Rho GTPase controls *Drosophila* salivary gland lumen size through regulation of the actin cytoskeleton and Moesin

Na Xu\(^1,2\), Gaiana Bagumian\(^2\), Michael Galiano\(^2\) and Monn Monn Myat\(^2,*\)

**SUMMARY**

Generation and maintenance of proper lumen size is important for tubular organ function. We report on a novel role for the *Drosophila* Rho1 GTPase in control of salivary gland lumen size through regulation of cell rearrangement, apical domain elongation and cell shape change. We show that Rho1 controls cell rearrangement and apical domain elongation by promoting actin polymerization and regulating F-actin distribution at the apical and basolateral membranes through Rho kinase. Loss of *Rho1* resulted in reduction of F-actin at the basolateral membrane and enrichment of apical F-actin, the latter accompanied by enrichment of apical phosphorylated Moesin. Reducing cofillin levels in *Rho1* mutant salivary gland cells restored proper distribution of F-actin and phosphorylated Moesin and rescued the cell rearrangement and apical domain elongation defects of *Rho1* mutant glands. In support of a role for Rho1-dependent actin polymerization in regulation of gland lumen size, loss of profilin phenocopied the *Rho1* lumen size defects to a large extent. We also show that Ribbon, a BTB domain-containing transcription factor functions with Rho1 in limiting apical phosphorylated Moesin for apical domain elongation. Our studies reveal a novel mechanism for controlling salivary gland lumen size, namely through Rho1-dependent actin polymerization and distribution and downregulation of apical phosphorylated Moesin.

**KEY WORDS:** *Drosophila*, Rho GTPase, Actin, Lumen, Salivary gland, Tube

**INTRODUCTION**

Epithelial tubes are the structural and functional components of many essential organs, such as the respiratory, circulatory and secretory organs. Tubular organs serve important physiological roles, including delivery of gases, nutrients and hormones, and removal of waste. Tube morphogenesis is a highly regulated process that requires dynamic cell shape changes, cell migration and cell rearrangements, as well as remodeling of cell adhesion junctions and select membrane domains (Andrew and Ewald, 2010; Jung et al., 2005; Lubarsky and Krasnow, 2003; Martin-Belmonte and Mostov, 2008). All tubular organs contain a lumen the size and shape of which is essential for organ function. Failure to achieve and/or maintain proper lumen size and shape can lead to pathological conditions. For example, polycystic kidney disease is characterized by lumen expansion whereas stenoses are characterized by abnormal narrowing of blood vessels.

The Rho family of small GTPases, which includes Rac, Cdc42 and Rho, are required for multiple cellular events, such as cell motility, proliferation and gene transcription. A crucial role for Rac and Cdc42 in lumen morphogenesis is well documented (Davis et al., 2007; Jaffe et al., 2008; Martin-Belmonte et al., 2007). Mammalian Cdc42 regulates micrulum formation and maintains cell polarity during pancreatic tube morphogenesis (Kesavan et al., 2009). In three-dimensional Caco-2 cell cultures, Cdc42 prevents multiple lumen formation by orienting cell divisions and directing apical membrane growth (Jaffe et al., 2008). We recently showed that in the *Drosophila* salivary gland, Cdc42 and the p21 activated kinase (Pak) 1 regulate gland lumen size (Pirraglia et al., 2010). In contrast to Cdc42 and Rac GTPases, the role of Rho in tube and lumen morphogenesis is poorly understood.

The only *Drosophila* Rho GTPase, Rho1, is required for invagination of the salivary gland and the posterior spiracles (Simoes et al., 2006; Xu et al., 2008). After invaginating from the ventral surface of the embryo, salivary gland cells migrate collectively as an intact tube, with the distal tip cells elongating and extending protrusions in the direction of migration (Bradley et al., 2003), and the proximal end cells changing shape from columnar to cuboidal (Xu et al., 2008). When the distal gland cells contact the overlying circular visceral mesoderm (CVM), the entire gland turns and migrates posteriorly (Bradley et al., 2003; Vining et al., 2005). Contact between the distal gland cells and the CVM is mediated through the integrin adhesion receptors; loss of the β2 integrin subunits results in glands that fail to turn and migrate posteriorly (Bradley et al., 2003). We previously showed that Rho1 controls salivary gland invagination and migration by regulating cell polarity and Rok-mediated cell contraction and that Rho1 activity is required in the gland cells as well as in the CVM (Xu et al., 2008).

The *Drosophila* embryonic salivary gland is a well-established model system for investigating lumen size control in a tubular organ. After salivary gland cells invaginate, they undergo a phase of robust apical surface membrane growth (Myat and Andrew, 2002) and the apical domain size of individual cells decreases and elongates to become anisotropic along the longest axis of the lumen (Pirraglia et al., 2010). Apical membrane growth is limited by the basic helix-loop-helix (bHLH) transcriptional corepressor Hairy and its regulation of Huckebein (Hkb), an Sp1/Egr-like transcription factor, and by target genes *klarsicht* (*klar*), which encodes a KASH-domain-containing regulator of organelle and nuclear transport (Fischer-Vize and Mosley, 1994; Fischer et al., 2004; Guo...
et al., 2005; Mosley-Bishop et al., 1999) and crumbs (crb), which encodes an apical membrane protein that is necessary for the establishment and maintenance of apical-basal polarity (Myat and Andrew, 2002; Tepass and Knust, 1993; Tepass et al., 1990). Apical membrane remodeling in salivary gland cells is also regulated by Ribbon (Rib), a Broad Tramtrack Bric-a-brac (BTB) domain transcription factor (Bradley and Andrew, 2001; Shim et al., 2001), which promotes Crb expression and limits apical activity of the ERM protein Moesin (Kerman et al., 2008). Based on mathematical modeling, it is thought that salivary gland lumens of rib mutant embryos fail to elongate because of increased apical surface stiffness and viscosity (Cheshire et al., 2008). We recently showed that apical domain elongation is regulated by Pak1 through differential localization of E-cadherin (E-cad; Shotgun – FlyBase) at the adherens junctions and at the basolateral membrane (Pirraglia et al., 2010). Here, we provide the first evidence that Rho1 controls lumen size in the Drosophila embryonic salivary gland through regulation of the actin cytoskeleton and Moesin.

MATERIALS AND METHODS

Drosophila strains and genetics
Canton-S flies were used as wild-type controls. The following flies were obtained from the Bloomington Stock Center and are described in FlyBase (http://flybase.bio.indiana.edu/): Rho1E3.10 (Rho1), Rho11B, Rho1E3.10, Rho11B, Rho1E3.10, armadillo (arm)-GAL4, UAS-Rok1E3.10, rib3, rok2, chic221, tsE(35)hs, UAS-Rab53455, UAS-Rab53455, UAS-rib11B, UAS-Ro1E3.10 and UAS-Dicer. UAS-Rok-RNAi was obtained from Vienna Drosophila RNAi Center (http://stockcenter.vdrc.at/), UAS-Moe2788 and UAS-Moe15501 were gifts from R. Feihon (University of Chicago, Chicago, USA). UAS Rib3 and UAS Rho1E3.10 were gifts from D. Andrew (Johns Hopkins University School of Medicine, MD, USA) and N. Harden (Simon Fraser University, Burnaby, Canada), respectively. UAS E-cadherin-GFP was obtained from H. Oda (JT Biochemistry Research Hall, Osaka, Japan).

fork head (fkh)-GAL4) was used to drive salivary gland-specific expression (Henderson and Andrew, 2000). UAS-Rok-RNAi expression was driven with arm-GAL4; fkh-GAL4. Twist-GAL4 was a gift from M. Baylies (Memorial Sloan-Kettering Cancer Center, NY, USA).

Antibody staining of embryos
Embryo fixation and staining were performed as previously described (Reuter et al., 1990). F-actin was stained with Phalloidin (1:20; Invitrogen) as described previously (Jani and Schöck, 2007). The following antiserum were used at the indicated dilutions: rat or rabbit (a gift from D. Andrew) dCREB-A antiserum at 1:10,000 for diaminobenzidine (DAB) staining and 1:250 for fluorescent antibody staining of embryos with F-actin and/or p-Moe was then calculated. Identical areas measuring 2.09 × 6.44 μm consisting of 1 z-series, each consisting of 1 μm-thick optical sections, were acquired by LSM confocal microscopy and the projected image of each z series was analyzed by ImageJ software (NIH). Identical areas measuring 3 μm in width and 3 μm in length were selected in the four proximal-most gland cells and the average fluorescence intensity (in pixels) of E-cad was normalized against the average fluorescence intensity of α-spectrin within the same area.

To quantification of the ratio of apical to basolateral F-actin or p-Moe, stage 12 WT, Rho1WT, Rho1E3.10, rib3, Moe15501, Rho11B,Rib1E3.10, Rho1E3.10, rib11B, rib11B, Moe2788, chic221, tsE(35)hs, tsE(35)hs, Rho11B or Rok-RNAi expressing embryos were stained with F-actin and/or p-Moe. A single z series acquired by LSM confocal microscopy was selected and analyzed by ImageJ software. Identical areas measuring 2.09 μm in width and 2.09 μm in length were selected in the middle of the apical or basolateral domain of the four proximal-most salivary gland cells. The ratio of the average fluorescence (in pixels) intensity of apical F-actin or p-Moe to that of basolateral F-actin or p-Moe was then calculated.

RESULTS

Rho1 controls salivary gland lumen size
We previously showed that Rho1 is required in salivary gland cells and in the surrounding mesoderm to regulate invagination and migration of the gland (Xu et al., 2008). In Rho11B mutant embryos, salivary gland cells invaginated and formed a tube with a central lumen but failed to migrate posteriorly, whereas in Rho1E3.10 mutant embryos, most gland cells did not invaginate and did not form a tube (Xu et al., 2008) (Table 1). Rho1F/K is a loss-
of-function allele with a P element insertion in the first intron (Magie et al., 1999). Rho11B is a loss-of-function allele in which the coding region C-terminal to amino acid 52 is removed by an imprecise P-element excision (Magie and Parkhurst, 2005). No Rho1 protein is detected by immunohistochemistry in Rho11B homozygous embryos (Magie and Parkhurst, 2005). The severity of the Rho1 allele is comparable to that of glands expressing dominant-negative Rho1 (Table 1). To determine a role for Rho1 in control of salivary gland lumen size, we analyzed three different alleles of Rho1, Rho11B, Rho1E3.10 and Rho172F (Table 1). The Rho1E3.10 allele is a loss-of-function allele in which the cysteine residue at position 189 is changed to a tyrosine residue (Halsell et al., 2000) and the Rho172F allele is a loss-of-function allele lacking part of the coding region including the translation start site (Strutt et al., 1997). Because the cysteine at position 189 is the first residue in the CAAX box and is the site of post-translational prenylation, the Rho1E3.10 mutant protein is unlikely to get prenylated and is likely to fail to localize to the plasma membrane to be activated. In embryos homozygous for Rho11B, Rho1E3.10 or Rho172F, all gland cells invaginated and formed a tubular organ (data not shown), allowing us to analyze Rho1 function in lumen size control.

In wild-type glands, the lumen diameter in the proximal region of the gland gradually decreased between embryonic stages 11 and 12 as the gland turned and migrated posteriorly, whereas lumen diameter in the medial and distal regions did not change (Pirraglia et al., 2010) (supplementary material Fig. S1). In Rho11B mutant embryos, lumen length was 60% of that of heterozygous siblings and lumen width in the proximal region was approximately twice that of heterozygous siblings (Fig. 1A-F). Embryos homozygous

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<td>Rho1K</td>
<td>P element insertion in the first intron (Magie et al., 1999)</td>
<td>Invagination and cell polarity defects (Xu et al., 2008)</td>
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<tr>
<td>Rho172O</td>
<td>P element insertion, lack of translation start codon (Strutt et al., 1997)</td>
<td>Invagination defects (data not shown)</td>
</tr>
<tr>
<td>Rho11B</td>
<td>Premature stop codon removing 2/3 coding sequence from C terminus (Magie and Parkhurst, 2005)</td>
<td>Migration defects and abnormal lumen size (Xu et al., 2008) (this study)</td>
</tr>
<tr>
<td>Rho1E3.10</td>
<td>Missense mutation in the CAAX box, reducing membrane-bound Rho1 (Halsell et al., 2000)</td>
<td>Migration defects and abnormal lumen size (this study)</td>
</tr>
<tr>
<td>Rho172F</td>
<td>P element insertion, lack of translation start codon (Strutt et al., 1997)</td>
<td>Migration defects and abnormal lumen size (Xu et al., 2008) (this study)</td>
</tr>
<tr>
<td>Rho1N19</td>
<td>Dominant negative (Strutt et al., 1997)</td>
<td>Invagination, migration and polarity defects (Xu et al., 2008)</td>
</tr>
<tr>
<td>Rho1V12</td>
<td>Constitutively active (Rangarajan et al., 1999)</td>
<td>Salivary gland cells invaginate simultaneously and form multiple lumens (Xu et al., 2008)</td>
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**Fig. 1. Rho1 controls salivary gland lumen size.**

(A-D) Drosophila embryos stained for E-cad (white) to label the lumen and dCREB-A (green) to label salivary gland nuclei. The gland lumen of Rho11B heterozygous embryos (A,C) is elongated (A, white) and of a distinct width (C, red arrow), whereas that of homozygous siblings (B,D) is shortened (B, white) and widened (D, red arrow). Scale bars: 5 μm in A; 2 μm in C. (E-G) Graphs depicting measurements of lumen length (E), lumen width (F) and apical domain elongation ratio (G) in Rho11B heterozygous and homozygous embryos and Rho1E3.10 and Rho172F homozygous embryos. ***P<0.001. Numbers on bars represent the number of glands (E,F) or gland cells (G) measured. Error bars represent s.d.
for Rho1E3.10 or Rho172F showed defects in gland lumen size of the same severity as those in Rho11B mutant embryos (Fig. 1E,F). To confirm that lumen size defects in Rho1 mutant embryos were not a consequence of changes in embryo size, we measured embryo length and height in Rho11B heterozygous and homozygous embryos. These measurements showed that embryo size was comparable in Rho11B heterozygous and homozygous embryos, demonstrating that salivary gland lumen size did not correlate with embryo size (data not shown).

Changes in salivary gland lumen length and width are normally accompanied by gradual elongation of the apical domain along the proximal-distal (Pr-Di) axis of the gland between stage 11, when the cells are internalized, and stage 12, when they migrate collectively (Pirraglia et al., 2010). Failure to elongate the apical domain can result in gland lumen size defects (Pirraglia et al., 2010). Therefore, we analyzed the extent of apical domain elongation in Rho11B homozygous gland cells compared with those of heterozygous siblings, and found that apical domains of Rho11B mutant gland cells did not elongate in the Pr-Di axis to the same extent as did apical domains of heterozygous siblings (Fig. 1G). We limited our analysis to the proximal gland cells, which showed the greatest reduction in lumen width (Pirraglia et al., 2010) and where Rho1 activity is predominantly required (Xu et al., 2008). Embryos homozygous for Rho1E3.10 or Rho172F also showed defects in apical domain elongation of the same severity as those in Rho11B homozygous embryos (Fig. 1G).

One mechanism for controlling apical domain elongation is through differential localization of E-cadherin (E-cad) at the adherens junctions (AJs) and at the basolateral membrane in a manner dependent on Pak1- and Rab5-mediated endocytosis (Pirraglia et al., 2010). In Pak1 mutant embryos, apical domains were expanded and failed to elongate in the Pr-Di axis concomitant with enhanced localization of E-cad at the AJs and reduced localization at the basolateral membrane (Pirraglia et al., 2010). In contrast to Pak1 mutant embryos, in Rho11B homozygous embryos, E-cad continued to be localized to the basolateral membrane and levels of E-cad at the AJs and at the basolateral membrane were similar in Rho11B homozygous and heterozygous gland cells (supplementary material Fig. S2). Similar to E-cad, localization of α-Catenin, another component of the AJs, was not affected in Rho11B mutant gland cells (data not shown). Moreover, expression of wild-type E-cad, encoded by shotgun (shg), specifically in gland cells of Rho11B mutant embryos, did not enhance or suppress the lumen size defects, or defects in cell shape change and apical domain elongation, and only slightly alleviated the cell rearrangement defects of Rho11B homozygous embryos (supplementary material Fig. S3). During gland invagination, Rho1 is required to maintain E-cad at the AJs; in Rho11B homozygous embryos where salivary gland cells did not maintain apical polarity (Xu et al., 2008), E-cad was lost from the apical-lateral membrane (supplementary material Fig. S4). Loss of E-cad in Rho11B mutant gland cells is possibly a secondary consequence of loss of apical polarity proteins, such as Crb, Stardust and atypical PKC (Xu et al., 2008). Thus, our analysis of Rho1 mutant embryos demonstrates that Rho1 regulates salivary gland lumen size without affecting levels of E-cad at the AJs or at the basolateral membrane.

**Rho1-dependent cell rearrangement is required for salivary gland lumen size control**

Rho-mediated signaling is known to control the cell rearrangements that drive elongation of the vertebrate gut tube (Reed et al., 2009). Thus, we hypothesized that Rho1 controls salivary gland lumen size, at least in part, by regulating cell rearrangement. To test this hypothesis, we first determined whether cell rearrangement normally occurred during elongation and narrowing of the gland lumen. We measured the extent of cell rearrangement in the proximal gland cells by counting the number of nuclei that surrounded the central lumen. In stage 11 wild-type glands, between ten and 12 cells (nuclei) surrounded the lumen in the proximal region of the gland (Fig. 2A,E). As the gland lumen elongated and narrowed proximally between stages 11 and 13, we observed a decrease in the number of nuclei surrounding the lumen, such that by stage 13, approximately half the number of nuclei surrounded the lumen compared with stage 11 (Fig. 2A-C,E). In contrast to wild-type glands, proximal gland cells of Rho11B mutant embryos failed to rearrange; in stage 12 Rho11B mutant glands, the
number of nuclei surrounding the gland was approximately twofold greater than that of heterozygous siblings (Fig. 2D,F). Embryos homozygous for Rho1E3.10 or Rho172F also showed defects in cell rearrangement (Fig. 2F). Thus, Rho1 function is required for the cell rearrangements that normally occur during salivary gland lumen elongation and narrowing.

**Rho1 controls salivary gland lumen size through Rho kinase**

We previously showed that Rho kinase (Rok), a key downstream effector of Rho GTPase, is required for gland migration, in particular for the proximal gland cells to flatten and change shape from columnar to cuboidal (Xu et al., 2008), which is quantified here as changes in apical-basal axis length. To test whether Rok was also required for the Rho1-dependent control of gland lumen size, we analyzed gland lumen size in embryos in which Rok function was specifically inhibited in the gland using RNAi knockdown. To achieve maximal knockdown of Rok, we co-expressed Rok-RNAi and Dicer with fork head (fkh)-GAL4 and armadillo-GAL4. Lumens of Rok-RNAi-expressing glands were widened like those of Rho11B mutant glands; however, lumen length in Rok-RNAi-expressing glands was only mildly affected (Fig. 3A,B). Rok-RNAi-expressing glands also had defects in cell shape change, apical domain elongation and cell rearrangement (Fig. 3C-E). Although we observed mild lumen length defects in Rok-RNAi-expressing glands, lumen length was shorter in glands of embryos homozygous for a loss-of-function allele of Rok, rok2 (Fig. 3A). rok2 mutant glands also showed defects in lumen width, cell shape change, apical domain elongation and cell rearrangement (Fig. 3B-E). Gland-specific expression of constitutively active Rok (RokCA) in Rho11B mutant glands was sufficient to partially restore lumen length and completely restore lumen width in Rho11B mutant glands (Fig. 3A,B). Expression of RokCA allowed Rho11B mutant gland cells to change shape, to rearrange and for the apical domains to elongate (Fig. 3C-E). These data demonstrate that Rok mediates Rho1-dependent cell shape change, apical domain elongation and cell rearrangement, all processes that collectively determine salivary gland lumen size.

**Rho1 functions cell-autonomously to regulate apical domain elongation and cell rearrangement**

We previously showed that Rho1 activity is required in the salivary gland, predominantly in the proximal gland cells, and surrounding mesoderm for gland migration (Xu et al., 2008). To

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**Fig. 3. Rho1 regulates salivary gland lumen size through Rok. (A-E)** Graphs depicting measurements of lumen length (A) and width (B), apical-basal axis length (C), apical domain elongation ratio (D) and number of nuclei surrounding the salivary gland lumen (E) of wild-type (WT) Drosophila embryos, Rho11B homozygous embryos, rok2 homozygous embryos, wild-type embryos expressing Rok RNAi in the gland, Rho11B homozygous embryos expressing RokCA in the gland and tsRho11B double mutant embryos. **P<0.01, ***P<0.001. Numbers on bars represent the number of glands (A,B,E) or the number of cells (C,D) measured. Error bars represent s.d.
test whether Rho1 function is required in gland cells for lumen size control, we expressed wild-type Rho1 (Rho1WT) in all gland cells with fkh-GAL4, in just the proximal gland cells with engrailed (en)-GAL4 or in the surrounding mesoderm with twist (twi)-GAL4. Expression of Rho1WT in either the salivary gland or surrounding mesoderm of Rho11B homozygous embryos had no effect on gland lumen length or width (supplementary material Fig. S5A,B). However, expression of Rho1WT in all gland cells or just the proximal gland cells of Rho11B homozygous embryos led to a partial but significant rescue of the apical domain elongation and cell rearrangement defects (supplementary material Fig. SSD,E). By contrast, expression of Rho1WT in the mesoderm only slightly alleviated the apical elongation defect and had no effect on the cell rearrangement defect of Rho11B mutant embryos (supplementary material Fig. SSD,E). Expression of Rho1WT in either the gland or the mesoderm had only a mild effect on cell shape change (supplementary material Fig. S5C). From these data we conclude that Rho1 functions predominantly in the proximal salivary gland cells to control apical domain elongation and cell rearrangement.

**Rho1 is required for actin polymerization and distribution in salivary gland cells**

Rho family GTPases are known to regulate the actin cytoskeleton, with mammalian RhoA being most directly linked to the formation of stress fibers (Ridley and Hall, 1992). Therefore, we tested whether the lumen size defects of Rho11B mutant glands could be due to defects in the actin cytoskeleton by analyzing the distribution of cortical F-actin at the apical and basolateral membranes. We quantified F-actin distribution by measuring the ratio of F-actin at the apical membrane to that at the basolateral membrane. In proximal gland cells of Rho11B heterozygous embryos, the apical membrane was slightly more enriched with F-actin compared with the basolateral membrane (Fig. 4A). In Rho11B mutant gland cells, the apical membrane was highly enriched with F-actin whereas F-actin was severely reduced at the basolateral membrane (Fig. 4B). Moreover, the apical to basolateral (A/B) F-actin ratio of Rho11B mutant gland cells was significantly higher than that of wild-type gland cells (Fig. 4K). Rho1E3.10 mutant glands and Rok-RNAi-expressing glands also had reduced basolateral F-actin and enriched apical F-actin (Fig. 4C,K; data not shown).

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**Fig. 4.** Rho1 regulates actin polymerization and distribution in salivary gland cells. (A) In Rho11B heterozygous Drosophila embryos, F-actin is slightly enriched in the apical membrane (arrow) and is localized along the basolateral membrane (arrowhead). (B) In Rho11B homozygous embryos, F-actin is highly enriched in the apical membrane (arrow) and is reduced from the basolateral membrane (arrowhead). (C) In Rok-RNAi-expressing glands, F-actin is enriched in the apical membrane (arrow) and reduced from the basolateral membrane (arrowhead). (D,E) In rib1 homozygous embryos (D) and wild-type embryos expressing Moe559D in the gland (E), F-actin is slightly enriched in the apical membrane (arrows) and is present along the basolateral membrane (arrowheads). (F,G) In Rho11B homozygous embryos expressing RibWT (F) or Moe559D (G), F-actin is highly enriched in the apical membrane (arrows) and is reduced from the basolateral membrane (arrowheads). (H) In chic221 homozygous embryos, F-actin is disorganized at the apical membrane (arrow) and is present at the basolateral membrane (arrowhead). (I,J) In tsrK homozygous embryos (I) and tsrK/Rho11B double mutant embryos (J), F-actin is slightly enriched in the apical membrane (arrow) and is present along the basolateral membrane (arrowheads). (K) Graph depicting the ratio of F-actin at the apical and basolateral membranes in wild-type (WT) glands, glands of Rho11B, Rho1E3.10, tsrK, chic221, tsrK, tsrK/Rho11B homozygous embryos, glands expressing Moe559D or Rok-RNAi and glands of Rho11B homozygous embryos expressing RibWT or Moe559D. Numbers on bars represent the number of cells measured. ***P<0.001. Error bars represent s.d. All embryos shown are at stage 12 and were stained for F-actin with phalloidin. Dashed lines in A-J outline the salivary gland. Scale bar: 5 μm.
shown); however, F-actin distribution between the apical and basolateral membranes was not as severely disrupted as in Rho1<sup>1B</sup> mutant glands (Fig. 4K). Thus, Rho1 and Rok promote F-actin localization at the basolateral membrane and limit F-actin at the apical membrane in salivary gland cells.

Because lumen size defects in Rho1<sup>1B</sup> mutant gland cells were accompanied by a reduction in basolateral F-actin, we hypothesized that promoting actin polymerization by preventing actin depolymerization might restore basolateral F-actin in Rho1 mutant gland cells. **Twinstar** encodes the only *Drosophila* homolog of Cofilin (Chen et al., 2001), an actin-binding protein, actin-depolymerizing activity of which is inhibited through phosphorylation by LIM-kinase, which, in turn, is regulated by ROCK/Rok (Maekawa et al., 1999). Mutations in *tsr* have been shown to affect border cell migration and planar cell polarity in *Drosophila* (Blair et al., 2006; Zhang et al., 2011). To inhibit *tsr* function in Rho1<sup>1B</sup> salivary gland cells, we used a *tsr* allele, *tsr<sup>k05633</sup>* (referred to here as *tsrk*) that was shown previously to have no effect on salivary gland development on its own (Chandrasekaran and Beckendorf, 2005). Loss of *tsr* function in Rho1<sup>1B</sup> homozygous embryos significantly suppressed the cell rearrangement and apical domain elongation defects of Rho1<sup>1B</sup> mutant glands, which, in turn, narrowed the expanded lumens (Fig. 3B,D,E). Loss of *tsr* in Rho1<sup>1B</sup> mutant glands had no effect on cell shape change or lumen length (Fig. 3A,C). Salivary glands mutant for *tsr*<sup>k</sup> alone showed no defect in lumen size or apical domain elongation (data not shown) or F-actin distribution (Fig. 4H,K). In *tsr<sup>p</sup>* Rho1<sup>1B</sup> double mutant salivary gland cells, F-actin was distributed normally and localized to the basolateral membrane (Fig. 4J,K). These data suggest that Rho1-mediated regulation of actin polymerization and distribution promotes cell rearrangement and apical domain elongation.

To test whether independent inhibition of actin polymerization can phenocopy the Rho1 lumen phenotype, we analyzed embryos mutant for chickadee (*chic*), encoding the *Drosophila* homolog of profilin, an actin-binding protein that promotes actin polymerization (Cooley et al., 1992). Loss of *chic* disrupts actin-dependent processes during *Drosophila* oogenesis and embryogenesis (Cooley et al., 1992; Verheyen and Cooley, 1994), and overexpression of *chic* in the salivary gland perturbs gland invagination and morphology (Maybeck and Roper, 2009). In *chic<sup>221</sup>* mutant embryos, gland lumens were widened and shortened, and gland cells failed to elongate their apical domains and rearrange, as was also observed in Rho1<sup>1B</sup> mutant embryos (supplementary material Fig. S6). In contrast to Rho1<sup>1B</sup> mutant embryos.
glands, in chic mutant glands, F-actin was present at the basolateral membrane and was disorganized at the apical membrane, resulting in an apical-basolateral F-actin ratio lower than that of wild-type glands (Fig. 4H,K). These data suggest that not only is Rho1 required for actin polymerization, but it is also required for the proper distribution of F-actin.

**Rho1 controls gland lumen size with Ribbon**

The salivary gland lumen size defects of Rho1 mutant embryos are similar to those of embryos mutant for ribbon (rib), which encodes a BTB domain transcription factor that is required for the development of multiple epithelial-based tubular organs, such as the salivary gland, Malpighian tubules and trachea (Blake et al., 1999; Bradley and Andrew, 2001; Jack and Myette, 1997; Kerman et al., 2008; Shim et al., 2001) and that is known to control gland lumen size (Kerman et al., 2008). Rib has been proposed to regulate gland lumen size by promoting Crb expression to facilitate lumen size independently of E-cad levels. Expression of RibWT in wild-type glands did not have any effect on gland lumen size (data not shown). However, expression of RibWT in Rho1 mutant glands completely suppressed the apical domain elongation defect but had little or no effect on cell rearrangement, cell shape change and lumen size (Fig. 5A-E). Expression of RokCA in ribi mutant embryos had no effect on apical domain elongation; however, RokCA did partially restore normal lumen width in ribi mutant glands possibly owing to the effect of RokCA on cell shape change (Fig. 5B,C). These data suggest that Rib contributes mainly to Rho1-dependent apical domain elongation and not to cell rearrangement.

**Rho1 limits apical phosphorylated Moesin**

Rho1 has been shown to regulate apical domain remodeling in gland cells by limiting apical Moesin (Moe) activity (Kerman et al., 2008). In ribi mutant embryos, levels of phosphorylated Moe (p-Moe), the active form of Moe, were elevated in the apical membrane (Fig. 6C) (Kerman et al., 2008). Rho11B mutant glands showed highly elevated levels of apical p-Moe, even higher than that of ribi mutant glands (Fig. 6A-C,F,G). Rho1E3.10 mutant glands showed a modest but statistically significant accumulation of p-Moe at the apical membrane (Fig. 6G). To test for a role for Moe in salivary gland lumen size control, we analyzed the effects of expressing a non-phosphorylatable form of Moe (MoeT559A), in which the conserved Threonine at 559 is changed to an Alanine (T559A), or a phosphomimetic form of Moe (MoeT559D), in which the conserved Threonine is changed to an Aspartic Acid (T559D) and has been shown to act in a constitutively active manner (Speck et al., 2003). Expression of MoeT559D in Rho1 mutant glands completely suppressed the apical domain elongation defect (Fig. 7D) but had no effect on the cell rearrangement defect (Fig. 7E), suggesting that the phosphorylated state of Moe at Thr 559 is important for Rho1 regulation of Moe and its effect on apical domain elongation specifically. Expression of MoeT559D alone in wild-type glands did not have any effect on gland lumen size (data not shown). However, expression of MoeT559D completely phenocopied loss of Rho1; in MoeT559D-expressing salivary glands, lumens were shorter and wider, cells failed to change shape, apical

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**Fig. 6. Rho1 and Ribbon limit apical phosphorylated Moesin in salivary gland cells.** (A-E) In wild-type Drosophila embryos (A), phosphorylated Moesin (p-Moe) is slightly enriched in the apical domain (A, arrow), whereas in Rho11B (B) and ribi (C) mutant gland cells, it is highly enriched in the apical domain (B and C, arrows). In Rok-RNAi-expressing gland cells (D) and ts3Rho1 double mutant gland cells (E), p-Moe is slightly enriched in the apical domains (D and E, arrows). All embryos shown are at stage 12. Embryos in A and B were stained for p-Moe and α-spectrin (not shown), whereas embryos in C-E were stained for p-Moe. Scale bar: 2 μm. (F) Graph depicting ratio of fluorescence intensity of apical p-Moe to α-spectrin in the proximal gland cells of Rho11B heterozygous and homozygous embryos. (G) Graph depicting ratio of apical to basolateral p-Moe in wild-type, Rho11B, Rho1E3.10, Rok-RNAi, ribi and ts3Rho118 mutant proximal gland cells. **P<0.01, ***P<0.001. Numbers on bars represent the number of gland cells measured. Error bars represent s.d.
domains failed to elongate and an increased number of nuclei surrounded the lumen (Fig. 7A-E). Similar to these data, it was previously reported that expression of MoeT559A in rib mutant trachea partially suppresses the tracheal defects and overexpression of MoeT559D phenocopies the rib mutant phenotype in the gland and trachea (Kerman et al., 2008). In contrast to Rho1, Rok was not required for limiting apical p-Moe; in Rok-RNAi-expressing salivary gland cells, p-Moe was not enriched apically (Fig. 6D,G). From these data we conclude that Rho1, independent of Rok, limits apical domain elongation but not for cell rearrangement.

In contrast to Rho1 mutant salivary gland cells, rib1 mutant gland cells and gland cells expressing MoeT559D showed a normal distribution of F-actin between the apical and basolateral membranes (Fig. 4D,E,K). Expression of either MoeT559A or RibWT in Rho11B mutant glands did not alter the apical enrichment of F-actin nor did it restore basolateral F-actin in Rho11B mutant gland cells (Fig. 4F,G,K). In tsr1Rho11B double mutant embryos, in which loss of tsr rescued the cell rearrangement and apical domain elongation defects of Rho11B mutant glands through proper distribution of F-actin, apical p-Moe was significantly reduced compared with Rho11B mutant glands (Fig. 6B,E,G). From these data we conclude that the enriched apical F-actin observed in Rho11B mutant gland cells is not due to the enriched apical p-Moe and that although Rib functions with Rho1 to limit p-Moe, Rib has no effect on F-actin distribution.

**Rab5- and Dynamin-mediated endocytosis and actin distribution**

We previously showed that Rab5-mediated endocytosis regulates differential localization of E-cad during apical domain elongation in salivary gland cells (Pirraglia et al., 2010). To test whether Rab5-dependent endocytosis plays a role in Rho1-mediated regulation of apical F-actin and/or p-Moe, we first determined whether loss of Rho1 affected the subcellular distribution of endocytic vesicles. In Rho11B heterozygous gland cells, F-actin and Avalanche (Aval), a Drosophila syntaxin that labels early endosomes (Lu and Bilder, 2005), colocalized at the sub-apical membrane and in intracellular puncta (Fig. 8A). By contrast, in Rho11B homozygous embryos, Aval and F-actin were localized at the apical membrane but not in intracellular puncta that lacked F-actin (Fig. 8B). We next tested whether independently inhibiting Rab5- and/or Dynamin-mediated endocytosis phenocopies the Rho1 gland lumen size defects. Gland-specific expression of
dominant negative Rab5 (Rab5DN) led to a moderate enrichment of F-actin at the apical membrane although F-actin continued to be localized at the basolateral membrane (Fig. 8C,E). Gland-specific expression of dominant negative shibire (shi), encoding Drosophila Dynamin, (ShiDN), resulted in the dramatic accumulation of F-actin at the apical and sub-apical membrane (Fig. 8D). Although F-actin accumulated apically in Rab5DN- or ShiDN-expressing gland cells, basolateral F-actin and apical p-Moe were not affected (Fig. 8C,D; data not shown). Apical domain elongation and gland lumen size were also not affected in ShiDN- and Rab5DN-expressing glands (Fig. 8F; supplementary material Fig. S7). Continued expression of ShiDN in the gland resulted in Aval-positive structures coated with F-actin that appeared tethered to the apical membrane (supplementary material Fig. S8). Thus, loss of Rho1 resulted in loss of F-actin from early endosomes, and inhibition of Rab5 or Shi led to apical enrichment of F-actin but did not affect gland lumen size.

DISCUSSION
We previously showed that Rho1 acts both in salivary gland cells and in the surrounding mesoderm to maintain apical polarity during gland invagination and to mediate cell shape change during gland migration (Xu et al., 2008). Here, we demonstrate a novel role for Rho1 in controlling salivary gland lumen size through regulation of actin polymerization and distribution and regulation of Moesin activity. By analyzing Rho1 alleles for which salivary gland cells invaginated and formed a gland, we showed thatygotic loss of function of Rho1 resulted in shortening and widening of the gland.
lumen, which was accompanied by defects in cell shape change and cell rearrangement and failure of apical domains to elongate along the Pr-Di axis of the gland. These effects of Rho1 are mediated through Rok, as inhibition of Rok completely phenocopied loss of Rho1 in these cellular events. Based on these studies, we propose a model for Rho1 control of salivary gland lumen size, in particular lumen width, which is determined by cell rearrangement and apical domain elongation. Rho1 and Rok, through inhibition of coflin, regulate cell rearrangement and apical domain elongation by promoting actin polymerization to localize F-actin at the basolateral membrane and by limiting the apical accumulation of F-actin (Fig. 9). In parallel to its role in actin polymerization and distribution, Rho1 acts independently of Rok to limit apical p-Moe with Rib by an unknown mechanism and this function of Rho1 is specific for apical domain elongation (Fig. 9).

Our data on coflin are consistent with those in cultured HeLa cells that showed that mammalian ROCK can inhibit coflin activity indirectly through LIMK-mediated phosphorylation of coflin (Maekawa et al., 1999).

Although manipulating Moe activity through gland-specific expression of MoeT559D was sufficient to completely phenocopy the Rho1 lumen defects, including cell rearrangement, it did so without disrupting actin polymerization or distribution. This is likely to be due to activated Moe strengthening the link between the actin cytoskeleton and the apical plasma membrane (without affecting levels of apical F-actin), which would increase apical membrane stiffness and remove the ability of gland cells to rearrange. Indeed, Moe511 has been shown to control cortical rigidity during mitosis of cultured Drosophila S2R+ cells (Kunda et al., 2008). Thus, Rho1 regulates cell rearrangement and apical domain elongation by controlling the actin cytoskeleton and Moe511 activity through distinct mechanisms.

Our observation that chic mutant glands phenocopied Rho1 mutant lumen defects to a large extent, suggests that Rho1 control of salivary gland lumen size is mainly dependent on a requirement for Rho1 in actin polymerization. However, as the chic and Rho1 gland lumen phenotypes are not identical, with chic mutant glands lacking the apical accumulation of F-actin and p-Moe observed in Rho1 mutant glands, Rho1 probably has an additional function in limiting accumulation of F-actin and p-Moe at the apical membrane. This function of Rho1, at least for limiting apical F-actin, might partly involve Rab5- or Shi-mediated endosome trafficking, because inhibition of Rab5 alone or Shi alone led to accumulation of F-actin at the apical membrane. Although Rab5DN- or ShiDN-expressing salivary gland cells were enriched with apical F-actin, lumen size was not affected. This could be due to Rab5DN and ShiDN affecting a pool of apical F-actin distinct from that affected by Rho1 and/or because Rab5DN-expressing gland cells retain basolateral F-actin and the ratio of apical to basolateral F-actin is not altered sufficiently to cause lumen size defects. In Rho11B mutant gland cells, some early endosomes were not coated with F-actin. Actin is known to contribute to multiple steps of the endocytic pathway, including movement of endocytic vesicles through the cytoplasm and their transport to late endosomes and lysosomes (Apodaca, 2001; Brown and Song, 2001; Merrifield et al., 1999; Qualmann and Kessels, 2002; Taunton et al., 2000; van Deurs et al., 1995). One possible mechanism by which Rho1 normally limits apical accumulation of F-actin is by promoting its removal from the apical membrane and accumulation on endocytic vesicles.

Currently, we do not know how Rho1 limits accumulation of apical p-Moe. Membrane localization and activity of Moe511 can be regulated via a number of mechanisms, such as its phosphorylation on a conserved Threonine residue (Matsui et al., 1998; Ohshiro et al., 1998), binding to phosphatidylinositol-(4,5)bisphosphate [PtdIns(4,5)P2] (Roch et al., 2010; Yonemura et al., 2002) and association with components of the sub-membrane cytoskeleton, such as Crb (Medina et al., 2002). Studies in cultured mammalian cells have demonstrated that Rho signaling activates Moe either through phosphorylation of Moe by ROCK (Matsui et al., 1998) or through ROCK-mediated inhibition of myosin phosphatase, which is known to dephosphorylate p-Moe (Fukata et al., 1998). Although it is possible that Drosophila Rho1 positively regulates Moe activity by one or more of these mechanisms, we show here that in the developing salivary glands Rho1 in fact negatively regulates Moe activity. In rib mutant embryos, in which p-Moe is enriched apically, salivary gland and tracheal cells showed decreased staining for Rab11 GTPase, which localizes to the apical recycling endosomes and to secretory vesicles destined for the apical membrane (Kerman et al., 2008). Thus, Rho1, like Rib might limit apical p-Moe through its membrane transport.

In Drosophila imaginal disc epithelia, Moe negatively regulates Rho1 activity to maintain epithelial integrity and to promote cell survival (Hipfner et al., 2004; Molnar and de Celis, 2006; Neisch et al., 2010; Speck et al., 2003). Our studies demonstrating that in the developing salivary gland Rho1 antagonizes Moe activity by limiting its localization at the apical membrane, shed novel insight into the functional relationship between Rho1 and Moe. It is possible that in a dynamic epithelium, such as the developing salivary gland, Rho1 contributes to the precise spatial and temporal regulation of Moe activity to fine-tune selective changes in apical domain shape. By contrast, in the imaginal disc epithelium, Rho1 regulation of Moe might not be necessary and, instead, Moe regulation of Rho1 activity is required to maintain epithelial integrity and cell survival. Thus, Rho and Moe can antagonize each other’s activities depending on the type of epithelia or cellular event.

Our rescue studies with Rho1WT demonstrated that Rho1 functions predominantly in the salivary gland cells to control apical domain elongation and cell rearrangement. Interestingly, expression of Rho1WT in the mesoderm with twi-GAL4 had no effect on cell rearrangement and had little effect on apical domain elongation.
domain elongation and lumen size (this study), whereas we previously showed that \textit{Rho1} expression in the mesoderm significantly rescued the gland migration defect of \textit{Rho1} mutant embryos (Xu et al., 2008). This suggests that gland migration and lumen size control are regulated by distinct mechanisms. In support of this conclusion, embryos mutant for \textit{multiple edematous wings}, encoding the cP31 integrin subunit, which was previously reported to have defects in gland migration (Bradley et al., 2003), showed no defects in gland lumen width (C. Pirraglia, J. Walters, N. Ahn, M.M.M., unpublished). Identifying the distinct and overlapping mechanisms by which salivary gland lumen width and length are controlled will help to elucidate the mechanisms by which lumen size is controlled in tubular organs.

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