

BRANCHING MORPHOGENESIS OF THE *DROSOPHILA* TRACHEAL SYSTEM

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■ **Abstract** Many organs including the mammalian lung and vascular system consist of branched tubular networks that transport essential gases or fluids, but the genetic programs that control the development of these complex three-dimensional structures are not well understood. The *Drosophila melanogaster* tracheal (respiratory) system is a network of interconnected epithelial tubes that transports oxygen and other gases in the body and provides a paradigm of branching morphogenesis. It develops by sequential sprouting of primary, secondary, and terminal branches from an epithelial sac of ~80 cells in each body segment of the embryo. Mapping of the cell movements and shape changes during the sprouting process has revealed that distinct mechanisms of epithelial migration and tube formation are used at each stage of branching. Genetic dissection of the process has identified a general program in which a fibroblast growth factor (FGF) and fibroblast growth factor receptor (FGFR) are used repeatedly to control branch budding and outgrowth. At each stage of branching, the mechanisms controlling FGF expression and the downstream signal transduction pathway change, altering the pattern and structure of the branches that form. During terminal branching, FGF expression is regulated by hypoxia, ensuring that tracheal structure matches cellular oxygen need. A branch diversification program operates in parallel to the general budding program: Regional signals locally modify the general program, conferring specific structural features and other properties on individual branches, such as their substrate outgrowth preferences, differences in tube size and shape, and the ability to fuse to other branches to interconnect the network.

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INTRODUCTION AND OVERVIEW OF TRACHEAL DEVELOPMENT

A major goal of developmental biology is to define the patterning principles and molecular programs that govern organ formation. The most common organ structural design is a branched tubular network, like those that make up many of our major organs such as lung, kidney, and vascular system. Each of these systems transports an essential gas or body fluid, and the branching pattern of the network and the proper sizes and shapes of the constituent tubes are critical for their transport function. How are these elaborate three-dimensional structures created? What specifies where branches bud, the directions they grow, and when they sprout again to form the next generation of branches? How do the migrating cells assemble into tubular structures of the appropriate size and shape for their position in the network? How do tubes fuse to form network connections? How are these processes regulated to produce an organ whose transport capacity matches physiological need? These questions are difficult to address for mammalian organs because their structures are extremely complex, and it has not been possible to systematically probe the genetic programs and cellular mechanisms that guide their development. In contrast, the *Drosophila melanogaster* tracheal (respiratory) system, with its simpler structure and accessible genetics, has emerged as a paradigm of branching morphogenesis and begun to provide answers.

The *Drosophila* larval tracheal system contains ~10,000 interconnected tubes that transport oxygen and other gases throughout the body (Figure 1*e,f*) (Manning & Krasnow 1993). Tracheal branches are simple tubes: just an epithelial monolayer wrapped into a tube surrounding a central lumen through which gases flow. Oxygen enters the network at the spiracular openings and passes through primary, secondary, and terminal branches to reach the target tissues. The tracheal network displays bilateral symmetry and a repeated segmental organization, reflecting its

developmental origin from 10 clusters (Tr1–Tr10) of ~80 tracheal precursor cells on each side of the embryo, to 1 cluster in every thoracic and abdominal hemisegment from T2 to A8 (Figure 1*a*).

Each cluster (tracheal placode) undergoes a similar sequence of developmental events to generate one hemisegment of the network (Figure 1*a–d, g*). This sequence begins 5 h after egg lay (AEL), as each placode invaginates from the ectoderm and forms an epithelial sac. Six primary tracheal branches bud and migrate out from each sac, followed several hours later by sprouting of about two-dozen secondary branches. Most secondary branches sprout terminal branches (tracheoles) throughout the several days of larval life, forming the hundreds of fine terminal branches in each hemisegment (Figure 1*h*). The pattern of primary and secondary branch budding is highly stereotyped and controlled by a hardwired developmental program, whereas terminal sprouting is variable and regulated by tissue oxygen need. The tracheal system is dramatically remodeled during metamorphosis to form the pupal and adult tracheal systems (Whitten 1980, Manning & Krasnow 1993), but only recently have these later processes begun to be analyzed genetically (Sato & Kornberg 2002). Our focus here is on embryonic and larval tracheal development.

Progress in this area began a decade ago with a description of the pattern of tracheal sprouting and cloning of the first tracheal genes (Klambt et al. 1992, Manning & Krasnow 1993). Since then systematic screens for tracheal markers and mutants and characterization of the process at cellular resolution have subdivided tracheal development into four general morphogenetic events, each representing a discrete step in the process and each associated with a specific set of genes: tracheal sac formation, and primary, secondary, and terminal branch sprouting (Figure 2). Genetic and molecular analysis of these genes and their products has begun to elucidate the molecular pathways that control and pattern each step, and how the patterning information interfaces with global patterning events in the animal, as well as with physiological processes such as oxygen homeostasis.

We begin below by describing the cellular events in tracheal development and the systematic genetic analyses that provided a genetic framework of the tracheal morphogenesis program and describe the genes and molecular pathways that control and couple each of the general steps (Figure 2, *middle*). We then discuss how regional and branch-specific genes locally modify the general program (Figure 2, *bottom*), conferring specific structural features and other properties on individual branches. These include substrate outgrowth preferences, the ability to fuse with other branches to interconnect the network, and differences in tube size.

CELLULAR AND GENETIC STUDIES DEFINE DISCRETE STEPS IN TRACHEAL BRANCHING

Cellular Analysis of Tracheal Branching

The initial description of the tracheal branching process revealed a series of sequential branching events that generated a network of successively finer tubes (Manning & Krasnow 1993) (Figure 2, *top*). Subsequent analysis of the underlying cellular

events showed that there are surprisingly diverse ways of forming tracheal tubes and that a different tubulogenesis mechanism is used at each level of branching; branching is thus not a strictly reiterative process (Samakovlis et al. 1996a). The tracheal sacs are the first tube-like structures to form. They arise by invagination of each ectodermal cluster of tracheal precursor cells between 5 and 7 h AEL (Figure 1*a,g*). As each ovoid cluster invaginates it seals off from the surface ectoderm and forms an elongate epithelial sac. The invagination is incomplete, however, leaving a short stalk (the spiracular branch) connecting the sac to the surface. The tracheal precursor cells undergo their final cell division as they invaginate, generating the ~80 cells of the tracheal sac. After this, no cell proliferation, cell death, or change in cell number is detected in the embryonic tracheal system (Samakovlis et al. 1996a). All the events that follow to create the larval tracheal network occur by cell migration and changes in cell size, shape, and intercellular contacts.

At 7 h AEL, primary branches begin to bud from the tracheal sac at stereotyped positions (Figure 1*b,g*). At each bud site, a pair of tracheal cells migrates out from the sac, and a small number of cells follow. As the cells migrate, they organize into tubes. Each of the six primary branches undergoes a similar budding process, with some differences. For example, a different but characteristic number of cells (from 4 to 20) is recruited into each branch. Also, most but not all primary branches subsequently undergo a 2-h period of cell elongation and intercalation in which cells initially arranged side-by-side interdigitate to assume an end-to-end configuration, with the resultant tubes only a single cell thick.

Secondary branches sprout from the ends of primary branches and at other select positions along the primary branches (Figure 1*d,g*). Secondary branches are formed by individual tracheal cells (Samakovlis et al. 1996a). Initially these cells are indistinguishable from other cells of the primary branch. But at 11 h AEL, they begin to extend and separate from their neighbors, forming a tube as they extend. The tube contains an autocellular junction along its length, suggesting that it forms by wrapping of the cell around its long axis until the edges of the cell meet and seal (Figure 2, *top*). These remarkable cells go on to elaborate another type of tube, tracheal terminal branches, beginning at 13 h AEL (Wigglesworth 1983, Guillemin et al. 1996, Samakovlis et al. 1996a). Terminal branches arise as cytoplasmic extensions that grow along the surface of tracheal target tissues, much like axonal outgrowth. Subsequently, an intracellular lumen forms within each extension (Figure 2, *top*), creating a fine (<1 μm in diameter) seamless tube continuous with the secondary branch from which it arises. This process of cytoplasmic extension and lumen formation repeats itself many times during the several days of larval life, generating individual terminal cells with complex branched structures and dozens of terminal branches (Figure 1*h*).

Although most tracheal branches follow this standard sequence of branching, specific branches cease branching and grow toward cognate branches in neighboring hemisegments, to which they fuse to interconnect the tracheal network. Branch fusion is mediated by individual cells at the tip of each branch, which contact a similar cell and undergo a coordinated series of morphogenetic events that create a bicellular fusion joint (see below).

Genetic Analysis of Tracheal Branching

An analysis of the expression patterns of several dozen tracheal enhancer trap markers (P[lacZ] transposon insertions) revealed striking molecular diversity among developing tracheal cells (Samakovlis et al. 1996a). Many of the markers are expressed in one of four common patterns that are activated in a defined sequence and in progressively restricted spatial domains that anticipate the morphogenetic events identified in the cellular studies. General tracheal markers turn on early and throughout the developing tracheal system, just before tracheal sac formation and primary branching begin. Secondary (“pantip”) markers turn on slightly later at the tips of growing primary branches, just before secondary branching initiates. Terminal markers begin to be expressed after secondary markers and in the subset of cells that sprout terminal branches. Fusion markers turn on about the same time as terminal markers, but in the complementary subset of secondary cells that cease branching and mediate branch fusion. The tight correlation between marker expression and the morphogenetic events suggested that the markers might represent genes with important functions in these events. Indeed, loss-of-function mutations in representative genes in each marker class specifically block the associated morphogenetic events. In this way, tracheal development has been subdivided into a series of genetically and molecularly distinct steps, each of which occurs by a distinct cellular mechanism (Figure 2).

The availability of these batteries of enhancer trap markers and representative mutants in each class allowed a test of the interdependence of gene expression between classes. Molecular epistasis experiments showed that the genes were organized into a regulatory hierarchy, with some genes in each expression class not only required to promote the cellular events of that stage of branching but also to activate expression of marker genes involved in the next stage of branching (see Figure 2 legend). Thus although the steps in tracheal development are genetically and cellularly distinct, they are coupled in a genetic regulatory hierarchy that ensures the steps occur in proper sequence.

In the following four sections we describe the current understanding of the genes and molecular pathways that control and pattern each step and how they trigger the next step in the general program.

THE TRANSCRIPTION FACTORS TRACHEALESS/TANGO AND VENTRAL VEINLESS SPECIFY TRACHEAL FATE AND DRIVE SAC FORMATION

The first step in tracheal development is specification of tracheal cell fate in the tracheal precursor cells of the ectoderm (reviewed in Affolter & Shilo 2000, Zelzer & Shilo 2000a). The heterodimeric transcription factor encoded by *trachealless* (*trh*) and *tango* plays a critical role. *trachealless* encodes a bHLH-PAS domain subunit expressed in all tracheal cells throughout development (Isaac & Andrew 1996, Wilk et al. 1996). *trachealless* turns on in tracheal precursor cells ~4 h AEL,

just before the first morphological signs of their specialization. It is the earliest tracheal-specific marker known. The other subunit, Tango (dARNT/HIF β), also contains a bHLH-PAS domain but is broadly expressed and has other partners (Ohshiro & Saigo 1997, Sonnenfeld et al. 1997, Zelzer et al. 1997). In *trachealess* mutants, many early tracheal genes (*btl*, *sms*, *rho*, *tdf*, *peb*) are not induced, and tracheal placodes fail to invaginate (Boube et al. 2000). Ubiquitous expression of *trachealess* can induce ectopic tracheal marker expression and invagination, although this ability is spatially restricted. This restriction, together with the finding that some tracheal genes (*vvl*, *kni*, *knrl*, *tkv*) are still expressed in *trachealess* mutants, suggests that additional, spatially restricted factors are required for tracheal cell fate specification. One such factor is *ventral veins lacking* (*vvl*), also called *ventral veinless* and *drifter*; *jing* may be another (Sedaghat et al. 2002).

vvl encodes a POU domain transcription factor (Anderson et al. 1995, 1996, de Celis et al. 1995, Llimargas & Casanova 1997). The gene turns on in tracheal precursors around the same time or just after *trachealess*. In *vvl* mutants, several early tracheal genes are not expressed properly (*btl*, *sms*, *rho*, *tkv*), although others are (*trh*, *tdf*, *hnt*, *kni*, *knrl*), and tracheal placodes partially invaginate but primary branches do not bud (Llimargas & Casanova 1997, 1999; Boube et al. 2000). Interestingly, *vvl* is also expressed in clusters of cells in three anterior segments that normally do not form tracheal placodes, which correspond to the sites where ectopic *trachealess* can induce tracheal marker expression. When both *vvl* and *trachealess* are ectopically expressed, *breathless* (*btl*) is induced throughout the ectopic expression domain (Boube et al. 2000). Thus, Trachealess/Tango and Vvl function combinatorially to specify tracheal fate, probably involving direct interaction between the proteins (Zelzer & Shilo 2000b; reviewed in Zelzer & Shilo 2000a).

What initiates expression of *trachealess* and *vvl* in tracheal precursor cells? Both genes are expressed in a specific anterior-posterior (A-P) and dorso-ventral (D-V) position within each segment, suggesting that the A-P and D-V coordinates established by the global patterning hierarchies specify these positions. Indeed, the segmentation genes *domeless*, an interleukin receptor homolog, and *stat92E*, a downstream transcription factor in the JAK/STAT pathway, are required to initiate *trachealess* expression (Brown et al. 2001, Chen et al. 2002), and *dpp* and *wingless* set dorsal and A-P limits on *trachealess* and *vvl* expression (de Celis et al. 1995, Isaac & Andrew 1996, Wilk et al. 1996). Maintenance of *trachealess* and *vvl* expression has been shown genetically to involve auto- and cross-regulation (Certel et al. 1996, Isaac & Andrew 1996, Wilk et al. 1996), which may provide robust maintenance of the tracheal fate decision.

Serine phosphorylation of Trachealess by protein kinase B (PKB/dAKT) promotes nuclear translocation of Trachealess (Jin et al. 2001). Although Trachealess is nuclear during normal development, it is possible that its nuclear localization and function could be arrested under physiological conditions that inhibit PKB activity.

It is important to identify the full set of targets of the Trachealess/Tango and Vvl transcription factors. One important class of targets mediates invagination of

tracheal cells. This class includes *rhomboid*, an EGF pathway activator required for complete invagination (Llimargas & Casanova 1999). *Trachealess/Tango* and *Vvl* also trigger expression of *breathless* and *stumps* (Wilk et al. 1996, Ohshiro & Saigo 1997, Boube et al. 2000), a fibroblast growth factor (FGF) receptor and downstream effector, respectively, that function in primary branching, the next step in tracheal development.

FGF SIGNALING DIRECTS PRIMARY BRANCH BUDDING AND OUTGROWTH

A major question in branching morphogenesis is how the pattern of branching is controlled: What specifies where branches bud and grow? Analysis of the primary branch genes *branchless* (*bnl*) and *breathless* (*btl*) has provided an answer. *branchless* encodes a homolog of mammalian FGFs (Sutherland et al. 1996), and *breathless* encodes a homolog of FGF receptors (FGFRs), classical receptor tyrosine kinases (RTKs) (Glazer & Shilo 1991, Klambt et al. 1992). Just before primary branching begins, the receptor gene *breathless* turns on in tracheal cells, driven by *Trachealess/Tango* and *Vvl*. The ligand gene *branchless* turns on at the same time, not in the tracheal cells themselves but in small clusters of epidermal and mesodermal cells arrayed around the tracheal sac, at each position where a primary branch will bud (Figure 3a). Tracheal cells migrate toward these FGF signaling centers, organizing into primary branches as they migrate. Expression of Branchless FGF is highly dynamic: As budding branches reach the FGF signaling centers, *branchless* expression turns off and migration ceases. In some areas a more distal cell cluster begins to express *branchless*, and the branch continues extending toward the new signaling center. In *branchless*- or *breathless*-null mutants, no primary branches form, whereas ectopic *branchless* expression attracts primary branches to new positions. These results demonstrate that Branchless FGF is a chemoattractant that guides primary branch budding and outgrowth and that the pattern of *branchless* expression sets the pattern of primary branching.

What controls the complex expression pattern of *branchless*? Each *branchless* expression domain lies in a specific A-P and D-V position within each segment, suggesting that the global A-P and D-V patterning systems specify these positions, as described above for *trachealess* (Figure 3b). But regulation of *branchless* expression is likely to be more complex than *trachealess* because there are multiple *branchless* expression domains in each segment and expression is highly dynamic. There might be multiple *branchless* transcriptional enhancers, each responsive to a different combination of A-P and D-V patterning genes and responsible for a specific expression domain (Krasnow 1997). Consistent with this idea, the dorsal domain of *branchless* expression, but not other domains, is eliminated in *dpp* mutants (Vincent et al. 1997).

Once *branchless* is expressed, the secreted FGF activates the Breathless receptor tyrosine kinase in nearby tracheal cells. In other FGF signaling pathways, heparan

sulfate proteoglycans (HSPGs) function as coreceptors that mediate formation of active FGF-FGFR signaling complexes (Pellegrini 2001). Genetic experiments imply a similar function in the Branchless FGF pathway. Mutations in *sugarless* (*sgl*) or *sulfateless* (*sfl*), which encode enzymes that catalyze HSPG biosynthesis (UDP-D-glucose dehydrogenase and heparan sulfate *N*-deacetylase/*N*-sulfotransferase, respectively), block activation of MAPK and outgrowth of primary branches (Lin et al. 1999). The requirement of *sgl* and *sfl* for Branchless signaling can be partially overcome by overexpression of the ligand, suggesting that HSPGs may be critical only when ligand is limiting. Another HSPG biosynthesis gene, heparan sulfate 6-*O*-sulfotransferase (dHS6ST), is selectively expressed during mid-embryogenesis in the developing tracheal system, and inactivation of the gene by RNAi blocks MAPK activation and impairs primary branch outgrowth (Kamimura et al. 2001). This suggests that the specific structure of the HSPG may be critical for Branchless signaling.

The downstream events following activation of the Breathless FGFR that lead to tracheal cell migration and primary branch budding are not well understood. Although the canonical Ras/MAPK cascade is activated and is critical for secondary branch sprouting (see below), the role of Ras/MAPK components in primary branch budding is unclear; only loss-of-function mutations in *corkscrew* (a homolog of mammalian SHP-2 tyrosine phosphatase) and activated forms of Ras and Raf have been shown to affect primary branching (Reichman-Fried et al. 1994, Perkins et al. 1996). Activation of the receptor causes dynamic actin-based filopodial extensions at the tip of budding primary branches, leading the epithelial migration (Shim et al. 2001, Ribeiro et al. 2002, Wolf et al. 2002). These extensions are similar to ones seen at the leading edge of migrating fibroblasts and growing axons, so similar mechanisms may be involved. One essential downstream component is *stumps* (*sms*), also called *dof* or *heartbroken* (*hbr*), which encodes a novel intracellular protein (Michelson et al. 1998, Vincent et al. 1998, Imam et al. 1999). The gene is expressed in the developing tracheal system, and in *stumps* mutants, filopodial extension and primary branch outgrowth are abrogated, just as in *branchless* and *breathless* mutants. *stumps* is also expressed in the developing mesoderm, and *stumps* mutations block mesodermal migrations that depend on the *heartless* FGFR. These genetic results, along with the observed enrichment of Stumps at the plasma membrane and the presence of potential docking sites on the protein for several intracellular signaling proteins (Vincent et al. 1998), suggest that Stumps may function as an adapter that couples FGFRs, but not other receptor tyrosine kinases (Dossenbach et al. 2001), to downstream effectors in cell migration. Identification of these effectors and how they drive tracheal cell movement are important goals for the field.

Two putative transcription factors involved in primary branching have also been identified, *tracheae defective* (also called *apontic*), which encodes a bZip protein with a Myb-type DNA-binding domain (Eulenberg & Schuh 1997), and *ribbon*, which encodes a BTB domain protein with a Pipsqueak DNA-binding domain (Bradley & Andrew 2001, Shim et al. 2001). Loss-of-function mutations in

either gene severely impair primary branch budding, and genetic analyses suggest that both function downstream or parallel to Branchless signaling. The *ribbon* phenotype is particularly intriguing because although the failure in primary branch outgrowth is grossly similar to the phenotype in *branchless*, *breathless*, or *stumps* mutants, *ribbon* tracheal cells are still able to extend cytoplasmic processes from the basal cell surface toward Branchless signaling centers (Shim et al. 2001); movement and morphogenesis of the cell body and apical surface are selectively impaired. Ribbon could be required to transmit the signal from *breathless* FGFR to the cell body and apical surface, or it could promote movement and morphogenesis of those parts of the cell once the signal reaches them. The target genes of Ribbon and Tracheae Defective that mediate these effects are unknown.

Activation of Breathless FGFR leads to activation of the canonical Ras/MAPK cascade and induction of gene expression in tracheal cells at the tips of growing primary branches (Sutherland et al. 1996, Gabay et al. 1997). One induced gene is *breathless* itself, which creates a positive feedback loop that maintains expression of the receptor in areas of active signaling (Ohshiro & Saigo 1997, Ohshiro et al. 2002). Signaling also induces expression of secondary branching genes that initiate the next step in tracheal development.

AN FGF-SPROUTY FEEDBACK LOOP PATTERNS SECONDARY BRANCH SPROUTING

Like primary branch sprouting, the pattern of secondary branch sprouting is stereotyped and controlled by *branchless* and *breathless*, but by a different molecular mechanism. Branchless FGF controls secondary sprouting by triggering expression of secondary branch genes at the tips of budding primary branches, in the cells located closest to the Branchless FGF signaling centers (Figure 3a) (Lee et al. 1996, Sutherland et al. 1996, Hacohen et al. 1998). This is promoted by phosphorylation and degradation of AOP/Yan, an ETS domain transcriptional repressor, and induction of *pointed*, another ETS domain transcription factor (Figure 3b) (Scholz et al. 1993, Samakovlis et al. 1996a, Hacohen et al. 1998, Ohshiro et al. 2002). In *branchless* and *breathless* mutants, primary branches do not grow out, and *pointed* and other secondary branch genes are not induced. Conversely, ectopic expression of *branchless* results in induction of *pointed* and other secondary and terminal branch genes throughout the tracheal system and a striking hyperbranching phenotype (Lee et al. 1996, Sutherland et al. 1996). Thus most or all tracheal cells have the potential to turn on secondary and terminal genes and form secondary and terminal branches, and it is the localized expression of Branchless FGF that restricts these events.

Not all cells that receive the Branchless FGF signal go on to form secondary branches because FGF signaling also activates expression of genes that limit secondary branching. One of these is *sprouty* (*spry*), which encodes an evolutionarily conserved protein with a novel cysteine-rich motif that antagonizes FGF and other

RTK signaling pathways (Hacohen et al. 1998, Casci et al. 1999, Kramer et al. 1999, Reich et al. 1999). *sprouty* expression is induced along with other secondary branch genes by the Branchless FGF pathway, and genetic mosaic analysis indicates that it functions cell non-autonomously to stop neighboring cells from inducing secondary branch genes. In *sprouty* mutants, the Branchless pathway is overactive, and cells in the stalk of the primary branch express secondary branch genes and go on to sprout ectopic secondary and terminal branches. Thus *sprouty* is part of a negative feedback loop that limits FGF signaling and thereby restricts secondary and terminal branch sprouting to the cells closest to the FGF signaling centers.

Although Sprouty's role in branch patterning is clear, the biochemical mechanism by which it antagonizes FGF signaling is not. Sprouty is a membrane-associated protein, and some studies suggest that it or its vertebrate homologs act upstream of MAP kinase by directly interacting with Drk/Grb2 and/or Gap1, canonical members of the RTK signaling pathway (Casci et al. 1999, Reich et al. 1999, Hanafusa et al. 2002). Other data suggest that Sprouty interacts with Cbl, a ubiquitin ligase (Wong et al. 2001) known to target RTKs for degradation (Levkowitz et al. 1998). None of these interactions, however, readily explains the cell non-autonomous action of *sprouty* in the tracheal system, so Sprouty may have other effects or a different mode of action in secondary branching.

Different numbers of cells are selected to form secondary branches at the tip of each primary branch, so there must be branch-specific differences in the strength of FGF signaling, or the response to it, or in the *sprouty*-dependent refinement process. Also, in some branches, a specific tip cell acquires an alternative fate: It mediates fusion with another tracheal branch. The mechanisms by which cells are singled out for fusion instead of secondary and terminal branching are discussed below, but evidence suggests that once fusion cells are selected some of these cells also produce an intercellular signal that inhibits terminal branch sprouting. The fusion gene *headcase*, which encodes cytoplasmic proteins of unknown biochemical function expressed specifically in fusion cells, functions cell non-autonomously to suppress terminal branching near the dorsal branch fusion cell (Steneberg et al. 1998). It will be important to elucidate the mechanisms by which the Branchless pathway and other signals are integrated at the end of each outgrowing primary branch to select the correct number of cells for each fate.

The transcription factor Pointed plays a central role in coordinating these different events. Not only does it maintain expression of the Breathless FGFR (Ohshiro et al. 2002) and induce expression of the FGF pathway antagonist *sprouty* (Hacohen et al. 1998), but presumably it also induces genes involved in the morphogenesis of secondary branches, such as cytoskeletal and cell surface proteins that drive formation of the unicellular tube or dictate its direction of outgrowth. Pointed also promotes the next stage of branching by inducing expression of *blistered*, a key regulator of terminal branching, and by inhibiting expression of the fusion regulator *escargot* (Samakovlis et al. 1996a). Because Pointed functions in RTK pathways in other tissues, it must work together with a tracheal-specific factor(s) to carry out its tracheal-specific effects.

OXYGEN CONTROL OF TERMINAL BRANCH SPROUTING IS MEDIATED BY BRANCHLESS FGF

Two aspects of terminal branching are of special interest and distinguish it from the earlier stages of branching. One is that terminal branches have a distinct cellular structure: They are extremely fine ($<1 \mu\text{m}$ diameter), lack junctional structures along the length of the branch, and are formed by individual cells that undergo a remarkable process of cellular morphogenesis. The cells undergo repeated episodes of cytoplasmic extension and intracellular lumen formation, creating ramified networks of terminal branches that contact almost every cell in the target area (Figure 1g) (Guillemin et al. 1996, Samakovlis et al. 1996a). The other distinguishing feature is that terminal branching is highly variable and regulated by oxygen physiology and is not stereotyped nor under fixed developmental control. Equivalent terminal cells in different larvae can form different numbers of branches and display different patterns of branching (Ruhle 1932), and they can supply different cells in the target tissue. Although variable, the pattern is not unorganized. For instance, branch points are regularly spaced and terminal branches do not cross over one another. Sprouting and outgrowth of terminal branches is carefully regulated to meet the oxygen needs of target tissues, much like angiogenesis in mammals. Low oxygen (hypoxia) stimulates terminal branch formation and high oxygen (hyperoxia) suppresses it (Wigglesworth 1954, Locke 1958, Jarecki et al. 1999). When the tracheal supply to a local region is eliminated, nearby terminal cells sprout extra branches that grow to supply the detraheated region. Oxygen-starved cells secrete a tracheogenic signal that can attract new terminal branches from as far as one segment away.

The tracheogenic signal generated by oxygen-starved cells was identified as Branchless FGF, the same signal used to regulate the earlier stages of branching (Jarecki et al. 1999). Although *branchless* expression shuts off as secondary branches begin to sprout and the first terminal genes begin to be expressed, it turns back on again several hours later at the beginning of larval life (Figure 3a), under different regulatory control. Early *branchless* expression is stereotyped and regulated by global patterning hierarchies in the embryo, whereas larval expression is regulated primarily by oxygen need (Figure 3b). Low oxygen stimulates *branchless* expression, and the secreted Branchless FGF functions as a terminal branch inducer and chemoattractant able to guide new terminal branches to each expressing cell. This supplies the signaling cells with extra oxygen, relieving the hypoxic crisis. The final pattern of terminal branching is thought to reflect the developmental history of the oxygen needs and *branchless* expression pattern of the target cells. There may also be factors that modulate Branchless action because some aspects of terminal branch distribution cannot be explained solely by the pattern of *branchless* expression.

The identification of Branchless as the tracheogenic factor induced by hypoxia raises new questions, such as how is low oxygen sensed by cells and how does this lead to induction of *branchless*? The effect of oxygen may be mediated by

the *Drosophila* cognate of mammalian hypoxia inducible factor (HIF), the central transcription factor in the mammalian hypoxic response (Semenza 2001). Mammalian HIF is a heterodimer of bHLH-PAS domain subunits. Under normal oxygen conditions, the alpha subunit is modified by a prolyl hydroxylase that targets it for recognition by the VHL ubiquitin ligase complex and destruction by the proteasome. Under low oxygen, the prolyl hydroxylase is inactive, which allows the transcription factor to accumulate and activate its targets, including glycolytic pathway genes, angiogenic factors, and other genes that serve to restore oxygen balance or help cells survive under low oxygen. *Drosophila* cognates of HIF pathway components including the HIF α (*sima*) and β (*tango*) subunits have been identified (Nagao et al. 1996, Adryan et al. 2000, Bruick & McKnight 2001, Lavista-Llanos et al. 2002), and the pathway is present in larvae and activated by hypoxia, as in the mammalian pathway (E. Johnson & M.A. Krasnow, unpublished results). Genomic analysis of HIF-responsive targets in larvae (E. Johnson & M.A. Krasnow, unpublished) and cell culture RNAi experiments (F. Karim, personal communication) have identified *branchless* as one of its targets. Thus the control regions of the *branchless* gene are likely to be complex with a HIF-dependent hypoxia-responsive enhancer as well as a variety of enhancers that drive its intricate expression in the embryo. Nitric oxide (NO) signaling might also influence *branchless* expression, as alteration of NO signaling during larval development can affect terminal branching in ways similar to hypoxia or hyperoxia (Wingrove & O'Farrell 1999).

The effects of Branchless on terminal branching are mediated by Breathless FGFR, the same receptor used in the earlier stages of branching (Reichman-Fried & Shilo 1995, Jarecki et al. 1999). The same ligand and receptor are used again later in development to direct sprouting and outgrowth of adult air sacs, but in this case cell proliferation accompanies branch budding (Figure 3b) (Sato & Kornberg 2002). These results raise a second question: How does activation of the same receptor by the same ligand at different times in development lead to such different branching outcomes? Analysis of the terminal branching gene *blistered* (also called *pruned*) provides an answer. *blistered* encodes the *Drosophila* homologue of mammalian serum response factor (SRF) (Affolter et al. 1994a, Guillemain et al. 1996, Montagne et al. 1996), a MADS domain transcription factor, which, along with the ETS domain protein Elk-1, forms part of a transcription complex whose activity is regulated by RTK signaling and the Ras/MAPK cascade (Figure 3b) (Treisman 1994). *blistered* SRF is specifically expressed in tracheal terminal cells, and loss-of-function mutations block cytoplasmic outgrowth and terminal branching, whereas constitutively active forms of SRF and Elk-1 drive excessive branch outgrowth. This suggests a model in which Branchless signaling leads to activation of a Blistered/Elk-1 transcription complex in terminal cells, which in turn induces expression of hypothetical target genes required for cytoplasmic outgrowth and terminal branch formation. Importantly, expression of *blistered* in terminal cells is triggered by Branchless signaling (via Pointed) in the embryo (Sutherland et al. 1996). Blistered protein is not made in time to participate in this first round of

Branchless signaling, which establishes terminal cell fate (Ribeiro et al. 2002), but expression of *blistered* persists, and the next time the cell receives the Branchless signal (during larval terminal branching), it responds differently because of this new pathway component. The first FGF signaling event changes the cells so that they respond differently the next time they receive the same signal.

The general tracheal branching program thus involves a core FGF pathway that is used repeatedly, from embryo to adult, to control branch budding. But at each stage the mechanisms controlling ligand expression and the downstream effector pathways change, altering the pattern and structure of the branches that form (Figure 3*b*) (Metzger & Krasnow 1999). In some cases, e.g., *blistered*, these changes are induced by earlier signaling events, ensuring that branching occurs in the proper sequence.

Despite substantial progress in understanding the signaling pathways and transcription factors that control terminal branching, the cellular and molecular mechanisms of cytoplasmic outgrowth, branching, and lumen formation remain largely unknown. Genes regulated by *Blistered* must be involved, but none of its targets has been identified. These targets are likely to encode cytoskeletal and/or cell surface proteins necessary to build or maintain a cytoplasmic projection, and proteins such as synaptobrevin needed to create and expand the lumen. Surprisingly, loss of function of the major nuclear lamin, encoded by *misguided*, leads to defects in the polarized outgrowth of the terminal branches, suggesting a role for the nucleus or nuclear envelope in guiding these outgrowths (Guillemin et al. 2001).

BRANCH-SPECIFIC PROPERTIES ARE CONTROLLED BY REGIONAL AND BRANCH IDENTITY GENES

As the sequence and general pattern of tracheal branching is set by the Branchless FGF pathway, individual branches acquire distinctive structure and other characteristics that distinguish them from other branches at the same level of branching. This diversity is easily seen among primary branches, which contain different numbers of cells, grow along different substrates, and form tubes of different sizes, shapes, and branching patterns. For example, the primary branches that give rise to the dorsal trunk (DT) have the largest diameter, 3 to 10 times the size of other mature primary branches. And, unlike other primary branches, DT branches neither undergo a period of cell elongation and intercalation after budding nor sprout again; rather, they grow toward a cognate branch in the neighboring segment and fuse.

The distinctive characteristics of individual primary branches are controlled by genes expressed in regional and branch-specific patterns. These function in parallel to the Branchless pathway to allocate cells to specific primary branches, establish boundaries between branches, and assign individual branch fates (Figure 2). This process begins at the onset of tracheal development when local signals establish different domains of cells within the tracheal placode; clonal analysis indicates that

final assignment of branch fates is made after the last tracheal cell division, which occurs as the placodes invaginate (Samakovlis et al. 1996a). Expression of the TGF β family member Dpp in ectodermal stripes dorsal and ventral to the tracheal placode assigns dorsal and ventral tracheal domains (Vincent et al. 1997), whereas Spitz/EGF (Wappner et al. 1997) and Wingless/Wnt signaling pathways (Chihara & Hayashi 2000, Llimargas 2000, Llimargas & Lawrence 2001) establish a central domain (Figure 4). Hedgehog signaling demarcates A-P differences (Glazer & Shilo 2001).

These signals induce expression of regional and branch-specific genes within the different domains or at their junctions. Some of the induced genes encode transcription factors that mediate cross-regulatory interactions, which refine the initial expression domains (Franch-Marro & Casanova 2002) and control expression of downstream genes that confer branch-specific properties (Boube et al. 2001). This gene regulatory hierarchy is conceptually similar to the early patterning hierarchy that partitions the A-P body axis into segments and specifies differences among the segments; the tracheal genes that control specific branch characteristics can be thought of as branch identity genes, analogous to segment identity (homeotic selector) genes in the A-P hierarchy.

Branch specification is best understood for the dorsal branch (DB) and dorsal trunk (DT) (Figure 4). Dpp emanating from a dorsal stripe of cells activates its receptors (Thickveins and Punt, type I and II TGF β R family members) in tracheal cells, which leads to phosphorylation of the Mad transcription factor (Affolter et al. 1994b, Ruberte et al. 1995, Vincent et al. 1997, Dorfman et al. 2002b). This induces expression in the dorsal-most tracheal cells of *knirps* (*kni*) and *knirps-related* (*knrl*), which encode highly similar and partially redundant zinc-finger transcription factors (Vincent et al. 1997, Chen et al. 1998). In the absence of Dpp signaling, *knirps* (and presumably *knrl*) are not induced, and DBs fail to form or are not maintained; the presumptive DB cells end up integrated into the DT (Vincent et al. 1997, Chen et al. 1998, Ribeiro et al. 2002). Similar effects are seen in the absence of *knirps* and *knrl*. Ectopic activation of the Dpp pathway, or ectopic expression of *knirps*, has the opposite effect: DT progenitors are recruited into the DB (Llimargas & Casanova 1997, Vincent et al. 1997, Chen et al. 1998). *knirps* and *knrl* can thus be considered dorsal branch identity genes.

Another zinc-finger gene, *spalt*, plays a critical role in DT identity (Kuhnlein & Schuh 1996). *spalt* loss-of-function mutations eliminate the DT and cause DT progenitors to assume a DB fate, just like ectopic activation of the Dpp pathway or ectopic expression of *kni*. *spalt* is expressed in the dorsal portion of the tracheal placode, initially encompassing DB and DT primordia; shortly thereafter its expression restricts to the DT. This downregulation of *spalt* in DB cells is mediated by Knirps/Knrl, likely through direct binding to a *spalt* regulatory element (Chen et al. 1998). The DT also does not form and may be transformed to other branch identities when Spitz/EGF or Wnt pathways are inactivated, perhaps through effects on *spalt* expression (Wappner et al. 1997, Chihara & Hayashi 2000, Llimargas 2000, Llimargas & Lawrence 2001; but see Llimargas & Casanova 1999, Bradley &

Andrew 2001). A current model proposes that opposing gradients and antagonistic interactions between different signaling pathways (e.g., Dpp versus Spitz/EGF, Dpp versus Wnt) establish the different expression domains and strict boundaries between them (Wappner et al. 1997, Llimargas 2000, Zelzer & Shilo 2000a).

Several lines of evidence suggest that branch specification requires additional factors other than *knirps/knrl* and *spalt*. *knirps/knrl* are expressed and required in other primary branch primordia besides DB, and tracheal expression of a *knirps* transgene in Dpp pathway mutants is not sufficient to rescue the tracheal defects (Chen et al. 1998). *spalt*, too, appears to function outside its principal domain, the DT (Franch-Marro & Casanova 2002). Other transcription factor genes expressed in overlapping domains may function combinatorially with *knirps/knrl* and *spalt* to distinguish specific branch identities such as *unplugged*, which promotes aspects of ganglionic branch (GB) fate (see below), and *elbow*, which can suppress aspects of visceral branch (VB) and DT fate (Dorfman et al. 2002a). Primary branch specification likely involves a combinatorial code of branch identity genes.

It is easy to envision how localized expression of branch identity genes in the tracheal placode and sac could allocate cells to different branch primordia and establish boundaries between them. But what are the downstream targets of these transcription factors that sort the cells into distinct branches and confer differences in substrate preference, tube size, and branching pattern? The only downstream target that has been identified is *mew* α -integrin, which mediates adhesion and spreading of the VB along its substrate (see below). Identification of other downstream targets is a priority for the field.

ROLES OF THE SUBSTRATE IN BRANCH OUTGROWTH

One aspect of branch identity is the migration path selected by individual branches. Each primary branch grows out in a different direction and traverses a different substrate to reach its target (Franch-Marro & Casanova 2000). DTa grows anteriorly and DTp posteriorly, DB courses dorsally, LTa and GB extend ventrally, and VB migrates internally toward the gut (Figure 1g). DBs migrate through grooves between groups of muscle precursor cells, whereas DT branches move through a contiguous field of mesodermal tissue, creating a groove as they migrate (Franch-Marro & Casanova 2000). Branch outgrowth is disrupted when the substrate is genetically altered or ablated, demonstrating the importance of substrate interactions (Franch-Marro & Casanova 2000, Boube et al. 2001). Substrates might provide special surface properties or physical constraints such as grooves that help shape or guide outgrowth of particular branches. They could also provide diffusible signals that synergize with Branchless.

Wolf & Schuh have provided an outstanding example of the role of the substrate in primary branch outgrowth (Wolf & Schuh 2000, Wolf et al. 2002). They discovered a specialized mesodermal cell, which expresses *hunchback*, next to the DTp bud site in each segment. After this mesodermal cell divides, one of its daughters,

called the bridge-cell, elongates and contacts both the posteriorly directed DTp bud and its fusion partner, the anteriorly directed DTa bud from the neighboring segment, forming a bridge between them. The DT branches traverse the bridge-cell as they extend out and fuse. In *hunchback* mutants that eliminate the bridge-cell, DT branches start to bud normally but subsequent outgrowth and fusion often fail. Branchless signaling is essential for DT budding and outgrowth, as it is for other primary branches, but the bridge-cell is necessary to complete the process efficiently. These results explain a puzzling set of earlier observations on the role of the *branchless* pathway in DT budding: Ubiquitous expression of constitutively activated Breathless FGFR or RAS in *breathless* or *branchless* mutants partially rescues outgrowth of DT branches but not other primary branches (Reichman-Fried et al. 1994, Sutherland et al. 1996). Also, outgrowth of DT branches is disrupted less than other primary branches by ubiquitous activation of the *branchless* pathway (Lee et al. 1996, Sutherland et al. 1996). These results had suggested there might be an auxiliary cue for migrating DT branches, and the recent work shows the bridge-cell provides it. Future studies should identify the molecular nature of the cue and how it functions with Branchless. Other substrates may also provide cues that enhance (e.g., Dorfman et al. 2002b, Englund et al. 2002) or inhibit (e.g., Sutherland et al. 1996) migration of other primary branches.

Outgrowth of specific secondary and terminal branches also involves specialized substrate adhesion systems and guidance cues in addition to Branchless FGF. Indeed, during outgrowth of most secondary branches and the first terminal branches, *branchless* expression is not detected in nearby tissues. Although the substrates and guidance cues for most of these branches are unknown, outgrowth of the secondary and terminal ganglionic branch in the CNS has been studied in some depth and shows cellular and molecular parallels to neuronal pathfinding (Englund et al. 1999, 2002). The primary GB initially migrates along the ventral epidermis toward the periphery of the CNS, attracted by a domain of Branchless expression. As Branchless expression declines, the leading cell of the GB enters the CNS and follows a complex migration path along specific neural and glial substrates, switching substrates five times before reaching its ultimate target. Genetic ablation of specific glial cells alters GB pathfinding, suggesting that specific and sequential changes in cell interactions between the GB and its substrates control its tortuous migration in the CNS, like neuronal pathfinding (Englund et al. 2002). The genes *unplugged* and *adrift* are expressed in the developing GB and required for efficient CNS entry (Chiang et al. 1995, Englund et al. 1999). *unplugged* encodes a homeodomain protein expressed throughout the GB, and *adrift* encodes a novel nuclear protein whose transcription is upregulated by Branchless signaling at the tip of the extending GB. One model is that the combination of Unplugged and Adrift leads to induction of specific cell adhesion or signaling molecules required for GB recognition of, or entry into, the CNS. Some of the subsequent steps in GB migration, including preventing the GB from crossing the midline, are under the control of the extracellular signal Slit and its receptor Robo (Englund et al. 2002), which play similar roles in neuronal pathfinding.

The importance of adhesion between specific tracheal branches and their substrates is also illustrated by an analysis of the role of integrin cell adhesion molecules in outgrowth of visceral branches on the gut (Boube et al. 2001). The $\alpha_{\text{mew}}\beta_{\text{mys}}$ integrin is selectively expressed in the budding primary VB under control of branch identity genes, whereas $\alpha_{\text{if}}\beta_{\text{mys}}$ integrin is expressed in the VB target, visceral mesoderm. In wild-type animals, the VB buds from the tracheal sac toward a Branchless FGF-signaling center in the visceral mesoderm, then secondary and terminal branches sprout and spread over the gut surface. In animals mutant for either $\alpha_{\text{mew}}\beta_{\text{mys}}$ or $\alpha_{\text{if}}\beta_{\text{mys}}$ integrin, migration to the gut is mostly normal, but subsequent spreading of branches on the gut does not occur properly, and in extreme cases contact is lost between VB and gut. Thus localized and complementary expression of two integrins contribute to adhesion and spreading of a tracheal branch along its substrate.

TRACHEAL TUBE FUSION

During tracheal development, specific branches cease branching and fuse to branches from neighboring hemisegments to form a continuous network, as occurs during formation of other tubular networks such as the mammalian vascular system and kidney. There are 50 fusion points in the tracheal network, with five participating branches in a typical hemisegment. Each fusion event is mediated by a specialized fusion cell at the tip of each participating branch (Samakovlis et al. 1996b, Tanaka-Matakatsu et al. 1996). A fusion cell extends a cytoplasmic process that grows out and contacts a similar projection from its fusion partner. This contact triggers cell surface changes that include enrichment of *shotgun* E-cadherin and formation of a ring of E-cadherin at the contact site and establishment of an intercellular junction. Contact also stimulates assembly of an actin-rich track, associated with the actin and microtubule-binding protein Short Stop, that runs from the contact site to the existing luminal surface of each branch and may act as a template for formation of new lumen in the fusion cells (Tanaka-Matakatsu et al. 1996, Lee & Kolodziej 2002). As lumen forms in each fusion cell, the cells shorten, drawing the flanking tracheal cells closer to the fusion site. The entire process takes ~ 3 h and creates a fusion joint consisting of two toroidal fusion cells, attached face-to-face, with a continuous lumen running through them.

The fusion process is controlled by a gene regulatory hierarchy involving the zinc-finger transcription factor Escargot (Samakovlis et al. 1996b, Tanaka-Matakatsu et al. 1996). *escargot* expression begins in fusion cells ~ 2 h before the first morphological events of fusion; it is the earliest fusion gene known. In *escargot*-null mutants, most fusion events are disrupted, and there is no expression of later fusion genes including *shotgun* and *members only*, which encode a nucleoporin Nup88 homolog (Uv et al. 2000) and *headcase* (Steneberg et al. 1998; see above). However, the Fusion-3 marker continues to be expressed. *escargot* is also required to repress terminal branching genes and suppress terminal branching in fusion cells (Samakovlis et al. 1996b, Tanaka-Matakatsu et al. 1996). Misexpression

of *escargot* can suppress terminal branching throughout the tracheal system and drive ectopic branch fusion. The fusion process and fusion gene regulatory hierarchy are similar but not identical in all fusion cells. For example, dorsal trunk fusion and expression of *shotgun* in DT fusion cells is little affected in *escargot* mutants, suggesting that there is another, partially redundant, fusion regulator in these cells.

The five fusion cells in each tracheal hemisegment arise at specific positions, all but one at the ends of outgrowing primary branches. The selection of fusion cells is a complex process involving interplay between several signaling pathways. Localized expression domains of Branchless and Wingless (and possibly other Wnts) in nearby tissues appear to provide combinatorial cues that initiate expression of *escargot* in individual cells or small numbers of cells at the ends of outgrowing primary branches (Ikeya & Hayashi 1999, Chihara & Hayashi 2000, Llimargas 2000). In DBs, the Dpp signaling pathway is also involved (Steneberg et al. 1999). These signaling pathways trigger Delta expression in the prospective fusion cell, which activates Notch and represses *escargot* expression in neighboring cells (Llimargas 1999, Ikeya & Hayashi 1999, Steneberg et al. 1999, Chihara & Hayashi 2000). This lateral inhibition pathway ensures that only a single cell at the end of each branch, the one with highest initial expression of *escargot* and *Delta*, becomes a fusion cell. The regulatory interactions are even more complex in branches where Branchless signaling also selects cells for secondary and terminal branching fate.

TUBE MORPHOGENESIS AND MAINTENANCE

In addition to their extensive migrations during branch budding and outgrowth, tracheal cells must form tubular structures with the correct size and shape for their position in the network. These steps are essential for efficient flow through a tubular network, as evidenced by the substantial human morbidity and mortality that result from narrow or occluded blood vessels and vascular aneurysms. Different tracheal tubes have different cellular structures: Some are multicellular tubes, some are unicellular tubes, and some are subcellular tubes that form within cytoplasmic extensions (Figure 2) (Manning & Krasnow 1993, Samakovlis et al. 1996a). The largest larval branches are $\sim 60 \mu\text{m}$ in diameter and the smallest are $0.1 \mu\text{m}$ or less. Even among tubes of the same cellular structure there are characteristic differences in tube size and shape, such as the differences in primary branch caliber and taper (Beitel & Krasnow 2000).

The critical step in tube morphogenesis is the creation of a lumen. Although little is known about this process, it appears to be a genetically distinct step in the tracheal morphogenesis program. Mutations in *ribbon* (Shim et al. 2001) and *synaptobrevin* (Jarecki et al. 1999) and expression of a dominant-negative RhoA (Lee & Kolodziej 2002) impair or prevent lumen formation but allow branch budding to proceed. Also, lumen formation is not always coincident with branch budding: The lumens of terminal and fusion branches form after the cells have extended and contacted their targets.

Once a lumen forms it must grow substantially to reach its mature size. Several lines of evidence imply that tube expansion is also a distinct step in tracheal

morphogenesis (Beitel & Krasnow 2000). When primary tracheal branches form, their lumens are all small and similar in caliber ($<2 \mu\text{m}$). Several hours later (stage 14) the lumens abruptly expand up to three times their original diameter, with different branches expanding to different extents. Two other brief periods of expansion occur during larval life, giving overall increases in tube diameter of up to 40-fold. Interestingly, tube length also increases during embryonic and larval life, but with different kinetics and to different extents than tube diameter, and tube length is affected differently than diameter by physiological signals and by the tube expansion mutations described below. This implies that there are different subprograms for tube elongation and dilation.

Tube size is not controlled by simply setting the number or overall size or shape of the constituent cells. As tracheal tubes expand, cell number does not change, and conversely, changing cell number with mutations in cell cycle genes does not affect tube size (Beitel & Krasnow 2000). Rather, the focus of tube size control appears to be the apical (inner) surface of the tubes. The apical surface of tracheal cells forms an even circular profile that increases dramatically during tube dilation, whereas the basal (outer) surface of the cells is irregular and changes little during the embryonic expansion. Also, the position of the apical surface within the tube is not tightly coupled to the position or size of the cell body and basal surface. Recent progress in other tube morphogenesis systems suggests that apical membrane biogenesis, fusion of cytoplasmic vesicles, and secretion across the apical surface play central roles in tube morphogenesis (O'Brien et al. 2002, Lubarsky & Krasnow 2003), and similar events may underlie tracheal lumen formation and expansion.

A number of tracheal molecular markers whose expression changes just prior to or during expansion have been found, and a dozen tube expansion genes have been identified, mutations that affect expansion and expression of expansion markers without disrupting earlier molecular and morphogenetic events (Beitel & Krasnow 2000, Wilk et al. 2000, Hemphala et al. 2003). These results outline a genetic hierarchy for tube formation and expansion, which may be triggered by Branchless during branch budding and modulated by branch identity genes to give branch-specific differences in diameter (Beitel & Krasnow 2000, Hemphala et al. 2003). Cloning and molecular characterization of tube expansion genes and other genes that specifically affect lumen formation and expansion should help elucidate these processes.

One characterized gene is *grainyhead*, which encodes a GRH-CP2-type transcription factor (Bray & Kafatos 1991). In *grainyhead* mutants, the initial formation and expansion of tracheal tubes is normal, but they continue to elongate and form convoluted tubes, apparently because of failure in terminating growth at the apical surface (Hemphala et al. 2003). Grainyhead activity is enhanced by Branchless signaling, suggesting that the Branchless pathway not only promotes branch budding and outgrowth but may also terminate branch elongation via its effect on Grainyhead and apical surface growth. Mutations in *fasII*, encoding a cell adhesion protein, and *ATP α* , encoding the α subunit of Na-K ATPase, also cause tracheal tube elongation and convolution; both proteins localize to the lateral surface of tracheal cells, suggesting that structures or events at the lateral surface

are also crucial for defining and/or maintaining tube structure (Hemphala et al. 2003). *hindsight* (also called *pebbled*), which encodes a zinc-finger transcription factor, has an even more severe phenotype (Wilk et al. 2000). During the embryonic expansion phase, tracheal tubes collapse or dilate abnormally, and ultimately fragment, perhaps because of a defect in the cuticle deposited along the apical surface at the end of the expansion period. A cellular and molecular understanding of how the tracheal tube lumens are created, expanded to the appropriate size and shape, and maintained is an important goal for the field.

FUTURE CHALLENGES

The studies reviewed here have provided an outline of the genetic program and have begun to elucidate the molecular pathways that control the major morphogenetic events of larval tracheal development. Many signaling molecules and transcription factors have been associated with specific steps. The major challenge now is to identify their targets and the molecular events that execute the epithelial cell migrations and rearrangements and create a lumen and expand it to the mature tube dimensions. One aspect of this will be to define the downstream events in the Branchless FGF-signaling pathway and the changes in these events at each stage of branching that give rise to tubes of different sizes and structures. It will also be necessary to understand how the FGF-signaling events interface with the branch-specific pathways and with the global patterning pathways and hypoxia response pathways that control the complex and changing expression of the FGF ligand. This will require dynamic *in vivo* imaging studies (see, for example, Ribeiro et al. 2002, Wolf et al. 2002) to visualize and study the cellular and molecular events, and the establishment of *in vitro* systems to probe the biochemical mechanisms. Saturation genetic studies and genomic studies are critical for identifying the full set of tracheal genes and for ordering them in the tracheal developmental program. As our knowledge of the program increases, it may be possible to begin engineering tracheal cells and recreating tracheal tubes and networks *in vitro*. This knowledge will also help guide studies of the more complex processes of branching morphogenesis of vertebrate organs.

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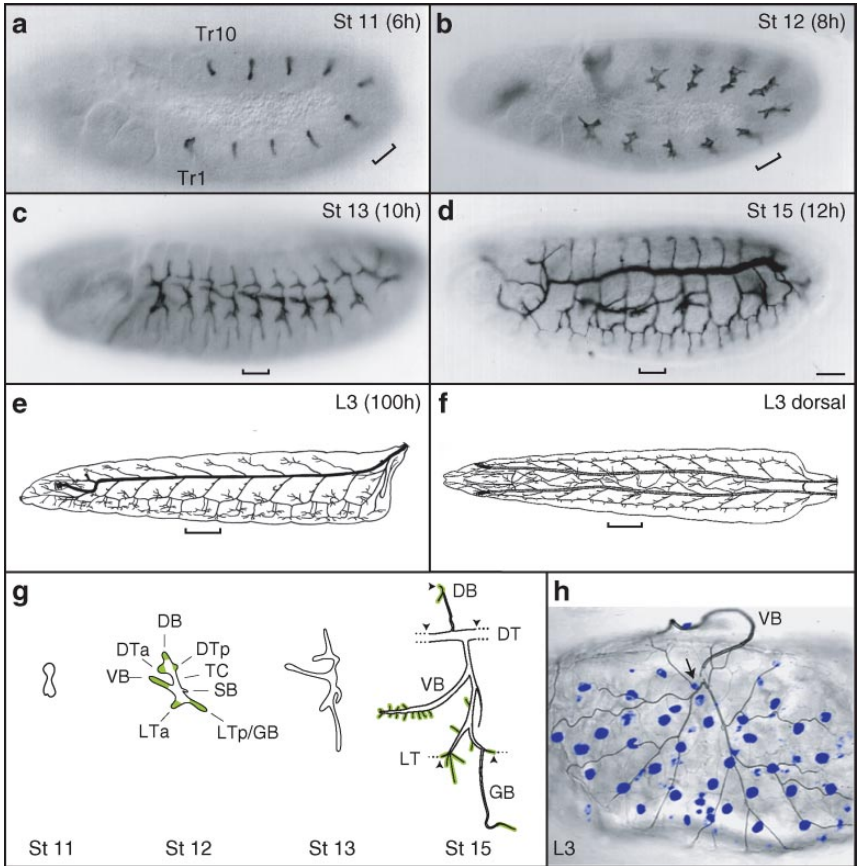
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Figure 1 The *Drosophila* tracheal system develops by sequential branching from a tracheal sac in each hemisegment. (*a-d*) Embryonic tracheal development visualized by immunostaining of tracheal lumen; the lumen of the tracheal sacs are seen in (*a*). Embryo stage and age (in hours) are indicated. The first (Tr1) and the tenth (Tr10) tracheal hemisegments are indicated in (*a*). Brackets indicate position of fifth tracheal hemisegment (Tr5). Lateral views, anterior left, dorsal up. (*e,f*) Lateral (*e*) and dorsal (*f*) views of tracheal system of third instar larva (L3). VBs and GBs are truncated, and only some terminal branches are shown. (*g*) Development of Tr5. The spiracular branch (SB), transverse connective (TC) and the six primary branches (DB, dorsal branch; DTa and DTp, anterior and posterior dorsal trunk; VB, visceral branch; LTa, anterior lateral trunk; LTp/GB, posterior lateral trunk/ganglionic branch) are indicated. Primary branches at stage 12 and secondary branches at stage 15 are highlighted green. Most secondary branches ramify to form terminal branches in the larval period (not shown). The branches that cease branching and fuse with branches in neighboring hemisegments are indicated (*arrowheads*). (*h*) Close-up of a secondary VB that has ramified to form dozens of fine terminal branches on the gut of an L3 larva. Propidium iodide-stained nuclei are pseudocolored blue. Arrow, nucleus of the VB terminal cell that forms all the terminal branches shown. Bar, 25 μm (*a-d*); $\sim 250 \mu\text{m}$ (*e,f*); 20 μm (*h*). Panels (*a-d,g,h*) are modified from Samakovlis et al. (1996a); (*e,f*) are from Ruhle (1932).

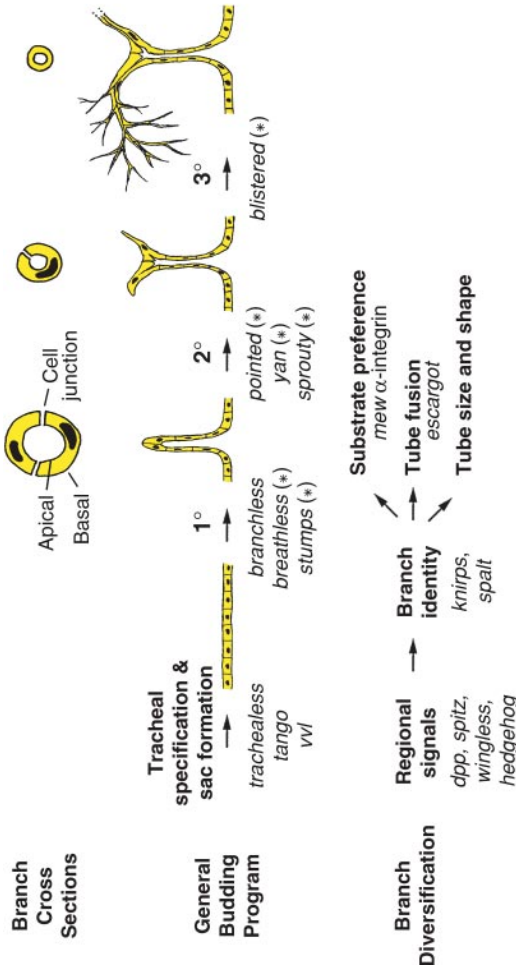


Figure 2 Steps in tracheal branching morphogenesis. A portion of the tracheal epithelium (yellow) is shown as the sac forms and sprouts 1°, 2°, and 3° (terminal) branches. Cross sections through each type of branch are diagrammed above to show their tubular structure; the apical epithelial surface lines the lumen and the basal surface is the outer surface of the tube. Genes that function in the general budding program, required throughout the developing tracheal system, are indicated at the first step affected by null mutations; some of the genes (e.g., *branchless* and *branchless**) are used again at later steps. Asterisks (*) indicate genes whose expression is triggered by genes that function in earlier steps in the program. Running in parallel to the general budding program is a branch diversification program, in which local signals induce branch identity genes in specific branches. These impart unique character to the branches, such as their substrate outgrowth preferences, ability to fuse with other branches, and tube size and shape.

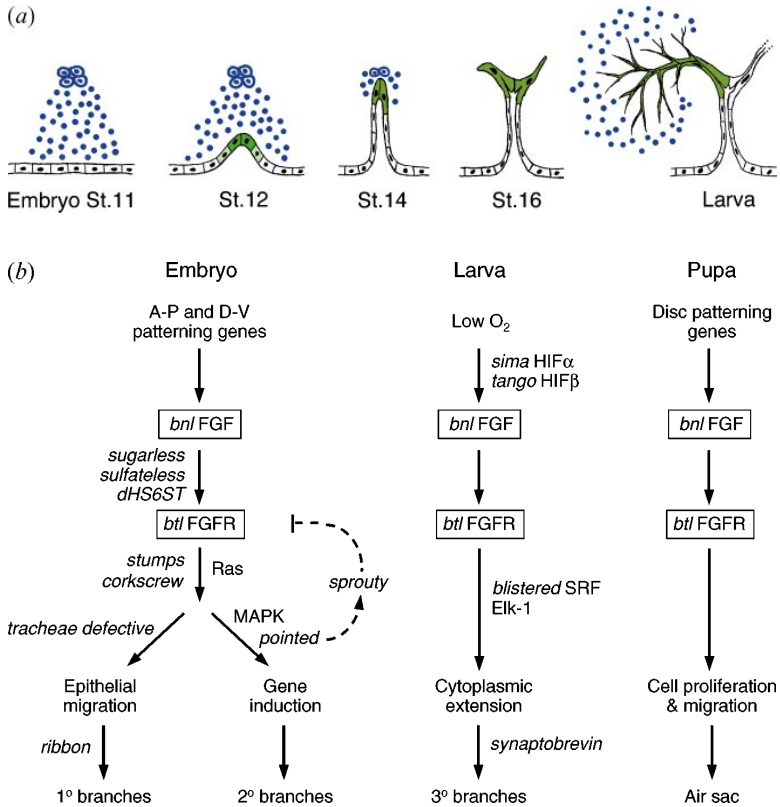


Figure 3 The Branchless FGF pathway controls each step of branching. (a) *branchless* FGF (blue) is expressed in clusters of cells surrounding the developing tracheal system, at each position where a primary branch will bud. The secreted growth factor activates the Breathless FGFR on nearby tracheal cells (black), and acts as a chemoattractant that guides outgrowth of primary branches. It also induces expression of secondary branch genes and triggers secondary branch sprouting at the ends of outgrowing primary branches (green; stages 12–16). *branchless* turns back on again, but in a completely different pattern, during larval life to control outgrowth of terminal branches. The gene is expressed yet again during pupal life where it controls budding of adult air sacs (not shown). (b) The genes that function upstream of Branchless and downstream of Breathless change during development, giving rise to different patterns and structures of branches at each step.

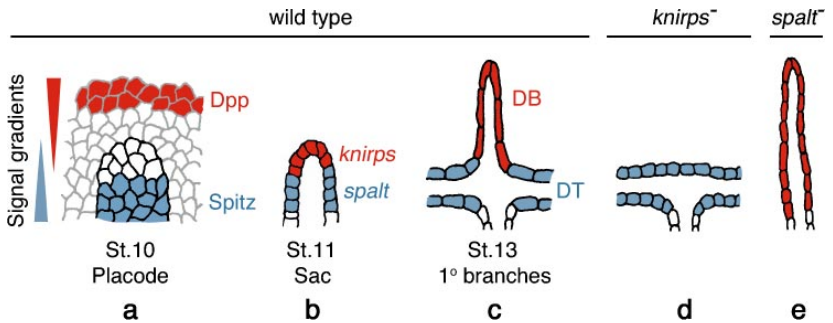


Figure 4 Model of dorsal branch (DB) and dorsal trunk (DT) specification. (*a–c*) At the onset of tracheal development (*a*), a gradient of Dpp/TGF β emanating from the dorsal ectoderm (*red*) signals to dorsal tracheal cells in the placode (*black outline*), inducing expression of *knirps* (*b*). A Spitz/EGF gradient emanating from central tracheal cells (*blue, panel a*), acting in conjunction with Wingless/Wnt signaling (not shown), maintains expression of *spalt*. Where *knirps* and *spalt* expression overlap, the transcription factor Knirps downregulates *spalt* expression, establishing distinct domains of *knirps* and *spalt* expression in DB and DT primordia, respectively (*b,c*). (*d*) In the absence of *knirps* (and the closely related gene *knrl*), DB does not form and the cells integrate into DT. (*e*) In *spalt* mutants, DT does not form and the cells integrate into DB.

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