

## Genetic control of epithelial tube fusion during *Drosophila* tracheal development

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### SUMMARY

During development of tubular networks such as the mammalian vascular system, the kidney and the *Drosophila* tracheal system, epithelial tubes must fuse to each other to form a continuous network. Little is known of the cellular mechanisms or molecular control of epithelial tube fusion. We describe the cellular dynamics of a tracheal fusion event in *Drosophila* and identify a gene regulatory hierarchy that controls this extraordinary process. A tracheal cell located at the developing fusion point expresses a sequence of specific markers as it grows out and contacts a similar cell from another tube; the two cells adhere and form an intercellular junction, and they become doughnut-shaped cells with the lumen passing

through them. The early fusion marker Fusion-1 is identified as the *escargot* gene. It lies near the top of the regulatory hierarchy, activating the expression of later fusion markers and repressing genes that promote branching. Ectopic expression of *escargot* activates the fusion process and suppresses branching throughout the tracheal system, leading to ectopic tracheal connections that resemble certain arteriovenous malformations in humans. This establishes a simple genetic system to study fusion of epithelial tubes.

Key words: tube fusion, epithelial morphogenesis, branching morphogenesis, trachea, *escargot*, *Drosophila*

### INTRODUCTION

Many networks of epithelial tubes develop from isolated branching units which interconnect by fusion of tubes from different units. In the development of the vertebrate vascular system, for example, blood islands form and then coalesce to establish the network of major vessels (Risau and Flamme, 1995). Later in development, new tubules sprout from these vessels and grow out and fuse with other vessels to further interconnect the vascular network. Fusion of epithelial tubes also occurs during development of the kidney: the developing nephron fuses with the extending ureteric duct system to establish continuity between these tubes, so that urine can flow from the nephron to the ureters (Bard et al., 1994). Fusion of epithelial tubes requires that growing tubes locate a fusion partner, adhere and establish a patent connection between them. Little is known of the cellular mechanisms or molecular control of such epithelial tube fusion events.

The *Drosophila* tracheal (respiratory) system is a branched tubular epithelial network that transports oxygen throughout the body. The twenty tracheal metameres arise independently from sacs of ~80 tracheal cells that undergo a series of sequential branching events (Manning and Krasnow, 1993; Samakovlis et al., 1996). While most tracheal branches continue forming new branches throughout embryonic and larval life, five branches in each metamere cease branching during embryogenesis and grow towards and fuse to branches from the neighboring hemisegments to interconnect the

tracheal network (Fig. 1A-D). Each fusion event is mediated by a single, specialized cell in each of the fusing branches, which expresses a set of fusion markers that were identified in a P[*lacZ*] transposon enhancer trap screen (Samakovlis et al., 1996; see below).

In this paper, we describe the cellular dynamics of a tracheal fusion event and identify a gene regulatory hierarchy that controls it. Each fusion cell and its partner undergo a sophisticated morphogenetic program involving cytoplasmic outgrowth, cell adhesion and formation of an intracellular lumen, generating a connecting joint composed of two doughnut-shaped cells. Fusion markers are expressed in a specific sequence that anticipates the cellular events of fusion. One of the two early markers is identified as the *escargot* gene, which encodes a zinc finger transcription factor that has previously been found to function in the development of imaginal histoblasts (Ashburner et al., 1990; Whiteley et al., 1992; Hayashi et al., 1993; Fuse et al., 1994). *escargot* is an activator of the fusion program, as well as a repressor of branching, that can drive ectopic tracheal fusion events and repress terminal branching when misexpressed.

### MATERIALS AND METHODS

#### *Drosophila* strains

The enhancer trap markers 1-eve-1 (Tracheal-1), Fusion-1 to 4, Fusion-7 (Branch-2), and Terminal-1 and 2 have been described

(Perrimon et al., 1991; Samakovlis et al., 1996). Fusion-1 alleles l(2)07082 and l(2)05730 were generated in the laboratory of Alan Spradling (Spradling et al., 1995), and the B7-2-22 allele was generated in the laboratory of Y. N. Jan (Hartenstein and Jan, 1992). The Fusion-5 and Fusion-6 markers are P[lacZ] insertions at cytological positions 92E and 99D, respectively. The *escargot* mutant strains used were the null *escargot* allele G66 (Whiteley et al., 1992) and the strong EMS-induced alleles VS2 and VS8 (Ashburner et al., 1990; Hayashi et al., 1993). The chromosomal deficiency used was *Df(2L)osp29* which removes the *escargot* locus.

### Embryo fixation and staining

Embryo fixation and staining, and light and confocal fluorescence microscopy were as described (Samakovlis et al., 1996). The lumen-specific antibodies used were TL-1 and mAb2A12. The anti-*escargot* antibody was a rat polyclonal antiserum from Shigeo Hayashi (Fuse et al., 1994). The anti-DSRF monoclonal antibody was mAb 2-161 from Michael Gilman (Ariad Corporation, Boston, MA) and the anti-oracle guinea pig polyclonal antiserum was from Rick Fehon (Fehon et al., 1994). Embryo staging was according to Campos-Ortega and Hartenstein (1985).

Tracheal cell counts and TUNEL staining to detect dead or dying cells in the lateral trunk were performed as described (White et al., 1994; Samakovlis et al., 1996). Cell counts were done on the lateral trunk of tracheal metameres Tr5 and 6 in an *escargot* hemizygote (*escargot*<sup>VS8</sup>/*Df(2L)osp29*) carrying the Tracheal-1 or Tracheal-2 markers; the comparison strain was an *escargot* heterozygote. TUNEL staining was followed by staining with the TL1 tracheal luminal marker to identify labelled cells in the lateral trunk.

### Electron microscopy

Specimens were prepared for electron microscopy as described (Tepass and Hartenstein, 1994) except that the fixative was 25% glutaraldehyde, 4% paraformaldehyde and 3% tannic acid in 0.1 M phosphate buffer. Specimens were viewed with a JEOL100CX electron microscope.

### Molecular biology

Molecular mapping of Fusion-1 alleles was carried out by polymerase chain reaction of genomic DNA prepared from the enhancer trap mutants using primers representing either positions 472-490 or 1484-1466 in the *escargot* sequence (Whiteley et al., 1992) in conjunction with a primer from the P element inverted repeat.

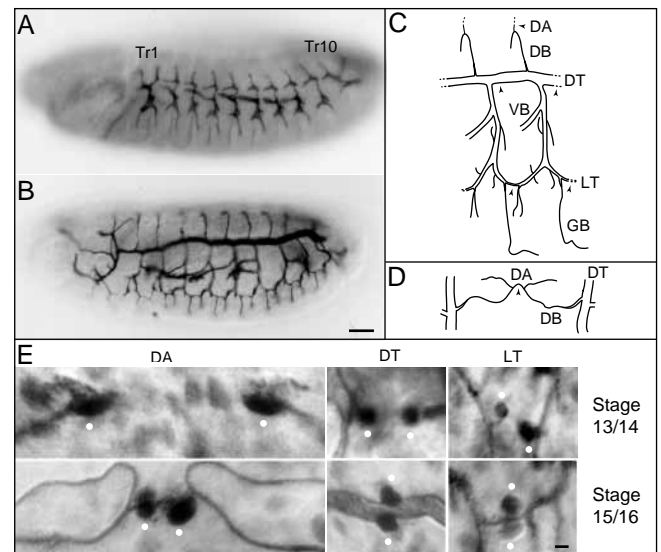
### GAL4 strains and ectopic expression of *escargot*

The driver strains used, TrGal4 and C38, express GAL4 in most tracheal cells beginning at stage 13 (Guillemin et al., 1996). The UAS-*escargot* construct has been described (Fuse et al., 1994). Embryos carrying a GAL4 driver and the UAS-*escargot* construct were collected at 18°C for 10 hours, aged at 22°C for 4 hours and then transferred to 29°C for 5 hours to maximize GAL4 activity. Embryos were fixed and stained with mAb2A12 alone or mAb2A12 and DSRF mAb 2-161 to examine tracheal morphology.

## RESULTS

### Cell dynamics of a tracheal fusion event

Fifty tracheal fusion events occur during embryonic development to interconnect the tracheal network. Dorsal and lateral trunk fusion events link up neighboring tracheal metameres on each side of the embryo, while dorsal and ventral anastomosis fusion events connect the two sides of the tracheal system (Fig. 1A-D). Each fusion event is mediated by a specialized cell at the tip of each fusion branch which expresses the Fusion-1

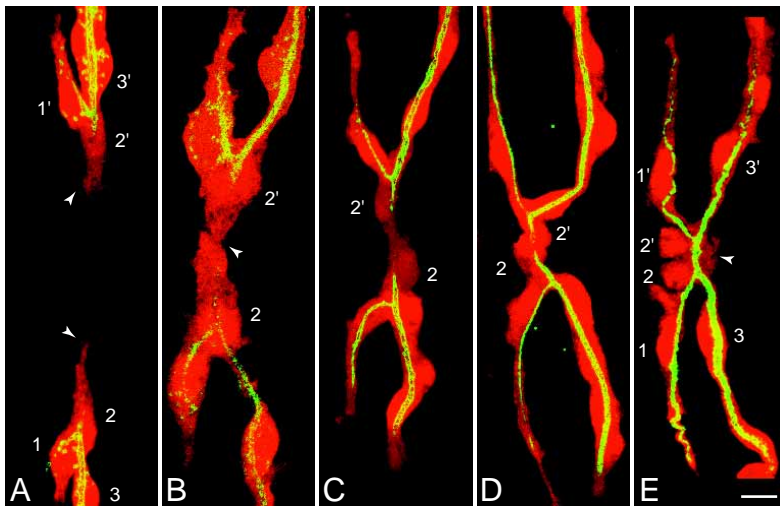


**Fig. 1.** Tracheal fusion and the expression of fusion markers at developing fusion joints. (A) The left side of the tracheal system at stage 13 before branch fusion (anterior left, dorsal up). There are ten independent tracheal hemisegments, Tr1 to Tr10. The embryo was stained with a lumen-specific antiserum. (B) Three hours later at stage 15 when specific branches in each hemisegment have fused to branches in the neighbouring hemisegments to form the dorsal and lateral tracheal trunks. The dorsal and ventral anastomoses (not shown), which interconnect the left and right sides of the tracheal system, form slightly later at stage 16. Bar, 25  $\mu$ m. (C) Schematic diagram of Tr5 and Tr6 (lateral view). The five positions where branches of Tr 6 have fused to branches in the neighboring tracheal hemisegments to form the dorsal anastomosis (DA), dorsal trunk (DT) and lateral trunk (LT) are indicated by arrowheads. Ventral anastomoses (not shown) arise from the ganglionic branches (GB) in Tr1 and Tr2. DB, dorsal branch; VB, visceral branch. (D) Schematic diagram of the left and right Tr6 showing the DA that connects them (dorsal view, anterior at top). (E) Expression of the Fusion-1 enhancer trap marker in both fusion cell partners before (top panels) and after (bottom panels) branch fusion to form the DA, DT and LT. Embryos carrying the Fusion-1 marker were double-stained for a tracheal luminal antigen (TL-1, top panels, mAb2A12, bottom panels) and the marker protein ( $\beta$ -galactosidase) which is expressed in the nucleus of all fusion cells. White dots are placed adjacent to the fusion cell nuclei. Bar, 2  $\mu$ m.

marker and other fusion markers and undergoes a similar morphogenetic program that results in formation of a bi-cellular fusion joint (Fig. 1E).

We analyzed in detail the cellular and molecular events of fusion of the dorsal branches (DB) to form a dorsal anastomosis (DA) in the second through ninth tracheal metameres. Initially each DB fusion cell (DB2 cell) lies at or near the end of the outgrowing DB and is indistinguishable from neighboring tracheal cells. The fusion cell extends a cytoplasmic process that grows out and contacts a similar outgrowth from its partner (Fig. 2A,B). After contact, the cells contract and their cell bodies move toward each other, and a lumen extends through each cell (Fig. 2C,D). The cells align their lumens and the lumens fuse end-on, forming a complete passage through each fusion cell and establishing the connection between branches (Fig. 2E). The entire process takes about 3 hours.

The two fusion cells do not form a syncytium in the process,



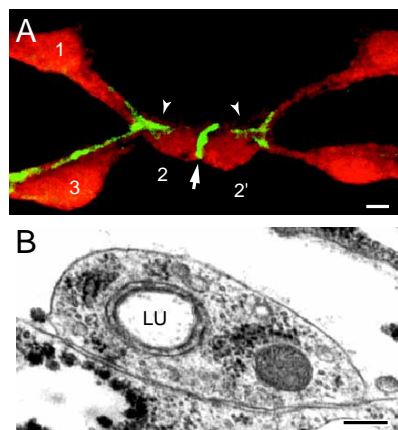
**Fig. 2.** Cell dynamics of a tracheal fusion event. Confocal images of DB fusion cells forming a dorsal anastomosis at successive stages of fusion, showing tracheal cells (1-*eve-1* marker, pseudocolored red) and lumen (mAb2A12; pseudocolored green) (dorsal view, anterior left). The cell bodies of the DB1, 2 and 3 cells in the two dorsal branches are labeled (1, 2, 3 and 1', 2', 3'). Each fusion cell (2 and 2') extends a cytoplasmic process towards its partner (arrowheads in A). After contact (arrowhead in B), their cell bodies move toward each other (C,D) and a lumen extends through each cell and connects to the lumen of its partner (E). The lumen expands slightly at the fusion point just prior to lumen fusion and the expansion persists afterwards (arrowhead in E). Bar, 5 µm.

but become tightly attached by an intercellular junction that can be visualised in electron micrographs or by staining with an anti-coracle antibody that labels septate junctions (Fig. 3A). The structure of the fusion cells is unlike other DB cells, which are curled up into a tube and have an autocellular junctional seam running along their length (Samakovlis et al., 1996). Junctional immunostains (Fig. 3A) and electron micrographs (Fig. 3B) show that the fusion cells lack a junctional seam and the tracheal lumen passes through each cell. In cross section, each fusion cell looks like a doughnut with the lumen forming the hole in the center (Fig. 3B).

**Fusion markers are expressed in a specific sequence during fusion**

During DB fusion, six fusion markers were activated in a fixed

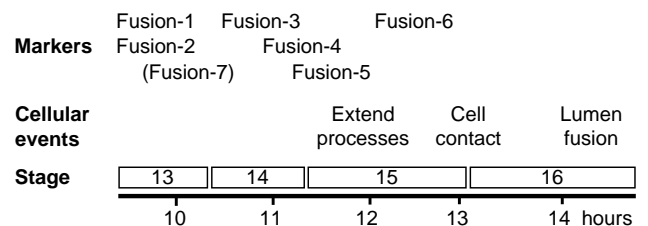
**Fig. 3.** Structure of a fusion joint. (A) Confocal projection through a fully formed DA fusion joint (dorsal view, anterior at top) showing tracheal cells (1-*eve-1* marker, pseudocolored red) and cell junctions (anti-coracle antiserum, pseudocolored green). The DB fusion cells (2 and 2') that form the fusion joint are attached by an intercellular junction (arrow); junctions with the DB1 and DB3 cells are also indicated (arrowheads). There is no coracle staining in the part of the fusion cell adjacent to the fusion point, indicating the absence of autocellular junctions. Analysis of serial confocal sections (not shown) confirmed that the DB2 fusion cells are seamless tubes. Bar, 2 µm. (B) Electron micrograph of a cross section through a DB2 fusion cell at stage 16. The cell is doughnut-shaped with the lumen (LU) forming a hole in the center of the cell. Note that there is no autocellular junction, which would appear as a double membrane connecting the plasma membrane to the membrane surrounding the lumen. Bar, 0.22 µm



sequence that anticipated the cellular events of fusion (Fig. 4). The earliest markers (Fusion-1 and Fusion-2) turned on at stage 13, about 2 hours before the first morphological events of fusion (Fig. 1E). The other fusion markers turned on over the next 1-3 hours, as the fusion partners approached each other. The order of fusion marker activation in other tracheal fusion events was similar, although two of the markers were not expressed in all fusion cells (see legend to Fig. 4). The correlation of fusion marker expression with the cellular events of fusion and the finding that mutations in these markers disrupt fusion (C. S., G. M., and N. H., unpublished results) suggested that the marker genes might comprise a hierarchical genetic regulatory program that controls the fusion process. Our molecular genetic analysis of Fusion-1, described below, supports this hypothesis.

**The Fusion-1 marker is *escargot***

Three Fusion-1 P[*lacZ*] inserts were mapped to cytological position 35C,D (Samakovlis et al., 1996). We carried out complementation tests with existing mutations in the region which showed that the homozygous lethal Fusion-1 alleles 1(2)07082



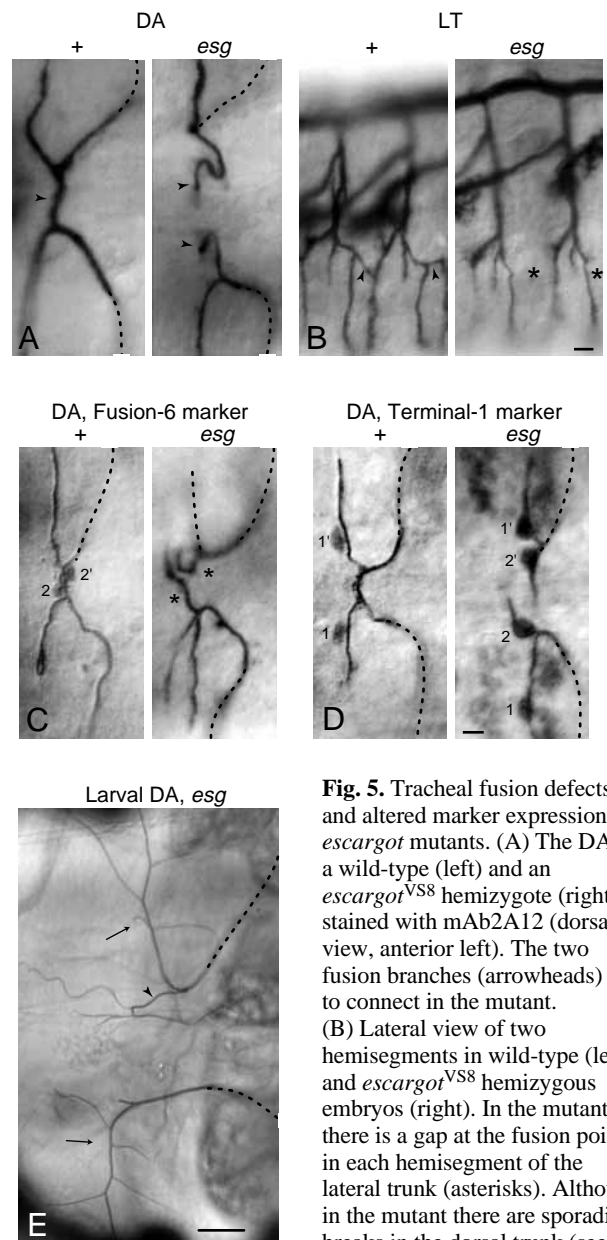
**Fig. 4.** Time course of fusion marker expression. The onset of expression of the different fusion marker markers in DB fusion cells during formation of the dorsal anastomosis was determined by immunostaining different enhancer trap strains for the beta-galactosidase marker and for a tracheal luminal antigen (TL-1 or mAb2A12) to accurately assess developmental stage. The results are represented on a developmental time line that also shows the timing of the cellular events of fusion (see Fig. 2). The same general order of marker activation and cellular events was observed for the other fusion cells. The Fusion-7 marker, however, was not expressed in DA or LT fusion cells and the Fusion-6 marker was not expressed in DT fusion cells.

and I(2)05730 failed to complement mutations in *escargot*. Molecular mapping of Fusion-1 alleles I(2)07082 and B7-2-22 by polymerase chain reaction (see Materials and Methods) demonstrated that the P[*lacZ*] transposon had inserted in an exon of the *escargot* gene, ~200 and ~400 base pairs, respectively, upstream of the assigned translation start site. Embryo staining with an anti-*escargot* antiserum and an mRNA probe confirmed that *escargot* is expressed in all fusion cells in the same pattern as the Fusion-1 marker, in addition to its previously described expression in imaginal histoblasts and in other embryonic tissues (data not shown). Fusion-1 is therefore allelic to *escargot*.

### ***escargot* is required for fusion and inhibits terminal branching by DB fusion cells**

The tracheal phenotype and the expression of fusion markers were examined in embryos homozygous and hemizygous (over *Df(2L)osp29*) for the null *escargot*<sup>G66</sup> allele (Whiteley et al., 1992) and the strong loss-of-function alleles *escargot*<sup>VS2</sup> and *escargot*<sup>VS8</sup> (Ashburner et al., 1990; Hayashi et al., 1993). The results for all three alleles were indistinguishable and are therefore described together. In the mutants, the DB cells that normally mediate fusion instead formed discrete branches that never met or joined (Fig. 5A). These cells went on to form additional branches that resembled normal terminal branches in the larva, unlike normal fusion cells which undergo no further branching (Fig. 5E). Marker expression studies showed that the mutant fusion cells failed to express the Fusion-4, 5 and 6 markers, while the Fusion-2 and 3 markers continued to be expressed normally (Fig. 5C and data not shown). The mutant cells also inappropriately expressed the terminal branching markers Terminal-1 (DSRF) and Terminal-2 (Fig. 5D); these genes are normally expressed and function only in tracheal cells that undergo terminal branching, such as the DB1 cell (Guillemin et al., 1996; Samakovlis et al., 1996). Thus, *escargot* is required to activate specific fusion genes, as well as to repress expression of genes that control terminal branching, in DB fusion cells.

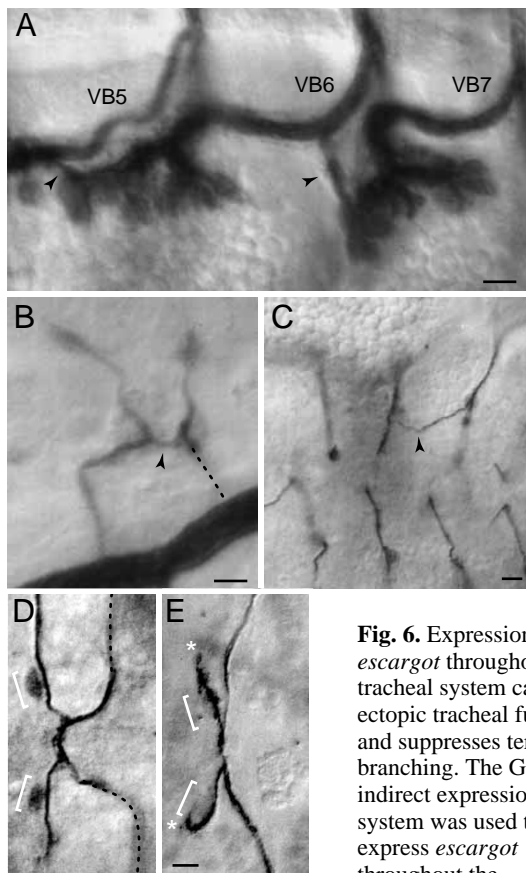
In ventral anastomosis fusion events, the *escargot* gene played a role very similar to the one just described for dorsal anastomosis fusion: it activated expression of the same fusion markers and repressed terminal marker expression in the fusion cells. The dorsal and lateral trunk fusion events, however, had different requirements for *escargot*. Lateral trunk fusion branches were completely missing in *escargot* mutants, leaving a gap in each segment of the lateral trunk (Fig. 5B). The cells (LTa4 and LTp8) that normally form these branches apparently died by apoptosis in the mutants, because (i) no expression of any fusion markers was detected in the lateral trunk, (ii) there were two fewer cells than normal in each segment of the lateral trunk ( $9.1 \pm 1.2$  cells in the *escargot* mutant ( $n=28$  metameres), compared to  $11.5 \pm 1.1$  cells in the control strain ( $n=36$ )), and (iii) TUNEL analysis (White et al., 1994), which transiently labels the DNA of apoptotic cells showed no staining in wild-type embryos ( $n=10$ ), but labelled the lateral trunk fusion cells in 6 of 13 mutant embryos. Unlike the defects in the lateral trunk, ventral anastomosis, and the dorsal anastomosis which were fully penetrant and expressive in the second through ninth tracheal metameres, dorsal trunk fusion proceeded almost normally with only sporadic breaks (~5% of fusion points) observed in the mutants.



**Fig. 5.** Tracheal fusion defects and altered marker expression in *escargot* mutants. (A) The DA in a wild-type (left) and an *escargot*<sup>VS8</sup> hemizygote (right) stained with mAb2A12 (dorsal view, anterior left). The two fusion branches (arrowheads) fail to connect in the mutant. (B) Lateral view of two hemisegments in wild-type (left) and *escargot*<sup>VS8</sup> hemizygote embryos (right). In the mutant there is a gap at the fusion point in each hemisegment of the lateral trunk (asterisks). Although in the mutant there are sporadic breaks in the dorsal trunk (see text), the portion of the dorsal trunk shown is intact. Bar, 5  $\mu$ m. (C) The Fusion-6 marker is expressed in the DB2 cells (2 and 2') of wild-type (left panel) but not in the DB2 cells (asterisks) of the mutant (right panel). (D) DSRF protein (Terminal-1 marker) is inappropriately expressed in the DB2 cell of the mutant (2 and 2', right panel). In the wild type (left panel), DSRF is expressed exclusively in terminal tracheal cells such as DB1 (1 and 1'), where it regulates formation of an extensive array of terminal branches (Guillemin et al., 1996). Bar (A,C,D), 5  $\mu$ m. (E) DA fusion branch (arrowhead) in an *escargot* mutant larva viewed by Nomarski optics (dorsal view, anterior left). Note the ramification into fine branches that resemble normal terminal branches like those formed by the neighboring DB1 cell (arrows). The *escargot* mutant shown is homozygous for a P element insert into an upstream region of the *escargot* locus; it is an apparent null allele for tracheal expression and function but leaves other *escargot* expression intact (C. S. and T. Heino, unpublished data). Other *escargot* alleles die earlier in development but the occasional larval survivor displays the same tracheal phenotype shown. Bar, 10  $\mu$ m. Dashed lines, continuations of tracheal branches out of the plane of focus.

### Ectopic expression of *escargot* causes ectopic tracheal fusion events

To further test the function of *escargot* in branch fusion, we analyzed the consequences of expressing *escargot* in tracheal cells that do not normally mediate fusion. The GAL4 indirect expression system (Brand and Perrimon, 1993) was used to drive *escargot* expression throughout the tracheal system beginning at stage 13, when expression of the gene is normally restricted to just the five fusion cells in each tracheal metamere. This resulted in ectopic expression of the two fusion markers analyzed and formation of ectopic tracheal connections, linking up tracheal branches that normally never join (Fig. 6A-C). Misexpression of *escargot* also repressed terminal marker expression and inhibited terminal branching throughout the tracheal system (Fig. 6D,E).



**Fig. 6.** Expression of *escargot* throughout the tracheal system causes ectopic tracheal fusions and suppresses terminal branching. The GAL4 indirect expression system was used to express *escargot* throughout the developing tracheal

system, starting at stage 13. (A-C) Arrowheads show ectopic connections between the stalk of visceral branch VB6 and the end of VB7 and between the stalk of VB5 and the end of VB6 (A), between the stalks of the Tr7 and Tr8 dorsal branches (B) and between the Tr5 and Tr6 ganglionic branches (C). None of these branches normally connect. (D,E) DSRF (Terminal-1 marker) expression in the DB of a wild-type embryo (D) and an embryo expressing *escargot* throughout the tracheal system (E). Note the inhibition of DSRF expression (brackets) and stunted terminal branches (asterisks) in the DB1 terminal cells in E. Suppression of terminal branching throughout the tracheal system and ectopic fusion events were observed in most embryos and were more pronounced in embryos carrying two copies of the UAS-*escargot* construct. Bars in A,B,E, 5  $\mu$ m; C, 10  $\mu$ m.

### DISCUSSION

The tracheal branch fusion process that we have described is a sophisticated morphogenetic program executed by a specialized cell in each fusing branch. The process is symmetric: both partners express the same markers and undergo the same morphogenetic program in concert. Initially the fusion cells extend a cytoplasmic process that grows out until finding a similar extension from its partner. After contact, the cells adhere and begin to establish an intercellular junction. As their cell bodies draw together, each cell develops an intracellular lumen. The net result is a connecting joint composed of two doughnut-shaped fusion cells, attached face to face, with the lumen running through them.

The initial stages of the fusion process are reminiscent of other oriented cytoplasmic outgrowths, such as shmoo formation during mating in yeast (Drubin and Nelson, 1996) and neurite extension in animals (Tanaka and Sabry, 1995). Although the signals that guide outgrowth of the fusion cells are unknown, it seems likely that the growing cells either signal each other or use a common guidepost provided by a surrounding tissue in order to find each other. The cellular events following contact of the fusion cells are more unusual, as the cells develop an intracellular lumen spanning the length of the cell. One appealing mechanism for this intracellular lumen formation is an oriented vesicular fusion process. Vesicles might line up in the cytoplasm and fuse, generating an elongate vesicle spanning the length of the cell and ultimately fusing with the plasma membrane to establish the external openings that allow air to flow through the cell. Whatever the mechanism, in order to establish a functional connection the lumen has to be directly in line with the lumen of its fusion partner and continuous with the lumen of the preexisting branch.

A gene regulatory hierarchy with seven extant members controls the fusion process. The genes are expressed sequentially in the fusion cells beginning two hours before the first morphological signs of fusion and anticipating the morphological events of fusion. *escargot*, the Fusion-1 marker, is one of the two earliest markers and lies near the top of the hierarchy. It is a key regulator that activates expression of some of the later fusion markers and can induce ectopic marker expression and ectopic branch fusion events when misexpressed. Since the *escargot* protein is a DNA-binding transcription factor, some of these regulatory effects may be direct. If so, then *escargot* can function as a transcriptional activator, as well as a transcriptional repressor as was previously shown (Fuse et al., 1994). The late genes in the regulatory hierarchy presumably execute the cellular events of fusion including cell adhesion and morphogenesis into doughnut-shaped cells. Further analysis of the hierarchy and the other fusion genes should reveal how they control and execute the remarkable cell dynamics of fusion and how the hierarchy is normally activated just in fusion cells.

The cessation of branching is another important aspect of the branch fusion program which is also regulated by *escargot*. In the absence of *escargot*, the DB fusion cells inappropriately expressed terminal cell markers and they went on to form fine branches that resembled the normal networks of terminal branches. Furthermore, when *escargot* was expressed throughout the developing tracheal system, it suppressed terminal

marker expression and terminal branching everywhere. *escargot* thus has two functions in fusion: it activates expression of fusion markers and promotes branch fusion, and it represses terminal marker expression and prevents terminal branching.

We have focused here on the fusion program controlling formation of the dorsal anastomoses in the second through ninth tracheal metameres. While the other pairs of tracheal fusion cells express a similar set of fusion genes and undergo a superficially similar morphogenetic process, their regulatory programs are not all identical. Two of the fusion markers were not expressed in some sets of fusion cells. Moreover, not every fusion cell responded to loss of *escargot* function in the same way as the dorsal branch fusion cells. Lateral trunk fusion cells died in *escargot* mutants and dorsal trunk fusion cells were only sporadically affected. Thus, other factors must influence the function of *escargot* and the fusion program at different fusion positions in the embryo.

The simple genetic system established here to study fusion of tracheal tubes may help understand other epithelial tube fusion processes and how they go awry, as in the human vascular system where there are sometimes anomalous connections between arteries and veins that resemble the ectopic tracheal fusions that we observed (Young, 1988). Blood vessel fusion also appears to occur by cytoplasmic outgrowth and cell adhesion (Flamme et al., 1993), and there is evidence that genesis of an intracellular lumen and formation of doughnut-shaped cells might also be involved (Wolff and Bär, 1972; Folkman and Haudenschild, 1980). And, since vertebrate homologs of *escargot* are known (Nieto et al., 1992) and can drive tracheal fusion in *Drosophila* (P. S. and C. S., unpublished data), the process could be controlled by similar genes.

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