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Rac function in epithelial tube morphogenesis

Carolyn Pirraglia, Rakhi Jattani, Monn Monn Myat *

Department of Cell and Developmental Biology, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021, USA

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Abstract

Epithelial cell migration and morphogenesis require dynamic remodeling of the actin cytoskeleton and cell–cell adhesion complexes. Numerous studies in cell culture and in model organisms have demonstrated the small GTPase Rac to be a critical regulator of these processes; however, little is known about Rac function in the morphogenic movements that drive epithelial tube formation. Here, we use the embryonic salivary glands of *Drosophila* to understand the role of Rac in epithelial tube morphogenesis.

We show that inhibition of Rac function, either through loss of function mutations or dominant-negative mutations, disrupts salivary gland invagination and posterior migration. In contrast, constitutive activation of Rac induces motile behavior and subsequent cell death. We further show that Rac regulation of salivary gland morphogenesis occurs through modulation of cell–cell adhesion mediated by the E-cadherin/β-catenin complex and that *shibire*, the *Drosophila* homolog of dynamin, functions downstream of Rac in regulating β-catenin localization during gland morphogenesis. Our results demonstrate that regulation of cadherin-based adherens junctions by Rac is critical for salivary gland morphogenesis and that this regulation occurs through dynamin-mediated endocytosis.

Keywords: *Drosophila*; Salivary gland; Epithelial morphogenesis; Tube; Rac; E-cadherin; Dynamin; Endocytosis

Introduction

Epithelial cells undergo dynamic morphogenic movements to build complex three-dimensional structures from simply organized epithelial sheets. Changes in the actin cytoskeleton and cell–cell adhesion are thought to be essential for such morphogenic movements although the molecular mechanisms that control the dynamics and precision of such changes are not well understood. The Rho family of small GTPases, which includes Rac, Rho and Cdc42, regulates a number of cellular events, including remodeling of cadherin-based adhesion junctions (Etienne-Manneville and Hall, 2002). Cadherins mediate cell–cell adhesion through homotypic binding of their extracellular domains and are indirectly linked to the actin cytoskeleton through the binding of β-catenin and α-catenin to their cytoplasmic tails (Tepass et al., 2001).

Rho GTPases were shown to alter both assembly and disassembly of cadherin-based adherens junctions. Studies performed on mammalian Madin–Darby canine kidney (MDCK) cells grown in culture and in the *Drosophila* embryonic epithelia showed that activated Rac1 can increase E-cadherin-mediated cell adhesion (Eaton et al., 1995; Harden et al., 1995; Ridley et al., 1995; Takaishi et al., 1997), whereas dominant-negative Rac1 can inhibit hepatocyte growth factor (HGF)-mediated disruption of junctions in MDCK cells (Potempa and Ridley, 1998). Similarly, Rac has been implicated in both the assembly and disassembly of junctions in cultured human epidermal keratinocytes (Akhtar et al., 2000; Braga et al., 1997, 1999). It is believed that the role of a particular GTPase in either the assembly or disassembly of adherens junctions depends on the cell type, the extracellular matrix and junctional maturity.

Rac function has been implicated in the development of numerous tissues in the *Drosophila* embryo. Rac is essential for axonal migration in the developing nervous system, epithelial sheet movement during dorsal closure, myoblast fusion and establishment of planar cell polarity (Eaton et al., 1996; Fanto et al., 2000; Hakeda-Suzuki et al., 2002; Kaufmann et al., 1998; Luo et al., 1994; Ng et al., 2002; Woolner et al., 2005).

* Corresponding author.
E-mail address: mmm2005@med.cornell.edu (M.M. Myat).

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However, little is known of Rac's role in epithelial tube morphogenesis, an essential step in the formation of most major organs where several different types of movements, such as invagination and migration, occur coordinately to form a three dimensional tube from a relatively simple epithelial anlage (Hogan and Kolodziej, 2002; Myat, 2005). A recent study of Rac in the *Drosophila* embryonic trachea showed that Rac negatively regulates cadherin-mediated adhesion (Chihara et al., 2003). Reduction of Rac activity increased the amount of E-cadherin/catenin complexes in the embryonic epidermis and tracheal epithelia and prevented tracheal cell rearrangements (Chihara et al., 2003). Furthermore, activation of Rac inhibited incorporation of E-cadherin into cell–cell junctions and led to loss of adhesion between tracheal cells and subsequent detachment from the epithelium. Although Rac might alter cadherin-based cell–cell junctions by several molecular mechanisms as suggested from studies on cultured mammalian cells (Briggs and Sacks, 2003; Lozano et al., 2003), it is still unknown how Rac regulates cadherin-mediated adhesion in the context of a developing organ or tissue.

Here, we analyze Rac function in the developing salivary gland, a pair of elongated tubes with a single layer of epithelial cells surrounding a central lumen (Myat, 2005). The *Drosophila* embryonic salivary gland is an ideal system to investigate the role of Rac in tube morphogenesis because of its relatively simple, unbranched structure and the genetic tools available in *Drosophila* that allow dissection of Rac function. The glands are formed from two placodes, or plates, of ectodermal epithelial cells that invaginate through a series of cell shape changes to form tubes (Myat and Andrew, 2000b). The transcription factor Fork Head (Fkh) is required for the cell shape changes that occur during salivary gland invagination and also for cell survival; in *fkh* mutant embryos, salivary gland cells remain columnar instead of becoming pyramidal and eventually die by apoptosis (Myat and Andrew, 2000a). Additionally, these cell shape changes need to be polarized in order to form an elongated tube, a process controlled by the transcription factors, Hairy and Huckebein, and their downstream targets, Crumbs, an apical membrane determinant, and Klarsicht, a mediator of microtubule-dependent organelle transport (Myat and Andrew, 2002). After invagination is complete, the salivary gland migrates posteriorly as an intact organ until it reaches its final position along the lateral body wall.

Here, we show that the Rac GTPases play an important role in regulating cadherin-mediated cell–cell adhesion during salivary gland morphogenesis. Our results further suggest that Rac regulates cell–cell adhesion in salivary gland cells by modulating dynamin-mediated endocytosis of E-cadherin.

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/): $Rac1^{J11}$, $Rac1^{J11}Rac2^{\Delta}$, $Rac1^{J11}Rac2^{\Delta}Mtl^{\Delta}$, $Rac1^{J10}Rac2^{\Delta}Mtl^{\Delta}$, Mtl^{Δ}, UAS-$Rac1^{L89}$, UAS-$Rac1^{N17}$, UAS-$Rac1^{V12}$, shi^{ts2} and UAS-shi^{K44A}. UAS-*DEcadherin-GFP* was a gift of H. Oda. UAS-*p35* was a gift from H. Stellar. *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). For staining of β-catenin and E-cadherin, embryos were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) instead of 3.7% formaldehyde. The following antisera were used at the indicated dilutions: rat dCREB-A antiserum at 1:10,000 for whole mount and 1:5000 for fluorescence; rat PH4αSG1 antiserum (a gift from E. Abrams and D. Andrew) at 1:10,000 for whole mount and 1:5000 for fluorescence; mouse monoclonal Crumbs antiserum (Developmental Studies Hybridoma Bank; Iowa City, IA) at 1:100; mouse monoclonal β-catenin (Armadillo) antiserum (Developmental Studies Hybridoma Bank) at 1:100; rat monoclonal DEcadherin antiserum (gift from H. Oda) at 1:20; mouse β-galactosidase (β-gal) antiserum (Promega; Madison, WI) at 1:10,000, rabbit polyclonal aPKC antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:1000 and rabbit polyclonal Croquemort antiserum at 1:500 (a gift from N. Franc). Appropriate biotinylated- (Jackson Immunoresearch Laboratories, Westgrove, PA), FITC- or Rhodamine- (Molecular Probes, Eugene, OR) conjugated secondary antibodies were used at a dilution of 1:500. Whole-mount-stained embryos were mounted in methyl salicylate (Sigma, St. Louis, MO) before visualization on a Zeiss Axioplan 2 microscope with Axiovision Rel 4.2 software (Carl Zeiss, Thornwood, NY). Fluorescently labeled embryos were mounted in 85% glycerol with 2.5% *N*-propylgalate. Thick (1 μm) fluorescent images were acquired on a Zeiss Axioplan microscope (Carl Zeiss) equipped with LSM 510 for laser scanning confocal microscopy at the Rockefeller University Bio-imaging Resources Center (New York, NY).

Analysis of recombinant lines

Recombinant lines carrying different UAS-transgene insertions were identified by PCR analysis of genomic DNA isolated from adult recombinant flies using one primer specific to the UAS sequence and another to the transgene of interest. Expression of the transgene was then confirmed by in situ hybridization with antisense digoxigenin-labeled RNA probes for the respective transgenes prepared as previously described (Lehmann and Tautz, 1994). *shotgun* (E-cadherin; Research Genetics), *Rac1* (Open Biosystems, Huntsville, AL) and *shibire* (Open Biosystems) cDNAs were used as templates for generating antisense digoxygenin-labeled RNA probes. Embryos were mounted in 70% glycerol before visualization as described above for antibody staining.

Results

Inhibition of Rac function disrupts salivary gland morphogenesis

The *Drosophila* genome encodes three *Rac* genes, *Rac1*, *Rac2* and *Mig 2-like* (*Mtl*). *Rac1* and *Rac2* share 92% amino acid sequence identity and *Mtl* is highly related. All three genes are expressed ubiquitously during development (Harden et al., 1995, 2002; Hariharan et al., 1995; Luo et al., 1994). To investigate the role of the Rac GTPases in salivary gland morphogenesis, we analyzed embryos carrying single, double or triple mutations in these *Rac* genes. In wild-type embryos, salivary gland cells form two placodes on the ventral surface of the embryo and begin their invagination at embryonic stage 11 (Fig. 1A). After invagination is complete, a dorsally-oriented tube has formed which then migrates posteriorly to form an elongated tube along the lateral body wall (Figs. 1B

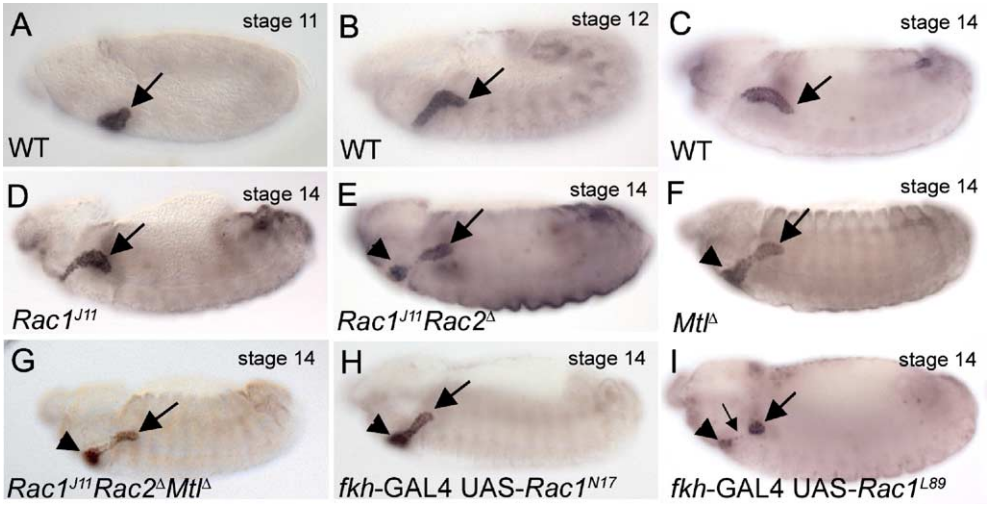

Fig. 1. Rac GTPases regulate salivary gland morphogenesis. In wild-type (WT, A–C) embryos, salivary gland cells invaginate from the ventral surface at stage 11 (A, arrow), and migrate posteriorly between stages 12 (B, arrow) and 14 (C, arrow). In $Rac1^{J11}$ mutants, the salivary gland does not complete its posterior migration (D, arrow). In $Rac1^{J11}Rac2^{\Delta}$ (E), Mtl^{Δ} (F) and $Rac1^{J11}Rac2^{\Delta}$ Mtl^{Δ} mutants (G), a cluster of cells remains at the ventral surface (E–G, arrowheads) and the gland fails to complete its posterior migration (E–G, arrows). In embryos expressing the dominant-negative *Rac1* mutations ($Rac1^{N17}$ or $Rac1^{L89}$) specifically in the salivary glands with *fkh*-GAL4, the salivary gland also fails to migrate posteriorly (H and I, arrows) and some cells remain at the ventral surface (H and I, arrowheads). The salivary glands of $Rac1^{L89}$ mutants are severely narrowed at their mid-points (I, small arrow). All embryos shown were stained for the transcription factor, dCREB-A to mark salivary gland nuclei.

and C). In contrast, in embryos homozygous for different combinations of the three Rac genes, salivary gland invagination and migration were perturbed. In $Rac1^{J11}$ mutant embryos, all cells invaginated but the gland did not complete its posterior migration and instead was abnormally shaped and appeared attached to the ventral surface (Fig. 1D). In $Rac1^{J11}Rac2^{\Delta}$ mutants, the salivary gland phenotype was enhanced, such that more cells remained at the ventral surface and the gland failed to migrate posteriorly (Fig. 1E). Thus, the functions of both *Rac1* and *Rac2* are required for proper invagination and migration of the salivary gland. In Mtl^{Δ} homozygous embryos (Fig. 1F), salivary gland invagination and migration were disrupted in a similar manner to $Rac1^{J11}Rac2^{\Delta}$ mutants (Fig. 1E). Embryos mutant for all three Rac genes, $Rac1^{J11}Rac2^{\Delta}Mtl^{\Delta}$, also showed defects in invagination and migration (Fig. 1G). These data suggest that all three *Rac* genes, *Rac1*, *Rac2* and *Mtl*, are individually required for salivary gland morphogenesis.

To confirm a function for *Rac1* in salivary gland morphogenesis and to determine cell autonomy, we expressed two types of Rac1 dominant-negative mutations, $Rac1^{L89}$ and $Rac1^{N17}$, specifically in the salivary glands of otherwise wild-type embryos using the UAS-GAL4 system (herein referred to as $Rac1^{L89}$ and $Rac1^{N17}$; Figs. 1H and I; Brand and Perrimon, 1993; Harden et al., 1995; Luo et al., 1994). In $Rac1^{L89}$ and $Rac1^{N17}$ embryos invagination and posterior migration of the gland were similarly affected to that of loss of *Rac1* function (Figs. 1H and I). These data demonstrate that the *Rac* genes are required in salivary gland cells during invagination and migration.

Expression of the $Rac1^{L89}$ dominant-negative mutation caused the most severe salivary gland phenotype; the gland appeared to be stretched or severed at its approximate mid-point (Fig. 1I). To better understand the $Rac1^{L89}$salivary gland phenotype, we visualized the lumen of the glands by staining for the apical membrane protein Crumbs and the transcription factor dCREB-A. In wild-type embryos, invagination of salivary gland primordial cells formed a tube with a contiguous, central lumen (Fig. 2A). In subsequent stages as the gland elongated and migrated posteriorly, the gland and its lumen remained intact (Figs. 2B and C). After migration was complete, the salivary gland lay along the lateral body wall with its longest axis in the anterior–posterior direction (Fig. 2D). In contrast, in $Rac1^{L89}$ embryos, the salivary glands were broken apart. Most salivary gland cells invaginated in $Rac1^{L89}$ embryos and formed a tube with a slightly expanded lumen (Fig. 2E). At the stage when wild-type salivary gland cells turned to migrate posteriorly (Fig. 2B), the $Rac1^{L89}$ salivary gland lumen was thinner at its midpoint (Fig. 2F) or broke completely (Fig. 2G) while the gland proper remained intact (white outline Figs. 2F and G). At later stages, the $Rac1^{L89}$ salivary gland proper was also separated into pieces with one segment at the surface and another internal (Figs. 2H and I). One hundred percent of glands scored at embryonic stage 14 displayed the broken gland phenotype (Fig. 5A). By the end of embryogenesis, the salivary glands of $Rac1^{L89}$ embryos were globular and consisted of several separated lumena (Fig. 2J). The salivary gland lumen was similarly disrupted in embryos homozygous for $Rac1^{J11}Rac2^{\Delta}$. During posterior migration of the salivary gland in $Rac1^{J11}Rac2^{\Delta}$ embryos, breaks in the lumen were detected (Fig. 2K). In the mature gland of $Rac1^{J11}Rac2^{\Delta}$ embryos, cyst-like lumena were found (Fig. 2L) similar to those of $Rac1^{L89}$ embryos (Fig. 2J). Therefore, these data demonstrate a crucial role for *Rac* in keeping the lumen and the gland intact during gland morphogenesis.

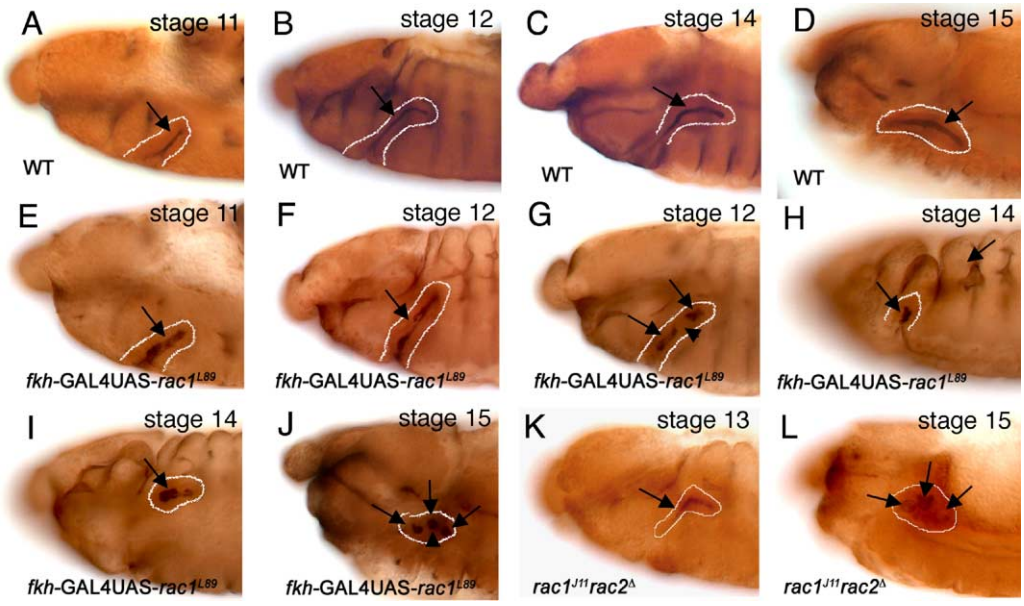

Fig. 2. Salivary gland lumen integrity is lost in $Rac1^{L89}$ mutants. In wild-type embryos (A–D), invagination forms a gland with a distinct lumen (A, arrow). Posterior migration of the WT gland is initiated at stage 12 (B, arrow), continues during stage 14 (C, arrow) and is completed by stage 15 (D, arrow). In $Rac1^{L89}$ embryos, a wider salivary gland lumen is formed (E, arrow). The lumen of $Rac1^{L89}$ glands severs (F and G, arrows) but the gland remains intact (G, arrowhead). $Rac1^{L89}$ mutant gland breaks apart into two pieces (panels H and I are different focal planes of the same embryo, arrows). The mature gland of $Rac1^{L89}$ mutants contains several lumena (J, arrows). In $Rac1^{J11}Rac2^{\Delta}$ embryos, the lumen of the invaginated salivary gland breaks apart (K, arrow) and several lumena are detected in the mature gland (L, arrows). All embryos were stained for the transcription factor, dCREB-A and for Crumbs to mark the lumen.

Constitutive activation of Rac causes loss of salivary gland cells

To better understand the function of Rac in salivary gland morphogenesis, we expressed the constitutively active form of *Rac1* ($Rac1^{V12}$) specifically in the salivary gland (herein referred to as $Rac1^{V12}$ embryos). In wild-type embryos, expression of the transcription factor dCREB-A is maintained throughout gland morphogenesis (Figs. 3A–C; Andrew et al., 1997). In contrast, in $Rac1^{V12}$embryos, we observed a gradual disappearance of dCREB-A beginning with cells in the distal tip of the gland (Figs. 3E, F and G). By late embryogenesis (stages 15–16), dCREB-A was detected in only a few cells or was completely lost (Fig. 3G and data not shown). Apical membrane proteins, such as Crumbs and atypical Protein kinase C (aPKC), also gradually disappeared from salivary gland cells of $Rac1^{V12}$embryos (Fig. 3H and data not shown). In wild-type embryos, Crumbs is localized to the apical domain and marks the lumen (Fig. 3D), whereas in $Rac1^{V12}$ embryos, Crumbs was found either in clumps or was absent from most salivary gland cells (Fig. 3H). An identical expression pattern was observed for aPKC (data not shown). These data demonstrate that Rac1 activation altered expression of dCREB-A and apical polarity markers.

To determine whether the expression of salivary gland marker proteins other than dCREB-A was similarly affected, we stained wild-type and $Rac1^{V12}$ embryos for Prolyl 4-hydroxylase alpha salivary gland 1 (PH4αSG1), which is expressed specifically in salivary gland cells beginning during embryonic stage 12 and continuing throughout embryogenesis (Figs. 3I and J; Abrams and Andrew, 2002). PH4αSG1 was expressed at reduced levels in $Rac1^{V12}$ embryos at stage 14 (Fig. 3K). Furthermore, PH4αSG1-expressing $Rac1^{V12}$ cells were dispersed and did not form an intact gland. By the end of embryogenesis, PH4αSG1-positive cells were detected in the head region and scattered in more posterior regions (Fig. 3L). The number of PH4αSG1-positive cells in $Rac1^{V12}$ mutant glands at stage 16 was consistently less than the normal number of salivary gland cells in wild-type embryos (data not shown). These data suggest that Rac activation induces migratory behavior and alters salivary gland specific gene expression. The reduced number of salivary gland cells in late $Rac1^{V12}$ embryos further suggests that a population of cells either dies or completely loses the salivary gland fate when Rac is activated.

Rac1 regulates cadherin-mediated adhesion during salivary gland morphogenesis

Previous reports have shown that in a number of different contexts Rac GTPases exert their cellular functions through regulation of E-cadherin-mediated adhesion. Therefore, we tested whether the salivary gland defects observed in *Rac1* mutants could be due to changes in cadherin-mediated cell–cell adhesion by analyzing the localization of the adherens junction proteins, β-catenin and E-cadherin. In salivary gland cells of wild-type embryos, β-catenin and E-cadherin were localized in the apical domain during gland morphogenesis (Figs. 4A–D). In invaginating $Rac1^{L89}$ mutant glands, β-catenin and E-cadherin expression was increased in the apical domain and β-catenin was also found along the basal–lateral membrane (Figs. 4E and F). At the point when $Rac1^{L89}$ salivary gland cells began to migrate posteriorly, β-catenin and E-cadherin remained

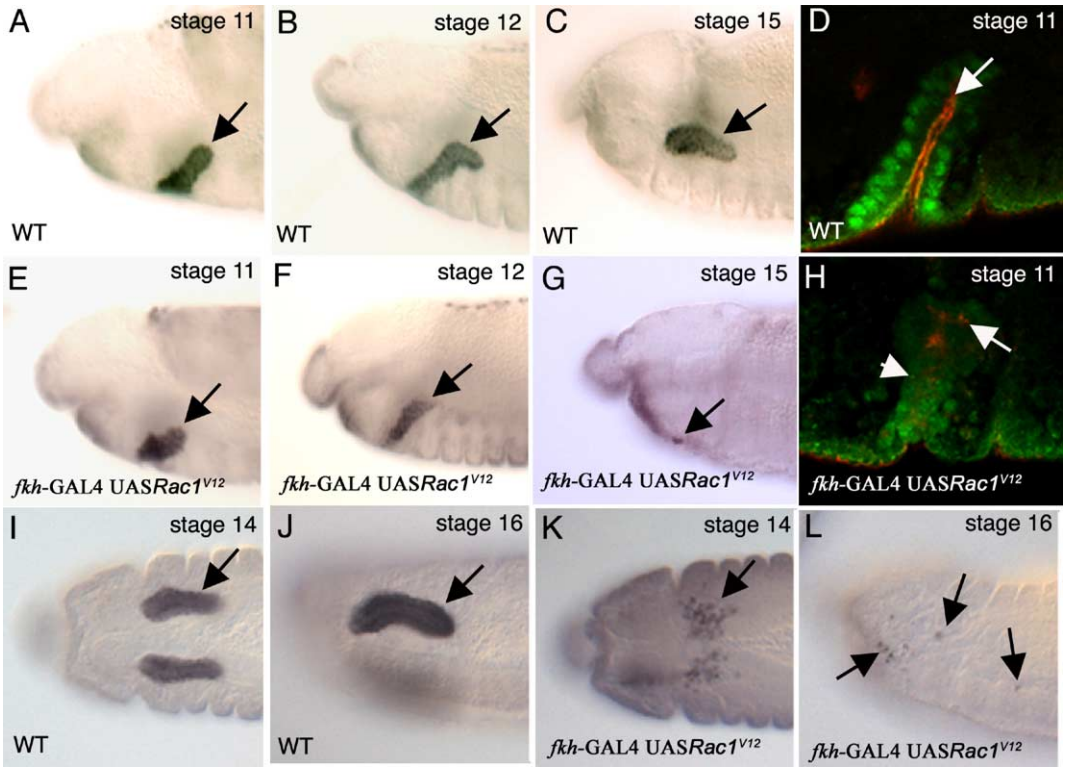

Fig. 3. Activation of Rac1 alters salivary gland gene expression. In wild-type embryos, dCREB-A is expressed in all salivary gland cells throughout gland morphogenesis (A–C). In $Rac1^{V12}$ salivary glands, dCREB-A staining reveals abnormal salivary gland morphology (E, arrow). dCREB-A expression begins to disappear at the time of posterior migration in salivary glands expressing $Rac1^{V12}$ (F, arrow). Few dCREB-A-stained cells are detected in late salivary glands expressing $Rac1^{V12}$ (G, arrow). In wild-type glands (D), the apical membrane protein, Crumbs (red), marks the lumen which is a contiguous structure (arrow). In $Rac1^{V12}$ glands, Crumbs staining is found in clumps (H, arrow) or is absent (H, arrowhead). Wild-type (WT) salivary glands express PH4αSG1 (I and J, arrows). In $Rac1^{V12}$ salivary glands, PH4αSG1-stained cells do not form a coherent tube (K, arrow) and become fewer and dispersed at late stages of embryogenesis (L, arrows). Embryos in panels A–C and E–G were stained for dCREB-A; embryos in panels D and H were stained for Crumbs (red) to mark the lumen and dCREB-A (green) to mark the salivary gland nuclei; embryos in panels I–L were stained for PH4αSG1 to mark the endoplasmic reticulum of salivary gland cells.

elevated in the distal and proximal portions of the gland, but were absent from the middle of the mutant glands coinciding with the thin or broken regions (Figs. 4G and H). We observed a similar increase in β-catenin expression and distribution in $Rac1^{J10}Rac2^{\Delta}Mtl^{\Delta}$ homozygous embryos and in wild-type embryos expressing the $Rac1^{N17}$ dominant-negative mutation in the salivary gland (data not shown).

In contrast to $Rac1^{L89}$, the constitutively active $Rac1^{V12}$ mutation caused loss of β-catenin and E-cadherin expression in salivary gland cells (compare Figs. 4I and J with E and F). At the invagination stage, only few $Rac1^{V12}$ salivary gland cells had apical localization of β-catenin and E-cadherin while most had cytoplasmic accumulation (Figs. 4I and J). By stage 12 when dCREB-A staining began to disappear in $Rac1^{V12}$ salivary glands, β-catenin and E-cadherin localization was cytoplasmic in all gland cells (data not shown). Therefore, expression of dominant-negative and constitutively active Rac mutations caused opposite effects on expression levels and localization of β-catenin and E-cadherin in salivary gland cells.

To determine whether the changes in localization of β-catenin and E-cadherin reflect changes in adhesiveness of the cadherin-based junctions, we attempted to rescue the *Rac1* mutant salivary gland phenotypes by increasing or decreasing the expression of *shotgun* (*shg*), which encodes *Drosophila* E-cadherin. We hypothesized that if the increased E-cadherin staining observed in $Rac1^{L89}$ mutant glands increases cell–cell adhesion and causes the glands to sever during migration, then reduction of E-cadherin levels by removing one copy of the *shg* gene should alleviate the $Rac1^{L89}$ salivary gland phenotype. Similarly, if loss of cadherin expression in $Rac1^{V12}$ mutant glands reduces cell–cell adhesion and leads to dispersal and cell death of salivary gland cells, then exogenous expression of wild-type E-cadherin may rescue the $Rac1^{V12}$ mutant salivary gland phenotype.

We expressed $Rac1^{L89}$ in salivary glands of embryos heterozygous for the shg^{2} mutation and scored embryos at stage 14 when the gland had completely severed in $Rac1^{L89}$ embryos. We observed a dramatic decrease in the $Rac1^{L89}$ salivary gland phenotype in $Rac1^{L89}$ *shg*/+ embryos from 100% to 16% (n = 88 glands; Fig. 5A), suggesting that increased cadherin-mediated adhesion is the cause for the break up of the gland in $Rac1^{L89}$ embryos. We also expressed wild-type full-length E-cadherin (*shg*) fused to GFP (*EcadGFP*) concomitantly with the $Rac1^{V12}$ mutation in salivary gland cells and scored dCREB-A-stained embryos at stage 14 when most dCREB-A expression is lost in $Rac1^{V12}$ embryos. Wild-type

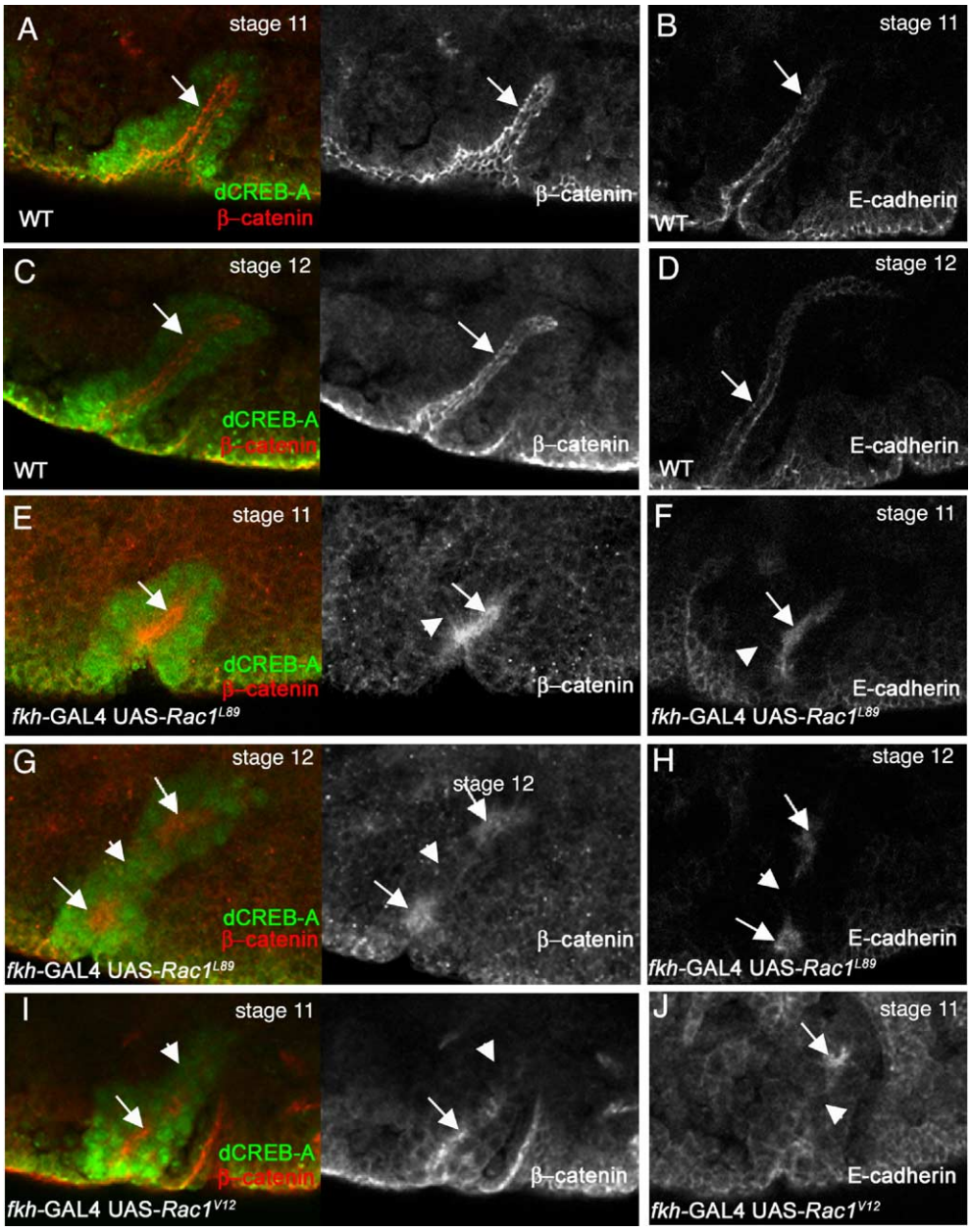

Fig. 4. β-catenin and E-cadherin localization is altered in *Rac1* mutant salivary glands. In wild-type embryos (A–D), β-catenin (A and C, arrows) and E-cadherin (B and D, arrows) are found exclusively in the apical domain during invagination (A and B) and during posterior migration (C and D). In $Rac1^{L89}$ salivary glands, β-catenin (E) and E-cadherin (F) are increased in the apical domain (E and F, arrows) and are also present along the basal–lateral membrane (E and F, arrowhead) of invaginating salivary gland cells. During posterior migration of $Rac1^{L89}$ glands, β-catenin (G) and E-cadherin (H) are present in the distal and proximal regions (G and H, arrows) but absent from the mid-region of the gland (G and H, arrowheads). In $Rac1^{V12}$ glands, β-catenin (I) and E-cadherin (J) are present in some cells (I and J, arrows) but lost from others (I and J, arrowheads). Embryos in panels A, C, E, G and I were stained for β-catenin (red) and dCREB-A (green); embryos in panels B, D, F, H and J were stained for E-cadherin. The right panels of panels A, C, E, G and I emphasize the β-catenin staining of the glands in the left panels.

embryos expressing *EcadGFP* in the salivary gland formed normal glands like wild-type embryos (Figs. 5B and C). Expression of *EcadGFP* in $Rac1^{V12}$ mutant salivary gland cells restored dCREB-A expression to normal levels and gland integrity in 40% of embryos (n = 150 glands; Figs. 5A and D). In some $Rac1^{V12}$ *EcadGFP* embryos, the salivary glands were slightly more elongated compared to wild-type (compare Figs. 5D with B). Altogether, these data demonstrate that *Rac* regulates epithelial integrity during salivary gland morphogenesis through modulation of cadherin function; loss of *Rac* function increases cadherin function whereas gain of *Rac* function decreases cadherin function.

Rac regulates cadherin activity through endocytosis

Mammalian cell culture studies have shown that one mechanism by which Rac controls cell–cell adhesion is through selective endocytosis of cell surface cadherins (Akhtar and Hotchin, 2001; Izumi et al., 2004). Therefore, we investigated the possibility that in $Rac1^{V12}$ salivary gland

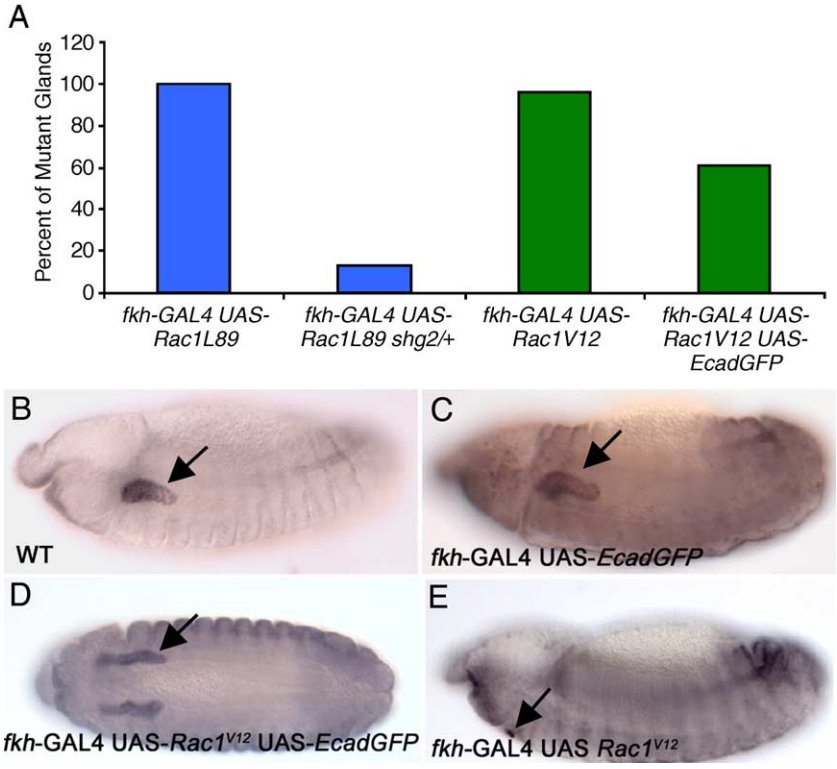

Fig. 5. Modulation of cadherin function rescues the Rac1 mutant salivary gland phenotypes. Graphical representation of embryos scored for salivary gland phenotypes at stage 14 (A). Loss of one copy of the *shg* gene ameliorates the $Rac1^{L89}$ salivary gland phenotype (A, *fkh*-GAL4; UAS-$Rac1^{L89}$; shg^{2}/+) whereas expression of wild-type full-length *EcadGFP* partially rescues the $Rac1^{V12}$ phenotype (A, *fkh*-GAL4; UAS-$Rac1^{V12}$-UAS-*EcadGFP*). Wild-type (WT) embryos (B) and WT embryos expressing *EcadGFP* (C) form normal glands (arrows). Concomitant expression of the $Rac1^{V12}$mutation and *EcadGFP* (D, arrow) prevents the loss of dCREB-A expression and loss of gland integrity induced by expression of the $Rac1^{V12}$mutation alone (E, arrow). Embryos in panels B through E were stained for dCREB-A.

cells changes in E-cadherin endocytosis may lead to the loss of E-cadherin from the apical–lateral membrane and subsequent dispersal and/or death of salivary gland cells. We first tested whether inhibition of endocytosis affected salivary gland morphogenesis in a similar manner to loss of *Rac* function. Different types of endocytosis have been shown to be regulated by the GTPase dynamin (Conner and Schmid, 2003), whose *Drosophila* homolog is encoded by the *shibire* (*shi*) gene (Chen et al., 1991; Obar et al., 1990). Therefore, we analyzed salivary gland development in embryos carrying a temperature-sensitive mutation of *shibire*, shi^{ts2}. At the permissive temperature (25°C), we observed normal salivary gland development; however, when the embryos were shifted to the non-permissive temperature (30°C) for 15 min and then allowed to recover at 25°C for an hour before being processed for immunocytochemistry, we observed embryos in which the salivary gland either failed to turn posteriorly or did not complete its posterior migration (Fig. 6B and data not shown). To determine whether the salivary gland migration defect observed in shi^{ts2} mutants was due to alteration of adherens junction proteins, we stained shi^{ts2} mutant embryos for β-catenin. To keep the experimental conditions and imaging parameters identical, we compared β-catenin expression of the same pool of shi^{ts2}embryos. We observed embryos with varying levels of β-catenin staining; some embryos had a level and pattern of β-catenin expression identical to that of wild-type salivary glands where β-catenin was concentrated in puncta at the apical–lateral membrane (Fig. 6C). In contrast, in other embryos, the level of β-catenin staining was dramatically elevated in the salivary gland where it was found as a continuous band throughout the apical domain (Fig. 6D), similar to the pattern observed in *Rac* loss of function mutants and wild-type embryos expressing dominant-negative *Rac1* mutations in the salivary gland (Fig. 4 and data not shown). Thus, compromised endocytosis leads to an increase in β-catenin levels which may account for the defects in salivary gland migration.

We hypothesized that if loss of E-cadherin in $Rac1^{V12}$ salivary gland cells is due to increased endocytosis of β-catenin/E-cadherin, then decreasing endocytosis of E-cadherin in $Rac1^{V12}$ mutant salivary gland cells may ameliorate the $Rac1^{V12}$ salivary gland phenotype. To disrupt dynamin-mediated endocytosis, we expressed a dominant-negative form of *shi*, shi^{K44A}, specifically in salivary gland cells of wild-type embryos and $Rac1^{V12}$ mutant embryos. In a few of the wild-type embryos expressing shi^{K44A} in salivary glands of wild-type embryos, the gland did not complete its posterior migration and resembled Rac mutants (Fig. 6E). Coexpression of $Rac1^{V12}$ and shi^{K44A} partially restored dCREB-A expression and gland integrity; however, the glands appeared narrower with fewer cells compared to their wild-type counterparts (Fig. 6F).

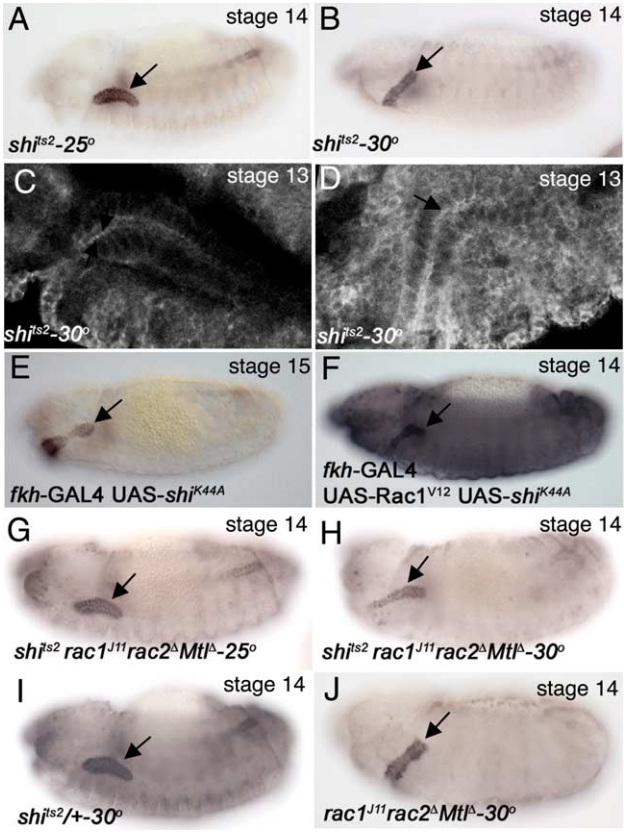

Fig. 6. Regulated endocytosis is required for salivary gland migration. In shi^{ts2} embryos at the permissive temperature (A), salivary gland development is normal (A, arrow) whereas in shi^{ts2} embryos at the non-permissive temperature (B), salivary glands fail to migrate posteriorly (B, arrow). In shi^{ts2} embryos at the non-permissive temperature (C and D), some embryos have a normal pattern of β-catenin expression (C, arrows) whereas others have elevated levels of β-catenin expression in the apical–lateral membrane (D, arrow). In embryos that express shi^{K44A} in just the salivary gland of otherwise WT embryos, the salivary gland fails to migrate properly (E, arrow). Coexpression of the shi^{K44A} and $Rac1^{V12}$ mutations partially restores dCREB-A expression (F, arrow), but the glands are smaller and appear to have less cells (F, arrow). In embryos heterozygous for shi^{ts2} and $Rac1^{J11}Rac2^{\Delta}$ Mtl^{Δ}, normal glands form at the permissive temperature (G, arrow) whereas glands fail to migrate at the non-permissive temperature (H, arrow). Embryos carrying one copy of shi^{ts2} at the non-permissive temperature form normal glands (I, arrow). In $Rac1^{J11}Rac2^{\Delta}$ *Mtl* homozygous embryos at the non-permissive temperature, salivary glands fail to migrate (J, arrow). All embryos were stained for dCREB-A except those in panels C and D which were stained for β-catenin.

We tested for a genetic interaction between *shibire* and the *Rac* genes during salivary gland morphogenesis. In trans-heterozygous embryos of $shi^{ts2}$and $Rac1^{J11}Rac2^{\Delta}Mtl^{\Delta}$ at the permissive temperature, all embryos formed normal salivary glands (Fig. 6G). However, in $shi^{ts2}$and $Rac1^{J11}Rac2^{\Delta}Mtl^{\Delta}$ trans-heterozygous embryos at the non-permissive temperature, posterior migration of the salivary gland was disrupted (Fig. 6H), indicating that *shi* and *Rac* genes interact genetically to regulate salivary gland migration. We confirmed that one copy of shi^{ts2}at the non-permissive temperature did not cause aberrant migration of the salivary gland (Fig. 6I). We also confirmed that $Rac1^{J11}Rac2^{\Delta}Mtl^{\Delta}$ homozygous embryos at the non-permissive temperature showed the salivary gland migration defect (Fig. 6J) and that heterozygous embryos formed normal glands (data not shown). In conclusion, loss of dynamin function resulted in increased β-catenin at the membrane and interrupted salivary gland migration. Furthermore, inhibition of dynamin function was sufficient to partially restore dCREB-A expression and gland integrity in $Rac1^{V12}$mutant embryos and dynamin interacts genetically with *Rac* during salivary gland morphogenesis. Thus, Rac-regulated dynamin-mediated endocytosis is critical for salivary gland formation.

Rac activation leads to cell death

In $Rac1^{V12}$mutant embryos at late stages of embryogenesis, we observed salivary gland cells dispersed throughout the head region (Fig. 3L). This observation raises the possibility that Rac1 activation induced cell migration. Alternatively, the perceived migrating cells could be apoptotic bodies of salivary gland cells being carried by migrating macrophages. To determine if either or both of these possibilities were true, we stained $Rac1^{V12}$ mutant embryos for PH4αSG1 and Croquemort (CRQ), a macrophage marker protein (Franc et al., 1999). In wild-type embryos, PH4αSG1-staining in the salivary gland was distinct from that of the nearby CRQ-positive macrophages (Figs. 7A, A′ and A″). However, in $Rac1^{V12}$ embryos, the residual PH4αSG1-staining colocalized with CRQ-positive macrophages. Indeed, some macrophages showed punctate PH4αSG1 staining which is likely to be ingested apoptotic salivary gland cells (Figs. 7B, B′ and B″). We were able to detect few PH4αSG1-stained salivary gland cells that did not stain for CRQ. These data suggest that, in $Rac1^{V12}$ mutant embryos at late stages, many scattered salivary gland cells die by apoptosis and are engulfed by macrophages. However, a subpopulation of salivary gland cells survives and continues to express salivary gland specific markers such as, PH4αSG1. Thus, activation of Rac1 leads both to cell migration and cell death.

The cell death observed in $Rac1^{V12}$ embryos raised the possibility that the dispersal of $Rac1^{V12}$ salivary gland cells is solely due to cell death and not to interference of cadherin-mediated cell adhesion. To determine whether the $Rac1^{V12}$ salivary gland phenotype was due to apoptosis or to loss of cell adhesion, we attempted to block apoptosis by coexpressing $Rac1^{V12}$ and the antiapoptotic *p35* gene from baculovirus (Grether et al., 1995) in salivary glands of wild-type embryos. Expression of *p35* in wild-type salivary gland cells did not affect gland morphogenesis (Figs. 7C, C′ and C″). In contrast, the glands of $Rac1^{V12}p35$ embryos were abnormally shaped and appeared to consist of a disorganized cluster of cells. Moreover, we did not detect a lumen in the glands of $Rac1^{V12}p35$ embryos (data not shown). Although expression of PH4αSG1 was markedly reduced in the $Rac1^{V12}p35$ embryos compared to that in wild-type embryos expressing *p35* alone (Figs. 7D, D′ and D″), it did not overlap with CRQ staining. Thus, inhibition of apoptosis by *p35* was able to keep all salivary gland cells from engulfment by macrophages. Although more PH4αSG1-expressing salivary gland cells survive in $Rac1^{V12}p35$ embryos due to inhibition of apoptosis, the abnormal development of the gland

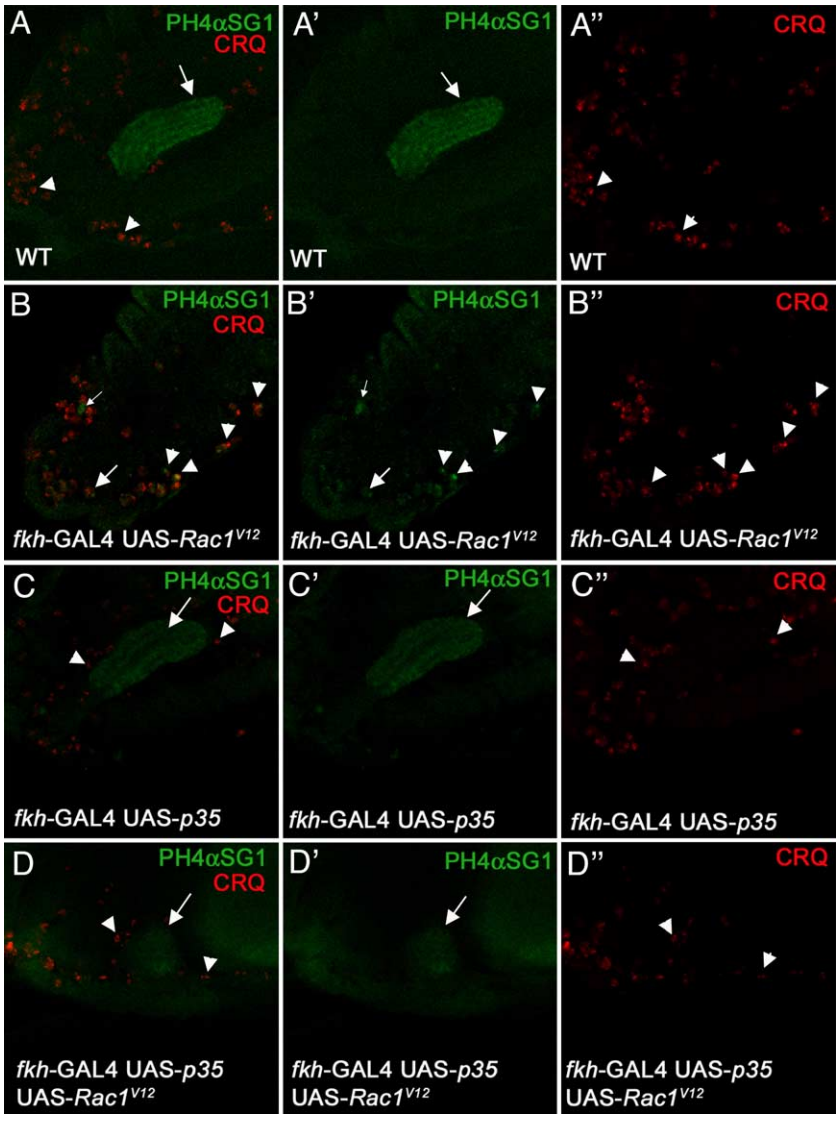

Fig. 7. *Rac1*^{V12} salivary gland cells die by apoptosis. In wild-type salivary glands (A and A′, arrows), and in salivary gland cells expressing *p35* (C and C′, arrows), PH4αSG1 is found in salivary gland cells and is distinct from surrounding macrophages labeled with CRQ (A″ and C″, arrowheads). In *Rac1*^{V12} mutant salivary glands (B and B′), faint PH4αSG1 staining (B′ arrow) and bright and punctate PH4αSG1 staining (B′, arrowheads) colocalize with CRQ-stained macrophages (B″, arrowheads). A PH4αSG1-stained salivary gland cell (B and B′, small arrow) that does not stain for CRQ. In embryos coexpressing *Rac1*^{V12} and p35 in the salivary glands (D, D′ and D″), faint PH4αSG1 staining is detected in the abnormally shaped glands (D and D′, arrows) and is distinct from CRQ-staining in the surrounding macrophages (D and D″, arrowheads). Salivary glands are detected with PH4αSG1 (green) and macrophages with CRQ (red). All embryos shown are at stage 16.

indicates that Rac activation interferes with proper gland morphogenesis.

Discussion

In this study, we show that the Rac GTPases regulate salivary gland morphogenesis through modulation of cadherin/catenin-based cell–cell adhesion, likely by dynamin-mediated endocytosis. Our characterization of the Rac mutant phenotypes suggests a model where Rac normally regulates cadherin-mediated cell–cell adhesion in salivary gland cells to allow enough plasticity for its invagination and migration yet keep the cells of the tube adhered to one another so that the gland can migrate as a cohesive tube. One mechanism by which cell surface cadherin levels are regulated is through selective endocytosis of E-cadherin from the apical–lateral membrane in a dynamin-mediated process (Akhtar and Hotchin, 2001; Paterson et al., 2003). When Rac function is compromised through loss-of-function mutations or expression of dominant-negative mutations, the balance between E-cadherin at the plasma membrane and internalized E-cadherin appears to be abrogated so that more E-cadherin remains at the plasma membrane resulting in increased cell–cell adhesion and causing the gland to sever. Our studies reveal the importance of precise regulation of adherens junction remodeling during cell migration in the context of a developing organ.

In all stage 14 *Rac1*^{L89} mutant embryos examined, the salivary gland broke apart close to its approximate mid-point. Reduction in cadherin levels rescues the mutant Rac severing phenotype, suggesting that severing occurs because loss of

Rac leads to an increase in cadherin-mediated cell–cell adhesion. We envision two possible explanations for the midpoint severing phenotype. In the first scenario, levels of cadherin remodeling may differ throughout the gland such that in $Rac1^{L89}$ embryos the cells in the distal tip are least affected and cells in the mid-region of the gland are most affected by the increase in cadherin function. In this situation, when the distal cells begin to migrate posteriorly, the increased adhesivity of the mid-region cells prevents their migration and causes the gland to sever in the middle. In the second scenario, movement of the mid-region and the distal region of the gland may occur through different mechanisms. It is possible that while the distal most cells migrate by undergoing cell shape change and extending prominent protrusions in the direction of migration, as was previously observed (Bradley et al., 2003), cells in the middle of the gland may follow the distal cells by rearranging their positions along the gland, such as occurs during the convergence extension movements observed in epithelial morphogenesis (Keller, 2002). Dynamic remodeling of E-cadherin may be particularly important for proper rearrangement of the mid-region cells and an inability to rearrange when E-cadherin adhesion is increased may cause severing of the gland and subsequent separation of the migrating distal portion from the rest of the gland. Alternatively, it is possible that both of these scenarios are at play during normal salivary gland migration. We currently cannot distinguish between these possibilities. In the developing tracheal tubes, Rac1 was recently shown to be required for cell rearrangements; in tracheal cells expressing a dominant-negative *Rac1* mutation, the dorsal branch was shorter than that of wild-type embryos (Chihara et al., 2003). Therefore, it will be important to determine whether cell rearrangement plays a role during salivary gland migration and to further elucidate the role of the Rac genes in this process.

When Rac1 function is over-activate, dynamin-mediated endocytosis of E-cadherin may be increased, resulting in decreased cadherin at the plasma membrane, and decreased cell–cell adhesion. The loss of adhesion leads to the dispersal of salivary gland cells and ultimately cell death. Preventing $Rac1^{V12}$-induced cell death led to the formation of abnormally shaped glands demonstrating that the $Rac1^{V12}$ salivary gland phenotype is primarily due to abrogation of gland morphogenesis and not to activation of the apoptotic pathway. Moreover, since wild-type full-length E-cadherin was sufficient to rescue the $Rac1^{V12}$ salivary gland phenotype, loss of cadherin function appears to be the primary cause for salivary gland defects. Thus, the Rac genes function in salivary gland cells to regulate E-cadherin-mediated cell–cell adhesion during tube morphogenesis.

Endocytosis allows temporal regulation of cell–cell adhesion

During salivary gland morphogenesis, gland integrity is kept intact while cells perform extensive cell shape changes and movements. Rac-regulated endocytosis of E-cadherin is one mechanism by which cell–cell adhesion is likely to be down-regulated temporarily. After E-cadherin is endocytosed, it can be recycled back to the cell surface, sequestered transiently inside the cell or routed to late endosomes and lysosomes for degradation (Bryant and Stow, 2004). Once salivary gland migration is complete and the gland has reached its final position, cell–cell adhesion may then need to be strengthened again in the mature gland and Rac activity may be down-regulated to promote increase in surface cadherins.

In addition to endocytosis, studies in mammalian cultured cells have shown that Rac can regulate levels of cell surface E-cadherin by other mechanisms, such as cleavage by presinilins and metalloproteinases (Egeblad and Werb, 2002; Marambaud et al., 2002), or tyrosine phosphorylation of the cadherin adhesion complex in a process involving reactive oxygen species (van Wetering et al., 2002). Thus, it will be interesting to determine whether additional mechanisms of E-cadherin regulation exist in salivary gland cells during gland morphogenesis.

Numerous studies in cell culture have demonstrated that recycling of E-cadherin occurs in both a clathrin-dependent and caveolin-dependent manner (Akhtar and Hotchin, 2001; Ivanov et al., 2004; Izumi et al., 2004; Le et al., 1999; Paterson et al., 2003). Since dynamin mediates both clathrin- and caveolin-dependent endocytosis, our studies do not allow us to distinguish which type is involved in cadherin endocytosis during salivary gland migration. Alternatively, both types of endocytosis may mediate Rac1 regulation of E-cadherin in salivary gland cells.

Rac activation and cell death

Expression of the $Rac1^{V12}$ mutation in salivary gland cells leads to loss of expression of salivary gland specific proteins, apical–basal polarity proteins and E-cadherin/β-catenin. Concomitant with changes in gene expression, $Rac1^{V12}$ mutant salivary gland cells lose adhesion to each other and subsequently migrate away or die by apoptosis. Our data suggest that overactivation of Rac1 primarily affects E-cadherin/β-catenin-mediated adhesion and salivary gland cell fate and that the observed cell death is a secondary consequence of these earlier changes. When cell death was prevented in $Rac1^{V12}$ embryos by expressing *p35*, more cells expressed the salivary gland specific protein PH4αSG1 than in $Rac1^{V12}$ embryos; however, the expression level was drastically reduced compared to wild-type, suggesting that even in the $Rac1^{V12}p35$ cells, cell differentiation was still mostly altered. Moreover, $Rac1^{V12}p35$ salivary gland cells did not form a normal gland, demonstrating a role for *Rac1* in gland morphogenesis. It is possible that apoptosis of $Rac1^{V12}$ cells is brought about by the loss or reduction of Fkh function. Fkh is expressed early in the salivary gland placode and its expression is maintained throughout embryogenesis (Myat and Andrew, 2000a). In the absence of *fkh* function, salivary gland cells die by apoptosis during the invagination stage. Since expression of dCREB-A and PH4αSG1 was reduced in $Rac1^{V12}$ mutant salivary gland cells, it is possible that Fkh expression was also similarly reduced, thereby, causing the cells to undergo apoptotic cell death.

Rac activation and cancer

Many human cancers are due to epithelial-derived tumors. When epithelial cells metastasize, they first undergo an epithelial to mesenchymal transition (EMT) before migrating away from the primary tumor to invade surrounding tissues. EMT is characterized by the loss of epithelial polarity and cell–cell adhesion (Thiery, 2003). When $Rac1^{V12}$ was expressed in salivary gland cells, expression of apical membrane proteins, Crumbs and aPKC and adherens junction proteins E-cadherin and β-catenin, was either lost or mislocalized. Based on these criteria, activation of Rac1 function induced features characteristic of early changes in EMT and metastasis. Interestingly, the expression levels of Rho GTPases are found to be elevated in a number of human cancers. For example, increased Rac protein levels and fast-cycling Rac mutations have been correlated with colorectal and breast tumors (Fritz et al., 2002). Expression of constitutively active Rac1 caused some salivary gland cells to lose polarity and adhesion to neighboring cells and migrate away in a manner similar to EMT. Our findings suggest that Rac1-regulated endocytosis of E-cadherin in the *Drosophila* salivary glands may be critical in maintaining epithelial character and preventing the loss of cell–cell adhesion and cell polarity. The *Drosophila* salivary gland might thus be powerful as a simple system to identify and characterize mechanisms that regulate cadherin-based cell–cell adhesion and certain aspects of EMT.

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