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# Tracheal branching morphogenesis in *Drosophila*: new insights into cell behaviour and organ architecture

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Our understanding of the molecular control of morphological processes has increased tremendously over recent years through the development and use of high resolution in vivo imaging approaches, which have enabled cell behaviour to be linked to molecular functions. Here we review how such approaches have furthered our understanding of tracheal branching morphogenesis in *Drosophila*, during which the control of cell invagination, migration, competition and rearrangement is accompanied by the sequential secretion and resorption of proteins into the apical luminal space, a vital step in the elaboration of the trachea's complex tubular network. We also discuss the similarities and differences between flies and vertebrates in branched organ formation that are becoming apparent from these studies.

## Introduction

Branching morphogenesis restructures epithelial sheets to give rise to organs of fascinating three-dimensional architecture, as exemplified by the adult lung, the kidney and the vasculature in humans. In *Drosophila melanogaster*, genetic studies have provided much insight into the regulatory networks that regulate the ordered formation of tracheal branches in the embryo, and into how the different branches coordinate their relative sizes, an issue that is of importance to the physical aspects of branched organ function (Affolter et al., 2003; Ghabrial et al., 2003; Uv et al., 2003). More recently, the development of live-imaging approaches in *Drosophila* have allowed researchers to take a deeper look at the behaviour of cells during the branching process, and have enabled events at the molecular level to be linked to the behaviour of individual cells or groups of cells.

This combination of molecular genetics and live-imaging techniques has provided investigators with a unique opportunity to understand the morphological processes that occur during branching morphogenesis. This review focuses and builds on some of these recent insights, and assesses how they have led to a better understanding of the cellular and molecular processes that contribute to the transformation of simple, two-dimensional epithelial sheets into fascinating, three-dimensional tubular structures that can perform important functions in development and homeostasis. We focus here on the *Drosophila* tracheal system because several cellular and molecular paradigms, such as cell migration, competition and rearrangement, as well as the elaboration of a complex apical luminal environment, have recently been uncovered in this system that might serve related roles in the formation of other branched organs or tissues; these similarities might help us in the future to gain an even better understanding of how morphogenesis in general is regulated during development.

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## Tracheal development in the fly embryo

The complex structure of the tracheal system consists of interconnected, metameric units of different-sized tubes that extend over the entire embryo shortly before hatching (Samakovlis et al., 1996b) (see Fig. 1, see also Movie 1 in the supplementary material). The metameric units begin their development independently during germ band extension, as groups of tracheal cells are set aside from the neighbouring epidermal cells and invaginate to form a sac-like tracheal structure. This structure generates the luminal cavity, which is subsequently expanded and remodelled during the branching process. Those tracheal cells in this sac-like invagination that are close to Branchless (Bnl)/Fibroblast growth factor (Fgf)-secreting (non-tracheal) cells adopt migratory properties and move toward the sources of Bnl/Fgf, while remaining attached to their tracheal neighbours. This results in the formation of interconnected, bud-like extensions.

Through extensive cell rearrangements and cell shape changes, these buds elongate to form branches of distinct cellular architecture, ranging from multicellular tubes to fine branches, in which cells are arranged in a head-to-tail-like fashion. Upon the interconnection of the metameric, branched units at distinct fusion points, specialized terminal tracheal cells at the periphery of the tracheal system extend the luminal space into individual cell extensions, which adopt tree-like structures and reach virtually every cell with a branch tip. It is at the tip of these fine, intracellular tubes that gas is exchanged with the surrounding tissue.

Research in the past few years has uncovered a series of interconnected cellular choreographies that are regulated by cell-cell signalling, by intracellular events controlling cell shape and motility, and by the sequential control of cell behaviour through apically secreted, luminal proteins. Similar scenarios might underlie branching morphogenesis in other organs systems, and we will mention a few such similarities that have recently emerged.

## Forming a sac from a planar epithelium

The formation of epithelial invaginations is of crucial importance and sets the stage for the ultimate development of the individual metameres of the tracheal system. Although considered to be a simple step, epithelial invagination is a process in which genes control the local formation and proper exertion of tissue forces, and its genetic dissection is a rather difficult task, as forces are already acting at earlier embryonic stages. Groups of epidermal cells are determined to become tracheal cells, in part, by their expression of a combination of transcription factors, including *trachealess* (*trh*), *ventral veins lacking* (*vvl*; also known as *ventral veinless*) and *knirps/knirps-related* (*kni/knrl*; also known as *knirps-like*) (reviewed by Affolter and Shilo, 2000). In an ordered choreography, these cells then invaginate while remaining attached to each other and to the neighbouring, non-tracheal cells.

As none of the cellular activities seen during invagination occur in *trh* mutants (Wilk et al., 1996), the identification and analysis of *trh* target genes and their effects on cell behaviour, protein

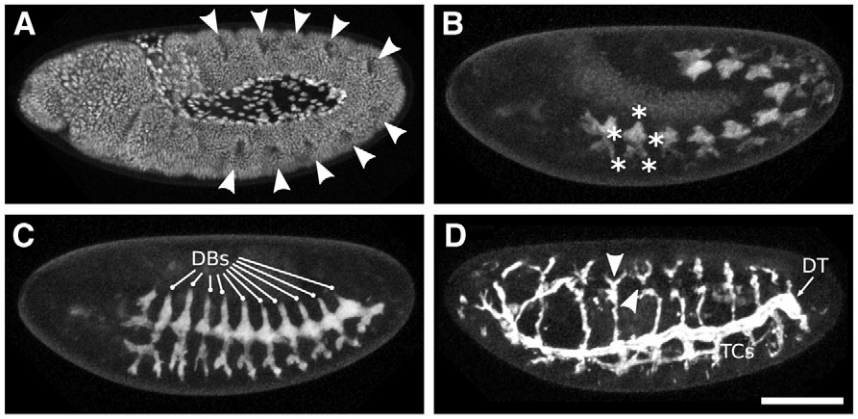


**Fig. 1. Branching morphogenesis of the *Drosophila* tracheal system during embryonic development.** Stills from time-lapse movies showing *Drosophila* embryos expressing either (**A**) *His2AvD::mRFP* ubiquitously or (**B-D**) *GFP::Actin* in the tracheal system (see Movie 1 in the supplementary material). (A) Stage 11 of embryonic development. Tracheal cells invaginate (arrowheads) and form tracheal sacs during the initial phase of germ band retraction. (B) Stage 12 of embryonic development. Tracheal sacs extend branches in stereotyped directions (asterisks). (C) Stage 14 of embryonic development. Tracheal branches elongate. (D) Stage 15 of embryonic development. Some branches are fusing to form an interconnected network of tracheal tubes (arrowheads). DBs, dorsal branches; DT, dorsal trunk; TCs, transverse connectives. Anterior is to the left and dorsal to the top. Scale bars: 100 μm.

localization, etc., should provide insight into how the flat epithelial sheet starts its transformation towards a branched organ. In a recent study, the careful analysis of cell behaviour in wild-type *Drosophila* embryos and in embryos mutant for Trh target genes did indeed lead to a two-step model in which *trh* induces and then organizes tracheal invagination (Brodu and Casanova, 2006) (see Fig. 2). In the first step, *trh* expression outlines an invagination field, a region of cells that acquire the competence to ingress or sink into the embryo. Relatively little is known about how this competence to 'invaginate' is molecularly brought about. However, Trh activity initiates a second step, the activation of Epidermal growth factor receptor (Egfr) signalling through the transcriptional activation of *rhomboid* (*rho*). *rho* encodes a transmembrane protein that specifically cleaves the Egf ligand and thus triggers the activation of the Egf pathway in tracheal cells. Egfr pathway activity appears to be tightly controlled within the placode. It is partly suppressed dorsally by the expression of *sal* (also known as *spalt*), which encodes a zinc finger transcription factor, leading to the observed asymmetry in invagination along the dorsoventral axis. In addition, the careful examination of cell behaviour and myosin localization during the invagination process has revealed that the Egf pathway is activated in a wave, which extends from the centre of the placode, where cells initially constrict apically, to the outer cells, coordinating the timing and positioning of intrinsic cell internalization activities (Nishimura et al., 2007). Egfr signalling eventually translates into an ordered apical distribution of actin and myosin, which presumably generates the forces that lead to invagination via actomyosin contraction. Another Trh target, *crossveinless c* (*cv-c*), which encodes a Rho-GAP enzyme, provides a key step in proper apical actomyosin distribution, presumably by regulating the activity of the small GTPase Rho1 (Brodu and Casanova, 2006). Loss of Cv-c results in a variably penetrant tracheal phenotype that resembles the

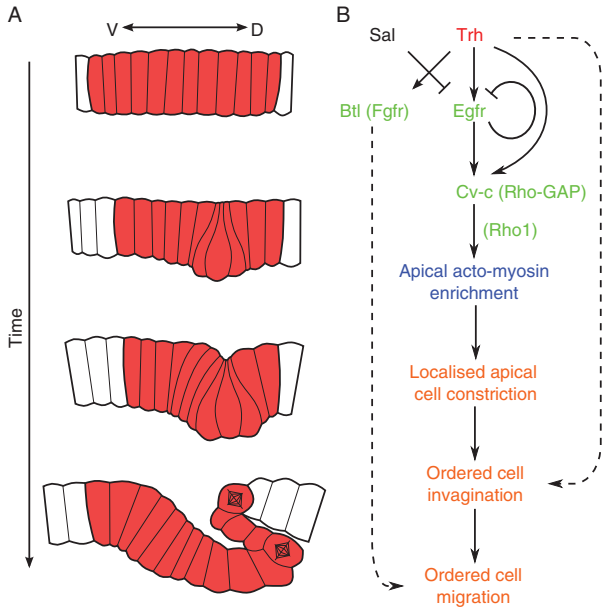


**Fig. 2. Tracheal cell invagination.** (**A**) A transverse representation of tracheal placode invagination. Tracheal placodes are clusters of ectodermal cells that express *trh* (red). These cells stop dividing shortly before apical constriction occurs and before the tracheal placodes invaginate into the embryo. They then re-enter mitosis and undergo one last round of cell division towards the end of the invagination process. The orientation of the cell division axis is biased towards the center of the tracheal pit and might help to direct cells to flow into the site of invagination (Nishimura et al., 2007). (**B**) A model of the signaling and cell remodeling events required for ordered tracheal cell invagination. The patterning information from *trh* (red) is translated through cell signaling processes (green) and cytoskeleton reorganization (blue) to cell remodeling events (orange; see text for details). Dashed lines indicate effects eventually caused by a factor. Adapted, with permission, from Brodu and Casanova (Brodu and Casanova, 2006).

phenotype caused by an absence of Egfr signalling. Because Egfr signalling is also required, in cooperation with Trh, for *cv-c* expression, it is likely that Egfr activity in tracheal invagination is, at least in part, mediated by the function of Cv-c in the apical localization of actomyosin.

Although still rather fragmentary, these studies clearly link cell fate specification (i.e. *trh* expression) to Egfr signalling, which presumably controls local actomyosin contractility in a complex spatiotemporal manner. This contractility is itself partly regulated by a Rho-GAP activity, and involves different feedback mechanisms. How it is, exactly, that the initial apical constriction of cells and the following ordered cell intercalation events eventually lead to the highly ordered invagination of tracheal cells, such as that which occurs in vivo, remains somewhat mysterious. More detailed investigations into where and how local forces act, and how these forces eventually translate into cell shape changes and cell displacements will be required to answer these questions. Such future studies must also be accompanied by computer modelling to determine whether such defined cell shape changes in an epithelial sheet are indeed potentially sufficient to translate into an ordered invagination process.

## Forming branches

### The role of cell migration

It has been known for several years that directed cell migration is the major cellular activity that underlies the branching process of the developing tracheal system. Tracheal cells express a Fgf receptor, encoded by the *breathless* (*btl*) locus (Klambt et al., 1992), as well as a Fgf-specific signalling adaptor protein encoded by *downstream-of-Fgfr* (*dof*; also known as *stumps*) (Vincent et al., 1998; Imam et al., 1999; Petit et al., 2004; Wilson et al., 2004). As for *rho* and *cv-c* expression (discussed above), *btl* and *dof* expression also require the Trh transcription factor, and the accumulation of *btl* and *dof* clearly distinguishes tracheal cells from neighbouring, non-tracheal, cells and prepares them for the branching process.

Upon invagination, tracheal cells migrate towards neighbouring cells or towards tissues that express the Btl ligand Bnl/Fgf; it is the spatial control of Bnl/Fgf secretion in the *Drosophila* embryo that ultimately controls the migratory behaviour and the direction of tracheal cell movement (Sutherland et al., 1996). Cells at the tip of tracheal branches (so-called tip cells) appear to be highly dynamic when visualized in live embryos with confocal microscopy; they send out filopodia and lamellipodia in response to Btl/Fgfr signalling. Stalk cells, which link the tip cells to the other tracheal branches, do not form such extensions (Ribeiro et al., 2002) (Fig. 3). In the complete absence of Fgfr signalling, cells remain in the sac-like configuration, and filopodia or lamellipodia are not seen (Ribeiro et al., 2002), demonstrating that Bnl/Fgf signalling regulates both the motility and the directionality of tracheal cell movement in the embryo.

Another important role of Fgf signalling during the branching process is the induction of Notch signalling. High levels of Fgf signalling in the tip cell of primary branches lead to the activation of the Notch (N) ligand Delta (Dl) (Llimargas, 1999; Steneberg et al., 1999; Ikeya and Hayashi, 1999). Dl expressed in tip cells activates N in neighbouring stalk cells. This N activation inhibits or reduces Fgf signalling in the neighbouring stalk cells, in part through the inhibition of activated Extracellular signal-regulated kinase (Erk) (Ikeya and Hayashi, 1999). Both Fgf and N signalling are involved in cell fate choice during the branching progress, and ensure that the correct number of fusion and terminal cells are generated at branch tips (reviewed by Zelzer and Shilo, 2000).

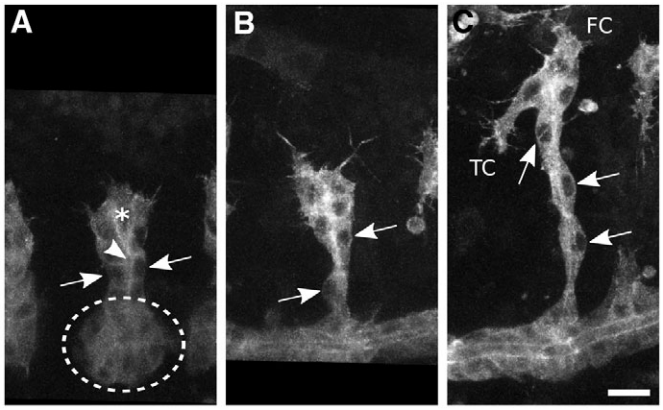


**Fig. 3. Formation of a dorsal branch. Dorsal branch development from embryonic stage 12 to stage 15.** Stills from a time-lapse movie showing an embryo expressing *GFP::Actin* in the tracheal system (see Movie 2 in the supplementary material). (**A**) A few tracheal cells at the tip of the tracheal bud (asterisk) form filopodial extensions under the control of FGF signalling. Tracheal cells in the dorsal branch stalk are initially paired (arrows), connecting the dorsal branch tip cells to the tracheal sac (dotted circle), and enclosing the dorsal branch lumen (arrowhead). (**B**) Gradually, as the dorsal branch elongates, the cells of the dorsal branch stalk adopt an end-to-end configuration (arrows). (**C**) In this image, stalk cells have fully intercalated (arrows). The terminal cell (TC) and the fusion cell (FC) continue to form filopodial extensions. Scale bars: 10 μm.

Thus, Fgf induces not only migratory cell behavior in tip cells, but also, via N activation, cell fate choices through lateral inhibition mechanisms.

Recent studies have shown that tracheal cells can take up positions within the developing branches in a rather dynamic fashion. Both in the embryo and in the larva (see also below), only cells with an intact Fgf signalling pathway can take up the leading position in outgrowing tracheal structures (Ghabrial and Krasnow, 2006; Cabernard and Affolter, 2005) (see Fig. 4). Genetic mosaic analysis has shown that cells with reduced Btl/Fgfr are found much less often at the tips of branches in the third instar stage than are cells with more receptor molecules (Ghabrial and Krasnow, 2006). Therefore, cells appear to compete for the leading position, such that those cells with the highest Bnl/Fgf receptor activity take the lead position, whereas cells with less activity assume subsidiary positions and form the stalk. In the same study, Ghabrial and Krasnow propose that this competition involves N signalling, leading to lateral inhibition that prevents additional cells from taking up the lead position (Ghabrial and Krasnow, 2006). Unfortunately, the N studies in this particular case did not involve a careful genetic mosaic analysis, but relied on the general expression of a dominant-active form of N and on the analysis of a N thermo-sensitive mutation. To link more definitively this competition for the leading tip cell position to N/Dl signalling requires more detailed genetic mosaic studies.

These and earlier studies have clearly attributed a key role to Fgf signalling in guiding branch budding and outgrowth in the embryo, and in particular they have linked Fgf signalling to directed cell migration and altered cytoskeletal dynamics. Furthermore, elegant studies have shown that Bnl also mediates the ramification of fine terminal branches in response to oxygen in *Drosophila* larvae (Jarecki et al., 1999). During larval life, oxygen deprivation stimulates the expression of Bnl, and the secreted growth factor

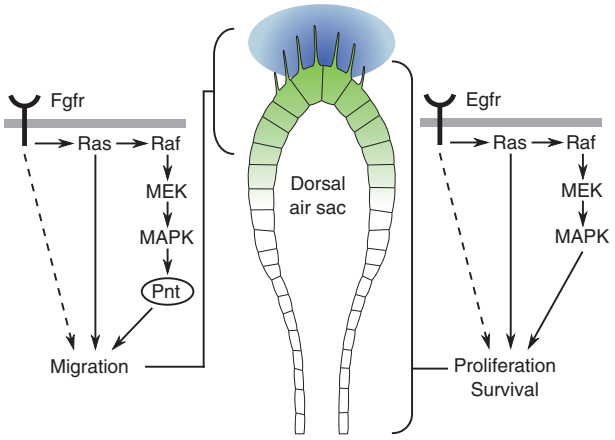


**Fig. 4. Signalling via two receptor tyrosine kinases, Fgfr and Egfr, controls the directed outgrowth of the primordium of the dorsal air sac in the *Drosophila* larva.** Fgf signalling (green), triggered by the presence of Bnl (blue) at the tip of the outgrowing air sac primordium, controls the direction of the outgrowth. Fgf signalling requires the Ras/MAPK cascade and Pointed (Pnt). Egf signalling is required more widely in the primordium, and regulates cell proliferation and cell death. Egf signalling does not require Pnt (for details, see Cabernard and Affolter, 2005).

functions as a chemoattractant that guides new terminal branches towards the expressing cells. Thus, a single growth factor is reiteratively used to pattern each level of tracheal branching, and the change in branch patterning results from a switch from developmental to physiological control of its expression. How the spatially restricted Fgf ligand Bnl regulates cytoskeletal dynamics at the molecular level is a key question that remains rather obscure. Only very few zygotic mutations with altered Fgf signalling levels in tracheal cells have been identified (Hacohen et al., 1998; Dammai et al., 2003). This is mostly because large maternal stores exist of many of the important components of this process, which hinder genetic approaches to identifying these components in embryos. However, many maternally provided transcripts and proteins have been used up by the third instar larval stages, and this has allowed researchers to use genetic mosaic analysis to investigate whether a given gene is specifically required for air sac primordia formation in an otherwise heterozygous animal, as discussed in more detail below.

In an earlier pioneering study, Sato and Kornberg showed that the formation of the primordia of the air sac of the dorsal thorax of the adult fly develops during the third instar larval stage under the control of Bnl/Fgf (Sato and Kornberg, 2002). Tracheal cells in close vicinity to Bnl-secreting cells in the wing imaginal disc start to proliferate and migrate towards Bnl-expressing cells. Because tracheal cells divide during this process, somatic genetic mosaic approaches can be used to study the requirement of various genes for Fgf signalling and/or air sac primordium formation (Cabernard and Affolter, 2005). Such mosaic analysis allows researchers to determine whether a given gene is specifically required for air sac primordia formation in an otherwise heterozygous animal, and, by using such an approach, Cabernard and Affolter showed that Fgf directs cell migration at the tip of the outgrowing branch (Cabernard and Affolter, 2005). Tracheal cells that lack Btl/Fgfr, or the downstream signalling component Dof, are never found at the leading front of the outgrowing air sac. Fgf signalling requires Ras function, the Map kinase pathway and the transcription factor Pointed. More recently it has been demonstrated that tracheal cells also require heparan sulphate proteoglycans (HSPGs) to respond to Bnl/Fgf; interestingly, HSPGs are not required for the secretion and distribution of Bnl/Fgf in the embryo, nor in the later developmental stages (Yan and Lin, 2007).

Although these analyses have shed some light on the molecular pathway involved in Fgf signalling in branching morphogenesis, unbiased screens for additional components will be essential to follow the Fgf signal as it propagates in responding cells. A first screen aimed at the identification of genes involved in Fgf-dependent cell migration that used genetic mosaic analysis in the developing air sac primordium has recently been published (Chanut-Delalande et al., 2007). Approximately 30 mutants have been recovered from this screen that show altered cell migration behaviour, an indication that the corresponding wild-type protein might play a role in, or downstream of, Fgf signalling. In this initial analysis, two genes were identified as being important for Fgf-dependent cell migration, Myosin heavy chain (Mhc) and Signal transducing adaptor molecule (Stam). Mhc has also been shown to be involved in border cell migration (Borghese et al., 2006), while the isolation and characterization of *Stam* suggests that trafficking of Fgfr-containing vesicles plays an important role in efficient cell migration. Previous studies have also shown that *Drosophila abnormal wing discs* (*awd*), the homologue of human *NM23*, regulates Fgf receptor levels and functions synergistically with *shibire* (also known as *dynamin*) during tracheal development (Dammai et al., 2003). The exact biochemical functions of Awd and the vertebrate homologues remain to be elucidated. Further studies of these genes, as well as the identification and characterization of the other genes mutated on those chromosomes that co-segregate with impaired tracheal cell migration efficiency in homozygous clones, will hopefully contribute to a much better understanding of the molecular aspects of Fgf signalling in branching morphogenesis.

## The role of cell intercalation

As mentioned above, the tracheal network consists of tubes of different size and architecture, ranging from multicellular tubes to unicellular tubes and intracellular tubes (Samakovlis et al., 1996b; Uv et al., 2003). We can thus reformulate the problem of tracheal morphogenesis and present it as a problem of epithelial remodelling, in which a flat epithelial sheet is transformed into different cellular arrangements that organize cells into tube structures in a stereotypic manner. Because each epithelial cell in a flat sheet has on average six neighbours, the formation of tubes, in which single cells are arranged in a chain-like, head-to-tail arrangement, requires extensive epithelial remodelling and the loss of junctional contact with four of the six initial neighbours (Fig. 5). To describe these cell rearrangements, a detailed characterization of the formation of the dorsal branch has been undertaken using high-resolution live imaging with a GFP-fusion protein that labels adherens junctions (AJs) (Ribeiro et al., 2004). These studies have shown that highly controlled cell intercalations, which require extensive AJ remodelling, are key to the formation of fine tracheal branches. Based on these studies and on single-cell analysis, a four-step model of tube remodelling has been proposed (Ribeiro et al., 2004) (see Fig. 5). In an initial step (pairing), tracheal cells appear to pair up along the bud axis. Subsequently, in the ‘reaching around the lumen’ step, individual cells reach around the lumen to establish contacts with themselves and start to form the first autocellular AJs. These autocellular AJs, which are made up of E-cadherin complexes anchored in the same cell, extend and zipper up as the two initially paired cells appear to slide past each other and intercalate (the

'zipping up' step). In order not to lose all intercellular AJs (such a loss would cause the cells to dissociate from each other), the two cells, which have rearranged from a side-by-side to a chain-like head-to-tail arrangement, need to stop the transformation of intercellular AJs into autocellular AJs (the 'termination' step). The entire intercalation process takes 20 to 30 minutes and occurs in most tracheal branches, except in the largest tube of the tracheal system, the dorsal trunk, which represents a multicellular tube.

Obviously, several questions come to mind when looking at this incredible epithelial remodelling. Where do the forces that trigger intercalation come from? How is AJ remodelling achieved such that the participating epithelial cells remain tightly attached to each other during intercalation? What is the role of junctional proteins and their upstream and downstream regulators during intercalation? Why does the transformation of intercellular AJs into autocellular AJs stop at some point? And how is intercalation regulated in the different tracheal branches to give rise to tubes of different cellular architecture?

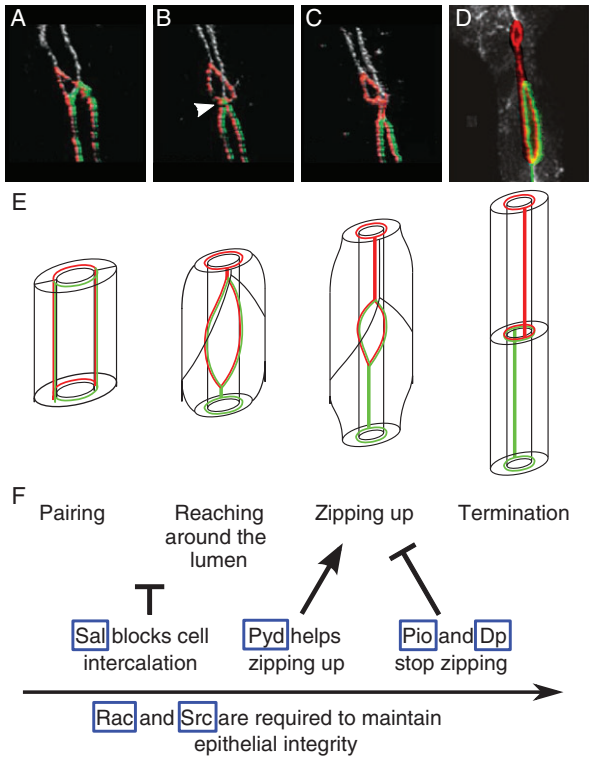


**Fig. 5. Genetic control of cell intercalation during branch outgrowth.** A four-step model of cell intercalation in a dorsal branch stalk. See also text for details. (**A-D**) Four representative micrographs, visualizing the AJs using *αCat::GFP* expression in the tracheal system, are shown in parallel with (**E**) four sketches representing the same steps. (**F**) The proteins involved in the genetic control of this process. (A-E) To better understand the 'topology' of the cells, the edges of two neighbour cells were colored in red and green, respectively. (A) Pairing: tracheal cells are in a side-by-side arrangement along the branch lumen. (B) Reaching around the lumen: individual cells establish contact with themselves and start to form the first autocellular AJs (arrowhead). (C) Zipping up: autocellular AJs extend as the two cells, which were initially paired, change their respective positions. (D) Termination: in order not to lose all intercellular AJs (and thus the adhesion between neighbouring cells), the transformation of intercellular AJs into autocellular AJs stops, with small intercellular AJ loops connecting adjacent cells to each other. Adapted, with permission, from Ribeiro et al. (Ribeiro et al., 2004). (F) The genetic control of these intercalation steps is beginning to be characterized. Proteins involved in this process are indicated with blue boxes.

So far, a clear molecular scenario has emerged only for the last of the four steps in the control of cell intercalation in tracheal branch formation, the termination step. Two mutants, *piopio* (*pio*) and *dumpy* (*dp*), were identified that display a rather specific tracheal defect. All tracheal branches that undergo cell intercalation to form fine tubes, which consist to a large extent of autocellular AJs in wild-type embryos, are disconnected from the network and form cyst-like structures in the mutants; only the dorsal trunk, in which intercalation does not occur, remains intact (Jazwinska et al., 2003). Both Pio and Dp proteins contain a zona pellucida (ZP) domain and are apically secreted into the luminal space of the developing tracheal system. Dp, in addition to its ZP domain, contains hundreds of Egf repeats interspersed with 21-residue-long, so-called, Dumpy (DPY) modules, and, at roughly 21,000 amino acids, it is the largest known *Drosophila* protein (Wilkin et al., 2000). As ZP domains form fibrillar structures (reviewed by Jovine et al., 2005), these studies strongly suggest that an apical extracellular matrix (ECM) that contains Pio and Dp provides a structural meshwork in the luminal space, around which cell rearrangements can take place in an ordered fashion and without losing interconnections. A physical barrier in the luminal space might be the most efficient molecular way to inhibit cells from transforming all of their intercellular AJs into autocellular AJs during intercalation, which would inevitably lead to a dissociation of neighbouring, fully intercalated cells. These studies represented the first identification of a luminal protein that has a key architectural role in tube formation.

The genetic analysis of cell intercalation during branch formation has also led to the identification of an important developmental regulator of cell intercalation. The only branches that never contain cells with autocellular AJs are those that eventually form the dorsal trunk, which, when interconnected, forms the largest tube of the tracheal system. The dorsal trunk is also the only tracheal tube in which all cells express the zinc finger transcription factor Sal (Kuhnlein and Schuh, 1996). Loss- and gain-of-function genetic studies, including single-cell analyses (Ribeiro et al., 2004), have demonstrated that Sal is indeed responsible for inhibiting cell intercalation and the formation of autocellular AJs in the dorsal trunk, and it can potently and completely inhibit intercalation when ectopically expressed in dorsal branches. How Sal inhibits intercalation remains unknown.

Additional genetic analyses have identified a few more molecular players that are involved in junctional remodelling during tracheal development. In mutants that lack the MAGUK protein Polychaetoid (Pyd; also known as ZO-1), the *Drosophila* homologue of the junctional protein ZO-1, cell intercalation is impaired in the sense that the zipping process, or the conversion of intercellular AJs into autocellular AJs, is incomplete (Jung et al., 2006). As Pyd localizes to the AJs in tracheal cells, this protein might play a direct role in the regulation of the dynamic state of the AJ during epithelial remodelling. In a different study, the strength of Egfr signalling in tracheal cells was proposed to regulate the maintenance of tissue integrity, partly by regulating the cadherin-based modulation of cell adhesion (Cela and Llimargas, 2006). However, the timing of Egfr signalling during branch outgrowth and the exact consequence of the signalling remain to be investigated in detail. In addition, Src and Rac have also been shown to play important roles in the maintenance of AJs during tracheal epithelial morphogenesis, through the regulation of E-cadherin levels (Chihara et al., 2003; Shindo et al., 2008). Src appears to play a dual role

during tracheal morphogenesis: it increases the rate of AJ turnover by reducing E-cadherin protein levels and, simultaneously, stimulates E-cadherin transcription (Shindo et al., 2008). These studies suggest that the opposing outcome of Src activation on E-cadherin facilitates the efficient exchange of AJs that is necessary during the remodelling and intercalation process. Together, these studies point to an important role for the proper regulation of adhesive strength in tracheal cells, and it will be interesting to see how the different pathways interact with each other. However, whether Sal inhibits cell intercalation via the regulation of these molecular modules acting at cell-junction complexes remains an open question.

## From branches to functional tubes: regulating the luminal space

In the past few years, much has been learned about how the epithelial cells that form the three-dimensional network of tubes control tube size, extension and length, and how the liquid-filled luminal space is emptied and ultimately filled with gas, allowing the trachea to carry out its assigned function, the efficient transport of air. In a genetic screen performed by Beitel and Krasnow, mutations that affect tube shape (e.g. size, length or diameter) were described (Beitel and Krasnow, 2000). The identification and cloning of the genes affected in these and other mutants has shed light on how the specialized apical secretion of ECM material contributes to tube expansion and tube length control.

Although the observed size differences between distinct tracheal branches is probably controlled by those genes that regulate branch identity (for example, by the presence or absence of Sal in the cells of the dorsal trunk and the dorsal branches, respectively) (Ribeiro et al., 2004; Neumann and Affolter, 2006; Casanova, 2007), the diameter of certain tubes expands rapidly in a relatively short time frame. The uniform expansion of tube diameter requires the formation of a transient luminal chitin-based matrix (Araujo et al., 2005; Moussian et al., 2006; Tonning et al., 2005; Devine et al., 2005) (reviewed by Swanson and Beitel, 2006) (see Fig. 6). In the absence of the chitin matrix, the lumen dilates in an uncoordinated fashion, resulting in cystic tubes. The matrix might provide either a physical scaffold that defines the shape of the tube cells surrounding it or it could signal to tracheal cells to adjust their shape in a uniform manner. In addition, the specific chemical modification of the luminal chitin matrix via apically secreted chitin deacetylases appears to be crucial for tracheal tubes to obtain the proper length (Luschnig et al., 2006; Wang et al., 2006), in line with the observation that tube length and tube dilation are under separate genetic control (Beitel and Krasnow, 2000). It is thought that one of these putative chitin deacetylases, encoded by a gene called *vermiform* (*verm*; *LCBP1* – FlyBase), is delivered to the apical lumen via a specialized secretory pathway that requires septate junctions; this is suggested by the observation that Verm is not properly secreted into the luminal space in several mutants of septate junction components, whereas Pio, for example, is normally secreted into the apical luminal space in the same mutants (Luschnig et al., 2006; Wang et al., 2006). The lack of Verm secretion in many septate junction mutants could indeed account for the tube-length-expansion defects that are observed in the absence of several septate junction components (such as Claudin, Megatrachea, Neurexin IV, Gliotactin and Neuroglian) (reviewed in Swanson and Beitel, 2006), but the exact role of septate junction components in secretion remains to be investigated.

A novel type of tracheal tube expansion gene is *polished rice* (*pri*; also known as *tarsal-less*), which is transcribed into a polycistronic mRNA that encodes multiple evolutionarily conserved open reading frames of 11 or 32 amino acids (Kondo et al., 2007). *pri* functions non-cell autonomously, indicating that these small peptides might travel through extracellular space to coordinate ECM organization. In the absence of *pri*, apical cuticular structures are completely eliminated, leading to defective tube expansion. Pri is essential for the formation of the specific F-actin bundles that prefigure the formation of the taenidial fold pattern of the tracheal cuticle. A novel formin with a similar role in actin organization has also been reported recently (Matusek et al., 2006).

Although the accumulation of discrete apical luminal matrices has clearly emerged as a key feature in the proper regulation of branching morphogenesis in the developing tracheal system, it is also clear that these matrices have to be removed or remodelled before the tracheal system can take over its assigned function. Based on an elegant study combining live imaging and genetic analysis, it has been proposed that tube maturation comprises three distinct epithelial transitions (Tsarouhas et al., 2007). First, a secretion burst deposits luminal proteins into the luminal space, leading to the rapid expansion described above. In a second step, the solid luminal material is rapidly cleared from the lumen, and, shortly afterwards, the liquid is removed. Genetic studies implicate exo- and endocytosis components in lumen expansion and solid matrix clearance, respectively (Tsarouhas et al., 2007; Behr et al., 2007).

## De novo lumen formation: interconnecting luminal networks

Thus far, we have only discussed how the initial luminal space is created and remodelled during the invaginations of the tracheal placodes during branching, and how it is cleared of protein and liquid material. However, for the trachea to be functional, new tubes and/or luminal spaces have to be generated de novo in specific cells,

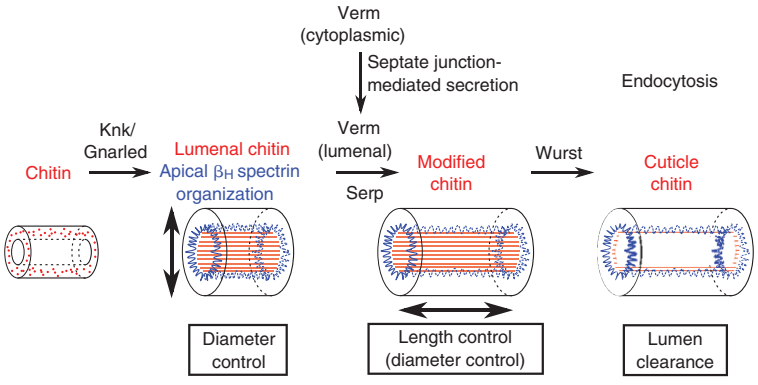


**Fig. 6. Sequential control of lumen expansion and lumen clearance in the *Drosophila* tracheal system.** At stage 14 of embryonic development, chitin (red) coordinates the radial expansion of the lumen by forming, upon secretion, a transient fibrillar chitin-based matrix (red lines). These chitin cables are also required for the normal organization of the apical $\beta_H$ spectrin cytoskeleton (blue). At stage 16 of embryonic development, septate junction proteins are required for the apical secretion of Verm, which, together with Serp, is required for modifying the chitin matrix to prevent the tracheal tubes from becoming too long (Wang et al., 2006). At stage 17 of embryonic development, Wurst is essential for remodeling the chitin matrix and for clearing the tracheal airway.

either to interconnect individual metameres via specialized fusion cells, or to allow the air to diffuse to the tip of the fine extensions formed by highly specialized terminal cells.

Although de novo lumen formation is an intriguing process, very little is known about the molecules that form extended luminal cavities inside cells in vivo during branching morphogenesis. The process is best understood during the fusion of tracheal tubes. The branches that ultimately form the dorsal trunk and the lateral trunk meet at specific locations at segment boundaries, and the dorsal and ventral (ganglionic) branches meet at the dorsal and ventral midline, respectively (Samakovlis et al., 1996b). The branch fusion process is initiated by specialized fusion cells at the tip of each of these branches. When two fusion cells of adjacent branches contact each other, they first establish a new AJ between them (Samakovlis et al., 1996a; Tanaka-Matakatsu et al., 1996). Inside these fusion cells, tracks of microtubules associated with F-actin and vesicles align in a pattern that prefigures the luminal axis (Lee and Kolodziej, 2002). Upon this alignment, the plasma membranes appear to invaginate along the future luminal axes from the pre-existing luminal sides toward the newly formed AJs. Finally, the lumen passes through the fusion cell, suggesting that localized plasma membrane fusion events have occurred. Recent studies have identified several fusion-cell-specific proteins with important roles in fusion, such as a formin protein (Tanaka et al., 2004) and an Arf-like 3 small GTPase (Jiang et al., 2007; Kakihara et al., 2008). In the absence of this Arf-like GTPase, the initial steps in the fusion process are normal (determination and adhesion of fusion cells, formation of F-actin bundles and vesicles), but the newly formed luminal cavities do not open up. Live imaging studies and genetic analysis suggest a failure in the localized assembly of the exocyst complex, implicating the targeting of the exocytosis machinery to specific apical domains as being a key step in lumen fusion.

De novo lumen formation is also essential for the formation of another specialized cell type in the tracheal system, the terminal cell (Guillemin et al., 1996). Terminal cells form the periphery of the tracheal system and extend numerous, long projections towards target tissues. A lumen then forms within each of these projections by a poorly understood process that creates an intracellular, membrane-bound channel without any associated AJs or septate junctions. Although genetic mosaic analyses have identified a number of mutations that result in the absence of a lumen in terminal cells (Baer et al., 2007) (see also Levi et al., 2006), the molecular mechanisms that underlie de novo lumen formation are not yet known, and await the further characterization of the mutants and identification of the affected gene products. Interestingly, a recent study has shown that integrin-talin complexes are necessary to maintain fine terminal tracheal branches and their luminal organization during larval life (Levi et al., 2006), suggesting that not only tube formation but also tube maintenance is under genetic control.

## Do general cellular principles underlie branching morphogenesis in different organs?

As outlined above, genetic analysis combined with live imaging approaches have revealed many of the cellular activities that are intimately linked to branching morphogenesis during tracheal development in *Drosophila*. The process relies on directed cell migration, controlled cell rearrangement and cell intercalation, the secretion of apical matrices to help keep branch cells together and to help shape the luminal space, and the de novo formation of luminal space in distinct cell types. Several genes that play important roles in branching have been identified, and the molecular events that underlie these cellular activities are beginning to be worked out.

An obvious question to ask at this point is whether similar cellular activities are involved in branching morphogenesis in other organisms (see also Lu et al., 2006). In the following section, we briefly discuss a few such similarities and differences.

The organ that is most often compared to the developing *Drosophila* tracheal system is the mammalian lung (Cardoso and Lu, 2006; Hogan and Kolodziej, 2002; Warburton et al., 2005). The finding that Fgf10 is expressed in a dynamic fashion at branch tips in the mammlian lung and is intimately linked to the branching process has highlighted that molecular similarities exist between branching in the mammalian lung and the insect trachea (Bellusci et al., 1997; Sekine et al., 1999; De Moerlooze et al., 2000). However, it remains unclear which cellular activities are controlled by Fgf signalling during lung budding. Although Fgf does affect cell proliferation at the bud tips, it has been argued, based on studies of lung bud explants, that these local changes in proliferation are not the triggering event that initiates lung budding, indirectly implying that the control of cell migration is a possible major player in branching (Nogawa et al., 1998). Indeed, Fgf can control cell migration in cultured mouse lung cells (Park et al., 1998). In the developing air sac in *Drosophila*, a branched organ that also grows tremendously during the branching process due to cell division, two different receptor tyrosine kinases, Egfr and Fgfr, have been linked to the control of cell proliferation and cell migration, respectively (Cabernard and Affolter, 2005) (see Fig. 4). Maybe the situation is somewhat similar in the developing lung buds. High-resolution live imaging and local loss-of-function analysis (for example, by creating chimeric mice; see below) will be necessary to address this issue in detail. It is possible that very limited cell movement or displacement (rather than extensive cell migration) induces an asymmetry in outgrowing buds that is sufficient to control the spatial organization of branches.

An emerging model system for the study of organ branching is the developing ureteric bud during mouse kidney development. The use of in vitro organ culture and live imaging, in combination with the analysis of genetically mosaic mouse kidneys, has begun to provide novel insights into the cellular processes that drive renal branching morphogenesis (Shakya et al., 2005) (reviewed by Costantini, 2006). It has clearly been shown that the tips of the ureteric buds are the growth centres. Similar to the lung, however, it remains to be seen whether Gdnf, a growth factor intimately linked to ureteric branching, controls the branching process via cell division or cell migration. It also remains to be investigated whether controlled cell rearrangement and cell intercalation, and whether the formation of apical matrices or the de novo formation of luminal space in distinct cell types, are intimately linked to certain stages of the branching process in the lung or ureteric bud. The same considerations apply to other organs that undergo excessive branching, such as the salivary gland or the mammary gland (Tucker, 2007; Lu et al., 2006; Cardoso and Lu, 2006).

A rather astonishing number of similarities between the developing tracheal system and the formation of blood vessels via angiogenesis have recently emerged. The vertebrate vasculature is first assembled from scattered endothelial precursor cells, and is then enlarged and remodelled by the sprouting, splitting and regression of branches, which shape a hierarchical vascular network that allows directional blood flow (Carmeliet, 2003). Members of the vascular endothelial growth factor (Vegf) family of signalling molecules are key regulators of blood vessel formation and function, and act through receptor tyrosine kinases of the Vgfr family (Ferrara et al., 2003). In an influential study, Gerhardt and colleagues have shown that Vegfa controls angiogenic sprouting by guiding filopodial

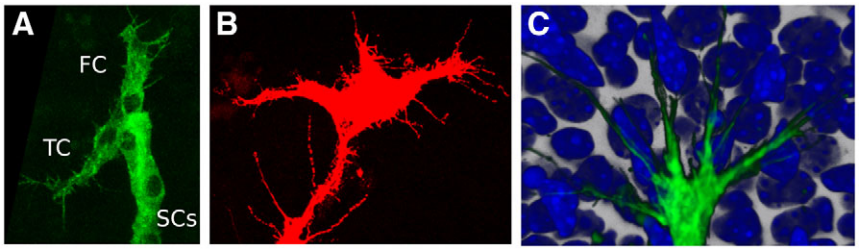


**Fig. 7. Similar functions of tip cells in the tracheal system and in the developing vasculature.** (**A**) A terminal cell (TC) and a fusion cell (FC) of the *Drosophila* tracheal system, which form the tip of a dorsal branch and express *GFP::Actin* (stage 15 of embryonic development). Note the presence of filopodial extensions in the TC and the FC, and their absence in stalk cells (SCs). (**B**) An endothelial tip cell, expressing *GFP* in a developing intersegmental vessel of an early zebrafish embryo (image courtesy of Y. Blum). (**C**) Isolectin B4-labelled endothelial tip cell (green) at the leading front of the developing vasculature in the retina of a 5-day postnatal mouse. Nuclei are labelled with DAPI (blue; image courtesy of H. Gerhardt).

extensions from specialized endothelial cells situated at the tip of vascular sprouts (Gerhardt et al., 2003) (Fig. 7). This is rather similar to how neural growth cones and tracheal cells navigate through the developing embryo. Further studies in different model systems, including in mouse and zebrafish, have shown that the distinction between the leading tip cell and the following stalk cells in growing angiogenic sprouts involves N signalling via the Delta-like 4 (Dll4) ligand (reviewed by Adams and Alitalo, 2007; Gridley, 2007; Roca and Adams, 2007). Reduced levels of Dll4 or reduced N signalling enhances the number of tip cells in a given branch, resulting in a dramatic increase in the sprouting of endothelial tubes; conversely, increased N signalling reduces angiogenic sprouting by inhibiting tip cell formation. Although the precise spatial regulation of N signalling in angiogenic sprouts remains to be analysed, the molecular control of the distinction between tip cells and stalk cells might be rather similar in angiogenic sprouts and tracheal branches, in which the control of tip cell formation is also linked to N-Dl signalling.

The morphological similarity between tip cells in angiogenic sprouts and those in growing tracheal branches and navigating axonal growth cones is paralleled by functional similarities. Although Vegf and Fgf play pivotal roles in guidance in angiogenesis and tracheal branching, respectively, branch outgrowth is also regulated by several ligand-receptor systems that were first associated with growth cone guidance. In the developing vasculature, important roles have been attributed to Semaphorin-Neuropilin/Plexin, Netrin-Unc5B and Slit-Robo4 signalling (Lu et al., 2004; Kamei and Weinstein, 2005; Suchting et al., 2006). Similarly, Robo signalling has been involved in tracheal pathfinding in the developing nerve cord in *Drosophila* (Lundstrom et al., 2004).

Do stalk cells of endothelial sprouts and tracheal branches also show similar cellular behaviour? Studies of tracheal cells have shown that stalk cells do not divide during branch formation but rearrange excessively, intercalate and form autocellular AJs in the fine branches of the trachea. The lumen is present from the onset of branching, although it is restructured during branch elongation. During the formation of the first angiogenic sprouts in the zebrafish embryo, the intersegmental vessels (ISVs), the cellular behaviour underlying the formation of a first, closed, vascular network has been analysed in detail using live imaging approaches (reviewed by Weinstein, 2002; Lawson and Weinstein, 2002; Childs et al., 2002; Kamei et al., 2004). It was discovered that during ISV formation, cells also rearrange extensively, but that sprouting is accompanied by endothelial cell divisions (Siekmann and Lawson, 2007; Leslie et al., 2007; Blum et al., 2008). Intercalation processes that generate cells in a chain similar to those observed in the fly tracheae have not been seen in the developing ISVs, although they might occur in capillaries formed in other regions of the embryo. It has initially been proposed that ISVs consist of three cells only, arranged in a chain-like structure (Childs et al., 2002), and that the lumen in this chain is established by the intracellular and intercellular fusion of vacuoles, resulting in a largely intracellular luminal space within the ISV network (Kamei et al., 2006). More recent studies suggest that more than three cells make up the ISVs, and that most of the luminal space is in-between cells and so is largely extracellular (Blum et al., 2008). Further studies are necessary before a definitive comparison of the cellular activities involved in ISV sprouting angiogenesis and tracheal branching can be made.

One of the most striking similarities between the development of the vertebrate vasculature and the tracheal system in *Drosophila* is the involvement of branch fusion, an essential process without which a continuous, functional tracheal or vascular network cannot be established. The fusion of adjacent branches has to be highly regulated in time and space, and involves de novo lumen formation processes. In the developing tracheal system, fusion involves a highly specialized cell type, the fusion cell, which eventually develops into a doughnut-like cell with two apical surfaces and a de novo generated luminal space (see above) (see also Uv et al., 2003). In the developing vasculature, only a few studies have addressed how vessels fuse in vivo (e.g. Blum et al., 2008), and the cellular events remain poorly characterized. It will be interesting to find out in future studies whether more similarities or differences are added to the cellular activities that underlie branching in different organ systems.

## Conclusion

What do we expect to learn in the next few years with regard to branching morphogenesis, and how will greater insights into this fascinating biological process be achieved?

Morphological processes in many cases involve the generation of force and the subsequent reaction to such forces. Very little is known still about the exact nature of the forces that control cell behaviour during branch formation in the *Drosophila* tracheal system. Future studies have to address this issue. Both in angiogenesis and in tracheal branching, cell rearrangements are linked intimately to the branching process, and it remains to be investigated how cell-cell junctions deal with the requirement to be constantly remodelled while providing permanent tissue integrity. Proteins secreted into the luminal space have emerged as being important and essential regulators of network formation and of tube length and expansion

control in the tracheal system. Are similar processes at work in branched organs in vertebrates? Are ZP proteins or chitin-like molecules involved? And, particularly with regard to the vertebrate vasculature and the invertebrate trachea, how are luminal spaces formed de novo, and how do branches fuse to establish integrated networks?

Live imaging will certainly play a key role in identifying the cellular activities linked to biological functions, and genetic analyses will have to provide evidence for the functional significance of molecular modules. The research approaches involved in trying to achieve a better understanding of branching morphogenesis have moved to cell biological analysis in vivo, and much progress can be expected in the near future from the integration of results obtained from cell biological studies in yeast and other systems with distinct cellular activities involved in organ formation in vivo.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/12/2055/DC1

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