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Novel mechanisms of tube-size regulation revealed by the *Drosophila* trachea

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Abstract

The size of various tubes within tubular organs such as the lung, vascular system and kidney must be finely tuned for the optimal delivery of gases, nutrients, waste and cells within the entire organism. Aberrant tube sizes lead to devastating human illnesses, such as polycystic kidney disease, fibrocystic breast disease, pancreatic cystic neoplasm and thyroid nodules. However, the underlying mechanisms that are responsible for tube-size regulation have yet to be fully understood. Therefore, no effective treatments are available for disorders caused by tube-size defects. Recently, the *Drosophila* tracheal system has emerged as an excellent in vivo model to explore the fundamental mechanisms of tube-size regulation. Here, we discuss the role of the apical luminal matrix, cell polarity and signaling pathways in regulating tube size in *Drosophila* trachea. Previous studies of the *Drosophila* tracheal system have provided general insights into epithelial tube morphogenesis. Mechanisms that regulate tube size in *Drosophila* trachea could be well conserved in mammalian tubular organs. This knowledge should greatly aid our understanding of tubular organogenesis in vertebrates and potentially lead to new avenues for the treatment of human disease caused by tube-size defects.

Keywords

Trachea; Tube size; Luminal matrix; Apical secretion; Cell polarity; Endocytosis; *Drosophila*

Introduction

The sizes of various tubes within tubular organs such as the lung, vascular system and kidney must be finely tuned for optimal function in delivering gases, nutrients, wastes and cells within the entire organism. Aberrant tube sizes lead to devastating human illnesses,

such as polycystic kidney disease (Steinman 2012), fibrocystic breast disease (Rinaldi et al. 2010), pancreatic cystic neoplasms (Garud and Willingham 2012) and thyroid nodules (Popoveniuc and Jonklaas 2012). However, the underlying mechanisms responsible for tube-size regulation have yet to be fully understood. Therefore, no effective treatments are available for disorders caused by tube-size defects. Recently, the *Drosophila* tracheal system has emerged as an excellent in vivo model to explore the fundamental mechanisms of tube-size regulation.

Overview of *Drosophila* tracheal development

The *Drosophila* trachea is a ramifying network of epithelial tubes with the apical surfaces facing the lumen and the basal surfaces facing the surrounding tissues or basement membrane. The fully developed trachea consists in four types of tubes that differ in size and architecture (Samakovlis et al. 1996; Fig. 1a, Table 1). Type I tubes are multicellular, such as the dorsal trunk (DT), the major artery of the trachea and the majority of the transverse connective (TC), the stalk connecting all of the tracheal branches within each metamere. In these tubes, several cells are connected by intercellular junctions and surround an extracellular central lumen. The narrower Type II tubes are unicellular, such as the lateral trunk (LT), ganglionic branch (GB), dorsal branch (DB) and visceral branch (VB). These tubes comprise a linear arrangement of single cells whose apical surfaces surround an extracellular lumen. Cells of unicellular tubes connect with themselves through autocellular junctions and with their neighbors through intercellular junctions. The main function of Type I and Type II tubes are to transport gases. Type III tubes are intracellular. In these tubes, two donut-shaped cells are arranged in a linear head-to-tail configuration and their apical surfaces span the inner wall of the donut, resulting in seamless tubes without intracellular junctions. Meanwhile, the two fusion cells connect themselves by intercellular junctions. Type III tubes connect Type I or Type II tubes of the adjacent or contralateral tracheal metameres. Type IV tubes, such as the lateral ganglionic (LG) branches, are highly branched intracellular cytoplasmic extensions that form in terminal cells at the tips of the unicellular tubes. Their main function is to contact target tissues for gas exchange.

The embryonic trachea is derived from a series of segmentally repeated clusters of tracheal precursor cells. The specification of trachea precursors depends on wingless (Wg) signaling, transforming growth factor- (TGF- ; DPP in *Drosophila* trachea) signaling and possibly epithelial growth factor (EGF) to limit tracheal formation to a subset of epidermal cells (de Celis et al. 1995; Isaac and Andrew 1996; Wilk et al. 1996). Additionally, JAK/STAT (Janus kinase/-signal transducers and activators of transcription) signaling induces tracheal formation by activating the expression of two of the earliest tracheal genes, *trachealess* (*trh*) and *ventral veinless* (*vvl*; Li et al. 2003; Sotillos et al. 2010). Trh and Vvl are transcription factors that regulate downstream target genes required for tracheal development (Chung et al. 2011). Subsequently, the activation of EGF signaling in tracheal precursor cells leads to the invagination of these cells to form tracheal sacs through an apical constriction mechanism (Llimargas and Casanova 1999). Thereafter, subsets of cells within each sac begin to migrate in stereotypical directions to form distinct branches. The cells that remain at the site of invagination become TC (Fig. 1b). Migration of all specific tracheal branches along distinct trajectories requires fibroblast growth factor (FGF) signaling and branch-specific signaling (Sutherland et al. 1996). Specifically, cells in the dorsal and ventral region of the tracheal sac, which are exposed to the DPP signals, migrate toward FGF signals from surrounding tissues either dorsally to populate the DB or ventrally to populate both the LT and GB (Affolter et al. 1994; Vincent et al. 1997; Myat et al. 2005; Chihara and Hayashi 2000; Llimargas 2000). In the central region of the tracheal sac, cells exposed to Wg signaling migrate toward FGF signals to form DT (Chihara and Hayashi 2000; Llimargas 2000); cells that do not receive Wg signaling migrate internally to form VB.

All tracheal branches begin as multicellular tubes. As the trachea develops, the cells within DB, LT, GB and VB intercalate to form unicellular secondary branches (Type II; Ribeiro et al. 2004), whereas DT and the majority of TC remain multicellular (Type I). Subsequently, both Type I and II branches from neighboring segments fuse to form interconnected tubes (Type III; Jiang and Crews 2006; Jiang et al. 2007; Lee and Kolodziej 2002; Tanaka-Matakatsu et al. 1996). Finally, terminal cells at the tips of unicellular branches send out cytoplasmic extensions to form highly branched intracellular tubes (Type IV; Ghabrial et al. 2003; Schottenfeld-Roames and Ghabrial 2012; Levi et al. 2006).

Blood vessel formation in an in vivo zebrafish model and in vitro human endothelial cells in a three-dimensional (3D) collagen matrix model have been used to reveal mechanisms of tube formation in vertebrates and clearly show differences and similarities compared with *Drosophila* trachea. In zebrafish embryos, Type I tubes are critical for blood circulation, such as the dorsal aorta (DA), the first blood vessel that forms *de novo* via vasculogenesis and the inter-somitic vessels, secondary blood vessels that form from vessels that arise from a pre-existing vessel such as DA via angiogenesis (Strilic et al. 2009; Lawson and Weinstein 2002). Similar to Type III tubes in *Drosophila* trachea, certain aortic endothelial cells sprout ventrally from the DA and connect with adjacent cells to form intersegmental blood vessels. However, unlike seamless fusion tubes produced by two donut-shaped cells, endothelial cells are not arranged in a linear head-to-tail configuration but overlap extensively and form a multicellular fusion tube (Blum et al. 2008). Consistent with Type IV seamless tubes in *Drosophila* trachea delivering oxygen to target tissues, capillary tubes, the smallest of all blood vessels, contact target tissues to exchange gases, nutrients and waste (Kamei et al. 2006). In addition, Type I and Type IV tubes can also be produced by human endothelial cells within an in vitro 3D collagen matrix (Davis et al. 2011). Nevertheless, unlike *Drosophila* trachea, Type II unicellular tubes are not common in vertebrates.

Model for tracheal tube maturation in *Drosophila*

The tubes in a mature tracheal tree acquire distinct sizes to carry out their biological functions. Tube size is tightly linked to branch identity. Once branch identities are specified, tubesize control is mediated by changes in the apical (luminal) surface of tracheal cells but not the number of cells in each branch (Beitel and Krasnow 2000). Immediately following the formation of a contiguous tube, tube diametric expansion (dilation) occurs within a defined time interval between embryonic stages 13 and 16 (Fig. 2a, b). During this interval, an apical secretory burst is initiated in the tracheal epithelium to provide components for apical membrane growth and the assembly of a chitin-based apical extracellular matrix. At mid-stage 17, the tracheal epithelium activates a pulse of endocytic activity that internalizes the solid matrix from the lumen (Fig. 2c). Subsequently, the luminal liquid is cleared from the lumen at late stage 17 and the entire trachea is filled with air (Swanson and Beitel 2006; Tsarouhas et al. 2007). In contrast to tube dilation, tube elongation is continuous throughout tracheal development (Beitel and Krasnow 2000). These mechanisms of tube-size regulation are mainly revealed by experiments carried out in multicellular branch DT.

Apical secretion and tube-size regulation

Epithelial tubes in the trachea need to expand their initially narrow lumens to attain their final dimensions. During the diametric expansion interval, apical secretion pulses drive tube dilation and the assembly of a luminal matrix. The luminal matrix is composed of chitin, chitin-modifying enzymes (Vermiform and Serpentine), structural zona pellucida domain proteins that maintain cell interconnections (Piopio and Dumpy) and other less well-characterized components, such as the antigen recognized by monoclonal antibody 2A12 (Jazwinska and Affolter 2004).

Generally, the secretory components pass through a series of membrane-enclosed organelles, including the endoplasmic reticulum (ER), the Golgi complex and various secretory vesicles, to be inserted into the apical membrane or be secreted into the lumen. The apical secretion of membrane and luminal components in tracheal cells is described in Fig. 3. The ante-rograde and retrograde transport between the ER and the Golgi depends on two distinct types of coated vesicles: COPII-coated vesicles that bud from the ER and shuttle nascent secreted proteins to the Golgi apparatus and conversely, COPI vesicles that retrieve luminal ER residential proteins from the Golgi and shuttle them back to the ER (Bonifacino and Glick 2004). Various post-Golgi vesicles are then trafficked by distinct mechanisms. For example, the secretion of the chitin-modifying enzymes, Vermiform and Serpentine, depends on basolateral septate junctions (SJ; Wang et al. 2006), which are analogous to apically localized tight junctions in vertebrates (Tepass et al. 2001). Vesicles delivered to the SJ are next trafficked to the apical lumen by an unknown mechanism. On the other hand, the secretion of luminal proteins 2A12 and Piopio depends on myosin V motor-mediated trafficking along the apical actin cable but not the SJ (Massarwa et al. 2009). The vesicle-tethering exocyst complex, annexin and membrane-specific SNARE (soluble N-ethylmaleimide-sensitive factor attachment receptor) proteins mediate the selective docking of these vesicles to target membranes and the subsequent fusion of these vesicles (He and Guo 2009; Wickner and Schekman 2008; Gerke et al. 2005; Jacob et al. 2004). Consistent with the role of the SJ in apical secretion in *Drosophila* trachea, Lethal giant larvae, a basolateral protein in vertebrates (an SJ protein in *Drosophila*), interacts with the secretory exocyst complex for polarized apical secretion in Madin-Darby canine kidney cells (Musch et al. 2002). Despite these discoveries, the underlying mechanisms that control the assembly of distinct post-Golgi vesicles and the trafficking of each vesicle to the apical side of the tracheal cell remain largely unknown.

In *Drosophila* trachea, mutants disrupting the coat assembly and vesicle budding of the COPII complex (*sec23*, *sec24*, *sar1*, *sec13*; Tsarouhas et al. 2007; Norum et al. 2010; Forster et al. 2010) or the COPI complex (*COP*, *COP*; Grieder et al. 2008; Jayaram et al. 2008; Armbruster and Luschnig 2012) lead to narrow tubes and luminal deposition failure. Similar defects in other tubular organs such as the salivary gland are also observed. In addition, mosaic analysis in *sec24* (*stenosis*) mutants reveals that single cells autonomously adjust apical membrane growth and secretion of luminal materials (Forster et al. 2010). Both COPI and COPII complexes are necessary for the maintenance of apical secretion pathways and thus, mutations in either cause similar tube-size defects.

The chitin-based matrix regulates both the diameter and the length of the trachea. Similar to the deposition of the luminal matrix in *Drosophila* trachea, luminal fluid influx occurs in the zebrafish gut and generates a distending force that drives the coalescence of narrow tubes into a single lumen (Bagnat et al. 2007). Therefore, one hypothesis is that the luminal matrix drives diametric expansion by providing a distending force in *Drosophila* trachea. Unexpectedly, mutations disrupting chitin biosynthesis (*krotzkopf verkehrt* and *mummy/cystic*) and matrix assembly (*knickkopf* and *retroactive*) grow over-dilated and elongated tubes (Araujo et al. 2005; Devine et al. 2005; Moussian et al. 2006). Moreover, the organization of subapical α -Spectrin, which crosslinks the apical cytoskeleton and membrane, is disrupted in the absence of chitin (Tonning et al. 2005). In addition, the modification of the chitin cable is specifically required to restrict tube over-elongation, as a lack of chitin modification by mutating genes encoding chitin deacetylases (*vermiform* and *serpentine*) leads to longer tubes (Luschnig et al. 2006). This evidence suggests that cues emanating from the chitin matrix are sensed by epithelial cells in order to coordinate cytoskeletal organization; subsequently, they prevent tube over-expansion in both diameter and length.

Experiments in *Drosophila* trachea clearly demonstrate that apical secretion is required for diametric expansion and luminal matrix assembly. The insertion of large amounts of apical membrane proteins is probably the driving force for tube dilation. On the contrary, the chitin luminal matrix restricts tube over-expansion in both diameter and length, possibly through interaction with the apical cytoskeleton. Interestingly, human endothelial cell capillaries contain a fibrillar luminal matrix, whereas mature blood vessels and lung epithelia are lined with an oligosaccharide-based “glycocalyx” (Folkman and Haudenschild 1980). This glycocalyx contains glycosaminoglycans analogous to chitin and plays an important role in maintaining tube stability (Reitsma et al. 2007). Investigation of whether the glycocalyx has a potential role in tube-size regulation, as suggested by the chitin matrix in invertebrates, will be of interest.

Endocytosis and tube-size regulation

In contrast to the apical secretion pathway that deposits massive amounts of membrane and luminal material during tube expansion, clathrin-mediated endocytosis regulates the internalization of the apical membrane and luminal components (Tsarouhas et al. 2007). In general, cargos are internalized by clathrin-coated vesicles (CCVs) that are cleaved by the GTPase dynamin, followed by fusion with the early endosome (containing the Rab family GTPase Rab5) and the late endosome (containing Rab7; Fig. 3). Then, these cargos take different routes including: (1) fusion with the lysosome for degradation; (2) fusion with the trans-Golgi network (Rab9-dependent) for apical secretion; or (3) fusion with the recycling endosome (containing Rab11) for recycling back to the apical membrane (Johannes and Popoff 2008; Bonifacino and Rojas 2006). Mutations in genes required for the formation of CCV (*clathrin heavy chain*, *dynamin* and a novel gene *wurst*) and the early endosome (*rab5*) show over-elongated tubes with defective apical matrix organization. In addition, the tubes are also slightly over-dilated with an extra apical membrane (Tsarouhas et al. 2007; Wingen et al. 2009). Hence, balanced internalization of the membrane or matrix components is critical for tube-size regulation by maintaining membrane size and matrix assembly. Interestingly, both mice and humans contain a single *wurst* ortholog to the *Drosophila* *wurst* and it is expressed in the lung and other tubular epithelia (Wingen et al. 2009). This indicates a putative role of the *wurst* ortholog and other components in the endocytosis pathway in clinically relevant lung syndromes, such as neonatal respiratory distress syndrome.

In addition to early steps in the endocytosis pathway, later steps, such as the trans-Golgi network retrograde pathway (pathway 2 in Fig. 3) that requires the Rab family GTPase Rab9, also regulate tube size (Lombardi et al. 1993; Barbero et al. 2002; Chia et al. 2011). In *rab9* mutants, the specific chitin-modifying enzyme Serpentine is depleted at later stages, an event that leads to longer tubes (Dong et al. 2013). Therefore, the later steps of the endocytosis pathway also play a role in tube-size regulation through retaining specific luminal proteins.

Whereas the apical secretion pathway is required for apical membrane expansion and matrix formation during tube dilation, the endocytosis pathway internalizes membrane and matrix components to sustain apical membrane size and luminal matrix assembly. Therefore, a proper balance between secretion and internalization is critical to control tube size.

Cell polarity and tube-size regulation

The *Drosophila* trachea is made up of polarized epithelial cells. Cell polarity plays a significant role in tube-size regulation, particularly tube length. In *Drosophila* trachea and in vertebrate epithelial cells, apical and basal polarity regulators are associated with distinct membrane domains. The adherens junction (AJ), containing E-cadherin and Armadillo (-

catenin in vertebrates), forms a belt around the apical pole of epithelial cells and segregates apical from basolateral membrane domains. The apical domain contains the Crumbs complex and the Par complex. The Crumbs complex contains the apical transmembrane protein Crumbs, which binds to Stardust (Pals1 in vertebrates), Patj, Lin7, the FERM proteins Moesin (Ezrin in vertebrates) and Yurt (EBP41L5 in vertebrates). The Par complex contains atypical protein kinase C (aPKC), its regulator subunit Par6, the small GTPase Cdc42 and the scaffolding protein Bazooka (Par3 in vertebrates). The basolateral SJ, analogous to the vertebrate tight junction (TJ), forms a diffusion barrier to prevent the exchange of solutes across epithelia (Tepass et al. 2001). The SJ contains Sinuous, Megatrachea, Kune-kune, Lethal giant larvae, Discs large, Scribble, Yurt, Coracle (Band 4.1 in vertebrate), the Na⁺/K⁺-ATPase, Neurexin IV, Lachesin (vertebrate ortholog unknown), and Varicose (Pals2 in vertebrates). Sinuous, Megatrachea and Kune-kune are homologs of vertebrate Claudins (Swanson and Beitel 2006; Laprise and Tepass 2011; Wu and Beitel 2004).

The establishment and maintenance of apical-basal polarity depends on the general secretion pathway and the endocytosis pathway. This includes the trans-Golgi-network-mediated sorting of newly synthesized proteins to specific domains and the recycling of endosomes responsible for polarized recycling or transcytosis of endocytosed proteins (Mellman and Nelson 2008). For example, the level of AJ component E-cadherin is maintained by a balance between lysosomal degradation and Rab11-mediated recycling of endocytosed E-cadherin (Shaye et al. 2008). How does apical-basal polarity affect tube length? As mentioned earlier, the luminal matrix restricts tube over-expansion. Loss of function mutations in most SJ components, except Yurt and Coracle, cause over-elongated tubes due to defects in the secretion of chitin-modification enzymes (Vermiform and Serpentine) (Wu et al. 2004; Moyer and Jacobs 2008; Paul et al. 2003; Llimargas et al. 2004; Behr et al. 2003). Therefore, one function of the SJ is to regulate the secretion of luminal proteins to restrict tube length. However, mutations in two other SJ polarity proteins, Yurt and Coracle, lead to tube over-elongation. This tube-elongation phenotype can be rescued by reducing the apical membrane protein Crumbs (Laprise et al. 2010), an indication that the phenotype is caused by an enlarged apical membrane, rather than the failed secretion of luminal chitin-modifying enzymes. Therefore, the SJ has two functions in tube length regulation: (1) to antagonize the over-expansion of the apical membrane to control tube length; (2) to control the secretion of chitin-modifying enzymes in order to restrict tube over-elongation (Fig. 4).

Interestingly, cell-cell interaction mediated by planar cell polarity (PCP) also plays a role in the regulation of tube length. PCP, orthogonal to the axis of apical/basal polarity, is required to orient epithelial cells in a directed fashion to generate polarized structure. In *Drosophila* and in vertebrates, a conserved PCP pathway, the Frizzled (Fz) pathway, mediates local cell-cell interactions that instruct neighboring cells to adopt appropriate polarity. The components in this pathway include: Fz, a seven-pass transmembrane receptor; Dishevelled (Dsh), an adaptor protein that acts downstream of Fz; Flamingo/Starry night (Fmi/Stan), a cadherin family member; Strabismus/Van Gogh (Stbm/Vang), a four-pass transmembrane protein; and Prickle (Pk) and Diego (Dgo), cytoplasmic proteins that are associated with the apical membrane during PCP signaling (Seifert and Mlodzik 2007; Klein and Mlodzik 2005; Adler 2002; Bastock and Strutt 2007). The role of the PCP pathway in tracheal tube size regulation is revealed by a mutation in *Serrano* (*sano*), a novel protein that directly binds to the core PCP component Dsh. Mutation in *sano* leads to an elongated tracheal DT, whereas over-expression of *sano* results in a shortened DT. Furthermore, null mutants of other core PCP genes, namely *fz*, *dsh* and *fmi*, show similar elongated tubes (Chung et al. 2009). PCP signaling has been implicated in directing asymmetric cytoskeletal reorganization and polarized cell morphology, partially through the downstream effector RhoA (Strutt et al. 1997). Consistently, *RhoA* mutants also show elongated tubes (Chung et al. 2009). This

indicates a potential role for PCP signaling in regulating the cytoskeleton through RhoA in order to control tube length in *Drosophila* trachea. However, the mechanisms of PCP proteins in tracheal tube-size regulation are yet to be determined. Although the PCP pathway has not been directly elucidated in *Drosophila* trachea, it is probably similar to the PCP pathway demonstrated in the wing and eye (Adler 2012; Jenny 2010). Therefore, we propose that, at the AJ in the *Drosophila* trachea, Fmi binds to Fz in one cell and that Fmi binds to Vang in the neighboring cell. Then, Fmi/Fz recruits Dsh and Dgo and Fmi/Vang recruits Pk. Subsequently, the communications between these complexes restrict tube elongation through RhoA-mediated cytoskeleton rearrangement. Interestingly, a role for PCP genes in regulating tube length and diameter by orienting cell divisions was demonstrated in vertebrate renal and gut epithelia (Matsuyama et al. 2009; Saburi et al. 2008). Therefore, PCP signaling could have multiple roles in regulating tube size, controlling cell division and potentially also affecting the cytoskeleton as suggested by *Drosophila* trachea (Fig. 4).

In conclusion, the maintenance of apical-basal polarity and PCP are critical for tube length regulation through effects on the apical membrane and/or apical cytoskeleton. Although no direct evidence shows that these two polarities interact with each other in *Drosophila* trachea, crosstalk between the PCP and apical-basal polarity determinants has been suggested. For example, Fz activity or stability is regulated by phosphorylation mediated by aPKC and antagonized by Bazooka, whereas Scribble is part of the Stbm/Vang PCP complex and might act as an effector of Stbm/Vang during PCP establishment (Djiane et al. 2005; Courbard et al. 2009). Therefore, apical-basal polarity and PCP probably interact with each other to maintain cell polarity and subsequently to restrict tube over-elongation.

Role of Src42A in the initiation of tube elongation

In contrast to the relatively broad knowledge of diametric expansion, tube-diameter restriction and tube-length restriction, little is known about the initial step to tube elongation. Recent work on Src42A (one of the two *Drosophila* homologs), which is a non-receptor tyrosine kinase, sheds light on this process. Immediately after the multicellular branch DT forms a continuous tube, Src42A mediates cell-shape changes by promoting AJ remodeling through recycling of DE-cadherin (Forster and Luschnig 2012); meanwhile, it also promotes axially oriented expansion of apical surfaces by interacting with Daam, a Diaphanous-related formin that has a role in cytoskeletal arrangement (Nelson et al. 2012). The combination of AJ remodeling and cytoskeleton rearrangement triggers the initiation of tube elongation. However, the specific AJ remodeling that is required and the way that the cytoskeleton is rearranged are still unknown. Consistent with this function, vertebrate Src also interacts with Daam (Aspenstrom et al. 2006) and phosphorylates several proteins associated with the cytoskeleton and AJ (Thomas and Brugge 1997; Thomas and Brugge 1997). Therefore, tube elongation is initiated by Src42A-mediated AJ remodeling and cytoskeleton rearrangement and afterward, tube elongation is a continuous process, maintained by cell polarity and the luminal matrix to ensure proper length.

Role of the receptor tyrosine kinase pathways in diametric expansion in unicellular and intracellular tubes

To date, the mechanisms of tube-size regulation have largely been revealed by studying the multicellular tube DT. These mechanisms might also be used by other types of tubes. However, as tube structures vary (Table 1), branch-specific mechanisms probably exist. Recent studies have shown that receptor tyrosine kinase (RTK) pathways control tube size in a branch-specific manner. The loss of type-III-receptor-linked protein tyrosine phosphatases (RPTPs), Ptp4E and Ptp10D, selectively alters tube size in unicellular and intracellular branches but not multicellular DT (Jeon and Zinn 2009). RPTPs are single-span transmembrane receptors that reverse tyrosine phosphorylation events catalyzed by trans-

membrane or membrane-associated tyrosine kinases (Johnson and Van Vactor 2003). Three growth factor receptors, including the EGF receptor (EGFR), the FGF receptor (FGFR) and the Pvr (PDGFR/VEGFR/CSF1R ortholog) are targets of RPTPs; all three have orthologs in vertebrates (Morrison et al. 2000). The over-activation of these RTKs in the trachea via a mutation in RPTPs leads to diametric over-expansion in unicellular and intracellular tubes (Jeon et al. 2012).

Why does over-active RTK signaling only affect unicellular and intracellular tubes but not multicellular tubes? Unlike multicellular branches, which do not change their shapes radically, unicellular and intracellular branches undergo dramatic cell-shape changes during branch outgrowth. In unicellular branches, the apical surface coordinates its growth with the remodeling of the cell as a whole, wrapping around the luminal matrix and forming an autocellular junction. In terminal cells, the apical surface and lumen extend in synchrony with the rest of the cell. The negative regulation of RTK signaling is necessary for synchronizing apical membrane expansion with the remodeling of the rest of the cells, possibly through the regulation of the interaction between the apical membrane and cytoskeleton (Jeon et al. 2012). However, the nature of the downstream targets of RTK signaling and the manner in which these targets regulate the apical membrane and cytoskeleton remain unknown.

Summary and future directions

Research in the *Drosophila* trachea has provided novel insights into the role of the apical luminal matrix, cell polarity, secretion pathways and signaling components in tube-size regulation in an in vivo organism. In summary (Fig. 5), in order to expand a narrower and shorter multicellular tube to its mature diameter and length, apical secretion pulses result in a large amount of apical membrane and matrix components being secreted at discrete time intervals to expand the tube diameter and to assemble a luminal matrix. The insertion of an apical membrane is probably the driving force in tube dilation. Conversely, the luminal matrix restricts tube over-dilation.

In contrast to tube diameter expansion, tube elongation is a continuous process. To initiate tube elongation, the non-receptor tyrosine kinase Src42A mediates cytoskeleton rearrangement and AJ remodeling to allow the expansion of the apical surface along the longitudinal axis. Meanwhile, apical secretion mediates the insertion of apical membrane proteins. Counter to apical secretion, balanced internalization of the apical membrane and luminal matrix by the endocytosis pathway is required to maintain proper tube size. This balance is also critical to establish and maintain cell polarity by sustaining apical-basal polarity and PCP polarity determinants. To ensure proper tube length, several mechanisms have been developed to antagonize tube over-elongation. First, a specifically modified luminal chitin matrix by Vermiform and Serpentine probably interacts with the apical cytoskeleton to restrict tube length. Second, the basolateral polarity SJ components either antagonize apical membrane over-expansion or mediate apical secretion of chitin-modifying enzymes to limit tube elongation. Third, PCP signaling possibly regulates the apical cytoskeleton to limit apical membrane expansion and restrict tube length. Fourth, balanced endocytosis maintains membrane size and luminal matrix assembly to prevent tube over-elongation.

These mechanisms have been discovered in multicellular tubes and might be generalized to other types. However, because of structural differences, branch-specific mechanisms do indeed exist. For example, RTK signaling specifically regulates tube diameter in unicellular and intracellular tubes. This is probably because these tubes require dramatic cell-shape change, junction remodeling and cytoskeleton rearrangement during tube maturation compared with multicellular tubes.

Despite recent progress in our understanding of the mechanisms of tube-size regulation, several intriguing questions remain to be addressed. Although we have learned the way that apical secretion, endocytosis, cell polarity, luminal matrix and signaling pathways control tube size in isolation, these processes are probably dependent upon each other during the execution of their functions. For example, the secretion pathway is required to establish the cell polarity, whereas cell polarity, such as SJ, regulates the apical secretion of the luminal matrix. In addition, Src42A mediates AJ remodeling and cytoskeleton rearrangement to initiate apical membrane expansion. Crosstalk is likely between the cytoskeleton and cell polarity during tube expansion. Moreover, the luminal matrix also interacts with the cytoskeleton through unknown signals. Therefore, the manner in which these processes are integrated and coordinated into a network remains a central challenge. Furthermore, as tracheal cells are connected with each other to form a continuous tube, we need to investigate the way that epithelial cells communicate with each other to form a tube with the desired size. Solving these fundamental problems will improve our understanding of organogenesis, tissue function and various pathological states.

The structural similarity and gene conservation between the *Drosophila* trachea and mammalian tubular organs and the availability of genetic tools make the *Drosophila* trachea a sophisticated model for studying tube-size regulation. Previous investigations of *Drosophila* trachea have provided a general insight regarding epithelial tube morphogenesis. Many mechanisms and molecules are shared between the *Drosophila* trachea and vertebrate tubular organs. For example, the neural tube, lung and liver of many vertebrates form through apical constriction that is similar to the invagination of tracheal primordia (Colas and Schoenwolf 2001). In addition, FGF signaling plays a similar role in the branching of the mammalian lung, kidney and mammary glands as in *Drosophila* trachea (Sternlicht et al. 2006; Kato and Sekine 1999; Lebeche et al. 1999). Therefore, the mechanisms that regulate tube size in *Drosophila* trachea could be well conserved in mammalian tubular organs. Determination of whether the apical luminal matrix, cell polarity, secretion pathways and RTK signaling play similar roles in the vertebrate system will thus be of interest.

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Fig. 1.

Development of the *Drosophila* trachea. **a** Cross-section of tracheal tubes (*red* lumen, *blue* nucleus). Type I multicellular tubes, such as the dorsal trunk (DT) and transverse connective (TC), consist in two or more cells surrounding a central lumen and are sealed by intracellular junctions. Type II unicellular tubes, such as the dorsal branch (DB), lateral trunk (LT), ganglionic branch (GB) and visceral branch (VB) consist in a single cell that is wrapped around the lumen and that forms an autocellular junction with itself. Type III tubes are intracellular tubes formed by two donut-shaped cells without intracellular junctions. Type IV tubes, such as the lateral ganglionic (LG) are intracellular tubes formed by cytoplasmic extensions without intracellular junctions. **b** The cells within each tracheal sac that receive fibroblast growth factor (FGF) signaling and branch-specific signaling migrate out to form stereotyped branches. Cells that do not receive such signals remain at the site of invagination to form TC (*white*). Cells that receive transforming growth factor- (TGF- , DPP) signal (*blue*) at the dorsal and ventral sides migrate toward the FGF signal (*red*) to form the DB, LT and GB branches. In the central region of each sac, cells that receive the wingless (Wg) signal (*green*) migrate out to form DT; cells that do not receive the Wg signal (*yellow*) migrate internally to form VB. As the trachea develops, cells in the DB, LT and GB branches intercalate to form Type II unicellular branches, whereas DT and TC remain Type I multicellular tubes. Eventually, DT branches fuse with their neighboring branches to form the fully connected DT at stage 14. LT branches fuse with their neighboring branches to form LT at stage 15. DB and GB fuse at the dorsal and ventral midlines, respectively, with sister branches that emanate from the other side of the embryo at stage 16 (DB dorsal branch, DT dorsal trunk, LT lateral trunk, GB ganglionic branch, VB visceral branch, LG lateral ganglionic branch, TC transverse connective)

Fig. 2.

A model for tracheal tube maturation in *Drosophila* embryos. Expansion of the tracheal diameter and length during late embryogenesis (**a**, **b**) is mediated by apical membrane growth (*green*), transient fibrillar chitin-based matrix (*red*) assembly and reorganization of the apical cytoskeleton (*blue*). When a tube reaches its mature size, the luminal material is cleared by endocytosis (**c**). Subsequently, the luminal liquid is cleared from the lumen and air (*blue circles*) fills the tracheal tube (**d**)

Fig. 3.

Apical secretion and endocytosis of luminal and membrane components in the *Drosophila* trachea. Secretory components pass through the endoplasmic reticulum (ER) and Golgi via COPI and COPII complexes (*COPI*, *COPII*). After being secreted from the Golgi, post-Golgi vesicles are trafficked by distinct mechanisms (red apical membrane, blue basolateral membrane, AJ adherens junction). *Pathway a* Vermiform (*Verm*, orange rectangle) and Serpentine (*Serp*, blue rectangle) vesicles are trafficked to the septate junctions (SJ) at the basolateral side of tracheal cells and then they are secreted to the lumen by an unknown mechanism. *Pathway b* Vesicles (green circles/oval) containing 2A12 and Piopio (2A12 & Pio) are carried by the myosin V motor (gray ovals) along the apical actin cable (row of maroon-colored circles) and are then secreted to the lumen. *Pathway c* The vesicles (light purple triangles) that carry unknown luminal (light purple) and membrane (red lines) components are trafficked to the apical membrane and to the lumen by unknown mechanisms. In contrast to apical secretion, the endocytosis pathway internalizes membrane and luminal components. The vesicles (blue, green and light purple circles) carrying internalized luminal and membrane components (red lines) fuse with the early endosome (Early Endosome (Rab5)) and the late endosome (Late Endosome (Rab7)). Consequently, these vesicles then take on different routes: route 1 they fuse with the lysosome for degradation; route 2 like Serp, they fuse with the Golgi (mediated by Rab9), followed by apical secretion to the lumen; route 3 they fuse with the recycling endosome (Recycling Endosome (Rab11)), followed by secretion to the apical membrane or to the lumen

Fig. 4.

Apical-basal cell polarity and planar cell polarity (*PCP*) control tube length. The basolateral septate junction (*SJ*) restricts tube elongation either by controlling the secretion of chitin-modifying enzymes (*Verm* Vermiform, *Serp* Serpentine), which in turn restricts tube elongation (*black line*), or by antagonizing the over-expansion of the apical membrane (*green line*). A model of *PCP* in restricting tube length is proposed (*maroon lines/arrows*). At the adherens junction (*AJ*), Flamingo (*Fmi*) is bound to Frizzled (*Fz*) in one cell and *Fmi* is bound to Van Gogh (*Vang*) in the neighboring cell, then *Fmi/Fz* recruits Dishevelled (*Dsh*) and Diego (*Dgo*) and *Fmi/Vang* recruits Prickle (*Pk*). Eventually, the communication between these complexes controls the apical cytoskeleton (*orange row of ovals*) rearrangement, possibly through a downstream effector (*RhoA*). This cytoskeleton rearrangement restricts the axially oriented expansion of apical surfaces to restrict tube elongation (*red* apical membrane, *blue* basolateral membrane)

Fig. 5.

Summary of tracheal tube-size regulation. In multicellular tubes, during diametric expansion, the general apical secretion pathway delivers vesicles carrying membrane proteins (*red circles*) to the apical membrane (*red*) to drive tube dilation and vesicles carrying luminal components Serpentine (Serp) and Vermiform (Verm, *yellow circles*), chitin (*gray triangles*) and other luminal proteins (*blue circles*) to assemble the luminal matrix. To initiate tube elongation, the non-receptor tyrosine kinase Src42A mediates adherens junction (AJ) and cyto-skeleton (*orange row of ovals*) rearrangement to allow apical membrane expansion (*green arrows*). In addition, apical secretion probably mediates the insertion of apical membrane proteins (*green arrows*). To sustain proper tube length, several mechanisms have been developed (*black lines*). *Mechanism 1* Luminal chitin matrix modified by Verm and Serp (*yellow circles*) probably interacts with the apical cytoskeleton to restrict tube length. *Mechanism 2* Basolateral polarity of septate junction (SJ) components either antagonizes apical membrane over-expansion or mediates apical secretion of chitin-modifying enzymes to limit tube elongation. *Mechanism 3* Planar cell polarity (PCP) signaling probably regulates the apical cytoskeleton to limit apical membrane expansion to restrict tube length. *Mechanism 4* Endocytosis antagonizes apical secretion to sustain proper membrane size and matrix assembly. Potential interactions between the luminal matrix, cell polarity components, apical secretion pathway, endocytosis pathway and the cytoskeleton are indicated by *pink dashed lines*

Table 1

Summary of tracheal tube types in *Drosophila* trachea. Four types of tube exist in the mature *Drosophila* trachea. These tubes differ in their cellular make-up surrounding the central lumen, connections between these cells and the lumen type (*DT* dorsal trunk, *TC* transverse connective, *DB* dorsal branch, *LT* lateral trunk, *GB* ganglionic branch, *DB* dorsal branch, *VB* visceral branch)

Type	Cellular make-up surrounding the central lumen	Tracheal tubes	Connections of cells surrounding the lumen	Lumen
I	Multicellular	Type I (DT, TC)	Intercellular junction	Extracellular
II	Unicellular	Type II (LT, GB, DB, VB)	Autocellular junction	Extracellular
III	Donut-shaped cell	Type III (Connect type I or II)	Seamless, no intracellular junction	Intracellular
IV	Intracellular cytoplasmic extension	Type IV (LG)	Seamless, no intracellular junction	Intracellular