Development 127, 4433-4442 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV2622

Control of tracheal tubulogenesis by Wingless signaling

Takahiro Chihara and Shigeo Hayashi[1,*]

Genetic Strain Research Center and The Graduate University for Advanced Studies, National Institute of Genetics, Mishima 411-8540, Japan
[1]RIKEN Center for Developmental Biology, National Institute of Genetics, Mishima 411-8540, Japan
*Author for correspondence (e-mail: shayashi@lab.nig.ac.jp)

Accepted 4 August; published on WWW 26 September 2000

SUMMARY

The tubular epithelium of the *Drosophila* tracheal system forms a network with a stereotyped pattern consisting of cells and branches with distinct identity. The tracheal primordium undergoes primary branching induced by the FGF homolog Branchless, differentiates cells with specialized functions such as fusion cells, which perform target recognition and adhesion during branch fusion, and extends branches toward specific targets. Specification of a unique identity for each primary branch is essential for directed migration, as a defect in either the EGFR or the Dpp pathway leads to a loss of branch identity and the misguidance of tracheal cell migration. Here, we investigate the role of Wingless signaling in the specification of cell and branch identity in the tracheal system. Wingless and its intracellular signal transducer, Armadillo, have multiple functions, including specifying the dorsal trunk through activation of Spalt expression and inducing differentiation of fusion cells in all fusion branches. Moreover, we show that Wingless signaling regulates Notch signaling by stimulating Delta expression at the tip of primary branches. These activities of Wingless signaling together specify the shape of the dorsal trunk and other fusion branches.

Key words: Wingless, Notch, Delta, Escargot, Spalt, Trachea, *Drosophila*

INTRODUCTION

The rapid locomotion of insects is supported by efficient aeration by the tracheal system, a network of finely branched, tubular epithelium throughout the body cavity. The branching pattern of this network is established during embryogenesis. Ectodermal epithelia first forms a sac-like primordium by invagination, and this is followed by localized activation of Breathless (Btl) receptor tyrosine kinase by the FGF homolog Branchless, triggering stereotyped branching and directed migration (Glazer and Shilo, 1991; Sutherland et al., 1996). Tracheal development involves the differentiation of cells with specialized functions, such as the fusion cells leading branch migration and fusion, and the terminal cells that put out long tracheolar extensions. The integrity of the tracheal epithelia is maintained throughout these processes, so that internal organs have access to air without exposure to the outside (reviewed by Manning and Krasnow, 1993).

Each primary branch has a unique identity indicated by its migration toward specific locations and branch-specific gene expression. Commitment of tracheal branches to specific fates is triggered by Decapentaplegic (Dpp) and EGF receptor (EGFR) signaling (Fig. 1G; Vincent et al., 1997; Wappner et al., 1997; Llimargas and Casanova, 1997; Zelzer and Shilo, 2000). Dpp is expressed in ectodermal cells abutting the dorsal and ventral sides of invaginated tracheal cells, promoting the development of the dorsal branch (DB), the lateral trunk (LT) and the ganglionic branch (GB). Those branches migrate along the dorsoventral axis and express the nuclear receptors Knirps (Kni) and Knirps-related (Knl) (Chen et al., 1998). EGFR is activated in the central subset of tracheal cells by localized transcription of the EGFR activator, *rhomboid (rho)*. EGFR activation is required for the development of the dorsal trunk (DT) and the visceral branch (VB) in cells migrating along the anterior-posterior direction (Wappner et al., 1997). These findings suggest that regional differentiation of the tracheal placode is induced by the dorsoventral positional cue.

Although DT and VB are derived from the same position and are induced by EGFR, they have distinct properties. DT expresses Spalt (Sal) (Kuhnlein and Schuh, 1996) and fuses to the DT from adjacent segments via fusion cells. VB expresses Kni/Knl and migrates to the interior of the embryo to ramify numerous terminal branches from terminal cells. The mechanism by which DT and VB acquire these different properties remains to be determined.

Further cellular differentiation in each branch contributes to tubulogenesis. A single fusion cell expresses the zinc-finger gene *escargot*, located at the tip of these migrating branches, and is required for the fusion event (blue circles in Fig. 1G; Tanaka-Matakatsu et al., 1996; Samakovlis et al., 1996). Notch-Delta signaling has an important role in limiting the number of fusion cells to one per branch (Llimargas, 1999; Ikeya and Hayashi, 1999; Steneberg et al.,

1999). Bnl stimulates the expression of Delta, helping to coordinate primary branching and fusion cell determination. It is not known, however, what induces fusion cell differentiation.

Wingless (Wg) is a secreted glycoprotein required for a variety of inductive signaling events during both embryonic and imaginal disc development (reviewed by Cadigan and Nusse, 1997; Martinez-Arias et al., 1999). The *Drosophila* β-catenin homolog, Armadillo (Arm), is a downstream component of Wg signaling (Noordermeer et al., 1994; Peifer et al., 1994; Siegfried et al., 1994). The severe tracheal phenotypes of *wg* or *arm* zygotic mutants (Uemura et al., 1996) suggest that Wg signaling is involved in tracheal development. We found that Wg signaling has two distinct roles in tracheal development, inducing DT fate and fusion cell fate. We also demonstrate that Wg signaling acts in concert with Bnl to regulate Notch signaling by stimulating Delta expression, limiting the number of fusion cells. This activity couples the additional function of Wg to promote fusion cell differentiation, specifying a single fusion cell to differentiate each fusion branch.

MATERIALS AND METHODS

Fly stocks

The following strains were used in this study: wg^{IL114} (Couso et al., 1994), arm^{YD35} and $arm^{H8.6}$ (Peifer et al., 1993; Peifer, 1995), *trachealess* enhancer trap line *1-eve-1* (Perrimon et al., 1991), *Delta-lacZ* (R. Ueda, personal communication), *UAS-wg* (Lawrence et al., 1995), *UAS-arm*^{S10} (Pai et al., 1997), *UAS-Daxin* (Hamada et al., 1999), *UAS-DTCFΔN* (van de Wetering et al., 1997), *UAS-Delta* (Doherty et al., 1996), *UAS-ECN* (a dominant negative form of Notch; Jacobsen et al., 1998), *UAS-gfpTTras* (a membrane-bound green fluorescent protein, GFP; S. H., unpublished material), *UAS-gfpnlacZ* and *btl-Gal4* (Shiga et al., 1996).

Embryo staining

The following primary antibodies were used: rat anti-Esg (Fuse et al., 1994), anti-Sal (Kuhnlein et al., 1994; a gift from R. Schuh), mouse anti-Delta 9B (Fehon et al., 1990; a gift from K. Matsuno), mouse anti-SRF (a gift from M. Gilman), rat anti-Kni (Kosman et al., 1998; East Asia Segmentation Antibody Center), anti-β-galactosidase (Cappel), mouse anti-Wg 4D4 (Brook and Cohen, 1996), anti-GFP (MBL) and 2A12, which recognizes an unknown luminal component (Developmental Studies Hybridoma Bank).

Immunostainings were performed as described by Hayashi et al. (1993). The secondary antibodies used were as follows: biotinylated anti-rat IgG, biotinylated anti-mouse IgM and Cy3-conjugated anti-mouse IgM (Jackson Laboratory), and Cy2-conjugated anti-rabbit IgG (Amersham). When biotinylated antibodies were used, signals were visualized by Cy2- or Cy3-conjugated streptavidin (Amersham). When necessary, weak signals were amplified by the use of biotinylated-tyramide (NEN Life Science Product) as a substrate followed by the use of an ABC elite kit (Vector Lab.). Fluorescent images were captured using confocal microscopy (Carl Zeiss LSM410), and image processing was performed with Photoshop software (Adobe).

In situ hybridization and antibody staining were performed as described (Tautz and Pfeifle, 1989) with minor modifications. An antisense RNA probe synthesized from a *bnl* cDNA clone (Sutherland et al., 1996; a gift from T. Ohshiro) was used. After detection of *bnl* mRNA expression, embryos were stained for β-galactosidase to visualize tracheal cells.

RESULTS

Spatial relationship between Wg expression and tracheal branching

To analyze the role of Wg signaling in tracheal development, we immunostained embryos bearing the *trachealess* enhancer trap insertion with anti-Wg antibody (Perrimon et al., 1991). The *trachealess* enhancer trap line (*trh-lacZ*) shows *lacZ* expression in all tracheal cells after stage 10, thus specifically marking tracheal identity. Late in stage 10, before the invagination of tracheal primordia, Wg was expressed in stripes of ectodermal cells located on the anterior and posterior side of each tracheal primordium (Fig. 1A,B,B′). At stage 11, after tracheal cells begin to invaginate into the embryo, Wg expression was downregulated along the lateral side (Fig. 1C). During stage 12 (Fig. 1D,D′), Wg protein was detected in ectodermal cells abutting the tracheal primordium but not, however, in the tracheal cells themselves. In later stages of tracheal development, Wg protein was still detectable in stripes abutting tracheal branches (Fig. 1E, stage 14, F, stage 15), indicating that Wg protein is expressed where it reaches a subset of tracheal cells.

Multiple requirement for Wg in tracheal development

Tracheal development is grossly disorganized in *wg* null mutants (Uemura et al., 1996). Due to the general requirement for Wg in ectoderm and mesoderm development prior to the specification of tracheal primordia (Cadigan and Nusse, 1997; Martinez-Arias et al., 1999), it is difficult to determine whether the tracheal defects in *wg* null mutants reflect a direct function of Wg in the trachea or are a consequence of secondary ectodermal defects at an earlier stage. To avoid this problem, we used a temperature-sensitive allele wg^{IL114}, which fails to secrete Wg protein at the restrictive temperature (30°C) (Couso et al., 1994). We categorized the defects in the tracheal system into three phenotypic classes by performing a series of temperature-shift experiments according to the time schedule shown in Fig. 2A.

Temperature-shift in the early stages of embryonic development revealed that Wg is required for invagination of some tracheal cells. In wild-type embryos, the tracheal placode invaginates into the inside of the embryo, followed by expression of the luminal antigen, detected by the monoclonal antibody 2A12. In wg^{IL114} embryos temperature-shifted 11 hours after egg laying (AEL) at 18°C (late stage 10), many tracheal cells were found to express 2A12 antigen while remaining in the outside cell layer (asterisk in Fig. 2C). These cells may be the descendants of ectopic tracheal cells observed in *wg* mutants (Wilk et al., 1996). Temperature-shift at 16 hours AEL (late stage 11) or earlier revealed a second class of defects: the DT of these embryos was partially lost (arrow in Fig. 2C,D) or failed to migrate and fuse (arrow in Fig. 2E), and fusion of LT was also inhibited. Migration of VB, however, was relatively normal. A third class of defects affecting migration of DB was caused by temperature-shift later than 17 hours AEL (early stage 12). DB normally migrates dorsally and fuses at the dorsal midline with DB from the other side of the segment. Also in wild-type embryos, terminal branches migrate ventrally from the fusion point to form the typical U-turn shape (Fig. 2F). In wg^{IL114} mutants, DB failed to fuse at the dorsal midline and often curved in the

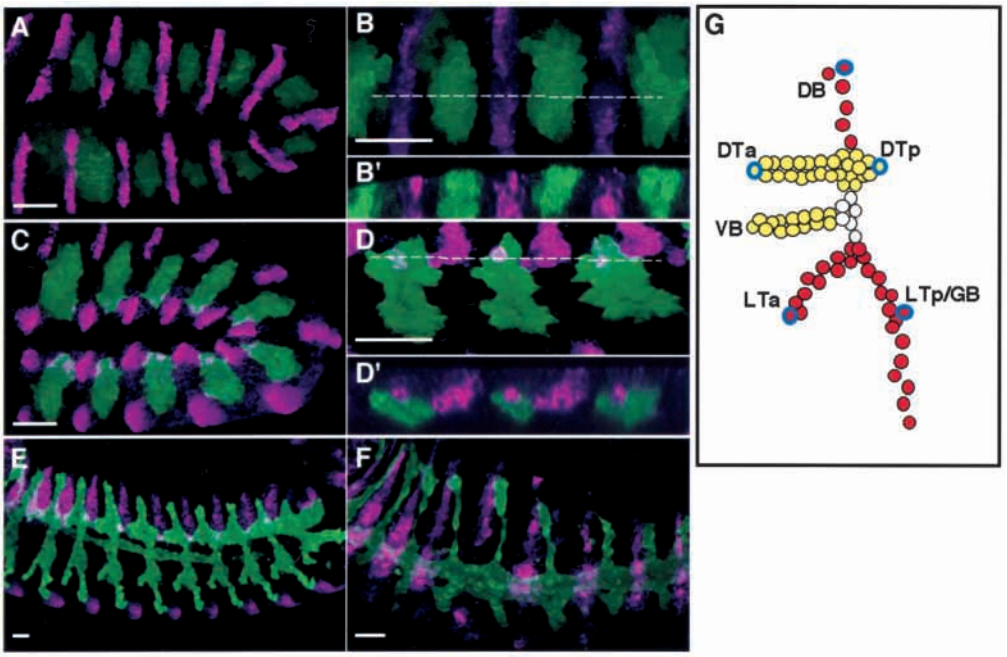

Fig. 1. The relationship between Wg expression and tracheal development. Tracheal cells were specifically labeled with *trh-lacZ* (green), and Wg with anti-Wg (purple). (A,B) Wg expression is seen in the extended germ band at stage 10 (lateral view). Wg is expressed in ectodermal cells flanking the tracheal placode in a striped pattern. (B′) The horizontal optical section through the broken line in B. Outer surface is up. (C) At stage 11, lateral expression of Wg becomes separated into dorsal and ventral stripes. (D) At stage 12, when primary branches have begun to migrate, Wg is detected in close proximity to tracheal cells. (D′) The horizontal section indicated by the broken line in D. At stages 14 (E) and 15 (F), the Wg protein is still detected in a striped pattern. (G) Schematic drawing of the tracheal branches. DB, dorsal branch; DTa, dorsal trunk anterior; DTp, dorsal trunk posterior; VB, visceral branch; LTa, lateral trunk anterior; LTp, lateral trunk posterior; GB, ganglionic branch. Each circle indicates the nucleus of a tracheal cell. EGFR-dependent branches (DT and VB) are yellow; Dpp-dependent branches (DB, LT and GB) are red. Blue circles indicate the positions of fusion cells. Scale bars: 30 μm.

anteroposterior direction to make contact with the tip of adjacent DB segments (arrowheads in Fig. 2G,H). These migration defects were often associated with loss or gain of terminal branches (arrowheads in Fig. 2I). These various defects in wg^{IL114} temperature-sensitive mutant embryos indicate that Wg is required at multiple steps of tracheal development, including (1) proper specification of tracheal primordia, (2) DT formation and (3) terminal morphogenesis at the tip of the developing tracheal branches. We investigated the latter two functions further.

Requirement for zygotic armadillo activity in determination of cell and branch identity

We next examined the tracheal phenotype of embryos mutant for *arm*, a downstream effector of Wg signaling. In zygotic arm^{YD35} null mutant embryos, invagination of tracheal primordia was normal, possibly due to a maternal supply of Arm. DT, however, was lost completely and branch fusion did not occur (Fig. 3G). The tracheal phenotype of *arm* mutants is very similar to that observed in *wg* temperature-sensitive embryos cultured at the restrictive temperature after 13 hours AEL (Fig. 2D).

Branchless (Bnl), expressed in ectodermal and mesodermal tissues flanking the future branching sites of tracheal primordium, is required for branch migration (Fig. 3B; Sutherland et al., 1996). Tracheal defects of arm^{YD35} mutants may be the result of altered expression of *bnl*. Examination of *bnl* mRNA levels in arm^{YD35} mutant embryos, however, did not reveal any significant alteration in expression (Fig. 3H).

The phenotype of arm^{YD35} mutants was also monitored by the molecular markers *spalt* (*sal*) and *knirps* (*kni*), expressed in complementary regions. *sal* is expressed in DT (Fig. 3C); *sal* mutant embryos have a specific defect in migration of DT (Kuhnlein and Schuh, 1996). *kni*, expressed in DB, LT, GB and VB, may function as a mediator in Dpp signaling (Fig. 3D; Vincent et al., 1997; Chen et al., 1998). In zygotic arm^{YD35} embryos, expression of Sal was lost, leaving Kni expression unaffected (Fig. 3I,J). These results confirm that the defect in primary branching of arm^{YD35} embryos is restricted to the formation of DT.

Because *wg* and *arm* mutants fail to fuse their branches and show abnormal terminal branching, we examined expression of Escargot (Esg), a marker for fusion cells (Fig. 3E; Tanaka-Matakatsu et al., 1996; Samakovlis et al., 1996), and serum response factor (SRF), a marker of terminal cells (Fig. 3F; Affolter et al., 1994). In arm^{YD35} mutant embryos, Esg was not expressed in tracheal cells (Fig. 3K). In contrast, we observed an increase in the number of SRF-expressing cells in DB (Fig. 3L). These results indicate that Arm is required for the expression of Esg in all tracheal cells, and for adjusting the number of terminal cells to one.

The arm^{YD35} mutant is functionally defective in both Wg signaling and the formation of adherens junctions (Peifer, 1995). Loss of *shotgun*, encoding the epithelial cell-cell adhesion molecule, *D*E-cadherin, caused a collapse of adherens junctions and severe defects in the tracheal system (Uemura et al., 1996). We speculated that the phenotype of zygotic arm^{YD35} mutants was due to defective cell-cell adhesion. To exclude this possibility, we analyzed $arm^{H8.6}$ hypomorphic mutant embryos. The $arm^{H8.6}$ mutation specifically blocks Wg signaling without affecting cell adhesion (Peifer et al., 1993). $arm^{H8.6}$ embryos demonstrate a phenotype similar to arm^{YD35} embryos in both tracheal branching and gene expression (data not shown), suggesting that the tracheal phenotype of arm^{YD35} is due to the loss of Wg signaling, not to problems in cell adhesion.

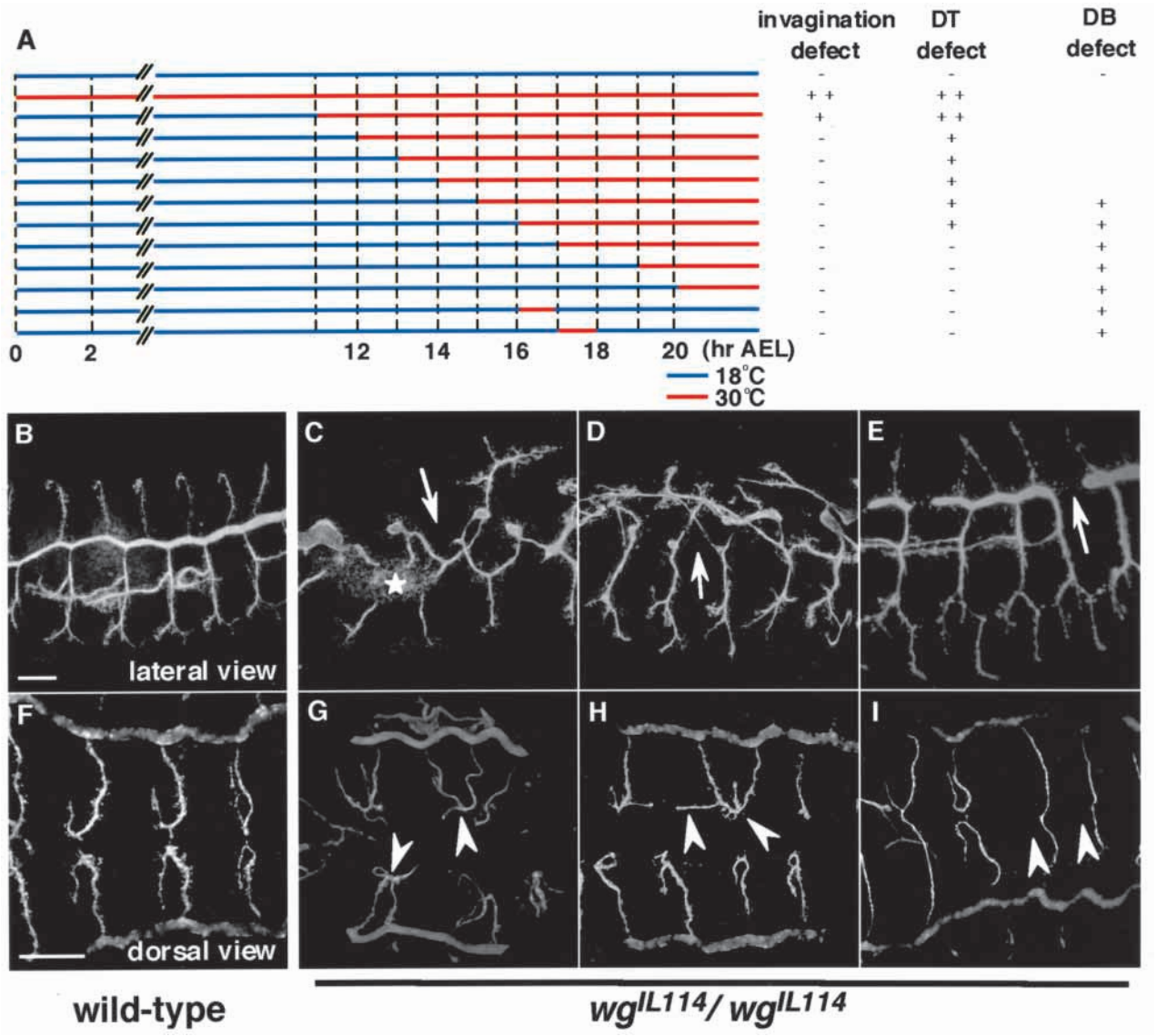

Fig. 2. Tracheal defects in *wg*^{IL114} temperature-sensitive mutants. (A) Schedule of the temperature-shift experiment. (B-E) Lateral and (F-I) dorsal views of stage-16 embryos were stained with mAb 2A12 to reveal the tracheal lumen. (B,F) Wild-type embryo. (C-E, G-I) *wg*^{IL114} homozygote embryos. Embryos of *wg*^{IL114} homozygotes were subjected to temperature-shift at 0 (C), 13 (D), 16 (E) and 20 (G,H) hours after egg laying at 18°C (AEL) and showed several tracheal defects, including failure of some tracheal cells to invaginate (asterisk, C), disruption of DT (arrows, C-E) and failure of DB morphogenesis (arrowheads, G-I). Scale bars: 30 μm.

Wingless signaling acts in tracheal cells

We modified Wg signaling within tracheal cells using *btl-Gal4* (Shiga et al., 1996) to drive expression of various regulators of Wg signaling in order to test whether Wg signaling is required in tracheal cells. When either *D*axin (Fig. 4D; Hamada et al., 1999) or a dominant negative *D*TCF (data not shown; van de Wetering et al., 1997) were expressed, DT formation was inhibited in a manner similar to *arm* mutants. In both embryos, expression of *bnl* mRNA was not altered (Fig. 4G and data not shown). These results indicate that Wg signaling acts in tracheal cells to promote DT formation. When Wg signaling was hyperactivated by expression of Wg (data not shown) or Arm^{S10} (the activated form of Arm, see Fig. 4H; Pai et al., 1997), however, VB was lost. In both cases, an increase in the number of Sal expressing cells was observed in the DT (Fig. 4K).

In *arm* mutant embryos, expression of Esg and SRF at the tip of the DB was altered (Fig. 3K,L). To analyze the role of Wg signaling in cell-fate specification, we examined the expression of Esg and SRF in tracheal cells of embryos with reduced or hyperactivated Wg signaling. As shown in Fig. 4E, Esg expression disappeared upon reduction of Wg signaling resulting from *D*axin overexpression. Expression of Arm^{S10}, however, increased the number of Esg-positive cells, especially in the DT (Fig. 4I). These results suggest that activation of Wg signaling in tracheal cells is required for expression of Esg. These results are consistent with the phenotype of *arm* mutants, suggesting that Wg signaling acts in the trachea both to induce DT fate and to specify fusion cells. Furthermore, the number of SRF expressing cells increased twofold in both types of embryos (Fig. 4F,J). How Wg regulates SRF

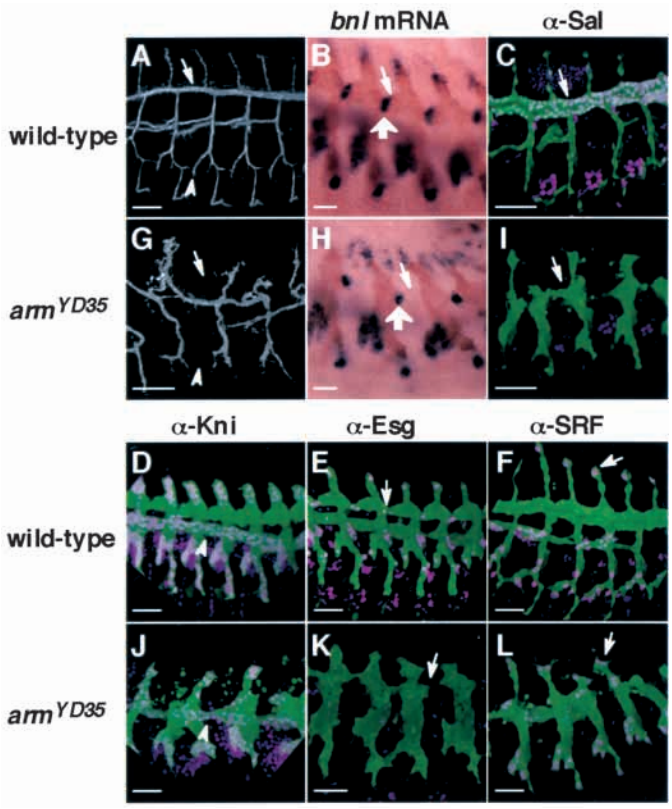

Fig. 3. Tracheal defects in zygotic *arm* mutants. Wild-type (A-F) or zygotic *arm*^{YD35} mutant (G-L) embryos. The tracheal lumen was detected with mAb 2A12 (A,G). Tracheal cells are stained brown (B,H) or green (C-F, I-L) with the *trh-lacZ* tracheal marker. Compared to wild type (A), in zygotic *arm*^{YD35} mutants (G), DT fails to develop (arrows) and branch fusion was not detectable (arrowheads). Lateral expression of *bnl* mRNA corresponding to DT migration was detected in both wild type and mutant (thick arrows, B,H), despite the lack of DT in zygotic *arm*^{YD35} mutants (thin arrows, B,H). In wild-type embryos, Sal is expressed in DT cells (arrow, C) and Kni is expressed in DB, LT, GB and VB (arrowhead, D). In zygotic *arm*^{YD35} mutants, the expression of Sal disappeared from tracheal cells (arrow, I); expression of Kni, however, remained in tracheal cells (arrowhead, J). Esg is expressed in fusion cells located on the tip of primary branches (arrow, E), and SRF is expressed in the terminal cells (arrow, F). In zygotic *arm*^{YD35} mutants, Esg expressing cells were not detectable in the trachea (K), whereas the number of SRF expressing cells increased (arrow, L). Scale bars: 30 μm.

expression is not clear at the moment, but it appears to be complex, involving *esg*, *headcase* and *pointed*.

To test if activation of Wg signaling is sufficient to promote autonomous development of DT and fusion cells, we expressed the activated form of Arm under the control of *btl-Gal4* on an *arm* mutant background. This treatment completely rescued DT formation and restored Sal expression (Fig. 4L,N) and Esg (Fig. 4M). These results strongly suggest that Bnl expression in *arm*^{YD35} mutants is sufficient to promote branch migration, if Wg signaling is active in the trachea.

Wingless signaling stimulates Delta expression

We observed that the tracheal phenotype of mutants defective in Wg signaling was reminiscent of mutants in Notch signaling, a pathway required for cell-fate specification at the tips of tracheal branches (Llimargas, 1999; Ikeya and Hayashi, 1999; Steneberg et al., 1999). Embryos lacking Notch signaling were similar to hyperactivated Wg signaling mutants; both demonstrated an increase in the number of Esg expressing cells and SRF expressing cells (Fig. 4I,J). Hyperactivation of Notch signaling reduced the number of Esg expressing cells and increased the number of SRF expressing cells, a phenotype similar to that observed in zygotic *arm* mutants (Fig. 3K,L). Similarly, disruption of DT formation was observed both upon hyperactivation of Notch (Ikeya and Hayashi, 1999) and in mutants with reduced Wg signaling. This reciprocal relationship of Wg and Notch signaling suggests that Wg signaling may inhibit Notch activation in tracheal cells.

To explore this possibility, we examined Delta expression in trachea of embryos overexpressing Wg. In wild-type embryos, Delta protein accumulates at the tips of primary branches (Fig. 5A). Upon hyperactivation of Wg signaling in the trachea, Delta expression became elevated in all tracheal cells (Fig. 5C,D). In zygotic *arm*^{YD35} embryos, however, Delta expression remained low throughout the trachea (Fig. 5E,F).

We also examined the activity of the Delta enhancer by monitoring expression of a *Delta-lacZ* enhancer trap insertion. In wild-type embryos at embryonic stage 13, *Delta-lacZ* is expressed at a low level in all tracheal cells, with high levels present in fusion cells (Fig. 5G; Steneberg et al., 1999). When an activated form of Arm was expressed in the trachea, the number of cells with strong *Delta-lacZ* expression increased (Fig. 5H). These results suggest that Wg signaling induces the transcriptional upregulation of Delta.

Epistatic analysis between Wg and Notch signaling

To further explore the interaction of Wg and Delta, we examined the epistatic relationship between these two signals. Overexpression of Delta in tracheal cells has a dominant negative effect on Notch, increasing the number of cells expressing Esg (Fig. 6A; Ikeya and Hayashi, 1999). If the inhibition of Notch resulting from Delta overexpression were the sole mechanism whereby Wg signaling induces Esg expression, expression of Delta would be expected to rescue some of the defects of *arm* mutants. When Delta was expressed in *arm*^{YD35} mutant embryos, however, there was no rescue of Esg expression or DT formation (Fig. 6C), suggesting that stimulation of Delta expression is not sufficient for the promotion of Esg expression by Wg signaling. These results suggest that although the regulation of Notch activity through Delta is one mechanism whereby Wg signaling regulates fusion cell differentiation, Wg signaling also directly induces Esg expression itself. We also confirmed that expression of Sal is dependent on Wg signaling, but not on the Notch-Delta pathway, as Sal expression was not affected by overexpression of Delta in either wild-type (Fig. 6B) or *arm*^{YD35} mutant embryos (Fig. 6D).

DISCUSSION

EGFR and Dpp are required for subdivision of the tracheal primordium into three dorsoventral domains (Dpp and EGFR domains; Figs 1G, 7A), conferring specific identities to primary branches (Vincent et al., 1997; Wappner et al., 1997).

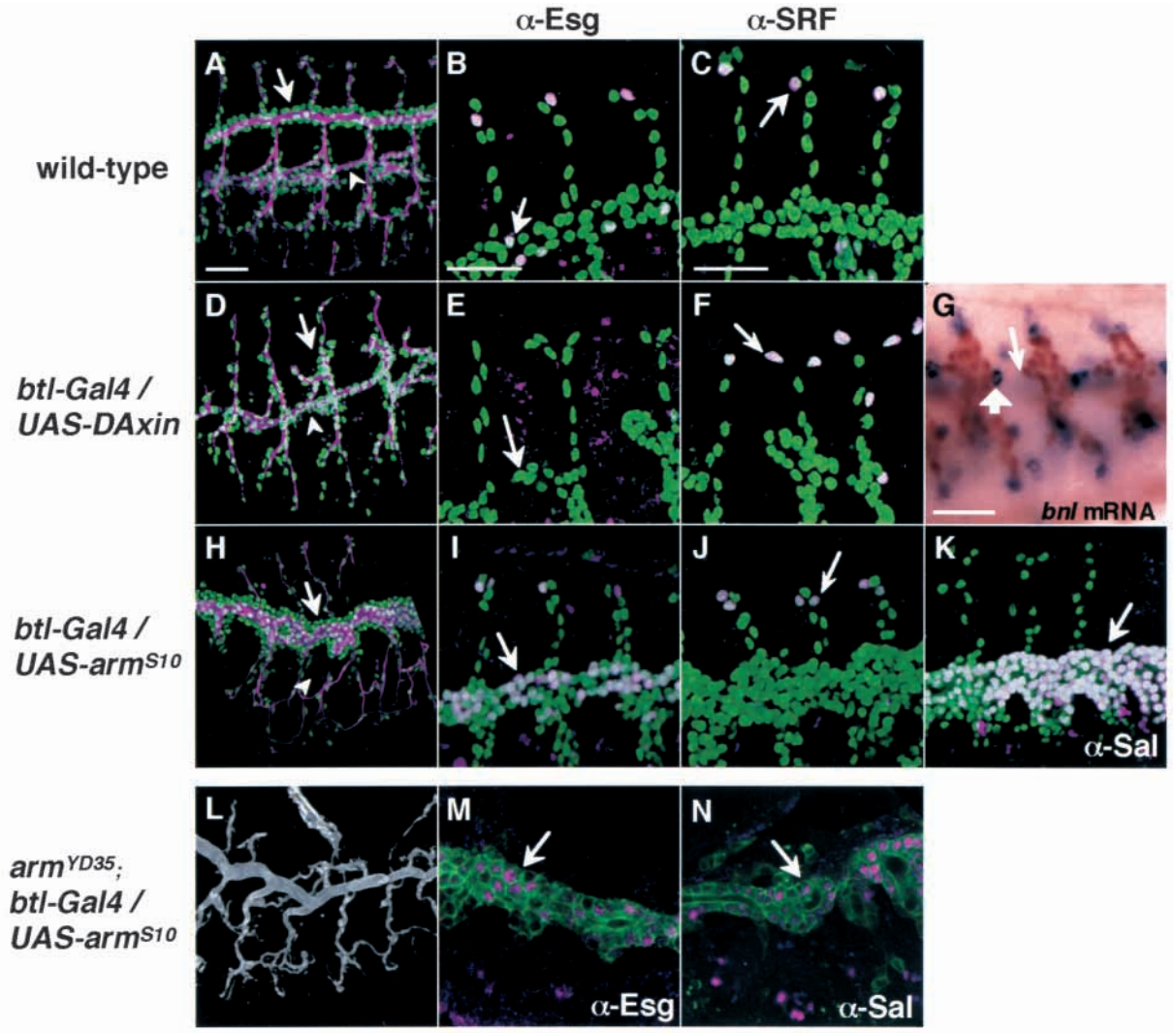

Fig. 4. Wg signaling has tracheal-autonomous roles for branching and cell differentiation. Tracheal nuclei were detected by the expression of nuclear GFP-β-galactosidase fusion protein in green (A-F, H-K) or brown (G). The tracheal lumen was stained with mAb 2A12, shown in purple (A,D,H) or gray (L). Tracheal cells were detected by the expression of membrane-bound GFP protein (*btl-Gal4 / UAS-gfpTTras*) visualized in green (M,N). In wild-type embryos, VB migrates to the inside of the embryo (arrowhead, A). Esg is expressed at the tip of migrating branches (arrow, B) and SRF is expressed in the terminal cells of DB (arrow, C). Ectopic expression of *D*Axin inhibited the formation of DT (arrow, D,G) and tracheal cell fusion at DB and LT (arrowhead, D). In these embryos, expression of Esg disappeared (arrow, E), together with an increase in the number of SRF-expressing cells (arrow, F), compared to wild type. However, expression of *bnl* (thick arrow in G) was detectable. Ectopic expression of Arm^{S10}, an activated form of Arm, inhibited the formation of VB (arrowhead, H). In these mutants, the number of Esg (arrow, I), SRF (arrow, J) and Sal (arrow, K) expressing cells increased. The effects observed upon the expression of Arm^{S10} in the developing tracheal system were similar to the effects observed following expression of the *UAS-wg* construct under the control of the *btl-GAl4* promoter (data not shown). In arm^{YD35} mutant embryos, Arm^{S10} overexpression induced complete DT formation (L) and expression of Esg (M) and Sal (N) in DT. Scale bars: 30 μm.

When Dpp signaling is hyperactivated in all tracheal cells, prospective DT or VB cells fail to migrate in the anterior-posterior direction, and instead migrate in the dorsoventral direction (Vincent et al., 1997). Although those signals account for many aspects of the initial subdivision of tracheal primordium, it was not clear how specific numbers of differentiated cells are assigned precisely to each branch and how branch identities are determined. This work demonstrates that Wg signaling plays a key role in inducing DT identity and fusion cell fate. Results from the analyses of *wg* temperature-sensitive mutants suggest that Wg itself is a major ligand activating Wg signaling in tracheal cells; however, a possibility remains that other *D*wnt ligands act on tracheal morphogenesis through activation of ARM.

Wg signaling induces the dorsal trunk cell fate

EGFR signaling at stage 10 induces the central region of the tracheal placode to give rise to DT and VB (Fig. 7A). DT then migrates beneath the ectoderm and forms the main anterior-posterior connecting tube. DT, expressing Sal, carries fusion cells at the tip, but lacks terminal cells. VB, however, migrates to the interior of the embryo, possessing multiple terminal cells

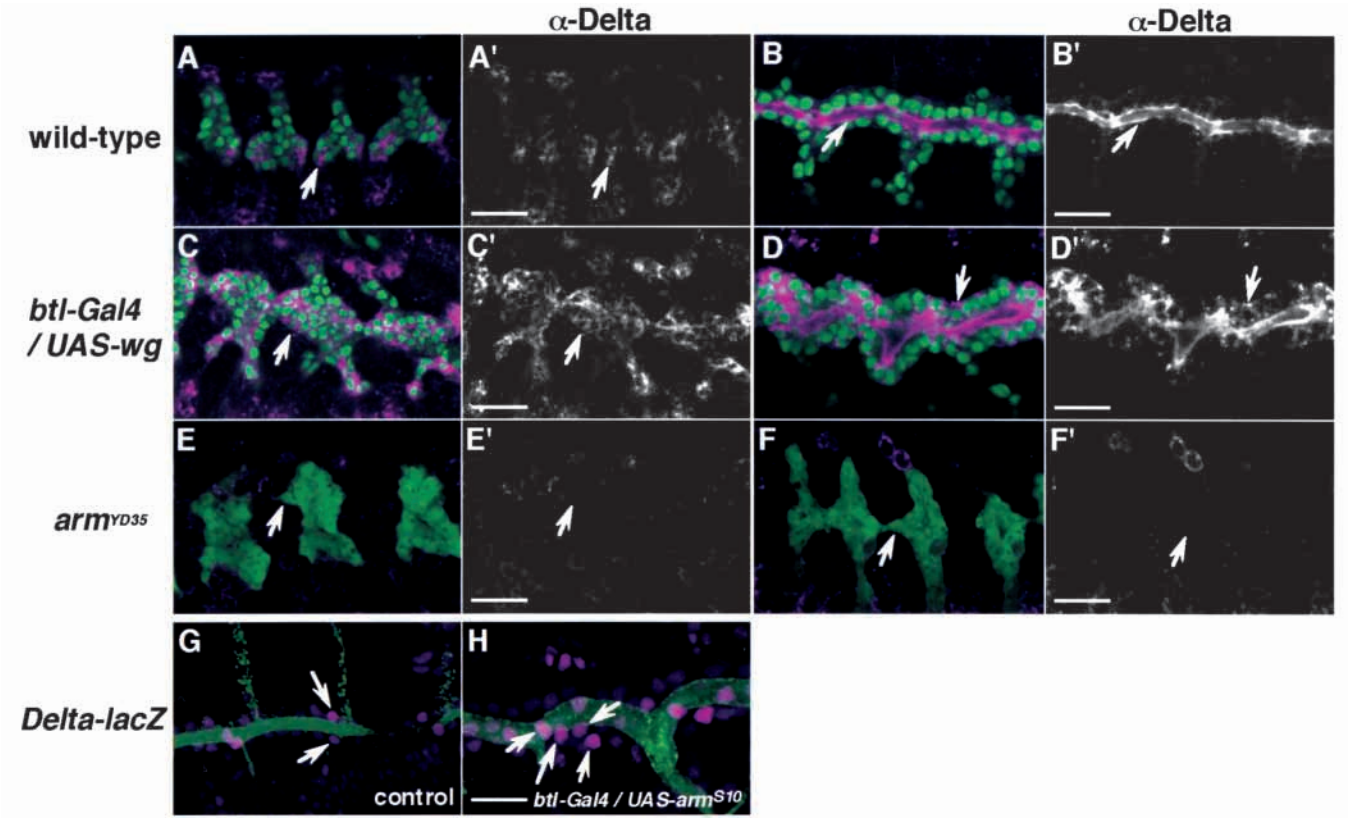

Fig. 5. Activation of Delta expression by Wg signaling. Tracheal nuclei (A-D; *gfpn-lacZ*), tracheal cells (E,F; *trh-lacZ*) and tracheal lumen (G,H; 2A12) were stained in green. Dl expression is shown in purple (A-F) or white (A′-F′). In wild-type embryos at stage 13, Delta protein localizes to the tips of the primary branches (arrows in A,A′). At stage 16, Delta specifically accumulates on the apical side of DT (arrows in B,B′). Ectopic expression of Wg causes high expression of Delta in all the tracheal cells (arrows, C,D). In zygotic arm^{YD35} mutant embryos, expression of Delta is not detected (arrows in E-F). Wild-type embryos showed strong expression of *Delta-lacZ* (purple) in fusion cells (arrows, G). When Wg was ectopically expressed in the tracheal cells, the *Delta-lacZ* expression became stronger in additional cells (arrows, H). Scale bars: 30 μm.

instead of fusion cells. We demonstrate that DT migrates in close proximity to the source of Wg and that Wg signaling is necessary for DT formation. Hyperactivation of Wg signaling in all tracheal cells forces prospective VB cells to express Sal and Esg and to participate in DT, without affecting other branches under the influence of Dpp. From these results, we propose that tracheal cells in the EGFR-induced cells close to a Wg source are instructed to adopt the DT fate. EGFR-induced cells distal to Wg take the fate of VB as a default. As ubiquitous activation of Wg signaling in tracheal cells during the migration phase has no effect on the direction of migration, we conclude that Wg signaling does not play a chemoattractive role during primary branching.

Wg signaling regulates fusion cell fate

Fusion cells are located at the tips of primary branches, and express a transcriptional factor, Esg, after stage 13. The mechanism of Esg induction is not known, although Dpp signaling has been implicated (Steneberg et al., 1999). Ectopic activation of Dpp in all tracheal cells can induce the expression of Esg in all branches (Steneberg et al., 1999; T. C. and S. H., unpublished data). In thick vein (Receptor for Dpp) null embryos, Esg expressing cells disappear from DB and LT but remain in DT (T. C. and S. H., unpublished data). These results suggest that Dpp signaling is required for the expression of Esg in DB and LT but not DT. We demonstrate here that Wg signaling is required for the expression of Esg in all fusion cells. We thus propose that Wg signaling is the primary inducer of fusion cell fate and that Dpp signaling provides an additional stimulus that is required to maintain fusion cell fate in a subset of branches.

Wg signaling regulates Notch signaling through Delta

During primary branching, Delta protein accumulates at the tips of primary branches, restricting the differentiation of excess fusion cells by stimulating Notch signaling (Ikeya and Hayashi, 1999; Llimargas, 1999). We demonstrate that Wg signaling is required for localized Delta expression. Ectopic expression of an activated form of Arm in all tracheal cells can activate Delta as well as *Delta-lacZ* expression (Fig. 5), suggesting that Wg signaling stimulates Delta expression at the transcriptional level. A similar conclusion was drawn from studies of Wg function in dorsoventral patterning of Wing imaginal discs (de Celis and Bray, 1997; Micchelli, et al., 1997). Another mechanism whereby Wg signaling interacts with Notch has been proposed. Dishevelled (Dsh), which is a transducer of Wg signaling acting upstream of Arm, inhibits

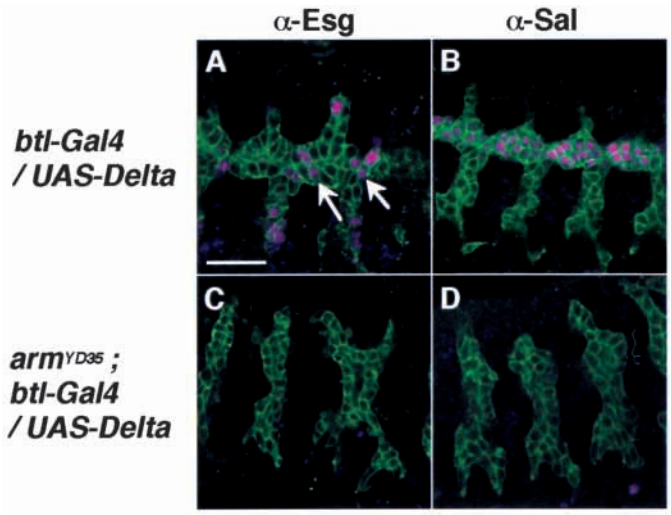

Fig. 6. The epistatic relationship between Wg and Notch signaling. Tracheal cells were detected by expression of membrane-bound GFP protein (*btl-Gal4 / UAS-gfpTTras*) visualized in green. The number of Esg expressing cells (purple) sporadically increased in Delta-overexpressing trachea (A), but no alteration of Sal expression was observed (B). In arm^{YD35} mutant embryos, the expression of Esg (C) and Sal (D) in Delta-overexpressing trachea was indistinguishable from that of arm^{YD35} mutant embryos. Scale bar: 30 μm.

Notch activity in *Drosophila* wing discs and interacts with the intracellular domain of Notch in yeast cells (Axelrod et al., 1996; Brennan et al., 1999). We consider that this mechanism is distinct from our proposed mechanism of Notch inhibition by Wg signaling, since activated Arm acting downstream of Dsh caused a phenotype of Notch inhibition. These mechanisms are not mutually exclusive, however, and may reflect a different aspect of complex self- and cross-regulatory interactions of the two signaling pathways.

How does the localized Delta induced by Wg signaling acts in tracheal cells? As revealed by the study on *Drosophila* wing disc development, Notch ligands have a cell-autonomous dominant-negative effect on Notch activity in addition to the well-established role of lateral inhibition of cell differentiation. Clones of cells lacking both Delta and Serrate showed a sign of Notch hyperactivation (Micchelli, et al., 1997) and clones of cells expressing high level of Delta autonomously inhibited Notch target genes (de Celis and Bray, 1997, 2000). The same relationship between Notch and Delta appears to exist in the trachea since overexpression of Delta showed phenotypes similar to loss of Notch function (Llimargas, 1999; Ikeya and Hayashi, 1999; Steneberg et al., 1999). Since Esg is expressed in cells with the highest Delta expression in primary branches and is normally inhibited by Notch signaling, it was proposed that Delta-dependent inhibition of Notch provides permissive conditions for fusion cell differentiation (Ikeya and Hayashi, 1999). Thus regulation of Delta by Wg signaling is an important mechanism of fusion cell-fate determination.

Wg and Bnl/FGF pathways converge on Delta

Delta expression in tracheal cells is also under the influence of Bnl/Btl signaling (Ikeya and Hayashi, 1999). Loss of Btl causes a reduction of Delta and overexpression of Bnl leads to excess Delta expression. These observations suggest that the localized expression of Delta in the developing trachea requires both Wg and Bnl signaling, implying that the two signals synergistically stimulate Delta expression. We propose that the two diffusible ligands Bnl and Wg, expressed in distinct special domains, separately exert an inductive influence on the tracheal primordium. *Delta* integrates the two inductive signals and elevates its expression in sharply defined regions at the tip of the primary branches, and initiates the cell-fate restriction

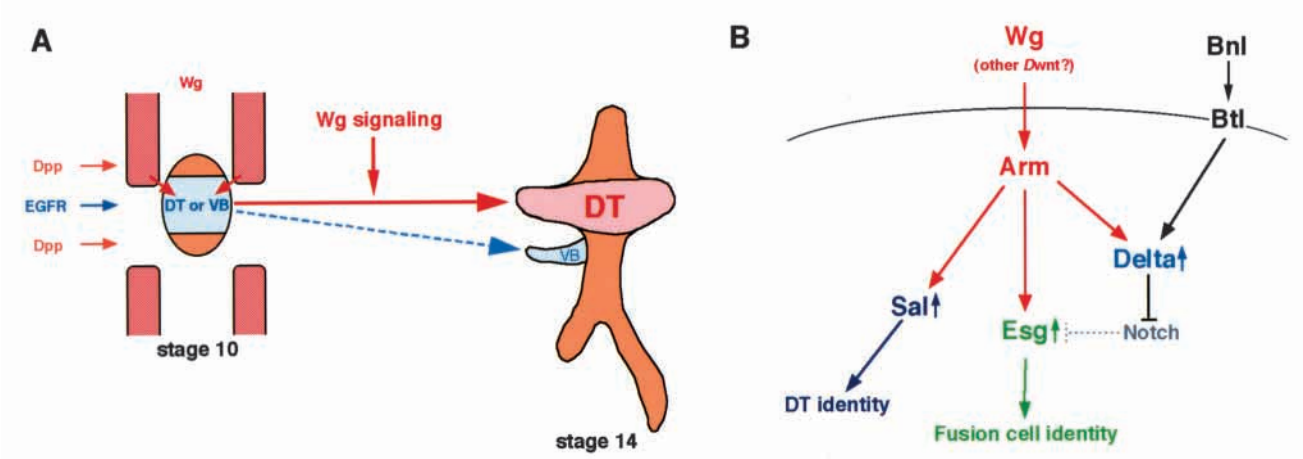

Fig. 7. A model for patterning the tracheal system by the Wg signaling. (A) At stage 10-11, EGFR signaling is activated at the central region of the tracheal placode, resulting in the production of anteroposterior migrating branches (DT and VB). On the other hand, Dpp signaling is activated at the dorsal and ventral regions of the tracheal placode, inducing the dorsoventral migrating branches (DB, LT and GB). Wg signaling stimulates EGFR-induced cells at the central region to differentiate into DT. At this time, if these EGFR-induced cells do not receive a signal from Wg, they become VB. (B) Schematic drawing of the interaction between Wg, Bnl and Notch signaling in the DT fusion cell. Wg signaling stimulates the expression of Delta, together with Esg and Sal. Elevated Delta expression in fusion cells inhibits Notch activity in a cell-autonomous manner and activates Notch in a non-cell-autonomous manner, resulting in repression of Esg in neighboring cells. The localized inhibition of Notch activity allows Esg expression in the fusion cell only. Sal expression is not influenced by Notch inhibition, resulting in a uniform pattern of Sal expression in DT.

program. This mechanism is likely to be useful for sharpening the response of cells to multiple diffusible ligands.

Roles of Wg signaling in specifying the shape of dorsal trunk

Wg signaling controls the formation of DT by regulating at least three target genes *sal*, *esg* and *Delta* in distinct ways. Sal is expressed in all DT cells and is required for directed migration along the anterior and posterior directions (Kuhnlein and Schuh, 1996). Most of the cells in the EGFR domain can respond to Wg signaling by expressing Sal (Fig. 4K), and the expression of Sal is not affected by excess Delta (Fig. 6B,D). We propose that Sal expression is regulated by Wg signaling but not by Notch signaling, and that it serves as a major mediator of Wg signaling in determining DT identity. Regulation of Esg is more complex. Although Esg expression is stimulated by Wg signaling, it is normally limited to a single cell on each branch due to repression by Notch. We have demonstrated that Wg signaling activates Esg expression independently of Delta (Fig. 6A,C). We propose that Wg signaling bifurcates after activation of Arm, activating Esg on the one hand, and Delta on the other. Elevated Delta activates Notch in nearby cells, leading to repression of Esg in the stalk of tracheal branches. These combinatorial effects limit Esg expression to the tip of fusion branches. Stimulation of both positive and negative regulation of Esg by a single inductive signal comprises a self-limiting process of cell-fate determination and accounts for the assignment of single fusion cells that mark the end of tracheal tubule. In combination with the specification of thick tubules through regulation of Sal, Wg signaling determines the shape of the tracheal tubule.

We thank Tadashi Uemura, Ben-Zion Shilo, Allan Shirras, Yash Hiromi, Reinhart Schuh, Tomokazu Ohshiro, Kenji Matsuno, Ryu Ueda, Makoto Nakamura, Yuh Nung Jan, Michael Gillman, Alfonso Martinez-Arias, the Developmental Studies Hybridoma Bank, the East Asian Distribution Center for Segmentation Antibodies and Umea and the Bloomington Stock Center for flies, DNA and/or antibodies. This work was supported by a grant to S. H. from the Japan Society for the Promotion of Sciences (Research for the Future) and from Ministry of Education, Science, Sports and Culture. T. C. is supported by the Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

REFERENCES

Affolter, M., Montagne, J., Walldorf, U., Groppe, J., Kloter, U., LaRosa, M. and Gehring, W. J. (1994). The *Drosophila* SRF homolog is expressed in a subset of tracheal cells and maps within a genomic region required for tracheal development. *Development* **120**, 743-753.

Axelrod, J. D., Matsuno, K., Artavanis-Tsakonas, S. and Perrimon, N. (1996). Interaction between Wingless and Notch signaling pathways mediated by Dishevelled. *Science* **271**, 1826-1832.

Brennan, K., Klein, T., Wilder, E. and Martinez-Arias, A. (1999). Wingless modulates the effects of dominant negative Notch molecules in the developing wing of *Drosophila*. *Dev. Biol.* **216**, 210-229.

Brook, W. J. and Cohen, S. M. (1996). Antagonistic interaction between Wingless and Decapentaplegic responsible for dorsal-ventral pattern in the *Drosophila* leg. *Science* **273**, 1373-1377.

Cadigan, K. and Nusse, R. (1997). Wnt signaling: a common theme in animal development. *Genes Dev.* **11**, 3286-3305.

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.

Couso, J. P., Bishop, S. A. and Martinez-Arias, A. (1994). The wingless signaling pathway and the patterning of the wing margin in *Drosophila*. *Development* **120**, 621-636.

de Celis, J. F. and Bray, S. (1997). Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the *Drosophila* wing. *Development* **124**, 3241-3251.

de Celis, J. F. and Bray, S. J. (2000). The Abruptex domain of Notch regulates negative interactions between Notch, its ligands and Fringe. *Development* **127**, 1291-1302.

Doherty, D., Feger, G., S., Y.-S., Jan, L. Y. and Jan, Y. N. (1996). Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in *Drosophila* wing formation. *Genes Dev.* **10**, 421-434.

Fehon, R. G., Kooh, P. J., Rebay, I., Regan, C. L., Xu, T., Muskavitch, M. A. T. and Artavanis-Tsakonas, S. (1990). Molecular interaction between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in *Drosophila*. *Cell* **61**, 523-534.

Fuse, N., Hirose, S. and Hayashi, S. (1994). Diploidy of *Drosophila* imaginal cells is maintained by a transcriptional repressor encoded by *escargot*. *Genes Dev.* **8**, 2270-2281.

Glazer, L. and Shilo, B. Z. (1991). The *Drosophila* FGF-R homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension. *Genes Dev.* **5**, 697-705.

Hamada, F., Tomoyasu, Y., Takatsu, Y., Nakamura, M., Nagai, S., Suzuki, A., Fujita, F., Shibuya, H., Toyoshima, K., Ueno, N. and Akiyama, T. (1999). Negative regulation of Wingless signaling by *D*-axin, a *Drosophila* homolog of axin. *Science* **283**, 1739-1742.

Hayashi, S., Hirose, S., Metcalfe, T. and Shirras, A. (1993). Control of imaginal cell development by the *escargot* gene of *Drosophila*. *Development* **118**, 105-115.

Ikeya, T. and Hayashi, S. (1999). Interplay of Notch and FGF signaling restricts cell fate and MAPK activation in the *Drosophila* trachea. *Development* **126**, 4455-4463.

Jacobsen, T. L., Brennan, K., Martinez-Arias, A. and Muskavitch, M. A. (1998). Cis-interactions between Delta and Notch modulate neurogenic signalling in *Drosophila*. *Development* **125**, 4531-4540.

Kosman, D., Small, S. and Reinitz, J. (1998). Rapid preparation of a panel of polyclonal antibodies to *Drosophila* segmentation proteins. *Dev. Genes Evol.* **208**, 290-294.

Kuhnlein, R. P., Frommer, G., Friedrich, M., Gonzalez, G. M., Weber, A., Wagner, B. J., Gehring, W. J., Jackle, H. and Schuh, R. (1994). *spalt* encodes an evolutionarily conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the *Drosophila* embryo. *EMBO J.* **13**, 168-179.

Kuhnlein, R. P. and Schuh, R. (1996). Dual function of the region-specific homeotic gene *spalt* during *Drosophila* tracheal system development. *Development* **122**, 2215-2223.

Lawrence, P. A., Bodmer, R. and Vincent, J. P. (1995). Segmental patterning of heart precursors in *Drosophila*. *Development* **121**, 4303-4308.

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

Llimargas, M. (1999). The Notch pathway helps to pattern the tips of the *Drosophila* tracheal branches by selecting cell fates. *Development* **126**, 2355-2364.

Manning, G. and Krasnow, M. A. (1993). Development of the *Drosophila* Tracheal System. In *The Development of* Drosophila melanogaster, Vol. 1 (ed. M. Bate and A. Martinez-Arias), pp. 2609-2686. New York: Cold Spring Harbor Laboratory Press.

Martinez-Arias, A., Brown, A. M. and Brennan, K. (1999). Wnt signalling: pathway or network? *Curr. Opin. Genet. Dev.* **9**, 447-454.

Micchelli, C. A., Rulifson, E. J. and Blair, S. (1997). The function and regulation of *cut* expression on the wing margin of *Drosophila*: Notch, Wingless and a dominant negative role for Delta and Serrate. *Development* **124**, 1485-1495.

Noordermeer, J., Klingensmith, J., Perrimon, N. and Nusse, R. (1994). *dishevelled* and *armadillo* act in the wingless signalling pathway in *Drosophila*. *Nature* **367**, 80-83.

Pai, L.-M., Orsulic, S., Bejsovec, A. and Peifer, M. (1997). Negative regulation of Armadillo, a Wingless effector in *Drosophila*. *Development* **124**, 2255-2266.

Peifer, M., Orsulic, S., Pai, L.-M. and Loureiro, J. (1993). A model system

for cell adhesion and signal transduction in *Drosophila*. *Development* **Supplement**, 163-176.

Peifer, M., Sweeton, D., Casey, M. and Wieschaus, E. (1994). wingless signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo. *Development* **120**, 369-380.

Peifer, M. (1995). Cell adhesion and signal transduction: The Armadillo connection. *Trends Cell Biol.* **5**, 224-229.

Perrimon, N., Noll, E., McCall, K. and Brand, A. (1991). Generating lineage-specific markers to study *Drosophila* development. *Dev. Genet.* **12**, 238-252.

Samakovlis, C., Manning, G., Steneberg, P., Hacohen, N., Cantera, R. and Krasnow, M. A. (1996). Genetic control of epithelial tube fusion during *Drosophila* tracheal development. *Development* **122**, 3531-3536.

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

Siegfried, E., Wilder, E. and Perrimon, N. (1994). Components of *wingless* signalling in *Drosophila*. *Nature* **367**, 76-80.

Steneberg, P., Hemphala, J. and Samakovlis, C. (1999). Dpp and Notch specify the fusion cell fate in the dorsal branches of the *Drosophila* trachea. *Mech. Dev.* **87**, 153-163.

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.

Tanaka-Matakatsu, M., Uemura, T., Oda, H., Takeichi, M. and Hayashi, S. (1996). Cadherin-mediated cell adhesion and cell motility in *Drosophila* trachea regulated by the transcription factor Escargot. *Development* **122**, 3697-3705.

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

Uemura, T., Oda, H., Kraut, R., Hayashi, S., Kotaoka, Y. and Takeichi, M. (1996). Zygotic *Drosophila* E-cadherin expression is required for processes of dynamic epithelial cell rearrangement in the *Drosophila* embryo. *Genes Dev.* **10**, 659-671.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M. and Clevers, H. (1997). Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene *dTCF*. *Cell* **88**, 789-799.

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. (2000). Cell fate choices in *Drosophila* tracheal morphogenesis. *BioEssays* **22**, 219-226.