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 dorso-ventral 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,J). This cell gives rise to daughter cells that maintain hb expression (Fig. 1E,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–O). Furthermore, tracheless [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 hbFR mutant embryos, which express a nonfunc-
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 \( hb^{B6} \) 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...
bridge-cells more directly, we ubiquitously expressed in $hb^{FB}$ mutant embryos the baculovirus P35 protein, a suppressor of apoptosis in Drosophila [Hay et al. 1994]. In contrast with $hb^{FR}$ 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 (Fig. 2G), is essential 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.
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-\( \beta \)-galactosidase antibody (Promega); anti-Hunchback antibody [gift from A. La Rose; MPI, Göttingen]; anti-Crumbs antibody [Tepass and Knust 1993]; Alexa 488 or Alexa 546 goat antirabbit IgG or antirabbit IgG; biotinylated antimouse IgM (Vector Laboratories); alkaline phosphatase-conjugated or biotinylated antirabbit IgG or antimouse IgG (Molecular Probes). 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); \( hh^{FB} \), \( hh^{9q} \), \( di^{EG} \), \( th^{S5} \), and \( hh^{P35} \) were obtained from the Tubingen Stock Center. \( btl/H82 \) drives Gal4 expression ubiquitously in the tracheal system from stage 10 onward [Shiga et al. 1996]. UAS-GFP-PNlacZ was used to detect nuclear \( \beta \)-galactosidase expression [Shiga et al. 1996]. The \( lacZ \) enhancer trap line 1-eve-1 reveals P-element integration in the \( thb \) gene and was used to mark tracheal cells by cytoplasmic \( \beta \)-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 homotopic cell nuclei [Whiteley et al. 1992]. We also used actin–Gal4 and twist–Gal4 flies [Greig and Akam 1993].

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 antibody staining of a stage 12 wild-type (A,B) and \( hh^{FB} \) mutant (C,D) embryos. Whole-mount antibody staining of a stage 12 wild-type (C) and a \( hh^{FB} \) mutant (D) embryo with anti-Crumbs antibodies. (G,H) Whole-mount antibody staining of a stage 15 wild-type (G) and a \( hh^{FB} \) 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 \( hh^{FB} \) mutant (H). Note that the \( hh^{9q} \) mutant embryos reveal an identical tracheal phenotype to that found in \( hh^{FB} \) mutant embryos. (I) Whole-mount antibody double staining of a stage 15 \( hh^{FB} \) mutant embryo bearing the \( G6 \) chromosome with 2A12 (brown) and anti-\( \beta \)-galactosidase (blue) antibodies reveals expression pattern in the dorsal trunk fusion cells of \( hh^{FB} \) mutant embryos. (J,K) Whole-mount in situ hybridization of a stage 12 wild-type (J) and a \( hh^{FB} \) mutant (K) embryo using \( bnl \) antisense RNA. The dynamic \( bnl \) expression pattern surrounding a single tracheal metamer [broken lines indicate \( bnl \) expression that guides dorsal trunk outgrowth] in either a wild-type (J) or \( hh^{FB} \) mutant (K) embryo is identical. (L) Whole-mount antibody double staining of a stage 15 \( bt^{H82\Delta3} \) mutant embryo with anti-\( \beta \)-galactosidase (brown) and anti-Hb [blue] antibodies. The \( bt^{H82\Delta3} \) mutant embryo reveals \( \beta \)-galactosidase expression in the tracheal nuclei [Reichman-Fried et al. 1994] and Hb expression in the bridge-cells [arrows].
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 hhP1 mutant embryo bearing UAS–bnl and btl–Gal4 (C). Note that the tracheal phenotype of amorphic bnlP1 mutant embryos bearing UAS–bnl and btl–Gal4 is indistinguishable from the phenotype shown in B. The lack of tracheal metameres in hhP1 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–hh, PO163–Gal4 and the 1-eve-1 chromosome with lacZ [red] and hh [blue] antisense RNA probes. PO163–Gal4 (Janning 1997) drives hh expression in SOP cells. (Arrow) Outgrowing dorsal trunk anterior branch that attaches to a hh expressing cell but lacks contact with the bridge-cell. [E] Whole-mount antibody staining of a stage 15 embryo bearing UAS–hh and PO163–Gal4 with antibody 2A12. Ectopic hh expression causes bottleneck-like lumen formation (arrowheads) and a lack of dorsal trunk interconnection [arrow]. [F,C] Whole-mount in situ double hybridization of a stage 12 embryo bearing the 1-eve-1 chromosome with lacZ [red] and hh [blue] antisense RNA probes. The same image is shown focused on the bridge-cell (F) and the tracheal cell extensions (G), respectively.
Tubular guidance by single cells

dation of the role of breathless, a Drosophila FGF receptor homolog, in tracheal cell migration. Genes & Dev. 8: 428–439.
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