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The plakin Short Stop and the RhoA GTPase are required for E-cadherin-dependent apical surface remodeling during tracheal tube fusion

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SUMMARY

Cells in vascular and other tubular networks require apical polarity in order to contact each other properly and to form lumen. As tracheal branches join together in *Drosophila melanogaster* embryos, specialized cells at the junction form a new E-cadherin-based contact and assemble an associated track of F-actin and the plakin Short Stop (*shot*). In these fusion cells, the apical surface determinant Discs Lost (Dlt) is subsequently deposited and new lumen forms along the track. In *shot* mutant embryos, the fusion cells fail to remodel the initial E-cadherin contact, to make an associated F-actin structure and to form lumenal connections between tracheal branches. Shot binding to F-actin and microtubules is required to rescue these defects. This finding has led us to investigate whether other regulators of the F-actin cytoskeleton similarly affect apical cell surface remodeling and lumen formation. Expression of constitutively active *RhoA* in all tracheal cells mimics the *shot* phenotype and affects Shot localization in fusion cells. The dominant negative *RhoA* phenotype suggests that RhoA controls apical surface formation throughout the trachea. We therefore propose that in fusion cells, Shot may function downstream of RhoA to form E-cadherin-associated cytoskeletal structures that are necessary for apical determinant localization.

Key words: Anastomosis, Discs lost, Cadherin, F-actin, Microtubules, *Drosophila*

INTRODUCTION

Drosophila tracheal development provides a powerful model for the study of lumen formation, a process that is integral to the development of tubular networks such as those found in vertebrate circulatory, respiratory and excretory organs. The *Drosophila* tracheal system arises from nests of cells that invaginate from the epidermis and undergo branching morphogenesis postmitotically within each embryonic hemisegment (Manning and Krasnow, 1993). The lumenal or apical surface originates as the surface of these tracheal pits and expands as tracheal cells progressively invaginate and form branches. Recent genetic analysis of tubulogenesis in *Drosophila* (Beitel and Krasnow, 2000) and *C. elegans* (Buechner et al., 1999) has identified mutations that affect lumenal morphology. However, the mechanism of apical surface regulation remains poorly understood.

The tracheal lumen is initially closed at branch tips. Concurrent with branching morphogenesis, specialized cells at branch tips, known as fusion cells, join branches into a continuous tubular network. This process of anastomosis requires each fusion cell to recognize its partner in the adjacent hemisegment and to form a lumen that connects the two branches (Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996). *Shotgun*, the *Drosophila* homolog of the cell adhesion molecule E-cadherin is integral to the initial fusion cell contact (Uemura et al., 1996). Mutations in *shotgun* affect tracheal branch extension and lumen formation at anastomosis sites, as do mutations in *armadillo*, the *Drosophila* homolog of its effector β-catenin (Uemura et al., 1996; Beitel and Krasnow, 2000). E-cadherin and β-catenin control cell polarity (McNeill et al., 1990) and tube extension in culture (Yap et al., 1995; Matsumura et al., 1997; Pollack et al., 1997), suggesting an evolutionarily conserved role for cadherin-mediated cell adhesion in apical surface regulation.

Tracheal cells face the lumen with their apical surfaces, suggesting that apically localized molecules play a role in lumen formation. Mutations in *discs lost* (*dlt*), which encodes an apically localized PDZ domain family protein (Bhat et al., 1999; Tanentzapf et al., 2000), disrupt epithelial cell polarity, as do mutations in *crumbs*, which encodes a Dlt-associated (Bhat et al., 1999), EGF repeat family transmembrane protein (Tepass et al., 1990). *crumbs* mutant embryos are defective in forming mature zonula adherens (ZAs) (Tepass, 1996), structures at apical/lateral contacts between cells that contain E-cadherin.

Several studies suggest that these apical surface determinants and E-cadherin regulate the cytoskeleton and therefore control lumen formation and morphology. For example, Crumbs may attach β_H-spectrin, an F-actin cross-linker, to the apical membrane in *Drosophila* (Wodarz et al., 1995) and mutations in a *C. elegans* β_H-spectrin moderately

enlarge the lumen of the excretory canal (Buechner et al., 1999). E-cadherin interacts with F-actin via multiple mechanisms (Gumbiner, 2000). These include binding to p120 catenin (p120ctn), a negative regulator of the RhoA GTPase (Anastasiadis et al., 2000; Noren et al., 2000). RhoA controls the formation of F-actin-containing focal adhesions and stress fibers in cultured cells (Ridley and Hall, 1992; Jou and Nelson, 1998). These interactions with RhoA also potentially regulate apical membrane protein targeting (Jou and Nelson, 1998), a process important for lumen development in culture (Lipschutz et al., 2000).

Little is known about the cytoskeletal structures required for lumen formation and how apical surface determinants are localized. We identify here an F-actin-rich track that is associated with E-cadherin-dependent contacts between fusion cells that appears to guide deposition of apical surface determinants and lumen formation. During anastomosis, Short Stop (Shot), an evolutionarily conserved plakin (Gregory and Brown, 1998; Strumpf and Volk, 1998; Leung et al., 1999; Lee et al., 2000a), accumulates at these contacts and transiently along the track. Mutations in *shot* and constitutively active alleles of the *Drosophila* RhoA (RhoA) GTPase specifically disrupt this contact and the associated track. Remarkably, the interactions of Shot with F-actin and its binding to microtubules are functionally redundant in organizing the track, suggesting that Shot acts with other pathways to organize F-actin and microtubules, rather than as an F-actin/microtubule cross-linker. We propose that in fusion cells, RhoA antagonizes Shot to regulate E-cadherin-associated cytoskeletal structures required for apical surface determinant localization and lumen formation.

MATERIALS AND METHODS

Immunohistochemistry and microscopy

Embryos to be stained with monoclonal antibody (mAb) 2A12 were fixed in B-5 (Kolodziej et al., 1995). Otherwise, embryos were fixed as described elsewhere (Uemura et al., 1996). The antibodies used in this study were rabbit anti-GFP (Boehringer Mannheim), rabbit anti-*lacZ* (Jackson Immunologicals), guinea pig anti-Shot (Strumpf and Volk, 1998), rabbit anti-Dlt (Bhat et al., 1999), rat monoclonal anti-E-cadherin (Uemura et al., 1996) and rat monoclonal anti-tubulin (Harlan Sera-Lab, UK), together with FITC-, Cy3- and Cy5-conjugated secondary antibodies (Jackson Immunologicals). Staged embryos (Campos-Ortega and Hartenstein, 1985) were filleted in 70% glycerol, stained and viewed under a Zeiss LSM 410 confocal microscope using a 100× PlanApo lens. For F-actin visualization, embryos were filleted live in Ringer's solution and fixed in Ringer's/4% formaldehyde for 20 minutes, before staining with fluorescently labeled phalloidin. To compensate for slight variations in specimen height, 1 μm confocal sections were obtained, channels from each section merged in Adobe Photoshop, and information from adjacent sections composed to yield a 1 μm sagittal section through the center of the tracheal lumen.

Molecular biology and genetics

pUAST-*C-Shot L-GFP* contains a DNA fragment that encodes a GFP fusion with the Shot long isoforms C-terminal microtubule binding domain (C-Shot L) (Lee and Kolodziej, 2002) in the GAL4 expression pUAST vector (Brand and Perrimon, 1993). pUAST-*Shot L(C)-GFP* expresses a GFP fusion with a full-length type C Shot long isoform (Lee and Kolodziej, 2002). Type C isoforms do not bind F-actin. pUAST-*Shot L(C)-ΔGAS2-GFP* also lacks DNA sequences encoding most of the GAS2 motif (amino acids 4859 to 4905; Accession Number, AAF24343).

Flies bearing these transgenes were obtained by standard methods (Ashburner, 1989); other transgenic flies have been previously described (Lee and Kolodziej, 2002). The *btl-GAL4* and *esg-GAL4* enhancer trap lines (chromosome II) obtained from Mark Krasnow (Stanford University) were used to drive pUAST transgenes in tracheal and fusion cells, respectively. In most cases, pUAST transgenes were expressed in $shot^3$ mutant embryos by crossing $shot^3$ pUAST recombinants to *btl-GAL4* $shot^3$ or *esg-GAL4* $shot^3$ stocks. To visualize tracheal microtubule organization in wild-type and $shot^3$ mutant embryos, $shot^3$/+; pUAST-*C-Shot L-GFP*/+ flies were crossed to *btl-GAL4* $shot^3/CyO^{276}$ flies. The $shot^3$ mutant embryos lack epidermal and CNS Shot protein (Lee et al., 2000a). Second chromosome pUAST-*actin-GFP* and pUAST-*GAP43-GFP* lines were obtained from Akira Chiba (University of Illinois, Urbana-Champaign); *esg-lacZ* lines were obtained from Shigeo Hayashi (National Institute of Genetics, Mishima, Japan). Homozygous third chromosome pUAST-$RhoA^{V14}$, pUAST-$Rac1^{N17}$ and pUAST-$Cdc42^{N17}$ lines were obtained from Liqun Luo (Stanford University) and pUAST-$RhoA^{N19}$ was obtained from Jeffrey Settleman (Massachusetts General Hospital). These flies were crossed to *btl-GAL4* pUAST-*actin-GFP* or *btl-GAL4/+;* pUAST-*C-Shot L-GFP/+* flies to visualize F-actin and microtubules in tracheal cells expressing dominant negative or constitutively active Rho family GTPases.

RESULTS

Mutations in *shot* selectively disrupt tracheal lumen formation at anastomosis sites

The wild-type tracheal system arises from segmentally repeated branched networks joined through anastomosis across segment boundaries (Manning and Krasnow, 1993). Anastomosis is complete in the dorsal trunk (Fig. 1A) by stage 14, in the lateral trunk (Fig. 1A) by stage 15, and across the dorsal midline (Fig. 1C) by stage 16 (Samakovlis et al., 1996b). In *shot* null mutant embryos, as well as in weaker $shot^1$ and $shot^2$ alleles (Lee et al., 2000a), these anastomoses are disrupted (Fig. 1 and data not shown). In stage 14 or later $shot^3$ null mutant embryos, the dorsal trunk lumen is discontinuous at 75% of anastomosis sites (n=273) (Fig. 1B,D). Lateral trunk connections (Fig. 1B) and all anastomoses at the dorsal midline (Fig. 1D) are also affected. However, the overall branching pattern within hemisegments appears normal, suggesting that *shot* does not affect branch formation (Fig. 1B,D). A few dorsal trunk connections form apparently normally (6% of dorsal trunk anastomoses) (Fig. 1D), or are constricted relative to wild type (19% of dorsal trunk anastomoses), suggesting that the role of *shot* overlaps that of other molecules.

These defects could reflect defects in the differentiation of fusion cells, the specialized cells that occupy anastomosis sites (Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996). At stage 14, vesicular structures containing lumenal antigens are detected in cells at anastomosis sites in wild-type and *shot* mutant embryos (Fig. 1E,F), suggesting that *shot* does not qualitatively affect the ability of these cells to synthesize and package lumenal antigens. A pair of fusion cells are present at anastomosis sites in wild-type and *shot* mutant embryos, and express all fusion cell specific markers examined (Fig. 1G,H, and data not shown). Thus, *shot* mutants appear normal with respect to fusion cell viability, and specific marker and lumenal antigen expression.

Mutations in *shot* disrupt apical cytoskeletal structures in tracheal cells

shot is allelic to *kakapo* (Gregory and Brown, 1998; Strumpf and Volk, 1998) and encodes a family of plakin-related proteins (Lee et al., 2000a). Long isoforms of Shot provide essential cross-links between F-actin and microtubules during axon extension (Lee and Kolodziej, 2002) and stabilize microtubule arrays in neuronal support and muscle attachment cells (Prokop et al., 1998). Our observation of lumen formation defects in *shot* mutant embryos led us to investigate the organization of the tracheal cell cytoskeleton after anastomosis in wild-type and *shot* mutant embryos. We focused on cells in the dorsal trunk, the largest tracheal branch and therefore the easiest in which to visualize cytoskeletal structures.

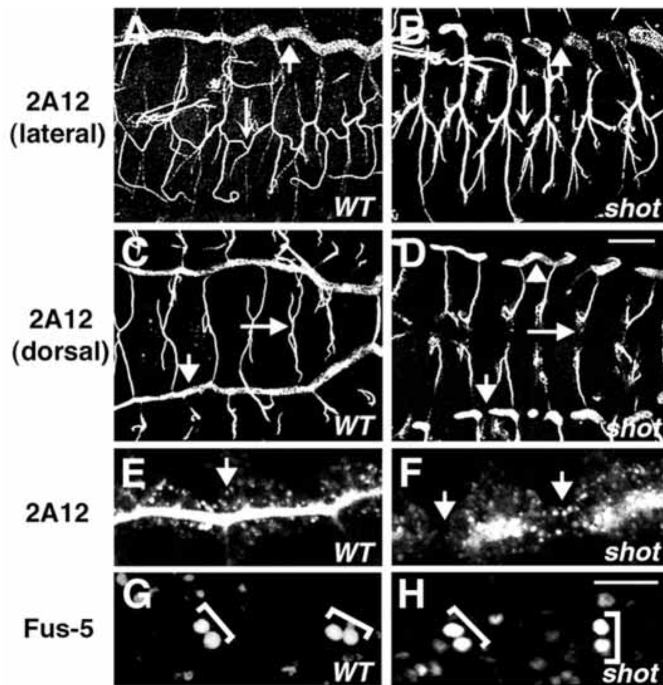


Fig. 1. *shot* mutants disrupt lumen formation at anastomosis sites. (A-D) 15 μm stacks of 1 μm confocal sections of stage 16 wild-type and $shot^3$ null mutant embryos stained with mAb 2A12, which recognizes a lumenal antigen (Samakovlis et al., 1996a). Anastomosis sites are indicated at hemisegment boundaries in the dorsal (short arrows) and lateral (concave arrows) trunks and the dorsal midline (long arrows). Anterior, leftwards; dorsal, upwards. (A) Lateral view, wild type. The lumen branches in a stereotyped pattern and is continuous at anastomosis sites. (B) Lateral view, $shot^3$. The lumen is discontinuous at anastomosis sites. (C) Dorsal view, wild type. The lumens of dorsal branches join together at the dorsal midline. (D) Dorsal view, $shot^3$. Dorsal branches have migrated towards the dorsal midline, but have not joined their lumens together. The dorsal trunk lumen is discontinuous at most (short arrow), but not all (arrowhead), anastomosis sites. (E-H) 1 μm confocal sections of dorsal trunk tracheal cells. (E) Stage 14, wild type. The mAb 2A12 lumenal antigen is transiently present in vesicular structures (arrow) within tracheal cells. Dorsal trunk anastomoses are complete. (F) Stage 14, $shot^3$. mAb 2A12 labels the lumen and vesicular structures (arrow) within tracheal cells. Vesicles are still present in cells at anastomosis sites that do not form lumen. (G) Stage 15, wild type. The *Fus-5* enhancer trap labels two fusion cell nuclei (brackets) with *lacZ* at each dorsal trunk anastomosis site. Other tracheal nuclei are also weakly labeled. (H) Stage 15, $shot^3$. The *Fus-5* enhancer trap labels two fusion cell nuclei at each anastomosis site. *shot* mutant embryos also express the fusion cell specific *Fus-2* and *Fus-3* enhancer trap markers normally (data not shown). Scale bars: in D, 25 μm for A-D; in H, 10 μm for E-H.

We visualized F-actin both by labeling dissected embryos with fluorescent phalloidin and by detecting actin-GFP (Verkhusha et al., 1999) specifically expressed in tracheal cells (Fig. 2). In wild-type tracheal cells at anastomosis sites, F-actin accumulates apically so that it appears as a continuous tube (Fig. 2A,G). In *shot* mutant tracheal cells at abnormal anastomosis sites, F-actin accumulates apically, but surrounds two blind-ended lumens (Fig. 2D,I).

We examined microtubules in tracheal cells using anti-tubulin. In wild-type tracheal cells, microtubules appear to accumulate apically (Fig. 2B,C). In *shot* mutant embryos, microtubules are no longer apically concentrated, though they remain largely at the cell periphery (Fig. 2E). Similar results are obtained with C-Shot L-GFP, a fusion between the microtubule binding domain of Shot and GFP that decorates all microtubules in transfected cells (Lee and Kolodziej, 2002). In wild type tracheal cells, C-Shot L-GFP accumulates apically (Fig. 2H), but in *shot* mutant tracheal cells, the protein is less clearly organized (Fig. 2J).

We investigated the identity of the cells that fail to form a lumen at anastomosis sites by labeling fusion cells with the *esg-lacZ* enhancer trap (Tanaka-Matakatsu et al., 1996) and labeling all tracheal cells with GAP43-GFP, a membrane-associated GFP derivative (Ritzenthaler et al., 2000). After anastomosis is complete in wild-type embryos (stage 14), the fusion cells form a compact doughnut around the lumen (Fig. 2K) (Samakovlis et al., 1996b) and flanking tracheal cells are drawn close to each other at the anastomosis site (Fig. 2K). The apically concentrated actin surrounds the tube and appears continuous through the fusion cells (Fig. 2K). In stage 14 *shot* mutant embryos, the fusion cells remain extended (Fig. 2L), creating a wide gap between flanking tracheal cells. The fusion cells are the only tracheal cells in the gap. The posterior cell often appears intercalated into the anterior branch, so that it stretches between the two branches (Fig. 2L). All tracheal cells accumulate F-actin apically, including the fusion cells, but the apical surface in each fusion cell does not develop into a bridging tube. Their apical surfaces remain facing the blind-ended lumens in their respective branches (Fig. 2L). Fusion cells therefore fail to remodel their F-actin cytoskeletons in *shot* mutant embryos.

Shot accumulates at E-cadherin containing contacts between fusion cells as it associates with an F-actin-rich track that marks the future axis of lumen formation

Antibodies against the long Shot isoforms (Strumpf and Volk, 1998) reveal that these proteins concentrate cortically in all tracheal cells, and also assemble in fusion cells into a transient track (Fig. 3A) that marks the future axis of the lumen bridging the two branches (Fig. 3D). Shot is not detected cortically in tracheal cells or in a track in fusion cells in $shot^3$ null mutant embryos (Fig. 3E,H). However, anti-Shot also non-specifically labels the tracheal lumen (Fig. 3E), precluding visualization of apically localized endogenous Shot with this reagent.

In order to investigate Shot localization during anastomosis in more detail, we expressed a Shot L(A)-GFP fusion in all tracheal cells, and used anti-GFP to follow its localization with

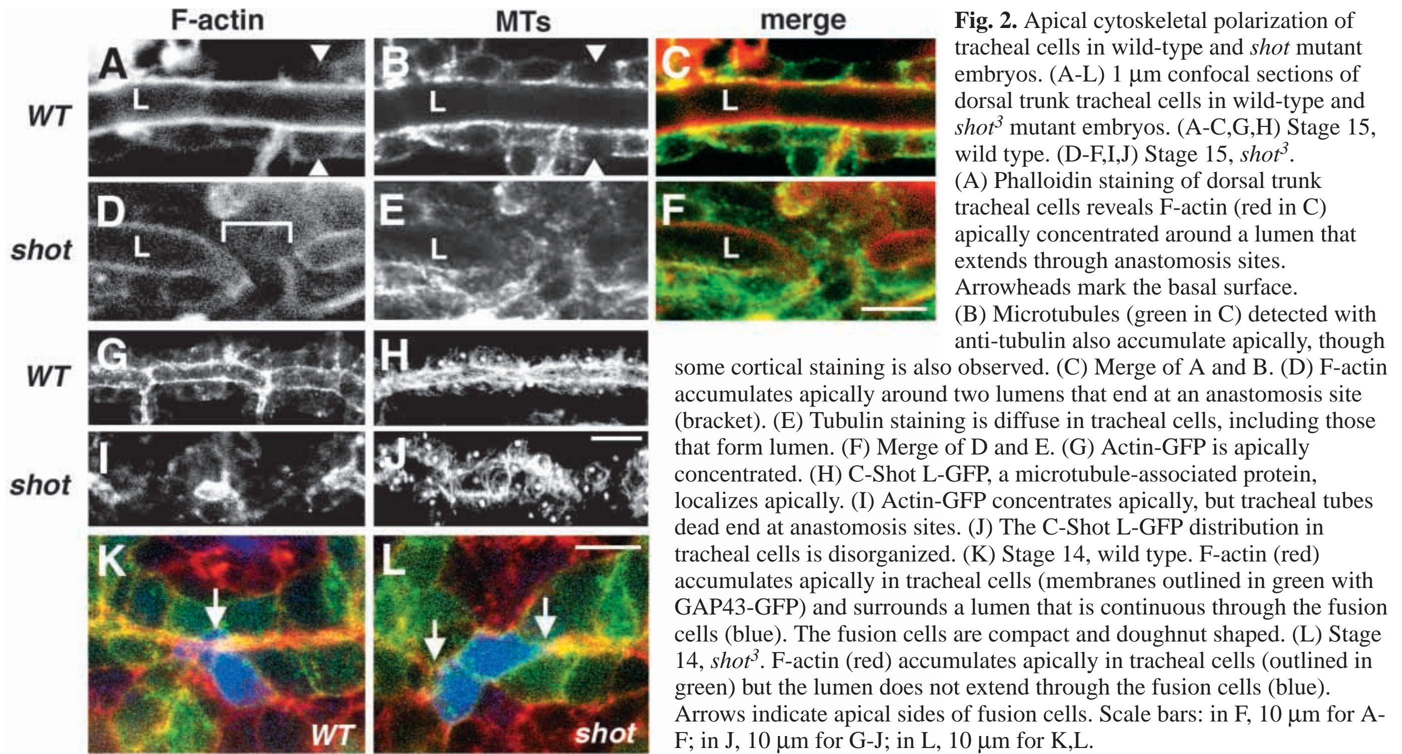


Fig. 2. Apical cytoskeletal polarization of tracheal cells in wild-type and *shot* mutant embryos. (A-L) 1 μm confocal sections of dorsal trunk tracheal cells in wild-type and *shot*^{3} mutant embryos. (A-C,G,H) Stage 15, wild type. (D-F,I,J) Stage 15, *shot*^{3}. (A) Phalloidin staining of dorsal trunk tracheal cells reveals F-actin (red in C) apically concentrated around a lumen that extends through anastomosis sites. Arrowheads mark the basal surface. (B) Microtubules (green in C) detected with anti-tubulin also accumulate apically, though some cortical staining is also observed. (C) Merge of A and B. (D) F-actin accumulates apically around two lumens that end at an anastomosis site (bracket). (E) Tubulin staining is diffuse in tracheal cells, including those that form lumen. (F) Merge of D and E. (G) Actin-GFP is apically concentrated. (H) C-Shot L-GFP, a microtubule-associated protein, localizes apically. (I) Actin-GFP concentrates apically, but tracheal tubes dead end at anastomosis sites. (J) The C-Shot L-GFP distribution in tracheal cells is disorganized. (K) Stage 14, wild type. F-actin (red) accumulates apically in tracheal cells (membranes outlined in green with GAP43-GFP) and surrounds a lumen that is continuous through the fusion cells (blue). The fusion cells are compact and doughnut shaped. (L) Stage 14, *shot*^{3}. F-actin (red) accumulates apically in tracheal cells (outlined in green) but the lumen does not extend through the fusion cells (blue). Arrows indicate apical sides of fusion cells. Scale bars: in F, 10 μm for A-F; in J, 10 μm for G-J; in L, 10 μm for K,L.

respect to E-cadherin and F-actin (Fig. 4), and also with respect to Discs Lost (Dlt), an apical surface determinant (Bhat et al., 1999) (Fig. 5). The Shot L(A) isoform contains an N-terminal F-actin binding domain and a C-terminal microtubule binding domain (Lee and Kolodziej, 2002), and Shot L(A)-GFP fully rescues anastomosis defects when expressed in tracheal cells in *shot* null mutant embryos.

In tracheal cells, E-cadherin localizes to an adherens junction network that encircles the lumen (Fig. 4F-J) (Uemura et al., 1996). During anastomosis, E-cadherin also localizes to an early site of contact between the fusion cells (Figs 4F, 6F) (Tanaka-Matakatsu et al., 1996). The spot of E-cadherin enlarges in one of the fusion cells, usually the anterior member of the pair (Figs 4G, 6G). Subsequently, E-cadherin forms a track extending in both cells (Figs 4H, 6H) (Tanaka-Matakatsu et al., 1996). This track is then remodeled into a ring (Fig. 4I,J) (Tanaka-Matakatsu et al., 1996). Later in anastomosis, the fusion cells become more compact and doughnut shaped (Figs 3, 6N,O) (Samakovlis et al., 1996b). Compaction of the fusion cells draws the adherens junctions that demarcate fusion cell

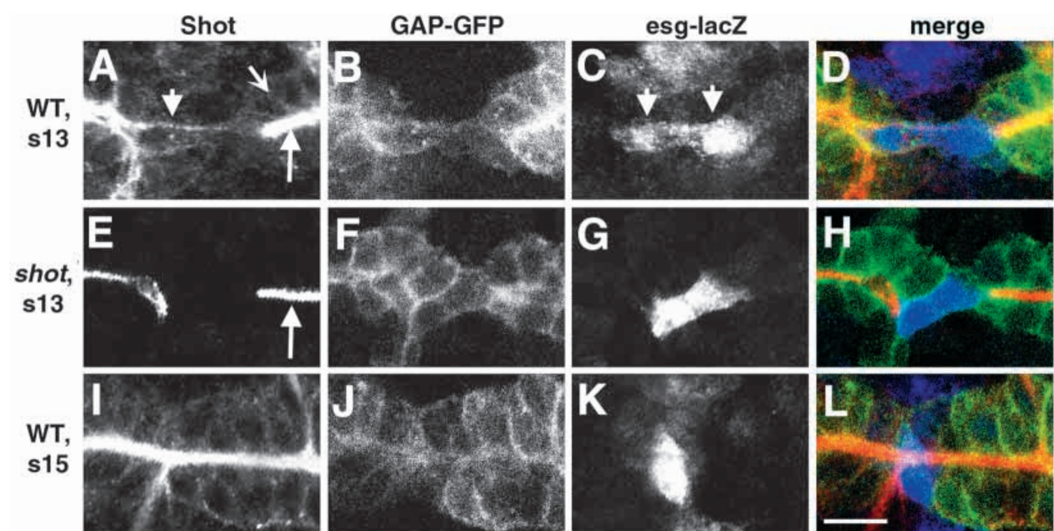


Fig. 3. Expression of Shot proteins in tracheal cells during anastomosis. 1 μm confocal sections. (A-D) Stage 13 wild-type embryo undergoing anastomosis. (A) Anti-Shot labels a track in fusion cells (short arrow). Shot proteins (red in D) are cortically concentrated in other tracheal cells (concave arrow). The lumen (long arrow) is labeled nonspecifically (see E). (B) GAP43-GFP expression (green in D) in tracheal cells labels cell membranes. (C) *esg-lacZ* enhancer trap expression (blue in D) labels the fusion cells (short arrows) that bridge the two dorsal trunk branches. (D) Merge of A-C. (E-H) Stage 13, *shot*^{3}. (E) Anti-Shot labels only the lumen (long arrow). (F) Tracheal cell membranes. (G) Fusion cells. (H) Merge of E-G. (I-L) Stage 15, wild type. Anastomosis is complete. (I) Shot is concentrated cortically in tracheal cells. Apically localized Shot cannot be distinguished from nonspecific lumenal staining. (J) Tracheal cell membranes. (K) Fusion cells are compact and encircle the lumen. (L) Merge of I-J. Scale bar: 10 μm.

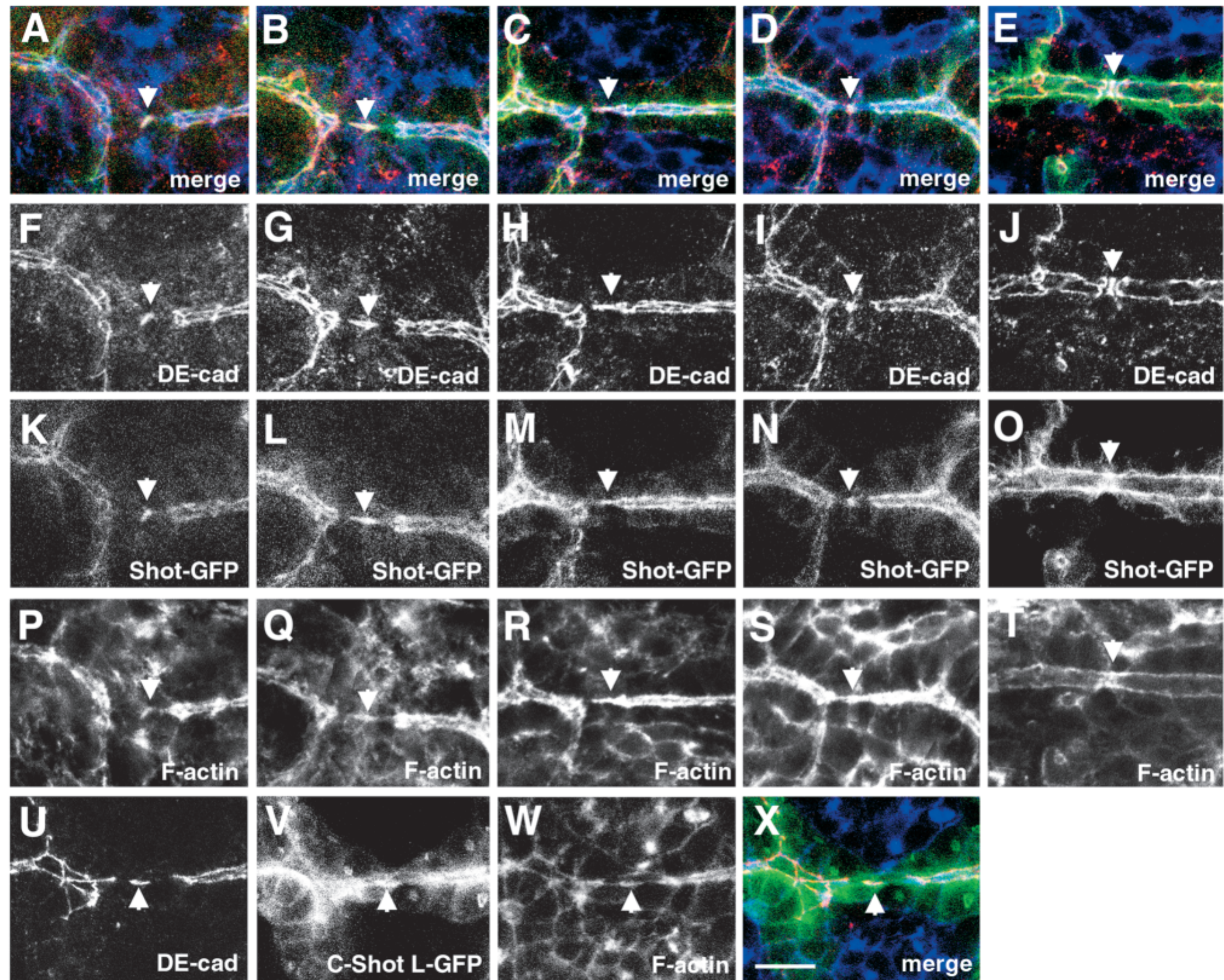


Fig. 4. Shot and F-actin form a cytoskeletal track associated with the E-cadherin (DE-cad) contacts between fusion cells. (A-T) Stage 13, wild-type embryos. (A-E) Remodeling of the fusion cell E-cadherin (red) contact (arrows) and associated Shot-GFP (green) and F-actin (blue) during anastomosis. (F) E-cadherin accumulates in a spot at the interface between the fusion cells. (G,H) Elongation of the E-cadherin spot. (I) Contraction of the E-cadherin contact into a ring. (J) The three rings of E-cadherin at the anastomosis site. (K-O) Shot L(A)-GFP is associated with the E-cadherin contact during anastomosis. (P-T) A track of F-actin forms, associated with the E-cadherin contact, that grows to span the two fusion cells (S). (T) The F-actin track becomes a ring. (U-X) C-Shot L-GFP (green in X) and F-actin (blue in X) accumulate at the E-cadherin (red in X) contact (arrow).

contacts with flanking tracheal cells closer to the central ring of E-cadherin. The central ring marks the anastomosis site and encircles the bridging lumenal connection (Figs 4I,J, 6J) (Tanaka-Matakatsu et al., 1996).

Shot L(A)-GFP and F-actin both accumulate in fusion cells at E-cadherin contacts (Fig. 4). As anastomosis proceeds, the F-actin forms a track that extends beyond the E-cadherin contact, and spans the two fusion cells (Figs 4S, 6Q,R). The track is a site for new membrane deposition (compare Fig. 6L with 6M). Shot L(A)-GFP also accumulates along this track (Fig. 5L), though it is more typically concentrated near E-cadherin contacts. These results are consistent with those obtained using anti-Shot (Fig. 3).

We investigated whether Shot L(A) can associate with E-cadherin contacts via its C-terminal domain (C-Shot L) or whether these associations primarily occur via its F-actin binding domain. C-Shot L-GFP expressed in tracheal cells accumulates at sites of E-cadherin localization (Fig. 4U-X), suggesting that it also mediates interactions with E-cadherin-associated cytoskeletal structures. As C-Shot L-GFP colocalizes with microtubules in cultured cells (Lee and Kolodziej, 2002), microtubules may also be concentrated near these sites. However, we were unable to visualize microtubules consistently during anastomosis using anti-tubulin staining and a variety of fixation methods, though we were able to detect microtubules in other tissues at this developmental stage (data not shown).

The Shot- and F-actin-containing track in fusion cells (Fig. 5L,Q) appears before apical surface determinants Dlt are detectable along the track (Fig. 5G). As the lumen forms within the fusion cells, Dlt accumulates along the track and then the new apical surface (Fig. 5H-J). Dlt levels are initially lower in the fusion cells relative to the flanking tracheal cells (Fig. 5I), suggesting that new apical surface is made in the fusion cells

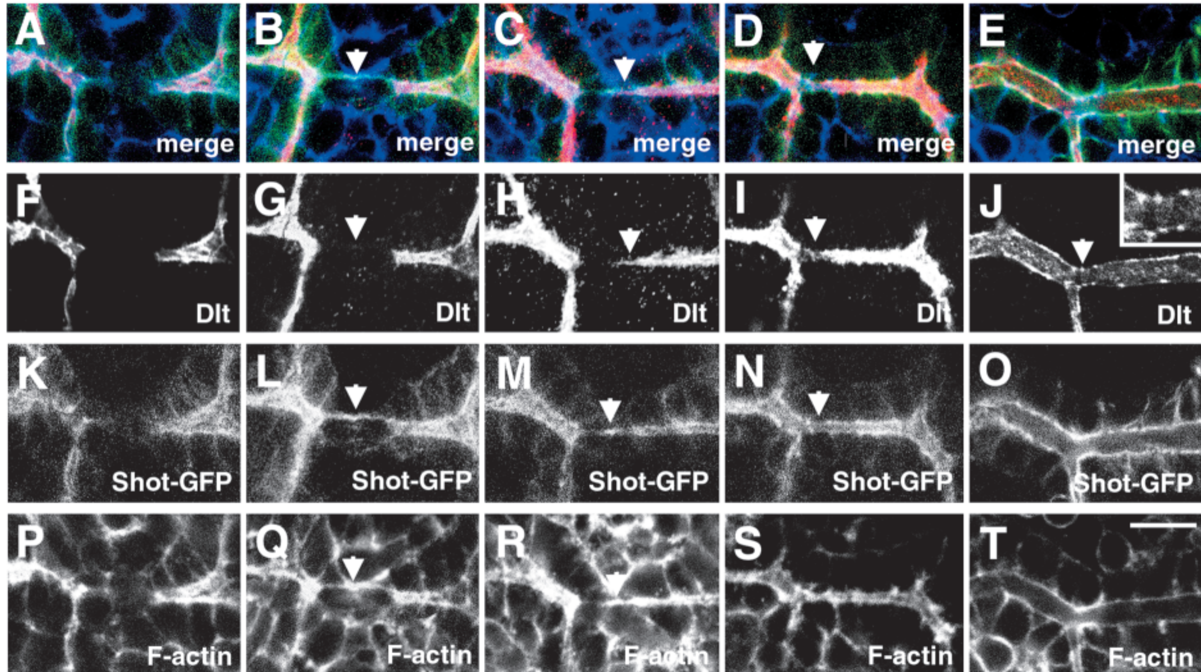


Fig. 5. Deposition of the apical surface determinant Dlt along the Shot/F-actin track. (A-T) Stage 13, wild-type embryos. (A-E) Dlt (red) accumulates in fusion cells along a track (arrows) containing Shot-GFP (green) and F-actin (blue). (F-J) Dlt is detected along the track after the track forms (compare G with H). Dlt concentration is initially lower in fusion cells along the apical surface connecting the existing lumens (I); it then accumulates in three rings (J, inset) at the anastomosis site (arrow). (K-O) Accumulation of Shot-GFP in fusion cells. (P-T) Formation of the F-actin track in fusion cells (P-R) and its association with new apical surface (S,T). Scale bar: 10 μm (5 μm for inset).

and existing apical surfaces do not simply stretch to connect with each other. Thus, a E-cadherin-associated track of F-actin and Shot appears to mark the site of new apical surface formation in fusion cells.

The interactions of Shot with the cytoskeleton are required for E-cadherin contacts that are essential for lumen formation in fusion cells

In *shot* mutant embryos, fusion cells frequently fail to form new E-cadherin contacts (Fig. 7E) and an associated F-actin track (Fig. 7G), but sometimes form inappropriate or misoriented E-cadherin contacts with tracheal cells other than their fusion partner (Fig. 7I,M). These aberrant contacts are not detectably associated with F-actin (Fig. 7K,N). These findings suggest that Shot is required to form or maintain the F-actin-containing structures associated with E-cadherin contacts, and to direct the formation of these contacts to the appropriate part of the fusion cells.

Rearranging the cytoskeleton during axon extension requires the presence of both the N-terminal F-actin binding domain and the C-terminal microtubule-binding GAS2 motif in the same Shot protein (Lee and Kolodziej, 2002). We therefore investigated whether these domains are required for lumen formation and cytoskeletal organization in fusion cells. Tracheal expression of Shot L(A)-GFP rescues anastomosis defects in the dorsal trunk (Fig. 8A), lateral trunk (Fig. 8A), and dorsal midline (data not shown). Fusion cell expression of Shot L(A)-GFP cells using an *esg-GAL4* enhancer trap also rescues these defects. However, these rescued embryos also express Shot L(A)-GFP at low levels in other tracheal cells (data not shown).

Surprisingly, tracheal expression of long Shot isoforms that lacked either the N-terminal F-actin binding domain (Fig. 8B) or the C-terminal microtubule-binding site (Fig. 8C) also restored lumen formation in fusion cells. Deleting both the F-actin binding site and the microtubule binding site abolished rescue activity (Fig. 8D), indicating that at least one cytoskeletal interaction domain must be present for fusion cells to form a connecting lumen. Expression of the Shot C-terminal domain-GFP fusion in tracheal cells does not rescue the anastomosis defects, suggesting that other domains in the Shot long isoforms are also required for activity (data not shown).

Shot L(A)-GFP accumulates apically in tracheal cells (Fig. 8E). Mutant derivatives that lacked either the F-actin (Fig. 8F) or the microtubule binding site (Fig. 8G) are largely apically concentrated, but are partially delocalized. However, Shot molecules that lack both the F-actin and microtubule-binding domains no longer accumulate apically (Fig. 8H). Thus, Shot concentrates apically via interactions with the cytoskeleton.

To investigate further the role of Shot in organizing the cytoskeleton, we examined F-actin and microtubule distributions in tracheal cells in rescued *shot* mutant embryos. Tracheal expression of Shot proteins that lack either the F-actin or the microtubule binding sites restores both normal F-actin distribution in fusion cells (Fig. 9A,B) and the apical accumulation of C-Shot L-GFP, a microtubule binding protein, in all tracheal cells (Fig. 9C,D). Normal Dlt localization in fusion cells was also restored in rescued embryos (data not shown). F-actin remodeling in fusion cells and apical accumulation of microtubules in tracheal cells can therefore occur provided that Shot can directly interact with either F-actin or microtubules. A Shot L(A) derivative that lacks both

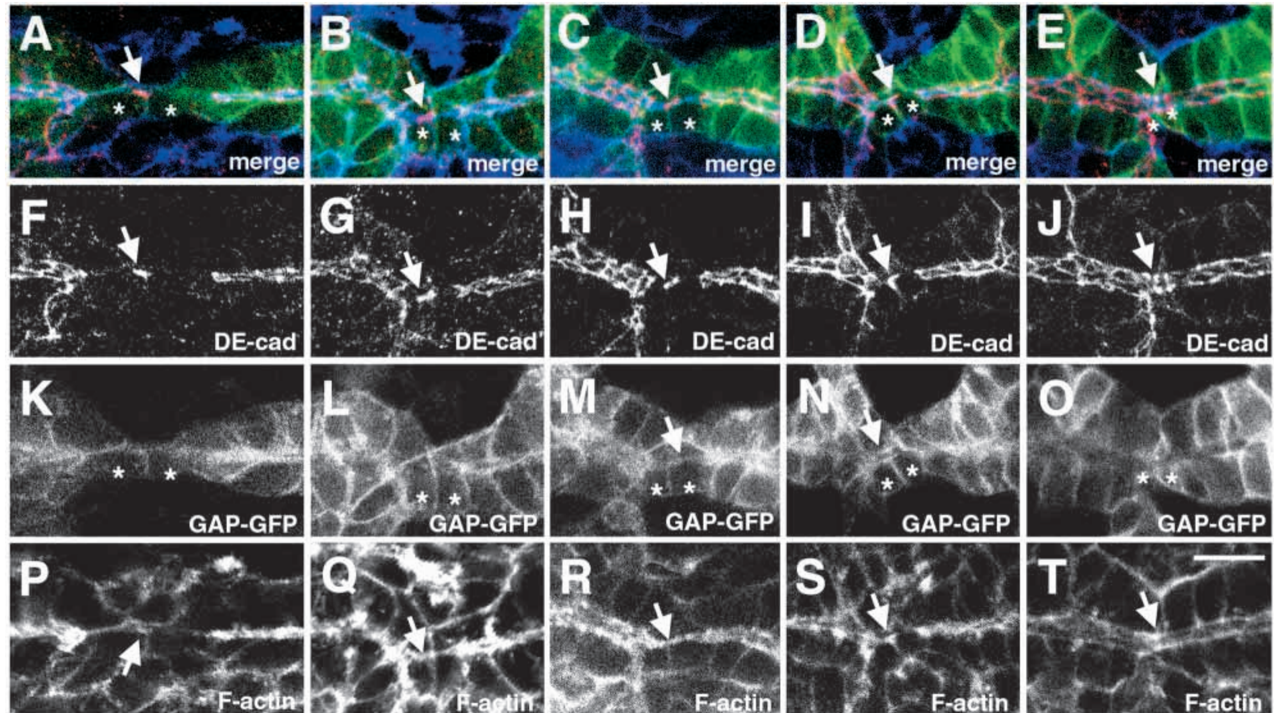


Fig. 6. Remodeling of E-cadherin (DE-cad) contacts and associated F-actin during anastomosis. (A-E) Tracheal cells (membranes outlined in green) in adjacent branches abut via the fusion cells (asterisks) and form a *D*E-cadherin contact (red, arrows) and associated F-actin (blue). (F-J) Remodeling of the E-cadherin contact (arrows) from a spot found in the anterior fusion cell (F,G) to one spanning both (M), and then a ring (I,J). (K-O) Membrane remodeling at the contact site. Initially (K,L), the fusion cells (asterisks) are round and their apical surfaces only face the existing lumens. As the contact matures (H), membrane is detected (arrow), spanning the fusion cells and connecting with these existing apical surfaces. This membranous compartment (arrow in N) expands as the cadherin contact becomes a ring (O). (P-T) An F-actin-rich track initiates at the site of E-cadherin contact (arrow in P) and lengthens to span the fusion cells (Q,R). As the central cadherin ring forms, the track disappears and F-actin is enriched at cadherin contacts (S,T). Scale bar: 10 μm.

the F-actin and the microtubule binding domains does not rescue F-actin remodeling in fusion cells (Fig. 9E), or apical accumulation of microtubules in all tracheal cells (Fig. 9F). Thus, the F-actin- and microtubule-binding domains of Shot are required for these processes, but appear functionally redundant. The interactions of Shot with the cytoskeleton may therefore facilitate additional organizing interactions between F-actin and microtubules.

RhoA regulates cytoskeletal organization, apical determinant localization and lumen formation in tracheal cells

Our findings that lumen formation defects are associated with the loss of E-cadherin-associated cytoskeletal structures led us to investigate the roles of other cytoskeletal regulators in lumen formation. E-cadherin signaling antagonizes the RhoA GTPase (Anastasiadis et al., 2000; Noren et al., 2000), which controls F-actin polymerization in many developmental contexts (Lu and Settleman, 1999). Constitutively active mutations in *Drosophila RhoA* reduce dendrite branching (Lee et al., 2000b), a phenotype also observed in *shot* mutant embryos (Prokop et al., 1998; Gao et al., 1999).

We therefore examined the effect of expressing constitutively active *RhoA*^{V14} in tracheal cells. Tracheal defects in these mutant embryos strongly resemble those in *shot* mutant embryos. Lumen formation is blocked at all anastomosis sites, but occurs normally in cells interior to branches (Fig. 10A). The effects of *RhoA*^{V14} on new apical cytoskeletal structures in fusion cells are also similar to those observed in *shot* mutant embryos, but more penetrant. In embryos expressing *RhoA*^{V14} in tracheal cells, F-actin (Fig. 10D) and Dlt (Fig. 10G) accumulate apically in tracheal cells, but surround blind-ended tubes terminating at anastomosis sites, rather than continuous lumenal connections. E-cadherin does not form a three ringed structure at anastomosis sites (Fig. 10J), and Shot L(A)-GFP is distributed apically around blind-ended tubes (Fig. 10L). Tracheal expression of *RhoA*^{V14} also disrupts apical accumulation of C-Shot L-GFP throughout the trachea (Fig. 10N), another similarity with *shot* mutant embryos (Fig. 2J). Fusion cells in embryos that express *RhoA*^{V14} in tracheal cells fail to form initial E-cadherin-containing contacts (Fig. 10P) and associated F-actin tracks (Fig. 10Q). *RhoA*^{V14} expression also modestly attenuates branch migration, but does not detectably affect the pattern of primary and secondary branching (Fig. 10A).

To investigate further the role of RhoA, we examined lumen formation in embryos expressing dominant negative *RhoA* (Lee et al., 2000b) in all tracheal cells. In these *RhoA*^{N19} embryos, cells in the dorsal trunk and elsewhere do not form a distinct lumenal cavity (Fig. 10B), though primary and secondary branching appears normal. Lumenal antigens recognized by mAb 2A12 (Fig. 10B) and anti-Shot (data not shown) accumulate around cells towards the center of the dorsal trunk, but not in a defined lumen. F-actin (Fig. 10E) is also more

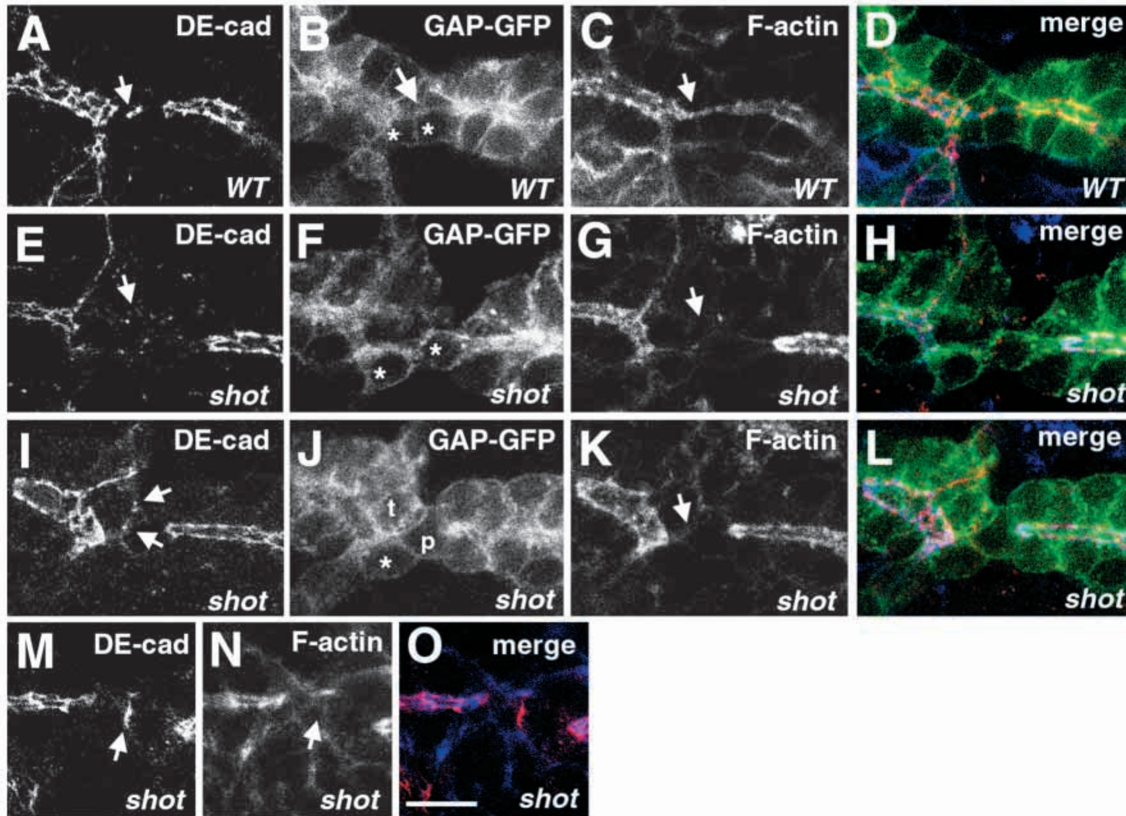


Fig. 7. *shot* mutants affect E-cadherin (DE-cad) contacts between fusion cells and F-actin associated with these contacts. (A-D) Stage 13, wild type. A E-cadherin (red in D) contact (arrow in A) between two fusion cells (asterisks in B) is associated with an F-actin (blue in D) containing track (arrow in C) spanning the two cells. Membrane (green in D)-associated with the track (arrow in B) bisects the fusion cells. (E-H) Stage 13, *shot*^{3}. E-cadherin contacts (arrow in E) between fusion cells (asterisks in F) fail to form, and no F-actin rich track is detected (arrow in G). Fusion cells were identified by their position (Figs 2,3). (I-L) Stage 13, *shot*^{3}. Weak E-cadherin contacts (arrows in I) form between the posterior branch fusion cell (p in J) and a tracheal cell (t) in the anterior branch. No F-actin-rich track is detected (arrow in K). (M-O) Stage 13, *shot*^{3}. Fusion cells occasionally form aberrantly oriented *D*E-cadherin contacts (arrow in M). These contacts lack detectable associated F-actin (arrow in O).

broadly and patchily distributed. Dlt is more extensively distributed, and is no longer restricted to a well-defined apical region (Fig. 10H). Surprisingly, E-cadherin localization appears normal (Fig. 10K), suggesting that these cells retain some polarity. Shot L(A)-GFP is distributed cortically, without any apical accumulation (Fig. 10M), and C-Shot L-GFP also fails to accumulate apically in tracheal cells expressing *RhoA*^{N19} (Fig. 10O). Cells in the dorsal trunk remain continuous (Fig. 10K,M,O) though they do not form a distinct lumen. These data therefore suggest that the formation of apical cytoskeletal structures and lumen require *RhoA*. *RhoA*^{N19} expression also modestly attenuates branch migration outside the dorsal trunk, suggesting an additional role in tracheal cell migration (data not shown).

RhoA, Rac and Cdc42 make up a family of related GTPases with specific roles in diverse F-actin-based morphogenetic processes (Hall, 1998). To determine whether the role of *RhoA* is specific, we also expressed wild-type *RhoA*, dominant negative *Drosophila Rac1* (Luo et al., 1994) or *Cdc42* (Luo et al., 1994) in tracheal cells and examined tracheal development. None of these alleles detectably affects lumen formation or apical structures (Fig. 10C,F,I and data not shown), though *Cdc42*^{N17} and *Rac1*^{N17} embryos exhibit modest defects in branch migration (data not shown). Thus, the role of RhoA role in controlling the formation of apical cytoskeletal structures is likely to be specific among these Rho family GTPases.

DISCUSSION

Organizers of the cytoskeleton are required for E-cadherin dependent apical surface remodeling in fusion cells

Lumen formation between tracheal branches requires fusion cells to make new E-cadherin-containing contacts with each other and to remodel their apical surfaces (Uemura et al., 1996). The results presented here provide further insights into how the cytoskeleton and associated proteins support contact formation and subsequent apical surface remodeling. The F-actin- and microtubule-binding domains of Shot are required to maintain and remodel E-cadherin contacts and to assemble a track of F-actin and Shot in fusion cells. This track initiates at the E-cadherin contact and extends outwards from it to connect with the existing apical assemblies of F-actin and Shot. We propose that the track guides new apical surface formation. Apical surface determinants and membrane appear

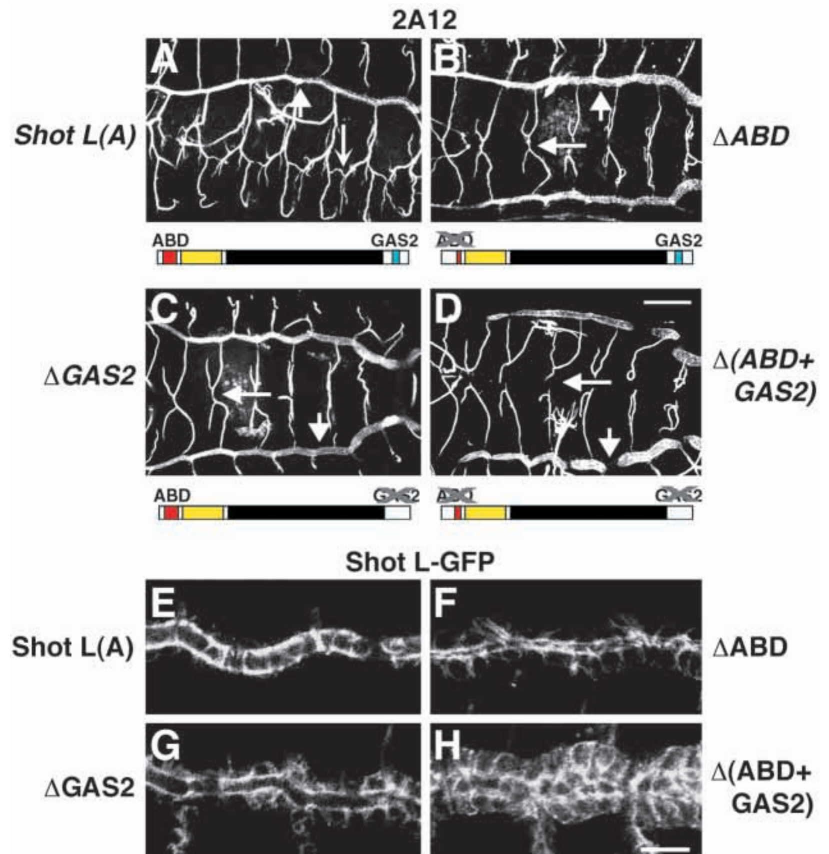
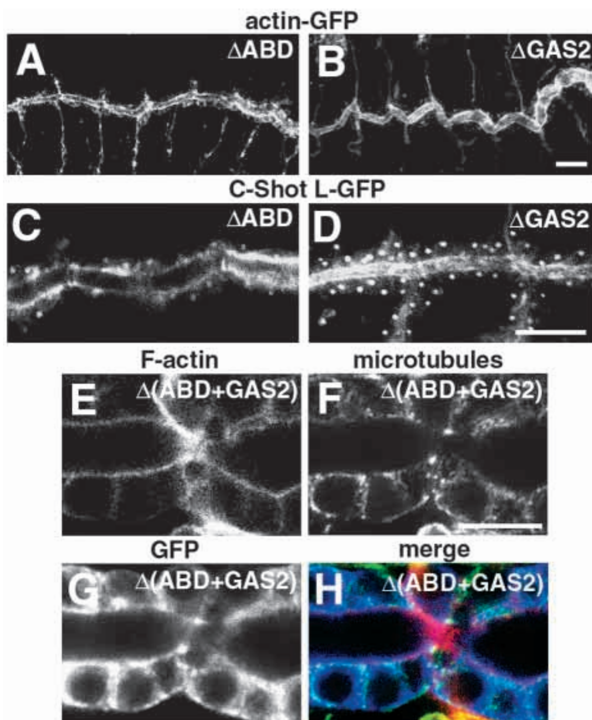


Fig. 8. The F-actin- and the microtubule-binding domains of Shot proteins redundantly promote lumen formation in fusion cells. These cells occupy anastomosis sites in the dorsal (arrows) and lateral (concave arrows) trunks and at the dorsal midline (long arrows). (A-D) Rescue activity of Shot L(A)-GFP and derivatives in stage 16 *shot*^{3} mutant embryos. Shot L(A)-GFP (schematic in A) contains an N-terminal F-actin binding domain (orange), central plakin-like (yellow) and spectrin repeat domains (black), and a C-terminal domain that binds microtubules via the GAS2 motif (blue). The gray X is used to indicate domains missing in Shot L(A)-GFP derivatives (schematics in B-D). (A) Lateral view. Tracheal expression of Shot L(A)-GFP restores lumen formation in fusion cells in the dorsal and lateral trunks. (B) Dorsal view. Tracheal expression of actin-binding defective Shot L(C)-GFP restores lumen formation in fusion cells in the dorsal trunk and at the dorsal midline. No gaps in the dorsal trunk were observed in ten rescued embryos, whereas all mutant embryos have multiple gaps. (C) Tracheal expression of a Shot L(A)-GFP derivative that lacks the microtubule binding GAS2 motif restores lumen formation in dorsal trunk and midline fusion cells. No gaps in the dorsal trunk were observed in ten rescued embryos. (D) Tracheal expression of a Shot L(A)-GFP fusion that lacks both cytoskeletal interaction domains does not restore lumen formation in dorsal trunk or midline fusion cells. (E-H) 1 μm confocal sections through the middle of the dorsal trunk of stage 15 wild-type embryos expressing Shot-GFP fusions. All Shot-GFP fusions produced green fluorescence when expressed in embryos and were expressed at comparable levels, approx. five- to tenfold higher than that of endogenous Shot proteins. GFP fusions were visualized with anti-GFP to enhance signal detection. (E) Shot L(A)-GFP accumulates apically in tracheal cells. (F) Shot L(C)-GFP accumulates apically, but is less apically concentrated than Shot L(A)-GFP. (G) Shot L(A)-GFP-ΔGAS2 accumulates apically, but is less apically concentrated than Shot L(A)-GFP. (H) Shot L(C)-ΔGAS2-GFP molecules lack both the F-actin- and the microtubule-binding domains, and are cortically and cytoplasmically distributed. Scale bars: in D, 25 μm for A-D; in H, 25 μm for E-H.



to accumulate along the track, possibly by spreading from existing apical concentrations. This track may also enable the fusion cells to contract and to draw the existing lumenal surfaces closer, as fusion cells appear notably less compact in *shot* mutant embryos.

Loss-of-function *shot* and gain-of-function *RhoA* alleles have similar phenotypes in fusion cells, and *RhoA* disrupts Shot

Fig. 9. Interactions between Shot and the cytoskeleton organize the tracheal cell cytoskeleton. (A,B) Stacks of five 1 μm confocal sections through dorsal trunk cells. (A) Stage 15, *shot*^{3}. Tracheal expression of Shot L(C) restores normal actin-GFP distribution in fusion cells. (B) Stage 16, *shot*^{3}. Tracheal expression of Shot L(A)-ΔGAS2 restores normal actin-GFP distribution in fusion cells. (C,D) Confocal sections (1 μm) of dorsal trunk cells. (C) Stage 15, *shot*^{3}. Tracheal expression of Shot L(C) restores apical accumulation of C-Shot L-GFP, a microtubule-binding protein. (D) Stage 15, *shot*^{3}. Tracheal expression of Shot L(C) restores apical accumulation of C-Shot L-GFP. (E-H) Stage 15, *shot*^{3}. Dorsal trunk tracheal cells express Shot L(C)-ΔGAS2-GFP, which lacks both cytoskeletal interaction domains. (E) Apical accumulations of F-actin (phalloidin staining) surround blind-ended lumens terminating at an anastomosis site. (F) Microtubules (detected with anti-tubulin) do not accumulate apically in tracheal cells. (G) Shot L(C)-ΔGAS2-GFP distribution. (H) E-G merge. Scale bars: in B, 25 μm for A,B; in D, 10 μm for C,D; in H, 10 μm for E-H.

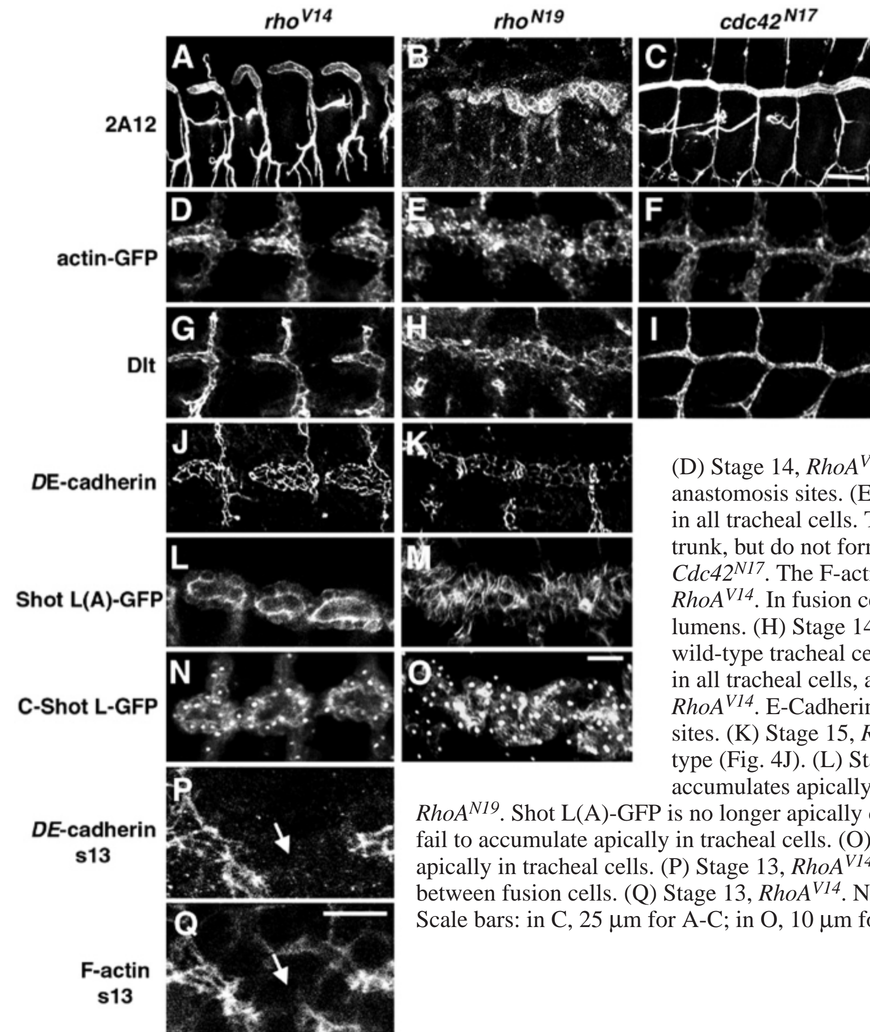


Fig. 10. *RhoA* mutations disrupt lumen formation, the organization of apical cytoskeletal structures and apical determinant localization in tracheal cells. (A-C) Stacks of eight 1 μm confocal sections; embryos stained with mAb 2A12 to reveal the lumen. (A) Stage 16 embryo that expresses $RhoA^{V14}$ in all tracheal cells ($RhoA^{V14}$ embryo). Fusion cells in the dorsal and lateral trunk do not form bridging lumenal connections. (B) Stage 15 embryo that expresses $RhoA^{N19}$ in all tracheal cells ($RhoA^{N19}$ embryo). 2A12 antigen does not accumulate in a discrete lumenal space, and is diffusely distributed extracellularly. (C) Stage 15 embryo that expresses $Cdc42^{N17}$ in all tracheal cells ($Cdc42^{N17}$ embryo). The lumen is continuous through fusion cells. (D-Q) Confocal sections (1 μm) through dorsal trunk cells. (D) Stage 14, $RhoA^{V14}$. F-actin surrounds blind-ended lumens at anastomosis sites. (E) Stage 14, $RhoA^{N19}$. F-actin accumulates in patches in all tracheal cells. The patches tend to be in the center of the dorsal trunk, but do not form a normal subapical structure. (F) Stage 14, $Cdc42^{N17}$. The F-actin distribution appears normal. (G) Stage 14, $RhoA^{V14}$. In fusion cells, Dlt accumulates apically around blind-ended lumens. (H) Stage 14, $RhoA^{N19}$. Dlt is more broadly distributed than in wild-type tracheal cells. (I) Stage 14, $Cdc42^{N17}$. Dlt accumulates apically in all tracheal cells, as in stage 14 wild-type embryos. (J) Stage 15, $RhoA^{V14}$. E-Cadherin fails to form a three-ringed structure at anastomosis sites. (K) Stage 15, $RhoA^{N19}$. E-cadherin distribution is similar to wild-type (Fig. 4J). (L) Stage 15, $RhoA^{V14}$. In fusion cells, Shot L(A)-GFP accumulates apically, but the lumens are blind-ended. (M) Stage 15, $RhoA^{N19}$. Shot L(A)-GFP is no longer apically concentrated. (N) Stage 15, $RhoA^{V14}$. Microtubules fail to accumulate apically in tracheal cells. (O) Stage 15, $RhoA^{N19}$. Microtubules fail to accumulate apically in tracheal cells. (P) Stage 13, $RhoA^{V14}$. No E-cadherin contact (arrow) is detectable between fusion cells. (Q) Stage 13, $RhoA^{V14}$. No F-actin track (arrow) is detectable in fusion cells. Scale bars: in C, 25 μm for A-C; in O, 10 μm for D-O.

localization. We therefore propose that RhoA negatively regulates track assembly and E-cadherin contact remodeling by Shot. Apically organized F-actin and adherens junctions in other tracheal cells appear to develop normally in *shot* mutant and $RhoA^{V14}$ embryos, suggesting specific requirements for *shot* and RhoA during new apical surface formation in fusion cells. We propose that Shot and RhoA regulate E-cadherin-dependent cell adhesion in selected developmental contexts.

Redundant interactions between Shot and cytoskeletal elements organize the fusion cell cytoskeleton

We and others have shown that *shot* is required in neurons for growth cone motility (Van Vactor et al., 1993; Kolodziej et al., 1995; Prokop et al., 1998; Gao et al., 1999; Lee et al., 2000a). We show here that *shot* is required to remodel E-cadherin-containing contacts between tracheal fusion cells. Surprisingly, Shot proteins perform these distinct morphogenetic roles using different combinations of the same cytoskeletal interaction domains. In fusion cells, the binding sites for F-actin and microtubules appear functionally redundant. The F-actin binding domain is essential when the GAS2 microtubule binding site is absent, and the GAS2 microtubule binding site is essential when the F-actin binding site is absent. By contrast, during axon extension, the Shot behaves as an F-actin/microtubule cross-linker because the cytoskeletal interaction domains are both individually essential and required in the same molecule (Lee and Kolodziej, 2002).

These observations suggest that direct interactions between Shot and cytoskeletal proteins organize the cytoskeleton in fusion cells. The F-actin and microtubule domains may directly enable the accumulation of their cytoskeletal partners at the E-cadherin contact. In support of this hypothesis, the structurally similar F-actin binding domain of plectin alters F-actin organization (Andra et al., 1998) and the GAS2 motif stabilizes associated microtubules against depolymerization (Sun et al., 2001) in cultured cells. Since Shot's interactions either with F-actin or with microtubules suffice to organize both cytoskeletal elements, binding to either F-actin or microtubules may then enhance other organizing interactions between F-actin and microtubules.

These other interactions may involve molecules required for E-cadherin signaling. E-cadherins are physically linked to F-actin via the β-catenin/α-catenin complex (Gumbiner, 2000) and to dynein, a microtubule-based motor, via β-catenin (Ligon et al., 2001). They can further regulate actin dynamics via association with p120, a RhoA antagonist (Anastasiadis et al., 2000; Noren et al., 2000); E-cadherins also stabilize microtubule minus ends in cultured cells (Chausovsky et al., 2000). E-cadherin signaling may therefore affect other proteins

mediating interactions between F-actin and microtubules. Candidates include other F-actin/microtubule cross-linkers (Fuchs and Yang, 1999; Goode et al., 2000), regulators of Rho family GTPases that bind to microtubules (Ren et al., 1998; Glaven et al., 1999; Waterman-Storer et al., 1999) and F-actin-based motors that form complexes with microtubule-based motors (Huang et al., 1999). Further analysis will be necessary to identify these other molecules in fusion cells; these other cytoskeletal regulators may permit residual anastomoses in *shot* mutant embryos.

RhoA controls the formation of apical cytoskeletal structures in tracheal cells

Our analysis also indicates that RhoA is required for lumen formation, most probably by regulating the apical cytoskeleton or by affecting the transport of lumenal antigens. Similarities between $RhoA^{V14}$ and *shot* mutant phenotypes suggest that RhoA could work either to antagonize Shot activity, or through parallel pathways acting on F-actin and microtubules. RhoA has many effectors that control F-actin distribution (Hall, 1998). $RhoA^{V14}$ has been reported to stabilize subsets of microtubules in fibroblasts in culture via an F-actin-independent pathway (Cook et al., 1998). Shot localizes apically via its interactions with the cytoskeleton, and either these interactions or the cytoskeletal structures themselves may be RhoA-regulated.

In cells throughout the trachea, reduced RhoA activity disrupts lumen formation and partially disrupts Dlt localization. Tracheal expression of $RhoA^{N19}$ does not appreciably affect E-cadherin localization. In cultured epithelial cells, E-cadherin localization is also resistant to $RhoA^{N19}$ (Jou and Nelson, 1998). These findings are consistent with RhoA functioning downstream of or parallel to E-cadherin. E-cadherin-associated p120ctn negatively regulates RhoA (Anastasiadis et al., 2000; Noren et al., 2000), but whether a similar pathway operates in *Drosophila* is unknown.

In fusion cells, RhoA can also function upstream of E-cadherin, as constitutively active $RhoA^{V14}$ affects E-cadherin localization selectively in these cells. E-cadherin distribution is more dynamic in fusion cells than in other tracheal cells (Tanaka-Matakatsu et al., 1996; Uemura et al., 1996), and may therefore be more sensitive to $RhoA^{V14}$. $RhoA^{V14}$ also affects new E-cadherin contacts in culture (Jou and Nelson, 1998). Further experiments will reveal whether Shot, RhoA and E-cadherin function in a common, evolutionarily conserved pathway to regulate apical surface remodeling in fusion cells.

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