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 β (TGFβ) 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)/β-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/β-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 Zeizer 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: wgC14 (Baker, 1987); Df(2L)IRF (Tiong and Nash, 1990); armXM19, which is a strong hypomorphic allele that impairs WG signal without affecting adherens junctions function (Cox et al., 1999); dTCE2 (van de Wetering et al., 1997); dsh256 (Geer et al., 1983); fzdH31 (Jones et al., 1996); fzdC1 (Chen and Struhl, 1999); Df(5), which removes both sal and sal-related (Barrio et al., 1999), porPB16 (Eberl et al., 1992) and tkfA (Nusslein-Volhard et al., 1984). wgIL114 (Bejsovec and Martinez Arias, 1991) is a conditional ts allele.

To remove the maternal contribution, germline clones were induced using the FRT/FLP/ovo method (Chou et al., 1993). Females carrying armXM19/FRT101/ovoD1/FRT101, dsh256/FRT101/ovoD1/FRT101, porPB16/FRT2.9/ovoD2/FRT2.9 or a doubly mutant chromosome fzdH31/fzdC1/FRT2A/ovoD1/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: UASgal (de Celis et al., 1996), UASwg (Lawrence et al., 1995), UASarm* (UAS-armS10; Pai et al., 1997), UASdTCEma (van de Wetering et al., 1997), UASnev* (UAS-tkvQ253; Lecuit et al., 1996) and UASEGFR* (UAS-tor4021-EGFR, Dominguez et al., 1998).

The bitGal4 line (Shiga et al., 1996) drives the expression of UAS constructs in the tracheal cells from stage 11; p127Gal4 (Stenberg et al., 1998) in the tracheal fusion cells, and LP39 (salGAL4, provided by M. Calleja and G. Morata) in a sal pattern of expression.

To visualise the tracheal cells embryos bearing the bitGal4-UAStauGFP in wild-type or other backgrounds were stained for α-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-fzt-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 mAβ2A12 (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 α-spalt (from R. Schuh) was used at 1:30 and the guinea pig α-knirps (developed by J. Revivitz and provided by M. Ruiz-Gomez) was used at 1:1000. The rabbit α-β-galactosidase (Cappel) was used at 1:1000 to 1:1500 to detect tracheal markers and blue balancers. The rabbit α-phosphorylated histone H3 (Upstate Biotechnology) was used at 1:500 to detect cell proliferation. The rabbit α-GFP (Molecular Probes) was used at 1:500 to visualise the tracheal cells. The purified mouse monoclonal α-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 NiCl2 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 Axiohot.

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, armXM19 (Cox et al., 1999; Peifer and Wieschaus, 1990), was used. Zygotic armXM19 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. wgts
(wg\textsuperscript{IL14}) 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\textsuperscript{CX4}) 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 bitGal4 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\textsuperscript{XM19} mutants. arm\textsuperscript{XM19} 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\textsuperscript{XM19} 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\textsuperscript{XM19}, 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 (dTCFDN). dTCFDN lacks its N-terminal domain and it is not able to bind to ARM (van de Wetering et al., 1997). The expression of dTCFDN 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\textsuperscript{XM19} mutants. This indicates that the pathway is required within the tracheal cells for these two tracheal events.

GBs were also misrouted (Fig. 2I) when dTCFDN was activated by the bitGal4, 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

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**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 (I) mutants.
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\textsuperscript{DN} was expressed in all tracheal cells or in arm\textsuperscript{XM19} 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; Guillemin et al., 1996) and \textit{adrift} (Englund et al., 1999) (data not shown). However it should be noted that the \textit{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**

\textit{arm} is required for \textit{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 \textit{knirps} (\textit{KNI}) (Fig. 4A,G), which is regulated by the DPP pathway (Chen et al., 1998; Vincent et al., 1997). Instead they express \textit{spalt} (\textit{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 \textit{sal} mutants, the DT fails to form a continuous branch (Kuhnlein and Schuh, 1996). In \textit{arm\textsuperscript{XM19}} mutants, the presumptive DT cells invaginated perfectly but did not migrate or join with other tracheal branches. Only occasionally they expressed low levels of \textit{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 \textit{sal} expression in precisely these presumptive DT cells (Fig. 4E). Remarkably, the early expression of \textit{sal} in broad regions overlapping with the dorsal part of all tracheal placodes (not shown) as well as \textit{sal} expression in other structures is maintained (Fig. 4E). This suggests that the WG/WNT pathway regulates DT formation by controlling \textit{sal} expression in DT cells. \textit{sal} is still expressed in the fragments of DT in \textit{wg\textsuperscript{CX4}} embryos (not shown).

To test whether ARM exerts part of its function through \textit{sal} in the DT, epistatic experiments were carried out. The misexpression of a wild type form of \textit{sal} in all tracheal cells (Fig. 5A) partially rescued the lack of DT produced by \textit{arm\textsuperscript{XM19}} 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 \textit{sal} function suppressed the
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} 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{sup-}, 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{sup-} conditions, as judged by the absence of α-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{sup-} 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,
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*, 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* and arm* were expressed simultaneously in all tracheal cells. The resulting phenotype is identical to that of arm* 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*, 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* or wg in a zygotic null tkv (tkv0) mutant background. In tkv mutants, only the DT expressed sal (Fig. 6A; Vincent et al., 1997); however, when ectopic wg or arm* 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* and arm* 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
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 \textit{escargot} (\textit{esg}) (Fig. 7A,B; Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996).

In zygotic \textit{arm} \textit{XM19} mutants, where no branch fusion occurs, no expression of the fusion markers \textit{esg}-\textit{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 \textit{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 \textit{sal} expression (\textit{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 \textit{wg} (Fig. 7L) or \textit{dTCF} mutants (not shown), also correlated with absence of \textit{esg}-\textit{lacZ} expression. Similarly, in \textit{wg} \textit{CX4} mutants, \textit{esg}-\textit{lacZ} expression was not detected in tracheal cells (Fig. 7M). A lack of \textit{esg} function could explain the loss of LT and DB fusion. However,
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 CX4 mutants was partially fused, indicating that they may express these other genes.

esg-lacZ expression and branch fusion are also impaired when dTCFDN is expressed in all tracheal cells (Fig. 7F,G,H). Similarly, prevention of many branch fusions also occurred when dTCFDN 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* 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
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\textsuperscript{C4}, 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 dTFC\textsuperscript{DN} in all tracheal cells impairs DT formation, indicating that there is an autonomous requirement within the tracheal cells. When the expression of dTFC\textsuperscript{DN} 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 expressed 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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