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From fate to function: the *Drosophila* trachea and salivary gland as models for tubulogenesis

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Abstract

Tube formation is a ubiquitous process required to sustain life in multicellular organisms. The tubular organs of adult mammals include the lungs, vasculature, digestive and excretory systems, as well as secretory organs such as the pancreas, salivary, prostate, and mammary glands. Other tissues, including the embryonic heart and neural tube, have requisite stages of tubular organization early in development. To learn the molecular and cellular basis of how epithelial cells are organized into tubular organs of various shapes and sizes, investigators have focused on the *Drosophila* trachea and salivary gland as model genetic systems for branched and unbranched tubes, respectively. Both organs begin as polarized epithelial placodes, which through coordinated cell shape changes, cell rearrangement, and cell migration form elongated tubes. Here, we discuss what has been discovered regarding the details of cell fate specification and tube formation in the two organs; these discoveries reveal significant conservation in the cellular and molecular events of tubulogenesis.

Keywords

Drosophila; salivary gland; trachea; tubulogenesis

Trachea development: overview

The *Drosophila* larval tracheal system is a ramified tubular network that provides oxygen directly to every cell in the organism. It forms during embryogenesis from primordia that are first visible as 10 groups of approximately 40 cells each on both sides of the embryo, corresponding to thoracic segment 2 (T2) through abdominal segment 8 (A8). A surface *en face* view of the primordia shows cells within each group arranged in an elongated rectangle, approximately four to five cells along the anterior–posterior axis and eight to 10 cells along the dorsal–ventral axis (Isaac and Andrew, 1996). Subsequently, cells within the primordia appear to acquire greater affinity for each other and form approximately oval-shaped tracheal placodes (Fig. 1A). Histological sections through the placodes at this stage reveal columnar cells with randomly positioned nuclei (M. M. Myat and D. J. Andrew, unpublished data). Through a series of coordinated cell shape changes, the primordia invaginate to form small epithelial sacs. The tracheal cells undergo their final round of mitotic divisions during invagination. Once internalized, each tracheal segment or metamere, referred to as Tr1–Tr10, contains approximately 80 cells, with slightly more in Tr1, and slightly fewer in Tr3. Thus, the entire trachea is comprised of approximately 1600 cells, which after the final mitotic division during invagination grow by polyploidization, as do most larval tissues in *Drosophila*. Once the tracheal cells are internalized, small groups of cells in distinct positions within each tracheal

sac begin to migrate away from the site of invagination in a stereotypical pattern to form the primary branches of the tubular trachea (Figs. 1B–1D). The major artery of the trachea is the dorsal trunk (DT), which connects all of the tracheal segments (or metameres) along the anterior–posterior axis (Fig. 1E). A thinner connection between all of the tracheal metameres forms more laterally and is known as the lateral trunk (LT). The transverse connective (TC) connects the DT and LT within each metamere. Additional primary branches include the dorsal branch (DB), which migrates dorsally to provide oxygen to the dorsal heart and epidermis, the visceral branch (VB), which forms from most but not all tracheal metameres and migrates internally to oxygenate the viscera, and the ganglionic branch (GB), which migrates ventrally to provide oxygen to the central nervous system (CNS). Cells remaining at the site of invagination form the spiracular branch (SB, not shown), which is continuous with the TC. This branch closes during embryonic stage 12 and subsequently collapses, opening again only during larval molts to expel the cuticular lining of the trachea (Manning and Krasnow, 1993).

The fully formed trachea is an air-filled network of tubes that spans the entire organism. Air enters through the posterior spiracles, which are attached to the posterior end of the DT, and is distributed throughout the tracheal network, ending in the fine tracheolar tubes that mediate gas exchange with target tissues. Four distinct types of epithelial tubes are found in the late embryonic/larval trachea (Fig. 2); each tube type is comprised of polarized epithelial cells that are arranged with their apical surfaces facing the internal luminal space and their basal surfaces facing surrounding tissues. Type I tubes comprise a major portion of the DT and some of the TC. These are multicellular tubes with two to four wedged-shaped cells surrounding the central lumen connected by intercellular epithelial junctions. Type I tube diameter ranges from 4 to 5 μm . Type II tubes comprise the DB, VB, LT, GB, and parts of the TC. These tubes are one cell in diameter, connecting with themselves through autocellular junctions and with their proximal and distal neighbors through intercellular junctions. Type II tubes have a luminal diameter of $\sim 1 \mu\text{m}$. Type III tubes include the fusion cells of the DT and LT, which connect tracheal metameres in adjacent segments, and the fusion cells of the DB, which connect each DB to the contralateral DB of the corresponding metamere on the other side of the embryo. Type III tubes are bicellular tubes that form by anastomosis, the fusion process that connects the individual tracheal metameres into a network. These tubes contain donut-shaped cells that connect to one another through intercellular junctions. Type III luminal diameter is linked to the bore size of the tubes the fusion cells connect, varying from ~ 0.5 to $5 \mu\text{m}$. Type IV tubes form in only the terminal cells of all branches. These tubes form the fine tracheolar extensions with hollow apical protrusions that are contiguous with the apical lumina of the terminal cells. These subcellular tubes make direct contact with the target tissues and are the sites of gas exchange; their luminal diameter is less than $0.5 \mu\text{m}$.

Trachea formation can be thus broken down into five developmental steps: (1) specification of the tracheal primordia within the ectoderm, (2) invagination of the primordia to form the internalized tracheal sacs, (3) general and branch-specific migration, (4) formation of the different tube types, and (5) tracheal tube growth. Tracheal morphogenesis involves processes that have been described in vertebrate epithelial morphogenesis, including regulated gene expression, cell shape change, cell intercalation, and cell migration.

Specification of the tracheal primordia

The trachea is specified through the combined activities of the global patterning genes, including the dorsal–ventral, homeotic, and segment polarity genes. These genes determine the anterior–posterior and dorsal–ventral location of the tracheal primordia. Among the earliest expressed tracheal genes are several encoding transcription factors, including *trachealess* (*trh*), which encodes a bHLH-PAS protein (Isaac and Andrew, 1996; Wilk et al., 2000), *ventral veinless* (*vvl*), also known as *drifter*, which encodes a POU-homeobox containing protein

(Anderson et al., 1995; de Celis et al., 1995), and *knirps* (*kni*) and *knirps-related* (*knrl*), two closely linked homologous genes that encode zinc finger proteins related to the vitamin D receptor (Chen et al., 1998). These early transcription factor genes, which are coordinately activated in the early trachea, act in combination to control tracheal morphogenesis (Isaac and Andrew, 1996; Boube et al., 2000). They also serve as excellent markers for revealing the roles of different patterning genes in the specification of tracheal cell fates.

The anterior and posterior limits on which segments form trachea is controlled by *spalt major* (*salm*), a gene that encodes a zinc finger transcription factor expressed early in broad anterior and posterior domains corresponding to segments in which trachea do not normally form (Kuhnlein et al., 1997; Boube et al., 2000). In *salm* mutants, expression of *trh* and *vvl* extends anteriorly into three additional segments and posteriorly into one additional segment. Similarly, *kni* expression is observed in two additional anterior and one additional posterior segment. Thus, *salm*, whose expression is controlled by the maternal terminal and zygotic gap genes (Kuhnlein et al., 1997), limits early tracheal gene expression to the 10 segments from T2 through A8 (Fig. 3). As will be discussed, *salm* is also involved in later steps of tracheal development.

Tracheal cells derive from a discrete anterior–posterior and dorsal–ventral domain within each segment. Double labeling experiments reveal that the primordia form between the stripes of *wingless* (*wg*) expression in each segment (de Celis et al., 1995) and, in *wg* mutants, both *vvl* and *trh* are expressed throughout each segment, from T2 through A8 (de Celis et al., 1995; Wilk et al., 1996). The dorsal limit on early tracheal gene expression is set by the dorsal patterning gene *decapentaplegic* (*dpp*). In embryos heterozygous for *dpp*, expression of *trh* expands slightly dorsally (Wilk et al., 1996), and in embryos null for *dpp*, expression of *trh* expands to include cells throughout the dorsal region of the embryo (Isaac and Andrew, 1996). The ventral limit on tracheal gene expression is set by the ventral patterning genes, although the specific signals that provide this limit have not been discovered to date. As with *salm*, *Wg* and *Dpp* signaling are also involved in later stages of tracheal development.

Based on the coordinate expression of the known early tracheal transcription factor genes, as well as the finding that the early expression of each of these genes is independent of the function of the others, it is likely that no single gene specifies tracheal fate (Isaac and Andrew, 1996; Boube et al., 2000). Instead, it is the coordinate activities of multiple early transcription factor genes such as *trh*, *vvl*, *kni*, and *knrl* that mediate tracheal development. These early transcription factor genes work together to regulate expression of key downstream target genes, such as *breathless* (*btl*), which encodes a fibroblast growth factor (FGF)-receptor essential for tracheal migration (Anderson et al., 1996; Zelzer and Shilo, 2000). They also work independently to regulate expression of other important genes, such as *thick veins* (*tkv*), a DPP receptor expressed to elevated levels in the trachea (Boube et al., 2000) and required for the specification of specific tracheal branches (Affolter et al., 1994a). Although the initial expression of the early tracheal transcription factor genes is regulated by global patterning genes, maintenance of their expression requires both auto- and cross-regulation in the case of *trh* and *vvl* (Boube et al., 2000; Zelzer and Shilo, 2000) and both *Dpp* (Chen et al., 1998) and FGF-signaling in the case of *kni* and *knrl* (Myat et al., 2005).

An additional argument that none of the early tracheal-expressed transcription factors functions as an “organ-specifying” gene is the observation that all of the known early genes are expressed in tissues outside the trachea. *trh* is expressed very early in the trachea primordia and continues to be expressed in all tracheal cells throughout the lifetime of the organ. It is also persistently expressed in other ectodermal tissues that form tubes and in a subset of cells in the CNS (Isaac and Andrew, 1996; Wilk et al., 1996). *vvl* is normally expressed at low levels in a small subset of the cells that form additional trachea in the absence of *salm* function, but is also expressed

in other ectodermal cells, both early and late (Kuhnlein et al., 1997 #27). *kni* RNA and protein are detected very early in a wide band of expression along the anterior–posterior axis, where *kni* serves a role as a zygotic gap gene controlling embryonic segmentation (Nauber et al., 1988; Oro et al., 1988). *knrl* is also expressed in other ectodermal cells at later stages, in cells for which a function has not yet been described. Both *kni* and *knrl* expression persists in the trachea beyond the tracheal placode stage, but expression of both genes becomes limited to the DB, VB, LT, and GB, branches that migrate further and form type II tubes from type I tubes (Chen et al., 1998). Other transcription factor genes that are expressed in the early tracheal primordia are also likely to play significant roles in tracheal development, although these roles await discovery (<http://www.fruitfly.org/cgi-bin/ex/insitu.pl>).

Invagination of the trachea

Tracheal cells internalize by an apical constriction mechanism, wherein the randomly positioned nuclei move to a basal position and the apical domains constrict to create wedge-shaped cells, a shape change likely contributing to the forces driving internalization. Two to three rows of cells along the anterior–posterior midline of each placode undergo apical constriction and internalize to form dorsal–ventrally elongated tracheal pits, with the more ventral cells internalizing slightly ahead of the more dorsal cells within each metamere. Subsequently, cells adjacent to the midline cells undergo apical constriction and internalize, with the process continuing until all of the primordia have internalized to form the tracheal sacs. During invagination, the tracheal cells undergo their final round of normal mitotic cell division (Manning and Krasnow, 1993).

Invagination of the tracheal primordia to form the tracheal sacs requires *trh*. Scanning electron micrographs of wild-type and *trh* primordia reveal that the tracheal cells fail to undergo the apical constriction events of invagination (Isaac and Andrew, 1996) and staining with phalloidin reveals a failure of the primordia to enrich F-actin at the apical surface (Llimargas and Casanova, 1999). Trh mediates invagination in part through epithelial growth factor (EGF) signaling. Trh, and to a lesser extent Vvl, are required for the tracheal expression of *rhomboid* (*rho*) (Boube et al., 2000; Zelzer and Shilo, 2000), which encodes an enzyme essential for processing of the EGF signal (Lee et al., 2001). In embryos mutant for *rho* or other components of the EGF signaling pathway, a subset of tracheal cells fail to invaginate (Llimargas and Casanova, 1999) and, as a consequence, there are fewer cells to populate the different branches of the trachea (Fig. 4; Wappner et al., 1997; Llimargas and Casanova, 1999; Bradley et al., 2001). As all of the cells fail to invaginate in *trh* mutants, whereas only a fraction fail to invaginate in *rho* mutants, Trh must regulate additional factors to mediate tracheal precursor internalization.

trh is also required for the formation of two other embryonic tubes: the salivary duct, which provides the conduit for the secretion of salivary gland proteins, and the filzkörper, which are the elongated cuticular air filters that attach to the posterior end of the trachea and prevent the entry of particulates into the trachea. In the absence of *trh*, the precursors to the salivary duct and filzkörper, both of which normally express *trh*, fail to invaginate to form tubes, although they continue to express markers specific to each cell type (Younossi-Hartenstein and Hartenstein, 1993; Isaac and Andrew, 1996). Thus, Trh has a more general role in early tube morphogenesis, where it is likely to function, at least in part, by regulating the expression of factors involved in organizing the actin cytoskeleton.

Tracheal cell migration

Tracheal cell migration or branching morphogenesis begins shortly after formation of the tracheal sac and absolutely requires the Breathless- (Btl) signaling pathway, one of the two FGF-signaling pathways known in *Drosophila*. In embryos mutant for the receptor Btl or the

downstream effector Stumps/Heartbroken/Downstream of FGF, both of which are expressed in tracheal cells, or the FGF ligand Branchless (Bnl), which is expressed in the target tissues toward which the tracheal cells migrate, the trachea remain as sacs of cells near their site of invagination (Klamt et al., 1992; Younossi-Hartenstein and Hartenstein, 1993; Reichman-Fried et al., 1994; Reichman-Fried and Shilo, 1995; Lee et al., 1996; Sutherland et al., 1996; Michelson et al., 1998; Vincent et al., 1998; Imam et al., 1999). Btl-signaling is also required in larvae to mediate branch migration in response to hypoxia (Jarecki et al., 1999). Btl-signaling induces the formation of filopodia at the growing tips of tracheal branches and thus functions, in part, to control cytoskeletal dynamics in the leading cells of the migrating tracheae (Ribeiro et al., 2002).

The FGF signaling components required in the trachea for tracheal branch migration, *btl* and *stumps*, require both Trh and Vvl to achieve robust tracheal-specific expression. Moreover, the ectopic expression of *btl* can be driven by ectopic expression of Trh, but only in cells that also express Vvl (Boube et al., 2000). Regulation of *btl* by Trh and Vvl is direct (Anderson et al., 1996; Ohshiro and Saigo, 1997) and requires function of the Trh partner protein known as Tango, which like Trh is a bHLH-PAS transcription factor (Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997), and Akt/protein kinase B phosphorylation of Trh, which mediates its nuclear localization (Jin et al., 2001). Btl levels at later stages of embryonic tracheal development are also regulated by a positive feedback loop wherein Btl signaling activates MAP kinase, which in turn activates *btl* expression in part through the phosphorylation of both an activator and repressor of *btl* expression (Ohshiro et al., 2002). Also, mutations in the *abnormal wing disc (awd)* gene, which disrupt the normal endocytosis of Btl, result in increased levels of protein and aberrant tracheal migration (Dammai et al., 2003). The importance of ligand-mediated Btl activation is further highlighted by the ectopic branching that occurs with the expression of constitutively activated forms of the receptor (Lee et al., 1996). Thus, tight regulation of Btl levels is crucial and is achieved through both transcriptional and post-transcriptional mechanisms.

Trh also regulates expression of a bZIP transcription factor gene, *tracheae defective (tdf)*, which like Btl-signaling, is required for migration of all tracheal branches (Eulenberg and Schuh, 1997). *tdf* does not affect the expression of the components of the Btl pathway that have so far been tested and its targets are not known. Thus, our understanding of where this gene fits into the regulation of branching morphogenesis is limited.

Another gene affecting migration of all branches of the trachea is *ribbon (rib)* (Bradley et al., 2001; Shim et al., 2001). *rib* encodes a BTB-POZ protein that localizes to the nucleus where it is likely to function as a transcription factor. In *rib* mutants, migration of all tracheal branches is delayed, although at later stages some branches appear almost normal. Notable exceptions are the DT, which fails to form, and the ventral branches, which fail to fully migrate. *rib* is expressed relatively ubiquitously with elevated levels in the trachea, salivary gland, and other tissues undergoing morphogenesis, consistent with the large number of tissues affected in *rib* mutants. DT formation can be fully rescued, however, by expression of *rib* specifically in the trachea (Bradley et al., 2001), supporting a cell-, or at least tissue-, autonomous requirement for the gene. The *rib* phenotypes in the trachea and other tissues, including the epidermis and salivary gland, suggest a role for Rib in linking multiple signaling pathways and apical-basal coordination of cell movement (Jack and Myette, 1997; Bradley et al., 2001; Shim et al., 2001).

Branch-specific identity and cell migration

Each branch of the embryonic trachea migrates in a stereotypical pattern to ultimately create a contiguous network that supplies oxygen to all tissues in the animal. The stereotypical

migration of each branch and the types of tubes formed within each branch are linked to distinct gene expression patterns and signaling pathways that function in subsets of cells within the trachea (Fig. 4). Among the key factors whose expression affect both migration and tube type is *salm*, the gene whose earlier role was to limit trachea formation to the ten metameres in the trunk region of the embryo. Early, when the tracheal primordia are still on the surface of the embryo, the dorsal tracheal cells (and surrounding ectodermal cells) express *salm* (Kuhnlein and Schuh, 1996). This early tracheal expression of *salm* is required to distinguish dorsal trachea from ventral trachea (Franch-Marro and Casanova, 2002). In *salm* mutants, not only are there additional tracheal metameres in both anterior and posterior segments, but the dorsal tracheae within each segment take on the appearance of ventral tracheae and express ventral tracheal markers. For example, the *unplugged* gene, which is normally expressed and required in only the GB, the most ventral branch (Chiang et al., 1995), is expressed in cells that should have formed DB, the most dorsal branch (Franch-Marro and Casanova, 2002). Also, DT marker genes are not expressed in dorsal tracheal cells in *salm* mutants. Conversely, ectopic expression of *salm* throughout the trachea converts ventral tracheae to dorsal tracheae based on morphology and the expression of dorsal tracheal markers in ventral tracheal cells (Franch-Marro and Casanova, 2002).

At later stages, *salm* expression is observed in only the DT, where it is required to prevent DT cells from either migrating dorsally with the DB or internally with the VB. *salm* expression becomes limited to the DT in part by DPP signaling. The most dorsal and ventral cells within the tracheal primordia are exposed to high levels of DPP, which just before and during tracheal invagination has a secondary pattern of expression in cells located ventral and dorsal to the trachea (Fig. 4; Vincent et al., 1997). Dpp signals through its receptors Thick veins (Tkv) and Punt (Put) (Ruberte et al., 1995) and downstream effectors to maintain late tracheal expression of both *kni* and *knrl*, either of which is sufficient to shut off *salm* (Kuhnlein et al., 1997). In embryos mutant for loss-of-function mutations in *tkv* or *put*, the DB cells fail to express *kni* and *knrl*, continue to express *salm* and stay with the DT precursors and fail to migrate dorsally (Vincent et al., 1997; Chen et al., 1998). Conversely, in embryos in which either Dpp or an activated form of the Tkv receptor is expressed throughout the trachea, the DT cells express both *kni* and *knrl*, do not express *salm* and migrate dorsally with the DB (Kuhnlein et al., 1997; Vincent et al., 1997). Similarly, ectopic high level expression of either *kni* or *knrl* throughout the trachea results in a loss of *salm* in DT cells and their dorsal migration with the DB (Chen et al., 1998). Thus, the most dorsal cells of the trachea, which require early function of *salm* to distinguish them from ventral tracheal cells, turn on *Kni* and *Knrl*, which shut off *salm* at later stages in order to form the DB and migrate dorsally. Interestingly, trans-heterozygous combinations of loss-of-function mutations in Dpp- and Btl-pathway components result in migration defects that are not seen with the individual heterozygous mutations, suggesting that the two pathways are interdependent (Myat et al., 2005). In further support of this idea, robust expression of both *kni* and *knrl* in DB precursors requires *btl* function, providing a molecular link between general and branch-specific tracheal migration (Myat et al., 2005).

Maintained expression of *salm* in the trachea requires Wg signaling (Fig. 4; Chihara and Hayashi, 2000; Llimargas, 2000). In embryos expressing a temperature-sensitive (ts) allele of *wg* that allows removal of its function after its earlier role in tracheal specification, the DT cells lose expression of *salm* and migrate internally with VB cells. Thus, active Wg signaling maintains *salm* expression in the DT precursors; this expression is required both for the migratory behavior of DT cells and for the formation of the wide bore type I tubes that typify this branch of the trachea. Correspondingly, in embryos in which either Wg or an activated form of *armadillo* (*arm*), the adherens junction protein/transcription factor that controls Wg target gene activation, is expressed throughout the trachea, VB cells express *salm* and migrate with the DT. In embryos in which both Dpp and Wg signaling is abrogated, all tracheal cells

behave as VB. In embryos in which Dpp signaling is abrogated and Wg is activated in all tracheal cells, a condition that allows persistent expression of Salm throughout the trachea, all tracheal cells behave as DT. Thus, Dpp signaling specifies DB through activation of *kni* and *knrl*, which shut off *salm*. Wg signaling distinguishes DT from VB by maintaining *salm* expression in the DT.

Although many of the signals that mediate migration of the tracheal branches remain to be discovered, genetic studies indicate that surrounding tissues play critical roles in tracheal migration (Englund et al., 1999; Franch-Marro and Casanova, 2000; Wolf and Schuh, 2000). For example, the DT migrates through a contiguous population of mesodermal precursors separating them into dorsal and ventral muscle, whereas the DB and ventral branches migrate through spaces created by the muscle precursors in either adjacent segments or in the lateral versus ventral muscle precursors. In *twist* mutants, in which no mesoderm forms, the DT completely fails to migrate and only limited migration of the dorsal and ventral branches occurs (Franch-Marro and Casanova, 2000). DT migration also requires signals from a single mesodermal cell within each segment, known as a bridge cell, that guides the DT cells to the point where fusion between individual metameres occurs (Wolf and Schuh, 2000). Bridge cells express *hunchback* (*hb*), which encodes a transcription factor required for their survival. If *hb* is missing or is ectopically misexpressed in other mesodermal cells, fusion of the DT is disrupted. Similarly, the GB, which normally tracks along the intersegmental (ISN) nerve and then switches to the segmental nerve (SN) to attain its correct final position in the CNS, fails to correctly switch to the SN in embryos mutant for a novel nuclear protein expressed in the terminal GB cell known as Adrift (Englund et al., 1999). A clear example where surrounding tissues provide a suitable substrate for tracheal migration is seen with the VB and the visceral mesoderm (VM), the layer of mesoderm that will form the visceral muscles of the gut (Boube et al., 2001). The VB expresses a specific α -integrin subunit known as α -PS1, whereas the VM expresses another specific α -integrin subunit known as α -PS2. In embryos mutant for either gene, the VB migrates toward and reaches the VM but fails to migrate along it. An identical phenotype is observed in the VB of embryos missing function of the β -integrin subunit, which is required for both α -PS1 and α -PS2 function, although in β -integrin subunit mutants, many embryonic tissues are affected. Thus, integrins are required in the VM to provide a suitable substrate for migration and in the VB to allow cells to migrate. VB expression of α -PS1 is controlled by *Kni*/*Knrl* and *Salm*, linking expression of molecules that directly mediate migration to the pathways that specify branch identity (Boube et al., 2001). Interestingly, salivary gland migration along the VM also requires integrins, with α -PS1 expression in the migrating salivary gland and α -PS2 expression in the substrate VM (Bradley et al., 2003).

One particularly well-characterized pathway known to direct tracheal migration along neighboring tissues is also involved in axonal guidance. Loss-of-function or overexpression of Slit, a secreted extracellular matrix protein, or either of two of its known receptors, Robo or Robo2, lead to mismigration of the DB, VB, DT, and GB (Englund et al., 2002; Lundstrom et al., 2004). Slit functions in both the attraction and repulsion of tracheal branches, whereas the Robo receptors act more specifically, with Robo primarily mediating repulsion and Robo2 primarily mediating attraction. Slit is expressed in the dorsal vessel (heart) where it appears to mediate Robo2-dependent migration of the DB. Slit is also expressed in a subset of cells in the VM, where it is required for Robo2-expressing VB cells to migrate along the VM. Overexpression of Slit in the VM leads to ectopic VBs forming from tracheal metameres that do not normally form VB. The overexpression of Slit in the VM of embryos also mutant for *robo* results in a threefold higher percentage of ectopic VBs than in otherwise wild-type embryos, suggesting that Robo is functioning as an antagonist of Robo2 in VB migration. Similar misexpression of Slit in wild type and mutant backgrounds indicates that DT and GB migration are also controlled by Slit signaling. Thus, the same molecules that mediate directed axonal outgrowth also figure prominently in directed migration of the trachea (Englund et al.,

2002) and, perhaps not surprisingly, the salivary gland (Kolesnikov and Beckendorf, 2005), as will be discussed later.

Formation of type II from type I tubes

Following invagination, subsets of cells within the tracheal primordia migrate out to form the primary branches, which include the DB, DT, TC, LT, GB, and VB (Fig. 1C). Primary branches are comprised entirely of multicellular type I tubes in which the cells are connected to one another through intercellular adherens junctions (Fig. 2). Subsequently, the DB, LT, GB, VB, and some of the cells of the TC undergo cell intercalation to form Type II tubes, which connect to themselves through autocellular junctions and to their proximal and distal neighbors through intercellular junctions (Fig. 2). This cellular rearrangement may be fueled in part by tension generated through Btl-mediated migration or by space compression imposed by adjacent tissues or both. The process involves one cell reaching around the lumen on the proximal side and contacting itself. At the same time, the distal end of the other cell reaches around the lumen and contacts itself (Fig. 5; Jazwinska et al., 2003). Subsequently, the autocellular junctions that are initiated at these self-contact points zipper up as the cells slide past one another until they stop with the distal end of the proximal cell contacting the proximal end of the distal cell through a persistent intercellular junction (Fig. 5). This process begins at the proximal ends of outgrowing tubes and requires an apical extracellular matrix containing at least two critical proteins: Piopio (Pio) and Dumpy (Dp) (Jazwinska et al., 2003). Pio, which contains a long ZP domain, appears to be secreted into the lumen, whereas Dp, which contains hundreds of EGF repeats interspersed with 21-residue long DPY modules and at 23,015 residues is the largest known *Drosophila* protein, may be tethered to the apical membrane through its C-terminal transmembrane domain. Pio and Dp are proposed to provide a tracheal luminal scaffold that functions to prevent the zippering of the autocellular junctions from continuing to such a point that the two cells separate, leading to disruptions in the normally contiguous tracheal network. Thus, *pio* and *dp* are required specifically for the remodeling of type I into type II tubes. *salm*, which is normally expressed in the DT and in a subset of the TC, inhibits the cell intercalation required to form type II tubes, allowing the DT and parts of the TC to remain large multicellular type I tubes with only intercellular junctions (Ribeiro et al., 2004).

Secondary branching and fusion and terminal cell fates

Within all of the major branches of the trachea, multiple cell types are observed. Typically, cells near the tips of the primary branches can either form fusion cells (type III tubes that connect tracheal metameres) or terminal cells (type IV tubes with subcellular capillary-like branches that directly mediate gas exchange with target tissues). The remaining cells in each branch form the stalk, either the type I stalks of the DT and TC or the type II stalks of the remaining primary branches. However, not all cell types are found in all branches. For example, the DT has only stalk and fusion cells, consistent with its role in forming the major artery connecting the tracheal metameres along the anterior-posterior axis of the embryo. The TC and VB have only stalk and terminal cells, although the numbers of each kind vary considerably between these branches. The DB, LTa, and LTp/GB form stalk, fusion and terminal cells. Based on the analysis of tracheal clones, the type of cell that is formed is not regulated by lineage but instead by the cell's relative position within the tracheal primordia and primary branch (Samakovlis et al., 1996); the position of a cell determines the level of critical ligands experienced by that cell. High levels of these ligands play important roles in the identity of cells within individual branches and in branch migration (Ikeya and Hayashi, 1999; Limargas, 1999; Steneberg et al., 1999; Chihara and Hayashi, 2000). For example, tracheal cells that experience the highest levels of Dpp, Wg, and Btl signaling in dorsal and ventral branches express higher levels of Delta, one of two well-characterized Notch ligands, as well as fusion cell specific markers, such as Escargot, Fusion-2 and Fusion-3 (two uncharacterized enhancer

trap lines). The high level of Delta activates Notch in neighboring cells to prevent them from forming additional fusion cells. Correspondingly, the expression of activated Notch throughout the trachea results in a loss of fusion cells and the expression of dominant-negative Notch throughout the trachea results in an increased number of fusion cells. Similar increases in the number of fusion cells are observed with a temperature sensitive *Notch* mutation brought to the restrictive temperature at critical stages of tracheal development. Thus, Notch signaling serves to limit the number of fusion cells to five per tracheal metamere, one each in the DTa, DTp, DB, LTa, and LTp (Fig. 6A; Ikeya and Hayashi, 1999; Llimargas, 1999; Steneberg et al., 1999). The finding that the expression of Dl in cells in which Wg signaling is blocked cannot rescue Esg expression suggests that although Dl has a role in limiting the number of fusion cells, it is not sufficient to induce fusion cell fates (Chihara and Hayashi, 2000). Finally, the observation that additional terminal cells are also sometimes observed in conditions where Notch function is abrogated suggests that Notch may also have an additional role in terminal cell fates (Llimargas, 1999; Steneberg et al., 1999).

Fusion cells form the doughnut-shaped cells that connect the tracheal primordia into a contiguous network through a process known as anastomosis (Figs. 2,6B). For this to occur, fusion cells must first recognize each other, make contact on their basal surfaces and then generate the apical lumen that bridges the junction between the two fusion cells. In embryos null for *escargot* (*esg*), which encodes a zinc-finger transcription factor expressed in fusion cells, the DT, LT, and DB completely fail to fuse (Tanaka-Matakatsu et al., 1996). In these embryos, the cells fail to adhere and continue to search for alternative targets. A similar failure to fuse is observed with mutations in *shotgun*, a transcriptional target of Esg encoding *Drosophila* E-cadherin (Tanaka-Matakatsu et al., 1996; Uemura et al., 1996). E-cadherin normally localizes to the adherens junctions of the apical cell surfaces (Tepass et al., 1996). During the process of fusion, E-cadherin also accumulates at the site of contact on the basal surfaces of fusion cells (Tanaka-Matakatsu et al., 1996; Uemura et al., 1996). This basal E-cadherin leads to the accumulation of several proteins that bind the cytoskeleton, including α -catenin, β -catenin, *short stop*, which encodes a plakin that binds both F-actin and microtubules, and Formin3, which encodes a regulator of several actin-based processes (Tanaka-Matakatsu et al., 1996; Uemura et al., 1996; Lee and Kolodziej, 2002a,2002b; Lee et al., 2003; Tanaka et al., 2004). These proteins mediate the formation and maintenance of an actin bridge that spans between the apical membrane surfaces of both fusion cells. Two models have been proposed for the function of this actin bridge. In one model, the actin bridge “pulls” apical surface within each fusion cell to the point of contact on the basal surface, where the apical membranes fuse to create a contiguous lumen. In the second model, the actin bridge serves as a site of fusion of apical membrane-containing vesicles; the docking and fusion of these vesicles eventually expands the apical surface through the site of initial contact, creating a contiguous lumen. The E-cadherin and associated proteins then expand to form a ring of the same diameter as the tubes the fusion cells connect.

The cells near the tips of the primary branches that also receive high levels of Bnl but do not form fusion cells instead form terminal branch cells. Nineteen of the approximately 80 cells of the typical tracheal metamere undergo terminal branching to form the blind-ended tracheoles, which serve as the interface for gas exchange (Fig. 6A; Guillemin et al., 1996). During early embryonic stage 15, the terminal cells of the DB, VB, TC, LT, and GB extend from one to three long cytoplasmic projections per cell into and over the surfaces of target tissues. This process occurs in a single terminal cell for the DB, TC, and GB, in four terminal cells for the LT and in 12 terminal cells for the VB, the source for gut oxygenation. During embryonic stage 16, small lumina begin to form discontinuously at various positions within the cytoplasmic extensions and continue to expand, eventually coalescing to form contiguous lumens by embryonic stage 17.

Two genes have been implicated in terminal branching, *sprouty* (*spry*) (Hacohen et al., 1998) and *pruned* (DSRF/blistered) (Affolter et al., 1994b; Guillemin et al., 1996). *sprouty* is expressed at the tips of primary branches and is induced by high levels of Bnl. Loss of *sprouty* function results in ectopic branching of the stalk cells located near the tips of tracheal branches, suggesting increased sensitivity to Btl signaling. In fact, genetic interaction studies confirm that Sprouty functions downstream of Btl signaling to antagonize the pathway and limit the number of stalk cells that take on a terminal cell fate. *pruned* encodes a transcription factor related to mammalian serum response factor (SRF) (Affolter et al., 1994b; Guillemin et al., 1996). Loss of *pruned* function results in terminal cell failure to elaborate cytoplasmic extensions. Conversely, embryos expressing constitutively active forms of Pruned or its presumed partner protein exhibit ectopic projections from the stalk cells near the tips of the primary branches. These ectopic projections can be aberrant, often invading territories normally supplied by other tracheal branches. Thus, Pruned is thought to respond to signals provided by target tissues to induce downstream target genes involved in regulated tracheolar outgrowth.

Tracheal tube size

Despite the fact that the control of tracheal tube size has only been studied over the past 5–6 years, considerable insight into this process has been obtained. The seminal paper in this area described the key features of tube size control in the embryonic trachea and identified mutations in eight genes affecting tube size without also affecting other, earlier aspects of tracheal morphogenesis (Beitel and Krasnow, 2000). From this work, we learn that tube size is linked to branch identity and is not controlled by the number, size or overall shapes of the cells that comprise the tracheal tubes but instead by coordinated regulation of the apical surface. The gene products corresponding to many of the mutations discovered in this original screen fall into two general categories. The first category consists of genes that when mutant cause an increase in tube length, most notably in the DT. These genes encode components of the pleated septate junctions (pSJ), membrane structures that are found just basal to the adherens junctions in ectodermally derived epithelia. Insect pSJs serve a subset of the functions of vertebrate tight junctions and contain a subset of the same proteins. pSJs serve both a fencing function to separate the apical and basolateral domains of the cell membrane and a barrier function to prevent the diffusion of water and solutes between epithelial cells. For example, two of the original tracheal tube size mutations *megatrachea* and *sinuous* encode claudins (Behr et al., 2003; Wu and Beitel, 2004); claudins are highly divergent proteins with four transmembrane spanning domains, which through homo- and heterophilic interactions form the paracellular barrier in vertebrate tight junctions (Tsukita and Furuse, 2000, 2002; Tsukita et al., 2001). *bulbous*, also known as *lachesin*, encodes a cell adhesion molecule that also localizes to the pSJ (Llimargas et al., 2004). Finally, *ectactic*, which turned out to be the same gene as *nervy2*, encodes the β -subunit of a Na^+/K^+ ATPase, which also localizes to the pSJ (Paul et al., 2003). Mutations in other pSJ protein genes were subsequently shown to affect tracheal tube size in a similar way, including mutations in *coracle*, *neurexin*, *gliotactin*, and the α -subunit of a Na^+/K^+ ATPase (Paul et al., 2003). Localization of many of these proteins to the pSJ requires the function of other tube size controlling genes, and most of the mutations affect barrier function as assayed by diffusion of small fluorescent molecules across the tracheal and salivary gland epithelial barriers. The finding that none of the mutations affect overall apical basal polarity casts doubt on a prior model wherein the pSJs would affect luminal expansion by regulating relative levels of apical membrane components such as Crumbs. Instead, recent evidence suggests that the pSJs indirectly control tube size through modifications of the apical luminal matrix.

The second category of tube size mutations consists of genes that when mutant cause dysregulation of tube diameter, most notably in the DT. These genes encode proteins involved

in chitin biogenesis, secretion, or maturation, and include *krotzkopf verkehrt* (*kkv*), which encodes one of the two chitin synthase genes in flies, *cystic* (*mummy*), which encodes a UDP-*N*-acetylglucosamine diphosphorylase required for the synthesis of the monomeric subunits of chitin, *gnarled* (*knickkopf*) and *retroactive*, which encode proteins required for the targeting and/or secretion of chitin or chitin synthesis enzymes (Araujo et al., 2005; Devine et al., 2005; Tonning et al., 2005, 2006; Moussian et al., 2006). Chitin is one of the most abundant polymers in nature; it forms the cell walls of fungi and the exoskeletons secreted by the epidermal cells of insects and other arthropods. Chitin is a carbohydrate polymer composed of $\beta(1-4)$ -linked *N*-acetylglucosamine arranged in parallel or antiparallel fibrils. Hydrogen bonding between the chitin fibrils increases the strength of chitin, and the repetitive structure of the chitin polymer is ideal for the formation of higher-ordered assemblies. In the *Drosophila* trachea, chitin is found in the lining of the mature trachea, but interestingly, it is also present at an earlier stage when it forms part of a transient luminal matrix that ensures uniform tracheal tube expansion (Uv et al., 2003; Devine et al., 2005). Loss of this transient chitin matrix, which forms long fibrillar cable-like structures, results in regions of tube dilation and tube constriction. Interestingly, the regions of tube constriction correspond to where the fusion cells meet to connect the tracheal metameres of the DT (Tonning et al., 2005).

Recent work suggests a model wherein the two mutant groups affecting tube size are related through the regulation of chitin activity. According to this model, the role of the septate junction is to mediate the secretion of additional structural components of the extracellular matrix (ECM) and of enzymes involved in further modification of the chitin matrix (Wang et al., 2006). This study identifies two chitin deacetylases that when mutant result in longer tubes without affecting tube diameter, and reveals that the luminal localization of both enzymes is disrupted in the pSJ mutants that affect tracheal length. The authors suggest that the assembly and growth of the chitin matrix is first required to coordinate uniform radial expansion of the tubes. Subsequent modification of secreted chitin by the chitin deacetylases, whose localization is dependent on pSJ function, is required to terminate tube elongation. In support of this model, the mutations that had the strongest effects on the uniformity of tube diameter with variable effects on tube length turned out to encode proteins more directly involved in chitin biogenesis and secretion, whereas the mutations primarily affecting tube length without affecting tube diameter encoded pSJ proteins. Overall, the study of the tube size control mutants suggests a critical role for a secreted apical ECM of chitin and probably other components in coordinating this process. The above studies in combination with the studies on the role of *pjo* and *dp* in the formation of type II from type I tubes indicate that apical matrix is critical at multiple steps in tracheal remodeling. A role for an apical ECM in controlling the uniformity of the salivary gland lumen has also recently been uncovered (Abrams et al., 2006), suggesting the exciting possibility that the organization and remodeling of luminal content may have more general roles in the regulation of size and shape of epithelial tubes.

Conclusions on tracheal morphogenesis

In the little over a decade that researchers have focused on the *Drosophila* trachea as a model for tube morphogenesis, much has been discovered regarding how this tubular organ forms. We now understand the role of patterning genes in specifying the tracheal primordia and the role of early-expressed transcription factors in the cell shape changes of invagination and branch migration. We have discovered that several signaling pathways and transcription factors function at multiple steps of tracheal organogenesis. We have learned how changes in cell contact and polarity allow the formation of a complex network of tubes that interconnect to deliver oxygen to all of the cells in the animal. As we begin to examine the progress that has been made in understanding the molecular and cellular events of tube formation in the *Drosophila* salivary gland, we will see that many of the features of organ formation are shared, suggesting more general paradigms for organogenesis in higher organisms, such as ourselves.

Salivary gland development: overview

The *Drosophila* larval salivary gland is a relatively simple tubular organ consisting of two unbranched secretory tubes connected through individual salivary ducts to a central common duct (Figs. 7,8; Abrams et al., 2003). Each secretory tube consists of approximately 100 large polarized epithelial cells specialized for secretion. The salivary duct is a Y-shaped tubular conduit of 55 relatively small polarized epithelial cells. The salivary gland forms during embryogenesis from a contiguous primordium on the ventral surface of parasegment 2 (PS2) (Fig. 8). The secretory glands form from the more lateral cells of this primordium (Fig. 7) and the duct forms from the central cells of this primordium, which flank the ventral midline (Fig. 8; Kuo et al., 1996). As with the trachea, the secretory portions of this primordium form placodes of columnar cells with randomly positioned nuclei that are easily seen in histological sections or in confocal images of glands stained with nuclear and membrane markers. Also like the trachea, the secretory cells invaginate by an apical constriction mechanism, although the pattern of invagination is slightly different in this tissue, allowing for the formation of a tube instead of a sac, as in the trachea (Myat and Andrew, 2000b). Unlike the trachea, the salivary gland cells cease mitotic divisions before invagination, thus simplifying analysis of this organ. Further growth of the salivary gland is achieved through the process of polytenization (DNA replication without strand separation or cell division) (Smith and Orr-Weaver, 1991). When the distal tips of the internalizing secretory tubes contact the layer of mesodermal cells known as the visceral mesoderm (VM), the gland turns and initiates posterior migration (Bradley et al., 2001,2003). The secretory glands contact several tissues as they navigate to their final position in the embryo, which is lateral to the ventral nerve cord and in direct contact with the thoracic body wall muscles, fat body (FB), and gastric caecae (GC) (Vining et al., 2005). The duct cells internalize after the last of the secretory cells have internalized to form tubes. The duct precursors form tubes through a “wrapping” process beginning with the posterior midline cells, which form the individual ducts, and ending with anterior midline cells, which form the shared common duct (Jones et al., 1998; B. Kerman and D. J. Andrew, unpublished).

Specification of the salivary gland primordium

Salivary glands are specified through the combined activities of the global patterning genes, including the genes specifying segment identity and dorsal–ventral identity (Fig. 9). Salivary gland formation absolutely requires the homeotic gene *Sex combs reduced* (*Scr*), whose initial expression is limited to PS2, and the homeotic cofactors encoded by *extradenticle* (*exd*) and *homothorax* (*hth*), which are expressed more globally (Panzer et al., 1992; Andrew et al., 1994; Henderson and Andrew, 2000). In embryos missing zygotic function of *Scr* or *hth*, or maternal and zygotic function of *exd*, salivary glands completely fail to form, and in embryos engineered to express *Scr* everywhere, ectopic salivary glands form in the anterior-most parasegments PS0 and PS1. Induction of salivary gland fates by ectopic *Scr* occurs only in more anterior segments because of the repressive activities of the trunk gene *teashirt* (*tsh*) and the homeotic gene *Abdominal-B* (*Abd-B*; Andrew et al., 1994). Correspondingly, ectopic expression of *Scr* in embryos missing *tsh* function results in salivary glands forming in PS0 through PS13 and ectopic expression of *Scr* in embryos missing *Abd-B* function results in salivary glands forming in anterior regions PS0–2 and in the most posterior parasegment PS14.

Salivary gland cell fates are normally limited to only the ventral ectodermal cells of PS2 by the dorsal patterning gene *dpp* (Fig. 9). In embryos mutant for *dpp* or any of the downstream components of Dpp signaling, salivary gland gene expression occurs in all ectodermal cells expressing *Scr* (Panzer et al., 1992; Henderson et al., 1999). Conversely, salivary glands fail to form when *dpp* is expressed throughout the embryo using heat shock-driven expression (K. D. Henderson and D. J. Andrew, unpublished). Thus, like the trachea, the salivary gland is

specified by the combined activities of the global anterior–posterior and dorsal–ventral patterning genes.

The secretory versus duct cell fate is controlled by another global patterning signal, EGF (Fig. 9; Kuo et al., 1996). *rhomboid* (*rho*), which encodes an enzyme required for processing of the EGF ligand Spitz, is expressed in cells along the ventral midline (Klamt, 2002). Secreted Spitz ligand then diffuses a few cell diameters from the midline to specify the most ventral ectodermal cell fates, which in the case of the salivary gland is salivary duct. In embryos missing function of *rho*, duct cells appear to be converted to secretory cells based on cell size and expression of secretory cell-specific genes (Kuo et al., 1996). Similarly, the expression of a dominant negative form of the EGF receptor or the loss of *pointed*, which encodes a transcription factor that mediates EGF signaling, results in duct cells taking on the appearance and gene expression patterns of secretory cells (Kuo et al., 1996; B. Kerman and D. J. Andrew, unpublished). Although duct gene expression requires EGF signaling, its role appears to be indirect and through the repression of secretory genes, which in turn repress expression of duct genes (Kuo et al., 1996; Haberman et al., 2003). For example, EGF signaling blocks expression of *fork head* (*fkh*), a secretory cell-specific transcription factor. In *fkh* mutants, at least three independently expressed duct-specific genes, including *trh*, *dead ringer* (*dri*), and *Serrate* (*Ser*) are expressed throughout the gland. Thus, in the most ventral cells of the salivary gland primordium, EGF signaling specifies salivary duct, in large part by repressing expression of secretory-specific transcription factor genes.

Notch signaling is required to establish a third cell type within the salivary gland, the imaginal ring cells, which are nestled between the individual duct and secretory tubes (Fig. 9). These are the precursors to the adult salivary gland, which forms during metamorphosis. Although the imaginal ring cells have no function in larvae, these diploid cells, which remain mitotically active until metamorphosis, are specified during embryogenesis. Duct precursors express *Ser* before secretory cell invagination and continuing through the remainder of embryogenesis (Kuo et al., 1996; Haberman et al., 2003). *Ser* encodes one of the two known *Drosophila* ligands for Notch (Fleming et al., 1990; Thomas et al., 1991), and secretory cells transiently express higher levels of *Notch* transcripts just before invagination (Kidd et al., 1989). In *Ser* mutants, the imaginal ring cells are absent, based both on the loss of marker gene expression in late embryos and on the absence of a diploid cell population between the duct and secretory cells in second instar larvae (Haberman et al., 2003). Thus, given the timing of *Ser* and *Notch* expression and the position of the imaginal cells relative to duct and secretory cells, it is likely that the imaginal cells derive from the subset of cells that directly contact the duct precursors before secretory tube invagination (Fig. 9). Imaginal precursors to the adult tracheal system are also established during embryogenesis and originate from the SBs near the junctions with the TC of metameres Tr2–Tr9, and from scattered sites in Tr1 and Tr2 (Manning and Krasnow, 1993). Although markers for the tracheal precursors are expressed as early as embryonic stage 13, the signaling pathways required to establish this cell population remain unknown.

Scr and its cofactors Hth and Exd are essential for salivary gland formation; the expression of these factors disappears early, however, before invagination of the secretory tubes (Henderson and Andrew, 2000). Genetic studies indicate that the Scr/Exd/Hth complex represses *hth* expression in the salivary gland (Fig. 10). As Hth is required for the nuclear accumulation of Exd, transcriptional repression of *hth* results in a loss of nuclear Exd. The coincident loss of *Scr* transcripts in the salivary gland at this stage indicates that Scr and/or its cofactors are required to maintain *Scr* expression; the finding that *Scr* transcripts are stable in a null *Scr* EMS mutant, however, suggests that only Exd and Hth are required to maintain *Scr* expression. Consistent with this model, engineered expression of *hth* in the late salivary gland results in persistent expression of *Scr* in this tissue. The early loss of Scr and its cofactors in the salivary gland indicates that the genes that specify the salivary gland cell fate can neither be directly

responsible for maintaining cell fate nor for the terminal differentiation of this tissue. Thus, the downstream target genes whose expression is regulated by Scr, Exd and Hth are likely to have more direct roles in salivary gland development. In fact, many of the earliest-expressed salivary gland genes encode transcription factors whose expression in the salivary gland persists throughout the lifespan of this organ.

As observed in the trachea, there are several secretory gland genes whose initial expression is both early and independent of other secretory gland genes. Among these genes are several encoding transcription factors, including the winged-helix DNA binding protein encoded by *fkh* (Weigel et al., 1989), the leucine zipper protein encoded by *CrebA* (Smolik et al., 1992), the SP1-like transcription factor encoded by *huckebein* (*hkb*) (Bronner et al., 1994), and the bHLH protein encoded by *sage* (Moore et al., 2000; Chandrasekaran and Beckendorf, 2003). Also among the early salivary gland genes is *pasilla*, which encodes an RNA binding protein whose mammalian homologs have been implicated in splicing (Seshaiah et al., 2001). As observed with the early tracheal genes, expression of the early salivary gland genes becomes dependent either on themselves and/or on other salivary gland transcription factors at later stages. Specifically, *fkh* is required to maintain its own expression (Zhou et al., 2001) as well as the expression of both *CrebA* and *sage* (Henderson, 2000; Myat and Andrew, 2000a; Abrams and Andrew, 2005; Abrams et al., 2006). Maintenance of high-level expression of *sage* is also partly autoregulatory (Abrams et al., 2006). *hkb*, which is expressed only transiently in the salivary gland primordium, requires down-regulation by *hairy*, which encodes a transcriptional repressor (Myat and Andrew, 2002).

Invagination of the salivary tubes

As with the trachea, the secretory cells of the salivary gland invaginate by an apical constriction mechanism, wherein the randomly positioned nuclei translocate to a basal position and the apical domains constrict to create wedge-shaped cells. Using histological sections and markers for subsets of secretory cells, we and others have found that invagination begins with cells located in the dorsal–posterior quadrant of the salivary gland and then spreads to include ventral posterior cells (Myat and Andrew, 2000b; Zhou et al., 2001). Subsequently, a second site of apical constriction and invagination initiates in the dorsal anterior quadrant. Finally, the ventral anterior cells are the last of the secretory cells to change shape and invaginate. Because only a limited number of cells are changing shape and invaginating at a given time, the secretory glands internalize as a growing tube. In contrast, the simultaneous invagination of multiple cells along the midline of each tracheal metamere causes the trachea to internalize as a dorsal–ventrally elongated pouch from which the primary branches arise. Thus, the pattern of internalization appears to largely dictate initial organ shape.

Invagination of the secretory primordium requires *fkh* (Weigel et al., 1989). Histological sections and transmission electron micrographs (TEM) of *fkh* mutant salivary glands show extensive evidence of apoptotic cell death, suggesting the possibility that secretory cells fail to internalize simply because the cells are dying (Myat and Andrew, 2000a). Indeed, these findings are corroborated by high-level expression of *reaper* (*rpr*) and *head involution defective* (*hid*), two well-characterized activators of cell death. However, when the secretory cells of *fkh* mutants are rescued from death by crossing in a deficiency removing *rpr*, *hid*, and other pro-apoptotic genes, they still fail to internalize, supporting roles for *fkh* in both secretory cell survival and invagination. Interestingly, although the secretory cell nuclei in the cell death-rescued *fkh* mutants translocate to a basal position with the same dynamics as in wild-type secretory cells, the cells fail to undergo apical constriction. This observation reveals that nuclear movement and apical constriction can be uncoupled and suggests that Fkh is likely to affect invagination through effects on the actin–myosin cytoskeleton. The transcriptional targets that link Fkh function to apical constriction await discovery; however, at least one target

gene that may contribute to its anti-apoptotic activity is known. The transcription factor encoded by *senseless* (*sens*) requires *fkh* for its expression in the secretory cells and loss of *sens* results in extensive secretory cell death (Chandrasekaran and Beckendorf, 2003). The observations that expression of wild-type *sens* in *fkh* mutants does not rescue secretory cell viability and that expression of *rpr* and *hid* is observed later in *sens* mutants than in *fkh* mutants, however, suggest that Fkh also controls other regulators of apoptosis in the secretory cells, perhaps even directly repressing expression of *rpr* and *hid*. Interestingly, Fkh regulates expression of more than a third of the known salivary gland genes, either directly or indirectly (E. Grevengoed and D. J. Andrew, unpublished data), indicating that Fkh is likely to have many roles in this tissue.

Recent studies have revealed a link between regulation of the endocycles (the DNA endoreduplication process that creates polytene chromosomes) and secretory cell invagination (Chandrasekaran and Beckendorf, 2005). Normally, the salivary gland begins its endocycles immediately following invagination in cells at the distal end of the tube, with a wave of endocycles subsequently moving proximally through the gland cells. Mutations in the non-receptor tyrosine kinase *Tec29* result in a subset of cells failing to internalize and remaining on the surface of the embryo. These mutants also show defects in the synchronized wave of endoreduplication, with cells still on the surface undergoing endoreduplication prematurely. Mutations in the actin-binding protein encoded by *chicadee* (*chic*) as well as in other non-receptor tyrosine kinase genes (*Src42A* and *Src64*) enhance the invagination defects of *Tec29* mutants and, in the two cases examined, *chic* and *Src42A*, the enhanced invagination defects correlate with increased premature endoreduplication. Moreover, the defects in salivary gland invagination in the *Tec29* mutants were suppressed in most embryos by blocking endoreduplication by expression of *cyclin E*. Thus, it appears that the primary defect in *Tec29* mutant salivary gland invagination is likely the premature initiation of endoreduplication. Similar defects in tissue morphogenesis have been observed with mutations that allow premature mitotic cycles during *Drosophila* ventral furrow formation (Grosshans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000) and during *Xenopus* paraxial mesoderm convergent extension (Leise and Mueller, 2004). Thus, cell division and morphogenesis may share key cytoskeletal components. Indeed, the abnormal accumulation of monomeric actin observed in the salivary glands of *Tec29* mutants, as well as the enhancement and suppression of the *Tec29* phenotypes with mutations in proteins that affect actin polymerization, suggest the specific involvement of the actin cytoskeleton in both processes (Chandrasekaran and Beckendorf, 2005).

Salivary tube elongation

Normal secretory cell invagination and subsequent tube elongation require apical membrane growth beginning very early, when the secretory primordium first internalizes. Proper regulation of *hkb*, which is dynamically expressed in the secretory cells, is key to this process (Myat and Andrew, 2000b, 2002). *hkb* encodes a transcription factor first expressed in a broad posterior domain within the placode, in the cells that internalize first (Myat and Andrew, 2000b). Slightly later, *hkb* mRNA is detected in a dorsal anterior domain corresponding to the second site at which apical constriction and invagination are observed. Only very low levels of *hkb* transcripts are ever detected in the ventral-most cells of the secretory tube, which are the last to internalize. *hkb* transcripts are normally quite transient, disappearing before internalization of any of the secretory cells. In *hkb* mutants, the secretory cells undergo apical constriction with the same dynamics as in wild-type glands (Myat and Andrew, 2000b). The cells do not, however, internalize in the normal pattern, perhaps because the limited apical membrane diminishes the ability of cells to invaginate locally. Correspondingly, although all of the secretory cells are eventually internalized in *hkb* mutants, the cells have smaller apical domains and less apical membrane than wild type, as assayed by confocal sections of salivary

glands stained with an antibody to the apical domain protein Crumb (Crb) and by direct measurements of apical membrane length from sagittal TEM sections (Myat and Andrew, 2002). As a consequence, *hkb* mutants form smaller, nearly spherical glands with less luminal space than the elongated secretory tubes of wild-type glands. Overexpression of *hkb*, using either a secretory-specific Gal4 driver or via mutations in *hairy*, a bHLH transcription factor that normally represses *hkb* expression, leads to increased apical surface area and overt defects in tube morphology. Gal4-driven overexpression of *hkb* results in the formation of large spherical glands, with increased overall luminal space. Certain alleles of *hairy* cause prolonged expression of *hkb* in its normal domain; such mutations most frequently result in branched tubes, with simultaneous internalization at the dorsal–posterior primary site and at the dorsal–anterior secondary site. Other alleles of *hairy* result in high levels of *hkb* expression throughout most of the placode; these alleles typically cause the large spherical glands observed with the engineered overexpression of *hkb*. Thus, the pattern and duration of *hkb* expression is critical to final tube morphology.

hkb affects tube morphology largely through its effects on two downstream target genes, *klarsicht* (*klar*) and *crb* (Fig. 11; Myat and Andrew, 2002). Klar is a pioneer protein thought to regulate organelle transport toward the minus ends of microtubules through interactions with the motor protein dynein (Welte et al., 1998; Mosley-Bishop et al., 1999). The minus ends of microtubules are oriented toward the apical surface in the salivary glands (Myat and Andrew, 2002). *klar* is transiently up-regulated in the cells of the salivary gland as they internalize, consistent with the membrane expansion that normally occurs at the stage. This transient expression of *klar* requires *hkb* and, in *hairy* mutants where *hkb* persists, *klar* expression persists longer than in wild type. Overexpression of *hkb* alone is not sufficient, however, to cause overexpression of *klar*, indicating that *hairy* may independently repress both *hkb* and *klar* expression. Compellingly, null mutations in *klar* lead to shorter secretory tubes with smaller apical surfaces, a phenotype very similar to that seen in *hkb* loss-of-function mutants, and overexpression of *klar* leads to tube expansion, very similar to that seen in *hairy* loss-of-function and *hkb* overexpression. Finally, *klar* is likely to be a major target of Hkb in this process as expression of *klar* in *hkb* mutants can partially rescue the phenotype from loss of *hkb* (Myat and Andrew, 2002).

hkb also affects the levels of *crb* mRNA accumulation in the secretory cells, although this effect is relatively minor. Instead, *hkb* and *klar* appear to be required for robust apical Crb protein levels. In wild-type embryos in which expression of *crb* mRNA is driven at high levels throughout the salivary gland, only moderately higher levels of Crb protein are observed and accumulation is limited to regions where *hkb* levels are high. However, when *crb* is co-expressed with *hkb* or *klar*, high levels of Crb protein are observed throughout the gland, suggesting that Hkb and Klar are required for robust apical Crb protein levels, affecting transport and/or stability of Crb protein. Interestingly, *hkb* overexpression on its own results in the fusion of all secretory vesicles with the apical membrane, based on confocal immunostaining of luminal markers and on TEMs. Altogether, these observations support a model wherein Hkb works through Klar to mediate the transport of Crb-containing vesicles to the apical surface, resulting in the membrane expansion required for tube elongation (Myat and Andrew, 2002).

Salivary gland migration

Through the series of regulated cell shape changes detailed above, the gland primordia are internalized to form dorsally oriented secretory tubes (Fig. 7). By midstage 12, the distal cells of the secretory tubes contact a layer of cells that will ultimately form the inner layer of gut muscle, known as the circular VM (cVM), and initiate posterior migration (Bradley et al., 2003). During this posterior migration, the salivary gland tip cells extend lamellipodial

protrusions like those seen in actively migrating tissue culture cells. Throughout most of this migration, the dorsal secretory cells remain in direct contact with the cVM and the ventral salivary gland cells contact two other cell types, the somatic muscle (SM) and FB precursors (Fig. 12; Vining et al., 2005). During late embryonic stage 13, the ingression of the mesodermal precursors that form the outer muscle layer of the gut, known as the longitudinal VM (IVM), further affect salivary gland positioning (Fig. 12). IVM cells derive from posterior mesoderm and migrate in two rows of cells to spread anteriorly over the cVM. The migrating IVM cells appear to separate the salivary glands from the cVM. The salivary glands subsequently shift anteriorly with respect to the gut, with contact ultimately maintained only between the distal ends of the secretory tubes and the tubular protrusions that arise from the most anterior part of the gut, known as the GC. Interestingly, the loss of intimate contact between the salivary gland and the cVM that normally occurs during late embryonic stage 13 requires the IVM (Vining et al., 2005). In mutants where the IVM fails to migrate, the salivary glands remain tightly attached to the anterior gut and become severely elongated at late embryonic stages (up to twice their normal length), suggesting that in the absence of an invading IVM, salivary gland-cVM contact is never severed. Whether the IVM normally disrupts this contact by its mere physical presence or by the secretion of enzymes that disrupt integrin-mediated cell adhesion remains to be determined. Whereas the tips of the salivary glands normally contact the GC, the lateral edges of the salivary glands maintain direct contact with a specific muscle in the third thoracic segment and the FB spreads over the distal portion of the salivary glands, establishing an intimate association that persists until metamorphosis. Although the glands do not directly contact the CNS, they are also in close proximity to this tissue, which also plays a role in guidance, during posterior migration.

The salivary glands migrate dorsally off course in embryos mutant for *heartless* (*htl*), which encodes the second *Drosophila* FGF receptor (the other being Btl), or mutant for *hbr*, a downstream effector of FGF signaling. Neither *htl* nor *hbr* are expressed in the salivary gland; instead they are expressed in the mesoderm in which they are required for migration. Among the mesodermal tissues most affected in these mutants are the dorsal vessel (heart) and VM, the tissues that migrate furthest. In both *htl* and *hbr* mutants, too few cells migrate far enough to populate the VM, leaving large gaps in what should be a contiguous monolayer. Salivary gland migration in *htl* and *hbr* mutants correlates with the position and integrity of the remaining VM, with the glands often migrating dorsally when they encounter gaps. The *htl* salivary gland migration defect is completely rescued by the mesodermal expression of *htl* using the Gal4-UAS system, indicating that *htl* is not required in the salivary gland for the cells to migrate normally. The dorsal mismigration phenotype is also not specific to Htl signaling; mutations in other pathways that affect VM integrity also affect migration, including mutations in the Dpp receptor gene *tkv* and mutations in *tinman*, a gene required to specify dorsal mesoderm fates (Bradley et al., 2003).

The VM is known to be a substrate for the migration of two tissues other than the salivary gland: the endoderm and the VB of the trachea (Martin-Bermudo et al., 1999; Boube et al., 2001). Both tracheal and endodermal cells require integrins for their migration along the VM substratum. α -PS1 integrin expression in the VB of the trachea and α -PS2 expression in the VM are required for proper migration of the VB. Similarly, α -PS1 and α -PS3 are required in endodermal cells and α -PS2 is required in the VM cells for endodermal migration. α -PS1 and α -PS3 are expressed in the salivary gland prior to and during its posterior migration in overlapping yet distinct temporal patterns (Zusman et al., 1993; Bradley et al., 2003). Mutations in either the α -PS1 or α -PS2 subunit genes lead to identical salivary gland defects. The cells reach the wild-type VM turning point yet fail to extend lamellipodia and fail to migrate posteriorly. The remaining secretory cells continue to internalize and the tube elongates. The distal tips of the glands remain stationary, however, and U-shaped tubes typically result. Mutations in the gene encoding α -1,2 laminin, which is expressed in the VM, give identical

salivary gland defects (C. A. Comeaux and D. J. Andrew, unpublished data). Laminins are secreted ECM proteins that bind integrins (Brown et al., 2000). The salivary gland phenotypes of integrin and laminin mutants suggest that although migration is required to position the salivary gland properly, overall tube dimensions (length and diameter) are independent of migration. Further, integrin signaling is not required for internalization of the salivary gland primordia as internalization is normal in embryos mutant for α -PS1, α -PS2, and α -PS3, either alone or in combination (Bradley et al., 2003). Similarly, salivary glands in embryos lacking both the maternal and zygotic contributions of β -PS, the partner for all non-gut α -subunits, have no defects in internalization or tube elongation though they have similar migration defects. In summary, the defects associated with the loss of integrin signaling suggest that, in the salivary gland, cell migration is not required for the cells to internalize and form elongated tubes. Migration is essential, however, for the final correct positioning of this organ.

The above studies on the role of the VM and integrin signaling in salivary gland positioning indicate that the VM acts as both a barrier to continued dorsal ingression as well as functioning as an appropriate substratum for salivary gland movement (Bradley et al., 2003). Additional spatial cues are likely to control the direction of salivary gland migration along the VM; the identity of most of these cues, however, remains to be discovered. Although genetic evidence suggests that signals from the VM, FB, SM, and CNS contribute to gland positioning (Bradley et al., 2003; Vining et al., 2005), only two signaling pathways have been specifically implicated: Netrin and Slit (Kolesnikov and Beckendorf, 2005). Netrins are expressed in the VM and CNS and appear to act as weak or partially redundant chemoattractants for the salivary glands. In embryos deficient for both *Drosophila* netrins, *netrinA* and *netrinB*, the distal ends of the mutant glands curve either laterally (23%) or medially (9%). Expression of either *netrinA* or *netrinB* in either the VM or CNS can largely rescue the mismigration observed with the netrin deficiency, although *netrin B* seems to be more effective. Loss-of-function mutations in the Netrin receptor that functions in chemoattraction, encoded by *frazzled* (*fra*), have only minor effects on migration, with 4% curving laterally and 2% curving medially. However, *fra* mutations do affect whether integrin mutant salivary glands buckle laterally or medially, introducing a strong lateral bias to buckling. Although the Netrin receptor that functions in chemorepulsion, encoded by *unc-5*, is not normally expressed in the salivary glands, its forced expression in the salivary glands results in highly penetrant lateral mismigration. Slit, which is expressed in the CNS midline, appears to act as a strong chemorepellent. In *slit* loss-of-function mutants, 90% of glands mismigrate medially toward the CNS (Kolesnikov and Beckendorf, 2005). Similar defects are seen with loss-of-function mutations in *robo1* and *robo2*, the genes encoding the Slit receptors expressed in the salivary gland, with mutations in both genes showing a much higher penetrance than mutations in either gene alone. Thus, the balance between Netrin and Slit signaling seems key to maintaining salivary gland migration parallel to the CNS, with one signaling pathway acting to attract and the other to repulse the salivary gland. Although the double mutants have phenotypes characteristic of slit mutants, the presence of *netrin* mutations reduces the penetrance of mismigration in *slit* mutants from 90% to 50%. Thus, as in the trachea, balancing chemoattraction and chemorepulsion is key to properly directed salivary gland migration.

Secretory activity in the salivary gland

The salivary gland is the largest secretory organ in *Drosophila*, making it an ideal system for learning not only how tubes are formed but also how secretory cells become specialized. The early secretory pathway, which includes the machinery required for endoplasmic reticulum (ER) targeting and translocation and transport between the ER and Golgi, has been well studied in yeast and in mammalian tissue culture cells. Clear homologs to most of the genes encoding the secretory pathway proteins exist in *Drosophila*. Interestingly, all of the 34 secretory pathway component genes (SPCGs) that have been examined are expressed to very high levels

in the secretory cells of the embryonic salivary gland, beginning shortly after the cells are first specified and continuing throughout embryogenesis (Abrams and Andrew, 2005). This finding suggests that salivary gland cells prepare for high-level secretory activity in large part by the transcriptional up-regulation of the protein components of the secretory machinery. The robust expression of the SPCGs requires two early salivary gland transcription factor genes: *CrebA* is required throughout embryogenesis and *fkh* is required only during later embryonic stages. It is likely that Fkh plays primarily an indirect role in SPCG expression, however, as Fkh is directly required to maintain *CrebA* expression in the salivary gland (after the disappearance of the Scr/Hth/Exd complex) (Fig. 11). Although it is likely that *CrebA* directly regulates the SPCGs based on the discovery of a conserved Creb-like binding site within 2 kb upstream of 32 of the 34 SPCGs, the critical tests for direct control have not been done (Abrams and Andrew, 2005). *CrebA* also controls the moderate level of expression of these genes in the epidermis, where secretion is important for cuticle formation. *CrebA* mutants have profound defects in the larval cuticle, further supporting a role for *CrebA* regulation of secretory activity. Interestingly, loss-of-function mutations in individual SPCGs cause the same cuticle defects, suggesting that the *CrebA* cuticle defects are entirely due to loss of secretory activity in the epidermis (Andrew et al., 1997; Abrams and Andrew, 2005). Secretory defects are also seen in the *dCrebA* mutant salivary glands, but they are only obvious at late embryonic stages, presumably because *CrebA* and the SPCG transcripts are provided maternally. Staining with an antibody to a salivary gland ER protein as well as with antisera to a secreted luminal protein is significantly reduced, suggesting a possible reduction in the size and function of secretory compartments in *CrebA* mutant salivary gland cells (Abrams and Andrew, 2005). The coordinate regulation of all of the SPCGs by a single transcription factor is an exciting finding that suggests an easy mechanism for controlling levels of secretory activity in different cell types. The tissues that require only low levels of secretory function would express low levels of *CrebA*, whereas tissues that require high levels of secretory function, like the salivary gland, would express high levels of *CrebA*. Whether such a simple mechanism is operative in other organisms with specialized secretory tissues awaits discovery.

Fkh, Sage and tube maintenance

Fkh is required to prevent the inappropriate expression of duct genes in secretory cells (Kuo et al., 1996; Haberman et al., 2003), to block secretory gland apoptosis (Myat and Andrew, 2000b), to internalize secretory gland precursors (Myat and Andrew, 2000b) and to maintain expression of itself and *CrebA* in the secretory cells (Fig. 11; Zhou et al., 2001; Abrams and Andrew, 2005). Two additional genes were identified in a preliminary screen for Fkh targets. These closely linked genes, *PH4aSG1* and *PH4aSG2*, which are expressed only in the salivary gland, encode the α -subunits of resident ER enzyme complexes that hydroxylate proline residues in collagen and other secreted proteins (Abrams and Andrew, 2002). Although both genes absolutely require *fkh* for their salivary gland expression, *in vitro* DNA binding studies in combination with *in vivo* expression assays of wild-type and mutant enhancers reveal that *PH4aSG1* and *PH4aSG2* regulation is complex and involves at least one additional transcription factor (Abrams et al., 2006). Fkh cooperates with the salivary gland-specific bHLH transcription factor Sage to directly regulate expression of *PH4aSG2*, as well as *sage* itself, and to indirectly regulate expression of *PH4aSG1*, likely through an additional unknown transcription factor (Fig. 11). Although the mammalian enzymes most related to *PH4aSG1* and *PH4aSG2* are best known for their roles in modifying collagens, which are secreted fibrillar proteins that are the major component of the ECM, it is unlikely that *PH4aSG1* or *PH4aSG2* modify collagen as the *Drosophila* collagen genes are not expressed in the salivary glands. Instead, genetic studies suggest that these enzymes modify apically-secreted proteins that normally form a fibrillar matrix in the lumen of the salivary gland (Abrams et al., 2006). Whereas wild-type salivary gland lumina from stage 15 and later are filled with a fibrillar matrix, the salivary glands from embryos homozygous for a deficiency that removes both

PH4aSG1 and *PH4aSG2* show significantly reduced secretory content with increased electron density (Fig. 13). The altered secretory content in the deficiency embryos correlates with regions of secretory tube dilation and constriction with intermittent tube closure, a phenotype reminiscent of the tracheal phenotypes observed with mutations in chitin synthesis and/or modification genes (Araujo et al., 2005; Devine et al., 2005; Tønning et al., 2005; Tønning et al., 2006). The salivary tube dilation and constriction defects observed in the deficiency animals are rescued with salivary gland-specific expression of either *PH4aSG1* or *PH4aSG2*, specifically implicating these genes in ensuring uniform luminal diameter. These phenotypes suggest *PH4aSG1* and *PH4aSG2* may alter the character of secreted apical proteins in much the same way as the mammalian enzymes alter collagen to allow these apical proteins to form fibrillar structures that maintain an expanded network of ECM. This expanded network would serve both a blocking function to prevent tube closure as well as some regulatory function to prevent tube dilation. Similar loss of tube uniformity, with regions of dilation and constriction, was also seen with mutations in *pasilla*, a gene encoding a splicing factor. The loss of tube uniformity in *pasilla* mutants correlated with a significant decrease in the number and size of secretory vesicles and in lumen volume (Seshaiah et al., 2001). Altogether, the luminal irregularities observed in the tracheal chitin synthesis mutants and in the salivary gland with the loss of *PH4aSG1* and *PH4aSG2* or *pasilla* suggest that an apical extracellular matrix may be a common critical component for maintaining tube size uniformity in other cellular tubes, not only in *Drosophila* but also in higher organisms such as ourselves.

Concluding remarks

From studies of the *Drosophila* trachea and salivary gland, it is clear that organs are specified by the combined activities of the global patterning genes: the dorsal–ventral, segmentation and segment identity genes, which include classical homeotic (or Hox) genes as well as other domain-specific genes such as *salm* and *tsh*. Combinatorial expression of the global patterning genes defines unique positions within the developing embryo where tissue-specific gene expression is induced. In both the trachea and salivary gland, multiple early tissue-specific genes are activated, which for the most part encode transcription factors whose expression persists. The early tissue-specific transcription factor genes, in turn, maintain their own and/or each other's expression and regulate the activities of the downstream genes more directly involved in tissue morphogenesis and specialization. From these and related studies on the mesoderm, it is clear that single “organ-specifying” genes do not exist in *Drosophila*. Although there may be genes that play major roles in the development of a specific organ, such as *trh* in the trachea and *fkh* in the salivary gland, these genes cannot on their own induce formation of either tissue, nor are they required for all tissue-specific gene expression. For example, although Fkh plays many key roles in salivary gland development, only about one third of the over 200 genes that have been tested require Fkh for their salivary gland expression. Fkh is required not to initiate but to maintain the expression of more than half of these genes, suggesting that, as with the SPCGs, regulation may be indirect and through other Fkh-dependent transcription factors. Thus, one should view with caution reports that Fkh homologs in other organisms function as organ-specifying genes (Gaudet and Mango, 2002; Lee et al., 2005). Such factors may affect the subset of organ markers being assayed or may be required for cell-type specific viability, resulting in the absence of an organ at a given developmental stage.

Studies of the *Drosophila* trachea and salivary gland reveal that cell shape change, rearrangement and migration underlie tube morphogenesis and that these processes are linked to changes in the cytoskeleton. However, many of the connections between the transcription factors that control morphogenesis and the consequent downstream morphological changes have not been made. For example, the intermediate molecules that function downstream of both Trh and Fkh to control actin dynamics and drive invagination are largely unknown. Similarly, the molecular changes that allow epithelial cells to slide past one another to form

type II tracheal tubes from type I tracheal tubes remain to be characterized. Nonetheless, great progress has been made in describing the cellular changes that transform a sheet of polarized epithelial cells into either the large simple tubes of the salivary gland, which specialize in secretion, or the multiple tube types that comprise the intricate interconnected network of the trachea, which specialize in oxygen transport.

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Fig. 1.

Tracheal morphogenesis in *Drosophila* embryos is largely complete by the end of embryogenesis. (A) A lateral view of a stage 10 embryo stained with antiserum to Ventral veins lacking (Vvl) reveals the ten tracheal placodes (Tr1–Tr10), which are present on both sides of the embryo. Lower levels of Vvl staining are also visible in corresponding regions in the two segments anterior to Tr1 and in one segment posterior to Tr10. (B) A lateral view of a stage 12 embryo stained with an antibody to the apical membrane protein Crumbs (Crb) reveals the primary branches of the internalized trachea. (C) A close up of tracheal metamere 4 (tr4) from a stage 11 embryo stained with Crb shows the different primary branches. (D) A close up of tracheal metamere 4 (Tr4) from a stage 12 embryo stained with Crb shows the different primary

branches and their proximity to the branches from the adjacent metameres, Tr3 and Tr5. (E) A lateral view of a stage 15 embryo stained with the luminal marker protein 2A12 shows the trachea after the dorsal trunk (DT) has fused. DB, dorsal branch; DTa, DT anterior; DTp, DT posterior; GB, ganglionic branch, LT, lateral trunk; LTa, LT anterior; LTp, LT posterior; TC, transverse connectives; Tr1–10, tracheal metameres 1–10; VB, visceral branch.

Fig. 2.

A cartoon representation shows the types of tubes that form in the *Drosophila* trachea, indicating which type of tube is found in each branch and the approximate diameter of the tracheal lumen. In the cartoons, the lumen is shown in pink, with the apical membrane in red. The adherens junctions are indicated in black with the remaining lateral membrane indicated in white. The basal surface is dark blue and the cytoplasm is lighter blue. Abbreviations are as described in the legend of Fig. 1.

Fig. 3.

Specification of the *Drosophila* tracheal primordia is controlled by global patterning genes. The Salm gene limits tracheal formation to the trunk region of the embryo, from the second thoracic (T2) segment, where the first tracheal metamere forms (Tr1), through abdominal segment 8 (A8), where the last tracheal metamere forms (Tr10). Wg signaling limits tracheal formation to a subset of cells within a limited anterior-posterior portion of each segment. Dpp blocks tracheal formation in the dorsal ectoderm, whereas the ventral patterning genes are predicted to block tracheal formation in the ventral ectoderm.

Fig. 4.

Several signaling pathways have been implicated in tracheal migration, including endothelial growth factor (EGF), fibroblast growth factor (FGF), Dpp, and Wg. EGF signaling is localized by the expression of *rho*, which encodes a protease essential for the processing of the EGF ligand. EGF signaling is required for the complete internalization of the tracheal primordia. FGF signaling is limited by the expression of the ligand Bnl in the tissues toward which the tracheal branches migrate. FGF signaling is required for all migration. Dpp signaling is limited by the expression of the ligand Dpp in cells dorsal and ventral to the trachea. Dpp signaling is required for migration of the dorsal branch, lateral trunk, and ganglionic branch. Wg signaling is limited by the localization of the Wg signal in ectodermal cells near the tracheal primordia. Wg signaling is required to maintain *salm* expression in the dorsal trunk (DT) and to prevent the DT from migrating with the visceral branch. Green indicates the spatial position of the signal relative to the trachea. Blue indicates the tracheal primordia in the drawing along the top. Blue filled circles indicate that the cells have migrated to their correct positions in wild type and in the various signaling pathway mutants. White circles indicate that cells are missing in particular branches in the mutants.

Fig. 5.

The formation of type I from type II tubes requires the formation of autocellular junctions from intercellular junctions. Formation of type II tubes requires that paired cells within the type I tube reach around and contact themselves at each end to initiate the formation of an autocellular junction. The autocellular junctions then zip up from the sites of initiation as the two cells slide past each other. Zipping terminates at the point where the two cells contact each other only at their ends. Apical proteins such as Pio and Dp are important in preventing the zippering from continuing to the point where the two cells would separate.

Fig. 6.

Different types of specialized cells form within each tracheal metamere. **(A)** This diagram shows the different cell types that form within a typical tracheal metamere; the cell types include stalk cells (type I and type II tubes, blue circles), fusion cells (type III tubes, red circles) and terminal cells (type IV tubes, green circles). **(B)** Cartoon diagram of branch fusion, showing as an example fusion of the dorsal trunk posterior and dorsal trunk anterior. The fusion cells (red) seek each other out and make contact along their basal surfaces. During initiation, the fusion cells localize E-cadherin (light blue) and other cytoskeletal proteins (yellow) typical of adherens junctions to the basal surface at the site of contact between the fusion cells. Subsequently, an actin-rich structure bridging between the apical surfaces of the two fusion cells is formed. This actin-rich structure is proposed to either physically bring the two apical surfaces together for fusion or to act as a scaffold for the accumulation of apical membrane vesicles, which will eventually fuse to create the apical surfaces connecting the two tracheal metameres. After fusion, a continuous adherens junction containing proteins such as E-cadherin is found near the apical surfaces of the two fusion cells.

Fig. 7.

CrebA is expressed to very high levels in the secretory cells of the salivary gland. (A) and (E) Staining of CrebA, and other early salivary gland proteins, is first detectable during embryonic stage 10, when the cells are on the surface of the embryo. (B) Beginning with cells in a dorsal-posterior domain of the placode, the cells change shape and internalize during embryonic stage 11. (C) By early stage 12, the most distal cells in the secretory tube have contacted the layer of cells known as the visceral mesoderm (VM) and have initiated posterior migration. (D) As new parts of the tube contact the VM during later stage 12, these cells also turn and migrate posteriorly. (F) The secretory tubes are fully internalized by embryonic stage 13. (G) The distal ends of the tube contact anterior portions of the midgut, which will form the gastric caeca (GC), whereas the sides of the tube make direct contact with the somatic muscle (SM) underlying the epidermis. (H) The secretory tubes maintain contact with the GC and SM at later embryonic stages.

Fig. 8.

The duct forms from the most ventral cells of the salivary gland primordia and internalizes to form tubes after the secretory tubes have formed. **(A–D)** A P-element insertion in the *trh* gene drives β -gal expression throughout the salivary gland primordia in both duct and secretory (sec) cells. **(A)** Expression of the insertion is first visible when the salivary gland precursors are still on the surface of the embryo. **(B)** The more lateral secretory cells internalize to form tubes during embryonic stages 11 and 12. **(C)** The individual duct (id) tubes, which form from the more posterior duct primordia, form during embryonic stage 13 after the secretory cells have internalized and formed tubes. **(D)** The common duct (cd) tube forms from more anterior duct primordia and forms during embryonic stage 14.

Fig. 9.

Global patterning genes specify the salivary gland and cell types within the salivary gland. (A) The homeotic gene *Scr* is expressed in parasegment 2, where salivary glands normally form. Salivary glands form from ventral *Scr*-expressing ectodermal cells that do not express *Dpp*. Ectopic expression of *Scr* can result in additional salivary glands forming in segments that do not also express either *tsh* (in PS3–13) or *Abd-B* (PS14). Within the gland primordia, epithelial growth factor (EGF) signaling along the ventral midline specifies salivary duct by shutting off expression of secretory genes, such as *fkh*. In turn, *Fkh* blocks expression of duct-specific genes, and with other secretory-specific proteins, promotes development of the secretory tubes. (B and C) The duct-specific protein *Ser* signals its lateral neighbors to form

imaginal ring cells, the precursors to the adult salivary gland. **(D)** In the fully formed embryonic salivary gland, the imaginal ring cells are located between the secretory tubes and the distal ends of the individual ducts.

(Adapted from Henderson and Andrew, 2000)

Fig. 10.

Genes that specify the salivary glands disappear early through a negative feedback loop. The Scr/Exd/Hth complex induces expression of several early-expressed transcription factors (*fkh*, *hkb*, *CrebA*, and *sage*, and a splicing factor, *pasilla*). The complex also represses expression of *hth*, either directly or indirectly. As Hth is required for nuclear entry of Exd, repression of *hth* results in a loss of Hth and nuclear Exd. As Hth and Exd maintain *Scr* expression, *Scr* also disappears in the early salivary gland.

Fig. 11.

Scr/Exd/Hth complex induces expression of several early transcription factor genes, including *sage*, *fkh*, *CrebA*, and *Hkb*. Some of these transcription factors regulate their own expression as well as the expression of other early transcription factor genes. They also regulate downstream factors involved in morphogenesis and physiological function. Fkh is required to maintain its own expression as well as the expression of *Sage* and *CrebA*. Fkh regulates unknown targets to mediate the cell shape changes required for secretory cells to internalize and form tubes. It also regulates genes required for secretory cells to survive. Sage works with Fkh to maintain its own expression and the expression of two downstream enzyme-encoding genes, *PH4αSG1* (indirectly) and *PH4αSG2* (directly). These enzymes are required to modify target proteins that form the luminal fibrillar matrix that keeps the lumen open and uniform. CrebA regulates expression of the secretory pathway component genes (SPCGs), which are expressed to high levels in the salivary gland. High-level expression of the SPCGs mediates the high level of secretory activity characteristic of this tissue. Hkb regulates *klar* and *crb* to facilitate the apical membrane expansion required for tube elongation. Black arrows indicate regulatory interactions that are known or likely to be primarily transcriptional. Black dashed arrows indicate interactions that are primarily post-transcriptional.

Fig. 12.

During its posterior migration, the secretory tube (sec; red from PH4 α SG1 staining) directly contacts a number of other cell types, including the circular VM (cVM; blue green from overlapping α -FasIII and α -Titin staining), longitudinal VM (IVM; blue from α -Titin staining), somatic mesoderm (SM; blue from α -Titin staining) and fat body (FB; dark, unstained areas between the SM clusters).

Fig. 13.

Quality and quantity of luminal secretions are altered in embryos missing *PH4aSG1* and *PH4aSG2*. (**A** and **B**) A thin sagittal section from a wild-type salivary gland (wt) reveals a relatively large and uniform luminal space filled with fibrillar material (asterisks). As the salivary tube is bent, the thin section catches the apical surface near the middle of the lumen; the bend in the tube also makes the lumen appear slightly narrower near the middle. (**C** and **D**) A thin sagittal section of a *Df(3R)Exel6216* salivary gland (Df) reveals irregular, small luminal spaces with an electron dense matrix (asterisks). The number, distribution and electron density of the secretory vesicles are not different in the wild type versus deficiency salivary glands, suggesting that the differences in luminal content arise post-secretion. The reduced volume and increased electron density of the salivary secretions from the deficiency glands correlate with the luminal abnormalities seen in confocal images, with regions of tube dilation, constriction and closure. The *Df(3R)Exel6216* luminal abnormalities are rescued by salivary gland specific expression of either *PH4aSG1* or *PH4aSG2*.