The Notch pathway helps to pattern the tips of the Drosophila tracheal branches by selecting cell fates

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SUMMARY
The Drosophila tracheal system consists of a stereotyped network of epithelial tubes formed by several tracheal cell types. By the end of embryogenesis, when the general branching pattern is established, some specialised tracheal cells then mediate branch fusion while others extend fine terminal branches. Here evidence is presented that the Notch signalling pathway acts directly in the tracheal cells to distinguish individual fates within groups of equivalent cells. Notch helps to single out those tracheal cells that mediate branch fusion by blocking their neighbours from adopting the same fate. This function of Notch would require the restricted activation of the pathway in specific cells. In addition, and probably later, Notch also acts in the selection of those tracheal cells that extend the terminal branches. Both the localised expression and the mutant phenotypes of Delta, a known ligand for Notch, suggest that Delta may activate Notch to specify cell fates at the tips of the developing tracheal branches.

Key words: Drosophila, tracheal system, branch fusion, terminal branching, N signalling pathway

INTRODUCTION
The morphogenesis of branched tubular structures, such as the lung and the vascular system, involves the coordination of many cellular processes, such as the specification of cell fates, changes in cell shape and cell migration. In addition, specific sets of cells have to act together in order to mediate the fusion of the tubes. The tracheal system of Drosophila is an excellent model to analyse these processes at the cellular, genetic and molecular levels. The tracheal system originates from 10 clusters of ectodermal cells at each side of the embryo; these are specified at stage 10 by the action of the gene tracheless (trh) (Isaac and Andrew, 1996; Wilk et al., 1996). The tracheal cells invaginate inside the embryo and then undergo four morphological events without cell proliferation: these are primary branching, secondary branching, fusion and terminal branching. The result is a stereotyped network of epithelial tubules that oxygenate embryonic tissues (Manning and Krasnow, 1993; Samakovlis et al., 1996a; Fig. 1G). Many mutants that impair the development of the tracheal system at different stages have been isolated, allowing the identification of genes that control particular steps.

The heterogeneous expression of several enhancer trap lines in subsets of tracheal cells indicates that these cells are molecularly diverse (Samakovlis et al., 1996a). Differences in expression patterns correlate with morphology; for instance, the tracheal cells that give rise to a terminal branch each extend a cytoplasmic process and express the so-called terminal markers (Guillemin et al., 1996; Samakovlis et al., 1996a). Thus, it appears that tracheal cells have distinct fates and it is likely that the fixed pattern of the final structure relies on a rigid control of cell fate decisions.

But when and how is this cellular diversity achieved in the trachea? Clonal analysis suggests that tracheal cells are equivalent until the last division takes place; in fact, the first signs of differences between tracheal cells only become apparent during primary branching (Samakovlis et al., 1996a). Tracheal cell fates are determined by inductive signals, such as the branchless (bnl) gene product. bnl encodes a fibroblast growth factor (FGF) homologue that appears to bind to the Breathless (btl) receptor (Sutherland et al., 1996), an FGF receptor homologue expressed in the tracheal cells (Glazer and Shilo, 1991; Klämbt et al., 1992). bnl is expressed in clusters of cells near the tips of the developing primary branches, and seems to activate the expression (Oshiro and Saigo, 1997) and the function (Gabay et al., 1997) of its receptor in a gradient, with the cells closest to the Bnl source showing highest receptor activity. The localised activation of Btl leads to local expression of some tracheal markers at the tip of the primary branches (Samakovlis et al., 1996a). Thus, the acquisition of different fates may depend on the relative position of the tracheal cell with respect to the source of Bnl. In addition to external signals, interactions between the developing tracheal cells themselves also help specify cell fates. For instance, the headcase (hdc) (Steneberg et al., 1998) and sprouty (spry) genes (Hacohen et al., 1998) are expressed in some subsets of tracheal cells yet determine the fate of other tracheal cells nearby. Although some aspects of tracheal specification have been identified, we still lack a complete picture of the process.

The Notch (N) signalling pathway acts in many
developmental systems to specify cell fate and is therefore a good candidate to mediate interactions between tracheal cells. There are two different ways in which Notch acts. In the first, termed inductive interactions, the signalling and the receiving cell begin with different properties; the acquisition of cell fates depends on the timing and spatial arrangement of the interacting cells. The second, “lateral inhibition”, occurs between groups of initially equivalent cells and results in the singling out of individual cells; these cells then inhibit their neighbours from adopting the same fate. Examples of both types of interactions are found in Drosophila and Caenorhabditis elegans (reviewed in Muskavitch, 1994; Artavanis-Tsakonas et al., 1995; Simpson, 1997; Greenwald, 1998).

Genetic and biochemical studies have identified many components of the N pathway. In Drosophila, the products encoded by Delta (Dl) and Serrate (Ser) bind to the N receptor. After ligand binding, the activated receptor seems to be cleaved and the intracellular domain of N translocates to the nucleus, where, together with Suppressor of Hairless (Su(H)), it activates the transcription of several target genes. A direct target of N is the Enhancer of split (E(spl)) complex, which encodes seven related bHLH proteins that interact with Groucho (Gro) and act as transcriptional repressors (reviewed in Weinmaster, 1997; Greenwald, 1998).

This paper studies the role of the N pathway in the developing trachea. The consequences of changes in the activity of N, or of its downstream components, indicate that the N signalling is needed in the tracheal cells for the fusion and terminal branching programs. Evidence is prevented that N is first required to single out the cells that will mediate branch fusion from a group of competent tracheal cells. The N signalling acts again later in selecting the correct number of terminal branches arising from some primary branches. The results suggest that N is differentially activated in the tracheal cells. This differential activation correlates with differential expression of Dl at the time and in the cells in which N operates. The phenotypes of Dl overexpression in the tracheal cells and its pattern of expression suggest that Dl may act as a ligand for N to pattern the tips of the primary branches.

MATERIALS AND METHODS

Drosophila strains and genetics
N55ec1 is a null allele of N (Kidd et al., 1983) and 1(1)N5 (Shellenbarger and Mohler, 1975) is a temperature-sensitive allele. For the experiments with N5, N5/N5ec1 embryos were raised at 25°C and a single 2 hour pulse at 32°C was applied at the beginning of stage 11. The following P(lacZ) tracheal enhancer traps described in and a single 2 hour pulse at 32°C was applied at the beginning of stage 11 were used: Fusion-1 (escargot), Fusion-2, Fusion-3, Antifusion-1, Antifusion-2, Terminal-1 (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999a; Artavanis-Tsakonas et al., 1999b). To maximise the efficiency of the Gal4/UAS system, the embryo collections were done at 29°C.

To identify mutant embryos, blue balancers of the first, second and third chromosomes were used: FM7-frz-lacZ, Cyo-hb-lacZ, TM3-frz-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 rabbit anti-β-Gal (Cappel) was used at 1:1000 to 1:1500 to detect tracheal markers and to visualise the tracheal cells. Biotinylated secondary antibodies (Jackson) were used at 1:300. To optimise double stainings with mAb2A12 and β-Gal, embryos were first stained with NiCl2 depositing black in the lumen and then for β-Gal (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 Axioshot.

For fluorescent stainings, embryos were fixed in 4% paraformaldehyde in PBS and stained according to standard protocols. Rabbit anti-β-Gal (Cappel) was used at 1:800, guinea-pig anti-Coracle (Fehon et al., 1994) at 1:1000 and mouse mAb202 against Delta protein (Kooh et al., 1993) at 1:1000. To stain for CD2 antigen, a mouse anti-CD2 (Serotec) was used at 1:250 followed by a mouse biotinylated secondary antibody (Jackson) and Streptavidin-FITC. Embryos were mounted in Fluoromount-G (Southern Biotechnology Associates) and observed in the confocal microscope (MRC Bio-Rad 1024).

Whole-mount in situ hybridisation and antibody staining were performed as described (Lehmann and Tautz, 1994) with minor modifications. An antisense RNA probe synthesised from a DI cDNA clone (gift from J. F. de Celis) was used. After detection of DI expression, embryos were stained for β-Gal to visualise the tracheal cells. Embryos were processed as for HRP histochemistry.

Embryos were staged according to Campos-Ortega and Hartenstein (1997).

RESULTS

Notch is essential for the establishment of the tracheal cells and for the fusion and terminal branching programs

During tracheal development, the specification of cell fates is highly ordered. As the N signalling pathway is needed for cell fate specification in other systems, the effects of N and other members of its pathway on the pattern of tracheal branching was analysed.

Zygotic N mutant embryos show abnormalities in most of the ectodermal derivatives, including the tracheal system, where some of the tracheal cells are converted into neuroblasts (Hartenstein et al., 1992); the embryos exhibit only rudimentary branches, most probably due to the loss of tracheal cells (Fig. 1E). In addition, abnormalities in fusion and terminal branching are also detected when luminal or cellular markers (see Materials and Methods) are used (Fig. 1C,D).
These phenotypes could be due to early requirements for the Notch pathway before tracheal fate is allocated. To detect late Notch requirements, Notch mutant embryos (Notch\(\Delta\) over a null Notch allele) were shifted to the restrictive temperature at stage 11. The terminal branching and fusion phenotypes produced resemble those observed in zygotic null Notch mutants, yet the defects in the primary branching pattern are milder (Fig. 1F). The phenotypes observed both in Notch combinations and in null Notch mutants suggest that, in addition to its early role in tracheal specification, Notch acts later in both fusion and terminal branching programs.

But is Notch directly required in the tracheal cells or are the defects seen in Notch mutants secondary effects? To answer this question Notch function was specifically blocked in the tracheal cells by means of the Gal4/UAS system (Brand and Perrimon, 1993). The activation of Notch receptor results in the regulation of gene expression by the transcription factor Su(H). The activity of Su(H) is antagonised by Hairless (H), and therefore the overexpression of H results in Notch loss-of-function phenotypes (Schweisguth and Posakony, 1994; Bailey and Posakony, 1995). The btl-Gal4 line, which is specifically expressed from stage 11 onwards in all tracheal cells (Shiga et al., 1996), was used to drive the expression of a wild-type form of H protein and a dominant negative form of Notch receptor (DN\(\Delta\)). The resulting phenotypes of overexpressing the two constructs were indistinguishable, although the penetrance in the

Fig. 1. Notch is required for normal tracheal development. Lateral (A,C,E,F) and dorsal (B,D) views of stage 16 embryos stained for the tracheal lumen with mAb2A12 (A-D,F), or for the tracheal cells with \(\beta\)-Gal (E). Dorsal is up and anterior to the left in all the figures. The genotypes are indicated in the images. Zygotic null Notch mutants have a rudimentary tracheal tree (C), ectopic fusions between adjacent dorsal branches and absence of terminal branches (arrow in D); the number of tracheal cells is reduced, resulting in reduced branching (E), (F) Notch\(\Delta\) mutants raised at the restrictive temperature. Ectopic fusions and absence of terminal branching persists (arrows), although the primary branching is less affected than in null Notch mutants (compare F with C). (G) Diagrams of lateral views of two central tracheal metameres at stage 13 (top) and 16 (bottom) showing the primary branches: dorsal trunk, DT (subdivided into anterior, DTa and posterior, DTp); dorsal branch, DB; visceral branch, VB; transverse connective, TC; lateral trunk, LT (subdivided into anterior, LTa and posterior, LTp); ganglionic branch, GB. The fine lines at stage 16 represent the terminal branches and the arrows indicate the points of fusion (DBs fuse with contralateral DBs).

Fig. 2. Phenotypes produced by the reduction of Notch signal in the tracheal cells. Overexpression of H from stage 11 (A,C-E) or from stage 13 (B) using the Gal4 drivers indicated. Dorsal or dorsolateral views of stage 16 embryos stained for the tracheal lumen with mAb2A12 (A,B) or for the cell bodies with \(\beta\)-Gal in embryos bearing also UAS-tau-lacZ (C-E). (A) Ectopic fusions (arrows) between adjacent dorsal branches, DB, and absence of terminal branches (DT, dorsal trunk). (B and inset) An ectopic terminal branch arises from the stalk of one dorsal branch (arrowhead) while the next dorsal branch does not extend any terminal branch (arrow). (C) Ectopic fusion (arrow) at the cellular level. (D,E) Confocal section showing the cell junction attaching the cells that mediate an ectopic fusion (arrows) between two adjacent dorsal branches. One of the dorsal branches is out of focus and is represented by a dashed line. (D) Merged image of anti-Coracle (red) and \(\beta\)-Gal (green) stainings to visualise the cell junctions and tracheal cells, respectively. (E) Single image of anti-Coracle staining.
case of $N^{DN}$ was lower. Therefore, the overexpression of $H$ was chosen for further characterisation.

The overexpression of $H$ produces ectopic tracheal fusions between adjacent dorsal branches (DBs) and represses terminal branching (Fig. 2A,C). As with fusions that occur in the wild type, these ectopic fusions also involve two cells that become tightly attached by an intercellular junction that stains with anti-Coracle antibody (Samakovlis et al., 1996a; Fig. 2D,E). Terminal branches arising from the DB are stunted (Fig. 2A) and those arising from other primary branches appear decreased in number and shortened (not shown). However, in sporadic cases, DBs and ganglionic branches (GBs) extend ectopic terminal branches when overexpressing $H$. This is more frequent if a tracheal Gal4 driver, which starts its expression later than the $btl$-Gal4 line, is used (Fig. 2B).

These results indicate that $N$ signalling may be directly required in the tracheal cells for both the fusion and the terminal branching programs. Unlike null $N$ mutants, the reduction of $N$ function in the tracheal cells neither decreases the number of tracheal cells nor affects primary branching. This suggests that the primary branching defects observed in null $N$ mutants are not due to a direct requirement for $N$ in the tracheal cells or that they are due to such a requirement before stage 11.

**Notch is required for the diversification of the tracheal cells**

To analyse the requirements of $N$ in fusion and terminal branching, the identity of the tracheal cells was studied using the expression of several tracheal markers. In wild-type
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embryos, tracheal cells that mediate branch fusion express the fusion markers; a single fusion cell is found at the tips of the following branches: DB (Fig. 3A), lateral trunk anterior (LTa), lateral trunk posterior (LTp), dorsal trunk anterior (DTa) and dorsal trunk posterior (DTp). Conversely, the antifusion markers are generally expressed in all other tracheal cells (Fig. 3C). Finally, the terminal markers are expressed in about 20 cells per tracheal metamere. Those are the cells, either clustered or isolated, that will extend terminal branches from most primary branches (Fig. 3B) except the DT (Samakovlis et al., 1996a). The pattern of expression of these markers in the DB is shown in Fig. 3N and the general pattern of branching in Fig. 1G.

Zygotic null mutants of Notch show abnormal patterns of these molecular markers. In the mutants, Fusion-1 (escargot, Samakovlis et al., 1996b) (Fig. 3D), Fusion-2 and Fusion-3 (two unidentified enhancer traps used as tracheal markers) are ectopically expressed in almost all the cells forming the rudimentary DB (Fig. 3N), DT and LT. The expression of Terminal-1 (pruned, Guillemin et al., 1996) is mainly found in some cells along the transverse connective (TC) and the visceral branches (VB), but almost never in the DB (Fig. 3E,N). Antifusion-1 (Fig. 3F,N) and Antifusion-2 (two unidentified tracheal markers) are consistently expressed only in the TC.

Similarly, the overexpression of H in the tracheal cells also causes changes in the expression of these tracheal markers. In most embryos, there is an increase in the number of cells expressing fusion markers: DBs contain up to 4 cells expressing Fusion-1 (Fig. 3G,N) and Fusion-2 as compared to 1 cell in the wild type; and LT and DT contain up to 4 fusion cells, as compared to 2 cells in normal conditions. Conversely, there is a decrease in the number of cells expressing Terminal-1, mainly in the DBs (Fig. 3H,N) and in the GBs, which in wild-type embryos contain a single cell expressing the terminal markers. The pattern of expression of Antifusion-1 is similar to wild type, although there is one fewer cell on average in the DBs expressing the marker (Fig. 3I,N). Cell counts in the DBs of wild type and of embryos overexpressing Notch indicate that there is no change in the number of cells when H function is reduced (5.7 cells in wild type, n=52 metameres, compared to 5.6 cells in H overexpression, n=49 metameres). This suggests that the extra fusion cells observed may arise by a transformation of presumptive terminal or antifusion cells into fusion cells.

The formation of ectopic terminal branches observed sporadically when overexpressing H correlates with the expression of Terminal-1 in more than one cell in some DBs (Fig. 3I,N) and GBs. The presence of extra terminal branches suggests a second role of Notch in selecting the correct number of terminal cells (see Discussion).

Fig. 4. Gain of Notch function prevents branch fusion. Stage 16 embryos bearing btlGal4-UASN\textsuperscript{act} stained with mAb2A12 to show the tracheal lumen (A,B) or with β-Gal to show the tracheal cells using the UAS-tau-lacZ (C,D). (A,C) Lateral views of embryos with unfused dorsal trunk, DT, and lateral trunk, LT. No other tracheal defects are observed at the cellular level (C). (B,D) Dorsal views of embryos with unfused dorsal branches, DB, and excess of terminal branches (arrows in B) arising from the two tip cells (asterisks in D and inset).

Fig. 5. Expression of E(spl)mβ in the tracheal cells. Confocal sections showing 2 to 4 tracheal metameres of wild-type embryos at stage 12/13 or 13. (A,C,E) Merged images of anti-CD2 and β-Gal stainings to detect E(spl)mβ-CD2 (green) and Fusion-1 (red) expressions, respectively; (B,D,F) single channel for the expression of E(spl)mβ-CD2. Asterisks are close to one cell expressing Fusion-1 in the dorsal branch, DB (A,B), dorsal trunk, DT (C,D) and lateral trunk, LT (E,F). Note the lack or the weak expression of E(spl)mβ-CD2 in the cells expressing Fusion-1.
The loss of N function indicates that N is both required to single out the fusion cells, as some cells seem to undertake the fusion fate at the expense of the terminal or antifusion one, as well as to ensure the correct number of cells expressing the terminal markers.

**Activation of Notch impairs tracheal fusion**

Engineered forms of the N protein that eliminate the extracellular domain are active in the absence of ligand (see Greenwald, 1994; Weinmaster, 1997). If N is required for the execution of terminal branching and fusion programs, a constitutively activated form of N (N\textsuperscript{act}) could cause opposite transformations to the loss of N function.

When N\textsuperscript{act} is expressed in all the tracheal cells from stage 11, the pattern of primary branching is correct but all the branches remain unfused. In addition to the absence of contralateral or adjacent branch fusions, some DBs extend an extra terminal branch (Fig. 4). Consistent with this, no expression of Fusion-1 (Fig. 3K,N), Fusion-2 and Fusion-3 is detected. The pattern of expression of the antifusion markers is indistinguishable from the wild type (Fig. 3M,N). About 20% of the DBs (n=93 metameres analysed) contain two Terminal-1-expressing cells at the tip (Fig. 3L,N), suggesting that the presumptive fusion cell is converted into a terminal cell. In contrast, cell counts show that LTA, LTP and the rest of the DBs contain one fewer cell on average as compared to wild type (5.7 cells in wild type DBs, n=52 metameres, compared to 4.5 cells in the DBs of N\textsuperscript{act}, n=50 metameres). As no expression of the fusion markers is observed in these branches, while the antifusion and the terminal markers are normally expressed, it is likely that the missing cell is the fusion one.

Only the cells that mediate branch fusion are affected when N is indiscriminately activated. This suggests that, normally, N could be less active in the presumptive fusion cells. In support of this possibility a reporter for the E\textit{spl}m\textbeta gene is not expressed, or only weakly, in those cells that start to express the fusion markers, whereas it is expressed in the rest of tracheal cells (Fig. 5). E\textit{spl}m\textbeta is a direct target gene of N and the E\textit{spl}m\textbeta-CD2 reporter used here has been shown to reproduce the endogenous expression of E\textit{spl}m\textbeta in other systems (de Celis et al., 1998).

**Notch affects the expression of the pantip markers**

Cells expressing fusion and terminal markers arise from the tips of the primary branches. At early stages of tracheal morphogenesis, broad domains at the tips of each budding primary branch express the pantip markers (Samakovlis et al., 1996a). Their pattern of expression suggests that the tip cells are apparently equivalent before adopting individual fates.

I studied whether the N pathway affects the early diversification of the tip cells. pointed (pnt, Pantip-1) and spry (Pantip-2) are expressed in each DB in about 3 to 4 cells at stages 12 and 13, at stage 13/14 high levels are found in the two tip cells and at later stages their expression is restricted to the cell expressing the terminal markers (Samakovlis et al., 1996a; Hacohen et al., 1998; Fig. 6A-C). In N loss-of-function conditions, the expression of pnt (Fig. 6D-I) and spry in the DBs is frequently lost from stage 13 while in N gain-of-function high levels of pnt (Fig. 6J-L) and spry are maintained in more than one cell at stage 16. This suggests that activity of N allows the tip cells to diversify. They also suggest a possible repression of these pantip markers by the fusion genes, as their expression decays in the cells expressing the fusion factors.

**Delta accumulates at the tips of the primary branches**

How is the activity of N regulated in the tracheal system? Elsewhere Dl has been shown to act as a ligand for N. Zygotic null N mutants have a weaker tracheal phenotype than that of null Dl mutants, possibly because the maternal N product rescues the zygotically mutant embryos (Seugnet et al., 1997). The tracheal branches in Dl mutants are so truncated that it is not possible to determine effects on fusion or terminal branching (not shown). The overexpression of Dl driven by the btl-Gal4 line affects fusion and terminal branching in ways
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similar to both the gain and loss of N function (see Discussion). Thus, ectopic fusions and unfused branches are observed as well as missing or extra terminal branches with variable frequency. These phenotypes correlate with the lack or excess of cells expressing fusion and terminal markers (Fig. 7K-N).

The pattern of expression of DI in the tracheal cells was studied in detail using both in situ hybridisation and antibody staining to further clarify DI function. The DI protein accumulates in vesicles at higher levels in the tip cells of the primary branches from stage 12 to 13 (Fig. 7D-G). The localised accumulation of the protein is coextensive with a localised expression of the gene in the tip cells (Fig. 7A-C). At later stages, large amounts of transcripts and protein are found in the DT (Fig. 7J) as described by Haenlin et al. (1990) and Kooh et al. (1993).

The early accumulation of DI depends on N activity. DI accumulates at low levels at the tips of the branches in embryos with Nact while, in null N mutants, there is a broader accumulation of DI protein (Fig. 7H,I).
Fig. 8. Model for the diversification of the tracheal cells of the dorsal branches. The coloured circles represent the tracheal types indicated, and the green shading indicates the level of pnt expression, the palest green being the lowest expression. (A) In a first step the activation of btl selects a group of competent cells that express the pantip markers. The two cells closest to the Bnl source express higher levels of pnt. (B) Eventually, persistent activation by btl or biasing differences in one of the two tip cells would allow that cell to express the fusion markers. The lack of N activity and the inductive signal mediated by bnl/btl would be sufficient to overcome the repression of the fusion program mediated by pnt in this tip cell. The fusion genes would in turn repress the expression of pnt. The fusion cell would send a signal via Dl to its neighbours to repress the fusion fate. The second tip cell, although very close to the Bnl source, is inhibited from following the fusion program by two negative signals, N and pnt. (C) Later, the terminal program would be activated in this second tip cell by high levels of expression of the pantip genes and btl signalling. The already committed terminal cell would prevent (via Dl and spry) its neighbours from adopting the same fate. In addition, the fusion cell also sends a signal (mediated by hdc) to the stalk cells to repress the terminal program. The repression of the fusion genes in the stalk cells would allow them to express the antifusion markers.

The coincidence of the early Dl expression with the activity of the N pathway and the phenotypes of Dl overexpression, suggest that Dl activates N to diversify the tip cells of the primary branches.

**DISCUSSION**

**N** singles out the fusion cells

In the developing trachea, specialised cells that mediate fusion of tracheal tubes are found isolated at the tips of primary branches. Here evidence is provided that the N pathway acts to single out all these fusion cells. The expression of the pantip markers suggests that, at the tips of primary branches, there are initially groups of equivalent cells; then one of these becomes a fusion cell and N prevents its neighbours from taking the same fate. Thus, the absence of N results in the allocation of extra fusion cells and consequently the formation of inappropriate branch fusions. This function of N in the trachea is similar to the process of lateral inhibition mediated by N in the nervous system. There, N acts within a cluster of equivalent proneural cells to select out one neural cell (Simpson, 1990; Hartenstein and Posakony, 1990).

The development of the fusion cells seems to require the inactivity of the N pathway, as only those cells are affected when N is constitutively activated. This suggests that, normally, the pathway is less active or even inactive in the fusion cells. The restricted expression of an Espl mutant reporter supports this hypothesis: the reporter is not or weakly expressed in the presumptive fusion cells at the onset of their specification. Conversely, the reporter is expressed in neighbouring cells, suggesting that N represses the fusion fate. The effects of N act may be generated by the repression of the fusion genes. The gene esg (Fusion-1) has been shown to encode a transcription factor that activates the fusion program and regulates the expression of later fusion markers (Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996). The repression of esg by the constitutive activation of N could explain the lack of DB and LT fusions, the transformation of the putative fusion cell of the DB into a terminal cell and the loss of the putative fusion cell of the LT. The repression of other fusion genes independent of esg may cause the lack of DT fusion.

N is also required for the terminal branching program

The N pathway has two different requirements for terminal branching. First, N singles out the fusion cells, allowing the expression of the terminal markers in some of the non-fusion cells. Thus, when N function is eliminated, cells that should acquire terminal fates presumably become fusion cells instead. Second, N is also required to repress the terminal fate, as reduction of N function can produce ectopic terminal branches. The explanation for these apparently contradictory results could be that the acquisition of fusion fates precedes the allocation of terminal fates; this is illustrated by the onset of expression of terminal markers which show later than the fusion markers (Samakovlis et al., 1996a; M. L., unpublished data). Therefore, timing can explain the paradox: in null N mutants most tracheal cells become fusion cells and no cells initiate terminal branching. Conversely, in mild N loss of function or when N is removed late, the first requirement can be met revealing the second one. N could co-operate with other signals like spry and hdc (Hacohen et al., 1998; Steneberg et al., 1998) that are also required to limit the number of terminal branches (Fig. 8).

**Dl acts as a ligand for N in the tracheal cells**

In other systems, the overexpression of Dl can result in both activation and suppression of N activity (de Celis and Bray,
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1997). Likewise, the overexpression of Dl in the trachea produces phenotypes that resemble both the loss and the gain of N function. Activation of N by Dl overexpression occurs in the fusion cells, where Dl expression is normally high and the N pathway seems to be less active. Suppression of N may occur because of elevated levels of Dl in other cells in which the N pathway seems to be normally active. These contradictory results suggest that, in normal development, the restricted expression of Dl at the tips of the primary branches, at the time when N is acting, is functionally relevant.

It is possible that Dl is a target of the bnl/btl pathway, certainly the cells that express high levels of Dl are those where btl signalling is most active (Gabay et al., 1997; Oshiro and Saigo, 1997). If so, Bnl would be received by a group of cells and would activate Dl expression there to initiate the specification of individual fates within the group. Interestingly, regulation of Dl expression by another signalling pathway has been observed before: in the wing, activation of the epidermal growth factor (EGF) receptor leads to expression of Dl in the presumptive veins (de Celis et al., 1997). In addition, N activity also modulates Dl expression in the trachea, as in other systems (Heitler et al., 1996). A regulatory loop between N and Dl in the trachea could be mediated by the fusion genes, as broader domains of Dl accumulation are observed when most cells express the fusion factors. The regulation by the fusion genes could help to maintain high levels of signalling until cell fates are specified.

A model for the establishment of tracheal fates at the tips of the branches

bnl is expressed in clusters of cells outside the tracheal system and, in addition to guiding branch migration, it is necessary to pattern the tips of the primary branches (Sutherland et al., 1996). Bnl activates the Btl receptor in a gradient, leading to the expression of the pantip, terminal and fusion markers (Samakovlis et al., 1996a). Two pieces of evidence indicate that the tip cells are initially equivalent: first, they all express the pantip markers in response to bnl and, second, they can all behave in the same way in different mutant backgrounds (Hacohen et al., 1998; Stenberg et al., 1998; this work). The expression of the pantip markers becomes restricted to those cells at the leading ends of the branches, as the Btl activity is higher there due to proximity of the Bnl source. Among these tip cells, some become fusion cells while others can differentiate as terminal cells, even though they all receive similar amounts of Bnl. Therefore, the bnl/btl pathway is not sufficient to account for the diversification of the tip cells, and it is proposed that the N signalling acts to achieve this (Fig. 8).

The pnt gene (Pantip-1) has been shown to repress the fusion markers (Samakovlis et al., 1996a), yet, even though the tip cells all express pnt, one of them acquires the fusion fate (Fig. 8). A possible resolution of this paradox is that there might be a balance between the bnl/btl pathway, which promotes the fusion fate, and the N signal and pnt, which repress it. The N pathway shifts this balance: its presumed inactivity in one cell would allow that cell to overcome the repression by pnt, while its activity in the remaining cells would allow them to overcome the activation by bnl/btl. It is possible that biasing differences in the tip cells are also required to shift the balance.

Once the fusion cell is specified, the expression of some pantip markers decays in that cell, suggesting that the fusion factors repress the pantip genes. A piece of evidence supports this: in N mutants, where all the cells express the fusion markers, Pantip-1 and Pantip-2 expressions are extinguished. Simultaneously, the fusion cell seems to signal via Dl to its neighbours to repress the fusion genes, allowing them to express the antifusion or the terminal genes. The data suggest that the fusion genes repress the antifusion ones, because, in the wild type, the antifusion and the fusion genes are expressed in complementary patterns and, in null N mutants (where all the cells express the fusion markers), the antifusion genes are repressed.

Similarly, the specification of the terminal fate also depends on a balance between inductive signals, mediated by bnl/btl and the pantip genes, and repressing signals, mediated by N, spry and hdc (Fig. 8). Cells closest to the Bnl source that also express high levels of pantip markers and that do not acquire the fusion fate, can become terminal cells. The remaining cells receive negative signals from the already specified terminal cell (mediated by Dl and spry) and from the also specified fusion cell (mediated by hdc) to repress the terminal fate. Thus, the acquisition of fates amongst a group of cells located at the tips of each primary branch depends on the integration of positive and negative effects from different signalling pathways.

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