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Systematic expression and loss-of-function analysis defines spatially restricted requirements for *Drosophila RhoGEFs* and *RhoGAPs* in leg morphogenesis

Lina Greenberg[a] and **Victor Hatini**[a,b,*]

[a]Department of Anatomy and Cellular Biology, Program in Cell, Molecular and Developmental Biology

[b]Program in Genetics, Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, 136 Harrison Avenue, Boston MA 02111

Abstract

The *Drosophila* leg imaginal disc consists of a peripheral region that contributes to adult body wall, and a central region that forms the leg proper. While the patterning signals and transcription factors that determine the identity of adult structures have been identified, the mechanisms that determine the shape of these structures remain largely unknown. The family of Rho GTPases, which consists of 7 members in flies, modulates cell adhesion, actomyosin contractility, protrusive membrane activity, and cell-matrix adhesion to generate mechanical forces that shape adult structures. The Rho GTPases are ubiquitously expressed and it remains unclear how they orchestrate morphogenetic events. The Rho guanine nucleotide exchange factors (RhoGEFs) and Rho GTPase activating proteins (RhoGAPs), which respectively activate and deactivate corresponding Rho GTPases, have been proposed to regulate the activity of Rho signaling cascades in specific spatiotemporal patterns to orchestrate morphogenetic events. Here we identify restricted expression of 12 of the 20 *RhoGEFs* and 10 of the 22 Rho *RhoGAPs* encoded in *Drosophila* during metamorphosis. Expression of a subset of each family of RhoGTPase regulators was restricted to motile cell populations including tendon, muscle, trachea, and peripodial stalk cells. A second subset was restricted either to all presumptive joints or only to presumptive tarsal joints. Depletion of individual *RhoGEFs* and *RhoGAPs* in the epithelium of the disc proper identified several joint-specific genes, which act downstream of segmental patterning signals to control epithelial morphogenesis. Our studies provide a framework with which to understand how Rho signaling cascades orchestrate complex morphogenetic events in multicellular organisms, and evidence that patterning signals regulate these cascades to control apical constriction and epithelial invagination at presumptive joints.

Keywords

Epithelial morphogenesis; apical constriction; epithelial invagination; dAP-*2*; *RhoGAP68F*; *RhoGAP5A*

*Corresponding author, victor.hatini@tufts.edu.

INTRODUCTION

The *Drosophila* leg imaginal disc is composed of distinct cell populations that undergo spectacular, coordinated, rearrangements during larval development and metamorphosis to generate the complex morphology of adult legs and adjoining ventral thorax (Fristrom et al., 1978; von Kalm et al., 1995). During metamorphosis the pseudostratified epithelium of the leg imaginal disc everts and elongates by changes in epithelial cell shape (Fristrom and Fristrom, 1993) and by intercalation between cells (Taylor and Adler, 2008). Epithelial cells at presumptive joints constrict their apices and invaginate to promote joint morphogenesis (Mirth and Akam, 2002), while the distal tarsal joints of the leg are further sculpted by Jun-kinase (JNK)-*reaper* mediated apoptosis (Manjon et al., 2007).

The *Drosophila* leg harbors specialized cell populations that migrate extensively during metamorphosis. Some are specified in the disc proper, while others migrate into the leg imaginal disc from the trunk. The imaginal are connected to the larval epidermis by peripodial cells that form a hollow stalk. At the onset of metamorphosis, these peripodial stalk cells intercalate into the larval epidermis to facilitate disc eversion (Pastor-Pareja et al., 2004). Subsequently, the lateral margins of each disc, led by the peripodial stalk cells, crawl over the larval epidermis and fuse with the lateral margins of adjacent discs to “stitch together” the adult body wall in a process known as disc closure (Pastor-Pareja et al., 2004; Usui and Simpson, 2000). Underneath the disc proper the muscle founders fuse with surrounding myoblasts to form syncytial myotubes., These myotubes migrate and anchor at epidermal muscle attachment sites that are specified in the epithelium near presumptive joints to generate tendinous structures (Soler et al., 2004). A distinct population of tendon precursors invaginates and elongates distally from the distal tip of the leg to generate an internal hollow rod-like structure. To facilitate gas exchange, a primary tracheal tube invades into the leg imaginal disc from the trunk, elongates distally and forms an elaborate system of lateral branches. In the process, the tip cells of the tracheal branches migrate, elongate, and create tensile stresses that trigger tube elongation by stalk-cell intercalation (SCI) (Caussinus et al., 2008).

Many of the signals and transcription factors that pattern the leg, and the cellular machineries that generate mechanical forces that alter cell shape and motile behavior have been identified (reviewed in) (Kojima, 2004; Lecuit and Lenne, 2007; Montell, 2008). However, it remains unclear how patterning signals regulate these machineries to drive morphogenesis. It has been proposed that changes in adhesive properties and contractile behavior of epithelial cells can alter the topology of epithelial structures (Lecuit and Lenne, 2007). However, the precise mechanisms that can trigger these changes are only beginning to be unraveled. The rich repertoire of morphogenetic events that shape the developing *Drosophila* leg imaginal disc provides a highly tractable genetic model with which to identify morphogenetic regulators and characterize their mechanism of action in a complex multi-cellular environment.

Members of the Rho family of GTPases modulate processes that can affect cell shape and epithelial topology such as cell adhesion, actomyosin contractility, actin dynamics, and polarized vesicle transport (Fukata and Kaibuchi, 2001; Hall, 2005; Symons and Rusk, 2003; Van Aelst and Symons, 2002). The *Drosophila* genome codes for 7 *RhoGTPases*, including the canonical RhoGTPases *Rho1, Rac1* and *Cdc42*, whose morphogenetic roles remain poorly characterized. Legs with reduced *Rho1* activity form crooked and thickened proximal leg segments (Edwards and Kiehart, 1996; Ward et al., 2003) suggesting a role for *Rho1* and its upstream regulators and downstream effectors in control of axis elongation by cell shape changes and cell intercalation. RhoGTPases are activated by the RhoGEFs, which promote the exchange of GDP for GTP, and are inactivated by the RhoGAPs, which

promote the hydrolysis of GTP to GDP (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005). The RhoGEFs and RhoGAPs modulate the activities of target RhoGTPases in a variety of pathways and regulate their interactions with downstream effectors. During development, *RhoGEFs* and *RhoGAPs* that are expressed in restricted patterns could regulate locally the activities of their target RhoGTPases and their downstream effectors (Bernards, 2003; Rossman et al., 2005). Localized changes in activity of these effectors could in turn locally alter the mechanical properties of epithelial cells and thereby the morphogenesis of adult structures. Recent studies have uncovered essential roles for several *RhoGEFs* and *RhoGAPs* in shaping the morphology of diverse epithelial structures such as the ventral groove (Dawes-Hoang et al., 2005; Fox and Peifer, 2007; Hacker and Perrimon, 1998), the segmental groove (Mulinari et al., 2008), the spiracles (Brodu and Casanova, 2006; Simoes et al., 2006), the Malpighian tubules (Denholm et al., 2005), and the salivary glands (Xu et al., 2008). Signals that pattern these structures control the expression pattern of a subset of the *RhoGEFs* and *RhoGAPs* in these epithelial derivatives suggesting that patterning signals control epithelial morphogenesis, at least in part, by regulating the activity of Rho signaling cascades. The leg imaginal discs provides an excellent model with which to interrogate the genetic control of tissue morphogenesis, yet the role of the *RhoGEFs* and *RhoGAPs* has not been systematically investigated in this system.

In this study, we examined the expression pattern of 35 of the 42 *RhoGEFs* and *RhoGAPs* encoded in *Drosophila* by whole-mount *in situ* hybridization during early pupal stages of leg development. In conjunction, we depleted the function of 33 of the 42 *RhoGEFs* and *RhoGAPs* by expressing inducible hairpin RNAs in the distal part of the leg and examined the requirements of these genes in leg morphogenesis. We find unique expression patterns of over half of these regulators (12/20 *RhoGEFs*, 10/22 *RhoGAPs*) in morphogenetically active cell populations including in presumptive tarsal joints. Our data suggest that tarsal joint morphogenesis is achieved, at least in part, through the patterned regulation of *RhoGEFs* and *RhoGAPs* expression in the epithelium of the leg imaginal disc. We further identify a crucial role for several joint-specific RhoGEFs and RhoGAPs in tarsal joint morphogenesis downstream of the signals that organize segmental pattern, which implicates novel pathways in the control of apical constriction and epithelial invagination.

RESULTS

Genes expressed in motile cell populations

The developing *Drosophila* leg consists of several cell populations that migrate extensively during metamorphosis. We identified expression of 6 *RhoGEF* and 3 *RhoGAP* in these cell populations (Fig. 1 and Table 1). The *RhoGEF Cdep* was expressed within the leg shaft in a hollow tendinous structure, which invaginates and extends proximally from the distal tip during pupal stages (Fig. 1A). The *drm* gene is expressed similarly to *Cdep* in this tendinous structure (compare Fig. 1B with 1A; internal tendinous structure marked by arrowheads). *RhoGEF CG8557* was expressed in a series of inner rings below the epithelium of the disc proper in a subpopulation of muscle founders that coalesces in a segmental pattern (Fig. 1C). *dumbfounded-lacZ*, which marks the entire muscle founder population, is expressed in a broader pattern (Soler et al., 2004). Several *RhoGEFs* and *RhoGAPs* were expressed in tracheal tubes that arborize within the leg during metamorphosis including *RhoGEF4* (Fig. 1D), *Ephexin (GEF)*, *RhoGAP92B*, and *Graf* (Table 1). The tracheal 2A12 antibody highlights a similar structure (inset in Fig. 1D). Finally, several *RhoGEFs* and *RhoGAPs* were restricted to peripodial stalk (PS) cells and the disc margin between the peripodial epithelium and the disc proper. Cells that localize to the disc margin, led by PS cells, crawls over the larval epidermis during metamorphosis to mediates disc closure. *RhoGEF CG15611* was restricted to the disc stalk in legs, eyes, and wing imaginal discs, and to part of the disc margin (Fig. 1E, 1F, and 1G respectively). *RhoGAP16F* was restricted to the disc

stalk of leg discs, suggesting a leg-specific role for this gene (Table 1). The subset of *RhoGEFs* and *RhoGAPs* that are expressed in motile cell populations could act along interdependent pathways to orchestrate the coordinated movements of cell clusters, sheets and tubes in order to generate specialized structures in adult legs.

Joint-specific *RhoGEFs* and *RhoGAPs*

Joint morphogenesis is mediated by apical constriction and epithelial invagination, but the underlying mechanisms remain poorly understood. During joint morphogenesis the apical surface area of epithelial cells at presumptive joints decreases relative to those of adjacent cells that form the flanking leg segments (arrowheads in Fig. 5A-A' point to apically constricted epithelial cells in presumptive joints). We have been particularly interested in identifying joint-specific regulators that could govern the progression of the process downstream of the signals that organize segmental pattern to better understand this morphogenetic process (Greenberg and Hatini, 2009).

We found a total of 8 *RhoGEFs* and *RhoGAPs* restricted to all joints (Fig. 2A–D and Table 1) and 9 restricted to tarsal joints (Fig. 2E–J and Table 1). Available *lacZ* reporters for *RhoGEF2, RhoGAP71E*, and *cdGAPr* were expressed at presumptive joints in a similar pattern to the endogenous genes (insets in Fig. 2H, I, and J, respectively).

The segmental patterning system regulates the expression of the joint-specific *RhoGAPs* and *RhoGEFs*

The expression of a large number of *RhoGEFs* and *RhoGAPs* at presumptive joints suggested that the segmental patterning system controls the expression of this subset of genes to promote joint morphogenesis. The Notch (N) receptor ligands *Delta (Dl)* and *Serrate (Ser)* are expressed at the distal end of each leg segment and signal to adjacent distal cells to induce the expression of several transcriptional regulators including *dAP-2* (Ciechanska et al., 2007; Kerber et al., 2001) and *bowl* (Greenberg and Hatini, 2009). In turn, these transcriptional regulators promote leg segment growth and joint morphogenesis, though their downstream targets have not been identified. In addition, *Dl* and *Ser* induce low levels of target gene expression in adjacent proximal cells (Rauskolb and Irvine, 1999). *dAP-2* and *bowl* also act cell-autonomously to repress *Dl* and *Ser* expression in the "N-activated region" to maintain a stable N signaling interface between *Dl/Ser* expressing cells and adjacent distal cells (Ciechanska et al., 2007; Greenberg and Hatini, 2009). The maintenance of this interface is crucial for leg segment growth and joint morphogenesis. The joint-specific *RhoGEFs* and *RhoGAPs* were expressed strongly distal to the *Dl/Ser* domain (Fig. 2) and some were expressed weakly proximal to this domain (Fig. 2B and 2E; secondary stripes indicated by arrowheads). To test if the N signaling interface is required to promote the expression of the joint-specific *RhoGEFs* and *RhoGAPs* genes, we expressed an inducible *dAP-2* hairpin RNA transgene with *ptc-GAL4* to de-repress *Dl* and *Ser* in the N activated region and thereby disrupt the N signaling interface in a narrow sector across each segment (marked by the expression of *dpp* in Fig. 3C). This led to gaps in expression of several representative joint-specific genes including *RhoGEF64C* and *Ephexin* (Fig. 3D and 3E, gaps in expression are marked by arrows). Broad expression of the *dAP-2* RNAi transgene with *Dll-Gal4* recapitulated the *dAP-2* loss-of-function phenotype (Kerber et al., 2001), indicating that this RNAi transgene downregulates dAP-2 function specifically and effectively (Fig. 3B, compare to wild type in Fig. 3A). Likewise, the ectopic expression of *dAP-2* with *ptc-GAL4* to repress endogenous *Dl* and *Ser* expression led to gaps in *RhoGEF68F* and *Ephexin* expression in the Ptc domain (data not shown). Expression of a dominant negative *Notch* receptor (N^{ecd}) with *ptc-GAL4* to inhibit N signaling in the Ptc domain led to similar gaps in expression of several representative joint specific genes including *RhoGAP68F, RhoGAP5A* and *a lacZ* reporter for *RhoGAP71E*. The expression of

the *RhoGAP71E-lacZ* reporter was specifically downregulated in a narrow sector in the anterior compartment (marked by Ci expression) along the anteroposterior compartment boundary where Ptc is upregulated. Thus, the Notch pathway appears to promote the expression of this reporter cell autonomously. The expression of dominant negative *N* receptor led to similar gaps in expression of the *N* target dAP-2 in the Ptc domain. Taken together, these results indicate that N receptor signaling promotes the expression of the joint-specific *RhoGAPs* and *RhoGEFs* at presumptive joints either directly or indirectly.

A subset of the joint-specific *RhoGEFs* and *RhoGAPs* is required to promote tarsal joint morphogenesis

To identify novel regulators of epithelial morphogenesis and specifically those affecting apical constriction and epithelial invagination of presumptive joints, we depleted the function of individual *RhoGEFs* and *RhoGAPs* encoded in *Drosophila* by inducible hairpin RNA-mediated interference (RNAi) in the distal part of developing leg imaginal discs and assayed for phenotypes in adult legs (Bienz et al., 1988; Dietzl et al., 2007). In control experiments, we found that expression of hairpin RNAs to deplete a green fluorescent protein (GFP) caused no phenotypes (not shown). In contrast, depletion of several known regulators of leg development including *bowl, son of sevenless (sos), Dachsous (Ds),* and *flamingo (fmi)* by RNAi using leg-specific drivers recapitulated the respective mutant phenotype either fully or partially (Fig. 1 supplemental), thus validating the usefulness of this gene “knock down” strategy in the leg imaginal disc. Depletion of a subset of the *RhoGEFs* and *RhoGAPs* by RNAi, or by RNAi together with Dicer (Dicer RNAi) to enhance the production of short hairpin RNAs, resulted in several classes of reproducible phenotypes including distal leg truncations, necrosis of joints, bending and thickening of leg segments, and malformation and necrosis of internal structures (for data summary see Table 1 and Table 1 supplemental; for experimental protocol and scoring criteria of leg phenotypes see Materials and Methods). This range of phenotypes reveals important roles for these genes in leg development. Depletion of several tarsal joints-specific genes including *RhoGEF CG33275, RhoGAP5A* and *RhoGAP68F* (Table 1 and Figs. 2E and 2F, respectively) gave rise to a class of phenotypes characterized by missing or partially formed tarsal joints (Fig. 4B–D, compare to wild type in 4A) suggesting a specific role for this class of genes in apical constriction and epithelial invagination at presumptive joints. Consistent with this idea, depletion of these genes’ function had no adverse affects on the segmentation, size or differentiation of tarsal segments.

To further characterize this phenotypic class, we examined the role of *RhoGAP68F* in epithelial morphogenesis in further detail. First, we examined epithelial morphology at metamorphosis in leg imaginal discs stained for E-cad to highlight cellular outlines and tissue contours. We found that the epithelium of the leg imaginal disc elongated along the proximodistal axis at metamorphosis and formed a tube-like structure with shallow or missing invaginations at presumptive joints (Fig. 5C-C’, compare to wild type in Fig. 5A-A’, and data not shown). To determine if *RhoGAP68F* affects joint morphogenesis after the establishment of tarsal segments, we stained legs depleted for *RhoGAP68F* for dAP-2 and E-cad to mark tarsal segment boundaries and highlight cell outlines, respectively. We observed largely normal expression of dAP-2 in most pupariating leg segments despite a block to apical constriction and epithelial invagination (Fig. 5D-D’, compare to wild type in 5B-B’, and data not shown; asterisks in D indicate presumptive joints that failed to invaginate). To determine if *RhoGAP68F* promotes joint morphogenesis indirectly by affecting JNK-*reaper* mediated apoptosis, we stained leg discs depleted for *RhoGAP68F* in the Ptc domain for a *puckered (puc)-lacZ* reporter, which mark JNK signaling activity and observed normal expression of this reporter in the Ptc domain despite a modest inhibition of epithelial invagination at presumptive joints (Fig 5F). In contrast, the expression of a

dominant negative Notch receptor led to gaps in this reporters' expression in the Ptc domain (compare Fig. 5G to wild type in 5E). These findings strongly suggest that RhoGAP68F acts downstream of segmental patterning signals and parallel to the JNK-reaper pathway to promote joint morphogenesis.

Several recessive lethal P-element insertions in the 5' non-coding region of the *RhoGAP68F* gene have been identified including *P{EP}RhoGAP68F*^{EP3152}, *p{GSV7}GS20760/TM3* and *P{GSV6}GS11699*. We characterized the *P{EP}RhoGAP68F*^{EP3152} insertion in further detail. We find that 62% of *P{EP}RhoGAP68F*^{EP3152} homozygous animals (62 of 100 embryos analyzed) died during embryogenesis, while the remaining embryos that hatched died during the subsequent first larval instar stage. This lethal phase is consistent with a role for *RhoGAP68F* in facilitating epithelial morphogenesis during embryogenesis as has been previously reported (Sanny et al., 2006). The *P{EP}RhoGAP68F*^{EP3152} insertion contains UAS response elements that can be used to overexpress neighboring genes using the GAL4/UAS system. To examine the RhoGAP68F gain-of-function phenotype we crossed the *P{EP}RhoGAP68F*^{EP3152} line to *Dll-GAL4* to broadly overexpress RhoGAP68F across the distal part of the leg. We found that the broad overexpression of *RhoGAP68F* also partially blocked the formation of tarsal joints (Fig. 4E), indicating that reduced or excess levels of RhoGAP68F impair joint morphogenesis.

Overall, we conclude that *RhoGAP68F* promotes apical constriction and epithelial invagination downstream of the signals that organize segmental pattern and specify the presumptive joints. We propose that *RhoGAP68F* in concert with other essential joint-specific genes including *RhoGAP5A* and *RhoGEF CG33275* determine the mechanical properties of the epithelium at presumptive joints and thereby the topology of the epithelium in this region.

DISCUSSION

We identified restricted expression of a large number of *RhoGEFs* and *RhoGAPs* in regions and cell populations that are remodeled by changes in cell shape, cell-cell interaction, and cell motility, with several *RhoGEFs* and *RhoGAPs* typically expressed in any given region or cell type. These regulators are precisely positioned to control the modular mechanical forces (cell-cell adhesion, contractility, membrane protrusions, and cell-matrix interaction) generated by epithelial cells by regulating crucial downstream effectors to drive the morphogenesis of adult structure. N signaling promotes the expression of the joint-specific *RhoGEFs* and *RhoGAPs* suggesting that patterning signals drive joint morphogenesis, at least in part, by modulating the activity of Rho signaling cascades. A large number of *RhoGEFs* and *RhoGAPs* were expressed at presumptive joints and several of these genes were required for tarsal joint morphogenesis. We discuss the possible mechanism of action of these genes and their potential contribution to the process of apical constriction and epithelial invagination.

Control mechanisms of epithelial invagination at presumptive joints

Dl and *Ser* signal to adjacent distal cells to promote leg segment growth and joint morphogenesis. *Dl* and *Ser* induce the expression of several transcriptional regulators at presumptive joints including *dAP-2* and the *odd-skipped* family genes *drumstick (drm), oddskipped (odd), brother of odd and bowl with entrails limited (bowl), and sister of odd and bowl (sob). dAP-2* controls the formation of all the joints (Kerber et al., 2001; Monge et al., 2001), while the *odd-skipped* family genes appear to act redundantly to control the formation of non-tarsal joints, also termed true joints (Greenberg & Hatini, 2009). The mechanisms by which these signals and transcriptional regulator control epithelial morphogenesis have remained elusive. Localized JNK-reaper mediated apoptosis

contributes to the articulation of presumptive joint by the localized elimination of epithelial cells in this region (Manjon et al., 2007). The activation of actomyosin contractility at presumptive joints has been proposed to promote apical constriction and epithelial invagination (Hao et al., 2003). The literature concerning the genes identified in our study such as RhoGAP5A and RhoGAP68F and their vertebrate homologs (Bruinsma et al., 2007; Sanny et al., 2006) suggests that the invagination of the epithelium at presumptive joints depends on additional mechanisms affecting junctional dynamics through the regulation of junction stability and trafficking itineraries of junction proteins as discussed below.

The control of junctional dynamics at presumptive joints

Emerging results suggest that the adherens junctions are the primary determinants of epithelial morphology (reviewed in) (Fernandez-Gonzalez and Zallen, 2008; Lecuit and Lenne, 2007; Warner and Longmore, 2009). The homophilic cell adhesion molecule-E-cadherin (E-cad) concentrates at the zonula adherens (ZA) below the apical cortex and links epithelial cells into a continuum of interconnected cells. Interacting E-cad molecules form a structure termed the adherens junction (AJ) that links interacting E-cad molecules to the actin and actomyosin cytoskeletal networks via several adaptor proteins to stabilize the ZA. Changes in cytoskeletal structure and dynamics can in principle enable epithelial cells to remodel cell-cell contacts, cell shape and epithelial topology. Like other transmembrane proteins, a fraction of E-cad molecules is constantly endocytosed and recycled to the plasma membrane. The modulation of this constitutive recycling pathway can also enables epithelial cells to remodel cell-cell contacts, cell shape and epithelial topology (Georgiou et al., 2008; Harris and Tepass, 2008; Leibfried et al., 2008).

During apical constriction the perimeter of the ZA appears to shrink, though the mechanisms involved remain poorly understood (Martin et al., 2009). The joint-specific *RhoGEFs* and *RhoGAPs* could affect the stability, trafficking and degradation of AJs in lysosomes to promote apical constriction. Consistent with this notion, the formation of the ventral furrow by apical constriction is associated with a large increase in endocytic internalization of AJs (Oda et al., 1998). Indirect evidence from the literature suggests that *RhoGAP5A* and *RhoGAP68F* (Fig. 2E & F, respectively) regulate adhesive cell-cell contacts by two distinct yet interdependent mechanisms. RhoGAP5A specifically inhibits Rac1, whose key function in epithelial cells is to stabilize cell-cell contacts by promoting the accumulation of actin filaments at AJs (Braga et al., 1999; Eaton et al., 1995; Takaishi et al., 1997). It is plausible that RhoGAP5A inhibits Rac1 at the ZA to decrease the accumulation of actin filaments at AJs in order to decrease the stability and increase the endocytic internalization of AJs at presumptive joints. Consistent with such a role, Chimaerins, the vertebrate homologs of RhoGAP5A, are recruited to the plasma membrane by the signal transducers phosphoinositide 3-kinase (PI3K) and phospholipase C-γ (Plc-γ), which are enriched in the ZA in epithelial cells (Yang and Kazanietz, 2007). *RhoGAP5A* promotes apical constriction and tube elongation of the salivary gland (Kolesnikov and Beckendorf, 2007), and the remodeling of cell-cell contacts in the fly eye (Bruinsma et al., 2007), suggesting a general role for *RhoGAP5A* in junctional remodeling in epithelial cells.

RhoGAP68F, which specifically deactivates Rho1 (Sanny et al., 2006), could regulate a subsequent step in the process. p50RhoGAP, the vertebrate homolog of RhoGAP68F, localizes to several endocytic compartments via its Sec14 lipid binding domain where it regulate endocytic trafficking (Sirokmany et al., 2006). The Sec14 domains of p50RhoGAP and RhoGAP68F are 43% identical and 64% similar suggesting related roles for RhoGAP68F in endocytic control. The Rho1 GTPase plays an important role in promoting the formation and movement of endosomes between compartments (Derivery et al., 2009; Gomez and Billadeau, 2009; Liu et al., 2009) and RhoGAP68F could inhibit this role of Rho1. By this mechanism RhoGAP68F could affect the turnover of E-cad or other

junctional components and thereby cell shape and epithelial topology. *RhoGAP68F* promotes the formation of the ventral furrow during embryogenesis suggesting a general role for this regulator in apical constriction (Sanny et al., 2006).

The control of actomyosin contractility at presumptive joints

Although, we were unable to examine the role of RhoGEF2 by RNAi, it is plausible that this regulator acts either alone or with other regulators to promote actomyosin contractility. During ventral furrow formation, *RhoGEF2* concentrates apically and activates Rho1 in this region to promote the constriction of the apical actomyosin meshwork that is anchored at the ZA. Active Rho1 activates the formin-family protein Diaphanous to promote polymerization of linear actin filaments that assemble into a contractile actomyosin meshwork. In addition, it promotes the phosphorylation of the regulatory light chain of Myosin II termed Spaghetti squash (Sqh) to upregulate actomyosin contractility. RhoGEF2 promotes epithelial invagination in other tissues during embryogenesis suggesting a general role for this gene in apical constriction (Dawes-Hoang et al., 2005; Fox and Peifer, 2007; Grosshans et al., 2005; Hacker and Perrimon, 1998; Kolsch et al., 2007; Mulinari et al., 2008). *RhoGEF2* is upregulated at presumptive joints (Fig. 2H) and is thus positioned to upregulate actomyosin contractility in this region to initiate joint morphogenesis. RhoGEF64C colocalizes apically with RhoGEF2, and the two proteins have been proposed to activate Rho1 to promote apical constriction and epithelial invagination (Simoes et al., 2006). *RhoGEF2* and *RhoGAP64C* could act along the same pathway to promote actomyosin contractility at presumptive joints. We note however, that the depletion of RhoGEF64C as well as several other genes including *RhoGAP54D* and *RhoGAP71E* (Table 1) adversely affected leg developmental suggesting earlier and/or more general roles for this subset of genes in epithelial morphogenesis. Additional work will be required to assign specific roles for these regulators in epithelial morphogenesis.

Overall, we propose that the activities of essential genes identified in our screen are coordinated to constrict the apical cell cortex, destabilize and decrease the surface expression of apical junctional proteins to promote apical constriction and epithelial invagination at presumptive joints. It has been shown that apical constriction occurs by the pulses of actomyosin contractility (Martin et al., 2009). While a subset of the essential genes identified in our screen such as RhoGEF2 might promote contractile pulses, others such as RhoGAP5A and RhoGAP68F might act in the refractory phases between pulses to remove junctional components and membrane from the apical cortex in order to consolidate the constricted state.

The potential role of RhoGTPase regulators that are expressed in motile cell populations

Although we were particularly interested in genes that regulate apical constriction and epithelial invagination, we identified several *RhoGEFs* and *RhoGAPs* that are expressed in motile cell populations. These genes may influence, in addition to cortical tension and cell adhesion, the protrusive membrane activity and cell-matrix adhesion required for the polarization, membrane extension, and forward movement of motile cell populations (Friedl and Gilmour, 2009; Montell, 2008). A distinct set of experimental tools will be required to investigate the roles of these genes' function in morphogenesis of motile cell populations.

The results presented in this study indicate that the leg imaginal disc holds a great promise to reveal general mechanisms and regulatory logic of epithelial morphogenesis in multi-cellular organisms. Our findings expand the list of genes involved in control of apical constriction and epithelial invagination, and suggest the existences of novel pathways that contribute to the process. Additionally, our findings identify novel genes that could regulate epithelial sheet migration, disc closure, and tracheal tube elongation. The identification of

these genes' expression and function in leg development provides a resource with which to understand the role of Rho signaling cascades, their upstream regulators and downstream effectors in shaping the morphology of adult structures in multicellular organisms.

EXPERIMENTAL PROCEDURES

Fly Strains

The following fly lines were used in this study: *RhoGAP71E-LacZ, RhoGEF2-LacZ, sos-lacZ, cdGAPr-lacZ,* and *rtGEF-lacZ* (Bloomington and Szeged Stock Centers). *dumbfounded-LacZ* (gift from Sree Devi Menon) was used to mark muscle precursors. *dAP-2 RNAi* (VDRC 41130) and a dominant negative N receptor (N^{ecd}) were used to block leg segmentation. *ptc-GAL4* was used to express the *dAP-2* RNAi along the AP compartment boundary and *Dll-GAL4* in the distal part of the leg. RNAi transgenes used to deplete the function of each *RhoGEF* and *RhoGAP* are listed in supplemental Figure 1. *Dll-GAL4* and *Dll-GAL4; UAS-Dicer* lines were used to express each RNAi transgene in the distal region of the leg.

In situ hybridization and probe preparation

w^- pupae were dissected 4–6 hours after puparium formation (APF) and processed for *in situ* hybridization (ISH) (Sullivan et al., 2000). cDNA vectors were either linearized at the 5' multiple cloning sites or the cDNA was PCR amplified with generic primer sets appropriate for each vector. Digoxigenin-labeled antisense RNA probes were transcribed with appropriate RNA polymerase (SP6, T7 or T3) according to the manufacturer's protocol (Roche). Yields of synthesized RNA were estimated by gel electrophoresis and the optimal probe concentration for ISH was determined empirically for each probe. Stacks of bright-field images were obtained using a Zeiss Axioscope 2+ and composite projections were generated using compositeZP. Figures were assembled and adjusted using Adobe Photoshop CS3.

cDNA clones were obtained from the *Drosophila* Gene Collection (DGC, http://www.fruitfly.org/DGC/index.html). Fully sequenced clones were used when available; otherwise, clones were obtained from the EST collection. The following cDNA clones were used for probe synthesis:

RhoGAPs

pBS-*RhoGAP68F* (LD02491), pBS-*RhoGAP71E* (LD04071), pOT2-*RhoGAP5A* (SD02309), pOT2-*RhoGAP18B* (LD25711), pOTB7-*RacGAP84C* (AT12815), pFlc-*cv-c* (RE02250), pOT2-*Graf* (LD28528), pOT2-*RhoGAP15B* (SD08167), pOT2-*RhoGAPp190* (GH17919), pOT2-*RhoGAP16F* (SD04011), pOT2-*CdGAPr* (LD27836), pOT2-*RhoGAP93B* (SD01504), pOT2-*RhoGAP100F* (LP17760), pOTB7-*RhoGAP92B* (AT11177), pOTB7-*GEF26* (AT08279), pFlc-*RhoGAP19D* (RH60035), pFlc-*RhoGAP54D* (RE04485).

RhoGEFs

pOT2-*Cdep* (SD09116), pOT2-*CG30115* (GH16956), pOT2-*CG30440* (LD43457), pOT2-*vav* (LD25754), pOT2-*CG8557* (SD02996), pFlc-*CG15611* (RE34668), pOT2-*RhoGEF4* (LD45290), pBS-*CG33275* (GM01778), pOT2-*RhoGEF64C* (GH26207), pOT2-*RhoGEF2* (SD04476), pOT2-*sos* (GH01796), pOT2-*CG10188* (GH26723), pOT2-*CG15612* (SD09786), pOT2-*trio* (SD08659), pOT2-*Ephexin* (GH03693), pOT2-*CG7397* (GH19526), pFlc-*rtGEF* (RE32772), pBS-*RhoGEF3* (HL01913), pFlc-*CG7323* (RH56938), pOT2-*sif* (GH10341). *In situ* hybridization with pOT2-*drm* (LD 26791) was used to mark the internal tendon precursors.

RNAi screen and criteria to identify RNAi phenotypes

Each RNAi transgene was expressed with *Dll-GAL4* at 25°C either alone or in the presence of *UAS-Dicer* to reduce gene function further. In cases where the expression of the RNAi transgene resulted in embryonic or pupal lethality, the RNAi transgene was expressed at 18°C to deplete gene function to intermediate levels. For each cross, at least 20 prothoracic legs were mounted and analyzed using a compound light microscope by two independent observers. Each observer checked: 1) if all the leg segments where accounted for, if segments where missing or if the leg proximodistal axis was truncated; 2) if legs were shorter, thicker, or bent; 3) if joints were missing or if they were partially formed; 4) if legs formed necrotic structures or other anomalies in epithelial organization such as ectopic invagination or internal vesicular structures; and 5) if bristles formed and if the bristle pattern was disorganized. Phenotypes that were detected in at least 5/20 legs where scored as positive. In most cases, related phenotypes were detected in greater then 10 of the 20 legs scored.

Immunofluorescence and confocal imaging

Discs were fixed and stained according to standard protocols. The following antibodies were used: rabbit anti-β-galactosidase (Cappel), rat anti-Ci (DSHB)and mouse anti-tracheal system 2A12 (DSHB). Secondary antibody conjugated to Cy3 or Cy2 fluorophores (Jackson ImmunoResearch) were used at 1:150. Stained discs were scanned using a Zeiss LSM510 confocal microscope in multi-tracking mode.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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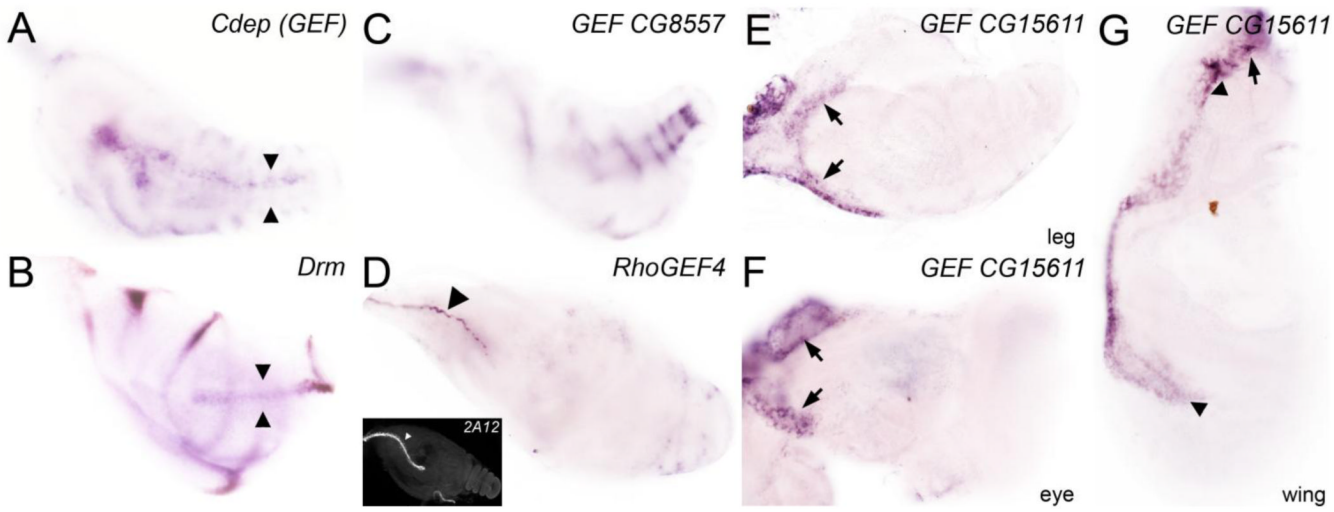

Figure 1. A subset of *RhoGEFs* and *RhoGAPs* is expressed in motile cell populations

(A) *Cdep (GEF)*; expression is restricted to internal tendon precursor cells. (B) *drm*; expression is restricted to each true leg joint and to the internal tendon precursors (marked by arrowheads). (C) *RhoGEF CG8557*; expression is detected in a subset of adepithelial cells that form muscle below the surface epithelium. (D) *RhoGEF4*; expression is detected in a primary tracheal tube at the periphery of the leg. Tracheal antibody 2A12 highlights a tracheal tube in inset. (E–G) *RhoGEF CG15611*; Expression is detected in the disc stalk of the (E) leg disc, (F) antenna, (F) and in part of the ventral pleura in the wing (marked by arrowheads). These cell populations connect the imaginal disc to the larval epidermis and contribute to disc eversion, migration and fusion during metamorphosis to promote disc closure.

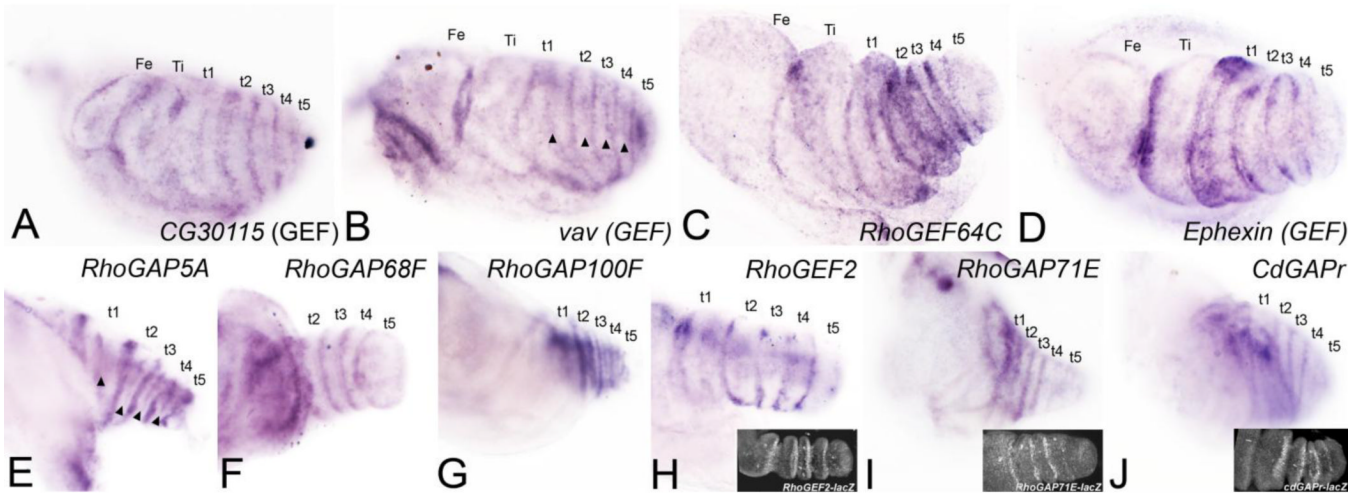

Figure 2. A subset of *RhoGEFs* and *RhoGAPs* is restricted to all presumptive leg joints or only to presumptive tarsal joints

The leg imaginal disc gives rise to five true segments moveable by muscle: the coxa, trochanter, femur, tibia and tarsus. The tarsus is further subdivided into five non-musculated tarsal segments and a distal claw. (A–D) Genes restricted to all leg joints. (E-J) Genes restricted to tarsal joints. (A) *CG30115 (RhoGEF)*. (B) *vav (RhoGEF)*. (C) *RhoGEF64C*. (D) *Ephexin (RhoGEF)*; expression spans the joint and several cell diameters proximal and distal to the joint. (E) *RhoGAP5A*. (F) *RhoGAP68F* (G). *RhoGAP100F*. (H) *RhoGEF2; RhoGEF2-lacZ* shown in inset. (I) *RhoGAP71E*; expression is stronger in proximal joints that are more articulated compared to distal joints that are less articulated, *RhoGAP71E-lacZ* shown in inset. (J) *CdGAPr; CdGAPrlacZ* shown in inset. A secondary stripe of expression is detected across each tarsal segment (marked by arrowheads) in B and E.

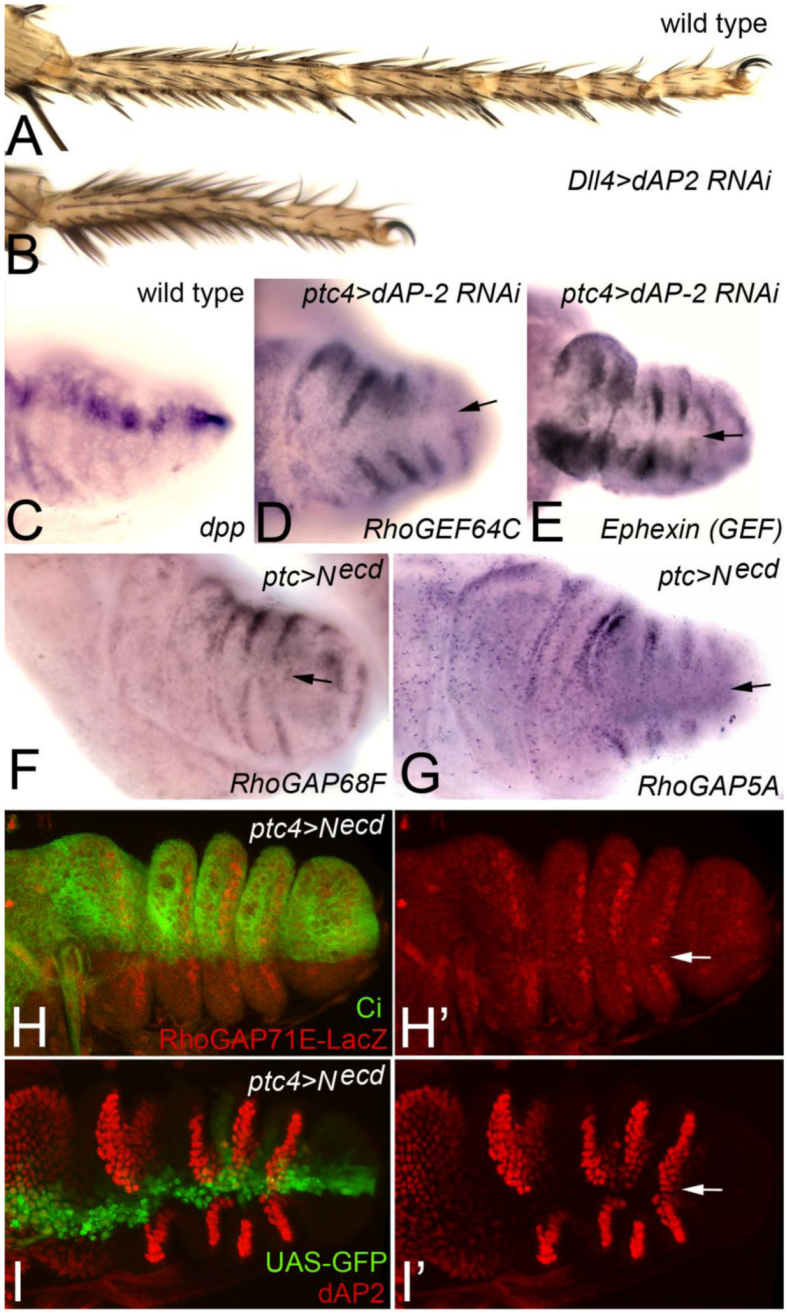

Figure 3. Expression of joint-specific *RhoGEFs* and *RhoGAPs* requires the proper patterning of tarsal segments

(A) Wild type adult tarsus. (B) *Dll>dAP-2 RNAi*; depletion of *dAP-2* function with *Dll-GAL4* resulted in shortened tarsus with fused joints that recapitulates a strong *dAP-2* loss-of-function phenotype. (C) *dpp* is expressed along the AP compartment boundary where the *ptc-GAL4* driver is active. (D–E) *ptc>dAP-2 RNAi*; depletion of *dAP-2* in the Ptc domain resulted in repression of (D) *RhoGEF64C* and (E) *Ephexin* in a narrow sector marked by arrows. (F–I') *ptc>N^{ecd}*; inhibition of N receptor signaling in the Ptc domain resulted in the repression of (F) *RhoGAP68F*, (G) *RhoGAP5A*, (H-H') a *RhoGAP71E-lacZ* reporter and (I-I') dAP-2 in a narrow sector marked by arrows. (H-H') The expression of *RhoGAP71E-lacZ*

was repressed in the anterior compartment (marked by Ci expression) along the AP compartment boundary. This narrow sector corresponds to the Ptc domain suggesting that N signaling promotes the expression of *RhoGAP71E* cell-autonomously. (I-I') dAP-2 was repressed cell-autonomously in the Ptch domain validating the efficacy of the N^{ecd} transgene used in this assay (marked by UAS-GFP expression).

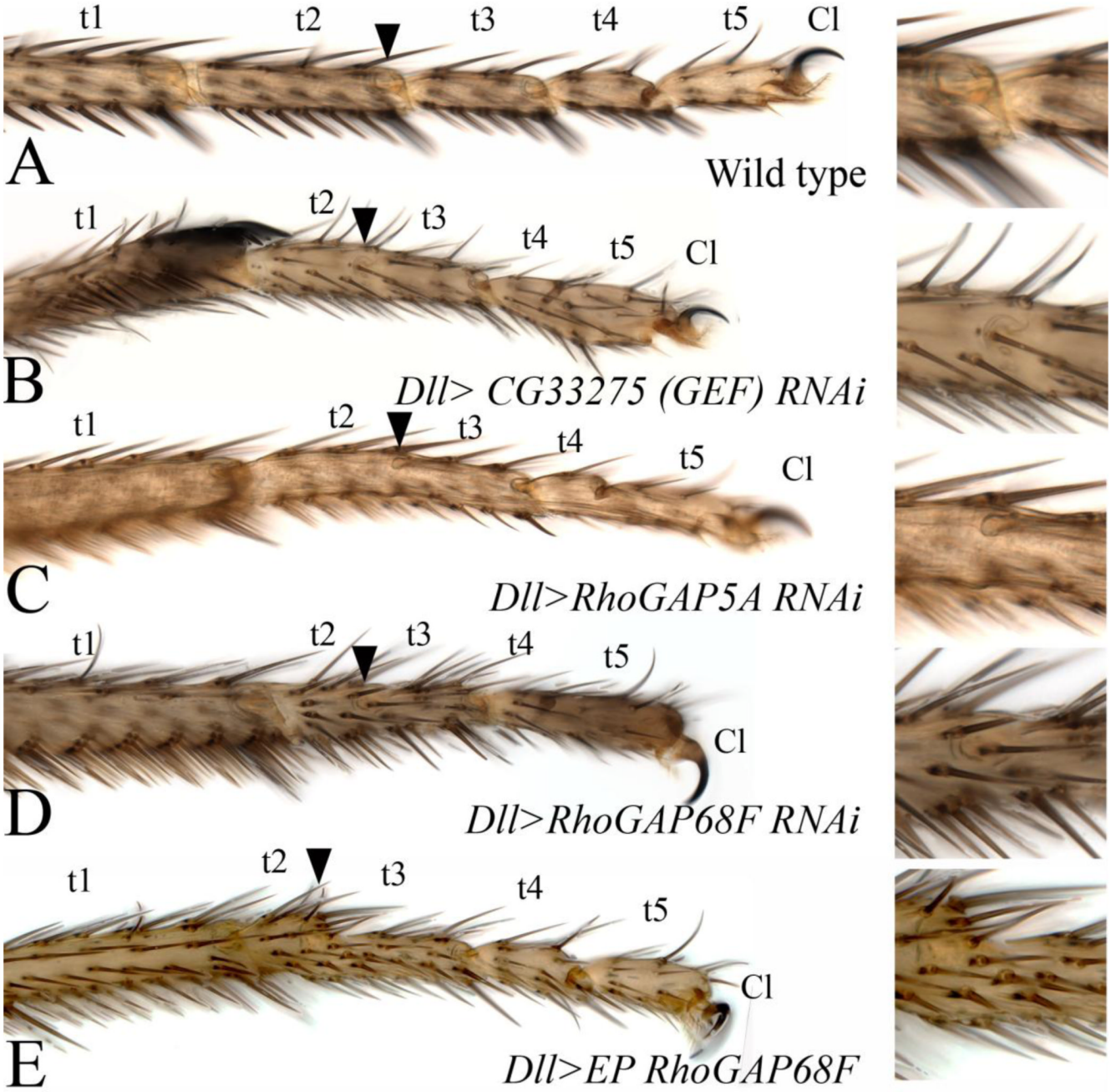

Figure 4. A subset of *RhoGEFs* and *RhoGAPs* is required for joint morphogenesis

(A–E) Adult tarsi; (A) wild type; the tarsal region of adult legs is subdivided into 5 tarsal segments (t1–t5) and the distal claw (Cl). (B) *Dll>CG33275* RNAi (*RhoGEF*), (C) *Dll>RhoGAP5A* RNAi and (D) *Dll>RhoGAP68F* RNAi. (B–D) Depletion of a subset of *RhoGEFs* and *RhoGAPs* by RNAi inhibits tarsal joint morphogenesis but does not adversely affect the growth and differentiation of leg segments. Arrowheads in B–D point to partially formed joints shown at higher magnification in insets. All the tarsal joints and tarsal segment (t1-t5) can be accounted for suggesting that the primary defect is in the progression of the process. Note that legs in B and D are slightly shorter and thicker than wild type reflecting a mild defect in axis elongation. (E) *Dll>P{EP}RhoGAP68F*^{P3152}, Ectopic expression of

RhoGAP68F also led to the formation of partially formed joints. Arrow in E points to such a joint.

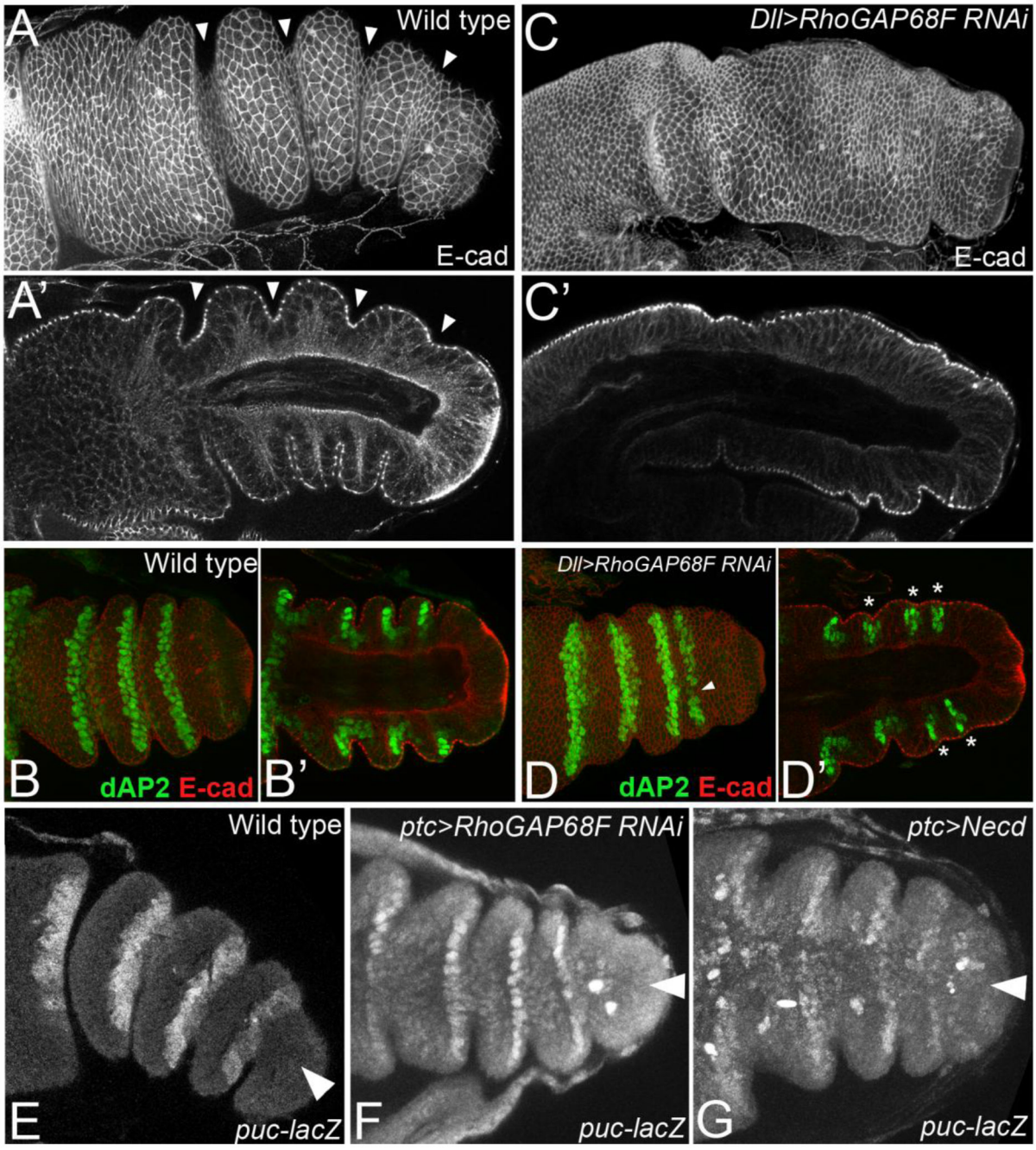

Figure 5.

RhoGAP68F acts downstream of *dAP-2* to promote apical constriction and epithelial invagination at presumptive joints. (A–B') Wild type and (C–D') *Dll>RhoGAP68F RNAi* leg imaginal disc stained for E-cad (white in A-A' and C-C', Red in B-B' and D'-D) to mark cell outlines, and for dAP-2 (green in B-B' and D'-D) to mark segment boundaries at ~4h after puparium formation (APF). (B and D) Grazing sections at the plane of the ZA (B' and D') and mid-saggital sections to reveal the apicobasal axis of the epithelium. (A-B') The concentrically folded leg imaginal disc telescopes-out along the proximodistal (PD) axis and the epithelium of presumptive joints invaginates by apical constriction to initiate joint morphogenesis. dAP-2 accumulates at high levels in the distal part of the joint and at lower

level in the proximal part. Note that proximal joints are more articulated compared to distal joints at this stage. (C–D') Legs depleted for *RhoGAP68F* elongate along the PD axis but form either shallow or no invaginations at presumptive joints (asterisks in D' indicate shallow invaginations). dAP-2 expression remains largely intact in these legs. In a small number of segments we detect small gaps or thinning (arrowheads in D) of the stripe of dAP-2 expression reflecting mild patterning defects. However, epithelial invaginations were either shallow or altogether missing despite the proper expression of dAP-2 in most segments indicating that *RhoGAP68F* acts downstream of *dAP-2* to promote apical constriction and epithelial invagination.

(E–G) RhoGAP68F acts in parallel to JNK-reaper mediated apoptosis to promote tarsal joint morphogenesis. Arrowheads point to the Ptc domain. Expression of a *puc-lacZ* reporter in (E) wild type, (F) *ptc>RhoGAP68FRNAi* and (G) *ptc>Necd* (dominant negative N receptor). (E) The *puc-lacZ* reporter is expressed at high levels in tarsal joints 2–5 (F) Expression of *RhoGAP68FRNAi* with *ptc-GAL4* led to a modest inhibition of epithelial invagination in the Ptc domain. However, *puc-lacZ* expression was not affected. . (G) In contrast, expression of *Necd* with *ptc-GAL4* to inhibit N signaling in the Ptc domain led to the downregulation of *puc-lacZ* expression in the Ptc domain.

Table 1

Summary of the expression pattern and loss-of-function phenotypes of the *RhoGEFs* and *RhoGAPs* analyzed in this study

For each gene, we describe the expression pattern in the leg and loss-of-function phenotypes induced by the expression of inducible hairpin RNAs with *Dll-GAL4* or *Dll-GAL4; UAS-Dicer*. *lacZ* reporters that recapitulate endogenous gene expression are marked with asterisk. NP indicates no phenotype.

Gene name	CG number	Leg Expression	RNAi phenotype
RhoGAP68F	CG6811	Tarsal joints only	Shortened fused tarsal segments; partial joints
RhoGAP71E	CG32149	Tarsal joints only *	Necrosis of distal leg
RhoGAP5A	CG3208	Tarsal joints only	Fused tarsal segments; partial joints
RhoGEF2	CG9635	Tarsal joints only *	RNAi N/A
RhoGAP100F	CG1976	Tarsal joints only	RNAi N/A
RhoGAP92B	CG4755	Tarsal joints only; trachea	Bent tibia & femur
RhoGEF	CG30115	All leg joints	Bent tibia & femur; tendon necrosis
vav GEF	CG7893	All leg joints	NP
RhoGEF64C	CG32239	All leg joints	Small leg stumps when raised at 18°C
RhoGAP15B	CG4937	All leg joints	Bent tibia
RhoGAPp190	CG32555	Ubiquitous with elevated expression in all leg joints	NP
trio GEF	CG18214	All leg joints	Bent tibia & femur; slight thinning of tarsus
RhoGEF	CG7397	All leg joints; trachea	NP
Ephexin GEF	CG3799	All leg joints; trachea	Bent tibia & femur; tendon necrosis
Cdep GEF	CG31536	Tendon precursor cells	NP
RhoGEF	CG8557	Muscle precursor cells	Bent tibia & femur; tarsus bent & thinner
RhoGEF	CG15611	Stalk and ventral pleura	NP
RhoGEF4	CG8606	Trachea	NP
Graf GAP	CG8948	Ubiquitous with elevated expression in tarsal joints; trachea	NP
RhoGAP16F	CG7122	Stalk and trachea	Bent tibia & femur; tendon necrosis
RhoGAP19D	CG1412	Ubiquitous	Bent tibia & femur
CdGAPr	CG10538	Ubiquitous with elevated expression in tarsal joints*	RNAi N/A
rtGEF	CG10043	Ubiquitous	Larval lethal
RhoGEF3	CG1225	Ubiquitous	Larval lethal
RhoGEF	CG7323	Ubiquitous	Bent tibia & femur
sif GEF	CG5406	Ubiquitous	NP; maybe embryonic lethal
sos GEF	CG7793	Ubiquitous with elevated expression in tarsal joints*	No claw; fused tarsal segments; partial joints
RhoGEF	CG10188	Low level ubiquitous	NP
RhoGEF	CG30456	Low level ubiquitous	Bent tibia & femur
RhoGAP54D	CG6477	Ubiquitous	Necrosis of tarsal joint

Gene name	CG number	Leg Expression	RNAi phenotype
RhoGAP93B	CG3421	Low level ubiquitous	NP
RhoGAP18B	CG7481	Low level ubiquitous	NP; maybe embryonic lethal
RacGAP84C	CG2595	Low level ubiquitous	NP
RhoGEF	CG33275	Ubiquitous	Larval lethal; fused tarsal segments when raised at 18°C
cv-c	CG34389	Ubiquitous	NP