Development 128, 1599-1606 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV2694

Hedgehog signaling patterns the tracheal branches

Lillian Glazer and Ben-Zion Shilo*

Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel
*Author for correspondence (e-mail: benny.shilo@weizmann.ac.il)

Accepted 14 February; published on WWW 5 April 2001

SUMMARY

The elaborate branching pattern of the *Drosophila* tracheal system originates from ten tracheal placodes on both sides of the embryo, each consisting of about 80 cells. Simultaneous cell migration from each tracheal pit in six different directions gives rise to the stereotyped branching pattern. Each branch contains a fixed number of cells. Previous work has shown that in the dorsoventral axis, localized activation of the Dpp, Wnt and EGF receptor (DER) pathways, subdivides the tracheal pit into distinct domains. We present the role of the Hedgehog (Hh) signaling system in patterning the tracheal branches. Hh is expressed in segmental stripes abutting the anterior border of the tracheal placodes. Induction of *patched* expression, which results from activation by Hh, demonstrates that cells in the anterior half of the tracheal pit are activated. In *hh*-mutant embryos migration of all tracheal branches is absent or stalled. These defects arise from a direct effect of Hh on tracheal cells, rather than by indirect effects on patterning of the ectoderm. Tracheal cell migration could be rescued by expressing Hh only in the tracheal cells, without rescuing the ectodermal defects. Signaling by several pathways, including the Hh pathway, thus serves to subdivide the uniform population of tracheal cells into distinct cell types that will subsequently be recruited into the different branches.

Key words: Hedgehog, Patched, Smoothened, Trachea, Branch migration, *Drosophila*

INTRODUCTION

The *Drosophila* tracheal system is comprised of an interconnected, stereotyped array of tubular structures (reviewed by Manning and Krasnow, 1993; Zelzer and Shilo, 2000b; Affolter and Shilo, 2000). Tracheal development is initiated after the anteroposterior and dorsoventral coordinates on the ectoderm have been specified. Ten segmental clusters of cells on each side of the embryo are designated as tracheal precursors, by the expression of the Trachealess (Trh) and Drifter/Ventral veinless transcription factors (Anderson et al., 1995; Wilk et al., 1996; de Celis et al., 1995; Llimargas and Casanova, 1997; Zelzer and Shilo, 2000a). Two more rounds of division take place at the placode stage, giving rise to ~80 tracheal cells in each placode. All subsequent events take place without any further cell division. When the tracheal placodes are formed, they appear to form a single tracheal equivalence group without an assignment of distinct fates (Samakovlis et al., 1996). Following invagination to form the tracheal pits, stereotyped migration is initiated simultaneously in six different directions. It is remarkable that in addition to the direction of migration being precise, the number of cells allocated to each branch is fixed. This is essential as, in the absence of further cell division, the cell number in each branch cannot be adjusted. This morphological observation indicates that prior to migration, a subdivision of cell fates in the tracheal placode must take place, assigning the cells to the future branches.

The simultaneous onset of branch migration is guided by localized activation of the fibroblast growth factor (FGF) receptor Breathless (Btl), expressed by all tracheal cells (Klämbt et al., 1992). The FGF ligand, Branchless (Bnl), is expressed dynamically in ectodermal and mesodermal patches, which prefigure the branching pattern (Sutherland et al., 1996). Localized activation of mitogen-activated protein (MAP) kinase is detected in the tracheal cells adjacent to the Bnl source (Gabay et al., 1997). While the pattern of Bnl expression is the key factor in determining the direction of migration, it cannot serve to determine the number of tracheal cells allocated to each branch. At the onset of migration, when the patches of Bnl-expressing cells abut the tracheal pit, a fairly coarse pattern of MAP kinase activation in the tracheal pit cells is observed (Michelson et al., 1998). This pattern cannot be responsible for the fine distinction between the number of cells recruited to each branch. Furthermore, there is no correlation between the intensity of activation in each future branch and the number of cells that will be recruited to that branch.

Additional mechanisms, which operate prior to the initiation of migration, are responsible for subdividing cell fates within the tracheal placodes and pits. The Dpp pathway specifies the fate of the tracheal branches that will arise from the dorsal and ventral parts of the tracheal pit. At stage 11, Dpp is expressed in two stripes, the ventrolateral stripe abuts the tracheal pit, while the dorsolateral stripe is positioned several cell rows above the tracheal pit. In the absence of signaling by the Dpp pathway at this stage, the dorsal branch, and lateral anterior and posterior branches fail to form (Affolter et al., 1994; Vincent et al., 1997; Wappner et al., 1997). Activation of the

EGF receptor (DER) pathway is initiated by transient expression of Rhomboid (Rho) within the placode. This leads to production of the active ligand, secreted Spitz, and induction of DER signaling in the tracheal cells. When the DER pathway is not functional, the branches originating from the center of the tracheal pit, namely the dorsal trunk and visceral branch, are not formed, in spite of the fact that Bnl expression is not perturbed (Wappner et al., 1997). Finally, activity of the Wnt pathway is necessary for specifying the fate of the dorsal trunk (Llimargas, 2000; Chihara and Hayashi, 2000).

Although several target genes for the above pathways have been identified in the trachea, the molecular basis for the capacity of these pathways to specify branch fates remains enigmatic. Several models have been proposed. For example, these pathways may trigger the expression of distinct receptor molecules that would respond to different cues associated with specific branches. Such cues would serve to refine the general Bnl guiding signal. Alternatively, it is possible that the leading cells in each branch are attracted by the common Bnl signal, but the cells that follow them and encounter only a low level of Bnl activation, must be tightly associated with the leading cell. The expression of distinct sets of homophilic cell adhesion molecules may be important in specifying cell number in each branch.

While the Dpp and DER pathways serve to subdivide the tracheal pit in the dorsoventral axis, additional intersecting signals must be operating in parallel, to provide a more elaborate specification. Both lateral trunk anterior and posterior branches are determined by Dpp pathway activation on the ventral side of the pit, but what generates the distinction between them is not known. Within the central part of the pit, the Wnt pathway serves to distinguish between the cells allocated to the dorsal trunk and the visceral branch.

In the course of a misexpression screen for new components in tracheal development, based on the EP system (Rorth, 1996), we identified an EP line inserted in reverse orientation within the coding region of the *smoothened* (*smo*) gene. Expression of *smo* antisense RNA in the tracheal system gave rise to lethality. Smo is a seven transmembrane domain protein that relays Hedgehog (Hh) signaling (van den Heuvel and Ingham, 1996; Alcedo et al., 1996). Hh signaling has been shown to regulate multiple developmental events in *Drosophila* and higher organisms (reviewed in Johnson and Scott, 1998; Ingham, 1998; Hammerschmidt et al., 1997). Hh binds Patched (Ptc), a multipass membrane molecule (Hooper and Scott, 1989; Nakano et al., 1989; Marigo et al., 1996; Stone et al., 1996). This binding may release a repression of Smo activity or increase Smo levels, and trigger downstream elements culminating in the activation of transcription factors belonging to the Ci (Gli) family of zinc-finger proteins (Aza-Blanc and Kornberg, 1999). A function for Hh in tracheal morphogenesis had not been previously identified. We describe the roles of the Hh signaling pathway in tracheal morphogenesis, most notably in patterning the primary tracheal branches.

MATERIALS AND METHODS

Fly strains

hh^8 is an amorphic allele of *hh* (Mohler, 1988). This line was used for all analyses. Other strains such as hh^3 and hh^4 (conditional temperature-sensitive alleles) were analyzed at the restrictive temperature (25°C) and similar phenotypes were obtained. In terms of phenotype severity, a gradation was observed: $hh^3 > hh^8 > hh^4$. smo^2 and smo^3 were analyzed for tracheal phenotypes. All mutant chromosomes were transferred to marked balancers, *CyO, ftz-lacZ* for the second chromosome or *SbTM3, ftz-lacZ* for the third. The following enhancer trap lines were used: P{*hh-lacZ*} (Tabata et al., 1992), P{A92}ptc^{H84} (Ingham, et al., 1991), l(2)01810 (Complex1) (Samakovolis et al., 1996), *rho-lacZ* R1.1 (provided by M. Levine), *kni-lacZ* (provided by R. Schuh) and *esg-lacZ* (provided by S. Hayashi).

The following UAS lines were used: *UAS-hh*, *UAS-ptc* and *UAS-smo* (provided by P. Ingham); and *UAS-Cice* and *UAS-CiAAA* (provided by K. Basler). EP(2)1220 has the P-element inserted in reverse orientation in the second exon of *smo* in codon 28. On the RNA this position is 389 bp 3′ to the transcription start site. Two tracheal GAL4 drivers were used, *btl-Gal4* (Shiga et al., 1996) and *tracheal-Gal4* (Brand and Perrimon, 1993), as well as the Maternal-Gal4 VP16 (obtained from D. St Johnston). Recombination between hh^8 and *UAS-hh* or *btl-Gal4* was carried out for the rescue experiment, so that all homozygous *hh*-mutant embryos would express *hh* in the trachea. Recombinations between *btl-Gal4* and *esg-lacZ* or *srf-lacZ* were carried out to follow the consequences of *hh* misexpression in the dorsal branches. Embryo collections were performed at 25ºC, unless otherwise stated.

Embryo fixation and immunostaining

Embryos were fixed in 3.7% or 10% formaldehyde-PBS for antibody and RNA in situ stainings, respectively. Staining was according to standard procedures. Hh rabbit antibody ATA7 was provided by P. Ingham. Rabbit anti-β-gal (Cappel) was used at 1:2,000 dilution. Rat anti-Trh was used at 1:100, to stain the entire tracheal system from stage 10. The mouse monoclonal antibody mAb2A12 (from C. Goodman) was used at a 1:5 dilution, to stain the tracheal lumen from stage 14. Monoclonal anti-dpERK (Sigma) was used at 1:50 and amplified using the TSA indirect kit (NEN). Rat anti-Dfr (from W. Johnson) was used at 1:100. All secondary antibodies (HRP-, Biotin-, CY2- and CY3-conjugated) were purchased from Jackson ImmunoResearch and used at a 1:150 dilution. Fluorescent staining was monitored with a BioRad 1024 confocal microscope. Whole-mount RNA in situ hybridization was carried out as described (Tautz and Pfeifle, 1989). Antisense RNA probes were prepared from plasmids containing cDNA to *btl* (Klämbt et al, 1992) and *bnl* (from M. Krasnow).

RESULTS

Expression of *smo* antisense RNA with a *btl-Gal4* driver, which is specific for the midline and trachea (Shiga et al., 1996), gave rise to lethality. Examination of these embryos showed defects in only the late stages of tracheal development (see Fig. 7G). This result points to a role for Hh signaling in tracheal development. However, the phenotype may not reveal the complete role for Hh in the trachea, as this driver is not expressed at the onset of tracheal morphogenesis. In addition, the EP insertion generates an antisense transcript of only 389 nucleotides. This work describes a systematic investigation of the roles played by the Hh pathway in tracheal development.

Early expression patterns of Hh pathway components

The expression pattern of Hh was studied from the onset of tracheal cell determination with a *hh-lacZ* enhancer trap line, and an anti-Hh antibody. Hh is expressed in two-cell wide

segmental stripes that abut, and occasionally overlap, the anterior border of the tracheal placodes at stage 10 (Fig. 1A,B). As the tracheal cells invaginate at stage 11 and initiate their migration at stage 12, Hh expression remains in the ectodermal cells above the anterior portion of the pit (Fig. 1C). Thus, Hh is temporally and spatially expressed in a pattern consistent with a role in early anterior patterning of cells within the tracheal placodes and pits. As tracheal morphogenesis proceeds, the cells of the dorsal trunk, visceral branch and lateral anterior branch migrate anteriorly away from the stripe of Hh-expressing cells. The cells forming the dorsal branch migrate dorsally under the Hh stripe (Fig. 1D).

ptc is a target gene for the Hh pathway (Hidalgo and Ingham, 1990). To define the domain of Hh signaling in the trachea, *ptc* expression was followed with a *ptc-lacZ* enhancer trap line. *ptc* is expressed at stage 10 in a Hh-independent manner as a broad band covering about three quarters of each parasegment and the posterior tracheal cells (Fig. 2A). The early function of Ptc in the ectoderm is unknown. The *ptc* expression pattern changes as the tracheal pits form at stage 11 and the cells begin migrating at stage 12. It is induced in response to Hh signaling and forms a stripe, four to five cells wide, in the anterior portion of the pit (Fig. 2B). This domain defines the tracheal region, showing a marked response to the Hh signal. Another stripe of *ptc* is induced in the cells lying between the pits. Expression of *ptc* in the anterior cells of the trachea, including the dorsal branch, dorsal trunk, visceral branch and lateral trunk anterior is clearly detected at stage 13 (Fig. 2C). When Hh is uniformly expressed in the trachea (in *btl-Gal4/UAS-hh* embryos), a concomitant expansion of *ptc-lacZ* expression is observed (not shown). By stage 15, *ptc* is expressed in an ectodermal stripe above the dorsal branch (Fig. 2D). It may also be expressed in low levels in the dorsal branch cells.

Hh induces expression of additional target genes in the anterior tracheal cells

Induction of *ptc* expression in the anterior tracheal cells defines the cells in which prominent Hh activation takes place. We wanted to test whether Hh signaling could regulate expression of additional genes within the same cells. An enhancer trap line expressed in all anterior tracheal cells at stage 13 has been previously identified and termed Complex1 (Samakovlis et al., 1996). This marker begins to be expressed at stage 12, and by stage 14 is detected in 10-15 cells in the dorsal trunk and lateral trunk anterior (Fig. 3A,B). The domain of expression of this line corresponds to the region of *ptc* expression (Fig. 2C). In *hh* mutant embryos, expression of this gene in the trachea is abolished, but is retained in other tissues (Fig. 3C). Conversely, in an embryo that expressed UAS-*hh* with the *btl*-Gal4 driver, we observed an expansion of the expression to most tracheal cells in the dorsal trunk, transverse connective, visceral branch and lateral trunk posterior (Fig. 3D). Thus, normal Hh activation, which is spatially restricted, provides a signal that is necessary and sufficient to determine a localized expression profile of target genes in the trachea.

hh phenotypes in the trachea

The early stages of tracheal morphogenesis were followed in *hh* mutant embryos, with anti-Trh antibodies, which mark all tracheal nuclei. The initial defects are observed in the determination of placode size. The majority of embryos exhibit a reduction in tracheal pit 3 (27/28 embryos). The number of cells expressing Trh in this pit is about half of the expected number of ~50 cells (Fig. 4A,B). In addition, 71% of the *hh* mutant embryos showed a reduction of tracheal cell number in pits 2, 4, 8 or 9.

In *hh* mutants, a small number of tracheal cells (~6-8) fail to invaginate at stage 11, and remain on the ectoderm. In the tracheal cell population that has invaginated, there is a distinct failure of migration of all tracheal branches at stage 12 (Fig. 4C,D). The phenotype is penetrant in all mutant embryos. More specifically, the dorsal and visceral branches are never formed. The dorsal trunk cells migrate partially, especially in the posterior segments. A reduced number of cells is allocated to the lateral trunk anterior branch. Conversely, the correct cell number is allocated to the lateral trunk posterior in most segments.

In wild-type embryos, the most pronounced tracheal cell migration takes place between stages 12 to 14 (Fig. 4E). In contrast, in *hh* mutants minimal migration is observed beyond the residual migration seen at stage 12, with the majority of the tracheal cells remaining in the transverse connective (Fig. 4F). *smo* mutant embryos exhibit a similar, but less severe phenotype, probably owing to a persistent maternal contribution (not shown).

Hh affects trachea independently of known pathways

Expression of Trh is retained in *hh* mutant embryos, although a reduced number of cells is occasionally observed in several

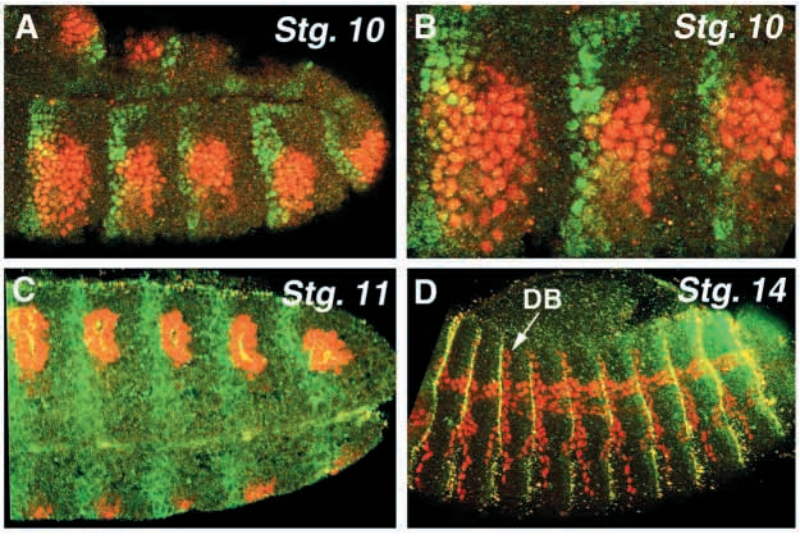

Fig. 1. Hh expression in the ectoderm abuts the tracheal placodes. (A,B) At stage 10, *hh* is expressed in ectodermal stripes of two to three cells, as followed with *hh-lacZ* (green). These cells abut and occasionally overlap the anterior border of the tracheal placode cells, marked by nuclear staining with anti-Trh (red). (C) At stage 11 after the tracheal pits have formed, Hh protein (followed by anti-Hh antibody, green) displays a punctate pattern that overlaps the anterior pit cells. (D) By stage 14, the Hh protein stripe is refined and lies above the tracheal dorsal branch (DB).

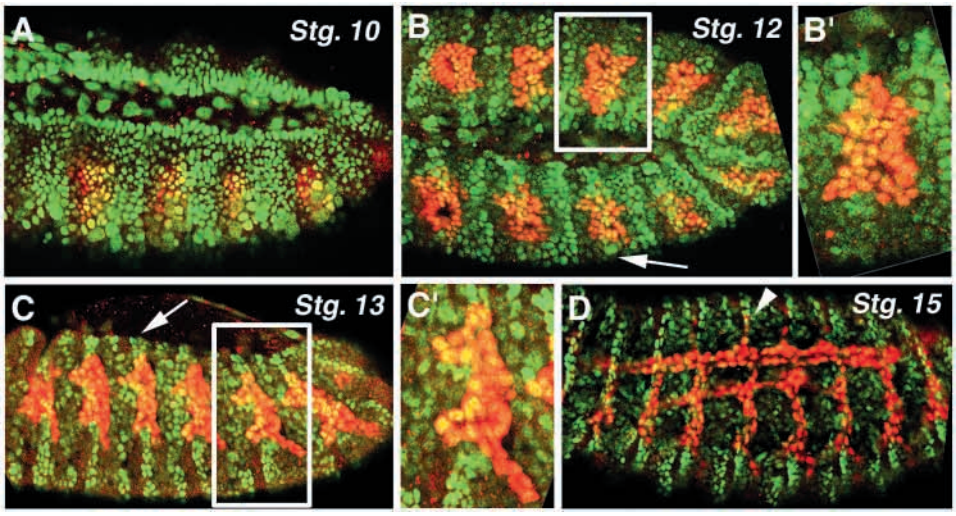

Fig. 2. Hh induces *ptc* expression in the anterior tracheal cells. Expression of *ptc* was monitored with a *ptc-lacZ* line (green). (A) At stage 10, *ptc* is expressed in all ectodermal cells (except the two rows of *hh*-expressing cells) in a Hh-independent manner. (B,B′) By stage 12, *ptc* expression is dependent on Hh signaling. In every segment it is observed in an ectodermal stripe above the anterior tracheal cells (arrow) and another stripe between the tracheal pits. Double labeling with anti-Trh antibodies (red) demonstrates that *ptc* is also expressed in the tracheal cells that lie below the ectoderm, in the anterior half of the pit. (C,C′) *ptc* expression in the trachea continues during stage 13 in all the cells recruited to the dorsal branch, dorsal trunk, visceral branch and lateral trunk anterior. (D) At stage 15, in accordance with the refined pattern of Hh expression, *ptc* is expressed in a narrow stripe of cells that lies above the dorsal branch (arrowhead).

pits. Another gene that is crucial to the early determination of tracheal cell fate is Drifter/Ventral veinless. Staining *hh* mutant embryos with anti-Dfr antibody shows normal expression of the protein (Fig. 5A,B).

Several pathways are known to converge in the trachea and affect early tracheal branching, including epidermal growth factor (EGF), FGF and Dpp (reviewed by Zelzer and Shilo, 2000b). To rule out the possibility that Hh is affecting the trachea indirectly by influencing signaling of the above pathways, downstream responses to these pathways were examined in *hh* mutant embryos.

Activation of DER in the trachea is triggered by expression of Rhomboid (Rho) in the tracheal placodes, leading to the accumulation of dpERK (activated MAP kinase) (Wappner et al., 1997). *rho-lacZ* expression and the dpERK pattern at stage 10, are detected normally in *hh* mutant embryos (Fig. 5C,D), indicating proper activation of the DER pathway in the tracheal cells. The FGF receptor Btl is triggered by localized expression of the ligand Bnl (Sutherland et al., 1996), and again can be directly followed by the resultant dpERK pattern of expression (Gabay et al., 1997). Expression of *bnl* is only slightly affected in *hh* mutants, with the lack of a dorsal cluster of cells in some of the segments (Fig. 5E). All other aspects of *bnl* expression are normal in *hh* mutant embryos. The resulting dpERK pattern at stage 12, shows activation within the tip cells of the different branches, which undergo partial or no migration (Fig. 5F). *knirps* (*kni*) expression defines the tracheal response to the Dpp pathway (Chen et al., 1998). *kni* expression is largely unaffected in *hh* mutants, and can be seen in the rudimentary dorsal, visceral and lateral branches (Fig. 5H). These results imply that Hh is acting independently of the EGF, FGF and Dpp pathways in early patterning of tracheal branches.

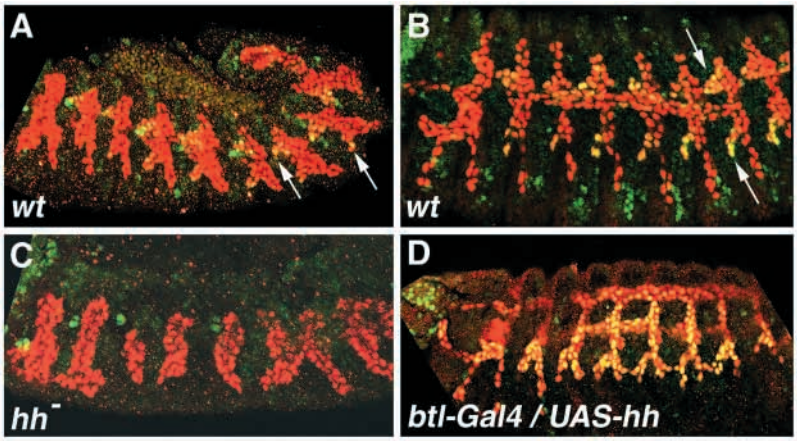

Fig. 3. Hh signaling is necessary and sufficient for expression of anterior tracheal markers. **(**A) The Complex1 *lacZ* line (green) begins to be expressed in the leading cells of the dorsal trunk (DT) and lateral trunk (LT) anterior at stage 12 (arrows). (B) By stage 14, expression in the DT and LTa is pronounced (arrows). (C) In *hh* mutant embryos, expression is abolished in the trachea, but retained in other tissues. (D) Uniform expression of Hh in the trachea with a *btl-Gal4* driver leads to expression of Complex1 in most tracheal cells.

Rescue of *hh* mutant tracheal phenotype

Two lines of evidence suggest that the signaling pathway induced by Hh is activated within the tracheal cells. First, is the expression of *ptc* and Complex1, which mark reception of the Hh signal in the anterior tracheal cells (Figs 2, 3). The second is that UAS-antisense *smo* leads to a tracheal phenotype when driven by *btl*-Gal4 in the tracheal cells. Additional attempts to inactivate the Hh pathway specifically in the tracheal cells at the onset of migration by expressing UAS-*ptc* or UAS-*ci* dominant negative, also gave rise only to more subtle and late phenotypes (see Fig. 7).

Another approach to test the effects of patterning the ectoderm by Hh on tracheal migration, was to provide Hh uniformly to all embryonic cells using the Maternal-Gal4 VP16 driver. While the overall morphology of these embryos is deranged, the tracheal cells are capable of forming all of the branches except the dorsal branch (Fig. 6A). The

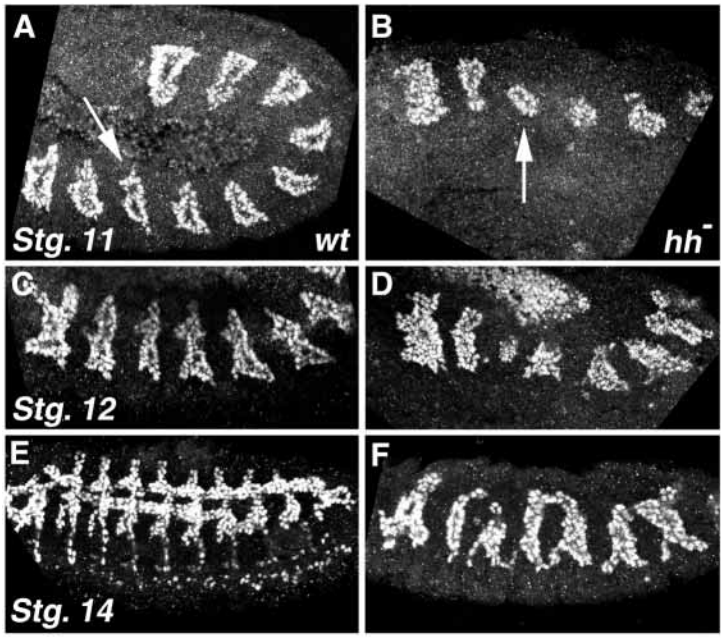

Fig. 4. *hh* tracheal phenotype. **(**A) Wild-type embryo at stage 11. Tracheal nuclei are visualized by anti-Trh antibodies. Arrow shows tracheal pit number 3. (B) In *hh* mutant embryos at the same stage, a reduction in the size of tracheal pit number 3 is observed (arrow). (C) Wild-type embryo at stage 12 shows migration of the six primary branches. (D) *hh* mutant embryos at the same stage show stalled migration of all branches. (E) By stage 14, pronounced migration is observed in wild-type embryos. (F) In contrast, *hh* mutant embryos retain only a rudimentary branching pattern.

morphology of the branches is slightly affected, but primary patterning proceeded normally. This experiment demonstrates that for most tracheal branches the migration cues do not rely on patterning the ectoderm by Hh. An alternative approach to activate the Hh pathway uniformly is to follow the phenotype of *ptc*-mutant embryos. However, the defects in tracheal branching were only marginal (not shown).

A more direct avenue to separate between the Hh effects on the ectoderm and the trachea would be to rescue the *hh* tracheal phenotype, without rescuing the ectodermal defects associated with the absence of Hh. Ideally, one would like to rescue the defect cell autonomously in the trachea. Constructs of UAS-*smo* were not sufficiently potent to overcome the absence of Hh. Instead, we resorted to ectopic expression of Hh in the trachea of *hh*-mutant embryos.

UAS-*hh* driven by *btl*-Gal4 in wild-type embryos shows almost normal branching of the primary and secondary branches, with occasional defects in the dorsal branch and lateral trunk posterior (Fig. 6B). This allowed us to examine rescue of the *hh* tracheal phenotype, by providing Hh in only the tracheal cells. Indeed, we saw partial rescue, in particular, restoration of the dorsal trunk, visceral branches and some lateral trunk anterior branches (compare 6C to 6D). The ectodermal defects of these *hh* mutant embryos were not rescued, as can be deduced from the overall defects in the structure of the embryo and from cuticle preparations (not shown). This experiment demonstrates that direct activation of the Hh pathway in the tracheal cells is sufficient for proper branch migration.

Late roles of Hh in the trachea

Hh expression is maintained as a stripe of ectodermal cells. By stage 14, this stripe is positioned above the dorsal branch cells. In all tracheal branches, the leading cells assume a terminal cell fate marked by expression of serum response factor (SRF) (Guillemin et al., 1996). Hh appears to be involved in the

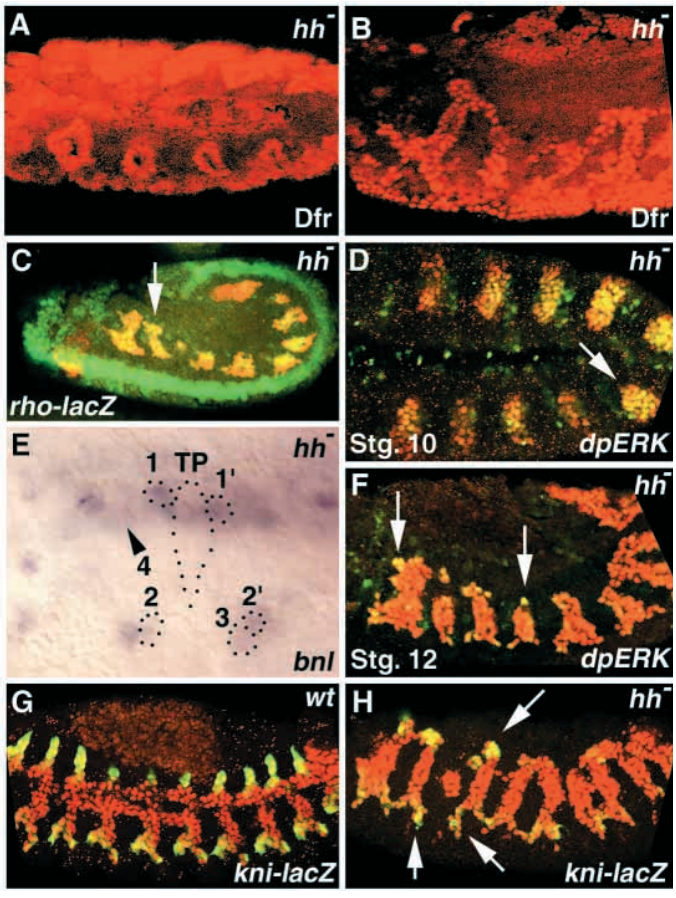

Fig. 5. *hh* mutant embryos retain normal signaling in the EGF, Btl and Dpp pathways. (A,B) Expression of Drifter in *hh* mutant embryos at stages 11 and 13 is normal. (C) *rho* is expressed in the tracheal placodes where it triggers processing of Spitz and DER signaling. A *rho-lacZ* line (green) shows normal expression in the tracheal pits (arrow) and midline cells of *hh* mutant embryos. The tracheal cells are labeled in red by anti-Trh. (D) Normal activation of the DER pathway is also evident by the appearance of dpERK (green) in the tracheal placodes at stage 10 (arrow). (E) The expression of *bnl*, which is responsible for triggering signaling by Btl in the trachea, is normal in *hh* mutants. Shown is a stage 12 *hh*-mutant embryo. TP, tracheal pit; 1, *bnl* patches directing dorsal trunk migration; 2, *bnl* patch guiding LTa migration; 3, *bnl* patch directing LTp migration; 4, expression of *bnl* in the visceral branch is seen out of focus (arrowhead). (F) Accordingly, the typical Btl-induced dpERK pattern in the leading tip cells is observed at stage 12 (arrows). (G) Expression of *kni-lacZ* (green) triggered by Dpp is observed in wild-type embryos in the DT, LTa, LTp and VB. (H) In *hh* mutant embryos, expression of *kni-lacZ* is retained (arrows), indicating that Dpp signaling is normal.

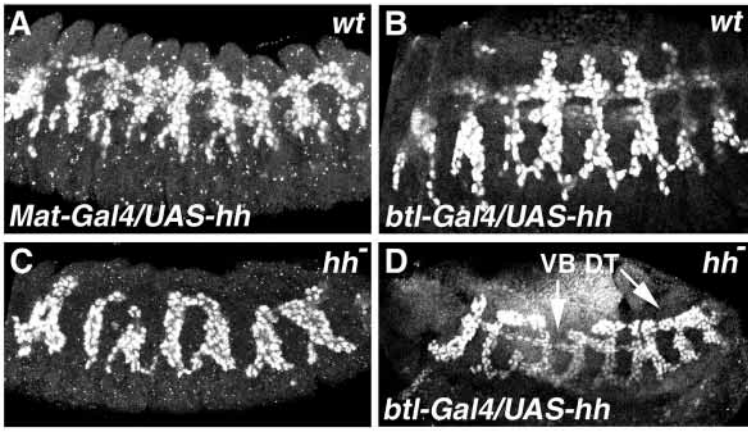

Fig. 6. Expression of Hh in the trachea rescues the migration defects. **(**A) Ubiquitous expression of *hh* by the Maternal-Gal4 driver disrupts ectodermal patterning, but not migration of the primary tracheal branches. (B) Expression of *hh* in the trachea of a wild-type embryo by the *btl-Gal4* driver does not give rise to marked defects in migration. (C) *hh* mutant embryo at stage 14. (D) Tracheal expression of Hh in a *hh* mutant embryo partially rescues the major migration defects, most notably in the dorsal trunk (DT) and visceral branch (VB), demonstrating that Hh signaling is required in the tracheal cells for normal migration. Tracheal nuclei are visualized using anti-Trh antibodies.

specification of terminal cells. When driven by *btl*-Gal4 or tracheal-Gal4, the positive effectors of the Hh pathway induce a change of cell fate in the dorsal branches. Embryos with UAS-*hh*, UAS-*smo* and UAS-*Ci activated* exhibit extra SRF-expressing cells that appear to arise from the branch cells located after the fusion cell (Fig. 7A-C). The fusion cells maintain their identity, as monitored by expression of *escargot*, a fusion cell marker (Fig. 7D). Thus, the branch cells have undergone a transformation of cell fate. However, overexpressing the negative components of the pathway (such as *ptc*, antisense *smo* or Ci inhibitory) leads to shortened terminal extensions. In these cases terminal cells retain normal SRF expression, but have lost some of the morphological features of terminal cells (Fig. 7E-G).

DISCUSSION

Multiple roles for the Hh pathway in tracheal patterning

This work has identified four distinct, consecutive roles for the Hh pathway in tracheal morphogenesis. The earliest function of Hh is to participate in allocating the correct cell number to several tracheal pits, most notably pit number 3, where only half the number of tracheal cells is observed in *hh* mutants. The determination of the position and number of cells in the tracheal placodes is highly regulated. Segmental differences in cell number are observed. Most placodes contain 80 cells, while the first placode contains 150 cells, and the third contains only 50 cells. In the dorsoventral axis, it appears that the activity of the DER pathway in the ventral ectoderm, and the Dpp pathway in the dorsal ectoderm, restrict the position of the placodes to the central region (Raz and Shilo, 1993; Isaac and Andrew, 1996). The cues for determination of the placodes in the anteroposterior axis are only partially known. Indications for repression of tracheal fates by Wingless have been reported (Wilk et al., 1996). As most of the placodes form normally in *hh* mutants, Hh signaling is not involved in providing global cues for the position of the placodes. The defects observed in tracheal cell number could imply that Hh collaborates with homeotic genes in the specification of segment-specific placode cell number, particularly in placode number 3.

The second defect is observed at stage 11 in all metamers, during the invagination of the tracheal pits. In *hh* mutants, six to eight cells remain on the ectoderm. Similar defects were observed in mutants for the EGF receptor pathway (Llimargas and Casanova, 1999). From stage 12 onwards, the most severe tracheal defect is the lack or significant impairment of migration. The reduction in cell number in the tracheal pits caused by the two earlier Hh functions cannot account for this severe defect. Tracheal pits containing half of the normal number were shown to migrate normally (Beitel and Krasnow, 2000). Thus, the migration defect represents a distinct function of Hh. The possible mechanistic basis for this role will be discussed below. In addition to defects in the migration of the branches anteriorly, we noted that in *hh* mutant embryos, the lateral trunk posterior branch is also stalled. Although we did not observe the induction of the Hh target genes *ptc* and Complex1 in the LTp cells, it is possible that these cells require a lower level of Hh signaling.

Finally, the Hh pathway is required for patterning the terminal cells in the dorsal branches. Compromising the activity of the pathway reduced the extent of terminal branching, while activation of the Hh pathway in the tracheal cells gave rise to an excess of terminal cells in the dorsal branch. It has previously been shown that high levels of Btl activation induce terminal cell fates, through the expression of Pointed P1 (Sutherland et al., 1996). Thus, the leading cells that are closest to the Bnl source become terminal cells. During the migration of the dorsal branch cells, Bnl activation is high in the leading cell and low in the trailing ones. Hh expression is poised directly over the migrating cells, and may provide a uniform level of activation in these cells. Hh signaling could provide a dorsal branch “context” for these cells, and function in conjunction with the signals elicited by Bnl. It is interesting to note that similar effects on terminal cell fates were observed in other branches such as the ganglionic branch, following expression of these constructs (not shown). Late Hh expression, however, is not positioned in close proximity to these branches, raising the possibility that these late abnormalities may reflect Hh influence at an earlier stage.

Direct role of Hh in tracheal patterning

Several observations point to a direct role for Hh signaling in patterning the tracheal branches, rather than an indirect effect on the ectoderm. The early expression of Hh in stripes abutting the anterior border of the tracheal pits has the potential to provide restricted signaling in the anterior portion of the pit, owing to limited diffusion of Hh. Induction of Hh-target genes in the trachea, such as *ptc* and the Complex1 marker can indeed

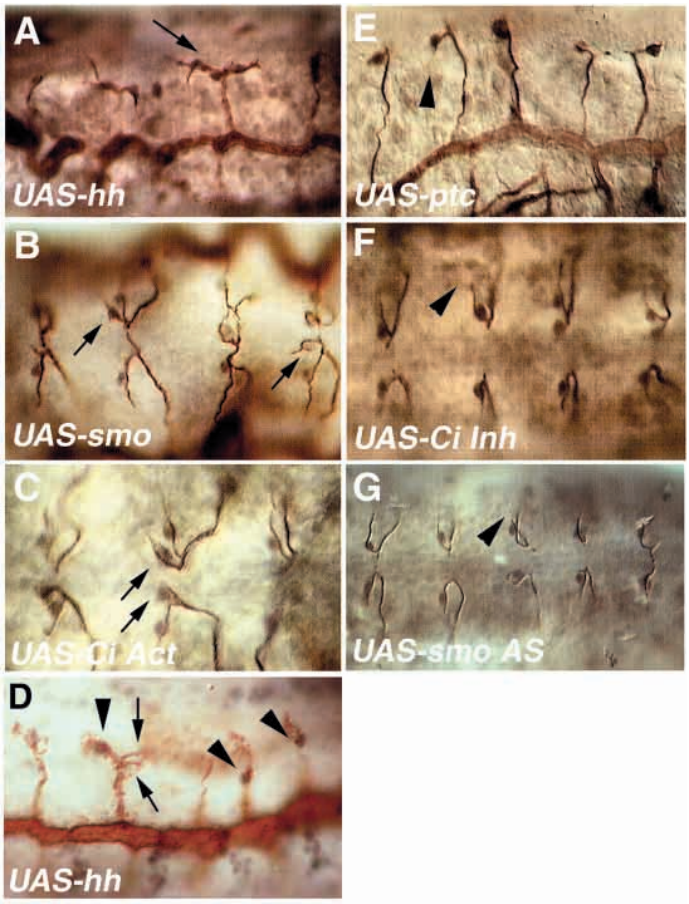

Fig. 7. Hh signaling participates in the determination of terminal cells in the dorsal branch. The late expression of *hh* in the ectoderm lies exactly above the migration path of the dorsal branch. Hh may facilitate signaling by Btl, which defines the terminal SRF-expressing cell in this branch. (A-C) Hyperactivation of the Hh pathway by ubiquitous tracheal expression (induced by *btl-Gal4*) of *hh* (A), *smo* (B) or activated *Ci* (C) did not alter the migration of the tracheal branches. However, it gave rise to an excess of terminal cells in the dorsal branch (arrows), as marked by expression of *srf-lacZ* (blue nuclear staining) and excess terminal branching (monitored by the 2A12 monoclonal antibody, which detects the tracheal lumen). (D) The fusion cell marker *escargot* continues to be expressed in the fusion cells after *hh* misexpression, marked by blue anti-β-gal nuclear staining (arrowheads). Extra terminal branches emanate from the tracheal branch cells located after the fusion cell (arrows). (E-G) Reduction of the activity of the Hh pathway in the trachea by ubiquitous tracheal expression of *ptc* (E), inhibitory *Ci* (F), or *smo* antisense (G) RNA, retained SRF expression in the terminal cell but resulted in absence of terminal branching by this cell (arrowheads). In wild-type embryos, low and uniform Hh activation in the dorsal branch may provide a 'context' for induction of the terminal cell fate, by collaborating with localized Btl activation in the leading cell.

be detected. Tracheal phenotypes can also be observed after partial removal of Smo activity only in the trachea, by driving expression of a *smo* antisense construct. No defects in ectodermal or tracheal markers that reflect the activity of other pathways, such as DER, Btl and Dpp were observed in *hh* mutants. The exception is the dorsal branch, where defects were observed in the dorsal Bnl signal in *hh* mutants. Finally, partial rescue of the tracheal migration phenotype could be obtained by providing Hh only in the trachea. The question arises, what mechanisms does Hh induce in the trachea, to allow proper cell migration?

Integration of signals patterning the tracheal branches

When the tracheal placodes are first established, all cells are equipotent and no lineage restrictions exist (Samakovlis et al., 1996). Prior to the onset of migration, however, a number of signaling pathways impinge on the tracheal placodes. They include the DER, Dpp, Wnt and Hh pathways. In some instances the signal emanates from the tracheal pits (e.g. DER is triggered by *rho* expression in the placode). Conversely, the Dpp and Hh pathways are triggered in the trachea by a ligand expressed in adjacent cells. Eventually, the combined outcome of these events is the allocation of a fixed number of cells to each of the branches, as migration is initiated. How are the different pathways integrated?

In two instances, a single pathway appears to be sufficient and instructive for specifying a distinct branch fate. Hyperactivation of the Dpp pathway redirects cells from the dorsal trunk towards the dorsal branch (Vincent et al., 1997). Similarly, ectopic Wnt activation enlarges the dorsal trunk at the expense of the visceral branch (Llimargas, 2000; Chihara and Hayashi, 2000). Conversely, in the case of the DER and Hh pathways, absence of signaling leads to dramatic migration defects, while hyperactivation does not significantly impair the migration pattern (Wappner et al., 1997; this work).

The Hh pathway is capable of inducing target genes in a restricted domain within the tracheal pit, i.e. in the cells of the anterior region. Induction of such genes may be essential for acquiring the proper branch fates by these cells, and is reflected in the *hh* mutant phenotype. However, uniform activation of Hh, which leads to the expansion in the expression of such genes, is not sufficient to divert the cells of the lateral posterior branch into the anterior branch. It is possible that combinatorial interactions with other pathways may be necessary in order to convert the cells from one branch to another. Such interactions could be manifested by an essential combination of expressed genes, induced by one pathway or another. Alternatively, the induction of genes that require a simultaneous input from more than one signaling pathway could also be important for branch specification.

The question of how tracheal cell fates are eventually manifested in the direction of migration of a given branch is still unanswered. Specifically, what are the crucial transcriptional targets of each of these pathways in the trachea? In the course of tracheal morphogenesis the cells maintain a close association with each other. This seems to be essential for migration, as activation of Btl by Bnl is restricted to the leading cell(s). One possible scenario is that the cells of each branch express distinct sets of homophilic cell adhesion molecules, allowing them to adhere to the leading cell and to each other. In addition, different branches may express distinct sets of membrane-spanning molecules, allowing them to recognize distinct migration cues or different migration milieus.

We thank K. Basler, C. Goodman, S. Hayashi, P. Ingham, W. Johnson, M. Krasnow, M. Levine, R. Schuh and D. St Johnston; and

the Bloomington, Szeged and Umea stock centers for providing strains, antibodies and cDNAs. We thank members of the Shilo laboratory and Talila Volk for critical reading of the manuscript. This work was supported by grants from The Israel Science Foundation, the Minerva Foundation and the HFSP to B.-Z. S.

REFERENCES

Affolter, M., Nellen, D., Nussbaumer, U. and Basler, K. (1994). Multiple requirements for the receptor serine/threonine kinase *thick veins* reveal novel function of TGFβ homologs during *Drosophila* embryogenesis. *Development* **120**, 3105-3117.

Affolter, M. and Shilo, B.-Z. (2000). Genetic control of branching morphogenesis during *Drosophila* tracheal development. *Curr. Opin. Cell Biol.* **12**, 731-735.

Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M. and Hooper, J. E. (1996). The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell* **86**, 221-232.

Anderson, M. G., Perkins, G. L., Chittick, P., Shrigley, R. J. and Johnson, W. A. (1995). *drifter*, a *Drosophila* POU-domain transcription factor, is required for correct differentiation and migration of tracheal cells and midline glia. *Genes Dev.* **9**, 123-137.

Aza-Blanc, P. and Kornberg, T. B. (1999). Ci: a complex transducer of the hedgehog signal. *Trends Genet.* **15**, 458-462.

Beitel, G. J. and Krasnow, M. A. (2000). Genetic control of epithelial tube size in the *Drosophila* tracheal system. *Development* **127**, 3271-3282.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Chen, C. K., Kuhnlein, R. P., Eulenberg, K. G., Vincent, S., Affolter, M. and Schuh, R. (1998). The transcription factors KNIRPS and KNIRPS RELATED control cell migration and branch morphogenesis during *Drosophila* tracheal development. *Development* **125**, 4959-4968.

Chihara, T. and Hayashi, S. (2000). Control of tracheal tubulogenesis by wingless signaling. *Development* **127**, 4433-4442.

de Celis, J. F., Llimargas, M. and Casanova, J. (1995). *Ventral veinless*, the gene encoding the Cf1a transcription factor, links positional information and cell differentiation during embryonic and imaginal development in *Drosophila melanogaster*. *Development* **121**, 3405-3416.

Gabay, L., Seger, R. and Shilo, B.-Z. (1997). MAP kinase in situ activation atlas during *Drosophila* embryogenesis. *Development* **124**, 3535-3541.

Guillemin, K., Groppe, J., Ducker, K., Treisman, R., Hafen, E., Affolter, M. and Krasnow, M. A. (1996). The *pruned* gene encodes the *Drosophila* serum response factor and regulates cytoplasmic outgrowth during terminal branching of the tracheal system. *Development* **122**, 1353-1362.

Hammerschmidt, M., Brook, A. and McMahon, A. P. (1997). The world according to hedgehog. *Trends Genet.* **13**, 14-21.

Hidalgo, A. and Ingham, P. (1990). Cell patterning in the *Drosophila* segment: spatial regulation of the segment polarity gene *patched*. *Development* **110**, 291-301.

Hooper, J. E. and Scott, M. P. (1989). The *Drosophila patched* gene encodes a putative membrane protein required for segmental patterning. *Cell* **59**, 751-765.

Ingham, P. W., Taylor, A. M. and Nakano, Y. (1991). Role of the *Drosophila patched* gene in positional signalling. *Nature* **353**, 184-187.

Ingham, P. W. (1998). Transducing Hedgehog: the story so far. *EMBO J.* **17**, 3505-3511.

Isaac, D. D. and Andrew, D. J. (1996). Tubulogenesis in *Drosophila*: a requirement for the *trachealess* gene product. *Genes Dev.* **10**, 103-117.

Johnson, R. L. and Scott, M. P. (1998). New players and puzzles in the Hedgehog signaling pathway. *Curr. Opin. Genet. Dev.* **8**, 450-456.

Klämbt, C., Glazer, L. and Shilo, B.-Z. (1992). breathless, a Drosophila FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. *Genes Dev.* **6**, 1668-1678.

Llimargas, M. and Casanova, J. (1997). ventral veinless, a POU domain transcription factor, regulates different transduction pathways required for tracheal branching in *Drosophila*. *Development* **124**, 3273-3281.

Llimargas, M. and Casanova, J. (1999). EGF signaling regulates cell invagination as well as cell migration during formation of tracheal system in *Drosophila*. *Dev. Genes Evol.* **209**, 174-179.

Llimargas, M. (2000). Wingless and its signaling pathway have common and separate functions during tracheal development. *Development* **127**, 4407-4417.

Manning, G. and Krasnow, M. A. (1993). Development of the *Drosophila* Tracheal System. In *The Development of* Drosophila melanogaster. Vol. 1, pp. 609-685. New York: Cold Spring Harbor Press.

Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M. and Tabin, C. J. (1996). Biochemical evidence that patched is the Hedgehog receptor. *Nature* **384**, 176-179.

Michelson, A. M., Gisselbrecht, S., Buff, E. and Skeath, J. B. (1998). Heartbroken is a specific downstream mediator of FGF receptor signalling in *Drosophila*. *Development* **125**, 4379-4389.

Mohler, J. (1988). Requirements for *hedgehog*, a segmental polarity gene, in patterning larval and adult cuticle of *Drosophila*. *Genetics* **120**, 1061-1072.

Nakano, Y., Guerrero, I., Hidalgo, A., Taylor, A., Whittle, J. R. and Ingham, P. W. (1989). A protein with several possible membrane-spanning domains encoded by the *Drosophila* segment polarity gene *patched*. *Nature* **341**, 508-513.

Raz, E. and Shilo, B.-Z. (1993). Establishment of ventral cell fates in the *Drosophila* embryonic ectoderm requires DER, the EGF receptor homolog. *Genes Dev.* **7**, 1937-1948.

Rorth, P. (1996). A modular misexpression screen in *Drosophila* detecting tissue-specific phenotypes. *Proc. Natl. Acad. Sci. USA* **93**, 18-22.

Samakovlis, C., Hacohen, N., Manning, G., Sutherland, D. C., Guillemin, K. and Krasnow, M. A. (1996). Development of the *Drosophila* tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. *Development* **122**, 1395-1407.

Shiga, Y., Tanaka-Matakatsu, M. and Hayashi, S. A. (1996). Nuclear GFP/β-galactosidase fusion protein as a marker for morphogenesis in living *Drosophila*. *Dev. Growth Differ.* **38**, 99-106.

Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J. E., de Sauvage, F. and Rosenthal, A. (1996). The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* **384**, 129-134.

Sutherland, D., Samakovlis, C. and Krasnow, M. A. (1996). *branchless* encodes a Drosophila FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell* **87**, 1091-1101.

Tabata T., Eaton S. and Kornberg T. B. (1992). The *Drosophila hedgehog* gene is expressed specifically in posterior compartment cells and is a target of engrailed regulation. *Genes Dev.* **12**, 2635-2645.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals transcriptional control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-85.

van den Heuvel, M. and Ingham, P. W. (1996). *smoothened* encodes a receptor-like serpentine protein required for *hedgehog* signalling. *Nature* **382**, 547-551.

Vincent, S., Ruberte, E., Grieder, N. C., Chen, C.-K., Haerry, T., Schuh, R. and Affolter, M. (1997). DPP controls tracheal cell migration along the dorsoventral body axis of the *Drosophila* embryo. *Development* **124**, 2741-2750.

Wappner, P., Gabay, L. and Shilo, B.-Z. (1997). Interactions between the EGF receptor and Dpp pathways establish distinct cell fates in the tracheal placodes. *Development* **124**, 4707-4716.

Wilk, R., Weizman, I. and Shilo, B.-Z. (1996). *trachealess* encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in *Drosophila*. *Genes Dev.* **10**, 93-102.

Zelzer, E. and Shilo, B.-Z. (2000a). Interaction between the bHLH-PAS protein trachealess and the POU-domain protein drifter, specifies tracheal cell fates. *Mech. Dev.* **91**, 163-173.

Zelzer, E. and Shilo, B.-Z. (2000b). Cell fate choices in *Drosophila* tracheal morphogenesis. *BioEssays* **22**, 219-226.