Distinct sites in E-cadherin regulate different steps in *Drosophila* tracheal tube fusion

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Summary

We have investigated how E-cadherin controls the elaboration of adherens junction associated cytoskeletal structures crucial for assembling tubular networks. During *Drosophila* development, tracheal branches are joined at branch tips through lumens that traverse doughnut-shaped fusion cells. Fusion cells form E-cadherin contacts associated with a track that contains F-actin, microtubules, and Shot, a plakin that binds F-actin and microtubules. Live imaging reveals that fusion occurs as the fusion cell apical surfaces meet after invaginating along the track. Initial track assembly requires E-cadherin binding to β-catenin. Surprisingly, E-cadherin also controls track maturation via a juxtamembrane site in the cytoplasmic domain. Fusion cells expressing an E-cadherin mutant in this site form incomplete tracks that contain F-actin and Shot, but lack microtubules. These results indicate that E-cadherin controls track initiation and maturation using distinct, evolutionarily conserved signals to F-actin and microtubules, and employs Shot to promote adherens junction-associated cytoskeletal assembly.

Key words: E-cadherin, *Drosophila*, Cytoskeleton

Introduction

Adherens junctions separate apical from basolateral surfaces in epithelial cells (Tepass, 2002; Perez-Moreno et al., 2003), enabling them to form sheets and tubes (Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003). E-cadherin and proteins associated with its cytoplasmic domain are key components of adherens junctions, and mediate homophilic, Ca^{2+}-dependent adhesion between cells. E-cadherin also plays essential roles in the dynamic developmental processes of migration (Niewiadomska et al., 1999), wound healing (Danjo and Gipson, 1998; Vaezi et al., 2002) and tracheal tube fusion (Tanaka-Matakatsu et al., 1996; Uemura et al., 1996) during *Drosophila* development. During these processes, new E-cadherin contacts must be made or broken for cells to change shape or move.

These remodeling processes require the adherens junction to organize the cytoskeleton. E-cadherin interacts with the F-actin cytoskeleton via the β-catenin/α-catenin complex (Rimm et al., 1995), and may also regulate F-actin assembly via Rho family GTPases (Kuroda et al., 1998; Anastasiadis and Reynolds, 2001; Kovacs et al., 2002a; Lampugnani et al., 2002) and the ARP2/3 complex (Kovacs et al., 2002b). E-cadherin can also organize and stabilize microtubules in cultured cells via an unknown mechanism (Chausovsky et al., 2000; Waterman-Storer et al., 2000). However, cytoskeletal structures associated with adherens junctions during development are not well characterized, nor are their functions understood.

Tracheal tube fusion in the *Drosophila* embryo provides a powerful system for investigating how cells remodel their E-cadherin contacts (Hogan and Kolodziej, 2002). During the fusion process, specialized cells at tracheal branch tips, fusion cells, meet their partners in the adjacent hemisegment at precise locations along segment boundaries (Samakovlis et al., 1996b) (Fig. 1A). A new E-cadherin contact forms between these fusion cells (Tanaka-Matakatsu et al., 1996; Uemura et al., 1996), and is associated with a track of F-actin and the plakin Short Stop (Shot) (Lee and Kolodziej, 2002). The evolutionarily conserved Shot proteins bind F-actin and microtubules to promote fusion (Lee and Kolodziej, 2002). Presumably, microtubules are also in the track, although they have only been detected indirectly (Lee and Kolodziej, 2002). This track then grows to span the two cells. These cells become doughnut shaped as lumenal connections between the branches form along the track (Lee and Kolodziej, 2002). Tracheal tube fusion may reveal mechanisms underlying tube formation in other systems. Similar doughnut-shaped cells exist in the mammalian vasculature (Wolff and Bar, 1972), and VE-cadherin is required for blood vessel formation (Lampugnani et al., 2002).

Like Shot, E-cadherin (Uemura et al., 1996) and the *Drosophila* β-catenin ortholog *armadillo* (*arm*) (Beitel and Krasnow, 2000) are required for tracheal tube fusion. However, the E-cadherin-dependent step has not been identified at the cellular level, and the signals from E-cadherin to other pathway components are not well characterized. Two regions in the E-cadherin intracellular domain have been implicated in

adhesion and other cadherin functions, such as migration. A juxtamembrane region binds to p120 (Reynolds et al., 1994; Yap et al., 1998), a substrate of the Src tyrosine kinase (Mariner et al., 2001), and the C-terminal region of the tail binds to β-catenin (Aberle et al., 1994; Jou et al., 1995). In cell culture and genetic assays, the β-catenin binding site appears essential for adhesion (Ozawa et al., 1990; Ozawa and Kemler, 1998; Yap et al., 1998). Recently, the single *Drosophila* gene encoding a p120 family member has been shown to be nonessential (Myster et al., 2003), though it modulates E-cadherin function. Moreover, a mutation in *Drosophila* E-cadherin that disrupts p120 binding does not detectably affect E-cadherin activity in cell adhesion and migration (Pacquelet et al., 2003).

We have further investigated how E-cadherin and Shot regulate track development during tracheal tube fusion. The formation of a Shot-containing track and its subsequent maturation are obligate intermediate steps in fusion. Surprisingly, E-cadherin controls both of these steps using distinct sites in its cytoplasmic domain. E-cadherin binding of β-catenin is required for fusion track formation. The juxtamembrane site, previously thought to be dispensable in *Drosophila*, controls track maturation. When expressed in wild-type tracheal cells, an E-cadherin bearing a mutation in this site causes fusion tracks to form that contain F-actin and Shot, but lack detectable microtubules. Fusion involves direct interactions between Shot and microtubules (Lee and Kolodziej, 2002). We propose that distinct sites in the E-cadherin cytoplasmic domain mediate the initial assembly of F-actin and recruitment of Shot to the fusion track, and subsequent microtubule-dependent track maturation.

Materials and methods

Fly strains

E-cadherin mutants *shg*^{2} and *shg*^{IH} were obtained from U. Tepass and T. Uemura. In null *arm*^{YD35}*; P[arm*$^{S14\text{-}C}$*]* mutant embryos, a mutant *armadillo* gene rescues β-catenin wingless signaling functions, but not E-cadherin association (Orsulic and Peifer, 1996). Transgenic flies expressing *E-cadherin* cDNAs were obtained by standard methods (Robertson et al., 1988). The *btl-GAL4* enhancer trap drives transgene expression in all tracheal cells (Samakovlis et al., 1996a). Track formation was visualized in *UAS-Shot L(A)-GFP; btl-GAL4 shg*^{2}*/shg*^{IH} mutant embryos. Fusion was filmed in *UAS-Shot L(A)-GFP; btl-GAL4* wild-type embryos or in *UAS-Shot L(A)-GFP; UAS-AAA-JXT /btl-GAL4* (*AAA-JXT*) mutant embryos.

Molecular biology

Using the Quikchange Multi kit (Stratagene), mutations were introduced into a full-length E-cadherin cDNA (Accession Number D28749) from T. Uemura (Kyoto University), that changed residues 1607-1609 to alanines (*AAA-JXT*) or deleted residues 1672-1696 (*Δ-arm*). Wild-type and mutant cDNAs were cloned into the *Eco*RI/*Not*I sites of pUASt, a P-element containing GAL4 expression vector (Brand and Perrimon, 1993).

Monoclonal production

A DNA fragment encoding residues 1454-1909 amplified from the Shot L(A) cDNA (Accession Number CAA09869), subcloned into pGEX6P1 (Amersham) was used to produce GST fusion protein for immunization. Mouse hybridomas (Vanderbilt Hybridoma Facility) were screened in pools of 10 for immunoreactivity with fly embryos and mAb Rod1 isolated by limiting dilution of a reactive pool.

Immunohistochemistry

Fusion tracks were scored in fillets using confocal microscopy. Fillets were staged using gut morphology prior to dissection and neuronal morphology after dissection. Scored embryos had well formed commissural and some longitudinal CNS axon tracts, and fusion cells had made contact by membrane-GFP staining. To visualize microtubules, wild-type *btl-GAL4 UAS-GAP43-GFP* or mutant *btl-GAL4 UAS-GAP43-GFP/+; UAS-E-cad-AAA-JXT* embryos were filleted in a silicone gel well on a glass slide and quickly fixed by exposure to 90% methanol, 5 mM sodium bicarbonate pH 9, 3% formaldehyde chilled to –70°C for 10 minutes (Rogers et al., 2002). The fillets were carefully rehydrated in PBS/0.1% Triton containing 0.2% BSA (bovine serum albumin). Otherwise, fillets were fixed in 4% formaldehyde in Ringer's solution at room temperature for 20 minutes and processed as described (Lee and Kolodziej, 2002). Schneider S2 cells were transfected with CellFECTIN (Invitrogen). pRmHA3-p120 was used to express p120 (Pacquelet et al., 2003), and pActin-GAL4 and pUASt-AAA-JXT-E-cadherin were used to express AAA-JXT E-cadherin.

Primary antibodies were: rabbit anti-EB1 (Rogers et al., 2002) (1:500), rabbit anti-CLIP190 (Lantz and Miller, 1998) (1:1000), rabbit anti-β-galactosidase (Sigma), rat anti-tubulin (Serotec), rat mAb anti-cadherin (1:5) (Oda et al., 1994), mouse mAb anti-Shot mAbRod1 (1:10), mouse mAb 2A12 (1:10), and mouse mAb 12CA5 (anti-hemagglutinen (HA) epitope tag, 1:200). Images were acquired on a Zeiss LSM 510 confocal microscope using filter sets and excitation frequencies specific for FITC, Cy3 or Cy5. Live images were acquired from dechorionated, late stage 12 embryos expressing *Shot L(A)-GFP (UAS-Shot-L(A)-GFP/+; btl-GAL4/+)* (Lee and Kolodziej, 2002) mounted laterally on a Matek coverslip/culture dish and covered with modified insect saline solution (Kim et al., 2002) containing 2% methylcellulose (Sigma). Argon laser power was set at an average of 10% (adjusted progressively downward to avoid saturating signal); a pinhole yielding 0.5 μm slices was used with a GFP filter set. Fluorescence signals were acquired in the linear range, and optimized for signal to noise by manually adjusting computer generated levels. To compensate for embryo movement or curvature, images were composited manually from serial sections using Adobe Photoshop 6.0 (Adobe Systems), and compared to 3D reconstructions made with Metamorph (Universal Imaging). A median filter was applied to remove noise.

E-cadherin was extracted for western blotting from embryos quick frozen on dry ice, and ground with a pestle in 1% NP40 extraction buffer (McNeill et al., 1993). Samples adjusted for equal amounts of protein were transferred to Immobilon-P (Millipore), western blotted and probed with rat anti-E-cadherin (Oda et al., 1994) at 1:1000. The membrane was further probed with HRP conjugated-anti-rat (Jackson Immunochemicals) at 1:5000 and developed with Supersignal (Pierce).

Results

Fusion proceeds via invagination of the existing apical surface along a cytoskeletal track that contains Shot

During tube fusion, the new adherens junction between fusion cells becomes associated with an F-actin rich, Shot-containing track that stretches across the fusion cells (Fig. 1A). The track appears before lumen forms and apical surface determinants are deposited (Lee and Kolodziej, 2002), so it appears to be an intermediate. However, the dynamic relationship between track and new lumen formation is unclear. For example, how long the track persists and how it may guide lumen formation are unknown.

To address these issues, we imaged Shot-GFP during

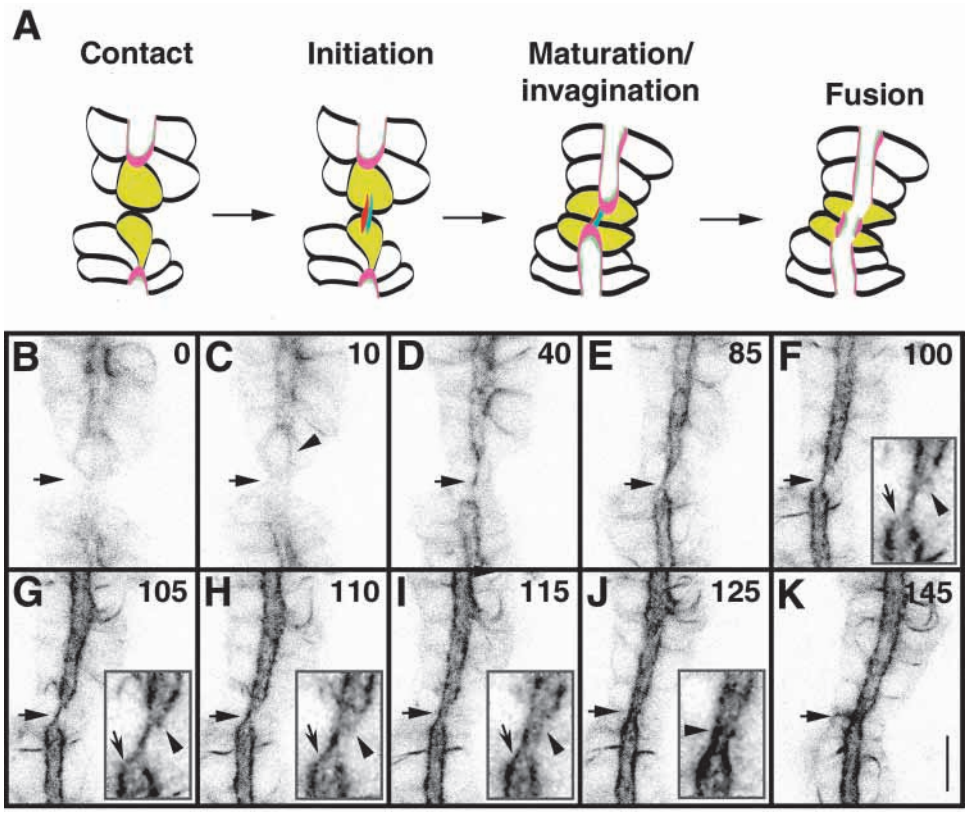

Fig. 1. Tube fusion proceeds through the formation and the maturation of a Shot containing track in fusion cells. **(**A) Schematic of morphological and cytoskeletal changes in fusion cells during fusion in an optical section along the plane of contact. Fusion cells (yellow) meet. They then form a new E-cadherin contact (blue) and initiate the assembly of a cytoskeletal track (red) that contains F-actin, Shot and microtubules (see Fig. 1B-K). This track matures, growing to span the two cells and guiding the invagination of the existing apical surfaces (light green) and associated cytoskeleton (pink). As the branches draw closer, the fusion cells also compress along the anteroposterior axis and elongate along the dorsoventral axis, bringing the existing apical surfaces closer. When the two apical surfaces meet, they fuse, leaving a ring of E-cadherin and associated cytoskeleton at the junction between the fusion cells. The two mature fusion cells are doughnut shaped. Other tracheal cells are wedge shaped. (B-K) Time lapse series of 0.5 μm confocal images of dorsal trunk tracheal cells in a live wild-type embryo expressing Shot L(A)-GFP in tracheal cells. Fusion sites are indicated by arrows, and are enlarged in F-J. Elapsed time is indicated in minutes (upper right corners). (B) At the start of the sequence (late stage 12), the tracheal branch tips are touching, but no track of Shot-GFP is visible at the future fusion site (arrow). (C) At 10 minutes, the anterior fusion cell accumulates Shot-GFP (arrowhead) along one side. (D) At 40 minutes, more Shot has begun to accumulate, and the track is centered more on the fusion cell contact. (D-G) A persistent track of Shot is visible from 40 minutes to 105 minutes. (F-I) The apical surfaces exhibit dynamic behavior (insets; concave arrow indicates an area that accumulates more Shot; the arrowheads indicate an area that loses and then regains Shot). (H) At 110 minutes, the apical surfaces of the two branches come closer together, forming a bottleneck structure. (I) At 115 minutes, a second track of Shot is clearly visible in this cross-section, indicating that the tubes are now joined. (J) The second track becomes darker, and the opening between the branches swells. A ring of Shot is visible (arrowhead, inset). (K) At 145 minutes, the opening assumes a diameter close to that of the adjacent branches. Scale bar: 10 μm. Anterior, upwards; dorsal, rightwards.

tracheal tube fusion in the dorsal trunk. Movies of several fusion events share similar kinetics and reveal details of the fusion process. In the first phase, contact, tracheal cells at branch tips touch, but there is no sign of Shot-GFP at the interface between the tips (Fig. 1B). The second phase, track initiation, begins ~10 minutes after initial contact. A faint track of Shot-GFP is detected in the anterior fusion cell, consistent with observations in fixed tissue that detect new E-cadherin (Tanaka-Matakatsu et al., 1996) and associated cytoskeletal assembly first in the anterior cell (Lee and Kolodziej, 2002) (Fig. 1C). Forty minutes later, this track is centered on the fusion cell contact (Fig. 1D). This cytoskeletal intermediate persists for ~1 hour, and accumulates progressively more Shot (Fig. 1D-G). Regions of the existing apical surface, defined by Shot-GFP accumulation, appear to change in intensity during this second phase (insets of fusion site in Fig. 1F-G).

In the third phase, track maturation and surface invagination, the existing apical surfaces invaginate and the track shrinks, eventually forming a bottleneck at the narrowest point (Fig. 1H). The track to bottleneck transition occurs within five minutes. In cross-section, the apical surfaces appear connected on one side and open on the other (Fig. 1H). In a final phase, fusion and expansion, the bottleneck expands to form a tube (Fig. 1I). In cross-section, two tracks of Shot-GFP are now clearly visible (Fig. 1I). Fusion and expansion is also very fast, occurring in five minutes or less, although expansion to the same diameter as the rest of the trachea takes longer (Fig. 1K). During this period, Shot becomes concentrated into a ring at the adherens junction between the fusion cells (Fig. 1J).

The new lumenal connection between the branches appears to develop as the existing apical surfaces approach closer along the track. We do not observe the development of a cavity internal to the fusion cells that subsequently fuses with its neighbors, an alternative possibility.

Shot colocalizes with dynamic microtubules in the fusion track

Our previous study suggested that the interactions of Shot with microtubules promote adherens junction and track assembly in fusion cells (Lee and Kolodziej, 2002). However, microtubules were not detected as fusion track components under the fixation conditions used in this earlier study. In order to determine whether microtubules are present, we dissected live embryos at early stage 13, a stage where fusion is ongoing in

the dorsal trunk, and fixed them rapidly in methanol/formaldehyde at –70°C, a treatment that preserves more dynamic microtubules. Under these conditions, microtubules are clearly detected apically in all tracheal cells even at these early stages (late stage 12 to late 13), and colocalize with the Shot/F-actin containing fusion track (Fig. 2A-F). Microtubules in tracheal cells at this stage of development appear especially sensitive to fixation conditions, as they were not observed using formaldehyde fixation at room temperature (Lee and Kolodziej, 2002).

To investigate the dynamic nature of these microtubules, we examined the distribution of two plus end microtubule binding proteins, EB1 and CLIP190, the *Drosophila* ortholog of mammalian CLIP170. Both of these proteins are associated with dynamic populations of microtubules in cells in culture (Perez et al., 1999; Nakamura et al., 2001; Komarova et al., 2002; Rogers et al., 2002), and like Short Stop, can link microtubules to cortical F-actin by binding to other proteins (Lantz and Miller, 1998; Korinek et al., 2000; Fukata et al., 2002). Both of these proteins concentrated apically in tracheal cells, and both of them were detected in the fusion track (Fig. 2A-B,G-H). Thus, the microtubules in the fusion track are probably highly dynamic.

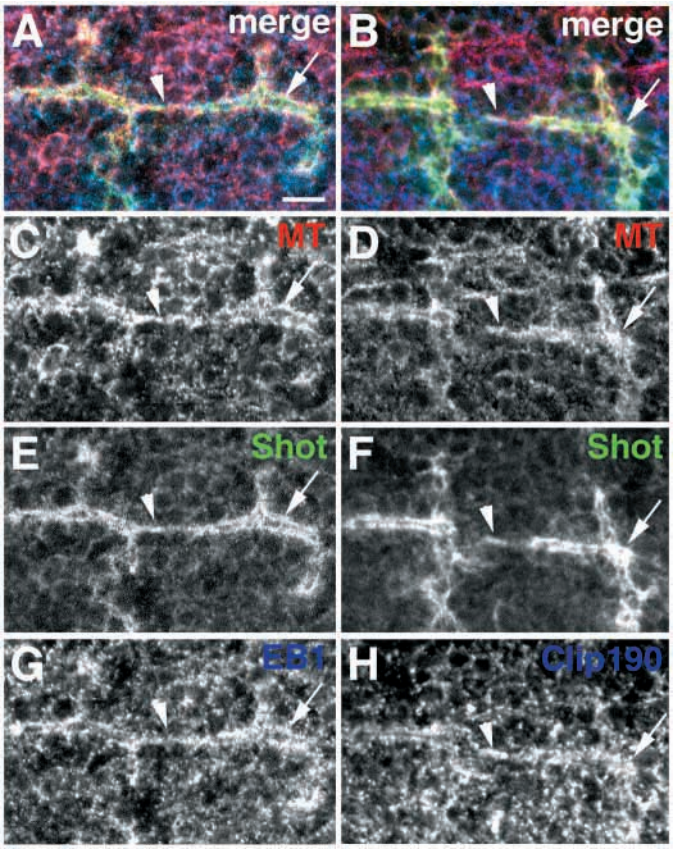

Fig. 2. Shot colocalizes with microtubules and the microtubule + end-binding proteins EB1 and CLIP190 in the fusion track. Anterior, leftwards; dorsal, upwards. (A,C,E,G) Before fusion; (B,D,F,H) after fusion. (A-H) Composited 1 μm confocal sections of stage 13 wild-type embryos. (A) Prior to fusion, a track (arrowhead) containing microtubules (red), Shot (green) and EB1 (blue) forms in fusion cells. The same proteins also colocalize apically (arrow). Merge of C, E, G. Scale bar: 10 μm. (B) The fusion track (arrowhead) contains CLIP190 (blue), as well as microtubules (red) and Shot (green, merge of D,F,H). (C,D) Microtubules in the fusion track (arrowhead). (E,F) Shot in the fusion track (arrowhead). (G) EB1 in the fusion track (arrowhead). (H) Clip 190 in the fusion track (arrowhead).

Short Stop is associated with adherens junctions

Short Stop (Shot) is required for fusion cells to form the new E-cadherin contacts that drive fusion, and a Shot-GFP fusion localizes to the cytoskeletal track in fusion cells that is associated with the new E-cadherin contact (Lee and Kolodziej, 2002) (Fig. 1). These data suggest that Shot may be associated with adherens junctions, at least in tracheal cells. To visualize the endogenous protein in tracheal cells, we raised a monoclonal antibody against a region of the rod domain, mAbRod1. mAbRod1 recognizes Shot protein in wild-type (Fig. 3A-H), but not in *shot*^{3} null mutant embryos (Fig. 3J-L). Co-staining of tracheal cells with a rat monoclonal antibody directed against E-cadherin (Oda et al., 1994) reveals that Shot colocalizes with adherens junctions (Fig. 3A-C). In *shot* mutant embryos, adherens junctions between tracheal branches frequently fail to form, and the branches therefore fail to fuse (Fig. 3J).

Shot also colocalizes with E-cadherin at other selected adherens junctions, most notably those associated with chordotonal neurons and their support cells (Fig. 3D-F). In *shot* mutant embryos, these contacts form, but are disorganized or morphologically abnormal (Fig. 3K). Microtubule organization is abnormal in these support cells (Prokop et al., 1998). In epidermal cells, 1 μm confocal sections show that both E-cadherin and Shot are cortically distributed, but E-cadherin is more concentrated apically than Shot (Fig. 3G-I and data not shown). In contrast to tissues where there is appreciable colocalization between Shot and E-cadherin, E-cadherin-dependent epidermal contacts appear normal in *shot* mutant embryos (Fig. 3L). In glial cells at the CNS midline, and in CNS axons, Shot does not detectably colocalize with E-cadherin (data not shown). These results suggest that the association of Shot with adherens junctions is cell type specific and developmentally regulated.

β-Catenin, but not p120, is required for track formation

As E-cadherin is required for fusion (Uemura et al., 1996), it is likely to recruit Shot to the fusion track. To investigate this possibility, we examined track formation in wild-type (Fig. 4A-C) and *E-cadherin*/*shotgun* (*shg*) (Fig. 4D-F) mutant embryos using phalloidin to label F-actin, and Shot-GFP. In these mutant embryos, some residual maternal E-cadherin function is present, and they are able to form trachea and in some cases, to complete fusion (Uemura et al., 1996). However, at segment boundaries where fusion did not occur, F-actin and Shot-GFP containing fusion tracks were absent (Fig. 4D-F). In these cases, fusion cells appeared to be in contact (Fig. 4D-F). These data therefore suggest that the failure to form adherens junction associated cytoskeletal structures in *shg* mutant embryos reflect a requirement for E-cadherin signaling in track formation, and not possible earlier roles for E-cadherin in enabling fusion cells to contact each other.

β-Catenin is a key partner of E-cadherin in adhesion and signaling. We therefore also compared adherens junction and

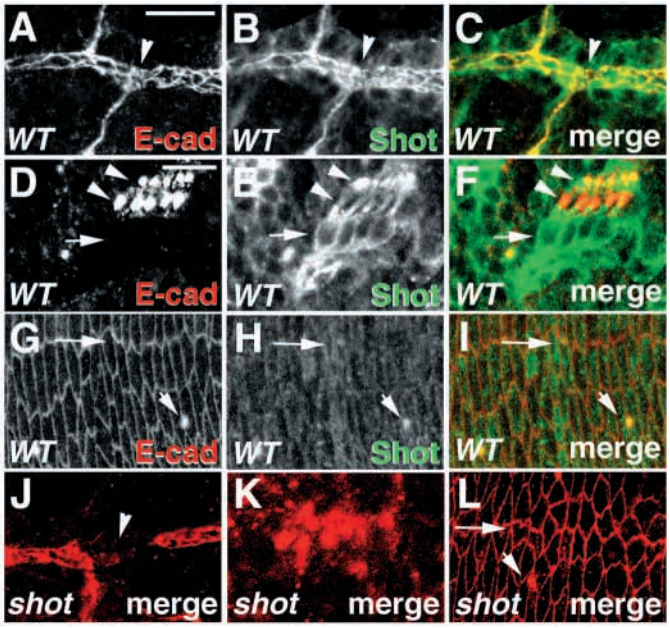

Fig. 3. Shot associates with E-cadherin and is required for adherens junction development in selected cell types. Anterior, leftwards; dorsal, upwards. E-cadherin, red; Shot, green. (A-C) Dorsal trunk tracheal cells in a stage 14 wild-type embryo form adherens junctions associated with Shot. (A) E-cadherin forms a web of junctions that gird the apical surfaces of tracheal cells, joined at segment boundaries (arrowhead). Scale bar: 10 μm in A-C. (B) Shot concentrates at adherens junctions. (C) Merge of A and B. (D-F) Lateral chordotonal (lch) neurons form highly organized adherens junctions with support cells. (D) In a stage 15 wild-type embryo, E-cadherin clusters in two rows of adherens junctions (arrowheads) formed by support cells contacting chordotonal dendrites. The arrow indicates neuronal cell bodies, which do not express E-cadherin. Scale bar: 10 μm in D-L. (E) Shot colocalizes with E-cadherin in the dorsalmost set of adherens junctions associated with lch neurons. (F) Merge of D and E. (G-I) Shot does not appreciably colocalize with epidermal adherens junctions. (G) Cadherin distribution at the dorsal midline (long arrow) of a stage 15 wild-type embryo. This section is taken 1 μm below the section where most E-cadherin is observed, and 1 mm above where most Shot can be detected. (H) Shot distribution in the same optical section. Shot is relatively diffuse, except in spots where it colocalizes with E-cadherin (short arrows). (I) Merge of G and H. E-cadherin and Shot overlap weakly. (J-L) E-cadherin and Shot distribution in *shot*^{3} null mutant embryos. (J) Cells in the dorsal tracheal trunk of a stage 14 *shot*^{3} mutant embryo. The branches fail to fuse at the segment boundary (arrowhead), and Shot is absent in all cells. E-cadherin appears normal in tracheal cells other than fusion cells. (K) E-cadherin contacts with chordotonal neurons in a stage 15 *shot*^{3} mutant embryo are abnormally organized and shaped. (L) E-cadherin contacts between epidermal cells appear normal at the dorsal midline (long arrow) in a stage 15 *shot*^{3} mutant embryo, including stereotyped spots where it colocalizes with E-cadherin (short arrows).

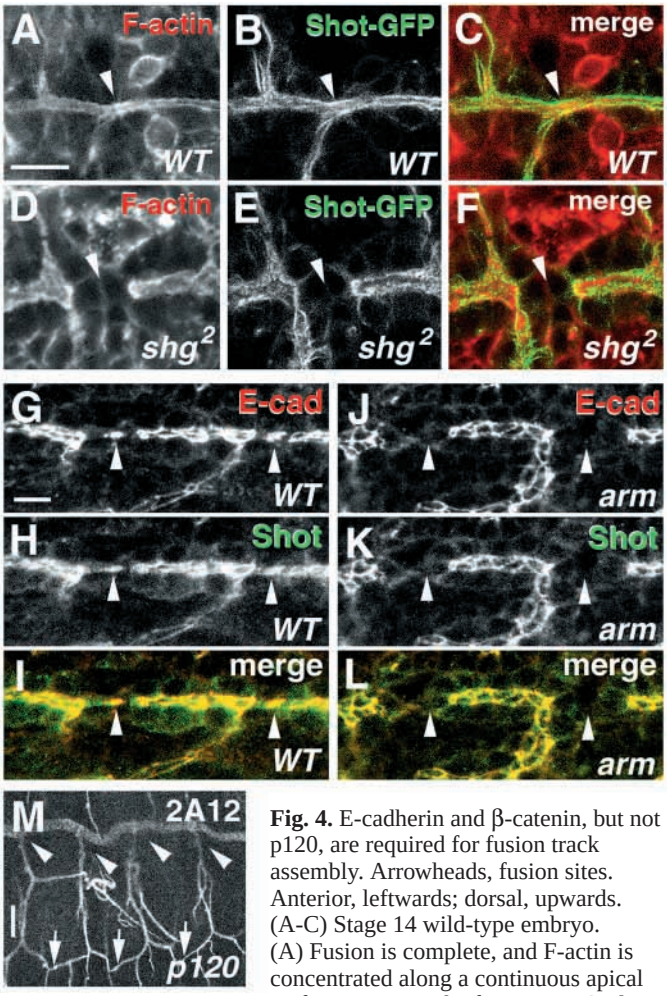

Fig. 4. E-cadherin and β-catenin, but not p120, are required for fusion track assembly. Arrowheads, fusion sites. Anterior, leftwards; dorsal, upwards. (A-C) Stage 14 wild-type embryo. (A) Fusion is complete, and F-actin is concentrated along a continuous apical surface spanning the fusion site. Scale bar in A: 10 μm for A-F. (B) Shot is also concentrated apically. (C) Merge of A and B. F-actin, red; Shot, green. (D-F) Stage 14 *shg*^{2} mutant embryo. (D) F-actin does not form a track in fusion cells and the tracheal tubes remain blind-ended. (E) Shot-GFP does not form a track in fusion cells. (F) Merge of D and E. F-actin, red; Shot, green. (G-I) Stage 13 wild-type embryo. (G) E-cadherin contacts form between fusion cells. Scale bar in G: 10 μm for G-L. (H) Tracks of Shot form at the new E-cadherin contacts. (I) Merge of G and H. (J-L) Stage 13 *arm*^{YD35}*; P[arm*$^{S14\text{-}C}$*]* embryo. (J) New E-cadherin contacts fail to form between fusion cells. K) Tracks of Shot fail to form in fusion cells. L) Merge of J and K. E-cadherin, red; Shot, green. M) A stage 15 *p120*^{308} mutant embryo stained with mAb 2A12, which reveals the tracheal lumen (Samakovlis et al., 1996a). Dorsal trunk (arrowheads) and lateral trunk (arrows) fusion appears normal, as does tracheal branching. Scale bar in M: 10 μm.

fusion track formation in wild-type (Fig. 4G-I) and β-catenin (*armadillo*) mutant embryos (Fig. 4K-L). In wild-type stage 13 embryos, E-cadherin (Fig. 4G) and Shot (Fig. 4H) co-localize in the fusion track (Fig. 4I) as well as in older tracheal adherens junctions. Dorsal trunk fusion is defective in the *arm* mutant embryos (Beitel and Krasnow, 2000), because fusion cells do not form E-cadherin contacts (Fig. 4J) and associated fusion tracks (Fig. 4K) at stage 13. The fragility of these mutant embryos precluded examination of the associated microtubules. Thus, β-catenin is required for new E-cadherin contacts to form between fusion cells and fusion track formation. E-cadherin localization in other tracheal cells was normal, most probably because sufficient β-catenin is provided maternally.

Recently, a null mutation in the single *Drosophila p120* related gene has been described (Myster et al., 2003). Though homozygous null mutant *p120* flies are viable and fertile, we

nonetheless examined tracheal fusion in these mutant embryos, as it is not known whether tracheal fusion is an essential process. Tracheal development appeared normal in all homozygous null mutant *p120* embryos examined (*n*=20) (Fig. 4M). Thus, *p120* is not essential for fusion or other aspects of tracheal development.

Mutations in the E-cadherin juxtamembrane domain dominantly interfere with track maturation and microtubule track assembly

We further investigated the signaling pathway required to localize Short Stop to the fusion track by structure/function analysis of E-cadherin. Two domains in E-cadherin affect cell adhesion in tissue culture models: the juxtamembrane domain and the C-terminal β-catenin-binding site (Ozawa and Kemler, 1998; Yap et al., 1998). We therefore constructed flies expressing wild-type E-cadherin (*UAS-WT*) (Uemura et al., 1996), or E-cadherins mutant in the β-catenin binding sites (*UAS-Δarm*) or in the juxtamembrane domain (*UAS-AAA-JXT*). These transgenes are under the control of a GAL4-dependent promoter, allowing expression to be targeted to particular tissues during development (Brand and Perrimon, 1993). The juxtamembrane domain mutant mutates the evolutionarily conserved residues 1607-1609 (ERD in fly; EED in mouse) to alanine. In mammalian E-cadherin, this mutation disrupts binding to p120 in tissue culture (Thoreson et al., 2000) and also affects activation of Rac by E-cadherin (Goodwin et al., 2003). These residues immediately follow the conserved glycine triplet required for p120 binding and recruitment to E-cadherin contacts in cultured *Drosophila* S2 cells, but not for E-cadherin function in *Drosophila* (Pacquelet et al., 2003).

Expression of wild-type (Fig. 5A) or *Δarm E-cadherin* (Fig. 5B) in wild-type tracheal cells did not detectably affect tracheal development. By contrast, expression of *AAA-JXT E-cadherin* from either of two independently derived transgenic lines dominantly disrupted fusion, without detectable effects on other aspects of tracheal development such as branch migration (Fig. 5C). Expression of the E-cadherin altered in the adjacent juxtamembrane glycine residues (Pacquelet et al., 2003) had no effect on fusion (Fig. 5D). Surprisingly, the AAA-JXT mutant E-cadherin concentrated at cell contacts when introduced into *Drosophila* S2 cells and colocalized with epitope-tagged p120 (Fig. 5E). As S2 cells do not normally express E-cadherin, these data suggest that the AAA-JXT mutant E-cadherin functions as a homophilic adhesion molecule and recruits p120 to cell contacts. E-cadherin mutant in the adjacent glycine triplet does not bind to p120 or recruit it to cell contacts (Pacquelet et al., 2003).

When expressed in tracheal cells, wild-type (Fig. 5F) or *Δarm* mutant E-cadherin (Fig. 5G) did not notably affect the normal pattern of adherens junctions as detected with anti-E-cadherin. However, E-cadherin was delocalized in tracheal cells expressing *AAA-JXT* mutant E-cadherin (Fig. 5H). As most of the E-cadherin detected in these experiments is due to the transgene (Fig. 5I), these data suggest that AAA-JXT mutant E-cadherin cannot localize efficiently to adherens junctions in tracheal cells, notwithstanding its ability to concentrate in cell contacts when expressed in S2 cells (Fig. 5E). Mutations in the juxtamembrane site affect E-cadherin clustering in mammalian cells (Yap et al., 1998). Wild-type, Δarm and AAA-JXT E-cadherins appear to be expressed at similar levels in wild-type embryos, to be processed to the same 150 kDa molecular weight, and to be similarly soluble in detergent, a measure of association with the cytoskeleton (McNeill et al., 1993) (Fig. 5I).

To investigate further the fusion defect in the embryos expressing AAA-JXT E-cadherin in tracheal cells, we examined track formation using phalloidin and anti-Shot. Confocal microscopy revealed that fusion cells in these *AAA-JXT* mutant embryos generally assemble F-actin and Shot at the site of cell contact (79%, *n*=41) (Fig. 5K,L,N). However, in these cells, F-actin appears abnormally organized (Fig. 5K; 5%) or forms weaker tracks (Fig. 5L,N; 74%) than in wild type, suggesting a defect in track maturation. Visualization of the plasma membrane (Fig. 5M) indicates that fusion cells still contact each other in *AAA-JXT* mutant embryos. Thus, these defects do not appear to reflect failure of fusion cells to contact each other.

We therefore used confocal microscopy to examine track development in live embryos expressing Shot-GFP and AAA-JXT in tracheal cells. The fusion track persisted longer in mutant fusion cells, and the Shot-GFP accumulations at existing apical surfaces failed to exhibit the remodeling (Fig. 5P-T) observed in wild-type (Fig. 1). In confocal sections along the plane of the track, persistent gaps are observed in the distribution of Shot-GFP along the existing apical surfaces (Fig. 5P-S). In other cases, the track resolved and the existing apical surfaces approached closer together, but remained blind-ended (Fig. 5T). In *AAA-JXT* mutant embryos, successful fusion events took 2-3 hours to complete after track initiation (data not shown), instead of ~1 hour in wild-type embryos (Fig. 1).

To address the basis of the maturation defect, we used confocal fluorescent microscopy to examine microtubules in fusion cell pairs in wild-type and mutant embryos. In stage 13 wild-type embryos, 97% (*n*=59) of fusion cell pairs had microtubules associated with the point of contact, often forming a track (Fig. 5U). By contrast, in 73% of fusion cell pairs examined in stage 13 embryos expressing AAA-JXT E-cadherin in tracheal cells (*n*=72), microtubule tracks are absent (Fig. 5V). In the remaining fraction, microtubules are often abnormally assembled (Fig. 5V). These data suggest that embryos expressing AAA-JXT E-cadherin can initiate F-actin, but not microtubule track assembly. Alternatively, they may be defective in capturing or stabilizing microtubules. We propose that microtubules are important for later steps in tube fusion, including track maturation, and possibly remodeling of the existing apical surfaces.

The cytoskeletal defects observed in *AAA-JXT* embryos may reflect defects in the localization of AAA-JXT E-cadherin. Delocalization of the receptor may delocalize signals that control F-actin and microtubule assembly. To test this possibility, we also examined tracheal fusion in embryos that express *AAA-JXT E-cadherin* under the control of *hairy-GAL4* (Fig. 5W-Y). *Hairy-GAL4* drives *AAA-JXT E-cadherin* expression in all cells in odd-numbered segments (T2, A1, A3, A5, A7) and at a lower level (Brand and Perrimon, 1993). Cells in tracheal branches expressing AAA-JXT E-cadherin appeared to localize most of their E-cadherin to adherens junctions, but still frequently failed to form fusion tracks (Fig. 5W-Y) or to complete fusion (data not shown). However,

dorsal closure, another E-cadherin dependent process involving epithelial migration, appeared normal in affected segments (data not shown). These results suggest that fusion defects do not arise solely from defects in E-cadherin localization or expression levels of the transgene, and that fusion cell development may be particularly sensitive to

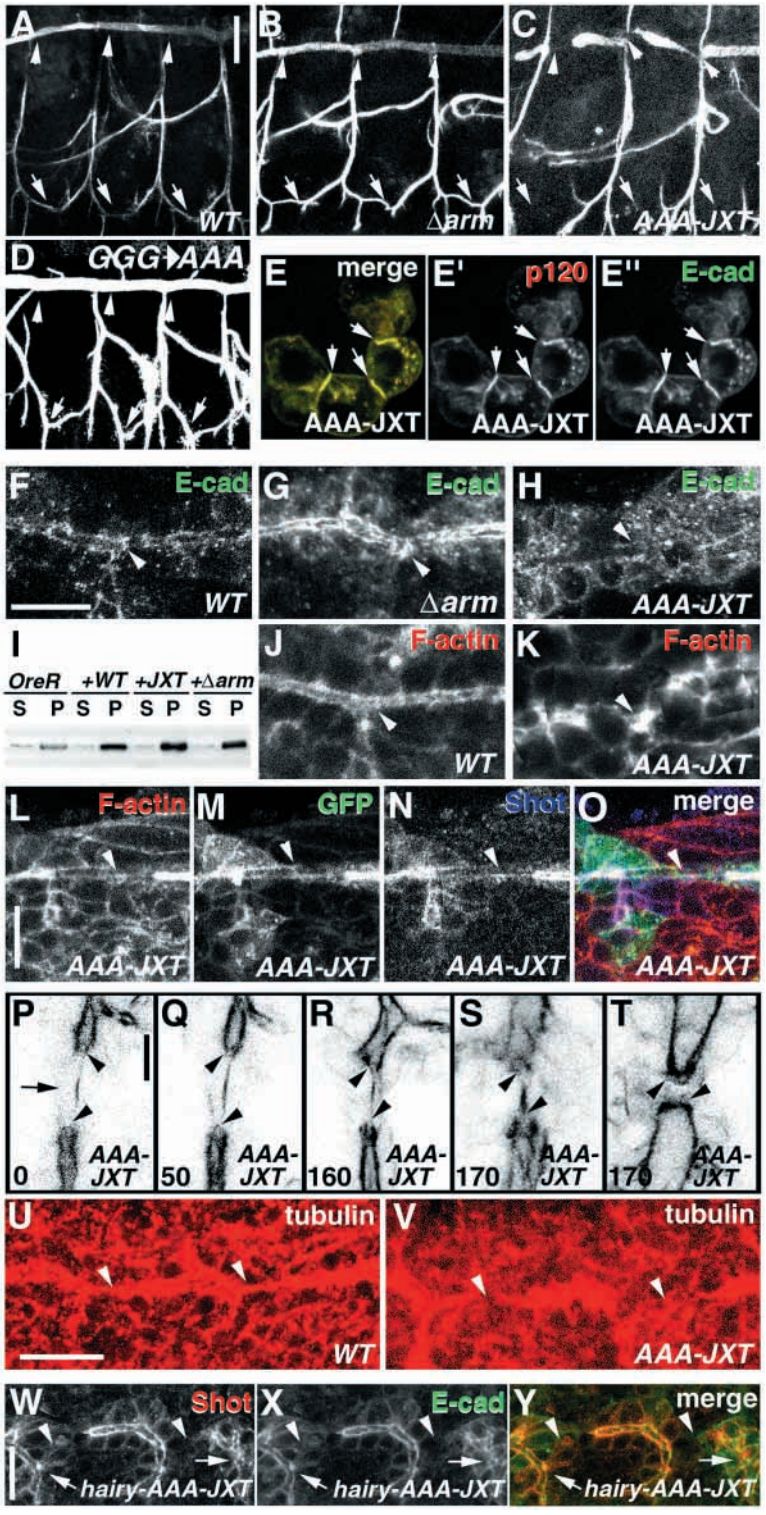

Fig. 5. A mutation in the E-cadherin juxtamembrane dominantly blocks tracheal tube fusion and disrupts microtubule tracks. Anterior, leftwards; dorsal, upwards (except in P-T where anterior is upwards and dorsal is rightwards. (A-D) Stage 15 embryos stained with mAb 2A12. (A) The lumen in a stage 15 wild-type embryo expressing wild-type E-cadherin in tracheal cells is continuous at fusion sites in the dorsal trunk (arrowheads) and lateral trunk (arrows). Scale bar: 10 μm in A-D. (B) Expression of Δ-arm E-cadherin in tracheal cells does not detectably affect fusion (arrowheads, arrows). (C) Expression of AAA-JXT E-cadherin in tracheal cells blocks fusion in the dorsal (arrowheads) and lateral (arrows), as well as at the dorsal midline (not shown). (D) Expression of E-cadherin mutant in the GGG juxtamembrane sequence in all cells (Pacquelet et al., 2003) has no effect on tracheal fusion or development (arrowheads, arrows). (E) *Drosophila* S2 cells expressing HA epitope-tagged p120 (red, E′) and AAA-JXT mutant E-cadherin (green, E″). E-cadherin and p120 colocalize at cell contacts (arrows). F) Stage 14 embryo expressing wild-type E-cadherin in tracheal cells. Scale bar: 10 μm in F-H,J,K. E-cadherin is localized largely in adherens junctions (arrowhead). (G) Stage 14 embryo expressing Δarm E-cadherin in tracheal cells. Adherens junctions appear normal (arrowhead). Somewhat more E-cadherin is found outside the adherens junctions than when wild-type is overexpressed in tracheal cells. (H) Stage 14 embryo expressing AAA-JXT E-cadherin in tracheal cells. E-cadherin is delocalized. (I) Western blot with anti-E-cadherin (Oda et al., 1994) revealing the relative amounts of NP40 soluble (S) and pelleted (P) E-cadherin in wild-type Oregon R embryos expressing no additional (OreR) E-cadherin or wild-type E-cadherin (+WT), AAA-JXT E-cadherin, or Δarm E-cadherin in tracheal cells. (J) Same embryo as in F. Fusion has occurred and F-actin accumulates apically in fusion cells (arrowhead). (K) Same embryo as in H. F-actin accumulates at the fusion site, but in an aggregate (arrowhead). (L-O) Stage 13 embryo expressing AAA-JXT E-cadherin in tracheal cells. Scale bar: 10 μm in L-O. (L) F-actin weakly accumulates at the fusion site (arrowhead). (M) GAP-43 GFP labels the membranes of the fusion cells (arrowhead), which are elongated. (N) A weak track of Shot is visible at the fusion cell contact (arrowhead). (O) Merge of L-N. F-actin, red; GFP, green; Shot, blue. (P-T) Frames from videos of fusion in *AAA-JXT* mutant embryos. Minutes elapsed, lower left. Scale bar: 10 μm in P-T. (P) A weak Shot fusion track (arrow) is visible at the start of the first sequence. Apical surfaces appear open (arrowheads). (Q,R) The track changes little even after 50 (Q) or 160 (R) minutes, becoming only moderately more intense. The apical surfaces remain open (arrowheads). (S) At 170 minutes in a second sequence, over 1 hour after fusion occurs in wild type, the track persists and the apical surfaces remain open (arrowheads). (T) At 170 minutes in a third sequence, the existing apical surfaces draw closer together after a track forms and shrinks (not shown), but remain blind-ended (arrowheads). (U) Microtubules in dorsal trunk tracheal cells form fusion tracks (arrowheads) in a stage 13 wild-type embryo. Scale bar: 10 μm in U,V. (V) The microtubule track is broken (left arrowhead) or missing in fusion cells (right arrowhead) in a stage 13 embryo expressing AAA-JXT E-cadherin in tracheal cells. (W-Y) A late stage 13 embryo expressing *AAA-JXT E-cadherin* under the control of *hairy-GAL4*. Segments expressing the mutant transgene (arrows). (W) Shot fails to accumulate in fusion tracks (arrowheads). (X) E-cadherin contacts fail to form between fusion cells (arrowheads). Adherens junctions appear abnormally arranged in segments expressing the transgene. However, E-cadherin remains largely in adherens junctions (arrows). Scale bar: 10 μm in W-Y. (Y) Merge of W and X. Shot, red; E-cad, green.

pathways disrupted by the juxtamembrane mutation or to reductions in E-cadherin function. Fusion tracks also frequently failed to form on both sides of the affected segment, suggesting that E-cadherin signaling must occur in both fusion cells for fusion to proceed.

Distinct sites in the E-cadherin cytoplasmic domain control track formation and maturation

Tracheal expression of wild-type and mutant E-cadherins in wild-type embryos suggested distinct functions for the juxtamembrane and β-catenin-binding domains. To determine the activity of these domains with respect to track formation and maturation, we examined tracheal fusion, track formation and maturation in *shotgun* (*shg*) mutant embryos expressing wild-type or mutant E-cadherin transgenes in tracheal cells. The allelic combination (shg^2/shg^{IH}) selected for these experiments represents a partial loss of cadherin function (Uemura et al., 1996), allowing both enhancement and suppression to be scored (Fig. 6A-E; Table 1). Tracheal expression of wild-type E-cadherin largely rescued fusion defects in *shg* mutant embryo (Fig. 6C; Table 1). Expression of either Δarm or AAA-JXT mutant E-cadherin enhanced the frequency and severity of fusion defects (Fig. 6D,E; Table 1). Thus, both the β-catenin binding site and the juxtamembrane site are required for fusion.

To determine whether these functions must be present in the same polypeptide chain for fusion to occur, we co-expressed the *Δ-arm* and *AAA-JXT* mutant transgenes in tracheal cells in *shg* mutant embryos (Fig. 6F). No rescue of fusion defects was observed. Moreover, co-expressed mutant E-cadherins did not localize to sites of normal adherens junctions. These data therefore suggest that the juxtamembrane and β-catenin-binding domains function in the same E-cadherin molecule to allow fusion and adherens junction localization.

We then investigated the ability of these mutant transgenes to localize Shot to the fusion track. In *shg* mutant embryos expressing Δarm mutant E-cadherins in tracheal cells, fusion cells fail to form Shot-containing tracks (Fig. 6G). Thus, the β-catenin-binding domain is required for cytoskeletal track assembly in the fusion cells. However, other tracheal cells

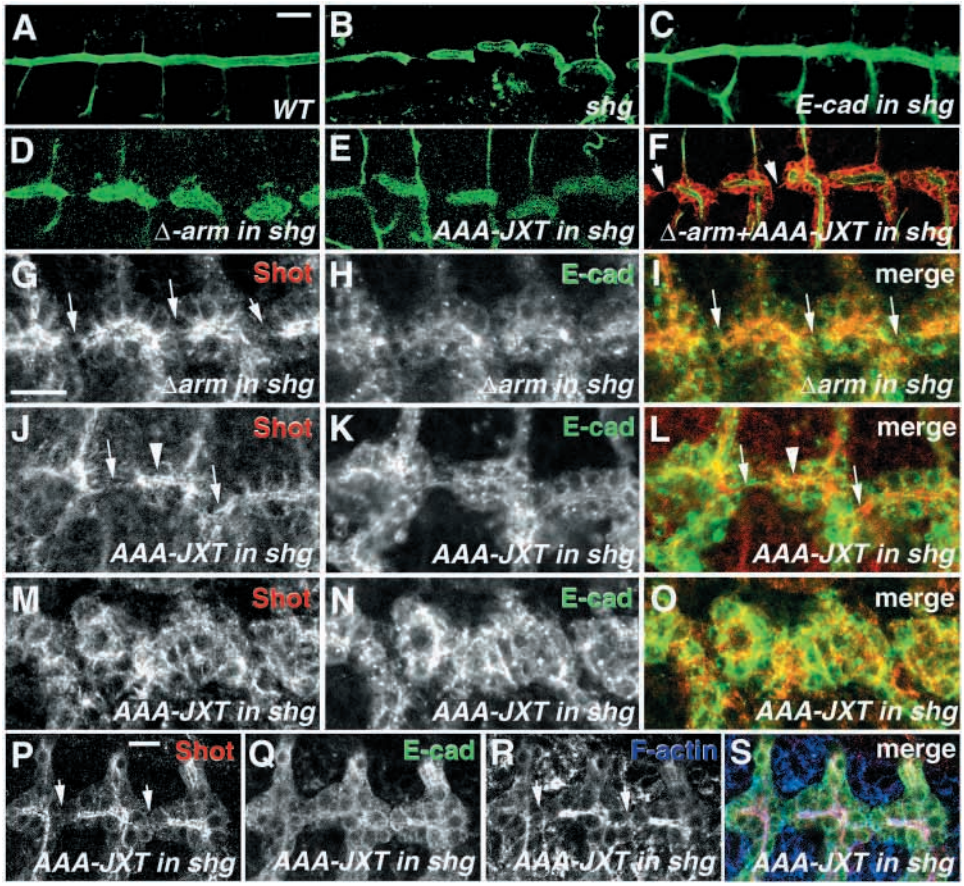

Fig. 6. The E-cadherin intracellular domain is required for fusion track assembly and maturation, and maintenance of apical cytoskeletal polarity in tracheal cells. Anterior, leftwards; dorsal, upwards. (A-F) Stage 15 embryos stained with 2A12 to reveal the tracheal lumen (green). In F, the embryo is also stained with anti-E-cadherin (red). Scale bar: 10 μm in A-F. (A) The dorsal trunk is continuous in a wild-type embryo. (B) Numerous breaks and constrictions in the dorsal trunk of a shg^2/shg^{IH} embryo. (C) Expression of a wild-type E-cadherin cDNA in tracheal cells restores fusion in a shg^2/shg^{IH} embryo. (D) Expression of a *Δarm* mutant E-cadherin in tracheal cells enhances fusion defects in a shg^2/shg^{IH} embryo. (E) Expression of *AAA-JXT* mutant E-cadherin in tracheal cells enhances fusion defects in a shg^2/shg^{IH} embryo. (F) Expression of both *Δ-arm* and *AAA-JXT* mutant E-cadherin in tracheal cells does not rescue fusion defects in a shg^2/shg^{IH} embryo. Mutant E-cadherins are delocalized and not organized into adherens junctions. Fusion cells maintain finger-like contacts (arrows) but do not form lumenal connections even as epidermal movements and shape changes pull tracheal branches apart. (I,L,O,S) Shot, red; E, cadherin, green; F-actin, blue (S only). (G-I) A stage 13 shg^2/shg^{IH} mutant embryo expressing a *Δarm* mutant E-cadherin in tracheal cells. (G) Shot tracks are not detectable at fusion sites (arrows). Scale bar: 10 μm in G-N. (H) E-cadherin is delocalized in tracheal cells. In these embryos, little endogeneous E-cadherin is detectable, so this represents transgene expression. (I) Merge of G and H. (J-L) A stage 13 shg^2/shg^{IH} mutant embryo expressing the *AAA-JXT* mutant E-cadherin in tracheal cells. (J) Shot tracks are weak (left arrow) or absent (right arrow) in fusion cells. Shot is apically concentrated in other tracheal cells (arrowhead). (K) E-cadherin is delocalized in tracheal cells. (L) Merge of J and K. (M-O) Stage ~15 shg^2/shg^{IH} mutant embryo expressing the *AAA-JXT* mutant E-cadherin in tracheal cells. (M) Shot is delocalized in tracheal cells. (N) E-cadherin is delocalized in tracheal cells. (O) Merge of M and N. (P-S) A stage 13 shg^2/shg^{IH} mutant embryo expressing the *AAA-JXT* mutant E-cadherin in tracheal cells. (P) Shot tracks are absent from fusion cells (arrows). Scale bar: 10 μm in P-S. (Q) E-cadherin is delocalized. (R) F-actin tracks are absent from fusion cells (arrows). (S) Merge of P-R.

Table 1. Frequency of tracheal fusion defects in mutant embryos

Genotype	Total segments	Break (%)	Constriction (%)
shg²/shg^IH	104	25	39.4
UAS-WT in *shg²/shg^IH*	176	6.8	5.1
UAS-AAA-JXT in *shg²/shg^IH*	112	62.5	15.2
UAS-Δarm in *shg²/shg^IH*	104	73	12.5
UAS-AAA-JXT in *WT*	128	47.6	11

Embryos of the indicated genotypes were scored at stage 14 or later by staining with staining with mAb 2A12, which recognizes tracheal lumen, and examining fusion sites at segment boundaries in the dorsal trunk for defects. Transgenes were expressed in tracheal cells using the *btl-Gal4* driver. Dorsal trunk breaks are very rare (<1%) in wild-type embryos.

localize Shot apically and continue to maintain this organization at later developmental stages (data not shown).

shg²/shg^IH mutant embryos express little detectable E-cadherin, so the E-cadherin observed in tracheal cells in these embryos is almost exclusively due to the transgene. In *shg²/shg^IH* embryos, Δ-arm E-cadherin was delocalized (Fig. 6H), suggesting that the β-catenin binding domain is required for the localization of E-cadherin to adherens junctions in tracheal cells.

In *shg* mutant embryos expressing AAA-JXT mutant E-cadherin in tracheal cells, Shot and F-actin fusion tracks are weak or abnormally oriented (Fig. 6J) or absent (Fig. 6P,R) from fusion cells. The fragility of these mutant embryos prevented us from examining microtubule track formation. At all stages (Fig. 6K,N), E-cadherin in these embryos appears delocalized. These data suggest that the juxtamembrane domain is required for E-cadherin localization to adherens junctions and fusion track maturation, which we define as a step occurring after initial track assembly. In these embryos, Shot initially localized apically in other tracheal cells (Fig. 6J,P). However, as development progressed, Shot became delocalized (Fig. 6M). Continued expression of the juxtamembrane site E-cadherin mutant disrupts the apical polarization of the cytoskeleton, suggesting that this site normally actively maintains this evolutionarily conserved feature of tubular epithelia.

Discussion

Shot connects the cytoskeleton to E-cadherin during adherens junction remodeling

Junctional contacts between cells are important for organizing the cytoskeleton and regulating cell polarity (Ohno, 2001; Tepass, 2002). The large size of plakins and their modular abilities to bind different cytoskeletal elements make them potentially well suited to play key organizational roles (Fuchs and Yang, 1999). However, except in the case of desmosomes, where the plakin desmoplakin appears to be a crucial for organizing junction-associated cytoskeleton (Pasdar et al., 1991), functional association of plakins with other cell-cell junctions has not been described.

We show here that in selected cell types, Shot localizes with proteins of the adherens junction and may play a role in adherens junction-mediated organization of the cytoskeleton. We propose that Shot and E-cadherin form a feedback loop in which E-cadherin, via β-catenin, recruits Shot to new contacts between the fusion cells and Shot stabilizes the contacts. The cytoskeleton organizes around these contacts because adherens junction associated Shot promotes the assembly of an F-actin/microtubule-rich track (Lee and Kolodziej, 2002). This track grows to span the fusion cells, extending the reach of the junctions through the cells. The recruitment mechanism may be indirect in that new adherens junctions in fusion cells are centers for cytoskeletal assembly, and Short Stop binds F-actin and microtubules. Alternatively, Shot may associate directly with E-cadherin or associated proteins. The assembly of Shot with F-actin and microtubules may stabilize E-cadherin contacts simply by bringing in cytoskeletal proteins that bind E-cadherin or associated proteins (Karakesisoglou et al., 2000). For example, EB1, which is present in the fusion track, co-immunoprecipitates with a C-terminal fragment of Shot in cultured cells (Subramanian et al., 2003) and associates with APC (Su et al., 1995). APC interacts with β-catenin to control tubulogenesis in vitro (Pollack et al., 1997).

Distinct sites in the E-cadherin cytoplasmic domain control F-actin and microtubule assembly

We propose that the assembly and maturation of a cytoskeletal intermediate are two E-cadherin-dependent steps in tracheal cell fusion (Fig. 7). Imaging of fixed and live embryos suggests that fusion proceeds through the assembly and maturation of a cytoskeletal track associated with adherens junctions. The track forms after contact between the fusion cells, and persists for ~1 hour before fusion occurs.

In this model, the β-catenin-binding site and the juxtamembrane site in the E-cadherin cytoplasmic domain

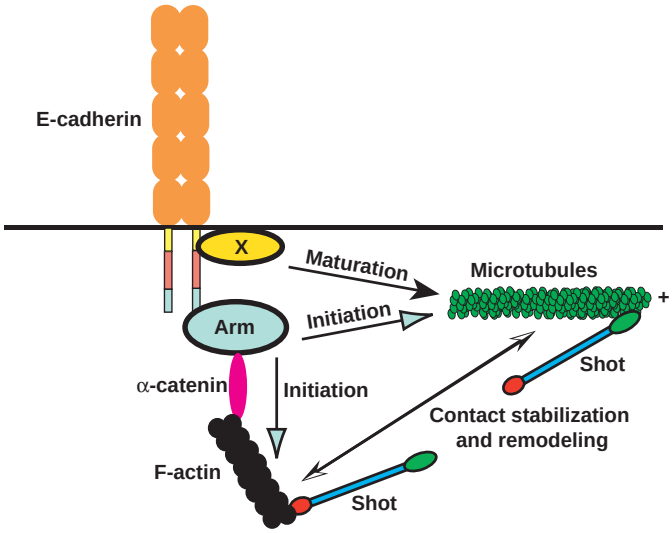

Fig. 7. A model for E-cadherin signaling during tube fusion. E-cadherin signals via β-catenin (Arm) to initiate track assembly. An unknown factor (X) associated with the juxtamembrane domain is required to assemble or stabilize track-associated microtubules. Microtubules are required for track maturation. Maturation events involve the subsequent reinforcement of initial F-actin/Shot assembly or the disassembly of apical cytoskeletal structures and the track itself prior to fusion. Shot functions to stabilize F-actin and microtubules associated with the track. The track components together stabilize the new E-cadherin contact and coordinate apical surface movements and remodeling.

operate sequentially and in the same E-cadherin molecule to promote fusion. In mutant embryos in which either β-catenin or its binding site is defective, fusion cells make contact but track assembly is not observed. These data suggest that E-cadherin may initiate track assembly via β-catenin. A mutation in the juxtamembrane site dominantly inhibits track maturation. Microtubules are generally absent from fusion tracks in these embryos, though some F-actin and Shot assembly occurs. In *E-cadherin/shotgun* (*shg*) mutant embryos, E-cadherin bearing this juxtamembrane mutation supports a low level of F-actin/Shot track formation, but the tracks do not mature. In addition, this juxtamembrane mutant E-cadherin causes progressive delocalization of the apical tracheal cytoskeleton in *shg* mutant embryos.

Both the β-catenin and juxtamembrane binding sites are required for E-cadherin localization to adherens junctions, although only the juxtamembrane mutation seems to interfere with endogeneous E-cadherin localization. Our results suggest that like mammalian E-cadherin, an evolutionarily conserved juxtamembrane site is required for some E-cadherin functions. Similar effects of mutations in the juxtamembrane site were observed in mammalian tissue culture cells (Yap et al., 1998). However, juxtamembrane site function in *Drosophila* E-cadherin probably does not require p120.

Dominant effects on localization appear sensitive to expression levels, whereas effects on fusion are less so, suggesting that defects in localization are not enough to explain the defects in track maturation. Possibly, effects on localization also reflect defects in organizing the cytoskeleton, as has been observed in studies in which dominant alleles of Rho family GTPases affect cadherin localization in culture (Braga et al., 1997; Jou and Nelson, 1998) and during tracheal development (Chihara et al., 2003).

We propose that the β-catenin-binding site and β-catenin are required for track assembly, and that the juxtamembrane site regulates other proteins involved in a later maturation step (Fig. 7). This later step likely requires microtubules. The microtubules or associated proteins may reinforce the initial F-actin assembly in the track, as F-actin in fusion tracks appears to be abnormally or poorly assembled in embryos expressing AAA-JXT mutant E-cadherin in tracheal cells. The microtubules appear also required for remodeling the fusion cell apical surfaces and also for bringing them together to fuse. In embryos expressing AAA-JXT mutant E-cadherin in tracheal cells, fusion cell apical surfaces do not develop or seal gaps at appropriate times, and fusion tracks persist substantially longer, if they resolve at all.

The microtubule regulated steps during fusion therefore likely involve effects on F-actin dynamics. Microtubule-associated factors that may regulate the F-actin cytoskeleton include Rac GTPase (Waterman-Storer et al., 1999) and exchange factors for Rho GTPase (Ren et al., 1998; van Horck et al., 2000). Rac1 affects E-cadherin dependent adhesion in tracheal cells (Chihara et al., 2003) and a mutation in the juxtamembrane site in mammalian E-cadherin analogous to the one described here affects Rac activation (Goodwin et al., 2003). RhoA activation inhibits fusion track assembly (Lee and Kolodziej, 2002). Downstream interactions between F-actin and microtubules, such as those mediated by Shot, may vary with cell type to produce distinct morphogenetic outcomes. Further studies of tracheal tube fusion, a genetic system in which adherens junction associated structures can be visualized in living embryos, promises to identify the regulatory molecules that allow E-cadherin to direct F-actin and microtubule assembly from the β-catenin binding and juxtamembrane domains.

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