Hedgehog and Decapentaplegic instruct polarized growth of cell extensions in the *Drosophila* trachea

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Summary

The migration of cellular extensions is guided by signals from tissues with which they contact. Many axon guidance molecules regulate growth cone migration by directly regulating actin cytoskeletal dynamics. Secreted morphogens control global patterns of cell fate decisions during organogenesis through transcriptional regulation, and constitute another class of guidance molecules. We have investigated the guidance roles of the morphogens Hedgehog and Decapentaplegic during directed outgrowth of cytoplasmic extensions in the *Drosophila* trachea. A subset of tracheal terminal cells adheres to the internal surface of the epidermis and elongates cytoplasmic processes called terminal branches. Hedgehog promotes terminal branch spreading and its extension over the posterior compartment of the epidermis. Decapentaplegic, which is expressed at the onset of terminal branching, restricts dorsal extension of the terminal branch and ensures its monopolar growth. Orthogonal expression of Hedgehog and Decapentaplegic in the epidermis instructs monopolar extension of the terminal branch along the posterior compartment, thereby matching the pattern of airway growth with that of the epidermis.

Key word: Guidance, Trachea, Hedgehog, Dpp, *Drosophila*

Introduction

During animal organogenesis, the formation of organ primordia is followed by innervations and angiogenesis. Molecules with organogenic functions, or morphogens, are often involved in guiding the subsequent migration of axons and blood capillary precursors, ensuring that different organ architectures receive appropriate neuronal input and adequate blood supply (Pola et al., 2001; Schnorrer and Dickson, 2004). One example is the patterning of the vertebrate central nervous system (CNS) in which Sonic Hedgehog (Shh) and Bmp2/4 families of signaling molecules specify multiple neuronal cell types along the dorsoventral axis. These molecules also promote attraction or repulsion of axonal extensions to facilitate the wiring of neural circuits according to the dorsoventral organization of the CNS (Augsburger et al., 1999; Charron et al., 2003). The powerful activities of Shh and Bmp2/4 as regulators of cell differentiation is based primarily on signal transduction to the nucleus where signaling molecules regulate transcription. However, the mechanism by which Shh and Bmp2/4 guide the migration of axons – which relies on growth cone motility far apart from the nucleus – is not understood. To understand the guidance function of morphogens, we used the *Drosophila* tracheal system as an experimental paradigm suitable for in vivo imaging of migratory cells of known identity.

The trachea is a respiratory organ consisting of a network of tubular epithelia that delivers outside air directly to target organs. The tracheal primordium forms six primary branches that migrate towards specific target tissues expressing Branchless (Bnl), a *Drosophila* homolog of FGF (Sutherland et al., 1996). Bnl activates Breathless (Btl), an FGF receptor, at the tip of primary branches and cell process formation (Gabay et al., 1997; Glazer and Shilo, 2001; Ribeiro et al., 2002). The dorsal branch (DB) migrates toward the dorsal midline, where it fuses with another DB from the contralateral side (Fig. 1A-C, see Movie 1 in the supplementary material). Two specialized cells are present at each DB tip (Fig. 1K). Fusion cells lead the migration and form anastomoses of tracheal tubules (Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996), whereas terminal cells extend a long cell process called a terminal branch (Samakovlis et al., 1996a). The terminal branch is also present in other branches such as visceral and ganglionic branches, and in all cases spreads over the surface of target tissues and serves as an interface for gas exchange by extending unicellular processes containing a dead-ended lumen, a structure known as the tracheole. Terminal branching in postembryonic stages is regulated by Bnl, which is induced as a hypoxic response (Jarecki et al., 1999). The regulation of terminal branch migration in the CNS uses the same molecules involved in axon guidance (Englund et al., 2002). Current knowledge is lacking, however, on the regulation of directed terminal branch growth over the

epidermis, as well as the mechanism by which it is positioned over the epidermis to maximize oxygen transfer.

A number of signaling molecules known to pattern the larval epidermis are involved in tracheal branching. The Bmp2/4 homolog Decapentaplegic (Dpp) is expressed in both the dorsal and ventral sides of tracheal primordia and specifies the fate of the dorsal and ganglionic branches (Vincent et al., 1997; Wappner et al., 1997). Wingless (Wg) expression abuts tracheal primordia from the anterior and posterior sides and triggers a genetic program specifying fusion and terminal cell formation (Chihara and Hayashi, 2000; Llimargas, 2000). Hedgehog (Hh) is expressed in the posterior (P) compartment and is implicated in determining terminal branch fate (Glazer and Shilo, 2001). Although Hh, Wg and Dpp regulate cell fate determination, the assessment of their roles in tracheal branch migration has been hindered by their requirement for early-stage tracheal cell specification.

We have assessed the roles of Hh and Dpp in directed outgrowth of tracheal terminal branch. Focusing on the terminal branches of the DB, we show that the terminal cells are located at a specific position in the dorsal epidermis (DE) defined by orthogonal stripes of Hh and Dpp expression, and that they extend terminal branches along a stripe of Hh expression. Hh acts on terminal cells to promote cell spreading over the epidermal surface. However, Dpp inhibits dorsal outgrowth, and thereby defines the monopolar shape of the terminal branch. We present a model that explains how Hh and Dpp signaling direct terminal branch outgrowth.

Materials and methods

Fly strains

The following strains were used in this study: *btl-Gal4* (Shiga et al., 1996), *ush-Gal4* (Hayashi et al., 2002), *en-Gal4* (Bloomington Stock Center), *UAS-gfp-moesin* (Chihara et al., 2003), *UAS-gfpTTras* (fusion of Ras1 C-terminal farnesylation signal to GFP S65T I167T), *UAS-gfpNLS* (Bloomington Stock Center), *UAS-D-axin* (Hamada et al., 1999), *UAS-ci*^{Act}, *UAS-ci*^{R} (Hepker et al., 1997), *UAS-hh* (Tabata et al., 1992), *UAS-dad* (Tsuneizumi et al., 1997), *UAS-dpp* (Staehling-Hampton and Hoffmann, 1994), *ptc-lacZ* (Bloomington Stock Center), dpp-lacZ=dpp^{10638} (Spradling et al., 1999) and 1-eve-1 [*lacZ* enhancer trap expressed in all tracheal cells (Kassis et al., 1992)]. *btl-gfp-moesin* was constructed by inserting the *gfp-moe* fusion gene (Edwards et al., 1997) downstream of the *btl* promoter (Ohshiro and Saigo, 1997), and was transformed into the germline. Egg were collected at 22°C. Under these conditions, about 80% of DB expressing Axin, Ci^{Act}, Ci^{R}, dpp or Dad reached dorsal midline and allowed us to analyze migratory behavior of the terminal branch. Larval phenotypes of those animals were examined at early third instar under bright field and fluorescence microscopy. Terminal branches of abnormal migration patterns characteristic for each genotype were identified and the formation of the tracheole was confirmed.

Embryo staining

The following primary antibodies were used: rabbit anti-GFP (MBL), anti-β-galactosidase (Cappel), mouse anti-Engrailed 4D9 and anti-Dlg (Developmental Studies Hybridoma Bank), anti-diphospho MAP kinase (Sigma), anti-pMAD (Kubota et al., 2000) and mouse monoclonal anti-mouse SRF (a gift from M. Gilman). The secondary antibodies used were as follows: Alexa 488-conjugated anti-rabbit IgG (Molecular Probes) and Cy5-conjugated anti-mouse IgG (Amersham Biosciences). Fluorescence images were captured using a confocal laser-scanning microscope (Olympus FV-500), and image processing was performed with the ImageJ application (http://rsb.info.nih.gov/ij/index.html).

Time-lapse observations

Unless otherwise noted, embryos heterozygous for *btl-Gal4* and *UAS-gfp-moe* insertions on the second chromosome were used for recording. Egg were collected at 22°C. Dechorionated embryos were mounted on a glass coverslip with rubber cement and were covered with halocarbon oil 700 (Sigma). GFP images were captured by a confocal laser-scanning microscope (Olympus FV-300) with a 60× oil immersion lens (NA 1.4) in a room maintained at 22°C. To minimize phototoxicity, we reduced the argon laser intensity (488 nm, 10 mW) to 1% and opened the pinhole to its maximum size (300 μm). Typically, 12 × 2 μm *z* stacks were taken every 1-5 minutes over a period of 6 hours. Images of the stacks were separated into single sections and manipulated using the ImageMagick software package (http://www.imagemagick.org/), and scripts were written in Perl and Ruby. Movies were encoded as MPEG-4 files.

Kinetic analysis of terminal cell movement

Images of terminal cells were aligned such that the anteroposterior and dorsoventral axes matched the *x*- and *y*-axes, respectively. The junction between fusion and terminal cells that was identified by the presence of the actin core was chosen as a reference point. Plots were constructed from measurements of the *y*-directed component of the distance to the tip of filopodia at the dorsal- and ventralmost positions as well as the *x*-directed component of the distance to the anteriormost position.

Results

The terminal branch of the DB migrates along the internal surface of the DE

We examined the spatiotemporal relationship between the DB and DE using time-lapse movies of each tissue. Green fluorescent protein (GFP) fused to the actin-binding domain of moesin was expressed using the trachea-specific breathless enhancer (Chihara et al., 2003) (Fig. 1A-C, see Movie 1 in the supplementary material). *engrailed* (*en*)-*Gal4* was used to additionally label the P compartment of the epidermis (Fig. 1D-F). The DE undergoes a morphogenetic process called dorsal closure, during which epidermal sheets move toward the dorsal midline to cover the dorsal part of the body. At the stage when the DE from each side meet at the midline (Fig. 1D), the DB tip (consisting of a pair of cells, the fusion cell and the terminal cell which are extending numerous filopodia) was positioned beneath the *en*-expressing region of the DE at two to three cells behind the leading edge (Fig. 1A,D). The position of the DB tip beneath the DE was constant up to 60 minutes (Fig. 1D,E). Sixty minutes after contacting the DE (Fig. 1B,E), fusion cells of the DB from each side made first contact at the midline via long filopodia. By 130 minutes, fusion of the DBs was complete (Fig. 1C). Transverse optical sections of similarly staged embryos expressing GFP in the trachea and other epithelial tissues revealed that the DB tip associated closely with the basal surface of the DE (Fig. 1G,H, arrows). The close association of the DB tip with the *en*-expressing region of the DE indicates that epidermal guidance signals may direct the precise placement of the DB tip and bring it to the midline by coupling its movement to dorsal closure of the epidermis.

Terminal cells of the DB started to send filopodia toward the ventral direction by 60 minutes (Fig. 1B, green arrowhead; see

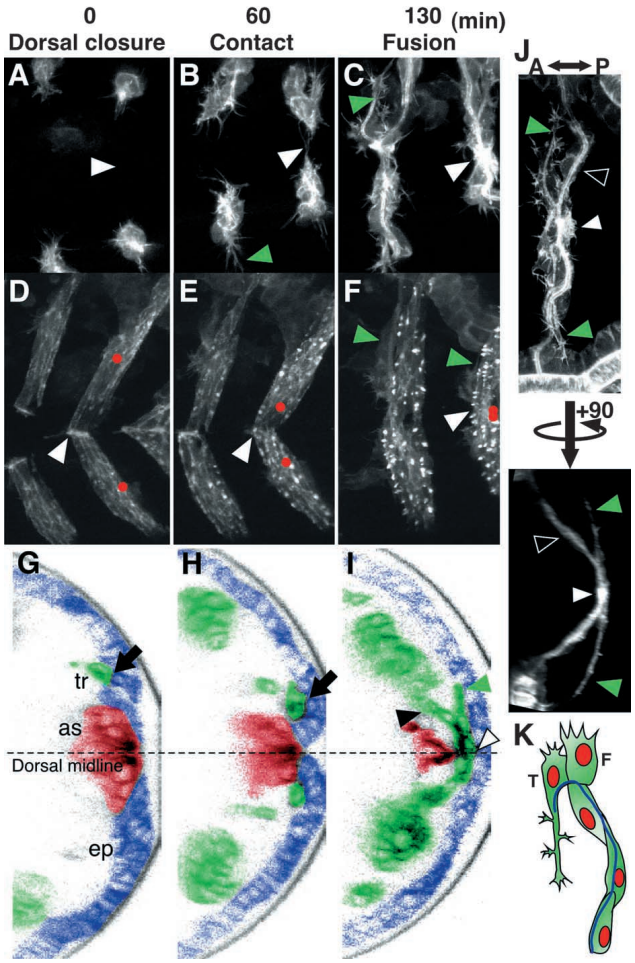

Fig. 1. Extension of the dorsal branch (DB) is coupled to dorsal closure of the epidermis. (A-I) Images of time-lapse recordings. White arrowheads indicate the fusion point of the DB (A-C,I,J) or dorsal epidermis (D-F). Green arrowheads indicate the terminal branch. Anterior is leftwards (A-F). (A-C) Dorsal view of DB8 and DB9 labeled with *btl>>gfp-moe* (*UAS-gfp-moe* driven by *btl-gal4*) taken from Movie 1. (D-F) Simultaneous labeling of the trachea and the P compartment of the epidermis with *gfp-moe*, expressed by *btl-gal4* and *en-gal4.* Tracheal cells are mostly hidden beneath the *en* stripe. Fusion cells are indicated by red dots. (G-I) Transverse optical sections obtained by *y-z* scanning of a *btl>>gfp-TT-ras* embryo. This combination of insertions caused fortuitous GFP expression in most epidermal cells. Trachea (green), epidermis (blue) and amnioserosa (red) were identified by their location and cell shape and were pseudo-colored. The contact point of the epidermis and the DB tip is indicated by an arrow. Dorsal is rightwards. (A,D,G) Time=0 minutes (early stage 15: the dorsal epidermis meets at the midline). The tip of the DB adheres to epidermal cells located two or three cells behind the leading edge (G), and follows dorsal closure prior to 0 minutes. (B,E,H) Time=60 minutes: contact of fusion cells. Fusion cells from opposing sides contact each other with numerous filopodia (B, white arrowhead). The terminal branch has begun ventral extension (B, green arrowhead). (C,F,I) Time=130 minutes (early stage 16): completion of branch fusion. Terminal branch extension is still in progress. (J) Dorsal (upper) and transverse (lower) views of a DB fusion point. The terminal branch follows the curved shape of the epidermis (green arrowheads), whereas the stalk does not associate closely with the epidermis (open arrowhead). (K) Schematic drawing of the DB. T, terminal cell; F, fusion cell.

also Fig. 2A), and later developed as terminal branches (Fig. 1C,I, green arrowhead). The actin core, a cytoplasmic structure that directs the formation of the lumen, was oriented ventrally (Fig. 1C, green arrowhead). Three-dimensional reconstruction confirmed that the terminal branch spread over the basal surface of the DE (Fig. 1I,J, green arrowhead), whereas stalk cells did not make close contact with DE (Fig. 1J, open arrowhead). In subsequent studies, we focused our analyses on the guidance mechanism of unidirectional cellular extensions of the terminal branch.

Selective cell spreading during terminal branching

We first analyzed the kinetics of terminal branch movement. At the DB tip at stage 14, terminal cells with numerous filopodia extending from all points on the cell can be distinguished from fusion cells by marking cell boundaries (Fig. 2A). Time-lapse recording revealed rapidly moving filopodia (Fig. 2B; see Movie 1 in the supplementary material). We measured the length of the dorsalmost, ventralmost and anteriormost tips of the filopodia at each time point and plotted these data versus time (Fig. 2C, see Materials and methods). Filopodia movement in each direction consisted of rapid extension and retraction, as reflected by the jagged appearance of the plot. Thus, random filopodial movement occurred in all three directions examined. Posterior extension appeared to be blocked by fusion cells. The length of anteriorly and dorsally directed filopodia ranged from 5-25 μm during the 70 minute period, whereas the length of ventrally directed filopodia increased constantly at 0.23 μm/minute. Ventral filopodial extension was accompanied by actin core formation (Fig. 2B, red arrowhead at 60 minutes). These results indicate that filopodial movement exhibits no strong directional bias and that extended filopodia may be selectively stabilized in the ventral direction.

Expression of Bnl-FGF during terminal cell differentiation

Bnl is a major signaling molecule that guides primary branch outgrowth (Sutherland et al., 1996). To ask whether Bnl plays a role in terminal branch guidance in the DB, we examined Branchless gene (*bnl*) expression patterns in stage 15 DE (Fig. 3A). *bnl* mRNA was expressed as spots abutting emerging DB during stages 13-14 (Sutherland et al., 1996). This expression decayed by stage 15 and was replaced by a short stripes that extended along the anteroposterior (AP) compartment boundary (Fig. 3A). Terminal cells that had begun to extend terminal branches were found beneath *bnl*-expressing cells, and they expressed activated MAP kinase, a downstream target of FGF signaling (Gabay et al., 1997) (Fig. 3B). Given the strong activity of Bnl to induce terminal cell differentiation (Sutherland et al., 1996), a localized source of Bnl in the DE probably promotes and/or maintains the differentiation of terminal cells at a specific location.

Does Bnl guide terminal branch elongation? The observation that terminal branches and their filopodia extend away from the region of highest Bnl expression (Fig. 3A) is not consistent with the proposed role of FGF as a chemoattractant for directed filopodial growth (Affolter et al., 2003; Ribeiro et al., 2002). Therefore, we examined other signaling pathways in this regard.

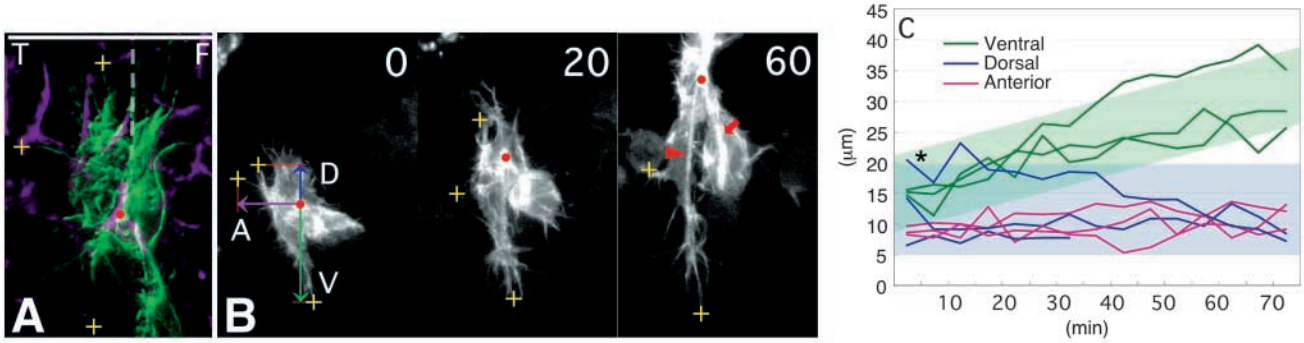

Fig. 2. Kinetics of terminal branch extension. (A) The tip of the DB is labeled with GFP-moesin (green) and Dlg (purple) labels the cell boundaries. The broken line indicates the signal of Dlg that accumulates in the boundary between the terminal cell (T) and the fusion cell (F). The junction between the fusion cell and terminal cell was identified by the high concentration of GFP-moe, and was designated as a reference point (red dot). The tips of filopodia are indicated by yellow crosses. (B) Time-lapse images of tracheal cells labeled with GFP-moe that were used for measurements. Elapsed time in minutes is indicated in the top right-hand corner of each image. In the 60 minute frame, the actin core in the terminal cell and stalk cell is indicated by a red arrowhead and arrow, respectively. (C) Plot of filopodial movement. The asterisk indicates an example of a terminal branch that transiently extended in the dorsal direction.

The DB tip localizes to a specific position marked by en and *dpp*

The DB arises from tracheal primordia in the anterior compartment and extends its tip dorsally and anteriorly to cross the segment boundary to reach an area beneath the En-expressing region, which is the source of Hh (Fig. 3C,D). Dpp expression at stage 14, as monitored with the *dpp-lacZ* reporter, manifested as a longitudinal stripe at the leading edge of the DE, mimicking the pattern of *dpp* mRNA expression (Fig. 3C) (see Jackson and Hoffmann, 1994). The tip of the DB was located two or three cells behind the region of *dpp-lacZ* expression (Fig. 3C) and accumulated phosphorylated MAD (pMAD), an indicator of Dpp signal transduction (Kubota et al., 2000; Tanimoto et al., 2000) (Fig. 3E). *dpp-lacZ* expression persisted at the completion of dorsal closure when outgrown terminal branch was observed (Fig. 3D). During stage 15 and thereafter, the terminal branch extended ventrally along the anterior edge of En-expressing cells and away from the leading edge of the DE (see Fig. 5A-C). These observations raise the possibility that Hh provides a positional cue that directs the terminal cells and that Dpp plays a role directing the orientation of terminal branch.

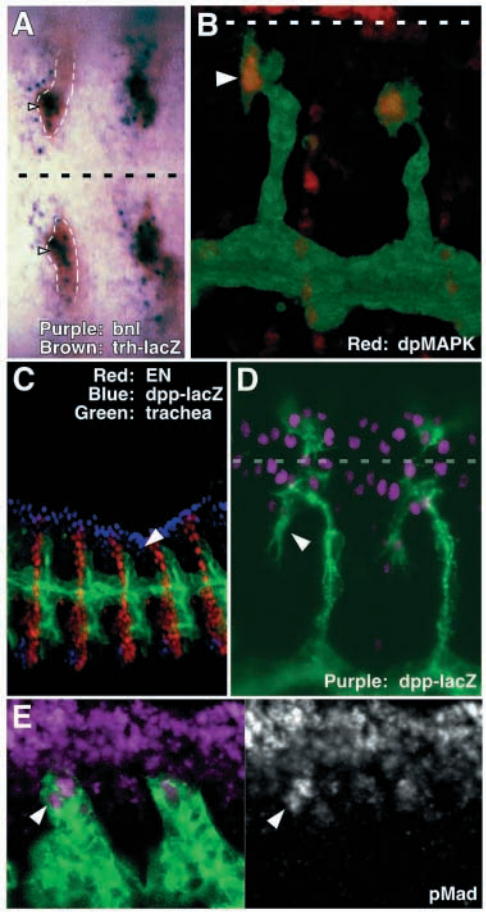

Fig. 3. Relationship between the tip of the DB and the expression domains of *dpp*, *en* and *bnl*. Anterior is leftwards. Broken lines in A,B,D indicate the dorsal midline. Tracheal cells are labeled with *lacZ* marker of 1-eve-1 tracheal reporter (brown in A, green in B) or with GFP-moe driven by btl-gal4 (green in C-E). (A) Dorsal view of two consecutive segments in a stage 15 embryo double-labeled for *bnl* mRNA in the epidermis (purple dot) and trachea (brown, outlined in one segment). Strong expression of *bnl* mRNA overlaps the tip of the DB, and weaker expression extends ventrally. (B) Activated MAP kinase expression (red, arrowhead) detected in the terminal cell. Lateral view. (C) During stage 14, the tip of the DB (white arrowhead) has entered the *en* stripe from behind and localizes immediately ventral to *dpp-lacZ* expression. (D) *dpp-lacZ* expression in dorsal midline at the completion of dorsal closure. Late stage 15. (E) pMAD expression (purple) in the DB (green) of a stage 14 embryo (white arrowhead). A grayscale image of pMAD expression is shown on the right.

Affinity of the terminal branch for the epidermal P compartment

To test whether the preferential association of the terminal branch with the En-expressing P compartment of the epidermis is a property of terminal cells, we observed the behavior of terminal cells when they were forced out of the P compartment. We used genetic means to increase the number of terminal cells. Axin overexpression downregulates Wg signaling in

tracheal cells (Hamada et al., 1999), causing the duplication of terminal cells at the expense of fusion cells (Chihara and Hayashi, 2000). Such supernumerary terminal cells were indistinguishable from normal terminal cells, as judged by the expression of the terminal cell marker SRF (serum response factor) (Affolter et al., 1994; Chihara and Hayashi, 2000; Guillemin et al., 1996) and the extensively ramified morphology observed in postembryonic stages (see Materials and methods). Those terminal cells were often displaced into adjacent segments (Fig. 4A). The fidelity of terminal cell placement over the P compartment was 96.8% in the cases examined (n=126). The frequent observation of a *y*-shaped DB suggests that one of the terminal branches of such DBs must have crossed a non-En-expressing region to reach the adjacent segment (Fig. 4A). The high fidelity of terminal cell placement over the P compartment suggests that Wg signaling is not essential for terminal cell placement in this compartment.

An example of a terminal cell captured by a time-lapse recording of a live embryo revealed interesting cell behavior (Fig. 4C; see Movie 2 in the supplementary material). Initially, this terminal cell appeared flat with a few filopodia and was extending in the AP direction (Fig. 4C, white arrowhead); its shape was strikingly different from another terminal cell that appeared to stay within the P compartment (red arrowhead). The misplaced terminal cell remained in the anterior compartment for about 60 minutes before moving backwards to the posterior compartment. The cell immediately began forming filopodia and extended a terminal branch (time 2:00, 3:00). These observations suggest that, relative to the anterior compartment, the P compartment constitutes a better environment for terminal branch formation.

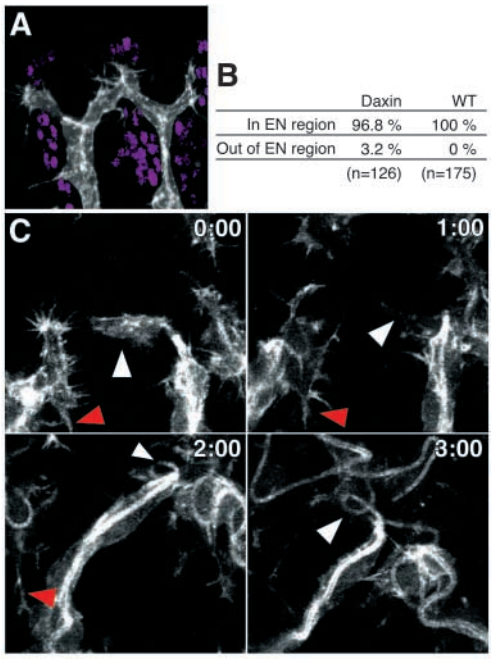

Fig. 4. Preferential association of terminal cells with the P compartment. Ectopic formation of a terminal cell was induced by expression of the *Drosophila* homolog of Axin. (A) Duplicated terminal cells associated with the P compartment (EN, purple). (B) Frequency of terminal cells located in the EN stripe. (C) Relocation of terminal cells from the anterior to the P compartment (Movie 2). A terminal cell located in the A compartment (white arrowhead) elongated along the AP axis and had fewer filopodia than cells located in the P compartment (red arrowhead). This cell moved to the P compartment and started to extend a terminal branch dorsally.

Involvement of Hh signaling in directed terminal branch outgrowth

We studied the function of Hh as a candidate guidance molecule secreted from the P compartment. First, we altered the pattern of Hh expression in the epidermis by driving Hh using the *ush* driver, which activated expression in a wide anteroposterior stripe along the dorsal midline (Fig. 5L). This treatment caused the duplication of terminal cells identified by SRF expression (data not shown). Some of terminal branches were misrouted along the AP direction or became stalled at the dorsal midline (Fig. 5K,K′, arrowhead), suggesting that extension of terminal branch outgrowth is misguided by alteration of Hh distribution in the DE.

We next attempted to alter Hh signal transduction activity specifically in tracheal cells by elevating or reducing the ability of tracheal cells to respond to Hh via expression of Hh-specific intracellular transducers. The nascent polypeptide of Ci155 is thought to constitute the inactive form of the protein in the cytoplasm (Aza-Blanc et al., 1997). In cells that do not respond to Hh signaling, Ci155 cleavage yields the 75 kDa fragment Ci^{R}, which represses several Hh target genes. Hh signaling is thought to generate another form of Ci, Ci^{Act}, which activates Hh target genes. We used truncated forms of Ci that mimic the activities of Ci^{Act} and Ci^{R} to alter Hh signaling (Hepker et al., 1997; Methot and Basler, 1999).

To confirm that terminal cells located on the basal side of the epidermis were affected by Hh signaling, we monitored Hh activity using a *patched-lacZ* (*ptc-lacZ*) reporter gene. *ptc-lacZ* was detected in terminal cells (Fig. 5M, yellow circle), and expression was significantly lower than that detected in epidermal cells abutting the P compartment, where Hh signaling was maximal (Fig. 5M, yellow arrowhead). *ptc-lacZ* was activated by Ci^{Act} and repressed by Ci^{R}, respectively (Fig. 5N,O), suggesting that those effectors may be used to modulate Hh signaling in the trachea.

We reasoned that tracheal cell-autonomous activation of Hh signaling by Ci^{Act} would allow terminal cells to migrate independently of external source of Hh. Tracheal cells expressing Ci^{Act} displayed aberrant DB migration (Fig. 5E-G, see Movie 3 in the supplementary material). These cells displayed abnormal morphology in that they produced multiple cytoplasmic extensions from a single SRF-expressing cell (100%, n=90, 98% of the DBs contained a single terminal cell, Fig. 5G) and were often displaced from the P compartment (Fig. 5E). Most notably, the orientation of the cytoplasmic extensions was not restricted to the ventral direction; rather, they were oriented in the anterior, and posterior directions (Fig. 5E-G, see Movie 3 in the supplementary material), suggesting that terminal cells and terminal branches must respond to external Hh to precisely localize to P compartment.

However, terminal cells expressing Ci^{R} often formed duplicated terminal branch (18.8%, n=250, compared with 3.6%, n=166 in the control strain). Time-lapse observation demonstrated that terminal branch apparently normal with respect to the initial direction of terminal branch extension form first (Fig. 5H-J, white arrowhead), followed by formation

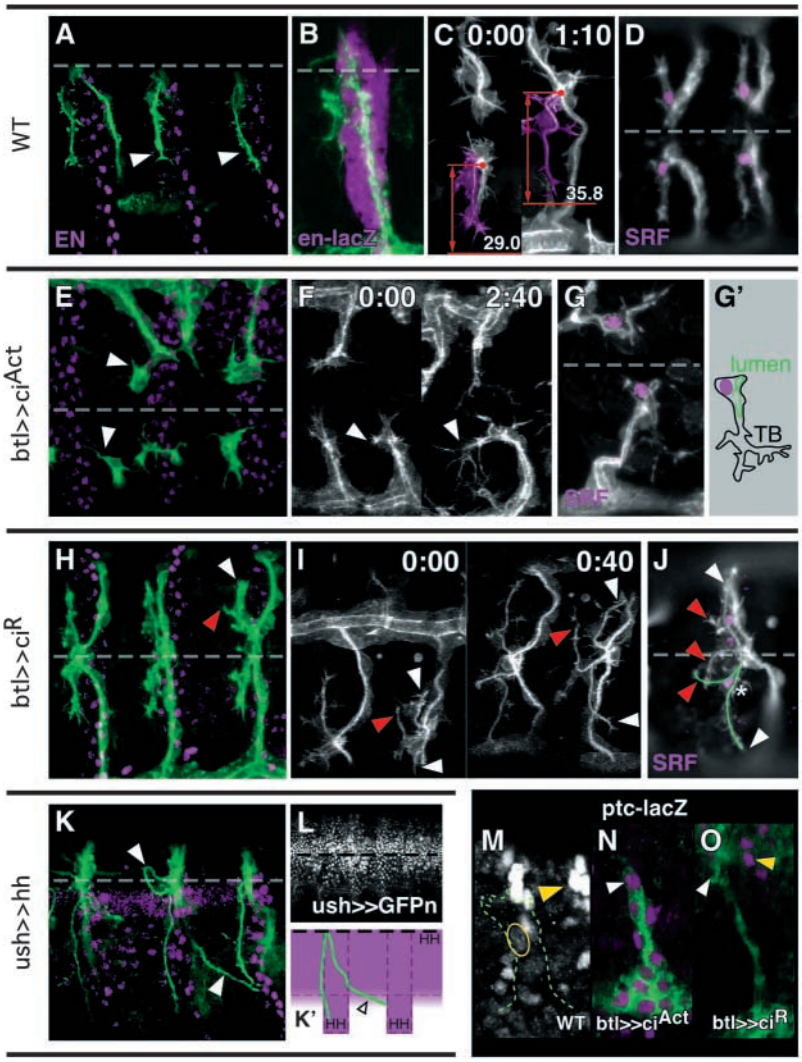

Fig. 5. Hh signaling restricts terminal branching to the P compartment. Broken lines indicate the dorsal midline, and white arrowheads indicate the terminal branch. (A,E,H,K) Stage 16 embryos labeled with anti-EN (purple), with the trachea shown in green (GFP-moe). Anterior is leftwards. (B) Double-labeling of *en-lacZ* (purple) and the trachea (green; GFP-moe). The terminal branch runs along the anterior border of the *en-lacZ* stripe. (C,F,I) Images taken from Movies 1 (C), 3 (F) and 4 (I) showing migration patterns of the terminal branch. The length (in μm) of one terminal branch (colored purple) is indicated in C. (D,G,J) Expression of SRF (purple). (A-D) Wild-type embryos. Terminal branches are aligned along the AP compartment borders. In C, terminal cells are pseudo-colored with purple to highlight the shape of terminal branches. (E-G) When Hh signaling is hyperactivated by Ci^{Act} in the trachea, terminal branches are not confined within the P compartment and cell extensions pointing in the AP direction are observed (arrowhead). (H-J) Downregulation of Hh signaling in the trachea by Ci^{R} expression results in the formation of secondary terminal branches that extend outside the P compartment (red arrowheads). Compare this with terminal branches that formed first (white arrowheads). An example of a single terminal cell extending two terminal branch (colored green) is labeled with asterisk in J. (K,K′) Hh expressed in a broad longitudinal band in the DE (using the *ush-gal4* driver) caused terminal branch misguidance. Tracheal cells were visualized with *btl-gfp-moe*. (L) Expression pattern of *ush;gfpNLS*. (M-O) Expression of *ptc-lacZ*. Expression in terminal cells (yellow circle) was low in control embryos (M) but was elevated when Ci^{Act} was ectopically expressed in the trachea using *btl-Gal4* (N) and was reduced by Ci^{R} (O). The yellow arrowhead indicates an epidermal cell expressing a high level of *ptc-lacZ*. Broken green line in M indicates the outline of the DB detected by GFP (for clarity, the GFP signal is not shown).

of secondary terminal branch that migrate anteriorly outside of EN stripe (Fig. 5H-J, red arrowhead; see Movie 4 in the supplementary material). Anti-SRF staining of such terminal branches revealed that approximately one-third (10/27) of duplicated branches arose from single terminal cells (Fig. 5J, asterisk). The result suggests that Hh signaling activity must be maintained to an appropriate level to restrict the number and the location of terminal branch.

These results suggest that terminal cells respond to Hh expressed in the epidermis and that the maintenance of Hh signal transduction activity at an appropriate level is essential for correct migration of the terminal branch.

Dpp inhibits terminal branch outgrowth

Terminal cells begin terminal branching by extending filopodia in both the dorsal and ventral directions, but they maintain only the ventral extension at later stages (Fig. 2B,C). As Dpp was expressed immediately adjacent to the leading front of the DB at the onset of terminal branch outgrowth (Fig. 3C), we reasoned that Dpp might constitute an inhibitory signal for terminal branch outgrowth. To test this idea, we observed terminal branching in cells with altered levels of Dpp signaling activity in the trachea. Because Dpp signaling is required for DB specification, it is important to separate the defects in terminal branching from those resulting from the earlier requirement for branch specification. In further analyses, we chose conditions in which most embryos had a normal number of DB cells. Under such conditions, terminal branches reached the dorsal midline, and their frequency remained one per DB as assessed by the expression of the terminal cell marker SRF (data not shown).

When Dpp signaling was constitutively activated by overexpression of *dpp* by the *btl* promoter, terminal branch extension stalled and was sometimes curtailed (Fig. 6A). The coiled appearance of the actin core in stalled terminal branches suggests that terminal branch failed to extend in a straight path while maintaining its growth, suggesting that cell-autonomous activation of Dpp signaling is inhibitory to polarized extension of terminal branch.

Conversely, terminal branch misdirection was frequently observed when Dpp signaling was prematurely downregulated by the expression of Dad, an inhibitor of Dpp signaling (Tsuneizumi et al., 1997) (Fig. 6B-F). Terminal branches often extended along the AP axis (Fig. 6D, class 2). Those terminal branches that reached the adjacent segment were often bent along the dorsoventral (DV) axis (Fig. 6D). Terminal branches

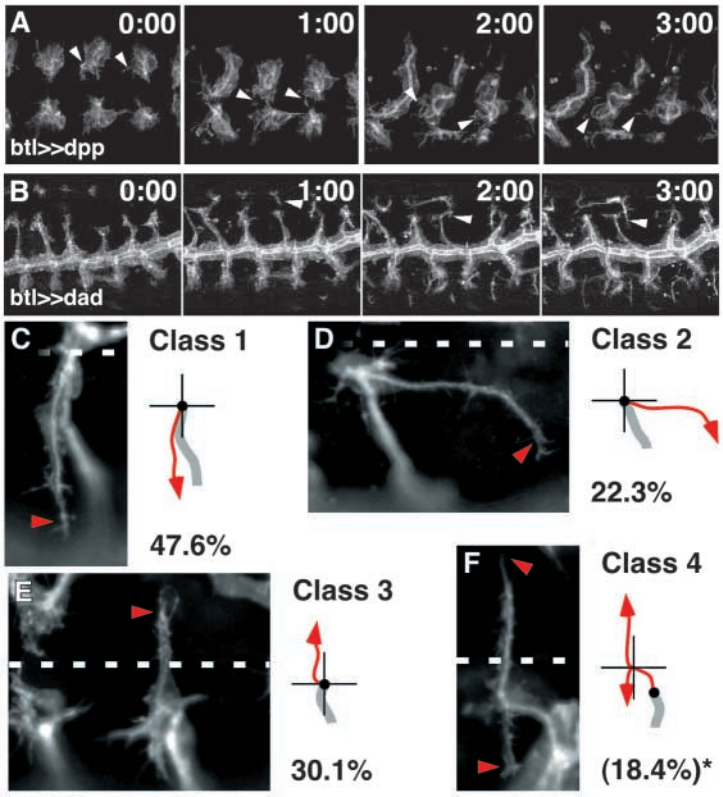

Fig. 6. DPP signaling inhibits terminal branch extension. (A) When a high level of Dpp signaling was maintained by expressing *dpp*, elongating terminal branches were misoriented and were stalled (white arrowheads). Compare this with the control terminal branch shown in Fig. 5C. Overexpression of Dpp promoted excess recruitment of tracheal cells into DB (Vincent et al., 1997), but the number of terminal cells expressing SRF remained one per DB (data not shown). (B-F) Premature reduction of Dpp signaling by overexpression of Dad causes misdirection of the terminal branch. (B) Time-lapse images of a terminal branch that crossed the dorsal midline (arrowhead). (C-F) Phenotypes of Dad expression in terminal branches were classified as either class 1 (C, normal), class 2 (D, drifting along the AP axis) or class 3 (E, midline crossing). Class 2 and 3 phenotypes included another subcategory, the class 4 phenotype (F, bipolar elongation). The asterisk indicates that this phenotype is a subset included within the class 2 and 3 phenotypes. A total of 103 terminal branches that reached dorsal midline (broken line) were scored.

also frequently extended dorsally and crossed the midline (Fig. 6E, class 3). In each class, bipolar-shaped terminal branches were observed (Fig. 6F, class 4). These observations suggest that Dpp signaling inhibits terminal branch elongation.

Discussion

Cell migration involves extension and retraction of cell processes accompanied by their adhesion to the substratum with subsequent spreading (Keller, 2002; Martin and Wood, 2002; Montell, 2003). Although neural-specific axon guidance molecules such as semaphorins repel axons by inhibiting growth cone motility (Luo et al., 1993), it is not known whether this principle extends to signaling molecules such as Shhs, Bmps and Wnts that have more general morphogenetic functions (Augsburger et al., 1999; Charron et al., 2003; Colavita et al., 1998; Lyuksyutova et al., 2003; Yoshikawa et al., 2003). We addressed this question by analyzing one type of *Drosophila* tracheal cell, the terminal cell of the dorsal branch. Our results suggest that Hh and Dpp, which are expressed as orthogonal stripes in the DE, contribute to the straight migration of the terminal branch. Hh functions as a permissive signal to allow migration along the P compartment, while Dpp functions as a repulsive cue that initially promotes monopolar growth of the terminal branch.

Hh guides terminal branch ourgrowth by promoting cell spreading

Terminal branches extend numerous cell processes that rapidly and repeatedly extend and retract in many directions. Although cell processes that extended anteriorly and dorsally were unstable, a subset of cell processes that extended ventrally along the P compartment became selectively stabilized (Fig. 2). The behavior of terminal cells in the anterior compartment was strikingly different from those in the P compartment (Fig. 4C). In the anterior compartment, the number and size of the cell processes was much smaller, suggesting that the P compartment constitutes the preferred substrate for terminal cell spreading.

Hh is important for promoting terminal cell spreading. Hh is secreted from the P compartment (Tabata and Kornberg, 1994) and forms symmetrical gradients of cellular responses in both the anterior and posterior directions within the epidermis (Struhl et al., 1997). We propose that Hh stimulates the adhesion of terminal cells to the epidermis by activating Ci. Because Hh signaling is submaximal in terminal cells (Fig. 5M), terminal branch filopodia that extend randomly would be preferentially stabilized near the source of Hh. Thus, terminal cell bodies are placed at the point of highest Hh concentration and terminal branches are stabilized at the apex of the Hh concentration gradient. Because terminal cell growth continues while the level of Hh signaling remains below maximum within terminal cells, terminal branches would be expected to extend along the P compartment (Fig. 7).

The mechanism of terminal branch guidance by Hh through regulation of Ci-dependent transcription differs from those that guide the behavior of the growth cone, which is primarily regulated at the level of cytoskeletal motility. The latter mechanism has the advantage of maintaining a small cell-surface area receiving guidance cues to minimize the chances of making aberrant connections during synapse formation. Terminal cells, however, use the entire basal cell surface to receive a guidance signal and to stabilize their association with the epidermis. This mechanism of terminal branching by cell

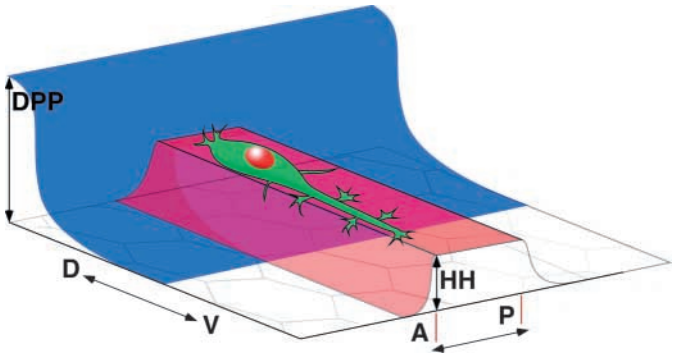

Fig. 7. A model of the terminal branch guidance mechanism. Hh is expressed in the P compartment and forms a concentration gradient that diminishes toward the anterior and posterior directions. Terminal cells adhere to the basal surface of the epidermis and receive Hh, which promotes cell adhesiveness and thereby maintains terminal cell localization at the top of the gradient. Dpp, which is expressed at the dorsal edge of the epidermis, acts as a barrier against terminal branching to ensure monopolar growth.

spreading meets the physiological requirement that the terminal branch serves as an interface for gas exchange.

Hh signaling has been implicated in another cell adhesion-related process, namely cell sorting behavior at the AP compartmental boundary in the wing imaginal disc (Blair and Ralston, 1997; Rodriguez and Basler, 1997). Because this behavior is regulated transcriptionally by Ci (Dahmann and Basler, 2000), there may be a common downstream target of Ci that acts in both wing disc cells and terminal cells.

Terminal branch extension is limited to the AP compartmental border, suggesting that there is an additional mechanism that shifts the terminal branch to the anterior side of the P compartment. Bnl was expressed as short stripes in the DE at the time of terminal branching. It was reported that mesodermal cells also contribute to correct patterning of DB (Franch-Marro and Casanova, 2000). It will be interesting to address guidance functions of those components on terminal branch outgrowth.

Negative control of terminal branch extension by Dpp

Dpp was expressed at the dorsal edge of the DE during dorsal closure (Fig. 3C). This corresponds to the time when terminal branch outgrowth in the DB started, suggesting that Dpp affects initial stage of terminal branch outgrowth. We observed that prolonged activation of Dpp signaling in terminal cells by expression of Dpp prevented its elongation (Fig. 6A). These observations suggest that in normal development Dpp prevents dorsally directed terminal branch extension at the onset of terminal branching (Fig. 2C, asterisk-labeled plot), thereby shunting terminal branch extension towards the ventral direction. We propose that Dpp converts the initial bipolar shape of a terminal branch into one that is monopolar. Once terminal branch extension is initiated, its direction may be maintained by localized Bnl expression at the AP compartment border. Whether this inhibitory effect of Dpp is mediated by direct signaling to cytoskeletons at the cell periphery or mediated by a nuclear transduction of the signal, remain to be determined.

Combinatorial control of terminal branching by FGF, Hh and Dpp

Terminal cells undergo an enormous increase in cell volume and surface area during terminal branching, which continues throughout embryonic and post-embryonic stages. FGF signaling promotes this process (Jarecki et al., 1999; Reichman-Fried and Shilo, 1995) in two ways, first by activating the target gene SRF, which is required for terminal branch growth (Guillemin et al., 1996), and second by stimulating rapid filopodial movement (Ribeiro et al., 2002). These two FGF signaling effects seem to be independent of Hh and Dpp. The FGF ligand Bnl is expressed in epidermal cells beneath terminal cells during terminal branching. Because this expression is limited to a relatively small region, we consider it unlikely that FGF signaling is sufficient to provide vectorial information for terminal branching. We suggest that FGF-driven growth and the motility of terminal branches are restricted to the P compartment by Hh signaling, wherein they are further limited by Dpp to establish a monopolar growth pattern.

Hh functions as both a cell fate determinant and a guidance molecule for terminal branch extension. But how are these two distinct Hh functions coordinated? Inactivation of Hh after initiation of terminal branching via a temperature shift of hh^{ts2} mutant embryos causes a loss of terminal cells (K.K., unpublished), suggesting that maintenance of tracheal cell fate also depends on a late Hh function. Thus, the striped expression of Hh in the epidermis is used simultaneously for epidermal patterning, tracheal cell fate determination and terminal branch guidance, exemplifying a simple strategy to coordinate the patterning of complex organs having multiple tissue types.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/131/21/5253/DC1

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