

Roles for Rac1 and Cdc42 in Planar Polarization and Hair Outgrowth in the Wing of *Drosophila*

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Abstract. The wing of *Drosophila melanogaster* is covered by an array of distally pointing hairs. A hair begins as a single membrane outgrowth from each wing epithelial cell, and its distal orientation is determined by the restriction of outgrowth to a single distal site on the cell circumference (Wong, L., and P. Adler. 1993. *J. Cell Biol.* 123:209–211.). We have examined the roles of Cdc42 and Rac1 in the formation of wing hairs. We find that Cdc42 is required for localized actin polymerization in the extending hair. Interfering with Cdc42 activity by expression of a dominant negative protein abolishes both localized actin polymerization and hair outgrowth. In contrast, Rac1 is important for restricting

the site at which hairs grow out. Cells expressing the dominant negative Rac1N17 fail to restrict outgrowth to a single site and give rise to multiple wing hairs. This polarity defect is associated with disturbances in the organization of junctional actin and also with disruption of an intricate microtubule network that is intimately associated with the junctional region. We also find that apical junctions and microtubules are involved in structural aspects of hair outgrowth. During hair formation, the apical microtubules that point distally elongate and fill the emerging wing hair. As the hair elongates, junctional proteins are reorganized on the proximal and distal edges of each cell.

SINGLE cells polarize their individual shapes and sub-cellular components to accomplish specific tasks. Axonal polarity in neurons and apical-basal polarity in epithelial cells are the two best understood examples of this behavior (Rodriguez-Boulan and Powell, 1992). However, to form a well-ordered tissue, epithelial cells must also polarize certain structural features with respect to an axis within the plane of the epithelium. Coordination of the planar polarity of cells within a tissue is called tissue polarization. It is apparent in a wide range of structures, from uniformly oriented leaf trichomes (Uphof, 1962) to cilia that beat coordinately in a wide variety of epithelial cells (Lemullois et al., 1988; Koenig and Hausen, 1993). A particularly well-studied example of tissue polarity is the cuticle of *Drosophila*. In *Drosophila*, the epithelial cells that secrete the cuticle form hairs and bristles that point posteriorly (in the thorax and abdomen) or distally (in the appendages). The mechanism by which tissue polarity is generated is not yet understood, but studying the process in *Drosophila* has provided some intriguing clues.

Each *Drosophila* wing epithelial cell forms a hair by extending a single process from its apical membrane at approximately 35 h after puparium formation (Mitchell et al., 1983; Wong and Adler, 1993; Fristrom et al., 1993). The emergence of a wing hair is presaged by the accumulation

of actin on the distal side of the cell. Outgrowth initiates from this site and is oriented distally. At this stage, the outgrowth is termed a prehair. Subsequently, the prehair elongates and tilts up out of the plane of the epithelium so that its base comes to lie in the center of the apical membrane (Wong and Adler, 1993). Genetic screens have identified a class of mutants, the tissue polarity mutants, that disorganize the orientation or number of hairs and bristles but leave their structure and other aspects of cellular polarity intact (for review see Adler, 1992). Wong and Adler (1993) have shown that the tissue polarity mutants interfere with the choice of the site at which actin begins to accumulate before wing hair formation.

The tissue polarity genes fall into three epistasis groups, the first of which includes *frizzled*, *disheveled*, and *prickle* (Wong and Adler, 1993). Because these genes act early in the polarization pathway, they are likely candidates for molecules that transmit the polarization signal. Frizzled is a seven transmembrane domain protein that localizes to cell boundaries; the nonautonomous behavior of *frizzled* clones suggests it is required for both reception and transmission of the signal (Vinson et al., 1989; Krasnow and Adler, 1994; Park et al., 1994). The Disheveled protein is present in both membrane and cytosolic pools (Yanagawa et al., 1995) and contains a PDZ/DHR motif that is shared by proteins that localize to cellular junctions (Klingensmith et al., 1994; Noordermeer et al., 1994; Theisen et al., 1994). The Frizzled signal transduction pathway is active before hair formation (Adler et al., 1994) and is also used

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to coordinate ommatidial orientation in the eye (Gubb, 1993; Theisen et al., 1994; Zheng et al., 1995), so it is unlikely to be involved in structural aspects of hair formation. Rather, it must polarize some feature of the cell that can later be used to determine either the site of hair outgrowth or cellular orientation. The tissue polarity genes *inturned*, *fuzzy*, and *multiple wing hair* act downstream of *frizzled* and are required only for hair (or hair and bristle) polarity. These genes may help translate the cellular polarity generated by the Frizzled pathway into polarized hair outgrowth, but the mechanism of their action remains unclear.

Since the only molecular event known to be associated with polarized outgrowth of hairs is the accumulation of actin at the distal side of the cell, we were intrigued to note that Rac1 and Cdc42 were required for the proper placement and formation of wing hairs (Eaton et al., 1995). These proteins are members of the *rho* family of small GTPases and have been implicated in a wide variety of cellular polarization processes that involve remodeling of the actin cytoskeleton. When expressed under the indirect control of the *patched* promoter, Rac1N17 caused a tissue polarity phenotype similar to that of *inturned* and *fuzzy*; cells that expressed this protein gave rise to duplicated or triplicated wing hairs. Cells that expressed the dominant negative Cdc42S89, on the other hand, often made no hair at all. Rac1 and Cdc42 affected both hairs and bristles; Rac1N17 caused multiple apolar bristles to form, whereas Cdc42S89 expression prevented bristle formation altogether (Eaton, S., unpublished observation). These results suggested that Rac1 and Cdc42 might control hair formation, at least in part, by modulating different subsets of the actin cytoskeleton during pupal development. In this paper we describe cytoskeletal changes that take place during hair formation and examine the cytoskeletal perturbations caused by expressing dominant negative Rac1 and Cdc42 proteins. These observations suggest models for hair formation and the planar polarization process that restricts hair outgrowth to a single site.

Materials and Methods

Crosses and Collection of White Prepupae

Flies were raised on a cornmeal, yeast, and molasses medium at 20°C. To generate flies expressing Cdc42S89 or Rac1N17 at the compartment boundary, we crossed females harboring either *UAS-Cdc42S89* or *UAS-Rac1N17* to males containing *Gal4* under the control of the *patched* promoter. Because *patched* is expressed continuously from early embryonic stages, we allowed embryogenesis and part of larval development to proceed at 18–20°C to maximize survival. At low temperatures, the GAL4 protein is less active. When third instar larvae began to emerge from the food, we shifted them to 25°C. White prepupae were subsequently collected and aged between 30 and 35 h at 25°C. Because cells that express *patched* are thought to be critical for wing development, we were initially puzzled to obtain viable adults from these crosses. Nevertheless, the mechanism by which *patched* expression is activated provides a sensible explanation. *patched* expression is induced in the anterior cells adjacent to the compartment boundary in response to the secretion of Hedgehog by posterior cells. Even if *patched* expressing cells were to die as a result of Cdc42S89 or Rac1N17, they would be continuously replaced by other anterior cells that in turn would activate the expression of *patched*.

Dissection and Fixation

For wings that were to be stained with antitubulin, alone or in combination with other antibodies, we dissected pupae in Shields and Sang M3 In-

sect Medium (No. S8398; Sigma Chemical Co., St. Louis, MO). Wings were separated from pupae, but no attempt was made to remove the cuticle, which has separated from the wing at this stage and forms a loose sac. Wings that had been stretched or otherwise damaged during dissection were discarded; stretching seemed to distort the morphology of the microtubules. Wings were then fixed for 5 min in methanol at –20°C and rehydrated through 75, 50, and 25% methanol into PBS at room temperature (Lajoie-Mazenc et al., 1994). The cuticle was then removed from the fixed wings before antibody staining. Similar staining was observed if cells were fixed for 10 min with freshly dissolved 4% paraformaldehyde + 0.2% glutaraldehyde in 0.1 M Pipes, 1 mM EGTA, 2 mM MgSO₄ (PEM); however, it was not as reproducible. Frequently large patches of the wing remained completely unstained, suggesting that permeability was a problem under these conditions.

For wing imaginal discs that were to be stained with antitubulin, alone or in combination with other antibodies, we followed a similar protocol to that described above with the exception that methanol fixation was carried out at 4°C and was preceded by fixation for 5 min with 4% paraformaldehyde in PEM. If imaginal discs were fixed in –20°C methanol alone, the peripodial membrane microtubules were very well preserved, but those in the wing pouch were sometimes only weakly detected even at high antibody concentrations, perhaps due to permeability difficulties.

For wings that were to be stained with rhodamine phalloidin, alone or in combination with antibodies, we dissected pupae in fixative that consisted of 8% paraformaldehyde, 100 mM K cacodylate, pH 7.2, 100 mM sucrose, 40 mM K acetate, 10 mM Na acetate, and 10 mM EGTA. Wings were completely dissected away from the surrounding cuticle and placed in PBS + 0.1% Triton X-100 (PBT). The total dissection time (time spent in fixative) varied between 5 and 7 min per pupa. After sufficient numbers of wings were obtained, they were permeabilized in PBS + 1.0% Triton X-100 for 1 h, and then rinsed twice in PBT. Although this fixation protocol preserves the actin cytoskeleton well, microtubules often appear clumped and indistinct.

Immunological Methods

Wings processed as described above were incubated overnight at 4°C in the following dilutions of antibody or phalloidin: rhodamine phalloidin (Molecular Probes, Eugene, OR), 2 U/ml; mouse anti- α -tubulin or anti- β -tubulin (T 5168 or T 5293; Sigma Chemical Co.), 1:200 (concentrations up to 1:50 give the same results); chicken anti-Cdc42 (Eaton et al., 1995), 1:20; rabbit anti-Rac1 (Eaton et al., 1995), 1:50; guinea pig anticoracle (Fehon et al., 1994), 1:1,000; rat anticadherin (Oda et al., 1994), 1:20; rabbit anti-discs large (Woods and Bryant, 1991), 1:500. All antibodies were diluted into PBT + 5% FCS. Binding of secondary antibodies was performed as described (Eaton et al., 1995).

Confocal Microscopy

Wings were observed either in a confocal microscope built at EMBL (Stelzer et al., 1989) or on a Zeiss confocal microscope (model LSM410; Carl Zeiss, Inc., Thornwood, NY).

Scanning Electron Microscopy

Flies were placed in ethanol and the wings were removed. Isolated wings were washed three times in 100% ethanol and subjected to critical point drying in CO₂. Dried wings were coated with gold-palladium and observed in a field emission scanning electron microscope at 5,000 V.

Results

Cdc42S89 Expression Produces Stunted and Malformed Wing Hairs

We had previously observed in the light microscope that Cdc42S89-expressing cells either failed to make wing hairs or made wing hairs that appeared stunted (Eaton et al., 1995). To get a more detailed picture of the abnormalities caused by Cdc42S89 expression, we examined the affected hairs using a scanning electron microscope and compared them to wild-type hairs. Fig. 1 shows regions of wings that expressed Cdc42S89 under the indirect control of the

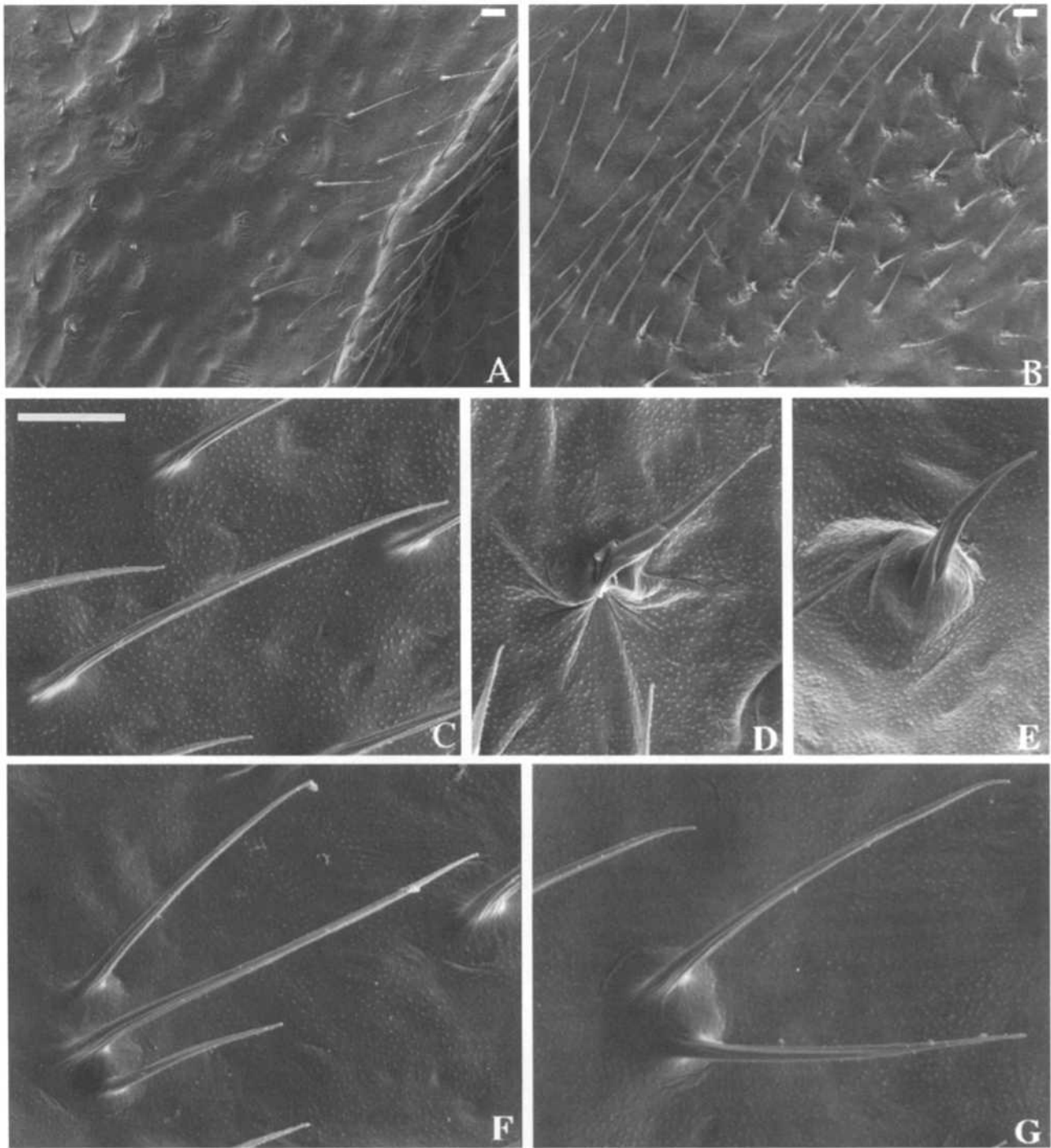


Figure 1. The effect of *Cdc42S89* or *Rac1* expression on hair ultrastructure. Scanning electron micrographs in *A–E* are of wings from flies that express *Cdc42S89* at the AP compartment boundary under the indirect control of the *patched* promoter. *A* shows a region near the site of the anterior crossvein (which is missing in wings expressing *Cdc42S89* at the AP boundary) containing both mutant and wild-type tissue. *B* shows a region near the posterior crossvein containing both mutant and wild-type hairs. *C* shows a wild-type hair. *D* and *E* show hairs deformed by *Cdc42S89* expression. *F* and *G* show the multiple wing hairs produced by *Rac1N17* expression. Bars: (*A* and *B*) 50 μm ; (*C–G*) 10 μm .

patched promoter. This promoter directs expression in a stripe of cells just anterior to the boundary between the anterior and posterior compartments of the wing. Fig. 1, *A* and *B*, shows *Cdc42S89*-affected tissue, along with adjacent wild-type tissue. These two wings have been affected by *Cdc42S89* to different degrees. The *Cdc42S89*-express-

ing cells in Fig. 1 *A* have completely failed in wing hair formation. The cells in Fig. 1 *B* have formed hairs, but they appear shorter than normal and have a malformed base relative to their normal neighbors. Variability in the expressivity of the *Cdc42S89* phenotype is probably due to differences in accumulation of the *Cdc42S89* protein; com-

plete absence of wing hairs is usually observed where accumulation of the Cdc42S89 protein is highest (data not shown). At higher magnification, it is clear that Cdc42S89-affected hairs are not only short, but they are also deformed. Wild-type hairs have longitudinal striations that remain parallel along their length and spread smoothly into the cuticle at the base (Fig. 1 C). Bristles have similar striations, and they correspond to the spaces between longitudinal actin bundles (Overton, 1967; Tilney et al., 1995). In Cdc42S89-deformed hairs, the striations are not as fine and the hairs frequently appear twisted. Often, the cuticle surrounding these hairs is puckered or otherwise deformed (Fig. 1, D and E). This illustrates clearly that the process of outgrowth is perturbed by Cdc42S89 expression. The abnormal appearance of the longitudinal striations suggests that the organization of actin in these hairs may be defective.

Cdc42S89 Expression Interferes with Actin Polymerization in Wing Hairs

To see whether the defect in hair formation correlated with specific disruptions of the actin cytoskeleton, we stained wings that expressed Cdc42S89 at the compartment boundary with both phalloidin and an antibody to Cdc42 and compared actin organization in mutant and wild-type cells. In wild-type cells before hair formation, actin is distributed around the cell periphery in the apical junctional region (Fig. 2 A). Actin fibers are observed running across the cells in many directions, often with a radial arrangement. The first sign of hair formation is the accumulation of actin on the distal side of each cell (Wong and Adler, 1993; Fig. 2 B). Fig. 2 B shows a projection of three optical sections through the apical region at this stage; when single sections are observed, it is apparent that the more diffuse actin accumulation is slightly apical to the junctional actin (data not shown). At the level of the junctions, bright filaments are often observed extending from the distal vertex into the center of the cell. As the hairs extend, actin disappears from the junctional region (Fig. 2 C).

Before the outgrowth of hairs, the only observable difference between Cdc42S89-expressing cells and their normal neighbors is that apical actin fibers are more abundant in the former (Fig. 2, D–F). Later, Cdc42S89 causes dramatic defects in the actin organization that correlates with prehair outgrowth. By the time their wild-type neighbors have extended actin-filled prehairs, cells that express high levels of Cdc42S89 have not polarized the distribution of actin filaments distally, and no sign of outgrowth is evident (Fig. 2, G–I). Cells expressing lower levels of the dominant negative protein make actin-filled projections that are normally placed, but stunted (Fig. 2 I, *asterisk*). These data suggest that Cdc42 is specifically required for actin polymerization in developing wing hairs and that actin polymerization is required for outgrowth.

We noted that before hair formation, the overexpressed dominant negative Cdc42S89 was distributed uniformly around cell boundaries and also localized to the superabundant apical actin fibers (Fig. 2, E and F). At later times, when the surrounding wild-type cells were extending wing hairs, the dominant negative protein appeared to be localized to the cytoplasm (Fig. 2, H and I). We wondered whether the endogenous wild-type protein had a similar distribution or whether overexpression of a dominant negative mutation might interfere with the ability of the protein to be localized normally. Although it is less abundant than the overexpressed protein, we were able to detect endogenous Cdc42 in the surrounding wild-type cells. Like the dominant negative protein, endogenous Cdc42 colocalizes with actin at cell contact sites before hair formation (Fig. 3, A–F). Unlike Cdc42S89 however, the endogenous protein relocates to emerging hairs (Fig. 3, G–L). Its distribution in the hair partially coincides with that of actin, but Cdc42 extends further distally and is absent from the actin-rich base of the hair. Taken together, these data suggest that distal relocation of functional Cdc42 may play a role in directing actin polymerization and hair outgrowth to the distal side of wing epithelial cells.

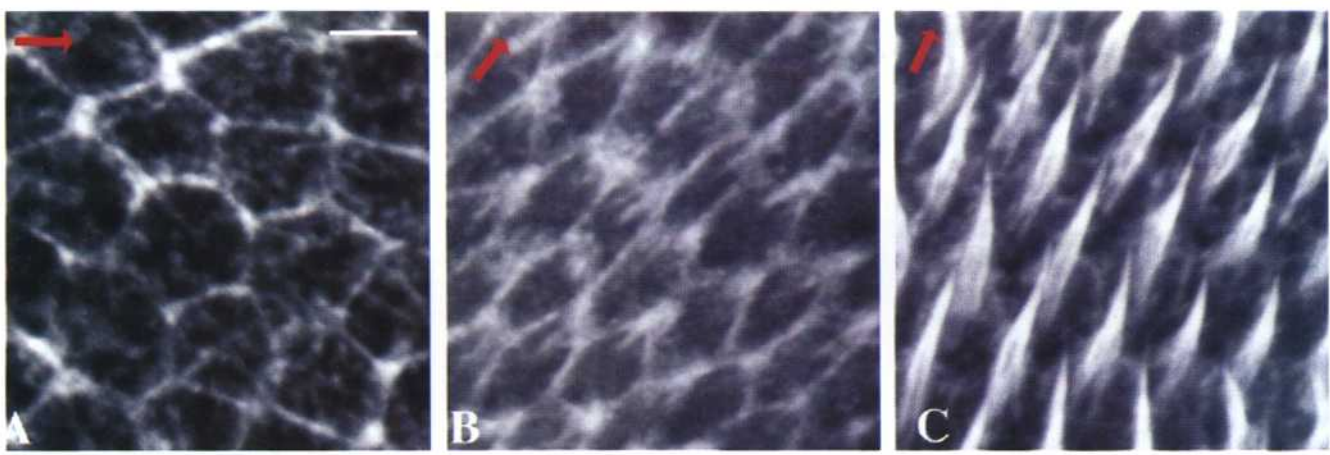
Rac1N17-expressing Cells Form Multiple Wing Hairs that Are Structurally Normal

While Cdc42 is critical for the outgrowth but not the placement of wing hairs, Rac1 is involved in placement but not outgrowth. We observed using the light microscope that expression of the dominant negative Rac1N17 caused duplication or triplication of wing hairs, but no defects in the appearance of the hairs themselves (Eaton et al., 1995). To verify that Rac1N17 affected the placement but not the structure of the hairs, we observed them more closely by scanning electron microscopy. Fig. 1, F and G, shows that duplicated or triplicated hairs are normally shaped and striated and do not appear to differ from the wild-type hair depicted in Fig. 1 C. This confirms that Rac1N17 expression does not interfere with hair outgrowth.

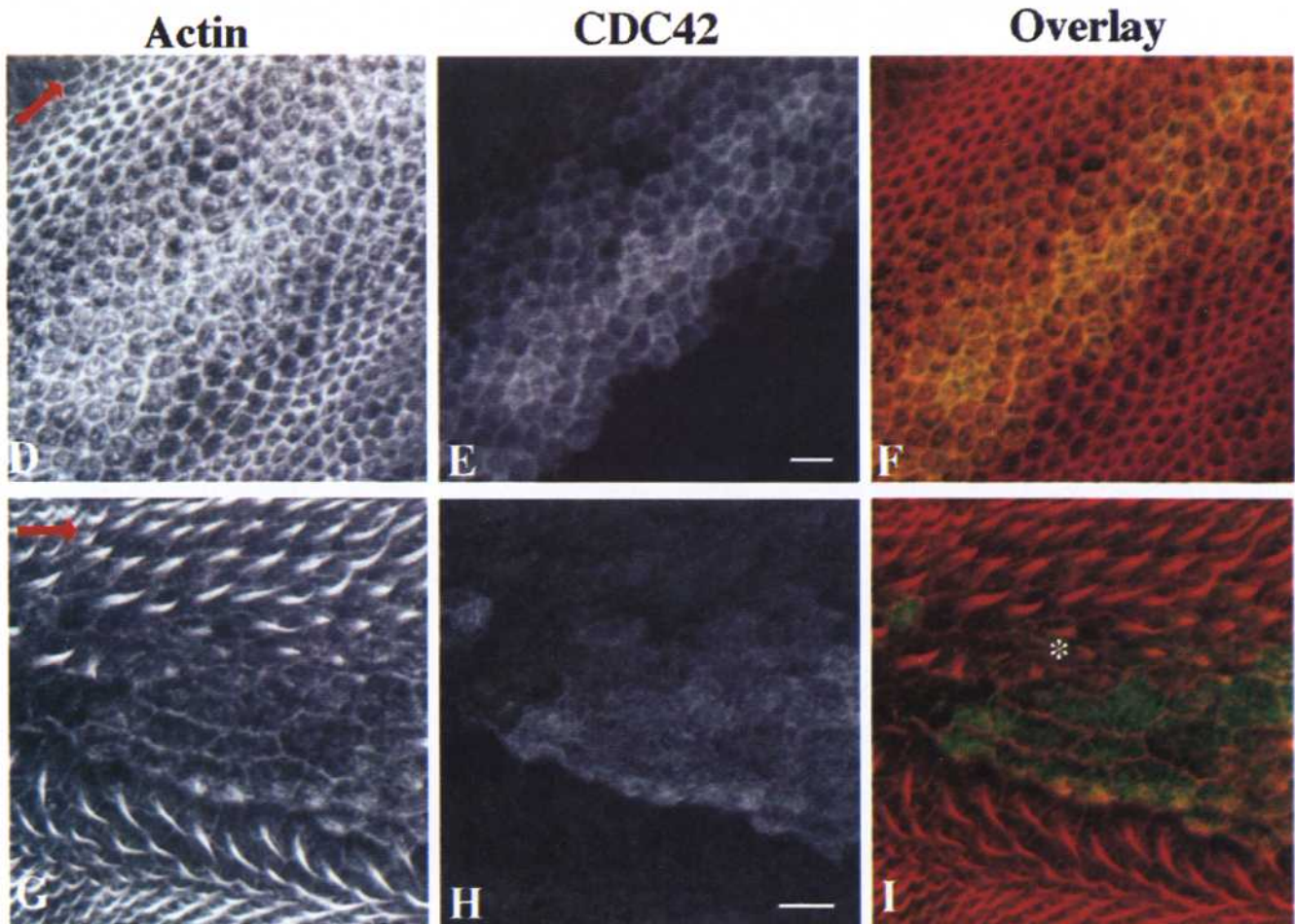
Rac1N17 Expression Causes Gaps in Junctional Actin

To see whether the inability of Rac1N17-expressing cells to restrict hair outgrowth to a single site correlated with specific perturbations of the actin cytoskeleton, we stained wings that expressed the dominant negative protein at the compartment boundary with phalloidin. We focused our attention on pupal wings aged between 29 and 35 h after puparium formation (at 25°C) because temperature shift experiments suggest that planar polarization occurs during this time (Adler et al., 1994). At 29 h, most wings show no sign of prehair formation, whereas at 35 h most wings have fully extended hairs. The time at which hair formation occurs is quite variable within this range, however.

Figure 2. The effect of dominant negative Cdc42 expression on the actin cytoskeleton. (A–C) Wild-type pupal wings aged between 30–35 h after puparium formation (apf) at 25°C are shown stained with rhodamine phalloidin. A–C are projections of three optical sections confined to the apical-most 1.0 μm (A and B) or 1.5 μm (C) of the cells. D–I depict pupal Cdc42S89-expressing wings either before (D–F) or during (G–I) hair formation. The wings are double stained with phalloidin (D, F, G, and H) and an antibody to Cdc42 (E, H, F, and I). In the overlay images F and I, phalloidin is shown in red and Cdc42 in green. D–I are projections of five optical sections, ensuring



Wild Type Actin



UASCdc42S89/PtcGAL4

that the apical regions of all cells in the field are included. The asterisk in *I* indicates a cell-expressing intermediate levels of Cdc42S89 that has accumulated lower than wild-type levels of actin in the wing hair. Red arrows indicate the distal direction. Bars: (A–C) 2 μm .; (D–I) 5 μm .

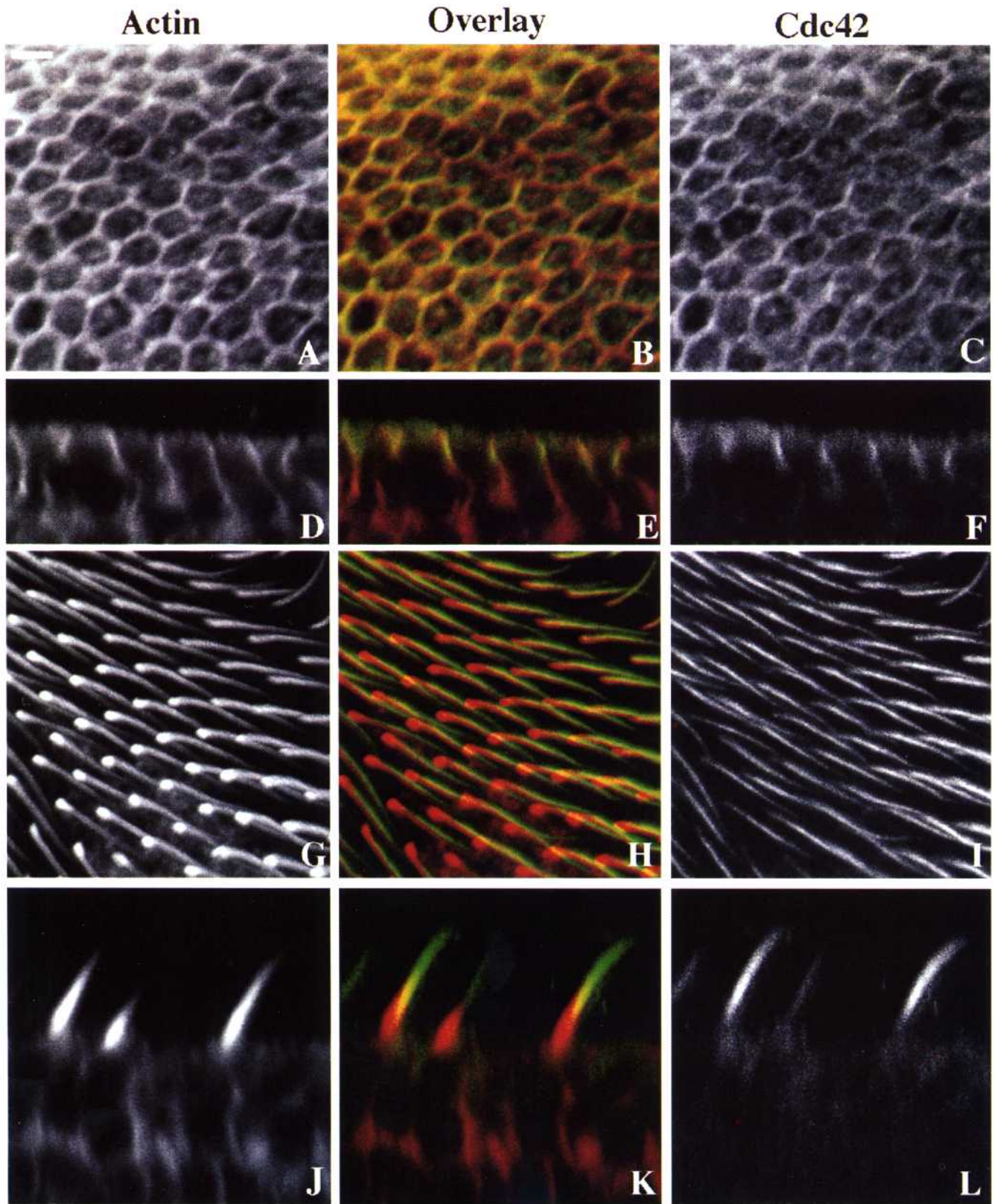
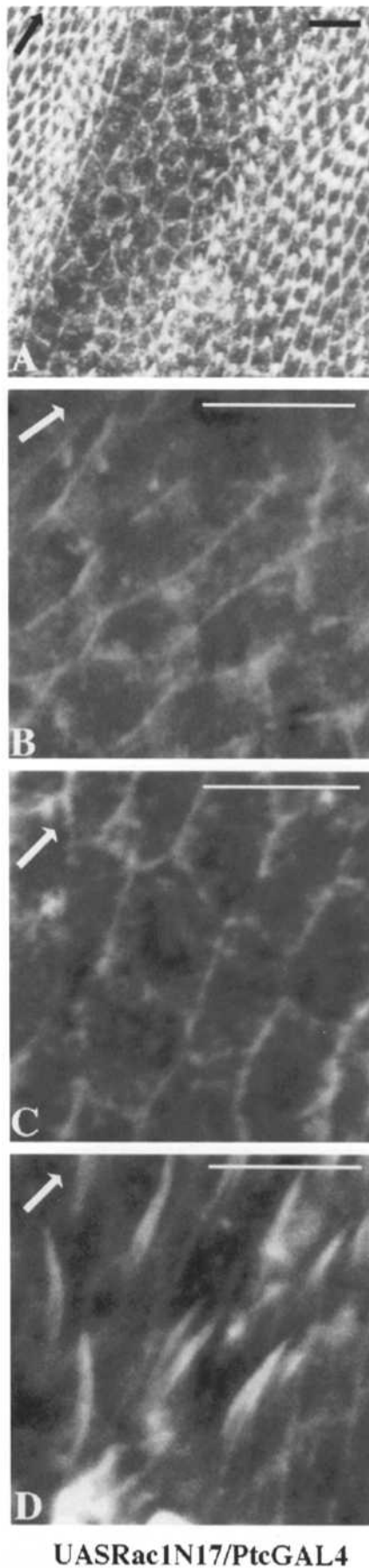


Figure 3. Localization of endogenous Cdc42. Pupal wings between 30 and 35 h apf are depicted double stained with phalloidin (A, B, D, E, G, H, J, and K) and an antibody to Cdc42 (B, C, E, F, H, I, K, and L). In the overlay images (B, E, H, and K), phalloidin staining is shown in red and Cdc42 in green. A–C are projections of three optical sections through the apical region of a wing that has not yet extended hairs. D–F are optical cross sections through the same wing. G–I are projections of four optical sections through extending hairs. J–L show an optical cross section through the same wing. Bar, 2 μ m.

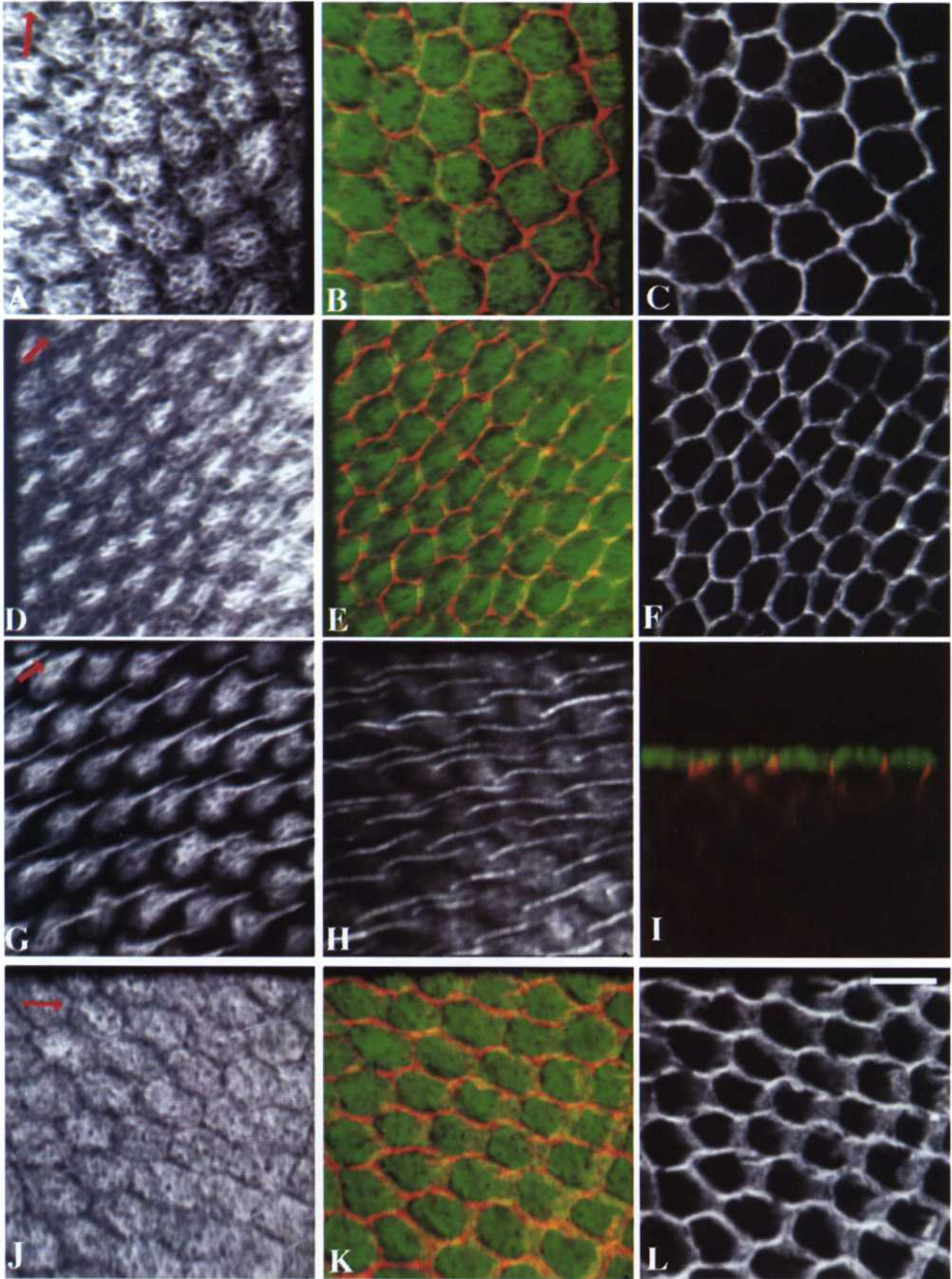


Cells within the region of the wing that expresses *Rac1N17* are clearly different from those in the surrounding normal epithelium even before hair outgrowth (Fig. 4 A). The normally continuous band of junctional actin is reduced in amount and fraught with gaps, especially in the row of cells closest to the compartment boundary (Fig. 4, A–C, compare with wild type cells in Fig. 2, A–C). The apical diameter of these cells is somewhat expanded. Furthermore, the distal sides of *Rac1N17*-expressing cells do not accumulate actin or extend prehairsts at the same time as their normal neighbors (Fig. 4 A). Close inspection reveals that these cells do accumulate some actin filaments, but these filaments are not concentrated on the distal side (Fig. 4 C). *Rac1N17* does not cause a general, nonspecific defect in actin polymerization because the duplicated hairs that *Rac1N17*-expressing cells eventually extend are filled with normal amounts of actin (Fig. 4 D). Nevertheless, this process is delayed with respect to wild-type cells. These data suggest that site selection may depend on proper organization of junctional actin, which is deranged by *Rac1N17* expression. In contrast, actin polymerization within the hair outgrowths and extension of the outgrowths proceed by a separable mechanism that is not disturbed by the expression of *Rac1N17*.

Microtubules in Pupal Wing Cells Are Organized in a Web at the Level of Apical Junctions

How might depletion of junctional actin lead to defects in planar polarization? Studies in MDCK cells suggest that the organization of microtubules depends on intercellular adhesion and actin at sites of cell–cell contact (Buendia et al., 1989). We wondered whether the apical junctions in pupal wing epithelial cells might help to organize microtubules in the plane of the epithelium. To begin to address this question, we examined the organization of microtubules and apical junctional proteins in wild-type cells both before and during hair outgrowth. Fig. 5 shows pupal wings stained with antibodies to tubulin and Coracle. Coracle encodes a homologue of band 4.1 and localizes to septate junctions (Fehon et al., 1994). Strikingly, optical sections along the apical basal axis reveal that most of the microtubules are localized at the level of apical junctions (Fig. 5 I), both before and just after the initiation of hair outgrowth. The array of microtubules appears somewhat dome shaped. Fig. 5, A–C, shows a projection of three optical sections

Figure 4. The effect of dominant negative *Rac1* expression on the actin cytoskeleton. Pupal wings aged between 30–35 h apf from pupae that expressed *Rac1N17* along the compartment boundary are shown stained with rhodamine phalloidin. The image in A is a projection of five optical sections ensuring that the apical regions of all cells in the field are included. Both wild-type and *Rac1N17*-expressing cells can be seen. The compartment boundary cells that express *Rac1N17* lie between veins L3 and L4. These veins can be identified by their elevated levels of junctional actin and smaller cross section. B shows *Rac1N17*-expressing cells at the same stage as in Fig. 2 A. C shows *Rac1N17*-expressing cells at the same stage as Fig. 2 B. D shows *Rac1N17*-expressing cells at the same stage as Fig. 2 C. B–D are projections of three optical sections through the apical region of the cell. Arrows indicate the distal direction. Bar, 5 μ m.



comprising the dome of microtubules in cells where outgrowth has not yet occurred. The microtubules are oriented parallel to the plane of the epithelium, and their organization resembles that of a spider web connected to the cell boundaries by a set of spokes. The microtubule spokes appear to actually make contact with the circumferential band of Coracle protein.

Microtubules and Junctional Proteins Rearrange during Hair Formation

Apical microtubules and junctional proteins undergo dramatic rearrangements during hair outgrowth. The initiation of hair outgrowth correlates with the selective accumulation and elongation of microtubules that run along the proximal–distal axis (Fig. 5 *D*). Comparing the patterns of microtubule and Coracle staining reveals that these microtubules lie between the center of the cell and its distal side (Fig. 5, *D–F*). We cannot order these microtubule rearrangements precisely with respect to the distal accumulation of actin because the fixation conditions that preserve actin well are not optimal for the preservation of microtubules. Elongating microtubules subsequently bundle together and fill the extending wing hair (Fig. 5 *G*). As elongation continues, the hair begins to tilt apically (Wong and Adler, 1993). Microtubules are detectable, both in the wing hair (Fig. 5 *H*), and in the underlying apical web (Fig. 5 *J*) throughout the process of hair elongation. During elongation, the Coracle protein changes its distribution. It acquires a diffuse, reticular appearance at proximal and distal cell boundaries, while remaining tightly localized on lateral boundaries (Fig. 5 *L*). Comparing the pattern of Coracle staining with that of the apical microtubule web (Fig. 5 *K*) suggests that the majority of the reticular staining spreads into the distal region of each cell. Fig. 5, *J–L*, shows projections of three optical sections through the apical microtubules. When single sections are observed, it is apparent that the majority of the reticular Coracle protein is located at the basal-most level of the dome-shaped microtubule web. Two other proteins that localize to apical junctions, E-cadherin and Discs Large, exhibit similar behavior (data not shown).

Formation of the Apical Microtubule Web Is Developmentally Regulated

We were surprised to find the majority of microtubules spanning the cell at the level of apical junctions because 30 h later, after hairs have formed, most microtubules comprise a longitudinal array that runs from the apex to the base of each cell (Tucker et al., 1986; Mogensen and Tucker, 1987; Fristrom et al., 1993). This suggested that microtubule organization was developmentally regulated during wing

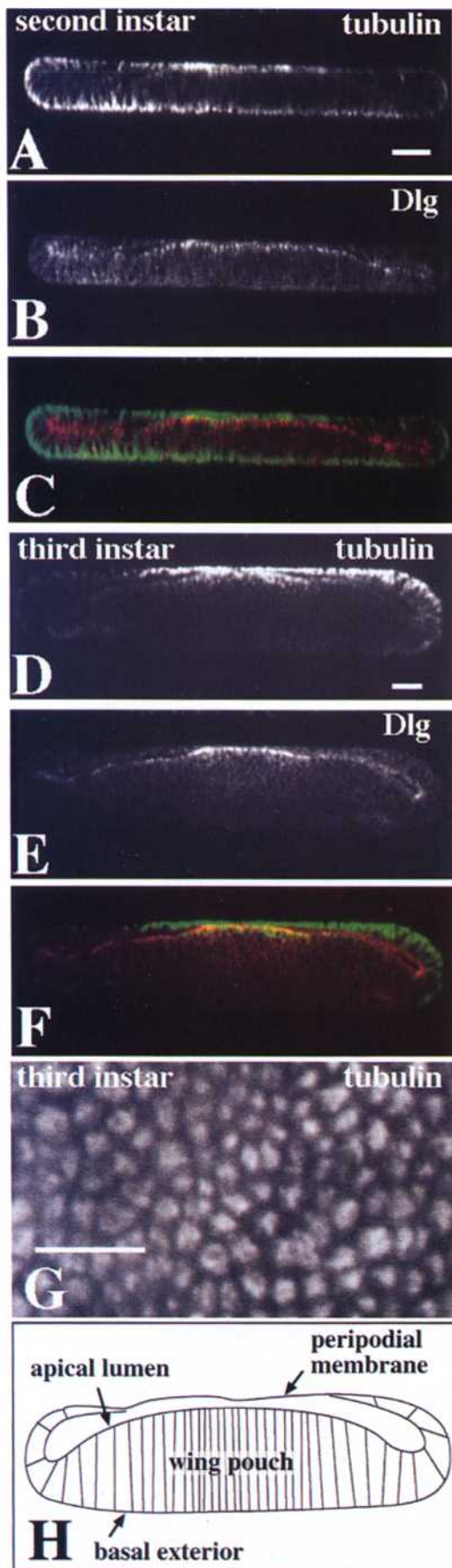
formation. To find out when the apical web arose, we stained wing discs at earlier developmental stages with antibodies to both tubulin (Fig. 6, *A, C, D, F* and *G*) and Discs Large (Fig. 6, *B, C, E*, and *F*). At the larval stages shown in Fig. 6, the wing disc is inside out relative to pupal wings; the cells form an epithelial sac of nonuniform thickness with their apical sides facing the lumen (see the schematic diagram in Fig. 6 *H*). Discs Large brightly stains the apices of the tightly packed cells of the wing pouch (which gives rise to the wing proper). It appears less bright in the shorter, columnar cells on either side of the wing pouch and is barely observable in the squamous peripodial membrane cells that overlie it (Fig. 6, *B* and *E*). We see abundant microtubules at all stages in the peripodial membrane. In second instar larvae, the microtubules in the wing pouch and in the lateral cells on either side of it are more abundant towards the basal side (Fig. 6, *A–C*). At the beginning of the third instar, the wing pouch cells begin to accumulate apical microtubules (Fig. 6, *D–F*), and the more lateral cells do not. The extremely narrow width of imaginal cells makes it difficult to examine the microtubules in any detail in the light microscope, but, viewed in plane of the epithelium, their organization has some similarity to that in pupal wing cells (Fig. 6 *G*). Ultrastructural observation of late third instar discs reveals two populations of apical microtubules: large bundles of microtubules parallel to the plane of the epithelium that appear to run directly into adherens junctions and longitudinally oriented microtubules that associate with the apical membrane at electron dense plaques (Fristrom and Fristrom, 1975). Taken together, these data indicate that microtubule organization varies greatly during development and show that the apical microtubule web is present in a subset of epithelial cells within a particular developmental window.

Rac1N17 Expression Disorganizes the Apical Microtubule Web

After determining the arrangement of microtubules in wild-type cells, we next examined whether microtubule organization was perturbed by Rac1N17 expression. Fig. 7 *A* shows the strip of Rac1N17-expressing cells at the AP compartment boundary with wild-type cells on either side. Compared to those in wild-type cells, the microtubules in Rac1N17-expressing cells appear disorganized. We conclude that Rac1 is needed to maintain the proper organization of the apical microtubule web at the time planar polarization is thought to occur, before prehair formation.

To investigate the site of action of Rac1N17, we determined the localization of the overexpressed protein. Staining wings that expressed Rac1N17 at the compartment boundary reveals that this protein is enriched apicolater-

Figure 5. Polarized rearrangement of microtubules during wing hair extension. Pupal wings between 30–35 h apf are shown stained with antibodies to tubulin (green) and Coracle (red). All panels except *H* and *L* show projections of three optical sections through the apical microtubule web-containing region. *A–C* show microtubules and Coracle before prehair formation: (*A*) microtubules, (*B*) microtubules + Coracle, and (*C*) Coracle. *D–F* show microtubules and Coracle when distally oriented microtubules begin to accumulate: (*C*) microtubules, (*D*) microtubules + Coracle, and (*E*) Coracle. *G* shows microtubules beginning to elongate distally. *H* depicts a single section that is predominantly apical to the microtubule web showing the outgrowing hairs filled with microtubules. *I* is an optical cross section of a 30-h apf wing. *J–L* show the microtubules and Coracle protein that underlie the hairs in *H*: (*J*) microtubules, (*K*) microtubules + Coracle, and (*L*) Coracle. Arrows indicate the distal direction. Bar, 5 μ m.



ally at sites of cell–cell contact, although it is also present throughout the cytoplasm (Fig. 7, *B* and *C*). This suggests that Rac1N17 acts at the junctions themselves, although it does not rule out its ability to act at other sites in the cell or to influence the organization of microtubules directly. Taken together, these data raise the possibility that the planar polarization defects caused by Rac1N17 expression derive from the disorganization of junctional actin and microtubules.

Discussion

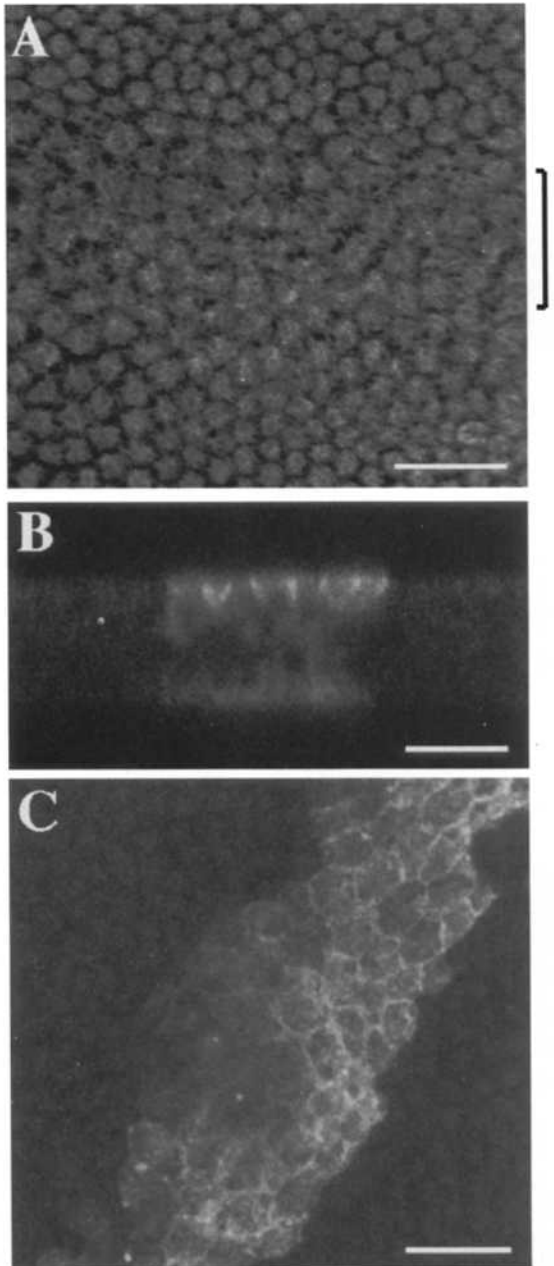
We have analyzed the cellular events leading to the formation of a single wing hair on the distal side of each cell. To that end, we have described changes in actin and microtubule organization that are associated with the polarized formation of wing hairs and examined the requirement for Cdc42 and Rac1 activity in this process.

Reorganization of the Cytoskeleton during Wing Hair Formation

Before hairs form, the actin in the apical region of wing cells is organized in a circumferential ring typical of other epithelial cells (Fig. 2). The first sign of hair formation is the accumulation of actin at the distal vertex of each cell (Wong and Adler, 1993; and Fig. 2). We observe that this actin is diffuse apical to the circumferential band of junctional actin, with a spike-like fiber extending into the cell at the junctional level. The hairs begin to extend from the distal vertex and are filled with filamentous actin.

This reorganization of the actin cytoskeleton is accompanied by a striking rearrangement of microtubules in the vicinity of apical junctions (Fig. 5). Before wing hairs form, microtubules in pupal wing epithelial cells are arranged in a web-like structure that appears to connect to the junctional region by an array of radial spokes. The construction of this web is developmentally regulated and first appears in the cells that will give rise to the wing at the beginning of the third larval instar (Fig. 6). During wing hair formation, the apical microtubule web undergoes a polarized reorganization; those microtubules that run between the center of the cell and its distal side accumulate and begin to elongate. Microtubules fill the extending wing hair throughout its elongation. Microtubule rearrangements occur in the same time window as those of actin, but we cannot order them precisely with respect to one another because of the incompatibility of fixation conditions.

Figure 6. Microtubule organization in second and third instar larval wing discs. Second and third instar discs were double stained with antibodies to Discs Large (*B*, *C*, *E*, and *F*) and tubulin (*A*, *C*, *D*, and *F*), or with an antibody to tubulin alone (*G*). (*A–C*) Optical cross section of a second instar larval disc through the wing pouch region. (*D–F*) Optical cross section through the wing pouch region of a third instar larval disc. Electron microscopic observation confirms the abundance of apical microtubules seen here but also reveals less abundant longitudinal microtubules in the central region of the cells (Eaton, S., and K. Simons, unpublished observation). (*G*) Tangential section through the apical region of a third instar larval disc. (*H*) Schematic representation of a third instar larval wing disc. Bar, 6 μm .



UASRacN17/PtcGAL4

Figure 7. Disorganization of microtubules by Rac1N17 and its localization. Pupal wings aged between 30–32 h apf from pupae that expressed Rac1N17 along the compartment boundary are shown stained with an antibody to tubulin (*A*) or Rac1 (*B* and *C*). *A* is a projection of five optical sections, ensuring the inclusion of the apical regions of all cells in the field. *B* is an XZ section through the region of the pupal wing overexpressing Rac1N17. *C* is a projection of three apical optical sections. Bar, 10 μ m.

Cdc42 Is Required for Distal Actin Polymerization and Hair Extension

Cdc42 and Rac1 control different aspects of hair formation by regulating different subsets of the cytoskeleton. Wing cells that express the dominant negative Cdc42S89

either fail to make wing hairs or make wing hairs that are deformed and stunted, depending on the level of dominant negative protein (Fig. 1). The stunted wing hairs are never duplicated and are normally placed. Endogenous Cdc42 protein is enriched in the distal part of extending hairs, suggesting that it is active there (Fig. 3). In Cdc42S89-expressing cells that do not make hairs, actin fails to accumulate distally and there is no sign of any actin-filled outgrowth (Fig. 2).

Cdc42 activity is required for generating polarized membrane outgrowths in a variety of cell types. It is required for polarized budding in *Saccharomyces cerevisiae* (Adams et al., 1990), and its activation produces filopodium extension in fibroblasts (Kozma et al., 1995; Nobes and Hall, 1995). In *Drosophila*, it is required for neurite extension (Luo et al., 1994) and apico-basal elongation of epithelial cells (Eaton et al., 1995), as well as wing hair formation. All of these processes involve localized Cdc42-dependent actin polymerization, but the nature of the connection between actin polymerization and membrane outgrowth is still controversial (for review see Mitchison and Cramer, 1996).

Filopodia are protrusive structures that form around actin bundles whose barbed ends are oriented in the direction of outgrowth. According to one model for filopodial extension, protrusive force derives from the elongation of actin bundles at their barbed ends (Mitchison and Cramer, 1996). Like filopodia (Nobes and Hall, 1995), wing hairs contain bundles of actin whose formation is controlled by Cdc42. By analogy, these actin bundles may provide force for wing hair extension. This model is supported by the finding that actin-bundling proteins are critical for hair formation (Cant et al., 1994; Petersen et al., 1994) and by the observation that drug-induced actin depolymerization produces deformed wing hairs (Tucker, C., and P. Adler, personal communication).

An additional mechanism is suggested by the role of Cdc42 in T cell polarization (Stowers et al., 1995). In these cells, the actin-rich cortical site organized by Cdc42 may interact with and reorient the microtubule cytoskeleton. Reorientation of microtubules and the Golgi complex is thought to direct secretion to the cortical site (Geiger et al., 1982; Kupfer et al., 1994). Perhaps Cdc42-dependent actin polymerization in the wing promotes the accumulation of distally oriented microtubules. Microtubules might subsequently stabilize extension by elongating or translocating into the hair or provide a route for the addition of new membrane to the growing hair. In support of this idea, lipid probes detect an extensive tubulovesicular membrane network in the vicinity of apical microtubules, a subset of which moves into the hair as it elongates (Eaton, S., and K. Simons, unpublished observation).

Rac1 Organizes Junctional Actin and Microtubules and Helps Limit the Site of Hair Formation

Wing cells that express the dominant negative Rac1N17 fail to restrict hair outgrowth to a single site (Fig. 1). These cells make multiple wing hairs that are otherwise structurally normal, unlike those made by Cdc42S89-expressing cells. Rac1N17-expressing cells have gaps in junctional actin (Fig. 5), and their apical microtubule web is disorganized (Fig. 7). These observations suggest that junctions

and microtubules may be structurally linked. A variety of other observations are consistent with this possibility. Direct association between planarly oriented microtubules and adherens junctions has been observed at the ultrastructural level (Fristrom and Fristrom, 1975). Furthermore, reorganization of microtubules in polarizing MDCK epithelial cells depends on cadherin-mediated contact and actin (Bacallao et al., 1989; Buendia et al., 1989). Finally, the product of the APC gene has recently been shown to associate with α -catenin in the vicinity of apical junctions (Miyashiro et al., 1995) and to bind to and promote the assembly of microtubules (Munemitsu et al., 1994; Smith et al., 1994). Rac1 may play a pivotal role in these interactions; in vitro experiments have shown that Rac1 can both promote actin polymerization through phosphoinositide synthesis (Hartwig et al., 1995) and bind to GTP tubulin (Best et al., 1996).

Rac1 is a component of a number of different signal transduction pathways, not all of which impinge on the polymerization of actin (Coso et al., 1995; Hill et al., 1995; Minden et al., 1995; Chou and Blenis, 1996). It is therefore possible that Rac1 helps select the site of hair formation in a manner that is completely unrelated to its role in organizing junctional actin and microtubules. Nevertheless, because reorganization of actin and microtubules mediates such a wide range of other cellular polarization events, they are credible targets for the Frizzled signal transduction pathway. Furthermore, experiments in tissue culture have shown that drugs that promote either the polymerization or depolymerization of microtubules cause pupal wing cells to form multiple wing hairs (Tucker, C., and P. Adler, personal communication), suggesting that modulation of microtubule dynamics is essential for site selection. For these reasons, we think it likely that Rac1N17 expression perturbs the site of prehair formation via its effects on the cytoskeleton.

We do not yet know whether Rac1 is directly involved in restricting the site of hair formation, or whether it might act further upstream in the reception or transmission of the Frizzled signal. Determining whether Rac1N17 acts autonomously or nonautonomously will help to address this question. The ability of Rac1 to organize junctional actin and microtubules might be important at any of these levels. Modulation of the interactions between apical junctions and microtubules in response to Rac1 activity might be directly involved in repressing inappropriate hair initiation. Interaction of microtubules with the junctional region might affect their stability or growth properties in such a way as to prevent accumulation and outgrowth. On the other hand, intact apical junctions and microtubules might be important for the reception or transmission of the Frizzled signal. Junctions have been proposed to play important roles in intercellular signaling (Woods and Bryant, 1993). Many proteins involved in reception of intercellular signals localize to junctions, and at least in some cases, junctional localization is critical for the function of these proteins (Simske et al., 1996). Furthermore, the array of microtubules associated with apical junctions might serve to target the secretion of signaling molecules to the junctional region.

The junctional microtubule web is present in wing epithelial cells from the beginning of the third larval instar

through much of pupal development (Fig. 7). During this time, a wide variety of intercellular signaling events are engaged in regulating the growth and patterning of the wing. In the future, Rac1N17 expression will be a useful tool with which to disturb the organization of this structure and thereby probe its function in intercellular signaling.

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