Role of Wingless signaling during *Drosophila* tracheal development

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ABSTRACT

Cell differentiation and cell migration are key processes in the development of multicellular organisms. In the nervous system, for instance, the pattern of cell differentiation and cell migration is tightly regulated in order to form an elaborate neural network. For studies of regulatory mechanisms of cell differentiation and cell migration, the *Drosophila* tracheal system has become an appropriate model system. 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 due to an induction by the FGF homolog Branchless (Bnl), differentiates into cells with specialized function such as fusion cells that perform target recognition and adhesion during branch fusion, and extends branches toward specific targets. Specification of an unique identity for each primary branch is essential for directed migration, as a defect in either the EGF receptor (EGFR) or Decapentaplegic (Dpp) signalings leads to a loss of branch identity and the misguidance of tracheal cell migration.

To elaborate the stereotyped pattern of tracheal network, cell differentiation and cell migration must be tightly regulated during embryogenesis. Following cell differentiation of primary branches, direction of tracheal cell migration must be also regulated so that tracheal branches reach specific targets precisely. However, little is known about what kind of signal in addition to EGFR and Dpp induces differentiation of primary branches and how migration of tracheal cells is guided.

Wingless (Wg) is known as a secreted signaling protein essential for segmentation events. The *Drosophila* β-catenin homolog, Armadillo (Arm), is a downstream component of Wg signaling. Previously it was reported that *wg* and *arm* mutant embryos exhibit the severe tracheal defects with loss of specific branch (dorsal trunk) and all tracheal fusion events. Although these phenotypes indicate that Wg signaling is essential for specification of cell and branch identity in the tracheal system, little is known about how Wg signaling acts in the tracheal cells.

To investigate the role of Wg signaling in the specification of cell and branch identity in the tracheal system, I first observed expression pattern of Wg during tracheal development, and found that Wg is expressed in stripes of ectodermal cells located on the anterior and posterior side of each tracheal primordium. The tracheal phenotypes in *wg* temperature-sensitive mutants and zygotic *arm* mutants were categorized into the following three phenotypes. First one is the invagination defect. In wild-type embryos, tracheal placodes invaginate into the inside of the embryo. But in *wg* temperature-sensitive mutants invagination failed, and descendants of tracheal placode were retained in the ectodermal layer. Second, dorsal trunk (DT) was lost. DT

normally expresses Spalt (Sal) which is essential for the DT identity, but in *arm* zygotic mutants Sal expression was lost. Finally, tracheal fusion was defective and Escargot (Esg) expression was lost at all fusion points. Esg is a zinc-finger type DNA binding protein, and its expression is required for fusion cell identity. The latter two phenotypes were further investigated, and I demonstrate that Wg signaling is required within tracheal cells for expression of Sal and Esg in tracheal cells. From these results, I show that Wg and its intracellular signal transducer, Arm, have multiple functions, one specifying dorsal trunk through activation of Sal expression and the other inducing differentiation of fusion cells in all fusion branches.

Moreover, I demonstrate that Wg signaling regulates not only Esg expression itself, but also singling-out of Esg expressing cell at the tip of migrating branches by regulating Notch activity. Notch is a transmembrane receptor stimulated by a ligand Delta. A single fusion cell expressing Esg always locates at the tip of each migrating branch. High level of Delta expression by Bnl signaling is limited to fusion cell at the tip of tracheal branches. The Delta expression in fusion cell activates Notch signaling in nearby cells and that activated Notch signaling represses the fate of fusion cell. As a result, fusion cell is singled out at the tip of tracheal branches by Notch-dependent lateral inhibition. I here show that expression of Delta is also up-regulated by Wg signaling at the transcriptional level, and the high accumulation of Delta permits Esg expression only in fusion cell. Because Notch activity does not affect Sal expression, expression of Sal is permitted in all DT cells.

From these results, I propose that Wg signaling controls the formation of DT by regulating three genes *sal*, *esg*, and *Delta* in distinct ways. Wg signaling activates Sal expression in tracheal cells, and the expression of Sal is required to render DT an identity to become thick tubule and to migrate in anteroposterior direction. Wg signaling also activates Esg in all DT cells. In addition, Wg and Bnl signals are combined to activate Delta expression at the tip of DT. Elevated Delta activates Notch in nearby cells, leading to repression of Esg in the stalk of tracheal branches. Stimulation of both positive and negative regulation of Esg by Wg signaling comprises a self-limiting 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.

In addition to the studies described above, I investigated the guidance mechanism of tracheal cell migration. I focused on well-studied dorsal branch (DB), which migrates dorsally and fuses at the dorsal midline with DB from the other side of the same segment during embryogenesis. Previous studies reported that several signaling molecules (Bnl, Dpp and Notch) affect DB formation by inducing cell differentiation, but little is known about the guidance mechanism of

DB migration. I found that DB fusion points are precisely located posterior to Wg stripes, suggesting that the direction of DB migration must be also tightly regulated.

To obtain insight into the guidance mechanism of DB migration, I first observed migration pattern of fusion and terminal cells, a pair of cells that occupy the tip of DB. Fusion cells were identified by expression of Esg and were initially located anterior to terminal cells labeled with SRF expression. After the initial expression of these genes, their relative positions switched. In this process, Esg expressing cells continue to contact Engrailed expressing ectodermal cells. I also analyzed the distribution of actin cytoskeleton in migrating DB, and found that fusion and terminal cells have filopodia and lamellipodia similar to those found in neuronal growth cone.

Based on these observations, I next investigated which signaling guides DB migration. I show that planar cell polarity (PCP) signaling has a role in the guidance of DB migration. It is known that PCP signaling regulates small GTPases Rho/Rac1 activity, and controls cytoskeletal rearrangements within the plane orthogonal to their apical-basal axis. I also show that a component of PCP signaling, Drac1 has an essential role on the guidance of DB migration, but not motility of tracheal cells. As it has been reported that axon guidance is regulated by Drac1 activity, these results imply that there is a common guidance mechanism in tracheal cell migration and axonal outgrowth.

In summary, I demonstrate that Wg signaling controls the shape of tracheal tubule in concert with Notch signaling. In addition, I show the guidance mechanism of tracheal cell migration is similar to that of axon guidance, and demonstrate that PCP signaling has effects on guidance of tracheal cell migration. From these results, I propose that Wg acts on both cell differentiation and the guidance of cell migration via distinct signaling pathway to elaborate *Drosophila* tracheal network.

CONTENTS

INTRODUCTION

Morphogenesis of branched tubular network is an essential element of the development of several organ systems, including lung and vascular systems. Formation of branched tubular structures involves determination of multiple cell fates, cell migration, and extensive cell shape changes. The mechanisms by which these diverse biological processes are coordinated to establish a complicated network are poorly understood. In *Drosophila*, a combined cellular and genetic approach is possible and has begun to elucidate the cellular mechanisms and molecules that govern formation of several organs. The *Drosophila* tracheal system is an attractive model organ to study the mechanism of branching morphogenesis.

The branching pattern of the *Drosophila* tracheal network is established during embryogenesis (Fig. 1A-D). Tracheal development initiates as ten tracheal placodes on each side of the embryo. Decapentaplegic (Dpp) and EGF receptor (EGFR) signalings subdivide these tracheal placodes into two cell fates along the dorsoventral body axis (Fig. 2, left; Vincent et al., 1997; Wappner et al., 1997; Llimargas and Casanova, 1997; Zelzer and Shilo, 2000). Mutations of EGFR signaling primarily affect anteroposterior migration whereas mutations of Dpp signaling selectively affect dorsal and ventral migrations of tracheal cells. Following this dorsoventral specification, invagination of tracheal placode leads to the formation of a sac-like primordium, which is followed by localized activation of Breathless (Btl) receptor tyrosine kinase by the FGF homolog Branchless (Bnl) to trigger stereotyped branching and directed migration (Fig. 1E, F; Glazer and Shilo, 1991; Sutherland et al., 1996). Tracheal development also 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. Throughout these processes, integrity of the tracheal epithelia is maintained, allowing internal organs to have an access to air without exposure to the outside (reviewed by Manning and Krasnow, 1993).

Members of Wnt/Wingless (Wg) family of secreted glycoproteins direct cell fates in both insects and vertebrates (reviewed by Cadigan and Nusse, 1997; Martinez-Arias et al., 1999). Genetic studies revealed many of the genes required for Wg signaling in *Drosophila* (Fig. 3). Mutations in several of these, including armadillo (Arm), were first identified in a screen for genes whose zygotic expression is required for embryonic viability and proper patterning of the larval cuticles (Nusslein-Volhard and Wieschaus, 1980). Previously it was reported that the severe tracheal defects with loss of specific branch (dorsal trunk) and all tracheal fusion events in

wg and *arm* mutant embryos (Uemura et al., 1996). Although these phenotypes indicate that Wg signaling is essential for specification of cell and branch identity in the tracheal system, little is known about how Wg signaling acts in the tracheal cells.

To elaborate the stereotyped pattern of tracheal network, cell differentiation and cell migration must be tightly regulated during embryogenesis. Following cell differentiation of primary branches, direction of tracheal cell migration must be also regulated so that tracheal branches reach specific targets precisely. However, little is known how the further differentiations of primary branching are induced in addition to EGFR and DPP signalings and how migration of tracheal cells is guided.

In this thesis, I describe two different approaches to get more insights into the mechanism of tracheal cell fate determination. In part 1, I investigated the role of Wg signaling during tracheal development. In part 2, I analyzed how tracheal cell migrate to specific targets.

PART 1

Control of tracheal cell fate determination by Wingless signaling

INTRODUCTION

Epithelial tubulelar networks seen in various organs (lung, kidney, blood vessel etc.) are very important structures to fulfill the function of organs. Tubule formation during embryogenesis is genetically regulated at the levels of both cell differentiation and tubular branching. But the molecular basis for those processes are poorly understood. The *Drosophila* tracheal system is a tubular network of ectodermal epithelia supporting gas exchange, and is known as a good model system to investigate the mechanism of tubule formation (reviewed by Manning and Krasnow, 1993).

The *Drosophila* tracheal system arises from the tracheal placodes, clusters of ectodermal cells that invaginate and migrate in different and stereotyped directions to originate each of the primary tracheal branches (Fig. 1A-D). Each primary branch has an unique identity indicated by migration toward specific locations and branch-specific gene expression. Commitment of tracheal branches to specific fates is triggered by Dpp and EGFR signaling (Fig. 2, left; 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 dorsal branch (DB), lateral trunk (LT) and 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 anteroposterior 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) (blue circle in Fig. 2, right; 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 (green circles in Fig. 2, right; Tanaka-Matakatsu et al., 1996; Samakovlis et al., 1996). Notch-Delta signaling plays an important role in limiting the number of fusion cells to one in each 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.

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 (Fig. 3; 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. Studying the function of Wg signaling in tracheal development, I found that Wg signaling has two distinct roles in inducing DT fate and fusion cell fate. I 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.

RESULTS

Spatial relationship between Wg expression and tracheal branching

To analyze the role of Wg signaling in tracheal development, I performed anti-Wg antibody staining of embryos bearing the *trachealess* enhancer trap insertion (Perrimon et al., 1991). The *trachealess* enhancer trap line (*trh-lacZ*) shows *lacZ* expression in all tracheal cells after stage 10, 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. 4A, B, B'). At stage 11, after tracheal cells begin to invaginate into the embryo, Wg expression was down-regulated along the lateral side (Fig. 4C).

During stage 12 (Fig. 4D and D'), Wg protein was detected in ectodermal cells abutting the tracheal primordium, not, however, in the tracheal cells themselves. In later stages of tracheal development, Wg protein was still detectable in stripes abutting tracheal branches (Fig. 4E; stage14, 4F; stage15), 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 distinguish whether the tracheal defects in *wg* null mutants reflect the direct function of Wg in the trachea or secondary ectodermal defects in a prior stage. To avoid this problem, I used a temperature-sensitive allele wg^{IL114}, which fails to secrete Wg protein at the restrictive temperature (Couso et al., 1994). I categorized the defects in the tracheal system into three phenotypic classes by performing a series of temperature-shift experiments in accordance with the time-schedule shown in Fig. 5A.

Temperature-shift in early stages revealed a requirement of Wg 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℃ (late stage 10), many tracheal cells were found to express 2A12 antigen while remaining in the outside cell layer (asterisk in Fig. 5C). 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 the second class of defects; the DT of these embryos was lost partially (arrow in Fig. 5C, D) or failed to migrate and fuse (arrow in Fig. 5E). In addition, fusion of LT was also inhibited. The migration of VB, however, was relatively normal. The third class of the defects affecting migration of DB was caused by temperature-shifts 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-shape (Fig. 5F). In wg^{IL114} mutants, DB failed to fuse at the dorsal midline and often curved in the anteroposterior direction to make contact with the tip of adjacent DB segments (arrowheads in Fig. 5G, H). These migration defects were often associated with loss or gain of terminal branches (arrowheads in Fig. 5I). These various defects in wg^{IL114} temperature-sensitive mutant embryos indicate that Wg is required in 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. I investigated the latter two functions further.

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

I 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. 6G). The tracheal phenotype of *arm* mutants was very similar to that observed in *wg* temperature-sensitive embryos cultured at restrictive temperature after 13 hours AEL (Fig. 5D).

Branchless (Bnl), expressed in ectodermal and mesodermal tissues flanking the future branching sites of tracheal primordium, is required for branch migration (Fig. 6B; 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 a significant alteration in expression (Fig. 6H).

The phenotype of arm^{YD35} mutants was also monitored by the molecular markers, *spalt* (*sal*) and *knirps* (*kni*), expressed in a complementary regions. *sal* is expressed in DT (Fig. 6C); *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. 6D; Vincent et al., 1997; Chen et al., 1998). In zygotic arm^{YD35} embryos, expression of Sal was lost, leaving Kni expression unaffected (Fig. 6I, 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, I examined expression of Escargot (Esg), a marker for fusion cells (Fig. 6E; Tanaka-Matakatsu et al., 1996; Samakovlis et al., 1996), and serum response factor (SRF), a marker of terminal cells (Fig. 6F; Affolter et al., 1994). In arm^{YD35} mutant embryos, Esg was not expressed in tracheal cells (Fig. 6K). In contrast, I observed an increase in the number of SRF-expressing cells in DB (Fig. 6L). 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 defective in the function of both Wg signaling and the formation of adherence junctions (Peifer, 1995). A loss of *shotgun,* the epithelial cell-cell adhesion molecule, *D*E-Cadherin, caused a collapse of adherence junctions and severe defects in the

tracheal system (Uemura et al., 1996). I speculated that the phenotype of zygotic arm^{YD35} mutants may be 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 problems in cell adhesion.

Wingless signaling acts in tracheal cells

I modified Wg signaling within tracheal cells using *btl-Gal4* (Shiga et al., 1996) to drive expression of various regulators of Wg signaling to test whether Wg signaling is required in tracheal cells. When either *D*Axin (Fig. 7D; Hamada et al., 1999) or a dominant negative form of *D*Tcf (data not shown; van de Wetering et al., 1997) was expressed, DT formation was inhibited in a manner similar to *arm* mutants. In both embryos, expression of *bnl* mRNA was not altered (Fig. 7G 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} (= activated form of Arm, Fig. 7H; 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. 7K).

In *arm* mutant embryos, expression of Esg and SRF at the tip of the DB was altered (Fig. 6K, L). To analyze the role of Wg signaling in cell fate specification, I examined the expression of Esg and SRF in tracheal cells of embryos with reduced or hyperactivated Wg signaling. As shown in Fig. 7E, Esg expression disappeared upon reduction of Wg signaling resulting from *D*axin overexpression. Expression of Arm^{S10}, in contrast, increased the number of Esg positive cells, especially in the DT (Fig. 7I). 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 to both induce DT fate and to specify fusion cells. I also examined the expression of SRF in these mutants. The number of SRF expressing cells increased two fold in both types of embryos (Fig. 7F and J). How Wg regulates SRF expression is not clear at the moment, but it appears to involve complex regulation involving *esg, headcase* and *pointed.*

To test if activation of Wg signaling is sufficient to promote autonomous development of DT and fusion cells, I expressed the activated form of Arm under the control of *btl-Gal4* in an *arm* mutant background. This treatment completely rescued DT formation and restored Sal

expression (Fig. 7L, N) and Esg (Fig. 7M). 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

I 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 in phenotype to those with hyperactivated Wg signaling; both demonstrated an increase in the number of Esg expressing cells and SRF expressing cells (Fig. 7I, J). Hyperactivation of Notch signaling reduced the number of Esg expressing cells and increased the number of SRF expressing cells, a similar phenotype observed in zygotic *arm* mutants (Fig. 6K, 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, I examined Delta expression in the trachea of embryos overexpressing Wg. In wild type embryos, Delta protein accumulates at the tips of primary branches (Fig. 8A). Upon hyperactivation of Wg signaling in the trachea, Delta expression became elevated in all tracheal cells (Fig. 8C, D). In zygotic arm^{YD35} embryos, however, Delta expression remained low throughout the trachea (Fig. 8E, 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. 8G; 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. 8H). 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, I examined the epistatic relationship between these two signals. The overexpression of Delta in tracheal cells has a dominant negative effect on Notch, increasing the number of cells expressing Esg (Fig. 9A; Ikeya and Hayashi, 1999). If the inhibition of Notch resulting from Delta overexpression is the sole mechanism whereby Wg signaling induces Esg expression, the 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. 9C), 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 though Delta is one mechanism whereby Wg signaling regulates fusion cell differentiation, Wg signaling also directly induces Esg expression. I also unveiled that the expression of Sal was dependent on Wg signaling, but not the Notch-Delta pathway, as Sal expression was not affected by overexpression of Delta in either wild-type (Fig. 9B) or arm^{YD35} mutant embryos (Fig. 9D).

Dpp/Tkv and Bnl/Btl signaling regulate Esg expression in tracheal cells

Wg signaling is essential for expression of Esg, because all of Esg expressing cells are lost in *arm* mutant embryos (Fig. 6K). Ectopic expression of Wg or Arm^{S10} expressing trachea showed increase the number of Esg expressing cell, however this effect was limited only to DT (Fig. 7I), suggesting an additional signal is required to induce Esg expression in other branches. How are the Esg expression in other branches (DB, LTa, and LTp) regulated? Dpp is expressed in two ectodermal stripes abutting the tracheal placode, and cells adjacent to the Dpp stripes become DB, LTa, and LTp (Fig. 2), I speculated that Dpp/Tkv signaling also regulates Esg expression, and analyzed its effects on Esg expression in tracheal cells. In mutant embryos for *thick vein* (*tkv*) which is a receptor for Dpp, Esg expressions of DB, LTa and LTp, but not DT were lost (Fig. 10A). When daughters against dpp (dad) was expressed to inhibit Dpp/Tkv signaling in tracheal cells, Esg expressions disappeared from DB, LTa and LTp (Fig. 10B). On the other hand, when dpp or constitutive-active form of Tkv were expressed in tracheal cells, the number of Esg expression increased in DB, LTa and LTp (Fig. 10C and data not shown). These results suggest that Dpp/Tkv signaling is essential for Esg expression in DB, LTa and LTp, but not in DT.

Because Bnl is expressed at the position where each branch will grow out (Fig. 1E, F), I next analyzed the effects of Bnl/Btl signaling on Esg expression in tracheal cells. In *btlΔOh10* mutant embryos (*btl* null mutant), tracheal cells did not migrate at all. In those embryos, Esg expressing cells were detected in tracheal cells (Fig. 10D), but it was difficult to discriminate to which branch these Esg expressing cells belong. When Bnl was expressed in tracheal cells, a number of Esg expression were detected only in DT, but not DB, LTa and LTp (Fig. 10E). From these results, I concluded that although Bnl/Btl signaling can induce Esg expression in DT, Bnl/Btl signaling is not absolutely necessary for Esg expression.

DISCUSSION

EGFR and Dpp are required to subdivide the tracheal primordium into three dorsoventral domains (Dpp and EGFR domains; Fig. 2 and Fig. 11A), conferring specific identity to primary branches (Vincent et al., 1997; Wappner et al., 1997). When Dpp signaling is hyperactivated in all tracheal cells, prospective DT or VB cells failed to migrate in the anterior-posterior direction, instead migrating in the dorsoventral direction (Vincent et al., 1997). Although those signals account for many aspects of initial subdivision of tracheal primordium, it was not well known how specific numbers of differentiated cells are assigned precisely on each branch and how branch identities are determined. This work demonstrated 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 (Llimargas, 2000).

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. 11A). 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 instead of fusion cells. We demonstrated 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, I 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, I 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, expressing a transcriptional factor, Esg, after stage 13. The mechanism of Esg induction is not known. I demonstrate here that Wg

signaling is required for the expression of Esg in ALL fusion cells. I thus propose that Wg signaling is the primary inducer of fusion cell fate and that Dpp and/or Bnl signaling provides an additional stimulus 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 differentiation of excess fusion cells by stimulating Notch signaling (Ikeya and Hayashi 1999; Llimargas, 1999). I demonstrate that Wg signaling is required for the 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. 8H), suggesting that Wg signaling stimulates Delta expression at the transcriptional level. Similar conclusion has been drawn from the studies of Wg function in dorsoventral patterning of wing imaginal discs (de Celis and Bray, 1997; Micchelli, et al., 1997). Another mechanism that Wg signaling interacts with Notch has been proposed. Dishevelled (Dsh), which is a transducer of Wg signaling acting upstream of Arm, inhibits 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). I consider this mechanism 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 interaction 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; de Celis and Bray, 2000). The same relationship between Notch and Delta appears to exist in the trachea since overexpression of Delta showed phenotypes similar to the loss of Notch function (Llimargas, 1999; Ikeya and Hayashi, 1999; Steneberg et al., 1999). Since Esg is expressed in cells with highest Delta expression in primary branches and Esg is normally inhibited by Notch signaling, it was proposed that Delta-dependent inhibition of Notch provides a permissive condition for fusion cell differentiation (Ikeya and Hayashi, 1999). Thus the 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 influence 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. I propose that the two diffusible ligands Bnl and Wg expressed in distinct special domains separately exert inductive influence on the tracheal primordium. *Delta* integrates the two inductive signals and elevates its expression in sharply defined regions at the tip of primary branches and initiates the cell fate restriction program. This mechanism is likely to be useful in 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 manners. Sal is expressed in all DT cells and is required for the directed migration along anterior and posterior direction (Kuhnlein and Schuh, 1996). Most of cells in EGFR domain can respond to Wg signaling by expressing Sal (Fig. 7K) and the expression of Sal is not affected by excess Delta (Fig. 9B, D). I propose that Sal expression is regulated by Wg signaling but not by Notch signaling, and 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. I have demonstrated that Wg signaling activates Esg expression independently of Delta (Fig. 9A, C). I propose that Wg signaling bifurcates after activation of Arm and activates Esg on one hand, and activates 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 signaling 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.

PART 2

Guidance mechanism of tracheal cell migration

INTRODUCTION

Guidances of cell migration and axon outgrowth are important steps in the development of neuronal network. However, studies of those processes at a single cell level have been difficult because of the complexity of the neuronal network. The *Drosophila* tracheal system might serve as a simple alternative for the study of cell migration because it consists of much fewer number of cells and it is formed in a stereotyped manner. During embryogenesis, tracheal cells must migrate to appropriate targets in order to form the network. Many studies addressed the mechanism of cell differentiation in the trachea (Vincent et al., 1997; Wappner et al., 1997; Llimargas and Casanova, 1997; Zelzer and Shilo, 2000), but little is known about guidance mechanism of cell migration. To unveil how cells recognize their environment and find the direction to which they should migrate, I here analyze the guidance mechanism of tracheal cells.

Dorsal branch (DB), which is the focus of this section, is a well-studied branch that migrates dorsally, and fuses at the dorsal midline with DB from the other side of the same segment. At stage 12, DB consisting of about 5 cells begins to migrate dorsally in response to Bnl (Fig. 1E, F; Sutherland et al., 1996). At stage 14, two specialized cells, fusion and terminal cell are detected by the expression of Esg and SRF, respectively (Fig. 12B; Tanaka-Matakatsu et al., 1996; Samakovlis et al., 1996; Affolter et al., 1994; Guillemin et al., 1996). Fusion cells contact with other fusion cells from the other side by forming cadherin-dependent intercellular adhesion (Tanaka-Matakatsu et al., 1996), whereas the terminal cell extends their cytoplasmic processes to supply the target with oxygen (Guillemin et al., 1996). By stage 16, DB shows typical U-shape with a dorsal fusion point and terminal branches (Fig. 12B).

The *Drosophila* ortholog of human β-catenin (β-cat), Armadillo (Arm) is an essential component for well-known canonical Wg signaling (Fig. 3). Arm is found associated with cell-cell adherens junctions, in the cytoplasm and in nuclei. Wg signaling elicits a cellular response by regulating the free pool of Arm/β-cat. In the absence of Wg signaling, cytoplasmic Arm is rapidly degraded via a proteosome-mediated pathway (reviewed in Polakis, 1999). Cells of the *Drosophila* epidermis that receive Wg signaling accumulate Arm in the cytoplasm and nucleus

(reviewed in Wodarz and Nusse, 1998). This depends upon the binding of Wg to the cell surface receptors of the Frizzled (Fz) family, the activation of Dishevelled (Dsh) and subsequent inactivation of Zeste white-3 (Zw3, the *Drosophila* ortholog of glycogen synthase kinase-3β). From studies in mammals, two additional proteins, APC and Axin, were implicated in regulating Arm/β-cat stability (reviewed in Polakis, 1999). Members of TCF/LEF family of transcription factors are also required for Wg signaling (reviewed in Wodarz and Nusse, 1998). TCF/LEF transcription factors bind DNA, and recruit Arm as a co-activator, thus activating Wg signaling responsive genes. Therefore, Wg signaling regulates cell fate choices directly by altering the pattern of gene expression.

In *Drosophila*, upstream components of the Wg signaling are also required during the establishment of planar polarity, the process whereby epithelial cells acquire positional information relative to the body axes of the animal. For example, both Fz and Dsh are required to coordinate the proximal-to-distal orientation of actin-based wing hairs (reviewed in Shulman et al., 1998). Many studies have suggested that planar polarity is independent of canonical Wg/Arm signaling, while genetic and biochemical studies suggest that the small GTPases signaling functions downstream of Fz and Dsh to establish planar polarity (Fig. 21; Boutros et al., 1998).

In the process of studies in previous part, I found that the migration of DB is always fixed relative to overlying ectoderm because DB fusion points are always located posterior to Wg stripes, and terminal branches migrate parallel to the Wg stripes, but never cross them. (Fig. 12A, B). These observations indicate that the direction of DB migration must be tightly regulated. In this part, I show fusion and terminal cells contact ectodermal cells, having filopodia and lamellipodia structure similar to those found in the axonal growth cone. Furthermore, I show that PCP signaling contributes to the guidance of DB migration. I also show that a down stream component of PCP signaling, small GTPase Drac1 has an essential role on guidance of DB migration, but not on outgrowth itself. As it has been reported that axon guidance is regulated by Drac1 activity, these results imply that there are common guidance mechanisms for cell migration between tracheal cell migration and axonal outgrowth.

RESULTS

Fusion and terminal cells switch their relative position during DB migration.

In wild-type embryos, fusion and terminal cells are located at the tip of DB. To investigate the

guidance mechanism of DB migration, I first observed the expression pattern of Esg and SRF, and checked their relative positions during DB migration in detail. Esg, a marker gene for fusion cells, initially begins to be expressed at stage 13, and its expression was always located at the anterior position in DB tip (Fig. 13A, B). On the other hand, SRF, a marker for terminal cells, is expressed at the posterior position in DB tip (Fig. 13D). After stage 16, their relative position switched. Esg expression was detected at the posterior position of DB tip (Fig. 13C), whereas SRF expression was detected at the anterior position of DB tip (Fig. 13E).

Fusion cells always contact with Engrailed stripes.

To obtain more insights into the mechanism of DB migration, I analyzed the spatial relationships between DB and ectodermal cells during DB migration. Epidermal cells were marked by anti-α-Spectrin antibody to recognize outline of cells. At stage 14, tips (including fusion and terminal cell) of DB contact with epidermal cells with pointed shape (data not shown). The pointed shape of DB tip become flattened after stage 15 (arrow in Fig. 14A, B). After tracheal fusion, fusion cells and terminal cells (including cytoplasmic extensions) keep in contact with epidermal cells (Fig. 14E, F, I). On the other hand, stalk cells do not contact with epidermal cells during DB migration (Fig. 14A, B, E, H). Furthermore, I found that fusion cells always contact with Engrailed expressing epidermal cells (En-stripes). At early stage 14, fusion cells begin to contact with the posterior side of En-stripes (Fig. 15A). When fusion cell and terminal cell switch their relative position, fusion cells continue to contact with En-stripes (Fig. 15B). At stage 16, although terminal branches are located anterior to En-stripes, fusion cells continue to contact with Engrailed stripe (Fig. 15C). From these observations, I speculate that the contact between fusion cells and En-strips may be important for guidance of DB migration.

Lamellipodia and filopodia structure in fusion and terminal cell

Previous studies demonstrated that cell migration requires coordinated changes of cytoskeletalon of membrane morphology as well as cell-cell junction (reviewed in Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Especially, localized actin polymerization is an essential event for cell migration. To study the distribution of F-actin in tracheal cells, I took advantage of a chimeric protein of GFP fused to the C-terminal actin binding domain of moesin reported by Edwards et al. (1997). To express GFP-moesin in specific tissues, I newly generated a strain bearing *UAS-GFP-moesin*, and crossed it to a strain bearing *btl-Gal4*. As shown in Fig. 16C, GFP-moesin signals were distributed around plasma membrane, and were highly accumulated at the apical membrane in all of tracheal cells (Fig. 16E). Broad, flat and sheet-like structures of

actin meshwork in lamellipodia were detected in fusion and terminal cells, but not in stalk cells (Fig. 16A-C). In addition to lamellipodia, filopodia with thin, cylindrical and needle-like projections in migrating tips of DT, fusion and terminal cells were observed (Fig. 16A, B, D).

DB migrates dorsally and anteriorly in close contact with anterior Bnl stripe.

Bnl-dependent activation of the Btl receptor is essential for migration of tracheal cell (Glazer and Shilo, 1991; Sutherland et al., 1996). Therefore, there is a possibility that the migration pattern of DB can be explained by Bnl expression pattern. Previously expression pattern of *bnl* mRNA until embryonic stage 14 was reported, but little is known about the pattern in later embryonic stage. To determine whether the expression pattern of *bnl* mRNA correlates with the migration pattern of DB, I observed the expression pattern of *bnl* mRNA during DB migration in wild-type embryo from stage 11 to 16. From stage 11 to 14, as reported by Sutherland el al., *bnl* mRNA is expressed at the position where DB will grow out in patch-like pattern (Fig. 1E, F). The patch-like patterns of *bnl* mRNA disappeared at stage 15, 16, and began to be expressed in a stripe pattern overlapping with Wg stripes (Fig. 17A-D). DB migrated dorsally and anteriorly in close contact with anterior *bnl* mRNA stripe, but not posterior one.

Activation of Btl signaling activates MAPK in tracheal cells. This activation of MAPK can be detected by anti-diphospho-MAPK (dpMAPK) antibody (Gabay et al., 1997). To know activation pattern of MAPK during DB migration at a single cell level, I stained wild-type embryo (*btl-Gal4/UAS-gfpTTras*) by using anti-dpMAPK antibody. As shown in Fig. 17E, dpMAPK signals were detected in all of tracheal cells until stage 12. At stage 13, the dpMAPK signals were restricted to 3 to 4 cells at the tip of DB, and its highest signal was detected in the anterior cell of DB tip (Fig. 17F). In later stage embryo, dpMAPK-positive cells were restricted to a single cell (Fig. 17G). Although fusion and terminal cells switch their relative position, the highest MAPK activation was always located at the anterior cell of DB tip. In other words, at early stage (stage 13) dpMAPK signal is strong in fusion cells, whereas at later stage (after stage 15) its signal is detected only in terminal cells. From these observations, I conclude that the expression pattern of *bnl* mRNA accounts for the pattern of MAPK activation in tracheal cells. However, the expression pattern of *bnl* mRNA was not sufficient to explain the pattern of DB migration.

Ectopic expression of Wg causes misrouting of DB accompanied with cell fate changes.

As shown in Fig. 12A, DB never crosses Wg stripes in wild-type embryo, whereas in *wg* temperature-sensitive mutants DB occasionally migrates to wrong direction and crossed Wg

stripes (Fig. 5G, H). From these results, I hypothesized that Wg stripes may restrict the region of DB migration by acting as a repulsive factor of DB migration. To test the hypothesis, I investigated the effects of Wg for guidance of DB migration. At first, I expressed Wg under control of *5133-Gal4* which is specifically expressed in dorsal epidermal cells (Fig. 18A, B). If Wg acts as a repulsive factor for tracheal cell migration, tracheal cells should avoid the regions where Wg ectopically expressed. I found misrouting of DB in embryos in which Wg was expressed in dorsal epidermal cells (Fig. 18C, D). In these embryos, alteration of tracheal cell fate similar to that found in embryos in which arm^{S10} was expressed in all tracheal cells was detected (Fig. 7J and data not shown). Because changes in cell fate were always observed in branches with defective migration, it was not possible to distinguish the misrouting phenotype was a direct consequence of Wg signaling or reflect an indirect effect of cell fate defect. I therefore tried to separate effects on cell fates and cell migration by modulating activities of components of Wg signaling.

Canonical Wg/Arm signaling is not essential for guidance of DB migration.

To determine whether the misrouting defects seen in *wg* temperature-sensitive mutants are due to a loss of Wg signaling within tracheal cells, I next expressed dominant-negative form of *D*Tcf, *D*TcfΔN under control of *btl* promoter (*btl-Gal4*) to inhibit canonical Wg/Arm signaling only in tracheal cells. *D*Tcf is a transcription factor, acting in canonical Wg/Arm signaling (Fig. 3; van de Wetering et al., 1997). I found that *D*TcfΔN caused the loss of Esg expression in tracheal cells and the failure of DB tracheal fusion as found in *esg* null mutants (Fig. 19A, B). However, migration to the dorsal midline, and positioning at the En-stripes, of DB proceeded normally (Fig. 19A, B). This result suggests that canonical Wg/Arm signaling is not required for guidance of DB migration. From these observations, I speculate that Esg expression in tracheal cells is not required for guidance of DB migration.

Ectopic expression of *D*Axin in tracheal cells causes misrouting of DB migration with cell fate changes.

I next expressed another component of Wg signaling, *D*Axin under control of *btl-Gal4* to inhibit Wg signaling at the level upstream of *D*Tcf. It was reported that *D*Axin is a negative regulator of Wg signaling by promoting Arm degradation (Fig. 3; reviewed in Polakis, 1999). *D*Axin also binds to *D*E-APC and colocalized with actin cytoskeleton, and this activity may be linked to an alternative pathway to regulate cytoskeletal rearrangement (McCartney et al., 1999; Townsley and Bienz, 2000). *D*Axin caused misrouting of DB with characteristic branching pattern (Fig.

20C, D). In wild-type embryo, DB finally migrates to anterior *bnl* mRNA stripe (Fig. 20A, A'). However in *D*Axin expressing trachea migration of DB occasionally shifted posteriorly to cross posterior *bnl* mRNA stripes (Fig. 20B, B'). This migration pattern of *D*Axin-expressing trachea is different from the *D*TcfΔN-expressing or *esg* mutant trachea (compare Fig. 19C with Fig. 20D). Thus, the misrouting phenotype observed in *D*Axin-expressing trachea is unlikely to be due to the cell fate change caused by defect of canonical Wg signaling, but rather due to a failure of actin rearrangement.

Planar cell polarity signaling affects guidance of DB migration.

Planar cell polarity (PCP) signaling controls the polarity of epithelial cells within a plane orthogonal to their apical-basal axis, and its components include Frizzled (Fz), Dishevelled (Dsh) and Drac1 (Fig. 21; Boutros et al, 1998). One manifestation of this cellular polarity in the epithelia is the oriented organization of trichomes (cell hairs). In wild-type flies, the cell hair arising from each cell's distal points to one direction, forming parallel arrays. On the other hand, PCP signaling defects disrupt the polarity of trichomes, resulting in swirls and distortions of the hair polarity pattern (reviewed in Shulman et al., 1998). Thus, PCP signaling is a key signaling for cells to recognize the direction of tissue. PCP signaling shares several of its components with Wg signaling. Fz is a putative receptor for Wnt pathway, whereas Dsh is a component of Wg signaling (Fig. 21). To test whether PCP signaling is involved in tracheal development, I measured the frequency of misrouted DB in dsh^{1} mutants. dsh^{1} behaves as a genetically null for PCP signaling, but is fully functional in Wg signaling (Fig. 21; Axelrod et al., 1998; Boutros et al., 1998). As shown in Table. 1, dsh^{1} mutants showed a misrouting phenotype, although its frequency was low. In these embryos, Esg and SRF expression in tracheal cells were completely normal (data not shown). This result suggests that PCP signaling specifically affects on guidance of DB migration.

To inhibit the activity of PCP signaling in tracheal cells, I next expressed DEP domain deleted Dsh, Dsh(ΔDEP+) in tracheal cells. Expression of Dsh(ΔDEP+) can specifically inhibit PCP signaling without affecting Wg signaling (Axelrod et al., 1998). The Dsh(ΔDEP+) expressing trachea showed normal DB migration with cell fate changes (data not shown). Furthermore, I analyzed other PCP signaling component, Flamingo (Fmi) which is a cell adhesion molecule and is essential for establishment of PCP (Usui et al, 1999). But fmi^{E45}/fmi^{E59} zygotic mutant embryos did not show any misrouting phenotype of DB (data not shown). These results suggests that, althrough PCP signaling is required for guidance of tracheal cell migration,

its contribution seems to be weak.

Dominant-negative form of Drac1 causes misrouting of DB migration.

The small GTPase Drac1 controls actin cytoarchitecture in leading edge structure of axonal growth cones (reviewed in Luo et al., 1997; Luo, 2000). Drac1 is also known as a down stream component of PCP signaling (Fig. 21; Boutros et al., 1998). To investigate the role of Drac1 in tracheal development, I expressed a dominant-negative form of Drac1, Drac1N17 in tracheal cells (Luo et al., 1994). As shown in Fig. 22, Drac1N17 caused severe misrouting defect in DB. Interestingly, defects of DB outgrowth or cell differentiation judged by the expression of Esg and SRF were not detected (data not shown). These results suggest that Drac1 activity in tracheal cells is required for the guidance of tracheal cell migration, but not for the motility of tracheal cells.

Appropriate level of Dcdc42 activation is required for DB development

As I showed in Fig. 16, numerous filopodia were seen at the tip of migrating DB. Because a member of small GTPases, Dcdc42 is known as an effecter for filopodia formation (reviewed in Hall, 1998), I speculated that Dcdc42 functions in DB migration by inducing filopodia. To analyze the role of Dcdc42 during DB migration, I expressed constitutive-active (V12) or dominant-negative (N17) form of Dcdc42 under control of *btl-Gal4* in tracheal cells. Tracheal cell-specific expression of Dcdc42V12 caused severe tracheal defects, including truncation of DT and DB, and misrouting of DB (Fig. 23A, A'). On the other hand, Dcdc42N17 expression in tracheal cells caused extra and malformed terminal branches in DB (Fig. 23B, B'). These results suggest that appropriate level of Dcdc42 activation is required for DB development.

DISCUSSION

Guidance of cell migration plays an important role for development of many organism. For example, neuron extends axon and dendrite to specific targets in order to form elaborate nervous systems. Although numerous studies addressed the problem of how the migratory direction of neuronal projection is regulated, little has been known about its mechenism. Like the nervous systems, the tracheal system in *Drosophila* is also constructed by directed cell migration. To

unveil the guidance mechanism of tracheal cell migration, I investigated temporal and spatial migratory pattern of wild-type or mutant DB tracheal cells. I showed that Drac1 activity is essential for the guidance of DB migration. Furthermore I showed that PCP signaling may be involved in regulation of Drac1 activity to guide tracheal migration.

Guidance mechanism of tracheal migration by contact with epidermal cells.

Expression pattern of Bnl is restricted in order to guide the migration of tracheal cell to specific targets, but is not sufficient to explain the migration pattern of DB which migrates dorsally and anteriorly (Fig. 12). For example, all of the branches grew anteriorly toward an ectopic patch of Bnl expressing cells, and none of the branches grew posteriorly toward the next segmental patch, although it was a similar distance away in the embryo (Sutherland et al., 1996). Therefore, the direction of tracheal cell migration does not seem to be absolutely guided by the expression pattern of Bnl. Here I suggest another mechanism of guiding DB migration: contact of DB fusion cell to Engrailed expressing epidermis (En-stripe). As fusion cells of DB continue to contact En-stripe, and final fusion points of dorsal midline are also located on En-stripe, En-stripe may act as a substrate for DB migration (Fig. 24). I speculate that non-tracheal cells (En-stripe) may provide a positional information for the guidance of tracheal cell migration. Consistent with this idea, it was recently reported that contact between tracheal cell and non-tracheal cell is essential for directed migration of tracheal cells. *hunchback* gene expressing mesodermal cell (bridge-cell) contact dorsal trunk, serving as an essential guide post (Wolf and Schuh, 2000). In addition, it has been also reported that DB migration is channeled by the groove between muscle precursors of adjacent metameres (Franch-Marro and Casanova, 2000). In summary, I propose that the final pattern of tracheal branching is highly constrained by non-tracheal tissues including epidermis (En-stripes) and mesodermal cells (bridge cells and muscle precursors).

Similarity of guidance mechanism between tracheal cell migration and axon development.

In neural development, neuronal growth cones must navigate though complex cellular environments in response to various guidance cues to reach specific targets (Tessier-Lavigne and Goodman, 1996). The neuronal growth cone is internally supported by a complex cytoskeletal architecture largely composed of actin microfilament, lamellipodia and filopodia (reviewed in Hall, 1998). Previously it has been shown that loss of Dcdc42 activity inhibits axon elongation, and disruption of Drac1 interferes with directional axon guidance in *Drosophila* motor neuron (Kaufmann et al., 1998). Tracheal cells of DB may also use these small GTPases in order to migrate to dorsal fusion points. Like axonal growth cones, fusion cells contact En-stripe with

lamellipodia and filopodia (Fig. 16A, B, Fig. 24). Furthermore, the requirement for proper regulation of Dcdc42 and Drac1 in fusion cell may be analogous to those in growth cone. Dcdc42V12 blocks DB and DT migration (Fig. 23A, A'), whereas Dcdc42N17 causes malformation of DB (Fig. 23B, B'). As cdc42 is known as an inducer of filopodia formation in cell culture system (reviewed in Hall, 1998), it is likely that cdc42 also regulates filopodia formation in tracheal cells. Furthermore, Drac1N17 causes a very conspicuous phenotype. In Drac1N17 expressing trachea, DB migrates dorsally, but misrouting of DB takes place in anteroposterior direction (Fig. 22). This phenotype is similar to guidance defects seen in Drac1N17 expressing motor axon (Kaufmann et al., 1998) in which outgrowth is normal but the direction is abnormal. As Rac1 activity is essential for lamellipodia formation in axonal growth cone, I suggest that Drac1 activity in tracheal cells may regulate lamellipodia formation in order to migrate to appropriate targets. Consistent with this idea, Drac1N17 did not cause any defect in DT which has filopodia, but not lamellipodia (Fig. 16D, E).

PCP signaling has effects on guidance of DB migration.

Planar cell polarity in the *Drosophila* wing epithelium is oriented by cell-cell communication and is regulated by PCP signaling. Here I showed that PCP signaling has effects on guidance of DB migration, because PCP signaling null mutant, dsh^1 showed a misrouting phenotype in DB (Table. 1). As Drac1 is known as a down stream component of PCP signaling, I speculate that PCP signaling activates Drac1 to guide the direction of tracheal cell migration. Another PCP signaling defective mutant, zygotic *fz* and *fmi* did not show any misrouting phenotype. However *fz* and *fmi* may have some maternal effects in tracheal developing stages, it is necessary to investigate maternal and zygotic mutant for *fz* or *fmi*.

Which signaling regulates Drac1 activity in order to guide DB migration?

Appropriate level of Drac1 activation in tracheal cells is essential for its directed migration. I showed that PCP signaling null mutant, dsh^1 has some effects on guidance of tracheal cell migration, but the frequency of misrouting phenotype in dsh^1 was notably weaker than Drac1N17 expressing embryos. This difference suggests a possibility that Drac1 activity is also regulated by another signaling in addition to PCP signaling. Which signaling regulates Drac1 activity in tracheal cells? As Drac1 activity regulates guidance of tracheal cell migration in anteroposterior direction, the candidate signaling must have a positional information along the anteroposterior body axis. From this consideration, I speculate that possible candidates are Wg, Hedgehog (Hh) and Bnl, which are expressed in striped pattern (Fig. 24). I here showed effects

of Wg on guidance of tracheal cell migration, and demonstrated that canonical Wg/Arm signaling can't affect on guidance of tracheal cell migration. But the possibility remains that Wg regulates Drac1 activity in tracheal cells. Because Wg can bind to Fz which is a component of PCP signaling, Wg may regulate Drac1 activity via PCP signaling. The effects of Hh and Bnl remain to be investigated.

CONCLUDING REMARKS

The *Drosophila* tracheal system has become an appropriate model for study of the mechanisms that how the cells differentiate to precise cell fate, and migrate to specific targets. In this thesis, I demonstrate that Wg signaling controls the shape of tracheal tubule in concert with Notch signaling. In addition, I show the guidance mechanism of tracheal cell migration is similar to that of axon guidance, and demonstrate that PCP signaling which bifurcates from canonical Wg signaling has effects on guidance of tracheal cell migration. This finding may shed light on the universal guidance mechanism of cell migration. From these results, I propose that Wg acts on both cell differentiation and the guidance of cell migration via distinct signaling pathway to elaborate *Drosophila* tracheal network.

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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), *btlΔOh10* (Ohshiro and Saigo, 1997), fmi^{E45}, fmi^{E59} (Usui et al. 1999), dsh^{1} (Perrimon and Mahowald, 1987), *trachealess* enhancer trap line *1-eve-1* (Perrimon et al., 1991), tkv^{str-11} (Nusslein-Volhard et al., 1984), *Delta-lacZ* (R. Ueda, personal communication), *UAS-wg* (Lawrence et al., 1995), $UAS\text{-}arm^{S10}$ (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-btl* (Glazer and Shilo, 1991), *UAS-DracN17, UAS-DracV12, UAS-Dcdc42N17, UAS-Dcdc42V12* (Luo et al., 1994), *UAS-dsh43-6A (full length), UAS-dshΔC18-111 (ΔDEP+), UAS-dshΔB21-1 (ΔbPDZ)* (Axelrod et al., 1998), *UAS-fmi* (Usui et al., 1999), *UAS-bnl* (Sutherland et al., 1996), *UAS-dpp*, *UAS-dad* (Tsuneizumi et al., 1997), *UAS-hh* (Tabata and Kornberg, 1994), *5133-Gal4* (NP consortium), *UAS-gfpTTras* (a membrane bounded GFP; S. Hayashi, 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), mouse diphospho-MAP kinase (Sigma), 2A12 which recognizes an unknown luminal component, mouse anti-Engrailed 4D9 and mouse anti-α-Spectrin 3A9 (Developmental Studies Hybridoma Bank). Immunostaining with anti-diphospho-MAPK was performed as described by Gabay et al (1997). Other antibodies used were as 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 by use of

Photoshop software (Adobe).

In situ hybridization and antibody staining were performed as described (Tautz and Pfeifle, 1989) with minor modification. 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.

Establishment of UAS-GFP-moesin flies

To generate *UAS-GFP-moesin* flies, I digested the sGMCA (*spaghetti squash* promoter-GFP-moesin without the coiled coil but with the actin binding site, Kiehart et al., 2000; a gift from D. P. Kiehart) DNA by SpeI and XbaI, and isolated 2.0 kbp DNA fragment. The DNA fragment was inserted into the XbaI sites of pUAST (Brand and Perrimon, 1993). The resultant plasmid, pUAST-GFP-moesin, was introduced into *w* animals by P element transformation using pΔ2-3 helper plasmid.

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I would like to greatly thank Shigeo Hayashi for instructions in this study. I thank Yasushi Hiromi, Isao Katsura, Hiroyuki Takeda, Tadashi Uemura, Tatsumi Hirata, and Hayashi's lab members for helpful comments and discussions. I also thank 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, Daniel P. Kiehart, Jeffrey D. Axelrod, Tetsuya Tabata, Developmental Studies Hybridoma Bank, East Asian Distribution Center for Segmentation Antibodies and Umea and 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.

At last, I greatly and heartly thank my parents for supporting my graduate research life.

Photoshop software (Adobe).

In situ hybridization and antibody staining were performed as described (Tautz and Pfeifle, 1989) with minor modification. 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.

Establishment of UAS-GFP-moesin flies

To generate *UAS-GFP-moesin* flies, I digested the sGMCA (*spaghetti squash* promoter-GFP-moesin without the coiled coil but with the actin binding site, Kiehart et al., 2000; a gift from D. P. Kiehart) DNA by SpeI and XbaI, and isolated 2.0 kbp DNA fragment. The DNA fragment was inserted into the XbaI sites of pUAST (Brand and Perrimon, 1993). The resultant plasmid, pUAST-GFP-moesin, was introduced into *w* animals by P element transformation using pΔ2-3 helper plasmid.

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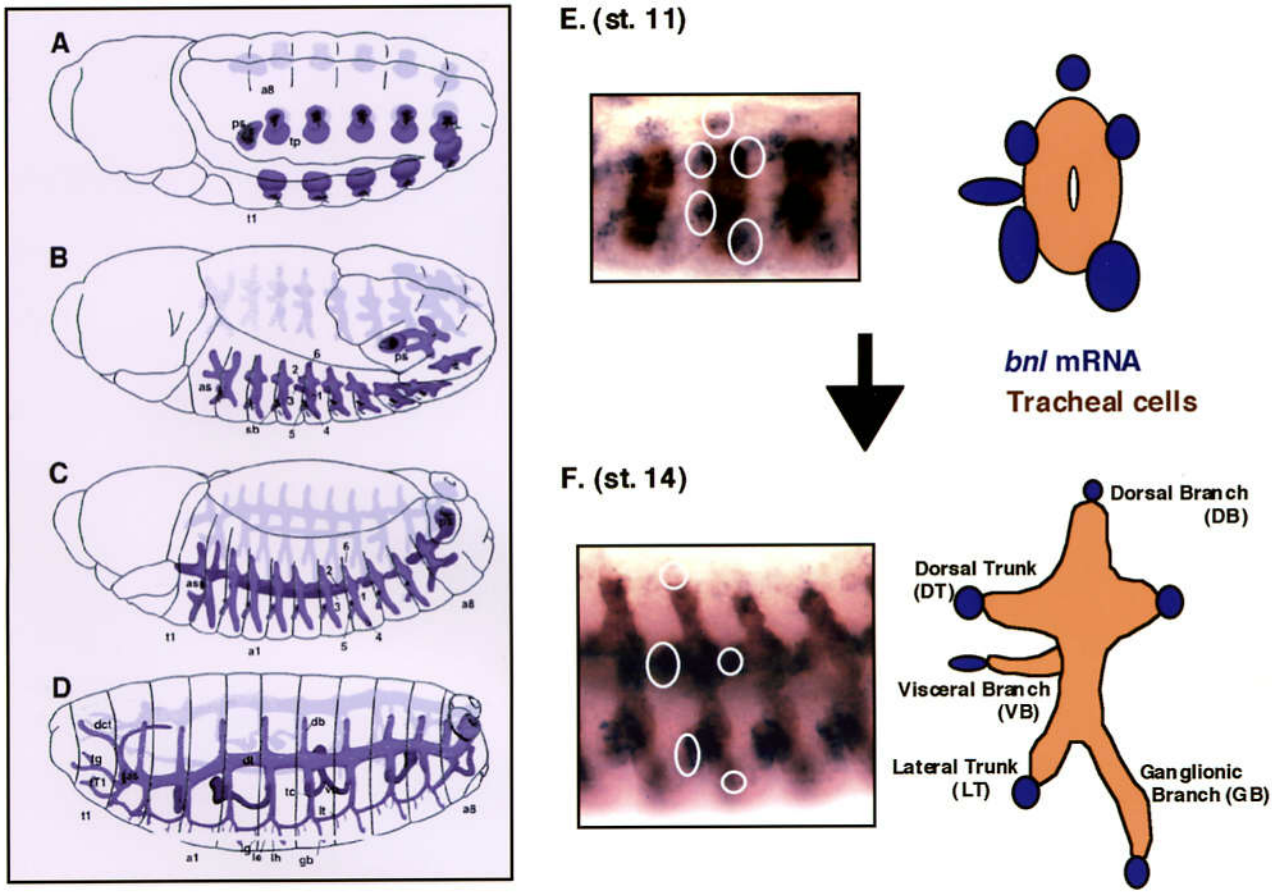

Fig. 1. General pattern of tracheal branching in embryogenesis.
(A-D) Stage 11, 12, 13 and 17 embryos, respectively. The tracheal system originates from 10 clusters of ectodermal cells at each side of the embryo. At stage11, the tracheal cells invaginate inside the embryo. During stage12 tracheal cells elongate to form six primary branches and each branch fuses with another branche from neighboring segments. (E, F) In tracheal development, cell migration and cell differentiation are induced by Bnl, an FGF homologue. Bnl (purple) is expressed in clusters of cells near the tip of the developing primary branches, and activates Btl (brown), an FGF receptor homologue expressed in tracheal cells.

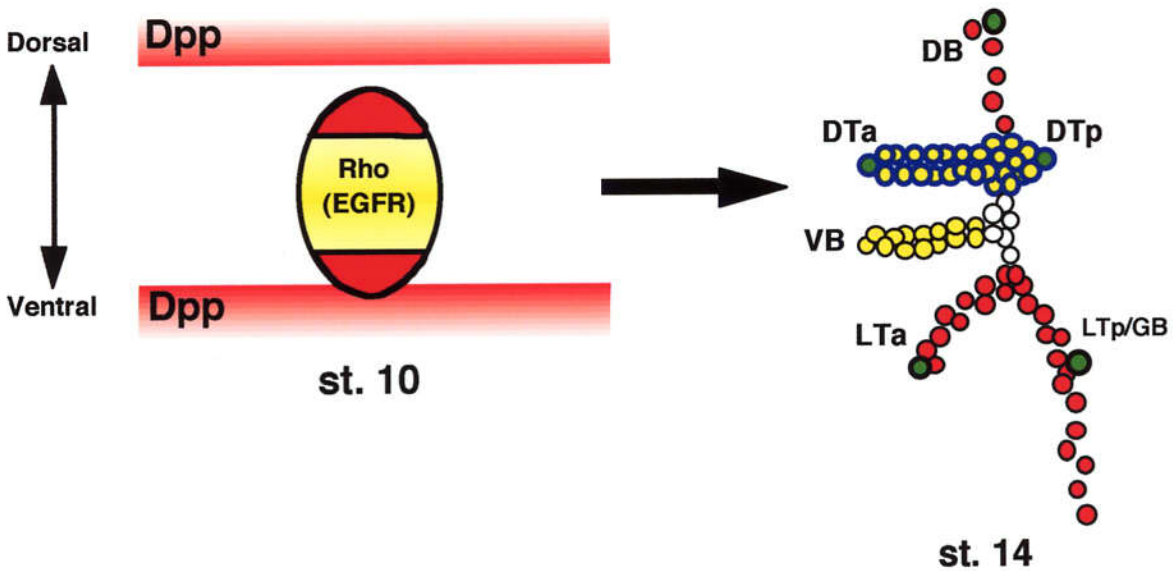

Fig. 2. Dorsoventral signals regulate tracheal branching.
(Left) At stage 10, the EGFR signaling is activated in the central part of the tracheal placode. Due to Rhomboid (Rho) expression in the central part of the placode and restricted diffusion of secreted Spitz, which is a ligand for EGFR, stronger activation levels of EGFR are observed in the central part (yellow region). In contrast, Dpp is expressed on the ectoderm in two stripes abutting the tracheal placode. Thus, stronger activation of Dpp signaling may be induced in the dorsal and ventral parts of the placodes. As a result, two different cell populations are determined in the trachea. (Right) The schematic drawing of the tracheal branches designates dorsal branch (DB), dorsal trunk anterior (DTa), dorsal trunk posterior (DTp), visceral branch (VB), lateral trunk anterior (LTa), lateral trunk posterior (LTp), and the ganglionic branch (GB). Each circle indicates the nucleus of a tracheal cell. EGFR-dependent branches (DT and VB) and Dpp-dependent branches (DB, LT and GB) are indicated by yellow and red respectively. Spalt expressing cells (DT) are indicated by blue circles. The position of fusion cells is indicated by green circles.

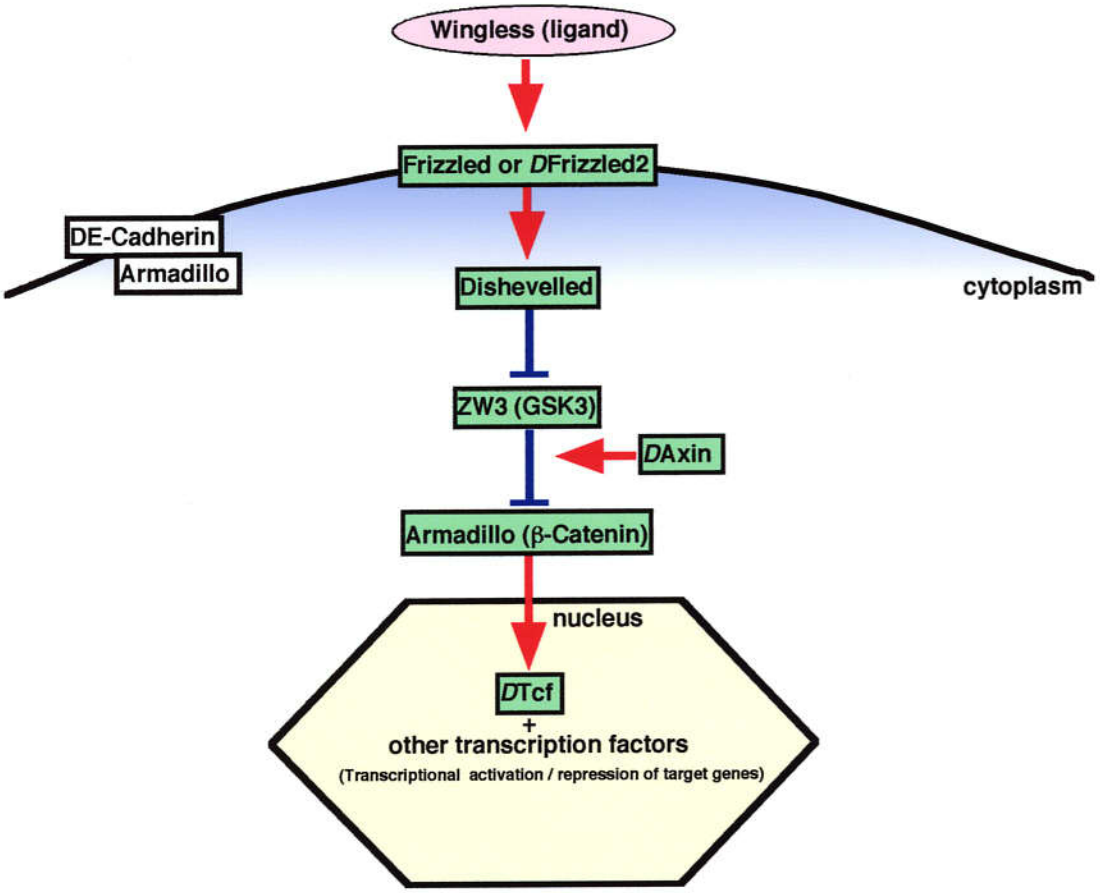

Fig. 3. Schematic drawing of canonical Wg / Arm signaling

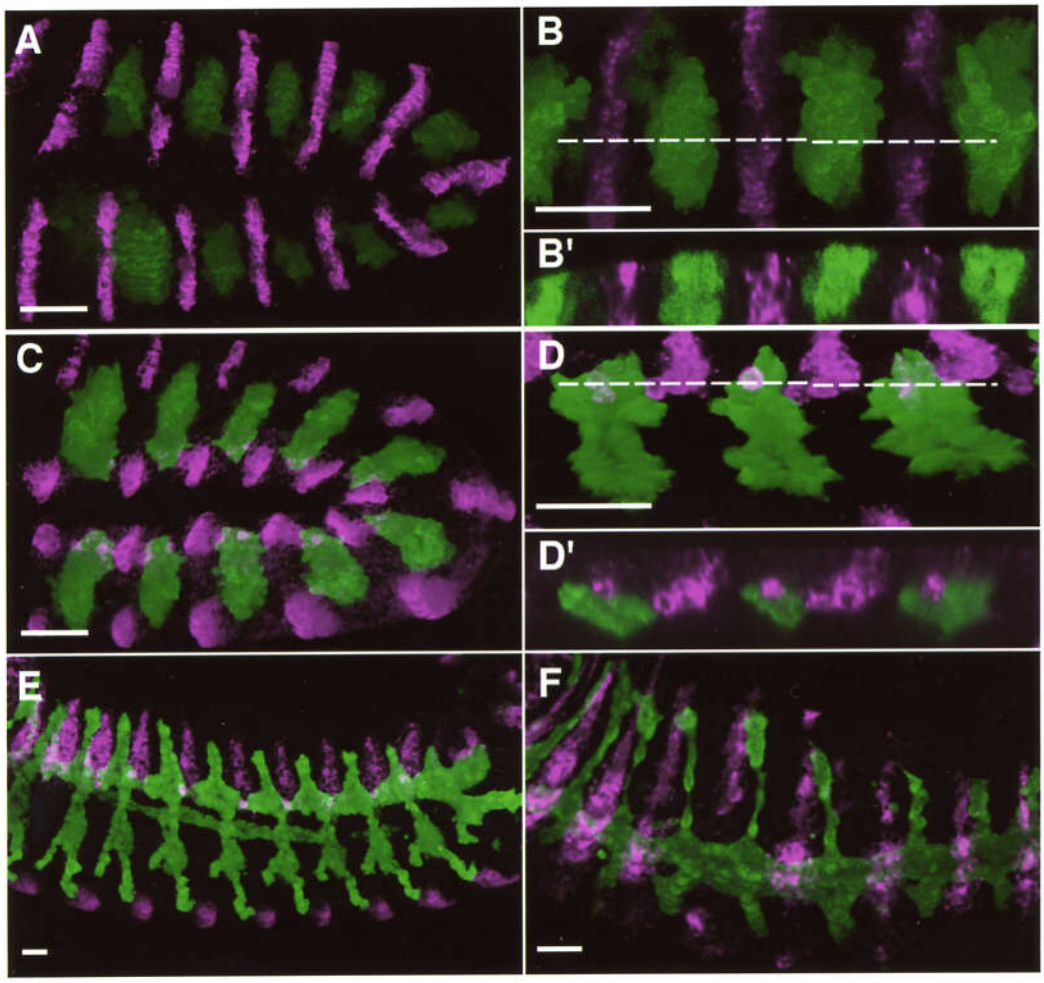

Fig. 4. The relationship between Wg expression and the tracheal development. Tracheal cells were labeled with *trh-lacZ* (green). Wg was labeled with anti-Wg (purple). (A, B) Wg expression is seen in the extended germ band at stage 10 (Lateral view). Wg is expressed in ectodermal cells flanking the tracheal placode in a striped pattern. B' is an image of the horizontal section through the broken line in (B). Outer surface is up. (C) At stage 11, lateral expression of Wg become 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' is an image of the horizontal section through the broken line in (D). At stage 14 (E) and stage 15 (F), the Wg protein is still detected in a striped pattern.

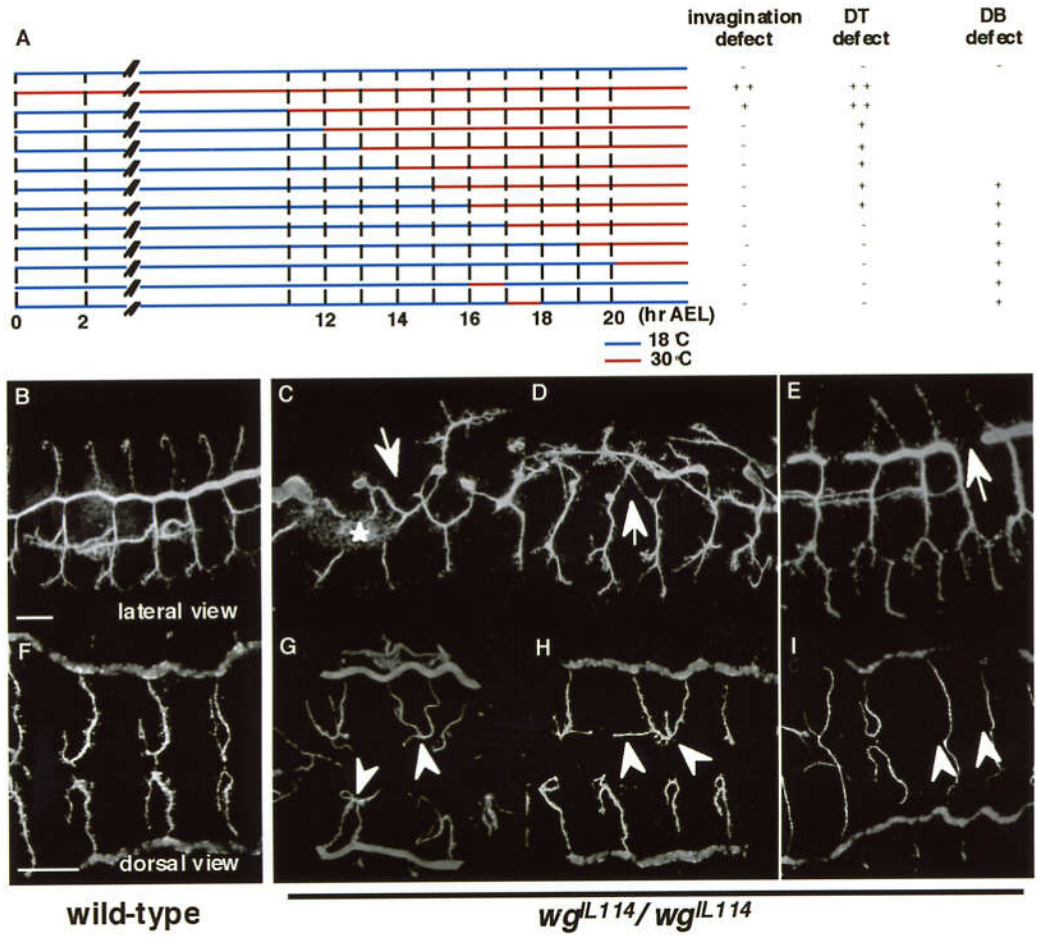

Fig. 5. Tracheal defects in *wg*^{IL114} temperature sensitive mutants.
(A) The schedule of temperature shift experiment. The time scale was equivalent to 18℃. Lateral (B-E) and dorsal (F-I) views of stage 16 embryos were stained for the tracheal lumen with mAb 2A12. (B and F) Wild-type embryo. (C-E, G-I) *wg*^{IL114} homozygotes embryos. Embryos of *wg*^{IL114} homozygotes showed several tracheal defects including failure of some tracheal cells to invaginate (star in C), disruption of DT (arrows in C-E) and failure of DB morphogenesis (arrowheads in G-I). Embryos subjected to temperature-shift at 0, 13, 16 and 20 hours after egg laying at 18℃ (AEL) are shown in C, D, E and G-H, respectively.

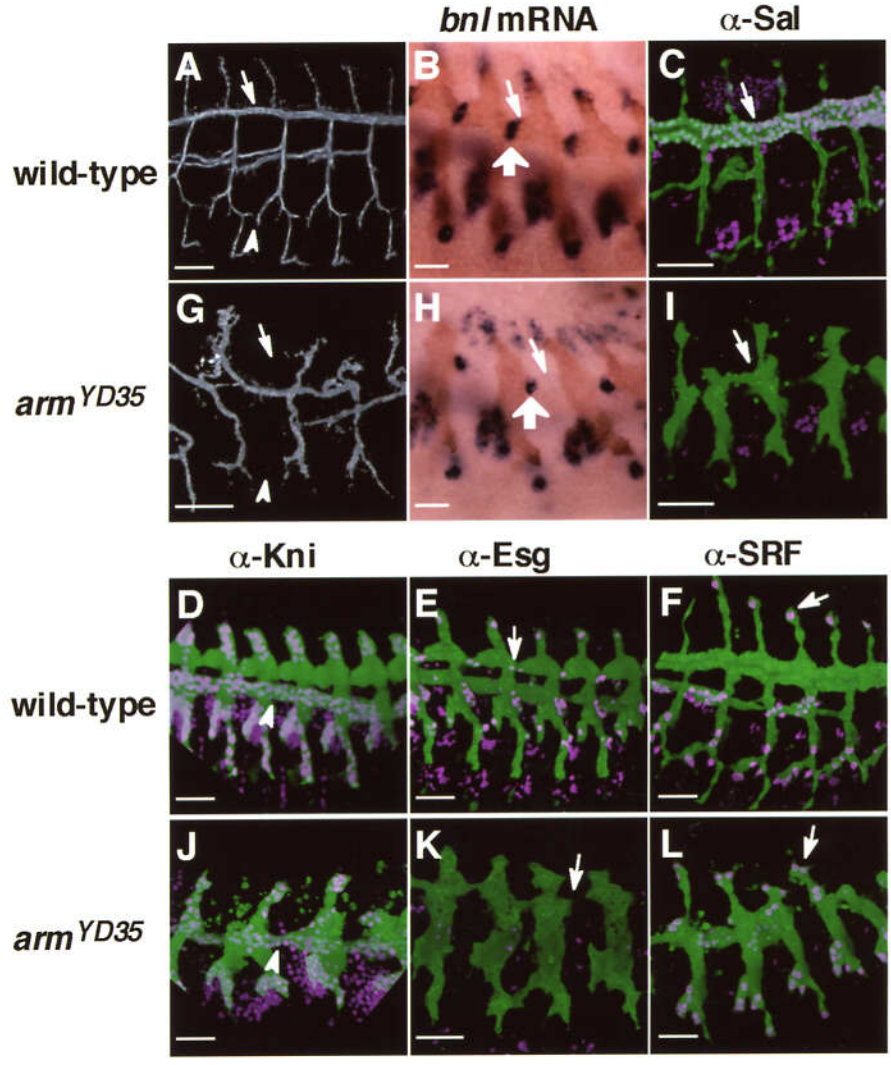

Fig. 6. Tracheal defects in zygotic *arm* mutants.
Embryos shown are wild-type (A-F) or zygotic arm^{YD35} mutants (G-L). The tracheal lumen was detected with mAb 2A12 (A, G). Tracheal cells are stained brown (B, H) or green (C-F, I-L) with the *trh-lacZ* tracheal marker. In zygotic arm^{YD35} mutants, DT fails to develop (arrow in G); branch fusion was not detectable (arrowhead in G). Lateral expression of *bnl* mRNA corresponding to DT migration was detected (thick arrow in H) despite the lack of DT in zygotic arm^{YD35} mutants (thin arrow in H). In wild-type embryos, Sal is expressed in DT cells (arrow in C) and Kni is expressed in DB, LT, GB and VB (arrowhead in D). In zygotic arm^{YD35} mutants, the expression of Sal disappeared from tracheal cells (arrow in I). Expression of Kni, however, remained in tracheal cells (arrowhead in J). Esg is expressed in fusion cells located on the tip of primary branches (E), and SRF is expressed in the terminal cells (F). In zygotic arm^{YD35} mutants, whereas Esg expressing cells were not detectable in the trachea (K), the number of SRF expressing cells increased (L).

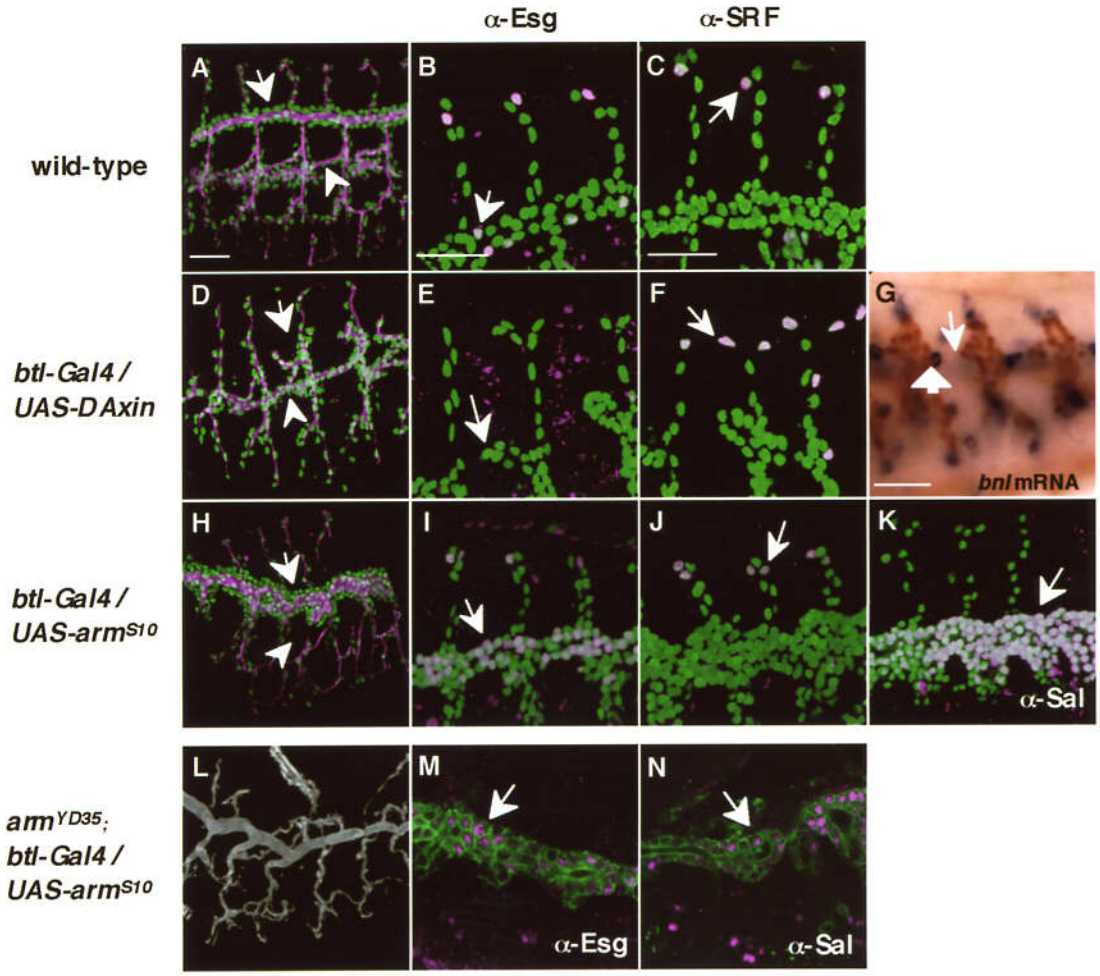

Fig. 7. Wg signaling has tracheal-autonomous roles for branching and cell differentiation

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 in A). Esg is expressed at the tip of migrating branches (arrow in B) and SRF is expressed in the terminal cells of DB (arrow in C). Ectopic expression of *D*Axin inhibited the formation of DT (arrow in D) and tracheal cell fusion at DB and LT. In these embryos, expression of Esg disappeared (arrow in E), together with an increase in the number of SRF expressing cells (arrow in F). Ectopic expression of Arm^{S10}, an activated form of Arm, inhibited the formation of VB (arrowhead in H). In these mutants, the number of Esg (arrows in I), SRF (arrow in J) and Sal (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.

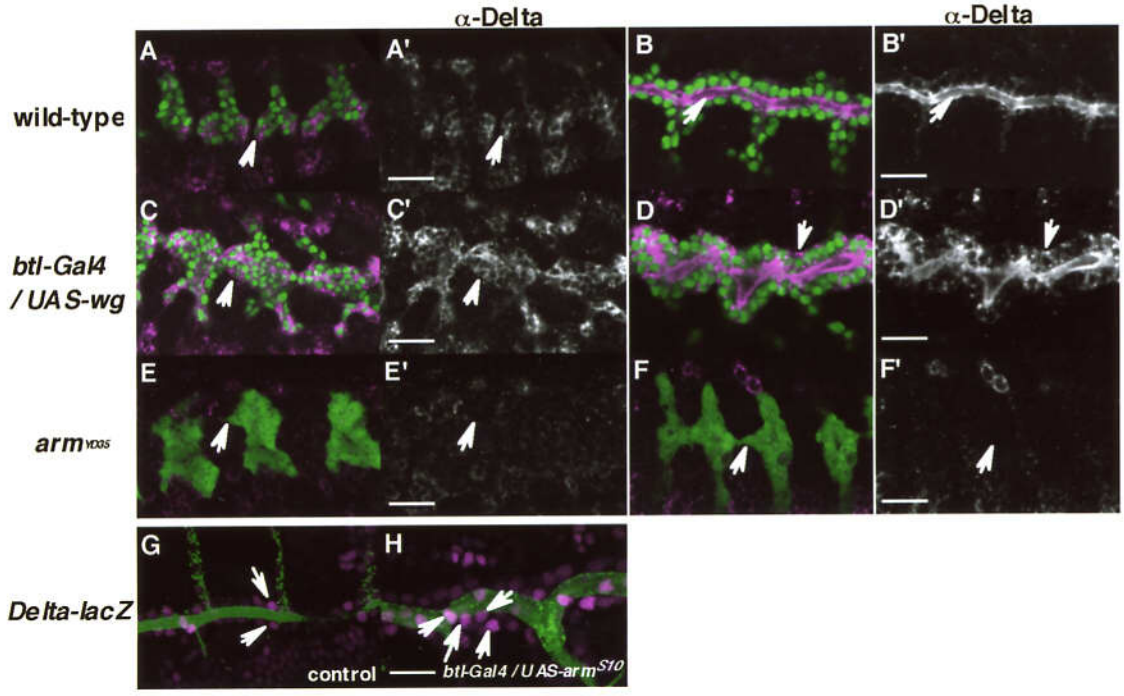

Fig. 8. Activation of Delta expression by Wg signaling

The tracheal nuclei (A-D; *gfpn-lacZ*), tracheal cells (E, F; *trh-lacZ*) and tracheal lumen (G, H; 2A12) were stained in green. In wild type embryos at stage 13, the 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 in 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* in fusion cells (arrows in G). When Wg was ectopically expressed in the tracheal cells, the *Delta-lacZ* expression became stronger in additional cells (arrows in H).

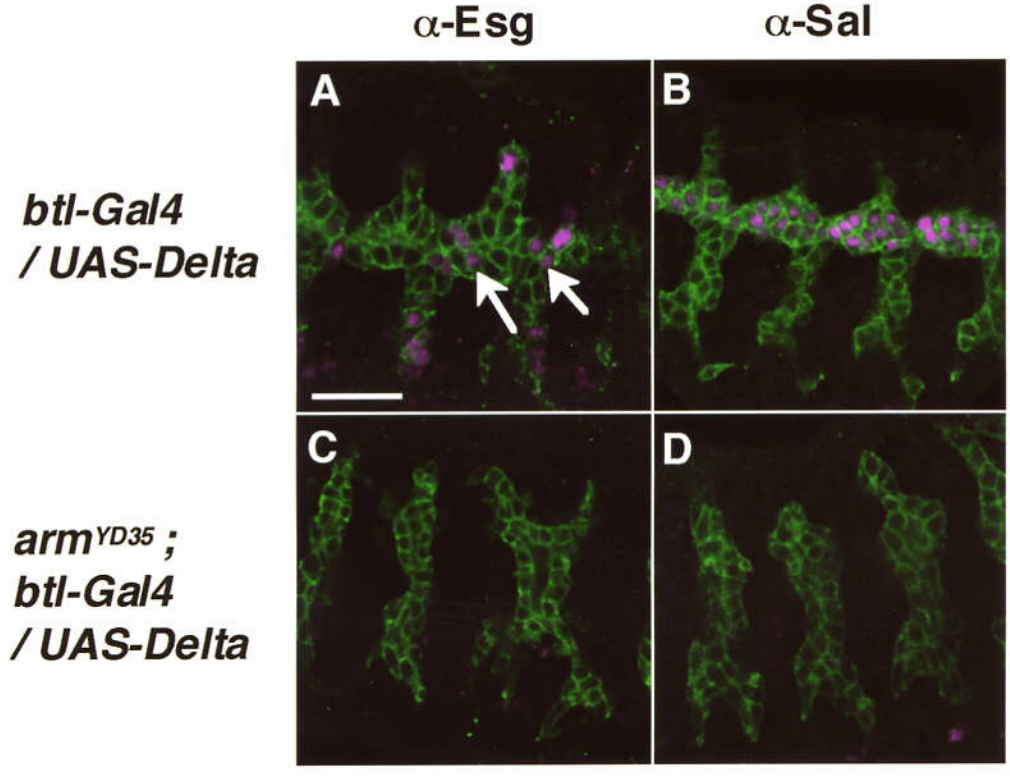

Fig. 9. Epistatic relationship between Wg and Notch signaling
Tracheal cells were detected by expression of membrane bounded GFP protein (*btl-Gal4 / UAS-gfpTTras*) visualized in green. The number of Esg expressing cells sporadically increased in Delta-overexpressing trachea (A), but no alteration of Sal expression was observed (B). The expression of Esg (C) and Sal (D) in Delta-overexpressing trachea was indistiguishable from that of arm^{YD35} mutant embryos.

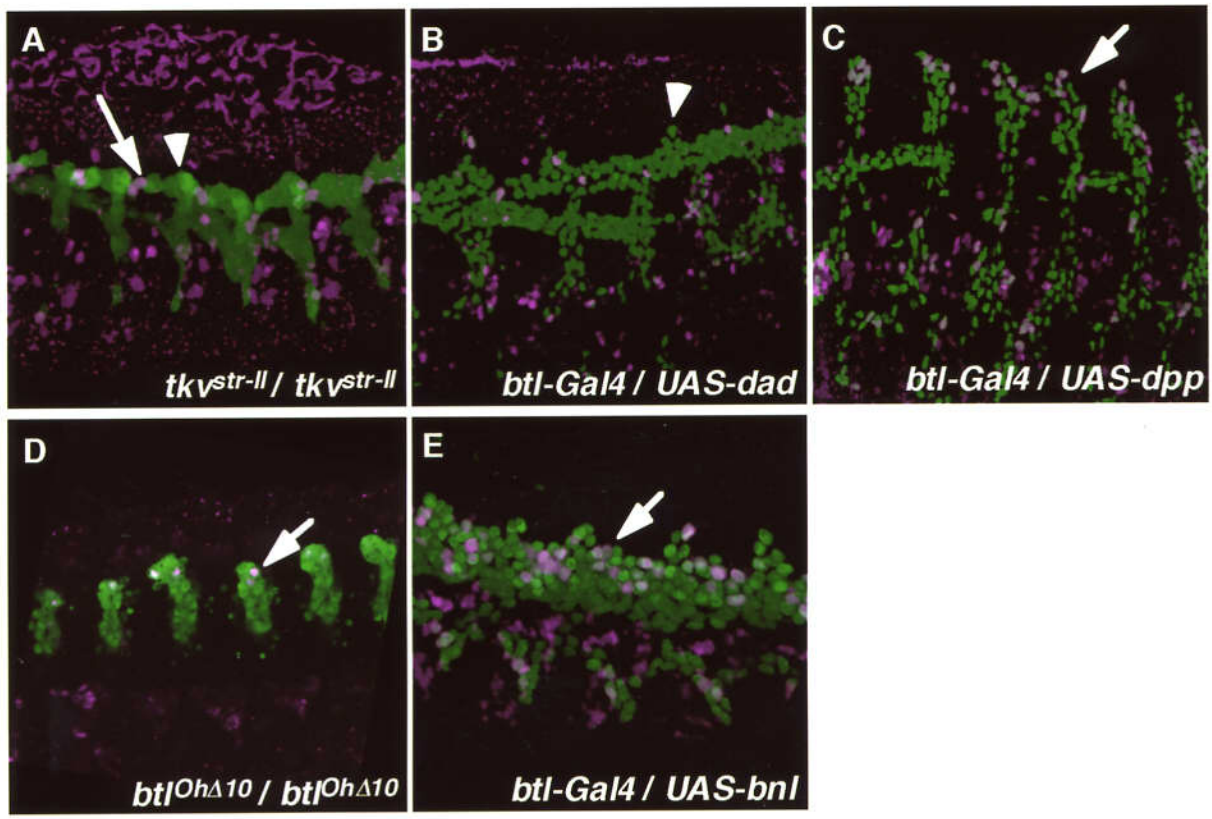

Fig. 10. Dpp and Bnl signaling regulate Esg expression in tracheal cells.
(A, D) Tracheal cells were specifically labeled with *trh-lacZ* (green). (B, C and E) Tracheal nuclei were detected by the expression of nuclear GFP-β-galactosidase fusion protein in green. (A-E) Esg was labeled with anti-Esg (purple). In $tkv^{str\text{-}II}$ / $tkv^{str\text{-}II}$ or *btl-Gal4 / UAS-dad* mutants, Esg expressing cells were detectable in DT (arrows in A), but not DB and LT (arrowheads in A and B). On the other hand, hyperactivation of Dpp signaling induced Esg expression in DB (arrow in C) and LT. In $btl^{OhΔ10}$ / $btl^{OhΔ10}$ mutants, Esg expressing cells remain in tracheal cells (arrow in D). The Bnl-expressing trachea showed increased number of Esg expressing cells in DT (arrow in E).

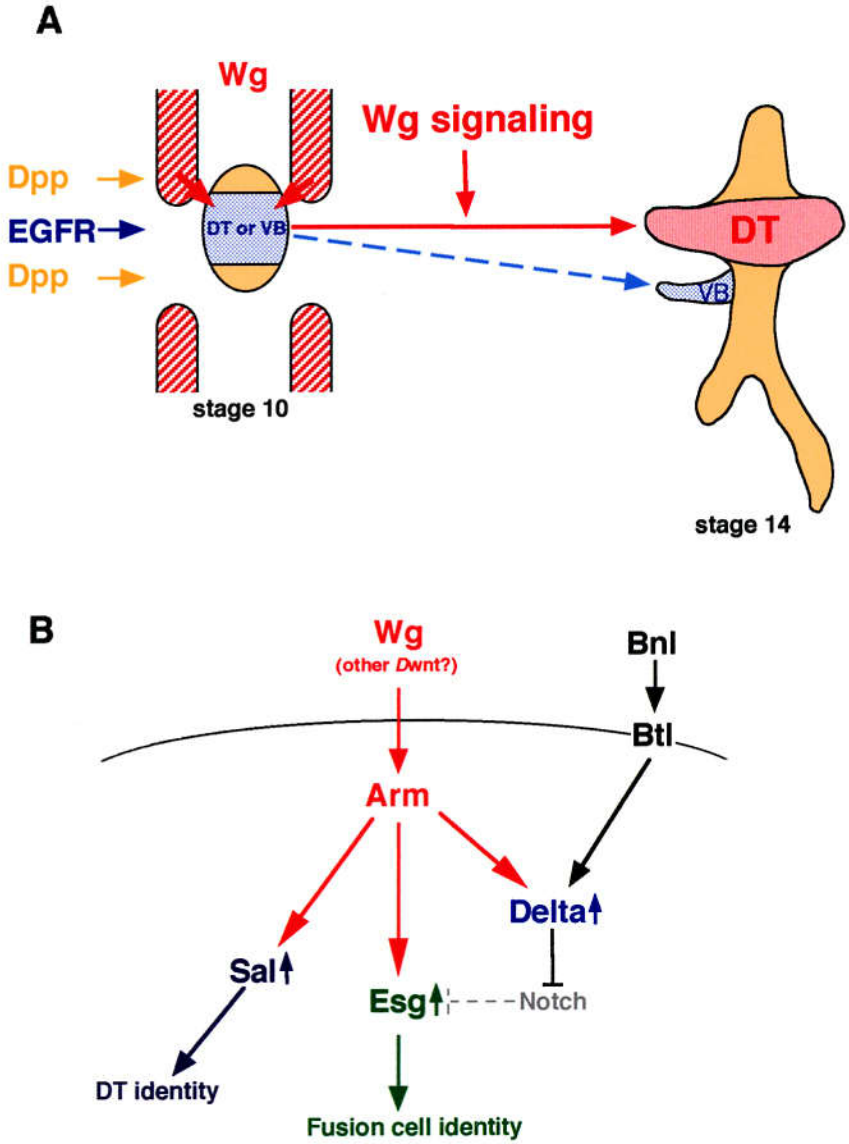

Fig. 11. A model of tracheal patterning by Wg signaling
(A) At stage 10-11, EGFR signaling is activated at the central region of the tracheal placode, specifying cells that give rise to the two anteroposterior migrating branches (DT and VB). Wg signaling stimulates EGFR-induced cells at the central part to differentiate into DT. At this time, if these EGFR-induced cells do not receive a signal from Wg, they become VB. On the other hand, Dpp signaling is activated at the dorsal and ventral region of the tracheal placode, inducing the dorsoventral migrating branches (DB, LT and GB). (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. The elevated Delta expression in fusion cells inhibits Notch activity in a cell autonomous manner and activates Notch in 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 uniformed pattern of Sal expression in DT.

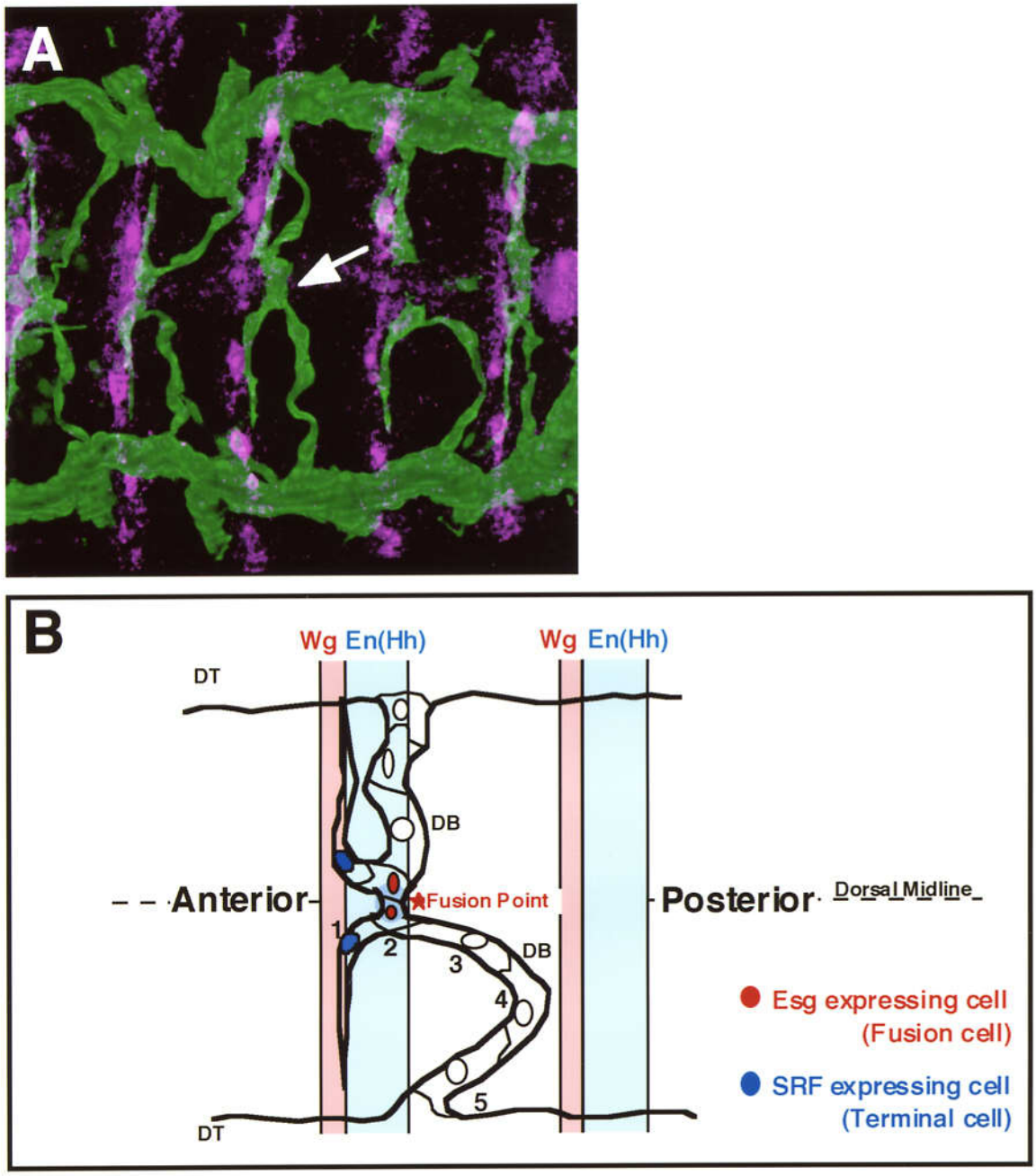

Fig. 12. The relationship between Wg expression and migration pattern of DB.
(A) Tracheal cells were labeled with *trh-lacZ* (green), and Wg with anti-Wg (purple). The dorsal fusion point (arrow in A) is always located at the posterior position of Wg expressing cells. (B) Schematic drawing of DB migration. Red and blue circles indicate the expression of Esg and SRF expressing cells, respectively.

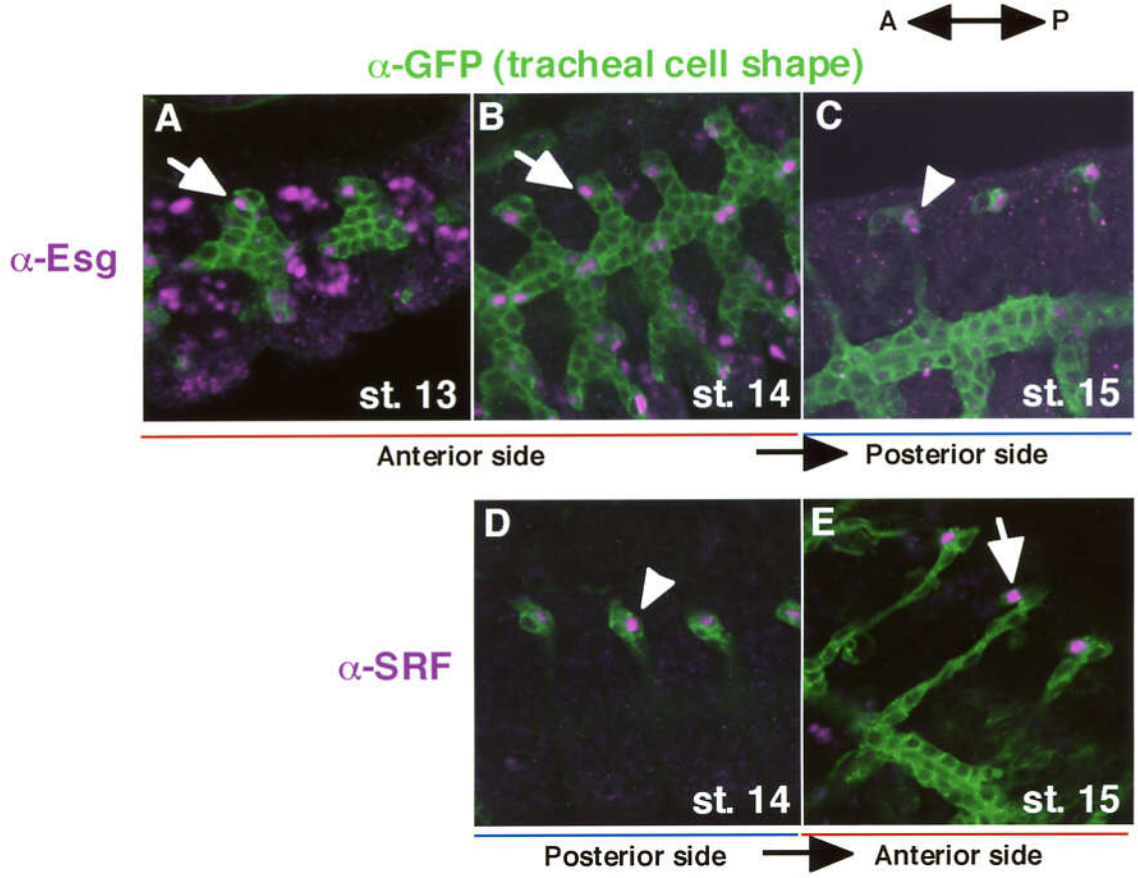

Fig. 13. Patterns of fusion and terminal cell markers during DB migration.
(A-E) Tracheal cells were detected by expression of membrane-bound GFP protein (*btl-Gal4 / UAS-gfpTTras*) visualized in green. Esg and SRF were detected by anti-Esg (A-C) and anti-SRF (D, E) antibody (purple), respectively. Escargot (Esg), a marker for fusion cells, initially begins to be expressed at stage 13, and its expression was located at the anterior side of DB tip (arrows in A, B). On the other hand, *Drosophila* serum response factor (SRF), a marker for terminal cells, is expressed at the posterior side of DB tip (arrowhead in D). After stage 15, the relative position of Esg and SRF expressing cells switched. Esg expression was detected at the posterior side of DB tip (arrowhead in C), and SRF expression was detected at the anterior side of DB tip (arrow in E).

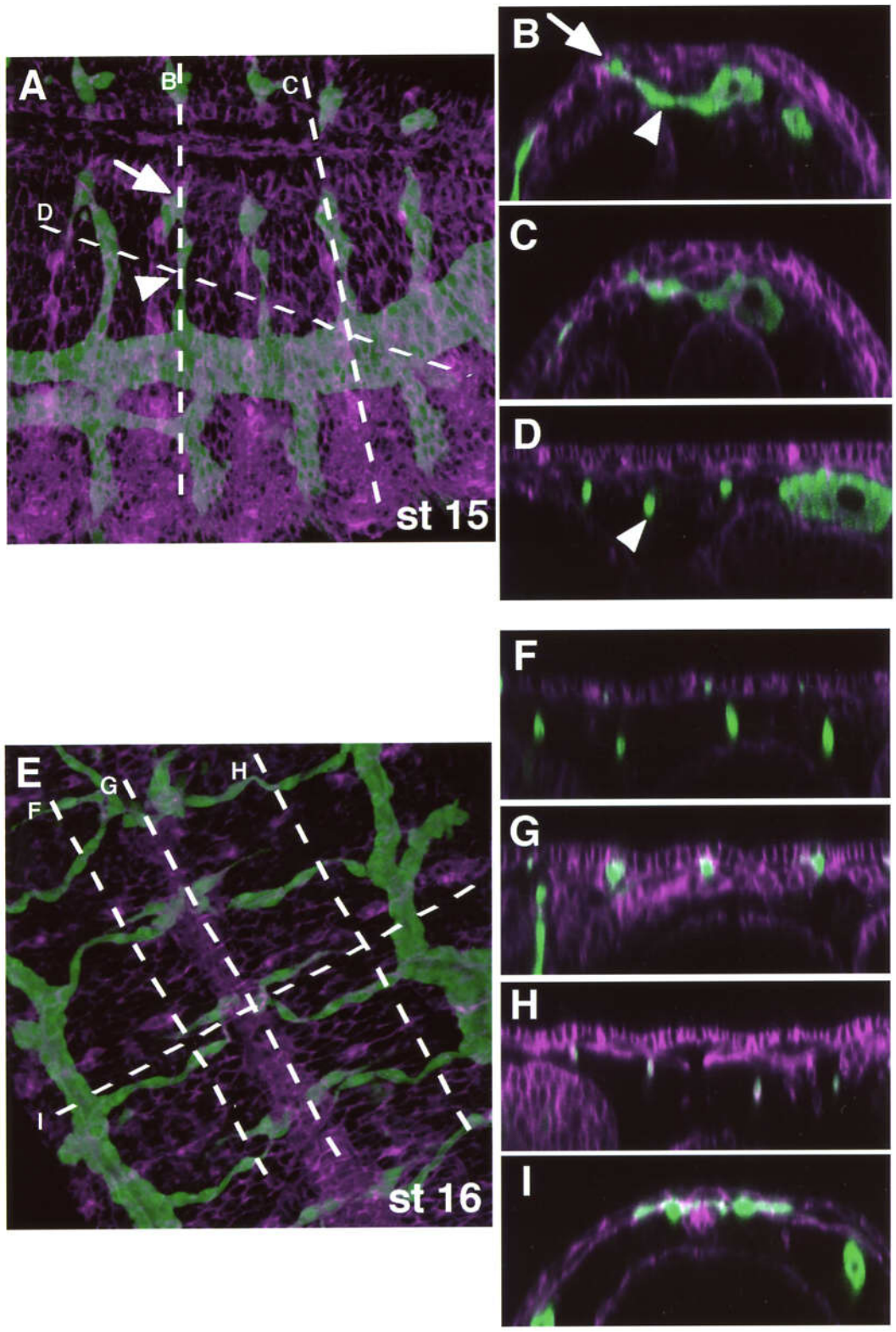

Fig. 14. Spatial relationship between DB and epidermal cells.
Tracheal cells were labeled with *trh-lacZ* (green). Outline of cells was labeled with anti-α-Spectrin (purple). B, C and D are the horizontal optical sections through the broken lines in A. F, G, H and I are also horizontal optical sections through the broken lines in E. Tips of DB including fusion and terminal cells are flattened in shape and contact with the basal surface of the epidermis. Stalk cells of DB do not contact with epidermal cells (arrowheads in A, B and D).

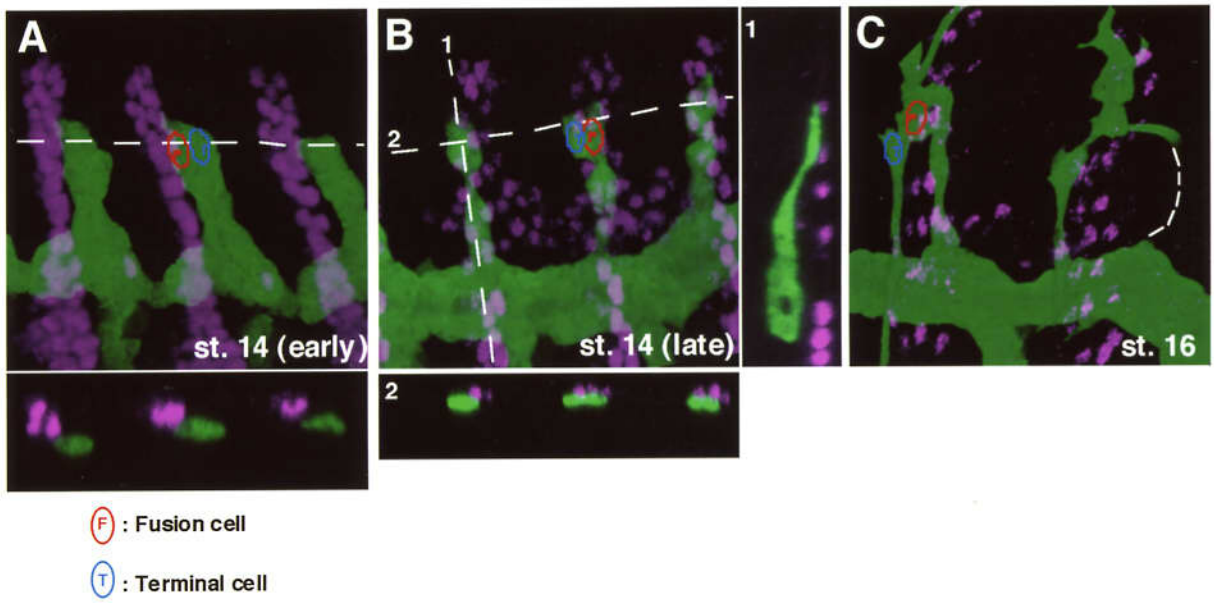

Fig. 15. Fusion cell always contacts with Engrailed expressing epidermal cells.
Tracheal cells were labeled with *trh-lacZ* (green). Engrailed was detected with anti-Engrailed (purple). At early stage 14, fusion cells contact with the posterior side of Engrailed expressing epidermal cells (En-stripes). After fusion cells switch their position with terminal cells, they continue to contact with Engrailed stripe. At stage 16, when terminal branches are located anterior to En-stripes, fusion cells continue to contact with En-stripes.

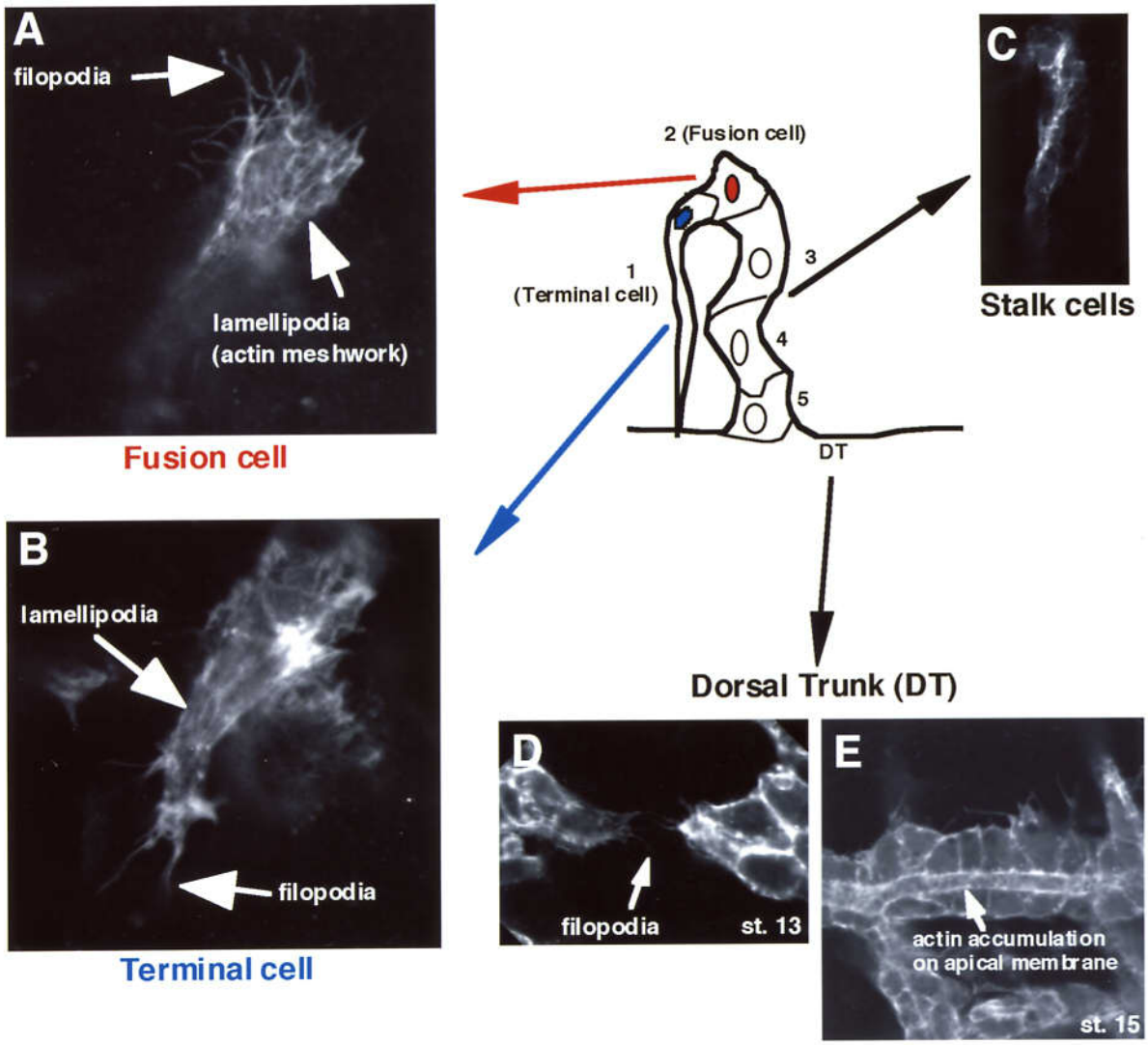

Fig. 16. Actin cytoskeleton in lamellipodia and Filopodia of fusion and terminal cells.

To check the distribution of F-actin in tracheal cells, GFP-moesin (F-actin binding GFP) was expressed in tracheal cells under control of *btl-Gal4*, and was detected by anti-GFP antibody. Lamellipodia (actin meshwork) and filopodia (needle like projection) structure were observed in fusion (A, D) and terminal cells (B), but not in stalk cells (C). Furthermore, GFP-moesin was also distributed around plasma membrane, and was specifically detected high accumulation at the apical membrane in all of tracheal network (E).

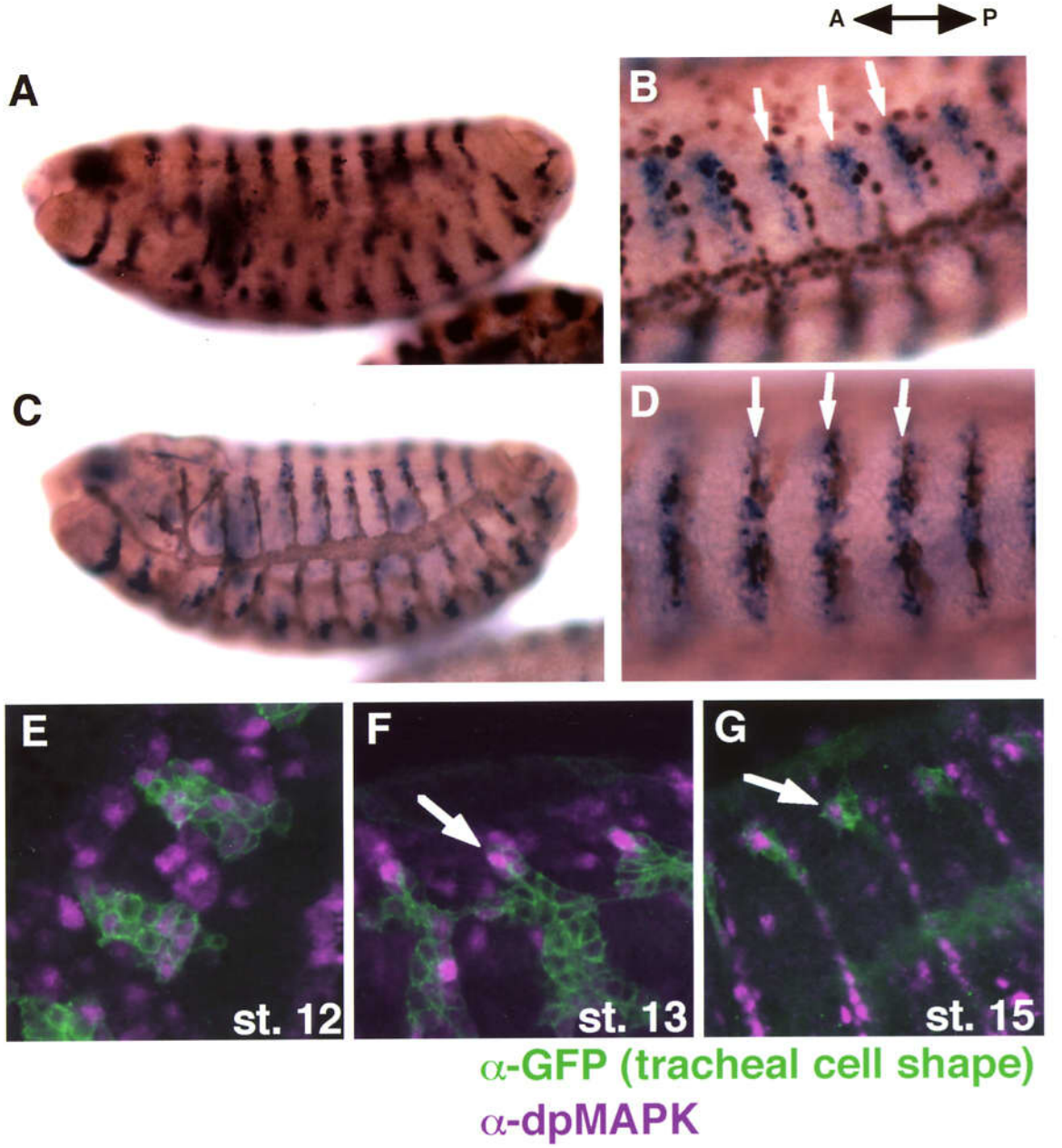

Fig. 17. *bnl* mRNA is expressed in stripe pattern on Wg stripe, but its pattern can't explain the DB migration pattern.

(A-D) *bnl* mRNA was visualized in purple. (B) Tracheal nuclei were detected by the expression of nuclear GFP-β-galactosidase fusion protein in brown. (C, D) Tracheal cells were specifically labeled with *trh-lacZ* (brown). (E-G) Tracheal cells were detected by expression of membrane-bound GFP protein (*btl-Gal4 / UAS-gfpTTras*) visualized in green. Activation of MAPK was detected by anti-dipohspho-MAPK (dpMAPK) antibody (purple). From stage 11 to 14, as previously reported, *bnl* mRNA is expressed at the position where DB will grow out in patch-like pattern (see Fig.1E, F). The patch-like pattern of *bnl* mRNA disappeared at stage 15, 16, and interestingly began to be expressed in stripe pattern on Wg stripe (arrows in B, D). DB migrated dorsally and anteriorly in close contact with anterior *bnl* mRNA stripe, but not posterior one. Although fusion and terminal cells switch their relative position, high accumulation of dpMAPK signal was always seen in the anterior side of migrating tip (arrows in F, G).

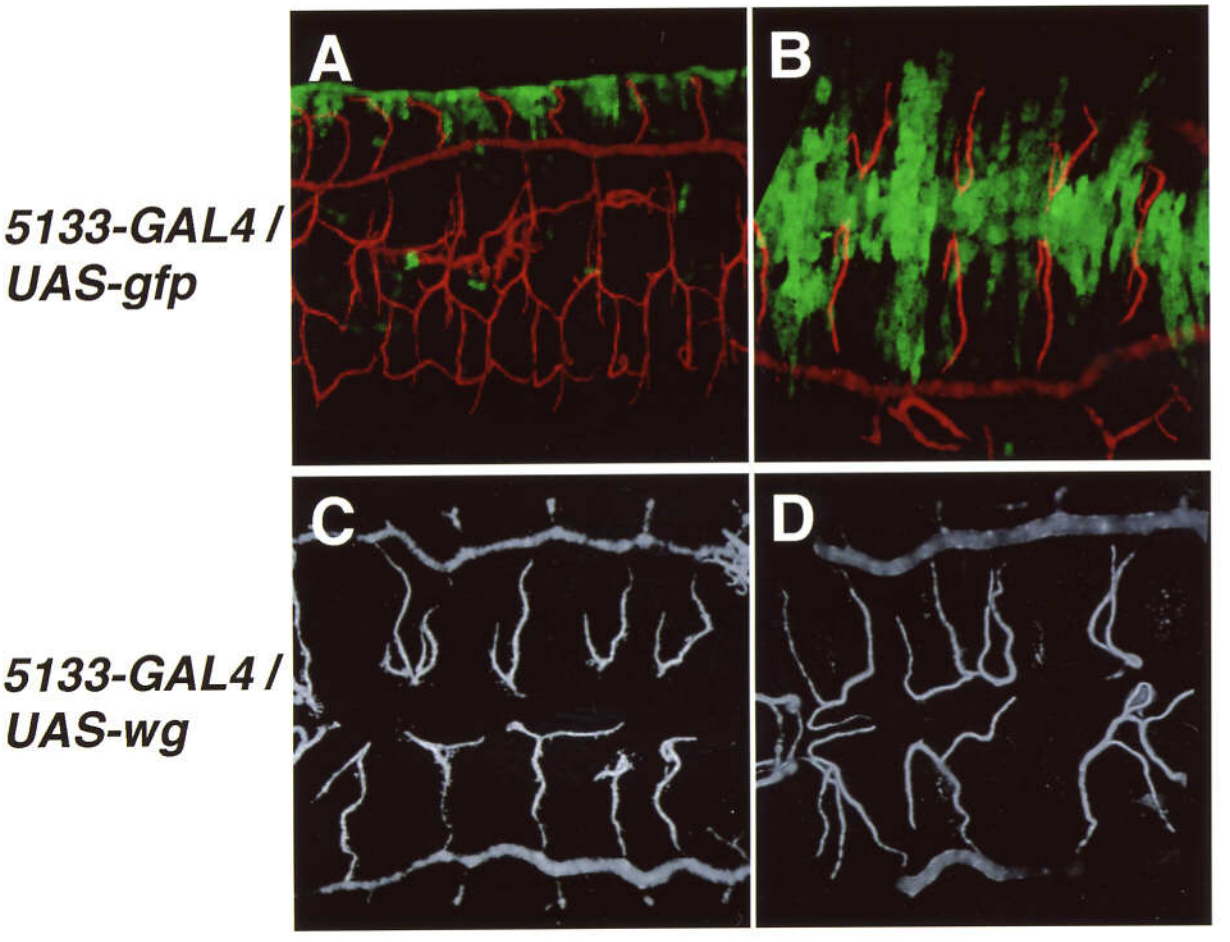

Fig. 18. Misexpression of Wg in the dorsal epidermis causes changes in cell fate and migration pattern of tracheal cells.
The tracheal lumen was detected with mAb 2A12 shown in red (A, B) or grey (C, D). The region of *5133-Gal4* expression was detected by anti-GFP antibody shown in green (A, B). As shown in C and D, the expression of Wg under control of *5133-Gal4* caused misrouting phenotype (C, D) with cell fate changes including extra expression of SRF (data not shown).

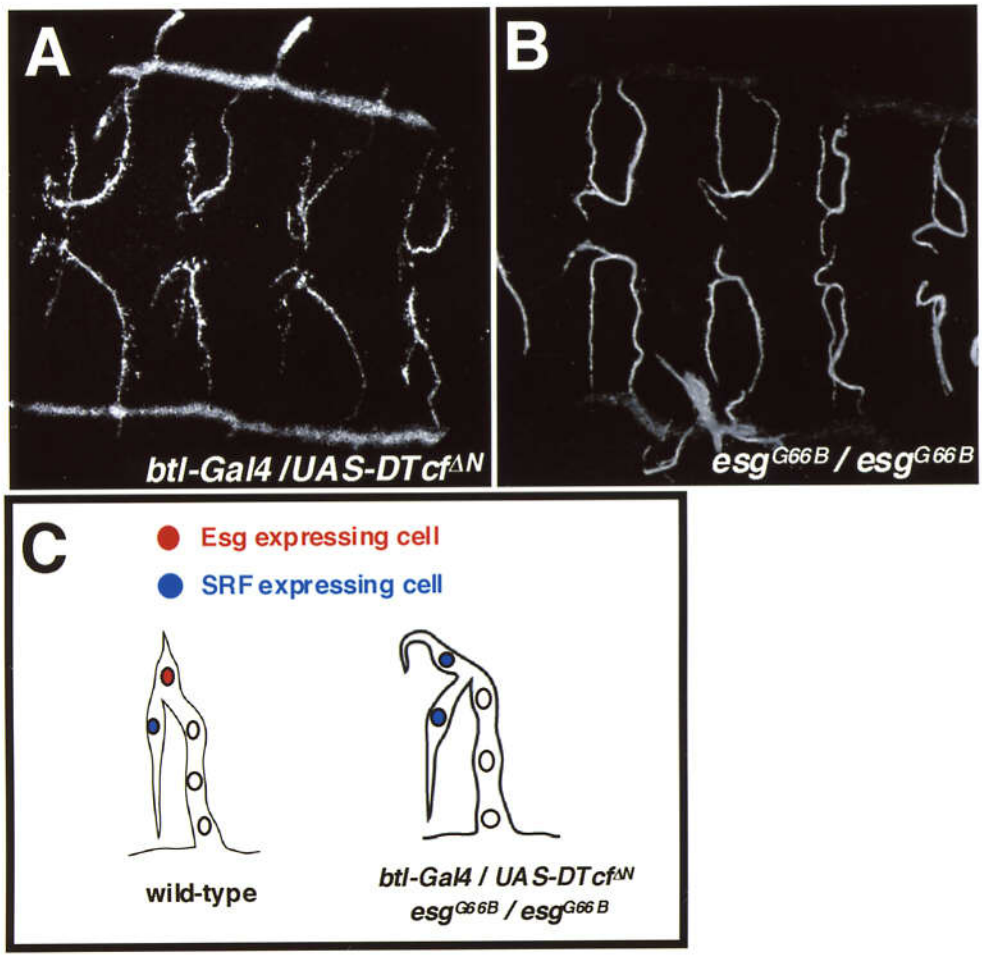

Fig. 19. Canonical Wg signaling and Esg expression in tracheal cell are not required for guidance of DB migration.

(A, B) The tracheal lumen was stained with mAb 2A12 shown in grey. (C) Schematic drawing of DB branching pattern seen in wild-type, *btl-Gal4 / UAS-DTcf$^{\Delta N}$* and *esg^{G66B} / esg^{G66B}* embryos. Dispite of tracheal cell fate changes, guidance of DB migration was normal in *btl-Gal4 / UAS-DTcf$^{\Delta N}$* or *esg^{G66B} / esg^{G66B}* embryos.

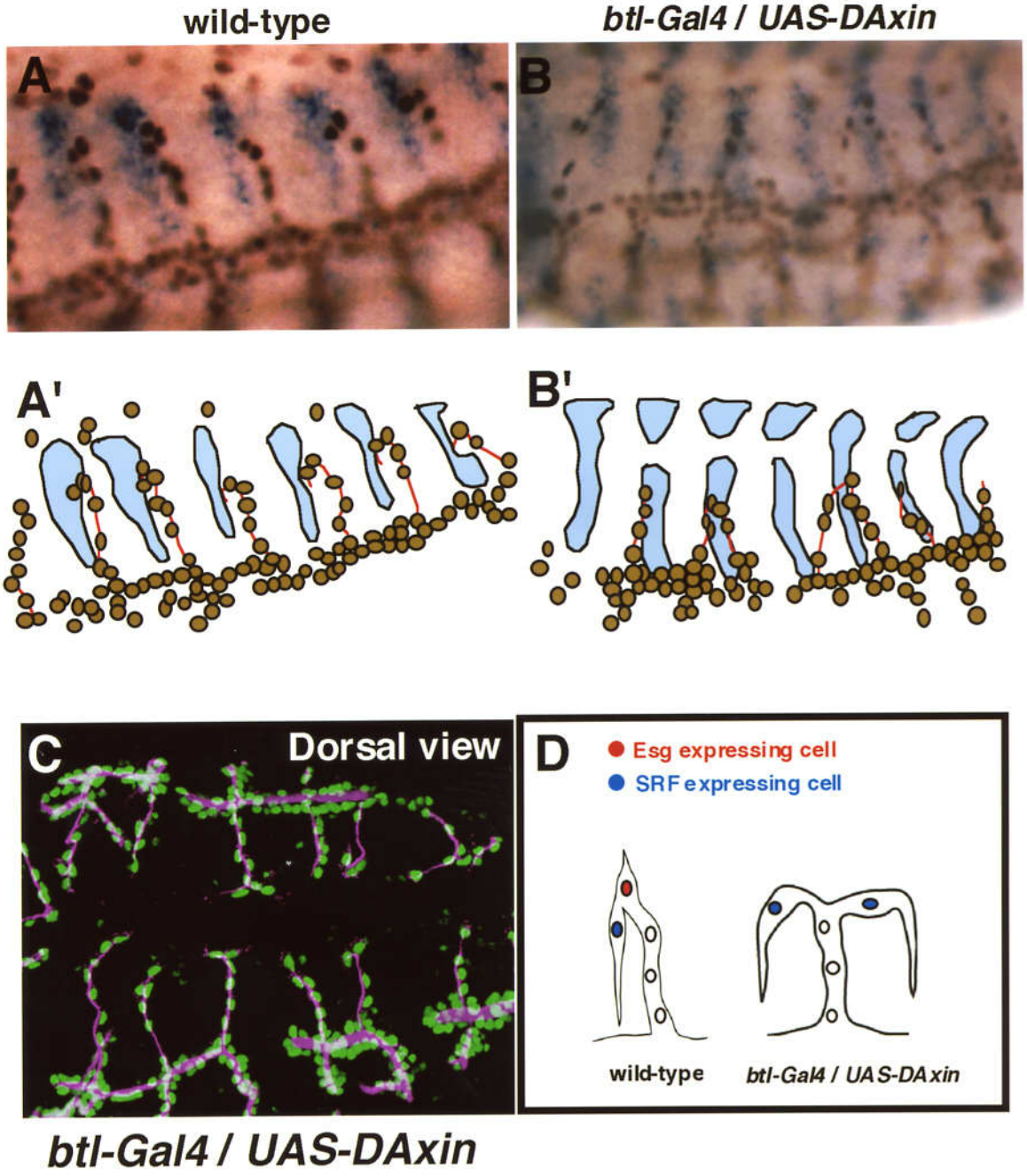

Fig. 20. *D*Axin-expressing trachea showed DB wrong migrations with tracheal cell fate changes.

(A, B) *bnl* mRNA was visualized in purple. Tracheal nuclei were detected by the expression of nuclear GFP-β-galactosidase fusion protein (*btl-Gal4 / UAS-gfpn-lacZ*) in brown (A, B) or green (C). The tracheal lumen was stained with mAb 2A12 shown in purple (C). (A', B' and D) Schematic drawing of DB migration pattern seen in wild-type or *btl-Gal4 / UAS-DAxin* embryos.

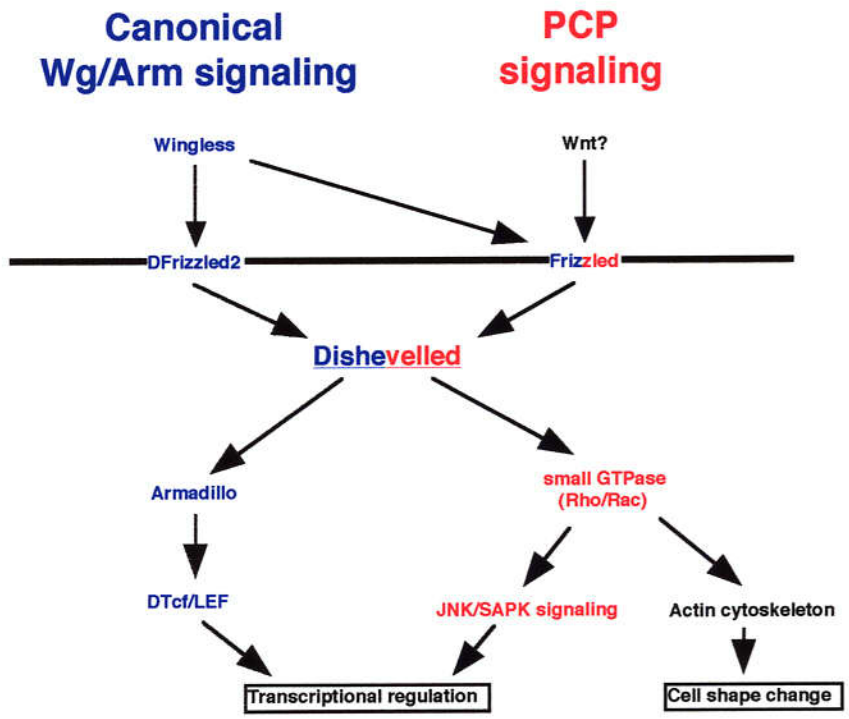

Fig. 21. Crosstalks between canonical Wg/Arm signaling and PCP signaling.
Canonical Wg/Arm signaling and PCP signaling are indicated by blue and red characters, respectively.

The number of misrouted DB (per embryo)	0	1	2	3
control (n=162)	161(99%)	1(1%)	0	0
dsh^{1} (n=187)	131(70%)	41(22%)	11(6%)	4(2%)

"n" is the number of embryo observed.

Table. 1. Dishevelled activity is required for guidance of DB migration.

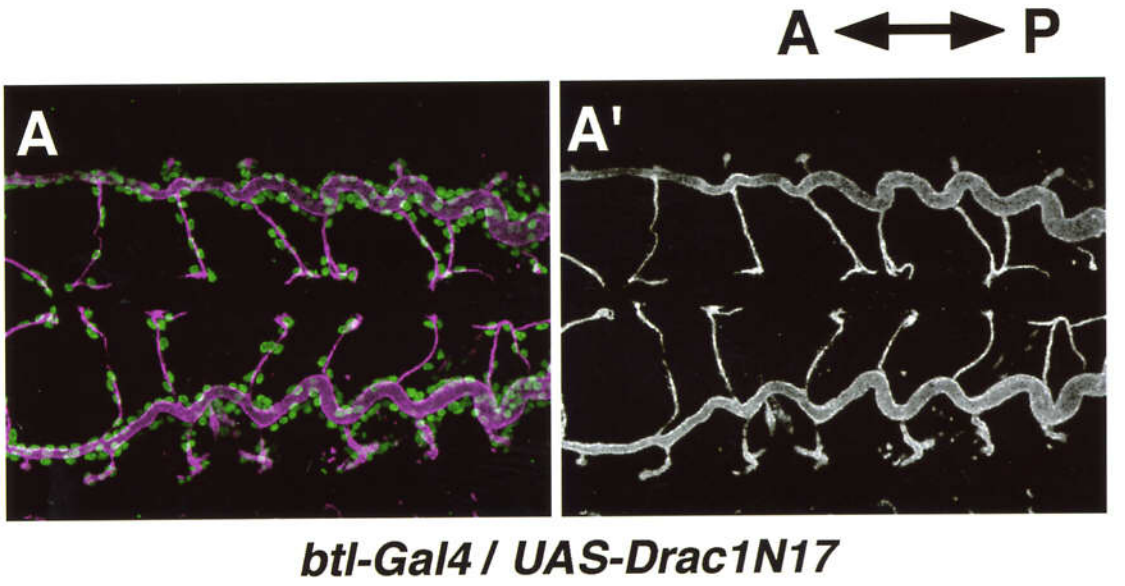

Fig. 22. Drac1 activity is required for directed tracheal migration but not migration itself.

(A) Tracheal nuclei were detected by the expression of nuclear GFP-β-galactosidase fusion protein (*btl-Gal4 / UAS-gfpn-lacZ*) in green. The tracheal lumen was stained with mAb 2A12 shown in purple (A) or gray (A'). Outgrowth of DB took place normally, but the direction of DB migration was abnormal in anteroposterior direction (compare with Fig. 12A). Expressions of Esg and SRF in tracheal cells were normal (data not shown).

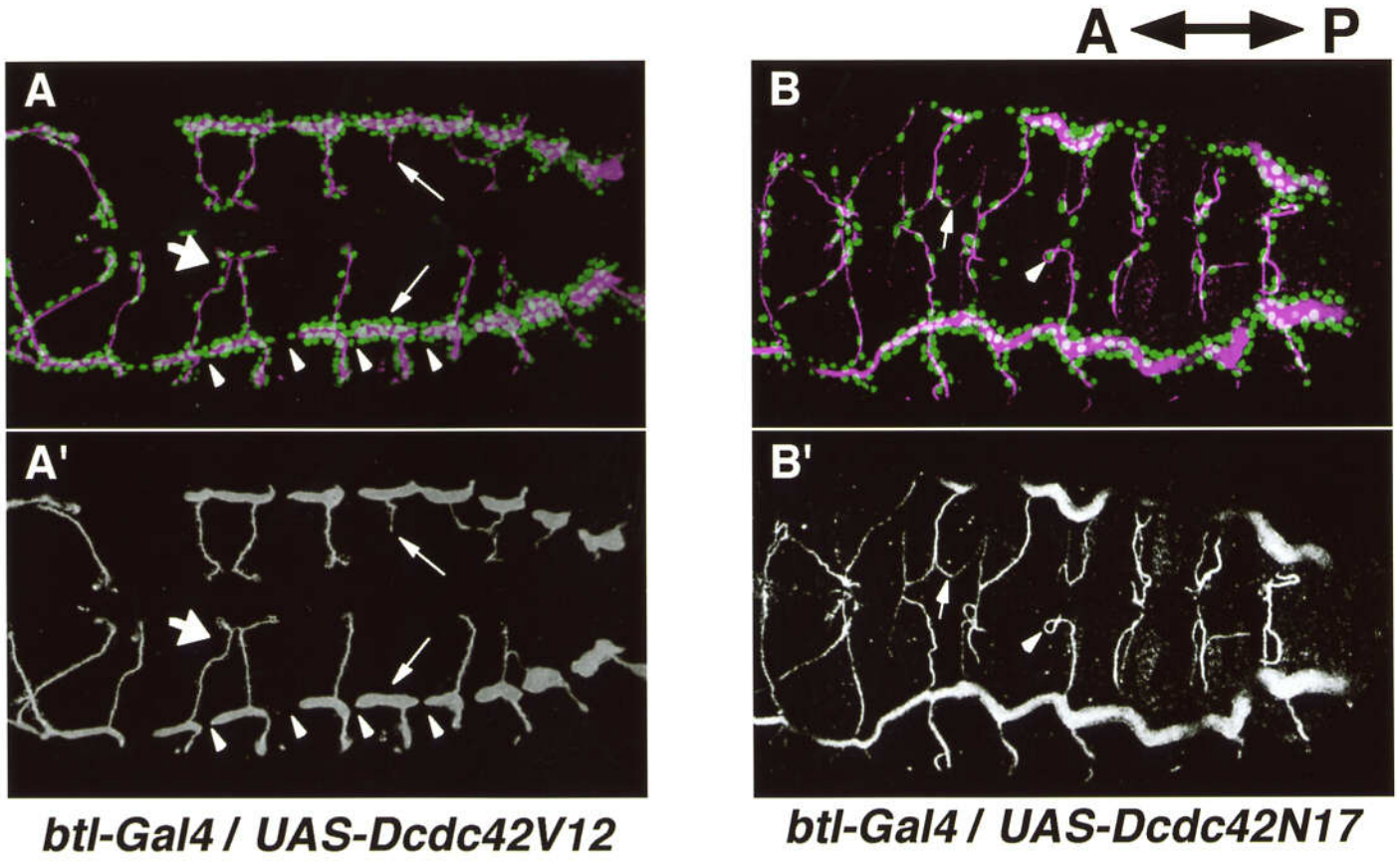

Fig. 23. Appropriate level of Dcdc42 activation is required for DB development.

(A, B) Tracheal nuclei were detected by the expression of nuclear GFP-β-galactosidase fusion protein (*btl-Gal4 / UAS-gfpn-lacZ*) in green. The tracheal lumen was stained with mAb 2A12 shown in purple (A, B) or gray (A', B'). (A) Constitutive-active form of Dcdc42, Dcdc42V12-expressing trachea showed severe phenotype including stalled DB (thin arrows in A, A'), misrouting of DB (thick arrow in A, A'), failure of DT fusion (arrowheads in A, A'). (B) Dcdc42N17, Dominant-negative form of Dcdc42 caused morformation of DB including extra (arrow in B, B') or coiled (arrowhead in B, B') terminal branches.

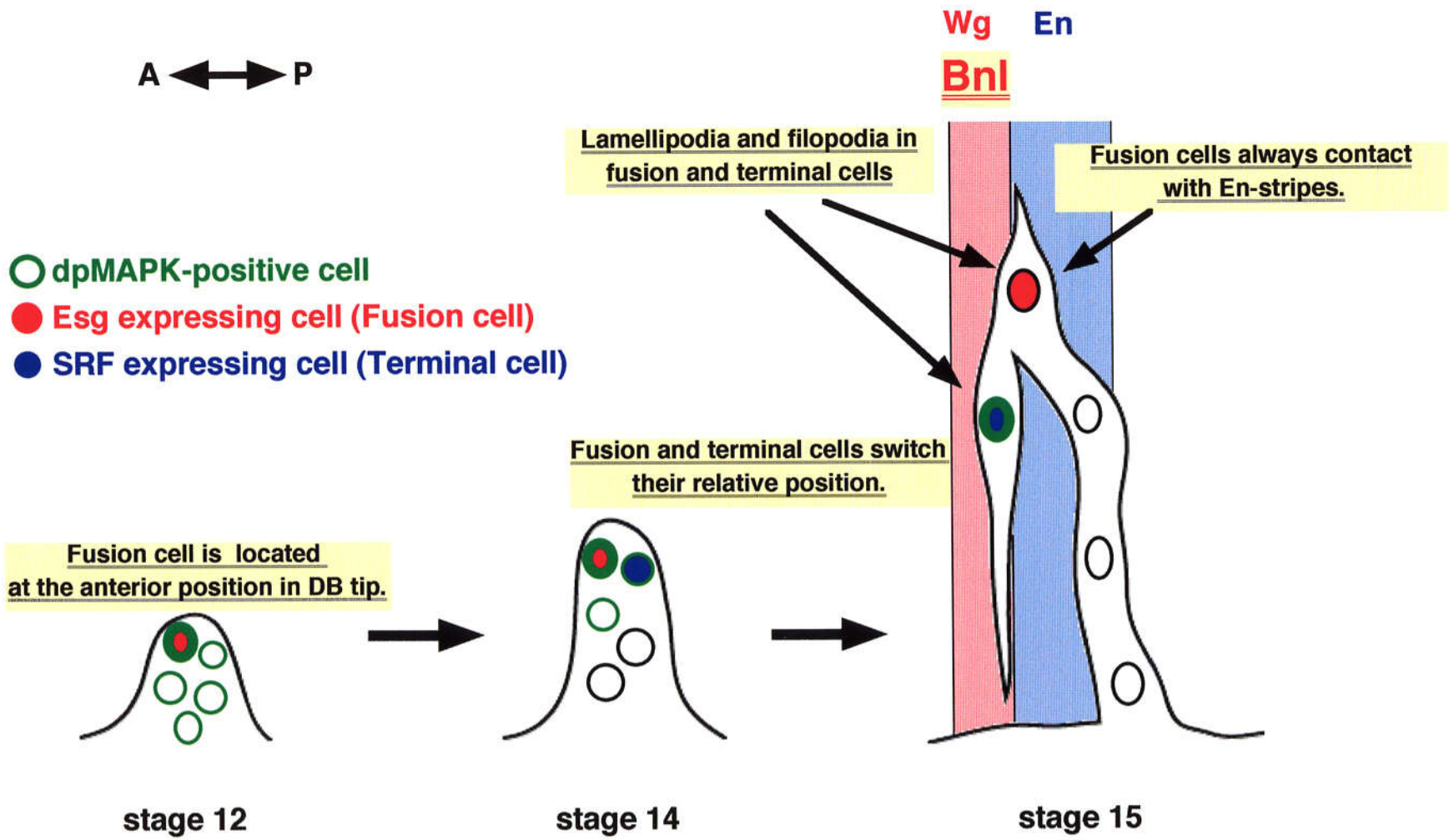

Fig. 24. Summary for DB development