Interplay of Notch and FGF signaling restricts cell fate and MAPK activation in the \textit{Drosophila} trachea

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Accepted 6 August; published on WWW 27 September 1999

SUMMARY

The patterned branching in the \textit{Drosophila} tracheal system is triggered by the FGF-like ligand Branchless that activates a receptor tyrosine kinase Breathless and the MAP kinase pathway. A single fusion cell at the tip of each branch expresses the zinc-finger gene \textit{escargot}, leads branch migration in a stereotypical pattern and contacts with another fusion cell to mediate fusion of the branches. A high level of MAP kinase activation is also limited to the tip of the branches. Restriction of such cell specialization events to the tip is essential for tracheal tubulogenesis. Here we show that Notch signaling plays crucial roles in the singling out process of the fusion cell. We found that Notch is activated in tracheal cells by Branchless signaling through stimulation of Delta expression at the tip of tracheal branches and that activated Notch represses the fate of the fusion cell. In addition, Notch is required to restrict activation of MAP kinase to the tip of the branches, in part through the negative regulation of Branchless expression. Notch-mediated lateral inhibition in sending and receiving cells is thus essential to restrict the inductive influence of Branchless on the tracheal tubulogenesis.

Key words: Lateral inhibition, Cell migration, MAP kinase, Notch, branchless, escargot

INTRODUCTION

Tubular epithelial networks such as the vascular system, lung buds, kidney tubules and mammary glands are essential elements in the body plan of higher metazoans. Signaling molecules controlling the development of these systems have recently been elucidated (Risau and Flamme, 1995; Hogan, 1999; Robinson et al., 1996). However, how those molecules coordinate cellular events in the morphological change from a sac to tubules during branching is not known.

The tracheal system in \textit{Drosophila} is a simple model to study the genetic circuitry underlying the tubule formation, because branching, migration and cell shape change including ramifications of fine cytoplasmic extensions (tracheoles) over target tissues proceed in a stereotyped pattern without cell proliferation (Manning and Krasnow, 1993). The tracheal primordia invaginates from the lateral ectoderm of the embryo to form an epithelial sac and subsequently undergoes primary branching (Fig. 1J). Immediately after the beginning of the branch migration, single cells at the tip of branches that are going to fuse express the Zn$^{2+}$ finger protein Escargot (Esg; Fuse et al., 1994). These cells later become fusion cells that join tracheal branches by forming cadherin-dependent intercellular adhesion and by undergoing a unique cell-shape change to form doughnut-like cells (Tanaka-Matakatsu et al., 1996; Samakovlis et al, 1996b). The key event in primary branching is the expression of \textit{branchless} (\textit{bnl}) in subsets of the ectoderm and mesoderm surrounding the primordium (Sutherland et al., 1996). \textit{bnl} encodes a putative fibroblast growth factor (FGF; Sutherland et al., 1996) that activates the receptor tyrosine kinase Breathless (Btl) expressed in all tracheal cells (Glazer and Shilo, 1991; Ohshiro and Saigo, 1997), leading to the stimulation of the mitogen-activated protein kinase (MAPK) pathway (Reichman-Fried et al., 1994). If Bnl is diffusible as predicted, it potentially influences several tracheal cells. However, the activated form of MAPK was detected only at the tip of the branches (Gabay et al., 1997b), suggesting that Bnl-Btl signaling is programmed to limit MAPK activation to the tip of tracheal branches.

A well-known mechanism of cell fate restriction is Notch (N)-dependent lateral inhibition (Greenwald, 1998). N is a transmembrane receptor that has 36 EGF and 3 cysteine-rich Notch/Lin-12 repeats in the extracellular region and 6 tandem CDC10/ankyrin repeats and a potential PEST sequence in its intracellular region (Wharton et al., 1985; Kidd et al., 1986). The transmembrane proteins Delta (Dl) and Serrate (Ser) are known as N ligands (Kopczynski et al., 1988; Fleming et al., 1990), and their expression and potentiation play key roles in the activation of N (Heitzler and Simpson, 1991; Panin et al., 1997). A significant feature of the N pathway is to amplify a small difference in the level of N activity among equivalent cells by a cross-talk between neighboring cells, leading to a selection of one or a few cells taking a distinct developmental fate.

In this work, we studied the regulation and function of N in tracheal development of \textit{Drosophila} and found that localized
activation of N is required to limit the number of esg-expressing cells to one in each fusion branch. N signaling in

the tracheal primordium is evoked by the Bnl-Btl pathway, which stimulates Dl expression at the tip of the primary branches where N appears to be suppressed. Moreover, MAPK activation in the trachea was found to be restricted by N, in part through downregulation of Bnl expression. Therefore, N-mediated negative regulation of Bnl and cell fate decision ensure that MAPK activation and differentiation of the fusion cell takes place only in a single cell at the tip of each branch.

**Fig. 1.** Expression of Dl, N and Ser in tracheal cells. (A) Dl expression in early stage 11 embryos. Two to three cells at the tip of primary branches express high level of Dl (green; enlarged in inset). Tracheal cells are labeled with trh-lacZ (red). (B) Double labeling of N (green) and trh-lacZ (red). (C) Same as B, showing only N expression, which is uniform. (D-F) Expression of Dl (green, E) and esg-lacZ (red, F) in late stage-11 embryo. High level of Dl expression was limited to single cells at the tip of each DT branch, when esg-lacZ was also expressed. (G,H) Dl expression in a stage-14 embryo (green, H), showing high expression in DT. Note that cells flanking fusion points (arrows) expressed the highest level of Dl. Tracheal cells were labeled with btl:NZ. (I) Ser expression in a stage 15 embryo (red), showing high accumulation in apical membrane of DT after completion of fusion. No Ser expression was detectable in tracheal cells in the prior stage.

**MATERIALS AND METHODS**

**Fly stocks**

The following strains were used in this study: Nts1 (Grimwade et al., 1985), N264-39 (Dietrich and Campos-Ortega, 1984), bs l(2)03267 (Guillemin et al., 1996), Dl 6B37 (Lehmann et al., 1983), Ser RX106 (Speicher et al., 1994), tracheless enhancer trap line 1-eve-1.
Fig. 3. Tracheal phenotypes in $N^{ts1}$. (A,C,E) control, (B,D,F) $N^{ts1}$. (A,B) Dorsal view of embryos showing misrouting of DB and aberrant contacts of DBs from the same side (arrows). (C,D) Lateral view of embryos showing defects in fusion by DT and LT which made contact but failed to deposit a luminal component at fusion points; D also shows defective migration of GB (arrow). (E,F) High magnification image of DB extending from DT (right) to dorsal midline (left). Nuclei are numbered. Excess nuclei are located at the tip of DB in $N^{ts1}$. In this and the other figures, dorsal midline is represented by broken line, fusion points by arrows and DTs by arrowheads. Anterior, up (A,B,E,F). Anterior is to left and dorsal is up (C,D). Tracheal nuclei were labeled with nuclear β-galactosidase (red) and the lumen was labeled with 2A12 immunostaining (green).

Fig. 4. $N$ mutation changes cell fate and cell adhesion property. Anti-Esg (green) and tracheal cell (labeled by $btl: NZ$; red) staining in control (A,C,E) and in $N^{ts1}$ (B,D,F) embryos. (A,B) Dorsal view of DB. (C-F) Lateral view of fusion points of DT (C,D) and LT (E,F). Each branch of control embryos contains single Esg-positive cell. Asterisk in C indicates non-tracheal cell. In $N^{ts1}$, each fusion point contained 2-4 extra Esg-positive cells (B,D,F), and made contact with neighboring branches from the same side (B). Intense NZ signal was associated with the apical surface of control tracheal cells but no such signal was found at DT contact points in $N^{ts1}$(D). Thin broken line indicates tracheal branch that runs out of focus. (G,H) Expression of a terminal cell marker DSRF-lacZ in DB (green). Tracheal lumen was traced by 2A12 staining (red). DSRF expression was lost or strongly reduced in the $N^{ts1}$ mutant (arrow in H). Non-tracheal expression of DSRF is seen in background. (I,J) Expression of DE-cadherin (green) and $shg$-lacZ (red) in DT fusion points. In fusion cells of control embryos, high accumulation of DE-cadherin was detected on the apical side (arrow in I) and $shg$-lacZ was expressed at a higher level than in other tracheal cells (asterisk in I, the other fusion cell nucleus is out of focus). In $N^{ts1}$ mutants (J), no elevation of DE-cadherin was observed (arrow), and the expression of $shg$-lacZ had not appreciably become elevated.
Embryo staining

The following primary antibodies were used in this work: rat anti-Esg (Fuse et al., 1994), mouse anti-Delta 9B and anti-Notch (Fehon et al., 1990; a gift from K. Matsuno), anti-Ser (Thomas et al., 1991; a gift from T. Uemura), which recognizes an unknown luminal component (Developmental Studies Hybridoma Bank), and anti-beta-galactosidase (Cappel). Immunostaining with dpMAPK was performed as described by Gabay et al. (1999a). Other antibodies were used as described by Hayashi et al. (1993). The secondary antibodies used were as follows: biotinylated-anti-rat-IgG, biotinylated-mouse-IgM and Cy3 conjugated-mouse-IgM from Jackson Laboratory, and Cy3-diphospho-MAPK (Gabay et al., 1997a; purchased from Sigma), anti-E-cadherin (DCAD2; Oda et al., 1993; a gift from T. Uemura), 2A12, which recognizes an unknown luminal component, and anti-Ser (Thomas et al., 1991; a gift from T. Uemura), mouse anti-Delta 9B and anti-Notch (Fehon et al., 1990; a gift from K. Matsuno), anti-Ser (Thomas et al., 1991; a gift from T. Uemura), which recognizes an unknown luminal component (Developmental Studies Hybridoma Bank), and anti-beta-galactosidase (Cappel). Immunostaining with dpMAPK was performed as described by Gabay et al. (1999a). Other antibodies were used as described by Hayashi et al. (1993). The secondary antibodies used were as follows: biotinylated-anti-rat-IgG, biotinylated-mouse-IgM and Cy3 conjugated-mouse-IgM from Jackson Laboratory, and Cy3 conjugated-rabbit-IgG from Chemicon. When biotinylated antibodies were used, signals were visualized by Cy2- or Cy3-conjugated streptavidin (Amersham). Weak signals were sometimes amplified by the use of biotinylated-tyramide (NEN Life Science Product) as a substrate followed by the use of an ABC elite kit (Vector Lab.). Fluorescent images were taken by confocal microscopy (Carl Zeiss LSM410), and image processing was performed by use of Photoshop software (Adobe). In situ hybridization was performed essential as described (Tauz and Pfeifle, 1989).

RESULTS

Elevated expression of Delta at the tip of migrating branches

Six primary branches form in the tracheal primordia (Fig. 1Ja), among which the dorsal branch (DB), anterior and posterior dorsal trunk (DTa, DTp), and anterior and posterior lateral trunk (LTa, LTp) migrate along a stereotyped path to be connected with other branches from adjacent primordia (Fig. 1Jb). These fusion branches are capped with fusion cells that express Esg (marked red in Fig. 1J). The remaining visceral branch (VB) migrates to reach the internal organs. Terminal cells expressing Drosophila serum response factor (DSRF, marked blue in Fig. 1Jb; Affolter et al., 1994; Guillemin et al., 1996) are formed in each primary branch except in DTs and later differentiate multiple tracheoles.

Previous studies reported high expression of DI mRNA and protein in the DT of stage-15 embryos (Haeinlin et al., 1990; Kooh et al., 1993), but the pattern of its early expression was not known. To know the temporal and spatial expression pattern of early DI, we used a biotinylated-tyramide amplification method to detect weak antibody signals (Materials and Methods). Cells in the tracheal primordium just after invagination expressed DI uniformly (data not shown). At early stage 11, DI expression started to be elevated in 2-3 cells at the tip of the branches in which outgrowth had begun (Fig. 1A) and the number of the DI-expressing cells was reduced to one at late stage 11 (Fig. 1D). At stage 14, high DI expression remained only in the DT, which had completed fusion (Fig. 1G,H; Kooh et al., 1993). Ser protein also accumulated at the apical side of the DT cells at the same stage (Fig. 1; Thomas et al., 1991). It is known that the expression of a N in pupal proventine region is activated by DI (Huppert et al., 1997). However, in the case of the trachea, the level of N protein expression remained uniform (Fig. 1B,C), suggesting that the expression level of DI, or its potentiation, must be crucial for N activation. We next monitored the expression of esg using a lacZ reporter (esgGal4 = esg-lacZ). esg-lacZ was initially expressed in 2-3 cells at the tip of the fusion branches in mid stage 11 embryos (data not shown), and was downregulated to be maintained in only a single cell at the tip of each fusion branch at late stage 11 (Fig. 1D,E). These cells also expressed a high level of DI (Fig. 1D,E). Therefore, localized elevation of DI expression in stage 11 correlates well with the selection process of a single fusion competent cell. The roles of late expression of DI and Ser in DT after stage 15 are currently not known.

FGF signaling stimulates Delta expression

The localized DI expression at the tip of the branches suggests the possibility that DI responds to a signal controlling primary branching. We therefore studied the regulation of DI by the Bnl/Btl pathway, the key regulator of primary branching. When tracheal cells became unresponsive to Bnl due to btlB10h10 mutation (Ohshiro and Saigo, 1997), no sign of primary branching and DI upregulation was observed (Fig. 2A,B). On the contrary, when Btl was hyperactivated by overexpression of Bnl in all the tracheal cells, primary branching was severely inhibited and DI expression was elevated (Fig. 2C,D). These results suggest that elevation of DI expression is triggered by the external signal Bnl. The results also suggest that the N/DI pathway may mediate the Bnl signal to control cell migration and cell fate decision. We therefore examined tracheal phenotypes of N mutants.

Defective tracheal patterning in Notch mutants

Hartenstein et al. (1992) showed that tracheal development is grossly disorganized in N null mutants. However, because of the general requirement for N in ectoderm and mesoderm development in prior stages, it was difficult to distinguish whether the tracheal defects in N null mutant reflect the function of N in the trachea per se, or the secondary effect of the ectodermal defects in prior stages. To avoid this problem, we used a temperature-sensitive allele Nts, which has a mutation at the 32nd EGF repeat and fails to transport the protein to the apical cell surface at the restrictive temperature (Xu et al., 1992). We used a temperature-shift condition that allows normal development up to stage 11 when primary branching is initiated. N was then inactivated and the phenotype was examined by staining tracheal nuclei and lumen (Fig. 3). Under this condition, distinct phenotypes were observed.

Temperature-shift condition

Nts was recombined with X chromosome insertion of btl-Gal4 and was crossed to males carrying marker strains. The y w 1ts21 chromosome was used as a control. The temperature-shift protocol for the Nts and Df(2R)B2a2 cultures was as follows: after 3 hours of egg laying at 18°C on apple juice-agar plates, the plates were kept at 18°C for 10 hours, and then transferred to a water bath at 30°C and incubated for an additional 6 hours before fixation. When we studied the effect of the inactivation of N on MAPK activation, embryos were collected at 18°C for 10 hours and immediately placed at 30°C for 2 hours before fixation.
First, a misrouting defect was observed in DB, which normally elongates to the dorsal midline where it meets its counterpart from the other side of the metamere (Figs 1Jb, 3A). In \( N^{ts1} \) embryos, DBs were often curved in the anteroposterior direction and made contact with the tip of DB from the same side (Fig. 3B). The misrouted DBs accumulated a luminal component detectable by 2A12 antibody at the ectopic contact sites, but did not appear to fuse properly (see below). Second, cell migration defect was also observed in DB. DB consists of a total of 5-7 cells in the case of Tr5 (Fig. 3A; Samakovlis et al., 1996a), of which two specialized cells are located at the tip. One is the terminal cell (marked as 1 in Fig. 3E), from which a thin terminal branch sprouted, the other is the fusion cell (marked as 2 in Fig. 3E). The remaining stalk cells (marked as 3-7 in Fig. 3E) are located between the tip and DT at regular intervals. In \( N^{ts1} \) mutants, the number of cells at the tip was increased (1'–4' in Fig. 3F) with a corresponding decrease in the number of stalk cells, the latter having become unusually elongated (6',7' in Fig. 3F). The total number of cell nuclei did not change compared to the control, so no additional mitosis had probably occurred. We also noted that the fine luminal extensions characteristic of terminal branch (marked as 1 in Fig. 3E) were often absent in DBs of \( N^{ts1} \) embryos (Fig. 3F), suggesting that the fate of terminal cells had changed.

**Notch restricts the fate of fusion cells**

The cell migration phenotype described above suggests that stalk cells and terminal cells acquired a property of fusion cells to become localized at the tip of DB. To extend this observation, we used cell-type-specific markers for fusion and terminal cells. Expression of \( esg \) is induced by a complex combination of signaling activities (T. Chihara and S. H., unpublished observation), and is limited to one cell on each fusion branch. The fusion points of DB, DT and LT contained two \( esg \)-positive cells in the control embryos, each derived from individual fusion branches (Fig. 4A,C,E). In \( N^{ts1} \) embryos, the number of \( esg \)-positive cells was increased at the tip of DB, DT and LT that made abnormal contact (Fig. 4B,D,F). No extra \( esg \)-positive cells were seen in the VB or GB (ganglionic branch), which normally contain only terminal cells (not shown). Consistent with the disappearance of terminal branches (Fig. 3F), expression of the terminal cell marker DSRF was reduced or lost (Fig. 4G,H) in \( N^{ts1} \) embryos. Since it was previously shown that \( esg \) represses DSRF expression and terminal branching (Samakovlis et al., 1996b), the loss of \( esg \)-expressing cells was the consequence of ectopic \( esg \) expression. Similar defects in \( esg \) and DSRF expression were also observed in null mutation \( N^{264-39} \) (data not shown). These results suggest that in \( N \)-embryos, several terminal and stalk cells are recruited to the fate of fusion cells.

**Localized Notch-Delta interaction is required in trachea**

We examined tracheal phenotypes of \( Delta \) (Dl) and \( Serrate \) (Ser) that encode transmembrane ligands of N (Artavanis-Tsakonas et al., 1995). The null allele \( Ser^{RX/100} \) did not show any abnormality in the overall pattern of the tracheal system nor any increase in the number of \( esg \)-positive cells (not shown). On the contrary, we found that the number of \( esg \)-positive cells was increased in temperature-sensitive \( Delta^{6B37} \) (Parody and Muskavitch, 1993) embryos and in \( N^{ts1} \) embryos (Fig. 5A), suggesting that Dl is a major ligand of N in the trachea.

To determine whether N activity is required autonomously in the trachea, we modified N activity within tracheal cells by expressing various effector genes under the control of \( btl-Gal4 \) (Shiga et al., 1996). When the dominant negative form of N was expressed, the number of \( esg \)-expressing cells at the tip of the DB increased up to four (Fig. 5C). This result indicates that Notch signal is required autonomously in the trachea to restrict the fusion cell fate. In contrast, when the activated N construct (Nact) was expressed, all \( esg \)-expressing cells disappeared from the tip of the fusion branches and no sign of a contact of fusion branches was observed (Fig. 5D). In addition, the DT became short and thick (Fig. 5G). The total number of tracheal cells per metamere was not significantly different from the control (for Tr5, Nact: 78.0, \( n=9 \); control: 79.6, \( n=3 \)), suggesting that the loss of \( esg \)-positive cells by Nact is due to a change in cell fate. These results suggest it is likely that N activity is required in tracheal cells adjacent to the tip of DB, but it must not be activated in the fusion cell itself.

Ectopic expression of Dl resulted in an unexpected finding. Contrary to our prediction that an excess of a ligand would cause a hyperactivation of N and a loss of \( esg \)-positive cells, we found an increase in the number of \( esg \)-expressing cells in DT (Fig. 5E), suggesting a possibility that a high dose of Dl represses the N activity. These results demonstrated that N is required in tracheal cells to limit the fate of the fusion cells. However, the expression of the dominant-negative N did not reproduce the misrouting and migration defects observed in \( N^{ts1} \) embryos, suggesting that N is also required outside of the trachea. This question was addressed in the next experiment.

**Negative regulation of FGF signaling by Notch**

Recent studies have shown that activation of MAPK by the Bnl/Btl pathway is limited to the tip of branches (Gabay et al., 1997b). As shown in Fig. 6A, each fusion branch in late stage 11 embryos contained a single dpMAPK-positive cell at its tip, dpMAPK expression was rapidly downregulated prior to the onset of \( esg \) expression in fusion cells and later reactivated in terminal cells during secondary branching (data not shown). Judging from the location, it is likely that the dpMAPK-positive cells are identical to the cell that later coexpressed \( esg \)-lacZ and Dl (Fig. 1D). We found that, in \( N^{ts1} \) mutants, the number of dpMAPK-expressing cells was increased at the tip of the branch (Fig. 6B). More severe phenotype was observed with a N null allele (Fig. 6C,D). In those embryos, a dpMAPK signal was detected in most of the tracheal cells and persisted for a longer period until stage 13. To clarify whether the effect of N mutations on MAPK activation is due to a change in bnl expression, we examined the expression pattern of bnl mRNA. In N null mutants, the number of bnl-expressing cells and the intensity of bnl mRNA were increased compared to control embryos (Fig. 6E,F). Therefore, N regulates the pattern of bnl expression and MAPK activation, thereby refining FGF signaling.

**Notch is required for branch fusion**

Finally, we considered the capability of N mutant cells to mediate fusion. In \( N^{ts1} \) embryos, fusion branches carrying extra \( esg \)-expressing cells migrated and contacted with other fusion branches (Fig. 4B,D,F). However, fusion was incomplete in
these branches. Many of the DB (79.4%), DT (33.3%) and LT (58.5%, n=54) branches that had made contact with their target branches failed to form tubes as evidenced by discontinuous deposition of a luminal antigen (Fig. 3B,D). When the timing of the temperature-shift was delayed, the penetrance was decreased (data not shown). To characterize cytoskeletal changes in tracheal cells, we used nod-lacZ (NZ; Clark et al., 1997) as a marker for the microtubule network. Nod is a kinesin-like, plus-end directed motor protein, and the fusion protein of its motor domain with β-galactosidase is concentrated at the apical side of epithelial cells and distal tips of dendrites (Clark et al., 1997). When NZ was expressed in all tracheal cells under control of btl-Gal4, it was highly concentrated at the apical membrane facing the luminal side with a weaker signal in the cytoplasm (Fig. 4A,C,E). At the fusion points of the control embryos, a strong NZ signal runs through the two fusion cells that are tightly attached (Fig. 4A,C, arrows), consistent with the formation of the apical membrane.

In Nts1 embryos, however, no continuous accumulation of NZ was detected in the fusion cells (Fig. 4B,D, arrows).

Two opposing fusion cells accumulate DE-cadherin at the contact interface in a ring pattern as a consequence of upregulation of the promoter activity of the DE-cadherin gene shotgun (shg; arrow and asterisk in Fig. 4I; Tanaka-Matakatsu et al., 1996). At the same time a luminal cavity was formed in each fusion cell and the lumen passed through the ring of DE-cadherin to complete the fusion and the formation of doughnut-like cells (Tanaka-Matakatsu et al., 1996). But in the Nts1 mutants, no accumulation of DE-cadherin was observed at the contact interface (arrow in Fig. 4J) and we did not observe a...

Fig. 5. Requirement for Dl and localized activation of N in fusion cell determination. (A) Phenotype of a temperature-sensitive allele Delta6B37. Two to three Esg-expressing cells (green) were seen at the tip of the DB. Tracheal lumen was stained with 2A12 (red). DT and DB are seen from the lateral side in progenies from crosses between btlGAL4; UAS-gfpmlacZ and y w (B), UAS-ECN (dominant negative form of Notch, D), UAS-Nact (active form of Notch, E) and UAS-Dl (F) are shown. Extra Esg-positive cells appeared by overexpression of ECN and Dl. However, all Esg-positive cells disappeared by expression of Nact. (EG) Nact inhibited anteroposterior migration of DT, caused thickening of DT and inhibited branch fusion. Arrows indicate tips of fusion branches.

Fig. 6. Suppression of MAPK activation by N. (A-D) dpMAPK expression (green) in stage 11 embryos. (A) Control embryos expressed dpMAPK in single cells at the tip of primary branches, which appeared to overlap with Dl-expressing cells (compare with Fig. 1D). (B) The number of dpMAPK-expressing cells was increased to 2-3 cells in the Nts1 mutant. (C) Overview of a N264-39 mutant embryo showing high level of dpMAPK throughout the trachea. (D) High-magnification view of C. Tracheal cells are marked with trh-lacZ (A) or btl:NZ (B-D), respectively. (E,F) Expression of bnl mRNA in control (E) and N264-39 (F) embryos. Clusters of a few cells express bnl in control (F). In N264-39 embryos, the clusters were irregular, slightly enlarged and the intensity of bnl expression was increased.
clear case of shg-lacZ upregulation. Taken together, although the data show that fusion cells in Nts1 embryos made contact with their targets, they failed to accumulate DE-cadherin and to undergo the characteristic cell shape change to allow fusion of the tube.

DISCUSSION

During tubulogenesis of the tracheal system, cell migration, rearrangement and differentiation are well coordinated. Because the cells at the tip of each branch are the only ones that respond to FGF signaling to activate MAPK (Fig. 5A; Gabay et al., 1997b), they are likely to play organizing roles in tubulogenesis, and directed migration in response to FGF. Having an excess of such cells by the hyperactivation of the FGF receptor Btl was deleterious to primary branching (Fig. 2C). Furthermore, cells at the tip of the fusion branches later became fusion cells that joined the branches by changing to a doughnut-like shape (Tanaka-Matakatsu et al., 1996; Samakovlis et al., 1996b). This mode of epithelial fusion requires 1-on-1 cell contact during the fusion. These considerations suggest that the selection of a single fusion competent cell that responds to FGF is an essential requirement for the tubulogenesis by fusion branches. This and a recently published work by Llimargas (1999) have shown that Notch plays a crucial role in this singling out process. This work has further demonstrated the regulatory relationships between N and FGF signaling that appears to be essential for the tracheal tubulogenesis (Fig. 7).

Roles of FGF and N signaling in tracheal tubulogenesis

Localized expression of FGF-like molecule Bnl in cells adjacent to the tracheal primordium is a crucial step in primary branching (Sutherland et al., 1996). We have shown here that DI expression is elevated early in primary branching in a Bnl-dependent manner. Since localized DI expression was essential to activate the N pathway, we concluded that the N pathway in the trachea is triggered by the exogenous FGF signal through activation of DI (Fig. 7, Induction). The transcription factor Pointed is expressed in response to Bnl in several cells at the tip of the tracheal branches (Samakovlis et al., 1996a; Sutherland et al., 1996). In pnt mutants, extra fusion cells differentiate as was also seen in Nts1 (Samakovlis et al., 1996a). This similarity of the phenotypes suggests that pnt mediates the Bnl signal to activate N. One possible reason for this is that DI transcription is stimulated in a pnt-dependent manner. The elevated expression of DI in fusion cells activates N in neighboring cells and represses transcription of esg (Fig. 7, Lateral inhibition).

Another key finding in this study was that N downregulates branchless transcription (Fig. 6E,F). This function of N is likely to account for its negative effect on MAPK activation in the trachea. We think that two aspects of the N-mediated lateral inhibition, one being the restriction of Bnl expression, and the other the restriction of the fusion cell fate, are essential in the singling out of cells with high dpMAPK and esg expression. Such cells are likely to change cell shape and/or motility which is the first step of primary branching (Fig. 7, Tubule formation). The role of MAPK in this stage should be distinguished from its role in secondary branching where MAPK is activated in the terminal branch. Although the detail is not yet available, the regulation and function of MAPK during terminal branching is different from those of primary branching.

The unique feature of N system in the trachea is its activation by an exogenous signal, FGF. In most of the known cases, N is activated as a consequence of differential gene expression within the same tissues. For example, DI-expressing domains in wing vein primordium are determined by the EGF receptor signaling (de Celis et al., 1997), and the activation of N along the wing margin is controlled by the border of fringe expression (Panin et al., 1997). A situation similar to the case of the trachea was observed in the vulval development in C. elegans in which the choice of cell fates is regulated by the Notch homolog LIN-12 (reviewed in Greenwald, 1998). In this case, activation of LET-23 receptor kinase and the MAPK pathway by EGF-like ligand LIN-3 biases the choice of two alternative vulval precursor cell fates. It was speculated that activated LET-23 governs the expression of the ligand for LIN-
Local activation and repression of Notch selects a single Delta-expressing cell

For an understanding of the function of Notch, it is important to know which cells activate N. Phenotypes of a loss-of-function mutation of N (Figs 4, 6) and uniform expression of dominant-negative form of N (Fig. 5) suggest that activation of N is required in cells adjacent to the tip of branches to repress Esg expression and activation of MAPK. On the contrary, uniform activation of N repressed esg (Fig. 5D), indicating that N activity is needed to be low at the tip of fusion branches. These observations suggest that the fusion cell must be equipped with a mechanism that activates N in adjacent cells and simultaneously represses N in itself. We suggest that high-level expression of DI in the fusion cell is responsible for this mechanism. The overlap of esg-lacZ and DI expression (Fig. 1D) suggests that fusion cells activate the N receptor in adjacent cells by presenting DI as an agonist. Overproduction of DI inhibited N activity in the development of the trachea, leg and bristle (this and our unpublished work; see also Llimargas, 1999; de Celis et al., 1998; Jacobson et al., 1998; Doherty et al., 1996). We thus propose that high-level expression of DI in fusion cells provides a cell-autonomous, dominant-negative effect on N within the cells to allow esg expression. It is unlikely that DI represses expression of N in the trachea, since the level of N was not changed in cells with high expression of DI (Fig. 7). It is more likely that the proposed negative effect of DI acts upon N signaling. It was previously shown that activated N represses expression of DI in the wing disc (Huppert et al., 1997; de Celis and Bray, 1997). The recent work by Llimargas (1999) indicates that such interaction also operates in the trachea. Cell autonomous, mutual inhibition between N and DI, and intercellular activation of N by DI would in principle constitute a regulatory circuit that sharpens a small difference in N activity among an equivalent cell group to select out a single cell with high expression of DI (Fig. 7, Lateral inhibition).

Possible role of N in branch fusion

Another phenotype in the Nmutant embryo was a failure of fusion. Whereas fusion cells in the wild-type trachea adhere to each other by DE-cadherin-mediated adhesion (Uemura et al., 1996; Tanaka-Matakatsu et al., 1996) and change to a doughnut-like cell shape to accommodate connection of the lumen, fusion cells in Nmutant failed to accumulate DE-cadherin after making initial contact and remained in their original shape. It was proposed earlier that the contact between fusion cells triggers some signaling event that stimulates expression of DE-cadherin through an esg-dependent mechanism (Tanaka-Matakatsu et al., 1996). The phenotype in Nmutant was very similar to the phenotype in shg (DE-cadherin gene) and esg mutants, suggesting an interesting possibility that N is involved in contact-mediated signaling between fusion cells. An alternative possibility is that recruitment of extra fusion cells due to a loss of N in a prior stage results in incomplete cell specification of fusion cells that subsequently fail in fusion.

Role of Notch in tubulogenesis in vertebrates

Recent studies show relationships between the N signaling and the tubulogenesis in vertebrates. Alagenle syndrome is an autosomal dominant disorder caused by haploinsufficiency of human Jagged1, which encodes a putative N ligand (Oda et al., 1997; Li et al., 1997). One major pathological feature is the paucity of intrahepatic bile ducts, indicating a requirement for N in bile duct development. We noted that a loss of N function in the trachea resulted in a failure of cuticle deposition and its function as an airway (our unpublished observation). Notch4 is expressed in endothelial cells of mice (Uyttendaele et al., 1996) and its spatial and temporal pattern correlate very well with that of the flk-1 gene encoding a receptor tyrosine kinase essential for endothelial cell proliferation (Millauer et al., 1993; Shirayoshi et al., 1997). It will be interesting to know whether the activity of flk-1 is modulated by Notch4 or its oncogenic form int-3.

We thank Elisabeth Knust, Tadashi Uemura, Alfonso Martinez-Arias, Yuh Nung Jan, Yash Hiromi, Ben-Zion Shilo, Ira Clark, Mark Krasnow, Tomokazu Ohshiro, Kenji Matsuno, Developmental Studies Hybridoma Bank, and Umea and Bloomington Stock Center for the flies and/or antibodies. We are grateful to Yash Hiromi for the critical reading of the manuscript. This work was supported by a grant to S. H. from the Japan Society for the Promotion of Sciences (Research for the Future). T. I. acknowledges the support from the Center of Excellence program of NIG.

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