RESEARCH COMMUNICATION

Single mesodermal cells guide outgrowth of ectodermal tubular structures in *Drosophila*

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The *Drosophila* tracheal system, a tubular network, is formed from isolated ectodermal metameres by guided branch outgrowth and branch fusion. Branch outgrowth is triggered by the localized and transient activity of Branchless (Bnl/dFGF). Here, we report the discovery of a mesodermal cell that links the leading cells of outgrowing main branches 2.5 hr before they fuse. This bridge-cell serves as an essential guidance post and needs Hunchback (Hb) activity to exert its function. The bridge-cell provides cues acting in concert with Bnl/dFGF signaling to mediate directed branch outgrowth that ultimately leads to position-specific branch fusion.

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Formation of three-dimensional tubular structures, such as the insect tracheal system (Manning and Krasnow 1993; Samakovlis et al. 1996), the vertebrate vascular system (Risau 1997), and the lung (Hogan et al. 1997), involves the guided outgrowth of epithelial cells. In *Drosophila*, the tracheal system is generated from 10 isolated lateral cell clusters on each side of the embryo (Fig. 1A). These cell clusters, which are each composed of about 80 ectodermal cells, invaginate in a strictly coordinated manner into the underlying mesoderm, where they establish a pattern of six primary tubular branches (Fig. 1B). Some of these branches grow along the dorsoventral body axis to form the dorsal, the lateral, and the ganglionic branches. Additional primary branches extend along the anteroposterior axis to generate the visceral and dorsal trunk anterior and posterior branches. The individual tracheal cell clusters connect by fusion of the dorsal trunk and the lateral trunk branches (Fig. 1C). The two halves of the network interconnect by anastomosis formation, and the three-dimensional system starts with the transport of gases during larval development (for details, see Manning and Krasnow 1993; Samakovlis et al. 1996).

Tubular branch outgrowth is guided by the local and complex expression pattern of a *Drosophila* FGF homolog, Branchless (Bnl/dFGF), emanating from cell clusters surrounding each tracheal metamere (Sutherland et al. 1996; Metzger and Krasnow 1999). However, although mutant analysis shows that Bnl/dFGF is necessary for primary branch outgrowth, the restricted Bnl/dFGF expression seems not to be essential for the directed outgrowth of all primary branches. This conclusion is based on the observation that the constitutive activation of Bnl/dFGF signaling in *bnl* mutant embryos partially restores outgrowth of the main tracheal tube, the dorsal trunk, whereas the other primary branches are not generated. Thus, it was proposed that additional guidance cues might be necessary for the outgrowth of dorsal trunk branches (Sutherland et al. 1996).

Results and Discussion

We noted a single cell that is marked by expression of the gene *hunchback* (*hb*; Lehmann 1985; Tautz et al. 1987; Hülskamp 1991) at the posterior lateral margin of each tracheal metamere (Fig. 1D,I). This cell gives rise to daughter cells that maintain *hb* expression (Fig. 1E,J,K). The more ventrally located daughter cell maintains a round morphology and remains in position, whereas the dorsal daughter cell connects to the posterior bud of the tracheal metamere, termed the dorsal trunk posterior branch (Fig. 1E). Subsequently, the dorsal daughter cell elongates and extends posteriorly and thereby contacts to the anterior bud, termed the dorsal trunk anterior branch, of the adjacent posterior tracheal metamere (Fig. 1E,F). In this way, the dorsal daughter cell bridges the leading cells of the dorsal trunk anterior and posterior branches of two adjacent metameres (Fig. 1F), which then fuse about 2.5 hr later to form the continuous dorsal trunk. Thus, we refer to the dorsal daughter cell as the bridge-cell. The cell remains at this position until fusion between the dorsal trunk anterior and posterior branches occurs (Fig. 1G). During this fusion process, the bridge-cell becomes displaced and *hb* expression starts to fade (Fig. 1H).

To trace the origin of the bridge-cell, we performed double-staining experiments with tracheal-specific markers and *hb*. β-Galactosidase expression in nuclei of dorsal trunk fusion cells and in nuclei of tracheal cells revealed a lack of colocalization with bridge-cell *hb* expression (Fig. 1L–1O). Furthermore, *trachealess* (*trh*; Isaac and Andrew 1996; Wilk et al. 1996) mutant embryos, which lack tracheal cell identity, show *hb*-expressing bridge-cells as found in wild-type embryos (Fig. 1P,Q). Thus, these results indicate that the bridge-cell is of nontracheal origin. Finally, double-staining of *hb* and a mesodermal marker (Greig and Akam 1993) revealed coexpression of *hb* and the marker in bridge-cell precursors (Fig. 1R,S). Therefore, the bridge-cell is a nontracheal cell and of mesodermal origin.

To understand the function of bridge-cells in dorsal trunk formation, we first asked whether bridge-cell development is affected in *hb* mutant embryos. Homozygous hb^{FB} mutant embryos, which express a nonfunc-

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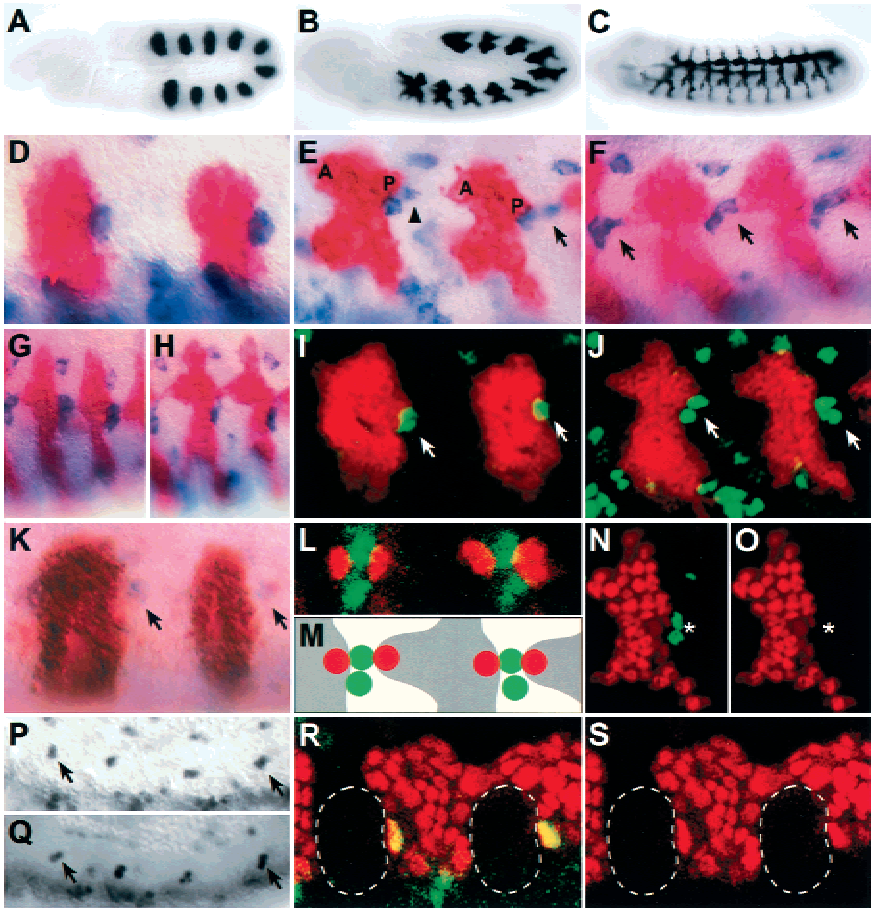

Figure 1. A non-tracheal mesodermal cell connects outgrowing tracheal dorsal trunk branches 2.5 hr prior to their fusion. (*A–C*) Whole-mount in situ hybridization of 1-eve-1 embryos at stage 11 (*A*), stage 12 (*B*), and stage 13 (*C*) with a *lacZ* antisense RNA probe. 1-eve-1 embryos reveal *lacZ* marker gene expression in the tracheal cells. (*D–H*) Whole-mount in situ double hybridization of 1-eve-1 embryos at stage 11 (*D*), early stage 12 (*E*), late stage 12 (*F*), stage 13 (*G*), and stage 14 (*H*) with *lacZ* (red) and *hb* (blue) antisense RNA probes; lateral view focusing on tracheal metameres 2 and 3. Arrowhead in *E* points to a bridge-cell before and arrows in *E* and *F* point to bridge-cells after connection to dorsal trunk anterior branches. (A) Dorsal trunk anterior branch; (P) dorsal trunk posterior branch. (*I,J*) Whole-mount antibody double staining of 1-eve-1 embryos at stage 11 (*I*) and stage 12 (*J*) with anti-β-galactosidase antibodies (red) and anti-Hb antibodies (green). The nuclear stainings with anti-Hb antibodies reveal a single nucleus at stage 11 (arrows in *I*) and two nuclei at stage 12 (arrows in *J*) posterior to the adjacent tracheal metameres. (*K*) Whole-mount in situ double hybridization of 1-eve-1 embryos at stage 11 with *lacZ* (red) and *string* (blue) antisense RNA probes. *string* (*stg*) expression (arrows), a marker for cell proliferation (Edgar and O'Farrell 1990), corresponds to the localization of the Hb-expressing nuclei (see *I*). (*L*) Whole-mount antibody double staining of stage 13 embryos bearing *esg–lacZ* with anti-β-galactosidase antibodies (red) and anti-Hb antibodies (green). The *esg–lacZ* enhancer trap line was used to mark the dorsal trunk fusion cell nuclei by β-galactosidase expression (Whiteley et al. 1992). Merged images of red and green pattern reveal no co-expression of β-galactosidase and Hb. (*M*) Scheme of tracheal branch formation (grey) and the localization of dorsal trunk fusion cell nuclei (red) and Hb-expressing nuclei (green) at stage 13. (*N,O*) Whole-mount antibody double staining of stage 12 embryos bearing *UAS–GFPNlacZ* and *btl–Gal4* with anti-β-galactosidase antibodies (red) and anti-Hb antibodies (green). Merged images (*N*) of β-galactosidase expression in the tracheal cell nuclei (red) and nuclear Hb expression (green) as well as single-channel image for β-galactosidase expression (*O*; asterisks indicate Hb expression) reveals no co-expression of β-galactosidase and Hb. (*P,Q*) Antibody staining of a stage 11 wild-type (*P*) and a *trh* mutant (*Q*) embryo with anti-Hb antibodies. (Arrows) Bridge-cell precursors that correspond to tracheal metameres 1 and 4. (*R,S*) Whole-mount antibody double staining of stage 11 embryos bearing *UAS–GFPNlacZ* and *twi–Gal4* with anti-β-galactosidase antibodies (red) and anti-Hb antibodies (green). (*R*) Merged images of nuclear β-galactosidase (red) and Hb expression (green) reveal co-expression of Hb and β-galactosidase (yellow) showing Hb expression in mesodermal cells. (*S*) Single channel image for *twi*-driven β-galactosidase expression in mesodermal cell nuclei (red); broken lines indicate tracheal placodes.

tional Hb protein because of a premature stop codon mutation (Hülskamp 1991), express the *hb* transcript only transiently in bridge-cell precursors (not shown), raising the possibility that these cells may die. In fact, TUNEL staining suggests cell death is occurring at positions that correspond to those of bridge-cell precursors in hb^{FB} mutants but not in wild-type embryos (Fig. 2A–D). This finding implies that the lack of *hb* activity causes bridge-cell precursors to undergo apoptosis. To show apoptosis as the underlying event of transient *hb* expression in

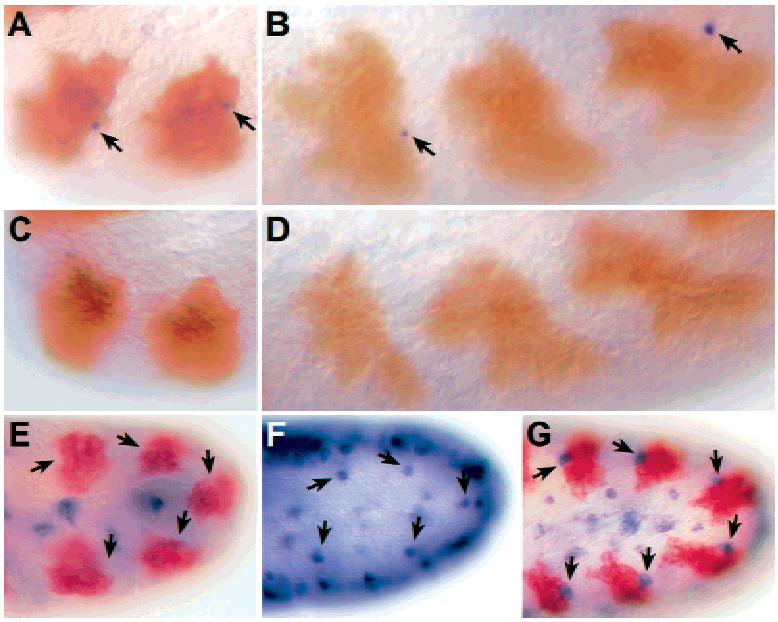

Figure 2. *hb* mutant embryos reveal apoptosis of the bridge-cell. (*A–D*) Whole-mount staining with anti-β-galactosidase antibody (brown) and TUNEL (blue) analysis of late stage 11 (*A,C*) and early stage 12 (*B,D*) hb^{FB} mutant (*A,B*) and wild-type (*C,D*) embryos bearing the 1-eve-1 chromosome. Apoptotic nuclei are detectable in hb^{FB} mutant embryos at posterior lateral positions of the tracheal placodes (arrows in *A* and *B*) but not in wild-type embryos (*C,D*). (*E*) Whole-mount in situ double hybridization of a stage 12 hb^{FB} mutant embryo bearing the 1-eve-1 chromosome with *lacZ* (red) and *hb* (blue) antisense RNA probes. No *hb* expression is detectable at the expected positions of the bridge-cells (arrows in *E*). (*F*) Whole-mount in situ hybridization of a stage 12 hb^{FB} mutant embryo bearing *actin–Gal4* and *UAS–P35* with a *hb* antisense RNA probe. *hb* expression is detectable in corresponding cells (arrows in *F*) after suppression of apoptosis by ubiquitous expression of P35 protein (Hay et al. 1994). (*G*) Whole-mount in situ double hybridization of a stage 12 wild-type embryo bearing the 1-eve-1 chromosome with *lacZ* (red) and *hb* (blue) antisense RNA probes. *hb* expression is detectable in the bridge-cells (arrows in *G*).

bridge-cells more directly, we ubiquitously expressed in hb^{FB} mutant embryos the baculovirus P35 protein, a suppresser of apoptosis in *Drosophila* (Hay et al. 1994). In contrast with hb^{FB} mutants, which lack *hb* expression in the bridge-cells at stage 12 (Fig. 2E), hb^{FB} embryos expressing P35 protein maintain *hb* expression in bridge-cells (Fig. 2F) as is found in wild-type embryos (Fig. 2G). Thus, expression of *hb* serves as a marker for bridge-cells, whereas its product, a transcription factor (Tautz et al. 1987; Hoch et al. 1991), is essential for bridge-cells viability. Therefore, analysis of tracheal development in hb^{FB} mutant embryos would allow us to study bridge-cell function in dorsal trunk formation directly.

In hb^{FB} mutant embryos initial tracheal development, including primary branch outgrowth, appears normal up to the end of stage 12 (Fig. 3A–D). Subsequently, the dorsal trunk branches become stalled and misrouted, whereas the other primary branches are formed as in wild-type embryos (Fig. 3E–H). Despite the strong dorsal trunk phenotype, the dorsal trunk branches occasionally fuse in hb^{FB} mutant embryos and form dorsal trunk rudiments (Fig. 3F,H). This observation and normal expression of the *escargot* (*esg*) fusion cell marker (Whiteley et al. 1992) in hb^{FB} mutant embryos (Fig. 3I) suggest that the fusion process, required for dorsal trunk formation, is not impaired in hb^{FB} mutant embryos. These results indicate that *hb* is not necessary for the initial outgrowth but for the subsequent outgrowth of dorsal trunk branches. Thus, the results also suggest that the *hb*-dependent bridge-cells are involved in the outgrowth of dorsal trunk branches toward their fusion partners.

Recent studies have shown that Bnl/dFGF is necessary for the primary tracheal branching, including the formation of the dorsal trunk (Sutherland et al. 1996; Metzger and Krasnow 1999). Therefore, we asked whether the absence of bridge-cells might interfere with *bnl* expression. We found that the expression pattern of *bnl* was unaffected in *hb* mutant embryos (Fig. 3J,K). Also, *hb* expression in the bridge-cells was not affected in *bnl* mutant embryos and in embryos that lack the activity of *breathless* (*btl*), which codes for the Bnl/dFGF receptor (Fig. 3L; data not shown). Thus, bridge-cells do not interfere with the proper expression of Bnl/dFGF around the developing tracheal branches, and *hb*-expression in the bridge-cells is independent of Bnl/dFGF signaling.

Because localized Bnl/dFGF signaling is not necessary for dorsal trunk formation (Reichman-Fried et al. 1994; Lee et al. 1996; Sutherland et al. 1996), we asked whether the bridge-cell mediates the proposed additional guidance mechanism for dorsal trunk branch outgrowth (Sutherland et al. 1996). By use of the Gal4/UAS-system (Brand and Perrimon 1993), we expressed Bnl/dFGF ectopically in tracheal cells to impede the spatial cues that are normally derived from the local arrangement of cell clusters expressing Bnl/dFGF. In contrast with wild-type embryos (Fig. 4A), embryos with ectopic expression of Bnl/dFGF develop complete dorsal trunk structures but lack the other primary branches (Fig. 4B). However, hb^{FB} mutant embryos that express Bnl/dFGF ectopically had no signs of dorsal trunk branch outgrowth at all (Fig. 4C). These results indicate that the bridge-cell is necessary and essential for dorsal trunk formation, suggesting that this cell provides guidance cues specifically during the anterior–posterior dorsal trunk branch outgrowth. Thus, the bridge-cell, in combination with Bnl/dFGF signaling, directs outgrowth of the main tracheal tube and may mediate the proposed additional guidance mechanism.

To test the above inference, we expressed *hb* ectopically via the Gal4/UAS-system (Brand and Perrimon 1993) in sensory organ precursor (SOP) cells in positions close to the bridge-cells. The outgrowing dorsal trunk anterior branches were seen in contact with the cells

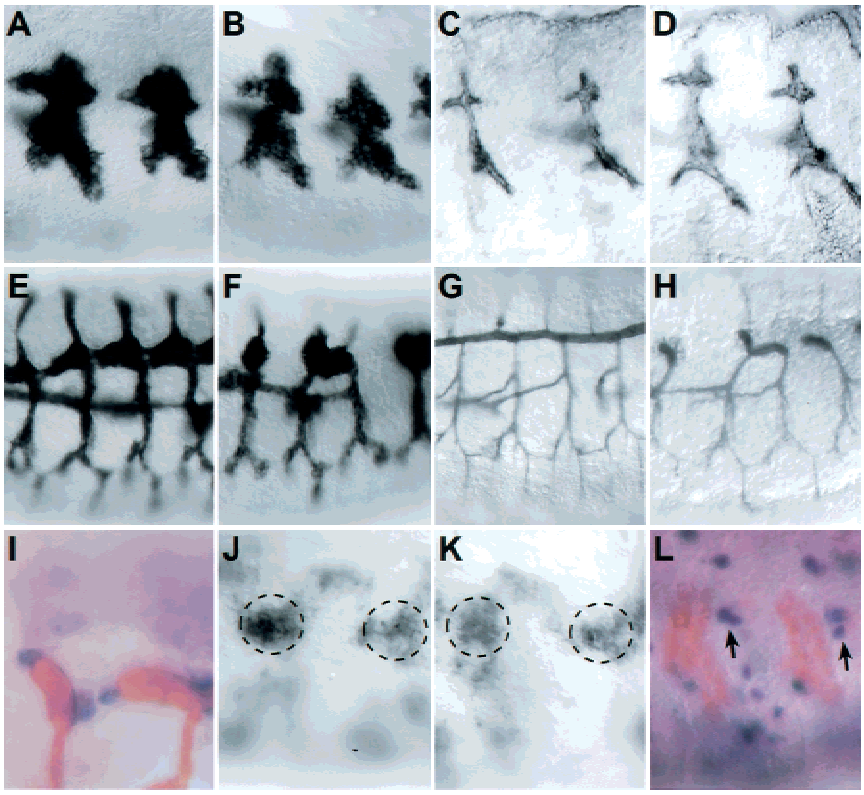

Figure 3. *hb* expression in the bridge-cell is essential for directed outgrowth of dorsal trunk branches and does not interfere with dFGF signaling. Whole-mount in situ hybridization with a *lacZ* antisense RNA probe of wild-type (*A,E*) and hb^{FB} mutant (*B,F*) embryos bearing the 1-eve-1 chromosome at stage 12 (*A,B*) and stage 15 (*E,F*). (*C,D*) Whole-mount antibody staining of a stage 12 wild-type (*C*) and a hb^{FB} mutant (*D*) embryo with anti-Crumbs antibodies. (*G,H*) Whole-mount antibody staining of a stage 15 wild-type (*G*) and a hb^{FB} mutant (*H*) embryo with antibody 2A12. This antibody specifically stains the tracheal lumen and reveals a normal lateral trunk but a lack of dorsal trunk formation in the hb^{FB} mutant (*H*). Note that the hb^{9Q} mutant embryos reveal an identical tracheal phenotype to that found in hb^{FB} mutant embryos. (*I*) Whole-mount antibody double staining of a stage 15 hb^{FB} mutant embryo bearing the G6 chromosome with 2A12 (brown) and anti-β-galactosidase (blue) antibodies reveals *esg*-driven β-galactosidase expression in the dorsal trunk fusion cells of hb^{FB} mutant embryos. (*J,K*) Whole-mount in situ hybridization of a stage 12 wild-type (*J*) and a hb^{FB} mutant (*K*) embryo using *bnl* antisense RNA. The dynamic *bnl* expression pattern surrounding a single tracheal metamere (broken lines indicate *bnl* expression that guides dorsal trunk outgrowth) in either a wild-type (*J*) or hb^{FB} mutant (*K*) embryo is identical. (*L*) Whole-mount antibody double staining of a stage 12 $btl^{H82\Delta3}$ mutant embryo with anti-β-galactosidase (brown) and anti-Hb (blue) antibodies. The $btl^{H82\Delta3}$ mutant embryo reveals β-galactosidase expression in the tracheal nuclei (Reichman-Fried et al. 1994) and Hb expression in the bridge-cells (arrows).

that ectopically express *hb*, even in the presence of the normal bridge-cells (Fig. 4D). As a consequence of the ectopic *hb* expression, the dorsal trunk of the embryos show interruptions and abnormal bottleneck-like fusion points (Fig. 4E). Thus, *hb* expression in ectopic cells close to bridge-cells triggers a differentiation program that interferes with the directed outgrowth of the dorsal trunk branches suggesting that *hb* activity is required not only for the viability but also for the identity of the bridge-cell. Whether the differentiation program involves local and short-range signals and/or provides a migration matrix by cell adhesion is unknown. However, we prefer the hypothesis that the bridge-cell serves as an adhesion-dependent guiding post, as we observed tracheal cell extensions along the bridge-cell directly after the initial contact (Fig. 4F,G).

Our discovery of the bridge-cell and previous studies on Bnl/dFGF signaling provide a coherent model of how dorsal trunk formation may occur. After invagination of the tracheal placodes, budding of the tracheal metameres is triggered by localized Bnl/dFGF activity (Sutherland et al. 1996). This signal apparently does not always have the necessary precision on its own to guide the leading cells. The bridge-cell provides this precision by serving as a guidance post to properly position the budding dorsal trunk branches. The results also demonstrate an interplay of cells deriving from two different germ layers, mesoderm and ectoderm, which is necessary to establish the interconnected tubular tracheal network during embryogenesis. The identification of a key player in bridge-cell differentiation, namely the transcription factor Hb, provides an entry point to unravel the molecular targets of *hb*. Their analysis may also contribute to gaining further insights into the function of the bridge-cells during tubular network formation, possibly in organisms other than *Drosophila*.

Materials and methods

Materials

We used the following antibodies: monoclonal antibody 2A12 to stain tracheal lumen (DSHB, Iowa); anti-β-galactosidase antibody (Promega); anti-Hunchback antibody (gift from A. La Rosée, MPI, Göttingen); anti-Crumbs antibody (Tepass and Knust 1993); Alexa 488 or Alexa 546 goat antirabbit IgG or goat antimouse IgG (Molecular Probes); alkaline phosphatase-conjugated or biotinylated antirabbit IgG or antimouse IgG; biotinylated antimouse IgM (Vector Laboratories); anti-digoxigenin- and anti-fluorescein-AP, Fab fragments (Roche).

We used a number of alleles and fly strains: *UAS-hb* flies (Wimmer et al. 2000); hb^{FB}, hb^{9Q}, dfr^{E82}, trh^{5D55}, and $btl^{H82\Delta3}$ were obtained from the Tübingen Stock Center. *btl-Gal4* drives Gal4 expression ubiquitously in the tracheal system from stage 10 onward (Shiga et al. 1996). UAS-GFP*nlacZ* was used to detect nuclear β-galactosidase expression (Shiga et al. 1996). The *lacZ* enhancer trap line 1-eve-1 reveals P-element integration in the *trh* gene and was used to mark tracheal cells by cytoplasmic β-galactosidase (Perrimon et al. 1991). PO163 drives Gal4 in a subset of peripheral nervous system precursor cells (Janning 1997). *UAS-P35* was provided by H. Steller (MIT, Cambridge). The P-element of the *lacZ* enhancer trap line G6 is integrated in the *esg* gene and marks dorsal trunk homotip cell nuclei (Whiteley et al. 1992). We also used *actin–Gal4* and *twi–Gal4* flies (Greig and Akam 1993).

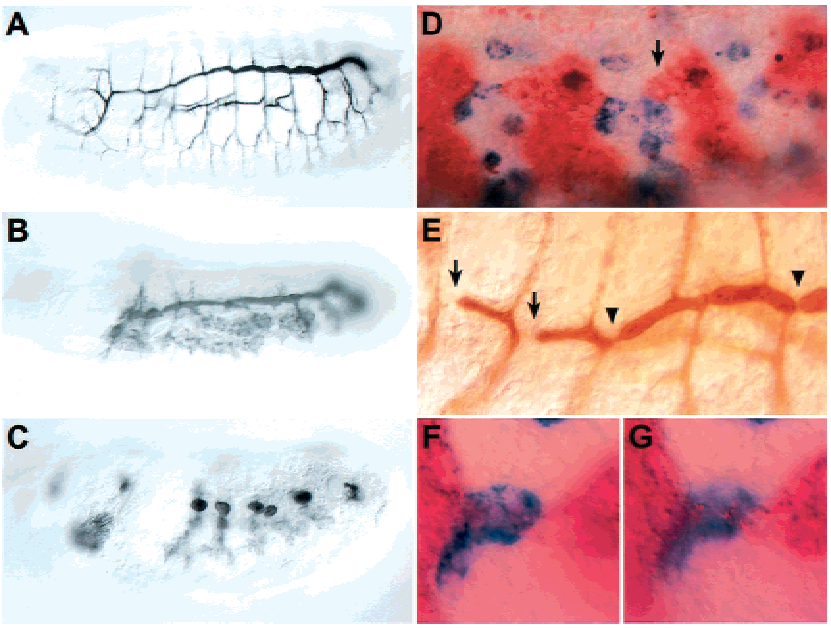

Figure 4. The bridge-cell is necessary for dorsal trunk branch outgrowth. (*A–C*) Whole-mount antibody 2A12 staining of a stage 15 wild-type embryo (*A*), an embryo bearing *UAS–bnl* and *btl–Gal4* (*B*), and a hb^{FB} mutant embryo bearing *UAS–bnl* and *btl–Gal4* (*C*). Note that the tracheal phenotype of amorphic bnl^{P1} mutant embryos bearing *UAS–bnl* and *btl–Gal4* is indistinguishable from the phenotype shown in *B*. The lack of tracheal metameres in hb^{FB} mutant embryos is caused by the lack of segmental anlagen in such embryos (Lehmann 1985; Tautz et al. 1987). (*D*) Whole-mount in situ double-hybridization of a stage 12 embryo bearing *UAS–hb*, *PO163–Gal4* and the 1-eve-1 chromosome with *lacZ* (red) and *hb* (blue) antisense RNA probes. *PO163–Gal4* (Janning 1997) drives *hb* expression in SOP cells. (Arrow) Outgrowing dorsal trunk anterior branch that attaches to a *hb* expressing cell but lacks contact with the bridge-cell. (*E*) Whole-mount antibody staining of a stage 15 embryo bearing *UAS–hb* and *PO163–Gal4* with antibody 2A12. Ectopic *hb* expression causes bottleneck-like lumen formation (arrowheads) and a lack of dorsal trunk interconnection (arrow). (*F*,*G*) Whole-mount in situ double hybridization of a stage 12 embryo bearing the 1-eve-1 chromosome with *lacZ* (red) and *hb* (blue) antisense RNA probes. The same image is shown focused on the bridge-cell (*F*) and the tracheal cell extensions (*G*), respectively.

TUNEL assay

TUNEL analysis of embryos was done by RNA in situ hybridization with the following modifications: After the second fixation step, the embryos were washed with TUNEL reaction mixture (In Situ Cell Death Detection Kit, AP; Roche) and incubated with 100 µl of TUNEL reaction mixture for 120 min at 37°C; the embryos were washed with PBT and incubated with anti-fluorescein antibody (Roche) for 12 hr at 4°C.

Immunostainings and in situ hybridizations

RNA in situ hybridizations and immunostainings to whole-mount embryos were performed as described (Goldstein and Fryberg 1994). The RNA probes used in our experiments were derived from *bnl* (Sutherland et al. 1996), *stg* (Edgar and O'Farrell 1990), *lacZ*, and *sal* (Kühnlein et al. 1994). Immunostained embryos were viewed with a Zeiss Axiophot microscope. Embryos stained with fluorescent antibodies were analyzed by laser scanning microscopy as described (Kühnlein and Schuh 1996).

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