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Rac promotes epithelial cell rearrangement during tracheal tubulogenesis in *Drosophila*

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SUMMARY

Cell rearrangement, accompanied by the rapid assembly and disassembly of cadherin-mediated cell adhesions, plays essential roles in epithelial morphogenesis. Various in vitro and cell culture studies on the small GTPase Rac have suggested it to be a key regulator of cell adhesion, but this notion needs to be verified in the context of embryonic development. We used the tracheal system of *Drosophila* to investigate the function of Rac in the epithelial cell rearrangement, with a special attention to its role in regulating epithelial cadherin activity. We found that a reduced Rac activity led to an expansion of cell junctions in the embryonic epidermis and tracheal epithelia, which was accompanied by an increase in the amount of *Drosophila* E-Cadherin-Catenin complexes by a post-transcriptional mechanism. Reduced Rac activity inhibited dynamic epithelial cell rearrangement. Hyperactivation of Rac, on the other hand, inhibited assembly of newly synthesized E-Cadherin into cell junctions and caused loss of tracheal cell adhesion, resulting in cell detachment from the epithelia. Thus, in the context of *Drosophila* tracheal development, Rac activity must be maintained at a level necessary to balance the assembly and disassembly of E-Cadherin at cell junctions. Together with its role in cell motility, Rac regulates plasticity of cell adhesion and thus ensures smooth remodeling of epithelial sheets into tubules.

Key words: Morphogenesis, Cadherin, Cell adhesion, *Drosophila*
Movies available online

INTRODUCTION

Tightly connected cells in the epithelia protect internal tissues from the external environment. Epithelial cells are connected by junctional structures such as the adherence junction (AJ) and tight junctions that separate the apical and basolateral domains of cell membranes by acting as barriers for small molecules and ensure the polarization of the epithelium. The first signs of AJ formation and cell polarization are observed at the eight-cell stage in mammalian embryos (Shirayoshi et al., 1983) and at the blastoderm stage of insect embryos (Schejter and Wieschaus, 1993), with apical accumulation of E-Cadherin, an epithelial cell-cell adhesion molecule, localized at the AJ. Although junctional structures are being maintained, the overall shape of the epithelia changes drastically to allow morphogenetic movement during embryogenesis.

Junctional structures are essential because epithelial breakdown causes internal fluid to leak, which is lethal to organisms. However, during organogenesis, epithelia undergo drastic remodeling, as is observed in vertebrate neurulation, in which the neuroectoderm forms the neural tube, and in angiogenesis, where a meshwork of blood vessels forms. During this process, cells must transiently loosen their adhesions and rearrange their relative positions to reshape tissues and organs. It is therefore important to understand the mechanism that regulates turnover of junctional structures during epithelial morphogenesis. However, little is known about how the turnover of cell adhesion is regulated.

A Rho GTPase family member, Rac is likely to fulfill the task of coordinating cell adhesion and actin-based cytoskeletal motility because it has been strongly implicated in the regulation of both processes (Hall, 1998; Kaibuchi et al., 1999; Van Aelst and D'Souza-Schorey, 1997). The roles of Rac in developmental events involving extensive cell-shape changes, such as axon and dendrite development in the nervous systems, closure of dorsal epidermis and muscle development, are well documented (Hakeda-Suzuki et al., 2002; Luo et al., 1994; Ng et al., 2002). Rac has also been implicated in the regulation of cadherin-dependent cell adhesion in cultured epithelial cells, but its exact role is controversial. For example, whereas several

investigators have reported that Rac promotes E-Cadherin-dependent cell adhesion (Kuroda et al., 1998; Takaishi et al., 1997), others have reported that Rac inhibits it (Braga et al., 2000; Gimond et al., 1999; Potempa and Ridley, 1998). It appears that the results have been influenced greatly by the experimental settings and the way in which Rac activity was manipulated. To clarify the in vivo roles of Rac in the context of epithelial morphogenesis during development, we have been studying the phenotypes of *Drosophila* embryos with mutations in *Rac* genes.

We chose the *Drosophila* tracheal system as a model to study the roles of Rac in epithelial cell rearrangement. The tracheal system is derived from segmentally repeated epithelial cell clusters consisting of ~80 ectodermal cells that undergo branching and migration processes to form a network of tubular epithelium (Samakovlis et al., 1996). Tracheal cell migration and differentiation are triggered by Branchless (Bnl)/FGF, which is expressed in the positions to which tracheal cells will migrate (Sutherland et al., 1996) (Fig. 2A). Bnl/FGF activates Breathless (Btl) receptor tyrosine kinase (Klambt et al., 1992), which is expressed in all tracheal cells. Localized activation of Btl induces primary branching that transforms sac-like tracheal primordia into tubules consisting of multiple cells surrounding the circumference (multicellular tubules). While the primary branches are extended toward target sites, cell rearrangement takes place to change these branches into thin unicellular tubules consisting of cells with autocellular junctions. Because these processes take place without cell division, cell-shape changes and cell rearrangement play major roles in the formation of the tracheal network. Tracheal branch migration requires zygotically expressed E-Cadherin (Tanaka-Matakatsu et al., 1996; Uemura et al., 1996), which suggests that a de novo supply of E-Cadherin is essential for tracheal cell rearrangement. However, little is known about how cell adhesion and cell rearrangement are coordinated in this process.

In this study, we found that Rac was required for cell rearrangement in the tracheal epithelium. Reduced Rac activity was associated with the phenotype of high accumulation of cadherins and its associated molecules. Activation of Rac, however, transformed the tracheal epithelium into mesenchyme, suggesting an essential role of Rac in controlling cell adhesion.

MATERIALS AND METHODS

Genetics

rac1^{J11} *rac2*$^{\Delta}$, *rac1*^{J11} *rac2*$^{\Delta}$ *mtl*$^{\Delta}$, *pak*^{6}, *pak*^{11}, *bnl*^{P1} and *btl*$^{\Delta Oh10}$ were balanced over *TM6B*, *abdA-lacZ*. *btl-Gal4* (Shiga et al., 1996) was used to drive transgene expression in tracheal cells. Genotypes were identified by β-galactosidase expression. *UAS-E-Cadherin-GFP* on the second chromosome (Oda and Tsukita, 1999) was recombined into a chromosome containing *btl-Gal4* and was balanced with CyO. *UAS-gfp-moesin* was constructed by introducing the *gfp-moesin* fusion gene (Edwards et al., 1997) into pUAST (Brand and Perrimon, 1993), and this vector was used for germline transformation.

Time-lapse observation

Dechorionated embryos were mounted on a glass coverslip with rubber cement and were covered with halocarbon oil 700 (Sigma). GFP fluorescence was captured by a confocal microscope (Olympus, FV-300) attached to an upright microscope (Olympus BX51) with a 60× oil immersion lens (NA 1.4). To minimize phototoxicity, we reduced the Ar laser intensity (488 nm, 10 mW) to 1% and opened the pinhole to its maximum size (300 μm). Typically, 13 × 2 μm *z* stacks were taken every 5 minutes over a period of 6 hours. Images of the stacks were projected and were displayed as MPEG format movies.

Immunohistochemistry

Primary antibodies used were the following: rabbit anti-β-galactosidase (Cappel), rabbit anti-GFP (MBL), mouse anti-actin (Sigma), mouse anti-SRF (provided by M. Gilman), rat anti-E-cadherin (Oda et al., 1994), rat anti-N-cadherin (Iwai et al., 1997), rat anti-α-catenin (Oda et al., 1993), anti-rat PP2AA (Uemura et al., 1993), mouse 2A12, mouse anti-Armadillo N2 7A1, rabbit anti-Rac1 (Eaton et al., 1995), mouse anti-Crumb Cq4 and mouse anti-Fasciclin 3 7910 (Developmental Studies of Hybridoma Bank, University of Iowa). Fluorescent images were captured with a confocal microscope (Olympus, FV-500).

Western blotting

Protein extracted from 15 embryos was separated by SDS-PAGE (9% polyacrylamide) and transferred to a PVDF membrane. After the blots had been incubated with primary antibodies and HRP-linked secondary antibodies, antibody signals were detected by use of Super Signal West Pico (PIERCE). Blots were repeatedly probed according to the manufacturer's instructions. Intensities of the bands were quantified by densitometric scanning of the film exposed to chemiluminescence.

mRNA quantification

Poly A+ mRNA was isolated from approximately 30 embryos with a QuickPrep Micro mRNA purification kit (Pharmacia Biotech). The *rac1, rac2* double mutant chromosome was balanced over the *TM3, Kr-Gal4 UAS-gfp* chromosome (Casso et al., 2000), and mutant homozygotes were identified by the lack of GFP fluorescence. An amount of mRNA equivalent to three embryos was reverse transcribed by AMV reverse transcriptase (Life Science) with random hexamers used as primers. mRNA was quantified by using a Prism 7000 Sequence Detection System (ABI) with a SYBRE Green PCR Master mix according to the manufacturer's instructions. Control reactions without reverse transcriptase for each mRNA preparation revealed no significant genomic DNA contamination. PCR primers were designed by using Primer Express Version 2.0 software (ABI), the sequences of which are available upon request. Specificity of PCR reactions was confirmed by measuring dissociation temperature, agarose gel electrophoresis and DNA sequencing of the PCR products.

RESULTS

Rac mutations reveal the requirement to maintain epithelial architecture

In the *Drosophila* genome there are three *Rac* homologs, *Rac1*, *Rac2* and *Mig-2-like* (*Mtl*), that perform functionally redundant roles (Hakeda-Suzuki et al., 2002; Ng et al., 2002). Rac1 protein detected by a specific polyclonal antibody was ubiquitously expressed in all embryonic cells and is associated with plasma membranes (data not shown). We analyzed the epithelial phenotypes of embryos that were zygotically null for *Rac1* and *Rac2* (*Rac1, 2*), which, despite the presence of *Mtl* and maternal gene products, showed severe defects in their nervous system and various other tissues (Hakeda-Suzuki et al., 2002; Ng et al., 2002).

To analyze the state of epithelial cell adhesion in *Rac1, 2*

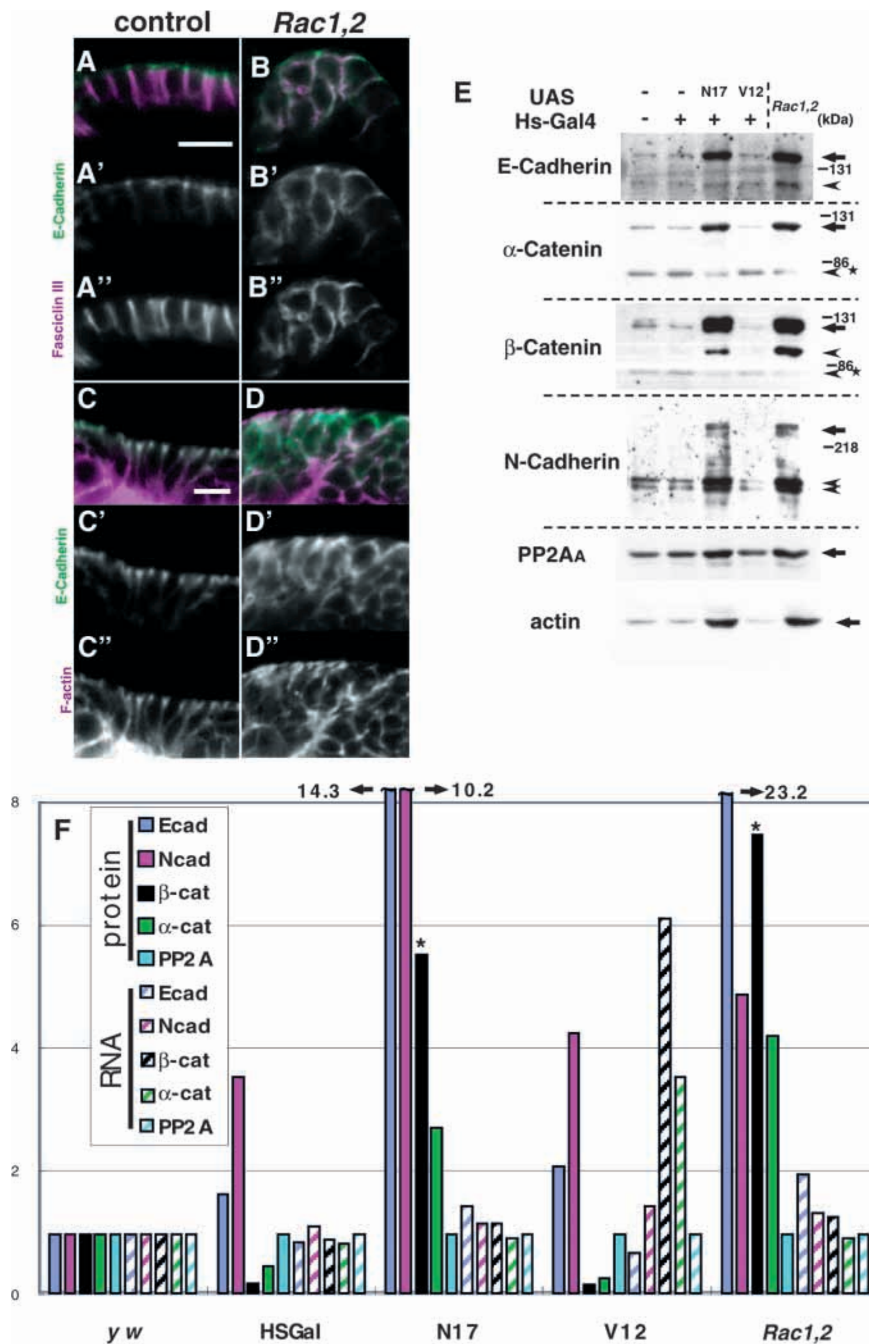

Fig. 1. Rac is required for apical localization of E-Cadherin and negatively regulates protein levels in the cadherin cell adhesion system. (A-B) Optical sections of the ventral epidermis of stage 15 embryos labeled for E-Cadherin (green) and Fasciclin 3 (purple). Grayscale images of each channel are shown below. In wild-type embryos, the apical concentration of E-Cadherin marks a distinct Fasciclin 3-free domain (A). In embryos zygotically null for *Rac1* and *Rac2* (*Rac1, 2*) this distinction is lost and many cells become flattened, with E-Cadherin localized over the entire cell surface (B). (C-D) Head epidermis of stage 6 embryos stained for E-Cadherin (green) and F-actin (purple). Cells in D have formed multiple cell layers and E-Cadherin is mislocalized. Grayscale images of each channel are shown below. (E) Western blot analysis of cadherins and their associated molecules in embryos with different levels of Rac activity. Arrows and arrowheads indicate mature and degradation products of each molecule, respectively. Arrowheads with an asterisk indicate degradation products of catenins, which were decreased in amount when Rac activity was reduced. Protein extracts of 15 embryos of each genotype were analyzed, and protein loading was monitored by Coomassie Brilliant Blue staining of the gel, which varied within a range of 10%. (F) Protein and RNA quantification. Protein was quantified by densitometric scanning of the films exposed to chemiluminescence. mRNA amounts were measured by quantitative RT-PCR. The amounts of cadherins and catenins were expressed as fold increase from the values for the control *y w* strain and were standardized by assuming that the amount of $PP2A_A$ remained constant. Asterisks on the bars for the amounts of β-Catenin protein indicate that the measurement was underestimated because of overexposure of the chemiluminescence. Scale bars: in A, 10 μm for A-B; in C, 10 μm for C-D.

mutants, we first observed the subcellular localization of *Drosophila* epithelial-cadherin (E-Cadherin) in the embryonic epidermis. E-Cadherin is a key component of adherence junctions (Oda et al., 1994; Tepass et al., 1996; Uemura et al., 1996). Its intracellular domain binds to β-catenin, which associates with α-catenin. This cadherin-catenin complex is concentrated at the apical side of the cell-cell junction of columnar epithelial cells and is essential for cell adhesion (Tepass et al., 2000; Yagi and Takeichi, 2000). Double labeling of the normal embryonic epidermis with the septate junction marker Fasciclin 3 (Patel et al., 1987) revealed that the apical domain of the cell-cell contact sites was uniquely labeled with E-Cadherin and that the basolateral domain had accumulated Fasciclin 3 and a low level of E-Cadherin (Fig. 1A). However, in the *Rac1, 2* mutant embryos at stage 15, E-Cadherin was highly and more broadly accumulated; and the distinction between the apical and basolateral domains of the cell contact site was lost in a subset of the epidermis (Fig. 1B). In stage 16 embryos, the columnar cells became shorter, and parts of the epidermis became multilayered, as revealed by F-actin staining (Fig. 1C,D).

Upregulation of cadherin systems in Rac mutants

Consistent with the broader distribution of E-Cadherin, we found that the level of E-Cadherin was increased by 23-fold in *Rac1, 2* mutant embryos, as measured by western blot analysis

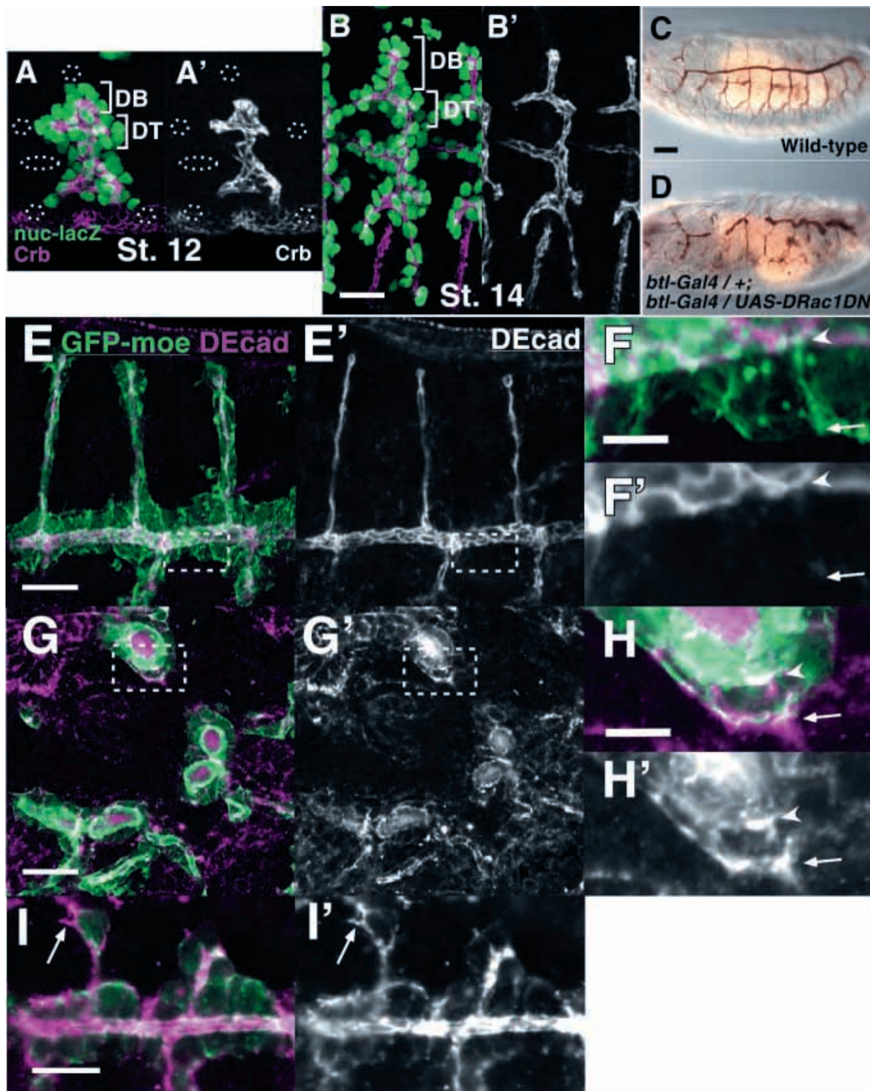

Fig. 2. Mislocalization of E-Cadherin in tracheal cells that have reduced Rac activity. (A-B) Wild-type embryonic trachea at stage 12 (A) and stage 14 (B). Cell nuclei are marked with nuclear localized β-galactosidase expressed by *btl-Gal4* (green in A,B). The apical cell surface is labeled with anti-Crumbs (purple in A,B). Grayscale images of anti-Crumb are shown in A′ and B′. Tracheal branches migrate toward the positions labeled with broken circles in which Bnl/FGF is expressed (Sutherland et al., 1996). While tracheal branches extend, cells of the dorsal branch (DB) and dorsal trunk (DT) rearrange their relative positions and form thinner tubules. (C,D) Wild-type (C) and *btl-Gal4*/+; *btl-Gal4*/*UAS-DRac1N17* (D) embryos were stained with monoclonal antibody 2A12, which labels the tracheal lumen. In Rac1N17-expressing trachea, phenotypes of zigzagged and/or truncated DT were similar to those of *Rac1, 2* mutants (compare D with G). (E-H,I) Wild-type (E,F), *Rac1, 2* mutant (G,H) and *btl-Gal4*/+; *btl-Gal4*/*UAS-DRac1N17* (I) embryos were stained with anti-E-Cadherin (purple), and tracheal cells were marked with GFP-moesin (green) to reveal F-actin. Grayscale images of anti-E-Cadherin are shown separately. F and H are high-magnification images of the broken frames shown in E and G. Arrows and arrowheads indicate basal and apical cell membranes of tracheal cells, respectively (F,H). E-Cadherin have accumulated at the apical side of the lateral membrane in wild-type trachea (E,F). In *Rac1, 2* mutant and *btl-Gal4*/+; *btl-Gal4*/*UAS-DRac1N17* embryos, E-Cadherin has abnormally accumulated at the basal membrane of tracheal cells (G-I). In I, E-Cadherin accumulation in cell protrusions is also evident (arrows). Scale bars: 20 μm in B,E,G,I; 5 μm in F,H; 40 μm in C.

(Fig. 1E,F). We also tested the effect of the dominant-negative form of Rac1 (Rac1N17) (Luo et al., 1994) driven by the *hsp70* heat shock promoter. To avoid nonspecific effects of heat shock and excess production of mutant proteins, we kept the incubation temperature at 25°C. At this temperature *rac1* mRNA was expressed within the range of the wild-type level (data not shown). Even under this mild induction condition, we observed an increase in E-Cadherin to a level comparable with that in the *Rac1, 2* mutant embryos. Furthermore, the amounts of α- and β-catenins, and of another type of cadherin, N-Cadherin (mainly expressed in neuronal systems and in mesodermal tissues (Iwai et al., 1997), were also increased in *Rac1, 2* mutants and in Rac1N17-expressing embryos. Although an increase in β-Catenin sometimes activates Wingless signaling, *Rac1, 2* mutant phenotypes did not resemble those of trachea in which Wingless signaling is activated (Chihara and Hayashi, 2000; Llimargas, 2000). This observation suggests that accumulated β-Catenin is prevented from entering the nucleus because it is tightly associated with the cell membrane. Consistent with the expanded F-actin distribution (Fig. 1D), the amount of cytoplasmic actin was also increased. We also measured the amounts of cadherin and catenin mRNA and found that their levels did not change significantly in *Rac1, 2* mutant or Rac1N17 embryos (Fig. 1F). These results suggest that reduced Rac activity leads to a general increase in the amount of cadherin-catenin complexes by a post-transcriptional mechanism, and to a change in the epithelial architecture. Some of lower molecular weight species of α- and β-catenin that are presumably degraded proteins are decreased in amount when the amounts of full-length proteins are increased upon reduction of Rac activity (bands labeled with arrowhead and asterisk in Fig. 1E), indicating that Rac may antagonize a catenin-degrading process. Despite such defects, the epidermis in *Rac1, 2* mutant embryos maintained its apical-basal polarity, as revealed by secretion of cuticles (Hakeda-Suzuki et al., 2002).

We also examined the effects of the constitutively active form of Rac1 (Rac1V12). Although the mRNA levels of cadherins and catenins increased to some extent, their protein levels did not change (Fig. 1F), suggesting that Rac activation

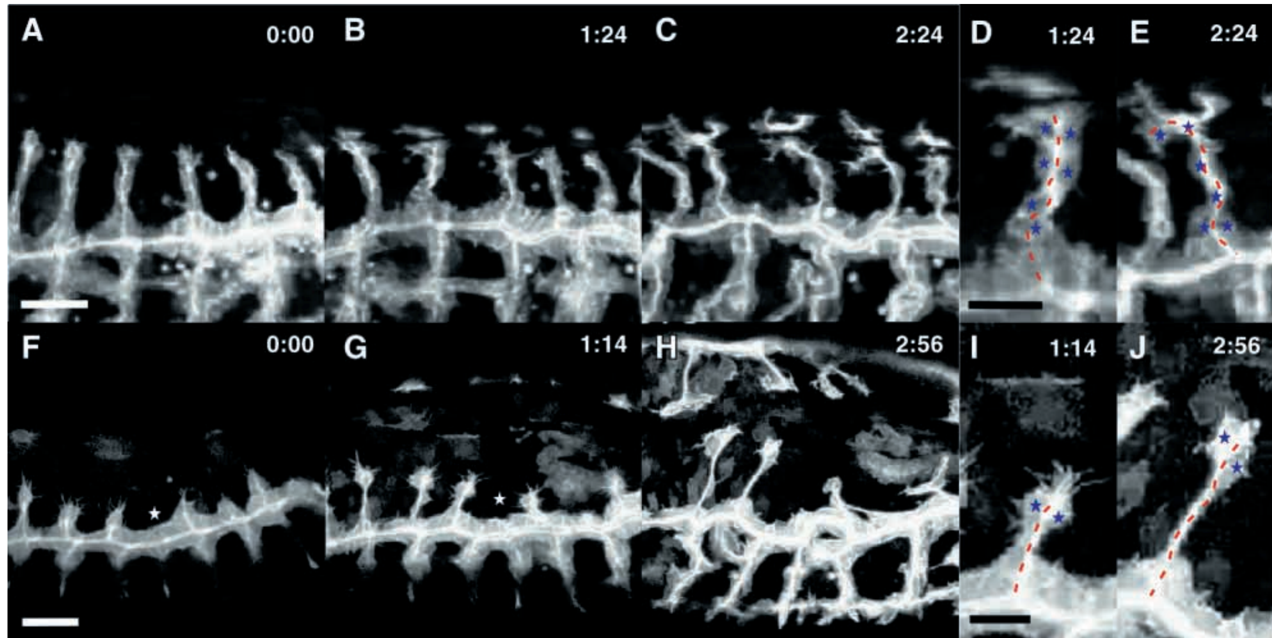

Fig. 3. Reduction in Rac activity inhibits tracheal cell rearrangement. Time-lapse observations of *btl-Gal4, UAS-GFP-moesin*/+ (A-E) and *btl-Gal4, UAS-GFP-moesin*/*UAS-DRac1N17* (F-J) embryos. High-magnification views are shown for DB3 (D,E) and DB5 (I,J). White asterisks in F and G indicate absence of DB sprouts. Blue asterisks and broken red lines in D,E,I,J indicate DB cell nuclei and lumens, respectively. In an example shown in I and J, two cells at the tip of DB extend numerous filopodia and lead migration, but no additional cells follows, leaving only a thin stretch of the cytoplasm. Scale bars: in A, 40 μm for A-C; in F, 40 μm for F-H; in D, 20 μm for D,E; in I, 20 μm for I,J.

does not directly leads to a decrease of the amount of E-Cadherin. The role of Rac on E-Cadherin expression will be further discussed below.

Rac is required for tracheal morphogenesis

Drosophila tracheal development provides a valuable model for studying epithelial cell rearrangement. At stage 12, tracheal cells have already stopped their cell division; and their cell number is about ~80 (Fig. 2A). To achieve the elaborate pattern of the tracheal network (Fig. 2C), tracheal cells drastically change their relative location, together with cell differentiation and cell-shape changes (Fig. 2A,B). It is worth noting that the trachea consists of highly polarized epithelial cells/tubules, as shown by the localization of Crumbs, which is an apical marker of epithelial cells (Fig. 2A,B).

To obtain insight into the role of Rac in the regulation of cell adhesion during epithelial cell rearrangement, we next examined the subcellular localization of E-Cadherin in tracheal cells. In the wild-type embryo, E-Cadherin protein was normally localized at the apical side of the lateral membrane in tracheal cells (Fig. 2E,F). In *Rac1, 2* mutant embryos, E-Cadherin (Fig. 2G,H), as well as α-Catenin and β-Catenin (data not shown), was no longer limited to apical cell contact sites, but was distributed to the lateral and basal sides; in addition, these embryos showed various levels of defects including a truncated dorsal trunk and failure of germband retraction (see Fig. 6). Furthermore, the amount of F-actin, as revealed by a green fluorescence protein fused to the actin binding domain of moesin (GFP-moesin) (Edwards et al., 1997), became abnormally high in the *Rac1, 2* mutants (Fig. 2G,H).

As shown in Figs 1, 2, *Rac1, 2* mutants had defects in several tissues. To remove the secondary effects of defects in tissues other than trachea, we inhibited the activity of Rac by expressing Rac1N17 specifically in tracheal cells. In those embryos, we found that E-Cadherin was sometimes localized at the lateral and basal membranes, as in the case of *Rac1, 2* mutant embryos (Fig. 2I). Such embryos also showed phenotypes of a truncated and/or zigzagged dorsal trunk (DT), misguided dorsal branch (DB) and no terminal branches (Fig. 2D; data not shown) that were similar to the mild class of defects observed in *Rac1, 2* or *Pak* mutants (see Fig. 6). Thus, autonomous Rac activity may be required for apical localization of E-Cadherin in tracheal cells and may contribute to tracheal morphogenesis.

Time-lapse analysis revealed a role for Rac in tracheal cell rearrangement

Tracheal branching is a coordinated process of cell migration and cell rearrangement. To investigate the role of Rac in the dynamic aspects of tracheal branching, we performed time-lapse analysis of tracheal cells labeled with GFP-moesin. Primary branching of tracheal primordia generated six multicellular branches consisting of cells joined by intercellular junctions (Fig. 3A-E, see Movie 1 at http://dev.biologists.org/supplemental/). While the DB, consisting of five to seven cells, extended toward the dorsal midline, the tracheal cells changed their position relative to each other to form thinner unicellular tubules with intracellular junctions (Fig. 3D,E). Such cell rearrangement was greatly inhibited by Rac1N17 (Fig. 3F-J; see Movie 2 at http://dev.biologists.org/supplemental/). Although paired cells with extensive movement of cell protrusions were attempting to migrate out of the DT, the DB was shorter than that of the

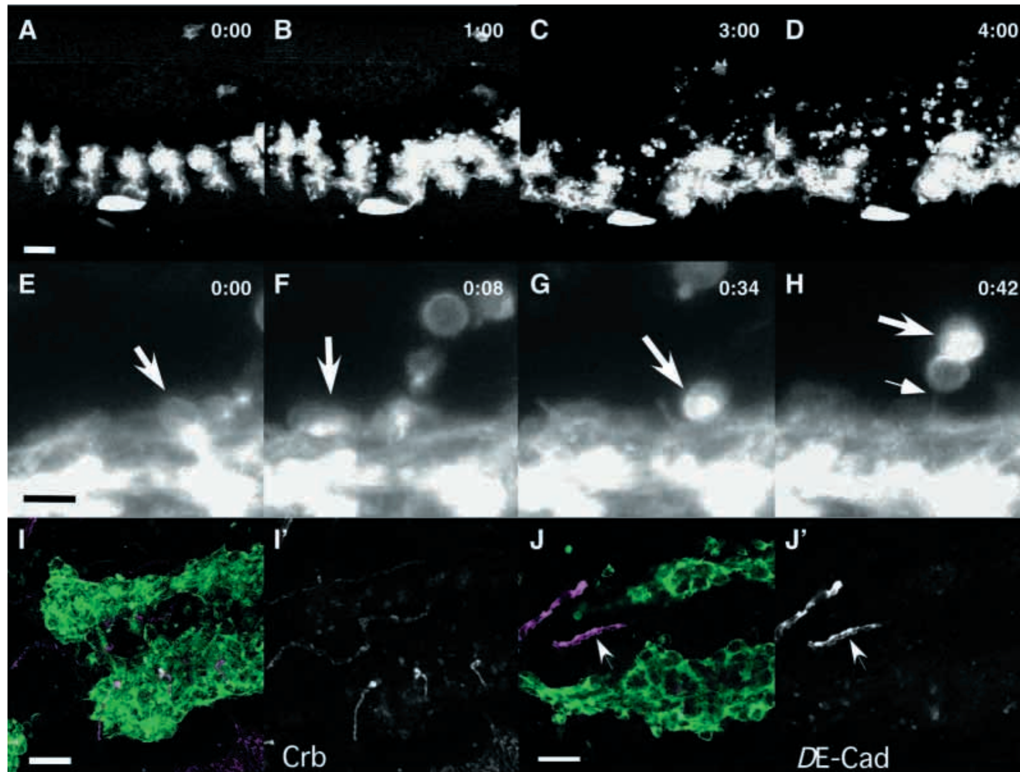

Fig. 4. Elevated Rac activity disrupts tracheal cell adhesion. (A-D) Time-lapse observations of a *btl-Gal4, UAS-GFP-moesin*/*UAS-DRac1V12* embryo. By stage 12, tracheal branching is already delayed (A), and in the next 4 hours many of the tracheal cells are expelled in clusters of one to five cells. (E-H) High-magnification images of a *btl-Gal4, UAS-GFP-moesin*/*UAS-DRac1V12* embryo. Tracheal cells have become spherical and detached from the tracheal cell cluster. Arrows follow the same tracheal cell. The thin arrow in H indicates a thin stalk of cytoplasm. (I,J) Activated Rac1 transforms the tracheal epithelium into a mesenchymal state. *gfp-moesin*-labeled cells (green) form large cell aggregates in which expression of apical cell marker Crumbs (purple in I, grayscale in I′) and E-Cadherin (purple in J, grayscale in J′) were greatly reduced. In J, E-Cadherin is undetectable in the trachea, although its expression in the malpighian tubule appears to be normal (arrows). Scale bars: in A,I,J, 40 μm for A-D,I,J; in E, 5 μm for E-H.

control embryos (Fig. 3F-H). The number of cells incorporated into the stalk of the DB was reduced to 0 or 1, when compared with three to five cells in wild-type embryos, leaving only thin cytoplasmic extensions (Fig. 3I,J). Occasionally, there was no sprout of the DB at all (Fig. 3F,G; asterisk), although cytoplasmic extensions from the site of DB outgrowth were still visible. Reduced Rac activity may block the cell rearrangement needed to allocate tracheal cells into the DB. Our observations also suggest that the rapid movement of cell protrusions of tracheal tip cells is insensitive to this level of change in Rac activity.

Hyperactivation of Rac disrupts epithelial architecture

To elucidate further the role of Rac in the regulation of E-Cadherin, we analyzed the phenotypes of tracheal cells expressing the constitutively active form of Rac1 (Rac1V12; Fig. 4A-D; see Movie 3 at http://dev.biologists.org/supplemental/). After late stage 12, tracheal primordia lost their branched morphology and expelled cells into the body cavity. By stage 15 the remaining tracheal primordia had fused together to form one or two large cell clusters on each side of the body. As shown in Fig. 4E-H, cells that had budded out from the tracheal primordia were held together by thin stalks of cytoplasm for about half an hour before detaching freely into the body cavity (Fig. 4H, thin arrow). Judging from their rounded shape and the uniform distribution of GFP-moesin, those cells were non-polarized ones. Localization of the apical cell membrane marker Crumbs (Tepass et al., 1990) (Fig. 2A,B) became disorganized at stage 13 (data not shown), and was nearly undetectable at stage 16 (Fig. 4I). These results suggest that Rac activation disrupts the epithelium.

Activated Rac prevents apical localization of E-Cadherin

We followed the expression of E-Cadherin to determine how its behavior correlates with the tracheal morphology upon activation of Rac. The amount of E-Cadherin was decreased and by stage 16 it was lost completely from the tracheal cells (Fig. 4J). We noted that the time course of the decrease was slow, taking more than 5 hours from stage 11 to stage 16 and was preceded by the loss of epithelial integrity. To clarify the effect of Rac1V12 on turnover of E-Cadherin, it is important to distinguish newly synthesized E-Cadherin from the pre-existing one because the maternal supply of the E-Cadherin gene product has a significant contribution to the formation of tracheal cell adhesion (Tanaka-Matakatsu et al., 1996; Uemura et al., 1996). Therefore, we used the functional E-Cadherin-GFP fusion protein (Oda and Tsukita, 1999) driven by *btl-Gal4*

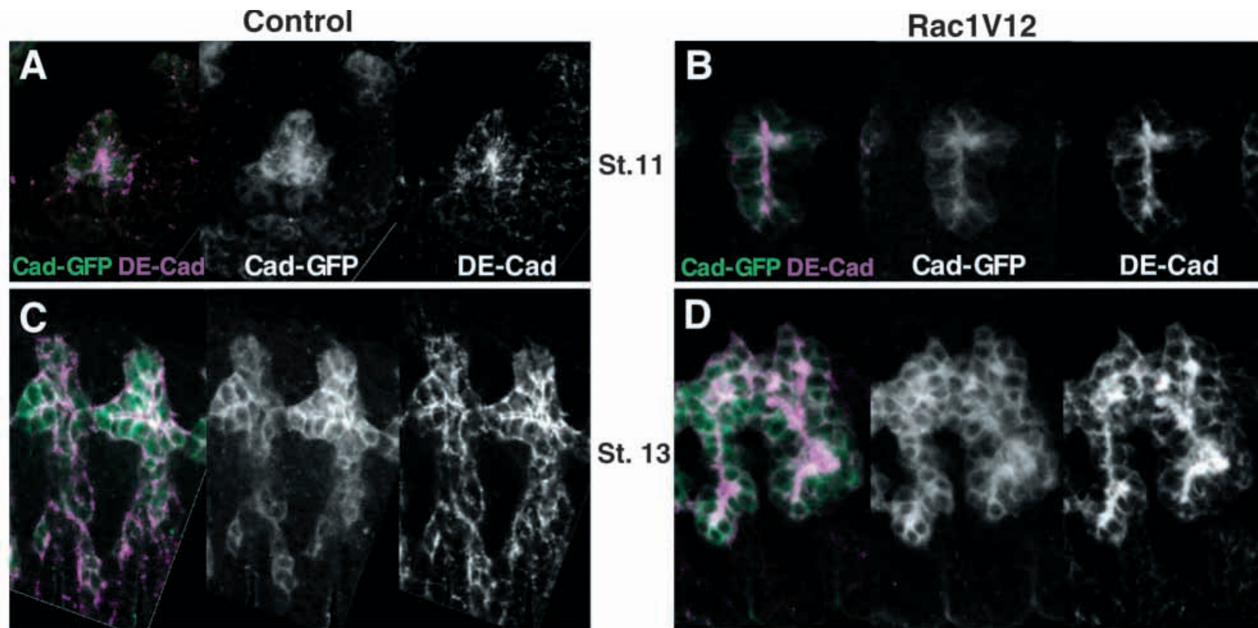

Fig. 5. Rac1V12 prevents newly synthesized E-Cadherin from accumulating at the apical cell membrane. (A-D) Embryos with the genotypes of *btl-Gal4, UAS-E-Cadherin-GFP / +* (control: A,C) and *btl-Gal4, UAS-E-Cadherin-GFP / +; UAS-Rac1V12 / +* (Rac1V12: B,D) at two different stages were labeled with anti-GFP (green) to reveal E-Cadherin-GFP and with DCAD2 (purple) to reveal both E-Cadherin-GFP and endogenous E-Cadherin. Note that in the control (A,C) and in a stage 11 Rac1V12 embryo (B), anti-GFP and DCAD2 signals overlap, but in D the signals are distributed differently.

to label the protein newly synthesized after the onset of tracheal invagination. E-Cadherin proteins were also detected with the monoclonal antibody DCAD2, which recognizes the extracellular domain. DCAD2 staining should reveal both E-Cadherin-GFP and endogenous E-Cadherin. It should be noted that mature E-Cadherin is a heterodimer, formed by proteolytic cleavage of the nascent polypeptide at the extracellular domain (Oda and Tsukita, 1999). Anti-GFP and DCAD2 recognize the C-terminal and N-terminal polypeptides of E-Cadherin-GFP, respectively (Oda and Tsukita, 1999). Anti-GFP and DACD2 staining of tracheal cells expressing E-Cadherin-GFP revealed essentially the same localization to the cell membrane (Fig. 5A,C), suggesting that E-Cadherin-GFP is incorporated into cell junctions. The only notable difference in their localization was cytoplasmic signals unique to anti-GFP. We speculate that the C-terminal E-Cadherin-GFP fragment stripped from the N-terminal fragment resides in the cytoplasm.

Rac1V12 dramatically changed the colocalization of anti-GFP and DCAD2 signals. At stage 11, when the tracheal primordia still appeared normal, both signals were colocalized (Fig. 5B). However, at stage 13, when the tracheal cells began to show signs of disorganization, DCAD2 signals became highly accumulated at what appeared to be the apical cell surface. This DCAD2 apical signal contained little anti-GFP signal, and thus should have consisted mostly of endogenous E-Cadherin. Rac activation possibly prevents newly synthesized E-Cadherin from being recruited to the apical cell membrane.

Rac signaling genetically interacts with FGF signaling

In addition to the defects in cell adhesion and rearrangement described above, *Rac1, 2* mutant embryos showed various defects in tracheal cell migration and differentiation. A wide range of tracheal defects was observed in *Rac1, 2* mutants (Fig. 6A,B, see figure legends for criteria to classify tracheal phenotypes). In embryos showing the weak class phenotype, misrouting of the DB toward the anteroposterior direction was often observed (Fig. 6A). In intermediate-class embryos, the number of truncated DTs increased and the germband did not retract completely (Fig. 6B). The severity of the defects and their frequency increased when the gene dose of *rac* was progressively reduced (Fig. 6F, lanes 1-5). When the maternal expression of Rac1 and Rac2 was reduced by half, the tracheal defects occurred in 25% of the embryos (Fig. 6F, lane 3). We also analyzed *Rac1 Rac2 Mtl* triple mutants laid by *Rac1 Rac2 Mtl* heterozygous mother and found that the mutants showed higher frequency of severe tracheal defects similar to those found in *Rac1, 2* mutants (data not shown). Those results suggest that a change in Rac activity within the physiological range significantly affects morphogenetic movement of the tracheal system.

p21-activated kinase (Pak) is known as a mediator of the activity of Rac GTPase. We found tracheal defects similar to those of *Rac1, 2* mutants in *pak* mutants (data not shown and lanes 6-8 in Fig. 6F). Furthermore, *Rac1, 2* and *Pak* mutations synergistically enhanced tracheal defects (compare lane 9 with lanes 2 and 7 in Fig. 6F). Such results suggest that Rac and Pak are required for directed movement of tracheal branches.

The loss of Rac activity also caused a defect in cell differentiation. Tips of DB 1-9 are normally capped with terminal cells that extend terminal branch in the ventral direction (Guillemin et al., 1996). In *Rac 1, 2* mutant embryos, the loss of terminal branches was observed with high

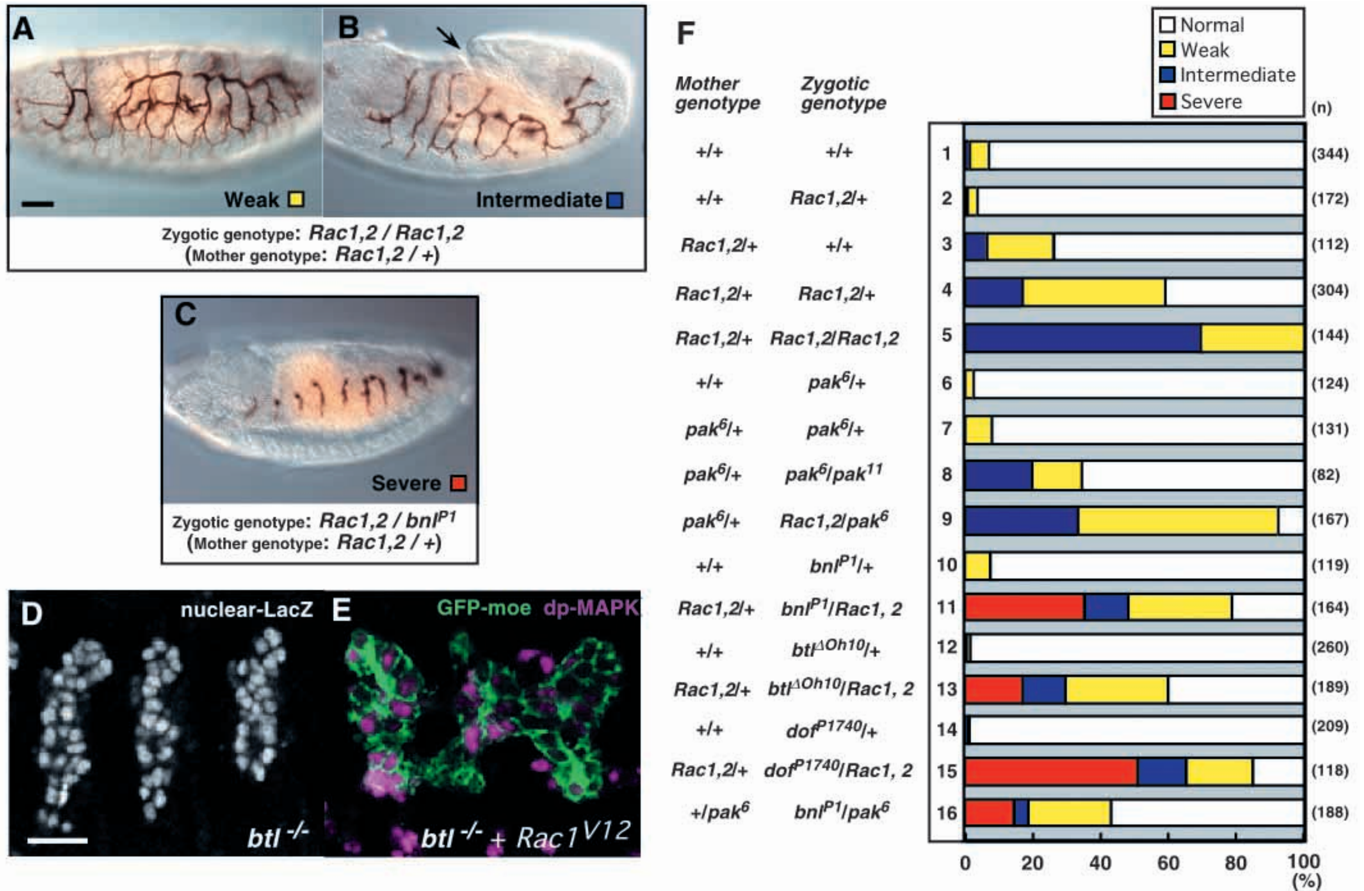

Fig. 6. Genetic interaction of Rac and FGF signaling. (A-C) Phenotypes of embryos deficient in Rac and FGF signaling classified into three classes. *Rac1, 2/Rac1, 2* (A, mild), *Rac1, 2/Rac1, 2* (B, intermediate) and *Rac1, 2/bnl^{P1}* laid by *Rac1, 2/+* mother (C, severe) were stained with monoclonal antibody 2A12 to label the tracheal lumen. In *Rac1, 2* mutants, the DT is disrupted (A,B) with a defect in germband retraction (arrow in B). In *Rac1, 2/bnl^{P1}* mutants, no tracheal cell migration has taken place (C). (D,E) Partial rescue of the *btl* mutant phenotype by the expression of the constitutively active form of Rac1. (D) A *btl$^{\Delta Oh10}$* mutant embryo at stage 12. Tracheal cells are labeled with a nuclear β-galactosidase marker. No sign of branching is apparent. (E) A *btl$^{\Delta Oh10}$* mutant embryo at stage 12 expressing Rac1V12 by the *btl* enhancer. GFP-moesin and dp-MAPK are shown in green and purple, respectively. Tracheal cells were able to move. (F) Genetic interactions involving Rac. Maternal and zygotic genotypes of scored embryos are indicated. Tracheal phenotypes were classified into 'normal' (white bar), 'weak' (yellow bar) and 'intermediate' (blue bar), according to the number of truncated DT of none, one to three, and four to nine, respectively, per one side of embryos. The 'severe' class (red bar) corresponds to the phenotype of no tracheal migration at all. 'n' is the number of embryos observed. Scale bars: in A, 40 μm for A-C; in D, 20 μm for D,E.

penetrance. Consistently, serum response factor (SRF) (Guillemin et al., 1996), a marker protein for the terminal cell, also disappeared (data not shown), suggesting that terminal cell differentiation did not occur.

As directed cell migration and terminal cell differentiation are processes requiring FGF signaling, we asked whether Rac is involved in FGF signaling and found a strong genetic interaction. Although tracheal patterning was only mildly affected by half dose reductions of *bnl* (ligand), *btl* (receptor) and *dof* [intracellular effector (Vincent et al., 1998)], the phenotype was strongly enhanced by introducing one copy of *Rac1, 2* mutant chromosome from mothers (compare lanes 10, 12, 14 with 11, 13, 15 in Fig. 6F). We also found a similar genetic interaction between *pak* and *bnl* (lane16 in Fig. 6F). These genetic interactions suggest that Rac and Pak are required for the migration of tracheal branches in response to FGF signaling.

To determine the epistatic relationship between Rac and FGF signaling, we tested the effect of constitutive activation of Rac in *btl* mutants. In the *btl* mutant, tracheal branching does not proceed beyond the invagination at stage 11 (Fig. 6D), and MAP kinase activation is absent (Gabay et al., 1997). Expression of Rac1V12 partially restored the movement of tracheal cells, and activated MAP kinase, as revealed by staining with the antibody against the diphosphorylated form of MAP kinase (dp-MAPK, Fig. 6E). These results suggest that Rac activation is an essential downstream event of tracheal cell motility induced by FGF signaling.

DISCUSSION

Essential function of Rac in epithelial remodeling

Cadherin-based cell adhesions are vital to maintain the

morphological and functional features of the epithelium of multicellular organisms. During morphogenesis of the epithelia, cell adhesions must be disrupted and re-assembled in a regulated manner to allow movement of individual cells in the epithelia. Our in vivo analyses demonstrated that a reduction in Rac activity prevented cell rearrangement. This phenotype was associated with an increase in the level of E-Cadherin and its associated molecules, and expansion of E-Cadherin localization to the basolateral membrane. We infer that increased E-Cadherin expression consolidates cell adhesiveness. Hyperactivation of Rac prevented incorporation of newly synthesized E-Cadherin into cell junctions and reduced cell adhesiveness, transforming the tracheal epithelium into mesenchyme. We suggest that switching of Rac between active and inactive states promotes turnover of the complex containing E-Cadherin at the cell junction, and maintains the plasticity of the tracheal epithelium to allow branching morphogenesis.

We demonstrated that expression of a dominant-negative form of Rac1 greatly reduced cell rearrangement required for partitioning cells into the stalk of the DB. Overproduction of this form, Rac1N17, would shift the cellular pool of Rac toward the inactive GDP-bound state. We suggest that turnover of E-Cadherin at a proper level requires a high level of Rac activity. However, highly active movement of cell extensions in the cells at the tip was still visible, suggesting that the ability of those cells to move toward their target was mostly intact. A stronger reduction in Rac activity might be required to demonstrate a role for Rac in promoting cell extensions, as proposed from studies on tissue culture cells (Hall, 1998; Kaibuchi et al., 1999; Van Aelst and D'Souza-Schorey, 1997).

Post-transcriptional control of E-Cadherin by Rac

Our time-lapse analysis demonstrated that reduced Rac activity inhibited cell rearrangement during branching of tracheal tubules. Under this condition, the amounts of cadherins and catenins were increased and filled the cell membrane. This phenotype was different from the phenotype of E-Cadherin-GFP overexpression, which does not inhibit cell rearrangement (Oda and Tsukita, 1999). We suggest that a reduction in Rac promotes the association of cadherin-catenin complexes with the cell membrane and stabilization of these complexes. Activation of Rac resulted in an opposite phenotype characterized by the loss of E-Cadherin and cell dissociation, and in prevention of E-Cadherin-GFP from accumulating at apical cell junctions. All of these observations are consistent with a hypothesis that Rac regulates the formation of cadherin-catenin complexes at the cell junction. Incorporation of a cadherin-catenin complex into the cellular junction would explain stabilization of the complex when Rac activity is reduced. Possible modes of Rac action on cadherin include apical transport and assembly/stabilization of the complex. We suggest that the inhibitory action on the cadherin cell adhesion system is a general property of Rac in the *Drosophila* embryo.

Relationship between Rac and FGF signaling

Extracellular signals that promote tracheal branching are good candidates for regulators of Rac in tracheal cells. In this regard, the strong genetic interaction between Rac and FGF signaling components that we observed suggests an intriguing possibility that FGF signaling activates Rac within tracheal cells to promote both cell motility and cell rearrangement. In support of this idea, we found that activated Rac1 partially rescued tracheal cell motility and MAP kinase activation in *btl* mutants (Fig. 6E). Involvement of Rac in FGF-dependent events may not be limited to cell motility. We found that expression of SRF, the product of one of the target genes activated by FGF signaling in the tracheal system, was lost in the mutant trachea with reduced Rac activity because of *Rac1, 2* mutation or Rac1N17 (data not shown). This result suggests that Rac also regulates transcription.

Several lines of evidence suggest that FGF signaling is activated locally at the tip of branches (Ikeya and Hayashi, 1999; Ohshiro et al., 2002), and activation of FGF signaling in all tracheal cells was shown to prevent branching (Ikeya and Hayashi, 1999), suggesting that localized activation of FGF signaling is essential for branching. Therefore the proposed function of Rac in transducing FGF signaling must be localized at the tip of branches. How does the proposed function of Rac in transducing FGF signaling relate to the Rac function in regulating cell rearrangement? As the effect of Rac1N17 was most clearly observed in cells destined to become tracheal stalk cells, the location of tracheal cells requiring two of the Rac functions appears to be different. One idea is that FGF signaling activated at the tracheal tip is transmitted to tracheal stalk cells by a secondary signal that activates Rac to promote cell rearrangement. It will be important to identify the upstream signal regulating Rac in stalk cells.

Use of the *Drosophila* tracheal system to investigate the mechanism of cell signaling and motility

This study provided in vivo evidence for the role of Rac in epithelial morphogenesis, which role was suggested previously from work in tissue culture systems. The tracheal system seems to be particularly sensitive to alteration of Rac activity compared with another well-studied system of *Drosophila* epithelial morphogenesis, i.e. closure of the dorsal epidermis. Dorsal closure is driven by extensive cell stretching and accumulation of F-actin, but does not involve much cell rearrangement. Studies on tracheal branching and dorsal closure would complement each other to reveal the rich spectrum of Rac functions.

We have shown that filopodial movement remains active in tracheal cells with reduced Rac activity, indicating that the control of cytoplasmic extension and cell adhesion appears to involve two distinct processes that require different levels of Rac activity. Recently, it was revealed that FGF signaling affected the formation of dynamics of filopodia at the tip of migrating branches (Ribeiro et al., 2002). How FGF signaling regulates filopodial formation and cell rearrangement through regulation of Rac and MAP kinase is still not clear at the moment. Further investigation using the tracheal system should provide useful insight into functions of intracellular signaling molecules involved in regulating cell adhesion previously indicated from studies in vitro.

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