Development and Function of the *Drosophila* Tracheal System

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ABSTRACT The tracheal system of insects is a network of epithelial tubules that functions as a respiratory organ to supply oxygen to various target organs. Target-derived signaling inputs regulate stereotyped modes of cell specification, branching morphogenesis, and collective cell migration in the embryonic stage. In the postembryonic stages, the same set of signaling pathways controls highly plastic regulation of size increase and pattern elaboration during larval stages, and cell proliferation and reprograming during metamorphosis. Tracheal tube morphogenesis is also regulated by physicochemical interaction of the cell and apical extracellular matrix to regulate optimal geometry suitable for air flow. The trachea system senses both the external oxygen level and the metabolic activity of internal organs, and helps organismal adaptation to changes in environmental oxygen level. Cellular and molecular mechanisms underlying the high plasticity of tracheal development and physiology uncovered through research on *Drosophila* are discussed.

KEYWORDS trachea; development; tubulogenesis; branching; physiology; FlyBook

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INSECT locomotion is fueled by the efficient exchange of gases through a dedicated respiratory organ, the trachea, to power muscles for flight and walking, the gut for taking in nutrition, and the central nervous system for neural processing. The evolutionary innovation of the tracheal system helped terrestrial arthropods and annelids expand their habitat and morphological diversity. The tracheal system, which consists of a network of branched tubules extending throughout the body cavity, allows air flow through openings in the body wall, called spiracles, and delivers it to highly ramified tracheal termini, called tracheoles. The exchange of gasses through the large surface area of the tracheole lumen causes waste gas (carbon dioxide) to diffuse from the terminus to the spiracles and fresh air (oxygen) to diffuse in the opposite direction (Wigglesworth 1972).

Tracheal system development has been studied extensively in the fruit fly *Drosophila* (Whitten 1980; Manning and Krasnow 1993; Samakovlis *et al.* 1996a; Affolter and Caussinus 2008; Schottenfeld *et al.* 2010; Loganathan *et al.* 2016). During embryogenesis, the tracheal primordia appear as 10 pairs of tracheal placodes that invaginate into the body cavity while maintaining epithelial integrity, and undergo stereotyped branching and fusion processes to form a network of tubular epithelium (Figure 1). The largest tubule is the dorsal trunk, which serves as the major airway connecting the open posterior spiracle and the anterior spiracle, which opens in the second larval instar. The dorsal trunk is a multicellular tubule, with multiple cells contributing to the luminal surface except at the fusion point, which is formed by a pair of torus-shaped fusion cells surrounding the lumen. Tubules branching off the dorsal trunk are thinner and consist mostly of tracheal cells with autocellular junctions (unicellular tubules).

The last embryonic mitosis (cycle 16) occurs during the invagination of the tracheal placode cells into the body. In the larval stage, most tracheal cells grow by endocycling. In the pupal stage, polyploid larval tracheal cells undergo apoptotic cell death, and the remaining diploid tracheal cells proliferate and remodel the tracheal system into the adult pattern. In this review, we summarize findings from the past three decades of research on the genetic and developmental features of the *Drosophila* tracheal system.

Placode Specification and Branching

Tracheal development begins during stage 10 of embryogenesis, about 5 hr after egg laying, with the specification of 10 pairs of tracheal placodes located in the lateral part of the second thoracic (T2) to eighth abdominal (A8) segments (Campos-Ortega and Hartenstein 1997) (Figure 1A). The first metamere in T2 consists of ~80 cells, and the remaining metameres have ~50 cells. During the G2 interphase after mitosis 15 (Hartenstein and Campos-Ortega 1985), tracheal placodes appear as a flattening of the apical surface, and cells in the center of each placode begin apical constriction to form the tracheal pit (Isaac and Andrew 1996; Wilk *et al.* 1996; Nishimura *et al.* 2007; Kondo and Hayashi 2013) (Figure 1C). This slow invagination phase, which lasts ~30 min, is followed by a rapid invagination phase that internalizes all of the tracheal placode cells within ~60 min (Kondo and Hayashi 2013) (Figure 1C). Tracheal cells undergo cycle 16 mitosis during invagination and are then arrested in the G1 phase. Like most larval somatic cells, the majority of tracheal cells resume the cell cycle during the larval period, growing by endocycling and becoming polyploid (see section *Larval Trachea and Metamorphosis*).

Early research focused on identifying the determinants of tracheal cell fate and tracheal tissue-specific characteristics. The transcription factor Trachealess (Trh), which is expressed in all tracheal cells from the onset of tracheal placode specification through adulthood (Isaac and Andrew 1996; Wilk *et al.* 1996), regulates a multitude of other tracheal genes, most notably *breathless* (*btl*), which encodes the FGF receptor Breathless (Btl) (Ohshiro and Saigo 1997; Chung *et al.* 2011). The tracheal system does not develop if *trh* function is absent, suggesting that *trh* is a central regulator of tracheal cell identity. Trh expression is stimulated in the anterior compartment by JAK/STAT signaling acting directly on the *trh* enhancer element (Brown *et al.* 2001; Sotillos *et al.* 2010), but is repressed in the posterior compartment and in dorsal ectoderm by *wingless* (*wg*) and *dpp*, respectively (Isaac and Andrew 1996; Wilk *et al.* 1996). Although *trh* mutants retain some early characteristics of tracheal cell identity, these characteristics are mostly lost in the *Stat92E* mutant (Li *et al.* 2003), suggesting that tracheal cell identity requires additional transcriptional factors such as *apontic* (Liu *et al.* 2003) and the POU-domain protein encoded by *ventral veinless* (Anderson *et al.* 1995; Llimargas and Casanova 1999; Boube *et al.* 2000; Zelzer and Shilo 2000).

The process of tracheal placode invagination involves several mechanisms. The constriction of apical cell surfaces in the middorsal placode shifts the apical surface of the epithelium basally to form the tracheal pit (Figure 1C). This process advances through EGF receptor (EGFR) signaling controlled by the EGF ligand activator molecule Rhomboid (Llimargas and Casanova 1999) and the intracellular signal transducers ERK and MEK, and is regulated by Rho GTPase signaling (Brodu and Casanova 2006). In a parallel process, an accumulation of the subapical protein Crumbs also contributes to apical constriction (Letizia *et al.* 2011). EGFR signaling coordinates the formation of circular supracellular myosin cables around the tracheal pit, and these cables apply centripetal compression (Nishimura *et al.* 2007; Kondo and Hayashi 2013). A similar circular arrangement of cells and myosin cables is found in salivary gland primordia undergoing invagination, although the circles in the latter case remain in the same cell population (Röper 2012). How the circular patterns of contractile myosin cables emerge in the ectodermal field under the control of anterior–posterior (Wg and Hh) and dorsal–ventral (Dpp and EGFR) coordinated systems remains to be explained.

In addition, tracheal placode cells undergo spatiotemporally regulated mitosis 16. During the apical constriction

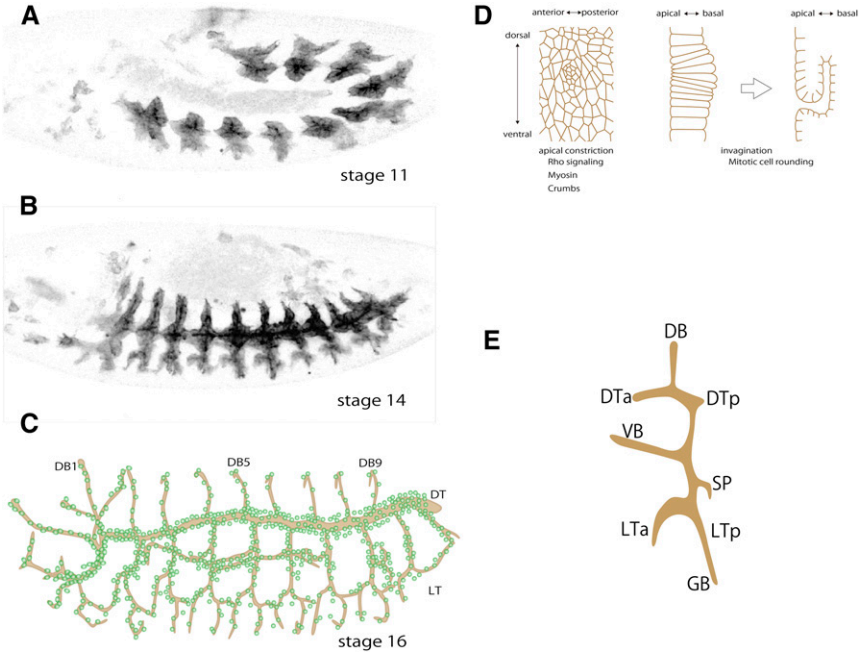


Figure 1 Embryonic tracheal development. (A) Tracheal placodes are specified in stage 10 and invaginate to form 10 pairs of primordia undergoing primary branching in stage 11. (B) Stage 14 embryos undergoing dorsal trunk branch fusion. (C) Trace of the tracheal system in a stage 16 embryo labeled with markers for nuclei (green spheres) and lumen (light brown). DT (dorsal trunk) is a longitudinal large-diameter tube connected to a posterior spiracle opening at the posterior end. LT (lateral trunk) is another longitudinal tube with smaller diameter. Dorsal branch (DB) 1, 5 and 9 are labeled. Note that the number of nuclei contributing to each DB is different (seven cells for DB5 and four cells for DB9). DB10 is out of focus of the original image. (D) Schematic of tracheal placode invagination. Left: during slow phase, cells in the central area of tracheal placode sharply constrict their apices to form the tracheal pit. Right: after rapid invagination, tracheal cells are fully internalized. (E) Primary branches in stage 12. Internalized tracheal primordia form stereotyped primary branches labeled DB, DTa (dorsal trunk anterior), DTp (dorsal trunk posterior), VB (visceral branch), LTa (lateral trunk anterior), LTp (lateral trunk posterior), and GB (ganglionic branch). SP (spiracular branch) is the remnant of tracheal invagination and connects the epidermal surface to the rest of the tracheal cells. (A and B) are taken from a time-lapse confocal movie of a *btl-Gal4 UAS-GFP-moesin* embryo (available in https://www.youtube.com/watch?v=agW1gYCz-Yo). Anterior: left, dorsal: up.

stage, all of the placode cells are arrested in the G2 interphase of cycle 15. In most cases, the first cells to reenter cycle 16 are in or around the tracheal pit. Mitotic cell rounding triggers buckling instability in the densely packed placode epithelia; the cells in the tracheal pit are rapidly internalized, followed by the surrounding tracheal cells (just entering cycle 16), forming the completely internalized, sac-like tracheal primordium (Kondo and Hayashi 2013) (Figure 1D). Although both mitotic cell rounding and EGFR-dependent Myosin force are important in coordinating the site, timing, and cell shape changes of invagination, embryos with a single mutation blocking EGFR signaling or cycle 16 mitosis still formed invaginated tracheal primordia. However, the invagination of the tracheal placode fails severely in triple-mutant embryos lacking cycle 16 mitosis, FGF signaling (which is required for cell migration and plays a backup role in invagination), and EGFR signaling (Kondo and Hayashi 2013), demonstrating that these cellular processes provide high redundancy and robust support for tracheal placode invagination.

Primary Branching and Guidance

Genetic mosaic analysis indicated that tracheal primordial cells are not committed to any position or cell type at the time of placode specification (Samakovlis *et al.* 1996a). Therefore, the cell and branch type specification must be determined under the influence of the surrounding environment. Once internalized, the tracheal primordia start expressing the FGF receptor tyrosine kinase Btl (Glazer and Shilo 1991; Klämbt *et al.* 1992; Shishido *et al.* 1993), which is activated by one of the three FGF ligands in *Drosophila*, Branchless (Bnl) (Sutherland *et al.* 1996), and the process of stereotyped primary branching begins. Each branch migrates toward a specific target tissue; for example, the dorsal branch toward the dorsal epidermis, the visceral branch toward the intestine, and the ganglionic branch toward the ventral nerve cord. Branch migration is guided by Bnl, which is expressed dynamically in a number of nontracheal tissues (Sutherland *et al.* 1996; Ohshiro *et al.* 2002; Du *et al.* 2017) (Figure 2A). At stage 15 and later, Bnl–Btl signaling stimulates the differentiation and outgrowth of a tracheal cell subtype called terminal cells, which send cytoplasmic branches out toward target tissues to support respiration (Figure 2, B and C).

The receptor tyrosine kinase Btl forms a complex with the FGFR adaptor protein Dof (Michelson *et al.* 1998; Vincent *et al.* 1998; Imam *et al.* 1999) and, when activated by Bnl, turns on the RAS pathway intracellular signaling cassette to activate ERK MAP kinase (Reichman-Fried *et al.* 1994; Gabay *et al.* 1997). The importance of the RAS–ERK pathway in tracheal branch migration was shown by the partial rescue of defective tracheal migration in *btl* mutants by constitutively active forms of Ras, Raf, and ERK (Reichman-Fried and Shilo 1995; Dossenbach *et al.* 2001).

Bnl/FGF is important for guiding branch migration, and two models have been proposed to explain its influence. The chemical guidance model suggests that some upstream-facing cells sense and respond locally to a chemical gradient of guidance cues, as observed in the migration of solitary cells in culture (Artemenko *et al.* 2014). Tracheal tip cells exposed to Bnl/FGF extend actively moving filopodia and lamellipodia in a Btl-dependent manner (Ribeiro *et al.* 2002), and this activity is further stimulated by ectopic Bnl/FGF. However,

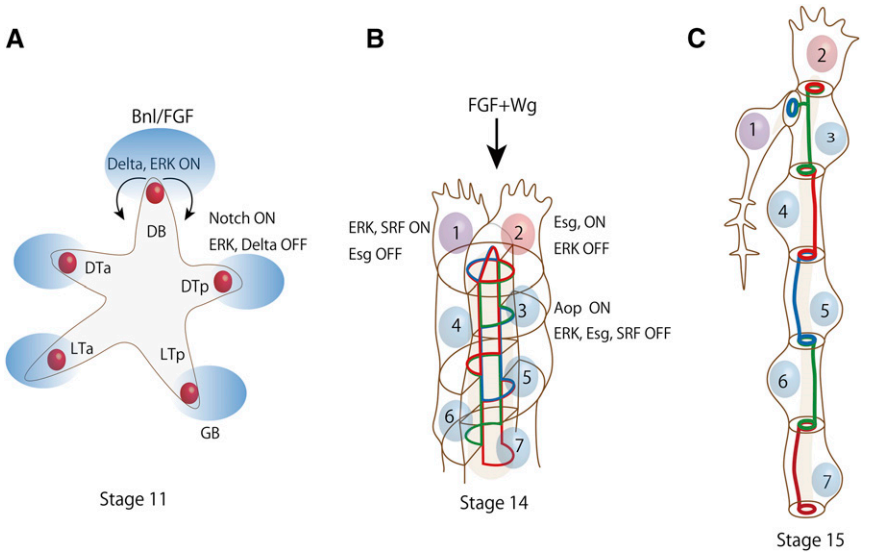


Figure 2 Cell specification and cell rearrangement during branching morphogenesis. (A) Schematic image of stage 11 tracheal primordia undergoing primary branching. Bnl/FGF (blue ovals) is expressed by groups of cells near invaginated tracheal primordia and activates Btl/FGFR and ERK activity in tracheal cells. One cell near the source of Bnl/FGF elevates Delta expression and ERK activity to the highest level and becomes a tip cell (red circle), and stimulates Notch signaling in the rest of the cells to reduce Delta expression and ERK activity to become stalk cells. Tip cells become motile and track Bnl/FGF to elongate tubules. VB (visceral branch) is not shown in this picture for clarity. (B) Schematic image of a stage 14 DB (dorsal branch) at the beginning of secondary branching. Under the influence of Wg, tip cells turn on Esg and other genes to acquire FC (fusion cell) fate (cell number 2). Esg turns off ERK signaling and permits activation of ERK signaling in the neighboring number 1 cell, which acquires TC (terminal cell) fate by activating ERK and SRF (serum response factor) gene expression. Other cells (numbers 3–7) turn on Anterior open (Aop) expression, turn off ERK, Esg, and SRF, and turn off FC and TC fate. In this stage, all cell interfaces are formed between different cells (adherens junction marked with blue, red, and green lines). One typical cell configuration is shown. Other configurations are possible. (C) Schematic image of stage 15 dorsal branch before anastomosis formation. Terminal cells (number 1) extend long cellular extension to ventral direction. Fusion cells (number 2) migrate dorsally. Stalk cells intercalate with each other (example: number 4 cell in stage 14 intercalated between cells numbered 3 and 5) and form an autocellular junction. DTa, dorsal trunk anterior; DTp, dorsal trunk posterior; GB, ganglionic branch; LTa, lateral trunk anterior; LTp, lateral trunk posterior.

the direction of the filopodia does not correlate well with the location of the Bnl/FGF (Okenve-Ramos and Llimargas 2014), arguing against the idea that the filopodia sense and extend along the Bnl/FGF gradient. Alternatively, the collective guidance model (Rørth 2007) posits that Btl signaling is activated only in one or two cells at the branch tip; in this model, only the tip cells respond to migratory cues, while the rest of the tracheal cells follow the tip. In late-stage tracheal branches, *btl* transcription is limited to the tip by positive feedback (Ohshiro *et al.* 2002). Genetic mosaic assays showed that *btl* activity is required in tip cells but not in stalk cells (Ghabrial and Krasnow 2006), and that expressing a constitutively active form of Btl in a single cell is sufficient to specify leader cell properties and induce branch migration (Lebreton and Casanova 2014). These findings suggest that limiting FGF signaling activity to a subset of tracheal cells is sufficient to differentiate two classes of cell types: tip cells, which sense Bnl/FGF and migrate toward the source, and tube-forming stalk cells that follow the tip cells. These findings favor the collective guidance model for FGF-guided primary branch migration. Although there is no decisive evidence for the chemical guidance model in primary branching, this mechanism is likely to be active in terminal branching, since localized ectopic Bnl expression attracts excessively elongated terminal branches in the embryo (Miao and Hayashi 2015) and larva (Jarecki *et al.* 1999).

The primary branches have unique identities that specify tube shape, cell composition, and target organ specificity. These branch identities are specified at least in part by the transcription factors Knirps/Knirps-like (Kni/Knrl) and Spält (Sal), known as the gap-class segmentation genes, in the blastoderm (Kuhnlein and Schuh 1996; Chen *et al.* 1998; Franch-Marro and Casanova 2002). During tracheal placode specification, these genes are expressed in a complementary pattern: Sal is expressed in the dorsal region, which gives rise to the dorsal trunk and dorsal branch, and Kni/Knrl is expressed in the ventral region, which gives rise to the lateral trunk and the ganglionic and visceral branches. In the dorsal region, Sal is expressed in the dorsal trunk but is repressed in the dorsal branch by Dpp-induced Kni/Knrl (Chen *et al.* 1998). The dorsal trunk is unique in that the lumen surface (except for the fusion point) is covered by cells with heterocellular junctions, producing a multicellular tubule with a relatively large diameter. Other branches are small-diameter unicellular tubules, formed of single cells with autocellular junctions (Ribeiro *et al.* 2004). Autocellular junctions in the dorsal branch are formed by cell intercalation, which is promoted by enhanced E-cadherin turnover (Shindo *et al.* 2008) and is inhibited in the dorsal trunk by enhanced endosomal recycling (Shaye *et al.* 2008) stimulated by Sal (Ribeiro *et al.* 2004). Self-adhesion interfaces do not form in most animal tissues, and are seen only in rare cases of tubular tissues such as blood vessels (Yu *et al.* 2015) and in fish gill pillar cells (Kato *et al.* 2007). The extent to which cell-autonomous myosin contractility and external pulling forces contribute to the formation and stabilization of tracheal autocellular junctions is a matter of some debate (Caussinus *et al.* 2008; Ochoa-Espinosa *et al.* 2017). Interestingly, the tracheal system in *Tribolium* larvae consists exclusively of unicellular tubules with a spiracle opening in each metamere. Sal is not expressed in the *Tribolium* dorsal trunk, which probably reflects an adaptation to the relatively dry environment found in flour

as opposed to the more humid environments inhabited by *Drosophila* larvae (de Miguel *et al.* 2016).

Successful branch migration requires guidance from other signals in addition to Bnl/FGF. Hedgehog, which is expressed in stripes in the posterior compartment, inhibits the migration of the dorsal and terminal branches, effectively confining their position to the anterior compartment of each hemi-segment (Kato *et al.* 2004). This segregates the branches from their anterior and posterior neighbors and assures a precise metamere-by-metamere arrangement. As the tip of the dorsal branch reaches the margin of the dorsal epidermis, Dpp emanating from the leading edge of the epidermis inhibits the dorsal elongation of the terminal branch and promotes the typical "U-turn" shape. The combined inhibitory effects of Hh and Dpp restrict the extent of branch migration in each half-metamere, which avoids overlap between branches from different segments. Slit-Robo signaling, which repels ganglionic branch migration in the CNS, has varying effects on dorsal branch migration (Englund *et al.* 2002).

The visceral branches migrate toward the midgut and spread over the surface of the visceral mesoderm. This migration requires two integrin subunits, αPS1 expressed in the visceral branch and αPS2 expressed in the visceral mesoderm, respectively (Boube *et al.* 2001). These integrin-α subunits both associate with the common βPS subunit, and are thought to assemble basal lamina for cell migration by capturing specific laminin molecules (Martin *et al.* 1999; Urbano *et al.* 2009). In addition, visceral branch migration requires extracellular matrix remodeling by AdamTS-A metalloprotease (Ismat *et al.* 2013).

Terminal Branching and Internal Lumen Formation

During secondary branching, tracheal branch termini become further elaborated through the appearance of the terminal branch, which is a single-cell extension that contains intracellular lumen and undergoes repeated elongation and branching, accompanied by lumen growth, to spread over the target organ (the epidermis, CNS, gut, or another internal organ). Cytoplasmic extensions from terminal cells depend on SRF (serum response factor) (Affolter *et al.* 1994; Guillemin *et al.* 1996), indicating that Bnl/FGF induces terminal cell differentiation. Bnl/FGF overactivation induces SRF expression and excessive terminal cell differentiation (Lee *et al.* 1996; Sutherland *et al.* 1996; Miao and Hayashi 2015). During the larval period, terminal cells undergo extensive growth and branching, covering increasingly larger areas, to keep up with the growth of the larva. Terminal cell growth is controlled by local oxygen demand, which stimulates Bnl/FGF expression in target tissues (Jarecki *et al.* 1999) to promote terminal cell outgrowth, and by trachea-autonomous stimulation of FGF receptors (Centanin *et al.* 2008). Both mechanisms are controlled by Sima, a Hypoxia-Inducible Factor-α homolog (Centanin *et al.* 2008).

Lumen formation in terminal cells is unique in that it forms at a distance from the cell–cell adhesion interface (Sigurbjörnsdóttir *et al.* 2014), and occurs by the inward growth of the apical plasma membrane at the interface of the terminal branch and stalk cells (Gervais and Casanova 2010). Lumen growth occurs in the direction of terminal branch outgrowth, most likely by following microtubule tracts (Gervais and Casanova 2010). Mutants with supernumerary centrosomes form multiple terminal branches in single-terminal cells, suggesting that microtubule organization is the key initial step in terminal branching (Ricolo *et al.* 2016). In mutants with defective actin filament organization, lumen growth fails to follow the direction of cell growth, resulting in misrouted lumen in the terminal branches as seen in *ikkε* mutants (Oshima *et al.* 2006), or in poor lumen formation as seen in *btsz* or *Slik* mutants (JayaNandanan *et al.* 2014; Ukken *et al.* 2014). Larvae require a continuous supply of apical membranes as they grow, and the elongation of terminal branch lumen is supported by dynein-dependent transport (Schottenfeld-Roames and Ghabrial 2012) and restricted by endocytosis (Schottenfeld-Roames *et al.* 2014).

Tip Cell Specification and Branch Fusion

Tracheal tip cells are ERK-active single cells, located at the tip of each primary branch, that lead tubule formation and branch migration, much like the tip cells in vascular sprouting in vertebrates (Herbert and Stainier 2011). The combined action of Bnl/FGF and Wingless increases the expression of the Notch ligand Delta in multiple cells (Chihara and Hayashi 2000; Llimargas 2000); from these, a single Delta-high cell is selected as the tip cell for the branch through lateral inhibition (Figure 2A). The remaining Delta-low, Notch-active cells assume the stalk cell fate (Ikeya and Hayashi 1999; Llimargas 1999). The tip cell remains at the leading position throughout branch migration and fusion, unlike vertebrate endothelial tip cells, which are frequently exchanged with stalk cells (Jakobsson *et al.* 2010). Activated Notch suppresses ERK signaling and restricts Bnl/FGF activity to the Delta-high tip cell (Ikeya and Hayashi 1999); this self-restrictive Notch mechanism limits the response of nontip cells to Bnl/FGF and shapes the tubular geometry. Anterior open, an ETS-domain transcriptional repressor, suppresses the tip cell fate in stalk cells by antagonizing ERK and Wingless signaling (Caviglia and Luschnig 2013).

Tip cells adhere to target tissues and follow sources of dynamically changing Bnl expression (Sutherland *et al.* 1996; Du *et al.* 2017). Under the influence of Wingless signaling, the tip cells then begin expressing genes that specify a fusion cell fate, *escargot* (*esg*) (Samakovlis *et al.* 1996b; Tanaka-Matakatsu *et al.* 1996; Chihara and Hayashi 2000; Llimargas 2000), followed by *dysfusion* (*dysf*) (Jiang and Crews 2003). At the same time, ERK expression diminishes in the tip cells while increasing in neighboring terminal cells, which begin to express SRF, a transcription factor encoded by *blistered* (Affolter *et al.* 1994; Guillemin *et al.* 1996), and take on a characteristic elongated shape that spreads out along the internal surface of the epidermis (Figure 2B). The switch

from the first stage of FGF signaling in primary branch migration to the second stage of FGF signaling in terminal cell differentiation is controlled by *esg*, which suppresses ERK activity in prospective fusion cells (Miao and Hayashi 2016). This allows a second tip cell to respond to Bnl/FGF and activates the secondary branching program.

Branch fusion (anastomosis) is mediated by contact between fusion cells and *de novo* lumen formation (Caviglia and Luschnig 2014). Dorsal branch fusion at the dorsal midline has been studied extensively, because this is the most accessible region for high-resolution imaging (Samakovlis *et al.* 1996b; Tanaka-Matakatsu *et al.* 1996; Gervais *et al.* 2012; Kato *et al.* 2016). Fusion cells attached to the basal surface of the leading edge of the epidermis migrate toward the dorsal midline after closure of the dorsal epidermis. Fusion cells from contralateral sides contact each other as they approach the dorsal midline by extending numerous filopodia, and they establish adhesion by accumulating E-cadherin at the contact interface. Escargot stimulates the *de novo* synthesis of E-cadherin, which is essential for adhesion between fusion cells (Tanaka-Matakatsu *et al.* 1996). Although the filopodia from fusion cells come into contact with neighboring epidermal and mesodermal cells, and sometimes with tracheal cells from the same side, stable E-cadherin adhesion is established only between fusion cells from contralateral sides. The mechanism of this selectivity is currently unknown. Once a stable attachment is established, the paired fusion cells create new lumen at the contact interface by directing Golgi-mediated secretion to the contact site (Kato *et al.* 2016). Fusion cell plasma membranes are fused internally to create their characteristic torus-like shape through a process requiring the endosomal protein ARF-like 3 (Jiang *et al.* 2007; Kakihara *et al.* 2008), which acts through the secretory lysosomal protein Staccato/Unc-13-4 (Caviglia *et al.* 2016). Interestingly, this conversion of cell surface topology always occurs simultaneously in paired fusion cells. Paired fusion cells at each fusion point produce CG13196, a specialized ZP (zona pelucida) domain-containing protein (Jaźwińska and Affolter 2004), and form a uniquely patterned cuticle that lacks taenidial folds (Matusek *et al.* 2006). During larval molt, tracheal cuticles are broken at the fusion point and are extruded through a transient reopening of the tracheal pit. Although ARF-like 3 and Staccato/Unc-13-4 are required for all tracheal fusion events, *esg* and *dysf* are dispensable for fusion of the dorsal trunk (Tanaka-Matakatsu *et al.* 1996; Jiang and Crews 2006), suggesting that the regulation of fusion cells is different in this branch. It should be noted that the conversion of fusion cells into a torus is not the sole method of joining tracheal branches in insects; in the *Manduca* moth, tracheal fusion points are formed by multiple proliferative cells (Nardi 1990).

Tube Geometry: Control of Length and Diameter

Researchers have conducted detailed morphometric analyses of the tracheal tubes (Beitel and Krasnow 2000). For tracheal tubes to serve as airways, the length of each branch must fit the size of the body. The number of cells in each tracheal branch is not strictly determined, and the number of cells varies even more when tracheal cell subsets are removed by apoptosis (Baer *et al.* 2010). This variability in cell number is compensated for by a flexibility in individual tracheal cell shape. In dorsal branches, for example, the number of cells in each branch is not predetermined (Samakovlis *et al.* 1996a), and in branches with fewer cells, each cell covers a greater length of lumen (Figure 1C). Experiments that increased or decreased the number of tracheal cells demonstrated this flexibility even more markedly (Caussinus *et al.* 2008). While branch connections and tube fusion are nearing completion in stage 15, the second phase of tube maturation begins, and each branch further elongates and takes on a sinusoidal shape. Cell shape analysis revealed that the axial elongation of dorsal trunk cells contributes to tube elongation (Förster and Luschnig 2012; Nelson *et al.* 2012). Dorsal trunk elongation is limited by the core planar cell polarity pathway (Chung *et al.* 2009; Nelson *et al.* 2012; Warrington *et al.* 2013) and Yorkie, a target of Hippo pathway-dependent repression (Robbins *et al.* 2014). Axial polarity in the larval trachea is specified by a transient transversal cell junction accumulation of atypical PKC that targets RhoA signaling to longitudinal cell junctions, and this planar polarization aligns cortical actin cytoskeletons into parallel arrays to support axial elongation (Hosono *et al.* 2015).

The dorsal trunk's largest tube diameter is at its posterior end, where it connects to the posterior spiracle that opens to take up air when the larva hatches. Although the dorsal trunk has a multicellular composition, the trunk diameter gradually decreases toward the anterior due to the differential effects of Hox genes in each metamere (Matsuda *et al.* 2015). Cross-sectional views show that dorsal tube sections with a similar diameter can be formed by one to five cells, demonstrating the great flexibility of cell size and shape. Various unicellular branches also maintain a near-regular luminal diameter unique to the branch type. These observations indicate that tube geometry is controlled, at least partly, at the tissue level.

Mutations that affect tracheal tube shape reveal three classes of genes that regulate tracheal tube geometry: those that primarily affect tube diameter (class I), tube length (class II), or both (class III) (Wu and Beitel 2004; Schottenfeld *et al.* 2010; Dong and Hayashi 2015). Class I mutations identify genes that affect the size and uniformity of tube diameter (Beitel and Krasnow 2000), including genes that affect components of chitinous fibers in the apical extracellular matrix (aECM) (Araújo *et al.* 2005; Devine *et al.* 2005; Tonning *et al.* 2005, 2006; Moussian *et al.* 2006; Wang *et al.* 2006) or the intracellular processing and secretion of the aECM (Tsarouhas *et al.* 2007; Grieder *et al.* 2008; Jayaram *et al.* 2008; Förster *et al.* 2010; Norum *et al.* 2010; Tiklová *et al.* 2013). Chitin, the N-acetylglucosamine polymer, which starts accumulating in the dorsal trunk lumen at stage 14 and in other branches at later stages, forms axially oriented fibers that fill the lumen (Tonning *et al.* 2005).

Massive luminal accumulations of chitin and other secreted macromolecules are thought to supply the force for tube expansion. In response to this secretory activity, F-actin accumulates on the apical plasma membrane facing the lumen in the form of multiple parallel cables, oriented in a circumferential direction, which act as a platform for myosin-dependent constrictive force (Matusek *et al.* 2006; Kondo *et al.* 2007; Öztürk-Çolak *et al.* 2016a,b). This expansion-triggered mechanical feedback and the viscoelastic property of the aECM (Förster *et al.* 2010; Dong *et al.* 2014a) prevent excess tube expansion throughout the length of the tubules. The absence of chitin causes a loss of stability, and uncontrolled cycles of constriction and relaxation in the cortical membrane, resulting in irregular tube diameter (Hannezo *et al.* 2015; Öztürk-Çolak *et al.* 2016b). How this mechanical feedback loop is equalized along the length of a tracheal tubule to create a uniform luminal diameter is not fully understood. Biophysical analysis of the aECM is necessary to identify mechanisms related to the tissue-level coordination of cell contractility.

Class II mutations cause excessive dorsal trunk elongation. These mutations can be grouped into three subclasses. The first subclass causes excessive apical membrane biosynthesis. Shrub/Vps32 and the epithelial polarity proteins Yurt and Scrib regulate the localization and activity of the apical polarity protein Crumbs (Laprise *et al.* 2006, 2010; Dong *et al.* 2014a). Activated Crumbs promotes apical membrane biogenesis (Tepass *et al.* 1990). The deregulation of Crumbs in *shrub*, *yurt*, and *scrib* mutants promotes excessive apical membrane synthesis and excessive tubule length. The second subclass affects aECM elasticity. Serpentine and Verm, which are chitin-binding proteins with domains homologous to bacterial and fungal chitin deacetylase, are required to negatively control tube length (Luschnig *et al.* 2006; Wang *et al.* 2006). Deacetylation converts chitin into the more water-absorptive form chitosan. Therefore, Serp and Verm might increase the aECM elasticity. Another aECM component, Dumpy, is a large elastic protein that helps to restrict dorsal trunk length (Dong *et al.* 2014a) and taenidial fold patterning (Wilkin *et al.* 2000), and maintains the dorsal trunk's mechanical strength under pulling force (Jaźwińska *et al.* 2003). The third subclass affects the apical membrane–aECM bond. ER-resident O-GlcNAc transferase (EOGT) modifies the extracellular domains of secreted and membrane proteins, including the EGF repeats of Dumpy. EOGT is required for maintaining connections between apical membranes and the aECM (Sakaidani *et al.* 2011). The chitin-binding protein Obst-A stabilizes chitin fiber organization, maintains cuticle–epidermis adhesion, and restricts tracheal tube length (Petkau *et al.* 2012). Taken together, the products of these three subclasses of genes that regulate tube length constitute two coupled elastic modules, stretchable aECM bound by an expanding apical plasma membrane. A physical model of these coupled elastic modules reproduced a sinusoidal-curving, overelongated tube in mutant embryos (Dong *et al.* 2014a).

Class III mutants include septate junction proteins that are required for both tube length and diameter control (Behr *et al.* 2003; Genova and Fehon 2003; Paul *et al.* 2003; Wu *et al.* 2004; Nelson *et al.* 2010), and are thought to control tube diameter by regulating the secretion of the aECM through mechanisms that are independent of the epithelial barrier function of the septate junctions (Wu and Beitel 2004).

The epidermis of the body wall develops independently of the trachea and grows larger than the size of the egg shell, so that the mature epidermis is tightly folded under the vitelline membrane. After the larva hatches, the epidermis and the trachea stretch out to matching lengths, suggesting that the two axial tissues undergo systemic-level coordination. Serp is initially transcribed mainly in the trachea and later in the epidermis, where it affects the aECM properties and cuticle rigidity (Luschnig *et al.* 2006; Wang *et al.* 2006). Serp is also produced in the mesodermal-tissue fat body, where excessive Serp accumulation was observed in a mutant with defective endocytosis and endosomal recycling (Dong *et al.* 2014b). Fat body-derived Serp travels through the body cavity, reaches the tracheal lumen by transcytosis, and is retained by chitin binding (Dong *et al.* 2014b). This systemic regulation of the aECM may help to coordinate the elongation of the trachea and epidermis to matching lengths.

Tube Maturation

Once tracheal tubules reach their final pattern and size, cuticle layers begin forming on the apical surface with a circumferential ridge, called a taenidial fold, with a pattern corresponding to the cortical F-actin cable arrays formed at an earlier stage (Matusek *et al.* 2006; Öztürk-Çolak *et al.* 2016a,b). Inside the lumen, the aECM is degraded and absorbed by tracheal cells to clear a large fraction of the luminal macromolecules (Behr *et al.* 2007; Tsarouhas *et al.* 2007). Finally, a gas is generated independently of the outside atmosphere on part of the cuticle surface, and the gas quickly spreads over the entire tracheal lumen to completely fill the tube with gas. Although the mechanism of gas generation is not well understood, it requires the apical membrane protein Uif (Zhang and Ward 2009). The identification of fatty acyl-CoA reductase as a critical requirement for gas generation suggests that the formation of a hydrophobic wax layer on the cuticle surface may contribute to bubble generation from gas saturated in the luminal liquid (Jaspers *et al.* 2014), previously suggested as cavitation-bubble formation on a hydrophobic surface (Wigglesworth 1953).

Spiracles

Spiracles, the epidermal structures that connect the internal tracheal network to the outside environment, serve as gatekeepers to control gas flow and water loss, and protect the luminal environment. Although spiracles can form in every metamere from which tracheal primordium is derived, only the posterior spiracle is functional in first-instar *Drosophila* larva. Extensive studies have determined that the posterior spiracle and the trachea develop independently of each other

(Castelli Gair Hombría *et al.* 2009). However, their primordia are juxtaposed and develop simultaneously, producing structurally interconnected mature tissue (Hu and Castelli-Gair 1999). The posterior spiracle, which is positioned in A8, consists of two parts: an internal tubule (called the spiracular chamber or filzkörper) that connects the trachea to the outside and the external stigmatophore. There are four sensory hairs at the exit of the spiracular chamber. Reflecting the mature structure, the posterior spiracle primordia develop in two parts: the central spiracular chamber part, marked by expression of the transcription-factor genes trh (Isaac and Andrew 1996), *cut* (Blochlinger *et al.* 1990), and *empty spiracle* (Dalton *et al.* 1989), and the outer stigmatophore part, which expresses *sal* (Hu and Castelli-Gair 1999). The central part also expresses the Unpaired ligand, which activates the JAK/STAT pathway (Brown *et al.* 2001) to promote invagination by the basolateral expansion and inward movement of prospective spiracular chamber cells (Lovegrove *et al.* 2006; Simões *et al.* 2006; Tsikala *et al.* 2014). The cell behaviors and mechanisms involved in posterior spiracle invagination appear to be distinct from those used in tracheal placode invagination. Furthermore, posterior spiracles utilize a distinct mechanism of air-filling that requires a scavenger receptor class B molecule (Wingen *et al.* 2017).

Larval Trachea and Metamorphosis

After larval hatching, most tracheal cells stop proliferating and begin endocycling, increasing ploidy and cell size, and the tracheal tubules elongate and increase in diameter to match the growth of the larval body. During molting, tracheal cells renew their apical surface by planar polarization and by reorganizing cortical F-actin (Hosono *et al.* 2015), and they secrete cuticles with taenidial folds at a spacing of $\sim$0.8 μm at the beginning of the second and third instar (Glasheen *et al.* 2010). Taenidial fold spacing increases up to twofold during each instar as the tracheal tube elongates, and this cuticle plasticity depends on apically secreted matrix metalloprotease 1 (Glasheen *et al.* 2010).

Tracheal histoblasts (tracheoblasts), which are derived from *cut*-positive cells, populate the spiracular branch that connects the tracheal tubule to the epidermal region at the site of the initial tracheal pit formed at the beginning of tracheal development (Pitsouli and Perrimon 2010, 2013). These unique cells, which proliferate extensively in the third instar, replace larval tracheal cells and form adult-specific tracheal branches during metamorphosis. The spiracular branch does not have an open lumen and does not function as an airway except during the molt, when the branch opens and sheds the tracheal cuticles from the previous instar. Therefore, tracheal histoblasts are considered to be undifferentiated, dedicated adult precursor cells.

An additional source of adult precursor cells is found among the functional tracheal cells. Dorsal branch stalk cells in the second to fifth tracheal metameres (Tr2–Tr5) (Weaver and Krasnow 2008) and in the dorsal trunk in Tr2 (Guha *et al.* 2008; Sato *et al.* 2008) enter the cell cycle at late L3 and proliferate to become adult tracheal cells. These cells enter quiescence after embryonic cycle 16 mitosis. While other tracheal cells start endocycling, these precursor cells retain their mitotic capacity. The nuclear proteins Htx and Exd, along with their binding partner Ubx, are implicated in maintaining cell cycle arrest and multipotency in these cells (Sato *et al.* 2008) by inhibiting the endocycle-promoting gene *fzr* (Djabrayan *et al.* 2014).

Air sacs are adult-specific tracheal tissues encased in the notum that supply air to flight muscles to meet their high oxygen demands during flight. The trachea becomes associated with the wing disc immediately after the wing disc invaginates in stage 15 of embryogenesis (Inoue and Hayashi 2007). In third-instar larvae, the air sac primordia are found as a part of the Tr2 tracheal metamere attached to the basal side of the presumptive notum of the wing imaginal disc, where the disc cells express *bnl*. Tracheoblasts in the primordia express *btl*, and the activation of FGF signaling promotes the migration of tracheoblasts toward the posterior (Sato *et al.* 2002) through an invasive process involving the extensive remodeling of basement membranes (Guha *et al.* 2009). FGF signaling also stimulates secretion of the ligand molecule Vein (Cruz *et al.* 2015), which activates tracheoblast proliferation and survival (Cabernard and Affolter 2005).

Future Challenges

The genetic pathways that specify the basic grand plan of the embryonic tracheal system have been described extensively. One prominent discovery is that a surprisingly simple set of signaling pathways, most notably FGF signaling, are used repeatedly at multiple stages of tracheal development to model and remodel tracheal pattern and functions, keeping pace with the ever-changing respiratory needs of the larva, pupa, and adult. These discoveries set the stage for investigating even more complex unexplored fields.

The structure of the tracheal terminus is finely tuned to the specific morphology and function of the target tissue. This fine tuning requires tracheal cells to sense and respond to local conditions and structures, and to adjust the developmental patterns accordingly. Dynamic changes in Bnl/FGF expression in the wing imaginal disc (Sato *et al.* 2002), in larval tracheal cells undergoing metamorphic apoptosis (Chen and Krasnow 2014), and in photoreceptor neurons (Chu *et al.* 2013) guide the migration, proliferation, and association of tracheoblasts with target tissues. The complex pattern of Bnl/FGF expression is regulated transcriptionally, as revealed by its transcript levels (Sutherland *et al.* 1996) and a genomic LexA knock-in construct (Du *et al.* 2017). Bnl/FGF expression is fine-tuned by its localization to specific subcellular regions. Transverse (T)-tubules are a network of plasma membrane invaginations in muscles that conduct membrane excitation to the calcium storage organelle, the sarcoplasmic reticulum. In adult flight muscles, the T-tubule network is associated with secreted

Bnl/FGF, which attracts invasive tracheal growth into the T-tubule through openings at the plasma membrane (Peterson and Krasnow 2015). Thus, the reactivation of FGF signaling competence in tracheal cells, along with various Bnl/FGF expression patterns, form the basis for tracheal remodeling and tissue-specific tracheal morphologies. The transcriptional and post-transcriptional regulation of Bnl remains to be elucidated.

Studies of adult midgut development reveal indications of reciprocal cross talk between the trachea and target tissues. Terminal branch growth associated with part of the larval intestine is regulated by insulin and by vasoactive intestinal peptide-like peptides produced by the intestine (Linneweber *et al.* 2014), indicating that tracheal growth is coupled to nutrient-dependent neuroendocrine signaling. Conversely, tracheal cells associated with adult midgut progenitors in the larval intestine supply the Dpp ligand to maintain a population of undifferentiated proliferative intestinal stem cells (Li *et al.* 2013). Further studies of tracheal associations with adult tissues should provide rich details on the mechanisms of interorgan communication and how they are modulated by environmental conditions.

Studies of the tracheal system may be particularly useful in the field of tissue mechanics. Excellent imaging tools are available for *Drosophila*, and the tracheal system is of particular interest, because it is possible to measure cellular signaling (Kamiyama and Chiba 2009) and perturb various forces (Caussinus *et al.* 2008). This is an attractive model system for addressing questions about the role of ECMs in organ-level shape control. Analyses of the aECM in the control of embryonic tracheal tube length and diameter has set the stage for biophysical investigation for the tissue-level coordination of organ shape. Although mechanical properties of aECM is crucial for specifying the shape of organs, biomechanical information on the aECM and the role of its molecular components remains poorly understood. Therefore, biophysical investigation of aECM properties and molecular genetic analysis of its protein components would open a new research field of tissue-level control of organ shape. Because the aECM of the trachea shares its components with exoskeletons (cuticles) that cover the outer surface of the insect and define its body shape, this line of investigation has the potential to develop into a wider spectrum of research on insect body shape and its variation.

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