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Craig Robert Magie

Roles of the Rho1 small GTPase during development in *Drosophila melanogaster*

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A dissertation submitted in partial fulfillment of the requirements for the degree of

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Abstract

Roles of the Rho1 small GTPase during development in *Drosophila melanogaster*.

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Rho, Rac and Cdc42 are small GTPases in the Ras superfamily initially shown to regulate the actin cytoskeleton. Subsequent work linked them to many cellular processes. The diversity of functions exhibited by Rho suggests that it may operate within multiple pathways in different contexts, although how directly Rho affects these functions is not clear in all cases.

We identified a mutation in *Drosophila Rho1*, allowing an investigation of Rho1 function in an organismal and developmental context. Interestingly, loss of *Rho1* results in phenotypes distinct from those resulting from overexpression of dominant-negative forms of Rho1. Zygotic *Rho1* mutants exhibit severe defects in head involution and imperfect dorsal closure. Despite its dorsal closure phenotype, *Rho1* does not activate genes downstream of the JNK signaling pathway or interact genetically with its components. Consistent with a role in actin cytoskeletal regulation, *Rho1* interacts genetically and physically with the *Drosophila* formin homologue, *cappuccino*. *Rho1* also interacts genetically and physically with *concertina*, a Gα protein.

Reduction of maternal *Rho1* results in the disruption of the actin cytoskeleton during oogenesis, and embryos display patterning defects as a result of failure to maintain expression of the segmentation gene *engrailed.* Signaling by Wingless is required for maintenance of Engrailed expression. Wingless is found in vesicular structures thought to be important in the proper formation of the Wingless protein gradient. Maternal Rho1 mutants exhibit general defects in endocytic processes, resulting in a reduction of the number of Wg-positive vesicles.

Rho1 is expressed ubiquitously, but accumulates at particular subcellular structures, including cadherin-based adherens junctions. Localization of cadherins and catenins, proteins involved in linking cadherins to the actin cytoskeleton, is aberrant in zygotic *Rho1* mutants. Significantly, Rho1 binds directly to α-catenin and $p120^{ctn}$ *in vitro* and *in vivo.* These interactions map to distinct surface-exposed regions of the protein not previously assigned functions. These observations suggest that α-catenin and $p120^{ctn}$ are key players in a mechanism of recruiting Rho1 to its sites of action.

Our data, along with recent data from other labs, suggests that Rho may be acting primarily as a cytoskeletal regulator, and its links to other cellular functions are indirect.

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DEDICATION

For my parents,
for giving me the life that so fascinates me.

CHAPTER 1

Introduction

Embryogenesis, or the transition from a single cell to a complex, differentiated organism, is an extremely intricate process that requires the proper regulation of many different events. These include the localization of maternal determinants in the egg, spatial and temporal regulation of gene expression, and a variety of cell movements and shape changes. Many of these processes are controlled by cell-cell signaling events. In this context, it is crucial for cells to respond properly to these cues and adopt behaviors necessary for the development of proper form. In addition to the receptors at the plasma membrane, the pathways activated by such signaling events involve a diversity of cytoplasmic factors vital for the transduction of the signal.

1.1. Rho GTPase biology.

One class of proteins that have been shown to be involved in interpreting extracellular cues into cellular behavior is the Ras superfamily of small monomeric G-proteins. The Ras superfamily can be divided into 5 families of small GTPases: Ras, Rab, Arf, Ran and Rho, which are grouped on the basis of sequence homology and share similar roles (reviewed in: Symons and Takai, 2001; Takai et al., 2001). As the founding member of the superfamily, and owing to its strong oncogenic activity, Ras has been extensively studied and linked to myriad signaling events. The Rho family has also received much attention since its discovery. Rho proteins have been found in a wide variety of eukaryotic species and are extremely well conserved, indicating the importance of their role in the regulation of development (Burridge and Wennerberg, 2004; Etienne-Manneville and Hall, 2002; Settleman, 1999). The founding members of this family are Rho, Rac and Cdc42 (Hall, 1998; Mackay and Hall, 1998). Since

the initial characterization of these proteins, a number of less conserved family members have also been identified in particular organisms such as TC10 and the Rnd proteins in mammalian systems (Sahai and Marshall, 2002), and Rop and Arac proteins in plants (Valster et al., 2000).

1.1.1. Rho GTPases function as molecular switches.

Rho proteins function as molecular switches, cycling between an active, GTP-bound state and an inactive, GDP-bound state. Their position in this cycle is modulated by their intrinsic GTPase activity and the action of associated regulatory enzymes of 3 major classes: the guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and GDP dissociation inhibitors (GDIs) (see Fig. 1.1) (Maruta, 1998). GEFs activate Rho proteins by promoting the exchange of GDP for GTP. GAPs increase the rate at which Rho hydrolyzes GTP, and hence inactivates itself. GDIs bind Rho-GDP in the cytoplasm and keep it in a GDP-bound, inactive state. Regulation by GDIs is unique to Rho family proteins among the Ras superfamily (Maruta, 1998).

The prevailing model for the mechanism of Rho activation is that extracellular cues initiate signal transduction pathways involving a number of different cell surface receptors, leading to activation of a particular GEF and the subsequent activation of Rho through binding to GTP (reviewed in: Kjoller and Hall, 1999). A large number of RhoGEFs have been identified to date. Some of these act specifically on a particular Rho family member, while others can act on multiple members (Hornstein et al., 2004), and this specificity is likely important in determining which cellular behaviors are elicited.

1.1.2. Rho activation of downstream pathways.

Once Rho binds to GTP through its phosphate binding loop, other conserved domains within the Rho protein change conformation, allowing it to bind effector

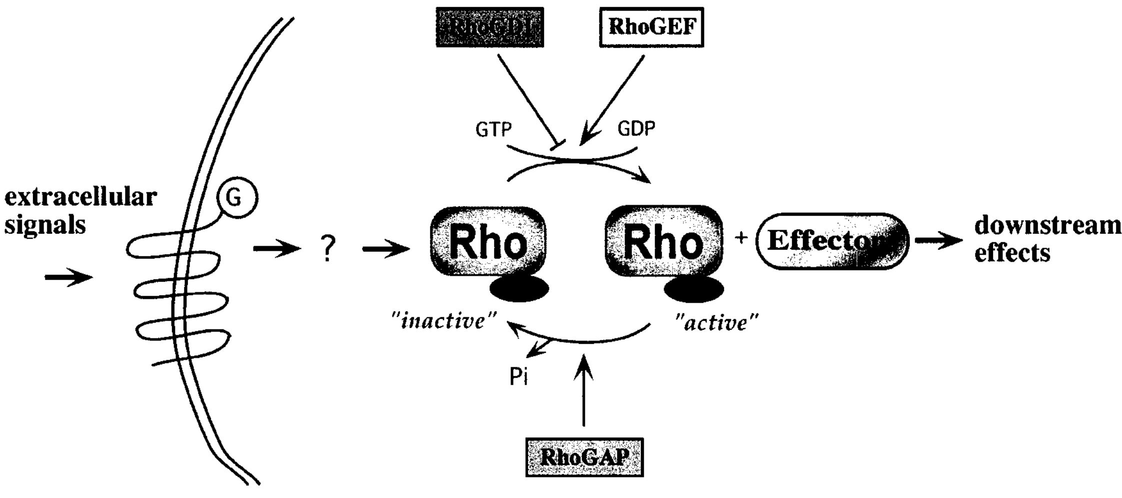


Figure 1.1. The Rho GTPase cycle. Rho GTPases function as molecular switches, with active, GTP-bound and inactive, GDP-bound forms. Associated regulatory enzymes modulate their position in this cycle, with guanine-exchange factors (GEFs) activating Rho by catalyzing the exchange of GDP for GTP, GTPase-activating proteins (GAPs) inactivating Rho by increasing the rate at which it hydrolyzes GTP, and guanine-dissociation inhibitors (GDIs) keeping Rho in a GDP-bound, inactive state. The activation of G-protein coupled receptors is thought to lead to the activation of particular GEFs and the subsequent activation of Rho. Once Rho is active, it can bind effector molecules and carry out downstream functions.

molecules and stimulate downstream pathways. The major domains in Rho to undergo this GTP-induced conformational change include the Effector (or Switch I) and Switch II domains. The Effector domain is so named because it has been shown to be required for binding of Rho to many of its effectors through analysis of point mutations within the domain, although the binding of some effectors cannot be abrogated by Effector domain mutations (Sahai et al., 1998). The function of the Switch II domain has not been as well characterized, however it has been shown to be involved in the binding of some GEFs to Rho proteins (Karnoub et al., 2001; Worthylake et al., 2000). In addition to the Effector and Switch II domains Rho proteins share other conserved motifs, including the Loop 6 domain, important in specificity of effector binding, the Insert Helix, required for regulation by GDIs, and a carboxy-terminal CAAX box that allows Rho GTPases to be isoprenylated and inserted into the plasma membrane. Unlike Ras proteins, which are constitutively localized to the plasma membrane, translocation of Rho GTPases from the cytoplasm to the plasma membrane is controlled by their activation state: in mammalian tissue culture cells Rho is predominantly cytosolic but translocates to the membrane upon ligand stimulation, with different ligands leading to differential localization (Fleming, 1996). Interfering with this translocation event affects the ability of Rho to carry out a subset of its functions, indicating that membrane localization is necessary for some, but not all, Rho functions (Kranenburg et al., 1997).

1.1.3. Rho has been linked to disparate cellular processes.

The nature of Rho GTPases as molecular switches allows for the construction of dominant-negative (dn) and constitutively-active (ca) versions of these proteins. Overexpression of these altered GTPases, primarily in tissue culture experiments, led to the identification of roles for Rho GTPases in a wide variety of cellular functions, including transcriptional activation, cell cycle progression, cell-cell and cell-substrate adhesion, secretion, endocytosis, phospholipid turnover, cytokinesis, apoptosis, and

Table 1.1. Rho effectors. Many Rho effectors identified to date are kinases or scaffold molecules involved in actin cytoskeletal regulation.

effector	protein class	functions
Rho-kinase (ROCK)	ser/thr kinase	actin/myosin interactions
Protein kinase N (PKN) /PRK1, PRK2	ser/thr kinase	actin regulation actin/myosin interactions cell adhesion vesicle transport
Citron kinase	ser/thr kinase	cytokinesis
Phosphatidylinositol-4-phosphate 5-kinase (PI-4-P5K)	lipid kinase	PIP_2 levels actin regulation
Diacylglycerol (DAG) kinase	lipid kinase	PA levels cytokinesis
Phospholipase-D (PLD)	lipase	PA levels actin regulation
Rhophilin	scaffold	actin regulation
Rhotekin	scaffold	unknown
mDia1, mDia2	scaffold	actin/microtubule regulation

others (see Table 1.2) (Braga, 1999b; Coleman and Olson, 2002; Ellis and Mellor, 2000; Fukata and Kaibuchi, 2001; Hall, 1998; Mackay and Hall, 1998). The diversity of functions Rho proteins have been implicated in suggests that they operate through many different pathways in different contexts. A number of effector molecules have been identified (Table 1.2) that act downstream of Rho activation (reviewed in Bishop and Hall, 2000), though the mechanisms underlying the specificity of Rho action are currently unclear. Many of the Rho effectors identified to date are kinases, which presumably act by phosphorylating targets following activation by Rho. These include protein kinase N (PKN) (Amano et al., 1996), Rho-associated kinase (ROCK) (Kimura et al., 1996) and Citron kinase (Madaule et al., 1995), as well as the lipid-modifying kinase phosphatidylinositol-4-phosphate 5-kinase (PI-4-P5K) (Ren et al., 1996). In addition, a number of Rho effectors are scaffold molecules whose functions are either unknown (i.e. Rhophilin, Rhotekin) (Reid et al., 1996; Watanabe et al., 1996) or important in actin cytoskeletal remodeling (i.e. mDia1, mDia2) (Watanabe et al., 1999; Watanabe et al., 1997). Many of these effectors share a common motif called the Rho effector homology (REM; also called the Rho binding domain, RBD) region that contains a number of conserved residues important in mediating binding to Rho (Bishop and Hall, 2000). This domain is distinct from the GTPase binding domain for effectors of the Rho relatives Rac and Cdc42 (Bishop and Hall, 2000).

The mechanisms through which Rho activates its effectors upon binding to GTP are beginning to be elucidated. The most common identified to date is the disruption of autoinhibitory intramolecular associations (reviewed in Bishop and Hall, 2000). ROCK, PKN, and mDia all contain autoinhibitory domains whose inhibitory activity is at least partly relieved upon Rho binding. In the case of mDia, autoinhibition is achieved by binding of the C-terminal diaphanous autoinhibitory domain (DAD) to the N-terminal Rho binding domain (RBD). Binding of Rho to the RBD displaces the DAD, activating mDia and allowing it to carry out its downstream functions. Consistent with this proposed mechanism, removal of the N-terminal RBD results in a constitutively active protein (Palazzo et al., 2001).

1.1.4. Rho regulation of the actin cytoskeleton.

The founding members of the Rho family, Rho, Rac, and Cdc42, were initially identified as potent regulators of actin cytoskeletal organization. Injection of fibroblasts with activated forms of Rho, Rac, or Cdc42 results in alterations in actin structure in a manner specific to each GTPase: Rho causes the formation of stress fibers, contractile cables composed of actin and myosin (Ridley and Hall, 1992), Rac causes the formation of lamellipodia, or membrane ruffles (Ridley et al., 1992), and Cdc42 causes the formation of filopodia, or actin-based spike-like projections (Fig. 1.2) (Kozma et al., 1995; Nobes and Hall, 1995). In fibroblasts there appears to be a hierarchical relationship between the Rho GTPases, with Cdc42 activating Rac, which in turn activates Rho (Nobes and Hall, 1995). In other cell types, however, this relationship has not been observed, and the effects Rho, Rac and Cdc42 have on actin cytoskeletal remodeling appear to be not only qualitatively different, but in many cases opposite in effect. For example, in neuronal precursors Rac and Cdc42 are involved in outgrowth of neurite extensions, whereas Rho is involved in their retraction (Kozma et al., 1997). Rho is thought to mediate this effect through its regulation of actin-myosin interactions and therefore its regulation of contractile activity.

There are a number of ways in which Rho has been shown to affect cytoskeletal organization. One of the most well characterized signaling pathways downstream of Rho is that which leads to activation of the regulatory light chain of nonmuscle myosin-II (MLC) by ROCK. ROCK mediates this effect both through direct phosphorylation of MLC and inhibitory phosphorylation of the MLC phosphatase, allowing the association of myosin and actin filaments and the formation of contractile structures such as stress fibers (Kimura et al., 1996). Another ROCK target is LIM kinase (LIMK), which acts to inhibit the actin severing protein cofilin following activation by ROCK, leading to stabilization of actin filaments (Maekawa et al., 1999). Rho can also influence actin structures through its association with PI-4-P5K, as regulation of phosphatidylinositol-2-phosphate (PIP_2) levels has been shown to

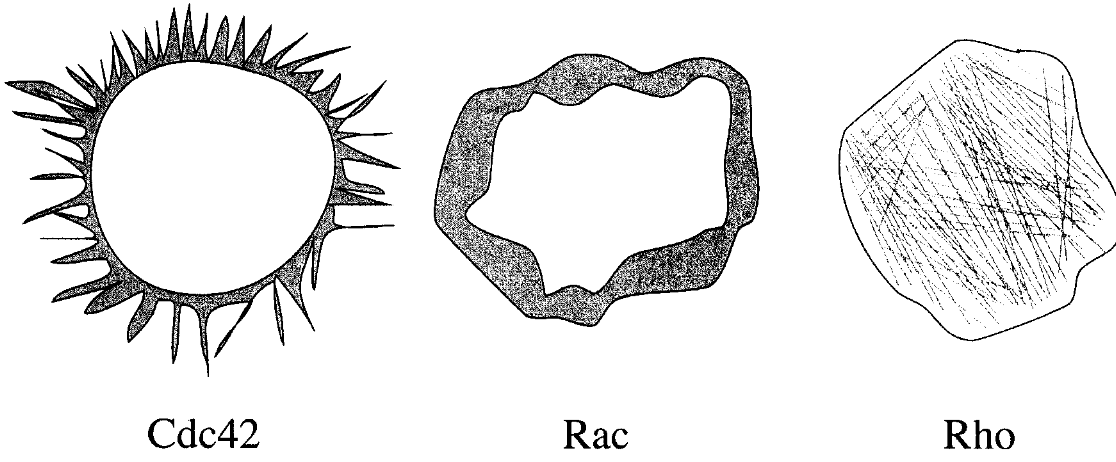


Figure 1.2. Rho GTPase effects on the actin cytoskeleton. Activated versions of Rho, Rac and Cdc42 cause distinct changes in actin morphology in fibroblasts (represented by the gray shading). Cdc42 causes the formation of filopodia, spike-like projections from the cell body. Rac causes the formation of lamellipodia, membrane ruffles around the cell periphery. Rho causes the formation of stress fibers, cables containing actin and myosin.

affect actin cytoskeletal rearrangements (Tolias et al., 2000). These roles for Rho in cytoskeletal remodeling position it as a key regulator of cell morphology in response to extracellular signals.

Classical work investigating Rho function was mainly done in tissue culture, however, utilizing overexpression of dn or ca versions of Rho proteins or bacterial toxins purported to specifically inhibit Rho GTPases such as C3 transferase (C3) from *Clostridium botulinum*, specific for Rho, and Toxin B from *Clostridium difficile*, which inhibits Rho, Rac, and Cdc42 (Boquet, 1999). While a great deal has been learned from these studies regarding the biochemical nature of Rho signaling, studies using pharmacological inhibition or ectopic expression of dominantly acting mutants are vulnerable to nonspecific effects. In addition, they leave open the question of how Rho functions in a multicellular context. Developmental model systems, such as *Drosophila melanogaster*, have recently emerged as potent systems in which to study Rho GTPase function in an organismal and developmental context.

1.1.5. Rho family members in *Drosophila*.

In *Drosophila*, seven Rho family members have been identified to date: one Rho gene (*Rho1*), three Rac genes (*Rac1, Rac2* and *mig-2-like,* or *mtl*) and one Cdc42 gene (*Cdc42*), along with two other Rho family members, *RhoL* and *RhoBTB*, that are equally similar to Rho, Rac, and Cdc42. The GTPases for which mammalian homologs can be assigned are 86% - 92% identical to their mammalian counterparts, indicating a high degree of evolutionary conservation (Fig. 1.3). Initial studies investigating the function of these proteins during *Drosophila* development, excluding *RhoBTB*, for which there has not yet been a functional study, involved ectopic expression of dn or ca forms of these proteins. Data from these studies linked these genes to a variety of morphogenetic processes in *Drosophila* oogenesis and embryogenesis (see Fig. 1.4) (Glise et al., 1995; Harden et al., 1996; Harden et al., 1995; Murphy and Montell, 1996; Noselli, 1998; Riesgo-Escovar et al., 1996). Loss-

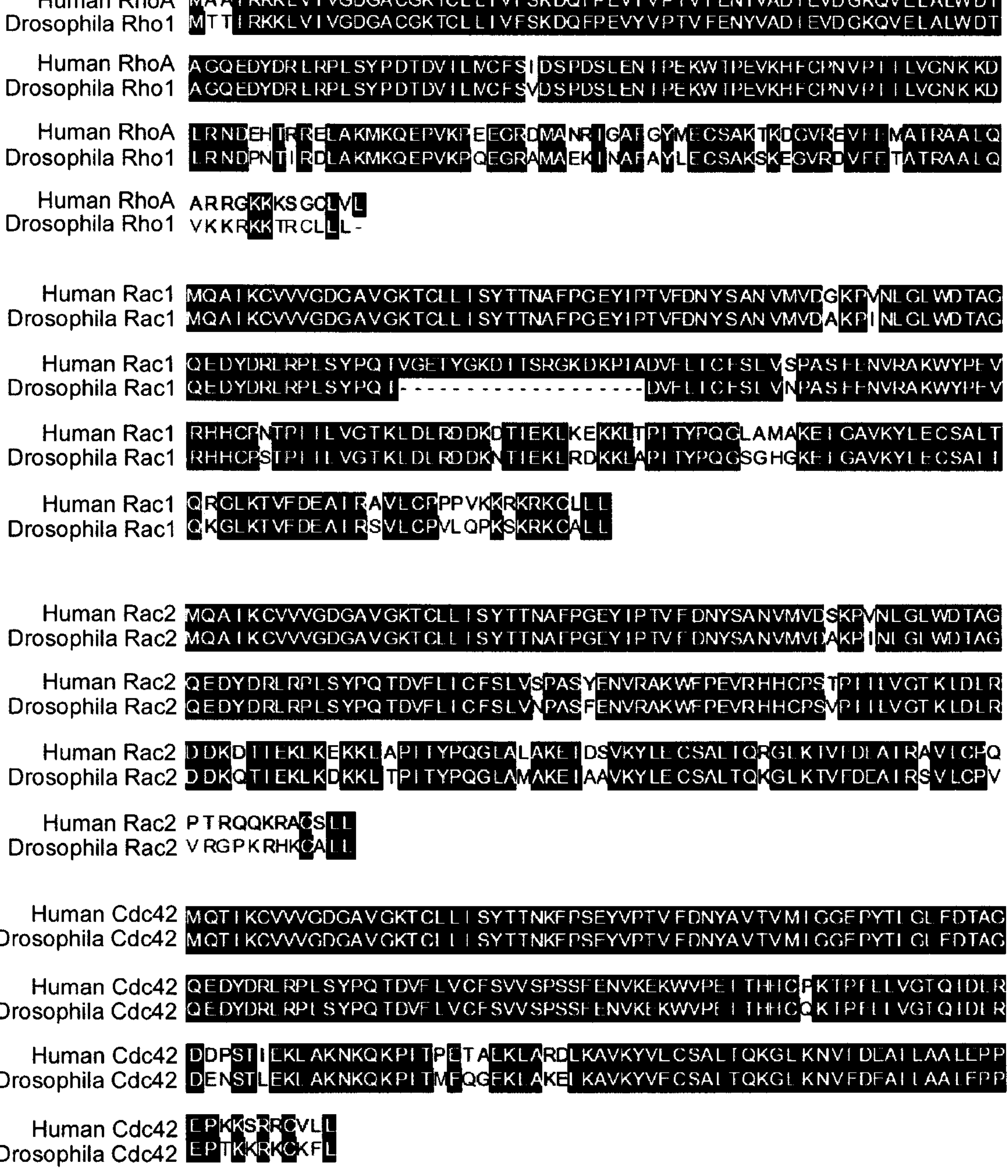


Figure 1.3. Comparison of human and *Drosophila* Rho GTPases. The four founding human and *Drosophila* Rho GTPases shown here are 86-92% identical, indicating a high degree of evolutionary conservation.

cuticle phenotype	gene	processes affected
wild-type	—	—
Hs-GAL4^{M4} DRhoAN19	**Rho1**	dorsal closure ventral furrow formation ommatidial polarity
[illegible]L4^{M4} Drac1N17	**Rac1, Rac2, mtl**	dorsal closure border cell migration axonal outgrowth myoblast fusion
Hs-GAL4^{2077} Dcdc42N17	**Cdc42**	axonal outgrowth nurse cell actin
—	**RhoL**	nurse cell actin
—	**RhoBTB**	?

Figure 1.4. Rho GTPases in *Drosophila*. Overexpression of constitutively-active (ca) or dominant-negative (dn) forms of these proteins has linked them to a number of morphogenetic processes (listed on right). Interestingly, ubiquitous expression of dnRho, dnRac and dnCdc42 all result in a similar dorsal open phenotype (compare bottom 3 cuticles to wildtype, top). Cuticles on left from Harden et al., 1999. (Reprinted with permission of Company of Biologists, Ltd.)

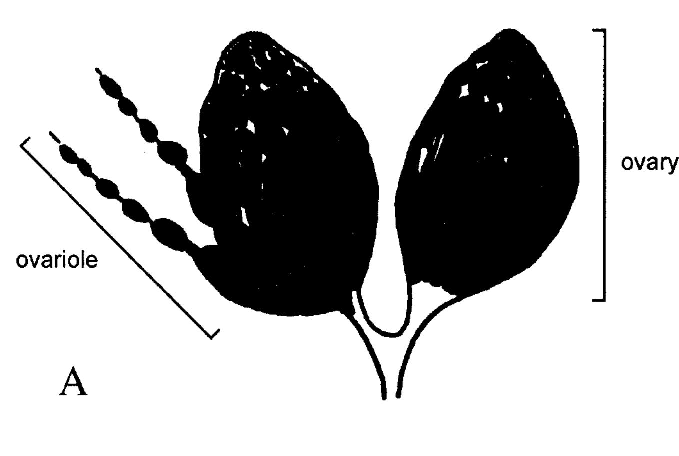
of-function mutations have subsequently been identified in *Rho1* (Magie et al., 1999; Strutt et al., 1997), all three *Rac* genes (Hakeda-Suzuki et al., 2002; Ng et al., 2002), and *Cdc42* (Genova et al., 2000). Given the involvement of Rho in many of the cell biological processes necessary for morphogenesis to occur, it is not surprising that analysis of the phenotypes associated with these mutants also links Rho function to many developmental events.

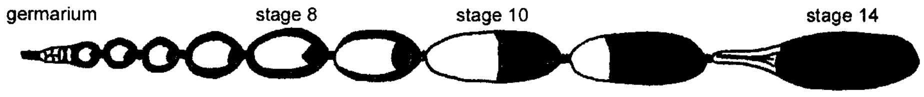
1.2. *Drosophila* morphogenesis.

Development in *Drosophila*, as in other organisms, requires a number of patterning events and stereotypical cell movements and shape changes to construct the egg and effect the transition from a single cell to a complex, differentiated organism. A schematic representation of oogenesis is shown in Fig. 1.5, and the embryonic stages of development are depicted in scanning electron micrographs (SEMs) of *Drosophila* embryos in Fig. 1.6. The complexity of *Drosophila* development offers numerous contexts in which to investigate particular aspects of Rho function.

1.2.1. Oogenesis.

Ovaries in *Drosophila* are organized as bundles of ovarioles (see Fig. 1.5). Each ovariole consists of a series of egg chambers arranged like beads on a string, with each egg chamber from anterior to posterior of a successively later stage. An egg chamber consists of 16 germline cells (15 nurse cells and 1 oocyte) surrounded by an epithelium of somatic follicle cells. The germline cells are descended from one precursor cell that undergoes 4 division cycles. Their cytoplasm is connected by cytoplasmic bridges known as ring canals, actin-rich structures that are the result of incomplete cytokinesis and allow for the transport of materials from the nurse cells to the oocyte. Actin is also concentrated in the cortices of individual cells, both in the germline and somatic cells.





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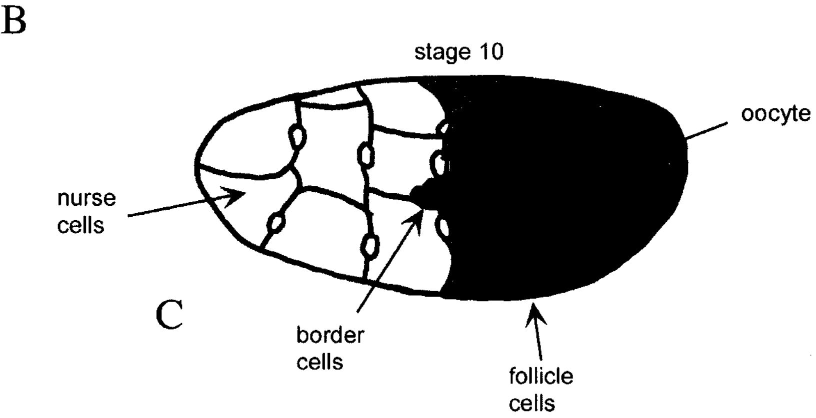

Figure 1.5. Oogenesis in *Drosophila*. (A) *Drosophila* ovaries consist of bundles of ovarioles. Anterior is up. (B) Each ovariole consists of a series of egg chambers of progressively later stages. (C) Egg chambers consist of the germline nurse cells (yellow) and oocyte (red), surrounded by somatic follicle cells (blue). The nurse cells and oocyte are connected by ring canals, the result of incomplete cytokinesis that allow for the transport of materials from cell to cell. The border cells are a specialized subset of follicle cells that migrate to the anterior of the oocyte and are responsible for forming the micropyle. In (B) and (C), anterior is left. (After Cooley and Theurkauf, 1994)

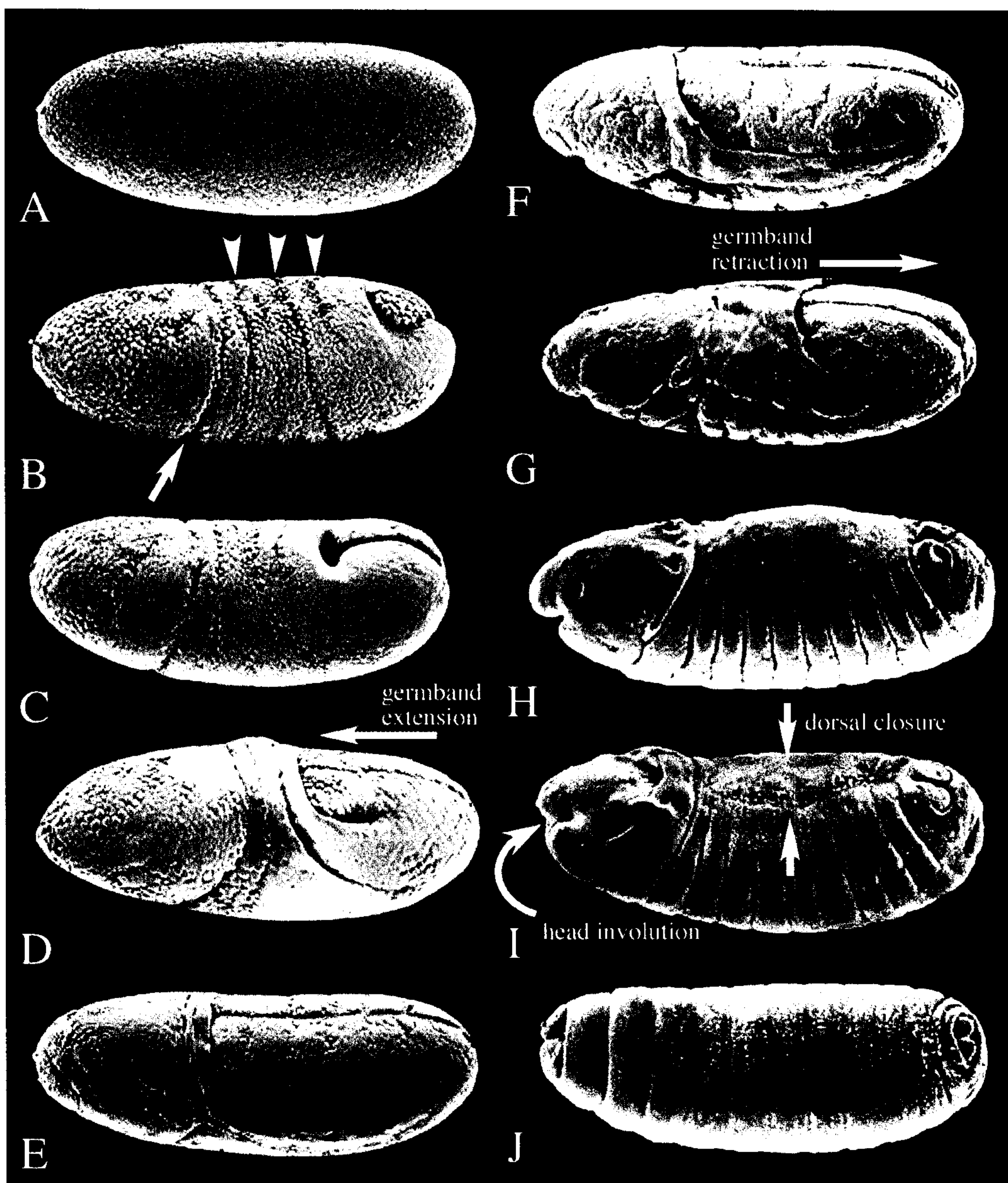

Figure 1.6. SEMs of the various stages of *Drosophila* development. (A) Cellular blastoderm. (B) Gastrulation begins with ventral furrow formation (not shown) and germband extension. Arrowheads indicate transverse folds, arrow indicates the cephalic furrow. (C-F) Germband extension continues until it is fully extended along the dorsal surface of the embryo. (G-H) Germband retraction results in a dorsal hole in the embryonic epithelium. (I) Dorsal closure zips the lateral epithelia together at the dorsal midline, while head involution internalizes anterior head structures. (J) A fully-formed larva ready for hatching. (M. Meyer and S.M. Parkhurst, unpublished)

Morphogenesis during oogenesis involves a number of follicle cell migration events. Between stages 9-10 of oogenesis most of the follicle cells transition from an even epithelial layer surrounding all the germline cells to one concentrating around the oocyte, accompanied by their conversion from a cuboidal to columnar shape (Fig. 1.5). The few follicle cells that do not encase the oocyte become squamous and stretched over the nurse cells. Also during this time a specialized subset of follicle cells known as border cells migrates as a cluster from the anterior pole of the egg chamber between the nurse cells to the anterior pole of the oocyte (arrow in Fig. 1.5C) (reviewed in Montell, 2003).

1.2.2. Early development.

The early stages of development occur in a syncytium, in which nuclei divide within a common cytoplasm. The first nuclear division cycles are rapid (approximately 10 minutes each) and synchronous, and do not involve cytokinesis. By cycle 10 the nuclei have migrated from their initial position in the interior of the embryo to the surface, just below the plasma membrane (Fig. 1.7). This migration involves a cytoplasmic streaming event that requires the function of the actin and microtubule cytoskeletons (Baker et al., 1993; Ji et al., 2002; von Dassow and Schubiger, 1994). The resulting embryo is known as a "syncytial blastoderm". The somatic nuclei continue to divide synchronously until cycle 14. With each division, pseudocleavage furrows form between the nuclei between interphase and metaphase, and disappear during anaphase (Foe et al., 2000). These furrows are the precursors of the cleavage furrows that will divide the nuclei from one another at cellularization. Cellularization is essentially a specialized form of cytokinesis, and like the contractile ring that forms during that process, the pseudocleavage and cleavage furrows exhibit rich accumulations of actin and myosin. The first cells to form are the pole cells, which pinch off from the posterior pole of the embryo and will go on to form the cells of the germline. Cellularization of the somatic cells involves the growth and extension

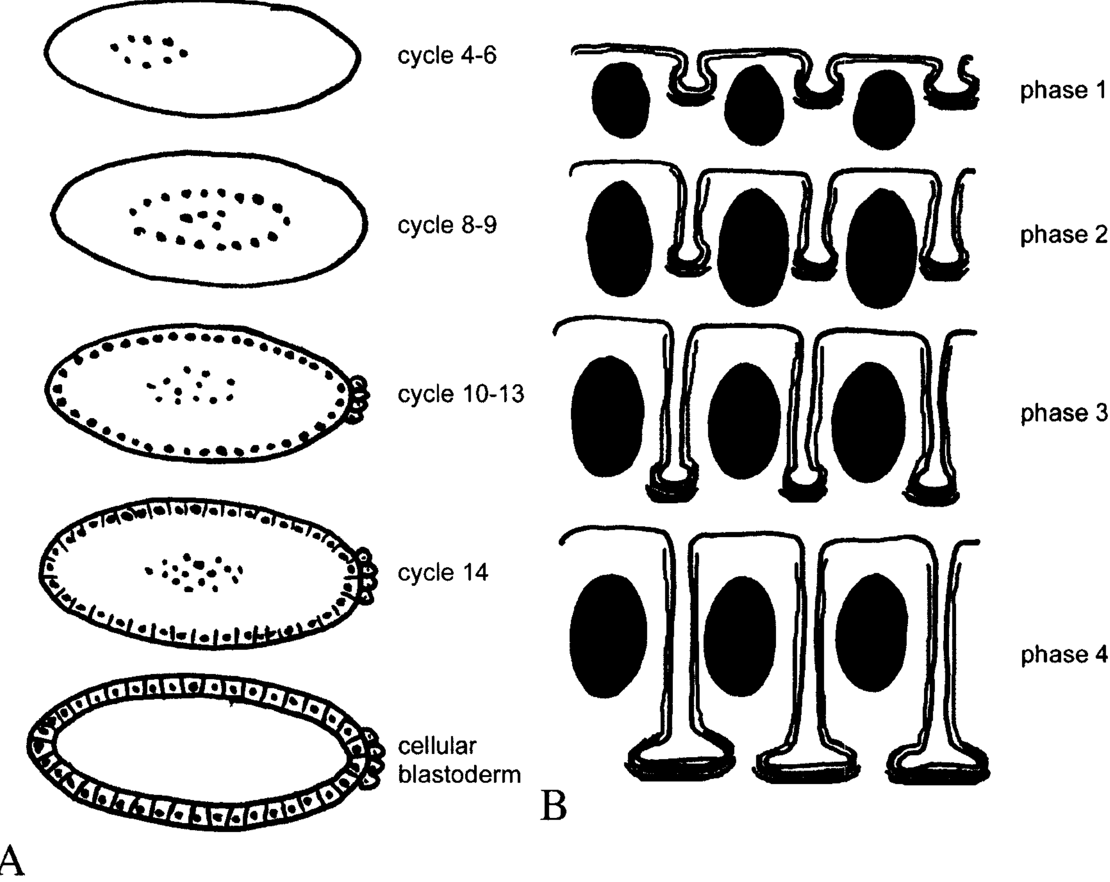

Figure 1.7. Early development in *Drosophila*. (A) The stages of nuclear migration, proceeding from early (top) to late (bottom). The nuclear cycles comprising each stage are indicated to the right. Cleavage divisions occur within a syncytium. Nuclei begin in the anterior cytoplasm and migrate to the poles as they divide. Once nuclei reach the surface (cycle 10), the pole cells form (3rd image from top). Cellularization occurs during the 14th cell cycle following fertilization. Anterior is left. (B) The phases of cellularization. Cellularization involves the invagination of the plasma membrane between nuclei (blue). This involves the coordinated regulation of actin (red) and myosin (green). (After Mazumdar and Mazumdar, 2002)

of plasma membrane between the nuclei toward the interior of the embryo, resulting in a monolayer of cells now termed the "cellular blastoderm" (Fig. 1.7) (reviewed in: Mazumdar and Mazumdar, 2002). A number of both maternally and zygotically acting genes have been identified that are required for cellularization. Maternal gene products include cytoskeletal components and associated proteins generally required for cytokinesis such as *twinstar*, the *Drosophila* homolog of the actin severing protein cofilin that is required for the formation of the contractile ring (Gunsalus et al., 1995), and *anillin*, another actin binding protein implicated in cytokinesis (Oegema et al., 2000). The zygotic genes identified are involved in regulating particular aspects of cellularization. Organization of the cellularization front, the leading edge of the invaginating membrane, requires Nullo, which acts to stabilize adhesive contacts between the forming cells (Hunter et al., 2002). In *nullo* mutants, the actin network localized to the cleavage furrows is disrupted and many multinucleate cells form. Another zygotically required protein is Slow-as-molasses (Slam), which is involved in the polarized insertion of membrane in the invaginating furrow and required for the progression of the cellularization front (Lecuit et al., 2002).

1.2.3. Gastrulation.

Gastrulation is the first time in development that the cells of the embryo change position relative to one another, and results in the formation of the 3 major germ layers: ectoderm, mesoderm and endoderm. In *Drosophila*, gastrulation begins with the formation of the ventral furrow, an invagination along the ventral midline of the embryo, resulting in the internalization of mesodermal precursor cells. This process involves the apical constriction of a ventral domain of cells approximately 18 cells wide, which pulls cells which are lateral to the ventral domain toward the ventral midline. The nuclei and cytoplasm of the constricting cells are pushed basally, causing the cells to elongate. Following elongation, the cells shorten leading to an expansion of their basal surfaces and the formation of the initial invagination (Fig. 1.8A) (reviewed in Costa et al., 1993).

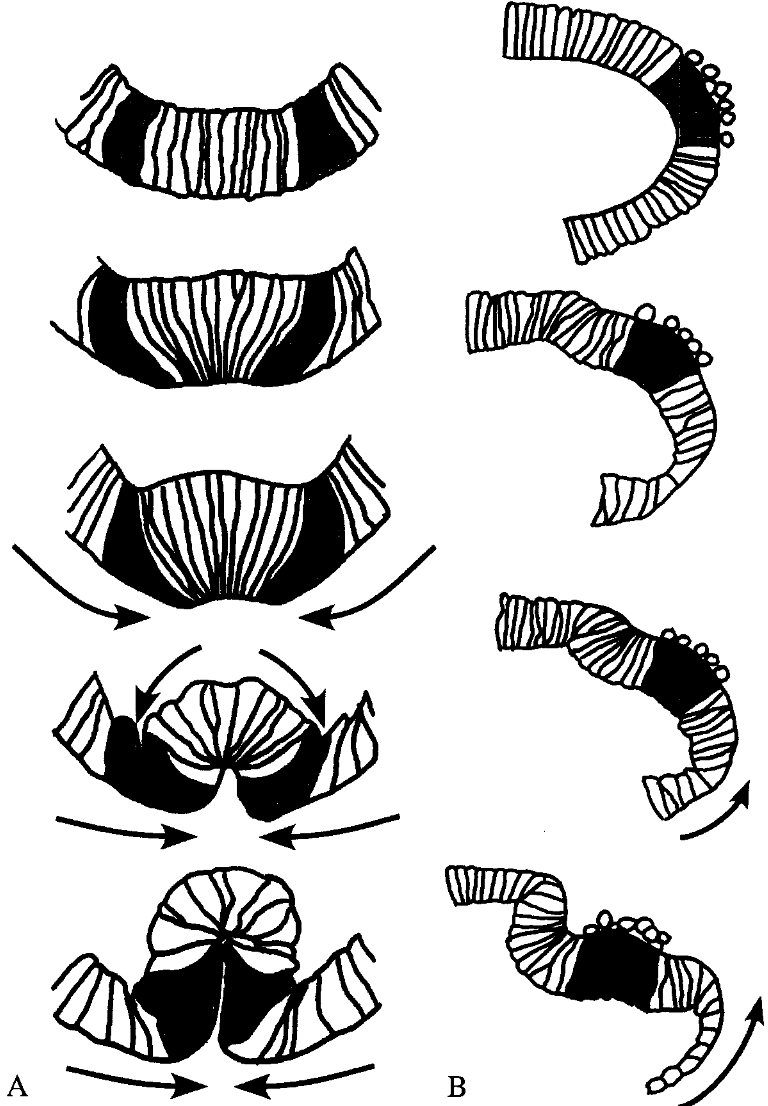

Figure 1.8. Cell shape changes during gastrulation in *Drosophila*. Cells undergoing apical constriction are highlighted in yellow. Red cells are marked for reference. (A) Cross-section diagram of an embryo during ventral furrow formation. Cells at the midline undergo apical constriction, pulling lateral cells toward the midline. The expanding basal surfaces of the yellow cells push down on the red cells, resulting in the closing and deepening of the furrow. Ventral is down. (B) Saggital section diagram of an embryo during posterior midgut formation. The yellow cells undergo apical constriction as germband extension is beginning, resulting in the formation of a depression containing the pole cells. Anterior is left, dorsal is up. (After Costa et al., 1993)

Following the initiation of the ventral furrow, the posterior midgut invagination forms and then germband extension occurs. The posterior midgut invagination is a cup-shaped invagination at the posterior pole that results in the internalization of the midgut and hindgut primordia along with the pole cells (Fig. 1.8B). This requires cell-shape changes very similar to those that occur during ventral furrow formation, including apical flattening and constriction of the cells that will form the interior of the invagination (reviewed in Costa et al., 1993).

The germband consists of the cells in the posterior two thirds of the embryo, from the cephalic furrow (arrow in Fig. 1.6) to the posterior pole. Germband extension involves the intercalation of cells along the anterior-posterior axis of the embryo, resulting in the lengthening of the embryo approximately two-fold (Irvine and Wieschaus, 1994). Because the embryo is encased in a hard vitelline membrane, the germband extends from the posterior pole of the embryo along the dorsal surface, folding back on itself. As germband extension begins, a reproducible pattern of dorsal transverse folds forms (arrowheads in Fig. 1.6). These folds deepen as germband extension progresses, but their initiation seems to involve cell shape changes very similar to those involved in ventral furrow formation (Costa et al., 1993). Cell apices constrict and their nuclei move basally, followed by a shortening of the central-most cells along their apical-basal axes. Formation of the cephalic furrow also involves cell shape changes of this sort (Vincent et al., 1997).

Following the completion of germband extension, the embryo begins the process of germband retraction (Fig 1.6). The end of the germband returns to the posterior pole in a process very different from that which drove its extension, in that it does not involve cell movements relative to one another, but instead relies primarily on cell shape changes (Schock and Perrimon, 2002). This process results in a hole in the dorsal embryonic epidermis where the extraembryonic amnioserosa is located (Fig. 1.6H). Mutants that affect germband retraction specifically have been described, including genes important in the development and maintenance of the amnioserosa such as *hindsight*, *u-shaped*, *serpent*, and *tailup* (Frank and Rushlow, 1996), as well as

the more pleiotropic loci *Egfr*, which encodes the *Drosophila* homolog of the epidermal growth factor (EGF) receptor (Schejter and Shilo, 1989), and *Cdc42* (Genova et al., 2000).

1.2.4. Dorsal closure.

The closure of the hole left in the embryonic epidermis upon completion of germband retraction is accomplished by the dorsalward migration and zippering together of the lateral epithelial sheets at the dorsal midline, a process known as dorsal closure (DC; Fig. 1.9) (reviewed in: Harden, 2002; Jacinto et al., 2002b). The signals that initiate DC are not well understood, however once the process is triggered, activation of the mitogen-activated protein kinase (MAPK) signaling pathway leading to Jun N-terminal kinase (JNK) activation is soon apparent. JNK signaling is required in the leading edge cell's for the expression of its targets *decapentaplegic (dpp),* a *Drosophila* transforming growth factor-β (TGF-β) homolog, and the JNK phosphatase *puckered* (*puc*) (Glise and Noselli, 1997). One major group of mutants that result in DC failure is composed of those that compromise JNK signaling such as *hemipterous* (*hep*), *basket* (*bsk*), and *kayak* (*kay*), which encode JNKK, JNK and Fos, respectively. Bsk and Hep activate Jun, which together with Fos forms the AP-1 transcriptional activation complex that activates the transcription of *dpp* and *puc*. JNK signaling is a central regulator of DC (Noselli, 1998). In wildtype embryos, cells of the leading edge (LE), the dorsalmost row of cells, undergo an elongation in the dorsal-ventral axis. This elongation is subsequently seen in more ventrally located cells. LE cells in mutants that compromise JNK signaling, such as *bsk* and *hep,* initially elongate but this shape change is not maintained and they revert to a cuboidal shape (Glise et al., 1995; Riesgo-Escovar et al., 1996). *PKN* mutants fail to maintain elongation of the LE cells in a manner very similar to JNK pathway mutants (Lu and Settleman, 1999). Expression of JNK targets, however, is unaffected in *PKN* mutants, indicating that these two pathways are separate, but both required for the proper regulation of DC.

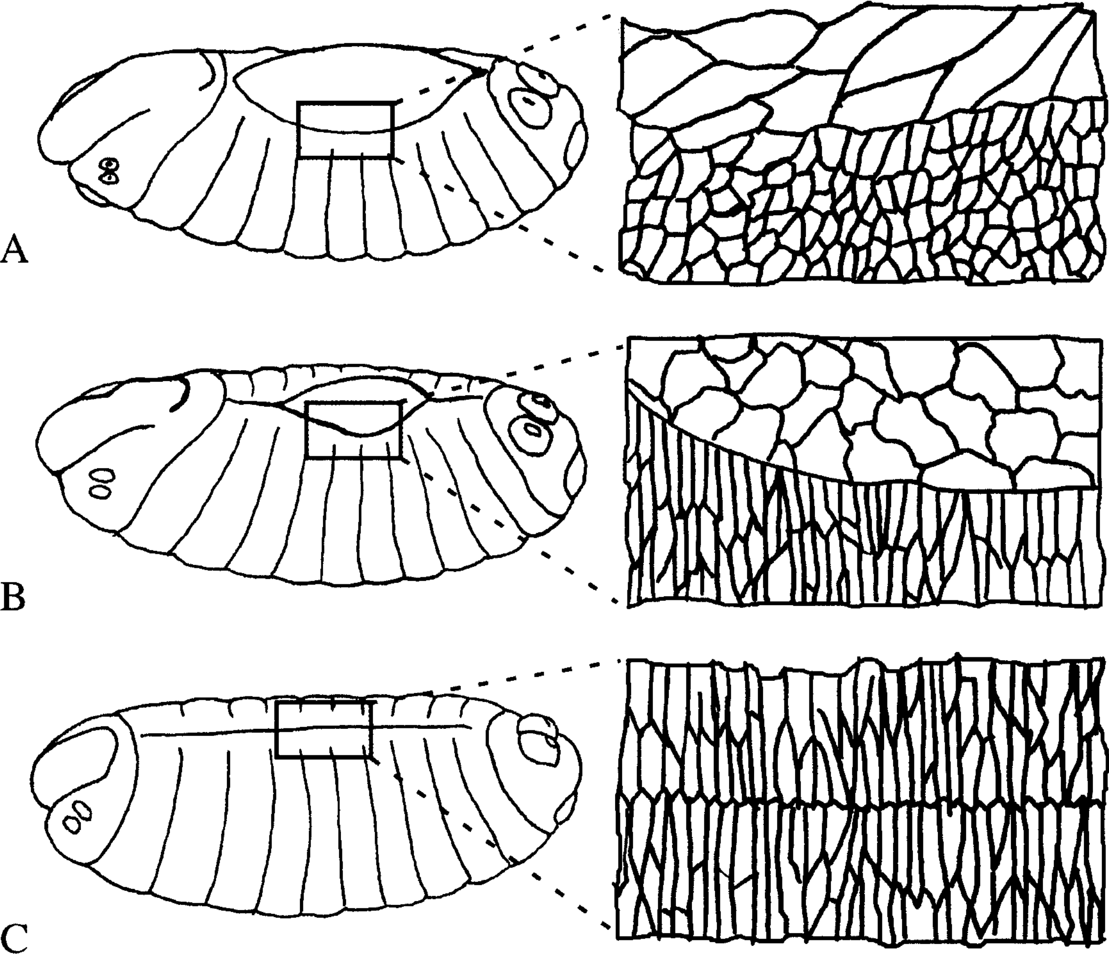

Figure 1.9. Dorsal closure (DC). (A) Prior to the initiation of DC, cells at the leading edge and lateral epithelia exhibit a cuboidal morphology. (B) As DC proceeds, these cells elongate along their D-V axis. (C) Cells from the opposing epithelia meet and zip together at the dorsal midline, internalizing the amnioserosal cells. Anterior is left and dorsal is up in all images. (After Harden, 2002)

One important feature of the LE cells later in the process of DC is the formation of an actomyosin cable at their dorsalmost edge. This cable was initially thought to act as a purse string, providing contractile force to pull the lateral epithelia together (Young et al., 1993). The cable clearly does provide some contractile force, as the organization of the LE cells into a smooth line and the beginning of their elongation coincides with the formation of the cable, and laser ablation experiments indicate that the LE is under tension (Kiehart et al., 2000). Additionally, analysis of mutant phenotypes indicates that contractile machinery is required for DC to occur normally. Mutations in the *Drosophila* homolog of the nonmuscle myosin-II heavy chain, *zipper,* fail to undergo normal cell shape changes in the lateral epithelium and cannot complete DC (Young et al., 1993). Subsequent work has indicated that in addition to providing a contractile force the cable also acts to constrain the leading edge cells and keep them organized, allowing for the formation of a neat seam at the dorsal midline. The tension present in the cable keeps the LE cells taut, and inhibits the formation of protrusions at their dorsal edge. Disrupting the cable in a subset of LE cells results in cells with hyperactive lamellipodial and filopodial protrusions and a consequent migratory advantage over their wildtype neighbors (Jacinto et al., 2002a).

Zippering together of the lateral epithelial sheets requires stable adhesive contacts between opposing cells. This involves the formation of epithelial cadherin (E-cadherin) -based adherens junctions (AJs) as cells meet at the dorsal midline. AJ formation in mammalian epithelial cells in culture occurs through the interdigitation of filopodial extensions from neighboring cells, resulting in the recruitment of E-cadherin components at the filopodial tips and the formation of an immature "adhesion zipper" with 2 rows of punctate accumulation of AJ components (Vasioukhin et al., 2000). This "adhesion zipper" then resolves through the retraction of the filopodia, resulting in mature AJs (Fig. 1.10). During DC, cells in the LE extend lamellipodia and filopodia as they migrate toward the midline (Jacinto et al., 2000). As they approach cells in the opposing LE, their filopodia interdigitate in a manner very similar to that seen in mammalian tissue culture. They then form AJs and close the seam. This

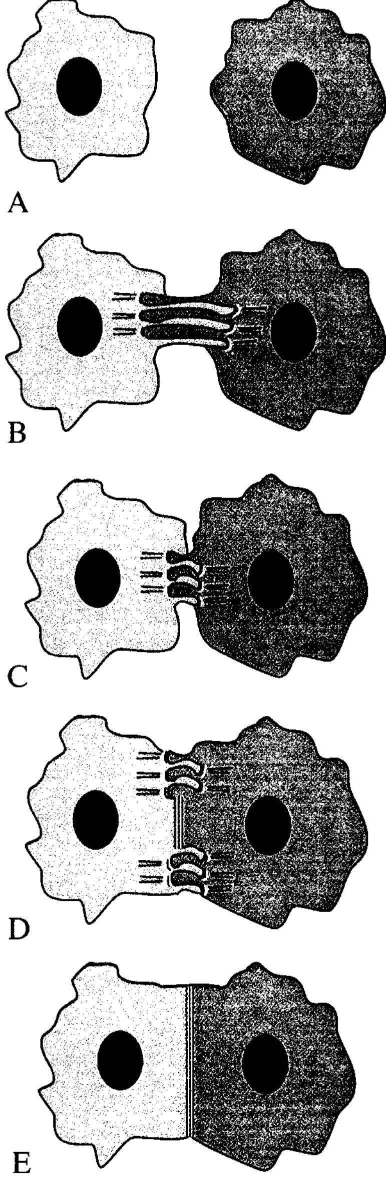

Figure 1.10. Adhesion zipper formation in fibroblasts. (A-B) Adjacent cells extend filopodia toward each other, which interdigitate and push into the opposing cell body. Actin (red), catenins (yellow) and cadherins (green) localize to these points of contact. (C) The filopodia shorten, drawing the cells together. (D) The filopodia resolve into mature junctions (center), and the zippering is propagated to the edge of the cell. (E) All filopodia resolve and mature adherens junctions form between the cells (After Vasioukhin et al., 2000).

zippering begins from the anterior and posterior aspects of the dorsal hole, and proceeds toward the center. As the zippering reaches its final stages, the gap between the opposing epithelia becomes quite small, and zippering occurs randomly along the length of the remaining hole.

1.2.5. Head involution.

At the same time DC is occurring, head involution is occurring in the anterior of the embryo. Head involution is required for the proper formation of larval head structures, and results in the internalization of the anterior portion of the embryo. The extensive cell movements and shape changes required for involution have been investigated using SEM (Fig. 1.11) (Turner and Mahowald, 1979). Following gastrulation, the head region forms a number of morphologically distinct lobes. The large procephalic lobes are located dorsally. Adjacent to the procephalic lobes at the anterior pole of the embryo is the clypeolabral lobe. Ventral to the clypeolabral lobe can be found, from anterior to posterior, the hypopharyngeal, mandibular, maxillary and labial lobes. The stomodeum, or the opening through which head structures will be involuted, forms between the clypeolabrum and the hypopharyngeal lobe. Head involution begins with the internalization of the hypopharyngeal lobe, which will form the floor of the pharynx. As involution proceeds the remaining lobes move anteriorly and are internalized through the stomodeum in turn, with the labial lobes and clypeolabrum coming last. While these events are occurring on the ventral surface of the embryo, a ridge forms on the dorsal surface between the procephalic lobes and the first thoracic segment. This "dorsal ridge" then stretches over the head segments to cover the entire head capsule, forming the "dorsal sac". Interestingly, while head involution clearly requires complex cell movements and shape changes, most mutants identified that specifically affect this process are involved in the regulation of apoptosis, as a stereotypical pattern of programmed cell death is necessary to allow head involution to occur normally (Nassif et al., 1998). Mutations in the genes *reaper*,

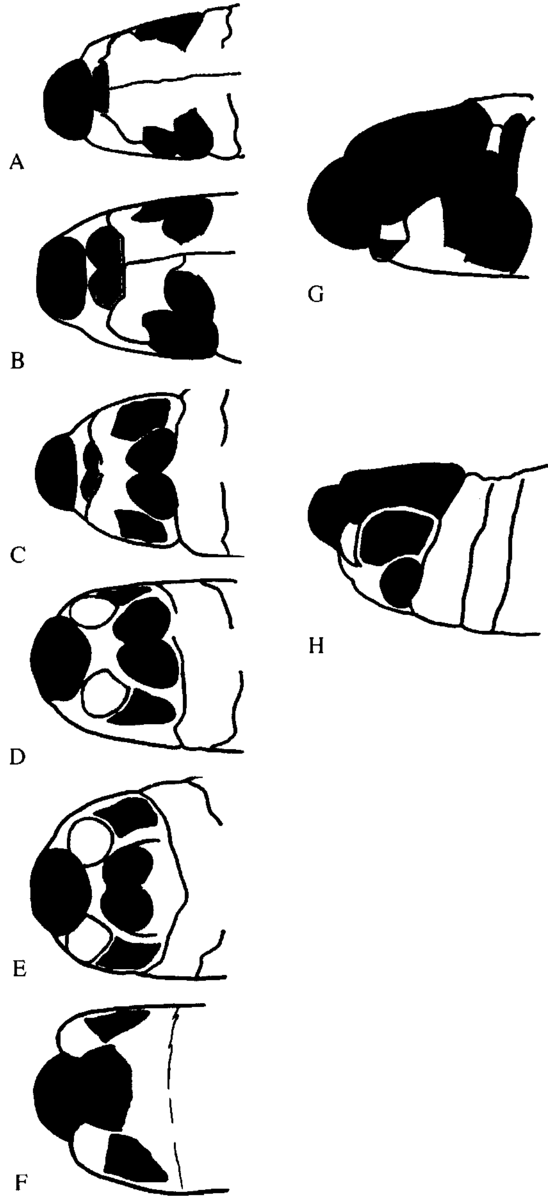

Figure 1.11. Head involution. (A) The clypeolabral (blue), hypopharyngeal (orange), mandibular (yellow), maxillary (green) and labial (red) lobes are visible from the ventral side of an embryo about to undergo head involution. (B-F) The hypopharyngeal lobe is the first to enter the stomodeum. The others then move anteriorly and sequentially follow. (G-H) The procephalic lobe (purple) and dorsal ridge (turquoise) are visible in lateral views. Anterior is left in all images (After Turner and Mahowald, 1979).

grim, *sickle*, and *head involution defective* (*hid*), which are necessary for apoptosis in *Drosophila*, all result in head involution phenotypes (Lee and Baehrecke, 2000).

1.3. Rho functions during development in *Drosophila*.

Following the identification of Rho GTPases in *Drosophila* initial investigations into their function were accomplished using ectopic expression of dn or ca versions (Eaton et al., 1995; Fanto et al., 2000; Harden et al., 1995; Harden et al., 1999; Luo et al., 1994; Murphy and Montell, 1996). In recent years loss-of-function mutations have been isolated in many *Drosophila* Rho homologs (Genova et al., 2000; Hakeda-Suzuki et al., 2002; Ng et al., 2002; Strutt et al., 1997), allowing the developmental consequences of specific removal of particular GTPases to be assessed. Not surprisingly, Rho proteins function in many different developmental contexts.

1.3.1. Rho functions in oogenesis.

Expression of dnRac, dnCdc42, and dnRhoL during oogenesis all result in defects involving actin cytoskeletal regulation. dnRac inhibits border cell migration, dn- and ca- Cdc42 both result in defects in cortical actin and nurse cell fusion, and dn- and ca-RhoL causes defects in nurse cell–follicle cell contacts, nurse cell fusion, and defects in the outer follicle cell monolayer (Murphy and Montell, 1996). However, this phenotype may be misleading as expression of endogenous RhoL in the embryo is restricted to hemocytes and hemocyte precursors and has not been reported during oogenesis (Casal and Leptin, 1996).

Cdc42 mutants have also been reported to have defects in oogenesis (Genova et al., 2000). Generation of follicle cell clones that are mutant for *Cdc42* results in their loss of cuboidal shape and epithelial integrity. Clones comprising the entire follicle cell epithelium exhibit fusion of adjacent egg chambers due to the loss of stalk cells. Germline clones result in defects in actin cytoskeletal structure and a delay in the transfer of material from the nurse cells to the oocyte.

1.3.2. Rho functions in early development.

Rho has been shown to be important for cytokinesis in systems such as early *Xenopus* and echinoderm embryos, as well as mammalian tissue culture cells. Not surprisingly given the similarities between cytokinesis and *Drosophila* cellularization, injection of *Drosophila* embryos with C3 exoenzyme results in cellularization defects, as does the injection of dnRho protein (Crawford et al., 1998).

Following cellularization, a series of mitoses occur at stereotypical times and in stereotypical locations, termed "mitotic domains", which continue through the later stages of embryogenesis (Foe, 1989). In *Drosophila* Rho has also been shown to play a role in mitosis through its effects on cytokinesis, mediated by the involvement of the Rho-specific GEF encoded by *pebble*. Mutants lacking Pebble function exhibit cytokinesis defects and an accumulation of multinucleate cells (Prokopenko et al., 1999). The basis for this phenotype is the failure to form a proper contractile ring at the presumptive cleavage furrow. Subsequent analysis has demonstrated that loss of Rho1 activity through mutation or expression of dnRho1 results in a similar cytokinesis defect (Prokopenko et al., 1999).

1.3.3. Rho functions in gastrulation.

A role for Rho in gastrulation has been suggested through the study of mutations in *DRhoGEF2*, a gene encoding a GEF with sequence homology to Rho-specific GEFs from other systems. Mutations in *DRhoGEF2* can genetically suppress a rough eye phenotype resulting from overexpression of Rho1, arguing for their function in a common pathway (Barrett et al., 1997). *DRhoGEF2* mutants fail to form a ventral furrow, and exhibit little of the apical flattening of cells in the ventral furrow region that is observed in wildtype embryos, likely due to a failure to form the contractile machinery necessary for apical flattening and constriction (Barrett et al., 1997; Hacker and Perrimon, 1998). Additionally, expression of dnRho1 results in a delay in ventral

furrow formation and the failure of some mesodermal precursors to be internalized properly, whereas dnRac or dnCdc42 do not have this effect. Significantly, *DRhoGEF2* mutants and embryos expressing dnRho1 also fail to form the posterior midgut invagination.

Whether the cell shape changes associated with the formation of the transverse folds and the cephalic furrow are compromised when Rho function is impaired has not been looked at in detail, although in *DRhoGEF2* mutants furrows do still form as the embryos try to initiate germband extension (Barrett et al., 1997; Hacker and Perrimon, 1998). Embryos expressing dnRac1 also show defects in germband retraction (Harden et al., 1995), though this is not recapitulated in embryos triply mutant for all 3 *Rac* genes (Hakeda-Suzuki et al., 2002).

1.3.4. Rho functions in dorsal closure.

Expression of dominant-negative Rho, Rac, or Cdc42 all result in DC defects, indicating an important role for Rho GTPases in this process (Harden et al., 1999). Rho GTPases have been linked to JNK pathway activation in tissue culture systems (Coso et al., 1995; Minden et al., 1995). In particular, there is some evidence that Rac and Cdc42 can activate JNK signaling through their activation of the Rac/Cdc42 binding PAK kinases (Bagrodia et al., 1995). During DC, expression of caRac1, or caCdc42 results in ectopic activation of JNK signaling (Glise and Noselli, 1997). *Cdc42* mutants, however, do not show a loss of *dpp* expression, suggesting that endogenous Cdc42 is not required for JNK signaling (Genova et al., 2000). Though analysis of the *Cdc42* phenotype indicates defects in the elongation of epithelial cells in other contexts (notably oogenesis; Genova et al., 2000), the morphology of LE cells in these mutants has not been examined closely. In *Rac* mutants, however, LE cells do not elongate properly and those cells do not exhibit the protrusive activity seen in wildtype cells (Hakeda-Suzuki et al., 2002). Another signaling pathway acting downstream of *Rho1* and required for cell shape changes at the LE is that mediated by PKN, which is also an effector of Rac1 (Lu and Settleman, 1999).

Rho1 and *zipper* (nonmuscle myosin II) interact genetically, indicating that the Rho-ROCK-MLC phosphatase pathway utilized by Rho to organize contractile machinery in mammalian cells is also utilized in *Drosophila*, and that Rho1 functions similarly in the context of DC (Halsell et al., 2000). Rho1 is required to properly organize the actomyosin cable, as expression of dnRho1 in a subpopulation of cells at the leading edge disrupts its formation in a cell autonomous manner (Jacinto et al., 2002a). The cells lacking a cable due to expression of dnRho1 acquire hyperactive lamellipodial and filopodial protrusions and a migratory advantage over neighboring, wildtype cells, resulting in their overrepresentation at the dorsal midline.

Expression of dnCdc42 in LE cells prevents the extension of filopodia from these cells, and adhesion of the opposing epithelium fails, indicating that the interdigitation of filopodial processes is crucial to proper zippering (Jacinto et al., 2000). In addition, filopodia have been proposed to be required for the proper matching of segments at the dorsal midline, as the expression of dnCdc42 also results in mismatched segments.

1.3.5. Potential Rho functions in head involution.

Involvement of Rho GTPases in head involution has not been specifically investigated to date, though given the extensive cellular movements and shape changes involved in this process it would be surprising if they were not involved. Morphogenetic cell death is also crucial for head involution to proceed normally (Nassif et al., 1998). Significantly, Rho GTPases have been implicated in the control of apoptosis in other systems, primarily through their effects on the cell shape changes associated with regulated cell death. Rho is thought to be involved in the cell contraction that accompanies apoptosis through activation of ROCK, whereas Rac is involved in the cell shape changes required for engulfment of the cell corpse (Coleman and Olson, 2002).

1.3.6. Rho functions in other developmental contexts.

Rho GTPases have also been linked to other developmental contexts, notably neuronal morphogenesis and the planar cell polarity (PCP) pathway. In the development of the nervous system, regulation of neuronal morphology is crucial to establishing the proper synaptic contacts. Regulation of the actin cytoskeleton by Rho GTPases has been shown to be important in this process. Expression of either dnRac1 or caRac1 in *Drosophila* causes abnormal filamentous actin accumulation and defects in axon outgrowth in peripheral neurons, while dendrites are not affected (Luo et al., 1994). Defects in axon morphology are also seen with expression of dnCdc42. *Cdc42* mutants, in contrast, do not show defects in axon outgrowth (Genova et al., 2000). Embryos triply mutant for the Rac genes *Rac1*, *Rac2*, and *mtl*, however, do exhibit defects in both guidance and growth of axons (Ng et al., 2002). Generation of *Rho1* mutant clones in the CNS has indicated a role for Rho1 in neuroblast proliferation and dendritic complexity (Lee et al., 2000), as neurons lacking Rho1 overextend their dendrites. Axon outgrowth, however, is unaffected.

Planar cell polarity (PCP) is the process through which epithelial cells, which exhibit apical-basal polarity, orient relative to one another and thereby acquire an additional axis of polarity in the plane of the epithelium. PCP is a developmental mechanism common to many organisms. In *Drosophila* examples of PCP include the orientation of hairs on the adult wing and of the ommatidia in the adult eye (Adler, 2002). In the wing, each cell orients itself proximal to distal, with an actin-based hair located at the distal edge. Whereas in the wing single cells are independently involved in forming the overall polarity of the tissue, in the eye each group of cells comprising an ommitidium adopts polarity as a unit. In both cases, however, the genes involved in controlling the process are similar. These "primary polarity genes" include the cell surface receptors *frizzled* (*fz*), *Van Gogh* (*Vang*), and the cadherin-like *starry night* (*stan*), along with the cytoplasmic factors *disheveled* (*dsh*), *prickle* (*pk*), and *diego* (*dgo*) (reviewed in Mlodzik, 2002). Overexpression of dnCdc42 in the developing wing results in the failure of the distal hairs to form normally, and expression of

dnRac1 under the same conditions indicates a role for Rac in specifying the location of the hair (Eaton et al., 1996). Expression of dnRac1 or caRac1 in the eye also results in polarity defects, and Rac activity in this context was determined to be required downstream of Fz and Dsh (Fanto et al., 2000). Analysis of *Rac1*, *Rac2*, *mtl* triple mutants, however, does not support a role for Rac in PCP (Hakeda-Suzuki et al., 2002). Similarly, overexpression of Rho1 in the eye results in polarity defects (Hariharan et al., 1995). In the eye caRho1 expression can rescue the *dsh* phenotype (Strutt et al., 1997), and reduction of Rho1 dosage can partially rescue the phenotype associated with overexpression of Fz or Dsh, indicating a role for Rho1 downstream of Fz signaling.

1.4. Rho is also involved in morphogenetic processes in other organisms.

Recently Rho GTPases have also begun to be studied in other organismal contexts, where they have been linked to morphogenetic processes. Convergent extension, the process through which cells intercalate and extend a structure during development, has been shown to share many features of the PCP signaling pathway in *Drosophila* (Mlodzik, 2002), suggesting that Rho GTPases may play a role in this process as well. Indeed, during development in *Xenopus* Rho GTPases have been shown to be involved in cell motility and convergent extension during gastrulation, as well as in the development of head structures (Habas et al., 2003; Habas et al., 2001). Injection of embryos with mRNA encoding dn or ca Rho and Rac results in delays in mesoderm involution and reduced or absent head structures. Similarly, in the zebrafish *Danio rerio*, overexpression of dominant-negative Rho kinase 2 (dnRok2) during gastrulation, which presumably acts downstream of a zebrafish Rho protein, disrupts convergent extension (Marlow et al., 2002). In the chick, bone morphogenetic protein (BMP) signaling induces neural expression of *rhoB*, and treatment of chick neural tube explants with C3 exoenzyme to inhibit Rho prevents the delamination of neural crest

cells (Liu and Jessell, 1998). In mice, inhibition of RhoA, Rac1 and Cdc42 by overexpression of RhoGDIα in the developing heart disrupts the normal morphogenesis of the heart and inhibits cell proliferation (Wei et al., 2002).

A large body of literature has emerged linking Rho GTPase function to neuronal development (reviewed in Luo, 2000). For example, Rac and Cdc42 promote neurite outgrowth, while Rho is involved in their retraction (Kozma et al., 1997). Rho GTPases are also involved in the formation of neuronal polarity, or the choice between the formation of an axon or dendrite. This process is thought to involve localized actin polymerization and depolymerization, allowing polymerized microtubules to enter particular neurites and form axons (Bradke and Dotti, 1999). Treatment of cultured hippocampal neurons with Toxin B, thereby inhibiting Rho, Rac, and Cdc42, prevents the establishment of neuronal polarity, as does treatment with actin depolymerizing factors such as cytochalasin D and latrunculin B. Tissue culture studies have also indicated that the balance of GTPase function is important in growth cone guidance (Yuan et al., 2003). Expression of dn- or ca- Cdc42 in *Xenopus* spinal neurons interferes with chemoattraction of growth cones, and expression of dnRhoA blocks the chemorepulsion caused by a gradient of lysophosphatidic acid (LPA), a ligand shown to activate Rho signaling.

1.5. Summary.

Recent discoveries regarding Rho GTPase function in organismal contexts clearly indicate that they and the genes that regulate them are essential mediators of morphogenetic processes. In many cases this can be attributed to their ability to affect actin cytoskeletal organization, as they have been shown to do in tissue culture systems. Similar roles for Rho have been identified in diverse organisms, and the continued study of Rho function during the many facets of *Drosophila* development promises to provide important insight into the regulation of Rho GTPases in general.

The powerful genetic tools of the *Drosophila* system and the in-depth analysis of loss-of-function mutant phenotypes will aid in this process, allowing the fleshing-out of the pathways involved in Rho regulation and more precise elucidation of its mechanisms of action.

CHAPTER 2

Mutations in the Rho1 small GTPase disrupt morphogenesis and segmentation during early *Drosophila* development

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2.1. ABSTRACT

Rho GTPases play an important role in diverse biological processes such as actin cytoskeleton organization, gene transcription, cell cycle progression, and adhesion. They are required during early *Drosophila* development for proper execution of morphogenetic movements of individual cells and groups of cells important for the formation of the embryonic body plan. We isolated loss-of-function mutations in the *Drosophila Rho1* gene during a genetic screen for maternal-effect mutations, allowing us to investigate the specific roles Rho1 plays in the context of the developing organism. Here we report that Rho1 is required for many early events: loss of Rho1 function results in both maternal and embryonic phenotypes. Embryos homozygous for the Rho1 mutation exhibit a characteristic zygotic phenotype, which includes severe defects in head involution and imperfect dorsal closure. Two phenotypes are associated with reduction of maternal Rho1 activity: the actin cytoskeleton is disrupted in egg chambers, especially in the ring canals, and embryos display patterning defects as a result of improper maintenance of segmentation gene expression. Despite showing imperfect dorsal closure, Rho1 does not activate downstream genes or interact genetically with members of the JNK signaling pathway, used by its relatives dRac and dCdc42 for proper dorsal closure. Consistent with its roles in regulating

actin cytoskeletal organization, we find that Rho1 interacts genetically and physically with the *Drosophila* formin homologue, *cappuccino.* We also show that Rho1 interacts both genetically and physically with *concertina*, a Gα protein involved in cell shape changes during gastrulation.

2.2. INTRODUCTION.

During embryogenesis in *Drosophila*, morphogenetic movements of individual cells and groups of cells are important for the proper formation of the three germ layers and for the structures that are subsequently derived from them. These activities are driven in part by differentiation of specific cells followed by cell shape changes and coordinated cell movements, many of which have been shown to be a direct result of signaling pathways whose targets are capable of effecting the reorganization and relocalization of various cytoskeletal components.

One family of signaling proteins shown to be important in mediating cytoskeletal changes in response to extracellular cues is Rho and its relatives, Rac and Cdc42. Rho, Rac and Cdc42 are small GTPases in the Ras superfamily initially shown through work on fibroblasts to affect the organization of the actin cytoskeleton (Nobes and Hall, 1995; Ridley and Hall, 1992; Ridley et al., 1992). Specifically, microinjection of constitutively active Rho causes the rapid formation of stress fibers, actin-myosin filaments anchored to integrin complexes in the cell membrane (Ridley and Hall, 1992), while activated Rac induces lamellipodial (membrane ruffle) formation (Ridley et al., 1992) and activated Cdc42 results in filopodial extension (Kozma et al., 1995; Nobes and Hall, 1995). Moreover, Cdc42 can induce Rac, which in turn induces Rho, suggesting that although each family member mediates particular aspects of cytoskeletal function, they are linked hierarchically (Nobes and Hall, 1995). In addition to its role in mediating actin-myosin interactions, Rho GTPases have been implicated in many seemingly disparate cellular processes such as cell cycle

progression, cadherin assembly, MAP kinase signaling, and phospholipid turnover through activation of PI-3 kinase (Hall, 1998; Mackay and Hall, 1998; Narumiya, 1996; Van Aelst and D'Souza-Schorey, 1997). The diversity of functions exhibited by Rho and its relatives suggests that they may operate within a number of different pathways at particular times and places during development. The ability of Rho family members to regulate the cytoskeleton, in particular, makes them excellent candidates for genes involved in morphogenesis.

To date five Rho family members have been identified in *Drosophila*: Rho1, RhoL (Rho-like), dRacA, dRacB, and dCdc42 (Hariharan et al., 1995; Murphy and Montell, 1996). Studies using constitutively activated (ca) and dominant-negative (dn) forms of these proteins indicate roles for these GTPases in a variety of processes linked to cell shape changes and actin cytoskeleton regulation during *Drosophila* development. During oogenesis, expression of dCdc42(dn) and RhoL(dn) causes nurse cell collapse and subcortical actin breakdown, while expression of RhoL(ca) disrupts nurse cell-follicle cell contacts (Murphy and Montell, 1996). dRac(dn) was also shown to affect the migration of border cells, a specialized subset of follicle cells in the oocytes (Murphy and Montell, 1996). In the embryo, ectopic expression of dRac(dn) and dCdc42(dn) reveals roles for these proteins in dorsal closure, by affecting the JNK signaling pathway (Glise et al., 1995; Harden et al., 1996; Harden et al., 1995; Noselli, 1998; Riesgo-Escovar et al., 1996). Disruptions are also seen during neurogenesis and myogenesis where dRac(dn) affects axonal outgrowth of the peripheral neurons and fusion of the myoblasts respectively (Luo et al., 1994). Other family members play roles later in development such as during the formation of the compound eye where Rho1(dn) was shown to cause a collapse of the actin network important for proper ommatidial morphogenesis (Hariharan et al., 1995). Because there is a high level of conservation among Rho family members, the specificity of each member for the different developmental processes is not yet clear.

In *Drosophila*, a single RhoA homologue (*Rho1*) has been identified molecularly (Hariharan et al., 1995; Sasamura et al., 1997). Consistent with what is known in

mammalian cells, studies of Rho1(dn) and Rho1(ca) suggest that *Rho1* is involved in regulation of actin cytoskeleton, transcriptional regulation, and cell cycle progression/cell proliferation. We have identified P-element insertions within the *Drosophila Rho1* gene during a genetic screen for maternal-effect mutations, allowing us to investigate the specific roles Rho plays in the context of the developing organism. These same P-element-induced mutations were also identified in a genetic screen for rough eye mutants where they were shown to affect ommatidial polarity during eye development and were placed downstream of a Frizzled receptor (Strutt et al., 1997). Here we report that loss of *Rho1* function also results in both maternal and embryonic phenotypes. Embryos homozygous for mutant *Rho1* exhibit a characteristic zygotic phenotype, which includes imperfect dorsal closure and severe defects in head involution. The *Rho1* maternal effect phenotypes consist of disorganized actin cytoskeletal structures in ovaries, especially the outer ring canals, and defective segmentation gene expression. Together, these phenotypes suggest that a Rho-mediated signaling cascade is required for the proper execution of many morphogenetic events during *Drosophila* oogenesis and embryogenesis.

2.3. RESULTS.

2.3.1. Molecular characterization of the *Rho1* gene.

We have identified two P-element insertions within the *Drosophila* homologue of the mammalian RhoA gene from a collection of lethal P-element insertion lines (Allele designations: $Rho^{l(2)k07236}$ and $Rho^{l(2)k02107}$; (Hariharan et al., 1995; Sasamura et al., 1997; Torok et al., 1993). Plasmid rescue and sequence analysis of the genomic DNA flanking the P-elements indicated that the $Rho^{l(2)k07236}$ P-element is inserted in an intron

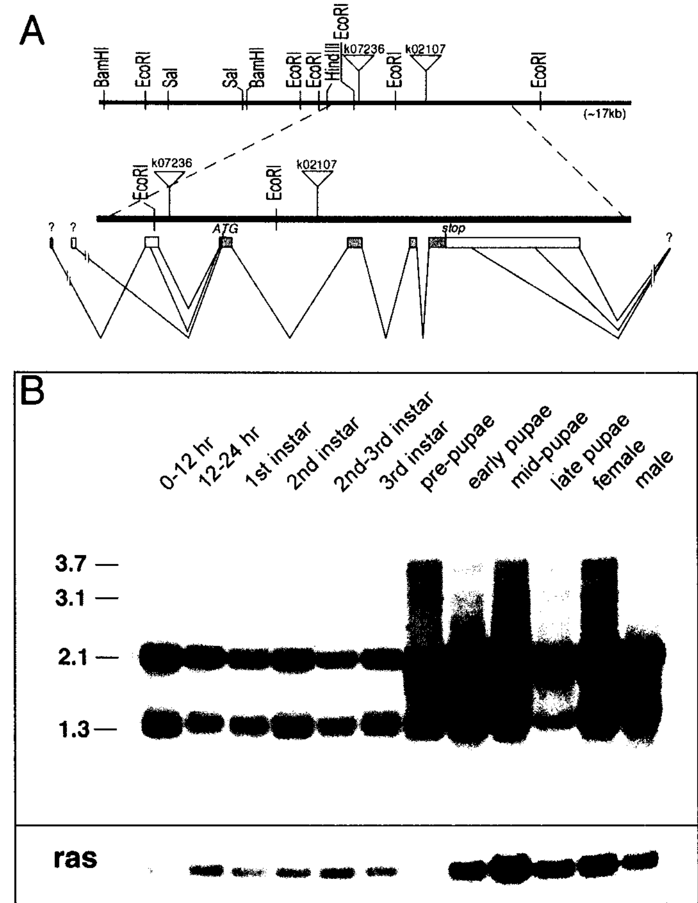

Figure 2.1. A molecular characterization of the *Rho1* locus. (A) Schematic diagram of the locus showing the restriction map of the region and a more detailed view of the area containing exons from the *Rho1* cDNAs identified to date, all of which contain the same ORF. The precise location of the 5' and 3' most non-coding exons is not known. Genomic and cDNA sequences are available (see Methods). (B) Developmental Northern documenting the temporal pattern of *Rho1* expression. Poly-A^+ RNA from successive developmental stages was hybridized to a probe generated against a cDNA fragment containing the *Rho1* coding region. The sizes of the four transcript classes identified are indicated in kb. *DRas* was used as a loading control. The relative abundance of particular transcript classes varies with developmental stage, indicating temporal regulation of *Rho1* expression.

33 bp upstream of the ATG, whereas the *Rho*$^{l(2)k02107}$ P-element is inserted in an intron within the coding sequences (see Fig. 2.1A). These P-elements localize to cytological region 52E (Hariharan et al., 1995; Strutt et al., 1997).

Genomic libraries were screened using probes from the sequences flanking the P-element, and a restriction map of the genomic region was generated. This map was confirmed by sequencing roughly 17 kb of genomic DNA surrounding the *Rho1* locus and is shown in Fig. 1 (see Methods). The same fragment was then used to screen embryonic and ovarian cDNA libraries (see Methods), and cDNAs ranging in size from 1.2 to 2.1 kb were recovered. Sequence analysis of these cDNAs revealed that they encompass at least six different classes of transcripts, all of which share an identical ORF encoding a putative protein of 192 amino acids. The variation in sizes among the cDNAs results from alternative splicing at both the 5' and 3' untranslated ends of the message (Fig. 2.1A and data not shown).

Using a cDNA fragment encompassing the entire *Rho1* coding region as a probe on a developmental Northern identified four transcripts. Two major transcript classes, corresponding to 1.3 and 2.1 kb, are expressed at all stages of development, although relative levels of each vary between stages (Fig. 2.1B). In addition, there are two transcripts of 3.1 and 3.7 kb, which are first expressed at pupariation (day 6) and persist until adulthood in females (but are not detected in males). The sizes of the cDNAs identified suggest that each of the major transcript bands on the Northern represent multiple transcript forms. In the future it will be important to determine the dynamic temporal or spatial changes associated with each *Rho1* splice form and the relative contribution/developmental consequences associated with each form.

2.3.2. Disruption of zygotic Rho1 function is associated with defects in morphogenetic processes.

Rho1 is essential for zygotic function as progeny that are homozygous for either the *Rho*$^{l(2)k07236}$ or *Rho*$^{l(2)k02107}$ mutations die as embryos. *Rho*$^{l(2)k07236}$ fails to complement

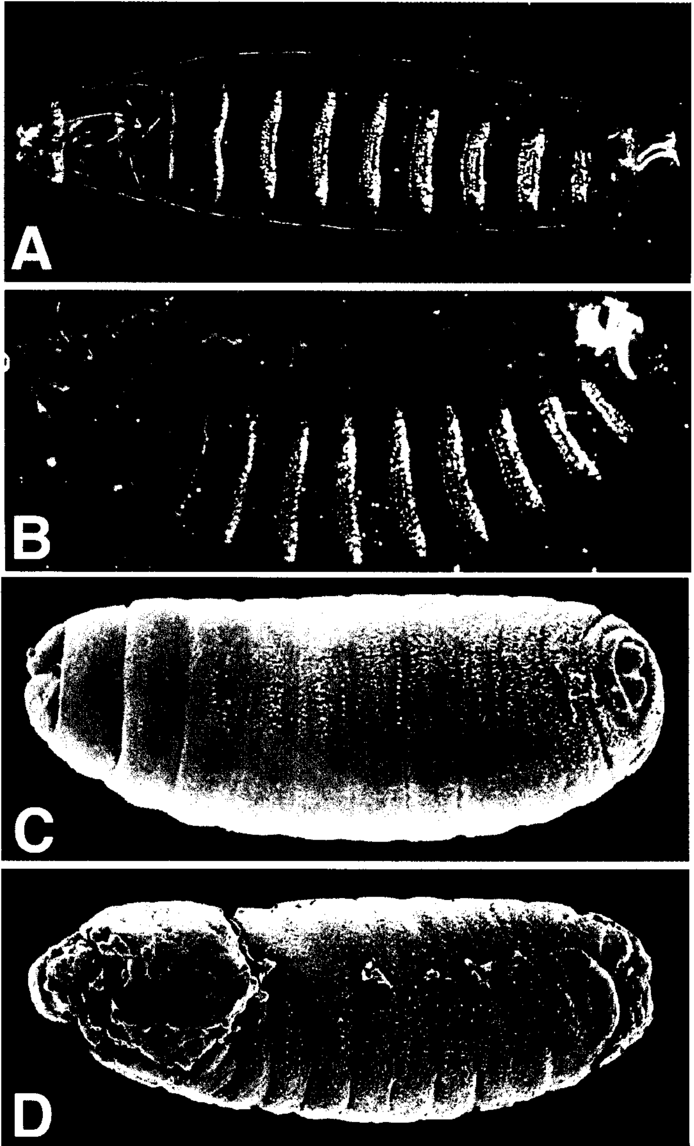

Figure 2.2. *Rho1* zygotic phenotypes. Cuticle preparations (A-B) and Scanning Electron micrographs (C-D) of a wildtype (A, C) and homozygous *Rho1* mutant embryo (B, D). The dorsal anterior hole in the larval cuticle (B) is a result of failed internalization of head structures on the *Rho1* embryo (D), resulting in the brain remaining on the anterior dorsal surface with the subsequent failure of this region to secrete cuticle. Anterior is left and dorsal is up in all images.

Rho$^{l(2)k02107}$, and their maternal and zygotic phenotypes are identical. Both *Rho*$^{l(2)k07236}$ and *Rho*$^{l(2)k02107}$ are strong alleles as imprecise excisions of the P-element do not differ greatly in their phenotypic severity. All subsequent analyses were done using an imprecise excision allele derived from the *Rho*$^{l(2)k07236}$ allele (*Rho*^{rev220}; from this point on referred to as *Rho1*; see Methods).

Heterozygous *Rho1* embryos are viable and have no embryonic cuticle defects, while homozygous *Rho1* embryos die with holes in the dorsal anterior region of the cuticle and a disruption of the dorsal surface that stretches the ventral surface and causes the cuticles to bow slightly (Fig. 2.2B). To better identify the processes leading to such disruptions, scanning electron microscopy (SEM) was used to visualize embryonic morphology throughout gastrulation. The most striking defects occur late in gastrulation when the embryos fail to undergo head involution, a process whereby the anterior structures of the embryo are internalized through dramatic cell shape changes and movements. The procephalon, which cannot secrete cuticle, remains on the exterior, leading to a characteristic dorsal anterior hole in the larval cuticle preparations (Fig. 2.2D). Additionally, the *Rho1* mutant embryos display a puckered dorsal midline suggesting improper dorsal closure.

During dorsal closure in wildtype embryos, actin and myosin localize along the leading edge of the dorsal lateral epidermis as they extend dorsally and have been proposed to act as part of the driving force of the cell shape changes occurring at this time (Edwards et al., 1997; Young et al., 1993). We examined dorsal closure in wildtype and *Rho1* mutants using phalloidin to visualize actin structures at successive developmental time points. Actin is enriched in the cortices of individual cells, allowing the cellular morphology during dorsal closure to be documented. As the two leading epithelial edges meet in wildtype embryos, they fuse from either end to form a straight, seamless dorsal midline (Fig. 2.3A,E,I,M). Embryos homozygous for the *Rho1* mutation undergo dorsal closure, in that the lateral epithelia do come together at the dorsal surface of the embryo (Fig. 2.3B,F,J,N), however, this process is disorganized compared to wildtype. At higher magnification, cells along the dorsal

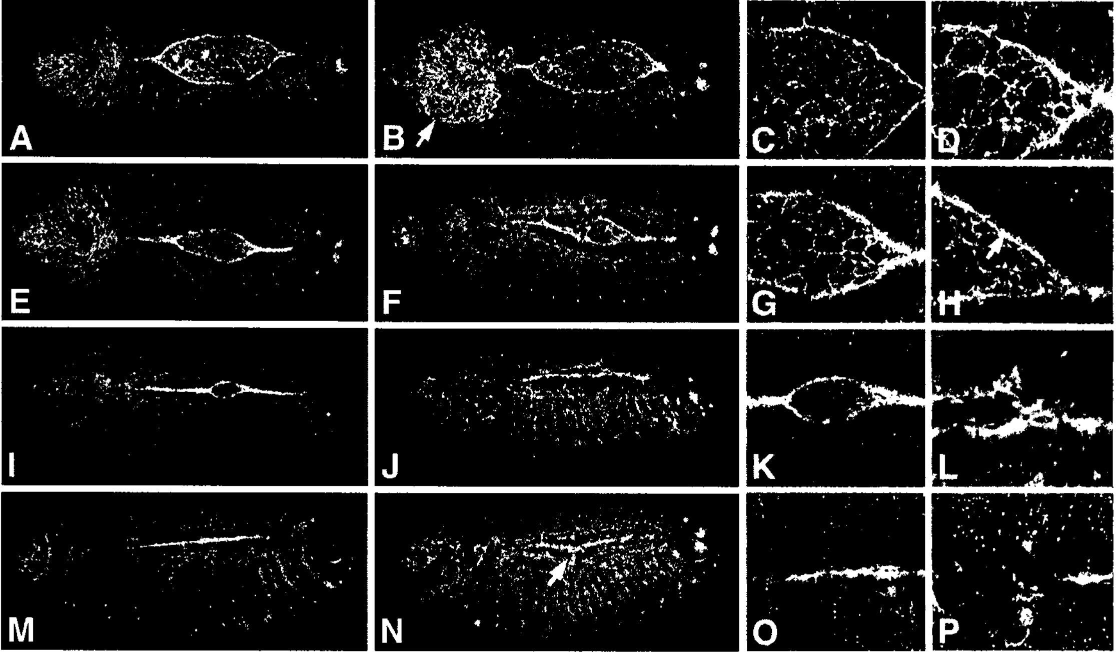

Figure 2.3. Developmental time course of dorsal closure and head involution in wildtype and *Rho1* zygotic mutant embryos. (A, E, I, M) Wildtype embryos undergoing dorsal closure. (B, F, J, N) Homozygous *Rho1* mutants at comparable time points. Even from the earliest time point shown, embryos homozygous for the *Rho1* mutation show increased actin staining in the head region (arrow in B) where the abnormally shaped cells do not undergo the coordinated movements of head involution (compare to A). (C, G, K, O) Higher magnification of the wildtype embryos undergoing dorsal closure. The morphology of the cells in the advancing dorsal epithelia is very well organized in these embryos. (D, H, L, P) *Rho1* mutants at higher magnification. Cells in the advancing dorsal epithelia of these embryos have abnormal morphologies (arrow in H) compared to wildtype (G), suggesting a misregulation of cytoskeletal components necessary for the proper regulation of cell shape. *Rho1* mutant embryos do complete dorsal closure, however actin organization at the dorsal midline is disrupted (arrow in N, P) compared with wildtype (M, O). Anterior is left, and all images are taken from a dorsal perspective.

midline in wildtype embryos are well ordered and columnar in shape (Figs. 2.3C,G,K,O and 2.4A). In *Rho1* mutant embryos, cells along the dorsal midline are inappropriately shaped, pinched together in some regions and stretched out in others (Figs. 2.3D,H,L,P, and 2.4B). This is especially clear in the later stages of dorsal closure, after the epithelia have come together (Figs. 2.3N,P and 2.4B). We do not observe any disruptions to the microtubule network as visualized by ß-tubulin staining (Fig. 2.3C,D), suggesting that *Rho1* primarily affects actin cytoskeletal components.

Harden et al. (1999) have reported disruptions to the leading edge cytoskeletal components using dominant negative and constitutively active transgenes of UAS-DRhoA or UAS-Dcdc42 expressed with Hs-GAL4. They observed segmentally reiterated splaying of cells and loss of myosin staining and phosphotyrosine nodes in leading edge cells flanking the segment border. While we observe aberrant cell shapes and inappropriate constriction of cells along the leading edge, we do not observe the segmental reiteration of these losses in the *Rho1* loss-of-function mutation (Fig. 2.4). In addition, nodes of phosphotyrosine expression are still visible in splayed out cells (Fig. 2.4F).

To look further at cell shape along the leading edge, we looked at the expression of fasciclin III (fas III). While fas III is present in the leading edge cells of wildtype embryos, it is not present in the dorsalmost edge of these cells (Fig. 2.4G,I) until they are touching the cells of the opposing leading edge epithelia (Fig. 2.4I). In *Rho1* mutant embryos, fas III accumulates in the dorsalmost edge of the leading edge cells prematurely (Fig. 2.4H). When cells along the leading edge in *Rho1* mutations constrict inappropriately, they appear to recognize their lateral neighbors as those on the opposing leading epithelial edge and elicit downstream events prematurely. Consistent with this possibility, we sometimes observe the appearance of secondary "midlines" perpendicular to the major midline (Fig. 2.4F).

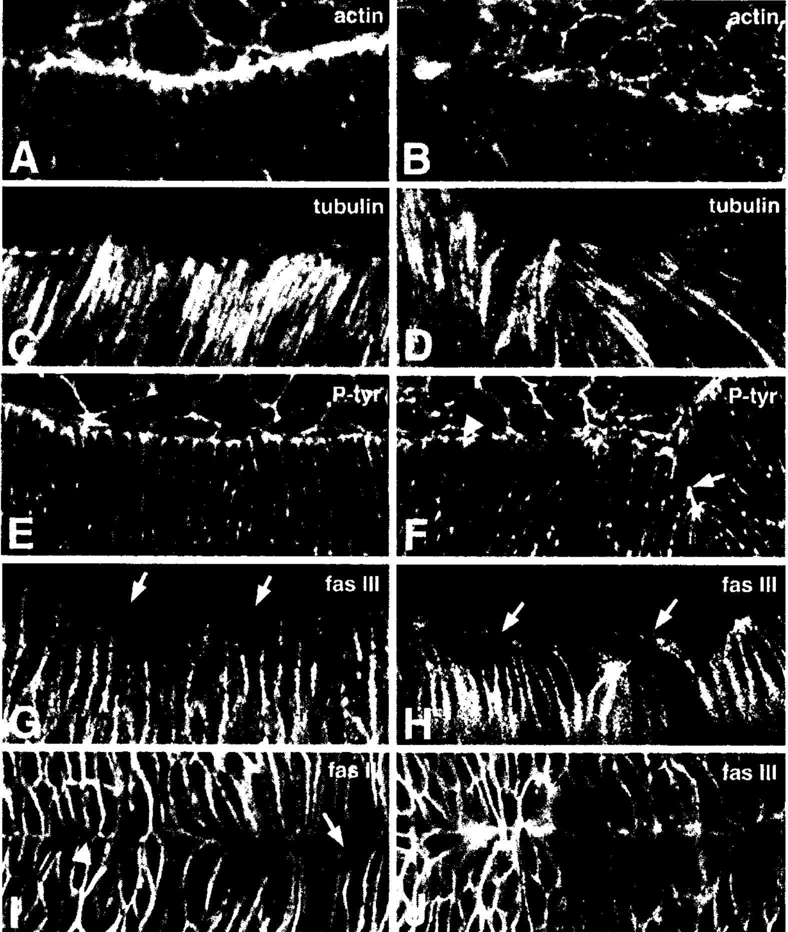

Figure 2.4. Localization of leading edge components in wildtype and *Rho1* zygotic mutant embryos. (A, C, E, G, I) Wildtype embryos undergoing dorsal closure. (B, D, F, H, J) Homozygous *Rho1* mutants at comparable time points. Confocal fluorescent micrographs of the boundary between the leading epithelial edge (bottom) and amnioserosa (top) from embryos stained with phalloidin to visualize F-actin (A, B), anti-ß-tubulin antibodies (C, D), anti-phosphotyrosine antibodies (E, F), and anti-fasciclin III antibodies (G-J). Despite the irregular cell shapes in the leading edge epithelial cells and misregulation of actin cytoskeletal components (B), ß-tubulin expression is largely normal in *Rho1* mutants (D). Nodes of phosphotyrosine staining present in wildtype (E) are also present in the leading edge cells of *Rho1* mutants, even in cells that are stretched out along their dorsalmost surface (arrowhead in F). Inappropriate cell constriction brings the dorsalmost surface of cells from the same leading edge together leading to the appearance of secondary midlines (arrow in F). The dorsalmost side of leading edge cells do not express fas III (arrows in G, J) until the two opposing epithelial sheets meet along the midline (arrowhead in J). In *Rho1* mutants, the leading edge cells prematurely express fas III along their dorsalmost edge (arrows in H).

2.3.3. Reduced maternal *Rho1* function disrupts actin cytoskeletal structure in ovaries.

Previous studies utilizing ectopic expression of dominant-negative and constitutively-active forms of *Drosophila* Rho family members have identified a role for these proteins in regulation of actin structure in the ovary (Murphy and Montell, 1996). The Rho1 mutation was initially identified in a genetic screen for maternal genes whose activity is essential for embryonic development (S. Dawson, M. Meyer, and S.M. Parkhurst, unpublished). In this screen, a change-of-function mutation in an RNA polymerase II subunit, *wimp*, was used to reduce, but not eliminate, Rho1 maternal contribution (cf. Parkhurst and Ish-Horowicz, 1991; Poortinga et al., 1998). It is not possible to completely eliminate maternal Rho1 function: germline clones of Rho1 cannot be generated due to inviability of the clonal cells (Strutt et al., 1997).

In order to examine whether reduction of maternal Rho1 function had any effect on the actin cytoskeleton in ovaries, we stained ovaries from mothers trans-heterozygous for Rho1 and wimp with phalloidin. In wildtype egg chambers, actin structures are well organized (Fig. 2.5A-D). This includes the ring canals, portals consisting of two tightly bundled concentric rings of actin resulting from incomplete cytokinesis that allow for the exchange of cytoplasmic material between nurse cells and the developing oocyte. Egg chambers from females with reduced Rho1 function show a general disruption of the actin cytoskeleton, particularly in the outer ring canals and oocyte cortex (Table 2.1; Fig. 2.5E-H). The inner ring canals appear relatively normal and oogenesis is able to proceed in these females leading to inviable embryos with patterning defects (see below).

2.3.4. Reduced maternal *Rho1* function also disrupts segmentation.

The maternal effect of the Rho1 mutation on embryos is distinct from its zygotic effects: embryos derived from mothers with reduced maternal Rho1 activity die with

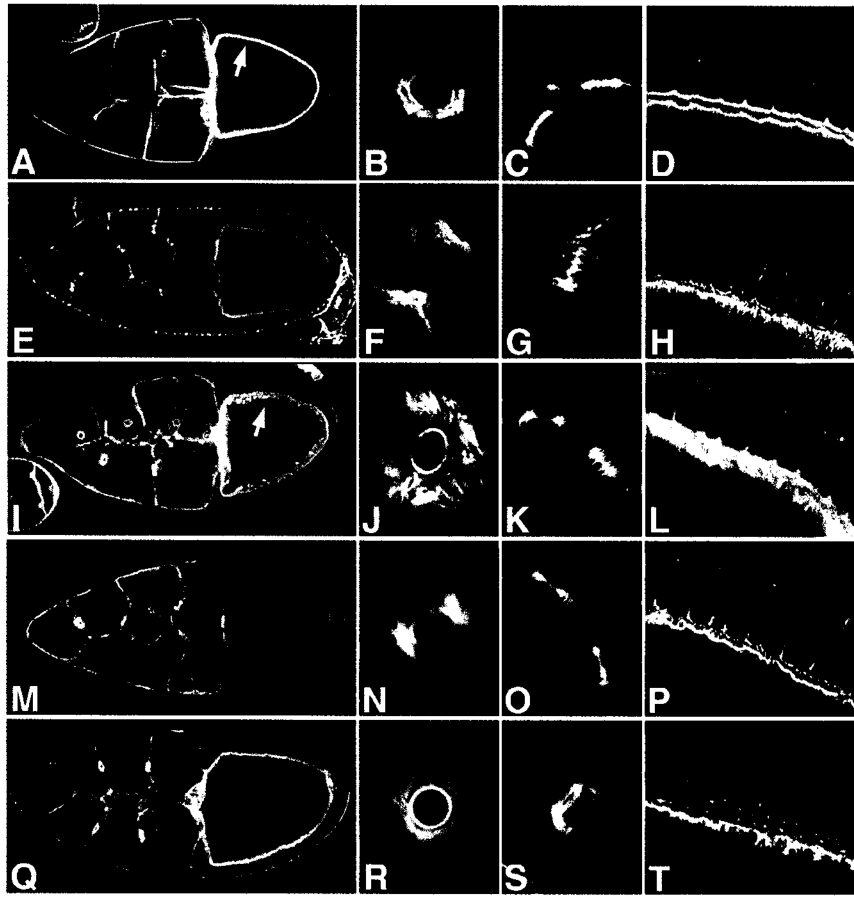

Figure 2.5. Ovarian actin phenotypes visualized with phalloidin. (A, E, I, M, Q) Stage 10a egg chambers of wildtype (A), *Rho1/+;+/wimp* (E), *capu Rho1/++* (I), *cta Rho1/+ +* (M), and *cta/cta* (Q). Anterior is left. Frontal (B, F, J, N, R) and edge (C, G, K, O, S) views of a ring canal or the oocyte-follicle cell boundary (D, H, L, P, T) in wildtype (B-D), *Rho1/+;+/wimp* (F-H), *capu Rho1/++* (J-L), *cta Rho1/+ +* (N-P), and *cta/cta* (R-T). Egg chambers derived from mothers with reduced *Rho1* maternal activity (E-H) or trans-heterozygous for either *Rho1* and *capu* mutations (I-L) or *Rho1* and *cta* mutations (M-P) display disorganized actin structure at the nurse cell boundaries, along the cortex of the developing oocyte (compare arrows in A and G), and especially in the outer ring canals, cellular bridges formed by incomplete cytokinesis of the germline cells. Egg chambers from homozygous cta^{RC10} mutations (Q-T) show similar, albeit weaker, disruptions to the actin cytoskeleton. In addition, aberrant accumulation or disruption of actin as viewed by punctate phalloidin staining within the follicle cells is visible in egg chambers from reduced maternal *Rho1* (H), *cta Rho1* trans-heterozygotes (P), and homozygous *cta* (T) mothers compared with wildtype (D) or *capu Rho1* trans-heterozygotes (L).

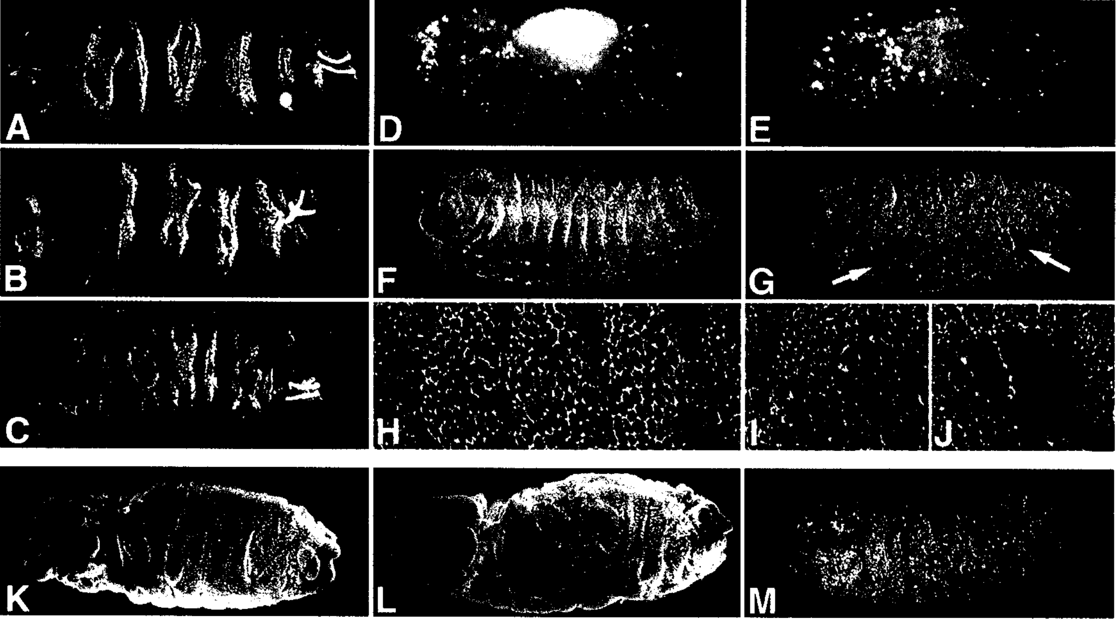

Figure 2.6. Phenotypes associated with reduced maternal *Rho1* activity. (A-C) Cuticle preparations demonstrating segmentation phenotypes associated with reduced maternal *Rho1* activity. Compare with wildtype cuticle in Figure 2A. Note the variation in phenotypic severity. Approximately 10% of embryos also show holes in their cuticles (C). Wildtype embryos (D, F, H) and embryos with reduced maternal *Rho1* activity (E, G, I, J) were stained with acridine orange (D, E) to visualize cell death or anti-phosphotyrosine (F-J) to outline cells. While there is no increase in cell death (compare D and E), embryos with reduced maternal *Rho1* activity exhibit defects in cell morphology (G, I, J). Wildtype embryos are mostly uniform in their cellularization (F, H), whereas patches of irregular cell shapes and aberrant cellularization are found in embryos with reduced maternal *Rho1* activity (arrows in G and close-ups in I-J). (K-M) Phenotypes associated with reduced maternal and zygotic *Rho1* activity. Scanning electron micrographs (K, L) and confocal fluorescent micrograph (M) of homozygous *Rho1* mutant embryos derived from mothers with reduced maternal *Rho1* activity. Embryo in (M) has been stained with anti-phosphotyrosine antibodies to outline cells. Both the maternal segmentation phenotype (compare to A, B above) and the zygotic morphogenetic phenotypes can be seen (compare to Fig. 2D). Maternal *Rho1* activity does not contribute to the *Rho1* zygotic phenotype: the zygotic phenotype is not more severe in this background. Anterior is left.

moderate segmentation defects as seen by the fusion of adjacent denticle bands in larval cuticles (Fig. 2.6A-C). In addition, approximately 10% of the dead embryos have cuticular holes (Fig. 2.6C). These cuticular holes are randomly placed and are not the same as holes on the dorsal surface resulting from failed dorsal closure. These holes could be the result of inappropriate cellularization or cell fate specification, cell death or other morphogenetic processes whereby cuticle is not properly secreted. To begin distinguishing among these possibilities, we stained embryos derived from mothers with reduced maternal Rho1 activity with acridine orange, a marker for cell death (Abrams et al., 1993). While the staining pattern is temporally and spatially dynamic, acridine orange staining is similar in Rho1 compared to wildtype embryos (Fig. 2.6D,E). We also stained embryos derived from mothers with reduced maternal Rho1 activity with antibodies to phosphotyrosine to outline the cell shapes (Fig. 2.6F-J). Patches of irregularly shaped cells can be seen throughout the embryo (Fig. 2.6G,I,J), suggesting that Rho1 is likely to be required for proper cellularization and additional morphogenetic processes.

2.3.5. Maternal *Rho1* activity does not contribute to the *Rho1* zygotic phenotype.

The *Rho1* loss-of-function mutation does not exhibit all the phenotypes expected if it is the primary target of genes such as DRhoGEF2: whereas DRhoGEF2 mutations and ectopic expression of dominant negative *Rho1* block gastrulation (Barrett et al., 1997; Hacker and Perrimon, 1998), we do not observe this phenotype with the *Rho1* loss-of-function zygotic mutation (Fig. 2.2). To determine if maternal *Rho1* activity was masking this phenotype, we looked at the *Rho1* zygotic phenotypes when maternal *Rho1* activity was reduced. Homozygous *Rho1* mutant embryos derived from mothers with reduced maternal *Rho1* activity exhibit both the maternal segmentation phenotype and the zygotic morphogenetic phenotypes (Fig. 2.6K-M).

New phenotypes are not uncovered and the zygotic *Rho1* phenotype is not enhanced in this background (compare to Fig. 2.2D).

2.3.6. Engrailed and Wingless expression is disrupted in maternal *Rho1* mutants.

The segmentation phenotype in embryos with reduced maternal *Rho1* activity indicates a maternal role for *Rho1* in patterning events that establish the embryonic body plan. To identify the developmental stage at which *Rho1* is necessary, we stained embryos derived from mothers with reduced maternal *Rho1* activity with a collection of antibodies recognizing segmentation gene products, including Bcd (maternal), Hb and Kr (gap), Eve and Ftz (pair rule), and En (segment polarity). All of the antibodies tested show that segmentation products are set up properly in *Rho1* maternal embryos (data not shown). However, while the En segment polarity protein is initially expressed properly (Fig. 2.7D), it fails to maintain its proper expression and shows severe aberration in pattern by stage 9 (Fig. 2.7E,F). Since Wingless (Wg) signaling is necessary for the maintenance of En expression (DiNardo et al., 1988), we next examined Wg expression in these embryos. Like En, Wg expression is initiated correctly (not shown), but fails to be maintained properly (Fig. 2.7H).

2.3.7. *Rho1* does not interact with members of the JNK pathway.

Because Rho GTPases have been linked to a number of pathways in cellular systems, we looked at *Rho1* in the background of other mutations to see whether we could genetically link *Rho1* to similar pathways in *Drosophila* (cf. (Noselli, 1998) and thereby correlate the numerous Rho pathways to specific roles in organismal development. We tested *Rho1* with putative interacting mutations (pim) using trans-heterozygous (*Rho1/+;*pim/+), dose sensitive (*Rho1/Rho1*;pim/+ or *Rho1*/+;pim/pim) and double mutant (*Rho1/Rho1*;pim/pim) combinations. No genetic interactions were detected with *hemipterous* and *basket* (members of the JNK signaling pathway),

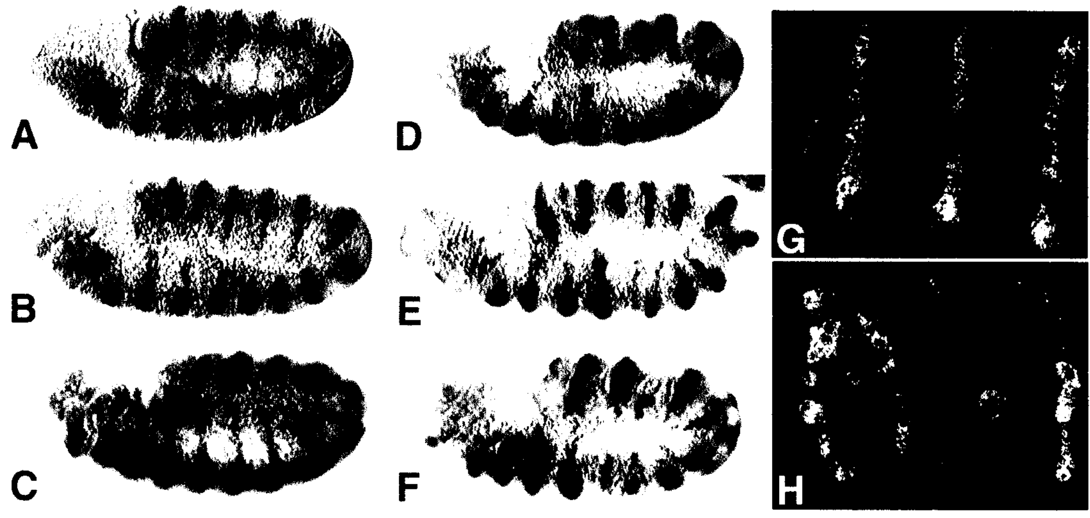

Figure 2.7. Disruption of Engrailed and Wingless expression in maternal *Rho1* mutants. (A-C) Wildtype embryos at progressively older stages stained with anti-*en* antibodies. En stripes are initiated and maintained in these embryos. (D-F) Embryos from mothers with reduced *Rho1* activity stained with anti-*en* antibodies. While En expression is initiated properly in these mutant embryos (D), some stripes are not apparent in late stage embryos, indicative of a failure in En maintenance. (G) Wildtype embryo stained with anti-*wg* antibody showing the normal pattern of Wg expression in stripes 2, 3, and 4 of late stage embryos. (H) A similarly staged embryo with reduced maternal *Rho1* activity demonstrating abnormal Wg expression in these same stripes, suggestive of a failure in Wg autocrine signaling. Anterior is left in all images.

chickadee (cytoskeletal protein), *puckered* (dorsal closure mutant), *wingless* and *armadillo* (Wingless signaling), *anterior open* (*ras* signaling), or *EGFR* (EGF receptor) (Baker, 1988; Cooley et al., 1992; Glise et al., 1995; Glise and Noselli, 1997; Lai and Rubin, 1992; Martin-Blanco et al., 1998; Peifer et al., 1991; Riesgo-Escovar et al., 1996; Rogge et al., 1995). We could not detect a genetic interaction with *DRhoGEF2* (Barrett et al., 1997), however, we only examined trans-heterozygous interaction since *DRhoGEF2* and *Rho1* map next to each other (<1 map unit) and we have not yet recovered a recombinant double mutant chromosome.

The lack of genetic interactions of *Rho1* with Wg and JNK signaling components during oogenesis and early embryonic development was unexpected in light of *Rho1*'s requirement for these factors during eye development (Strutt et al., 1997) and the classical dorsal closure defects described for dominant negative and constitutively active *Rho1* transgenes (cf. Harden et al., 1999). Activation of the JNK signaling pathway by the Rac and Cdc42 GTPases results in the induction of *dpp* and *puckered* (*puc*) expression in the leading edge cells (cf. Glise and Noselli, 1997; Hou et al., 1997). Since inability to detect genetic interactions does not rule out a role for *Rho1* in JNK signaling, we also examined *dpp* and *puc* expression in embryos homozygous mutant for *Rho1* (Fig. 2.8). Consistent with the lack of genetic interactions, *dpp* and *puc* expression in *Rho1* homozygous mutant embryos are indistinguishable from wildtype. These results suggest that *Rho1* mediates a pathway for epidermal cell shape changes that is independent of the previously reported Rac-mediated JNK cascade required for dorsal closure (Glise et al., 1995; Harden et al., 1995; Noselli, 1998; Riesgo-Escovar et al., 1996) and does not share all the properties attributed to it based on ectopic expression of its dominant negative and constitutively active versions (cf. Barrett et al., 1997; Harden et al., 1999; Lu and Settleman, 1999).

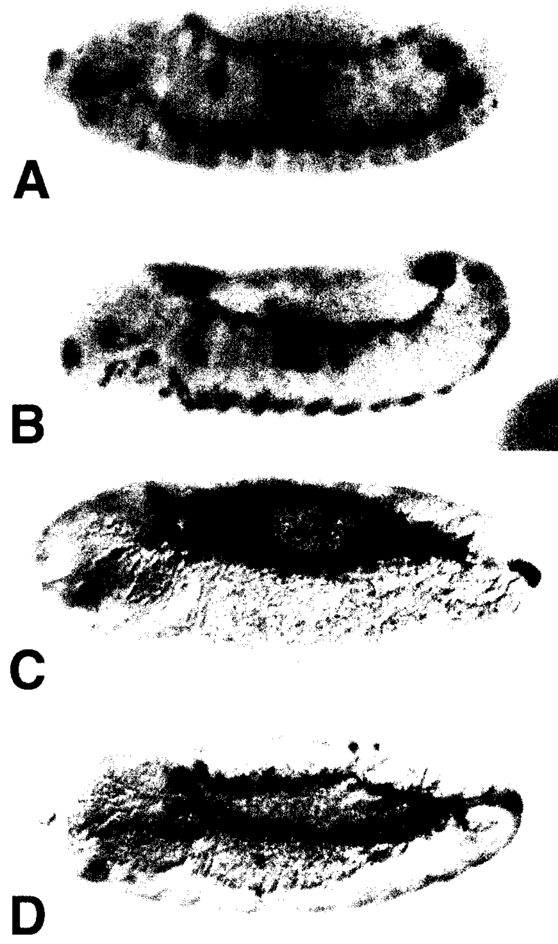

Figure 2.8. *Rho1* functions independently of the JNK pathway during dorsal closure. *dpp* and *puckered*, two genes activated by the JNK signaling pathway are expressed normally in *Rho1* mutations. (A-B) Whole mount in situ hybridizations with *dpp* in wildtype embryos (A) and homozygous *Rho1* mutant embryos (B). (C-D) X-gal staining of the *puckered-lacZ* enhancer trap line in wildtype embryos (E) and homozygous *Rho1* mutant embryos (F). Homozygous *Rho1* mutants exhibit normal levels of *dpp* mRNA and *puckered-lacZ* expression in the leading edge cells.

2.3.8. *Rho1* interacts genetically and physically with *concertina* and *cappuccino*.

We did identify genetic interactions of *Rho1* with two mutations, ca*ppuccino* (*capu*; formin homologue; Emmons et al., 1995) and *concertina* (*cta*; Gα protein; Parks and Wieschaus, 1991). Egg chambers from mothers heterozygous for *Rho1* (*Rho1/+*) exhibit normal actin morphology (Table 2.1). Egg chambers from mothers trans-heterozygous for *Rho1* and *capu* (*Rho1 capu/+ +*) or *cta* (*Rho1 cta/+ +*) exhibit disruptions of the ovarian actin cytoskeleton (Table 2.1; Fig. 2.5I-P), similar to that in females with reduced maternal *Rho1* activity (*Rho1/+; wimp/+*; Table 2.1; Fig. 2.5E-H). While *capu* has been shown to affect actin integrity during oogenesis, similar studies have not been reported for *cta*. We examined phallodin staining in egg chambers from homozygous *cta* mothers and find similar, albeit weaker, defects in the actin cytoskeleton (Fig. 2.5Q-T). The interaction of *Rho1* with *capu* is more severe than with *cta*: the subsequent embryos from the *Rho1/cta* interaction survive, whereas the embryos from *Rho1/capu* are inviable and exhibit severe patterning defects (not shown).

We also used an in vitro binding assay to examine the interaction specificity between the Rho1 and Cta or Capu proteins. Rho1 fused to glutathione S-transferase (GST-Rho1) was expressed in bacteria and immobilized on glutathione-Sepharose beads. Rho1 was then tested for its ability to bind ^{35}S-labeled full-length Cta or Capu proteins. Consistent with the observed genetic interactions, Rho1 specifically pulls down full length Cta or Capu (Fig. 2.9). Interestingly, Capu preferentially interacts with GTP bound Rho1, whereas Cta interacts equally with GTP or GDP bound Rho1.

Table 2.1. Ring canal morphology is disrupted in egg chambers with reduced *Rho1* maternal activity.

genotype	%wildtype	%mutant	#scored
wimp/+	67	33	359
Rho1/+	62	38	194
Rho1/+;*wimp*/+	39	61	615
Rho1 $capu^{RK12}$/+ +	25	75	203
Rho1 cta^{QB37}/+ +	33	67	166
Rho1 cta^{RC10}/+ +	26	74	197
Rho1 $chic^{1320}$/+ +	76	24	123
hep^{r75}/+;*Rho1*/+	81	19	155
arm^{XP33}/+;*Rho1*/+	76	24	168

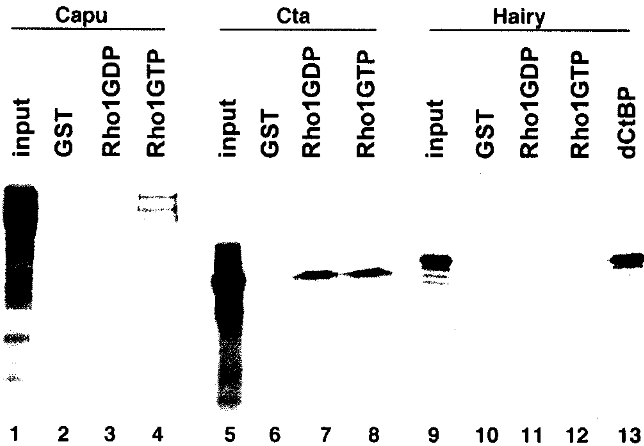

Figure 2.9. in vitro interaction of Rho1 with Capu and Cta. ^{35}S-labeled full-length Capu (25% input, lane 1) binds to GST-Rho1 preferentially when in its GTP bound state (lane 4), compared to its GDP bound state (lane 3) or to GST alone (lane 2). ^{35}S-labeled full-length Cta (25% input, lane 5) binds to GST-Rho1 regardless of its association with GTP (lane 8) or GDP (lane 7), but not to GST alone (lane 6) or to an unrelated protein, Hairy (not shown). Neither the GDP- nor the GTP- bound GST-Rho1 binds these proteins nonspecifically. An unrelated protein, Hairy (25% input, lane 9), does not bind to GST alone (lane 10) or to the GDP- (lane 11) or GTP- (lane 12) bound forms of GST-Rho1, but does bind to its interaction partner, dCtBP (lane 13).

2.4 DISCUSSION

Rho GTPases play a central role in diverse biological processes such as actin cytoskeleton organization, gene transcription, cell cycle progression, and adhesion. It is not yet clear however, if the same or different biochemical mechanisms are used by Rho family proteins to regulate these activities. Rho GTPases are required during early *Drosophila* development where morphogenetic movements of individual cells and groups of cells are important for the proper formation of the embryonic body plan. Our characterization of the *Rho1* maternal and zygotic phenotypes suggests that while *Rho1* is likewise mediating diverse biological pathways during embryonic development, the underlying mechanism used for each process may be similar.

2.4.1. *Rho1*'s zygotic phenotypes appear to result from direct effects on the cytoskeleton.

Dorsal closure involves the migration of the lateral epithelial sheets to cover the dorsal region of the embryo, a process requiring the proper localization of actin and myosin along the leading edge. Mutants that fail to undergo this process appropriately can not secrete cuticle on the dorsal surface and exhibit a large dorsal hole or "dorsal open" cuticular phenotype. Dorsal closure mutants can be divided into two main classes: those that affect cellular architecture such as the cytoskeleton (e.g., *capu*, *zipper*) or cell junctions (e.g., *coracle*, *canoe*), and those that are involved in signaling pathways (e.g., *basket* and *hemipterous*), which encode components of the MAPK-like JNK pathway (reviewed in: Noselli, 1998).

Studies of dominant negative and constitutively active Rho1, dRac and dCdc42 suggest they play roles in dorsal closure: the resulting embryos display large dorsal holes and puckering of the dorsal surface (Barrett et al., 1997; Harden et al., 1995; Harden et al., 1999; Hariharan et al., 1995; Strutt et al., 1997). More recently, activated forms of dRac and dCdc42 have been linked genetically with components of the p38 MAPK and JNK signaling pathways during dorsal closure (Glise et al., 1995;

Glise and Noselli, 1997; Harden et al., 1996; Harden et al., 1995; Harden et al., 1999; Hou et al., 1997; Noselli, 1998; Riesgo-Escovar et al., 1996), suggesting a signal transduction mechanism leading to proper control of cell morphology.

The dorsal closure phenotype that the *Rho1* loss-of-function mutations display is distinct from that observed with ectopic expression of its dominant negative or constitutively active forms and from that of other dorsal closure mutants as well. While cells in *Rho1* loss-of-function mutations are inappropriately shaped and do not form a straight seamless dorsal midline, they complete dorsal closure. However, *Rho1* loss-of-function mutations do exhibit severe defects in head involution. Head involution is a complex and well-ordered rearrangement of cells that results in the internalization of anterior head structures. The anterior dorsal hole observed in *Rho1* homozygous mutant larval cuticle preparations is a consequence of failed head involution, rather than aberrant dorsal closure (see Fig. 2.2). In addition, we find that *Rho1* does not activate downstream genes (*dpp* or *puc*) or interact genetically with *basket* or *hemipterous,* suggesting that Rho is not utilizing the same JNK signaling pathways as its relatives dRac and dCdc42 to effect changes in cell morphology at the dorsal midline.

Work in fibroblasts suggests that Rho proteins act hierarchically (Cdc42 > Rac > Rho; Nobes et al., 1995). Cdc42 can induce Rac (filopodia are usually present with lamellipodia), and Rac, in turn, can induce Rho, albeit weakly. In neuronal cells, however, the existence of a linked pathway is less clear. In these cells, the consequences of activated Rac or Cdc42 expression appears to act opposite to that of Rho: Rac or Cdc42 expression results in formation of lamellipodia and filopodia along neurite extensions, whereas Rho expression induces neurite retraction and cell rounding (Hall, 1998; Kozma et al., 1997). Recent work on *Drosophila* PKN, and ectopically expressed dominant negative and constitutively active *Rho1* suggests these proteins are not likely functioning in a hierarchy and mediate a pathway that is independent of JNK signaling (Harden et al., 1999; Lu and Settleman, 1999). Our results are consistent with *Rho1* acting in a different fashion than that reported for

dRac and *dCdc42*: *Rho1* appears to have a direct effect on regulation of cytoskeletal components necessary for proper coordination of cell shape changes, rather than as part of a signaling pathway, during early *Drosophila* development.

2.4.2. Phenotypes resulting from reduced maternal *Rho1* activity may result from direct effects on cytoskeletal components.

Ectopic expression studies using dCdc42(dn) and dRhoL(dn) indicate that these *Rho1* relatives affect actin structure in the ovary: the expression of these proteins results in nurse cell collapse and subcortical actin breakdown (Murphy and Montell, 1996). Reducing *Rho1* function maternally also affects the integrity of the actin cytoskeleton. Egg chambers derived from females with reduced *Rho1* activity exhibit generally disorganized actin cytostructure, as well as disruptions to more specialized cytoskeletal structures such as the ring canal morphology. While reduced *Rho1* maternal activity affects ring canal morphology, especially that of the outer ring canal, it does not affect their generation or growth. Some of the molecules involved in ring canal morphogenesis have been identified (Cooley and Theurkauf, 1994; Robinson and Cooley, 1997). Phosphorylation events seem to play an important role, and recent studies have implicated two tyrosine kinases, *Src64* and *Tec29*, in normal ring canal development (Cooley, 1998; Dodson et al., 1998; Roulier et al., 1998). Mutations in these genes show defects in ring canal growth that we do not observe in *Rho1* mutants. *Hu-li tai shao (Hts)* encodes a protein localized to ring canals following the increase in phosphotyrosine levels that may direct the addition of actin filaments to the developing ring canal. *Kelch* encodes another protein localized to ring canals after Hts, which may bundle actin filaments (Robinson et al., 1994). Most of these mutations lead to a “dumpless” phenotype: ring canals are blocked by nuclei not properly held in place by the disordered actin filaments, thus preventing the transport of cytoplasm to the oocyte from the adjoining nurse cells. We do not observe a

dumpless phenotype in egg chambers from mothers with reduced *Rho1* activity. Despite the actin disorganization observed, oogenesis proceeds in these ovaries.

Consistent with Rho interacting directly with cytoskeletal proteins, we observe a strong genetic interaction between *Rho1* and *capu*, suggesting that *capu* is a downstream effector of *Rho1*'s organization of the actin cytoskeleton of the ovary. *capu* encodes a member of the formin homology (FH) class of proteins, which includes *diaphanous* in *Drosophila, cyk-1* in *C. elegans, BNI1* and *BNR1* in budding yeast, *cdc12* in fission yeast, and the founding member of the family, the *limb deformity* locus in mice (Castrillon and Wasserman, 1994; Emmons et al., 1995; Mass et al., 1990; Petersen et al., 1998; Swan et al., 1998; Watanabe et al., 1997). These genes have been implicated in processes involving cytoskeletal regulation such as cytokinesis and the establishment of cell polarity. The yeast formin, BNI1p, has been shown to interact with a yeast Rho gene, Rho1p (Imamura et al., 1997; Watanabe et al., 1997). Similarly, we find that Rho1 and Capu interact physically in an *in vitro* binding assay.

Embryos obtained from mothers with reduced *Rho1* activity are able to proceed through embryogenesis, however, they do not hatch and show severe patterning defects, suggestive of effects on transcriptional regulation. We have shown that the early steps of the transcriptional cascade controlling segmentation are normal in embryos with reduced *Rho1* maternal activity. The observed segmentation defects are first manifested by the failure to maintain Engrailed (En) expression. En maintenance has been shown to be dependent on the presence of the Wingless (Wg) protein (reviewed in: Martinez-Arias, 1993). Wg is also required for its own maintenance (DiNardo et al., 1988). Wg is made in a cell adjacent to the cell expressing En. It is then secreted and taken up by the En-expressing cell (paracrine signaling) and by the secreting cell itself (autocrine signaling). The segmentation phenotype associated with reduced maternal *Rho1* activity could result from different biochemical mechanisms. The segmentation phenotype could be due to direct effects of the actin cytoskeleton: secretion or endocytosis could be disrupted whereby the Wg- and/or En-expressing

cell fail to take-up Wg protein. Consistent with this possibility, a role for Rho in receptor-mediated endocytosis has been described (Lamaze et al., 1996). Alternatively, *Rho1* could be a component of the Wg signaling pathway that affects transcription of segmentation genes in the En-expressing cell. While further experiments will be required to distinguish between these possibilities, this segmentation phenotype either provides an *in vivo* system to look at *Rho1*'s effects on transcriptional regulation or complements *Rho1*'s other phenotypes providing an additional process in which to investigate Rho's effects on cytoskeletal components.

2.4.3. Rho GTPase regulatory pathways.

The numerous seemingly distinct biological responses of the Rho GTPase suggest that its activation must be both temporally and spatially regulated. Part of this regulation is likely to come from interaction of Rho with different GEFs. The mechanisms that lead to activation of Rho family proteins by extracellular signals are thought to be similar to that of Ras: mediated by GEFs linked to heterotrimeric G protein coupled membrane receptors (reviewed in: Machesky and Hall, 1996; Van Aelst and D'Souza-Schorey, 1997; Zohn et al., 1998). A large family of RhoGEFs have been identified in mammalian systems, some of which are specific for a particular family member (e.g., Lbc for Rho, Tiam1 for Rac), while other GEFs act on all members (Michiels et al., 1995; Olson et al., 1996; Zheng et al., 1995). Three RhoGEFs have been identified in *Drosophila*, but little is known about their specificity. No mutations corresponding to DRhoGEF1 have been reported (Werner and Manseau, 1997). DRhoGEF2 (*shar pei*) has been shown to affect many of the morphogenetic movements associated with gastrulation and suppress genetic phenotypes associated with overexpression of wildtype or constitutively active *Rho1* (Barrett et al., 1997; Hacker and Perrimon, 1998). However, while both *Rho1* and *DRhoGEF2* loss-of-function mutations affect gastrulation, their phenotypes are very different and we do not detect trans-heterozygous genetic interactions between *DRhoGEF2* and loss-of-function *Rho1* mutations. The third *Drosophila* RhoGEF,

Pebble, does interact genetically with *Rho1* loss-of-function mutations (Prokopenko et al., 1999). Since the identified RhoGEFs do not have completely overlapping phenotypes with *Rho1* loss-of function mutations, it is likely that additional RhoGEFs exist. Similarly, since phenotypes associated with loss-of-function mutations in dRac, dCdc42 and dRhoL have not yet been reported, the specificity of the existing RhoGEFs is not yet known.

Work in fibroblasts suggests a role for subunits of the heterotrimeric Gα proteins ($G\alpha_{12}$ and $G\alpha_{13}$) in Rho-mediated signaling. While the exact link between the G proteins and Rho family proteins has not been described, a physical interaction between specific RhoGEFs and Gα proteins was recently reported (Hart et al., 1998). GEFs are thought to act immediately upstream of Rho family proteins. *concertina* is a Gα-like G protein shown to be important in transducing signals necessary to appropriately organize cell shape changes during *Drosophila* gastrulation (Morize et al., 1998; Parks and Wieschaus, 1991). Ectopic expression studies utilizing dominant-negative *Rho1* led to its implication in the cell shape changes leading to proper ventral furrow formation, consistent with studies showing disruption of *Drosophila* cellularization after microinjection of the botulinum C3 exoenzyme Rho-specific inhibitor (Barrett et al., 1997; Crawford et al., 1998). While *Rho1* loss-of-function mutations do not show the same severe cellularization or gastrulation phenotypes of DRhoGEF2, *Rho1* does interact both genetically and physically with *cta*, suggesting that *Rho1* is likely to be a downstream effector of the Cta Gα protein in the ovary. Interestingly, Cta interacts equally with the GTP and GDP bound forms of Rho1 and may form a complex including GEFs.

Proper oogenesis and morphogenesis in *Drosophila* are dependent on *Rho1* activity. Because these are complicated developmental processes involving multiple cellular events, it is expected that a large number of genes are involved in regulating and executing them. To understand the biochemical mechanisms through which Rho family proteins regulate the organization of the actin cytoskeleton, gene transcription, and their other associated activities, identification of regulatory factors and cellular

targets is essential. *Drosophila* offers a genetically amenable system in which to systematically identify components of the Rho pathway required for the proper execution of these events. Future genetic screens with loss-of-function *Rho1* mutations should also help in identification of regulators and effectors, an important step in describing the pathways through which Rho acts in the organism.

2.5. APPENDIX.

2.5.1. Rho1 and Cappuccino interact *in vivo*.

We detected a physical interaction between Rho1 and Cappuccino *in vitro* through the use of a GST-pulldown assay, suggesting that the genetic interaction we observe is indicative of a direct association between these proteins *in vivo*. In order to determine whether this is the case, we used a monoclonal antibody to Rho1 to immunoprecipitate Rho1-containing complexes from ovary lysates (see Chapter 3 for a description and characterization of the Rho1 antibody). Consistent with the *in vitro* binding observed between Rho1 and Cappuccino, western analysis of proteins in these complexes indicates the presence of Cappuccino (Fig. 2.10).

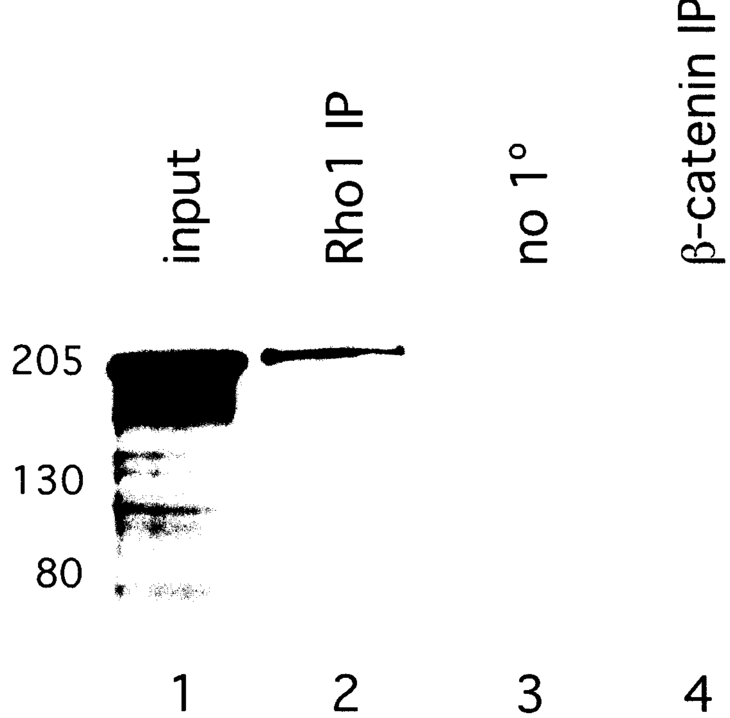

Figure 2.10. Capu co-immunoprecipitates with Rho1-containing complexes present in ovary lysates.
Antibodies against Rho1 and β-catenin were used to immunoprecipitate complexes from ovary lysates. The resulting proteins were separated by SDS-PAGE and analyzed via Western blotting for the presence of Capu. Capu protein was detected in the input (lane 1) as well as the Rho1 IP (lane 2), but not when 1° antibody was omitted from the IP reaction (lane 3) or with immunoprecipitation of β-catenin (lane 4).

CHAPTER 3

Rho1 interacts with p120^{ctn} and α-catenin, and regulates cadherin-based adherens junction components in *Drosophila*

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3.1. ABSTRACT

Rho GTPases are important regulators of cellular behavior through their effects on processes such as cytoskeletal organization. Here we show interactions between *Drosophila* Rho1 and the adherens junction components α-catenin and p120^{ctn}. We find that while Rho1 protein is present throughout the cell, it accumulates apically, particularly at sites of cadherin-based adherens junctions. Cadherin and catenin localization is disrupted in *Rho1* mutants, implicating Rho1 in their regulation. p120^{ctn} has recently been suggested to inhibit Rho activity through an unknown mechanism. We find that Rho1 accumulates in response to lowered p120^{ctn} activity. Significantly, we find that Rho1 binds directly to α-catenin and p120^{ctn} in vitro, and these interactions map to distinct surface-exposed regions of the protein not previously assigned functions. In addition, we find that both α-catenin and p120^{ctn} co-immunoprecipitate with Rho1-containing complexes from embryo lysates. Our observations suggest that α-catenin and p120^{ctn} are key players in a mechanism of recruiting Rho1 to its sites of action.

3.2. INTRODUCTION.

Members of the Rho family of small GTPases have been shown to be important regulators of cellular behavior, especially actin cytoskeletal organization and acto-myosin based contractility (Hall, 1998). Rho proteins function as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state. Their activation state is controlled by regulatory proteins such as guanine dissociation inhibitors (GDIs), which inhibit the release of GDP and keep Rho inactive, guanine exchange factors (GEFs), which catalyze the exchange of GDP for GTP and activate Rho, and GTPase activating proteins (GAPs), which increase the rate at which Rho hydrolyzes GTP and hence becomes inactivated (Maruta, 1998; Symons and Settleman, 2000). Rho GTPases have been implicated in many seemingly disparate cellular processes such as cell cycle progression, MAP kinase signaling, and cell-substrate and cell-cell adhesion (Braga, 1999a; Mackay and Hall, 1998; Narumiya, 1996; Van Aelst and D'Souza-Schorey, 1997). Understanding the biochemical mechanisms through which Rho is activated and carries out its various functions has been the focus of much recent work, and a number of downstream targets have been identified from studies in cell culture which suggest that Rho acts through multiple pathways (Bishop and Hall, 2000; Narumiya et al., 1998).

The subcellular localization of Rho proteins has been shown to play an important role in their function (Symons and Settleman, 2000). Rho is predominantly cytosolic but translocates to the membrane upon ligand stimulation, with different ligands leading to differential localization (Fleming, 1996). Membrane localization is dependent on a carboxy-terminal CAAX motif which allows the protein to be isoprenylated (Fleming, 1996; Kranenburg et al., 1997). Interfering with this translocation event affects the ability of Rho to carry out a subset of its functions. Prenylation of mammalian RhoB is required for its ability to transform NIH3T3 cells (Lebowitz et al., 1997), while the RhoA CAAX domain is needed for cytoskeletal contraction in response to ligand stimulation in N1E-115 neuroblastoma cells (Kranenburg et al., 1997). However, RhoB's ability to activate transcription from the

c-fos serum response element and RhoA's stress fiber induction is not compromised in these systems, indicating that membrane localization is not necessary for all Rho functions.

In epithelial and endothelial cells, Rho is enriched at sites of actin accumulation and membrane ruffling, where it co-localizes with the ezrin/radixin/moesin (ERM) family of proteins (Takaishi et al., 1995). ERM family proteins are involved in mediating association of the plasma membrane and the actin cytoskeleton. The colocalization of Rho with ERM proteins suggests a role for both in the regulation of adhesive complexes. Consistent with this, the formation of cadherin-based adherens junctions (AJs) has been shown to be regulated by Rho and Rac in mammalian cell culture (Braga, 1997).

In addition to the cadherins themselves, AJs contain a number of associated proteins including members of the catenin family: β-catenin (Armadillo), α-catenin, and p120 catenin ($p120^{ctn}$) (Hatzfeld, 1999; Steinberg and McNutt, 1999). While β-catenin and α-catenin are involved in linking cadherins to the actin cytoskeleton, $p120^{ctn}$ appears to play more of a regulatory role and has been proposed to both positively and negatively regulate adhesion, although the precise mechanisms involved are not yet known (Anastasiadis and Reynolds, 2000; Noren et al., 2000). In addition to their roles at adherens junctions, β-catenin and $p120^{ctn}$ function in the nucleus in conjunction with transcription factors to regulate gene expression (Behrens et al., 1996; Daniel and Reynolds, 1999). Recently $p120^{ctn}$ has been suggested to negatively regulate the activity of Rho when present in the cytoplasm by acting as a GDI and preventing the exchange of GDP for GTP, although $p120^{ctn}$ has no sequence homology to other GDIs (Anastasiadis et al., 2000). $p120^{ctn}$ could also be involved in activating Rho by recruiting it to sites of cadherin localization, where it can become accessible to GEFs and its downstream effectors.

Cadherin-based AJs are responsible for many of the cell-cell contacts found in the *Drosophila* embryo (Tepass et al., 1996; Uemura et al., 1996). We have previously characterized a mutation in the *Drosophila* RhoA homolog, *Rho1* (Magie et al., 1999).

This mutation results in a number of maternal and zygotic defects in morphogenetic processes consistent with a role for Rho1 in regulating cytoskeletal dynamics and cell shape changes, as well as in patterning events involving transcriptional activation. Here we show that Rho1 protein accumulates at AJs in the *Drosophila* embryo and ovary and that cadherin and catenin localization is aberrant in *Rho1* mutants. We find that Rho1 interacts physically with $p120^{ctn}$ and α-catenin, components of adherens junction complexes. These data suggest a role for the catenins in recruiting Rho1 to the plasma membrane, and a subsequent role for Rho1 in the regulation of proper AJ formation.

3.3. RESULTS.

3.3.1. Characterization of monoclonal antibodies to *Drosophila* Rho1.

A hybridoma fusion was conducted to recover monoclonal antibodies from mice immunized with a full length *Drosophila* Rho1-glutathione S transferase (GST) fusion protein. Western analysis of GST fusion proteins of the *Drosophila* Rho family members Rho1, RhoL, Rac1, and Cdc42 identified one line that recognized Rho1 with high specificity (P1D9; Fig. 3.1A). This line, P1D9, recognizes an epitope within the C-terminal 55 amino acids of Rho1, which is the region most dissimilar with other Rho family members (data not shown). Western blots prepared with whole cell and nuclear embryo extracts indicate that P1D9 recognizes one major band of the predicted size, 21 kDa, in whole cell, but not nuclear, extracts (Fig. 3.1B). We intermittently observe a 130kDa band in the nuclear extract, the identity of which is not known.

To examine the specificity of P1D9 for Rho1 in vivo we examined P1D9 staining in embryos lacking dRho1. Embryos homozygous for a deficiency that includes the Rho1 locus exhibit no staining above background, compared with sibling controls (Fig. 3.1C, D). We also overexpressed Rho1, Rac1, and Cdc42 in embryos using the

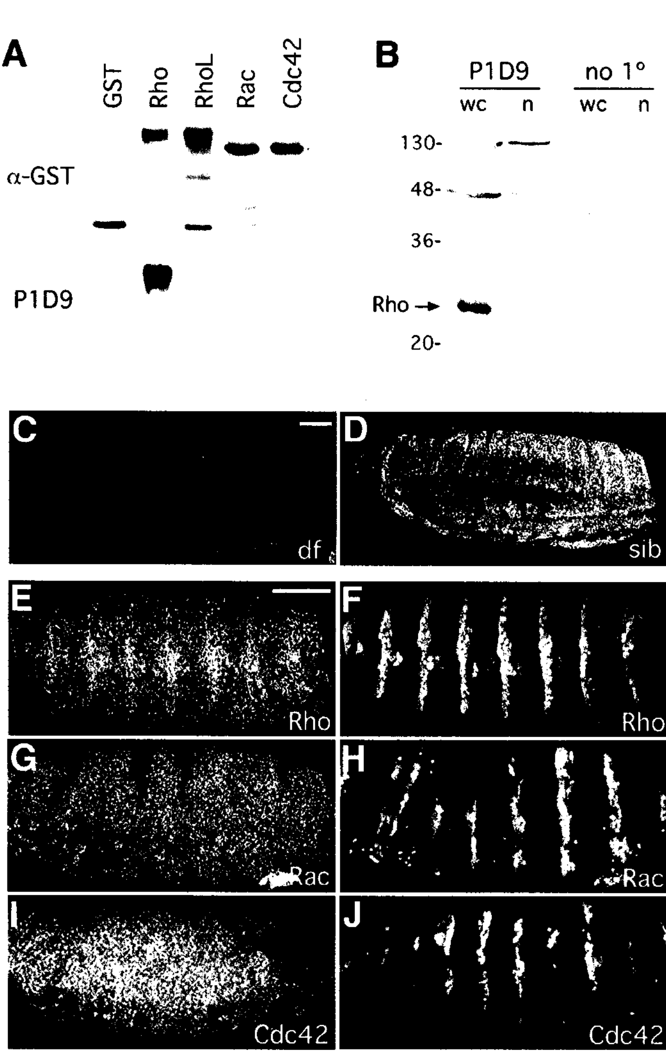


Figure 3.1. P1D9 monoclonal antibody is specific for Rho1 protein *in vitro* and *in vivo.*

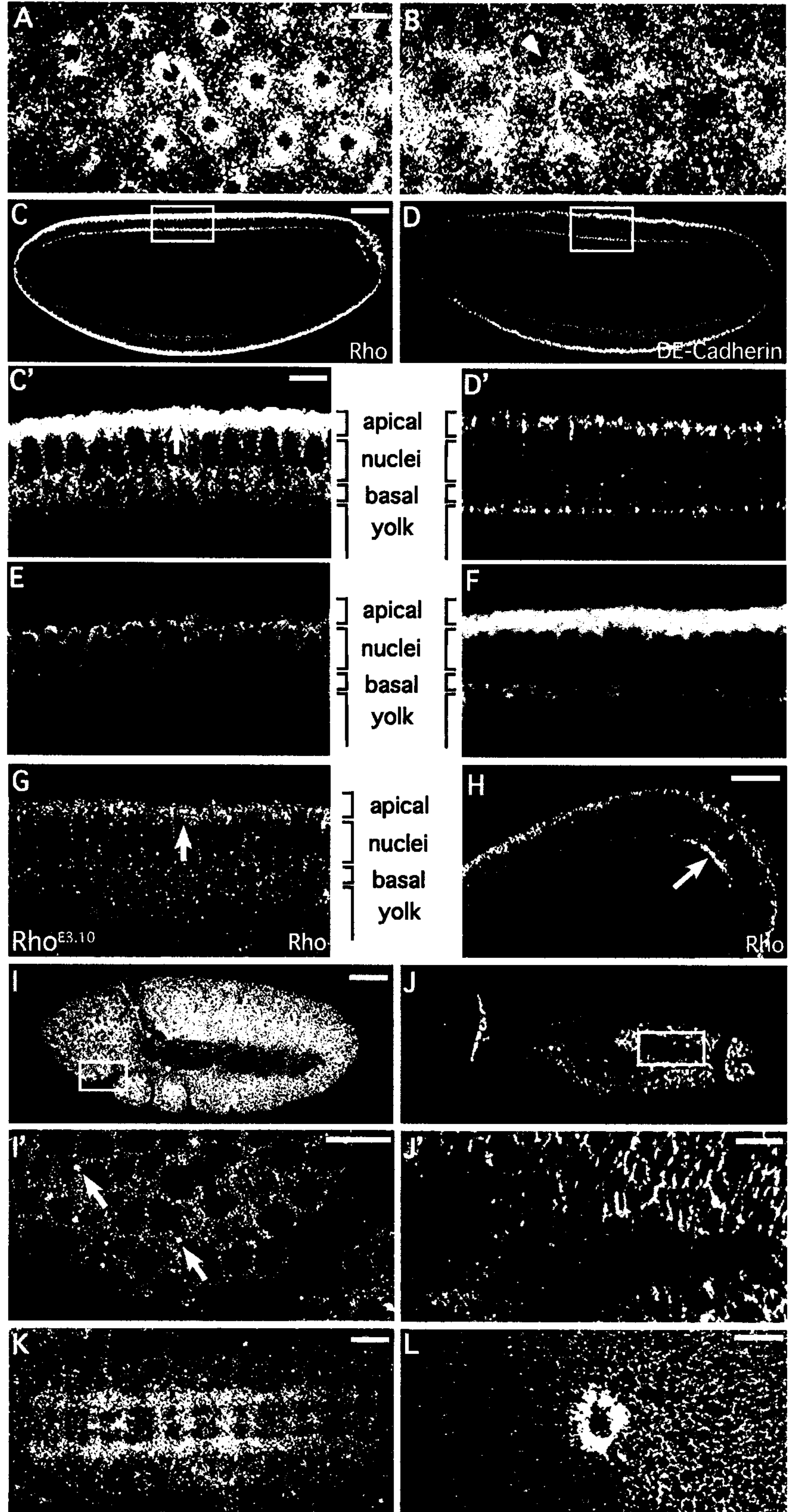
(A) Western analysis of *Drosophila* Rho family GST fusion proteins hybridized with α-GST (top) and P1D9 monoclonal (bottom) antiserum. (B) Western analysis of whole cell (wc) and nuclear (n) lysates prepared from 0-2hr and 0-12hr embryos, respectively. P1D9 recognizes a single major band in whole cell but not nuclear lysates. This band is not detected when 1° antibody is omitted. (C-D) P1D9 staining of a stage 14 embryo homozygous for a deficiency that includes the *Rho1* locus (C) relative to a sibling control (D). The relative intensity of the staining in these embryos can be directly compared, as both embryos were photographed in the same visual field. (E-J) P1D9 recognizes ectopically expressed Rho1 (E), but not Rac1 (G) or Cdc42 (I). Each protein was overexpressed in the Engrailed domain, as highlighted by green fluorescent protein expression (F,H,J), and embryos were examined at stage 14. For all embryos, anterior is left and dorsal is up. Scale bars: 50 μm.

conditional Gal4-UAS system. An Engrailed-Gal4 driver was used to express the Rho proteins in stripes, along with green fluorescent protein to visualize the overexpression domains. Stripes of ectopic protein accumulation could be seen above the uniform endogenous Rho1 levels in embryos expressing ectopic Rho1 (Fig. 3.1E), but not in embryos expressing ectopic Rac1 (Fig. 3.1G) or Cdc42 (Fig. 3.1I), confirming the in vivo specificity of P1D9.

3.3.2. Rho1 protein is expressed ubiquitously during development.

Previous studies in cell culture have suggested that the subcellular localization of Rho can have important effects on some of its functions, particularly those involving cell-cell adhesion. We used the P1D9 monoclonal antibody to examine the localization of Rho1 during *Drosophila* embryogenesis. During the syncytial blastoderm stages, Rho1 subcellular localization changes in a cell-cycle dependent manner. During interphase, Rho1 surrounds nuclei asymmetrically (Fig. 3.2A), while in metaphase Rho1 accumulates at the transient furrows that form around each nucleus (Fig. 3.2B). At the cellular blastoderm stage Rho1 protein is present throughout the cytoplasm of all cells, but concentrated apically (Fig 3.2C, arrow in Fig. 3.2C'). This pattern of apical localization is similar to that of the cell-cell adhesion molecule DE-cadherin, which is concentrated at AJs (Fig. 3.2D, D') (Oda et al., 1994). Rho1 is excluded from nuclei (Fig. 3.2E) and localizes to the cytoplasm, as indicated by the overlap in staining with the cytoplasmic protein Lava Lamp (Fig. 3.2F) (Sisson et al., 2000). High levels of protein are also seen at the basal surface of cells underlying the pole cells in the posterior of the embryo (Fig. 3.2H). At later stages Rho1 protein is also located throughout the cytoplasm of cells, but in addition to showing a diffuse, uniform pattern, it is concentrated in occasional punctate spots (Fig. 3.2I-J'). Rho1 protein is enriched in the neural commissures of the central nervous system (Fig. 3.2K), and also accumulates at wound sites, such as those resulting from the microinjection of early embryos (Fig. 3.2L), implicating Rho in the wound healing response.

Figure 3.2. Rho1 protein is ubiquitously expressed, but concentrated apically, in blastoderm embryos.
(A-B) Confocal micrograph showing Rho1 localization at interphase (A) and metaphase (B) in an early syncytial blastoderm stage. Note that the accumulation of Rho protein (arrows) relative to nuclei (arrowheads) changes with cell cycle phase. (C-D') Confocal micrograph showing P1D9 (C,C') and DE-cadherin (D,D') staining of embryos at the cellular blastoderm stage. Note apical accumulation of Rho1 protein localization at this stage (arrow in C'). (E-F) Rho protein is localized cytoplasmically. (E) Double staining with P1D9 (green) and propidium iodide (red) to label nuclei. (F) Double staining with P1D9 (green) and an antibody against Lava Lamp (red), a protein present in the cytoplasm. (G) P1D9 staining of $Rho1^{E3.10}$ homozygous embryos at the cellular blastoderm stage. Note the lack of apical Rho1 accumulation (arrow, compare with C'). (H) Close-up of an embryo at the cellular blastoderm stage showing basal accumulation of Rho1 protein in cells underlying the pole cells (arrow). (I-J') P1D9 staining in stage 11 (I,I') and stage 14 (J,J') embryos. (I',J') Higher magnification views of the portion of the embryos boxed in I and J, respectively. Note subcellular punctate spots of Rho1 accumulation (arrows in I'), and that Rho1 protein does not accumulate in cells at the leading edge during dorsal closure (J'). (K) Neural commissures of a stage 14 embryo stained with P1D9. (L) Grazing section of a cellular blastoderm stage embryo showing accumulation of Rho1 protein at a puncture wound site. In all images except (L), anterior is left and dorsal is up. Scale bars: A, C', I': 10 μm; C, I: 50 μm; H, J', K, L: 25 μm.



A
B
C
Rho
D
DE-Cadherin
C'
D'
apical
nuclei
basal
yolk
E
F
apical
nuclei
basal
yolk
G
Rho$^{E3.10}$
Rho
apical
nuclei
basal
yolk
H
Rho
I
J
I'
J'
K
L

We observe aberrant Rho1 localization in *Rho1* mutants, particularly in embryos homozygous for $Rho^{E3.10}$, a point mutation that disrupts the C-terminal isoprenylation site involved in tethering Rho to the plasma membrane (Fig. 3.2G). In these mutant embryos cytoplasmic staining is present, however apical accumulation of Rho protein is no longer detectable (compare Fig. 3.2G to 3.2C').

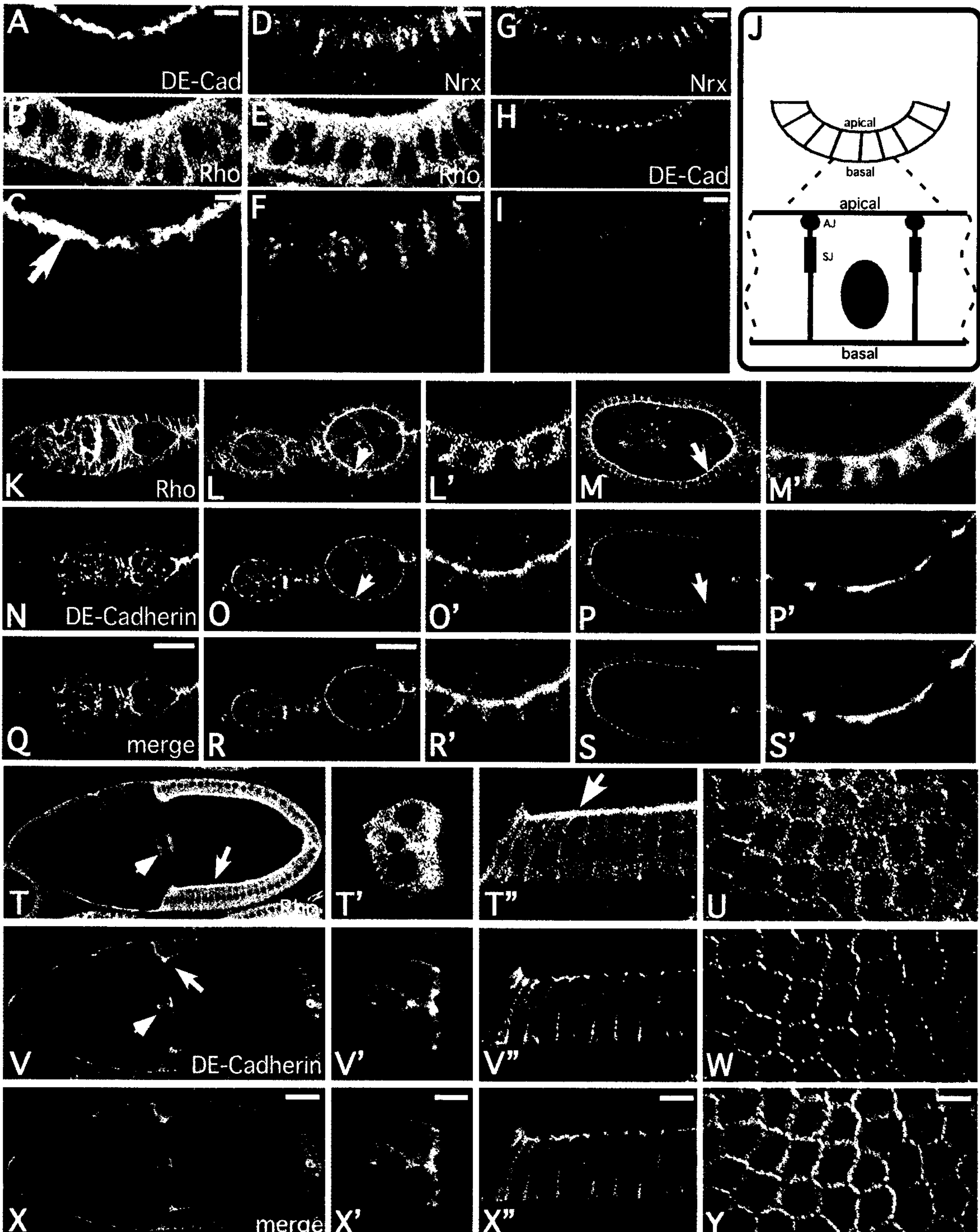
3.3.3. Rho1 protein accumulates at sites of adherens junction formation in the embryo.

Rho has been implicated in the regulation of cell adhesion through its effects on a number of different types of cellular junctions, including integrin-based focal adhesions (Schwartz and Shattil, 2000) and cadherin-based AJs (Kaibuchi et al., 1999). We examined Rho1 localization relative to DE-cadherin, a component of AJs located around the apical margin of cells and Neurexin, a component of septate junctions (SJs) which are thought to be analogous to tight junctions in mammalian cells (Baumgartner, 1996). Since AJs and other cell-cell contacts are not yet fully formed at the cellular blastoderm stage, we examined cells of the gut epithelium, which show a clear apical-basal polarity with AJs located at the apical end of the cell and SJs more basal (Fig. 3.3J) (Tepass and Hartenstein, 1994). As in blastoderm embryos (Fig. 3.2), Rho1 protein is ubiquitously cytoplasmic but concentrated apically. Significantly, the apical cytoplasmic accumulation of Rho1 protein overlaps with the sites of cadherin localization (Fig. 3.3C). Neurexin is localized basal to the apical accumulations of Rho1 and does not show substantial overlap (Fig. 3.3F).

3.3.4. Rho1 accumulates at sites of cadherin localization in ovaries.

Previous studies have indicated a role for Rho1 in the regulation of the actin cytoskeleton during oogenesis (Magie et al., 1999). As in the embryo, Rho1 is present throughout the cytoplasm of all cells in the ovary, but accumulates in the apical

Figure 3.3. Rho1 accumulates at sites of DE-cadherin localization.
(A-C) Confocal microscopy showing double labeling of a hindgut tube from a stage 14 embryo for both DE-cadherin (A) and Rho1 (B). A higher magnification view of the merged image is shown in (C). In all merged images, Rho1 staining is red and the staining for other molecules is green. Apical is up in all images. Note the apical accumulation of Rho1 protein that coincides with DE-cadherin expression (arrow in C). (D-F) Double labeling of a stage 14 hindgut tube for the septate junction protein Neurexin (D), Rho1 (E), and the merged image (F). Note that the Neurexin staining (green) is largely exclusive of Rho1 apical accumulation (red). (G-I) Control staining documenting DE-Cadherin (green) localization relative to Neurexin (red). (J) A schematic diagram of the gut showing the relative locations of the adherens junctions (AJ) and septate junctions (SJ). (K-M', T-U) Expression of Rho1 protein during oogenesis. Rho1 protein is expressed in all stages, including the germarium (K). Note Rho1 accumulation in the apical regions of follicle cells (L', M'; arrows in L, M). Rho1 expression is upregulated in border cells (T') and accumulates at lateral follicle cell contacts (cross-section T", grazing section U) and in the cortex of the oocyte (arrows in T, T"). (N-P', V-W) Localization of DE-cadherin during oogenesis. Cadherin is upregulated in the border cells (R') and also localized to lateral follicle cell contacts (R", W). (Q-S', X-Y) Merged images of Rho1 and DE-cadherin staining (red: Rho1, green: DE-cadherin). Stages of oogenesis shown are the germarium (K,N,Q), stage 3 and 5 (L,O,R), stage 8 (M,P,S) and stage 10a (T,V,X). Scale bars: A, D, G: 0.6 μm; C, F, I: 0.2 μm; Q, R, S, X: 30 μm; X', X", Y: 10 μm.



A
DE-Cad
D
Nrx
G
Nrx
J
apical
basal
apical
AJ
SJ
basal
B
Rho
E
Rho
H
DE-Cad
C
F
I
K
Rho
L
L'
M
M'
N
DE-Cadherin
O
O'
P
P'
Q
merge
R
R'
S
S'
T
T'
T"
U
V
DE-Cadherin
V'
V"
W
X
merge
X'
X"
Y

regions of follicle cells (Fig. 3.3L-M'). Rho1 also accumulates in specialized subsets of follicle cells such as the border cells (Fig. 3.3T, T'), in addition to regions of contact between follicle cells (Fig. 3.3T", U) and the oocyte cortex (Fig. 3.3T, T"). As is the case in the embryo, Rho1 apical accumulation overlaps DE-cadherin localization (Fig. 3.3N-P'; V-W). Cadherin protein is localized to AJs at the apical end of follicle cells (Fig. 3.3O-P'), and at lower levels to lateral follicle cell contacts and nurse cell-nurse cell contacts (Fig. 3.3V", W). Cadherin is also up-regulated in those same populations of follicle cells that show accumulation of Rho1 protein, including the border cells (Fig. 3.3V') (Niewiadomska et al., 1999).

3.3.5. Cadherin and catenin localization is disrupted in Rho1 mutants.

To determine whether the accumulation of Rho1 protein at sites of DE-cadherin localization has a functional role, we examined DE-cadherin expression in *Rho1* mutants. In wildtype embryos, DE-cadherin protein is localized to AJs in the apical cortex of epithelial cells, resulting in an antibody staining pattern that outlines cells distinctly (Fig. 3.4A-B, E-F). In zygotic *Rho1* mutant embryos, however, this pattern of cadherin localization is disrupted (Fig. 3.4C-D, G-H). Rather than being restricted to cell-cell contacts, cadherin protein is diffusely distributed across the cell. This is especially evident in the cells near the leading edge of the epithelia undergoing dorsal closure (arrow in Fig. 3.4H), the process whereby the lateral epidermal cells zip together at the dorsal midline. The mislocalization of DE-cadherin in *Rho1* mutants does not appear to be simply the result of cellular disorganization, as localization of the septate junction protein Neurexin is not disrupted (compare Fig. 3.4J with Fig. 3.4L). In addition, expression of other proteins such as β-tubulin and those detected by anti-phosphotyrosine antibodies are not disrupted in *Rho1* mutants (Magie et al., 1999). We do not observe a similar disruption of DE-cadherin staining in *Rho1* mutant ovaries, likely because we can only reduce, but not eliminate, *Rho1* activity during oogenesis.

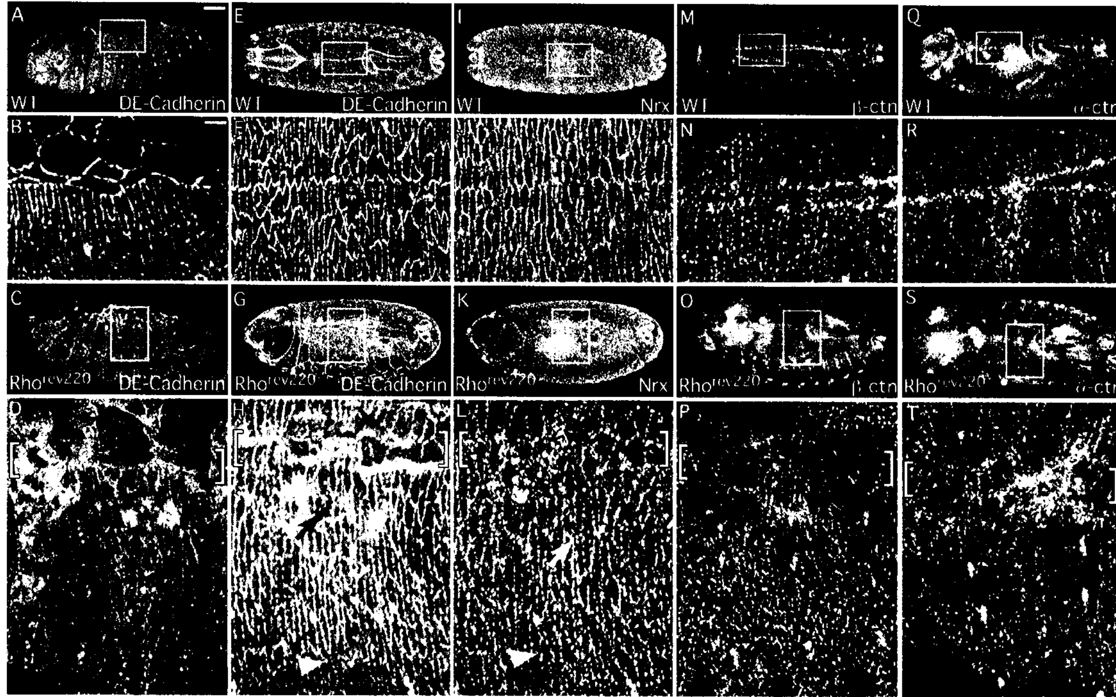


Figure 3.4. Embryos mutant for Rho1 show aberrant DE-cadherin and catenin localization.

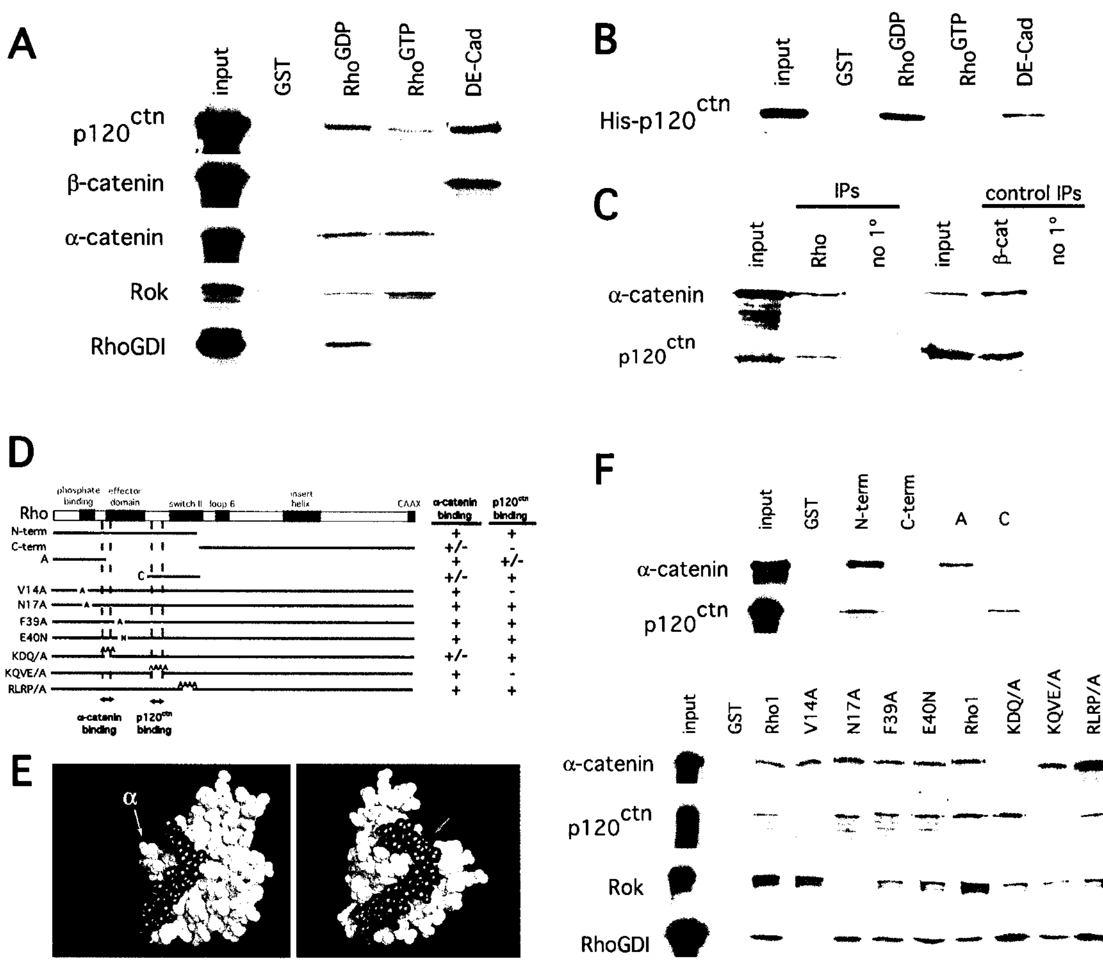
Confocal micrographs showing junction protein expression in wildtype and Rho1 mutant embryos. (A-B, E-F) DE-cadherin expression in stage 14 (A,B) and stage 15 (E,F) wildtype embryos. (C-D, G-H) DE-cadherin expression in stage 14 (C,D) and stage 15 (G,H) Rho1 mutant embryos. Note the disruption of DE-Cadherin localization near the leading edge (arrow in H), but not in more lateral regions (arrowhead in H). (I-L) No difference in Neurexin expression (septate junctions) is observed in the stage 15 Rho1 mutant embryo (K, L) compared to wildtype (I, J). DE-Cadherin and Neurexin were simultaneously imaged in the same wildtype and Rho1 mutant embryos. Brackets indicate the leading edge in D, and the dorsal midline in H,L,P,T. (M-P) β-catenin expression is disrupted in stage 15 Rho1 mutants (O, P) compared to wildtype embryos (M, N). (Q-T) α-catenin expression is disrupted in stage 15 Rho1 mutants (S, T) compared to wildtype embryos (Q, R). In all images, anterior is left. Dorsal is up in A-D, E-T are dorsal views. Boxes in A,C,E,G,I,K,M,O,Q,S indicate the region of the embryo shown in B,D,F,H,J,L,N,P,R,T, respectively. Scale bars: A: 50 μm, B: 10 μm.

We also examined the localization of the AJ proteins α- and β-catenin in *Rho1* mutants. β-catenin binds to the cytoplasmic domain of DE-cadherin, and α-catenin binds to β-catenin. Both proteins show expression patterns similar to DE-cadherin in wildtype embryos, although not as tightly localized to the plasma membrane (compare Fig. 3.4M-N and Q-R with Fig. 3.4E-F). Both catenins are less precisely organized in *Rho1* mutants (Fig. 3.4O-P, S-T), especially in cells on the dorsal surface near the leading edge. As expected given the mislocalization of DE-cadherin, loss of Rho1 also affects the localization of α- and β-catenin.

3.3.6. Rho1 interacts directly with p120^{ctn} and α-catenin.

To determine if the localization of Rho1 at AJs is due to direct interactions between Rho1 and components of the junctional complex, we examined the ability of Rho1 to bind the catenins using an in vitro GST-pulldown assay. As reported previously, we find that in vitro translated (IVT-) p120^{ctn} and β-catenin, but not α-catenin, bind to the intracellular domain of DE-cadherin (Fig. 3.5A) (Aberle et al., 1994; Daniel and Reynolds, 1995; Herrenknecht et al., 1991; Hulsken et al., 1994). While Rho1 does not bind β-catenin, we were surprised to find that Rho1 binds directly to both p120^{ctn} and α-catenin. As would be expected for a protein reported to keep Rho in a GDP-bound state, p120^{ctn} binds preferentially to GST-Rho1^{GDP}. Unexpectedly, Rho1 also binds directly to α-catenin independently of the phosphorylation state of the nucleotide Rho1 is associated with, suggesting a role for α-catenin in either recruiting Rho1 to AJs, or tethering it there once recruited by p120^{ctn}. We also examined the binding of Rho-kinase (Rok), a known Rho effector, and a putative RhoGDI identified by the Berkeley *Drosophila* Genome Project (BDGP) based on sequence homology, as controls. As expected, Rok bound preferentially to GST-Rho1^{GTP}, while RhoGDI bound exclusively to GST-Rho1^{GDP} (Fig. 3.5A).

Figure 3.5. Rho1 interacts directly with p120^{ctn} and α-catenin.
(A) GST pulldown experiments assessing binding among Rho1, the DE-cadherin intracellular domain, p120^{ctn}, β-catenin and α-catenin. ^{35}S-labelled in vitro translated (IVT) α-catenin (third panel from top) binds GST-Rho1 independently of the phosphorylation state of its associated nucleotide, IVT-p120^{ctn} binds preferentially to GST-Rho1^{GDP} (top panel), and IVT-β-catenin does not interact with either form of GST-Rho1 (second panel from top). Rok and a putative RhoGDI are included as binding controls. 5% input is shown. (B) GST pulldown experiment utilizing purified bacterially-expressed His-p120^{ctn}. His-p120^{ctn} also binds preferentially to GST-Rho1^{GDP}. (C) Immunoprecipitations showing in vivo interaction between Rho1 and α-catenin, and Rho1 and p120^{ctn}. β-catenin immunoprecipitations were performed as a positive control. 5% input is shown. (D) Diagram of the protein fragments, substitutions and point mutants used to map interaction domains on Rho1. (E) Computer model of GDP-bound RhoA crystal structure. Residues required for α-catenin binding are highlighted in yellow and those required for p120^{ctn} binding are highlighted in red. For reference the effector domain is highlighted in green. (F) GST pulldown experiments demonstrating the regions of Rho1 required for binding of α-catenin (top panel) and p120^{ctn} (second panel from top). α-catenin binds to region A and its binding is disrupted by replacing aa 27-29 (KDQ) with alanines, whereas p120^{ctn} binds to region C and its binding is disrupted by replacing aa 51-54 (KQVE) with alanines. Rok and RhoGDI bound preferentially to constitutively active (V14A) and dominant negative (N17A) forms of Rho1, respectively, but equally well to all other forms of Rho1 tested. V14A protein was exchanged with GTP and N17A with GDP in all experiments. All other forms of Rho1 in the α-catenin, p120^{ctn} and RhoGDI binding experiments were exchanged with GDP, while the Rho1 proteins used to test Rok binding were exchanged with GTP.



A
input
GST
Rho GDP
Rho GTP
DE-Cad
p120 ctn
β-catenin
α-catenin
Rok
RhoGDI
B
His-p120 ctn
C
IPs
control IPs
Rho
no 1°
β-cat
D
Rho
α-catenin binding
p120 ctn binding
N-term
C-term
V14A
N17A
F39A
E40N
KDQ/A
KQVE/A
RLRP/A
E
α
F
N-term
C-term
Rho1

To test whether the interactions we observe between Rho1 and the catenins are direct or mediated by bridging proteins within the IVT lysate, we purified bacterially-expressed His-tagged p120^{ctn}. Consistent with the results using IVT-p120^{ctn}, His-p120^{ctn} binds directly to GST-Rho1 with preference for GST-Rho1^{GDP} (Fig. 3.5B). To verify that the in vitro interactions we observed occur *in vivo*, we co-immunoprecipitated Rho-containing complexes from *Drosophila* embryo lysates. The P1D9 monoclonal antibody immunoprecipitates endogenous Rho1 (data not shown). Western blot analysis of these Rho1-immunoprecipitated complexes reveals the presence of α-catenin and p120^{ctn} (Fig 3.5C).

We mapped the domains of Rho1 required for interaction with the two catenins by using a series of Rho1 protein fragments followed by targeted amino acid substitution mutations in the context of full length Rho1 (Fig. 3.5D). The Rho1 binding domains for the two catenins are distinct. α-catenin maps to a surface-exposed region between the phosphate binding loop and the effector domain that has not previously been assigned a function (Fig. 3.5E). α-catenin binding can be greatly reduced by substituting alanines for 3 amino acids (aa 27-29) within this domain (KDQ/A; Fig. 3.5D,F), but is not affected by other small substitutions (Fig. 3.5D,F). We also tested point mutations within the effector loop previously shown to affect binding of RhoA to particular effectors in mammalian cell culture (Sahai et al., 1998), but none of these affected α-catenin binding.

p120^{ctn} binds preferentially to a region between the effector domain and the switch II domain (Fig. 3.5D-F). This binding can be disrupted by the KQVE/A substitution (Fig. 3.5F). These residues are part of a surface-exposed loop distinct from that to which α-catenin binds (Fig. 3.5E). This region changes conformation depending which nucleotide is bound, consistent with the preference of p120^{ctn} for GDP-bound Rho1. Also consistent with this preference, p120^{ctn} binds to dominant negative Rho1 (N17A) but not to constitutively active Rho1 (V14A) (Fig. 3.5F). As controls, we examined the binding of Rok and RhoGDI to these Rho mutants. Rok binding is

disrupted by dominant negative Rho1, and RhoGDI by constitutively active Rho1, as expected given their nucleotide preference. Both bind as well to all other mutants tested as they do to wildtype Rho1 (Fig. 3.5F).

3.3.7. Rho1 localization is disrupted in catenin mutants.

$p120^{ctn}$ has recently been proposed to negatively regulate Rho in the cytoplasm (Anastasiadis et al., 2000; Noren et al., 2000). We find that embryos homozygous for a deficiency that uncovers $p120^{ctn}$ show a severe dorsal open phenotype and, unlike wildtype (Fig. 3.2J'), an accumulation of Rho1 protein in the leading edge cells (Fig. 3.6A-B'; arrows in A', B'). Since this deficiency removes several genes, and specific $p120^{ctn}$ mutations have not yet been reported, we used dsRNA interference (RNAi) to specifically disrupt $p120^{ctn}$ function (Kennerdell and Carthew, 1998). RNAi-generated $p120^{ctn}$ mutant embryos exhibit severe morphogenetic defects, particularly in head involution. This phenotype is more severe than homozygous deficiency embryos, likely due to removal of maternal as well as zygotic $p120^{ctn}$ by the RNAi method. In cases where the leading edge cells were detectable, an accumulation of Rho1 protein was observed in and around those cells. To confirm this result, we injected $p120^{ctn}$ dsRNA into embryos expressing β-galactosidase under the control of the *puckered* promoter, a gene expressed in the leading edge cells. Rho1 protein accumulates in and around the cells expressing β-gal (Fig. 3.6C-F'). This accumulation is not seen in uninjected embryos or in embryos injected with a control dsRNA (*ftz*; Fig. 3.6G-H'). Accumulation of Rho1 protein at the leading edge is not a general property of dorsal closure mutants, as Rho1 accumulation is not observed in the dorsal closure mutants *basket* (Fig. 3.6I, K) and *hemipterous* (Fig. 3.6J, L). Since neither deficiencies covering the locus nor specific α-catenin mutations have been reported, we also used RNAi to remove α-catenin activity. RNAi-generated α-catenin mutant embryos cannot carry out the cell movements that accompany the early stages of gastrulation, and fail to form recognizable structures (Fig. 3.6M-O'). Antibodies

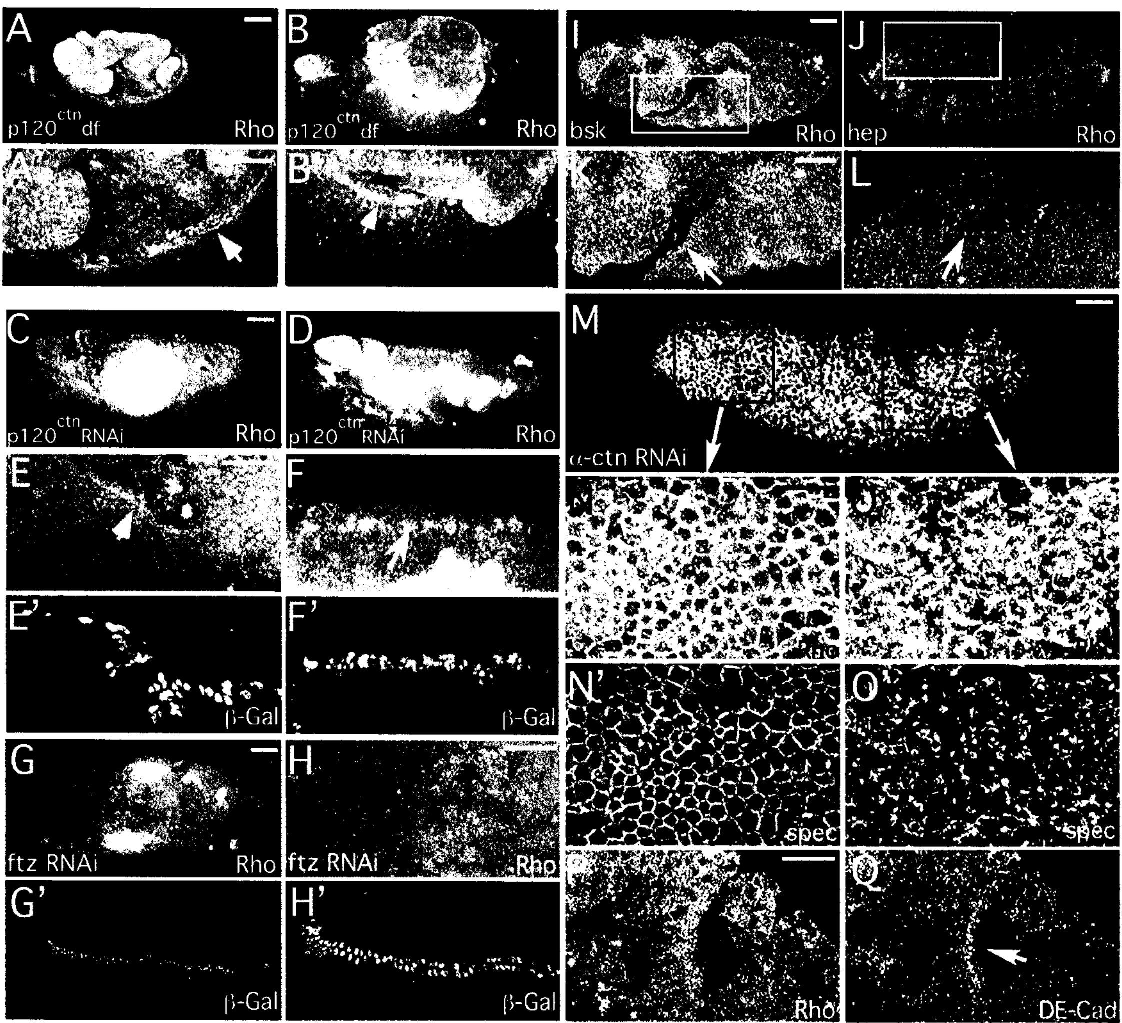


Figure 3.6. Rho1 localization is aberrant in catenin mutants.
(A-B') Rho1 expression in stage 15 embryos homozygous for a deficiency that removes the p120^{ctn} locus. (C-F') Rho1 (C-F) and β-galactosidase (E', F') expression in stage 15 *puc-lacZ*/TM3 embryos injected with p120^{ctn} dsRNA. Note the accumulation of Rho1 protein at the leading edge in both deficiency and RNAi embryos (arrows in A', B', E, F), not observed in either *ftz* RNAi embryos (G-H') or other dorsal closure mutants *basket* (I,K) and *hemipterous* (J,L) (arrows in K, L; compare to A', B'). (M-O') Rho1 (M, N, O) and α-spectrin (N', O') expression in stage 6 embryos injected with α-catenin dsRNA. (P, Q) Rho1 (P) and DE-cadherin (Q) expression in embryos injected with α-catenin dsRNA. Note apical localization of DE-cadherin protein in some cells (arrow in Q). In all images, anterior is left. Scale bars: A,C,G,I,M: 50 μm; A',E,H,K,N,P: 25 μm.

recognizing α–spectrin, a component of the membrane cytoskeleton, provide a means of visualizing cell architecture, and α-catenin has been shown to interact with the spectrin cytoskeleton (Pradhan et al., 2001). We therefore examined Rho1 and α-spectrin localization in embryos injected with α-catenin dsRNA. In early stages, cells in the posterior of the α-catenin mutant embryo that would normally be involved in extending over the dorsal surface show a breakdown of the spectrin cytoskeleton, although cells in the anterior remain relatively normal (Fig. 3.6N', O'). The Rho1 staining pattern mirrors that of α-spectrin, showing disrupted localization in the posterior regions of the embryo (Fig. 3.6N, O). At later stages, all regions of the embryo show severely reduced DE-cadherin expression, although occasional attempts at forming a polarized epithelium can be seen (Fig. 3.6Q).

3.3.8. Overexpression of $p120^{ctn}$ or α-catenin enhances the *Rho1* phenotype.

The observation that localization of the catenins is disrupted in embryos lacking Rho1 and Rho1 localization is aberrant in embryos with reduced $p120^{ctn}$ or α-catenin suggests that the balance of Rho1 to the catenins is important to their proper function. If this is the case, then perturbing this balance by overexpressing the catenins should show effects in a *Rho1* mutant background. We therefore generated transgenic flies containing $p120^{ctn}$ or α-catenin under the control of the Gal4-inducible UAS promoter that would allow us to ectopically express the catenins. One copy of UAS-$p120^{ctn}$ or UAS-α-catenin overexpressed in a wildtype background using an actin-Gal4 driver results in approximately 15% lethality, with a third of these embryos exhibiting very mild segmentation defects. 25% of *Rho1* homozygous mutant embryos show segmentation defects (20% mild, 5% severe; Fig. 3.7A). $p120^{ctn}$ and α-catenin enhance both the incidence and severity of the segmentation defects observed when overexpressed in a *Rho1* mutant background, with α-catenin exerting the stronger effect (Fig. 3.7A). The enhancement effects of $p120^{ctn}$ and α-catenin are specific, as

overexpression of GFP-tagged actin using this method had no effect on the severity of the *Rho1* phenotype (Fig. 3.7A). 42% of Rho1 mutants overexpressing $p120^{ctn}$ (26% mild, 16% severe) and 51% of Rho1 mutants overexpressing α-catenin (17% mild, 34% severe) show segmentation defects. Significantly, 22% of embryos overexpressing α-catenin in a *Rho1* mutant background show cuticular holes, whereas only 2% of *Rho1* mutants and 3% of those overexpressing α-catenin in a wildtype background display this phenotype.

3.4. DISCUSSION.

Subcellular localization of Rho protein has been shown to be important for its proper function. Such localization could bring Rho into close juxtaposition with either activating molecules such as GEFs or downstream effectors that can then carry out local functions. We have used a monoclonal antibody that specifically recognizes *Drosophila* Rho1 to examine its localization in an organismal and developmental context. Our results indicate that in addition to Rho1's ubiquitous cytoplasmic expression, it accumulates at adherens junctions and is involved in regulating the proper localization of AJ components. Further, we have identified direct physical interactions between Rho1 and the catenins, $p120^{ctn}$ and α-catenin. Isoprenylation at the C-terminal CAAX motif is involved in regulating the subcellular localization of Rho (Fleming et al., 1996; Kranenburg et al., 1997), however, binding to the catenins may represent another mechanism of recruiting Rho1 to its sites of action.

We find that Rho1 activity is required to properly localize DE-cadherin during development, consistent with data from mammalian cell culture experiments implicating Rho and Rac in cadherin assembly and maintenance (Braga, 1999b; Braga, 1997). The defects we observe in cadherin localization are most prevalent in and around the leading edge (LE) cells undergoing dorsal closure. Previously Rho1 had

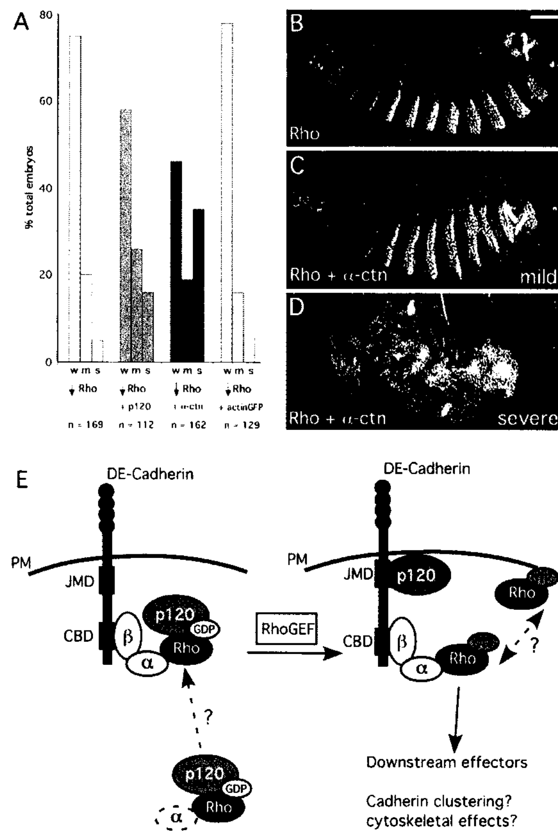


Figure 3.7. Overexpression of p120^{ctn} or α-catenin enhances the Rho1 phenotype. The graph shows % total embryos (y-axis) with segmental patterning in each phenotypic class (w=wildtype, m=mild, s=severe) for the following genotypes: *Rho1* homozygous mutants (↓Rho), *Rho1* mutants with one copy of UAS-p120^{ctn} overexpressed with the actin-Gal4 driver (↓Rho + p120^{ctn}), *Rho1* mutants with one copy of UAS-α-catenin overexpressed with the actin Gal4 driver (↓Rho + α-ctn), and *Rho1* mutants with one copy of UAS-actin-GFP overexpressed with the actin Gal4 driver (↓Rho + actinGFP). The number of embryos scored is indicated beneath each genotype. (B-D) Cuticles depicting representative examples of each phenotypic class, (B) homozygous *Rho1* mutant phenotype exhibiting an anterior dorsal hole but relatively normal anterior/posterior (A/P) segmentation, (C) mild disruption of A/P segmentation, (D) severe disruption of A/P segmentation. (E) Model depicting the relationship of Rho1 to components of adherens junctions. (PM: plasma membrane, CBD: catenin binding domain, JMD: juxtamembrane domain). For cuticles, anterior is left. Scale bar: 50 μm.

been implicated in dorsal closure via its regulation of the LE actin cytoskeleton in cells flanking the segment borders (Harden et al., 1999). However, the disruption we observe in cadherin distribution suggests that regulation of cell-cell adhesion may play a role in the dorsal closure phenotype observed in these embryos. Thus Rho1's effects on cadherin localization could be the result of a direct role in DE-cadherin clustering, or an indirect effect on the cortical actin cytoskeleton. The process of AJ formation in keratinocytes has been shown to require actin polymerization and the interdigitation of filopodia from neighboring cells (Vasioukhin et al., 2000). A similar interdigitation of filopodia is seen during dorsal closure in *Drosophila* and is likely involved in forming adhesive contacts between the two epithelial fronts (Jacinto et al., 2000). Since Rho and Cdc42 have been shown to act antagonistically in the formation of cellular processes in neurons (Kozma et al., 1997), it is possible that disrupting the balance of Rho1 and Cdc42 function in LE cells results in inappropriate regulation of filopodial extensions. This could partially explain the disruption of DE-cadherin localization we observe in *Rho1* mutants. Alternatively, Rho1's primary role could be in directly regulating the adhesion of cells near the LE, with Rac and Cdc42 acting as the major organizers of the acto-myosin network.

In addition to the accumulation of Rho1 protein at sites of cadherin localization, we observe a direct physical interaction between Rho1 and both $p120^{ctn}$ and α-catenin. The catenin family of proteins is important in regulating cadherin-based adhesion and linking cadherins to the actin cytoskeleton (Kemler, 1993). β-catenin binds to the catenin-binding domain of the cadherin molecule as well as to α-catenin. α-catenin, in turn, acts as a link to the actin cytoskeleton, either by directly binding actin filaments or through association with other actin-binding proteins. α-catenin also has been shown to bind spectrin, a major component of the membrane skeleton underlying the plasma membrane involved in stabilizing it and determining cell shape. Human colon carcinoma Clone A cells that contain mutant α-catenin have defects in spectrin assembly (Pradhan et al., 2001). Consistent with this, we observe a breakdown of the

α-spectrin cytoskeleton in embryos injected with α-catenin dsRNA, especially in morphogenetically active cells early in gastrulation. α-catenin protein is enriched at adherens junctions, but is not as strictly localized to them as is DE-cadherin. Binding of α-catenin to Rho1 may be a general mechanism through which Rho1 is recruited to the plasma membrane.

$p120^{ctn}$ regulates the adhesive properties of cadherin complexes through its binding to the juxtamembrane domain of the cadherin molecule, although the precise mechanisms underlying this function are not known. $p120^{ctn}$ also acts in the cytoplasm where it has been proposed to negatively regulate Rho activation in a manner similar to the GDI proteins, which prevent Rho from exchanging GDP for GTP, although it shares no sequence homology with them (Anastasiadis et al., 2000). The binding of $p120^{ctn}$ to cadherins and its effects on Rho function have been shown to be mutually exclusive, such that once $p120^{ctn}$ binds a cadherin molecule, it is no longer capable of inhibiting Rho activity or function (Anastasiadis et al., 2000). Rho would then be accessible to activating regulatory proteins such as GEFs, and could carry out its downstream functions. The physical interaction we observe between Rho1 and $p120^{ctn}$ suggests that this negative regulation of Rho1 is due to direct binding of $p120^{ctn}$ to GDP-Rho1. Interestingly, this is the same face of the Rho protein that has been shown to bind to classical GDIs (Hoffman et al., 2000), consistent with the idea that despite the lack of sequence homology, $p120^{ctn}$ may be acting in a similar way. Overexpression of $p120^{ctn}$ in mammalian cells leads to an inhibition of Rho activity (Anastasiadis et al., 2000; Noren et al., 2000). Overexpression of $p120^{ctn}$ in our system enhances the *Rho1* mutant phenotype, as would be expected for a negative regulator. We find that embryos homozygous for a deficiency uncovering the $p120^{ctn}$ locus show an accumulation of Rho1 protein at the leading edge and exhibit a severe dorsal open phenotype. A similar accumulation of Rho1 protein is observed in embryos injected with $p120^{ctn}$ dsRNA. A positive feedback mechanism may be functioning whereby the relief of $p120^{ctn}$-mediated regulation in those cells results in the upregulation of Rho1 protein or an increase in

Rho1 stability. It has recently been shown that overexpression of a RhoGDI in the hearts of mouse embryos results in the upregulation of RhoA expression, suggesting the existence of a negative feedback mechanism in the regulation of RhoA levels (Wei et al., 2002), although we are not aware of any other instances in which a positive feedback mechanism has been linked to Rho expression. Excess Rho activity disrupts cellular migration (Nobes and Hall, 1999); cells at the leading edge in embryos that lack $p120^{ctn}$ function remain cuboidal, rather than elongating as they would during normal dorsal closure, suggesting that Rho1 may be involved in regulating these cell shape changes. Alternatively, $p120^{ctn}$ has been suggested to activate Rac and Cdc42 in the cytoplasm through an interaction with the GEF Vav2, and this could account for some of its effects on cell morphology (Noren et al., 2000). The observation that Rho1 can bind both $p120^{ctn}$ and α-catenin and that their binding sites are not overlapping suggests that either could be involved in recruiting Rho1 to AJs or the plasma membrane in general. Our data indicating that overexpression of α-catenin enhances the *Rho1* mutant phenotype to a greater degree than $p120^{ctn}$ suggests an important role for α-catenin in Rho1 function, perhaps as a factor generally involved in localizing Rho1 to its sites of action, while $p120^{ctn}$ plays a more specific role at AJs.

Our data suggest a model (Fig. 3.7E) in which $p120^{ctn}$ or α-catenin or both are involved in recruiting Rho1 to sites of cadherin localization, where it can then be activated and carry out its functions, including proper AJ formation. If Rho1 is not recruited properly, as in the case of a *Rho1* mutant, this results in mislocalization of AJ components. The binding of $p120^{ctn}$ to Rho1, either in the cytoplasm or while Rho1 is tethered at AJs through its interaction with α-catenin, inhibits the exchange of GDP for GTP and keeps Rho1 in an inactive state. The binding of $p120^{ctn}$ to the juxtamembrane domain may release Rho1, allowing it to be activated by GEFs. GTP-Rho1 could then bind its downstream effectors and either directly regulate DE-cadherin assembly or maintenance, or indirectly affect AJ formation through its effects on the actin cytoskeleton. Rho1 localization at AJs could then be mediated either

through continued association with α-catenin or through isoprenylation and insertion into the plasma membrane. Mutational analysis aimed at distinguishing between these models will provide further insight into this important feature of Rho1 function during morphogenesis.

3.5. APPENDIX.

3.5.1. Induction of ectopic pole cells results in the ectopic accumulation of Rho1 protein.

The basal accumulation of Rho1 protein in cells underlying the pole cells in the posterior of the embryo is unusual, as Rho1 protein is present in higher levels apically in most other cell types (Fig. 3.2). In addition, this localization identifies the somatic cells underlying the pole cells as distinct from the rest of the somatic cells, the only known marker to do so. This is suggestive of a functional relationship between Rho1 and some aspect of pole cell biology, such as their formation or migration. In order to investigate the link between the pole cells and this basal Rho1 accumulation, we examined Rho1 protein levels in embryos that either lacked pole cells or possessed ectopic pole cells. *oskar* mutant embryos lack normal pole plasm and cannot form pole cells. These embryos also lack the posterior basal accumulation of Rho1 protein (Fig. 3.8). Importantly, the apical accumulation of Rho1 is not affected. If pole cells are required to induce Rho1 expression, then this would predict that ectopic pole cells would result in ectopic accumulation of Rho1. To test this, we induced ectopic pole cells by taking advantage of embryos that express *oskar* mRNA fused to the bicoid 3'UTR (*osk-bcd*), which results in anterior accumulation of oskar and ectopic pole cells at the anterior pole of the embryo. Embryos that possess these ectopic anterior pole cells also show ectopic accumulation of Rho1 protein (Fig. 3.8). Interestingly, the levels of Rho1 that accumulate in the anterior are higher than those at the posterior, which obscure whether the ectopic Rho1 accumulates apically or basally.

These data indicate that either pole plasm assembly or some signal from the pole cells themselves is instrumental in causing the accumulation of Rho1 protein.

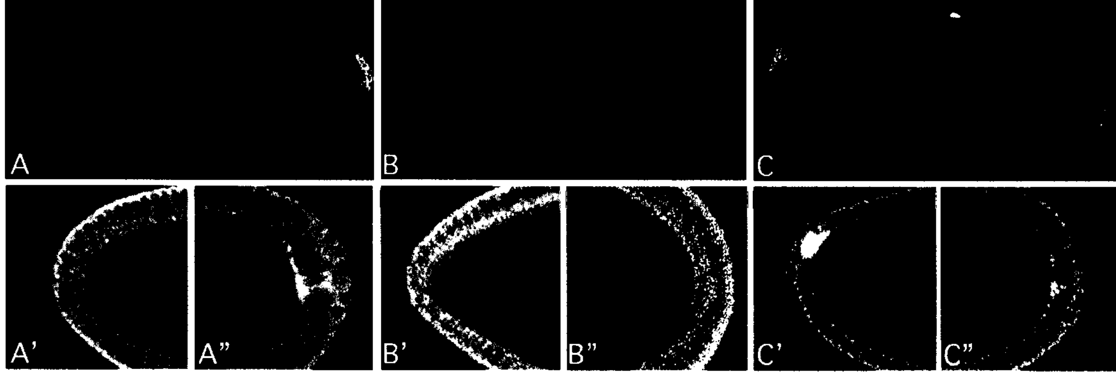


Figure 3.8. Pole cell presence correlates with basal Rho1 protein accumulation. Rho1 expression in stage 5 wildtype (A-A"), *osk* (B-B"), or *osk-bcd* (C-C") embryos. Pole cells are marked by staining against Vasa (red in A, B, C). In wildtype embryos, Rho1 accumulates at the posterior pole of the embryo basal to the pole cells (A, green; A"). Note absence of basal accumulation in the anterior (A'). In *osk* mutant embryos, no pole cells are formed (note absence of signal in the red channel) and no basal accumulation is seen either in the anterior or posterior (B, green; B'-B"). Embryos expressing the *osk-bcd* transgene possess ectopic pole cells at the anterior pole (C, red). These are accompanied by the ectopic accumulation of Rho1 protein (C, green; C'). Rho1 accumulates normally at the posterior in these embryos (C").

CHAPTER 4

Rho1 regulates endocytic processes that affect early *Drosophila* development

4.1. ABSTRACT

The Rho GTPase has been implicated in a wide variety of cellular functions, including actin cytoskeletal regulation, transcriptional activation, and cell cycle progression. It is not clear in all cases, however, how directly Rho affects the processes to which it has been linked. *Rho1* mutants exhibit a general endocytosis defect, as signaling pathways whose activity is down-regulated by endocytosis such as those mediated by EGFR and the Torso receptor tyrosine kinase are hyperactivated in maternal *Rho1* mutants. Here we show that the segmentation phenotype associated with loss of maternal Rho1 in *Drosophila melanogaster* results from a failure to maintain expression of the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*). Formation of a Wg protein gradient is required to maintain En expression and involves the internalization of Wg into vesicles that spread from the Wg-expressing cells. The number of these vesicles is reduced in maternal *Rho1* mutants, suggesting a defect in endocytosis and/or secretion required for their formation. In addition, the extent of cytoplasmic Armadillo accumulation in response to Wg signaling in maternal *Rho1* mutant embryos is spatially restricted. The maternal segmentation phenotype, with its disruption of the segmentation gene transcriptional cascade, appears superficially to indicate a role for Rho1 in transcriptional activation. Our observation that the underlying basis for this phenotype is an endocytic defect, however, suggests that Rho may indeed be acting primarily as a cytoskeletal regulator, and the other functions it has been linked to are secondary consequences.

4.2. INTRODUCTION.

The specification of pattern during embryonic development requires the proper regulation of the various signaling pathways necessary to determine cell fate. In this context, overactive signaling can be as deleterious as a lack of signaling, necessitating mechanisms to control signaling activity. Downregulation of a number of signaling pathways has been known for some time to involve the endocytosis, and subsequent degradation, of receptor-ligand complexes (reviewed in: Di Fiore and Gill, 1999; Gonzalez-Gaitan, 2003). Recently, data has accumulated linking endocytosis not only to downregulation of signaling, but also to activation of signaling and dispersal of ligands. Notch signaling, for example, is regulated by endocytosis in an unusual way. The receptor Notch and its ligand Delta are both transmembrane proteins. Upon binding to Delta, Notch is cleaved and its cytoplasmic domain enters the nucleus of the receiving cell and activates target gene expression. The Notch extracellular domain, in complex with Delta, is internalized into the ligand-presenting cell through endocytosis, and this is critical to Notch signaling as Delta mutants that cannot be endocytosed cannot activate Notch signaling (Parks et al., 2000; Piddini and Vincent, 2003).

Roles for endocytosis in ligand dispersal are particularly evident in the formation of morphogen gradients. For example, in the *Drosophila* wing disc, the formation of the gradient of Decapentaplegic (Dpp), a TGF-β homolog, requires the Dpp receptor as well as Dynamin, indicating a requirement for receptor-mediated endocytosis (Entchev et al., 2000). In addition, the distribution of the morphogen Wingless (Wg), the *Drosophila* Wnt-1 homolog, has been suggested to involve its encapsulation within vesicular structures termed argosomes, which are then transported from cell to cell through the endocytic compartment (Greco et al., 2001).

In the early *Drosophila* embryo, signaling mediated by Wg, a member of the segment polarity class of segmentation genes, is crucial to proper patterning of the

embryonic epidermis. Wg is a secreted protein that binds to the seven-pass transmembrane receptor *Dfrizzled-2* (*Dfz-2*; Bhanot et al., 1996; Bhanot et al., 1999). Wg protein is expressed in 14 stripes that are 1-2 cells wide. In addition to diffuse cytoplasmic staining, Wg can be detected in punctate structures identified through EM studies as multi-vesicular bodies (MVBs; (van den Heuvel et al., 1989). These vesicles spread out from the cells actively expressing Wg, and are thought to be important in the formation of the proper Wg protein gradient (Dierick and Bejsovec, 1998). Endocytosis is required for their formation, as these Wg-containing vesicles are absent in embryos mutant for *shibire* (*shi*), the *Drosophila* homolog of Dynamin, which cannot undergo endocytosis. Posterior to each Wg stripe, these vesicles are thought to influence the shape of the Wg protein gradient by targeting Wg to lysosomes and degradation (Dubois et al., 2001). An alternative model for how these vesicles may be involved in regulating Wg protein distribution is that they are instrumental in the transport of Wg from one cell to another through transcytosis (Pfeiffer et al., 2002).

One class of proteins demonstrated to have a role in the regulation of endocytosis is the Rho family of small GTPases, including Rho and its relatives Rac and Cdc42. Expression of constitutively active (ca) Rho or Rac in mammalian tissue culture cells has been shown to inhibit transferrin-receptor-mediated endocytosis (Lamaze et al., 1996). Treating dendritic cells with Toxin B, a nonspecific Rho GTPase inhibitor, or expressing dominant-negative (dn) Cdc42 greatly inhibits endocytic activity in these cells (Garrett et al., 2000). Microinjection of the Rho-specific inhibitor C3 exoenzyme blocks constitutive endocytosis in *Xenopus* oocytes, whereas injection of caRho stimulates it (Schmalzing et al., 1995). Overexpression of mammalian RhoB, which localizes to endosomes, inhibits the trafficking of EGFR-positive vesicles in tissue culture cells (Adamson et al., 1992). Mammalian RhoD also localizes to early and recycling endosomes and is involved in their trafficking (Murphy et al., 1996; Murphy et al., 2001).

In addition to its roles in endocytosis, Rho has been linked to a wide variety of cellular functions, including transcriptional activation, cell cycle progression, cell-cell and cell-substrate adhesion, secretion, phospholipid turnover, cytokinesis, apoptosis, and others, though it is not clear in all cases how direct Rho's involvement is (reviewed in: Hall, 1998; Mackay and Hall, 1998). These studies have been largely done in tissue culture using overexpression of dn- or ca- forms of Rho or treatment with pharmacological inhibitors of Rho GTPases. A great deal has been learned from studies of this type regarding the biochemical mechanisms of Rho function. In *Drosophila*, however, overexpression of dnRho1 results in phenotypes that are not identical to the loss-of-function phenotypes, suggesting that overexpression of dnRho1 non-specifically inhibits other GTPase pathways (Harden et al., 1999). One of the phenotypes associated with mutations in *Rho1*, the fly Rho homolog, is a disruption of segmentation resulting from the failure to maintain expression of the segment polarity genes *engrailed* (*en*) and *wg* (Magie et al., 1999). This segmentation phenotype could result from different biochemical mechanisms. One possibility is that Rho affects Wg signaling indirectly, through its functions in a general cellular process such as actin cytoskeletal regulation or endocytosis, and this phenotype provides a context in which to investigate how what is apparently a transcriptional readout could be due to this primary effect. Alternatively, Rho1 could be a classical component of the Wg signal transduction pathway. Genetic evidence places Rho1 downstream of *frizzled* (*fz*) and *disheveled* (*dsh*) signaling in eye development, as reduction of Rho1 levels can rescue the rough eye phenotype induced by overexpressing *fz* or *dsh* (Strutt et al., 1997). Examination of the loss-of-function phenotypes associated with *Rho1* in model organisms such as *Drosophila* allows the identification of functions specific for Rho in a developmental context.

In this chapter, we describe a general role for *Rho1* in endocytosis during early development in *Drosophila*, one consequence of which is aberrant Wg protein localization. This defect in Wg protein localization leads to defects in the activation of the Wg signaling pathway and the failure to maintain expression of En, as well as Wg

itself. Further, we can recapitulate these effects in *Drosophila* tissue culture cells, indicating that this effect is a general aspect of Rho function.

4.3. RESULTS.

4.3.1. Maternal *Rho1* mutants exhibit a segmentation defect that is the result of failure to maintain segment polarity gene expression.

It is not possible to completely eliminate maternal *Rho1* function, as germline clones of *Rho1* cannot be generated due to cell inviability (Magie et al., 1999). To examine the phenotypes associated with loss of maternally-deposited Rho1 we utilized a change-of-function mutation in an RNA polymerase II subunit, *wimp*, to reduce, but not eliminate, expression of maternal *Rho1* (Parkhurst and Ish-Horowicz, 1991). This is done by generating females doubly-heterozygous for *wimp* and *Rho1*, then mating them to wildtype males. The resulting embryos will hereafter be referred to as maternal *Rho1* mutants. Embryos with reduced maternal Rho1 exhibit a segmentation phenotype due to improper maintenance of segment polarity gene expression (Magie et al., 1999). In particular, expression of the segment polarity gene *engrailed*, while initiated normally, is not maintained properly, leading to the fusion or absence of stripes (Fig. 4.1A-B"). Since maintenance of En expression requires Wg signaling, we also examined expression of Wg in maternal *Rho1* mutants. Similar to En, Wg expression fails to be properly maintained in maternal *Rho1* mutants (Fig. 4.1C-D"). In embryos of later stages some stripes have disappeared completely, indicating a failure to properly maintain expression of both En and Wg (Fig. 4.1A', B', C', D'). Because we cannot make clones due to the requirement of Rho1 for cell viability, we

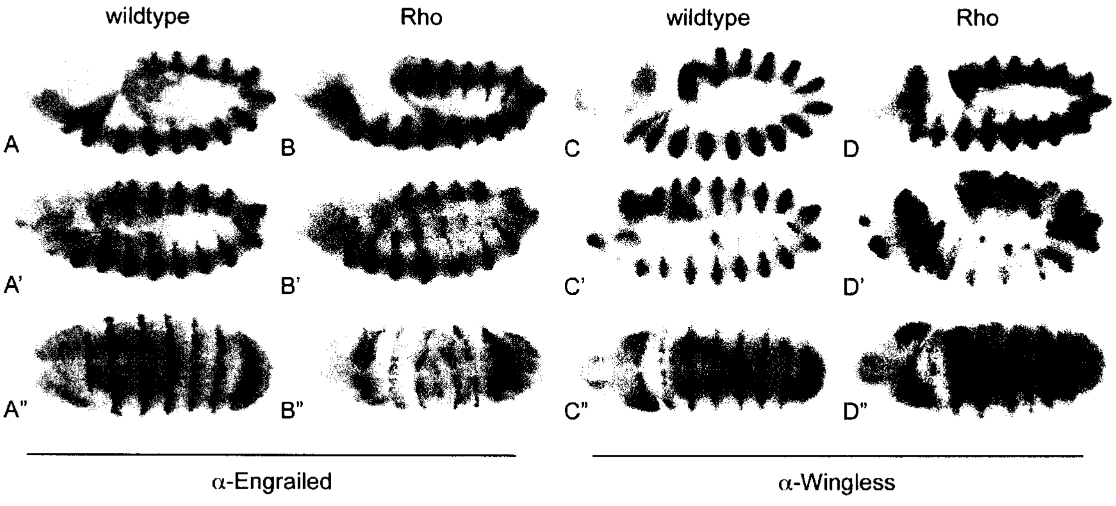


Figure 4.1. En and Wg expression is not maintained properly in maternal *Rho1* mutants. Wildtype (A-A" and C-C") or maternal Rho1 mutant (B-B" and D-D") embryos stained with an α-Engrailed (A-B") or α-Wingless (C-D") antibody. Whereas at early stage 9 En expression is normal (compare A and B), as development proceeds some stripes become reduced, others disappear entirely by stage 10 (compare A' with B'). Likewise, stage 9 (C, D) Wg expression in maternal *Rho1* mutants looks normal, but by stage 10 (compare C' with D') Wg stripes also become reduced or eliminated. (A", B", C", D") Ventral views of stage 10 embryos showing stripe morphology, all other images are lateral views. Anterior is left in all images.

are not able to directly assess whether the Wg- or En-expressing cell or both require Rho1 function for proper maintenance.

4.3.2. Wg protein distribution is aberrant in maternal *Rho1* mutants.

The segmentation phenotype associated with reduced maternal *Rho1* could result from different biochemical mechanisms. One possibility is that Rho1 impinges on the Wg signal transduction pathway through its effects on a general cellular process such as regulation of the cytoskeleton or endocytosis, and the readout we observe in transcriptional activation is a secondary consequence of this primary effect. Alternatively, Rho1 could be a component of the Wg signal transduction pathway. To distinguish between these possibilities, we examined Wg protein distribution in maternal *Rho1* mutants relative to wildtype (Fig. 4.2). In wildtype stage 9 embryos Wg is expressed in a stripe 2-cells wide and can be detected in multi-vesicular bodies up to a distance of several cells away. The formation of these vesicular structures has been shown to require endocytosis, as they are absent in embryos mutant for *shibire,* which cannot undergo endocytosis (Fig. 4.2G-J). Maternal *Rho1* mutants also exhibit fewer Wg positive vesicles than wildtype embryos. This phenotype is more variable than in *shibire* mutants, likely due to the fact that we cannot completely eliminate maternal Rho1 (Fig. 4.2L-O). Thus maternal *Rho1* mutants exhibit phenotypes similar to mutants defective for endocytosis.

4.3.3. Maternal *Rho1* mutants exhibit general endocytosis defects.

To determine whether the defect in Wg vesiculation in maternal Rho1 mutants is due to a general role for Rho1 in endocytosis or is specific to Wg, we examined other factors known to require endocytosis for their proper function (Fig. 4.3). Epidermal growth factor receptor (EGFR) signaling is under feedback control whereby activation of EGFR signaling leads to endocytosis of active receptor and attenuation of the signal, allowing the total amount of signaling to remain within optimal parameters

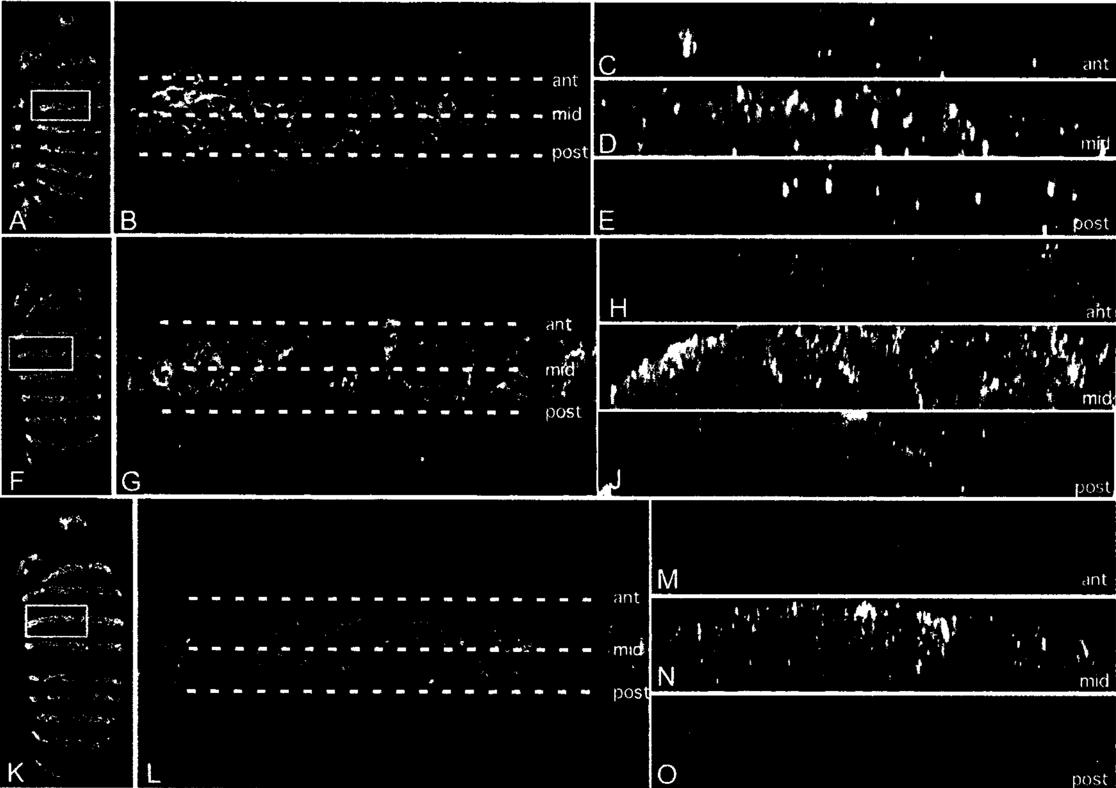


Figure 4.2. Wingless protein distribution is aberrant in maternal *Rho1* mutants. Z-series projections of wildtype (A, B), *shibire* (*shi*, F, G), and maternal *Rho1* (K, L) stage 9 embryos stained with antibodies against Wingless (Wg, green) and Discontinuous Actin Hexagon (Dah, red) serves as a marker for cell boundaries. The boxed regions of embryos in A, F, K are shown at higher magnification in B, G, L. (A-E) In wildtype embryos Wg protein can be found in vesicles spreading away from the Wg-expressing cells, as shown by cross-sections through the z-series in B anterior to (C), through the middle of (D), or posterior to (E) the Wg –expressing cells at the positions indicated by the dotted lines. Note the punctate accumulations of Wg protein in C and E. (F-J) *shibire* mutants are defective in endocytosis. Because of this, cross-sections through G (H, I, J) show a lack of Wg-containing vesicles outside the Wg-expressing cells (compare H and C, J and E). (K-O) Maternal *Rho1* mutants exhibit a phenotype resembling, though not as severe as, *shi* embryos, with a reduction in the number of Wg-containing vesicles (L; compare M with H and C, O with J and E).

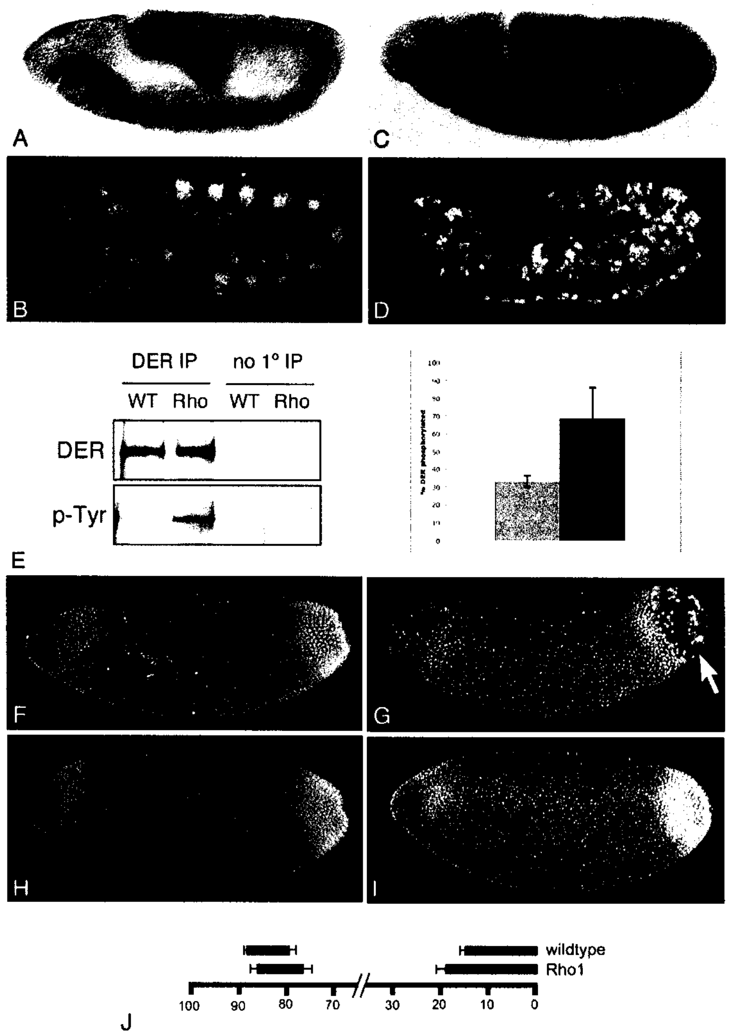


Figure 4.3. Maternal *Rho1* mutants are generally defective in endocytosis. (A-D) Wildtype (A-B) and maternal *Rho1* mutant (C-D) embryos labeled with antibodies to dpERK and visualized with either immunohistochemistry (A, C) or immunofluorescence (B, D). Note the ectopic accumulation of dpERK in *Rho1* mutants relative to wildtype. (E) *Drosophila* EGFR (DER) was immunoprecipitated from wildtype and maternal *Rho1* mutant embryo lysates. Western analysis was used to identify the overall amount of DER protein immunoprecipitated and its phosphorylation state. The graph indicates the amount of phosphotyrosine detected relative to the total amount of DER protein. (F-I) Wildtype (F, H) and maternal *Rho1* mutant (G, I) embryos labeled with antibodies against Tll. Brackets indicate the extent of the anterior and posterior expression domains. Note the posterior cellularization defect indicated by the arrow in G. (J) Quantitation of the extent of Tll expression domains, as expressed as % embryo length. For all embryos, anterior is left, dorsal is up.

(Sturtevant et al., 1994). The accumulation of dual-phosphorylated ERK (dpERK) in response to phosphorylation by EGFR has been used as a marker for EGFR signaling, and mutants in genes such as *hepatocyte growth factor-related tyrosine kinase substrate* (*hrs*) that are unable to properly endocytose activated EGFR show higher levels of dpERK accumulation (Lloyd et al., 2002). Similar to other mutants defective in endocytosis, maternal Rho1 mutants exhibit ectopic accumulation of dpERK (Fig. 4.3A-D). To verify that this effect is indeed due to the failure to attenuate EGFR signaling, we immunoprecipitated the *Drosophila* EGFR from wildtype and maternal *Rho1* mutant embryo lysates. If active receptor is not endocytosed and degraded properly, the phosphorylated form of EGFR accumulates to higher levels (Boni-Schnetzler and Pilch, 1987). In maternal *Rho1* mutants we observe higher levels of phosphorylated EGFR relative to wildtype, indicating that the dpERK accumulation is likely due to overactive EGFR signaling (Fig. 4.3E).

Another signal transduction pathway whose activity is regulated by endocytosis is that of the receptor tyrosine kinase (RTK) Torso, which is involved in the specification of terminal pattern in *Drosophila* (Sprenger and Nusslein-Volhard, 1993). Activation of the Torso signaling pathway at the poles of the embryo results in the activation of its downstream targets. Similar to the effects on EGFR signaling, mutants defective in endocytosis exhibit ectopic accumulation of Torso targets, one of which is *tailless* (*tll*, Lloyd et al., 2002). Tll is expressed at the posterior pole of the embryo, and in a stripe near the anterior pole (Fig. 4.3F, H). In endocytosis mutants the anterior stripe is shifted posteriorly and the posterior domain is expanded. A similar effect on Tll expression is seen in maternal *Rho1* mutants, as indicated by the embryos in Fig. 4.3G and I and the bottom bars in Fig. 4.3J ($n=10$, $p < 0.002$ for the anterior stripe, $n=10$, $p < 0.0001$ for the posterior domain). Additionally, maternal *Rho1* mutants exhibit terminal cellularization defects that have also been linked to endocytosis defects in *hrs* mutants (Lloyd et al., 2002) and mutants affecting Torso

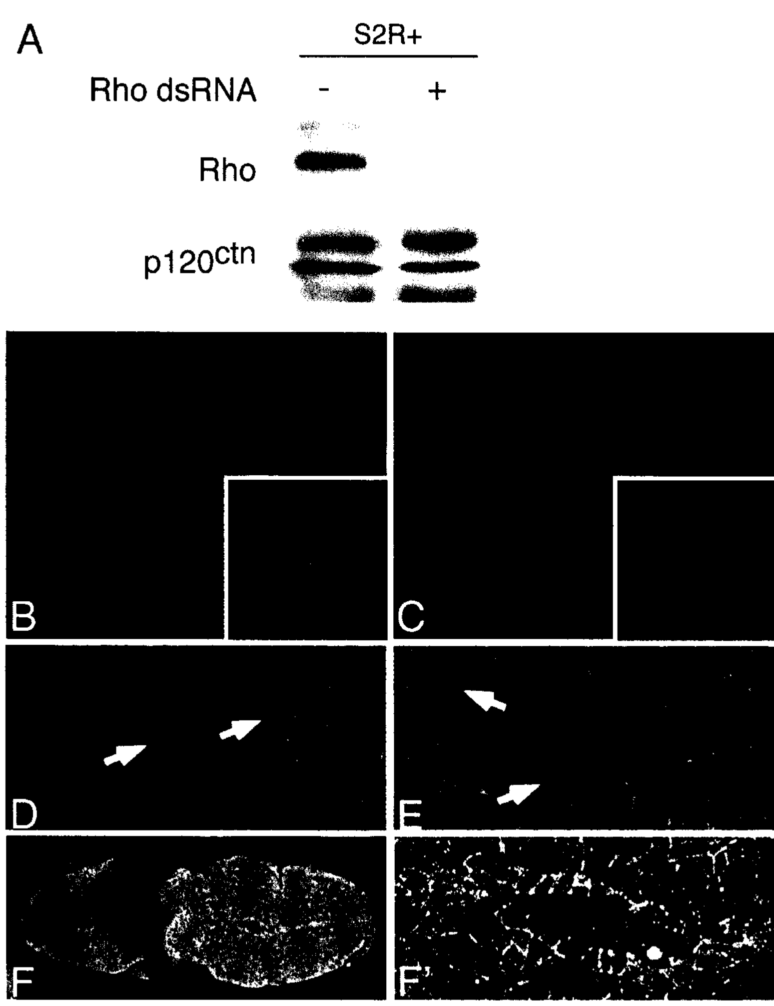


Figure 4.4. *Drosophila* S2R+ cells respond to treatment with *Rho1* dsRNA. (A) Western analysis of lysates made from untreated S2R+ cells and those treated with *Rho1* dsRNA indicates that Rho1 protein levels are greatly reduced, while levels of other proteins such as p120^{ctn} are unaffected. (B, C) Cells untreated (B) or treated with *Rho1* dsRNA (C) stained with antibodies against *Rho1* (green) and the nuclear marker propidium iodide (PI, red). Note the lack of staining for Rho1 and the multinucleate phenotype in the treated cells. (D, E) Stage 5 maternal *Rho1* mutant embryos labeled with antibodies against phosphotyrosine (green) to outline cells and PI (red) to label nuclei. Note the multinucleate cells (arrows in D, E), which indicate a defect in cellularization. (F, F') Maternal *Rho1* mutants stained with an α-DE-Cadherin antibody demonstrating the defect in ventral furrow formation observed in some embryos.

signaling (Perrimon et al., 1986), providing further evidence that maternal *Rho1* mutants are generally compromised in endocytosis.

4.3.4. Rho1 RNAi is effective in *Drosophila* S2R+ cells.

To investigate the mechanism of Rho1's involvement in Wg signaling, we examined the effect of loss of Rho1 on Wg signaling using *Drosophila* tissue culture. S2R+ cells express the Wg receptor *D-frizzled2* (*Dfz2*), making them capable of responding to Wg signaling, and are susceptible to RNAi. Treatment of these cells with *Rho1* dsRNA results in the loss of Rho1 protein, while levels of unrelated proteins such as $p120^{ctn}$ are unaffected (Fig. 4.4A). Staining the cells with antibodies to Rho1 also reveals a decrease in Rho1 protein levels with RNAi (Fig. 4.4B, C).

Under Rho1 RNAi conditions, the cells exhibit a multinucleate phenotype indicative of a failure in cytokinesis. Consistent with this, maternal *Rho1* mutant embryos exhibit defects in cellularization (Fig. 4.4D, D'), a specialized form of cytokinesis. These results suggest that Rho1 functions identified in tissue culture are biologically relevant to Rho1 function in the embryo.

4.3.5. S2R+ cells treated with *Rho1* dsRNA cannot respond properly to Wg signaling.

In many systems, Wg signaling results in the cytoplasmic accumulation of β-catenin, which then enters the nucleus, binds transcription factors of the TCF/LEF family and activates transcription from target genes (reviewed in: Barker et al., 2000; Novak and Dedhar, 1999). While S2R+ cells do not express Wg, treatment of S2R+ cells with Wg-conditioned medium (see Methods) results in the cytoplasmic accumulation of Armadillo (Arm), the *Drosophila* homolog of β-catenin (compare Fig. 4.5A and B) (Yanagawa et al., 1998). Treatment of cells with *Rho1* dsRNA attenuates the accumulation of Arm, particularly in those cells exhibiting a multinucleate phenotype (Fig. 4.5C). Another target of Wg signaling in S2R+ cells

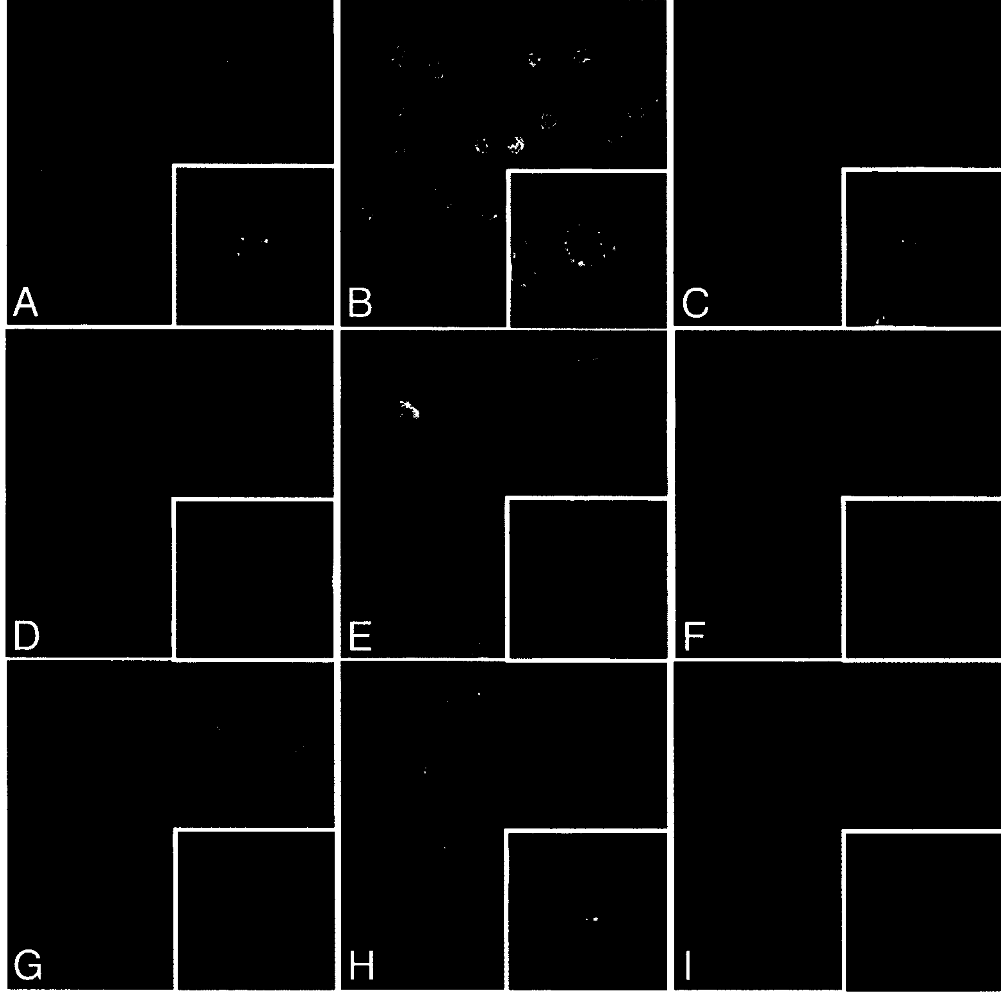


Figure 4.5. S2R+ cells treated with *Rho1* dsRNA do not respond properly to Wg signaling. (A-C) S2R+ cells labeled with antibodies against Armadillo (Arm, green) and PI (red). Untreated, wildtype cells (A) show little Arm staining. Upon treatment with Wg-conditioned medium, cells accumulate cytoplasmic Arm (B). Treatment of cells with *Rho1* dsRNA reduces the amount of this accumulation (C). (D-F) S2R+ cells labeled with antibodies to DE-Cadherin (DE-Cad, green) and PI (red). Similar to Arm, S2R+ cells normally express little DE-Cad (D). DE-Cad expression is upregulated upon treatment with Wg-conditioned medium (E), and this upregulation is largely lost from cells treated *Rho1* dsRNA (F). (G-I) S2R+ cells labeled with antibodies to Wg (green) and PI (red). Upon treatment with Wg-conditioned medium, Wg-positive punctate structures can be seen in wildtype cells (H), but not those treated with *Rho1* dsRNA (I).

is the cell-cell adhesion molecule *DE-Cadherin* (*DE-Cad*; Yanagawa et al., 1998). Prior to Wg treatment, S2R+ cells express little DE-Cadherin (Fig. 4.5D). Upon treatment with Wg-conditioned medium, levels of DE-Cad increase (Fig. 4.5E). Similar to the effect seen with Arm, DE-Cad levels do not increase to the same levels in cells treated with *Rho1* dsRNA (Fig. 4.5F). To determine whether we could observe defects in Wg protein localization in S2R+ cells similar to those we observe in embryos, we stained cells with antibodies to Wg. Cells that are not exposed to Wg-conditioned medium show little staining (Fig. 4.5G). Upon treatment with Wg, punctate staining could be seen in some cells (Fig. 4.5H), presumably due to the internalization of Wg in these cells. Treatment of cells with *Rho1* dsRNA abolishes this punctate staining pattern, particularly in multinucleate cells (Fig. 4.5I). The overall level of Wg staining in dsRNA-treated cells is lower than wildtype, and appears to be more restricted to the cell surface than in wildtype cells.

4.3.6. Cytoplasmic Arm accumulation is aberrant in maternal *Rho1* mutants.

Activation of Wg signaling in the embryo also leads to accumulation of cytoplasmic Arm (Peifer et al., 1994). In stage 9-10 embryos stripes of cytoplasmic Arm accumulation can be seen in cells responding to Wg signaling (Fig. 4.6). These stripes are narrower in *shibire* mutants relative to wildtype or *wimp* controls, due to the failure to form a proper Wg protein gradient (compare Fig. 4.6A,B with Fig. 4.6C; (Bejsovec and Wieschaus, 1995);. Similarly, maternal *Rho1* mutants also exhibit narrower overall stripe width (Fig. 4.6D). Quantitation of the widths of stripes 2-7 indicates that these differences are statistically significant ($n=10$, $p<0.001$ for *shibire*, $n=10$, $p<0.001$ for maternal *Rho1*). The similarity between the *shibire* and maternal *Rho1* mutant phenotypes suggests that the general endocytosis defect seen in maternal

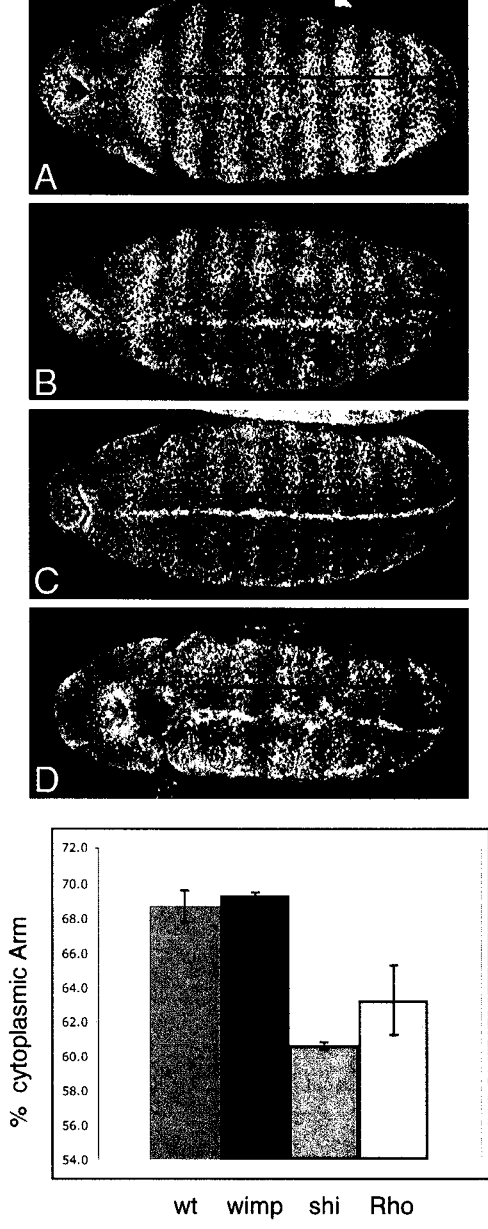


Figure 4.6. Maternal *Rho1* mutants exhibit aberrant cytoplasmic Armadillo accumulation. (A-D) Representative stage 9 wildtype (A), *wimp* (B), *shibire* (C), or maternal *Rho1* mutant (D) embryos stained with antibodies to Armadillo (Arm). Stripes of cytoplasmic Arm accumulate in response to Wg signaling. The widths of stripes 2-7 are indicated by the red dashes. (E) The percentage of the embryo length in this region that consists of cells with elevated levels of cytoplasmic Arm for each of the genotypes listed above. *shibire* and maternal *Rho1* mutants show decreased overall stripe width relative to wildtype or *wimp* controls (n=10, $p<0.001$).

Rho1 mutants, and their aberrant Wg protein distribution, results in the failure to properly activate Wg signaling in the embryo.

4.4. DISCUSSION.

Rho GTPases have been linked to a wide variety of cellular processes, ranging from actin cytoskeletal regulation to cell cycle progression and transcriptional activation (Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002; Hall, 1998; Mackay and Hall, 1998). In this paper we show that the segmentation phenotype associated with loss of maternal Rho1, while on the surface suggests defects in transcriptional activation e.g. the failure to maintain En expression, is due to a general defect in endocytic processes in these mutants. This observation has 2 major consequences: endocytic processes play a major role in governing proper patterning during *Drosophila* development, and the phenotypes resulting from ectopic expression of dn or caRho can result from unexpected biochemical mechanisms.

Our results indicate that maternal Rho1 plays a general role in the regulation of endocytic processes, which is required for the proper activity of a number of signaling pathways during *Drosophila* development. Endocytosis is an important aspect of the regulation of many developmental signaling pathways (Gonzalez-Gaitan, 2003). Initial links between endocytosis and signal transduction were shown to involve the attenuation of signal by internalizing receptor-ligand complexes and targeting them for lysosomal degradation, as in the case of EGF signaling (Dickson et al., 1983). Our observation that there is hyperactive EGF and Torso signaling in maternal Rho1 mutants suggests that Rho1 is involved in this signal attenuation process.

Recent evidence suggests that endocytosis can also play a role in the activation of signaling (Gonzalez-Gaitan, 2003). We find that treatment with Rho1 dsRNA (RNAi) inhibits the ability of S2R+ cells to respond to Wg signaling. In addition, Wg-positive punctate structures are no longer observed in RNAi treated cells, which implies that internalization of Wg protein may be important in the activation of downstream signaling events, at least in this context. Activation of signaling downstream of EGF

has been shown to require endocytosis in some cell types, with different scaffold complexes forming on the endosomal compartment versus the plasma membrane (Vieira et al., 1996). This could be important in providing output specificity, with signaling from each compartment resulting in distinct downstream events. There is currently no data suggesting that Wg signal transduction occurs from endosomes in this manner, however. Indeed, the observations that Wg signaling occurs in *shibire* mutants, albeit in a restricted domain, and that mutant forms of Wg that cannot activate signaling are still internalized into vesicular structures argue against the idea that endocytosis is required for the transduction of Wg signaling (Bejsovec and Wieschaus, 1995).

Endocytic processes are also involved in the dispersal of ligands (Entchev et al., 2000; Greco et al., 2001; Strigini and Cohen, 2000). This is particularly important in the context of morphogen gradient formation, both for morphogens that act long range such as Dpp, as well as short range morphogens such as Hedgehog (Hh) and Wg. In the *Drosophila* embryo, the presence of Wg protein in vesicular structures spreading out from cells that actively express it indicates the importance of endocytosis in the formation of the Wg protein gradient. The lack of Wg-positive vesicular structures observed in maternal Rho1 mutants suggests that the formation of a proper Wg gradient is crucial for proper regulation of En expression, and that Rho1 is important in mediating this effect (see Fig. 4.7). Whether these vesicles are primarily involved in transcytosis of Wg protein or targeting of Wg to lysosomes for degradation is still controversial. During wing development in *Drosophila*, Wg protein has been found in vesicular structures termed argosomes, which have been proposed to act as shuttles carrying Wg protein from cell to cell (Greco et al., 2001). It is not known whether the vesicles seen in the embryo are argosomes. Interestingly, it has recently been reported that *hrs*, which is generally involved in regulating multi-vesicular body formation during early *Drosophila* development, also has effects on Wg signaling levels (Gonzalez-Gaitan and Stenmark, 2003).

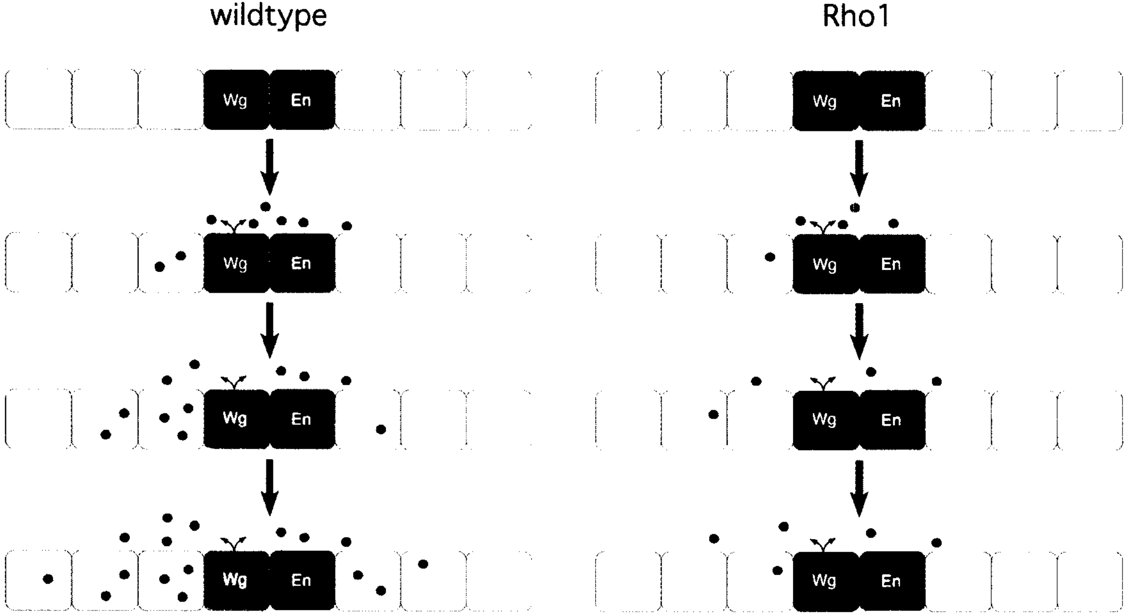


Figure 4.7. Schematic representation of the effects of Rho1 on Wg protein distribution. In wildtype embryos (left), Wg (green) is produced in Wg-expressing cells and spreads outward from these cells as development proceeds (top to bottom). Wg can be found both in the extracellular space and in vesicular structures within cells. In *Rho1* mutants (right) the number of vesicles is reduced, and the Wg protein gradient does not form properly.

Wg activates signaling through its receptor Dfz-2. Both genetic and molecular evidence has placed Rho downstream of fz receptors in other contexts. During planar cell polarity in *Drosophila*, for instance, reduction of Rho1 levels is able to partially suppress a rough eye phenotype caused by overexpression of Fz (Strutt et al., 1997). Wnt/Fz signaling during gastrulation in *Xenopus* activates Rho and Rac, and this is important to properly regulate the convergent extension cell movements required for gastrulation to proceed (Habas et al., 2003). These observations, together with our data, suggest that Rho1 may generally act downstream of Wnt/Fz signaling.

The biochemical mechanisms through which Rho proteins affect endocytosis and membrane trafficking are currently unclear. One possibility is that the function of Rho1 in this process is a byproduct of its regulation of the actin cytoskeleton. In yeast there is evidence that the actin cytoskeleton is important in endocytosis, as mutations in actin and some actin-binding proteins inhibit endocytosis (Lanzetti et al., 2001; Munn, 2001). In mammalian cells, treatment with pharmacological agents that perturb actin structure can affect endocytosis in a cell type specific way. In polarized epithelial cells, for example, treatment with the actin-depolymerizing drug cytochalasin D inhibits endocytosis specifically at the apical surface, but not the basolateral (Gottlieb et al., 1993). In *Drosophila*, Rho1 has clear roles in actin cytoskeletal regulation during oogenesis and embryogenesis, consistent with the notion that Rho1 may be acting primarily through actin (Magie et al., 1999). Alternatively, Rho proteins could interact directly with components of the endocytic machinery. In the case of Cdc42, its effector Ack binds to clathrin. While overexpression of Ack results in only mild effects on the internalization of EGFR, it may also be involved in trafficking of EGFR-containing endosomes to the lysosome (Teo et al., 2001). Future studies aimed at determining the precise molecular mechanisms underlying the regulation of endocytosis by Rho1 will be instrumental in answering this question.

Our observation that the segmentation phenotype is the result of an endocytosis defect and not a primary effect on transcriptional activation has important implications

for the interpretation of data linking Rho to disparate cellular processes. Rho may in fact have a small number of primary functions, and many of the process it has been linked to are secondary effects. Rho was initially thought to act upstream of a kinase cascade leading to transcriptional activation in much the same way its relative Ras is involved in activating MAPK signaling. The search for a pathway regulated by Rho led to data linking Rho to the activation of transcription by serum response factor (SRF), a MADS-box transcription factor that regulates expression of growth factor-responsive immediate-early genes (Arsenian et al., 1998). However, recent work has shown that levels of G-actin in the cell are instrumental in determining levels of transcription from this promoter, as drugs that perturb actin cytoskeletal organization affect transcription from an SRF-responsive promoter (Sotiropoulos et al., 1999). Rho has been shown to influence transcription by SRF through signaling pathways leading to the actin severing protein cofilin and the formin homology protein mDia1, a member of a class of proteins shown to regulate the actin and microtubule cytoskeletons (Geneste et al., 2002), indicating that the primary function of Rho in this context is regulation of the actin cytoskeleton and the transcriptional effects are secondary. Additionally, mechanical deformations have been shown to affect gene expression, both in isolated tissue culture cells (Wasserman et al., 2002) as well as *Drosophila* embryos (Farge, 2003). This suggests that the regulation of cell morphology through the actin cytoskeleton is another context in which proteins such as Rho that regulate actin dynamics could have profound, but indirect, effects on transcription. Current data cannot exclude the possibility, however, that Rho directly acts in transcriptional activation or through many disparate mechanistic pathways. Indeed, the number of Rho effectors identified to date argues for Rho's involvement in multiple pathways (reviewed in Bishop and Hall, 2000). In either case, identifying the molecular mechanisms underlying each of Rho's activities is crucial. Investigations of Rho GTPase function in genetically amenable model organisms are providing a diversity of developmental contexts in which to examine all aspects of Rho biology, and the ability to examine specific, loss-of-function phenotypes are aiding

identification of the mechanisms underlying Rho function. Future studies promise to provide continuing insight into Rho GTPase function.

CHAPTER 5

Targeted mutations within Rho1

5.1. INTRODUCTION.

The Rho GTPase transduces signals linked to a wide variety of cellular functions, including cytoskeletal regulation, adhesion, transcriptional activation and others. The diversity of functions exhibited by Rho suggests that it operates through distinct pathways in particular contexts, a conclusion supported by the multiple phenotypes associated with loss of *Rho1* function during development in *Drosophila* (see Chapters 2 and 3). A number of downstream effectors have been identified that are activated by Rho1 and are responsible for carrying out its functions (see Fig. 1.2), though the mechanisms that allow Rho to specifically activate particular pathways in different developmental contexts remain to be elucidated.

Rho GTPases contain a number of conserved domains that are shared among the various family members and are important for their proper function (Ihara et al., 1998). These include the Phosphate Binding Loop, Effector (or Switch I) and Switch II domains, Loop 6, Insert Helix, and the carboxy-terminal CAAX box (Fig. 5.1). The Phosphate Binding Loop is responsible for nucleotide binding. The Effector and Switch II domains are the regions of the protein that change conformation based on the nucleotide bound, adopting an "active" conformation upon binding of GTP, and reverting to an "inactive" conformation as the GTP is hydrolyzed to GDP. The Effector domain is so named because it is the most well-characterized region of the protein to bind downstream effectors, and is therefore crucial to allow GTP-bound Rho proteins to activate the pathways necessary to carry out their functions (Kozminski et al., 2000; Sahai et al., 1998; Van Aelst and D'Souza-Schorey, 1997). The Loop 6 region has also been shown to be important in mediating the specificity of

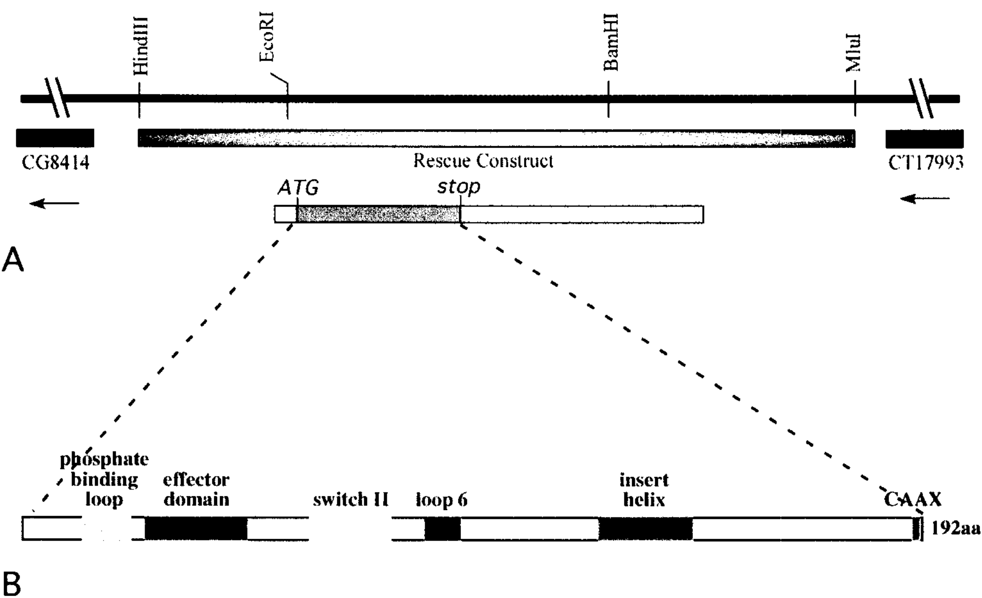


Figure 5.1: *Rho1* locus and protein domains. (A) Diagram of the *Rho1* locus indicating the region used to generate the transgenic described in this study. (B) Line diagram of the Rho1 protein with conserved domains highlighted.

effector binding between Rho family members. The Insert Helix is found only in the Rho subfamily of Ras proteins. Ras, in contrast, possesses a simple loop at the same location (Neuman-Silberberg et al., 1984). Although there is some evidence that the Insert Helix is required for the regulation of Rho GTPases by guanine dissociation inhibitors (GDIs), a class of negative regulators of Rho proteins that act by keeping them in a GDP-bound, inactive state (Wu et al., 1997), structural studies indicate that GDIs bind the face of Rho proteins opposite the Insert Helix (Hoffman et al., 2000). Finally, the carboxy-terminal CAAX box is required for the isoprenylation of Rho proteins, allowing them to be localized to the plasma membrane (Casey and Seabra, 1996). This membrane localization has been shown to be an important aspect of Rho function, as Rho translocates to the membrane upon activation (Takaishi et al., 1995).

Point mutations within the Effector domain have been shown in cell culture to differentially affect its ability to bind particular effectors and carry out particular functions (Sahai et al., 1998). Specifically, mutations have been characterized that affect the ability of mammalian RhoA to form stress fibers, activate transcription through serum response factor (SRF), or both. These mutations have also been shown to attenuate binding to particular effectors, allowing a correlation of effector binding with RhoA functions. An extension of this type of analysis to an organismal context promises to identify which pathways Rho1 acts through in various stages of development and to determine the relative contributions of the domains of Rho1 to its overall function. Because of the high degree of conservation between mammalian and fly Rho proteins (86% amino acid identity overall, 100% identity within the Effector domain; see Fig. 1.4), mutations characterized in mammalian RhoA could in many cases be translated directly to *Drosophila* Rho1. We generated targeted mutations within the Rho1 protein, including point mutations in the Effector domain previously characterized in mammalian systems, the catenin binding sites described in Chapter 3, and the other domains described above. By expressing these altered forms of *Rho1* in a *Rho1* mutant background under endogenous promoter control we can assay the resulting phenotypes and determine which of the null mutant phenotypes are linked to

V14A: constitutively active
N17A: dominant negative
KDQ/A: α-catenin binding

effector domain

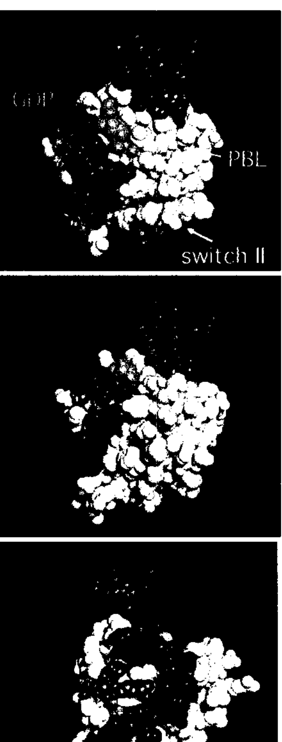
- T37A: Rok, Dia binding
- F39A: actin and transcription
- F39V: actin
- E40N: transcription
- N41A: C3 ribosylation
- Y42C: neither actin nor transcription

KQVE/A: $p120^{ctn}$ binding

D87V,D90A: loop 6

RhoΔRas: insert helix

C189R: isoprenylation



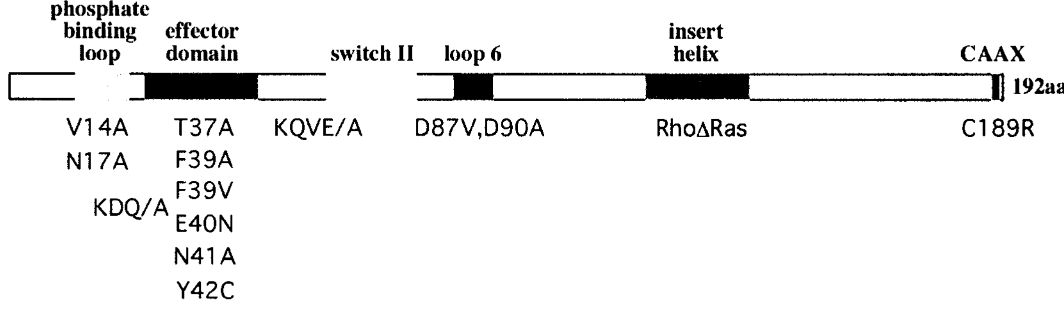


Figure 5.2: Targeted mutations within Rho1. The various mutations generated in Rho1 are listed above, and their positions within the Rho1 protein are indicated below the line diagram. Structural models of human RhoA with the relevant domains highlighted are indicated for reference.

each of the point mutants. This should indicate which pathways Rho is acting through at various times in development. In addition, *in vivo* data can be compared to the cell culture studies in the literature to gain insight into the differences between experimental results obtained in cell culture versus organismal contexts. As an example of this, an EMS-induced *Rho1* allele exists whose molecular lesion is a point mutation in the isoprenylation site required for plasma membrane localization (Halsell et al., 2000). In cell culture experiments this protein is partially functional. In embryos, however, the phenotype that results is very similar to null alleles.

5.2. RESULTS.

5.2.1. Construction of the Rho1 rescue construct.

In preparation for the generation of targeted mutations in Rho1, a rescue construct was constructed. To do so, a 7kb 5' HindIII, 3' MluI fragment of genomic DNA (see Fig. 5.1) containing the *Rho1* locus was subcloned into a version of the pCasper4 transformation vector lacking BamHI and EcoRI sites in the polylinker. The 3.7 kb 5' EcoRI, 3' BamHI fragment within the coding region was excised and replaced with the corresponding 1.2 kb fragment from the *Rho1* m7 cDNA. This resulted in the generation of a cassette that could be mutated as desired and easily replaced.

5.2.2. Point mutations target multiple domains of the Rho1 protein.

The point mutations chosen for analysis in the way outlined above include mutations identified in the literature as well as the catenin binding site mutants from my own work (see Chapter 3). The various domains of the Rho1 protein as well as the mutations generated in this study are shown in Figure 5.2.

Phosphate binding loop mutations. Within the Phosphate binding loop, V14A results in a constitutively active protein that cannot hydrolyze GTP, whereas N19A yields a dominant-negative form of Rho1 with decreased affinity for GTP.

Effector domain mutations. 6 mutations were made within the Effector domain. T37A abrogates binding of the Rho1 effector Rho-kinase (Rok) (Winter et al., 2001). N41A alters the residue upon which Rho is ADP-ribosylated by the C3 exoenzyme, a bacterial toxin that specifically inactivates Rho (Faure et al., 1999; Sekine et al., 1989). The 4 others were shown to compromise a subset of RhoA functions in mammalian tissue culture (Sahai et al., 1998). F39A attenuates RhoA's ability to both form stress fibers and activate transcription from the SRE. F39V affects stress fiber formation alone, E40N affects only SRE activation, and Y42C affects neither.

Loop 6 mutation. The Loop 6 mutation (D87V, D90A) is a conversion of Rho residues 87 and 90 to their Rac counterparts and results in loss of binding to the Rho-binding motifs of the Rho effectors PKN and ROCK (Zong et al., 1999).

Insert Helix mutation. There is some evidence that the Insert Helix domain is required for regulation of Rho proteins by GDIs (Wu et al., 1997). Ras proteins, unlike their Rho relatives, are not regulated by GDIs and do not possess an Insert Helix domain (Symons and Settleman, 2000). Instead that region of Ras is comprised of a simple loop. To investigate the role of the Insert Helix domain in Rho1 function, this domain was replaced with the loop region of *Drosophila* Ras.

Isoprenylation site mutation. Finally, the carboxy-terminal CAAX box, which is necessary for isoprenylation of Rho and its subsequent insertion into the plasma membrane, was disrupted with the C189R mutation (Takaishi et al., 1995).

Protein binding domain mutations. The KDQ mutation located just N-terminal to the Effector domain abolishes α-catenin binding, as described in Chapter 3. The KQVE mutation disrupts binding of Rho1 to $p120^{ctn}$, as described in Chapter 3.

5.2.3. Generation of Rho1 point mutant constructs.

Altered forms of the Rho1 protein containing the various point mutants were constructed by designing internal primers that contain the mutation of interest, then incorporating the mutation into a full-length form of the Rho1 protein through 2

Table 5.1. Primers used to generate targeted Rho1 mutants.

mutant			internal primers	
V14A	fwd	5'	GTCGGCGACGTTGCCTGCGGT	3'
	rev	5'	ACCGCAGGCAACGTCGCCGAC	3'
N19A	fwd	5'	CAGAAGGCGATTTTTACCGCA	3'
	rev	5'	TGCGGTAAAAATTGCCTTCTG	3'
KDQ	fwd	5'	GTCTTCAGCGCAGCTGCGTTCCCCGAG	3'
	rev	5'	CTCGGGGAACGCAGCTGCGCTGAAGAC	3'
T37A	fwd	5'	TATGTGCCCGCCGTATTCGAG	3'
	rev	5'	CTCGAATACGGCGGGCACATA	3'
F39A	fwd	5'	GTGCCCACCGTAGCCGAGAATT	3'
	rev	5'	ATTCTCGGCTACGGTGGGCAC	3'
F39V	fwd	5'	GTGCCCACCGTGAGTCGAG	3'
	rev	5'	CTCGACTACGGTGGGCAC	3'
E40N	fwd	5'	ACCGTATTCAACAATTAT	3'
	rev	5'	ATAATTGTTGAATACGGT	3'
N41A	fwd	5'	GTATTCGAGGCTTATGTCGCC	3'
	rev	5'	GGCCACATAAGCCTCGAATAC	3'
Y42C	fwd	5'	GAGAATTGTGTGGCCGAG	3'
	rev	5'	GTCGGCCACACAATTCTC	3'
KQVE	fwd	5'	GTGGATGGCGCAGCGGCGGCGCTGGCCTTG	3'
	rev	5'	CAAGGCCAGCGCCGCCGCTGCGCCATCCAC	3'
D87V,D90A (loop 6)	fwd	5'	TCAGTGGTTTCACCCGCTTCGCTA	3'
	rev	5'	TAGCGAAGCGGGTGAAACCACTGA	3'
Insert	5	5'	CACCTGCTGCTGGTGCTTCAAATCTTTCTT	3'
	3	5'	CACCAGCAGCAGGTGTCCCAGGAGGGTCGC	3'
C189R	fwd	5'	AAGAAGACCAGACGCCTTTTG	3'
	rev	5'	CAAAAGGCGTCTGGTCTTCTT	3'

rounds of PCR. The internal primers are summarized in Table 5.1. The external primers used correspond to the 5' EcoRI site and 3' BamHI site in the *Rho1* m7 cDNA. Once this 1.2 kb fragment was obtained for each mutant, they were cloned into the endogenous *Rho1* locus that was previously subcloned into the pCasper4 transformation vector.

5.2.4. Germline transformation.

pCasper4 constructs were injected along with the pTURBO helper plasmid into isogenic w^{1118} flies. Only insertions present on the 2nd chromosome were kept for recombination onto a *Rho1* null chromosome (Table 5.2). Interestingly, no transgenic lines were recovered for the V14A or N19A mutations, and in fact the injection of embryos with these constructs resulted in almost complete lethality. This is consistent with the dominant effects these forms of Rho have been shown to have. Additionally, this suggests that the other mutations made for this study do not exert dominant effects of their own.

5.2.5. Generation of a Rho1 null allele.

In order to generate a null allele of *Rho1*, the P-element insertion $Rho^{l(2)k02107}$ was excised. Imprecise excision events that resulted in embryonic lethality were balanced and kept as stocks for further analysis. Staining of embryos with the P1D9 antibody was used to determine whether any Rho1 protein was made, and Southern analysis was used to determine the extent of the excision. One allele, $Rho1^{1b}$, was determined to lack Rho1 protein. Southern analysis indicated an excision of approximately 3kb, which removed the majority of the Rho1 coding region.

5.2.6. Recombination and verification of recombinants.

Homozygous insertion lines for the mutations listed in Table 2 were recombined onto the chromosome carrying Rho^{1b}.

Table 5.2. 2nd chromosome transformant lines and putative recombinants. Transformant lines are designated by the male (m) or female (f) from which the line was started. Those recombinants tested for the presence of the wt locus are indicated.

Transformants		Recombinants		
mutant	2nd chr. Lines	line	hz?	wt allele
wt	m4	5)1b wt-m5	yes	yes
	m5	D)1b wt-m5	yes	no
	m7	A)1b wt-m7	yes	no
	m9	B)1b wt-m7	yes	no
	f2	C)1b wt-m7	yes	no
T37A	m2	D)1b wt-m7	yes	no
	m6	A)1b wt-m9	yes	no
	m8	D)1b wt-m9	no	
F39A	m1	B)1b KDQ-m4	no	
	m2	D)1b KDQ-m4	yes	yes
	m3/CyO	C)1b KDQ-m4	no	
	m4/CyO	C)1b KDQ-f2	no	
	f4	D)1b KDQ-f2	no	
	f5/CyO	A)1b T37A-m6	yes	
F39V	m4	C)1b T37A-m6	yes	
	m6	A)1b T37A-m8	yes	
	m8	B)1b T37A-m8	yes	
	m10	A)1b F39A-m1	yes	
	m12	C)1b F39A-m1	yes	
E40N	m7/CyO	D)1b F39A-m1	yes	
	m9/CyO	B)1b F39A-m2	no	
	m10	C)1b F39A-m2	yes	
	f1	D)1b F39A-m2	no	
	f10	A)1b F39A-f4	yes	
N41A	m6	B)1b F39A-f4	no	
	m8	A)1b F39V-m8	no	
	m10	A)1b F39V-m10	no	
	f6	D)1b F39V-m10	yes	
Y42C	m4/CyO	B)1b F39V-m12	no	
	m8	D)1b E40N-m10	yes	
KDQ	m4	C)1b E40N-f1	yes	
	m7/CyO	B)1b E40N-f10	yes	
	f2	C)1b N41A-m6	yes	
KQVE	m3	A)1b N41A-m8	no	
	m5	D)1b N41A-m8	no	
	m9	D)1b N41A-m10	yes	
	m12	C)1b Y42C-m8	yes	
	m14	D)1b Y42C-m8	no	
L6	m2	A)1b KQVE-m3	no	
	m5	B)1b KQVE-m3	no	
	f3	C)1b KQVE-m3	yes	yes
I	m4	A)1b KQVE-m5	yes	yes
	m5	B)1b KQVE-m5	no	
	f2	C)1b KQVE-m5	yes	yes
C189R	m4/CyO	D)1b KQVE-m14	no	
	m7	C)1b C189R-m7	yes	
	m8	D)1b C189R-m7	no	
	m9	A)1b C189R-f9	no	
	f9	B)1b C189R-f9	yes	
		C)1b C189R-f9	no	
		D)1b C189R-f9	yes	

Recombinants were scored for the presence of the transgene insertion by eye color. To determine whether recombinants that could be made homozygous possessed the wildtype allele, the forward Loop 6 and reverse C189R primers were used to perform PCR. Southern analysis indicated that this region of the locus is absent from the *Rho^{1b}* allele, so there should be no product resulting from it. The wildtype locus would result in a fragment of ~900bp, and the cDNA insertion would result in a fragment of ~300bp. Homozygous putative recombinant lines carrying wildtype, KDQ and KQVE inserts were assayed by this method, with the results shown in Table 2. Briefly, 6 of the 7 homozygous lines carrying the wildtype insertion were determined to lack the wildtype allele, whereas the 1 KDQ and 3 KQVE homozygous lines did possess the wildtype locus. The likely reason other putative recombinant lines cannot be made homozygous is that they possess the *Rho^{1b}* allele.

An analysis of these targeted mutations within Rho1 is just beginning. Preliminary data from one of the KQVE lines, which disrupt p120^{ctn} binding to Rho1, show different phenotypes from the *Rho1* mutant alone (Susan Parkhurst, personal communication), indicating that this will be a viable approach.

5.3. DISCUSSION.

Rho GTPases have been implicated in a wide variety of cellular processes, largely through studies done in tissue culture. These include such seemingly disparate processes as actin cytoskeletal regulation, transcriptional activation, cell cycle progression, and cellular adhesion. It remains an open question, however, whether Rho is directly involved in all of the functions it has been linked to or whether the effects seen in cell culture are secondary consequences of a primary function of Rho, for example in actin cytoskeletal regulation. Analysis of the various point mutants described here should address this question by identifying whether Rho acts through multiple pathways in various developmental contexts. If the phenotypes resulting from the various mutations are distinct, this would indicate that compromising Rho's ability to activate particular pathways has different effects depending on the

developmental context. In contrast, if all mutations result in the same phenotypes, this would suggest that any reduction of Rho1 function results in the same outcome and argue for Rho1's involvement in one major pathway. In addition, the data already present in the literature regarding the abrogation of effector binding to some of these mutants, particularly the catenin binding sites and those in the effector domain, will provide a good starting point to identifying precisely which pathways Rho1 is using in different contexts. For instance, an investigation of the RhoA Effector domain mutants indicated that the E40N and Y42C mutations, which bind the effector Rho-kinase (ROCK), are able to form stress fibers normally. F39A, however, which cannot bind ROCK, is not. Interestingly, the F39V mutation, which is capable of binding ROCK, is not capable of forming stress fibers, suggesting that one or more effectors in addition to ROCK are involved in stress fiber formation (Sahai et al., 1998). In *Drosophila* we have the added advantage of genetics to assist in unraveling pathways downstream of Rho1, and can use mutations in particular effectors to verify results obtained with the point mutations.

An example of the effects point mutations within Rho1 can cause, and a control for one of my targeted mutations, is provided by the $RhoA^{E3.10}$ allele (Halsell et al., 2000). This is a single amino-acid substitution induced by EMS that results in a conversion of aa189 from cysteine to tyrosine, disrupting the C-terminal CAAX box. Because this is the same effect predicted for the point mutant C189R, a comparison of the phenotypes resulting from C189R and $RhoA^{E3.10}$ is a useful control for the functionality and expression of the altered forms of Rho1. Analyses of the $RhoA^{E3.10}$ phenotypes to date indicate that they are very similar to strict loss-of-function phenotypes, suggesting that the subcellular localization mediated by isoprenylation at the CAAX box is crucial to the majority of Rho1 functions (Halsell et al., 2000). This is in contrast to data from tissue culture experiments, in which mutation of the CAAX box results in a form of RhoA that does not localize to the plasma membrane but is partly functional in that it is still capable of forming stress fibers (Kranenburg et al., 1997). In addition, prenylation of mammalian RhoB is not required for its activation of transcription from

SRF-responsive promoters (Lebowitz et al., 1997). It is this type of structure-function information that will result from the analysis of the other mutations and will provide important insight into the function of Rho1 in various developmental contexts.

CHAPTER 6

Conclusions and Perspectives

The mammalian Rho family consists of many family members. Since it was once thought that they were likely to act redundantly within the family (i.e. Rho, Rac, Cdc42) or subfamily (i.e. RhoA, B, C), mouse knockouts were not made. Therefore, the initial characterization of Rho GTPases was conducted in tissue culture cells utilizing overexpression of dominant-negative (dn) or constitutively-active (ca) forms of these proteins (Hall, 1998; Mackay and Hall, 1998; Narumiya, 1996; Van Aelst and D'Souza-Schorey, 1997). In this context, the dn form was considered equivalent to the loss-of-function. Studies of this sort provided a great deal of information into the biochemical mechanisms through which they act, and aided in identifying a number of interacting molecules (Bishop and Hall, 2000). Similarly, initial investigations into Rho function in organismal contexts also utilized ectopic expression of these dominantly-interfering forms of Rho proteins (Harden et al., 1995; Harden et al., 1999; Murphy and Montell, 1996). At the time this work was begun, Rho1 was the only Rho family member for which there was a specific loss-of-function mutation, making it an attractive target of study. Examination of loss-of-function phenotypes promised to identify those functions Rho1 was specifically required for, and for which no other small GTPase could substitute. Interestingly, the loss-of-function phenotypes in *Drosophila* are not identical to those resulting from overexpression of dnRho1, a finding that has important implications for the interpretation of studies based on overexpression of dominantly-interfering Rho proteins.

In *Drosophila*, expression of dnRho1, dnRac1 or dnCdc42 during embryogenesis all result in a dorsal-open phenotype indicative of failure to complete dorsal closure (see Fig. 1.4; Harden et al., 1999). *Rho1* and *cdc42* loss-of-function mutants,

however, do not exhibit this phenotype (Genova et al., 2000; Magie et al., 1999). *Rho1* mutants do have defects in dorsal closure, but are not severe enough to result in the complete failure indicated by the dorsal-open phenotype. *cdc42* mutants exhibit a number of morphogenetic defects, particularly in germband retraction and epithelial organization, but also do not exhibit dorsal-open phenotypes. Mutants lacking function of all three *Rac* genes, however, do fail in dorsal closure (Hakeda-Suzuki et al., 2002). Additionally, Rho proteins have been suggested to act upstream of Jun N-terminal kinase (JNK) pathway activation in dorsal closure based on genetic evidence utilizing ectopic expression (Glise and Noselli, 1997). This has also been suggested through analysis of the loss-of-function mutants to be an effect of Rac, but not Rho1 or Cdc42 (Genova et al., 2000; Magie et al., 1999). Thus the dnRho and dnCdc42 phenotypes likely represent a cross-inhibition of Rac signaling pathways.

During the course of our characterization of the *Rho1* mutant phenotypes, we utilized a candidate-gene approach to identify genes that interacted with *Rho1*, with the goal of identifying pathways through which Rho1 was acting during *Drosophila* development (Magie et al., 1999). We based our selection of likely candidates on published data from other systems linking Rho to particular functions. Surprisingly, we only found two interactors, the Gα-like gene *concertina* (*cta*) and the formin homology domain gene *cappuccino* (*capu*), out of more than a hundred tested. While the lack of a genetic interaction does not necessarily indicate that two genes do not act together, our failure to find interactions between *Rho1* and genes identified as likely interactors based on studies utilizing overexpression of dominant-interfering Rho proteins argues that the pathways identified through those studies and analysis of loss-of-function phenotypes may not be the same. Since our initial characterization of these interactions, other members of the lab have conducted a screen using a series of deficiencies that collectively delete approximately 80% of the genome, and have identified roughly twenty dominantly-interacting deletions, suggesting that our failure to see genetic interactions with the candidate genes tested was not simply because of sensitivity issues. The identification of regions of the genome that interact with Rho1

will allow future work to be aimed at determining which genes within those regions are specifically responsible for the interaction, which will allow us to begin building pathways through which Rho1 is acting in *Drosophila*. Taken together, these observations suggest that dominant-negative versions of Rho proteins are not as specific in their effects as has been assumed, and it is likely that they result in the cross-inhibition of multiple small GTPase pathways. The basis for this effect may involve the binding and sequestration of GEFs, some of which have been shown to affect more than one Rho signaling pathway (Kjoller and Hall, 1999).

Loss of Rho1 results in both maternal and zygotic phenotypes. Maternally *Rho1* mutants exhibit defects in actin cytoskeletal organization during oogenesis, and a segmentation defect in the embryo (Magie et al., 1999). Our identification of genetic interactions with *capu* and *cta* indicate that these genes are acting with Rho1 to regulate actin cytoskeletal structure in the oocyte, as females doubly heterozygous for *Rho1* and either *cta* or *capu* show defects in actin cytoskeletal organization in the ovary. Work in the lab investigating the nature of the interaction between *capu* and *Rho1* has shown that *capu* acts downstream of Rho1, and is important in cytoplasmic streaming events during oogenesis crucial for the proper localization of embryonic determinants. Cta is a Gα-protein, a member of a class of molecules involved in transducing signals from transmembrane receptors, and presumably acts upstream of Rho1 (Parks and Wieschaus, 1991). Our observation that Rho1 can bind directly not only to Capu but also to Cta has important implications regarding the mechanisms of the signaling events acting upstream of Rho proteins. Future work clarifying the nature of this interaction will provide insight into the biochemical details of these signaling events.

The maternal segmentation phenotype, with its disruption of the segmentation gene transcriptional cascade, appears superficially to indicate a role for Rho1 in transcriptional activation. Upon closer examination, however, we determined that this phenotype is actually due to a general defect in endocytic processes. This observation has important implications for Rho biology in general, as Rho has been linked to a

wide variety of cellular functions, though how directly it affects each of them is in many cases an open question. The mechanisms through which Rho proteins affect endocytosis and membrane trafficking are currently unclear. One possibility is that the function of Rho1 in this context is a byproduct of its regulation of the actin cytoskeleton. In yeast there is evidence that the actin cytoskeleton is important in endocytosis, as mutations in actin and some actin-binding proteins inhibit endocytosis (Lanzetti et al., 2001; Munn, 2001). In mammalian cells, treatment with pharmacological agents that perturb actin structure can affect endocytosis in a cell type specific way. In polarized epithelial cells, for example, treatment with the actin-depolymerizing drug cytochalasin D inhibits endocytosis specifically at the apical surface, but not the basolateral (Gottlieb et al., 1993). Alternatively, Rho1 could be acting directly on the endocytic machinery itself. Future studies addressing the biochemical mechanisms of Rho1 function in this context will resolve this question.

The primary defect associated with loss of zygotic Rho1 is the failure of head involution. This process involves highly complex cell movements and shape changes, in addition to morphogenetic cell death. Rho proteins have been linked to apoptosis primarily through their regulation of the cell shape changes associated with it, with Rho playing a role in the contraction of dying cells while Rac and Cdc42 are involved in the engulfment process (Coleman and Olson, 2002). Regulation of the cell shape changes necessary for head involution is also likely to involve the regulation of actin-myosin interactions and the formation of contractile structures, suggesting that this may be the primary function of Rho1 in this context.

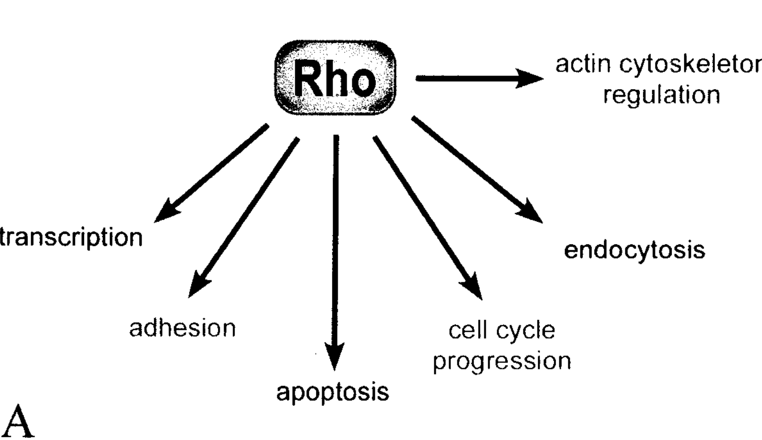
Rho1 mutants also exhibit dorsal closure defects, though these do not completely abrogate the ability of these embryos to close their dorsal hole. Regulation of the actin cytoskeleton is important in this context as well, as Rho1 is required for the proper formation of the actin cable at the leading edge of cells undergoing dorsal closure (Harden et al., 1999; Jacinto et al., 2002a; Magie et al., 1999). In addition, we find that DE-Cadherin localization is disrupted in *Rho1* mutants, suggesting a role for Rho1 in regulating cell-cell adhesion during DC (Magie et al., 2002). We identified direct

physical interactions between Rho1 and the adherens junction components, α-catenin and p120^{ctn}. The association between Rho1 and α-catenin suggests that α-catenin may be involved in either recruiting or tethering Rho1 to adherens junctions where it can then carry out its downstream functions. To investigate this relationship in more detail we have made targeted mutations within *Rho1* that disrupt α-catenin binding and generated transgenic flies in which this form of Rho1 is expressed under its endogenous promoter. Analysis of the phenotypes associated with this mutant promises to clarify the functional roles this binding event has in overall *Rho1* function.

p120^{ctn} has been shown to negatively regulate Rho in mammalian cells by keeping it in a GDP-bound, inactive state (Anastasiadis et al., 2000). Consistent with this, p120^{ctn} binds preferentially to GDP-Rho1, whereas α-catenin binding to Rho1 is nucleotide-independent. We found that inhibition of p120^{ctn} function through RNAi results in the accumulation of Rho1 protein near the leading edge of embryos undergoing DC. In addition, we observed significant morphogenetic defects in embryos injected with p120^{ctn} dsRNA. Since our study was published, a loss-of-function mutant in p120^{ctn} has been described (Myster et al., 2003). Interestingly, this mutant is reported to be viable, though reducing the gene dose of DE-Cad in a *p120^{ctn}* mutant background significantly reduces this viability. *p120^{ctn}* also interacts genetically with *armadillo*, the *Drosophila* β-catenin homolog. The fact that RNAi and genetic mutant phenotypes are not identical suggests that the method used to remove the function of some molecules is important in determining the effect that removal will have. In the case of p120^{ctn}, perhaps removal through genetic deletion allows the organism to compensate for its loss, whereas catastrophic removal through injection of dsRNA disrupts complexes that were constructed in the presence of p120^{ctn} and are therefore unable to function in its absence. Future work clarifying the details of this effect will be useful not only to provide insight into Rho and p120^{ctn} biology, but also in investigating the effects of gene abrogation by RNAi.

Similar to α-catenin, we have also made a targeted mutation within Rho1 that disrupts p120^{ctn} binding. Other labs have shown that mutation of the p120^{ctn} binding site in DE-Cadherin is dispensable for DE-Cad function (Pacquelet et al., 2003). Disruption of the p120^{ctn} binding site in Rho1, however, results in its inability to rescue *Rho1* mutants to viability. An analysis of the phenotypes associated with this version of Rho1 that cannot bind p120^{ctn} will be instrumental in identifying the processes that require the association between these proteins.

Rho has been linked to a large number of cellular processes. A question central to an accurate understanding of Rho biology, however, is how directly it affects each of these processes. Evidence is accumulating that Rho may in fact have one or two major functions, with an ability to affect others secondarily (Fig, 6.1). The ability of Rho to influence transcriptional activation, for example, is due to its effects on actin cytoskeletal regulation. Rho is a member of the Ras superfamily. Ras proteins have been shown to directly activate signaling downstream of receptor tyrosine kinases (RTKs), leading to activation of mitogen-activated protein kinase (MAPK) signaling pathways (Marshall, 1995). The similarity of Rho to Ras initially led researchers to look for the MAPK pathways they suspected were downstream of Rho, leading to the observation that Rho activity is necessary for the activation of transcription by the serum response factor (SRF; Hill et al., 1995). Difficulty in identifying the specific pathway leading from Rho to SRF, however, led Richard Treisman's group to show that Rho was affecting SRF function through its regulation of the actin cytoskeleton (Sotiropoulos et al., 1999). Specifically, SRF responds to the levels of G-actin in the cell, modulated by the Rho effectors mDia1 and ROCK. Studies investigating the links between Rho and cell cycle control have also implicated actin cytoskeletal regulation as the underlying mechanism. Disruption of stress fibers in fibroblasts results in a G_1 arrest due to the failure to sustain ERK signaling and hence CyclinD1 induction (Roovers and Assoian, 2003). Thus, the link between Rho and transcriptional activation may well be based solely on cytoskeletal mechanisms.



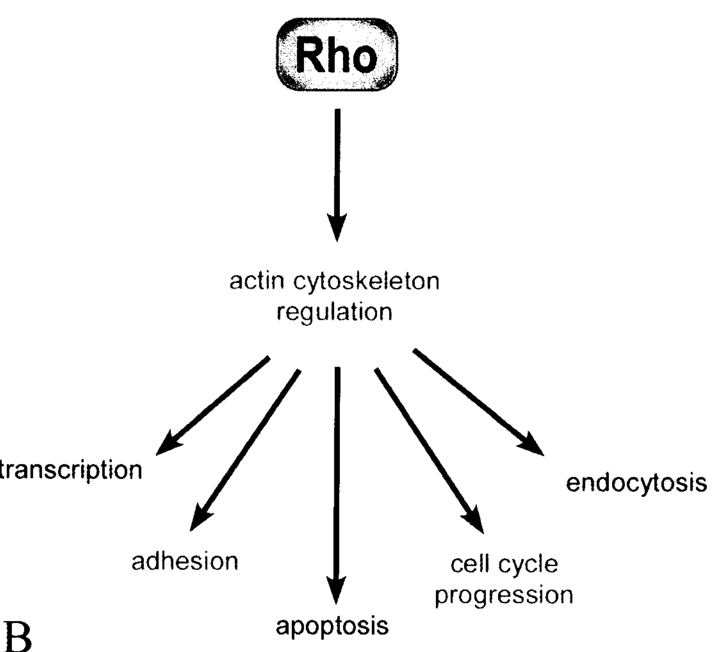


Figure 6.1. Possible mechanisms of Rho function. (A) Rho acts directly through a number of distinct signaling pathways to carry out its downstream functions. Actin cytoskeletal regulation is one function among many. This is the classical view of Rho function. (B) Rho acts primarily as a regulator of the actin cytoskeleton, and its links to other cellular functions are indirect. Data is accumulating that makes this scenario more likely.

The identification of a number of effector molecules that act downstream of Rho has been thought to indicate that Rho acts through distinct pathways in particular contexts. The function of these effectors, however, can in many cases be traced back to actin cytoskeletal regulation (see Table 1.2). ROCK is important in regulating actin/myosin interactions (Kimura et al., 1996). PKN is involved in DC and has been implicated in actin cytoskeletal regulation, cell adhesion and vesicle transport (Lu and Settleman, 1999; Mukai, 2003). Citron kinase localizes to the cleavage furrow of dividing cells and is involved in regulating cytokinesis, a process that also requires actin/myosin interactions (Yamashiro et al., 2003). Dia and Capu, as members of the formin homology class of proteins, can regulate both actin and microtubules (Wasserman, 1998). Rhophilin has recently been shown to affect actin cytoskeletal function (Peck et al., 2002). PI4,5K could also have effects on actin cytoskeletal function by producing $PI4,5P_2$, which can then uncap gelsolin from actin to allow filament elongation or promote a conformational change in vinculin to allow it to tether actin at focal adhesions (Gilmore and Burridge, 1996; Janmey and Stossel, 1987). Phospholipase D has been shown to be involved in stress fiber formation (Kam and Exton, 2001). Taken together, these data, along with our own, suggest that Rho may indeed be acting primarily as a cytoskeletal regulator, and the other functions it has been linked to are secondary consequences (Fig. 6.1).

A cell is a highly integrated system. An overly reductionist approach to investigating any particular part of it can be misleading, as any perturbation can have unintended and unforeseen secondary consequences. A precise determination of the signaling pathways activated by particular proteins and the relationships of those pathways to others in the cell is crucial to determining the biological function of any given molecule. Toward this end, our construction of targeted mutations in Rho1 will assist in identifying the pathways through which Rho1 is acting in particular developmental contexts. It is only with an accumulation of data from many researchers that we are acquiring a comprehensive picture of Rho biology, though we

still have a long way to go before we can truly be said to understand all aspects of how these proteins function.

CHAPTER 7

Methods

Fly Stocks

Flies were cultured and crossed on yeast-cornmeal-molasses-malt extract medium at 25°C. The Rho1 alleles used in this study are: *Rho*$^{l(2)k07236}$, *Rho*$^{l(2)k02107}$ (from I. Kiss, T. Laverty and G. Rubin) *Rho1*^{rev220}*/CyO*, an imprecise P-element excision line (Magie et al., 1999) and *Rho1*$^{E3.10}$*/CyO*, a point mutation within the C-terminal CAAX domain of Rho1 (Halsell et al., 2000). Other alleles used: *cta*^{RC10}, *cta*^{QB37} (S. Parks); *capu*^{RK12}, *capu*^{G7} (L. Manseau); *DRhoGEF2* (K. Barrett); *hep*^{1}, *hep*^{r75} (S. Noselli); *arm*^{XP33}, *arm*^{YD35} (M. Peifer); *puc*^{E69} (A. Martinez-Arias); *bsk*^{1}, *aop*^{IP}, *Egfr*^{E1}, *Egfr*^{F2}, *wg*^{NZ}, *shi*^{1}, *Actin3-Gal4/TM6B*, *en-Gal4*, *UAS-GFP/CyO*, In(2R)*bw*^{Vde2L}*Cy*^{R}/In(2LR) *Gla*, *wg*$^{Gla-1}$ (*p120*^{ctn} deficiency), and *w*^{a} *N*$^{fa-g}$; Df(2R) *Jp8*, *w*$^{+}$*/CyO* (*Rho1* deficiency) (Bloomington Stock Center); *UAS-dRho1* (Harden et al., 1999); *UAS-dRac*^{N17}, *UAS-dRac*^{L89}, *UAS-dCdc42*^{N17}, *and UAS-dCdc42*^{V12} (Murphy and Montell, 1996); *UAS-actin-GFP* (H. Oda). Revertants of the *Rho* alleles were generated as described in Török et al. (1993). One of these, *Rho*^{rev220}, was determined by Southern analysis to remove a portion of the coding region thereby affecting all *Rho1* transcripts.

Embryo/ovary phenotypic analysis

Embryos were prepared and immunohistochemical detection of proteins was performed as described previously (Parkhurst et al., 1990) using Alkaline Phosphatase coupled secondary antibodies (Jackson Labs) visualized with Substrate Kit II reagents (Vector Labs, Inc.), or Alexa 488 or 594 conjugated fluorescent secondary antibodies (Molecular Probes). Ovaries were fixed for 10' in 6% formaldehyde, 16.7 mM KPO_4 (pH 6.8), 75 mM KCl, 25 mM NaCl and 3.3 mM $MgCl_2$, then washed 3X in PBS +

0.1% Triton X-100. Ovarioles were dissected by hand, then blocked in 2% goat normal serum for 2 hours before incubation with primary antibody. Antisera used were as follows: anti-*hb* from D. Tautz; anti-*Kr* from S. Carroll; anti-*eve*, anti-*en* (1:5) and anti-*fasIII* (1:3) from N. Patel and C. Goodman; anti-*ftz* from H. Krause; anti-*wg* (1:100) from R. Nusse and the Developmental Studies Hybridoma Bank; anti-ß-tubulin (N357) from Amersham (1:500); anti-phosphotyrosine (clone 4G10) from Upstate Biotechnology Inc (1:1000); anti-DE-cadherin from H. Oda (1:100); anti-NeurexinIV from H. Bellen (1:15000); anti–α-spectrin from D. Branton (1:500); anti–β-galactosidase from J. Scully (1:1000); anti-Lava Lamp from J. Sisson (1:5000); anti-dpERK from Calbiochem (1:100); anti-Dah from T. Hsieh (1:50); anti-Tll from H. Rennitz (1:100); and anti-Armadillo from M. Peifer and the Developmental Studies Hybridoma Bank (1:100). anti-Rho1 P1D9 monoclonal antiserum was used at 1:50. Embryos were prepared and stained with acridine orange as described by Abrams et al. (1993). Propidium iodide staining was done by incubating embryos in 1 μg/mL propidium iodide and 50 μg/mL RNase for one hour following antibody staining, then washing as described (Parkhurst et al., 1990).

Embryos to be stained with phallodin were fixed for 3 minutes in a 37% formaldehyde/heptane bilayer. Embryos were then incubated in 400uL of DAPI (1 μg/mL) + 1 unit rhodamine- or Alexa 594- conjugated phalloidin (Molecular Probes) for 20 minutes, washed 3X in PBS + 0.025% Tween and mounted in 80% glycerol with 20mM sodium carbonate buffer (pH 9.5) + 4% n-propyl gallate to inhibit photobleaching. Ovaries were dissected by hand, then fixed in 4% formaldehyde in PBS + 0.05% Tween for 20 minutes. Following fixation, ovaries were washed 3X in PBS-Tween, permeabilized in PBS-Tween + 1% Triton X-100 for 1 hour, then stained with 3 units rhodamine- or Alexa 594- conjugated phalloidin in 400 μl DAPI (1 μg/ml) for 1-1.5 hrs.

Immunohistochemical whole-mount in situ hybridization was performed according to the protocol of Tautz and Pfeifle (1989). Digoxygenin-substituted probes were obtained by PCR amplification of the *dpp* cDNA.

Embryos for scanning electron microscopy analysis were fixed in a 25% glutaraldehyde/heptane bilayer for 15 minutes. The vitelline membrane was removed by replacing the glutaraldehyde layer with methanol. Embryos were hydrated in 1x PBS then incubated in 2% osmium (Polysciences) in 1x PBS for 30 minutes. Embryos were dehydrated in an ethanol series then placed in hexamethyldisilazane (HMDS; Ted Pella). The HMDS was allowed to evaporate then the embryos were mounted on carbon black tape and sputter-coated using gold-palladium.

Larval cuticle preparations were prepared and analyzed as described by Wieschaus and Nüsslein-Volhard (1986).

Genomic characterization

Isolation of the genomic DNA flanking the P-element insertion sites was carried out as described by Cooley et al. (1988). These flanking sequences were used to screen an EMBL3 *Drosophila melanogaster* genomic library (from R. Blackman). Restriction enzyme and Southern analyses were used to characterize and compare the DNA flanking the P-elements and of the overlapping genomic phage clones. The DNA flanking the k07236 P-element was also used to screen an ovary (Stroumbakis et al., 1994) and several early embryonic cDNA libraries (Novagen, Inc.).

Genomic and cDNA fragments were subcloned into Bluescript and overlapping subclones were generated using ExoIII nuclease (Henikoff, 1984). Sequencing was carried out manually using Sequenase (United States Biochemical) or with Taq DyeDeoxy terminator AutoSequencing (Applied Biosystems). *Rho1* genomic and three cDNA sequences (*Rho1 m3, Rho1 m7, Rho1 m8*) are available from the GenBank database under accession numbers AF177871, AF177872, AF177873 and AF177874, respectively.

Northern Analysis

Developmentally staged RNAs were prepared as previously described (Mozer et al., 1985). Northern production and hybridization was as described (Mozer et al., 1985), using 5 μg of poly-A^{+} selected mRNA per lane and Magnagraph membrane (Micro Separations Inc.). *DRas* is expressed ubiquitously during development and was used as a loading control (Mozer et al., 1985).

Antibody Production and Characterization

Balb/c BYJ Rb(8.12) 5BNR/J mice (Jackson Labs) were immunized with GST-Rho1. The P1D9 monoclonal line was generated in the FHCRC Hybridoma Production Facility as described (Hoffstrom and Wayner, 1994; Wayner et al., 1989), and has been sent to the Developmental Studies Hybridoma Bank (University of Iowa). Antibody specificity was tested via Western blot using bacterially expressed GST-dRho1, GST-RhoL (obtained by PCR and subcloned into pGEX-3X as a 5'BamHI-3'EcoRI fragment), GST-dRac1 and GST-dCdc42. Wildtype *Drosophila* whole cell (from 0-2hr embryos) and nuclear (from 0-12hr embryos) extracts were a gift from T. Tsukiyama. Polyclonal antiserum against *Drosophila* p120^{ctn} was generated by immunizing Balb/c BYJ Rb(8.12) 5BNR/J mice with a protein comprised of GST fused to the first 222 aa of *Drosophila* p120^{ctn} (CG17484).

In vitro interactions

The full length *cta* ORF (58A, Parks and Wieschaus, 1991) was obtained by PCR then subcloned into the BamHI-EcoRI site of pCite 4c^{+} (Novagen). *capu* was subcloned into the EcoRI-NotI sites of pCite 4a^{+} using the EcoRI-NotI *capu* fragment from the Capu-pEG202 expression plasmid described by Manseau et al. (1996). The Cta and Capu ORFs were expressed from the T7 promoter using the Promega TnT in vitro expression kit (^{35}S-methionine labeled). cDNAs corresponding to *DE-cadherin* (CG3722), *α-catenin* (CG17947), *armadillo* (CG11579), *p120^{ctn}* (CG17484), *rok*

(CG9774) and *RhoGDI* (CG7823) were obtained from the Berkeley *Drosophila* Genome Project (BDGP). The DE-cadherin cytoplasmic domain was amplified by PCR, then subcloned as a 5'BamHI-3'EcoRI fragment into pGEX-3X. A full-length $p120^{ctn}$ clone was generated by overlapping PCR with a truncated cDNA (lacking exon 4) and exon 4 obtained by PCR from genomic DNA. The *α-catenin, armadillo,* and *$p120^{ctn}$* ORFs were subcloned into pCite $4c^+$ (Novagen) as 5'SalI-3'NotI, 5'BamHI-3'XhoI, and 5'SalI-3'NotI fragments, respectively. *rok* and *RhoGDI* ORFs were subcloned into pCite $4a^+$ (Novagen) as 5'BamHI-3'NotI, and 5'BamHI-3'EcoRI fragments, respectively. They were then expressed from the T7 promoter using the Promega TnT in vitro expression kit.

The *Rho1* full-length cDNA was subcloned into the *Bam*HI-*Eco*R1 sites of pGEX-3X (AMRAD Corp.). The GST-Rho1 fusion protein was expressed at 30°C to improve protein solubility. Pieces of Rho1 were amplified by PCR from the Rho1 cDNA (N-term = aa 1-75; C-term = aa 76-192; piece A = aa 1-27; piece C = aa 50-75). Substitutions were made within the full-length Rho1 cDNA using primers that change the codons corresponding to the appropriate amino acids (V14A; N17A; F39A; E40N; KDQ/A, substituting alanines at aa 27-29; KQVE/A, substituting alanines at aa 51-54; RLRP/A, substituting alanines at aa 68-71). The Rho1 pieces and substitutions were cloned into pGEX-3X as 5'BamHI-3'EcoRI fragments. His-$p120^{ctn}$ was generated by subcloning the *$p120^{ctn}$* ORF into pRSetA (Invitrogen) as a 5'XhoI-3'KpnI fragment. The GST-Rho1 protein was incubated with GDP or GTPγS and the binding assays were performed as described by Lu and Settleman (1999), then analyzed by SDS-PAGE. The in vitro translated full-length Hairy protein and GST-dCtBP fusion protein used as a negative control were described previously (Poortinga et al., 1998).

Amino acid residues corresponding to the α-catenin and $p120^{ctn}$ binding domains were modeled on the GTP- and GDP-bound RhoA crystal structure using the Quanta protein modeling program.

Immunoprecipitations

Embryo or ovary lysate was prepared by homogenizing an overnight collection of embryos or dissected ovaries from one bottle of females in 0.5mL L-buffer (PBS + 0.1% NP-40 + protease inhibitors), followed by sonication and centrifugation to pellet debris. Lysate was incubated with primary antibody in 0.5 mL L-buffer for 1hr at 4° C. Protein G sepharose was then added and the reaction allowed to proceed overnight. For DER IPs, lysate was prepared in buffer with a higher concentration of detergent to aid in solubilization: 20mM Hepes pH 7.5, 10% glycerol, 1% Triton X-100, 1mM EGTA, 1.5mM $MgCl_2$, 150mM NaCl +protease inhibitors (aprotinin, leupeptin, PMSF) +phosphatase inhibitors (2mM $NaVO_3$, 2mM NaF). 500μg total protein in 500μL lysis buffer was incubated with anti-DER antibody from N. Baker (1:250) overnight (~15hrs). Protein A agarose was then added and the reaction allowed to proceed ~6hrs. Analysis was conducted using SDS-PAGE followed by Western blots. Quantitation was performed using ImageQuant (Amersham).

RNA interference (RNAi) in embryos

$p120^{ctn}$, α-catenin and ftz single-stranded RNAs (ssRNA) were transcribed from the T7 and SP6 promoters present on the pOT2A plasmids (BDGP) using the RiboMAX RNA production system (Promega). RNA preparation and injection was conducted as described (Kennerdell and Carthew, 1998). Embryos aged 15-45 minutes were injected at roughly 50% egg length with double-stranded RNA (dsRNA; 5 μM). The embryos were aged to the appropriate stage, then fixed and stained as described (Magie et al., 1999).

Construction of the Rho1 rescue construct and point mutants.

The Rho1 rescue construct was made by subcloning a 7kb 5' HindIII, 3' MluI fragment of genomic DNA containing the *Rho1* locus was into a version of the pCasper4 transformation vector lacking BamI and EcoRI sites in the polylinker. The

3.7 kb 5' EcoRI, 3' BamHI fragment within the coding region was excised and replaced with the corresponding 1.2 kb fragment from the Rho1 m7 cDNA. This resulted in the generation of a cassette that could be mutated as desired and easily replaced.

The targeted mutations were constructed using 2 rounds of PCR. The internal primers are summarized in Table 5.1. The external primers used correspond to the 5' EcoRI site and 3' BamHI site in the Rho1 m7 cDNA. This 1.2 kb fragment was then cloned into the endogenous Rho1 locus that was previously subcloned into the pCasper4 transformation vector

Germline transformation

The UAS-p120^{ctn} and UAS-α-catenin expression constructs were made by first amplifying the ORFs by PCR from the appropriate catenin cDNA, then cloning them into the pUASp vector (Rorth, 1998) as 5'Kpn-3'Xba fragments. The UAS-p120^{ctn}, UAS-α-catenin, and all Rho1 point mutation vectors (500 μg/mL) were injected along with the pTURBO helper plasmid (100 μg/mL) (Mullins et al., 1989) into isogenic w^{1118} flies as described (Spradling, 1986). Transgenics were scored by eye color and the insertions were mapped and balanced using standard genetic methods.

Cell culture

Drosophila cell lines used in this study: S2, S2R+ from S. Yanagawa, and S2hs-Wg from S. Cumberledge. Cells were grown in Schnieder's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 25mM glutamine, penicillin and streptomycin. Cells were grown at 25°C and passaged every 3-4 days.

RNAi in cells

In order to generate Rho1 dsRNA, the Rho1 ORF was cloned into pBluescript in both orientations. Templates were produced by linearizing the forward construct with

KpnI and the reverse construct with BamHI. ssRNA was transcribed from the T7 promoter of both templates using the Megascript RNA production kit (Ambion). The ssRNA was annealed at 75°C and purified as per the kit protocol. Cells were spun down, washed 1X in Schneider's medium without FBS, and resuspended in medium without FBS containing 45μg Rho dsRNA/mL (experimental) or no dsRNA (control). Cells were placed in 6-well culture dishes coated with gelatin and incubated at 25°C for 1hr. 2mL Schneider's medium with FBS was added to each well and the cells were incubated at 25°C for 3 days.

To assess the efficiency of the RNAi, cell lysates were made by spinning down cells from 1 well and resuspending them in 200μL L-buffer +protease inhibitors, sonicating 3x 10 sec and spinning out cellular debris. Lysates were separated by SDS-PAGE and analyzed by Western blot.

Wg treatment of S2R+ cells

Wg-conditioned medium was produced by culturing S2hs-Wg cells in Schneider's medium +FBS, heat shocking them at 37°C for 30min and allowing them to recover at least 3 hrs. Following this recovery period, the S2hs-Wg cells were spun down, and the medium added to S2R+ cells that were then incubated at least 3hrs at 25°C. The biochemical responses of these cells to Wg exposure were assessed by making lysates and analyzing them by SDS-PAGE and Western blots, or by fixing and staining cells (see below).

Immunofluorescence in cells

Cells were grown in 6-well culture dishes coated with gelatin and containing a coverslip. Following experimental treatment (RNAI +Wg exposure), the coverslips were removed and the cells fixed in 4% formaldehyde in PBS for 15min. They were washed 3X in PBS +0.1% Tween, then incubated in 1° antibody for 1hr. They were again washed 3X in PBS +0.1% Tween, then incubated in Alexa 488 2° antibody

(Molecular Probes) +propidium iodide and RNase for 1hr. Following the 2° incubation, they were washed 3X in PBS +0.1% Tween and mounted on slides for visualization via confocal microscopy.

The 1° antibodies used were: anti-Armadillo (1:50) and anti-Wg (1:100) from the Developmental Studies Hybridoma Bank; and anti-DE-Cadherin from H. Oda (1:10).

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Rotation in the lab of Dr. Gerold Schubiger. Zoology Department, University of Washington. Project: Interactions between *chickadee* (profilin) and cyclin B during nuclear migration in *Drosophila.*

2/96 - 7/97	Research Technician in the lab of Dr. Richard Nuccitelli. Section of Molecular and Cellular Biology, University of California, Davis. Projects: Investigation of calcium dynamics and phosphorylation patterns at fertilization in *Xenopus laevis*; voltage-dependent activation of frog eggs with a sperm surface disintegrin peptide.
9/95 - 12/95	Undergraduate researcher in the lab of Dr. Richard Nuccitelli.. Section of Molecular and Cellular Biology, University of California, Davis. Project: Examination of the phosphorylation state of PLC-γ upon fertilization in *Xenopus laevis.*
3/95 - 6/95	Undergraduate researcher in the lab of Dr. William Jeffery. Bodega Bay Marine Research Laboratory, University of California, Davis. Project: Regulative determination and unequal cleavage in the sand dollar, *Dendraster excentricus.*
1/93 - 3/93	Undergraduate researcher in the lab of Dr. Dorothy Gietzen. Section of Neurobiology, Physiology and Behavior, University of California, Davis. Project: Role of the vagus nerve in modulating consumption of nutritionally deficient diets in the rat.

Teaching Experience

9/98 – 12/98	Teaching Assistant, University of Washington, Biology 402 – Cell Biology
3/98 – 6/98	Teaching Assistant, University of Washington, Zoology 302 – Physiology
9/97 – 12/97	Teaching Assistant, University of Washington, Biology 202 – Introductory Biology: Development and Physiology

Awards and Fellowships

3/02	Best Student Poster, Northwest Developmental Biology Conference (Society for Developmental Biology)
3/00	Best Student Poster, Northwest Developmental Biology Conference (Society for Developmental Biology)
7/99 – 6/02	Molecular and Cellular Biology Training Grant
9/90	Eagle Scout Award

Memberships

1997 – present	member – Society for Developmental Biology
1999 – present	member – Genetics Society of America

Publications

Magie, C.R., Pinto-Santini, D., and Parkhurst, S.M. (2002) Rho1 interacts with p120ctn and alpha-catenin, and regulates cadherin-based adherens junction components in *Drosophila. Development,* 129(16): 3771-3782.

Magie, C.R., Meyer, M.R., Gorsuch, M.S., and Parkhurst, S.M. (1999) Mutations in the Rho small GTPase disrupt morphogenesis and segmentation during early *Drosophila* development. *Development,* 126(23): 5353-5364.

Shilling, F.M., **Magie, C.R.**, and Nuccitelli, R. (1998). Voltage-dependent activation of frog eggs by a sperm surface disintegrin peptide. *Developmental Biology,* 202(1): 113-124.