Rho GTPase controls invagination and cohesive migration of the *Drosophila* salivary gland through Crumbs and Rho-kinase

Na Xu 1, Benison Keung 2, Monn Monn Myat *

Department of Cell and Developmental Biology, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10065, USA

**Abstract**

Coordinated cell movements shape simple epithelia into functional tissues and organs during embryogenesis. Regulators and effectors of the small GTPase Rho have been shown to be essential for epithelial morphogenesis in cell culture; however, the mechanism by which Rho GTPase and its downstream effectors control coordinated movement of epithelia in a developing tissue or organ is largely unknown. Here, we show that Rho1 GTPase activity is required for the invagination of *Drosophila* embryonic salivary gland epithelia and for directed migration of the internalized gland. We demonstrate that the absence of zygotic function of Rho1 results in the selective loss of the apical proteins, Crumbs (Crb), *Drosophila* atypical PKC and Stardust during gland invagination and that this is partially due to reduced *crb* RNA levels and apical localization. In parallel to regulation of *crb* RNA and protein, Rho1 activity also signals through Rho-kinase (Rok) to induce apical constriction and cell shape change during invagination. After invagination, Rho-Rok signaling is required again for the coordinated contraction and dorsal migration of the proximal half of the gland. We also show that Rho1 activity is required for proper development of the circular visceral mesoderm upon which the gland migrates. Our genetic and live-imaging analyses provide novel evidence that the proximal gland cells play an essential and active role in salivary gland migration that propels the entire gland to turn and migrate posteriorly.

© 2008 Elsevier Inc. All rights reserved.

**Introduction**

Epithelial cells move cohesively to form functional tissues and organs during embryogenesis. The specification of an epithelial placode followed by distinct changes in cell shape leading to the invagination of cells into the underlying tissue is a prevalent morphogenetic movement observed during early stages of the formation of the lens and optic cup (Hilfer, 1983), otic vesicle (Alvarez and Navascues, 1990), neural tube (Schoenwolf and Smith, 1990), mammary gland, tooth and hair follicle (Mikkola and Millar, 2006). Similar changes in cell shape and movement are also observed in *Drosophila* and *Xenopus* gastrulation and *Drosophila* tracheal and salivary gland invagination (Hardin and Keller, 1988; Leptin, 1999; Myat, 2005). In addition to invagination, epithelial tissues and organs migrate as cohesive sheets or groups of cells. For example, *Drosophila* epidermal cells migrate as sheets during dorsal closure whereas border cells migrate as clusters of motile cells (Lecaudey and Gilmour, 2006). Despite the prevalence of epithelial invagination and migration in organogenesis, little is known about the molecular mechanisms that govern epithelial characteristics and movement.

Members of the Rho family of small GTPases are critical regulators of epithelial morphogenesis. They act as molecular switches to control epithelial cell polarity, cell–cell adhesion, cell–substratum adhesion and actin cytoskeleton organization (Jaffe and Hall, 2005). The small GTPase Rac has been shown to regulate cohesive movements of several epithelial tissues and organs in the *Drosophila* embryo such as dorsal closure migration (Woolner et al., 2005), tracheal cell rearrangement (Chihara et al., 2003) and salivary gland migration (Pirraglia et al., 2006). During *Drosophila* gastrulation, the small GTPase Rho signals through Myosin II to induce apical constriction and mediate invagination of mesodermal cells (Barrett et al., 1997; Nikolaidou and Barrett, 2004). Activators of Rho GTPase have also been shown to be required for invagination of the *Drosophila* embryonic salivary gland implicating Rho GTPase in this process (Kolesnikov and Beckendorf, 2007; Nikolaidou and Barrett, 2004); however, it is not known whether Rho GTPase controls salivary gland invagination solely through actin–myosin contraction or by multiple mechanisms.

Here, we analyze Rho GTPase function in morphogenesis of the *Drosophila* embryonic salivary glands. The salivary glands form by invagination of primordial cells from the embryo surface followed by cohesive migration of the gland along surrounding mesoderm (Myat, 2005). Gland cells invaginate by apical constriction and cell shape change from columnar to pyramidal, a process dependent on the
transcription factor, Fork head (Fkh) (Myat and Andrew, 2000a; Myat and Andrew, 2000b). Hairy and Hucklebein-dependent transcriptional regulation of the apical determinant protein, Crumbs (Crb) is necessary for apical membrane generation during gland invagination. After invagination is complete, the distal tip of the gland contacts the overlying circular visceral mesoderm (CVM) and migrates with the distal tip cells elongating and extending protrusions in the direction of migration (Bradley et al., 2003). The entire gland then turns to align itself along the anterior–posterior axis before migrating further posteriorly. In this study, we show that Rh1 GTPase regulates salivary gland invagination by maintaining apical localization of Crb, Drosophila atypical PKC (Dap1PKC) and Stardust (Sdt) and that this occurs partially through regulation of crb RNA level and apical localization of the transcript and by inducing apical constriction and cell shape change through Rho-kinase (Rok). The Rho–Rok signaling pathway is required again during gland migration for contraction and dorsal migration of the proximal half of the gland that allows the entire gland to turn and migrate posteriorly.

Materials and methods

Drosophila strains and Genetics

Canton-S flies were used as wild-type controls. The following fly lines were obtained from the Bloomington Stock Center and are described in FlyBase (http://flybase.bio.indiana.edu/): Rho1fl107b and UAS-Rho1V12 were gifts of N. Knust, UAS-Rho1fl141, Rho1fl72, wingless (wg)-GAL4, engrailed (en)-GAL4, armadillo (arm)-GAL4, UAS-rok′-GAL4, UAS-rok-1′-GAL4, rok′ and C57BL/6j strain for UAS-actinGFP (mCD8GFP), UAS-mCD8GFP, UAS-actinGFP were obtained from the Vienna Drosophila Research Center (VDRC), crb1fl122 and UAS-crb1vir were gifts of E. Knust, UAS-Rho1f105 and UAS-Rho1f112 were gifts of N. Perrimon, UAS-Rho1f105 was a gift of N. Harden, UAS-actinGFP, bagpipe (bag)-GAL4 and twist (twi)-GAL4 were gifts of M. Baylles and crb1fl122 Df(3L)H99 was a gift of D. Bilder, fork head (fkh)-GAL4 was used to drive salivary gland specific expression (Henderson and Andrew, 2000).

Antibody staining of embryos

Embryos were fixed and processed for antibody staining as previously described (Reuter et al., 1990). The following antibodies were used at the indicated dilutions: rat dCREB-A antiserum at 1:10,000 for DAB staining and 1:1250 for fluorescence; rabbit Fkh antiserum (a gift from M. Stern and S. Beckendorf) at 1: 1000; rabbit DaPKC antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:500; mouse Crumbs antiserum (Developmental Studies Hybridoma Bank, DSHB; Iowa City, IA) at 1:100 for DAB and 1:10 for fluorescence; rabbit Stardust antiserum at 1:500 (a gift from E. Knust); mouse Neurotactin antiserum at 1:10 (DSHB); mouse α-fodrin antiserum at 1:10 (DSHB); mouse Fasciclin III (FasIII) antiserum at 1:20 (DSHB); mouse β-galactosidase (β-gal) antiserum (Promega; Madison, WI) at 1:10,000 for DAB staining and 1:500 for fluorescence; rabbit phospho-myosin light chain (p-MLC) antiserum at 1:20 (Cell Signaling Technology, Danvers, MA), rabbit Bazooka antiserum at 1:1000 (a gift from A. Brand), mouse GFP antiserum at 1:20,000 (Roche Diagnostics, Indianapolis, IN) and Alexa488-conjugated anti-GFP at 1:50 (Invitrogen Molecular Probes, OR). Appropriate biotinylated–(Jackson Immunoresearch Laboratories, Westgrove, PA), AlexaFluor488– or Rhodamine–(Molecular Probes, Eugene, OR) conjugated secondary antibodies were used at a dilution of 1:500. F-actin was labeled with AlexaFluor488-phalloidin (Molecular Probes). Whole-mount stained embryos were mounted in methyl salicylate (Sigma, St. Louis, MO), 85% glycerol with 2.5% N-propylgalactate or Aqua Polymount (Polysciences, Inc., Warrington, PA). Embryos were visualized on a Zeiss Axioplan 2 microscope with Axiosvision Rel 4.2 software (Carl Zeiss, Thornwood, NY) and thick (1 μm) fluorescent images were acquired on a Zeiss Axioplan microscope (Carl Zeiss) equipped for laser scanning confocal microscopy at the Rockefeller University Bio-imaging Resources Center (New York, NY).

RNA in situ hybridization

In situ hybridization with antisense digoxigenin-labeled RNA probes for crumbs was performed as previously described (Lehmann and Tautz, 1994). crumbs and β-galactosidase cDNAs were used as templates for generating antisense digoxigenin-labeled RNA probes as previously described (Myat and Andrew, 2002). Embryos were mounted in 70% glycerol before visualization as described above for antibody staining.

Reverse transcription (RT) and real-time PCR analyses

UAS-Rho1f119 UAS-actinGFP/CFL flies were crossed to armadillo-GAL4 flies and heterozygous and homozygous embryos were manually selected with a Zeiss Stereo Discovery V12 Zoom Microscope (Carl Zeiss). Total mRNA was extracted according to manufacturer's instructions using the QiAshredder and RNeasy mini kit from Qiagen (Valencia, CA). Reverse transcription (RT) was performed according to manufacturer's instructions using the One-Step RT-PCR kit (Qiagen). Primer sequences used for quantification of crb transcript were Dcrb-53 primer (5′ CGGACCTTCTCCGTCTTCTTACTC′3′) and Dcrb-53 primer (5′ GTGTTGCGGAAATCAGTGTGGCC′3′). Reference control was rp49 amplified with specific primers, rp49-5-2 (5′ ATGACCTCCTGCCCTATTAGGG′3′) and rp49-3-2 (5′ CTTGGCTTTCTGAAGAAGCCGGCC′3′). All primers used were generated by Invitrogen (Carlsbad, CA). Intensity of the crb and control rp49 PCR products were measured with NIH's ImageJ software and the ratio was calculated. Real-time PCR was performed at the Weill Cornell Microarray Core Facility.

Live imaging

Live imaging analysis was performed on the LSM 5 LIVE confocal system (Carl Zeiss) equipped with a Diode 488–100 laser. Images were acquired using either a 20X or 40X lens objective every 5 min at a scan speed between 1 and 4 for the duration of the recording period. Embryos were adhered to double-sided tape, covered in Halocarbon oil (SIGMA) and maintained at 25 °C during the recording.

Scoring of salivary gland invagination and migration phenotypes

To score gland invagination phenotypes, stage 14 embryos stained for dCREB-A were scored for glands that did not invaginate at all (None), partially invaginated with some cells that formed a tube and other cells that remained at the ventral surface (Partial) or completely invaginated (Complete). To score gland migration phenotypes, stage 14 embryos stained for dCREB-A were scored for glands that had completely turned, incompletely turned, only the distal tip turned or distal tip did not turn at all.

Quantification of fluorescence intensity and extent of apical-basal contraction

For quantification of fluorescence intensity in salivary gland placodes (Figs. 7A and D), stage 11 en-GAL4 UAS-mCD8GFP and wg-GAL4 UAS-mCD8GFP embryos were double stained for GFP and DaPKC. Three sets of Z series each consisting of three to five 0.5 μm thick optical sections were acquired by LSM confocal microscopy and the projected image of each of the Z series was analyzed by ImageJ software. Identical areas measuring 6.07 μm in width and 4.95 μm in length were selected and the ratio of the mean total signal intensity
For quantification of p-MLC fluorescence intensity in migrating salivary glands (Figs. 7I and J), stage 12 embryos were double stained for p-MLC and Fkh. Pixel intensity measurements of an area 18 μm in width and 17 μm in length were performed as described above.

For quantification of extent of apical–basal contraction, live images of Rho11β heterozygous and homozygous embryos at stage 11 were first acquired as described above. The distance between the apical and basal membranes of proximal gland cells at the beginning and end of the recording were measured with LSM 510 software and the average calculated. P values were obtained by STATA software two-way ANOVA analysis (StataCorp, TX).

Results

Rho1 GTPase is required for salivary gland invagination and migration

To understand how Rho1 GTPase regulates salivary gland invagination, we analyzed embryos mutant for three different alleles of Rho1, Rho1K02107b (Rho1K), Rho11β and Rho172F and found that gland invagination was defective in all three alleles. In Rho1K homozygous embryos, majority of glands failed to invaginate and gland cells remained at the ventral surface of the embryo (Figs. 1D–F and J) in contrast to heterozygous embryos (Figs. 1A–C and J). Invagination defects in Rho1K mutant glands were first observed in late stage 11. In
those Rho1\textsuperscript{K} mutant glands that did invaginate, invagination always began in the correct dorsal–posterior position (data not shown). In Rho1\textsuperscript{18} homozygous embryos, majority of glands partially invaginated (Figs. 1H and J); however, the internalized portion of the gland failed to turn and migrate posteriorly unlike heterozygous glands that turned and migrated completely (Fig. 1G). Rho1\textsuperscript{22F} homozygous embryos showed an identical phenotype to Rho1\textsuperscript{18} homozygous embryos where majority of glands invaginated but failed to turn and migrate posteriorly (data not shown). Gland invagination and migration defects were also observed in embryos homozygous for Df(2R)p1, a deficiency that deletes the entire Rho1 gene and trans-heterozygotes of Rho1\textsuperscript{K} and Df(2R)p1 (data not shown). Furthermore, expression of the dominant-negative Rho1\textsuperscript{V12} mutation specifically in the gland with fkh-GAL4 phenocopied the Rho1\textsuperscript{K} loss of function phenotype with the majority of gland cells failing to invaginate (Figs. 1I and J).

To confirm that the gland invagination defects observed in Rho1\textsuperscript{K} mutant embryos were due to loss of Rho1 function in the gland, we expressed wild-type Rho1 (Rho1\textsuperscript{WT}) specifically in glands of Rho1\textsuperscript{18} homozygous embryos with fkh-GAL4 and obtained a substantial rescue; the percentage of non-invaginated glands decreased from 80% to 27% and the percentage of completely invaginated glands increased from 2% to 58% (n = 126 glands; Fig. 1J). Expression of Rho1\textsuperscript{V12} specifically in salivary glands of wild–type embryos with fkh-GAL4 had no effect on gland invagination (data not shown). Together, these data indicate that the invagination defects observed in Rho1\textsuperscript{K} homozygous embryos were due to lack of Rho1 function in salivary gland cells. The Rho1\textsuperscript{K} allele is due to a F-element insertion in the first intron within the coding region (Magie et al., 1999) whereas the Rho1\textsuperscript{18} allele is an imprecise excision removing the coding region C-terminal to amino acid 52. Although no Rho protein was detected in embryos (Magie and Parkhurst, 2005), the phosphate binding loop and S2). In early salivary gland placodes of Crumbs (Crb) and basolateral localization of Neurotactin (Nrt) (Fig. S1). In contrast, Baz maintained its apical localization in the non-invaginated gland cells of Rho1\textsuperscript{V12} homozygous embryos (Figs. 3A and A') as in the invaginating gland cells of heterozygous embryos (Figs. 3A and A') while Baz was lost in the homozygous gland cells (Figs. 3B and B') and maintained in the heterozygous gland cells (Figs. 3A and A'). We next tested whether basolateral polarity was maintained or lost in Rho1\textsuperscript{K} mutant embryos by staining for the basolateral protein, Neurotactin (Nrt). Nrt maintained its normal localization at the basolateral membrane in Rho1\textsuperscript{K} homozygous gland cells as in heterozygous cells (Figs. 3C–F). Thus, these data demonstrate that zygotic activity of Rho1 is required to maintain apical polarity of a subset of apical proteins, namely, Crb, DaPKC and Sdt in invaginating gland cells and had no effect on localization of the apical protein, Baz, and the basolateral protein, Nrt.

Expression of dominant negative Rho1\textsuperscript{V12} in all cells of the salivary gland placode with fkh-GAL4 also led to loss of apical Crb, as in Rho1\textsuperscript{18} homozygous embryos (Fig. S4B). Furthermore, expression of Rho1\textsuperscript{V12} in only a subset of gland cells, such as the posterior two rows of the placode with wingless (wg)–GAL4, resulted in loss of apical Crb specifically in this group of cells, whereas Crb localization was normal in the remaining gland cells (Fig. S4C). Loss of Crb, DaPKC and Sdt from the apical membrane was not accompanied by mislocalization to the basolateral membrane (Figs. 2E and G).

In Drosophila, crb is required for maintenance of epithelial polarity and proper positioning of adherens junctions (Grave et al., 1996; Izaddoost et al., 2002; Klebes and Knust, 2000; Pelllkka et al., 2002). In embryos homozygous for crb\textsuperscript{11A2}, salivary gland cells die (Fig. S5A and B) and small glands are formed due to degeneration of the epithelium as previously described (Grave et al., 1996; Tepass and Knust, 1993). Therefore, we analyzed embryos homozygous for crb\textsuperscript{11A2} and Df(3L)H99 (crb\textsuperscript{11A2} Df(3L)H99) that fail to undergo apoptosis due to the H99 deletion (Bilder et al., 2003). In crb\textsuperscript{11A2} Df(3L)H99 homozygous embryos, 68.7% of glands failed to invaginate, 25.6% partially invaginated and 5.7% completely invaginated (n = 102 glands; Figs. 1J and S5D). Salivary gland invagination defects in crb\textsuperscript{11A2} Df(3L)H99 mutant glands were accompanied by loss of A/B polarity. In the early gland epithelium of crb\textsuperscript{11A2} Df(3L)H99 heterozygous embryos, DaPKC was localized apically and Nrt was localized basolaterally (Fig. S5E) whereas in homozygous embryos, neither DaPKC nor Nrt showed a polarized localization and instead was diffused in the cytoplasm (Fig. S5F). These data demonstrate that proper polarizatation of salivary gland cells is required for invagination and that Rho1 GTPase plays an important role in maintaining apical polarity during invagination.

We previously showed that crb RNA becomes elevated in the apical domains of salivary gland cells prior to and during invagination in a manner partially dependent on the transcription factor,
Huckebein (Myat and Andrew, 2002). Moreover, it was recently reported that in *Drosophila* follicular cells, dynein transports Crb protein and RNA to the apical membrane (Li et al., 2008). To test whether the loss of Crb protein observed in *Rho1*K heterozygous embryos is in part due to reduced *crb* transcript levels and/or apical localization, we performed real time and RT-PCR analyses of wild-type embryos expressing dominant negative *Rho1N19* in the entire epidermis with arm-GAL4 and whole mount in situ hybridization (ISH) of *crb* RNA in *Rho1*K mutant embryos. We observed a 10% decrease in *crb* RNA levels in *Rho1N19* mutant embryos by real-time PCR and RT-PCR compared to control embryos suggesting that the loss of Crb protein observed in *Rho1*K mutant embryos is in part due to reduced *crb* transcription. In non-invaginating gland cells of *Rho1*K heterozygous embryos, *crb* RNA was elevated compared to surrounding non-gland cells, consistent with our previous findings (Fig. 4A) (Myat and Andrew, 2002). In contrast, in non-invaginating gland cells of *Rho1*K homozygous embryos with a similar apical domain size, *crb* RNA was not elevated in the apical domains of gland cells (Fig. 4B) or elsewhere in the cells. We observed a similar reduction of apical *crb* RNA specifically in gland cells expressing dominant negative *Rho1N19* (Fig. 4D) compared to control gland cells (Fig. 4C). Thus, these data demonstrate that the loss of Crb protein due to absence of zygotic Rho1 activity is in part due to reduced *crb* RNA levels and apical localization of the transcript.

**Fig. 2.** Rho1 activity is required for epithelial shape and Crb localization. Salivary gland cells of *Rho1*K heterozygous embryos are columnar with prominent F-actin (A, arrow) whereas cells of *Rho1*K homozygous embryos are mesenchymal-shaped with disorganized F-actin (B, arrow). In *Rho1*K heterozygous embryos (C), Crb (red) in salivary gland cells marked by Fkh (green) is localized in the apical membrane before (C, arrow) and during invagination (E, arrow). In *Rho1*K homozygous embryos (D and F), Crb (red) is localized in the apical membrane of gland cells before invagination (D, arrow) and in the internalized cells (F, arrow) during invagination but is lost in the gland cells that do not invaginate (F, arrowheads). Projected images of ten 1 μm Z optical sections of *Rho1*K heterozygous (G) and homozygous glands (H) show that Crb is lost in the homozygous glands (H, arrows) unlike in heterozygous glands (G, arrow). Embryos in panels A and B were stained for phalloidin to label F-actin (green) and dCREB-A (red) to mark salivary gland nuclei, embryos in panels C–H were stained for Crb (red) to mark the apical membrane and embryos in panels C–F were also stained for Fkh (green) to mark gland nuclei. All embryos were stained for β-gal to distinguish heterozygous from homozygous embryos. All panels shown are lateral views of embryos except for the embryos in G and H which are horizontal views. Scale bar in panel A represents 10 μm and scale bar in panel G represents 2 μm.
Rho1 regulates gland invagination through Crb and Rok

Since Rho1 activity regulated crb transcript levels, we tested whether expression of wild-type crb (crbWT) in Rho1K mutant glands through a heterologous promoter will rescue the polarity and/or invagination defects. In the early gland placode of Rho1K heterozygous embryos (Fig. 4E) and wild-type embryos expressing crbWT specifically in the gland (Fig. 4F), Crb was localized in the apical membrane, albeit Crb was more robust in the latter embryos. Crb was lost in gland cells of Rho1K homozygous embryos (Fig. 4G); however, Crb was restored to the apical membrane of Rho1K homozygous embryos expressing crbWT specifically in gland cells (Fig. 4H). Restoration of apical Crb in Rho1K homozygous embryos expressing crbWT also restored apical DaPKC (Fig. 4K) and Sdt (data not shown). In stage 14 wild-type glands (Fig. 4I), DaPKC was localized at the apical membrane and Nrt at the basolateral membrane. In stage 14 wild-type glands expressing crbWT, DaPKC was localized around the entire plasma membrane (Fig. 4K). These data show that Crb is necessary and sufficient to maintain apical polarity of DaPKC and Sdt in the gland epithelium downstream of Rho1 activity. Overexpression of crbWT in Rho1K homozygous embryos also rescued the gland invagination defect to a small extent; the number of non-invaginated glands decreased from 80% in Rho1K homozygous embryos to 68% (n=120 glands) with a concomitant increase in the number of partially invaginated glands from 18% to 29% (Fig. 5I).

In crbWT expressing Rho1K mutant glands that failed to invaginate, apical domains did not constrict even though apical localization of Crb and DaPKC was restored (Figs. 4H and K), suggesting that additional processes downstream of Rho1 were required to achieve apical constriction and invagination. To test this hypothesis, we determined whether Rho-kinase (Rok), a known regulator of apical constriction in Drosophila epithelia (Dawes-Hoang et al., 2005), mediated apical constriction downstream of Rho1 during gland invagination. In a small percentage of embryos mutant for rok2, a strong loss-of-function allele of rok (Winter et al., 2001), glands failed to invaginate or did not invaginate completely (9%, n=170 glands) and often began with anterior gland cells (Fig. 5A) instead of dorsal–posterior cells, as

Fig. 3. Baz and Nrt are maintained in invaginating Rho1 mutant glands. In Rho1K heterozygous (A) and homozygous (B) embryos, Baz (A, A′, B and B′, red) is localized at the apical membrane of invaginating heterozygous gland cells (A and A′, arrows) and noninvaginating homozygous gland cells (B and B′, arrows) whereas Crb (A′, B and B′, green) is maintained in heterozygous gland cells (A and A′, arrows) and is lost in homozygous gland cells (B and B′, arrows). In Rho1K heterozygous (C and E) and homozygous (D and F) embryos, Nrt is localized in the basolateral membrane of gland cells at stages 11 (C and D, arrows) and 14 (E and F, arrows). Embryos in panels A and B were stained for Crb (green) and Baz (red) and embryos in panels C–F were stained for Nrt (green) to mark the basolateral membrane. Heterozygous and homozygous embryos were distinguished by β-gal staining on the CFL balancer chromosome (not shown). Panels in A and B are horizontal views of gland cells whereas panels in panels C–F are lateral views of embryos. Scale bar in panel A represents 2 μm and scale bar in panel C represents 1 μm.
in wild-type embryos. rok2 mutants also showed gland migration defects (35%, n = 170 glands) where the distal tip cells turned but the proximal half of the gland did not turn and the gland failed to migrate posteriorly (Fig. 5B). Gland invagination and migration defects were also observed in glands where rok was inhibited in the entire epidermis with RNAi (Fig. 5C, D and Fig. S6). In stage 14 wild-type glands (I), DaPKC is localized at the apical membrane (I, arrow) and Nrt at the basolateral membrane (I, arrowhead). In stage 14 wild-type glands overexpressing crbWT (J), DaPKC is localized at the apical membrane (J, arrow) and in more basal regions (J, arrowheads), whereas Nrt is completely lost. In stage 14 glands of Rho1K homozygous embryos overexpressing crbWT (K), DaPKC (K, large arrow) is localized exclusively at the apical membrane and Nrt (K, arrowhead) at the basolateral membrane in most cells but in some cells DaPKC is localized around the entire plasma membrane (K, small arrow) and Nrt is lost. All panels shown are horizontal views except for panels I, J and K which are lateral views. Embryos in panels A–H are at stage 11, whereas embryos in panels I, J and K are at stage 14. In panels A–D, cells anterior to the salivary gland (A, C and D, small arrows) express high levels of crb RNA and serve as an internal control for ISH. Gland cells in panels A–D were identified by the position of the gland placode in parasegment 2 and gland cells in panels E–H were identified by double staining for dCREB-A (not shown). Scale bars in panels E and I represent 5 μm.

Fig. 4. Rho1 maintains apical polarity through crb. In Rho1K heterozygous (A) and fkh-GAL4 control (C) embryos, crb RNA is elevated in the apical domains of salivary gland cells (A and C, large arrows) compared to neighboring non-gland cells (A and C, arrowheads). In Rho1K homozygous embryos (B) and wild-type embryos overexpressing dominant negative Rho1K specifically in the gland (D), crb RNA is lost from the apical domain of gland cells (B and D, large arrows). In the early gland placode of Rho1K heterozygous embryos (E) and wild-type embryos overexpressing crbWT in the gland (F), Crb is localized at the apical membrane (E and F, arrows). Crb is lost in Rho1K mutant glands (G, arrows), whereas it is maintained in Rho1K mutant glands overexpressing crbWT (H, arrows). In stage 14 wild-type glands (I), DaPKC is localized at the apical membrane (I, arrow) and Nrt at the basolateral membrane (I, arrowhead). In stage 14 wild-type glands overexpressing crbWT (J), DaPKC is localized at the apical membrane (J, arrow) and in more basal regions (J, arrowheads), whereas Nrt is completely lost. In stage 14 glands of Rho1K homozygous embryos overexpressing crbWT (K), DaPKC (K, large arrow) is localized exclusively at the apical membrane and Nrt (K, arrowhead) at the basolateral membrane in most cells but in some cells DaPKC is localized around the entire plasma membrane (K, small arrow) and Nrt is lost. All panels shown are horizontal views except for panels I, J and K which are lateral views. Embryos in panels A–H are at stage 11, whereas embryos in panels I, J and K are at stage 14. In panels A–D, cells anterior to the salivary gland (A, C and D, small arrows) express high levels of crb RNA and serve as an internal control for ISH. Gland cells in panels A–D were identified by the position of the gland placode in parasegment 2 and gland cells in panels E–H were identified by double staining for dCREB-A (not shown). Scale bars in panels E and I represent 5 μm.
Rho1 mediates cell contraction during salivary gland migration

Our genetic analysis of Rho1 and rok mutants demonstrated that Rho–Rok signaling was required not only for gland invagination but also for migration (Figs. 5A–D and Fig. S6). Since Rok is an important regulator of actin–myosin contraction we sought to determine whether cell contraction plays a role in gland migration. We analyzed normal gland migration by live-imaging wild-type glands expressing either mouse CD8GFP in the entire gland with fkh-GAL4 (Fig. 6A and Movie 1) or actinGFP in a cluster of proximal gland cells with en-GAL4.
Rho1-dependent cell contraction is required for gland migration. Live-imaging of wild-type salivary gland expressing mCD8GFP in the entire gland with fkh-GAL4 (A) shows the proximal tip of the gland (A, arrows) contract and migrate away from the embryo surface (A, arrowheads) followed by posterior turning of the entire gland. Live-imaging of wild-type salivary gland expressing actinGFP in a cluster of proximal gland cells with en-GAL4 (B) shows apical–basal contraction, rounding and dorsal migration. Live-imaging of Rho11B heterozygous (C) and homozygous (D) glands expressing actinGFP in all gland cells with fkh-GAL4 shows that Rho11B heterozygous gland cells in the proximal half of the gland contract (C, arrows) resulting in detachment of the proximal gland from the embryo surface (C, arrowheads) whereas Rho11B homozygous gland cells do not contract (D, arrows) and the gland remains close to the embryo surface (D, arrowheads) and fails to migrate posteriorly. Asterisks in panels B and D mark the movement of a single cell during the recording period. Recording time (t) is shown in minutes. White line in the B panels outlines proximal gland cells. D: dorsal, V: ventral, A: anterior and P: posterior.

(Fig. 6B and Movie 2) and Rho11B heterozygous glands expressing actinGFP with fkh-GAL4 (Fig. 6C and Movie 3) at the stage when all gland cells had invaginated but the gland had not migrated yet. In wild-type glands, columnar cells in the proximal half of the gland contracted coordinately in the apical–basal axis to become round and moved dorsally away from the ventral surface of the embryo (Figs. 6A–C, Movies 1, 2 and 3). In contrast, Rho11B mutant gland cells did not contract in the apical–basal axis and did not migrate dorsally (Fig. 6D and Movie 4). We quantified the Rho11B cell contraction defect by measuring the distance between the apical and basal membranes of Rho11B heterozygous and homozygous proximal gland cells labeled with actinGFP (see Materials and Methods). During the 50 min of live recording, Rho11B heterozygous cells in the proximal half of the gland contracted from 12.5 μm to 8.6 μm in length (n = 18 cells, p = 0.0000). In contrast, Rho11B homozygous gland cells showed no contraction and measured 16 μm at the beginning and end of the recording (n = 12 cells, p = 0.7249). Thus, our live-imaging studies provide the first evidence for coordinated cell contraction and a rounded type of motility in the proximal half of the gland during migration and the identification of Rho1 GTPase as a key regulator of this process.

To confirm that the Rho11B gland migration defect was due to absence of Rho1 activity in gland cells, we attempted to rescue the Rho11B migration defect by expressing wild-type Rho1 (Rho1WT) in all gland cells. Expression of Rho1WT in all Rho11B mutant gland cells with fkh-GAL4 resulted in a partial rescue of the gland migration defects; percentage of glands that turned incompletely and completely increased in the rescue embryos with an accompanying decrease in the percentage of glands that did not turn at all and only the tip turned (Fig. 7G). Since our data showed contractile motility of the proximal gland cells which contrasted from the previously described elongated motility of distal tip cells (Bradley et al., 2003), we next tested whether Rho1 activity was required for both types of movement. We used the en-GAL4 and the wg-GAL4 lines to achieve transgene expression in a cluster of proximal gland cells or distal gland cells, respectively (Figs. 7A–F). Initial studies with mouse CD8GFP driven by either en-GAL4 or wg-GAL4 confirmed that en-GAL4 drove expression robustly in a...
Expression of dominant negative Rho1\textsuperscript{N19} in the proximal gland cells with en-GAL4 or in the distal gland cells with wg-GAL4 also showed that inhibition of Rho1 function in the proximal cells was more deleterious for gland migration than inhibition in the distal cells (Fig. 7H). We confirmed that en-GAL4 and wg-GAL4 mediated transgene expression at similar levels by embryonic stage 11 when the salivary gland placodes form by measuring the levels of GFP fluorescent intensity of gland cells expressing en-GAL4 driven or wg-GAL4 driven mCD8GFP (data not shown). Therefore, these studies show that normal Rho1 activity is more critical in the proximal cells than in the distal cells of the migrating gland and that Rho1-dependent contraction and dorsal migration of proximal cells is a key element of turning and migration of the entire gland.

To test whether Rho1 controlled proximal gland cell contraction and migration that inhibition of actin-myosin contraction, we stained Rho1\textsuperscript{F18} mutant embryos for p-MLC. In Rho1\textsuperscript{F18} heterozygous embryos, prominent p-MLC staining was found throughout the cells of migrating glands (Fig. 7I) whereas glands of Rho1\textsuperscript{F18} homozygous embryos showed decreased p-MLC staining (Fig. 7J). We quantified the level of p-MLC in glands of Rho1\textsuperscript{F18} heterozygous and homozygous embryos and found

Fig. 7. Rho1 function is required predominantly in the proximal gland cells for migration. In wild-type embryos where mCD8GFP expression is driven by en-GAL4 (A–C), GFP (A–C, red) is expressed in the anterior-most cluster of placode cells prior to gland invagination (A, arrow), in a cluster of proximal gland cells (B, arrow) as the gland begins to migrate and continues to be expressed in the proximal gland cells (C, arrow) during later stages of migration. In wild-type embryos where mCD8GFP expression is driven by wg-GAL4 (D–F), GFP (D–F, red) is expressed in the dorsal–posterior cells during gland invagination (D, arrow), wg-GAL4 driven mCD8GFP is later expressed in the distal cells including the elongated distal tip cells (E, arrow) as the gland begins to migrate and in the distal-half of the gland (F, arrow) during later stages of migration. A single wg-GAL4 UAS-mCD8GFP gland cell is occasionally observed in a more proximal location (F, arrowhead). (G) Graph depicts extent of gland migration in stage 14 wild-type embryos, Rho1\textsuperscript{WT} homozygous embryos and Rho1\textsuperscript{F18} homozygous embryos where Rho1\textsuperscript{F18} was expressed in all gland cells with fkh-GAL4, proximal gland cells with en-GAL4 or distal gland cells with wg-GAL4. (H) Graph depicts extent of gland migration in stage 14 wild-type embryos and wild-type embryos expressing Rho1\textsuperscript{N19} in proximal gland cells with en-GAL4 or in distal gland cells with wg-GAL4. In migrating glands of Rho1\textsuperscript{F18} heterozygous embryos (I), phosphorylated MLC (p-MLC) is prominent in all cells (I) whereas in cells of Rho1\textsuperscript{F18} homozygous glands, p-MLC is decreased (J). Embryos in panels A–F were stained for GFP (red) and Fkh (green) to label gland cells. Embryos in panels I and J were stained for p-MLC (red) and dCREB-A (not shown). White lines in panels I and J outline the gland. Salivary gland lumen in panels I and J is marked by L. Scale bar in panel A represents 10 μm.

cluster of proximal gland cells (Figs. 7A–C and S8A) and to a lesser extent in clusters of CVM cells (Fig. S8A) whereas wg-GAL4 drove expression robustly in the distal gland cells (Figs. 7D–F and S8B) and to a lesser extent in the entire CVM (Fig. S8B). Interestingly, rescue of the Rho1\textsuperscript{F18} migration defect with en-GAL4 driven expression of Rho1\textsuperscript{WT} led to a significantly better rescue than with wg-GAL4 (Fig. 7G) suggesting a greater requirement for Rho1 activity in the proximal gland cells than in the distal cells. en-GAL4 driven expression of Rho1\textsuperscript{WT} also led to a better rescue than with fkh-GAL4 which could be due to the earlier embryonic expression of Rho1\textsuperscript{WT} by en-GAL4 compared to fkh-GAL4. Alternatively, spatial regulation of Rho1 activity may be important for gland migration and uniform overexpression of Rho1\textsuperscript{WT} in the entire gland with fkh-GAL4 may have inhibitory effects on gland migration. To test this possibility, we expressed Rho1\textsuperscript{WT} in the entire gland with fkh-GAL4 of otherwise wild-type embryos and analyzed gland migration at different stages of embryogenesis. Gland migration was delayed at stage 12 in glands overexpressing Rho1\textsuperscript{WT}; however, this delay in gland migration was corrected by stage 14 (Fig. S9). Thus, spatial expression of Rho1 within the gland appears to be important for efficient gland migration.
that p-MLC fluorescence intensity in *Rho1*18 mutant glands was reduced to 78% of the intensity of heterozygous glands. These data suggest that Rho1 regulates gland migration in part through phosphorylation of MLC and subsequent actin-myosin contraction.

### Rho1 Function is required in both the salivary gland and CVM for gland migration

Expression of *Rho1WT* in all or a subset of salivary gland cells of *Rho1*18 mutant embryos did not completely rescue the gland migration defects (Fig. 7C). Furthermore, *en*-GAL4 and *wg*-GAL4 drove transgene expression in the CVM in addition to the gland (Fig. S8A and B). These data raised the possibility that Rho1 might regulate gland migration in a cell non-autonomous manner. Thus, we tested whether Rho1 activity is also required in the circular visceral mesoderm (CVM) upon which the gland migrates. The CVM is derived from the trunk visceral mesoderm primordia which ingresses into the interior of the embryo to form cell clusters that then expand along the anterior-posterior (A-P) axis to form a continuous layer (Fig. S10A and B) (Lee et al., 2005). In *Rho1*18 homozygous embryos, the CVM was discontinuous with clusters of cells at discrete intervals along the A-P axis (Fig. S10C and D). Furthermore, expression of dominant negative *Rho1N19* in the CVM with *twi*(*twi*)-GAL4 that drives transgene expression in the CVM and somatic mesoderm (SM) (Fig. S8C) resulted in failure of the glands to turn and migrate posteriorly (Fig. S10F). These gland migration defects correlated with failure to form a properly structured CVM where the cells were spindle shaped and did not elongate in the dorsal-ventral axis (Fig. S10F), as in control embryos (Fig. S10E). Due to these CVM defects observed upon loss of Rho1 function, we next tested whether expression of *Rho1WT* in the mesoderm could rescue the gland migration defects of *Rho1*18 homozygous embryos. Expression of *Rho1WT* in the entire CVM of *Rho1*18 homozygous embryos with *twi*-GAL4 (Fig. S8C) partially rescued the gland migration defect albeit to a weaker extent than with *en*-GAL4 (compare Fig. S10G to Fig. 7C). Similar level of rescue was observed when *Rho1WT* was expressed in clusters of the CVM of *Rho1*18 homozygous embryos with *hwp*-GAL4 (Figs. S8D and S10G). These data demonstrate that Rho1 activity is required for proper development of the CVM; however, Rho1 activity is required predominantly in the proximal gland cells for their contraction and migration.

### Discussion

In this study, we demonstrate that Rho1 activity controls salivary gland invagination through at least two distinct mechanisms; one, by maintaining apical polarity specifically of Crb, DaPKC and Sdt in the early salivary gland placode and two, by inducing apical constriction and cell shape change through Rok (Fig. 8A). Since simultaneous expression of Crb and Rok did not result in complete rescue of the Rho invasion defect, it is possible that Rho regulates gland invagination by other as yet unidentified mechanism(s) in addition to Crb and Rok. We show that Rho1 and Rok are required again during gland migration to control cell contraction and rounded movement of the proximal half of the gland (Figs. 8A and B). Our live-imaging and genetic analyses provide the first evidence that cell contraction and rounded motility of cells in the proximal half of the gland is essential for turning and subsequent posterior migration of the entire gland. Although Rho1 activity is required in the gland cells and in the CVM for gland migration, Rho1 activity is required predominantly in the proximal gland cells for contraction and migration.

We show that zygotic function of Rho1 is required to prevent loss of the apical proteins, Crb, DaPKC and Sdt in the invaginating salivary gland and not of the apical protein, Baz, demonstrating that Rho1 activity regulates a specific subset of apical proteins and not apical polarity in general. Rho1 activity maintains Crb protein in salivary gland cells in part by regulating *crb* RNA levels and apical localization of the transcript. Since Rho GTPases are important regulators of the actin and microtubule cytoskeletal systems which together with their respective motor proteins, are required for the proper delivery of mRNA to one membrane domain or the other of polarized cells (St Johnston, 2005), it is possible that *crb* transcripts fail to localize in the apical domain of Rho1 mutant cells because they are not transported to and/or stabilized at the apical membrane due to underlying defects in the apical cytoskeleton. Rho GTPases have been shown to regulate RNA stability such as those of the Na+/Ca2+ exchanger (Maeda et al., 2005) and endothelial nitric oxide synthase mRNAs (Laufs and Liao, 1998) although the mechanism is unknown.

Although loss of Crb, DaPKC and Sdt occurred simultaneously in Rho1 mutant glands, overexpression of *crbWT* from a heterologous promoter was sufficient to restore expression and apical localization not only of Crb but also of Sdt and DaPKC. These data suggest that apical Crb maintains DaPKC and Sdt in their proper localization at the apical membrane either directly or through Rho1-mediated stabilization of the sub-membrane cytoskeleton. A recent study reported that the Dynein motor localizes Crb in follicle cells through apical targeting of *sdt* RNA (Horne-Badovinac and Bildner, 2008). Therefore, it appears that the apical proteins, Crb and Sdt reciprocally regulate each other's apical localization in polarized epithelia. The proper apical localization of Crb, DaPKC and Sdt observed in *Rho1*18 homozygous embryos prior to gland invagination is likely due to the maternal contribution of Rho1. However, reduced levels of Rho1 due to zygotic loss of Rho1 function are insufficient to maintain these apical proteins during embryogenesis.

---

**Fig. 8.** Model for Rho1 function in salivary gland invagination and migration. During salivary gland invagination, Rho1 activity maintains Crb, DaPKC and Sdt in part by controlling *crb* RNA level and apical localization and induces apical constriction and cell shape change through Rok (A). Rho-Rok mediated cell constriction is also required for gland migration (A). Salivary gland turns posteriorly (B) through coordinated contraction and cohesive migration of proximal tip cells (B, red) and elongated motility of distal tip cells (B, blue). Diagrams are not drawn to scale.
The relevance of complete maintenance of A/B polarity during salivary gland invagination is reflected in Drosophila border cells that retain asymmetric distribution of polarity proteins during their migratory process (Pinheiro and Montell, 2004). One possible explanation for why it is necessary that gland epithelia maintain their apical polarity is that the glands are secretory organs that synthesize and secrete large quantities of digestive enzymes and glycoproteins that allow the larva to attach to its environment. Thus, proper maintenance of all aspects of apical–basolateral polarity is likely to be essential for proper delivery of such secretory products into the lumen of the gland.

Integrin expression in the gland and CVM is necessary to initiate the posterior turn of the salivary gland (Bradley et al., 2003), possibly to mediate contact of the distal tip of the gland to the CVM; however, the contribution of the proximal gland cells to gland migration was previously not known. Here, we demonstrate that salivary gland cells, particularly those in the proximal half, contracted and migrated in a rounded type of motility and that these events were dependent on Rho1 function and downstream actin proteins that allow the larva to attach to its environment. Thus, proper maintenance of all aspects of apical–basolateral polarity is likely to be essential for proper delivery of such secretory products into the lumen of the gland.

Our studies reveal that migrating salivary glands have an advancing “front” that extends membrane protrusions and a contracting “back.” Moreover, expression of GFP reporter genes in subpopulations of gland cells indicates that distal and proximal gland cells largely retain their positions throughout gland migration. These features of salivary gland migration are in contrast to collective migration of border cells in the Drosophila ovary that do not have an apparent “back” and there is more fluidity within the cluster, with the position of the leading cells being interchangeable (Bianco et al., 2007; Prasad and Montell, 2007). One obvious distinction between salivary glands and border cells is that salivary glands have to coordinate cohesive migration of approximately 100 cells whereas border cells move as a cluster of six to ten cells. We propose that in large populations of migrating cells where cell positions are fixed, cohesive migration is best achieved if the front and back of the migrating group are well defined and each subpopulation contributes a unique role to the overall migration of the group. Consistent with this proposal, during wound healing of cultured epithelial sheets, cells several rows behind the wound edge extend lamellipodia suggesting an active role in collective migration of the sheet (Faroqui and Fenteany, 2005).

Our studies reveal that salivary glands use a unique combination of elongated motility at the distal tip and contractile motility at the proximal tip during their cohesive migration. A contractile versus elongated type of motility has previously been observed in tumor cells migrating in 3D matrices where the contractile or rounded type of motility was dependent on Rho signaling through ROCK, and the elongated type of motility was associated with Rac-dependent membrane protrusions and did not require Rho or ROCK (Sahai and Marshall, 2003). Thus, the salivary gland provides a unique model system not only for studying how cells migrate cohesively during embryogenesis but also for studying different modes of cell migration common to tumor cell migration.

Acknowledgments

We thank the Bloomingston Stock Center, the Developmental Biology Hybridomma Bank, the Vienna Drosophila Research Center, the Rockefeller University Bio-imaging Center and our many colleagues for generously providing us with fly stocks and antisera, including S. Beckendorf, D. Bilder, A. Brand, N. Harden, E. Knust and N. Perrimon. We thank M. Schober, J. Zallen and members of the lab for critical reading of the manuscript. This work was supported by a Research Scholar Grant from the American Cancer Society to M.M.M.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.06.007.

References


