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Cadherin-mediated cell adhesion and cell motility in *Drosophila* trachea regulated by the transcription factor Escargot

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SUMMARY

Coordination of cell motility and adhesion is essential for concerted movement of tissues during animal morphogenesis. The *Drosophila* tracheal network is formed by branching, migration and fusion of tubular ectodermal epithelia. Tracheal tip cells, located at the end of each branch that is going to fuse, extend filopodia to search for targets and later change their cell shape to a seamless ring to allow passage of lumen. The cell adhesion molecule *D*E-cadherin accumulates at the site of contact to form a ring that marks the site of lumen entry and is essential for the fusion. *D*E-cadherin expression in tip cells of a subset of branches is dependent on *escargot*, a zinc finger gene expressed in all tip cells. Such *escargot* mutant tip cells failed to adhere to each other and continued to search for alternative targets by extending long filopodia. We present evidence indicating *escargot* positively regulates transcription of the *D*E-cadherin gene, *shotgun*. Overexpression of *D*E-cadherin rescued the defect in one of the fusion points in *escargot* mutants, demonstrating an essential role of *D*E-cadherin in target recognition and identifying *escargot* as a key regulator of cell adhesion and motility in tracheal morphogenesis.

Key words: epithelium fusion, cell shape change, filopodium, adherens junction, *Drosophila*, *escargot*, *shotgun*, cadherin

INTRODUCTION

During embryogenesis, multiple cell types are specified and are assembled to form functional organs. This process involves cell sorting, migration of individual cells and movement of cells as a group (Hynes and Lander, 1992). Cell-to-cell adhesion is essential (Takeichi, 1995) and is required to be efficiently coupled to cell motility during morphogenesis (Lauffenburger and Horwitz, 1996). Cell motility has mostly been studied by use of in vitro culture systems but more detailed assessment in the developmental context awaits genetic analyses at the single cell resolution.

The insect trachea is a network of tubular epithelium that serves as a respiratory system (Manning and Krasnow, 1993; Samakovlis et al.,1996a). In *Drosophila*, the tracheal system is derived from ten pairs of ectodermal invaginations that each extends six tubular branches without cell division. These branch further to generate terminal branches and fusion branches. Fusion branches migrate to find branches from an adjacent segment and fuse to form a network (Fig. 1A). There are eleven fusion points on the dorsal midline (one of cerebral branch and ten of dorsal branch [DB], Fig. 4B), eighteen on each side (nine each of dorsal trunk [DT] and lateral trunk [LT], Fig. 4A), and three on the ventral midline in the thorax (ventral branch [VB]), all of which are joined with remarkable precision. Genes essential for tracheal formation have been identified. These include genes required for invagination of the tracheal placode (Isaac and Andrew, 1996; Wilk et al., 1996), branch migration (Klämbt et al., 1992) and cell specification (Guillemin et al., 1996). However, no gene that is specifically involved in the fusion has been identified.

At the tip of migrating branches, there are specialized tip cells that express *lacZ* in several enhancer trap lines (Hartenstein and Jan, 1992; Samakovlis et al., 1996a). Since tip cells lead the migration of fusion branches and are the first cells to make contact during fusion, they are thought to play important roles in branch migration and in epithelial reorganization during fusion (Nardi, 1990; Samakovlis et al., 1996a). The *escargot (esg)* gene is expressed in tip cells (Uemura et al., 1996). Esg expression in the trachea is first detectable soon after the start of tracheal branch migration. Expression of Esg protein and mRNA becomes weaker after fusion but continues throughout the rest of embryogenesis. Esg is a zinc-finger type DNA-binding protein that regulates transcription of specific target genes (Whiteley et al., 1992; Fuse et al., 1994). *esg* has been shown to be essential for the maintenance of diploidy of imaginal cells (Hayashi et al., 1993; Fuse et al., 1994; Hayashi, 1996) and for specification of the wing primordium during embryogenesis (Fuse et al., 1996). Function of Esg in tracheal formation is not known.

Inside the trachea there is a lumen that is connected to the outside of the embryo. The tracheal cells are in tight contact with each other by the junctional complex in the lateral side of the cells that restricts the movement of ions and small

molecules between the apical (facing luminal side) and the basal (facing hemolymph) side. The junctional complex is composed of two parts. The basally located septate junction is the invertebrate equivalent of the tight junction and includes Fasciclin III (FasIII; Patel et al., 1987), a member of the immunoglobulin superfamily. The apically localized adherens junction includes *D*E-cadherin, which, like its vertebrate counterparts, mediates cell-to-cell adhesion by homophilic association (Oda et al., 1994). Its cytoplasmic domain is associated with Armadillo (Arm; a *Drosophila* homolog of β-catenin, reviewed in Peifer, 1995) and *D*α-catenin (Oda et al., 1993) that are linked to the actin network. When tracheal branches fuse, they first make contact at their basal side, which is normally devoid of the junctional complex. This situation poses a problem. Tracheal branches have to either break existing junctions and rejoin with their fusion partner, or reverse their apical-basal polarity prior to fusion to create new junctions. In either case, a smooth transition must occur since leakage of luminal contents is not observed. Epithelia encounter a similar situation many times during vertebrate development (for example, kidney development; Gilbert, 1994). How epithelial cells manage this problem is not understood.

The gene encoding *D*E-cadherin, *shotgun (shg)*, has been identified (Nüsslein-Volhard et al., 1984; Tepass et al., 1996; Uemura et al., 1996). In the case of an intermediate loss *shg* of function, the fusion branches migrate to contact their targets, but fusion does not occur. The effect of the loss of zygotic *D*E-cadherin appears to be specific to cell rearrangement and the formation of a new cell-cell junction.

In this study, we analyzed the migration and fusion of tracheal branches by following the morphology of and molecular marker expression in the tip cells. Expression of *D*E-cadherin was closely correlated with a dynamic cell shape change associated with epithelial reorganization during fusion. In *esg* mutants, tip cells of a subset of the tracheal branch failed to accumulate *D*E-cadherin and such branches did not fuse but became hyperactive in motility. This phenotype was occasionally rescued by expression of *D*E-cadherin from the heat-shock promoter, leading to complete restoration of fusion. These results suggest that Esg stimulates *D*E-cadherin accumulation in the tracheal tip cell and that this accumulation is sufficient to induce a series of events leading to tracheal branch fusion.

MATERIALS AND METHODS

Drosophila strains

The null mutation esg^{G66B} (Whiteley et al., 1992) and the strong hypomorphic mutation esg^{VS8} (Fuse et al., 1994) caused essentially the same tracheal phenotype in homozygous condition and in hemizygous condition over *Df(2L)A48* (Ashburner et al., 1982). esg^{G66B} is an enhancer trap line that express β-galactosidase in the cytoplasm in a pattern nearly identical to that of *esg*. Development of heterozygous esg^{G66B} embryos was indistinguishable from that of wild-type Oregon R embryos. shg^{E17B}, shg^{P2370} and *hsp-cadE* were described by Uemura et al. (1996). shg^{P2370} *(shg-lacZ)* expresses β-galactosidase in a pattern similar to that of *D*E-cadherin. shg^{2} was described by Nüsslein-Volhard et al. (1984), and *HS-esg* and *HS-esg ΔZF* lines were described by Fuse et al. (1994). 6-81a is a homozygous viable enhancer trap line of *btl* (Klämbt et al., 1992) that frequently shows failure of tracheal fusion.

Immunological procedures

Embryos and larval tissue were immunostained as described by Hayashi et al. (1993). The following primary antibodies were used for immunostaining and western blotting: rat anti-*D*E-cadherin (DCAD1 and 2; Oda et al, 1994), rat anti-*D*α-catenin (DCAT1; Oda et al., 1993), rat anti-protein phosphatase 2AA (Shiomi et al., 1994), mouse anti-FasIII (Brower et al., 1980), mouse anti-Crumbs (Tepaß et al., 1990), mouse anti-β-galactosidase (Boehringer Mannheim), and rabbit anti-β-galactosidase (Cappel). To stain the tracheal lumen, we employed lumen-specific antibody #55 (Klämbt et al., 1992) and rabbit anti-actin (Biomedical Technologies Inc. cat#BT-560), which fortuitously recognize a luminal antigen. The following secondary antibodies were used: FITC-conjugated antibodies against rat, mouse and guinea pig IgG (Jackson Laboratory), Cy3-conjugated anti-rabbit IgG (Chemicon), HRP-conjugated goat anti-mouse IgG (Jackson) and HRP-conjugated sheep anti-rat IgG (Amersham). Sometimes the mouse and the rat primary antibodies were detected with biotinylated secondary antibodies and FITC-streptavidin (Vector Lab). Photomicrographs were taken with a confocal microscope (Zeiss LSM410).

Heat-shock experiment

Stage 12-13 embryos collected on apple juice-agar plates were transferred to a plastic dish together with a slice of agar (1 mm thick) and covered with parafilm. Heat shock was applied by floating the dishes in a 37˚C water bath for 15 minutes and then incubating them at 25˚C for 30 minutes for recovery. After repeating this procedure, embryos were allowed to develop to appropriate stage and were fixed for immunostaining or mounted in 70% glycerol for direct observation under bright-field optics. For Western blotting, embryos were homogenized in SDS sample buffer (3% SDS, 10% glycerol, 65 mM Tris-HCl [pH 6.8], 5.25% 2-mercaptoethanol, 0.2 mM PMSF, 1 μg/ml aprotinin, 10 μg/ml chymostatin, 2 μg/ml leupeptin, 1 μg/ml pepstatin). Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories). Protein samples (120 μg) were separated in 7.5% SDS-PAGE, blotted as described by Sambrook et al. (1989) and detected with a chemiluminescence kit (NEN). Filters were reprobed according to the manufacturer's instruction and quantitated by use of densitometric analysis software (Quantity One, PDI).

RESULTS

In this study, we classified tracheal branches into two types, thin and thick. Ventral and dorsal branches were thin, as were those seen in the lateral trunk (Figs 4A,B, 5A). The diameter of these branches was small, and a single cell surround their circumference except for the tenth dorsal branch (DB10), the circumference of which consists of two cells. Tip cells in thin branches use filopodia to make initial contact as described in detail below. The dorsal trunk contained thick tracheal branches that consisted of multiple cells in their circumference. No filopodia were apparent on the tip cells in their branches. These two types of branches are described separately below.

Dynamic changes in cell shape and cadherin expression during tracheal branch fusion

According to the shapes and position of tip cells, and to the temporal sequence of *D*E-cadherin expression, the fusion of branches could be separated into five stages (Fig. 1). Since the tracheal development proceeds in the anterior-to-posterior direction along the body axis, it was possible to unambiguously determine the temporal order of the fusion. In stage 0, while tracheal branches were migrating to their targets, *D*E-cadherin accumulation was restricted to the apical side of the tip cell at

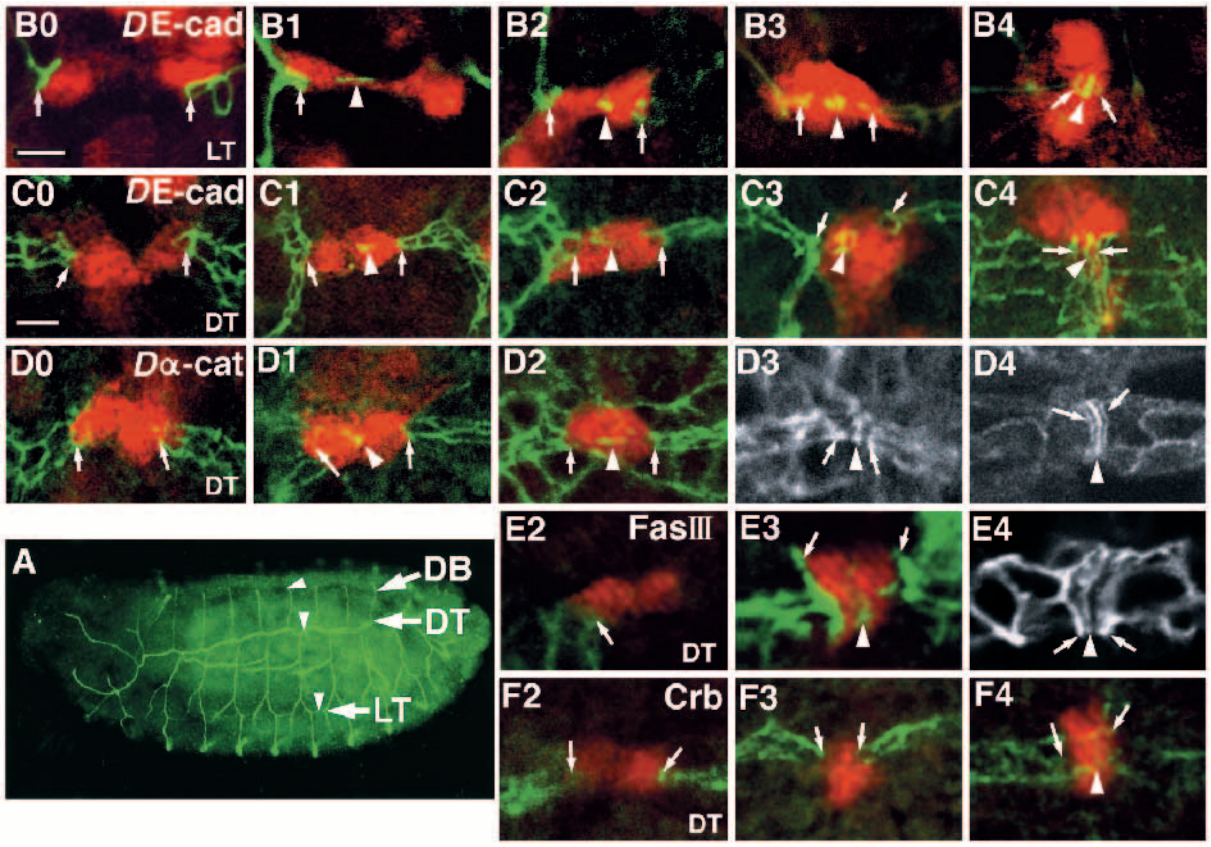

Fig. 1. Change in cell shape and the surface marker expression in tip cells during tracheal fusion. (A) Overall view of the tracheal system stained with the lumen-specific antibody. Arrows and arrowheads indicate dorsal branch (DB), dorsal trunk (DT) and lateral trunk (LT) and their fusion points, respectively. (B-F) *esg*^{G66B} heterozygotes were stained for β-galactosidase to reveal the shape of tip cells (red) and for cell-surface markers (green). (B) *D*E-cadherin expression during lateral trunk fusion. In stage I (B1), tip cells extended filopodia that contact each other and *D*E-cadherin accumulates in a line. Tip cells retracted their filopodia in stage II (B2) and the line of *D*E-cadherin assumed a dot shape. Subsequently, the dot of *D*E-cadherin changed to a ring in stage III (B3) as the lumen forms (Fig. 2C). In stage IV (B4), the major part of cell body was displaced laterally and three rings of *D*E-cadherin moved close to each other. The rings expanded to their final size in this stage. (C) *D*E-cadherin expression during dorsal trunk fusion. No filopodia were detected in this branch. *D*E-cadherin was first detected within one of the tip cells as a line (C1). The line of *D*E-cadherin subsequently extended to encompass both of the tip cells in stage II (C2). In stage III (C3), the *D*E-cadherin was expressed in a ring and underwent the same series of events as observed in the lateral trunk. *D*E-cadherin accumulated in the two tip cells in a seamless ring. (D) *D*α-catenin expression in the dorsal trunk. The pattern was indistinguishable from that of *D*E-cadherin. (E) FasIII expression in the dorsal trunk. Fas III between contact interface was first detected in late stage III (E3). (F) Crb expression in the dorsal trunk. Crb expression followed expansion of the lumen (F2 to F4). Its accumulation between tip cells was only detected in stage IV during expansion of the lumen. In this and other figures, arrows indicate the boundary between a tip cell and the adjacent tracheal cell. Arrowheads indicate contact sites between tip cells. Numbers corresponds to stage of fusion. Bar, 5 μm. Anterior, left; dorsal, up.

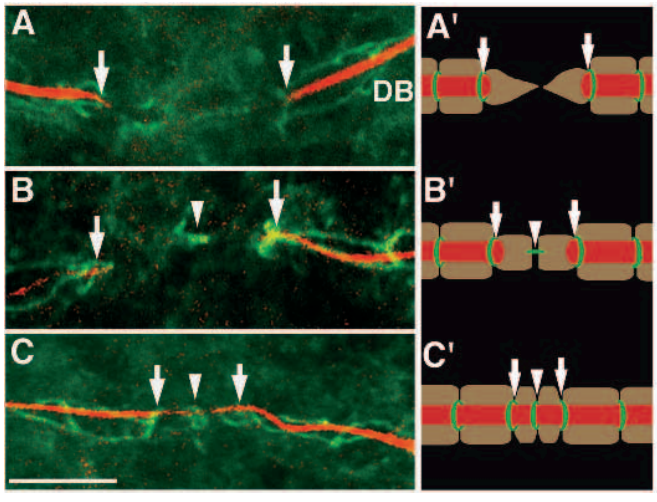

Fig. 2. A ring of *D*E-cadherin protects the site of lumen fusion. (A-C) Dorsal branch 10 stained for tracheal lumen (red) and *D*E-cadherin (green). Tracheal lumen was not detected within tip cell while DB10 was migrating (A; stage 0) or when the tip cells have just contacted each other (B; stage II). When *D*E-cadherin forms a ring in stage III (C), the lumen rapidly forms in the ring. (A′-C′) Schematic diagrams of tip cells during fusion. Note that rings of *D*E-cadherin (green) separate lumen (red) from hemolymph (dark background). Bar, 10 μm; anterior, top.

the interface between the tip cell and the tracheal cell next to it. When the thin tracheal branches had migrated to within 5 μm of their targets (stage I, Fig. 1B1), the tip cells started to extend filopodia to make contact with each other. This was the first time that a line of *D*E-cadherin accumulation was observed at the contact interface. Tip cells then moved closer in tandem as the filopodia shortened (stage II, Fig. 1B2) and the *D*E-cadherin-positive area become a dot in shape at the site of contact. In stage III, the dot became a ring (Fig. 1B3) and, shortly afterward, a lumen appeared in it (Fig. 2C). During stage IV, the ring of *D*E-cadherin expanded and the cell bodies were displaced laterally, leaving thin discs of cytoplasm between branches flanked by three rings of *D*E-cadherin (Fig. 1B4). Fusion of the thick tracheal branch was more straightforward. The tip cells of migrating branches contacted one another directly without extending filopodia and accumulated *D*E-cadherin in a dot shape (stage I, Fig. 1C1). In stage II, the pattern of *D*E-cadherin became a line parallel to the orientation of the branch (Fig. 1C2). It subsequently changed to a ring and the tip cells underwent a series of events similar to those that occurred in thin branches in stages III and IV. The final shape of the tip cell was a seamless ring as judged by the pattern of *D*E-cadherin localization. We next followed fusion of lumen in the tenth dorsal branch (DB10). Staining of luminal antigen was not detectable within the tip cell until stage II (Fig. 2B). In stage III (Fig. 2C) when a ring of *D*E-cadherin was first detectable, a thin lumen penetrated through each tip cell and both lumens become connected at the ring. The expression pattern of *D*α-catenin was very similar to that of *D*E-cadherin in the DT (Fig. 1D) and in thin branches (data not shown). Thus the ring of *D*E-cadherin and *D*α-catenin marking the site of the lumen connection is a common feature of tracheal branch fusion, but distinct steps are taken by thin and thick branches to reach this stage. In contrast to *D*E-cadherin and *D*α-catenin, which were detectable in the contact interface in fusion stage I, FasIII accumulated in the interface in late stage III when the tracheal lumens had already connected (Fig. 1E3). The transmembrane protein Crumbs (Crb) is normally localized to the apical cell membrane of epithelia (Tepaß et al., 1990). Crb accumulation appeared to follow expansion of the apical membrane (Fig. 1F).

Multiple roles of *D*E-cadherin during fusion

To understand the role of *D*E-cadherin in mediating fusion, we studied the effect of reducing *D*E-cadherin activity. Uemura et al. (1996) described tracheal defects of *shg* mutants that could be ordered in a phenotypic series according to severity. They ranged from complete failure of fusion in the null mutants to failure in expansion of the lumen in mild mutants. *D*E-cadherin expression was examined in two classes of mutants. In the 'mild' shg^{E17B} embryo, overall intensity of *D*E-cadherin staining was comparable to that of the control; however, the signal was not localized on the apical side but was more broadly distributed over all of the plasma membrane. Thus the amount of *D*E-cadherin available for adhesion at the adherens junction was reduced. In such embryos, the majority of tracheal branches fused, but did not expand to normal width and exhibited a 'bottleneck' appearance (Uemura et al., 1996; Fig. 3A). Such fusion points were arrested in stage III as a small ring of *D*E-cadherin with a thin lumen inside. A minor fraction of fusion points was arrested in stage 0; tip cells contacted one another but did not accumulate *D*E-cadherin at the contact interface. In no case was fusion arrested at any other stage. The 'intermediate' shg^2 embryos produced unstable *D*E-cadherin molecules as observed in Western blots (Uemura et al., 1996) and showed an increased cytoplasmic *D*E-cadherin signal in immunostained preparations. Tracheal cells in shg^2 embryo appeared rounded, indicating decreased cell-to-cell adhesiveness (Fig. 3B). Fusion points of such embryos showed one of the two phenotypes as seen in shg^{E17B} embryo; in this case, the majority of fusion points were arrested in stage 0 (Fig. 3B). It thus appears that tip cells in stage 0 and III with insufficient amount of *D*E-cadherin cannot continue to the next stage. These results indicate that *D*E-cadherin is not only essential for initial adhesion, but also for expansion of lumen.

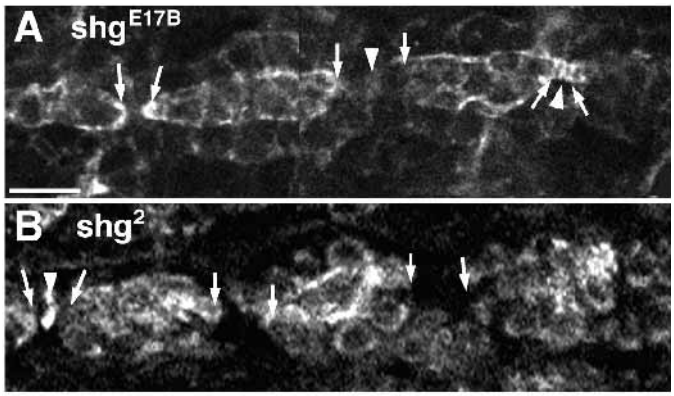

Fig. 3. Requirement of *D*E-cadherin for stabilization of initial contact and expansion of lumen during fusion. *D*E-cadherin staining of dorsal trunk in *shg* mutant embryos is shown. (A) The mild tracheal phenotype (shg^{E17B}). *D*E-cadherin localization was no longer restricted to the apical side. The fusion point at the left showed no *D*E-cadherin accumulation between tip cells. *D*E-cadherin accumulation between tip cells was detectable in the two fusion points at the right which remained in stage III. (B) The intermediate tracheal phenotype (shg^2). Most tracheal cells were rounded, but had migrated to make contact. Only a small fraction of them proceeded to stage III and the majority of them were arrested in stage 0. Arrows, interface between tip cell and adjacent tracheal cells; arrowheads, interface between two tip cells. Three fusion points are shown in each figure. Bar, 10 μm.

Loss of *D*E-cadherin accumulation and failure of tracheal branch fusion in *esg* mutants

Expression of Esg in tip cells during fusion makes it a good candidate for a regulator of tip cell function. In the null esg^{G66B} embryo, none of the fusion points in LT, DB and VB were connected (Figs 4, 5B, and data not shown), a phenotype reminiscent of that of intermediate *shg* mutants (Uemura et al., 1996, Fig. 3B). Tip cells of DB10 in the *esg* mutant migrated and contacted one another normally but never accumulated *D*E-cadherin and subsequently detached (Fig. 5B′). They developed a blind-ended lumen inside, as revealed by deposition of a cuticle in the mature embryo and staining of a luminal antigen (Fig. 5B, data not shown). Branches of DT expressed *D*E-cadherin and were in most cases successfully connected. However, the shape of the dorsal trunk appeared unusual after cuticle deposition. It was occasionally constricted at the tracheal node, suggesting that expansion of lumen was incom-

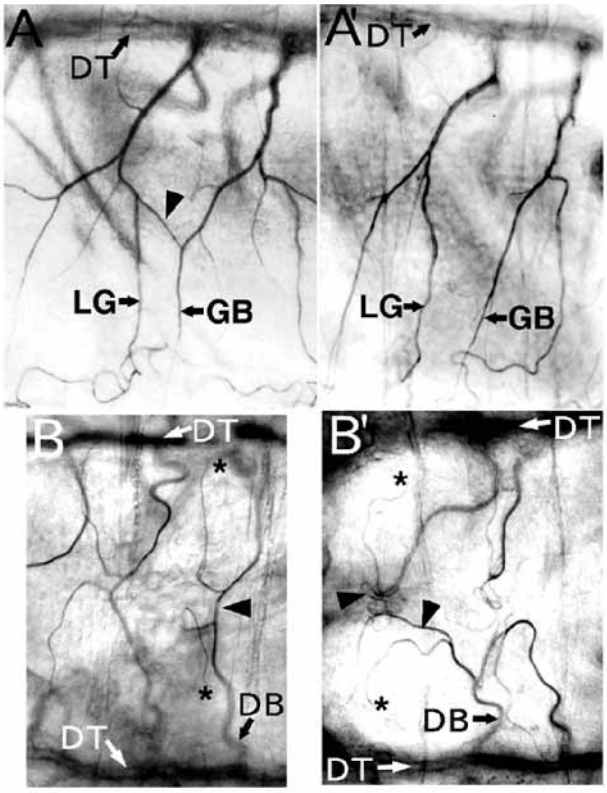

Fig. 4. Failure of tracheal fusion in *esg* mutants. Lateral (A,A′) and dorsal (B,B′) sides of newly hatched first instar larvae are shown. (A,B) esg^{G66B}/+; (A′,B′) esg^{G66B}. (A) Parts of lateral group branch G (LG) and ganglionic branch (GB) have grown out to fuse to form LT. Fusion point is indicated with solid arrowhead. (A′) In esg^{G66B}, fusion of LT did not occur. Unfused branches were still present but have migrated out of focus (see also Fig. 6C). (B) Fusion points of dorsal branch (DB). Terminal branches located next to the fusion branches have migrated to associate with tissue (asterisks). (B′) In esg^{G66B}, no fusion of DB is seen. Instead, long coiled branches freely floating in body cavity were often observed (arrowheads). These branches are distinct from terminal branches in the migration pattern and are assumed to be derived from the mutant tip cells. (A,A′) Anterior, left; dorsal, up. (B,B′) Anterior, left.

plete (Fig. 5B). *HS-esg* transgene was used to rescue the *esg* mutant defects. In the embryos heat shocked seven times, tip cells of DB10 often stably contacted one another and accumulated *D*E-cadherin at their interface (Fig. 5C′). After cuticle deposition, 26.7% (n=105) of DB10 were found to be connected (Fig. 5C). A regime of less than five heat shocks was not effective. Since the heat-shocked period had to cover the entire stage of dorsal branch migration and fusion, the continuous presence of Esg appears to be essential for fusion.

Hyperactive cell motility in *esg* mutant tip cells

Tip cells on thin branches showed an additional interesting phenotype. They migrated and extended filopodia in esg^{G66B} embryos, but did not accumulate *D*E-cadherin or stably adhere to each other. The mutant filopodia detached and continued to extend to various directions (Fig. 6A). They contained a lumen inside and deposited a cuticle in their blind-ended structure (Fig. 4B′), that sometimes reached more than 25 μm in length and displayed a coiled appearance with its terminus floating freely in the body. These features are distinct from the morphology of a terminal branch, which migrates in a relatively straight path and becomes fixed to the tissue. Furthermore, the *esg* mutant tip cells appeared larger than normal and occasionally extended additional lamellopodium-like cytoplasmic protrusions (Fig. 6A). This phenotype could be due to a general property of tip cells that fail to adhere to targets, or could be due to a specific effect of the loss of *esg* activity. To distinguish between these possibilities, we examined phenotypes of branches that did not fuse due to reasons other than *esg* mutations. In the wild-type embryo, fusion of tracheal branches sometimes fails and the frequency can be increased by heat shocks. Fusion of tracheal branches also failed in *breathless* $(btl)^{6\text{-}81a}$ and shg^{2} embryos. In such embryos, no extension of filopodia was observed (Fig. 6B, data not shown), suggesting that the elongated filopodia phenotype is specific to the *esg* mutations. In the dorsal trunk of *esg* mutants, the shape of the tip cells after fusion was unusual. They appeared larger and sometimes extended cytoplasmic protrusions. These phenotypes suggest that, in *esg* mutants, cell motility is hyperactivated in tip cells of both thin and thick tracheal branches.

Transcriptional regulation of *D*E-cadherin by Esg

The promoter activity of *D*E-cadherin was studied by use of the *lacZ* reporter under the control of the *shg* enhancer in shg^{P2370} *(shg-lacZ). shg-lacZ* was expressed uniformly within migrating tracheal precursors (data not shown). When tracheal branches started to fuse, apposed tip cells accumulated a higher amount of β-galactosidase in both thick and thin branches (Fig. 7A,B), suggesting that *shg* transcription is stimulated upon fusion. This stimulation in thin branches requires Esg; upregulation of *shg-lacZ* did not occur in any of thin branches of hemizygous esg^{VS8} embryos (Fig. 7C, data not shown). These results suggest that Esg is required for transcriptional upregulation of *shg* in thin branches to provide a sufficient amount of *D*E-cadherin to support fusion. *D*E-cadherin and *shg-lacZ* expression were unaffected in DT of esg^{VS8} hemizygotes (data not shown), indicating that a distinct mechanism regulates *D*E-cadherin transcription in thick branches.

The above observation suggests that Esg normally stimulates transcription of *D*E-cadherin in tip cells of thin branches. Western blotting experiments were performed to quantitate the effect *HS-esg* on *shg-lacZ* and *D*E-cadherin expression. We observed a consistent increase in β-galactosidase and *D*E-cadherin levels upon induction of *HS-esg* (Fig. 8A,B,D). The amounts of *D*α-catenin and protein phosphatase 2A did not vary significantly between different strains and were not affected by heat shock (Fig. 8C,D). Time-course experiments showed that the amount of *D*E-cadherin reached maximum 30 minutes after the end of a heat shock and then started to decline (Fig. 8E). *D*E-cadherin induced by *HS-esg* was shown to be localized in the cytoplasm (data not shown), suggesting that most of the induced *D*E-cadherin did not participate in cell adhesion and was rapidly degraded. These results indicate that Esg can stimulate transcription of *D*E-cadherin in non-tracheal cells when expressed in a large amount.

Rescue of the tracheal defect in *esg* mutant by *D*E-cadherin

To test whether the fusion defect in the *esg* mutant is due to the loss of *D*E-cadherin expression, we examined an effect of

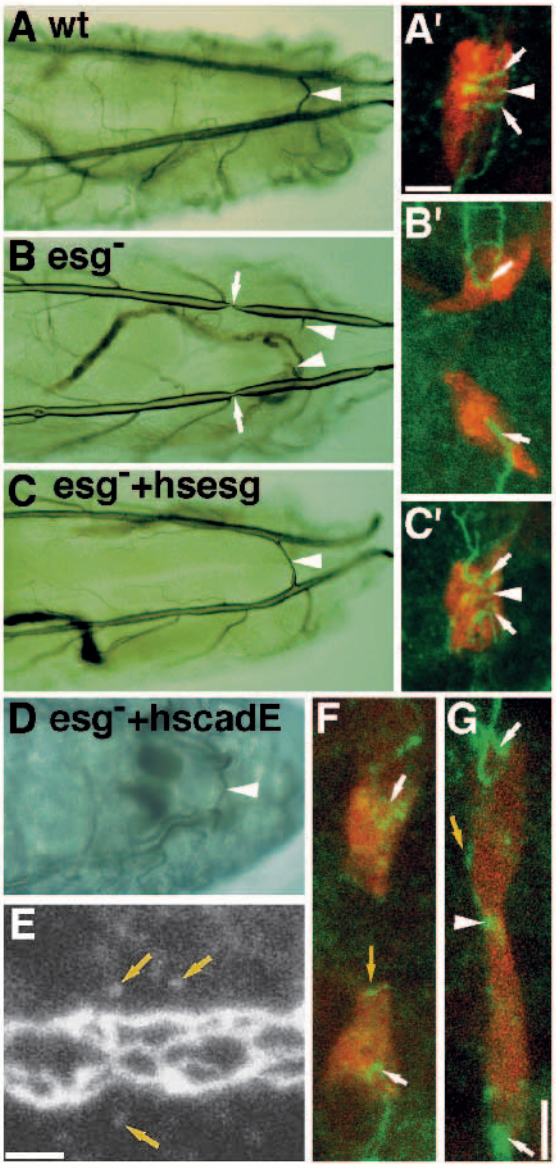

Fig. 5. Phenotypes of *esg* mutant tip cells and its rescue by *HS-esg* and *hsp-cadE*. (A-C) Whole-mount preparations of newly hatched first instar larvae. (A′,B′,C′,F,G) Fusion points of DB10 stained for *D*E-cadherin (green) and for β-galactosidase expressed by esg^{G66B} (red). (D) Whole-mount preparation of mature embryo. (A,A′) Normal morphologies of DB10 fusion points in esg^{G66B} / + embryo and larvae. (B,B′) In the esg^{G66B} embryo, tip cells did not accumulate *D*E-cadherin in their filopodia, which migrate in other directions and no fusion occurred. In addition, some fusion points in DT remain constricted (white arrows). (C,C′) esg^{G66B}*; HS-esg* embryo heat shocked seven times. *D*E-cadherin expression and adhesion were restored, and the larvae formed completely fused DB10. (D) *hsp-cadE*; esg^{G66B} embryo strongly heat shocked two times. Completely fused DB10 is indicated by the solid arrowhead. This photo is less clear than A′, B′, C′ because the embryo is still covered by the vitellin membrane. (E) Dorsal trunk of *hsp-cadE*; esg^{G66B} embryo that was mildly heat shocked (32˚C for 15 minutes) and immediately fixed. Note punctuated *D*E-cadherin stainings not normally seen in wild-type or esg^{G66B} embryos (yellow arrows). (F) The fusion point of DB10 of an embryo similar to that shown in E. Punctuated *D*E-cadherin staining is observed at the end of filopodium. (G) Similar to E, fixed 30 minutes after the end of a mild heat shock. Tip cells adhered to each other with high concentration of *D*E-cadherin (arrowhead). *D*E-cadherin staining on surface is also found in the other part of the tip cells (yellow arrows). Bars, 5 μm.

*D*E-cadherin expression from the heat-shock promoter on the esg^{G66B} background. *D*E-cadherin induced with a mild heat shock (32˚C, 15 minutes) appeared in a punctuated pattern in basolateral side of epitherial cells in dorsal trunk (Fig. 5E), no other difference in the pattern and intensity of *D*E-cadherin staining was observed. In tip cells of DB10 in esg^{G66B} embryos, punctuated staining of *D*E-cadherin was seen on the surface of filopodia, especially at the terminus which was yet to contact to the target (Fig. 5F). 30 minutes later, tip cells of DB10 in some of the treated embryo adhered to each other and *D*E-cadherin accumulated in the contact interface (Fig. 5G). With stronger induction conditions (37˚C 30 minutes heat shock repeated twice with 30 minutes interval), which induced a large amount of *D*E-cadherin filling the plasma membrane and the cytoplasm, the frequency of successful adhesion was increased and completely fused DB10 were frequently observed at the end of embryogenesis (26.7%, n=101; Fig. 5D). Therefore induced *D*E-cadherin localized to filopodia of tip cells and supported stable adhesion to complete fusion of DB10 in *esg* mutants.

DISCUSSION

Cell motility and cell adhesion play essential roles in the construction of organs during development of multicellular organisms. We studied the function of Esg in coordinating these two activities in the formation of the tracheal network in *Drosophila*. The tracheal tip cells were shown to dynamically change cell shape and apical-basal polarity, and activate transcription of the essential cell adhesion molecule *D*E-cadherin. In the thin tracheal branch of *esg* mutant, motility of filopodia was hyperactivated and *D*E-cadherin was not properly expressed. The rescue of fusion in DB10 by *hsp-cadE* suggests that *D*E-cadherin is a major target for Esg in regulating the fusion of thin branches.

The effects of *HS-esg* and *hsp-cadE* to rescue the fusion defect was limited to DB10, which has a circumference comprising two cells instead of one as usually seen in other thin branches, and it migrates the shortest distance among the dorsal branches. In addition, movement of DB is coupled to dorsal closure, which may make path finding less problematic. We think these properties hold tip cells on DB10 close together in the *esg* mutant condition and make the rescue more effective. However, we think it highly likely that the genetic interaction seen in DB10 is relevant to other thin branches. Samakovlis et al. (1996b) recently reported cellular analyses of tracheal fusion and its regulation by *esg*. Their results are basically in good agreement with the results in this paper, although they presented a somewhat different view on the role of *esg*, which remains to be clarified.

Dynamic changes in adhesion and polarity of tip cells during tracheal fusion

This study revealed the dynamic behavior of the tracheal tip cell. Once thin branches have migrated to within 5 μm of one another, the tip cells start to extend filopodia to contact each other. Their behavior is similar to mammalian epithelial cells

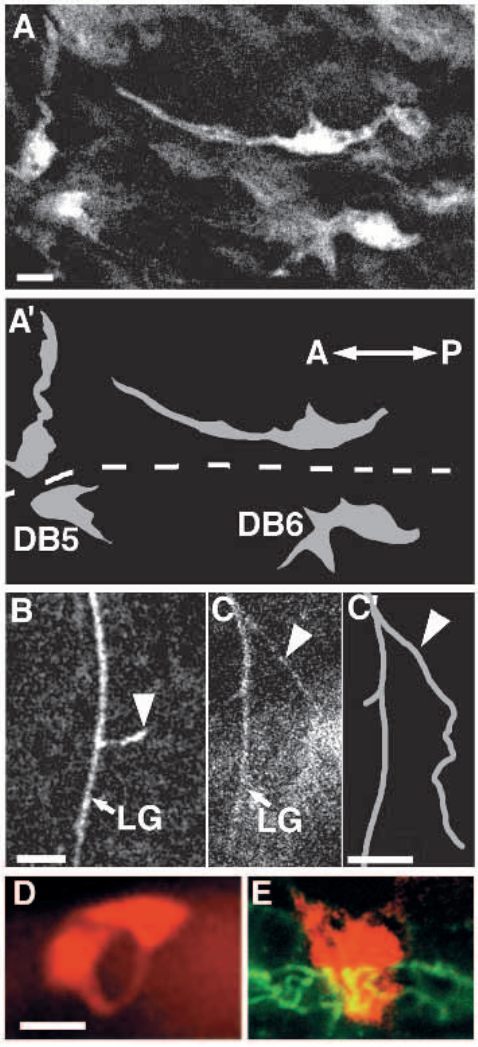

Fig. 6. Hyperactive cell motility in *esg* mutant tip cells. (A) Morphology of dorsal branch tip cells in esg^{G66B} embryo stained with anti-β-galactosidase. The tip cells extended a single long filopodium and additional lamellopodium-like extensions. (A′) Tracing of A. Dorsal midline (broken line) and orientation are indicated. (B) A rare example of unfused branch of LT in wild-type embryo stained with antibody against a luminal antigen. Length of the posterior branch of lateral trunk (LTp) (arrowhead) is 5 μm. (C) Unfused branch of LT in esg^{G66B} embryo. Length of LTp is 24.3 μm. (C′). Tracing of C. (D) Tip cells in DT in late esg^{G66B} / + embryo. Two tip cells adhered tightly together to form a ring. (E) DT of esg^{G66B} embryo of about the same age. Tip cells were still extending cytoplasmic protrusions. This sample is also stained for *D*E-cadherin. Bars, 5 μm.

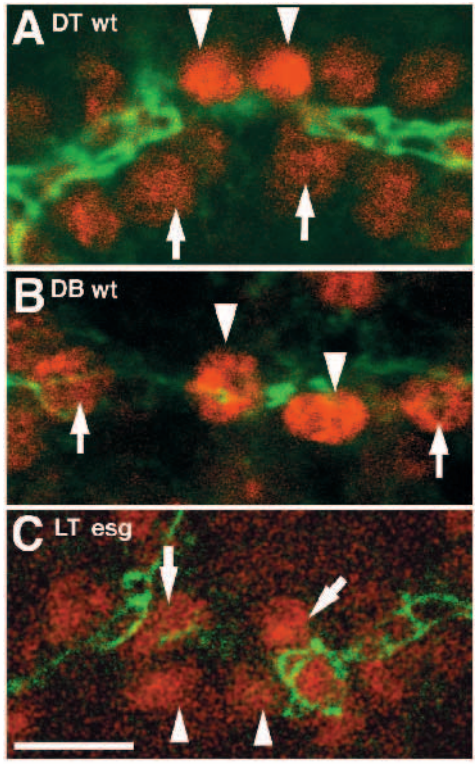

Fig. 7. Upregulation of the *shg* promoter upon fusion. Paternally supplied *shg-lacZ* expression is shown in red; and *D*E-cadherin, in green. Dorsal trunk (A), dorsal branch (B) and lateral trunk (C) fusion points in fusion stage II are shown. Tip cells (arrowheads) expressed a higher level of β-galactosidase than other tracheal cells (arrows) in the esg^{G66B} / *shg-lacZ* condition (A,B). This upregulation was not observed in the lateral trunk of esg^{VS8} *shg-lacZ / Df(2L)A48* embryos (C). Bar, 5 μm.

in culture, which extend filopodia to make multiple transient contacts with each other prior to stable adhesion via E-cadherin (Mcneill et al., 1993; Yonemura et al., 1995). The behavior of tip cells suggests that they search and recognize their target by direct contact with filopodia. Once in contact, the tip cells accumulate *D*E-cadherin at the contact interface. This accumulation probably occurs very rapidly after contact, since we rarely found any examples of contacted tip cells without accumulation of *D*E-cadherin. The polarity of the tip cell in this stage is unusual in that *D*E-cadherin, normally localized on the apical side, is expressed on both apical and basal sides of the cell. As this 'bipolar' tip cell retracts its filopodia, the pattern of *D*E-cadherin accumulation changes from a dot to a ring where lumen penetrated. This *D*E-cadherin accumulation is associated with *D*α-catenin and with F-actin (Fig. 1D, our unpublished observation). Inside the ring of accumulated *D*E-cadherin, the gap between the two adjacent tip cells is presumably separated from hemolymph, thus creating a space where the cell membrane can be opened, without leakage of luminal contents. We speculate that the ring of *D*E-cadherin and its associated proteins form a barrier to separate the lumen and hemolymph (see Fig. 2C′), minimizing the risk of mixing of the luminal content and hemolymph, since no breakage of existing junction occurs. FasIII and Crb are first detectable at the contact interface when the ring of *D*E-cadherin accumulation is expanding. These observations suggest that the tracheal lumen is already continuous prior to the full accumulation of FasIII and Crb, and raise the possibility that these two molecules are dispensable for the initial formation of the barrier. The function of FasIII may be to consolidate adhesion in later stage of fusion. The coincident pattern of Crb expression with lumen formation suggests that the function of Crb is to restore apical-basal polarity (Wodarz et al., 1995).

The observed temporal order of the cell-surface marker

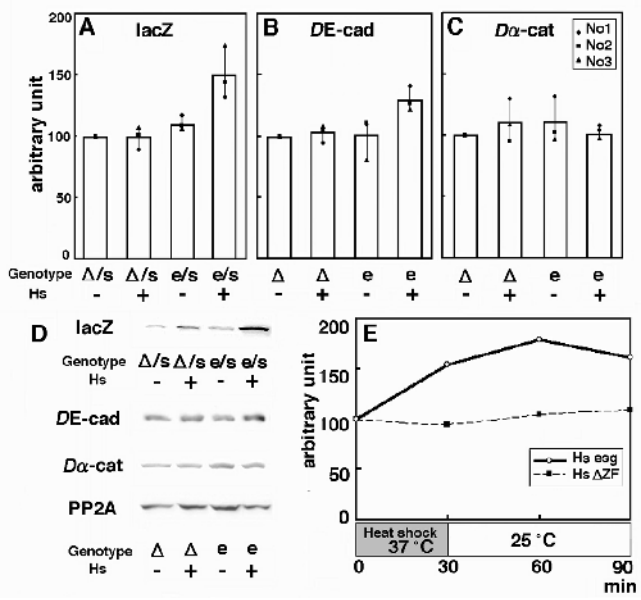

Fig. 8. Activation of the *shg* promoter and *D*E-cadherin expression by *HS-esg*. Embryos were identically treated as in the *HS-esg* rescue experiment and analyzed by western blotting 60 minutes after the end of the last heat shock. Abbreviations of genotype: Δ, *HS-esgΔZF*; e, *HS-esg*; s, *shg-lacZ*. (A-C) Relative amounts of β-galactosidase, *D*E-cadherin and *D*α-catenin in three independent experiments. Protein amount of each experiment was standardized for the sample of non-heat-shocked Δ or Δ / s, and is shown in arbitrary units (vertical axis). Values obtained from three independent experiments and their average (vertical bars) are shown. Genotype and the use or not of heat treatment are indicated below. (D) Representative autoradiograms. Data were collected by serially probing the same filter. (E) Time-course assay. Embryos were given a single heat shock (37˚C for 30 minutes) and then incubated at 25˚C for the indicated time and used for western blotting analysis. Relative amount of *D*E-cadherin normalized for *D*α-catenin is shown.

expressions suggests that *D*E-cadherin may be a key molecule that organizes the junctional complex. This idea was tested by ectopic expression of *D*E-cadherin in esg^{G66B} mutants. *D*E-cadherin not only restored adhesion of tip cells, but also restored fusion. The rescued branches secreted a continuous cuticle in the lumen suggesting that complete junctional complex was formed between tip cells. These results suggest that *D*E-cadherin expression in the tip cell induces the formation of the adherens junction, which in turn induced septate junction and apicalization by Crb, and completion of the junctional complex formation. Consistent with this idea, a similar role of E-cadherin-mediated cell adhesion in inducing epithelial polarization has been demonstrated (Watabe et al., 1994).

The expression of *hsp-cadE* in *esg* mutants revealed an interesting property of tip cells. Induced *D*E-cadherin accumulated in the filopodium of tip cells in a punctuated pattern before they adhere to each other (Fig. 5F), suggesting a sorting mechanism independent of cell contact and the *esg* function localizes *D*E-cadherin at the end of filopodia. We think it likely that reorganization of apical-basal polarity occurrs when tip cells start extending filopodia, and this process do not require *esg*.

Esg regulates cell motility and adhesion of the tip cells in the thin tracheal branches

The use of filopodia to make initial contact is a unique property of the thin tracheal branches. Cells in tissue culture migrate on a substratum by use of filopodia that repeat a cycle of protrusion, adhesion and traction (Lauffenburger and Horwitz, 1996). In the case of the tip cell, cell-cell adhesion replaces cell-substratum adhesion in the adhesion step. In *esg* mutants, tip cells of the thin branches extend filopodia normally but fail to adhere to their target and stay in a prolonged protrusion step. Furthermore, additional short protrusions appear in *esg* mutant tip cells. Hyperactivated tip cell motility is also evident in the fused dorsal trunk which showed numerous protrusions. We have shown that activated cell motility is unique to the *esg* mutations among other conditions that affect fusion, suggesting that *esg* regulates cell motility independent of its function to regulate cell adhesion. Cells injected with activated forms of the small GTP-binding proteins Rac and Cdc42 showed a phenotype similar to that of *esg* mutant tip cells (Nobes and Hall, 1995) suggesting that *esg* is involved in actin-based motility. However, it is not known how the transcriptional regulatory activity of *esg* is related to motility of a filopodium.

Differential requirement for Esg in the fusion branches

In contrast to the striking phenotype in the thin tracheal branches, the *esg* mutations did not block the fusion of the thick tracheal branch, which is different from the thin tracheal branch in several aspects. Thick branches contain multiple cells in their circumference and migrate in a straight path to their targets. No filopodia are apparent during fusion. In the absence of Esg, the tip cells of thick tracheal branches contact, accumulate *D*E-cadherin at the interface, upregulate *shg-lacZ* and fuse as in the wild-type embryo. This suggests that an *esg*-independent pathway activates *D*E-cadherin transcription upon fusion of DT.

Regulation of *D*E-cadherin by Esg

There was no apparent correlation between the temporal change of *esg* expression and the onset of the tracheal defect due to the *esg* mutations. *esg* RNA and protein were detectable in tip cells before fusion and their expression was maintained, albeit at a lower level, in later stages of embryogenesis. This continuous expression of *esg* is probably essential for tracheal fusion, since only repeated induction of *esg* starting prior to the contact between tip cells supported the fusion. We speculate that Esg is essential, but is not sufficient, for expression of target genes such as *shg* and genes regulating cell motility. Contact between tip cells may invoke a signal that activates a rate-limiting factor regulating motility and adhesion. Perhaps such a signal is also important for the switch from the adhesion to the traction step. In the case of thick tracheal branches, in which tip cells contact with large interface from the beginning, the contact-initiated signaling may be sufficient to stimulate *shg* transcription. In the thin tracheal branches, in which initial contact is made in a small region between filopodia, an additional boost from Esg may be essential to activate *shg*. The ability of *HS-esg* to stimulate *shg* transcription in many cell types suggests that Esg is one of many regulators that activate the *shg* promoter and that the amount of Esg is rate limiting in many cells. We have shown

that widely expressed *D*E-cadherin is under dynamic transcriptional control in a cell-type-specific manner. Given the enormous variety of cell types and morphogenetic processes requiring *D*E-cadherin, its regulatory mechanism must be very complex. Focusing on a single cell type in a defined developmental context should be a fruitful way to understand the function of cell adhesion molecules during morphogenesis.

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