

## wingless and its signalling pathway have common and separable functions during tracheal development

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### SUMMARY

The *Drosophila* tracheal tree consists of a tubular network of epithelial branches that constitutes the respiratory system. Groups of tracheal cells migrate towards stereotyped directions while they acquire specific tracheal fates. This work shows that the wingless/WNT signalling pathway is needed within the tracheal cells for the formation of the dorsal trunk and for fusion of the branches. These functions are achieved through the regulation of target genes, such as *spalt* in the dorsal trunk and *escargot* in the fusion cells. The pathway also aids tracheal invagination and helps guide the ganglionic branch. Moreover the wingless/WNT pathway displays

antagonistic interactions with the DPP (decapentaplegic) pathway, which regulates branching along the dorsoventral axis. Remarkably, the *wingless* gene itself, acting through its canonical pathway, seems not to be absolutely required for all these tracheal functions. However, the artificial overexpression of *wingless* in tracheal cells mimics the overexpression of a constitutively activated armadillo protein. The results suggest that another gene product, possibly a WNT, could help to trigger the wingless cascade in the developing tracheae.

Key words: *Drosophila*, wingless, WNT, Trachea, armadillo

### INTRODUCTION

The morphogenesis of branched tubular structures involves various cellular events, such as cell migration, cell shape changes and specification of different cell types to render a functional organ. The tracheal system of *Drosophila* has become an ideal model for tubulogenesis.

The *Drosophila* tracheal tree arises from ten pairs of segmental clusters of ectodermal cells, each invaginating to form a sac. From each sac, six primary branches, containing a fixed number of cells, organise into multicellular tubes by cell intercalation as they migrate towards stereotyped positions. Later on, most tip cells of the primary branches produce unicellular secondary branches. By the end of embryogenesis, some of the secondary branches make cytoplasmic processes that form the terminal branches. Other secondary branches fuse with each other, interconnecting the lumina to form a network. During tracheal morphogenesis, no cell division occurs; thus tracheal branching and fusion are both achieved by cell shape changes and by cell migration (Manning and Krasnow, 1993; Samakovlis et al., 1996a).

Each of the above steps is controlled by a set of genes. The different steps are genetically coupled, so that the genes that control an event also regulate the genes required for the next step (Samakovlis et al., 1996a). For instance, fibroblast growth factor (FGF) signalling first acts as a motogen to stimulate and guide branch pathfinding and then regulates target genes required for further branching (reviewed in Metzger and

Krasnow, 1999; Placzek and Skaer, 1999). The transforming growth factor  $\beta$  (TGF $\beta$ ) and epidermal growth factor (EGF) pathways are required for the guidance of specific branches (Llimargas and Casanova, 1997; Vincent et al., 1997; Wappner et al., 1997) while the Notch pathway helps to specify tracheal cell types (Ikeya and Hayashi, 1999; Llimargas, 1999; Steneberg et al., 1999). These signalling pathways are used repeatedly during the development of several structures of *Drosophila* and other organisms. They control cellular functions needed during tubulogenesis, not only in the tracheae but also in other tubular networks such as the mouse lung (reviewed in Metzger and Krasnow, 1999; Placzek and Skaer, 1999). Although much has been learnt about tracheal development in recent years, important questions still remain unresolved: for example, how do the tubes become so diverse and how are tracheal cells assigned to different branches?

As with these other signalling pathways, WNT signalling is also involved in a variety of developmental processes. For instance, it acts in the specification of embryonic axes in *Xenopus*, in segmentation in *Drosophila*, in mesoderm induction in *Caenorhabditis elegans* and in oncogenesis in mice (reviewed in Arias et al., 1999; Cadigan and Nusse, 1997; Klingensmith and Nusse, 1994; Willert and Nusse, 1998). wingless (WG)/WNT proteins encode secreted molecules. In *Drosophila* the secretion of WG depends on the activity of porcupine (POR). WG/WNT interact with the frizzled (FZ) family of transmembrane receptors activating the downstream protein dishevelled (DSH). This leads to the inactivation of a

protein complex formed by zeste-white 3 (ZW3), adenomatous polyposis coli (APC) and Axin (AXN) allowing the accumulation of armadillo (ARM)/ $\beta$ -catenin in the cytoplasm. Upon receipt of the WG signal, ARM is thought to enter the nucleus and interact with DNA-binding proteins of the TCF/LEF family, resulting in the activation of *wg* target genes. In the absence of WG, free cytoplasmic ARM is targeted for destruction by the AXN/APC/ZW3 complex, thus preventing activation of target genes. Then ARM becomes confined to adherens junctions where it associates with the intracellular domain of E-cadherin, a cell-adhesion molecule (reviewed in Bienz, 1999; Cadigan and Nusse, 1997; Cavallo et al., 1997; Cox and Peifer, 1998; Dierick and Bejsovec, 1999; Willert and Nusse, 1998). Thus, ARM/ $\beta$ -catenin has a dual function: it transduces the WNT signal that affects gene expression and is required for cell adhesion. Both roles are separable as there are mutations that affect each of these two functions independently (Orsulic and Peifer, 1996).

The *Drosophila wg* gene is the best known member of the Wnt family. *wg* and the members of its canonical pathway act in the specification of cell fates within several embryonic organs. Does the WG pathway help to specify cell fates in the tracheae? The tracheae are thought to begin as a homogeneous population of cells that then diversify (reviewed in Zelzer and Shilo, 2000). The results indicate that the WG/WNT pathway, from the membrane to the nucleus, is essential for different aspects of tracheal development. It is autonomously required in the tracheal cells to control at least two different tracheal events: formation of the dorsal trunk and specification of the fusion cells. WG/WNT signalling interacts with other pathways and modulates other genes known to affect these different branching events. Remarkably, *wg* itself does not seem to be essential for all the tracheal functions fulfilled by its canonical pathway. Nevertheless, in experimental situations, *wg* can affect them, suggesting that another *Wnt* gene could help to trigger the cascade.

## MATERIALS AND METHODS

### *Drosophila* strains and genetics

The following amorphic or loss of function alleles were used: *wg<sup>CX4</sup>* (Baker, 1987); *Dff(2L)RF* (Tiong and Nash, 1990); *arm<sup>XM19</sup>*, which is a strong hypomorph allele that impairs WG signal without affecting adherens junctions function (Cox et al., 1999); *dTCF<sup>2</sup>* (van de Wetering et al., 1997); *dsh<sup>v26</sup>* (Geer et al., 1983); *fz<sup>H51</sup>* (Jones et al., 1996); *fz<sup>2C1</sup>* (Chen and Struhl, 1999); *Dff(5)*, which removes both *sal* and *sal*-related (Barrio et al., 1999), *por<sup>PB16</sup>* (Eberl et al., 1992) and *tkv<sup>8</sup>* (Nusslein-Volhard et al., 1984). *wg<sup>LL114</sup>* (Bejsovec and Martinez Arias, 1991) is a conditional ts allele.

To remove the maternal contribution, germ line clones were induced using the FRT/FLP/ovoD method (Chou et al., 1993). Females carrying *arm<sup>XM19</sup>FRT101/ovo<sup>D1</sup>FRT101*, *dsh<sup>v26</sup>FRT101/ovo<sup>D1</sup>FRT101*, *por<sup>PB16</sup>FRT9.2/ovo<sup>D2</sup>FRT9.2* or a doubly mutant chromosome *fz<sup>H51</sup>fz<sup>2C1</sup>FRT2A/ovo<sup>D1</sup>FRT2A* were heat shocked at 37°C for 1 hour during the second or third instar larvae.

The following P(lacZ) tracheal enhancer traps described in Samakovlis et al. (1996a) were used: Fusion-1 (*esg-lacZ*), Fusion-2, Fusion-3, Terminal-1 (*pruned*) and Pantip-4 (*adrift*). *Repo-lacZ* was used to visualise the glial cells (Xiong et al., 1994). *sal-TSE-lacZ* contains the enhancer responsible for *sal* expression in the dorsal trunk (DT) (Kuhnlein and Schuh, 1996).

The following UAS-lines were used: *UASsal* (de Celis et al., 1996),

*UASwg* (Lawrence et al., 1995), *UASarm\** (UAS-armS10; Pai et al., 1997), *UASdTCF<sup>DN</sup>* (van de Wetering et al., 1997), *UAStkv\** (UAS-tkvQ253; Lecuit et al., 1996) and *UASEGFR\** (UAS-tor4021-EGFR, Dominguez et al., 1998).

The *btlGal4* line (Shiga et al., 1996) drives the expression of UAS constructs in the tracheal cells from stage 11; *p127Gal4* (Steneberg et al., 1998) in the tracheal fusion cells, and LP39 (*salGALA*, provided by M. Calleja and G. Morata) in a *sal* pattern of expression.

To visualise the tracheal cells embryos bearing the *btlGal4-UAStauGFP* in wild-type or other backgrounds were stained for  $\alpha$ -GFP. To maximise the efficacy of the Gal4/UAS system the embryo collections were carried out at 29°C.

To identify mutant embryos, 'blue balancers', of the first, second and third chromosomes were used: FM7-*ftz-lacZ*; Cyo-*hb-lacZ*, TM3-*ftz-lacZ*.

### Embryo fixation and staining

For HRP histochemistry embryos were fixed in PEM-FA (Patel, 1994) for 20-30 minutes and stained with the Vectastain-ABC kit according to standard protocols. The mouse monoclonal mAb2A12 (developed by N. Patel and C. Goodman and obtained from the Developmental Studies Hybridoma Bank) was used at 1:5 to stain the lumen of the tracheal system from stage 13/14 onwards. The mouse mAb22C10 (from N. Patel) was used at 1:10 to visualize the nerves. The rabbit  $\alpha$ -spalt (from R. Schuh) was used at 1:30 and the guinea pig  $\alpha$ -knirps (developed by J. Reivitz and provided by M. Ruiz-Gomez) was used at 1:1000. The rabbit  $\alpha$ - $\beta$ -galactosidase (Cappel) was used at 1:1000 to 1:1500 to detect tracheal markers and blue balancers. The rabbit  $\alpha$ -phosphorylated histone H3 (Upstate Biotechnology) was used at 1:500 to detect cell proliferation. The rabbit  $\alpha$ -GFP (Molecular Probes) was used at 1:500 to visualise the tracheal cells. The purified mouse monoclonal  $\alpha$ -wingless (Developmental Studies Hybridoma Bank) was used at 1:200. Biotinylated secondary antibodies (Jackson) were used at 1:300. To optimise double staining embryos were first stained in black with NiCl<sub>2</sub> and then in brown. Embryos were dehydrated, rinsed in acetone, and transferred to a 1:1 mixture of acetone and Durcupan (Fluka) overnight. After evaporation of acetone, embryos were observed and photographed in a Zeiss Axiophot.

Embryos were staged according to Campos-Ortega and Hartenstein, 1997. Images were processed in Adobe Photoshop.

## RESULTS

### The WG/WNT pathway is required for several aspects of tracheal development

As tracheal formation depends on the action of different signalling pathways, I asked whether the WG pathway is also required. Uemura et al (1996) already reported that null *arm* mutants exhibit a tracheal phenotype. To see which part of the phenotype (if any) was due to a block in WG transduction, a strong hypomorphic allele of *arm* that compromises the signalling activity without affecting the adhesion properties, *arm<sup>XM19</sup>* (Cox et al., 1999; Peifer and Wieschaus, 1990), was used. Zygotic *arm<sup>XM19</sup>* mutants displayed three main kinds of tracheal defects (Figs 1H and 2D-F). (1) The dorsal trunk (DT), which in the wild type connects the adjacent tracheal metameres, is completely missing or very much reduced. (2) There are no branch fusions and thus the ten tracheal metameres are completely disconnected. (3) The ganglionic branch (GB) is misrouted.

The above results prompted the analysis of the tracheal requirements of other members of the WG pathway. *wg<sup>LS</sup>*

(*wg<sup>L114</sup>*) mutants shifted at the restrictive temperature from early stage 11 to stage 13 displayed severe defects of lateral trunk (LT) and dorsal branch (DB) fusion, as well as defects in GB guidance. However, signs of DT differentiation were generally observed and the DT was continuous between some metameres (Fig. 1D). Similarly, in null *wg* mutants (*wg<sup>CX4</sup>*) or

in mutants with a deficiency that removes *wg* (*Df(2R) RF*; data not shown), there is no LT fusion but some fragments of DT are formed and often fused, even though the embryo and the tracheae are generally malformed. In addition, null mutants for *wg* also displayed tracheal invagination defects (Fig. 1C; Uemura et al., 1996). The results suggest that *wg* is necessary for GB guidance and fusion of LT and DB. However, although the DT is sometimes affected, *wg* does not seem to be absolutely essential for fusion and formation of the DT. On the other hand, *wg* misexpression in all tracheal cells, by means of the *btlGal4* line, produced a phenotype totally penetrant and reproducible: the DT is thicker than in the wild type, while the visceral branches (VB, branches that reach the gut) were missing or very much reduced (Fig. 1B). This DT hypertrophy can be regarded as opposite to the absence of DT in *arm<sup>XM19</sup>* mutants.

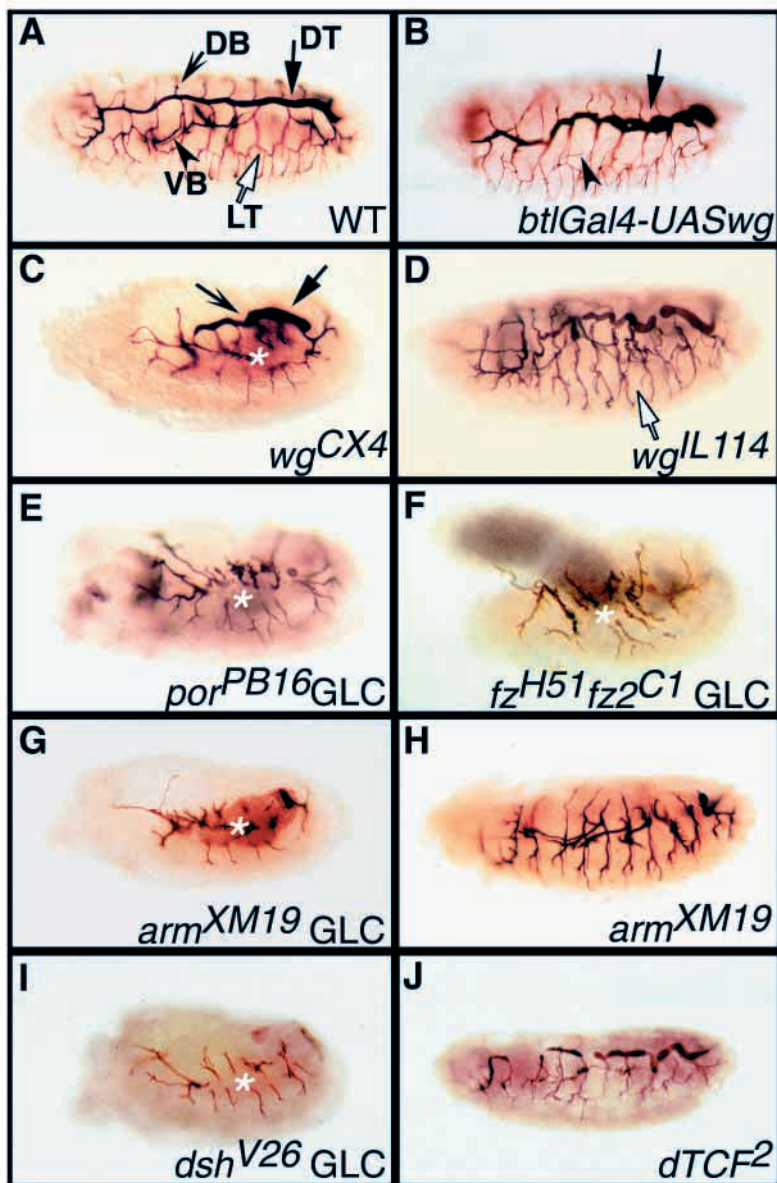
*arm<sup>XM19</sup>* germline clones also showed invagination defects, like *wg* mutants (Fig. 1G). Other members of the WG pathway, including *dsh*, *por* and the receptor complex formed by *fz/fz2*, have a tracheal phenotype comparable with that of *arm<sup>XM19</sup>* germ line clones: the embryos were defective in tracheal invagination, branch fusion and DT formation (Fig. 1E,F,I). It is difficult to assess the guidance of the GBs in these embryos because they are so malformed. Zygotic mutants for *dTCF* display similar tracheal defects to those of zygotic *arm<sup>XM19</sup>*, although weaker (Fig. 1J), probably due to a rescue by the strong maternal contribution of *dTCF*.

These data suggest that all members of the WG pathway are required for tracheal invagination, fusion of some branches and GB guidance. In addition, the WG pathway, excluding *wg* itself, is essential for formation and fusion of the DT. The phenotype of *por* mutants and the fact that *wg* is potentially able to affect DT formation, suggest that another gene, possibly a *Wnt*, could help to activate the cascade to form the DT. For this reason, in this work the pathway will be referred as the WG/WNT pathway.

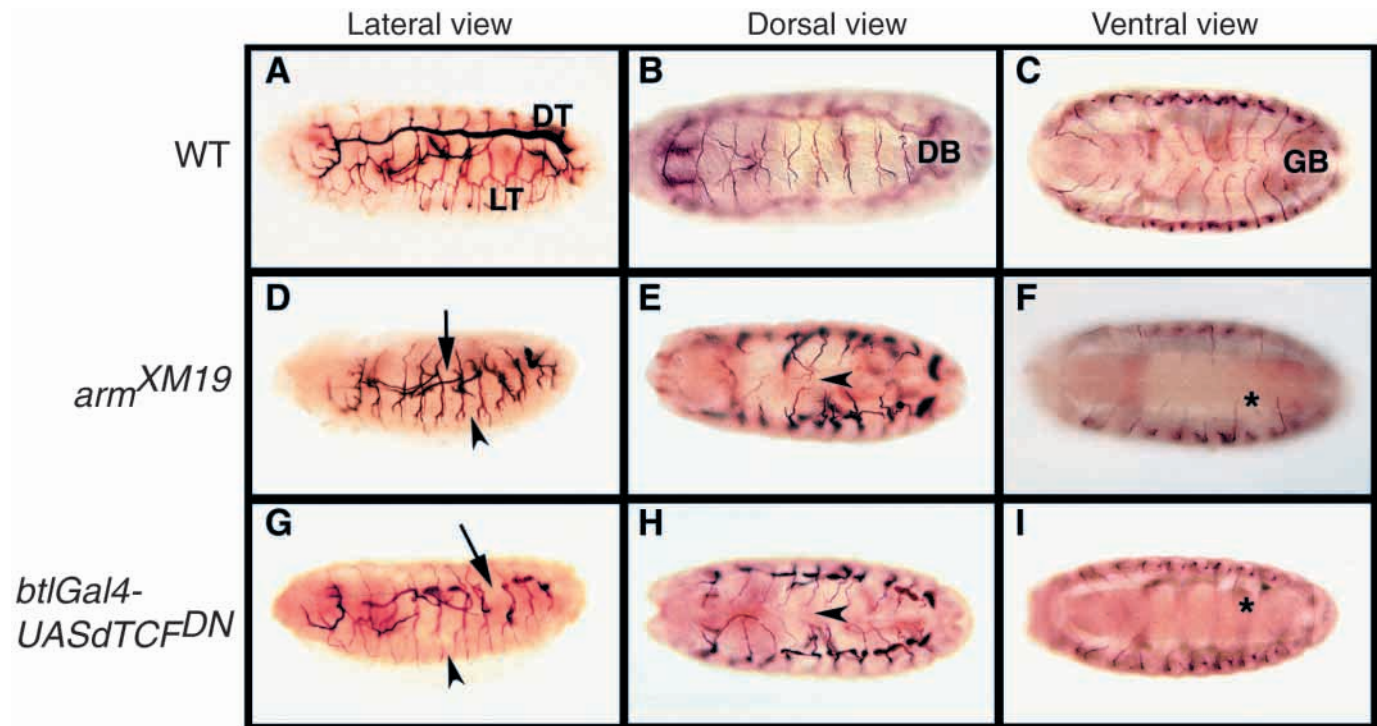
### The WG/WNT pathway is required within the tracheal cells

Are all these phenotypes due to failure of the WG/WNT pathway in the tracheal cells themselves or elsewhere in the embryo? To test for autonomous requirement in the tracheal cells the pathway was blocked by using a dominant negative form of *dTCF* (*dTCF<sup>DN</sup>*). *dTCF<sup>DN</sup>* lacks its N-terminal domain and it is not able to bind to ARM (van de Wetering et al., 1997). The expression of *dTCF<sup>DN</sup>* in all tracheal cells resulted in absence or reduction of DT and complete lack of branch fusion (Fig. 2G,H), as in the zygotic *arm<sup>XM19</sup>* mutants. This indicates that the pathway is required within the tracheal cells for these two tracheal events.

GBs were also misrouted (Fig. 2I) when *dTCF<sup>DN</sup>* was activated by the *btlGal4*, suggesting that there is also an autonomous requirement in the tracheal cells. In the wild type, the GB grows towards the ventral nerve cord (VNC) in association with the



**Fig. 1.** The WG/WNT pathway is required for tracheal development. Lateral views of embryos at late stages of embryogenesis stained with mAb2A12 to visualise the lumen of the tracheae. Anterior is to the left and dorsal is upwards in all the figures. (A) Wild-type embryo. *arm*, *fz+fz2*, *por* and *dsh* germline clones. The dorsal branch (DB), dorsal trunk (DT), lateral trunk (LT) and visceral branch (VB) are indicated by the different arrows and arrowheads. (B) Phenotypes after of ectopic expression of *wg* in all tracheal cells: the DT (arrow) is hypertrophied and the VBs (arrowhead) are totally or partially missing. In *wg* mutants (C,D) some DT fragments (arrows) are formed and often fused. The embryos display defects of tracheal invagination (white asterisk in C), branch fusion (white arrowhead in D) and loss of DBs (smaller arrow in C). (E-G,I) Defects of tracheal invagination (white asterisks), the DT is completely missing and there are no branch fusions. Similar defects are observed in zygotic *arm* (H) and *dTCF* (J) mutants.



**Fig. 2.** The reduction of *arm* or *dTCF* function causes tracheal defects. Stage 15 or 16 embryos stained for the tracheal lumen with mAb2A12. (D-F) In zygotic *arm* mutants, the dorsal trunk (DT) is missing (arrow in D) and the lateral trunk (LT) and dorsal branches (DB) fail to fuse (arrowheads in D and E). Only few ganglionic branches (GB) enter the VNC (asterisk in F) but they do not follow the normal stereotyped pathway (G-I) Expression of *dTCF*<sup>DN</sup> produces a similar tracheal phenotype. The DT is partially missing (arrow in G); the weakness of this phenotype compared to the *arm* phenotype might be due to late and/or insufficient accumulation of *dTCF*<sup>DN</sup>. LT and DBs are unfused (arrowheads in G and H) and GBs do not reach the proper position (asterisks in I).

intersegmental nerve. Just before entering the VNC, the GB changes substratum to associate with the segmental nerve; the exit glia is associated with this switch. The GB then migrates along the VNC following a stereotyped pathway in association with the segmental nerve and glial cells, such as the longitudinal glia (Fig. 3A,D; Englund et al., 1999). When *dTCF*<sup>DN</sup> was expressed in all tracheal cells or in *arm*<sup>XM19</sup> mutants, GBs associated normally with the intersegmental nerve as they grew towards the VNC, but they often failed to switch and associate with the exit glia and the segmental nerve before entering it. These GBs remained floating in the body cavity or inside the VNC without properly associating with the segmental nerve or the longitudinal glia (Fig. 3). The tip cells of these GBs express the correct markers, such as Terminal-1 (Affolter et al., 1994; Guillemain et al., 1996) and *adrift* (Englund et al., 1999) (data not shown). However it should be noted that the *btlGal4* line is also expressed in the midline glia. Thus, more experiments are needed to precisely determine where the WG/WNT pathway acts to help GB guidance.

In the following sections the role of the WG/WNT pathway during DT formation and branch fusion will be described in detail.

### Dorsal trunk formation

*arm* is required for *spalt* expression in the dorsal trunk

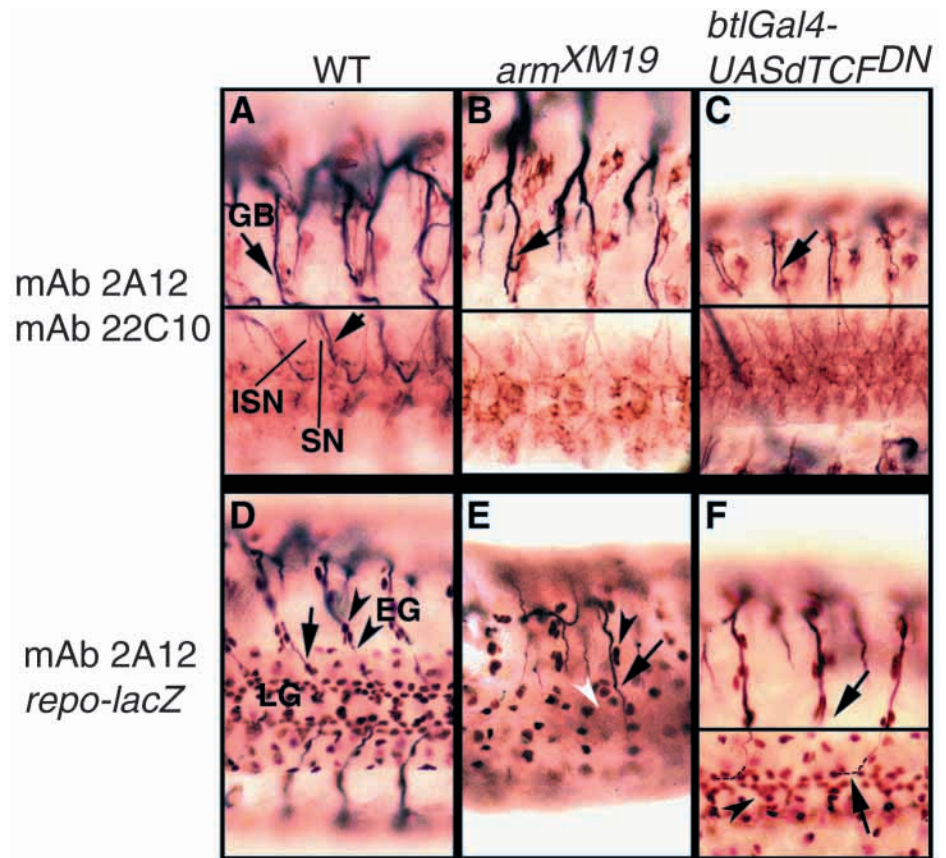
The DT is the main longitudinal branch and connects the tracheal tree to the exterior. DT cells remain cuboidal throughout development (Llimargas and Casanova, 1999;

Samakovlis et al., 1996a) and do not express the transcription factor knirps (KNI) (Fig. 4A,G), which is regulated by the DPP pathway (Chen et al., 1998; Vincent et al., 1997). Instead they express spalt (SAL) (Fig. 4A,D), which encodes a transcription factor required for DT formation (Kuhnlein and Schuh, 1996). Could the WG/WNT pathway be another cue that acts to distinguish the DT from the rest of tracheal branches?

In *sal* mutants, the DT fails to form a continuous branch (Kuhnlein and Schuh, 1996). In *arm*<sup>XM19</sup> mutants, the presumptive DT cells invaginated perfectly but did not migrate or join with other tracheal branches. Only occasionally they expressed low levels of *kni* (Fig. 4H). Most DT cells remained clustered in the position where the DT is normally formed (Fig. 4B). This correlates with the absence of *sal* expression in precisely these presumptive DT cells (Fig. 4E). Remarkably, the early expression of *sal* in broad regions overlapping with the dorsal part of all tracheal placodes (not shown) as well as *sal* expression in other structures is maintained (Fig. 4E). This suggests that the WG/WNT pathway regulates DT formation by controlling *sal* expression in DT cells. *sal* is still expressed in the fragments of DT in *wg*<sup>CX4</sup> embryos (not shown).

To test whether ARM exerts part of its function through *sal* in the DT, epistatic experiments were carried out. The misexpression of a wild type form of *sal* in all tracheal cells (Fig. 5A) partially rescued the lack of DT produced by *arm*<sup>XM19</sup> mutants. DT cells were able to migrate anteriorly and posteriorly, but the branches did not fuse (compare Fig. 5B with Fig. 2D). Moreover, loss of *sal* function suppressed the

**Fig. 3.** Pathfinding of the ganglionic branches (GBs). Each image shows three to four GBs of embryos at stage 15 or 16. To show the whole path, parts A-C and F are composed of two pictures (separated by a black line) of the same GBs. The upper part of the panel is focused on the lateral part of the embryo and the lower part on the ventral nerve cord (VNC). (A-C) Embryos stained for the tracheal lumen with mAb2A12 (black) and for the nerves with mAb22C10 (brown). The GBs (arrows) of WT (A) switch from the intersegmental nerve (ISN) to the segmental nerve (SN) just before entering the VNC. Many GBs (arrows) of *arm* and *dTCF<sup>DN</sup>* mutants (B,C) fail to switch from the ISN to the SN and to follow the SN inside the VNC. (D-F) Embryos bearing a *repo-lacZ* reporter stained for the tracheal lumen with mAb2A12 (black) and for the glia with  $\beta$ -gal (brown). (D) WT GBs (arrows) are in close contact with different glial populations such as the exit glial cells (EG, arrowheads) and the longitudinal glia (LG). GBs (arrows) of *arm* and *dTCF<sup>DN</sup>* mutants (E,F) normally associate with *repo* positive cells in the lateral part of the embryo (black arrowheads). However, in many cases these GBs fail to associate with the EG migrating erratically in a superficial plane of focus (white arrowhead in E indicates the position of the LG in a much lower plane of focus). In other cases the GB (highlighted with a broken line in F) enters the VNC but does not cross the LG ventrally (arrowhead in F).



effects of overactivating the WG/WNT pathway by means of a *UASwg*. The phenotype observed was almost indistinguishable from that of *sal* mutants (Fig. 5C), except for the VBs that remain partially reduced (compare Fig. 5D with Fig. 1B).

The WG/WNT pathway is required in *sal*-expressing cells to form the DT

The above results suggest that WG/WNT pathway regulates *sal* in DT cells. To see whether the pathway is needed precisely in the *sal* domain, *dTCF<sup>DN</sup>* was expressed by means of a *salGal4* line. *sal* is normally expressed in the dorsal part of the tracheal placodes but maintained only in DT cells throughout tracheal development (Kuhnlein and Schuh, 1996). Under these conditions the DT failed to form properly and to express detectable levels of *sal*. The rest of tracheal branches did not show major defects, and migrated and fused normally (Fig. 5E). These embryos, however, displayed other morphological defects that may be due to the expression of *sal* in non-tracheal tissues.

Thus, DT formation depends on the activity of the WG/WNT pathway in those tracheal cells that normally express *sal*, suggesting an autonomous requirement within the DT cells themselves.

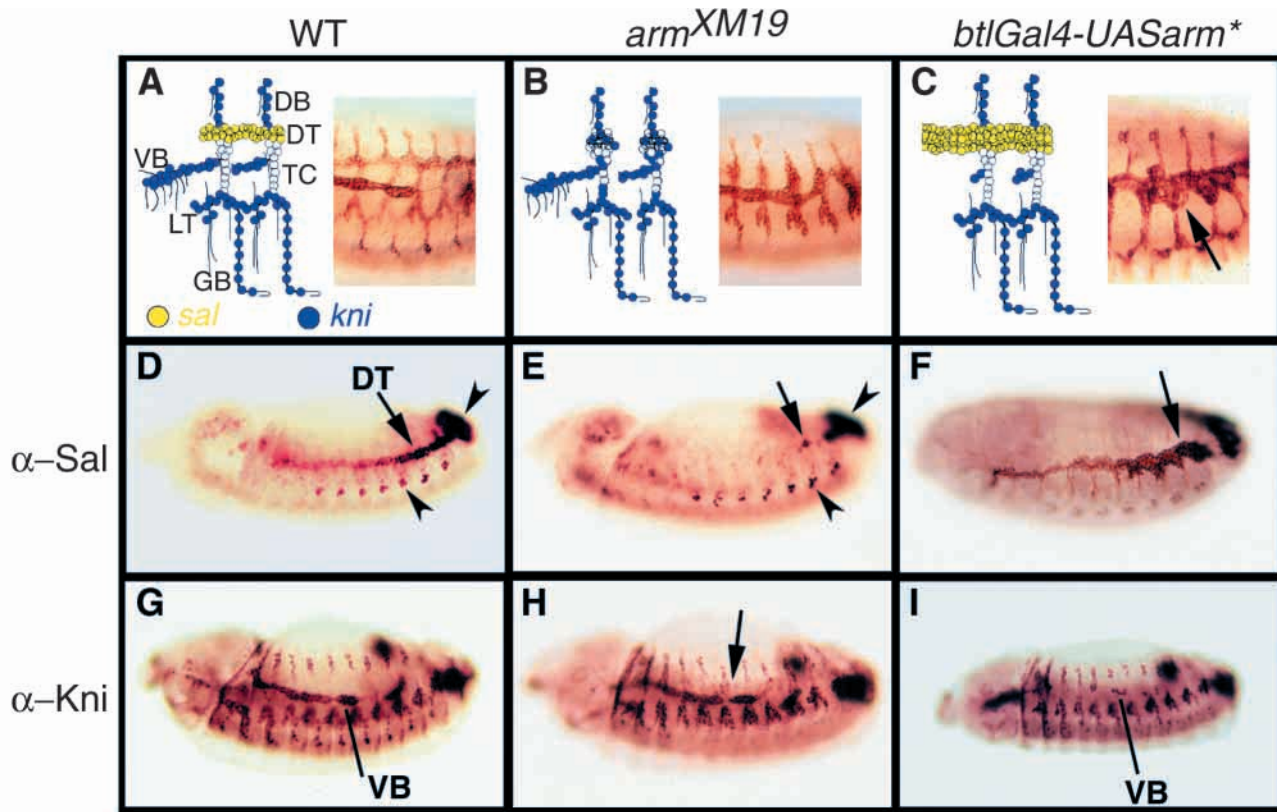
The WG/WNT pathway causes integration of VB into DT  
If the Wg/Wnt pathway is necessary for DT formation, one could expect that overactivating the pathway would increase the DT. That is the case when *wg* or a constitutively active

form of ARM, *arm\**, is expressed using the *btlGal4*: the DT is hypertrophied, often showing ectopic branches with DT features, and the VB are shortened or even completely missing (Figs 1B and 4C). More cells expressed *sal* (29 cells on average in the DT of the eighth tracheal metamere, Tr8, as compared with 17.9 in the wild type) and the levels of *sal* expression are higher than in the wild type (Fig. 4F). *kni* expression (Fig. 4I) revealed that the VBs contained fewer cells (six cells on average in the VB of Tr8 as compared with 23 in the wild type). The rest of the branches contain roughly the normal number of cells. These data do suggest that the VB cells have joined the DT and behave as DT cells. In further support of this conclusion, no extra tracheal cell proliferation was observed in *arm\** conditions, as judged by the absence of  $\alpha$ -phosphorylated histone H3 staining in the tracheal cells (not shown).

The *sal-TSE-lacZ* reporter reproduces *sal* expression exclusively in tracheal cells (Kuhnlein and Schuh, 1996). In *arm\** conditions, the reporter was expressed in the DT in a similar pattern to that of the antibody (Fig. 5F). This indicates that all the elements required for the regulation of *sal* by the WG/WNT pathway are contained within the tracheal-specific enhancer.

The WG/WNT and DPP pathways show antagonistic interactions

The misexpression of *sal* in all tracheal cells confers some DT features (Fig. 5A; Chen et al., 1998; Kuhnlein and Schuh,



**Fig. 4.** Dorsal trunk defects. Lateral views of embryos at stages 14 or 15. (A-C) Diagrams summarising the pattern of two tracheal metameres in wild type (A) and mutant conditions (B,C). Each circle represents a tracheal cell expressing *sal* (yellow), *kni* (blue) or neither (white circles). By the side of each diagram are shown four metameres of embryos bearing *btlGal4-UAStauGFP* in the different backgrounds, stained to visualise the tracheal cell bodies with  $\alpha$ -GFP. In *arm* mutants (B), the presumptive DT cells do not migrate. When *arm* is constitutively active (C) the DT is thicker, ectopic branches associated with it arise (arrow), and the VBs are generally missing or very much reduced. (D) Wild-type SAL expression. In *arm* mutants (E), *sal* is lost or very much reduced in the DT (arrow) but is maintained in other structures (arrowheads). In *arm<sup>\*</sup>* (F), more cells express *sal* (arrow) and the levels of expression are higher than in wild type. (G) Wild-type KNI expression. In zygotic *arm* mutants (H), *kni* is sporadically expressed by presumptive DT cells (arrow). When *arm* is constitutively active (I), the few cells forming the reduced VBs express *kni*. DB, dorsal branches; DT, dorsal trunk; GB, ganglionic branch; LT, lateral trunk; TC, transverse connective; VB, visceral branch.

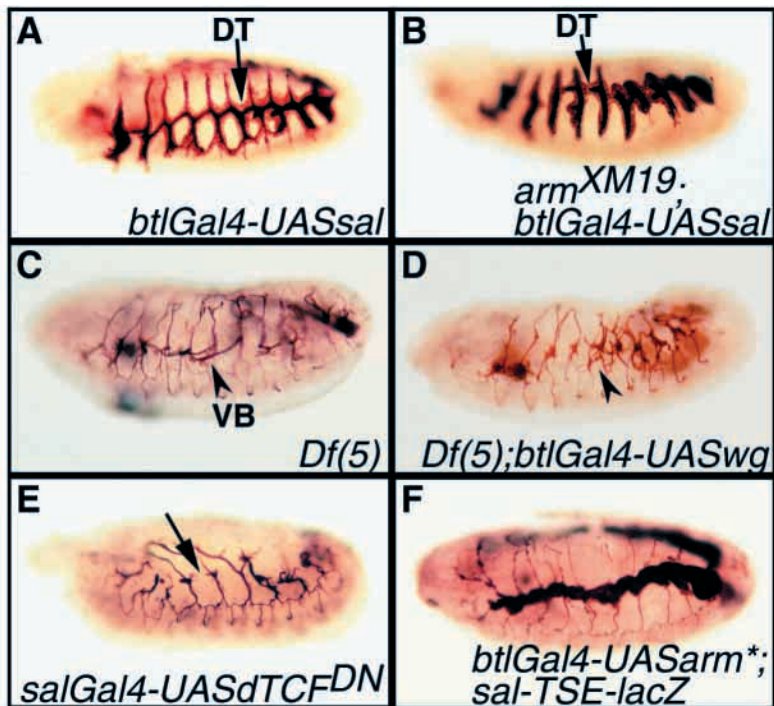
1996). The WG/WNT pathway is also sufficient to apparently confer DT identity to the VB; however, it is not sufficient to activate *sal* and recruit the other tracheal cells into DT. Several explanations could account for this.

One possibility is that the WG/WNT pathway cooperates with other factors to form the DT. A likely candidate is the EGF pathway, which has been proposed to control DT and VB formation and to maintain *sal* expression in DT cells (Chen et al., 1998; Wappner et al., 1997). The expression in all tracheal cells of a constitutively active EGFR, *EGFR<sup>\*</sup>*, is not able to induce *sal* expression outside the DT or to confer DT or VB features to other tracheal branches (not shown). To test whether the WG/WNT and the EGF pathways act together to activate *sal*, *EGFR<sup>\*</sup>* and *arm<sup>\*</sup>* were expressed simultaneously in all tracheal cells. The resulting phenotype is identical to that of *arm<sup>\*</sup>* alone (not shown).

Another possibility is that the WG/WNT pathway is antagonised by other factors. Indeed, *kni*, which is expressed in every tracheal branch except the DT and the transverse connective (TC) (Fig. 4A,D, Chen et al., 1998; Vincent et al., 1997), is responsible for repressing *sal* (Chen et al., 1998). The DPP pathway is responsible for *kni* activation in dorsal

and ventral branches. The misexpression of a constitutively activated thick veins receptor, *tkv<sup>\*</sup>*, leads to the generalised expression of *kni*; this causes integration of DT cells into the dorsoventral branches and repression of *sal* (Vincent et al., 1997). To test whether the DPP pathway is the factor that limits the activation of *sal* by the WG/WNT pathway, *sal* expression was monitored in conditions of misexpression in all tracheal cells of *arm<sup>\*</sup>* or *wg* in a zygotic null *tkv* (*tkv<sup>Δ</sup>*) mutant background. In *tkv* mutants, only the DT expressed *sal* (Fig. 6A; Vincent et al., 1997); however, when ectopic *wg* or *arm<sup>\*</sup>* were also present, all the tracheal cells observed expressed *sal*. These tracheal cells migrate only along the anteroposterior axis to form a huge DT, and no other branches are usually formed (Fig. 6B). Moreover, when *tkv<sup>\*</sup>* and *arm<sup>\*</sup>* were co-expressed, a partial restoration of DT is observed, although no detectable levels of *sal* expression were seen in this partially rescued DT (Fig. 6C). Finally, the absence of both zygotic *arm* and *tkv* impaired the formation of all tracheal branches except the VB and the TC (Fig. 6D).

Altogether these results indicate that the WG/WNT and the DPP pathways have antagonistic interactions during tracheal development. This antagonism through DPP seems to be at



**Fig. 5.** (A,B) Stage 15 embryos, bearing *btlGal4-UASstauGFP* in the indicated genotypes, stained for the lumen with mAb2A12 (black) and with  $\alpha$ -GFP to visualize the tracheal cells (brown). Overexpression of *sal* (A) confers dorsal trunk (DT) features to the tracheal tree. It is also able to partially restore DT migration in *arm* mutants (arrow in B). (C,D) Stage 16 embryos stained with mAb2A12. No DT forms in the absence of *sal*. The visceral branches (VBs, arrowheads) defects produced by *wg* overexpression are not rescued by the absence of *sal* (D). (E) Stage 16 embryo stained with  $\alpha$ -SAL (brown) and with mAb2A12 (black). The DT is completely or partially missing and *sal* expression is lost. (F) Stage 15 embryo bearing a *sal-TSE-lacZ* reporter stained for  $\beta$ -gal (black) and for the lumen with mAb2A12 (brown). The reporter responds to the constitutive activation of *arm*.

least one of the reasons for the inability of the WG/WNT pathway to confer DT identity to the entire tracheal tree.

### Branch fusion

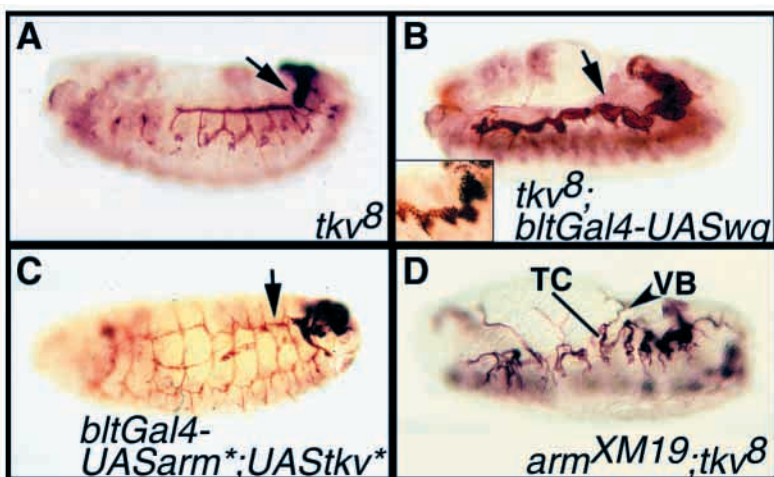
The WG/WNT pathway is necessary for the specification of the fusion fate

Fusion of branches is a complex process that involves cell shape changes, cellular reorganisations and cell adhesion (Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996). The tracheal cells that mediate branch fusions, the fusion cells, are found at the tip of every branch committed to fuse, that is the DB, DT and LT (Fig. 7C). They express specific markers, such as *escargot* (*esg*) (Fig. 7A,B; Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996).

In zygotic *arm<sup>XM19</sup>* mutants, where no branch fusion occurs, no expression of the fusion markers *esg-lacZ* (Fig. 7D,E,H),

Fusion-2 and Fusion-3 (two unidentified fusion genes; data not shown) was observed. Instead, the putative fusion cells seem to acquire other tracheal identities. DBs contain extra cells expressing Terminal-1 (Fig. 7N), suggesting that the fusion cell of the DB has adopted a terminal identity. In addition, one or two Terminal-1-expressing cells are often observed among the presumptive DT cells (Fig. 7N). These could correspond to the presumptive fusion cells of the DT that can now express terminal markers due to the absence of *sal* expression (*sal* has been shown to repress the terminal fate, Chen et al., 1998).

The absence of branch fusion observed in other mutants of the WG/WNT pathway, such as in *dsh* (Fig. 7L) or *dTCF* mutants (not shown), also correlated with absence of *esg-lacZ* expression. Similarly, in *wg<sup>CX4</sup>* mutants, *esg-lacZ* expression was not detected in tracheal cells (Fig. 7M). A lack of *esg* function could explain the loss of LT and DB fusion. However,



**Fig. 6.** Genetic interactions between the DPP and the WG/WNT pathway. Lateral views of embryos at stage 15 or 16 stained with mAb2A12 (A-D, brown) and with  $\alpha$ -SAL (A-C, black). In *tkv* mutants *Sal* is only expressed in the dorsal trunk (DT) cells (A). When *wg* is ectopically expressed at the same time, only a huge DT that expresses *sal* is formed (arrow in B and inset). *arm<sup>\*</sup>* and *tkv<sup>\*</sup>* partially rescue each other's phenotype (C), although *SAL* is not detected in the DT (arrow in C). In *arm; tkv* double mutants (D), only transverse connective (TC) and visceral branch (VB) form.

**Fig. 7.** The WG/WNT pathway regulates the fusion fate. Dorsal (A,D,F,I) and lateral (B,E,G,J,L,M,N) views of stage 15 or 16 embryos stained for the lumen with mAb2A12 (black) and for *esg-lacZ* (all panels except N) or Terminal-1 (N) with  $\beta$ -gal (brown). (C,H,K) Diagrams of two tracheal metameres to summarise the expression of fusion markers of the genotypes indicated. Each circle represents a tracheal cell; the fusion cells are highlighted in red. In mutant conditions of *arm* (D,E), *dTCF* (F,G), *dsh* (L) and *wg* (M) no expression of *esg-lacZ* is observed in any branch that normally contains fusion cells. *arm* mutants show Terminal-1-expressing cells in the DT (arrowhead in N) as well as extra terminal cells in the DBs (arrow in N). Conversely, in *arm*<sup>\*</sup> the DT and the LT contain more *esg-lacZ*-expressing cells (arrows in J); the DBs usually contain a normal number of fusion cells (I). DB, dorsal branches; DT, dorsal trunk; LT, lateral trunk.

DT fusion does not depend much on *esg* (Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996), indicating that it may depend on other fusion genes. The DT of *wg*<sup>CX4</sup> mutants was partially fused, indicating that they may express these other genes.

*esg-lacZ* expression and branch fusion are also impaired when *dTCF*<sup>DN</sup> is expressed in all tracheal cells (Fig. 7F,G,H). Similarly, prevention of many branch fusions also occurred when *dTCF*<sup>DN</sup> was selectively activated in the fusion cells by the *p127Gal4* line (data not shown). This suggests that the WG/WNT pathway is required autonomously in the fusion cells for their proper specification.

In agreement with this function of the WG/WNT pathway, the expression of *arm*<sup>\*</sup> in all tracheal cells produced more cells expressing *esg-lacZ* in the DT and the LT (Fig. 7J,K). This indicates that the pathway is necessary and sufficient to activate the fusion fate in these two branches. However, it rarely produced extra fusion cells in the DBs (Fig. 7I,K), suggesting that the levels of constitutive protein may not be high enough or that other cues are also necessary to positively or negatively regulate the fusion fate in the DBs.

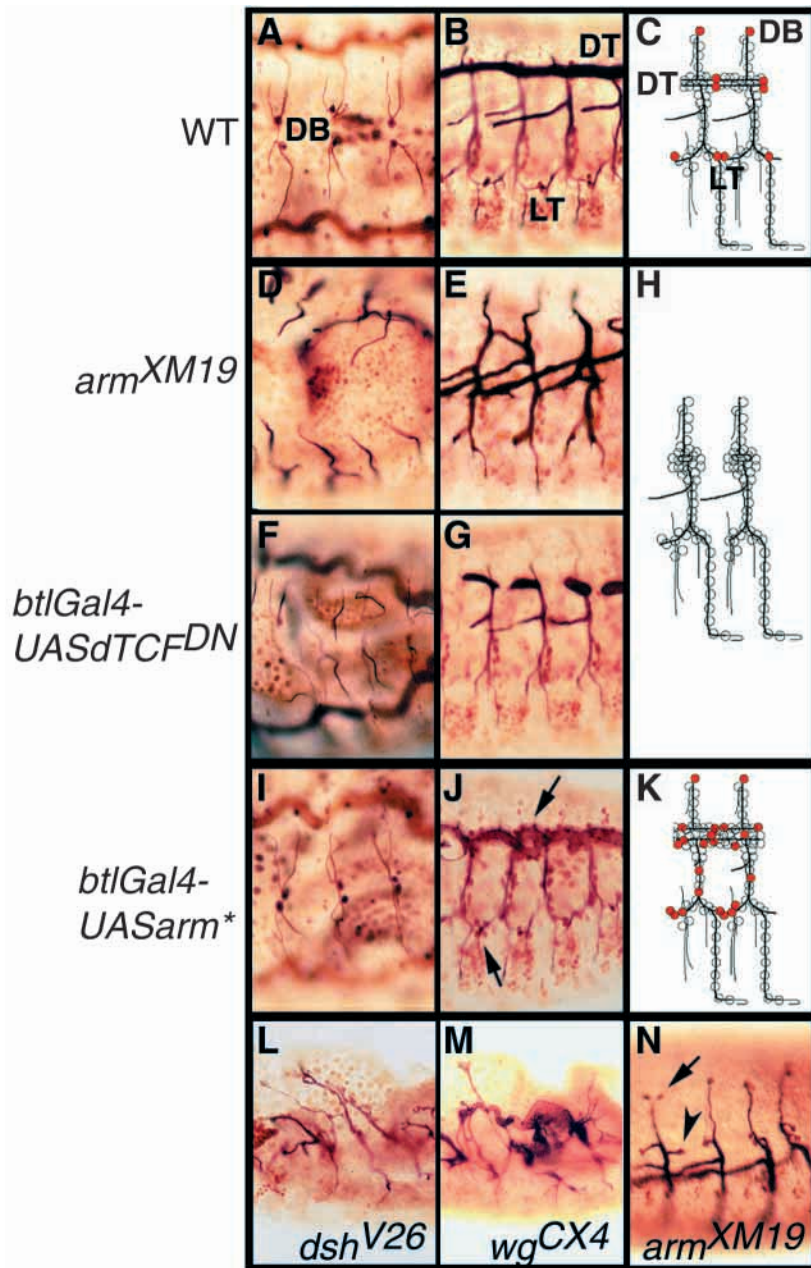
### **wg is expressed near the developing trachea**

During early stages of tracheal development, Wg was expressed in ectodermal cells that lie close to the tips of the DBs and the LT, where the fusion cells form (Fig. 8A). Later, WG was still expressed in ectodermal cells close to the growing GBs (Fig. 8B). This pattern of expression could fit with roles for *wg* in both GB guidance and the activation of fusion genes.

## **DISCUSSION**

### **Tracheal functions of *wg* and its signalling pathway**

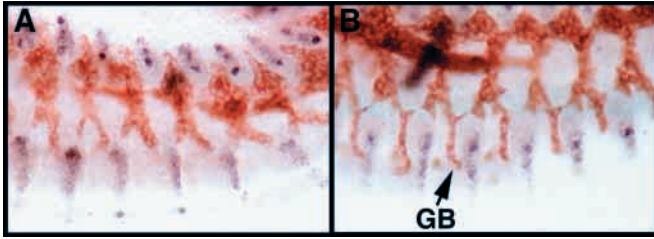
The WG/WNT pathway appears to be activated by *wg* itself during several tracheal events such as tracheal invagination,



activation of fusion genes and GB guidance. However, null *wg* mutants show a tracheal phenotype that is significantly weaker than is found when other members of the WG/WNT pathway are removed: in *wg* mutants, fragments of DT were formed and fused, yet, in other mutants of the pathway the DT is eliminated. This suggests, in acting during DT development, the WG/WNT cascade does more than just respond to *wg*. But whereas loss of function shows that *wg* is not absolutely required for DT formation, its misexpression in tracheal cells indicates that it is sufficient to confer DT identity. These unconventional results are difficult to reconcile with a simple model.

A possible explanation is that an additional factor or factors might help to trigger the WG/WNT cascade to form the DT, the other *Drosophila* *wg* genes being the most likely candidates. Intriguingly, in *por* mutants, the DT also failed to





**Fig. 8.** WG expression. Lateral views of dissected embryos bearing *bitGal4-UAStauGFP* stained with  $\alpha$ -WG (black) and with  $\alpha$ -GFP (brown) to show the tracheal cells. At stage 12 (A), WG is expressed in ectodermal cells that lie close to the tips of primary branches, and at stage 13-14 (B) near the ganglionic branches (GB).

form, suggesting that *por* mutants lack this factor/s. Therefore, if the activation of the pathway depends, at least in part, on other *Wnt* genes, the results indicate that *por* would be required for their secretion too. The WG/WNT cascade could be activated by a particular *Wnt* or *Wnt* combination or alternatively, *Wnt* genes could be all functionally redundant. *Wnt4* maps near *wg* and both genes are deficient in *Df(2R)RF*. However, the tracheal phenotype of this deficiency is almost identical to that of *wg<sup>CX4</sup>*, ruling out that zygotic *Wnt4* might act on its own or in combination with *wg* in forming the DT. *Wnt2* mutants are homozygous viable suggesting that it is dispensable in DT formation. However, it might contribute to DT formation in combination with *wg* or other *Wnts*. So far, there are no point mutations for *Wnt3*, and thus any possible tracheal phenotype could not be assessed. Other *Wnt* genes have recently been identified by the Genome Sequence, and these also remain candidates.

Indeed, one can envisage a model where the WG/WNT pathway has a basal activity stimulated upon binding of any *Wnt* or *Wnt* gene combination to the receptor complex. These low levels of WG/WNT pathway activity would be sufficient to allow DT formation and DT fusion (presumably through activation of some fusion genes). Tracheal invagination, activation of other fusion genes, and guidance of GBs would require higher levels of activity of the pathway – these would depend on binding of *wg* itself, sourced from nearby tissues.

### ***arm* induces tracheal fates through its function in signalling**

*arm* has been shown to play dual but separable roles: one in WG/WNT signal affecting gene expression and the other in cell adhesion (Orsulic and Peifer, 1996). Uemura et al (1996) reported that null *arm* mutants have a tracheal phenotype that is, in part, due to loss of its adhesive function. They also pointed out that this phenotype can not be simply explained by a decrease in cell adhesion.

The results of this work indicate that at least part of *arm* function in the trachea is indeed due to its action in WG/WNT signalling. First, an *arm* allele that specifically impairs WG/WNT signal produces a similar phenotype to that of an *arm* null allele. Second, some tracheal defects are related to a direct or indirect regulation of tracheal target genes. Third, other members of the WG/WNT pathway participate in the same tracheal events.

Which part of the *arm* tracheal phenotype is due to its adhesive role? *arm* has a role in cell adhesion by interacting

with *shotgun* (*shg*), which encodes DE-cadherin. *shg* and *arm* mutants are both defective in branch fusion (Uemura et al., 1996). However, the cause of these two phenotypes does not appear to be the same, as *esg* (a marker of fusion fate) is normally expressed in *shg* mutants (Uemura et al., 1996) but not in *arm* mutants. This indicates that the fusion cells are normally specified in *shg* mutants, whereas *arm* is required within the fusion cells themselves to regulate fusion markers. Therefore, *arm* might have a dual function during branch fusion: it is first required to activate the fusion fate through the WG/WNT signalling and later, through its interaction with *shg*, it is required for the cell reorganisations that lead to branch fusion.

### **The WG/WNT pathway is essential for DT formation**

The results indicate that the WG/WNT pathway acts in DT formation by regulating *sal* expression. Although this regulation could be direct, it is also possible that the WG/WNT pathway acts by regulating the number of cells that will later express *sal* under a WG/WNT-independent control. Two pieces of evidence show that normally the WG/WNT pathway is active only in the DT during primary branching. First, the constitutive activation of ARM cause integration of the VBs into DT. Such transformation would not be expected if the pathway were normally active in the VBs. Second, in the absence of *tkv*, the pathway must be constitutively activated to observe *sal* expression in every tracheal cell. If the pathway were normally active in all tracheal cells, the mere absence of *tkv* should be sufficient to allow *sal* expression. Moreover, the pathway seems to be required in the DT cells themselves. The expression of *dTFCD<sup>DN</sup>* in all tracheal cells impairs DT formation, indicating that there is an autonomous requirement within the tracheal cells. When the expression of *dTFCD<sup>DN</sup>* in the tracheae is maintained only in the DT cells, similar DT defects are observed.

The DPP pathway is required to form the dorsal and ventral branches (Vincent et al., 1997) while the WG/WNT pathway is required for DT formation. Accordingly, only the VBs and TCs, which are independent of these two pathways, form in their absence. The WG and the DPP pathways are known to act antagonistically in several structures, such as the wing or the leg disc (Brook and Cohen, 1996; Jiang and Struhl, 1996; Theisen et al., 1996). Similarly, the WG/WNT and the DPP pathways have opposing activities during primary branching. Only when the DPP pathway is not active is the WG/WNT pathway able to confer DT identity to the tracheal cells. This antagonism is likely to be mediated through the negative regulation of *sal* by *kni* (Chen et al., 1998). *sal* is positively regulated by the WG/WNT pathway and *kni* is activated by the DPP pathway (Vincent et al., 1997). However, when the WG/WNT signal is constitutive in a wild type background, the VBs (which express *kni*) seem to adopt a DT identity. Remarkably, in the VBs, the DPP pathway is neither active nor controls *kni* expression (Vincent et al., 1997). This indicates that *kni* expression is not sufficient to prevent *sal* regulation by the WG/WNT pathway and that other targets of DPP might also antagonise the WG/WNT pathway.

It has been proposed that the EGF pathway controls VB and DT formation (Wappner et al., 1997). If the action of the WG/WNT pathway is sufficient to confer DT identity when the DPP repression is released, what role remains for the EGF

pathway in DT formation, and how does that relate to WG/WNT activity? There are several possibilities that do not exclude each other. A first possibility is that the EGF pathway might regulate the WG/WNT pathway in the DT. However, against this possibility is the observation that a constitutive form of ARM is not able to rescue the DT defects of *rhomboid* mutants properly (M. L., unpublished), although it could still be argued that other targets of EGF could be necessary to complete this rescue. A second possibility is that both the EGF and the WG/WNT pathways might collaborate to form the DT. The constitutive activation of both pathways does not produce a tracheal phenotype different from *arm\** alone. This suggests that it is not the restricted activity of the EGF pathway that prevents a complete transformation into DT. However, the EGF pathway seems to be also antagonised by the DPP pathway (Wappner et al., 1997). A third possibility is that the EGF pathway might not be directly involved in DT formation. Indeed, we already suggested that those DT defects observed in mutants of the EGF pathway could be secondary and due to an inadequate invagination of the tracheal cells (Llimargas and Casanova, 1999).

In summary, this work suggests that the WG/WNT pathway is normally active in DT cells. The pathway shows antagonistic interactions with the DPP pathway. The action of the WG/WNT pathway seems to be sufficient to confer DT identity when the repression mediated by the DPP pathway is released.

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