and numerous other developmental processes across species$^{10}$. Due to its role in establishing asymmetry in dividing cells and subsequently controlling cell proliferation rates, Ric-8 has become of interest to the field of cancer biology$^{51,52}$. Our model cell culture system provides a streamlined approach for further investigation into parsing out the complicated signaling networks involved in establishing these disease states.

The role of Ric-8 as a non-canonical GEF has been established in a variety of biological systems$^{10}$. Our efforts to directly determine if Ric-8 was acting as a GEF for Cta were thwarted as our efforts to purify both Cta and Ric-8 resulted in insoluble, or aggregated protein fractions. If Ric-8 is acting as a GEF one might predict that overexpression of Ric-8 in our cellular assay would drive constriction, however, overexpression of Ric-8 in S2 or S2R+ cells does not elicit Fog pathway activation. The reason for this may be explained by data showing that Ric-8 is unable to interact with an intact heterotrimeric complex consisting
of Gaβγ⁴, and that Ric-8 and GPCRs potentially compete for the same binding sites on Ga⁵⁰. Therefore, it is possible that, in the absence of Fog-mediated receptor activation, Ric-8 is unable to bind Ga to stimulate (potentially as a GEF) downstream pathway components.

Previous work has implicated Ric-8 as a chaperone during Ga biosynthesis to stabilize nascent protein production, and in turn as an essential factor in Ga membrane targeting. This function of Ric-8 has been shown to affect the stability of all classes of mammalian Ga subunits⁹,⁵⁰. Given the necessity of Ric-8 in mammalian systems for Ga stabilization and membrane localization it is likely that Ric-8 acts similarly in Drosophila, as evidenced by the mis-targeting of Ga, and Cta, in the absence of Ric-8, to the cortex of the epithelium of Drosophila embryos⁷,²⁸,²⁹,⁵³. However, unlike Ga⁷, levels of Cta are not decreased in whole cell lysate in the absence of Ric-8 (Figure 4.2E); additionally, we see some rescue in cells depleted of endogenous Ric-8, overexpressing constitutively active Cta (Figure 4.2C), indicating that at least a small amount of Cta is localized correctly and functional. Therefore, while initial localization⁵³ of Cta to the plasma membrane and later localization of Cta is dependent on Ric-8, stabilization of Cta is independent of Ric-8 function (this study).

Many molecules are involved in the complex signaling networks downstream of receptor activation, and all of these components must be precisely regulated to interact and communicate in a specific way for effective signal transmission. Though signaling nodes involving GPCRs, Ga subunits, GDIs and Ric-8 have been extensively studied there is little known about the structure of Ric-8 and how it interacts with Ga subunits during these events. We used a predicted model⁴⁸ 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 provided information into the structural components important for comprising the Cta/Ric-8 binding interface, as well as the minimal mutations necessary to abrogate Fog-induced pathway activation.

We identified four cluster mutations, mutants 1, 9, 10 and 13 (Figure S4.4), that inhibited Myc-CtaGA binding, of which three: 1, 9, and 13, also failed to rescue constriction to wild-type levels. Of these four mutants we found that only mutant 1 (located in the N-terminus of Ric-8) had an inhibitory effect on binding to wild-type, constitutively active and constitutively inactive versions of Cta, while mutants 9, 10 and 13 (located in the C-terminus of Ric-8) were only deficient 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 G\(_{\alpha_q}\)\(^{54}\). In accordance with these data, we suggest that the residues in mutant 1 are important for non-nucleotide specific Cta interaction, while the residues in mutants 9, 10 and 13 confer nucleotide specific recognition of Cta.

Several mutants had effects in only the binding or contractile assay. Mutant 10 inhibited binding, while mutants 6-8 prevented Fog-induced constriction. Mutant 10 was able to rescue cellular constriction but exhibited decreased binding to Cta, implying 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 mutant 13 (Figure 4.5A). Mutants 6-8 are capable of binding Cta, but not rescuing Ric-8 function downstream of pathway activation. While the function of mutant clusters 6-8 is unclear, it is tempting to hypothesize that the region encompassing mutants 6-8 is a potential site for Ric-8 GEF activity. Our data is the first evidence of specific residues within Ric-8 facilitating interaction with a Ga.

In the early dividing C.elegans embryo\(^{26}\), Drosophila melanogaster neuroblasts and
epithelium\textsuperscript{7,28,29} and several mammalian tissue culture cell lines\textsuperscript{9,30,31} Ric-8 has been found to localize Ga subunits to the plasma membrane. Our data suggest there is an additional level of regulation of Ga localization that is dependent on the nucleotide-bound state of Ga. We have identified a cluster of residues that may facilitate this interaction with Cta. Clustered Ric-8 mutants, deficient in binding Cta\textsubscript{GA} in immunoprecipitation assays, when tagged with a sequence directing them to the mitochondria had varying effects in their ability to ectopically localize Cta\textsubscript{GA}. Mito-Ric-8 mutant 1 did not recruit Cta\textsubscript{GA} to its ectopic location at the mitochondria, while Mito-Ric-8 mutants 9, 10, and 13 triggered mis-localization of Cta\textsubscript{GA} to the mitochondria (Figure 4.4C). Interestingly, mutants 9, 10 and 13 exhibited preferential binding to constitutively inactive Cta, Cta\textsubscript{GA}, but not wild-type nor constitutively active Cta, Cta\textsubscript{QL} (Figure 4.5B, C and S5). This implies that these residues of Ric-8 may be important in conferring temporally regulated nucleotide specific recognition sites for Cta.

Based on our characterization of Ric-8, we propose the following model (Figure 4.4F). Ric-8 acts to initially chaperone the folding of Cta, allowing Cta, Gβ13F, and Gγ1 to form a complex which is then transported to the plasma membrane. Upon 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 facilitates stabilization of a nucleotide-free version of Cta. This allows Cta to bypass destruction and be re-inserted into the Fog pathway to activate downstream targets.

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on the manuscript. We thank D. Bosch and D. Siderovski for collaborative efforts to purify *Drosophila* Ric-8 and Cta. We thank J. Olate for the xRic-8 PDB file. We thank G. Rogers, W. Chia and E. Wieschaus for reagents. This work was supported by grants from the NIH (RO1-GM081645 to SLR) and the Arnold and Mabel Beckman Foundation (Beckman Young Investigator Award to SLR). KAP was supported by funding from the Lineberger Comprehensive Cancer Center.
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Figure 4.1 Recapitulation of Fog signaling in S2R+ cells. (A) Fog-Myc is secreted into the medium of a stable cell line expressing the construct, but not by un-transfected control S2 cells. Fog-Myc is recognized by anti-Myc and anti-Fog by immunoblot. (B) S2R+ cells undergo cellular shape changes in response to ectopic Fog application. RNAi-mediated depletion of Cta or Ric-8 prevents Fog-induced cellular constriction. Scale = 10 µm. (C)
Fog-induced S2R+ contraction is accompanied by an increase in active phosphorylated non-muscle Myosin II (pRLC). S2R+ cells were treated with either control or Fog containing media and stained for actin (red), pRLC (green), and DNA (blue). Scale: 100 µm. (D) S2R+ cells lose their responsiveness to Fog following RNAi against known pathway components, as well as Ric-8. Percentage of cells constricting in response to Fog was measured within a population of cells (±SEM).
Figure 4.2 Ric-8 regulates the function of Cta within the Fog signaling pathway. (A) Expression of Ric-8-GFP, but not GFP alone, rescues the ability of cells depleted of endogenous Ric-8 to respond to Fog. Scale = 20 µm. (B) The number of GFP or Ric-8-GFP transfected cells within a population depleted of endogenous Ric-8 were scored for their ability to contract in response to Fog (±SEM). (C) Cells depleted of endogenous Ric-8 or Cta
were transfected with constitutively active Cta (CtaQL), RhoGEF2-GFP or Ric-8-GFP+Fog treatment. Their ability to drive constriction was quantified as a percentage of the number of cells contracting within the population (±SEM). (D) Summary chart illustrating the epistatic relationship of Ric-8 in the Fog pathway. Transfected DNA and targeted dsRNA are indicated. (+) represents that ≥15% of transfected cells within a population constricted, (−) represents that ≤15% of transfected cells within a population constricted. (E) Cells were treated with control or Ric-8 dsRNA and transfected with a dual expression construct for both Cta (WT, constitutively inactive:GA, or constitutively active:QL) and mCherry under separate promoters. Immunoblotting revealed equal amounts of Cta in control and Ric-8 dsRNA treated cells, while anti-dsRed was used as a protein loading control and anti-Ric-8 to verify protein depletion.
Figure 4.3 Ric-8 and Cta physically interact, and Ric-8 preferentially binds the constitutively inactive Cta\textsubscript{GA}. Ric-8 binds to a lesser extent to the other two variants of Cta,
but not the control, α-actinin. (A) Cells were transfected with the three variants of Cta or α-actinin. IPs were carried out using anti-myc antibodies and probed with anti-Ric-8 and anti-Myc. (B) All cells were transfected with Ric-8-GFP and the three variants of Cta or α-actinin. IPs were performed with GFP-binding protein and probed with anti-GFP and anti-Myc. (C) Ric-8 preferentially binds to constitutively GDP-bound Cta. Quantification of IPs performed as outlined in (3B). Input: pulldown ratios were determined using quantitative densitometry, and normalized against Cta$_{GA}$. 
Figure 4.4 Ectopic localization of Ric-8 drives mis-localization of constitutively inactive Cta, and attenuates the efficacy of Fog signaling. (A) Targeting Ric-8 to the mitochondria causes CtaGA, but not Myc-Cta nor 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. Enlarged views of boxed images in Mito-Ric-8-GFP and Cta variants are shown in insets. Scale bar = 20 µm. (B) Mis-localized 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). (C) Evolutionarily conserved residues contribute to localization of CtaGA. Mito-tagged versions of Ric-8-GFP mutants that exhibit decreased Myc-CtaGA binding (see Figure 5B, C) were co-expressed with Myc-CtaGA. While mutants 9, 10 and 13 co-localized with Myc-CtaGA, mutant 1 did not. Enlarged images of boxed areas are shown in insets. Scale bar: 20 µm.
Figure 4.5 Evolutionarily conserved electrostatic residues are required for binding between Ric-8 and Cta\textsubscript{GA}. (A) Clusters of point mutants used in our screen are represented
by different colors on a model of Ric-8. (B-C) Mutant clusters within Ric-8 disrupt binding to CtaGA, and inhibit pathway activation downstream of Fog. (B) S2 cells were transfected with GFP, Ric-8-GFP or cluster Ric-8-GFP mutants and CtaGA. IPs were performed with GFP-binding protein and probed with anti-GFP and anti-Myc. (C) The pulldown:input ratios for Ric-8-GFP and Myc-CtaGA were quantified using densitometry, and normalized to Ric-8-GFP (±SEM) (black bars). S2R+ cells were depleted of endogenous Ric-8 and transfected with Ric-8-GFP and cluster Ric-8-GFP mutants, and then scored for the percentage of transfected cells constricting within the population (±SEM) (hatched bars).
Figure 4.6 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-Cta\(_{GA}\). Cells were transfected with GFP, Ric-8-GFP or individual Ric-8-GFP point mutants and Cta\(_{GA}\). IPs were performed with GFP-binding protein and probed with anti-GFP and anti-Myc. (B) Quantification of the IP experiments in 6A are presented (black bars). The pulldown:input ratios were determined using quantitative densitometry, and normalized to Ric-8-GFP (±SEM). 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). (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 prior to Cta association with β13F 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 bind either 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 re-activated by the GPCR (A) or through disassociation of GDP, which facilitates GTP binding, and subsequent pathway re-insertion directly upstream of RhoGEF2 (B).
Figure S4.1 Ric-8 is depleted by dsRNAs directed against the 5'/3' UTR of the gene. Protein levels were determined by immunoblot with anti-Ric-8 and an antibody to α-tubulin was used as a loading control.
Figure S4.2 Myc-tagged Cta functions as a proxy for wild-type and constitutively inactive Cta. (A) Expression of Myc-Cta rescues the ability of cells depleted of endogenous Cta to respond to Fog. Transfected cells were identified using an anti-Myc antibody. Scale bar: 20 µm. (B) Myc-Cta can rescue constriction in response to Fog in the absence of endogenous Cta, while constitutively inactive Myc-CtaGA cannot. Quantification shows percentage of S2R+ cells within a population transfected with Myc-Cta or Myc-CtaGA depleted of endogenous Cta able to contract in response to Fog application (±SEM).
Figure S4.3 Sequence alignment of Ric-8 across taxa reveals evolutionarily conserved residues. Residue clusters used for Ric-8 mutational analysis are highlighted in yellow. The number of the mutant cluster is indicated below its residues.
Figure S4.4 Location of individual point mutants comprising mutant clusters that strongly inhibit CtaGA binding are mapped onto a structural model of Ric-8. Note that glutamic acid-487 is buried within the predicted molecule.
Figure S4.5 Wild-type and constitutively active Cta exhibit differential binding to Ric-8 cluster mutants as compared to inactive Cta. (A-D) Similarly to Myc-CtaGA, both Myc-Cta and Myc-CtaQL are deficient in binding Ric-8-GFP cluster mutant 1. However, unlike Myc-CtaGA, Myc-Cta and Myc-CtaQL are capable of binding mutants 9, 10 and 13. (A) S2 Cells were transfected with GFP, Ric-8-GFP or cluster Ric-8-GFP mutants and wild-type Myc-Cta. IPs were performed with GFP-binding protein and probed with anti-GFP and anti-Myc. (B) Quantification of IPs presented in S5A. The pulldown:input ratios were determined using quantitative densitometry, and normalized to Ric-8-GFP (±SEM). (C) S2 Cells were transfected with GFP, Ric-8-GFP or cluster Ric-8-GFP mutants and Myc-CtaQL. IPs were performed with GFP-binding protein and probed with anti-GFP and anti-Myc. (D) Quantification of IPs presented in S5C. The pulldown:input ratios were determined using quantitative densitometry, and normalized to Ric-8-GFP (±SEM).
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**Table 4.1 Summary of data collected from Ric-8/CtaGA 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 pulldown 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 both strong affects in binding and contractility assays are high-lighted in yellow.
Chapter V

Discussion and Future Directions

Summary of presented work

The goal of this dissertation was to provide insight into the regulation and dynamics of the Folded gastrulation signaling pathway, specifically addressing the modulation of the \( \text{G}_{\alpha12/13} \) family member, Concertina. To achieve these goals we established a novel assay in \textit{Drosophila} tissue culture, utilizing the S2R+ cell line, to recapitulate Fog signaling events, as presented in Chapter II. The S2R+ system was invaluable to the research presented in Chapters III and IV of this dissertation. It allowed us to identify essential novel pathway components, establish epistatic relationships, determine localization patterns of proteins downstream of Fog pathway activation, and much more. The scope of this assay is not limited to these two projects. One could utilize this cell line to investigate several outstanding questions in the field, as well as address the general mechanisms of mechano-chemical signaling and subsequent contractile-based force production. This assay will be an essential tool for future research within our laboratory.

Using the tissue culture system we developed we were able to identify a singular GPCR, Mist, that binds to the Fog ligand, and confers Fog responsiveness to non-responsive cell lines. Additionally, we were able to transfec in modified, truncated versions of the receptor to investigate structural aspects of the protein and determine specific domains important for its function. Localization studies within the embryo revealed that Mist is transcriptionally
localized in a ventral/posterior stripe corresponding to the presumptive mesodermal and endodermal cells comprising the ventral furrow and posterior midgut, respectively. Protein localization of Mist is tightly regulated and expression is highest in cells undergoing contraction within the ventral furrow. Specific localization of Mist protein is first observed very early during ventral furrow formation, when cells start to move their nuclei basally and begin to constrict their apices, and reaches maximum expression when cells are at the peak of apical constriction and have formed a deep furrow. By the time the presumptive mesodermal cells have invaginated as a tube Mist protein is no longer present, indicating that Mist expression is very tightly spatially and temporally regulated during this gastrulation event. Mist RNAi and overexpression experiments in the *Drosophila* embryo showed that Mist is necessary for regulating epithelial folding in several different tissue types, including the Fog signaling pathways active during ventral furrow and posterior midgut (PMG) invagination, as well as in the wing imaginal disc. We found that Mist is transcriptionally regulated in the ventral furrow by the mesodermal zygotic transcription factor Snail. To our knowledge Mist is the first identified transcriptional target of Snail. However, while Snail itself is expressed in the tissue of the ventral furrow it is not expressed in the posterior midgut. This distinct patterning in Snail/Mist expression indicates that Mist expression is regulated by one or more, as yet to be determined, differing transcription factors during gastrulation. Thus, within this project, combinatorial studies using *Drosophila* tissue culture and the *Drosophila* embryo have yielded identification of a GPCR capable of binding Fog and facilitating Fog pathway activation.

The highly conserved cytosolic protein Ric-8 is a modulator of Ga dynamics and the work presented in Chapter IV establishes Ric-8 as an essential pathway component in the
Fog signaling pathway. In the absence of Ric-8 S2R+ cells are no longer able to respond to ectopic Fog application, which can be rescued with the expression of Ric-8-GFP. Ric-8 is essential in the Fog signaling pathway as Cta function is dependent on expression of Ric-8. Ric-8 preferentially binds to GTP-free Cta, and data from Ric-8 mis-targeting studies revealed that Ric-8 can localize the constitutively inactive version of Cta, Cta\textsubscript{GA}, but not the wild-type nor the constitutively active version of Cta, Cta\textsubscript{QL}. Further, we show that Ric-8 acts to localize inactive Cta downstream of Fog signaling, and is necessary for robust pathway activation. We made a series of fourteen point mutations in the \textit{Drosophila} Ric-8 protein, based on evolutionarily conserved residues found throughout species. We tested all of these mutants for 1) their ability to physically interact with Ric-8, and 2) their ability to drive Fog-induced cellular constriction in the absence of endogenous Ric-8.

Of the fourteen mutants we tested we found four that strongly inhibited binding between Ric-8 and Cta\textsubscript{GA} and six that inhibited the ability to rescue constriction in Fog-induced contractile experiments. Three mutants were found that strongly inhibited Cta binding as well as Fog induced cellular contraction. Two of these mutants (mutants 9 and 13 found in the C-terminal half of the protein) exhibited nucleotide specific recognition of Cta, while one mutant (mutant 1 found in the N-terminal half of the protein) showed non-nucleotide specific recognition of Cta. We made individual Ric-8 point mutants for each of these three clustered mutants and tested their ability to bind Ric-8 and rescue contraction. From these individual point mutants we found singular residues that negatively affected both binding and contractile assays. To connect the localization, binding, and contractility assay data we mis-targeted the Ric-8 mutants deficient in Cta binding to the mitochondria and examined their ability to co-localize with constitutively inactive Cta. Only one mutant
(mutant 1), of the three that had a strong affect in both pulldown and contractile assays, did not co-localize with ectopically localized Ric-8. We therefore propose that the N-terminal cluster mutant, mutant 1, facilitates non-nucleotide specific binding and localization of Cta; while residues found in mutants 9 and 13, found in the C-terminal half of the protein confer temporal, nucleotide specific binding and localization. Using Drosophila tissue culture we have demonstrated a role for Ric-8 in the Fog signaling pathway, further defined the mechanism of Ric-8/Gα function, and identified nucleotide specific residues important for Cta function as well as its interaction with Ric-8.

**Discussion and Future Directions**

**Chapter III**

While we have made major strides in examining modulators of the Gα, Cta, there are several aspects of the pathway that require further investigation. The identification of a GPCR, Mist, that transduces the Fog signal is a major boon to the field. Previously performed screens for zygotic genes essential for gastrulation used deficiencies that covered both the Rok and Mist genes\(^1\)\(^2\). Rok is an essential Fog pathway component and upon its depletion completely prevents invagination of the ventral furrow\(^3\). Our S2R+/Fog signaling assay was ideal for identification of Mist due to the ability to easily, and individually deplete cells of all known GPCRs, and test for abrogation of contraction in response to Fog.

Knowing the identity of Mist allows us to investigate how the receptor is regulated. Our lab is interested in addressing how GPCR signaling is regulated on a temporal level, as well as identifying the molecules necessary for receptor internalization and recycling. S2R+
cells are highly amenable to phase-contrast microscopy and time-lapse imaging. The ability to capture cells responding to Fog in real-time will address questions involving the temporal dynamics of normal Fog signaling pathway activation, and allow for investigation into molecules involved in receptor deactivation.

Although this dissertation has focused on how the Fog signaling pathway is activated, it is equally as important to understand how signaling events are terminated. Research performed by a graduate student in the Rogers Lab, Alyssa Manning, has made some headway into addressing these questions. The canonical view of receptor inactivation is that after a receptor has activated its effector Gα, a family of proteins, G protein receptor kinases (GRKs) phosphorylate a GPCR, causing recruitment of beta-arrestins and subsequent removal of receptors via clathrin coated pits. There are three beta-arrestins encoded in the Drosophila genome, two visual arrestins (Arr 1 and 2) and one non-visual arrestin Kurtz, as well as two GRKs (GPRK 1 and 2). Preliminary experiments have found that depletion of GPRK1 or Kurtz in S2R+ cells increases the percentage of constricting cells in a population downstream of Fog application, unlike GPRK-2, Arr-1 or Arr-2. Co-immunoprecipitation experiments performed in our lab have also found that Kurtz directly interacts with Mist. These results suggest that GPRK-1 and Kurtz are important signaling components involved in the deactivation of the Fog signaling pathway.

Interestingly, research has shown that beta-arrestins are able to bind non-phosphorylated GPCRS, and recently it has been found that beta-arrestins are capable of activating downstream effectors in a Gα independent manner. Further, beta-arrestins have been implicated in the activation of the small GTPase Rho, to form stress fibers in mammalian cells. Kurtz could play an, as yet, unidentified role in the Fog signaling
pathway in regulating the cytoskeleton. Overexpression and loss of function studies may yield interesting results in how Kurtz is not only involved in receptor deactivation but also force production or reorganization of the cytoskeleton.

We have shown in Chapter III that Mist is under the transcriptional activation of Snail in the ventral furrow. Snail is not expressed in the PMG\(^6\); so what then regulates Mist expression in presumptive endodermal cells? The formation of the ventral furrow and PMG utilize many of the same molecules, including Fog, Cta, RhoGEF2 and Rho. However, in the posterior midgut loss of Fog or Cta completely prevents invagination while in the ventral furrow fog or cta mutants have disorganized constriction of the presumptive mesoderm and delayed ventral furrow formation, but the furrow is still able to internalize\(^7\). This discrepancy in function highlights that while mechanically similar in function, there are different components necessary for pathway activation in each of these tissues. Overlapping patterns of zygotic transcription factors regulate gene expression prior to gastrulation. Snail expression is dependent on Twist, but Twist is expressed in the PMG while Snail is not. This is due to the anteriorly localized zygotic transcription factor Huckebein, which acts as a transcriptional repressor for Snail. In huckebein embryos the formation of the PMG is disrupted. Both Huckebein and another zygotic transcription factor, Tailless, are expressed in the presumptive endodermal cells of the posterior midgut\(^8,9\). Therefore, the best two candidates for Mist transcriptional regulation in the PMG are Tailless and Huckebein. *in situ* analysis of Mist expression in tailless and huckebein mutants could reveal if these potential regulators play a role in this process.

Chapter IV
An outstanding question involving Ric-8 in the Fog signaling pathway is whether it acts as a non-canonical GEF to activate Cta. While overexpression of Ric-8 does not drive constriction in S2 or S2R+ cells this may be due to overlapping GPCR and Ric-8 binding sites for Gα. Therefore to directly investigate GEF activity we wanted to perform GTPase exchange assays. We enlisted the help of Dr. David Siderovski’s lab, where this procedure is routinely performed. Dr. Siderovski first suggested to us that Cta may not express solubly due to its “floppy, unstructured N-terminal sequence”. He therefore provided us with an ideal sequence for an alternative N-terminally truncated (Δ amino acids 1-100) version of Cta. We made a tagged version of this construct in both Drosophila and E. Coli expression vectors. While a graduate student in the Siderovski lab, Dustin Bosch, had some success expressing this protein, we found that it did not pull down Ric-8 in immunoprecipitation experiments, and was therefore not useful for our study. The lack of binding in our system may have been due to the necessity of the N-terminal residues of Cta to create a binding interface with Ric-8 or due to misfolding of the protein. We therefore progressed with expression of full-length tagged Ric-8 and Cta in E. coli and found that upon protein expression Ric-8 and Cta were both insoluble. Previous methods used for Gα expression and purification produced a very low abundance of soluble Gα. However, it was recently found that co-expression of Ric-8 and Gα in insect cells using baculovirus dramatically increased the yield of purified, soluble Gα<sup>10</sup>. We therefore created Ric-8 and Cta vectors suitable for baculovirus expression in insect cells. Unfortunately, upon expression of these constructs we found that Cta and Ric-8 proteins were aggregated and insoluble. Thus, we found that even under ideal expression conditions Drosophila Ric-8 and Cta proteins were insoluble and were unable to perform GTPase assays and address the nature of Ric-8’s GEF activity.
Investigation of Ric-8 in the *Drosophila* embryo has mostly focused on its role in aligning the mitotic spindle during asymmetric cell division of the neuroblast, and SOP cells\(^{11-14}\). The G\(\alpha\) involved in these processes is the *Drosophila* G\(\alpha_i\). We have focused our attention on Ric-8’s function during gastrulation signaling events within the Fog pathway. While examining Ric-8’s role in the Fog signaling pathway in S2R+ cells fostered a more in-depth understanding of Ric-8/Cta dynamics, we would like to show that Ric-8 is functioning analogously in the *Drosophila* embryo. However, antibodies to Cta do not currently exist. We attempted to make two antibodies to two different peptides of Cta and upon testing both crude serum and affinity-purified antibody we found that neither one was specific for recognition of Cta. We also tried using commercially available antibodies that target other G\(\alpha_{12/13}\) family members, these did not recognize Cta either. To overcome this issue we have two options 1) attempt to make another antibody, or revisit purification of the previously made antibodies, or 2) make transgenic flies expressing a tagged version of Cta.

Ric-8 is essential for Cta function in the Fog signaling pathway, and the data presented in Chapter IV presents a role for Ric-8 in binding and localizing Ric-8 to its site of function. We hypothesize that localization of Cta to the plasma membrane is dependent on Ric-8. This is supported by the fact that Fog pathway activation is suppressed when Ric-8 ectopically targets Cta to the mitochondria. Our attempts to visualize Cta’s cellular localization using high-resolution scanning-disc confocal microscopy failed, as I was unable to differentiate the cytoplasmic and membrane bound pools or Cta and Ric-8 due to the small size of the cells. An alternative method we could use to address this question is to perform cellular fractionation experiments.

The research presented in Chapter IV provided a detailed understanding of the
functional relationship between Cta and Ric-8. However, there are additional inputs and effectors in this pathway that have not been as rigorously investigated. Ric-8 is unable to bind Ga, in Drosophila neuroblasts when Gai is part of the heterotrimeric complex. This is most likely the case for Ric-8 and Cta, as Ric-8 potentiates Cta signaling downstream of Fog pathway activation, but does not activate the pathway when it is overexpressed. To directly test for interaction we can perform pulldowns with an anti-Gβ13F antibody and probe for Ric-8. S2R+ cells depleted of Gβ13F or Gγ1 do not respond to ectopic Fog application. An intact heterotrimer of Gαβγ is needed for any of the subunits within the complex to localize to the plasma membrane\textsuperscript{15}, thus it is probable that Gβγ RNAi prevents pathway activation due to mislocalization of Cta. However, Gβγ subunits are capable of activating effectors downstream of GPCR activation\textsuperscript{16}. It is possible that Gβγ also drives signaling in a parallel or distinct pathway; overexpression studies would be informative to answer this question.

The RGS domain of RhoGEF2 binds to GTP-bound Cta, and catalyzes GTP to GDP hydrolysis. While several Ga effectors have RGS domains, many do not\textsuperscript{17}. How then is the RGS domain of RhoGEF2 regulating Cta behavior? Rapid GTP hydrolysis allows for quick turnover of Cta subunits, and provides a tightly spatially localized pool of inactive Cta. This temporal and spatial regulation of Cta localization by RhoGEF2 is ideal for the function of the Fog signaling pathway. In the Drosophila embryo gastrulation movements in the ventral furrow are completed in less than 20 minutes\textsuperscript{18}. Therefore, a GEF containing an RGS domain would be advantageous to produce a localized pool of rapidly recycled Cta ready for reactivation, potentially by Ric-8.

Two Gα binding GDIs in the Drosophila genome, Pins and Loco, have been found to function during Drosophila neuroblast spindle alignment to bind GDP-Gα and GTP-Gα
respectively, acting as both a GDI and a GAP, respectively\textsuperscript{11-14}. No GDI has been implicated in regulating the behavior of Cta in the Fog signaling pathway. It is possible that Pins and/or Loco proteins are involved in modulating Cta behavior, in an intermediary step between GDP-Cta release from RhoGEF2 and binding of Ric-8. However, due to the rapid turnover of Cta it would be somewhat surprising that a GDI would bind during this intermediary time period, unless perhaps it is needed for Cta stabilization. Using anti-Pins and anti-Loco antibodies we can test for interactions between Ric-8 and Cta and either of these GDIs using IP experiments.

While most research on Ric-8 has been preformed in \textit{C.elegans}, it is obvious that Ric-8 plays very similar roles throughout different species. It has been shown repeatedly to bind and localize Gα subunits, as well as regulate spindle positioning and neurotransmitter release in varying cell types and species investigated thus far\textsuperscript{19}. All of these functions have been described in \textit{Drosophila}, outside of neurotransmitter release. We therefore feel that \textit{Drosophila} Ric-8 acts similarly during Fog pathway activation, and that the research we have performed is applicable to Ric-8 biology in all systems.

There is little information about the mechanistic regulation of Ric-8. Some of the first data provided have come from our structure/function study investigating the role of evolutionarily conserved amino acids necessary for productive Cta signaling and binding. Several residues have been identified in phosphoproteomic screens that may be potential targets for kinase activity in Ric-8\textsuperscript{20-22}. Performing a structure/function assay of Ric-8 based on mutating evolutionarily conserved electrostatic amino-acids, revealed insight into the structural components important for comprising the Cta/Ric8 binding interface as well as the minimal mutations necessary to abrogate Fog-induced pathway activation. Mutant 13,
strongly inhibited both binding and Fog induced cellular constriction. This mutant ectopically localized Cta\textsubscript{GA} but failed to rescue constriction and had impaired binding to Cta\textsubscript{GA}, but not wild-type or Cta\textsubscript{QL}; indicating that this mutant may be important for regulating the activity of nucleotide specific GTP-free Cta, its preferred substrate. The location of the mutated residues within the C-terminal mutant 13 is proximal to an area previously identified as a phosphopeptide from a phospho-proteomics screen\textsuperscript{22}. Additionally, in Ric-8-GFP IPs this cluster mutant appeared to have a slight band shift as compared to the molecular weights of all other cluster mutants. Based on the multiple sequence alignment of the mammalian residues comprising the phosphopeptide from the proteomics screen I will make both phospho-mimetic and non-phospho-mimetic mutants of potential phosphorylation sites. I will then use these in the same experiments used for cluster and point mutant immunoprecipitations and contractile assays to determine if this area regulates Ric-8 phosphorylation, and activation. Mutant 9, also found in the C-terminus and found to strongly inhibit both Cta binding and functionality in the Fog-induced contractile assay did not show any molecular weight shift, or appear nearby any phospho-peptide hits in previous studies\textsuperscript{20-22}. Therefore, while residues surrounding mutant 13 may be important for regulating the active/inactive state of Ric-8, mutant 9 may only function in nucleotide specific recognition of Cta.

**Conclusions**

Using S2 and S2R+ *Drosophila* tissue culture cells we have established a system to study signaling events and cytoskeletal regulation. This system has allowed us to identify a canonical and non-canonical regulator of the G\textalpha, Cta; Mist and Ric-8, respectively. Future
use of this assay, and further investigation of Mist and Ric-8, will allow further advancement of our understanding of the dynamics of the Fog signaling pathway.
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