Regulation of Epithelial Morphogenesis by the *Drosophila* Folded gastrulation Signaling Pathway

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Abstract

ALYSSA JULIA MANNING: Regulation of Epithelial Morphogenesis by the *Drosophila* Folded gastrulation Signaling Pathway
(Under the direction of Stephen Rogers)

Understanding morphogenesis, the set of processes by which cells are rearranged and change shape to form organs and other higher-order structures, is crucial to our knowledge of biology. I have used the Folded gastrulation (Fog) signaling pathway necessary for *Drosophila* epithelial folding to study the principles of morphogenesis. During gastrulation, a signal from the secreted protein Fog is received by cells of the presumptive mesoderm. Then the Gα protein, Concertina (Cta), signals through the canonical Rho axis to induce actin-based apical constriction and invagination of these cells. This pathway is also active during several other epithelial folding events throughout development. We have developed a cell culture model to study Fog signaling and used it to discover a GPCR, Mist, which is a Fog receptor. *mist* RNA is specifically expressed in cells known to undergo Fog signaling. We show that the transcription factor Snail is necessary for *mist* expression in the mesoderm. We have also made a deletion allele which disrupts *mist* expression. This allele causes ventral midline defects and improper invagination of mesodermal cells, which shows that *mist* is also required for proper gastrulation movements. We also investigated GPRK2 and Kurtz which act as negative regulators of Mist signaling in cell culture. Alterations of Kurtz levels in wing discs disrupt their folding, similarly to core Fog pathway components. These data reveal that the GPCR Mist controls the location and timing of epithelial morphogenesis in *Drosophila* downstream of Fog.

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List of Abbreviations

Abl	Abelson kinase
Cta	Concertina
Fog	Folded gastrulation
GAP	GTPase activating protein
GBE	Germ band extension
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GTP	Guanine triphosphate
Krz	Kurtz
Mist	Mesoderm invaginating signal transducer
MT	Microtubule
PMG	Posterior midgut
Rok	Rho kinase
Sqh	Spaghetti squash
TF	Transcription factor
VF	Ventral furrow
VFF	Ventral furrow formation
Zip	Zipper

Chapter 1

Introduction
The Fog signaling pathway: Insights into signaling in morphogenesis

This chapter is in preparation as a review article.

Abstract

A complex interplay between many inter- and intracellular signaling molecules, along with extrinsic cues such as temperature and cellular tension, controls morphogenesis during animal development. The *Drosophila* Folded gastrulation pathway is one of the most extensively studied examples of signal transduction pathways controlling morphogenesis. It is used reiteratively during epithelial folding events and all of its core components are known. In this review, I discuss principles of morphogenesis and signaling gleaned through in-depth examination of this pathway. I also consider various regulatory mechanisms and the system's relevance to mammalian development. I propose future directions which will continue to broaden our knowledge of morphogenesis across taxa.

Introduction

Epithelial morphogenesis, or the process through which simple sheets of cells are rearranged and change shape to form mature structures and organs, has recently become an area of intense focus in the field of developmental biology, e.g. (Spear & Erickson, 2012; Nelson & Gleghorn, 2012; Suzuki, et al., 2012). A key morphogenetic movement which occurs in almost all multicellular organisms is the folding or bending of flat epithelial sheets to form more complex structures. These changes are often driven at least in part by actin- and myosin-based apical constriction (Sawyer, et al., 2010). One of the best-studied developmental signaling

pathways regulating this process is the *Drosophila* Folded gastrulation (Fog) pathway in which all the crucial steps are known, from initiation by transcription factors (TFs) to the mechanics of cell shape changes. This pathway, which drives apical constriction, therefore allows examination of some of the intricacies of protein signaling during development *in vivo*.

Many stereotypical signaling mechanisms exist in the Fog pathway, including patterned induction of gene expression by TFs, G-protein coupled receptor (GPCR) to G-protein signaling, and actin rearrangement induced by the Rho GTPase axis. The Fog pathway also reveals some novel insights, such as how multiple signaling pathways can be integrated into a single outcome and that GPCRs, among their many other functions, have morphogenetic roles. While certain aspects of the Fog pathway have been worked out in great detail, many questions still remain. What mechanisms recruit signaling components apically? How are Fog pathway components spatially and temporally patterned and what role does this patterning play in development? Which mechanisms regulate the attenuation of Fog signaling? We will explore these questions in this review.

The Fog pathway, diagrammed in Figure 1-1, begins with the specific expression of Fog in subsets of cells fated for actomyosin-based shape changes. Fog is a large secreted protein that is thought to signal primarily as an autocrine factor (Costa, et al., 1994). The Fog signal is transmitted across the plasma membrane by the GPCR Mesoderm invaginating signal transducer (Mist), a member of the secretin family of GPCRs, to a G-protein of the $G\alpha_{12/13}$ family, Concertina (Cta) (Parks & Wieschaus, 1991)(Chapter 2). In turn, RhoGEF2, a Dbl family RhoGEF; the small GTPase Rho1; and the Rho effector, Rho Kinase (Rok) are all activated (Barrett, et al., 1997; Dawes-Hoang, et al., 2007). Rok phosphorylates the regulatory light chain of non-muscle myosin II to induce contraction of the apical actomyosin network in the cells that receive the Fog signal. While the ligand, Fog, is not conserved outside of *Drosophila* and the receptor, Mist, is not conserved outside of insects, the axis of signaling from $G\alpha_{12/13}$ proteins through Rho to effect actin rearrangement is highly conserved and is important in human

development and disease (Figure 1-1) (Waterhouse, et al., 2011). For example, lysophosphatidic acid and sphingosine 1-phosphate are membrane lipid derivatives known to signal through GPCRs, the $G\alpha_{12/13}$ family, RhoGEFs, RhoA, and various effectors in mammals (Xiang, et al., 2013; Suzuki, et al., 2009). These pathways are able to modulate various cytoskeletal and cell

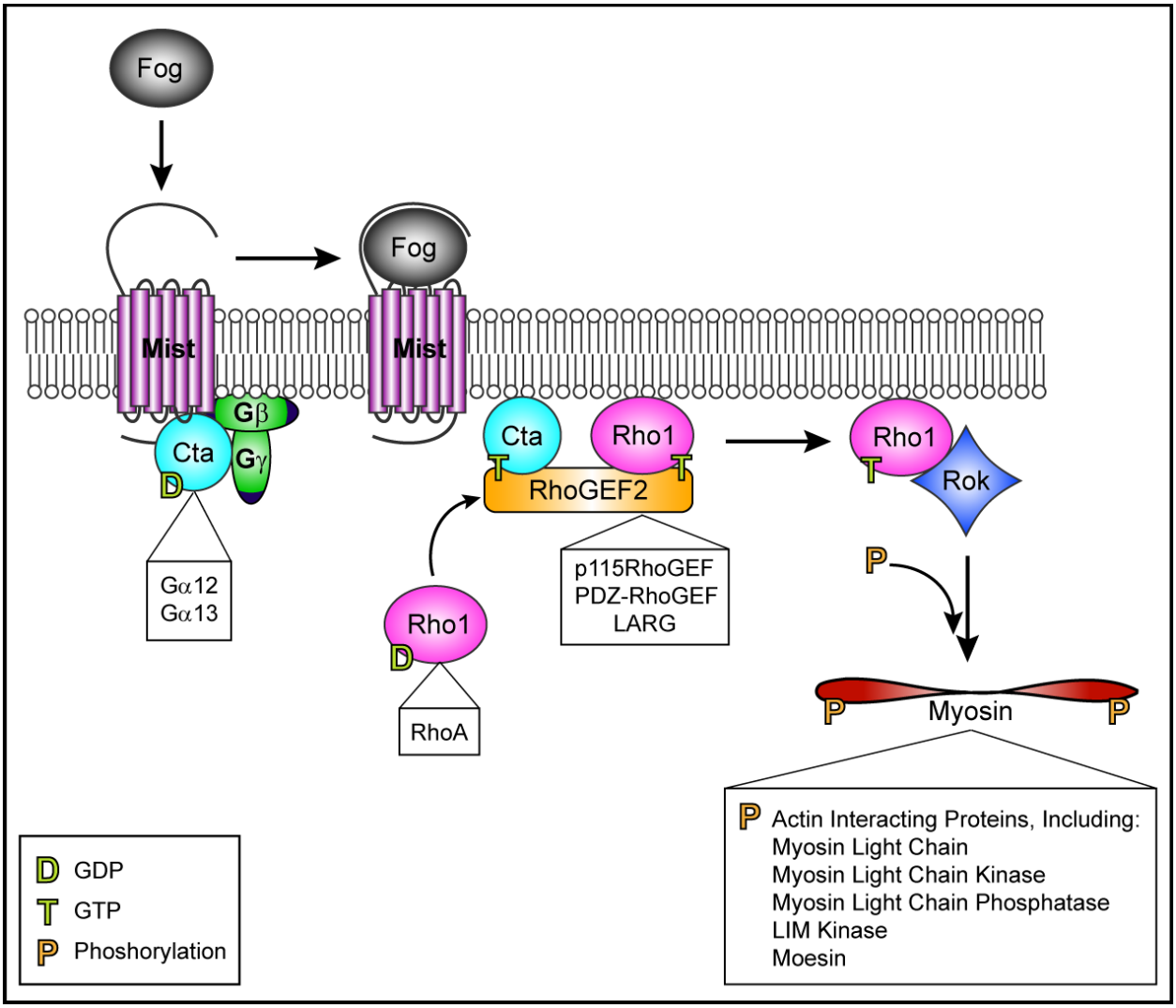


Figure 1-1. The Fog Signaling Pathway. Fog is a large secreted protein which acts as a ligand for Mist, a seven pass transmembrane GPCR. In its ligand-free state Mist is predicted to interact with inactive, GDP-bound Cta. Once Fog binds Mist, it likely stimulates Cta's exchange of GTP for GDP, which allows Cta to dissociate from its trimer partners, Gβ and Gγ. Cta-GTP binds to RhoGEF2 which can then act as a GEF for Rho1. In its GTP-bound form Rho1 then activates Rok. Finally, the regulatory light chain of non-muscle myosin II, Spaghetti squash, is phosphorylated by active Rok to induce apical actomyosin network contraction in the cells which receive the Fog signal. Boxed are vertebrate components of Rho axis signaling which act in a similar manner to induce actomyosin cytoskeleton rearrangements. In vertebrates, Rok is known to phosphorylate many proteins which interact with actin, activating some and inactivating others.

shape changes, including neurite outgrowth and retraction, tumor cell invasion, as well as angiogenesis.

The Fog pathway is active in many morphogenetic events in *Drosophila* development, with known roles in ventral mesoderm and posterior midgut (PMG) invagination during gastrulation, imaginal disc folding during larval development, salivary gland internalization in mid-embryogenesis, and morphogenesis of the central nervous system during late embryogenesis (Costa, et al., 1994; Nikolaidou & Barrett, 2004; Ratnaparkhi & Zinn, 2007). In all of these cases Fog induces apical constriction, except in the CNS where the cellular results of Fog's action are not known. Apical constriction, along with other concomitant shape changes, in cells of the ventral mesoderm, PMG, and salivary gland eventually results in complete internalization of these cell groups. The folds of imaginal discs only invaginate as far as to form U-shaped folds within the plane of the tissue.

During VFF there are two phases of apical constriction: a stochastic, nonproductive phase, when individual cells contract and relax without any overall reduction in apical area, and a concerted, coordinated phase, when individual cells undergo ratchet-like reductions in apical area which are much more stable (Sweeton, et al., 1991; Martin, et al., 2009). Actin and myosin periodically coalesce and these concentrations tend to move toward the center of a cell (Martin, et al., 2009). Via these actomyosin contractions, the plasma membrane is pulled inward. During random constriction the membrane relaxes to its original position when actomyosin coalescences are disassembled. Once the concerted phase of constriction begins, membrane deformations are stabilized to reduce apical cell area. This pulsatile mode of cellular constriction has also been observed in other contracting groups of cells in the *Drosophila* embryo (Solon, et al., 2009).

In addition to the conserved nature of the signaling components, these cell shape changes are similar to morphogenetic processes in mammals (Sweeton, et al., 1991; Schoenwolf & Franks, 1984). Internalization of the mesoderm during *Drosophila* gastrulation closely

resembles neural tube formation in vertebrates. In both cases, a subset of epithelial cells within a flat sheet undergoes apical constriction to invaginate and form a tube sealed off from the surrounding epithelium (Copp & Greene, 2010). When these processes are disrupted *Drosophila* eggs don't hatch; in humans debilitating congenital defects such as spina bifida or anencephaly can occur, sometimes leading to death. Working out the intricacies of the Fog signaling pathway and its resulting cell and tissue movements will ultimately lead to a more profound understanding of our own development and greater potential for medical interventions in disease states.

Interactions between Fog pathway members

Ligand and receptor

A discussion of the core components of the Fog signaling pathway must begin with Fog itself. Embryos lacking Fog, the secreted ligand initiating the pathway, display disorganized VF cell apical constriction, though most mesodermal cells do eventually internalize (Costa, et al., 1994). Major problems arise in the next steps of development since PMG cells do not invaginate and improper germ band extension (GBE) leads to a twisted body axis. All embryos mutant for *fog* die before emerging from the egg. Embryos lacking *fog* in subsets of cells that cross the VF have a distinct divide between apically constricting cells (wild-type) and non-constricting cells (*fog* mutant) (Costa, et al., 1994). This experiment suggests that the Fog signal does not diffuse farther than a couple of cell widths, consistent with Fog being a fairly large protein predicted to be glycosylated.

The most recent addition to our knowledge of Fog signaling is the discovery of a receptor, Mist, which can function downstream of Fog (Chapter 2). Mist is a GPCR with a large extracellular domain, appropriate for interacting with a large ligand such as Fog. This discovery was made possible by the development of a cell culture model for studying Fog signaling. *Drosophila* S2R+ cells plated on a substrate of Concanavalin A respond to exogenously added Fog protein by transitioning from a flat profile to a cone shape due to actomyosin constriction.

Many avenues of study not possible using whole animals have been opened by the development of this model system. The discovery of Mist both answers old questions and raises new ones. *fog* and *mist* transcription are both precisely regulated in space and seem to be under independent control, with overlapping but not completely coincident expression patterns (Dawes-Hoang, et al., 2007)(Chapter 2). This redundancy helps explain how the formation of Fog-induced epithelial invaginations is so regular within the complex developmental dynamics of wild-type animals.

Ubiquitous overexpression of Fog in the early embryo results in a normal VF and no precocious apical constriction (Dawes-Hoang, et al., 2007). This can now be explained by *mist's* restriction to ventral and posterior cells and its upregulation at the end of cellularization when VF invagination normally begins (Chapter 2). The opposite is also true, with ubiquitous Mist expression not significantly disrupting gastrulation presumably due to Fog's spatial restriction. Adding complexity to the situation, however, is that ubiquitous Fog overexpression results in apical flattening in cells outside the VF (Morize, et al., 1998; Dawes-Hoang, et al., 2007). Perhaps there is a low level of Mist in dorso-lateral cells which allows flattening but does not reach the threshold for full apical constriction. There is also the possibility of multiple Fog receptors working either redundantly with, in concert with, or differently from Mist in the same or different tissues. There may be a second receptor in cells outside the VF and PMG invaginations in the early embryo which responds to Fog by inducing apical flattening specifically. Another possibility is a redundant receptor in other tissues, though it is not likely in the VF and PMG given the similarities of *mist* and *fog* zygotic phenotypes (Chapter 2). Mist may have an obligate coreceptor, in which case missing either one of the pair would phenocopy a complete lack of receptor. One possibility for a receptor working with or in parallel to Mist is the GPCR CG31660, which was found by genetic screening to play a role during the morphogenetic movements of gastrulation (Mathew, et al., 2009). The precise actions of this receptor and its possible interactions with Mist or Fog have not yet been determined.

The recent discovery of a receptor connecting Fog and Cta across the plasma membrane in the well-studied Fog signaling pathway creates an easily manipulated system for examining GPCR activity *in vivo*. This pathway can be further studied in *Drosophila* cell lines to add to our picture of GPCR signaling. Combining the genetic malleability and drug susceptibility of the fly embryo with live imaging and other microscopic techniques will allow fine detail of the interactions between components of G-protein signaling pathways, and the developmental results thereof, to be examined. Mist being the primary example of G-protein signaling in morphogenesis, it will be extremely important to learn all that we can from this system.

Heterotrimeric G-protein signaling

Among all of the known Fog pathway components, Cta was discovered first and yet comparatively little is known about it (Parks & Wieschaus, 1991). Embryos lacking maternal Cta have very similar gastrulation phenotypes to *fog* or *mist* zygotic mutants. Cta is required to organize myosin apically in the contractile VF cells, though it is not necessary for apical actin (Fox & Peifer, 2007). However, Cta is expressed much more broadly throughout embryogenesis than are Fog and Mist, and likely has roles outside the VF and PMG. One possible Fog-independent role of Cta is in maintenance of cortical cytoskeletal stability throughout the blastoderm (Kanesaki, et al., 2013).

In the early embryo, ubiquitous expression of constitutively active Cta or injection of cholera toxin, which activates Cta, phenocopies ubiquitous expression of Fog, including apical flattening of all cells (Morize, et al., 1998). This result suggests that Fog-dependent apical flattening works through Cta, though as mentioned above it may not work through Mist. Receptor-specific Cta activation and subcellular localization in certain cells may help restrict which downstream effectors are activated and therefore which cellular pathways are tiggered. Unfortunately, no method for visualizing endogenous Cta has been developed, making it difficult to learn about this protein in more detail. A reliable antibody to Cta or replacement of the endogenous gene with a tagged version would be highly beneficial to the field. These would

open up a wealth of new information about how G-proteins function during development *in vivo*.

Gα proteins function with Gβs and Gγs in obligate heterotrimers. Gβ13f and Gγ1 have been suggested as partners for Cta during gastrulation, as embryos lacking either have the same gastrulation and cuticle phenotypes as those lacking Cta (Figure 1-2) (Schaefer, et al., 2001; Wang, et al., 2005; Izumi, et al., 2004). They are also the most widely expressed β and γ subunits during embryogenesis. Gα proteins are generally thought to be the primary signal transducing members of heterotrimeric G-proteins, but it is now well established that β and γ subunits can signal independently of Gαs (reviewed in (Clapham & Neer, 1997). Additionally, Gαs have been reported to require chaperone-like cofactors, such as Ric-8, for proper localization (Figure 1-2) (Wang, et al., 2005). Embryos lacking Ric-8 have disrupted VF apical constriction which results in similar cuticle phenotypes to embryos from *cta* mutant mothers (Wang, et al., 2005; Kanesaki, et al., 2013). Ric-8 is also necessary for apical myosin accumulation and cortical tension during VFF (Kanesaki, et al., 2013). It will be interesting to further investigate the roles of these three essential co-factors in epithelial morphogenesis.

<u>The Rho signaling axis</u>

The intracellular signaling components of the Fog pathway fit into the well-established Rho signaling axis which leads from activation of a $G\alpha_{12/13}$ family member to actin cytoskeletal rearrangement, e.g. (Somlyo & Somlyo, 2000). Some vertebrate members of this pathway are listed in boxes in Figure 1-1. Cta, RhoGEF2, Rho1, Rok, myosin, and actin are present in all cells in *Drosophila* early embryos and imaginal discs (Parks & Wieschaus, 1991; Barrett, et al., 1997; Hacker & Perrimon, 1998; Mizuno, et al., 1999; Kiehart, et al., 1990; Warn & Magrath, 1983). They are all supplied maternally to embryos, as well, which speaks to their importance during the early stages of development. However, these proteins are apically localized specifically in cells undergoing apical constriction. The presence and activity of their upstream activators and

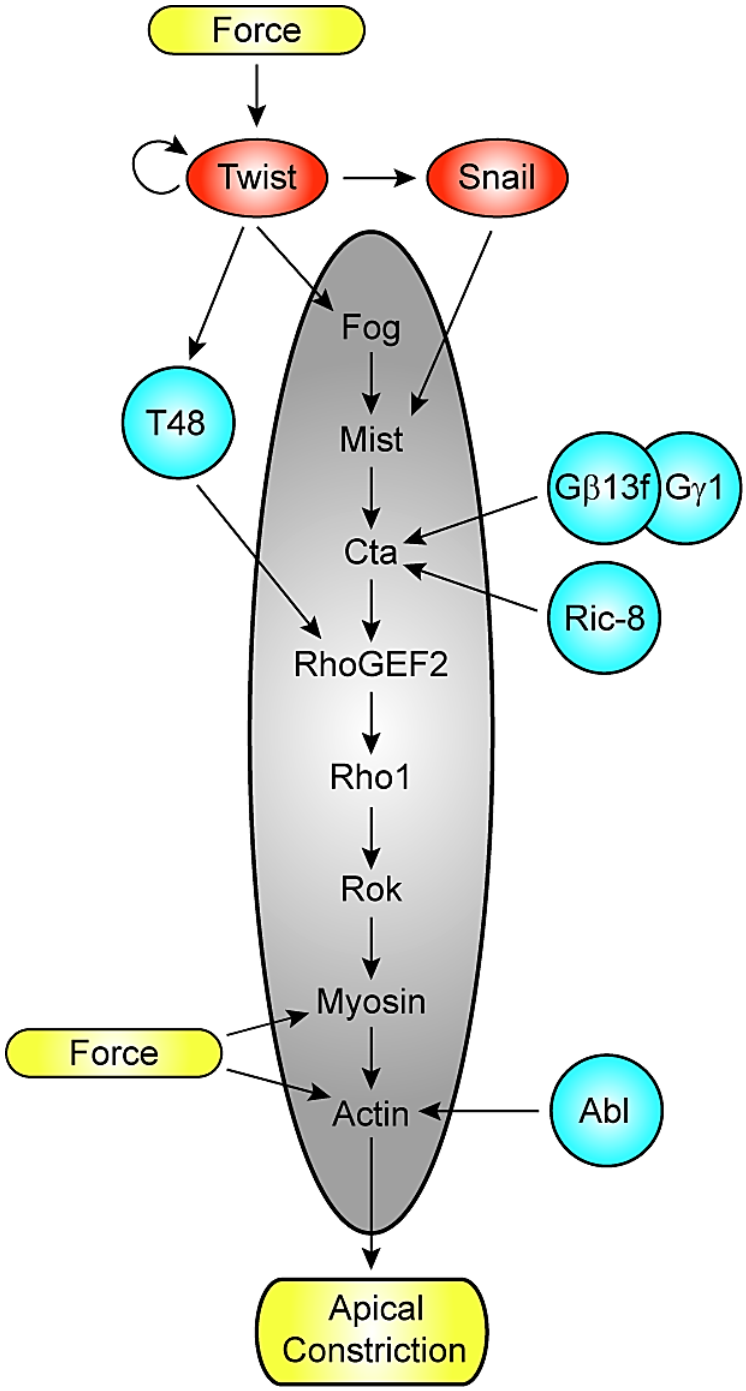


Figure 1-2. Known Inputs into the Fog Signaling Pathway. The core Fog signaling pathway components are shown in the central gray oval. Transcription factors are in red ovals. Accessory proteins are in aqua circles. Yellow bars denote physical changes. Physical forces act on Twist, myosin, and actin to change their abundance and localization, though the mechanisms of these functions and whether they are direct are not entirely clear. Twist induces transcription of *fog* and *T48* in VF cells. Similarly, Snail is necessary for *mist* transcription in the VF. T48, a single pass transmembrane protein, helps to localize RhoGEF2 apically in the VF. Gβ13f, Gγ1, and Ric8 are all required for Cta protein stability and function. Abl helps organize actin apically in contracting cells. All of these inputs, and likely more, help organize and activate Fog signaling in developmental time and space.

their limited subcellular localization help give developmental control to their downstream effects. This section aims to highlight some of the important points we have learned about how this pathway enacts cell shape changes from studying Fog signaling and what we can potentially learn from further examining Rho axis signaling in *Drosophila*.

RhoGEFs, and RhoGEF2 in our case, act as signal concentrators within the cell, specifying and amplifying the outcome of Rho activation. Maternal *RhoGEF2* mutant gastrulation phenotypes are much stronger than those of either zygotic *fog* or maternal *cta* mutants, with no mesoderm or posterior endoderm internalization at all (Hacker & Perrimon, 1998; Barrett, et al., 1997). Also unlike *fog* and *cta* mutants, *RhoGEF2* mutants have defects in both actin and myosin accumulation at the apical sides of VF cells (Fox & Peifer, 2007). There must be another pathway feeding into the activation of RhoGEF2 in the VF which is somewhat additive with the input from Fog-Mist-Cta. (Some possibilities will be discussed in the "Other inputs into Fog-induced cell shape change" section below.)

Rho1 acts in early embryos and cell culture to organize both the actin and myosin networks, with Cta upstream of its action on myosin (Fox & Peifer, 2007). Disruption of Rho1 function in early embryos by exogenous expression of an inactive version mimics the loss of RhoGEF2 (Barrett, et al., 1997; Hacker & Perrimon, 1998). Embryos with disruptions in *RhoGEF2* or *Rho1* do exhibit apical constriction but not in a coordinated or concerted fashion. However, *Rho1* and *RhoGEF2* maternal mutants have noticeably different phenotypes, with *Rho1* mutants having more and varied cell shape defects throughout embryogenesis (Barrett, et al., 1997; Magie, et al., 1999). These results are complicated by the requirement for Rho1 during egg formation, but do suggest that Rho1 can be activated by other RhoGEFs in addition to RhoGEF2 or by other mechanisms (Magie, et al., 1999). Overall, RhoGEF2 and Rho1 do not seem to be absolutely necessary for actin and myosin rearrangement but act to organize and maintain actomyosin structures and contractions.

Rho1, *RhoGEF2*, and *zipper* (encoding the heavy chain of myosin II) all interact genetically during leg and wing morphogenesis, during imaginal disc folding and/or limb eversion (Halsell, et al., 2000; Nikolaidou & Barrett, 2004). Fog, Mist, and Cta have all been implicated in these processes as well (Nikolaidou & Barrett, 2004)(Chapter 2). Improper expression levels or patterns of Fog pathway components in wing imaginal discs leads to stochastic folding of the epithelium. Proliferation, specification, and polarity of discs do not seem to be altered when the Fog pathway is disrupted, but the tissue's normal growth forces once flat epithelial sheets to fold within the confines of the disc without proper patterning information (Nikolaidou & Barrett, 2004). These data confirm again that patterning and specificity of Rho activation is crucial during morphogenesis. Techniques for imaging imaginal disc development live have been developed (Aldaz, et al., 2010). Additionally, Förster Resonance Energy Transfer (FRET) probes for the activity of the GTPase Cdc42 have been used in live *Drosophila* embryos (Kamiyama & Chiba, 2009). A combination approach could be taken using a Rho1 biosensor to further investigate the protein's activity downstream of Fog activation in wing discs. The prospect of using an initially flat tissue with increasing complexity and dynamics such as the imaginal disc for studying Rho activation at high resolution *in vivo* is exciting.

Induction of the Fog pathway

Transcription factors

There are several factors that contribute to the expression pattern of Fog pathway components, as well as initiation and organization of the pathway itself. First, transcriptional control of certain Fog pathway members can influence pathway activation within developmental space and time. We know the most detail about this topic relative to ventral furrow formation (VFF). During egg production, a nuclear gradient of the Dorsal TF is maternally set, with highest levels on the ventral side of the egg (Roth, et al., 1989). The cells that receive the highest concentration of Dorsal then zygotically transcribe the TFs Twist, a member of the basic helix-

loop-helix family, and Snail, a zinc finger TF (Leptin & Grunewald, 1990). Twist in the ventral mesoderm reinforces both its own expression and Snail expression (Ip, et al., 1992). Twist and Snail are each independently required for both mesoderm specification and the morphogenetic movements of gastrulation, though they have slightly different phenotypes (Figure 1-2)(Leptin, 1991). *twist* single mutants retain some ability to accumulate myosin and constrict VF cells, though they are never able to transition to the coordinated, productive phase of apical constriction (Martin, et al., 2009). Twist is required to stabilize actomyosin-based constrictions, perhaps due in part to an ability to respond to force (see "Mechanical inputs" below). *snail* mutants do not undergo visible myosin coalescence, though some mesodermal cells are eventually internalized, suggesting that Snail is required for the initial stages and coordination of apical constriction (Martin, et al., 2009). In *snail twist* double mutants VF cells don't accumulate myosin apically, contract, or form an invagination suggesting that these two TFs together are necessary to transcribe key molecules involved in all steps of VF cell shape change (Leptin, 1991; Martin, et al., 2009).

Some of these transcriptional targets are known. Twist activates the transcription of *fog* and *T48*, a single pass transmembrane protein that acts to apically localize RhoGEF2 during VFF (see "Other inputs into Fog-induced cell shape change" below; Figure 1-2) (Morize, et al., 1998; Kolsch, et al., 2007). Snail's only known target necessary for gastrulation is *mist* (Figure 1-2; Chapter 2). *fog* mRNA and *mist* mRNA have similar localizations in wild type embryos, with enrichments along the ventral side and the posterior end of the embryo. One marked difference between them is that *mist* RNA is present in a continuous stripe while *fog* RNA exhibits a gap between its mesodermal and endodermal patches. *fog* RNA in *twist* mutant embryos and *mist* RNA in *snail* mutant embryos both lose expression in the ventral mesoderm while retaining it in the PMG (Seher, et al., 2006)(Chapter 2). An independent set of TFs is probably required in the PMG. These somewhat independent and overlapping patterns suggest that robust spatial control of apical constriction is important during this morphogenetic event.

Mist being a transcriptional target of Snail clarifies several previously unexplained results. First, ectopic Fog expression in wild-type or *fog* mutant embryos induces a VF to form in its normal location (Morize, et al., 1998). Twist is not required for this to occur. In *snail* mutants, though, ectopic Fog expression fails to induce flattening of VF cell apices (Morize, et al., 1998; Dawes-Hoang, et al., 2007). Mist may be the Snail target required for apical flattening, at least in VF cells. Second, the stochastic phase of VF apical constriction occurs in *twist* but not *snail* mutants (Martin, et al., 2009). Twist, T48, and, importantly, Fog are not required for random cellular constrictions, but a Snail target is. This could be explained by spontaneous agonist-free excitation of Mist, which is a property of many GPCRs (reviewed in (Smit, et al., 2007). Overlapping expression of Snail and Twist patterning expression of Mist and Fog is a novel mechanism for robustly controlling the location and timing of a developmentally important signaling pathway.

Outside of the VF we don't know the transcriptional regulators controlling Fog pathway members. The Fork head TF is necessary for salivary gland primordium apical constriction and invagination (Myat & Andrew, 2000). As Fork head is also expressed at the extreme ends of the early embryo, it may also be involved in PMG invagination, though it has not been specifically implicated in controlling Fog signaling in either of these processes (Weigel, et al., 1989). Fog and Mist expression patterns in the wing imaginal disc are complex and don't follow any known TF patterns (Chapter 2). They are likely under combinatorial control of many TFs in this tissue. Downstream players in the Fog pathway are maternally deposited in embryos and are widely expressed in other tissues. Their localized activity rather than expression is likely the determining factor in localized signal transduction.

Mechanical inputs

Another mode of control feeding into Fog signaling is mechanical force (Figure 1-2). As a flat sheet of cells folds the apically constricting cells produce force which pulls on neighboring cells. Therefore, cells within a folding sheet that aren't actively contributing to the deformation

can experience mechanical strain. We don't know all of the implications of these forces yet, but there are some ideas in the literature. For instance, stress across the apical surfaces of cells undergoing Fog signaling could increase the membrane tension enough to reduce endocytosis, leaving more competent or active Mist on the membrane for signaling (Driquez, et al., 2011). Conversely, apical-basal shortening toward the end of furrow invagination could result in a reduction in total cell volume, cell surface area, and membrane tension leading to an increase in endocytosis and termination of signaling.

As mentioned previously, VF cell contraction occurs in two phases: a random unproductive period of contraction and then a coordinated period that forms an epithelial fold (Sweeton, et al., 1991; Martin, et al., 2009). The trigger that allows for the change from the stochastic phase to the collective phase is not yet known, but it has been suggested that this transition occurs when a threshold of strain builds up across the tissue (Martin, et al., 2010). This mechanical strain may feed directly into the actomyosin network. It will be interesting to further study the interactions between signaling and mechanics during these contractions and to investigate their roles in other organisms.

Force could also feed less directly into Twist, Fog, and T48 expression, as Twist protein expression seems to be positively correlated with the mechanical deformation of cells during GBE (Farge, 2003). Just after gastrulation, large scale tissue rearrangements comprising GBE produce compressive forces on the dorsal side of the embryo and stretching forces on the ventral side. Physically disrupting GBE movements reduces Twist expression, but artificial force on these disturbed embryos can rescue Twist levels (Desprat, et al., 2008). (While Twist is no longer required for Fog signaling at GBE, it is still necessary for proper mesoderm differentiation (Leptin, 1991).) Similarly, Snail is required for apical myosin localization in the VF, but artificial indentation of *snail* mutant embryos can rescue myosin localization and promote complete mesoderm invagination (Pouille, et al., 2009).

There is evidence for mechanical strain influencing RNA transcription, cytoskeletal dynamics, and tissue movements in many systems. For instance, formation of the head fold in the chick embryo, an epithelial folding event, exerts significant forces on the surrounding tissues (Varner, et al., 2010). Application of ectopic forces to embryo explants undergoing this process alters their morphogenetic movements. We don't yet know how forces are involved in most tissues where Fog signaling is active, but we can use this pathway and its resulting epithelial invaginations to investigate the problem in a very detailed manner. The early *Drosophila* embryo and imaginal discs can be mechanically manipulated and methods have already been developed to do so, e.g. (Farge, 2003). The embryo is a relatively simple, yet 3-dimentional *in vivo* system in which we can modulate gene activity and mechanical stress in combination. Insights about the interaction between these two inputs into the Fog signaling pathway will likely be broadly applicable to many developmental processes.

Subcellular localization

We know that much of the signal transduction within the Fog pathway must occur at or near the apical surface of contractile cells in order to restrict actomyosin contraction to cell apices, but we know very little about how this is achieved. *fog* mRNA is focused apically in the PMG and imaginal discs, and *mist* mRNA is apical in imaginal discs (Dawes-Hoang, et al., 2007)(Chapter 2). Fog protein localizes to puncta, presumably vesicles, in the apical portion of PMG cells during invagination, suggesting that it may be apically secreted (Dawes-Hoang, et al., 2007). Mist protein is present in discrete puncta on the apical surface of VF cells during invagination (Chapter 2). Localized translation and directional trafficking likely contribute to the apical localization of these proteins. Specific association of Cta with apically concentrated Mist in cells undergoing Fog signaling may help to restrict Cta to the apical domain, but this is not yet known.

Before gastrulation, RhoGEF2 localizes to the basal ends of cellularization furrows but is redistributed throughout the cytoplasm once cellularization is complete (Fox & Peifer, 2007). In

VF cells, but not in lateral and dorsal cells, RhoGEF2 then becomes apically concentrated before constriction occurs. This striking relocalization may, at least in part, be caused by directional transport of RhoGEF2 on dynamic plus-ends of microtubules (MTs) (Rogers, et al., 2004). Activation of Cta causes RhoGEF2 to dissociate from MTs, possibly allowing for RhoGEF2 to associate with Cta itself, interact with lipids in the plasma membrane, and activate Rho1. MTs in the blastoderm epithelium are generally thought to be oriented with their plus-ends basally, the reverse orientation to that which would bring RhoGEF2 to the apical surface (Harris & Peifer, 2005). The MT arrays in many interphase *Drosophila* cells are acentrosomal, however, so there may be mixed polarity MT arrays or short MTs along apical cell surfaces which may contribute to localization of Fog signaling components (Rogers, et al., 2008). Alternatively, RhoGEF2's association with MT plus-ends could be a mechanism for keeping it basally localized before Cta activation. The orientation and dynamics of MTs in contractile cells *in vivo* should be examined in greater detail in order to determine whether and how they play a role in localizing these signaling components.

Myosin localizes apically during VFF, PMG invagination, salivary gland invagination, and imaginal disc folding (Nikolaidou & Barrett, 2004; Zhang & Ward, 2011). Myosin is concentrated basally during early cellularization, but, unlike RhoGEF2, it is both lost from the basal surface and enriched apically only in VF cells. This accumulation during VFF does not occur in embryos lacking Fog, Mist, Cta, RhoGEF2, or Rok, suggesting that a complete Fog pathway is required for establishment or maintenance of the apical myosin network (Nikolaidou & Barrett, 2004; Dawes-Hoang, et al., 2007)(Chapter 2).

The major determinant of epithelial apical behavior in most organisms is the apical PAR complex, made up of Par-6, Par-3/Bazooka, and aPKC, which must be in place for apically restricted events to occur properly (reviewed in (Goldstein & Macara, 2007). These apical proteins likely have direct as well as indirect roles in organizing Fog. In the early *Drosophila* embryo cellular polarity is established during cellularization, immediately preceding VFF

(Müller & Wieschaus, 1996). Bazooka, through recruitment of several partner proteins, localizes Gα proteins apically in *Drosophila* neuroblast cells (Siegrist & Doe, 2005). A similar mechanism may help localize Cta. The PAR complex also interacts with the proteins that set up subapical adherens junctions in the early embryo. These cell-cell contacts are necessary for tissue cohesion during gastrulation (Müller & Wieschaus, 1996; Dawes-Hoang, et al., 2007). Adherens junction proteins move from their normal subapical localization to a more extreme apical localization in the VF cells just before apical constriction (Dawes-Hoang, et al., 2007). We do not know how much influence their location along the apical-basal axis has on the ability of cells to invaginate in the VF, though adherens junction migration is known to be a driving force in *Drosophila* dorsal epithelial folding (Wang, et al., 2012).

The transmembrane protein Crumbs is also a major player in apical membrane identity and recruitment of proteins to the apical region of cells (Assémat, et al., 2008). During salivary gland invagination, Rho1 activity in the invaginating cells is required for c*rumbs* transcription and for *crumbs* mRNA and protein apical localization (Xu, et al., 2008). Crumbs, in turn, helps to organize the apical domain of these cells, leading to proper actomyosin constriction downstream of Rho1. How Crumbs- and PAR complex-induced polarity interacts with other signaling complexes is a convoluted matter and will likely take years more work to figure out. The strict localization and restricted timing of Fog signaling offer a good system with which to study these interactions.

Other regulatory mechanisms

Negative regulation of Fog signaling

Several other signaling pathways or proteins have been shown or hypothesized to feed in to the Fog pathway at various points. One thoroughly unknown aspect of the Fog pathway is how the contractile signal is terminated. The mRNAs or proteins of pathway members may be turned over to terminate signaling. *mist* RNA is present in the presumptive mesodermal cells for quite a while after they have been internalized (Chapter 2). However, *fog* RNA is lost from

mesodermal cells shortly after the VF has invaginated (Costa, et al., 1994). If there is no activating ligand there should be no pathway activation, whether other pathway components are competent for signaling or not. Translational or transcriptional regulation may not be rapid enough for termination of the signal in VFF, as mesoderm internalization only lasts about ten minutes. Other Fog pathway-dependent morphogenetic processes probably occur on a longer time scale, though.

GPCR signaling is canonically terminated by phosphorylation of the C-terminal tail of ligand-bound GPCRs by G-protein Coupled Receptor Kinases (GRKs). Once phosphorylated, GPCRs are bound by β-Arrestins which can induce receptor internalization, cause receptor degradation, compete for GPCR binding with Gαs, and potentially activate independent signaling cascades. Vertebrate genomes encode many GRKs and β-Arrestins, some of which are visual system specific and some of which are utilized more generally across tissues. *Drosophila* only has one non-visual GRK and one β-Arrestin, GPRK2 and Kurtz (Krz) respectively (Cassill, et al., 1991; Roman, et al., 2000). GPRK2 is required maternally for egg production (Schneider & Spradling, 1997). However, some of the few eggs laid by *GPRK2* mutant mothers display disrupted gastrulation phenotypes suggesting a possible role in regulating VFF and PMG invagination. Eggs lacking Krz also display cuticle phenotypes suggestive of gastrulation defects (Tipping, et al., 2010). Alteration of levels of either protein in wings also causes morphological defects (Molnar, et al., 2011). These data raise the possibility that GPRK2 and/or Krz could play a role in termination of Fog signaling. Investigation of the roles of GPRK2 and Krz in this pathway could allow us to more precisely determine how and when signal termination is achieved during other morphogenetic signaling events.

There are a few canonical molecules which terminate Rho axis signaling in many contexts: Rho GTPase activating proteins (GAPs) and myosin phosphatase. RhoGAPs accelerate the inherent GTPase activity of Rho proteins, increasing the ratio of inactive to active Rho. During *Drosophila* posterior spiracle invagination, an apical constriction event not connected to

Fog signaling, Rho1 activity is restricted to the apical sides of cells (Simões, et al., 2006). In these cells RhoGEFs remain apical while RhoGAPs are baso-lateral. The complementary localization of these regulatory proteins organizes Rho1 activation and also allows for its deactivation promptly after termination of an activating signal. However, we don't know whether or which GAPs are acting in Fog signaling or how they may contribute to signaling dynamics.

Myosin phosphatase removes the activating phosphates from regulatory myosin subunits. Rok can phosphorylate both myosin light chain to activate it and phosphorylate myosin phosphatase to inactivate it, a twofold way of maintaining myosin activity (Amano, et al., 2010). When negative regulation is not exerted on myosin phosphatase, it can act to downregulate myosin activity. The role of this deactivation mechanism in Fog signaling is not yet known.

There may be other contributing factors to the termination of Fog signaling. For instance, Par-6 has been found to negatively regulate Rho in several contexts, and therefore Rho activation within an apical PAR domain must overcome this local downregulation (Goldstein & Macara, 2007). Also, changes in membrane trafficking could influence aspects of signaling such as Mist presentation on the apical plasma membrane and secretion of Fog. Alteration of membrane tension during cell shape change may also influence the ability of the actomyosin cytoskeleton to pull against the plasma membrane. These questions may be difficult to approach *in vivo*, but are ideal problems to solve using a cell culture model of apical constriction.

<u>Other inputs into Fog-induced cell shape change</u>

There are several other accessory proteins that have been shown genetically or mechanistically to influence Fog signaling but don't fit into a well-defined category. First, the single pass transmembrane protein T48, a Twist transcriptional target, is expressed along the ventral side of early embryos and is restricted to their apical membranes (Gould, et al., 1990;

Leptin, 1991). Interestingly, it is required for organized VF invagination but is not even expressed in the PMG (Kolsch, et al., 2007). T48 is necessary for proper apical localization of RhoGEF2 in the VF. It also helps to organize the transition of adherens junctions from subapical to apical localization in VF cells as constriction begins. Just as Fog and Cta aren't absolutely required for mesoderm internalization, neither is T48, but embryos lacking both Cta and T48 do not form a VF at all. T48 may act as an accessory protein in Fog signaling or in a parallel pathway, though the mechanism of its influence is not yet known.

MTs have been implicated in working with the actin cytoskeleton in order to enact cell shape changes during morphogenesis, potentially in nuclear positioning or membrane trafficking, (e.g. (Suzuki, et al., 2012). Within the cytoplasm actin regulatory proteins could also influence the organization or formation of the apical contractile array during Fog-induced cell shape changes. For instance, the formin Diaphanous (Dia) is an actin filament elongation factor which is also a Rho effector in several systems (reviewed in (Young & Copeland, 2010). Embryos lacking maternal *dia* have defects in coordinating apical constriction in the VF so that only a subset of cells constrict (Homem & Peifer, 2008).

One actin regulator with a more defined role in VFF is Ableson kinase (Abl), a non-receptor tyrosine kinase that interacts directly with the actin cytoskeleton (Figure 1-2) (Van Etten, et al., 1994). Abl is present apically in all cells during early embryogenesis and is enriched and activated in VF and PMG invaginations (Fox & Peifer, 2007). Embryos lacking Abl zygotically have similar gastrulation defects to those lacking Cta maternally. They have uncoordinated VF cell contraction with disorganized apical networks of actin, but do internalize most, if not all, mesodermal cells. The double mutant phenotype of *abl* and *cta* is much stronger than either alone, and resembles *RhoGEF2* mutants. Abl likely acts parallel to Cta to coordinate the actin and myosin networks in apically constricting cells. Loss of Abl and Abl-related gene in mice leads to strong neural tube closure defects, implicating a similar molecular mechanism of cell shape change in mammalian development (Koleske, et al., 1998). The

interaction between G-protein signaling and actin regulatory proteins in Rho activation and cell shape change should be more deeply studied, with VFF being a great model.

Conclusions

In this review I have summarized our current understanding of the Fog signaling pathway and discussed known and potential inputs into the ultimate cell shape changes which occur in cells undergoing Fog signaling. *Drosophila* morphogenesis, specifically VFF, has long been used as a simplified model for vertebrate morphogenesis and signaling for several decades. Many wide-reaching paradigms have been discovered and investigated in depth using this model, not the least of which is the complement of physical cell shape changes which occur during apical constriction. Additionally, quantification of different aspects of VF cellular contraction in wild-type and perturbed embryos has allowed us to analyze how physical forces are coupled to cellular contractions and ultimately to tissue-scale movements (Martin, et al., 2010; Driquez, et al., 2011). The intimate integration of multiple signaling pathways to trigger a single outcome has become clearer in recent years as well, with the study of how cell polarity affects cell shape and Rho signaling (Xu, et al., 2008). Fog signaling is also a pioneer model for GPCR-G-protein signaling in morphogenesis (Chapter 2).

The mechanistic interactions between known players in Fog-activated morphogenetic events do need more attention in the coming years. We still have a lot to learn from this system in terms of spatial and temporal regulation, for example. The complementary patterns of Fog and Mist expression throughout *Drosophila* development in combination with all of the accessory proteins required for normal tissue invagination give us a hint as to the level of robust control required by evolution for development. I predict that one of the main questions moving forward will be how the timing of Fog signaling is regulated, which will likely lead to the discovery of more auxiliary players. We still know nothing of Fog signal termination. Our current and future knowledge of Fog-induced cell shape changes in *Drosophila* has contributed

to the understanding of signaling and morphogenesis in our own development and will continue to do so.

Chapter 2

Regulation of epithelial morphogenesis by a G-protein coupled receptor, Mist, and its ligand, Fog

This chapter represents a manuscript in revision. The experiments were designed by me, Mark Peifer, and my advisor, Stephen Rogers, and were carried out by me, Kimberly Marston, and Mark Peifer. The manuscript was written by me, Kimberly Marston, Mark Peifer, and my advisor, Stephen Rogers.

Abstract

Epithelial morphogenesis is essential for shaping organs and tissues and for establishment of the three embryonic germ layers during gastrulation. Much of our understanding of how epithelial morphogenesis is governed by developmental patterning mechanisms has come from studies of gastrulation in Drosophila. We developed a novel assay to recapitulate morphogenetic shape changes in individual cultured cells, and used RNAi-based screening to identify Mist, a Drosophila G-protein coupled receptor, which acts as a receptor for the secreted ligand Folded gastrulation in cultured cells. Mist plays a role in Fog-dependent embryonic morphogenesis, and its zygotic expression is regulated by the transcription factor Snail. Our data show how a cell fate transcriptional program can act through a ligand-GPCR pair to provide spatial regulation of epithelial morphogenesis.

Introduction

During embryogenesis, the developmental program sculpts sheets of epithelial cells to build organs, define tissue compartments, and establish the embryonic body plan. Forces driving these tissue-level rearrangements are produced by the actin and myosin cytoskeleton acting within individual cells and are transmitted from cell to cell within epithelia by

adherens junctions (Pilot & Lecuit, 2005; Kasza & Zallen, 2011). Cell and tissue shape changes are regulated by a complex interplay between maternally supplied proteins and patterned zygotic gene expression. Understanding how developmental patterning organizes cytoskeletal processes with spatial precision is a key question in the field of developmental biology (Leptin, 1995).

G-protein coupled receptors (GPCRs) are one of the largest groups of proteins found in the human genome, yet there are few examples of GPCRs regulating morphogenesis. Genetic analyses in Drosophila have revealed a possible example involving a pathway that triggers epithelial folding via apical constriction during gastrulation and salivary gland invagination downstream of the secreted protein Folded gastrulation (Fog) (Costa, et al., 1994; Sawyer, et al., 2010). This pathway is thought to involve a GPCR, as the $G\alpha_{12/13}$ homologue Concertina (Cta) is an integral component of the pathway. GPCR-independent activities of G-proteins also can regulate the cytoskeleton (Parks & Wieschaus, 1991; Izumi, et al., 2004; Wilkie & Kinch, 2005); thus it is unclear whether GPCRs are involved in initiating apical constriction.

Downstream of Fog, Cta is thought to activate RhoGEF2, which is recruited to the apical membrane by the transmembrane protein T48 (Rogers, et al., 2004; Kolsch, et al., 2007). RhoGEF2 then activates the small GTPase Rho1 to recruit and stimulate cytoskeletal contractile machinery, including Rho kinase (Rok), non-muscle myosin II, and actin, thereby inducing apical constriction (Barrett, et al., 1997; Hacker & Perrimon, 1998; Dawes-Hoang, et al., 2007; Kolsch, et al., 2007). This pathway is best characterized during gastrulation where it initiates formation of both the ventral furrow (VF), to internalize mesoderm, and the posterior midgut (PMG), to internalize endoderm (Sweeton, et al., 1991). It has served as a powerful paradigm for morphogenesis from the level of gene expression to cytoskeletal regulation. Fog is thought to act as a ligand to initiate this signaling pathway, but a receptor for Fog has remained elusive despite 20 years of genetic and cell biological analysis (Costa, et al., 1994; Dawes-Hoang, et al., 2007).

Results

Mist acts as a Fog receptor in cell culture

We developed a novel functional genomic approach to identify Fog receptors by reconstituting the pathway in a cell-based assay. We previously found that activating the downstream effector Rho1 in cultured Drosophila S2 cells induces a characteristic contracted morphology (Rogers, et al., 2004). We engineered S2 cells to express Fog, and used conditioned medium from these cells to screen several immortalized Drosophila cell lines for a contractile response. S2R+ cells exhibited robust contraction in response to Fog, including actin rearrangement and increased levels of phosphorylated myosin regulatory light chain (Spaghetti squash; Sqh), while S2 cells and several other epithelial-derived cell lines failed to respond (Figure 2-1A). RNAi-mediated depletion of proteins known to act in the epithelial folding pathway, including Cta, RhoGEF2, or Rho1, prevented Fog-induced S2R+ cell contraction, indicating that we had recapitulated this morphogenetic cascade in cultured cells (Figure 2-1B).

To identify a receptor that acts downstream of Fog, we performed a targeted RNAi screen, individually depleting the 138 known and predicted GPCRs in the Drosophila genome (Table 2-S1) (Brody, 2000; Broeck, 2001) and looking for cell contraction in response to Fog. Among the candidates, only two independent dsRNAs corresponding to the uncharacterized gene CG4521 (*methuselah-like 1*) consistently blocked Fog-induced contraction (Figure 2-1C). This gene, designated here as *mesoderm-invagination signal transducer* (*mist*), encodes a predicted GPCR of the secretin family. Mist is predicted to have a large N-terminal extracellular domain characteristic of this family, seven membrane-spanning helices, and a cytoplasmic C-terminal domain (Figure 2-1D). We generated antibodies to Mist that recognized a single protein band on immunoblots of S2R+ cells that was depleted by treatment with mist dsRNA (Figure 2-S1A). Consistent with the hypothesis that Mist is a Fog receptor, S2R+ cells overexpressing Mist-GFP immobilize highly elevated

levels of Fog on the plasma membrane when treated at 4°C to block endocytosis when compared to the Fog trapped by S2R+ cells expressing GFP alone (Figure 2-1E, F).

We next addressed whether Mist is sufficient to confer Fog responsiveness to otherwise nonresponsive cells. S2 cells have undetectable levels of Mist and do not respond to Fog (Figure 2-1C, 2-S1B). However, ectopic expression of full-length Mist endowed these

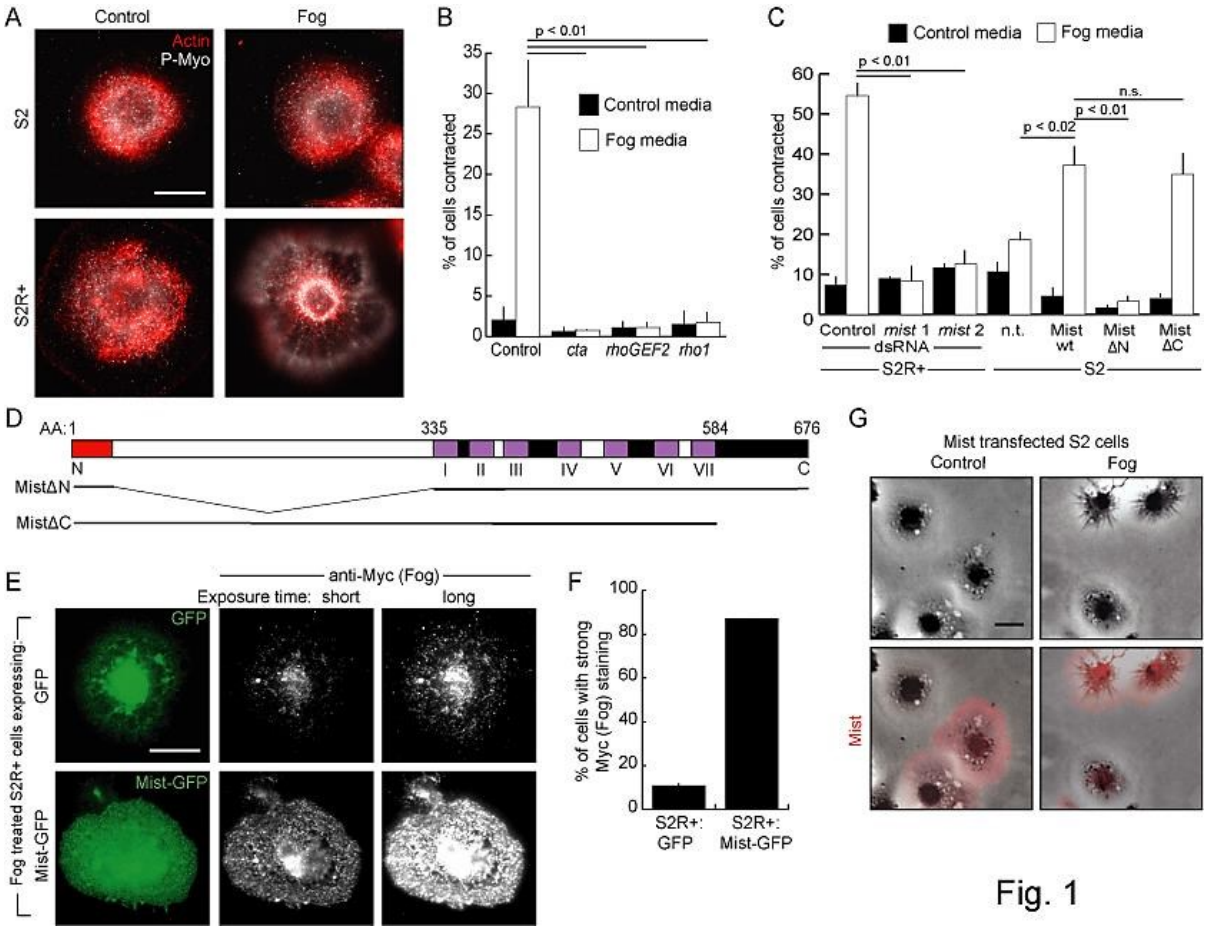


Figure 2-1. Mist acts as a Fog receptor in cell culture. **A.** S2R+ and S2 cells treated with control- or Fog-conditioned media and stained for actin (red) and phosphorylated myosin (Sqh) (P-Myo; white). **B.** Percentage of S2R+ cells contracted in response to treatment with control- or Fog-conditioned media after RNAi knockdown of known Fog pathway components. **C.** Percentage of cells contracted in response to control or Fog treatment after mist knockdown (in S2R+ cells) or overexpression of Mist constructs (in S2 cells). n.t.: not transfected. n.s.: not significant. **D.** Mist predicted structure. Top: 37aa signal sequence (red), 298aa extracellular domain (white), 7 predicted transmembrane domains (purple, numbered with Roman numerals), and a 93aa intracellular domain (black). Extracellular loops are white and intracellular loops black. Bottom: Mist truncations used in C. **E.** S2R+ cells expressing GFP or Mist-GFP were treated with Fog at 4°C and stained for Myc (Fog). Short and long exposures of Myc staining are shown. **F.** Percentage of GFP or Mist-GFP transfected S2R+ cells with strong Myc (Fog) staining after treatment with Fog at 4°C. **G.** S2 cells transfected with untagged Mist, treated with control or Fog media, and stained for Mist (red). Error bars B,C,F: standard deviation. Scale bars A,E,G: 20μm.

cells with the ability to contract upon treatment with Fog (Figure 2-1C, G). To define the domains required for Fog responsiveness we created Mist deletion constructs that retain the signal sequence but lack the predicted N-terminal extracellular domain (MistΔN), or lack the cytoplasmic domain (MistΔC) (Figure 2-1D). MistΔN failed to confer Fog responsiveness upon S2 cells, indicating that the extracellular domain of Mist is required for Fog signaling (Figure 2-1C). In contrast, MistΔC did confer Fog responsiveness on S2 cells, indicating the C-terminus is not essential for activating downstream effectors (Figure 2-1C, D). This result is not surprising, as it has been shown that some Gα subunits are primarily activated by intracellular loops of GPCRs (Cronshaw, et al., 2010). Together these data demonstrate that Mist is required for Fog signaling in cultured Drosophila cells and that the large extracellular domain of Mist is necessary, perhaps acting as a ligand-binding surface.

Mist is essential for *Drosophila* gastrulation

While these data indicate that Mist can act as a Fog receptor, they do not reveal whether Mist mediates the effects of Fog *in vivo*. Fog was originally identified as a secreted protein that triggers the early embryonic movements of gastrulation (Costa, et al., 1994). Thus, we tested the hypothesis that Mist acts as a Fog receptor to induce mesoderm invagination. We first examined whether *mist* is expressed at the right time and place to act in the Fog pathway. *mist* mRNA is present in the blastoderm, suggesting a maternal contribution (Figure 2-2A). Just prior to mesoderm invagination *mist* mRNA is strongly elevated specifically along the ventral side and posterior end of the embryo, corresponding to the VF and PMG primordia (Figure 2-2B-C'). It is also expressed in the cells between these two regions but remains at much lower levels in all other cells. *fog* RNA differs slightly in its expression pattern, notably lacking expression in the region between the VF and PMG (Figure 2-4F) (Costa, et al., 1994). *mist* mRNA expression remains strong in the mesoderm and endoderm after invagination (Figure 2-S2A). During VF invagination, Mist protein

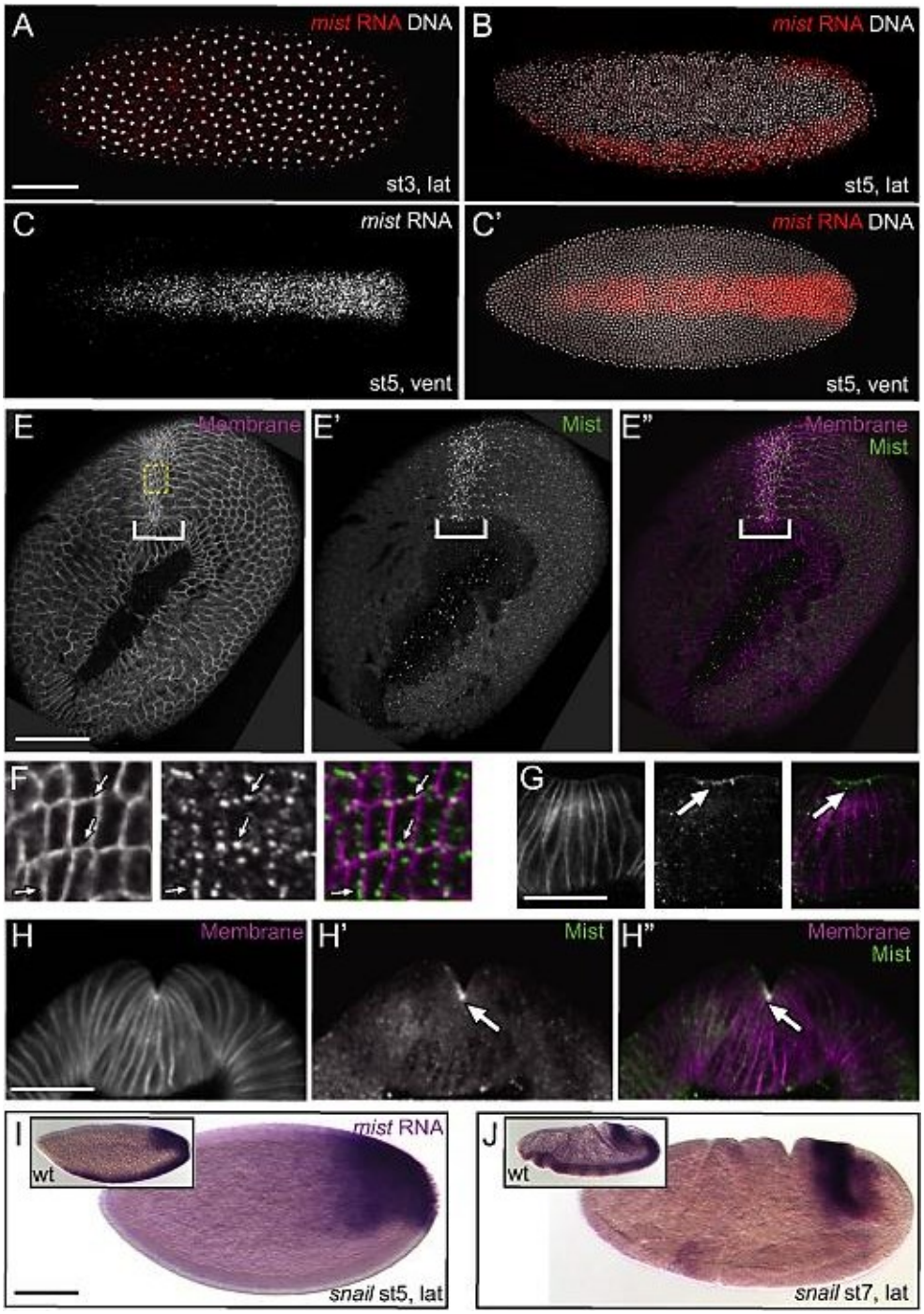


Figure 2-2. *mist* RNA is expressed specifically in the ventral furrow downstream of Snail**. A-C'.** Fluorescent *in situ* hybridization for mist RNA (red) in wild-type embryos counterstained for DNA (white). A. Pre-blastoderm stage embryo. B-C. Blastoderm stage embryos before VF apical constriction. C. mist channel alone from C'. Anterior is to the left in this and all other embryo figures. **E-H".** Mist protein (green) in cross sectioned embryos undergoing VF invagination--ventral is to the top and membranes are marked in magenta. E-E". Grazing apical cross section. F. Enlarged images of boxed area from E-E". G. Onset of apical constriction. H-H". Continuation of apical constriction. Arrows and brackets: Mist is enriched apically in cells of the ventral furrow. **I-J.** *in situ* hybridization to mist RNA in snail mutant embryos. I. Blastoderm stage embryo. J. Gastrulating embryo. Corresponding stages of wild-type embryos are shown in insets. lat: lateral view; vent: ventral view. Scale bar A, I: 100µm; E: 50µm; G, H: 25µm.

localized to the apical contractile surfaces of VF cells (Figure 2-2E-H"). Our antibody controls suggest that weak punctate cytoplasmic staining seen in all cells is likely background (Figure 2-S3). The elevation of mist mRNA in contractile cells of the VF and PMG primordia just prior to gastrulation is consistent with a role for Mist in the regulation of morphogenesis.

To investigate how the *mist* expression pattern is formed we looked to the embryonic dorso-ventral axis specification pathway, which is initiated by the maternally supplied Dorsal transcription factor. Dorsal acts through the zygotic transcription factors Twist and Snail, both of which are independently required for VF invagination (Zusman & Wieschaus, 1985; Leptin & Grunewald, 1990). *fog* is a known transcriptional target of Twist in early embryos, but Snail targets involved in VF invagination remain unclear (Simpson, 1983; Boulay, et al., 1987; Costa, et al., 1994; Seher, et al., 2006). We thus tested the hypothesis that *mist* might be a Twist or Snail target gene. Wild-type embryos exhibited robust expression of *mist* mRNA in the VF and PMG from cellularization through germ band extension (Figure 2-2A and 2-2F insets). When we crossed snail heterozygous parents, 25% of embryos, likely snail homozygous mutants, lacked mist expression in the VF but retained expression in the PMG (Figure 2-2F, 2-S2B). In contrast, most embryos from twist heterozygous parents exhibited wild-type mist expression with only a few lacking VF expression, presumably because Twist enhances Snail expression in the mesoderm (Figure 2-S2B, C) (Leptin, 1991). These data are consistent with *mist* being an embryonic target of Snail.

To test whether Mist functions *in vivo* during gastrulation, we created a mutant affecting *mist* expression by imprecise excision of a P-element inserted in the *mist* 5'UTR (Figure 2-S4A). This generated a small deletion, which we call $mist^{YO17}$ (Figure 2-S4B). $mist^{YO17}$ lacks the promoter, upstream regulatory region, and part of the 5'UTR of *mist*. It also disrupts the coding regions of adjacent genes: the small ribonucleoprotein particle

protein *SmG*, the ribosomal subunit *RpS19a*, the unannotated gene CG9777, and the maternally supplied Fog pathway component *rok* (see Supplemental Text for further description of the mutant). The *mist*^{YO17} mutation is zygotically embryonic lethal and embryos hemizygous for this mutation exhibit a significant reduction of mist mRNA throughout gastrulation (Figure 2-3A and 2-S4C). Further, like other Fog pathway mutants (Nikolaidou & Barrett, 2004; Dawes-Hoang, et al., 2007), *mist*^{YO17} mutant embryos have reduced apical recruitment of non-muscle myosin heavy chain (Zipper; Zip) within VF cells and uncoordinated VF apical constriction (Figure 2-S4D, E).

fog hemizygous mutants exhibit defects in internalization of mesodermal and PMG cells which result in changes to the morphology of the ventral midline (Figure 2-3C, E) (Costa, et al., 1994). These defects are not seen in wild-type embryos (Figure 2-3B, E). In crosses yielding 25% *mist*^{YO17} mutant embryos, we saw clear defects in the internalization of Twist-expressing mesoderm cells or morphology of the ventral midline in slightly more than a quarter of the embryos, suggesting that *mist* or one of the other genes deleted in *mist*^{YO17} is critical for this process (Figure 2-3D, E). We then used *in situ* hybridization for *mist* to genotype individual embryos. As expected, embryonic progeny of *mist*^{YO17} heterozygous females and wild-type males either had wild-type patterned *mist* expression or very little *mist* expression (presumptive *mist*^{YO17}/Y; Figure 2-4A, B and 2-S5A). More than 80% of embryos with wild-type *mist* expression showed no gastrulation defects, while 95% of embryos with weak *mist* RNA staining exhibited either a failure to fully invaginate mesoderm cells or a defect in the ventral midline, correlating *mist* expression with embryonic phenotype (Figure 2-4A, B, and D).

To test the hypothesis that *mist*^{YO17} gastrulation phenotypes are solely due to the lack of *mist*, we examined whether restoration of *mist* expression could rescue the observed defects. To do so, we took advantage of the fact that although the *mist*^{YO17} allele deleted the endogenous *mist* promoter, it retained from the P-element a GAL4-regulated Upstream

Activating Sequence (UAS) and minimal promoter directed toward the *mist* coding region (Figure 2-S4B). This allowed us to express *mist* under control of specific GAL4 drivers from the endogenous locus. We confirmed that this allele precisely expressed *mist* mRNA and

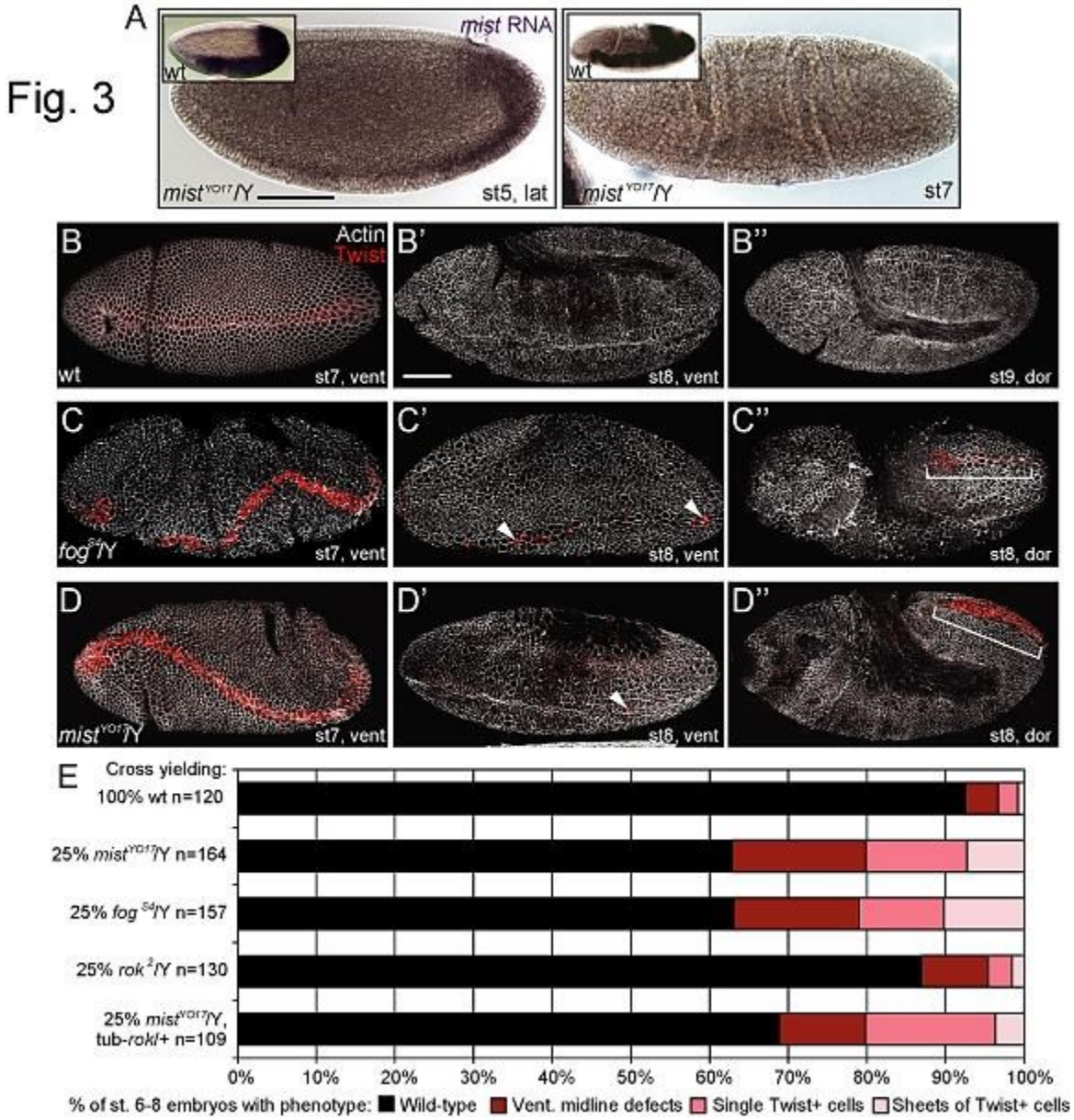


Figure 2-3. Mist is zygotically required for gastrulation. **A.** *in situ* hybridization for *mist* RNA in *mist*^{YO17}/Y embryos. Corresponding stages of wild-type embryos are shown in insets. **B.-D".** Actin (white) and Twist (red) stained embryos showing the range of gastrulation defects seen. B-B". Wild-type embryos. C-C". *fog*^{S4}/Y embryos. D-D". *mist*^{YO17}/Y embryos. C. and D. Ventral midline defects. C'. and D'. Single Twist positive cells not internalized (arrowheads). C". and D". Sheets of Twist positive cells not internalized (brackets). **E.** Quantification of gastrulation phenotypes for stage 6-8 embryos as pictured in B-D". *rok*^{2} gastrulation phenotype distribution is not significantly different from wild-type, while *mist*^{YO17}, *fog*^{S4}, and *mist*^{YO17};tub-*rok* distributions vary from wild type ($p<0.001$). n=number of embryos scored for each condition. vent: ventral view; dor: dorsal view; lat: lateral view. Scale bar A,B: 100μm.

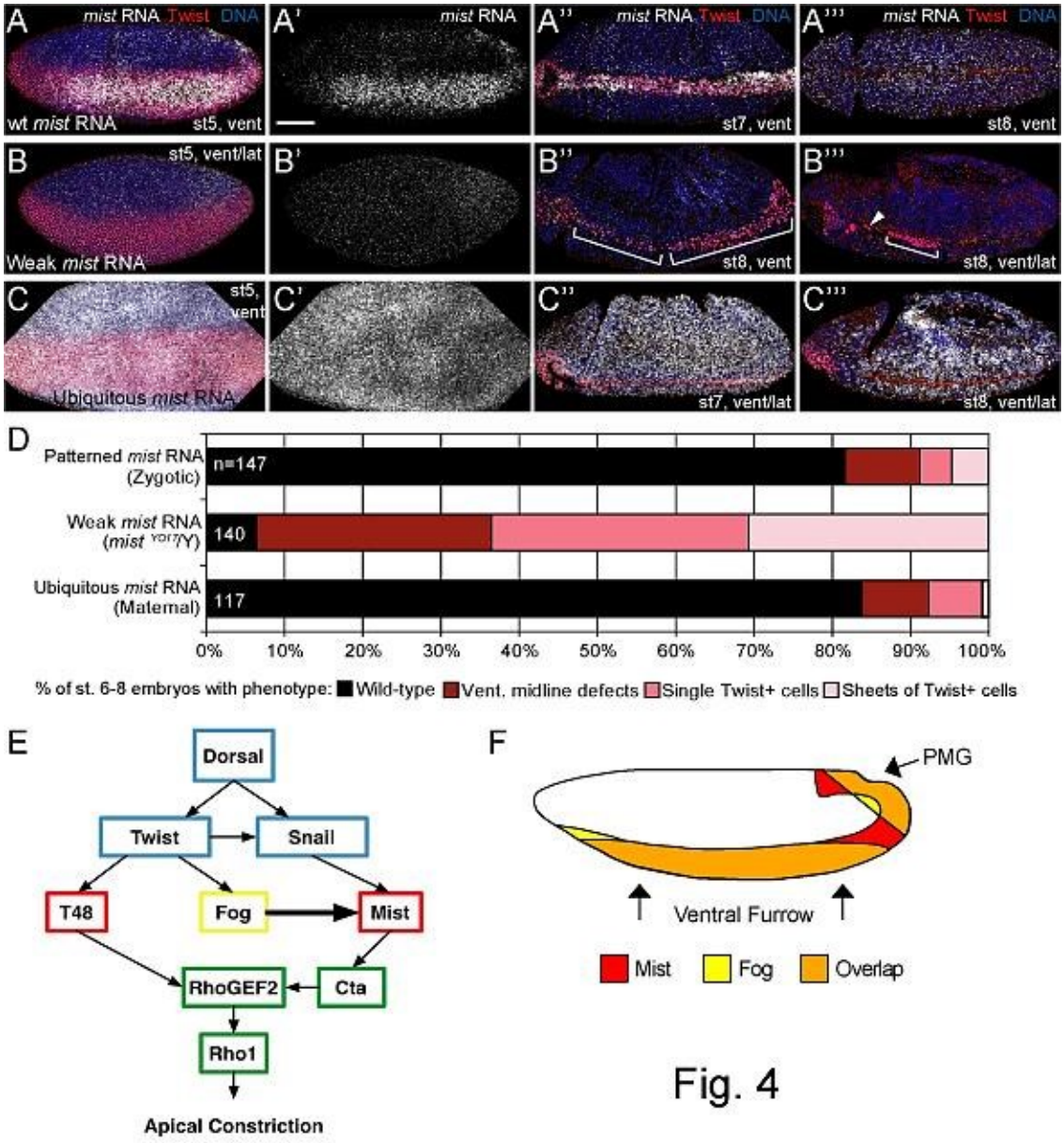


Fig. 4

Figure 2-4. *mist*^{YO17} gastrulation phenotypes are specific to Mist activity. **A-C'''.** Fluorescent *in situ* hybridization for *mist* RNA (white) seen in embryos from crosses shown in fig. S7. Twist antibody (red) reveals presumptive mesoderm and DNA is shown in blue. A.-A'''. Wild-type embryos showing patterned *mist* mRNA expression. B.-B'''. *mist*^{YO17}/Y embryos showing loss of patterned *mist* expression. C.-C'''. Embryos expressing ectopic *mist* mRNA uniformly (driven maternally). A.-C. Cellularization stages. A'.-C'. *mist* RNA alone from embryos in A.-C. A''.-C''. Early germ band extension stages. A'''.-C'''. Late germ band extension stages. **D.** Quantification of gastrulation phenotypes as pictured in Fig. 3, B-D''. Embryos with weak *mist* RNA expression have a higher frequency of gastrulation defects compared to wild-type embryos and embryos expressing ubiquitous *mist* ($p<0.001$). **E**. 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. **F.** Schematic of mist and fog RNA expression in cellularizing embryos. Areas of overlapping expression are where the VF and PMG invaginate. arrowhead: single Twist positive cells not internalized. brackets: sheets of Twist positive cells not internalized. n=number of embryos scored for each condition. vent: ventral view; lat: lateral view. Scale bar A: 100µm.

protein by using a driver which is activated in the posterior compartment of each segment in the later embryo (engrailed-GAL4; Figure 2-S3 and 2-S5B). We then crossed *mist*^{YO17} heterozygous females containing a maternally expressed GAL4 driver to wild type males (Figure 2-S5A). This cross results in GAL4 being loaded into eggs during their formation in the ovary and remaining into embryogenesis. The progeny of this cross had high level ubiquitous expression of *mist* RNA throughout most of embryogenesis (Figure 2-4C). Strikingly, these embryos showed normal gastrulation in proportions similar to those with wild-type *mist* RNA expression (Figure 2-4D). While these embryos had ubiquitous *mist* expression, they still have properly patterned Fog which presumably allows for the normal organization of their morphogenesis. Interestingly, embryos ubiquitously expressing *fog* but with presumed localized *mist* expression also form a fairly normal VF (Morize, et al., 1998).

To further confirm that loss of *mist* alone can cause gastrulation defects, we injected mist dsRNA into preblastoderm embryos and compared them to *fog* dsRNA- and control dsRNA-injected embryos. Control injected embryos rarely exhibited morphogenetic defects, while more than 50% of *mist* dsRNA injected embryos displayed disorganization of the ventral midline and/or failure of mesoderm invagination (Figure 2-S6A, D-F). These defects resemble those of *fog* dsRNA injected embryos, *fog* mutants, as well as *mist*^{YO17} mutants (Figure 2-S6B, C, and G, and 2-3C, D). Together, these data suggest that *mist* is the gene responsible for the *mist*^{YO17} gastrulation defects and that Mist is necessary for *Drosophila* gastrulation.

Of the other genes disrupted in *mist*^{YO17}, only *rok* has been shown to have a role in morphogenesis. Therefore, it was imperative to test whether gastrulation defects in *mist*^{YO17} embryos are due to *rok* loss of function. Previous analysis revealed that *rok* is not zygotically embryonic lethal, which suggests that *mist*^{YO17} defects are not solely caused by loss of Rok (Winter, et al., 2001). We found that flies hemizygous mutant for *rok* do not exhibit gastrulation defects and are indistinguishable from wild-type controls at the stages

examined (Figure 2-3E and 2-S7A). Finally, embryonic expression of *rok* under control of a ubiquitous tubulin promoter did not rescue the gastrulation defects of $mist^{YO17}$ mutants (Figure 2-3E and 2-S7B), though this construct can rescue *rok* mutant phenotypes (Winter, et al., 2001). These data suggest that *rok* disruption is not the sole cause of the $mist^{YO17}$ embryonic phenotype and further suggests that Mist is essential for *Drosophila* gastrulation.

Fog and Mist act together during wing and leg morphogenesis

The downstream Fog effector RhoGEF2 also plays an important role in another epithelial folding event, morphogenesis of the wing imaginal disc. Loss of RhoGEF2 in wing discs, precursors of adult wings, leads to aberrant folding patterns (Barrett, et al., 1997). The folds seen in discs with decreased levels of RhoGEF2 are stochastic in location, presumably due to spatial restriction of the growing epithelial layer. There is no evidence of aberrant proliferation or specification. *RhoGEF2* folding defects are enhanced by either *fog* or *cta* mutation (Nikolaidou & Barrett, 2004). Consistent with roles in wing imaginal disc folding, *fog* mRNA is expressed in this tissue and is enriched in cells forming the folds (Figure 2-5A). In contrast, past data suggest that adult wing morphogenesis can occur normally in the absence of Fog or Cta (Zusman & Wieschaus, 1985; Parks & Wieschaus, 1991). We believe there are likely two distinct issues which lead to these contrasting results. First, some defects in disc folding may be corrected later, and are thus not evident in the adult structure. Second, in the embryo, the Fog/Cta and T48 pathways converge on RhoGEF2 so that the *RhoGEF2* phenotype is stronger than that of any upstream single mutant (Kolsch, et al., 2007). It is possible that more than one pathway converges on RhoGEF2 in disc development, as well.

Despite these complications, the imaginal disc epithelium provided an opportunity to both explore whether Mist plays a role in adult morphogenesis and further explore the relationship between Fog and Mist. We compared mist and fog mRNA expression patterns in wild-type wing discs. In addition to *fog* mRNA expression in wing disc folds, it was also

present in the wing pouch (Figure 2-5A). Strikingly, *mist* mRNA levels were also elevated in stripes correlating with the folds of the wing disc (Figure 2-5B). Both mRNAs were specifically enriched on one side of the fold cells, presumably the apical end, as in the embryo where *fog* RNA is apically concentrated (Figure 2-5A and B right) (Dawes-Hoang, et al., 2007).

We next asked if altering normal expression of Fog pathway components affects wing disc morphogenesis by expressing different constructs using a wing disc-specific driver (A9-

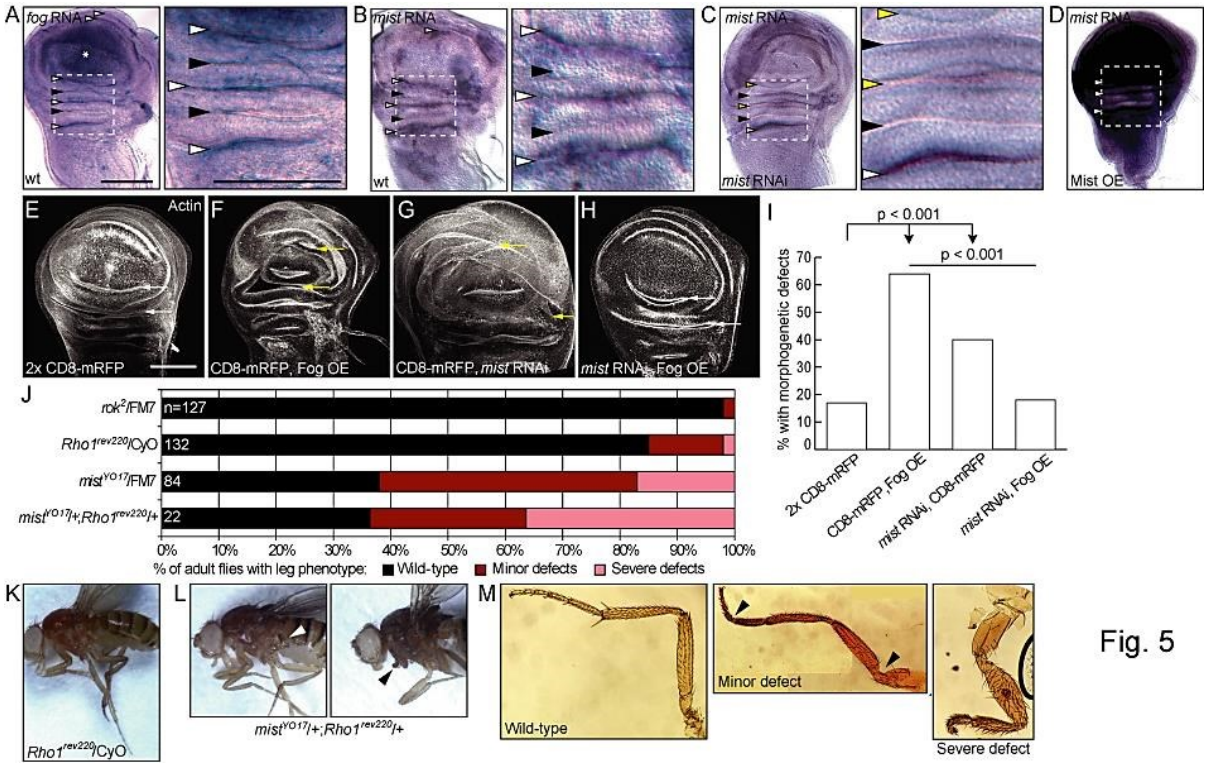


Fig. 5

Figure 2-5. Mist and Fog regulate wing and leg morphogenesis. **A, B.** Left panels: *in situ* hybridization for fog (A) or mist (B) RNA in wild-type wing imaginal discs. *: RNA in wing pouch. **C, D.** *in situ* hybridization for mist RNA in mist RNAi (C) or Mist overexpressing (D) wing imaginal discs. Right panel: Higher magnification of boxed areas in left panel. We chose a relatively morphologically normal wing disc for the *mist* dsRNA panels to allow comparison of *mist* expression in the folds to wild-type. White arrowheads: *mist* RNA in folds. Black arrowheads: basal sides of fold cells without RNA localization. Yellow arrowheads: fold regions without *mist* RNA. The fold with *mist* RNA in C is outside the expression domain of the driver. **E.-H.** Actin staining in wing imaginal discs. E. Control expression of two copies of CD8-mRFP. F. CD8-mRFP and Fog overexpression. G. mist dsRNA and CD8-mRFP. H. mist dsRNA and Fog overexpression. White arrows: proper folds; yellow arrows: misfolding. **I**. Percentages of wing imaginal discs with morphogenetic defects. Number of imaginal discs scored: 10-15 per condition. **J.** Quantification of leg defects in adults of given genotypes according to images in M. **K.** Adult *Rho1*^{rev220}/CyO fly with wild-type legs. **L.** Adult *mist*^{YO17}/+;*Rho1*^{rev220}/+ flies. **M.** Examples of legs with wild-type, minor defect, and major defect phenotypes. Arrowheads: malformed legs. Scale bar A, E: 100µm.

GAL4). A fluorescent control construct alone had no effect on imaginal disc morphology, but shows the domain of A9 expression (Figure 2-5E, I and 2-S8). In contrast, Fog overexpression led to disc and adult wing defects (Figure 2-5F; 21/49 adult wings malformed, 43.3%). To test whether Mist was important for these effects, we manipulated Mist levels in wing discs using transgenic RNAi. We confirmed strong reduction or increase of *mist* RNA expression compared to controls via *in situ* hybridization in *mist* RNAi or Mist overexpressing imaginal discs (Figure 2-5C, D). *mist* RNAi led to moderate stochastic, abnormal folding patterns in wing imaginal discs (Figure 2-5G, I). Next, we simultaneously expressed ectopic Fog and *mist* RNAi. Reducing levels of Mist rescued the misfolding phenotypes induced by Fog overexpression in both wing discs and adult wings (Figure 2-5H; 5/108 adult wings malformed, 4.7%; $p<0.001$). Together these data suggest that Mist can act in concert with Fog during wing disc morphogenesis. These data and those of Barrett, et al. are consistent with the idea that Fog, Mist, and Cta may provide a contributing, although not necessarily essential, input regulating RhoGEF2 in this tissue (Barrett, et al., 1997).

We further examined the role of Mist in epithelial morphogenesis using the $mist^{YO17}$ mutant. Reducing the levels of downstream Fog effectors including Rho, RhoGEF2, and Zipper disrupts leg morphogenesis (Halsell, et al., 2000; Patch, et al., 2009). We found that heterozygosity for $mist^{YO17}$ leads to a high frequency of defects in leg morphogenesis and that this phenotype is enhanced by heterozygosity for *Rho1* (Figure 2-5J-M). Importantly, heterozygosity for *rok* alone does not affect leg morphogenesis (Figure 2-5J). These suggest that Mist, perhaps in concert with Rok, may play a role in leg morphogenesis with other Rho pathway components. Together, our data are consistent with the hypothesis that Mist is an important player in the Fog pathway during gastrulation and also plays a supporting role in imaginal disc morphogenesis.

Discussion

The Fog pathway is one of the most well understood examples of how transcriptional programming is translated into cell behavior, but our picture of how this process is regulated was incomplete. Our data strongly support that the Drosophila GPCR Mist can act in the Fog morphogenetic pathway, providing a novel role for GPCRs in morphogenesis. Mist may be the sole receptor for Fog. Alternatively, there may be other receptors that act with Fog either as co-receptors with Mist or in different developmental processes. One possibility is the GPCR CG31660, which Matthew, et al. identified as a candidate by deletion mapping (Mathew, et al., 2009). Further detailed analysis of *fog*, *T48, mist* and *RhoGEF2* mutants, and cell biological and biochemical characterization of the relationship of Mist to other putative proteins in the pathway, will help define the precise array of cell behaviors controlled by each. The embryo also provides a venue to firmly establish the epistatic relationships of Fog and Mist by more extensive use of misexpression constructs of Fog pathway members.

Our data also allow us to complete the connection between the mesoderm transcriptional program driven by the transcription factors Twist and Snail and the cellular machinery involved in triggering epithelial folding (Figure 2-4E). Mist represents the first downstream transcriptional target of Snail required for VF invagination. Our data, in combination with data from others, provide a model for how the branches of the Twist and Snail regulatory pathway are ultimately integrated, by driving independently patterned, yet overlapping expression of a ligand-receptor pair (Figure 2-4F) (Costa, et al., 1994; Dawes-Hoang, et al., 2007). Twist activates production of Fog and T48 for VF invagination and reinforces Snail expression in the 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. Strikingly, the process can function correctly with either

ubiquitously expressed Fog or ubiquitously expressed Mist (our data) (Morize, et al., 1998). The dual localized expression of Fog and Mist may help make the patterned morphogenetic response of VF formation more robust, with possible subtle effects in timing or coordination. The broader implications of these concepts will be important and exciting to explore in the future.

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 (Rogers & Rogers, 2008). S2 cells were maintained in SF900 SFM (Invitrogen, Carlsbad, 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'-TAATACGACTCACTATAGG-3'). Control-fwd: 5'-TAAATTGTAAGCGTTAATATTTTG-3' and Control-rev: 5'-AATTCGATATCAAGCTTATCGAT-3' to amplify a region from the pBluescript plasmid; Cta-fwd: 5'- TGACCAAATTAACTCAAGAACGAAT-3', Cta-rev: 5'-TTCCAGGAACTTATCAATCTCTTTG-3'; RhoGEF2-fwd: 5'-ATGGATCACCCATCAATCAAAAAACGG-3', RhoGEF2-rev: 5'-TGTCCCGATCCCTATGACCACTAAGGC-3'; Rho-fwd: 5'-GTAAAACTTGCCTTCTGATTGTCT-3', Rho-rev: 5'-ATCTGGTCTTCTTCCTCTTTTTGA-3'; Mist1-fwd: 5'-AATTGCAAATTGAGGCCAAG-3'; Mist1-rev: 5'-AGAGCATTGATCGGCTGACT-3'; Mist2-fwd: 5'-CTCCATTGCCGGTGATTG-3'; Mist2-rev: 5'-GGAACGTCCACCAGATGTT-3'. For individual dsRNA treatments, cells at 50-90% confluency in 6- or 12-well plates were treated every other day for 7 days with 10μg/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). In Fog capture experiments all conditions were the same except Fog or control treatments were carried out at 4°C and HL3 and formaldehyde solutions were equilibrated to 4°C but used at room temperature. S2R+ cells were transfected using Effectene Transfection Reagent per the instructions (Qiagen, Dusseldorf, Germany). For dsRNA 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 μg 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). S2R+ cells were transfected using Fugene HD transfection reagent (Promega). For quantifying numbers of cells contracted, each condition was repeated at least in three times and ≥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 with pMT-Fog-Myc and 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 10min. Cleared medium was concentrated 40x in Amicon 30k

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 of cells:

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 $CaCl_2$-$2H_2O$; 20 mM $MgCl_2$-$6H_2O$; 10 mM $NaHCO_3$; 5 mM trehalose; 115 mM sucrose; 5 mM HEPES; pH to 7.2), and permeabilized with PBST, or TBST for phosphorylated myosin antibody staining. Cells were blocked with 5% normal goat serum (Sigma-Aldrich, St. Louis, MO) in PBST (or TBST) and stained with antibody diluted into the same solution. Following washing, cells were incubated with secondary antibodies and Alexa488-phalloidin (1:100 dilution; Invitrogen), washed again, and mounted in fluorescence mounting medium (Dakocytomation, Glostrup, Denmark). We acquired images using a CoolSnap HQ CCD camera (Roper Scientific, Ottobrunn, Germany) on a Nikon Eclipse Ti inverted microscope driven by Nikon Elements software (Tokyo, Japan). Photoshop CS4 (Adobe, San Jose, CA) was used to adjust input levels so that the main range of signals spanned the entire output grayscale and to adjust brightness and contrast.

Drosophila tissues:

Embryos were collected on apple juice plates supplemented with yeast paste at 25°C, fixed in 4% formaldehyde in PBS/heptane, methanol devitellinize, and stained as above without phalloidin. For Zipper staining embryos were fixed in NaCl/Triton at 100°C. DNA was stained with Hoescht 33342 diluted 1:10,000. In mutant embryo analysis all embryos in a population within a certain range of stages were scored. The percentages of mutant embryos expected and the range of stages examined are indicated in each figure legend.

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. Imaginal discs were

mounted by dissecting wing discs from the larval cuticles in 70% glycerol in PBS. We always used the same number of UAS-transgenes to eliminate the effects of GAL4 squelching. Wing disc abnormal morphology was defined by discs having at least two of the following characteristics: bifurcation of a usually single fold, a fold reduced to half or less of its normal length, an extra fold, intersection of two folds, or obvious bend in a normally straight fold. Adult wing abnormal morphology was defined by the presence of at least one distinct fold or blister in the wing blade. Statistics for Fig. 3E, 4D, 5I and J, and adult wings were determined by Chi squared test.

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 *(11)* 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). Photoshop CS4 (Adobe, San Jose, CA) was used to adjust input levels so that the main range of signals spanned the entire output grayscale and to adjust brightness and contrast.

Immunoblotting:

S2 or S2R+ extracts were produced by resuspending cell pellets in PBST. 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.

in situ hybridization:

Probe preparation and *in situ* hybridization for embryos and imaginal discs was performed as described previously (Kearney, et al., 2004). *mist* dsRNA 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 Axiophot microscope, Sony 3XDD CCD video camera, and Zeiss Axiovision software.

Embryo injection:

Embryos were prepared as previously described previously, unless noted below (Carthew, 2006). Primers used for dsRNA synthesis are as follows and are all preceded with the T7 sequence. Control-fwd: 5'-TAAATTGTAAGCGTTAATATTTTG-3' and Control-rev: 5'-AATTCGATATCAAGCTTATCGAT-3'; Fog-Fwd: 5'-ATATTTTTGAGAAGAAATTCCCCAC-3', Fog-Rev: 5'-CTGTGGTATACTCGTCTTCCTCACT; 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% paraformaldehyde, and hand-peeled to remove the vitelline membrane. Images were obtained using a Zeiss LSM 710 and LSM software.

Antibodies:

The following antibodies were used in this study: rabbit anti-Myc (Sigma), used at 1:300 dilution; mouse anti-α tubulin monoclonal DM1α (Sigma), used at 1:500; 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; mouse anti-actin (Millipore) used at 1:1000; rabbit anti-phosphorylated myosin II S19 (Cell Signaling, Danvers, MA) used at 1:100; rabbit anti-Zipper (Kiehart, et al., 1990) used at 1:1000. In addition, streptavidin-alkaline phosphatase (Jackson Immunoresearch, West Grove, PA) was used at 1:1000. Antibodies to Mist were raised in rabbit against recombinant GST fusions with the C-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). Secondary antibodies RhodamineX-conjugated goat anti-rabbit, Cy2-donkey anti-mouse, etc. were all diluted 1:1000 (Jackson Immunoresearch).

P-element excision and determination of mutation:

All procedures were carried out at 25°C. Homozygous P-element containing female flies were crossed to males with a marked chromosome expressing transposase. F1 males with mosaic eye and/or body color and the transposase chromosome were chosen and crossed to females with a balancer X chromosome. Single F2 females with the balancer X chromosome, no transposase, and altered eye and/or body color were selected. Each was crossed to males with the same balancer X chromosome to create balanced stocks of excised X chromosomes. These stocks were screened for the lethality of the excisions and for absence of at least one P-element end and non-wild-type *mist* region by PCR. Single dead embryos were chosen from this stock and screened for the absence of amplification of a balancer chromosome-specific product by single embryo PCR. Further PCRs were performed on this hemizygous embryo DNA to determine the extent of the lesion. Once breakpoints were determined, a fragment across the lesion was amplified and sequenced using an Applied Biosystems 3730xl Genetic Analyzer by the UNC – CH Genome Analysis Facility.

For initial screening genomic DNA was collected from adult flies as suggested by E. Jay Rehm (Berkeley Drosophila Genome Project). Single embryos were prepared for PCR by dispensing single fixed embryos into tubes with 10µl Single Embryo Buffer (10mM Tris-HCl, pH 8.2, 1mM EDTA, 25mM NaCl) and incubating at -20°C for 1 hour. These were thawed and Proteinase K was added to 4mM. The embryos were then incubated at 37°C for 30 min and 95°C for 2 min. All amplification was performed using Phusion HF Master Mix (Thermo Scientific, Waltham, MA).

Fly stocks:

The following fly lines were used in this study: UAS-*mist* dsRNA , UAS-*cta* dsRNA (Vienna Drosophila Resource Center), Ubi-*moesin*-GFP (Edwards, et al., 1997), *yellow white*,

fog^{S4}/FM7 *twist*-GFP, A9-GAL4, *twist*^{1}/CyO, *snail*^{18}/CyO, *rok*^{2}/FM7c, tub-*rok*, P{EPgy2}mthl1[EY16157], *Bc*/CyO H{w^{+},Δ2-3}, UAS-CD8-mRFP (II and III), *Rho1*^{rev220}/CyO, matα4-GAL4VP16 (II and III) (from Bloomington Drosophila Stock Center, Bloomington, Indiana), UAS-*fog* (Eric Wieschaus, Princeton University), *arm*^{XP33} FRT101/FM7 *twist*-GFP (Peifer & Wieschaus, 1990). 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. The stock used in these studies has UAS-*mist* inserted on the third chromosome.

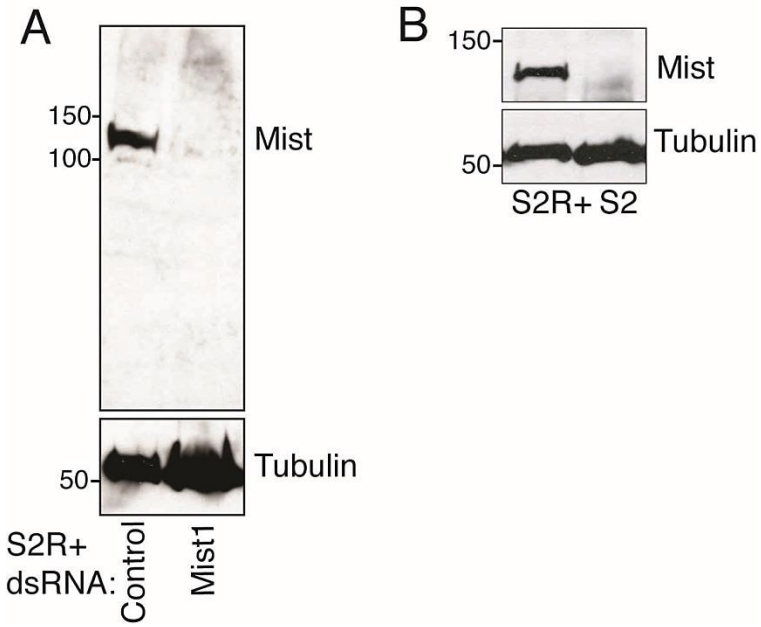
Supplemental text

Description of the *mist*^{YO17} excision allele:

We began with an EPgy2 P-element within the 5'UTR of mist, P{EPgy2}mthl1[EY16157]. This parental P-element strain does not exhibit any embryonic or other morphological or growth defects. We mobilized this, seeking imprecise excisions that deleted part or all of *mist*, by introducing one copy of transposase into the background of the P-element. We selected for mosaic eye and/or body color in the next generation and crossed in a balancer chromosome. Balanced lines were established from single flies with altered body and/or eye color from the original line, and these were examined. We focused on one candidate that was zygotically lethal and characterized it by PCR. PCR and sequencing of *mist*^{YO17} indicated that the right end of the P-element is intact and present in its parental location. This 4.4kb stretch of P-element includes a UAS (upstream activating sequence) driven promoter oriented towards mist, and 3.5kb of yellow and its flanking region. The rest of the P-element is missing. *mist*^{YO17} deletes the 92bp of the 5'UTR of *mist* to the left of the P-element, the entire promoter and upstream regulatory regions of *mist*, the genes encoding the small ribonucleoprotein particle protein SmG, the ribosomal protein RpS19a, the

unannotated gene CG9777, and approximately 7.9kb of the *rok* gene. The coding sequence of *mist* is not disrupted. Loss of SmG is embryonic lethal but has no described alterations in morphogenesis (Bellen, et al., 2004). Mutation of ribosomal subunits results in the well-described Minute phenotypes that include prolonged development, low fertility, and decreased body size (Marygold, et al., 2007). Consistent with our molecular analysis $mist^{YO17}$ heterozygotes have a Minute phenotype. rok^{2} zygotic mutants are embryonic viable and die during larval development (Winter, et al., 2001).

Supplemental figures



Supplemental Figure 2-1. *mist* RNAi reduces Mist protein levels in S2R+ cells and S2 cells do not express endogenous Mist. **A.** Western blot of S2R+ cell lysates for Mist, after control or *mist* dsRNA treatment. **B.** Western blot for Mist in S2R+ and S2 cells. α-tubulin is used as loading control for Western blots.

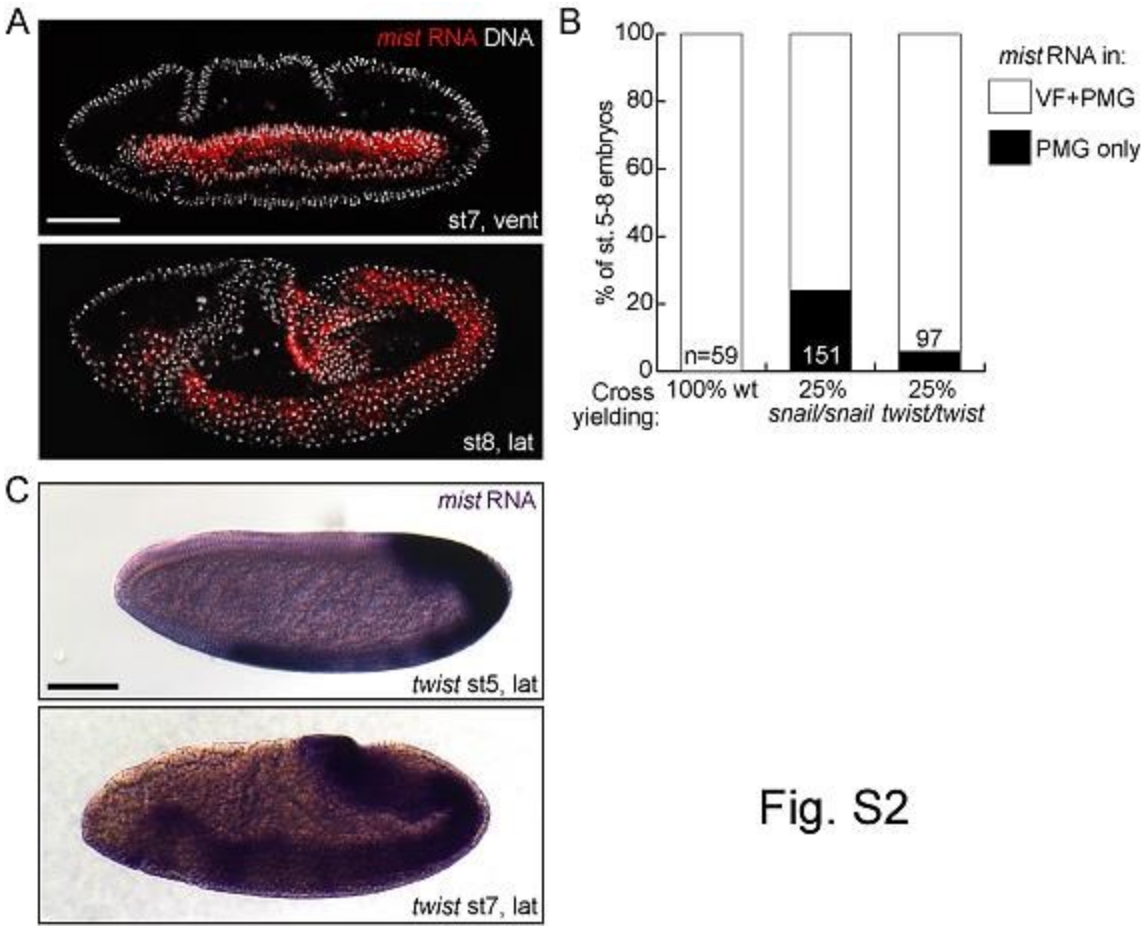
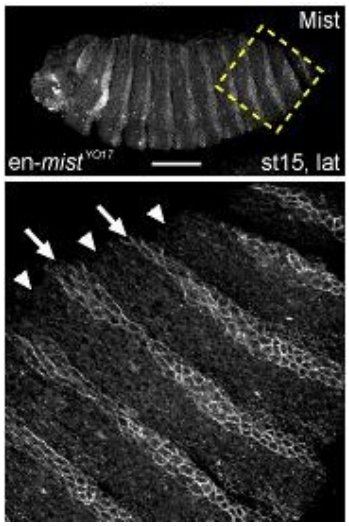


Fig. S2

Supplemental Figure 2-2. *mist* RNA remains in mesoderm after invagination and is only modestly affected by loss of *twist*. **A.** mist *in situ* hybridization (red) in wild-type embryos shows mesodermal mist expression after VF and PMG invagination. DNA is in white. B. Percentages of stage 5-8 embryos with PMG only or PMG and VF localization of mist RNA in wild-type, and *snail*, or *twist* mutants. **C.** mist *in situ* hybridization in embryos from twist heterozygous parents shows both VF and PMG expression. Scale bars A, C: 100µm. vent: ventral view; lat: lateral view.



Supplemental Figure 2-3. Mist antibody specifically recognizes Mist in embryos. Embryo from cross between *mist*^{YO17} heterozygous female and en-GAL4 homozygous male stained for Mist. The UAS-promoter in the P-element is driven by en-GAL4, leading to ectopic Mist expression in the posterior compartment of each body segment. Bottom panel is enlarged boxed area in top panel. Arrows indicate cortical Mist staining in the posterior compartment. Arrowheads indicate presumed antibody background in intervening regions. Scale bar: 100μm. lat: lateral view.

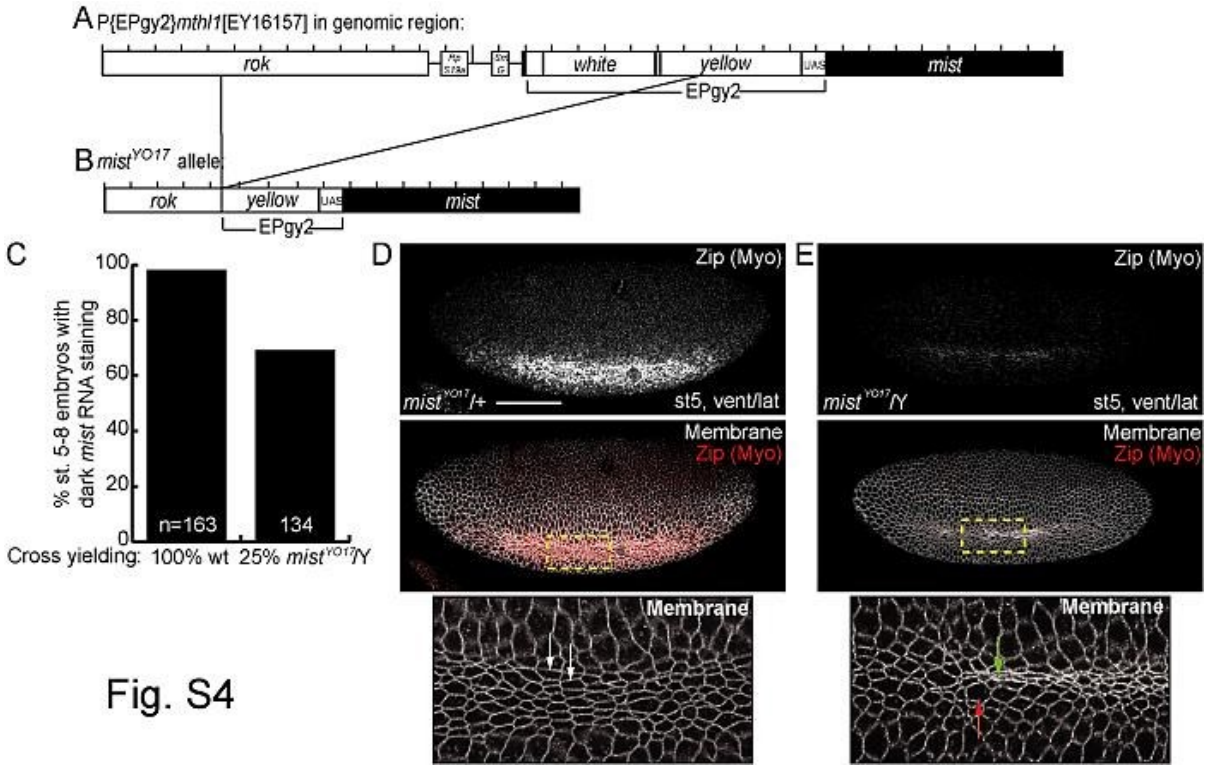


Fig. S4

Supplemental Figure 2-4. *mist*^{YO17} is a deletion disrupting both *mist* and *rok*. **A.** Diagram of the *mist* genomic region containing P{EPgy2}mthl1[EY16157] within the *mist* 5'UTR. **B.** Diagram of the genomic lesion in *mist*^{YO17}. Tick marks are placed 1kb apart. **C.** Quantification of stage 5-8 embryos with dark *mist* RNA staining in wild-type and *mist*^{YO17} embryos. n=number of embryos scored for each condition. **D, E.** Non-muscle myosin heavy chain (Zipper; Zip) staining (top), and Zip (red) and neurotactin (membrane, white) staining (middle) in embryos. Bottom panels show membrane staining only in cells within and surrounding the VF (boxed region of middle panels). D. *mist*^{YO17}/+ embryo with strong apical Zip staining and coordinated VF cell contraction. White arrows show contractile cells of the VF with similar apical area. **E.** *mist*^{YO17}/Y embryo with weak Zip staining and uncoordinated VF cell contraction. Green arrow shows a cell with more constricted apex and red arrow shows a cell with less constricted apex than average. Scale bar D: 100µm. vent: ventral view; lat: lateral view.

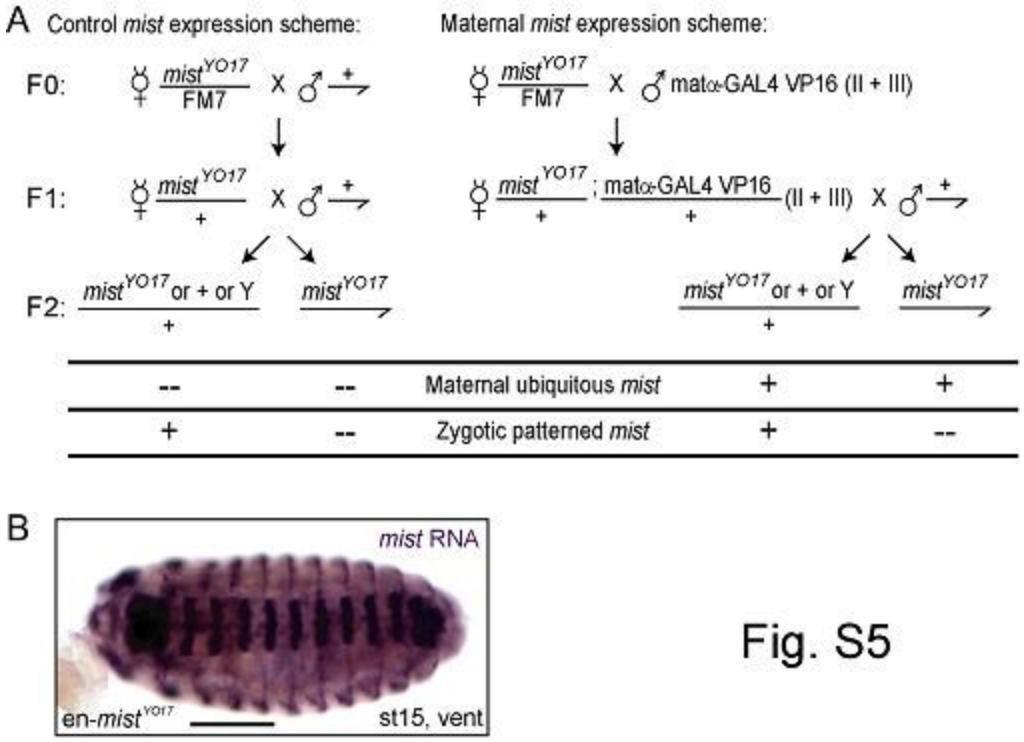
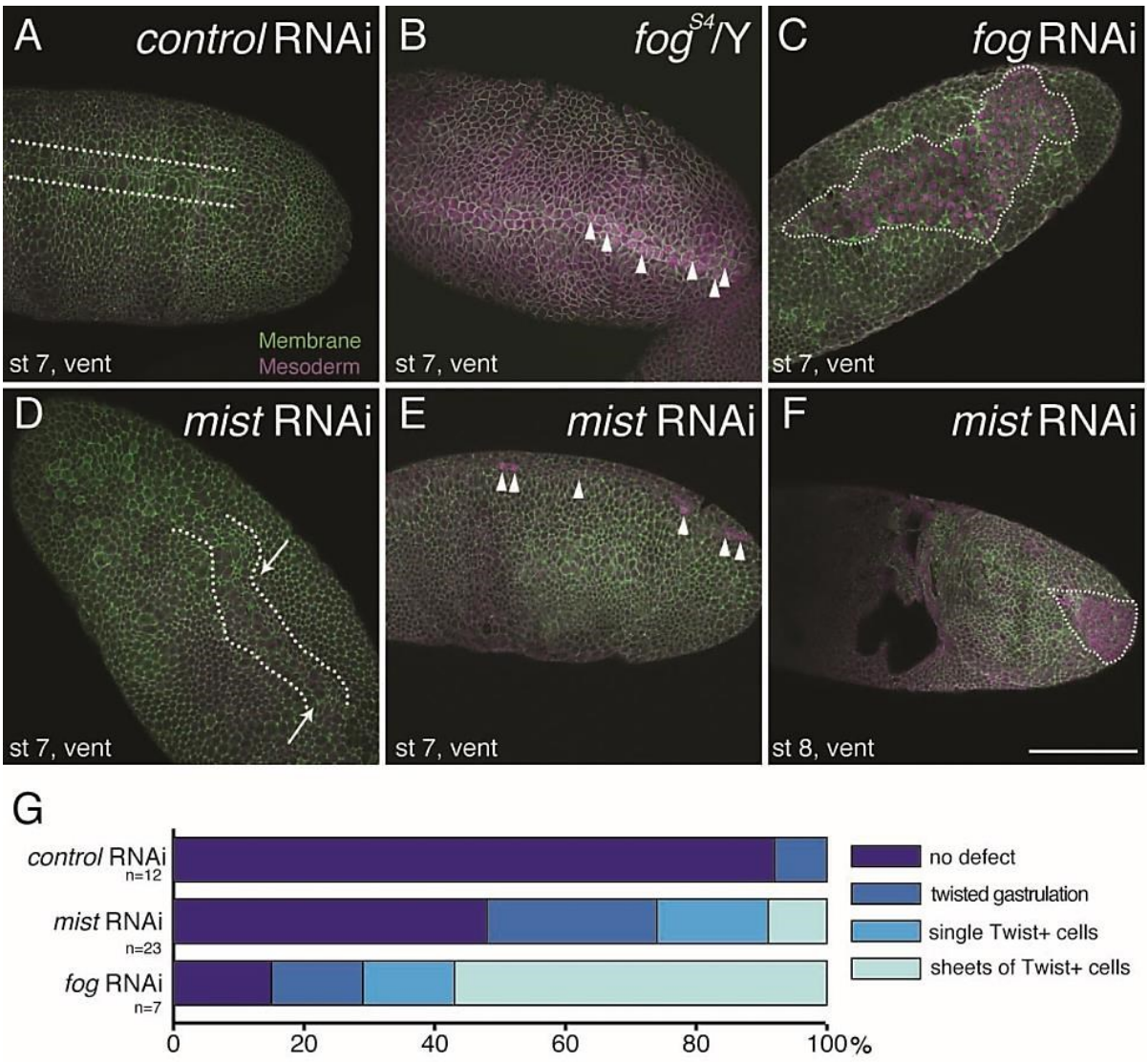
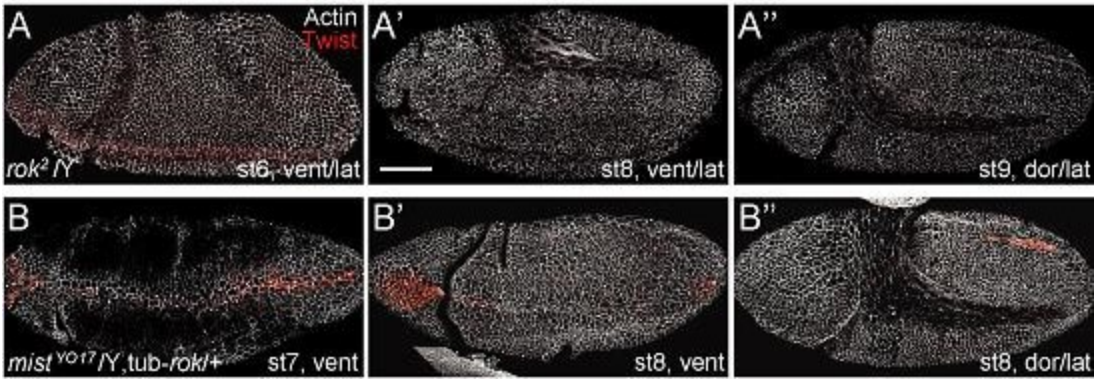


Fig. S5

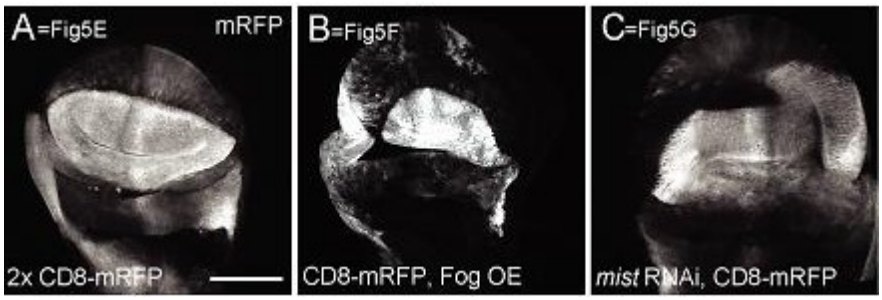
Supplemental Figure 2-5. The *mist*^{YO17} allele expresses *mist* RNA under UAS control. **A.** Cross schemes to examine the phenotypes of embryos expressing different patterns of *mist* RNA. Left: F2 embryos collected from this scheme are expected to have either wild-type patterned *mist* RNA expression or no *mist* RNA expression. Right: F2 embryos collected from this scheme are expected to have ubiquitous maternal *mist* RNA expression. Some have *mist* RNA expressed from a wild-type allele as well, but this was not visible due to high levels of ubiquitous RNA. Bottom: Indication of the expected embryonic *mist* RNA expression pattern for each genotype listed above. **B.** *in situ* hybridization for *mist* RNA in *mist*^{YO17} embryos carrying an engrailed-GAL4 driver which expresses in the posterior compartment of each segment. Scale bar B: 100μm. vent: ventral view.



Supplemental Figure 2-6. *mist* RNAi disrupts mesoderm invagination. **A.** Moesin-GFP expressing embryo injected with control dsRNA has a straight ventral midline (flanked by dotted lines). **B.** *fog* hemizygous mutant with improperly internalized mesoderm (arrowheads). **C.** *fog* dsRNA injected embryo has 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 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. Scale bar: 100μm. **G.** Quantification of morphological defects in dsRNA injected embryos. n=number of embryos scored for each condition.



Supplemental Figure 2-7. *rok* disruption does not cause *mist*^{YO17} gastrulation phenotypes. Actin (white) and Twist (red) staining in embryos. **A.-A".** *rok*^{2}/Y embryos with normal gastrulation. **B.-B".** *mist*^{YO17}/Y;tub-*rok* /+ embryos with gastrulation defects as in Fig. 3C-C" and D-D". Scale bar A: 100μm. vent: ventral view; dor: dorsal view; lat: lateral view.



Supplemental Figure 2-8. A9-GAL4 drives exogenous construct expression in a subset of wing disc cells. **A.-C.** mRFP expression in the same wing imaginal discs as in Figure 5E-G showing the A9 expression domain. Scale bar: 100μm.

5-HT1A	CG13802	CG4313	ETHR	mthl13	Rh4
5-HT1B	CG13995	CG4395	FR	mthl14	Rh5
5-HT2	CG14593	CG4875	Fsh	mthl2	Rh6
5-HT7	CG15556	CG7431	fz	mthl3	Rh7
AdoR	CG15614	CG7497	fz2	mthl4	rho
AlCR2	CG18208	CG7536	fz3	mthl5	rho-4
AlstR	CG2061	CG7918	fz4	mthl6	rho-5
AR-2	CG2901	CG7994	GABAB-R1	mthl7	rho-6
boss	CG30106	CG8007	GABAB-R2	mthl8	rho-7
capaR	CG30340	CG8784	GABAB-R3	mthl9	rk
CcapR	CG31660	CG8795	GRHR	mXr	ru
CCKLR17D1	CG31720	CG9643	GRHRII	NepYr	SIFR
CCKLR17D3	CG31760	CG9918	kek3	ninaE	smo
CG10481	CG32447	Cirl	Lgr3	NPFR1	SPR
CG10483	CG32547	cry	Lkr	NPFR76F	stan
CG11318	CG32843	Cyp1	mAcR-60C	oa2	star1
CG11910	CG33310	D2R	mGluRA	Oamb	stet
CG12290	CG33639	Dh44-R1	moody	Octbeta2R	Takr86C
CG12370	CG33696	DmsR1	mth	Octbeta3R	Takr99D
CG12796	CG34372	DmsR2	mthl1 (Mist)	pdfr	Tre
CG13229	CG34381	DopEcR	mthl10	Proc-R	Tre1
CG13575	CG34411	DopR	mthl11	Rh2	TyrR
CG13579	CG4168	DopR2	mthl12	Rh3	TyrRII

Supplemental Table 2-1. List of genes targeted with dsRNAs in cell culture screen. Each was targeted by at least one dsRNA. *mist* (*mthl1*) is highlighted in red.

Acknowledgments

We thank S. Crews, R. Duronio, 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).

Chapter 3

Negative regulation of Fog signaling by GPRK2 and Kurtz

This chapter represents a manuscript in progress. Experiments were designed by me and my advisor, Stephen Rogers. They were carried out by me and two undergraduate researchers under my mentorship, Haley Simpson and Emily Simon.

Abstract

The *Drosophila* Folded gastrulation pathway is a major model used for studying signaling in morphogenesis and contains as a crucial component Mist, a G-protein coupled receptor (GPCR). All of the major components of the pathway have been discovered but little has been done to investigate the termination of cellular contraction downstream of Fog. Signaling by GPCRs, a major class of proteins involved in signal transduction, is canonically attenuated by GPCR kinases and β-Arrestins. The sole members of these regulatory protein classes expressed outside the eye in *Drosophila* are GPRK2 and Kurtz (Krz). We hypothesized that they may play a role in terminating Fog signaling. We used a cell culture model of Fog-induced actomyosin constriction to show that Krz and GPRK2 both act negatively on cell contraction. Altering Krz levels also interferes with wing imaginal disc morphogenesis, which relies on Fog signaling. These results suggest that the Fog signaling pathway is an ideal model for further examining mechanisms of GPCR regulation in morphogenesis.

Introduction

G-protein coupled receptors (GPCRs) are a major class of transmembrane signaling proteins which allow cells to sense and respond to their environments (Bockaert & Pin, 1999). GPCRs can respond to a broad range of signals, from light to lipids to peptides. They play roles in processes as wide-ranging as sensory perception, neurotransmission, and cell migration and

are the targets of hundreds of medicines. One major aspect of biology in which a GPCR was only recently implicated is morphogenesis, the process by which cells and simple tissues change shape and orientation to form complex and mature structures.

The secretin-family GPCR Mist has been implicated in the Folded gastrulation (Fog) signaling pathway during *Drosophila* development (Chapter 2). Fog is a secreted protein which acts through Mist to activate the $G\alpha_{12/13}$ family member Concertina (Cta) (Chapter 2 (Parks & Wieschaus, 1991; Costa, et al., 1994). Cta activates a signaling cascade which results in actin- and nonmuscle myosin II-based constriction in the areas of the cells in which the Fog signal was received. This apical constriction event mainly occurs in subsets of cells within flat epithelial sheets, producing folds or invaginations. Fog signaling is crucial for gastrulation, axon guidance, and salivary gland invagination during embryogenesis, and imaginal disc morphogenesis during larval development (Leptin & Grunewald, 1990; Nikolaidou & Barrett, 2004; Ratnaparkhi & Zinn, 2007). The overall process of ventral furrow (VF) invagination as well as the mechanisms of cell shape change during gastrulation in *Drosophila* have been compared to vertebrate neurulation (Schoenwolf & Franks, 1984; Sweeton, et al., 1991; Copp & Greene, 2010). Defects in *Drosophila* gastrulation leave mesodermal and endodermal cells on the exterior of the embryo and also result in a twisting of the body axis (Sweeton, et al., 1991). These defects are lethal. Neurulation defects in vertebrates similarly lead to developmental abnormalities such as spina bifida (Copp & Greene, 2010).

The activation of the Fog signaling pathway has been well studied, but how signaling is terminated has not. GPCR signaling is canonically attenuated by the action of G-protein coupled receptor kinases (GRKs) and β-Arrestins. The C-terminal tail of an activated GPCR is phosphorylated by GRK from one time up to tens of times (Premont & Gainetdinov, 2007). β-Arrestins then bind the phosphorylated GPCRs. This interaction may have many effects on the receptor's signaling potential, including competing for receptor binding sites with Gα proteins, activating Arrestin-dependent downstream effectors, or recruiting clathrin-mediated

endocytosis machinery to internalize receptors. Internalization of receptors can lead to their degradation and also to their relocalization to an area of the cell where their effectors are not present, thereby inhibiting signaling temporarily but allowing receptors to be recycled.

The *Drosophila* genome encodes only one GRK and one β-Arrestin, GPRK2 and Kurtz (Krz), with roles outside visual perception (Cassill, et al., 1991; Roman, et al., 2000). They are both expressed in early embryos and imaginal discs and so are present in the right places to function in the Fog pathway (Schneider & Spradling, 1997; Roman, et al., 2000). GPRK2 is necessary in adult females for egg production, but G*PRK2* mutants do lay a few eggs (Schneider & Spradling, 1997). These GPRK2-deficient embryos show a range of phenotypes which include twisted gastrulation and ventral defects, the hallmark Fog pathway phenotypes. Similarly, embryos lacking maternal *krz* have phenotypes which include a twisted body axis and ventral cuticle holes (Tipping, et al., 2010). *GPRK2* and *krz* are also each active and genetically interact during wing morphogenesis, though their roles are complicated by their functions in various pathways, including tissue patterning and cell specification (Molnar, et al., 2011). These phenotypes lead us to believe that they may also play a role in attenuating Fog signaling in these tissues.

Our lab previously established a cell culture system to study the Fog pathway (Chapter 2). We engineered S2 cells to produce Fog and concentrate the secreted protein from the cells' media. Application of concentrated Fog protein to S2R+ cells, an S2-derived cell line, induces characteristic actin- and myosin-based constriction and transformation of cells from a flat pancake-like shape to a volcano-like shape (Chapter 2). We are now using this system to further investigate the dynamics of Fog-induced cellular constriction and to determine the roles of GPRK2 and Krz in this process. We also determined that altering levels of Krz in wing imaginal discs disrupts their folding. Alterations of GPRK2 and Krz levels in both cell culture and imaginal discs influences apical constriction in ways consistent with their being players in Fog signal termination.

Results

In order to investigate potential signal termination we first needed to know how cells normally respond to Fog. We first utilized phase contrast time lapse imaging of S2R+ cells to explore their contraction (Figure 3-1A). S2R+ cells in control media were flat, with smooth lamellipodia and nuclei centered within the cell. Fog treated cells quickly changed shape, with ridges forming in their lamellipodia, and generally remained that way for the course of the time lapse. The percentage of cells contracted was counted at 30 second time points over the course of 30 minutes in the presence of Fog or in control media concentrated from S2 cells not expressing Fog (Figure 3-1B). Cells were counted as contracted if they had identifiable ridges throughout at least half of their lamellipodial surface area. Control cells occasionally looked contracted, but this phenotype was seen less than 10% of the time. In the presence of Fog, the number of contracted cells increased rapidly over the course of about five minutes and then began to level off. The percentage continued to increase slowly for the rest of the time course to a maximum of around 70%. A large percentage of S2R+ cells in Fog can stay contracted for several hours (data not shown). Since the majority of the contraction occurred within five minutes, we examined this time period more closely counting the percentage of cells contracted every ten seconds (Figure 3-1C). The rate of cell contraction was approximately linear over the five minute period.

The data collected so far will allow us to examine the effects of disrupting the system on cell contraction. However, we hypothesize that GPRK2 and Krz are involved in termination of signaling and any effect they may have could be more easily seen upon removal of Fog, the activating signal. We performed more time lapse imaging of S2R+ cells treated with Fog for five minutes then washed with excess Schneider's media to remove any unbound Fog protein (Figure 3-1B). These cells continued to contract for about two minutes after Fog washout. Then there was a ten minute period of fairly rapid loss of contracted cells followed by a slow decrease to about 10% contracted over the rest of recording. It took a few minutes after Fog washout for

signaling termination to be seen by our readout of percent cells contracted, but after that cells relaxed fairly rapidly. The initial delay could indicate the time it takes for signal termination at the top of the pathway, Fog binding to Mist, to reach the bottom of the pathway, rearrangement of the actomyosin cytoskeleton. Ventral furrow formation (VFF) takes less than ten minutes all told. Comparing that to the timing we see suggests that there only needs to be a brief pulse of Fog expression *in vivo* to induce this large scale movement. On the other hand, imaginal disc folding occurs over a longer period and must remain in place over the initial steps of limb

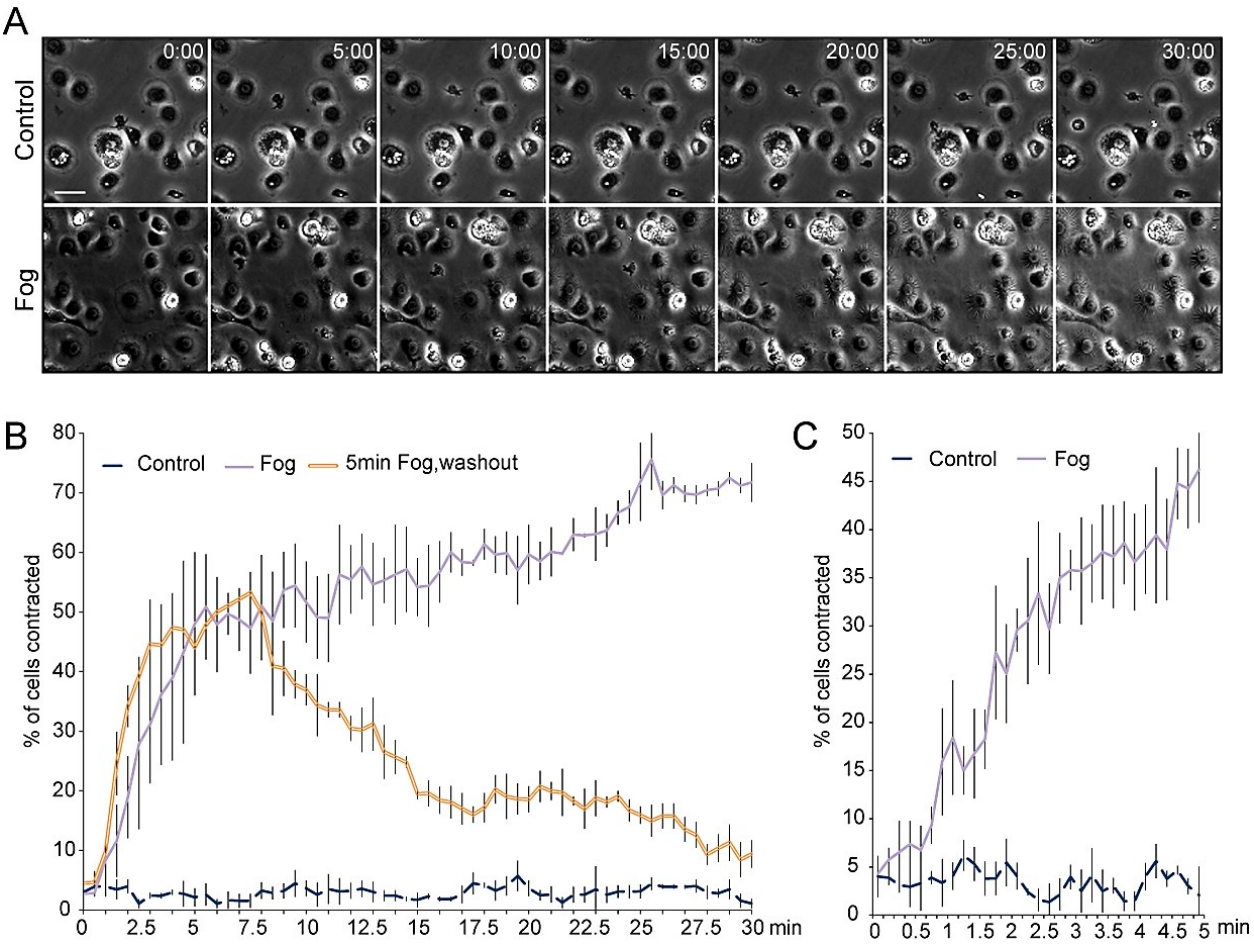


Figure 3-1. S2R+ cells respond quickly to Fog treatment. **A.** Frames from phase contrast time lapse image series of S2R+ cells treated with control media or Fog media at time 0:00. Times are shown as minutes:seconds in the top panels. Scale bar: 20μm. **B.** Percentage of S2R+ cells contracted at 30 second time points for 30 minutes. Cells were treated with control or Fog media at time 0:00, or treated with Fog at time 0:00 and then washed with excess Schneider's media at time 5:00 (5min Fog, washout). **C.** Percentage of S2R+ cells contracted at 10 second time points for 5 minutes. Cells were treated with control or Fog media at time 0:00. Best fit line was determined for Fog condition (not shown) and R^2=0.93 indicating a linear fit of the data. For B and C data was collected from at least 50 cells in each of 3 independent trials. Error bars show standard deviation.

eversion. Fog signaling is likely controlled differently in these two systems, though further study will be necessary to discover what these differences are.

Now that we established a baseline for comparison, we can examine the effects of perturbation of the system. We treated S2R+ cells with dsRNA, treated them with Fog for 10 minutes and counted the percentage of cells contracted at that fixed time point. Knocking down known members of the Fog pathway, such as Cta, in S2R+ cells blocks cellular contraction (Figure 3-2A; Chapter 2). We hypothesized that dsRNA treatment for Krz or GPRK2 would reduce the potential for relaxation in Fog-responding cells and so may result in a higher percentage of contracted cells at a given time point. *GPRK2* and *krz* RNAi showed a consistent, but only minor, increase in the number of cells contracted after a ten minute Fog treatment (Figure 3-2A). For *krz*, we used one dsRNA targeted to the coding sequence and one to the 3'UTR. We confirmed that the dsRNAs are functional by expressing Krz-GFP in cells treated with *krz* dsRNA. Blotting for GFP showed a significant decrease in Krz-GFP protein compared to cells treated with negative control dsRNA (Figure 3-2C). We have no way currently to ensure that the *GPRK2* dsRNA is functional, but this will be confirmed when possible. While there is great variation in the data, the trend is that reduction of either *krz* or *GPRK2* does increase cell contraction.

Next, we wanted to test the effect of overexpressing Krz, thinking it may have the opposite effect. Expression of Krz-GFP in S2R+ cells did slightly decrease the percentage of cells contracted after ten minutes of Fog treatment when compared to cells expressing GFP only, though not significantly (Figure 3-2B). However, expression of Krz-GFP rescues the knockdown of the endogenous RNA using *krz* 3'UTR dsRNA. In other words, absolute expression level of Krz is negatively correlated with percentage of cells contracted after Fog treatment. Krz overexpression and knockdown phenotypes are consistent with a role for it in termination of Fog signaling in S2R+ cells. Next, time course experiments will be done in cells with altered GPRK2 or Krz levels.

We also wanted to test for an interaction between Mist and Krz, as we hypothesized that Krz would only bind Mist after Mist has been activated by Fog and phosphorylated by GPRK2. We performed immunoprecipitation for GFP in S2R+ cells expressing Krz-GFP after the cells were treated with control or Fog media for seven minutes (Figure 3-2D). Mist could only be detected in the Fog treated IP sample, though it was present in both inputs. Therefore, Krz interacts with Mist in a Fog-dependent manner in S2R+ cells, supporting our hypothesis. The same experiments will be performed with GPRK2 to test for an interaction with Mist.

By wide field fluorescence microscopy Krz-GFP expressed in S2R+cells localized diffusely in the absence of Fog (Figure 3-3A). After a ten minute Fog treatment, the majority of Krz-GFP was still diffuse but some cells had bright puncta of Krz-GFP. These puncta could indicate Krz-GFP coating vesicles of internalized Mist receptor in response to Fog treatment. Together these and

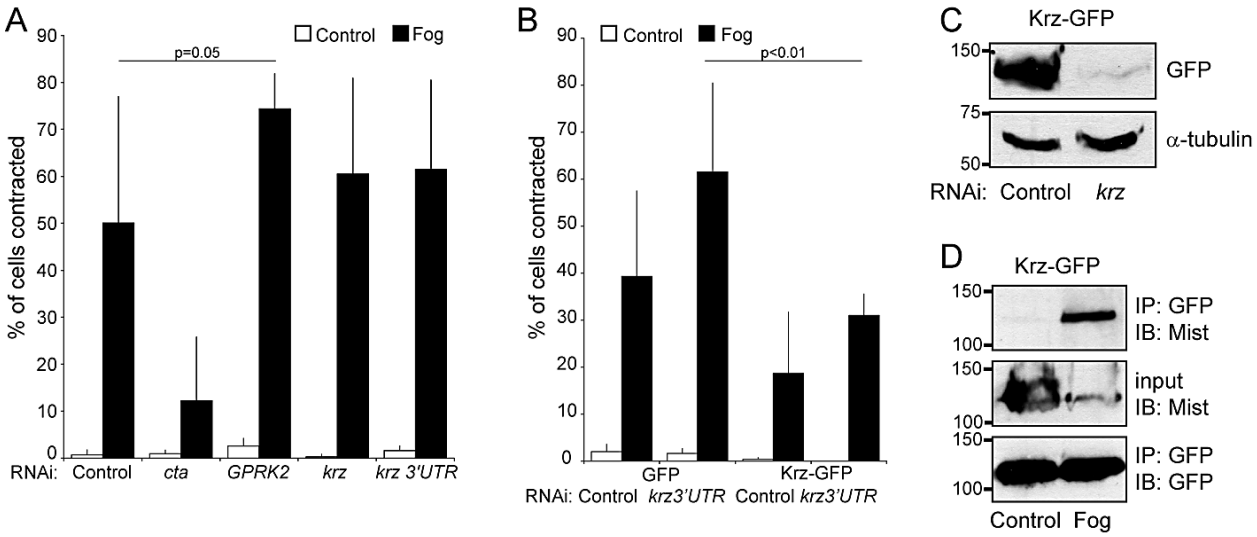


Figure 3-2. Krz and GPRK2 have a modest attenuating effect on Fog-induce S2R+ cell contraction and Krz interacts with Mist in a Fog-dependent manner. **A.** Cells were treated with the indicated dsRNAs, treated with control or Fog media for ten minutes, and counted for contractile phenotype as above. *GPRK2* or *krz* RNAi slightly increases the percentage of cells contracted. **B.** Cells were transfected with the indicated construct, treated with the indicated dsRNAs, treated with control or Fog media for ten minutes, and counted for contractile phenotype. *krz* RNAi slightly increases the percentage of cells contracted and expression of Krz-GFP reduces it. For A and B data were collected from at least 100 cells in each of at least 3 independent trials. Error bars show standard deviation. **C.** Western blot for GFP in S2R+ cells expressing Krz-GFP and treated with control or *krz* dsRNA. *krz* RNAi reduces expression of Krz-GFP. α-tubulin was used as a loading control. **D.** Immunoprecipitation for GFP was performed in S2R+ cells expressing Krz-GFP after control or Fog treatment. Blotting for endogenous Mist shows that Krz-GFP only interacts with Mist in the presence of Fog.

the previous data suggest that GPRK2 and Krz proteins have a small but consistent negative effect on Fog-induced cellular contraction.

To further investigate the localization of Krz in S2R+ cells, we examined them by time lapse total internal reflection (TIRF) microscopy. S2R+ cells stably expressing Krz-GFP and Mist-mCherry were treated with control or Fog media for ten minutes (Figure 3-3B). On or near the plasma membrane, both Krz-GFP and Mist-mCherry are present in small puncta with a general background of fluorescence. In both control and Fog conditions, Krz-GFP localization hardly changes over the course of ten minutes. Mist-mCherry bleaches a fair amount over ten minutes, but its localization is also stable in the control treatment. However, in the presence of Fog, Mist-mCherry was frequently seen to accumulate in several bright puncta which move directionally toward the center of the cell, possibly vesicles of internalized Mist. No Krz-GFP

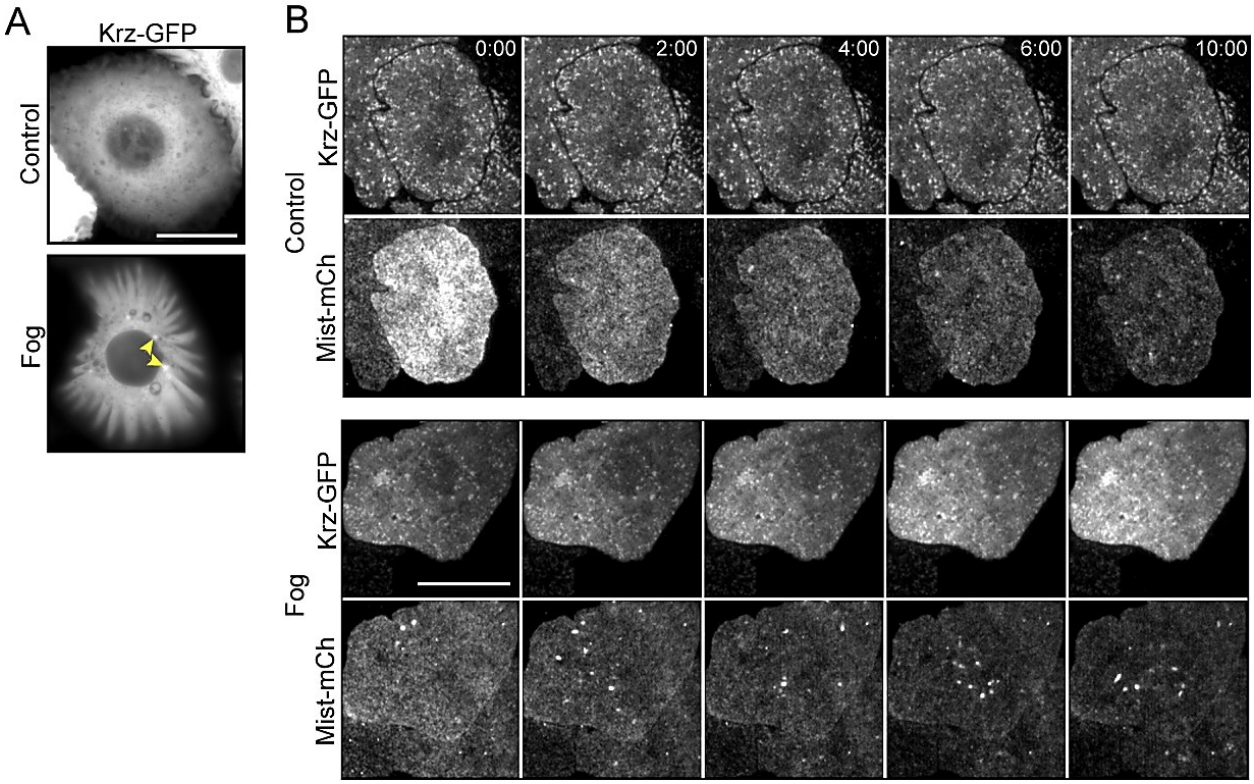


Figure 3-3. Krz and Mist may be internalized following Fog treatment. **A.** Wide field fluorescence microscopy of S2R+ cells expressing Krz-GFP. Cells were treated with control or Fog media for ten minutes. Yellow arrowheads: bright puncta of Krz-GFP following Fog treatment. **B.** Frames from TIRF time lapse image series of S2R+ cells expressing Krz-GFP and Mist-mCherry and treated with control or Fog media at time 0:00. Times are shown as minutes:seconds in the top panels. Scale bars: 10μm.

was seen in these puncta. There are several explanations which could help explain this discrepancy. Krz-GFP could be removed from many Mist-containing vesicles as they are internalized or transported. Alternatively, Krz-GFP coated vesicles may move away from the plasma membrane and be lost from the TIRF imaging plane. Additionally, mCherry attached to the C-terminus of Mist may affect how Krz can interact with the GPCR. However, by wide field and TIRF microscopy Krz and Mist, respectively, were seen in vesicles after the addition of Fog. A subset of Mist is probably internalized after being activated, and this may be mediated by Krz.

We have established that GPRK2 and Krz can play a role in regulating Fog signaling in our cell culture assay so we next moved to the fly to determine if they can act on Fog-induced morphogenesis during development. To do this we examined wing imaginal discs. It has been previously shown that Fog, Mist, Cta, RhoGEF2, and Rho are involved in wing imaginal disc folding and that many of the components genetically interact in this process (Chapter 2; (Nikolaidou & Barrett, 2004). We overexpressed Krz or a *krz* RNAi hairpin in the wing disc using the A9 driver and examined the morphology of the resulting discs. A control construct (the fluorescent membrane marker CD8-RFP) did not affect wing disc morphology, but shows the expression domain of the A9 driver (Figure 3-4A,D). Folds were all present in the expected locations and were straight and organized. Krz overexpression and *krz* RNAi both affected the folding of wing discs (Figure 3-4B-D). Some discs had folds located where there should not be folds, folds intersecting others, or bifurcated folds (all categorized as misfolded; Figure 3-4B). Other discs had folds in the correct location, but those folds were not the same width along their lengths as is seen in wild-type discs (categorized as nonuniform folds; Figure 3-4C). In these cases, it seems as if folds are initiated in the correct locations but their formation is misregulated as would be expected if a signal termination molecule were misexpressed. Thus, among its other roles in wing development Krz is also able to regulate wing disc folding. It will be interesting to

test whether GPRK2 has the same phenotypes and if *krz* genetically interacts with known Fog pathway members in this tissue.

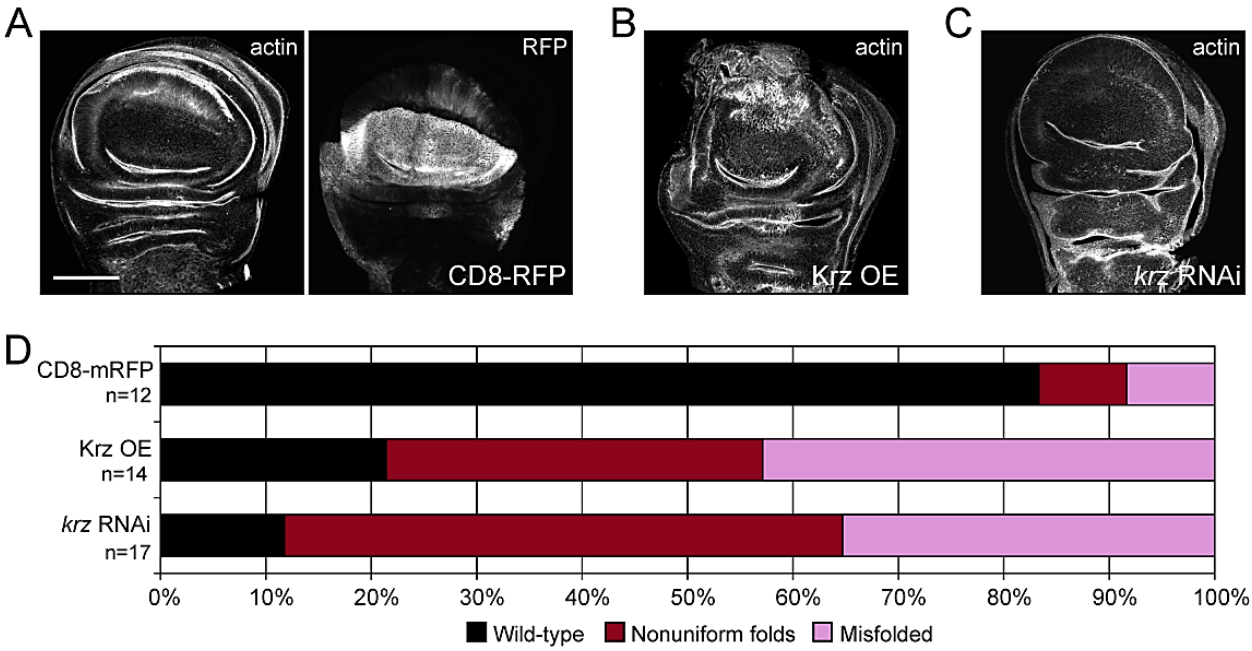


Figure 3-4. Krz is involved in wing imaginal disc folding. **A.** A wing imaginal disc expressing the CD8-RFP control construct has normal folds as shown by actin staining (left). RFP shows the expression domain of the A9 driver used in these experiments (right). Scale bar: 100μm. **B.** A wing imaginal disc exogenously expressing Krz has folds in improper locations as shown by actin staining. **C.** A wing imaginal disc expressing *krz* RNAi has folds with nonuniform widths as shown by actin staining. A-C are all maximum projections of 6μm confocal stacks. **D.** Quantification of percentages of phenotypes seen in wing imaginal discs with the given expression constructs. n=the number of discs scored for each condition.

Discussion

In this work, we have shown that there is great potential for studying the regulation of GPCR signaling in morphogenesis using the *Drosophila* Fog pathway. The Mist GPCR was recently discovered to be necessary for Fog-induced cellular contractility through the use of cell culture, wing imaginal discs, and embryos. Here, we have shown that the GRK GPRK2 and the β-Arrestin Krz can act to attenuate actomyosin contractility downstream of Fog, but there is much work to be done to fill out the data presented thus far.

The dynamics of cell contraction and relaxation over time following Fog application and removal were examined in wild-type S2R+ cells. These experiments need to be followed up by

comparison with the same methods performed in cells with increased or reduced levels of GPRK2 and Krz. The timing of relaxation will be of primary interest, as these proteins are suspected to play a role in Fog signal termination. Overexpression and reduction of GPRK2 and Krz had modest but consistent effects on cellular contraction at a fixed time point and it is likely that a dynamic analysis will be more revealing.

We previously showed that S2 cells are not normally able to respond to Fog because they do not express Mist (Chapter 2). Expression of exogenous Mist endows them with the ability to contract in response to Fog. A version of Mist lacking its C-terminal tail (MistΔC) was also able to transmit the Fog signal in S2 cells. We hypothesize that the C-terminus of Mist is the portion able to interact with Krz and GPRK2. If we are correct, MistΔC would not be able to interact with these proteins and would have different signaling properties than full length Mist. Cell contraction and relaxation in S2 cells expressing full length Mist or MistΔC will be examined in future studies.

The potential for GPRK2 and Krz to be acting to internalize Mist is an intriguing possibility brought up by our TIRF experiments. While we only saw significant accumulation of Krz-GFP or Mist-mCherry in potential vesicles after Fog treatment, more work will have to be done to investigate the identity of these puncta. Mist and Krz localization should be examined in control and Fog treated cells with confocal microscopy to be able to capture the whole 3-dimentional space of the cells. This is especially important in the case of Fog treated cells which become very tall during Fog-induced contraction. Additional TIRF experiments could be done using cells expressing fluorescent tubulin with Mist to see if the directional Mist puncta are moving along microtubules.

Excitingly, Krz plays a clear role in wing imaginal disc folding. While Krz has been shown to be involved in wing patterning and explicitly in wing vein specification, the late third instar imaginal discs examined here would not likely have such robust morphological defects from the effects of misregulating those pathways (Molnar, et al., 2011). We will also be

examining fly legs, the products of leg imaginal disc morphogenesis, for similar morphogenetic defects as well as genetic interactions with *mist* and other Fog pathway members. It will also be exciting to look for a genetic interaction between *GPRK2* and *krz* with Fog pathway members during embryogenesis. This is the first work examining a possible mechanism of Fog signaling termination, even though the Fog pathway has been a major model of morphogenesis for many years. However, further studies are needed to determine exactly how GPRK2 and Krz are functioning to regulate Fog signaling.

Methods

Cell culture:

Cells were maintained as in Chapter 2. Fog protein was produced and collected as in Chapter 2. Cells were RNAi treated, Fog treated, and transfected as in Chapter 2. Negative control and *cta* dsRNAs are the same as Chapter 2. Primers for production of others are all preceded by the T7 sequence and are as follows: GPRK2fwd: GCTGCCTGCTGTACGAGATGA; GPRK2rev: ACGAATCCGACACGAATTTCT; Krzfwd: CTTATAGTGGACCATAATACC; Krzrev: CTCACCGACTTTGGATGGTGC; Krz3'UTRfwd: CACCATTTTAACAAATAAGAA; Krz3'UTRrev: AGTATTTTCTTCATATGAATT.

Microscopy:

Phase contrast and wide field fluorescence microscopy were carried out using a CoolSnap HQ CCD camera on a Nikon Eclipse Ti inverted microscope driven by Nikon Elements software as in Chapter 2. For live phase contrast imaging S2R+ cells were plated on ConA-coated glass bottomed 35mm dishes and allowed to spread at least 45 minutes. Just before imaging was begun, the media on the cells was removed and replaced with control or Fog media diluted 1:1 in Schneider's media. For washout experiments, Fog media was added as usual at the beginning of imaging. After the 5:00 frame was acquired, imaging was paused and an excess of Schneider's was added to the cells. The majority of this wash was removed and a fresh wash of Schneider's

was added. Most of the liquid was removed again and imaging was resumed with the next frame taken immediately. Images were acquired every ten or 30 seconds for five or 30 minutes.
For quantification of cellular contraction, all whole cells with visibly spread lamellipodia and the subset of these with at least half of their lamellipodial surface area with ridges were counted in each frame.
TIRF microscopy was carried out using an Andor camera on a Nikon Eclipse Ti inverted microscope driven by Nikon Elements software. S2R+ cells stably expressing Krz-GFP and Mist-mCherry were plated and treated as above. Frames were acquired every ten seconds for ten minutes.

Immunoprecipitation and immunoblotting:

S2R+ extracts were produced by resuspending cell pellets in PBST. SDS-PAGE sample buffer was then added and samples were boiled for 5 minutes.
Immunoprecipitation was performed using GFP binding protein, a portion of a single chain llama antibody made to GFP, conjugated to a human Ig domain (Rothbauer, et al., 2008). Protein was covalently conjugated to Protein A Sepharose Fast Flow beads. S2R+ cells stably expressing Krz-GFP were plated densely in a 35mm dish with 100μM $CuSO_4$ to induce expression overnight. Media was removed and 1ml control or Fog 1:1 with Schneider's media was added and incubated 7min. Cells were resuspended, transferred to 1.5ml microcentrifuge tubes, and pelleted. Cell pellets were washed twice with cold PBS and then resuspended in cold Lysis Buffer for Transmembrane Proteins (50mM HEPES pH7.5, 100mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mg/ml BSA, 1% Nonidet P-40, 0.2mM PMSF) and incubated on ice for 15min. Cell debris was pelleted. A sample of this supernatant was set aside as the Input for each condition. ~30ul GFP Binding Protein conjugated to Sepharose Beads was used per sample. Just before using, beads were washed trice with cold Lysis Buffer. The rest of the supernatant from each sample was added to the beads, and these were rocked at 4°C for 1hour. Beads were pelleted and washed thrice with Lysis Buffer with rocking at 4°C for 30min in between each.

Beads were pelleted for the final time and all liquid removed. To input tubes and beads, SDS-SB was added and samples were incubated at 65°C for 7min.

SDS-PAGE and Western blotting were performed using standard procedures. Antibodies used were mouse anti-GFP JL-8 (Clontech) used at 1:5000; rabbit anti-Mist (see Chapter 2) used at 1:500; mouse anti-α-tubulin DM1α (Sigma) used at 1:1000.

Drosophila culture:

Drosophila were reared as in Chapter 2. Imaginal discs were collected, stained, and imaged as in Chapter 2. Fly lines used were: A9-GAL4, UAS-CD8-mRFP (II), UAS-Krz (Bloomington *Drosophila* Stock Center) , UAS-*krz* RNAi (Vienna *Drosophila* Resource Center).

Chapter 4

Conclusions and Future Studies

A model for Fog signaling in cell culture

In this work I have described the development of a cell culture model of the *Drosophila* Folded gastrulation (Fog) signaling pathway. The Fog pathway has been well studied during gastrulation, and has also been implicated in salivary gland invagination, axon guidance, and imaginal disc morphogenesis (Costa, et al., 1994; Nikolaidou & Barrett, 2004; Ratnaparkhi & Zinn, 2007). This pathway has mainly been studied using traditional genetic approaches, which have been enlightening in establishing ventral furrow formation (VFF) during *Drosophila* gastrulation as a model for the morphogenetic events of vertebrate neurulation as well as many other developmental events (Leptin & Grunewald, 1990; Schoenwolf & Franks, 1984; Copp & Greene, 2010; Sawyer, et al., 2010). However, some pieces of information have eluded us in these methods.

Drosophila S2R+ cells in culture respond to exogenous application of Fog protein. This actomyosin-based contractile response requires all of the known members of the Fog pathway and allowed us to discover some unknown members as well as some mechanistic information about how Fog signaling works. The G-protein coupled receptor (GPCR) Mist has now been established as a crucial member of the pathway and GPRK2 and Kurtz (Krz) were found to influence it. The Fog signal has been thought to act through a GPCR since Concertina (Cta), a Gα protein, is a known signaling component, but our cell culture model made it possible to discover that receptor. My work has also established that this GPCR signaling pathway is likely regulated in a canonical way, with termination of receptor signaling by the actions of a GRK and

a β-Arrestin. Further work with Mist deletion constructs, GPRK2, and Krz will elucidate much more about how Fog signaling is regulated on a cellular level. These cell culture results can then be brought back into fly tissues to allow us to understand Fog regulation on a tissue level.

New insights into Fog pathway actions *in vivo*

The work presented in this dissertation shows that Mist not only acts in cell culture during Fog-induced contraction, but also in limb and embryo development. *mist* genetically interacts with *fog* and *Rho* in wing disc and leg morphogenesis, lending support to its role in the Fog pathway. *mist* mutants also have the same distribution of embryonic phenotypes as *fog* mutants. Krz also plays a role in wing disc morphogenesis, though genetic interaction experiments will reveal more details of its role there and test whether it is acting downstream of Fog.

This work, through *in vivo* experiments, has also revealed several interesting new ideas which may be broadly applicable throughout development. *mist* is the first known Snail transcriptional target necessary for ventral furrow (VF) invagination. It has been long thought that Snail primarily acts as a transcriptional repressor in the mesoderm, but *mist* transcription requires its presence (Leptin, 1991). These data force us to rethink the characterization of Snail as a transcriptional repressor. Snail regulation of *mist* is also the first evidence to explain the differences in VF phenotypes between *twist* and *snail* mutants (Leptin, 1991; Martin, et al., 2009). *twist* mutant embryos can initiate apical constrictions but can't stabilize them, possibly because Mist is still present in these cells and may have some stochastic activation potential (Martin, et al., 2009). *snail* mutants, which lack Mist, cannot apically constrict at all even though Fog is present, suggesting that Mist is an essential Fog receptor in the mesoderm. This can be further tested by expressing Mist in a *snail* mutant.

In both wing imaginal discs and in embryos *fog* and *mist* mRNA expression patterns are similar and overlapping, yet distinct. As discussed above, this is due to separate transcriptional control of the ligand-receptor pair during gastrulation but it is not known how this is achieved

during imaginal disc folding. Twist- and Snail-dependent induction of *fog* and *mist* could represent a new paradigm for spatially controlled morphogenetic events whereby multiple transcription factors independently pattern the expression of signaling molecules within the same pathway. The places where these multiple expression patterns overlap can help determine which cells achieve signal transduction.

Future studies

This work answers some long open questions, but also raises some new ones and opens the door for many future studies. The cell culture model of Fog signaling we have established can be manipulated in ways the whole animal cannot and allows for translational studies moving from single cells to whole tissues. The work on roles for GPRK2 and Krz in Fog signaling presented here is far from complete. Many future experiments along these lines were mentioned in Chapter 3. Once the roles of GPRK2 and Krz are known, mutant versions of Mist could be introduced into the system in order to further examine how the receptor interacts with its signaling partners. Additionally, there are probably other mechanisms of Fog signal regulation that are still unknown. Broad screens in S2R+ cells could lead to the identification of other players in this pathway.

Screening also brings up the idea of possible high-throughput studies using Fog signaling. For instance, one of the hallmark outcomes of Fog activation is phosphorylation of non-muscle myosin II (Spaghetti squash in *Drosophila*) and a qualitative increase in phospho-myosin is seen in Fog treated S2R+ cells (Halsell, et al., 2000). Overall localization of myosin also drastically changes in S2R+ cells after Fog addition. Myosin phosphorylation or relocalization could possibly be quantified in high-throughput studies over time after Fog treatment and washout. These measurements could also be examined after overexpression or reduction in regulatory proteins such as Krz. We have only measured the contractile response in a binary manner so far which may not be the best readout for discovering how regulatory proteins act in Fog signaling. Measurement of phospho-myosin levels could also allow us to

resolve more detail by using instead a readout of the degree of cellular contraction. The timing of contraction can be better addressed using these approaches, as well.

It will be imperative to investigate the dynamics of cellular contraction in embryos lacking Mist, GPRK2, or Krz during VFF and compare these to previous results (Martin, et al., 2009; Martin, et al., 2010). This type of analysis will shed light on how each of these proteins affects apical constriction *in vivo*. I also propose that examination of Rho1 activation during Fog signaling events be carried out in cell culture and in the fly using Rho FRET probes or fluorescent GTP-Rho binding domains (Hodgson, et al., 2010). In cell culture, these methods could also allow us to measure the degree of pathway activation at the single cell or high-throughput level. In the fly, they will allow us to get a sense of the subcellular location of pathway activation. We may also be able to determine whether Fog signaling in different tissues is activated to different extents.

The importance of studying Fog signaling

The mechanisms of protein signaling, developmental patterning, and morphogenesis discovered or elaborated using the Fog pathway in *Drosophila* have already contributed much to our understanding of biology. In the past we have learned about how cell shape changes translate into tissue level changes, how cytoskeletal rearrangements influence those, the role of interaction between maternal and zygotic transcription in embryonic development, the fact that some cells and proteins can respond to force, and much more from the Fog pathway. The work presented here has allowed us to fill in some of the gaps in this pathway and elucidate new developmental control mechanisms. Yet we still have much more to learn.

This work presents the discovery of Mist, a GPCR which acts within the Fog signaling pathway to induce cell shape changes during *Drosophila* development. It also highlights the regulatory molecules GPRK2 and Krz as potential actors in Fog signal termination. The ligand and receptor in this pathway are not conserved in mammals, but signaling pathways from extracellular cue to GPCR to Rho to cytoskeletal rearrangement is one used repeatedly

throughout biology. The flexibility and malleability of the Fog pathway allow us to manipulate signaling both in the new cell culture model developed and in a living organism in ways which may not be possible in other systems. These include both physical and genetic manipulations which will continue to lead to new insights into the interactions of forces, genes, and proteins in development. This is also the first instance of a role for a GPCR in morphogenesis, indicating that we have not exhausted the possible knowledge of the applications of this large family of important molecules. The breadth of knowledge we have been able to gain from this system will continue to be built on in the future.

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