Airway maturation in *Drosophila*

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The cover shows a scanning electron micrograph of a *Drosophila* embryo merged with the embryonic trachea visualized by the luminal antigen 2A12.
“Man cannot discover new oceans unless he has the courage to lose sight of the shore.”
Andre Gide

To my family
ABSTRACT

Tubes are a fundamental unit of organ design. Most of our major organs like the lung, kidney and vasculature are composed primarily of tubes. To identify fundamental biological principles of tubular organ formation we used the respiratory organ of *Drosophila melanogaster*, the trachea.

This work dissects embryonic trachea maturation. Three precise epithelial transitions occur during airway maturation. A secretion burst deposits proteins into the lumen; then luminal material is cleared and finally liquid is removed. We identified the cellular mechanisms behind these transitions. Sar1 and γCOP are required for protein secretion, matrix assembly and tube expansion. Rab5-dependent endocytic activity internalizes and clears luminal contents. The data show how programmed transitions in cellular activities form functional airways, and may reflect a general mechanism in respiratory organ morphogenesis.

We further focused on tube size regulation. We identified Melanotransferrin, a new component of septate junctions that limits tracheal tube elongation. MTf is a lipid-modified, iron-binding protein attached to epithelial cell membranes, similarly to its human homologue. We show that septate junction assembly during epithelial maturation relies on endocytosis and apicolateral recycling of iron-bound MTf. Mouse MTf complements the defects of *Drosophila* MTf mutants. This provides the first genetic model for the functional dissection of MTf in epithelial morphogenesis.

In the last part, we describe two genes, which are selectively involved in tube diameter expansion. Obst-A and Gasp are closely related proteins with characteristic chitin-binding domains. They are strongly expressed in the trachea at the time of lumen expansion. The single and double mutants cause a tube diameter reduction, whereas their overexpression leads to its increase. We propose that Obst-A and Gasp organize luminal matrix assembly and thereby regulate the extent of tube diameter expansion.
LIST OF PUBLICATIONS


IV. Tiklová K., and Samakovlis C.  
Control of tube diameter expansion by secreted chitin-binding proteins. **Manuscript**
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<td>ANF</td>
<td>Atrial Natriuretic Factor</td>
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<td>atypical protein kinase C</td>
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INTRODUCTION

Tubular organization is a common feature of many tissues. Tubes can also represent a transient phase of organ development. The vertebrate neural tube initially forms as a simple columnar epithelium, but ultimately gives rise to the complex architecture of the brain and spinal cord. Tubular organization is quite useful and can serve many important physiological roles, including: control and delivery of gases, nutrients, waste and hormones. Many respiratory, circulatory, and secretory organs are built of networks of interconnected tubes. Tubular epithelial organs arise from each of the germ layers, ectoderm (e.g. mammary gland), mesoderm (e.g. kidney), and endoderm (e.g. lung). Many of the same cellular and molecular mechanisms are utilized during the formation, elongation and elaboration of endothelial tubes (Ellertsdottir, Lenard et al. 2010).

Studies of tube formation in complex vertebrate systems are difficult due to the large number of cells, specialisations along the tubes and variation in size along the tube (Buechner 2002). In order to identify fundamental biological principles for regulation of tube formation it is therefore advantageous to use simpler model systems, which are genetically favourable, such as the respiratory organ of the fruit fly, Drosophila melanogaster.

The Drosophila respiratory organ as a model system for epithelial tube morphogenesis

Several features of the tracheal system make it an excellent system for investigating the mechanisms of tubulogenesis and tube size control. First, the tubes are simple in structure. They are an epithelial monolayer and hence lack complexities introduced by the multilayer structure of most vertebrate tubes including blood vessels and the bronchial tubes of the lungs. Second, each tube is composed of only a small number of cells, each of which can be followed during development (Samakovlis, Hacohen et al. 1996). Finally, powerful genetic and genomic approaches can be applied to identify and analyze tubulogenesis genes.

Because fundamental mechanisms controlling epithelial tube outgrowth and branching are similar in Drosophila and vertebrates (Metzger and Krasnow 1999), it seems likely that other aspects of the process, including tube formation and size regulation will also be conserved.
**Tracheal morphology and development**

The respiratory organ of the fruit fly is called the trachea. The trachea is an air filled network of tubes that spans the entire organism. Air enters the network through specialized openings, called spiracles, and becomes distributed to all branches of the network, ending in narrow capillary-like, blind-ended tubes, which facilitate gas exchange with target tissues. To optimize the air supply, the tracheal branches must extend into all tissues and the tubes have to reach specific dimensions. This is achieved by a two-step process: first, a stereotyped larval tracheal network forms during embryogenesis, later, during larval growth, the network expands by the enlargement of the pre-existing branches and ramification of the blind-ended capillaries that infiltrate the new targets (Manning 1993).

The tracheal network is highly stereotyped, both in branch pattern and tube size. In contrast to more complex vertebrate systems, development of tracheal tubes does not involve cell proliferation or cell death, but relies on cell migration and changes in cell shape and cellular junctions (Samakovlis, Hacohen et al. 1996; Beitel and Krasnow 2000). Thus, the *Drosophila* trachea is well suited for elucidating the molecular and genetic mechanisms that underlie epithelial tube morphogenesis.

**Trachea branching**

Trachea originates from 20 independent units with similar branching pattern, 10 of each side of the embryo. Each tracheal metameric unit (named Tr1-Tr10) is initially an epithelial pocket formed by invagination of epidermal cells during stage 10, and contains approximately 80 cells. Tracheal precursors are specified in the ectoderm by the combined action of two transcription factors, Trachealess (Trh) and Ventral veinless (Vvl) (de Celis, Llimargas et al. 1995; Boube, Llimargas et al. 2000). Trachealess expression identifies the clusters of epidermal tracheal precursor cells and binds to its ubiquitous co-factor Tango (Tgo) (Ohshiro and Saigo 1997). In the next 12 hours and without further cell division the tracheal cells form invaginating pockets and undergo a synchronized program of branch outgrowth and branch fusion to generate a continuous tubular system (Samakovlis, Hacohen et al. 1996). During the branching process cells migrate and change their shape and relative positions.

Tracheal morphogenesis can be divided into the following phases: invagination, primary branch formation, secondary branch formation, terminal branch formation and branch fusion.
During the invagination the epithelial cells of the ectoderm form ingrowing pits towards the underlying mesoderm. After invagination the tracheal pits close, but remain connected to the epidermis through the cells of the spiracular branch. Each pit forms six buds, which will give rise to the primary branches. The six primary branches are termed dorsal trunk anterior (DTa), dorsal trunk posterior (DTp), dorsal branch (DB), visceral branch (VB), lateral trunk anterior (LTa) and lateral trunk posterior/ganglionic branch (LTp/GB) (Fig. 1). This commitment of cells to different branches is determined by the spatial expression of Decapentaplegic (Vincent, Ruberte et al. 1997), Rhomboid (Wappner, Gabay et al. 1997), Wingless (Wappner, Gabay et al. 1997; Chihara and Hayashi 2000; Llimargas 2000) and Hedgehog (Glazer and Shilo 2001) in the early embryo. All primary branches start growing as multicellular tubes. Outgrowth is initiated by the extension of broad cellular protrusions from the tip cells in each bud. DB, GB and VB continue to extend, developing into narrow primary branches. The conversion in the structure of the tubes involves changes in cell contacts and shapes.

As primary branches navigate towards the target, a distinct number of cells, typically located at the tip of each primary branch, sprout off and form smaller unicellular branches (Samakovlis, Hacohen et al. 1996). Some of them grow towards target tissues and form terminal branches.

The key inducer of tracheal primary branch growth is a member of the secreted fibroblast growth factor (FGF) family, Branchless (Bnl) (Sutherland, Samakovlis et al. 1996). Bnl is temporarily expressed in cell patches around each tracheal sack and as a chemoattractant promotes branch migration towards its source. Bnl activates the tracheal receptor tyrosine kinase Breathless (Btl; a Drosophila FGF receptor) (Klambt, Glazer et al. 1992), and the intracellular protein Downstream-of-FGF-receptor (Dof, Stumps) is specifically required for FGF signal transduction in Drosophila (Vincent, Ruberte et al. 1997; Imam, Sutherland et al. 1999). In mutants for bnl, btl or dof, only rudimentary primary branches form, whereas ectopic expression of Bnl in wild-type embryos can redirect primary branch growth towards its new site of expression. The cells that actively migrate are called the tip cells. Tip cells migrate and pull trailing stalk cells along behind. Cells must communicate with each other to sort into leading tip cells and trailing stalk cells, and this communication takes the form of competition. Tip cells are postulated to generate a lateral inhibitory cue proportional to the level of FGFR activity, and thus enforce the
follower stalk cell fate upon their neighbors. Notch is required cell autonomously in the stalk cells to restrict tip cell number (Ghabrial and Krasnow 2006).

In addition, the Rho-family GTPases, which are instrumental in the regulation of the cytoskeleton during cell migration, have been implicated in primary branching; overexpression of constitutively active Cdc42 in the trachea induces filopodial extensions, whereas expression of a dominant negative form of Cdc42 impedes branch extension (Wolf, Gerlach et al. 2002).

The subsequent movement of the cell body and growth of apical cell surface during primary branching appear to require transcriptional gene regulation involving Ribbon (Rib) and Grainyhead (Grh). Rib is a putative transcription factor expressed in all tracheal cells and, although basal filopodia towards the Bnl source still form in rib mutants, cell-body movement and lumen growth are aborted (Bradley and Andrew 2001; Shim, Blake et al. 2001). grh mutants on the other hand, complete branching morphogenesis but show overelongated and tortuous tubes. This defect is accompanied by a selective expansion of the apical membrane of the tracheal cells.

As primary branching proceeds, the individual branches grow towards, along and across different cell types, and some, including the DB GB and VB, develop into narrow extended type-II branches.

Secondary branching initiates at stage 14, several hours after initiation of primary budding. In each metamere, 23 of the 80 cells of the typical tracheal metamere form unicellular sprouts.

During secondary branching, Branchless/Breathless signalling is again used, but in a different manner. In this cases, it determines the acquisition of specific cell fates among the cells at the tip of each primary branch by inducing the expression of pantip markers such as the transcription factor Pointed (Pnt) at stage 13 (Scholz, Deatrick et al. 1993). The Pnt protein plays an essential role in the selection and identity of cells that undergo sprouting. It promotes the next stage of branching by activation of transcription of Drosophila serum response factor (DSRF) and inhibiting the expression of the fusion gene Escargot, in terminal cells (Samakovlis, Manning et al. 1996). Fusion cells expressing transcription factor Escargot (Esg) form the anastomoses that interconnect the hemisegments to the network (tube type III) (Samakovlis, Manning et al. 1996). Terminal cells expressing DSRF continue their journey towards target organs, which they penetrate by forming capillary tubes (tube type IV) (Guillemin, Groppe et al. 1996). Ramification of the terminal branches
during larval life is highly variable and regulated by the oxygen-needs of the tissue. Oxygen-deprived cells upregulate the expression of *branchless* and thereby induce branch migration, similar to VEGF-induced (Vascular endothelial growth factor) chemotaxis during angiogenesis in vertebrates (Shweiki, Itin et al. 1992; Jarecki, Johnson et al. 1999).

To form the functional tracheal network, branch fusion events have to interconnect the twenty independent metameric units into a continuous network. During stage 13 the DTa and DTP from neighboring ipsilateral metamers meet and form continuous dorsal trunk. Fusion cells form doughnut-shaped connections that lack autacellular junctions. The fusion event begins as the fusion cells extend broad cytoplasmic processes towards each other. Fusion cells recognize each other and establish an initial contact point. They then move closer to each other broadening their contact surface, and simultaneously forming a new lumen continuous with the primary branch lumen (Samakovlis, Manning et al. 1996). The earliest fusion cell-specific protein identified is the transcription factor Escargot. It is expressed in all five-fusion cells of the tracheal metamere and is required for three of five fusion events to occur (DB, LTA, LTP). During the fusion there is deposition of *DE*-cadherin (*DE*-cad) at the contact point between the two fusion partners (Tanaka-Matakatsu, Uemura et al. 1996). *DE*-cad is upregulated in fusion cells by Esg.
Figure 1. The Drosophila tracheal system
(a) Lateral view of a Drosophila embryo at stage 16 with the anterior part to the left. The tracheal lumen is visualised by the luminal marker 2A12. (b) Each tracheal branch is made up of six primary branches: the dorsal trunk (DT), dorsal branch (DB), visceral branch (VB), spiracular branch (SB), lateral trunk (LT) and ganglionic branch (GB).

Tube architecture
The fully developed embryonic trachea consists of a continuous single-layered epithelium of around 1600 cells. The apical cell surface is facing the lumen and the basal surface facing the internal tissues. The luminal surface is lined by a cuticle and function as barrier against dehydration and invading microorganisms.

We can distinguish four different tubular types, each of them associated with a distinct cellular architecture, diameter and length (Samakovlis, Hacohen et al. 1996; Uv, Cantera et al. 2003) (Fig. 2). Type I tubes are composed of flattened, wedge-shaped cells connected by intercellular cell junctions (dorsal trunk). The type II tube is also multicellular, but narrower and is represented by the dorsal, visceral and ganglionic branches. Each tracheal cell is placed in a “head to tail” arrangement along the length of these branches. A characteristic feature of these tubes are autocellular
junctions that run along the proximal-distal axis of the cells. Type III tubes are represented by the bicellular fusion-branches (anastomoses) that connect the individual metameres to a network. These tubes consist of a pair of doughnut-shaped cells, connected by intercellular junctions. Those tubes are seamless, without intracellular junctions. Type IV tubes are represented by the blind-ended tracheoles. These branches are extended, hollow protrusions emanating from a single terminal cell, and become increasingly narrower along their length. For comparison to other tubular organs, the first tube type is found in lungs, blood vessels and many glandular organs, whereas seamless tubes and tubes with autocellular junctions have been found in the vertebrate vasculature (Wolff and Bar 1972) and the *Caenorhabditis elegans* excretory cell (Buechner 2002).

**Figure 2. Tracheal tubular types**

In type I branches wedge-shaped cells connected by intercellular junctions surround the lumen circumference. Type II branches are formed by cells wrapped around the lumen and sealed with autocellular junctions. The cells are connected to the neighbours by intracellular junctions. Type III branches consists of two doughnut-shaped cells, connected by intercellular junctions. Type IV branches are single cell tubes without junctions. Adapted from (Uv, Cantera et al. 2003).
Lumen formation
Lumen can form by different mechanisms, including the wrapping, budding, cavitation, cord hollowing or cell hollowing (Lubarsky and Krasnow 2003).

Wrapping occurs when a portion of an epithelial sheet invaginates and curls until the edges of the invaginating region meet and seal, as during neural tube formation in many vertebrates (Colas and Schoenwolf 2001).

During budding, a group of cells in an existing epithelial tube (or sheet) migrates out and forms a new tube as the bud extends. The new tube is a direct extension of the original tube. This is how tubes arise during branching morphogenesis of many organs, including the mammalian lung and the major branches of the Drosophila tracheal (respiratory) system (Metzger and Krasnow 1999; Hogan and Kolodziej 2002).

In both of these mechanisms, tubes arise from a polarized epithelium. In the other three mechanisms described below, tubes arise from cells that are not epithelial, and the cells polarize as the tubes form.

During cavitation, cells organized in a mass create a central cavity by eliminating cells in the center of the mass, as occurs during salivary gland morphogenesis (Borghese 1950; Melnick and Jaskoll 2000).

In cord hollowing, a lumen is created de novo between cells in a thin cylindrical cord, without cell loss. Examples include the Caenorhabditis elegans gut (Leung, Hermann et al. 1999), the Drosophila heart and Madin-Darby canine kidney (MDCK) cells (Pollack, Runyan et al. 1998).

In cell hollowing, a lumen forms within the cytoplasm of a single cell, spanning the length of the cell. For instance, some capillary endothelial cells (Wolff and Bar 1972).

Tubes are generally very small when they form and must grow to achieve their mature size and shape. The expansion process can begin as soon as the lumen forms or can occur at specific times later in development.

The tracheal tubes of the tracheal branches are all narrow and of similar diameter when they form. Several hours later, they undergo an expansion that can triple the diameter of some of the branches. Just before expansion begins, multiple vesicle-like structures carrying an apical membrane antigen appear in the cytoplasm. These are presumably membrane vesicles, like those of MDCK and endothelial cells, which target and fuse to the apical surface, driving the apical membrane expansion that occurs during lumen growth. As the apical surface expands, the basal surface changes
little if at all, demonstrating that lumen expansion is not a process of generalized cell growth but rather a specific growth and remodeling of the apical surface. Two additional expansions later in development increase lumen diameter up to 40 times its original size. Expansion is not accompanied by cell proliferation, only by a large increase in apical surface area and dramatic thinning of the cells. Although apical membrane biogenesis is required for both lumen formation and expansion, it is not sufficient. There must also be a mechanism that drives apical membranes apart to keep the lumen open. Liquid secretion into the lumen may play such a role. Although tracheal tubes function in oxygen transport, liquid accumulates in the lumen when the tubes first form and during each expansion, only to be cleared after each expansion cycle so that oxygen can flow (Manning and Krasnow, 1993).

**Cell organization**
The tracheal epithelium derives from the blastoderm epithelium, which forms by a process known as cellularization. During cellularization the common plasma membrane that surrounds the syncytial embryo becomes subdivided into domains. Subdivided regions or domains fulfill specialized roles in cell organization and physiology. The main subdivisions of the plasma membrane are the apical, lateral and basal domain (Tepass, Tanentzapf et al. 2001).

In a tubular organ the apical domain faces the external environment of the lumen, the lateral domain contacts neighboring cells within the epithelial layer, and the basal domain is anchored to the basement membrane. Cohesion between epithelial cells relies pre- dominantly on the zonula adherens, a belt-like adherens junction (AJ) located at the interface between the apical and lateral membrane (Meng and Takeichi 2009). Other junctional complexes limit paracellular diffusion to support the barrier function and the selective permeability of epithelia. In most invertebrates, this function is mediated by the septate junction (SJ), which is most often located basal to the AJ (Tepass, Tanentzapf et al. 2001) (Fig. 3). In chordates, the intercellular space is sealed by tight junctions, which are apical to the zonula adherens (Tsukita, Furuse et al. 2001).
Figure 3. **Epithelial cell structure**
A schematic drawing of an epithelial cell with intercellular junctions. Right brackets indicate the positions of cuticle, MZ (marginal zone), AJ (adherens junctions), SJ (septate junctions) and basement membrane. Cuticle is composed of epicuticle and procuticle. Adapted from (Tepass, Tanentzapf et al. 2001).

**a) Apical domain**
The apical domain contains several polarity regulators, which form two complexes: Crumbs and Par complex.

The **Crumbs complex** is build around the transmembrane protein Crumbs (Crb), which binds to Stardust (Sdt; mammalian PALS1), Patj, Lin7, the FERM proteins Moesin and Yurt (Yrt; EBP41L5 in mammals) and βHeavy-Spectrin (Bulgakova and Knust 2009; Tepass 2009).

Both Crb and Sdt are essential for the maintenance of epithelial-cell polarity in the embryo (Tepass, Theres et al. 1990; Tepass and Knust 1993; Bachmann, Schneider et al. 2001), whereas other factors either have a more restricted function in epithelial differentiation (e.g. Yrt, Lin7) or their function within the Crb complex needs to be further clarified (e.g. Moesin, Patj, bHeavy-Spectrin) (Laprise and Tepass 2011).
The Crumbs complex is concentrated in a distinct region of the apical plasma membrane adjacent to the ZA; this region is called the subapical or marginal region in epithelial cells (Tepass 1996).

There are four main components of Par complex: atypical protein kinase C (aPKC), adaptor protein Par6, the small GTPase Cdc42, and the scaffolding protein Bazooka (Baz; the Drosophila homolog of the mammalian protein Par3) (Suzuki and Ohno 2006; Goldstein and Macara 2007). These proteins usually function together. However, Baz can also act independently of aPKC, Par6 and Cdc42 in epithelial polarity. The Par complex is a major determinant of apical-basal polarization and in epithelial cells it is required for apical localization of Crb (Bilder, Schober et al. 2003).

There are multiple interactions between members of the Crumbs complex and the Par complex. The PDZ domain of the scaffolding protein Par-6 can bind to Crb (Kempkens, Medina et al. 2006), to the evolutionarily conserved regions of Sdt (Wang, Hurd et al. 2004), and to the PDZ domain of PATJ (Nam and Choi 2003). Both of those complexes are needed both for the establishment of the apical-basal polarity and for formation of AJPs (Muller and Wieschaus 1996).

b) AJ Complex

Adherens junctions (AJs) are cell–cell adhesion complexes that provide a defining feature of epithelial tissues and make important contributions to homeostasis (Gumbiner 2005; Halbleib and Nelson 2006). Cadherin adhesion molecules are core AJ components (Takeichi 1991). Most classic cadherins operate as homophilic adhesion receptors to facilitate cell–cell recognition and adhesion. All cadherins contain two or more extracellular cadherin domains. Classic cadherins additionally have a highly conserved cytoplasmic tail that interacts with a defined set of cytoplasmic proteins — the catenins. p120 catenin and β-catenin (called Armadillo in Drosophila melanogaster) bind the cytoplasmic tail of cadherins, and β-catenin binds to α-catenin to form the cadherin–catenin complex (Pokutta and Weis 2007; Nishimura and Takeichi 2009). A second protein complex, the nectin–afadin complex, can also be an integral part of AJs. Cadherin clustering could be promoted by lateral interactions between cadherin extracellular regions or through cytoplasmic interactions that link cadherins into a supramolecular complex (Troyanovsky 2005; Kovacs and Yap 2008). The catenins
make key links from cadherins to actin and microtubules. The relationship is mutual in both cases, actin and microtubules support AJ formation and stability, and AJs can organize actin and microtubules.

\textit{β-catenin (Armadillo).} The cytoplasmic tail of classic cadherins becomes structured only when bound to \(β\)-catenin (Huber, Stewart et al. 2001), an association that begins in the endoplasmic reticulum and is required for the effective surface transport of cadherin through the biosynthetic pathway (Chen, Stewart et al. 1999; Lock and Stow 2005). Once at the plasma membrane, the cadherin–\(β\)-catenin complex rapidly recruits \(α\)-catenin (Bajpai, Correia et al. 2008).

\textit{p120 catenin.} p120 catenin is a positive regulator of cadherin function. The cadherin–p120 catenin interaction counteracts cadherin endocytosis and degradation, and thus enhances the surface abundance of cadherin (Ireton, Davis et al. 2002; Davis, Ireton et al. 2003; Ishiyama, Lee et al. 2010). p120 catenin also links cadherin to microtubules.

\textit{α-catenin.} \(α\)-catenin is essential for AJ formation and function and operates at the interface of the cadherin–catenin complex and the actin cytoskeleton (Hirano, Kimoto et al. 1992; Kobielak and Fuchs 2004). How \(α\)-catenin acts at AJs is currently not well understood. \(α\)-catenin binds to \(β\)-catenin and can, at least \textit{in vitro}, directly bind actin filaments (Rimm, Koslov et al. 1995).

There is a general model for the interplay between actin and cadherin–catenin clusters during AJ assembly. First, actin produces cell protrusions that initiate cadherin clustering. Second, actin polymerization and reorganization are coupled to cell contact growth and the aggregation of cadherin–catenin clusters into AJs. Third, multiple actin networks associate with cadherin–catenin clusters at mature AJs to hold them in place.

AJs also associate with microtubules (Stehbens, Akhmanova et al. 2009). In \textit{Drosophila}, microtubules have a central role in AJ assembly. During cellularization, cells are forced into contact by the invagination of lateral membranes from the embryo surface, forming the first embryonic epithelium (Harris, Sawyer et al. 2009). Cadherin–catenin clusters first form between apical microvilli and then shift to the apicolateral domain to form spot AJs (Tepass and Hartenstein 1994; McGill, McKinley et al. 2009). Bazooka plays a role in relocalization of the cadherin–catenin clusters to the apicolateral domain (Muller and Wieschaus 1996; McGill, McKinley et al. 2009).
In addition to forming AJs, cells must disassemble and remodel AJs during tissue morphogenesis. Endocytosis is a major mechanism of AJ remodeling (Delva and Kowalczyk 2009; Wirtz-Peitz and Zallen 2009).

Figure 4. **Organization of Adherens junction and its cytoskeletal relationship**

The main cytoplasmic binding partners of cadherins are p120 catenin, β-catenin and α-catenin. The main cytoplasmic binding partner of nectin is afadin. The relationship of these proteins to the cytoskeleton is shown. Adapted from (Harris and Tepass 2010).

c) **SJ complex**

A second type of insect epithelial junctions is localized on the lateral membrane and is called septate junctions (SJ). SJs first appear midway through embryogenesis, after cellularization is completed, epithelial polarity has been established, and the ZA has formed (Tepass, Tanentzapf et al. 2001). The SJ lies just basal to the ZA in epithelial cells, and within the SJ the membranes of adjacent cells maintain a constant distance of approximately 15 nm. In the pleated SJ (found in ectodermally derived epithelia and the glia sheets), regular arrays of electron-dense septae span the intermembranal space. The septae form circumferential spirals around the cell much like the threads of a screw, and increase the distance that molecules must travel to pass between the apical and basolateral compartments of the epithelial sheet. This regular alignment pattern of both the membranes and the septae gives the junction a ladder-like appearance (Tepass and Hartenstein 1994). Smooth SJs, which lack these ladder-like structures, are found only in the midgut and its derivatives.

Apart from their role in transepithelial diffusion barrier formation (Lamb, Ward et al. 1998), SJs have been proposed to play a role in cell adhesion (Tepass, Tanentzapf et al. 2001), in the formation of the blood–brain barrier (BBB) (Schwabe, Bainton et al.
SJs may play also a role in the maintenance of cell polarity, because loss of the Dlg component in the imaginal cells results in the loss of polarity (Woods and Bryant 1991). However SJs are not required for the initial polarization of the epithelial cells in the embryos as evidenced by the normal epithelial polarization in embryos that lack SJ genes, such as mega (Behr, Riedel et al. 2003) and nrv2 (Paul, Ternet et al. 2003). Insect SJs have a function analogous to vertebrate tight junctions. TJs also form a diffusion barrier that prevents water and solute exchange across epithelia (Tepass, Tanentzapf et al. 2001; Tsukita, Furuie et al. 2001). Tight junctions are located apical of the adherens junctions, whereas SJs are basal. Despite morphological and molecular differences between SJs and TJs (Anderson 2001; Tepass, Tanentzapf et al. 2001), the identifications of two claudin-like Drosophila SJ proteins, Sinuous (Sinu) and Megatrachea (Mega), suggests that the barrier functions of septate and tight junctions could have a common biochemical basis and evolutionary origin (Behr, Riedel et al. 2003; Wu and Beitel 2004).

Identified SJ components are summarized in Table 1. Among these is the Lethal giant larvae/Discs large/Scribble (Lgl/Dlg/Scrib) complex (Tepass, Tanentzapf et al. 2001), which has an early role in establishing apical/basal polarity. The SJ components have been recently subdivided to the core complex formed by at least 8 members: ATPç, Cora, Mega, Nrg, Nrv2, Nrx IV, Sinu and Vari. The SJ proteins are initially highly mobile on the lateral membrane at stage 12, but in stage 13 they form the stable core complex (Oshima and Fehon 2011). Loss of SJ components dramatically affects the mobility of SJ core complex (Laval, Bel et al. 2008; Oshima and Fehon 2011). Lys-6 family proteins (Boudin, Crooked, Coiled, Crimped), which are essential for SJ formation and maintenance but do not localize to the SJ, have also a function in the formation of the SJ core complex. Loss of them increases the mobility. The endocytosis and the apical-basal polarity Lgl-group proteins do not affect the mobility of the SJ core components. This divides the SJ assembly to two processes: the formation of SJ core complex and its localization to the apico-lateral membrane. Endocytosis and Lgl group proteins play function in the localization part (Oshima and Fehon 2011).
<table>
<thead>
<tr>
<th>Gene product</th>
<th>Protein domains</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
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<td><strong>Drosophila</strong></td>
<td><strong>Vertebrate</strong></td>
</tr>
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<td>P-type ATPase</td>
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<tr>
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<td>Cell-adhesion, SJ formation</td>
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<td>Contactin</td>
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</tr>
<tr>
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<td>PDZ, SH3, GUK</td>
<td>SJ formation, tumor suppressor</td>
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<td>PDZ</td>
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</tr>
<tr>
<td>Varicose</td>
<td>PALS2</td>
<td>HOOK, L27, PDZ, GUK, SH3</td>
<td>SJ formation</td>
</tr>
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</table>

Table 1. List of Septate junction related molecule
d) Basal membrane
Most information about the basal membrane of epithelial cells and ECM, which is in contact with basal membrane, comes from oocyte follicle cells and cell cultures studies. Interactions between the epithelial cells and BM are mediated by a variety of cell surface receptors including integrins and Dystroglycan (Yurchenco, Amenta et al. 2004).

The basement membrane (BM) is a highly specialized form of extracellular matrix found on the basal side of polarized animal epithelia (Quondamatteo 2002). The BM is composed primarily of the secreted glycoproteins Laminin, Collagen IV, and Nidogen and the heparan sulfate proteoglycan Perlecan. Evidence from genetic analyses in model organisms and experiments in cultured cells has indicated an important role for the BM in generating epithelial cell polarity (O'Brien, Zegers et al. 2002; Li, Edgar et al. 2003). The accumulation of BM on the basal side of the epithelium is crucial to convey the positional information to maintain a polarized architecture.

e) Apical extracellular matrix and tracheal lumen
The apical surface of the tracheal cells is covered by the cuticle, which is deposited during late embryogenesis. The cuticle is an ECM produced by the epidermis at its apical site and is characterized by a layered organization.

The first step in cuticle deposition is the formation of an outer impermeable envelope. The envelope protects the tissue from dehydration. Subsequently, the middle protein-rich epicuticle responsible for cuticle stiffness is formed and, finally, an innermost procuticle loaded with lamellar linear chitin is assembled to confer stability and elasticity to the cuticle (Locke 2001).

Tracheal cuticle forms prominent ridges that are called taenidial folds and are projected inside the lumen. They form annular rings or run a helical course around the tracheal lumen. The taenidial pattern can differ between branches and even along the length of the branch. Taenidia are believed to allow the tubes to be flexible while simultaneously providing strength against collapse. Taenidial spacing expands as the tube elongate (Glasheen, Robbins et al. 2010).

Next to the taenidial chitin, there is also a transient chitin accumulation inside the growing tracheal tubes. Already at the stage of tube expansion the tracheal lumen...
contains chitin fibers running along the length of the tubes. The uniform growth of this tracheal luminal filament has been shown to assist the coordinated tracheal tube dilation (Tonning, Hemphala et al. 2005). At the end of embryogenesis chitin fibers start loosing their continuity (Moussian, Seifarth et al. 2006) and the luminal contents will be endocytosed (Tsarouhas, Senti et al. 2007). At stage 17 the lumen of the trachea is completely cleared and the trachea will fill with air.

Chitin is synthesized by chitin synthase, which is called Krotzkopf verkehrt (kkv) in flies. Kkv is a transmembrane enzyme that links cytosolic UDP-N-acetylglucosamine (UDP-N-GlcNAc) into long GlcNAc polymers (chitin) and extrudes them across the plasma membrane (Tonning, Hemphala et al. 2005). After chitin is synthesized it has to be arranged to stereotypic structure. An essential chitin-organizing factor is Knickkopf (Knk) that is inserted via a GPI-anchor in the apical plasma membrane. The proposed function of Knk is the release of chitin fibers from the chitin synthase complex (Moussian, Tang et al. 2006). Another membrane-inserted protein needed for correct chitin organization is Retroactive (Rtv). Rtv may bind chitin and assist orienting chitin microfibrils at the surface of chitin producing cell (Moussian, Soding et al. 2005). Chitin is also modified by chitin deacetylases Serpentine (Serp) and Vermiform (Verm), which are secreted molecules (Luschnig, Batz et al. 2006; Wang, Jayaram et al. 2006). Presumably the modification of chitin including deacetylation is a prerequisite for its abilities to interact with luminal proteins and to form filaments, as the phenotypes of serp and verm double mutant and knk or rtv mutants are similar.

**Protein secretion**

The analysis of many mutants in chitin biosynthesis indicates that the complex intraluminal matrix acts as a mold for tube growth. How are all those matrix components delivered to the right place to form the functional trachea?

The synthesis of all proteins begins on ribosomes in the cytosol, except for the few that are synthesized on the ribosomes of mitochondria. Their subsequent fate depends on their amino acid sequence, which can contain sorting signals that direct their delivery to specific locations. Transmembrane and secreted proteins have signal sequences mostly at the N-terminus. When the ER signal sequence is synthesized, it directs the ribosome to the ER. The ER membrane contains translocators, which form
pores in the membrane through which the polypeptide is translocated into the ER lumen. As they are synthesized, these proteins translocate into the ER lumen, where they are glycosylated and acquire their three dimensional folding. Misfolded proteins are identified and retrotranslocated to the cytosol, where they become degraded by the proteasome. The vesicles containing the properly folded proteins then enter the Golgi apparatus. In the Golgi apparatus, the glycosylation of the proteins is modified and further posttranslational modifications may occur.

Transport between the ER and Golgi compartments is facilitated by specialized vesicles. These vesicles are surrounded by distinctive coating proteins called coatomer protein complex-I (COP1) and COPII. COPII mediates anterograde transport from the ER to either the ER-Golgi intermediate compartment (ERGIC) or the Golgi complex (Barlowe, Orci et al. 1994), while COP1 is involved in intra-Golgi transport and retrograde transport from the Golgi to the ER (Letourneur, Gaynor et al. 1994) (Fig. 5).

The core **COPII** components are the small Ras-like GTPase Sar1, the Sec23/Sec24 subcomplex, and the Sec13/Sec31 subcomplex (Barlowe, Orci et al. 1994). The COPII coat assembles by the stepwise deposition of Sar1GTP, Sec23/Sec24, and Sec13/Sec31 onto sites, where newly synthesized proteins exit from the ER. Sar1GTP together with Sec23/Sec24 forms pre-budding complex (Lederkremer, Cheng et al. 2001). Sec23 makes direct contact with Sar1GTP (Bi, Corpina et al. 2002), while Sec24 participates in cargo recognition. Sec23 stimulates the GTP hydrolysis activity of Sar1 (Yoshihisa, Barlowe et al. 1993). This activity of Sec23 as a GTPase-activating protein (GAP) is increased approximately ten-fold by addition of Sec13/Sec31 (Antonny, Madden et al. 2001).

The majority of cargo proteins are actively concentrated in COPII-coated vesicles prior to export from the ER (Malkus, Jiang et al. 2002). Most transmembrane cargo proteins exit the ER by binding directly to COPII (Aridor, Weissman et al. 1998; Kuehn, Herrmann et al. 1998), but some transmembrane and most soluble cargo proteins bind indirectly to COPII through transmembrane export receptors (Muniz, Nuoffer et al. 2000; Powers and Barlowe 2002). Export receptors leave the ER together with their ligands, unload their cargo into the acceptor compartment, and recycle back to the ER. The sorting signals recognized by the COPII coat are found in
the cytosolic domains of transmembrane cargo proteins. These signals are quite diverse (Barlowe 2003).

**COPI** vesicles are involved in intra-Golgi transport and retrograde transport. Their assembly involves activation and membrane recruitment of GTPase ADP ribosylation factor (Arf) (Donaldson, Cassel et al. 1992). During COPI coat assembly; ArfGTP simultaneously recruits the membrane-proximal $\beta\gamma\delta\zeta$ and the membrane-distal $\alpha\beta\varepsilon$ subcomplexes (Hara-Kuge, Kuge et al. 1994), in contrast to the stepwise assembly of COPII. COPI entry requires specific signals in the cytosolic domains of transmembrane cargo proteins. These signals function to retrieve proteins from the ERGIC or the Golgi complex to the ER (Cosson and Letourneur 1994).

Once the proteins reach the trans-Golgi network (TGN), they are again sorted into vesicles by intrinsic sorting motifs and cytoplasmic adaptor complexes, and are transported along cytoskeletal elements directly to the apical or basolateral plasma membrane.

The adaptor complex crucial for the transport between the Golgi, the plasma membrane and endosomes is called the **adaptor protein (AP)–clathrin complex** (the AP–clathrin complex). Four AP complexes (AP-1–4) have been identified, and these localize to different membrane compartments between the Golgi complex, the plasma membrane and endosomes (Robinson 2004). Each AP complex is composed of four subunits. One subunit mediates the binding to the target membrane, the other one recruits clathrin through the clathrin binding sequence (Brodsky, Chen et al. 2001), the third subunit is responsible for cargo recognition and the last subunit is involved in the stabilization of the complex (Collins, McCoy et al. 2002).

**Vesicle transport**

Each vesicle transport event can be divided into four essential steps that include vesicle budding, transport, tethering, and fusion (Bonifacino and Glick 2004).

Vesicle budding is mediated by protein coats (Kirchhausen 2000; Bonifacino and Lippincott-Schwartz 2003). Protein coats are dynamic structures that cycle on and off membranes. They are recruited from the cytosol onto donor membranes by small GTPases that regulate their assembly (Springer, Spang et al. 1999). Coats deform flat membranes into round buds, which lead to the release of coated vesicles. Coat proteins
also participate in cargo selection through the recognition of sorting signals present in the cytoplasmic domain of transmembrane cargo proteins. Clathrin was the first coat to be identified (Pearse 1975). After budding, vesicles are transported to their final destination by diffusion or by motor-mediated transport along a cytoskeletal track (microtubules or actin). The molecular motors kinesin, dynein, and myosin have all been implicated in this process (Hammer and Wu 2002; Matanis, Akhmanova et al. 2002).

The third step in vesicle-mediated membrane traffic is tethering. Tethering is a term used to describe the initial interaction between a vesicle and its target membrane. It precedes the pairing of transmembrane SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) on apposing membranes, an event that leads to membrane fusion (Sollner, Whiteheart et al. 1993). Together with Rabs, small GTPases of the Ras superfamily, tethers play a critical role in determining the specificity of vesicle targeting. Rabs continuously cycle between the cytosol and membranes. In the cytosol, the GDP-bound form of the Rab is complexed with GDI (guanine nucleotide dissociation inhibitor). Rabs are recruited to membranes with the aid of a GDF (GDI displacement factor) (Dirac-Svejstrup, Sumizawa et al. 1997). The membrane-bound Rab is then activated by a specific GEF (guanine nucleotide exchange factor) through the exchange of GDP for GTP (Ullrich, Horiuchi et al. 1994). Rabs in their GTP-bound form appear to facilitate the recruitment of tethers to specific locations. Activation of the Rab is terminated when GTP hydrolysis is stimulated by a specific GAP (GTPase-activating protein) (Rybin, Ullrich et al. 1996).

To date more than 60 Rab proteins have been identified in mammalian cells. The last step in vesicle-mediated transport is the fusion of the vesicle with its target membrane. Fusion is thought to occur by the pairing of SNAREs, a family of membrane proteins that are related to three different neuronal proteins: synaptobrevin, syntaxin, and SNAP-25. A SNARE on a transport vesicle (v-SNARE) pairs with its cognate SNARE-binding partner (t-SNARE) on the appropriate target membrane (Sollner, Whiteheart et al. 1993).

Protein trafficking is crucial for building the polarized cells. Like other transmembrane proteins, cadherins are synthesized in the ER, modified in the Golgi, and trafficked to the basolateral cell surface by the exocytosis. Once at adherens junctions, cadherins
can be endocytosed into Rab5-positive early endosomes, and either recycled back to adherens junctions via Rab11-positive recycling endosomes, or sent via the Rab7- and Hrs-positive multivesicular body to lysosomes for destruction (Duncan and Peifer 2008). When the exocyst function is reduced, cadherin accumulates in Rab11 recycling endosomes (Langevin, Morgan et al. 2005). This is an example how important is the protein trafficking through the right vesicles, to get the functional cell with proper junctional complex.

Figure 5. Post-translational pathway for protein trafficking to the plasma membrane
After synthesis in the endoplasmic reticulum (ER), membrane proteins are sorted into vesicles by the coatomer protein complex-II (COPII) machinery and delivered to the Golgi complex by vesicle-tethering and SNARE machineries. Intra-Golgi transport and retrograde transport from the Golgi to the ER are regulated by the COPI machinery. At the trans-Golgi network (TGN), proteins are sorted into different vesicles by intrinsic sorting motifs and cytoplasmic adaptor complexes, and are transported along cytoskeletal elements to the plasma membrane or different compartments. Clathrin coats are heterogeneous and contain different adaptor and accessory proteins at different membranes. Additional coats or coat-like complexes exist but are not represented in this figure. EE - early endosome, RE – recycling endosome, LE –late endosome, SG – secretory granule, PM – plasma membrane. Adapted from (Bonifacino and Glick 2004).
Control of tracheal tube size

An important step in trachea tube morphogenesis is the acquisition of the proper length and diameter. Tube size is critical for organ function therefore it must be controlled not only during tube formation but also throughout development in order to maintain functionality as the animal grows. The diameter and length of the tracheal branches are controlled independently. The tube diameter expansion occurs step-wise, whereas the length of the branches increases continuously through development.

Tube diameter grows at three brief periods of development, during which the branches expand to their characteristic sizes (Beitel and Krasnow 2000). These sizes are genetically programmed, and some of the genes in the program have been identified. Cell number does not directly determine tracheal tube size. There is not a correlation between the size of a tracheal tube and the number of cells in it, and altering cell number using cell cycle mutations did not affect tube size. Furthermore, the dramatic expansions of the tracheal tubes in the embryo and larva occurred without changing cell number, and tube sizes were altered in tube expansion mutants without affecting cell number. Tracheal cells in a given branch must collectively adjust their shapes to form a tube of the specified size. One idea is that the program dictates branch size by controlling the amount of apical membrane biogenesis and secretion for each branch. To this group belongs transcription factor Grh. In grh mutants, excessive apical membrane forms, resulting in elongated and convoluted tubes, whereas tracheal over expression of Grh prevents lumen extension (Hemphala, Uv et al. 2003).

The next group of genes involved in tube size regulation is the genes involved in luminal matrix formation. The matrix is transiently secreted into the lumen, and is composed of fibrillar chitin. Mutations of genes involved in chitin biogenesis and assembly (krotzkopf verkehrt, mummy/cystic, knickkopf, retroactive) result in irregular diametric expansion of the tracheal tubes with local constrictions and dilations (Araujo, Aslam et al. 2005; Tonning, Hemphala et al. 2005; Moussian, Tang et al. 2006). In addition, these mutants develop overelongated tracheal branches. Also two putative chitin deacetylases, Vermiform and Serpentine, which are secreted into the lumen and assumed to modify chitin structure, regulate tracheal length (Luschnig, Batz et al. 2006; Wang, Jayaram et al. 2006). This suggest that the chitin matrix
provides either a physical scaffold for the underlying epithelia or it sends signals to the tracheal cells to adjust tube shape and size.

Another group of tube size mutants includes genes encoding septate junction components. These mutants develop over-elongated tubes with irregular cell shapes. Most of the septate junction mutants show impaired secretion of Vermiform and Serpentine and a defect in the assembly of the luminal chitin filaments. Given that \textit{verm} and \textit{serp} mutants also show tortuous tubes, the function of SJ proteins in tube size control may be to facilitate the apical secretion of chitin modifying enzymes. An additional mechanism by which SJ proteins may control tube size is, through the regulation of apical polarity proteins such as Crumbs and aPKC (Wu and Beitel 2004). The reduction in the levels of the apical membrane determinant Crumbs suppresses the tube length defects in \textit{cora} mutants. In addition, \textit{crb} mutations suppress \textit{cora} length defects without restoring Verm secretion. Similarly, mutations in \textit{yurt} and \textit{scribble} cause an expansion of tracheal tubes but do not disrupt Verm secretion. Reducing the activity of Crumbs suppresses the length defects in \textit{yurt} but not in \textit{scribble} mutants, suggesting that Yurt acts by negatively regulating Crumbs. Yrt, Cora, Crb, and Scrib operate independently of the Verm pathway, where Cora and Yrt act through Crb to regulate epithelial tube size (Laprise, Paul et al. 2010).

The tight control of tube expansion suggests that tubes can sense their size and inhibit expansion when a specified size is reached. The sensor and output pathway have to be still identified in the tracheal system.
Figure 6. **Model of the pathways involved in tube size regulation by SJ proteins**

Interplay between Yrt, Cora, and Crb modulates the dimensions of the apical surface of tracheal cells to control tracheal tube size. This mechanism acts independently of and in parallel to a pathway depending on the apical secretion of the matrix-modifying enzymes Verm and Serp, which requires several SJ-associated proteins. There can be one more mechanism regulating the tube size, acting through Scrib. *scrib* mutants have long trachea with normal Verm levels and the defects are not suppressed by loss of Crb, like in *cora* and *yrt* mutants. Adapted from (Laprise, Paul et al. 2010).
AIMS

The aim of the work described in this thesis was to:

1) Dissect respiratory organ maturation in the embryonic trachea
2) Identify genes, which are required for tracheal tube size control
3) Address the mechanism of septate junction assembly
RESULTS AND DISCUSSION

Paper I: Sequential Pulses of Apical Epithelial Secretion and Endocytosis Drive Airway Maturation in Drosophila

To gain insight into Drosophila tracheal maturation, we used a combination of live imaging and genetic analysis. Those methods allowed us to analyze the diametric expansion of the narrow tracheal branches to their final size and the final clearance of the tubes from matrix and liquid at the end of embryogenesis.

We used live imaging of secreted GFP-tagged proteins and analysis of mutants with defects in gas filling. These experiments revealed the distinct steps of airway maturation: 1) secretion burst followed by tube diameter expansion, 2) clearance of solid luminal material, 3) replacement of luminal liquid by gas.

1) Sar-1 mediated luminal deposition of secreted proteins, drives tube expansion

ANF-GFP (rat Atrial Natriuretic Factor fused to GFP) was used as a heterologous sand Gasp-GFP as an endogenous secreted marker. Both of them were predominantly localized in the cytoplasm at early stage 13. At late stage 13 there was a sudden secretion burst that deposited those markers into the tracheal lumen. 30 minutes after the secretion burst tube diameter began to expand.

We found that the small GTPase Sar1 is required for efficient secretion into the tracheal lumen. In the absence of Sar1 luminal secretion of ANF-GFP, Gasp-GFP, Gasp and other endogenous proteins like 2A12 and Verm was incomplete and secreted proteins remained in the cytoplasm. The consequence of incomplete secretion was the failure of tube diameter expansion.

Sar1 regulates COPII vesicles budding from the ER to the Golgi complex (Bonifacino and Glick 2004; Abrams and Andrew 2005). sar1 mutant showed disrupted ER structures and loss of Golgi structures. To test if there is general requirement of COPII complex, two additional COPII coat subunits sec13 and sec23 were analyzed for protein secretion and tube expansion phenotypes (Abrams and Andrew 2005). Both mutants had the same phenotypes like sar1 mutant. This showed the requirement of secretion apparatus for efficient protein secretion, which consequently drives tube
expansion. We proposed three ways by which the secretion burst drives tube expansion: 1) by deposition of apical membrane, 2) by deposition of a secreted expanding matrix that molds the growth of the tubes 3) by targeting of apical channels or other regulators that may inflate the lumen. The secretory burst depends on COPII complex, which can be important for transport of unknown regulators of tube expansion, or add additional membrane to the growing luminal surface. The other hypothesis is that the luminal deposition of chitin binding proteins during the secretory burst can organize the construction and swelling of luminal matrix, which in turn induces lumen diameter expansion.

2) A Rab-5 dependent endocytic wave clears the lumen of solid material
At stage 16 the dorsal trunk lumen contains multiple proteins and a chitinous cable, which have been deposited during the secretion phase. To follow the fate of the luminal proteins, we used live imaging of ANF-GFP and Gasp-GFP. At stage 16 the lumen contained high levels of ANF-GFP. At mid-stage 17 luminal ANF-GFP rapidly decreased to very low levels. The same was observed for Gasp-GFP, which was first accumulated in the lumen and than was removed similarly to ANF-GFP. We also tested if the endogenous protein Gasp and the fluid-phase marker dexran were taken up into tracheal cells during the clearance. For the dextran we established a live endocytosis assay. Dextran was injected inside the hemocoel of stage 13 embryos, which SJs are not formed yet, and accumulated inside the tracheal lumen. Both endogenous Gasp and dextran were cleared from the DT lumen. This confirmed the ANF-GFP and Gasp-GFP results. To prove that the dextran detected inside the cell during the luminal clearance is coming from lumen and not from the hemocoel, we injected rhodamine-labeled dextran into the hemocoel of late stage-16 embryos. At stage 16 paracellular barriers are formed and prevent the luminal access of the 10 kDa dextran (Lamb, Ward et al. 1998). We did not find any dextran inside the tracheal cells at stage 17. This demonstrates that the transient intracellular dextran puncta in embryos preloaded at stage 13 indeed derive from the lumen. Transmission Electron Microscopy also showed that the luminal chitinous cable disappeared within the interval of ANF-GFP clearance.
What is the mechanism of protein clearance? We found that Rab5, dynamin and clathrin are required to remove the luminal contents, indicating that the endocytosis is required for this process. Dynamin encodes the GTPase that clips Clathrin-coated pits off the membrane to form endocytic vesicles (van der Bliek and Meyerowitz 1991). Rab5 encodes GTPase that regulated this process (Bucci, Parton et al. 1992).

In all three mutants ANF-GFP, Gasp-GFP, endogenous Gasp, Verm, 10 kDa dextran and chitin have been retained inside the tracheal tubes. In the wild type situation the material, which is endocytosed during the wave of endocytosis, colocalized with the endocytic markers inside tracheal cells. The most pronounced colocalization was with clathrin coated vesicles, late endosomes and lysosomal markers. The lysosomal localization indicated that the endocytosed proteins from the tracheal lumen might be degraded inside the tracheal cells during the protein clearance. In rab5 mutant we observed defects in those endocytic compartments during the period of protein clearance but the secretion burst and the diameter expansion have been not affected. The apically localized dotted pattern of Clathrin heavy chain was lost, GFP-FYVE endosomal apical vesicles were absent and also the Rab7 late endosomal structures were decreased in intensity. Those defects argue for role of endocytosis in direct internalization of luminal material.

3) The transition from liquid- to gas-filled trachea

After the protein clearance step, the liquid has to be removed from the lumen to allow the trachea to be gas filled and become functional. To follow this process, late stage 17 embryos have been recorded by live imaging. In a stereotypic way, a bubble of a so far unknown gas appeared in one of the dorsal trunk metamers (4-6) and quickly expanded first posteriorly and then anteriorly. Within 10 min, the entire trachea was filled with gas. Approximately 80 min later the mature larva hatched.

The tracheal maturation steps are interdependent in a sequential manner. Efficient secretion is a prerequisite for the endocytic wave. Similarly, protein endocytosis is a condition for luminal liquid clearance. This suggests a hierarchical coupling of the initiation of each pulse to completion of the previous one and provides an example of how pulses of epithelial activity drive distinct developmental events to form functional tracheal tube.
**Paper II: COPI Vesicle Transport Is a Common Requirement for Tube Expansion in *Drosophila***

The second paper follows the studies from the first paper, where we analyzed the function of anterograde COPII transport in the tracheal formation. COPII anterograde transport mediates luminal deposition of secreted proteins, what drives tube expansion. Here we focused our analysis on the retrograde transport which is mediated by COPI vesicles.

COPII vesicles bud from the ER and shuttle nascent secreted proteins to the Golgi apparatus. COPI coated vesicles retrieve escaped luminal ER enzymes and recycling cargo adaptor proteins from the Golgi back to the ER (Bonifacino and Glick 2004). The coatomer complex of COPI vesicles is composed of two different layers. The tetrameric subcomplex of the inner layer is formed from four subunits: β, δ, γ, ζ. The β- and γ- subunits bind to active Arf1-GTP, γCOP also binds to p23. The outer trimeric coat layer contains three subunits: α, β’, ε (Bethune, Wieland et al. 2006).

**Tube diameter expansion is dependent on COPI vesicles**

In the P-element screen for tracheal tube size mutants we identified the γCOPP1 mutant. By imprecise excision we generated a null allele, which we used for our analysis. In γCOP mutants the tracheal dorsal trunk diameter was reduced by ~50% compared to wild type embryos. To follow the diameter expansion defects in time we imaged live wild type and γCOP mutant embryos expressing btl>GFP-CAAX. Wild type embryos initiate tracheal tube diameter expansion at stage 14. γCOP mutant embryos already showed a narrower tube diameter at stage 14 and later expanded their tubular diameter with a slower rate compared to wild type.

γCOP is also expressed in other ectodermally derived tissues: salivary glands and epidermis from stage 11, hindgut and foregut from stage 16. To see if γCOP is required for diameter expansion in other tubular organs, we measured tube diameter of salivary glands. Like in tracheal tubes, the salivary glands of γCOP mutants were thinner compared to wild type. Both trachea- and salivary glands- tube diameter phenotype can be rescued by re-expression of γCOP in the mutants. Those results
suggest that \( \gamma \text{COP} \) is crucial for tube expansion in tubular organs and there is a common cellular mechanism, which expands tubular organs.

**COPI dependent secretion drives the tube diameter expansion**

Diametric expansion is mediated by the secretory burst of luminal proteins at stage 13 in tracheal tube (Tsarouhas, Senti et al. 2007). We tested if the tracheal diameter defects in \( \gamma \text{COP} \) mutant are caused by defects in luminal protein secretion. Three tested secreted proteins 2A12, Gasp and Verm were localized to the tracheal lumen in wild type embryos at stage 15, but strongly retained inside tracheal cells in \( \gamma \text{COP} \) mutants. Those proteins are binding and modifying the luminal chitin. Transmission electron microscopy and luminal chitin visualization in the tracheal tube revealed the change of chitin structure in \( \gamma \text{COP} \) mutant. The chitin was denser in mutant compared to the wild type embryos at stage 16.

The salivary gland lumen is also filled with a luminal matrix. The composition of this matrix is not well known; we know that it contains glycans with a single N-acetyl-Galactosamine O-linked to Serine or Threonine (Tian and Ten Hagen 2007), which can be visualized by antibodies recognizing the Tn antigen. First, the Tn antigen is localized inside the cells, followed by luminal localization at stage 12 and 13, when the salivary gland diameter expansion occurred. \( \gamma \text{COP} \) mutants showed reduced cellular accumulation and luminal deposition of the antigen. Similar defects were detected in \( \text{sar1} \) mutants. Transmission electron microscopy analysis confirmed the change of salivary gland luminal matrix in \( \gamma \text{COP} \) mutant, where the material was deformed and abnormally dense. Those results suggest the requirement of COPI and COPII for efficient luminal deposition and diameter expansion.

COPI vesicle coat consists next to the \( \gamma \text{COP} \) of another six subunits. We tested if another COPI vesicle coat subunit: \( \delta \text{COP} \), plays similar function in the protein secretion and tube expansion. Both Verm and Gasp retained in the tracheal cells in \( \delta \text{COP} \) mutant and tube expansion failed both in tracheal- and salivary gland tube. The secretion defects of COPI vesicle mutants can be indirectly caused by defects in epithelial polarization. The tested apical membrane, adherens- and septate- junctions
were not affected in those mutants arguing that the tube diameter reduction is due to insufficient secretion of luminal components.

**Defects in the secretion apparatus underly the tube size defects**

We examined the cellular localization of γCOP in *Drosophila* cells and the integrity of the secretion apparatus in γCOP mutant. A γCOP antibody showed punctuate cytoplasmic signal in S2 cells. Those punctuated structures were identified as ER and cis-Golgi units by co-localization of γCOP with ER markers KDEL (the ER retention signal found in many ER proteins) and Calreticulin, and cis-Golgi markers GM130 and Lava lamp.

Because of the localization of γCOP in the ER and cis-Golgi, we analyzed those structures in γCOP mutant. γCOP mutant embryos showed a substantial reduction of the ER markers: KDEL, Calreticulin, the transmembrane adaptor, p23 and a cis-Golgi marker, Lava lamp both in the trachea and salivary glands. TEM analysis additionally revealed bloated ER structures in the γCOP mutants. Those defects are indicative of an activated unfolded protein response (UPR). The UPR activation was further confirmed by the presence of processed *xbp1* transcripts in γCOP mutants.

Our analysis indicated that COPI vesicles are required for ER integrity. Once the ER integrity and vesicle transport are disrupted, the luminal proteins cannot be secreted leading to a tube diameter reduction. The same cellular defects were detected in the mutants affecting COPII vesicles (Tsarouhas, Senti et al. 2007). The double mutant of COPII and COPI components: *sar1*, γCOP did not show any additive phenotype in the trachea, indicating that anterograde COPII- and retrograde COPI-mediated transports are equally important to maintain both ER and Golgi structures. The bidirectional ER-Golgi traffic is essential for secretion and assembly of luminal matrixes during tube expansion.
Paper III: Epithelial Septate Junction Assembly Relies on Melanotransferrin Iron Binding and Endocytosis in Drosophila

Analysis of Drosophila mutants revealed two groups of tracheal tube length mutants. One group of mutants relates to apical membrane growth and includes two transcription factors: ribbon (rib) and grainy head (grh). rib mutants fail to extend the tracheal lumens, as the apical membrane growth is limited (Shim, Blake et al. 2001), grh mutants have excessive apical membrane, resulting in elongated and convoluted tubes, whereas tracheal over-expression of Grh prevents lumen extension as in rib mutants (Hemphala, Uv et al. 2003).

Another group of tube size mutants, disrupt genes encoding septate junction components. The tubes of these mutants become overelongated and convoluted with irregular cell shapes. The first septate junction mutant discovered to have a tracheal tube size phenotype was ATPα, which lacks a functional α-subunit of the Na+/K+ ATPase (Hemphala, Uv et al. 2003). Subsequently, mutations in other 14 genes have been found which showed a similar tracheal tube size phenotype. Much has been learned about the components of the septate junctions, but the mechanism of their assembly into functional junctions was unknown.

Melanotransferrin is a new septate junction component

Melanotransferrin (MTf) is expressed in all ectodermal epithelial tissues and is phylogenetically conserved between mammals and Drosophila. MTf mutants have overelongated tracheal tubes, resembling the defects of mutants lacking septate junctions. Expression of the Drosophila or the mouse MTf in the trachea rescued the tracheal phenotype of mutant Drosophila embryos arguing that the function of the protein is conserved. Further analysis of the cellular phenotypes of MTf mutant embryos showed that the septate junction epithelial barrier function was disrupted, the secretion of Vermiform failed and that septate junction components were mislocalized.

To confirm our hypothesis that MTf is a new septate junction component, we raised antibodies against the MTf protein. MTf colocalized with other septate junction markers. Its localization was dependent on other septate junction components since
MTf was spread basolaterally in cora and ATPα mutants. Finally, we showed that MTf forms complexes with Nrx, Cont and Nrg during late embryogenesis. We concluded that MTf is a septate junction component required for junction integrity.

**Endocytosis is required for functional septate junction formation**

During our analysis of MTf function we found that MTf and other septate junction components were localized along the lateral and basal epithelial cell membranes and partially in the vesicular structures inside the cell during stage 13, before septate junctions form. In contrast to stage 13, there is restricted apicolateral localization at stage 16. We wanted to know how are septate junctions assembled into functional junctions and what is the mechanism that drives their formation.

Substantial portion of MTf already formed complexes with Cont and Nrx at early stages. MTf and another septate junction component, Nrg colocalized with endosomal markers, and were also co-purifying with Rab5 early- and Rab11 recycling-endosomes at stage 13. At stage 16 when septate junctions were fully functional, we did not detect this co-localization.

The potential role of endocytosis in septate junction formation was confirmed when the septate junction components were found mislocalized in endocytic mutants (like clathrin heavy chain, dynamin, and in embryos expressing dominant negative forms of rab5 and rab11). The results suggested that septate junction components are relocalized from basolateral to the apicolateral membrane through early and recycling endosomes.

**MTf iron binding is crucial for septate junction assembly**

To elucidate the function of MTf in septate junction assembly, we examined its conserved motifs. By using phoshatidylinositol-phospholipase Cγ we confirmed that MTf is a GPI-linked membrane protein. The significance of the GPI-modification motif was tested in over-expression experiments by expressing MTf with deleted GPI-motif in MTf mutant embryos. This construct rescued septate junction defects in MTf mutants, suggesting that GPI-motif is not essential for MTf function.
MTf has two iron binding domains with conserved residues, which were expected to mediate the iron binding. The iron binding capacity of MTf was tested by two different methods: atomic absorption spectroscopy and electron paramagnetic resonance. Both methods confirmed the iron binding of wild type MTf. The mutations of the conserved residues Y231F in the N-terminal lobe reduced iron binding, whereas the Y533F in the C-terminal lobe abolished it. Expression of the Y231F construct rescued the MTf mutant phenotypes including the septate junctions defects, but the iron-deficient Y533F construct did not rescue those defects. This indicates that iron binding is critical for MTf function and septate junction maturation.

We tested if iron homeostasis is defective in MTf mutants, by monitoring ferritin levels. Ferritin is an iron storage molecule and its levels indicate aberrant iron concentrations (Hentze, Muckenthaler et al. 2004). MTf mutants did not show changes in ferritin levels suggesting that MTf has not a major role in iron uptake; its iron binding is specifically required for septate junction maturation.

**Uptake of MTf induces septate junction assembly**

Requirement of endocytosis for septate junction formation, made us to investigate if endocytosis of iron bound MTf is sufficient to induce septate junction assembly in epithelial cells lacking MTf. Wild type MTf, MTfΔGPI and MTfY533FΔGPI were expressed in epidermal stripes of MTf mutant using en-GAL4 driver. We could detect MTfΔGPI also in cells not expressing en-GAL4, but not the wild type MTf. Cells, lacking MTf, were binding and internalizing exogenous secreted MTfΔGPI. In those cells MTfΔGPI localized along the lateral membranes and in recycling endosomes, suggesting that it is endocytosed. The iron depleted form of MTf the MTfY533FΔGPI was not detected in non expressing en-GAL4 cells, indicating that MTf internalization requires iron bound MTf. MTfΔGPI, which localized in the en-GAL4 not expressing MTf mutant cells, also rescued the Nrx mislocalization in those cells. The results indicate that endocytosis of the iron binding form of MTf is sufficient to induce septate junction assembly in MTf mutant cells.
Paper IV: Control of Tube Diameter Expansion by Secreted Chitin-binding Proteins

The Obstructor multigene family

Despite that tube size is critical for the organ function, the cellular mechanisms of tube expansion are not clear. Tracheal tube expansion involves apical secretion and deposition of luminal matrix, but the mechanistic role and the nature of the forces involved are not clear.

In our studies we analyzed two members of the Obstructor multigene family, Obst-A and Gasp. Phylogenetic analysis showed, that Obst-A and Gasp are the most closely related genes among the members of the Obstructor gene family. Also their protein motif arrangement is the same: a signal peptide, followed by three chitin-binding domains type 2. This suggests that Obst-A and Gasp can have similar function. Obst and Gasp are expressed in the trachea, at the time of diametric tube expansion and cuticle deposition; therefore we analyzed their function in cuticle formation and tracheal maturation.

The Gasp expression pattern is very similar to the pattern detected with the widely used 2A12 antibody, which recognizes the unknown antigen. Gasp and the unknown antigen are first localized in the cells and become later secreted into the lumen. The similarity made us to test if the 2A12 antibody shows staining in gasp mutant embryos. We did not detect any signal both by 2A12 and Gasp antibodies in the mutants. When we ectopically expressed Gasp protein in the dorsal part of the hindgut, and found that both 2A12 and Gasp antibodies could recognize this protein. This argues that the unknown antigen which 2A12 antibody recognizes is Gasp.

Body size, cuticle and tracheal luminal matrix are disrupted in obst-A and gasp mutants

For our analysis we used null mutants of both genes. We found that single obst-A and gasp mutants showed reduced larval length: 8% in obst-A mutant and 14% in gasp mutant. The phenotype became enhanced in the obst-A, gasp double mutant to 25% length reduction compared to the wild type. The enhanced defects of obst-A, gasp
double mutants were also detected when we examined cuticle preparations of first instar larva. Single mutants showed normal cuticular structures but the cuticles of the double mutant were dilated. Those results suggest that Obst-A and Gasp have overlapping partially redundant functions.

The presence of the chitin binding domains in Obst-A and Gasp proteins led us to examine the luminal and taenidial chitin in the trachea. Labeling with a chitin-binding probe revealed that the single mutants showed a reduced luminal chitin cable compared to the wild type embryos. In double mutants the reduction in intensity of the chitin labeling was more severe than in the single mutants. We also detected a reduction in the luminal stainings for the chitin-modifying enzyme Verm. This showed that the luminal chitinal matrix is defective in obst-A, gasp mutants.

Do also the taenidia, which contain chitin, display any phenotypes? We analyzed the mutant taenidia and their chitin containing procuticular structure by TEM. This revealed an irregular shape and size of the taenidial folds and a defective procuticular layer in the obst-A, gasp double mutant. Collectively, the structure of both luminal chitin and taenidial procuticle are affected in the mutants lacking Obst-A and Gasp. These two chitin-binding proteins may help to form and assemble the chitin filaments into higher order structures.

**Obst-A and Gasp regulate the tracheal tube diameter**

Both Obst-A and Gasp are secreted into the tracheal lumen, during diameter tube expansion. Is luminal diameter expansion influenced by the amount of secreted proteins and chitin structure? To address this question, we measured the tracheal tube diameter both in obst-A and gasp single mutants, and obst-A, gasp double mutant. We labeled the apical and lateral membranes with Crumbs and α-Spectrin. Both obst-A and gasp single mutants showed a minor reduction in DT diameter. obst-A mutant 5% and gasp mutant 10% reduction compared to the wild type embryos at stage 16. This phenotype became enhanced in the double mutant to 15%. This suggests, that Obst-A and Gasp proteins are required for tube diameter expansion. The two proteins presumably modify the structure of the luminal chitin filament and provide a mold or an inflating force for tube expansion.
To test if an increase in luminal levels of chitin binding proteins may influence tube expansion, we overexpressed *obst*-A or *gasp* in the trachea. The measurements showed a minor increase of tube diameter, 7% in Obst-A and 13% in Gasp overexpressed embryos. This supports the idea that the tube expansion can be regulated by the amounts of secreted proteins.

**CONCLUSIONS AND PERSPECTIVES**

In our work we made several important findings. In paper I, we dissected the process of airway maturation and identified three precise epithelial transitions: deposition of proteins into the tracheal lumen which we named secretion burst, this is followed by luminal protein clearance and shortly thereafter by luminal liquid clearance. We also identified genes, which are required for those transitions. Sar1 (paper I) and γCOP (paper II) are required for secretion burst and Rab5 (paper I) for protein clearance. All three transitions are prerequisites for gas filling and functional trachea. We still have to find out the genes, which drive the third step of trachea maturation the luminal liquid clearance, which is followed by gas filling. To identify such genes we could perform screens for mutants that fail in liquid clearance without showing defects in the secretion burst and the protein clearance processes. This way we will specifically find genes regulating only gas filling process.

There is a class of proteins that likely regulate the liquid clearance and gas filling process, the epithelial sodium channels (ENaCs). In the lung the influx of sodium through these channels drives water from the lumen into the epithelial cells. RNAi inactivation of *Drosophila* homologs leads to liquid clearance phenotypes. This suggested that a similar process could be involved in the embryonic liquid clearance of the airways.

The three epithelial transitions are highly coordinated and occur at specific time points. What is regulating this sequence in such precise manner? Probably there are some signaling pathways activated at every step of the maturation. The secretion burst can be activated by signals from the apical membrane. Once the apical membrane reaches a certain size, which can be monitored by the apical cytoskeleton, the lumen needs to also expand. A sudden secretion of luminal proteins drives this expansion. Once the lumen reaches certain size, we believe that ECM sensors on the apical membrane initiate clearance of the luminal material to allow the tube to be gas filled. In this case again the apical membrane triggers the luminal
clearance, by sensing the tube size and activating the Rab5-dependent endocytic activity, which internalizes and clears luminal contents.

An important step in testing these models is the identification of tube size regulators. Here, we identified mutants in MTf (paper III), which show overelongated tracheal tubes. Melanotransferrin is in complex with other septate junction components and is required to form the epithelial barrier. Melanotransferrin binds iron and the iron binding is required for septate junction assembly.

We explored the mechanism by which septate junctions assemble to functional complex. The assembly relies on endocytosis and apicolateral recycling of septate junction components. First, septate junction components are localized along the basolateral membrane where they form complexes, later they are endocytosed and relocalized to apicolateral region, where they form mature functional junctions. The relocalization of septate junction components is iron dependent. We speculate that the iron triggers the SJ complex formation and later has a function in stabilization of the complex. Once the iron binds to MTf, MTf can increase its affinity to other SJ molecules and this initiates the formation of the complex. The assembled complex can trigger endocytosis and its re-localization to apicolateral membrane. The targeting to apicolateral membrane can be guided by signals from AJs or apical membrane. The next possibility is the presence of specific SNARES at the vesicles and targeted membrane domain. The identification of such targeting molecules could be accomplished by the isolation and biochemical analysis of the vesicles containing SJ proteins. We also found that mouse Melanotransferrin can replace the Drosophila Melanotransferrin function. It will be of a great interest to investigate if the tight junctions, the homolog of septate junctions, assemble in a similar fashion like septate junctions.

The diametric expansion of the tubes during development is critically important for airway function. In our studies we found several genes, which are required for tube diameter expansion. During the secretion burst, Sar1 and γCOP facilitate protein transport from ER to Golgi and vice versa. In both sar1 and γCOP mutants secretion is disrupted, which causes narrow tracheal tubes. It has been shown that sec24, a cargo-binding subunit of the COPII complex can support secretion and local tube expansion in a cell-autonomous fashion.

Another class of genes, which encode regulators of tube expansion, is Obst-A and Gasp, which we identified in paper IV. Those genes belong to Obstructor multigene family, are
strongly expressed in the trachea and encode proteins that are secreted into the tracheal lumen during the secretion burst. Impairment of their secretion leads to tracheal diameter reduction. We showed that secreted molecules are regulating tube expansion, which could be based on the generation of intra-luminal forces. The next step would be to measure the intra-luminal forces and find the receptors, which respond to those forces. The forces could be measured by visualization of the apical cytoskeleton and its localized changes. Forces are probably generated by luminal chitin, which expands after being modified. It could be possible to reconstitute chitin modification in vitro, by applying chitin-modifying proteins on chitin. This will allow testing directly if the chitin-modifying proteins play a role in inducing changes in the biophysical properties of the polymers and in local force generation.
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Sequential Pulses of Apical Epithelial Secretion and Endocytosis Drive Airway Maturation in Drosophila

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SUMMARY

The development of air-filled respiratory organs is crucial for survival at birth. We used a combination of live imaging and genetic analysis to dissect respiratory organ maturation in the embryonic Drosophila trachea. We found that tracheal tube maturation entails three precise epithelial transitions. Initially, a secretion burst deposits proteins into the lumen. Solid luminal material is then rapidly cleared from the tubes, and shortly thereafter liquid is removed. To elucidate the cellular mechanisms behind these transitions, we identified gas-filling-deficient mutants showing narrow or protein-clogged tubes. These mutations either disrupt endoplasmic reticulum-to-Golgi vesicle transport or endocytosis. First, Sar1 is required for protein secretion, luminal matrix assembly, and diametric tube expansion. Subsequently, a sharp pulse of Rab5-dependent endocytic activity rapidly internalizes and clears luminal contents. The coordination of luminal matrix secretion and endocytosis may be a general mechanism in tubular organ morphogenesis and maturation.

INTRODUCTION

Branched tubular organs are essential for oxygen and nutrient transport. Such organs include the blood circulatory system, the lung and kidney in mammals, and the tracheal respiratory system in insects. The optimal flow of transported fluids depends on the uniform length and diameter of the constituting tubes in the network. Alterations in the distinct tube shapes and sizes cause pronounced defects in animal physiology and lead to serious pathological conditions. For example, tube overgrowth and cyst formation in the collecting duct are intimately linked to the pathology of Autosomal Dominant Polycystic Kidney Disease. Conversely, stenoses, the abnormal narrowing of blood vessels and other tubular organs, are associated with ischemias and organ obstructions (Lubarsky and Krasnow, 2003; Sadler, 1985).

While the early steps of differentiation, lumen formation, and branch patterning begin to be elucidated in several tubular organs (Affolter et al., 2003; Hogan and Kolodziej, 2002), we only have scarce glimpses into the cellular events of lumen expansion and tubular organ maturation. De novo lumen formation can be induced in three-dimensional cultures of MDCK cells. Recent studies in this system revealed that PTEN activation, apical cell membrane polarization, and Cdc42 activation are key events in lumen formation in vitro (Martin-Belmonte et al., 2007). In zebrafish embryos and cultured human endothelial cells, capillary vessels form through the coalescence and growth of intracellular pinocytic vesicles (Kamei et al., 2006). These tubular vacuoles then fuse with the plasma membranes to form a continuous extracellular lumen. Salivary gland extension in Drosophila requires the transcriptional upregulation of the apical membrane determinant Crumbs (Crb) (Myat and Andrew, 2002), but the cellular mechanism leading to gland expansion remains unclear.

The epithelial cells of the Drosophila tracheal network form tubes of different sizes and cellular architecture, and they provide a genetically amenable system for the investigation of branched tubular organ morphogenesis. Tracheal development begins during the second half of embryogenesis when 20 metameric placodes invaginate from the epidermis. Through a series of stereotypic branching and fusion events, the tracheal epithelial cells generate a tubular network extending branches to all embryonic tissues. In contrast to the wealth of knowledge about tracheal patterning and branching (Ghabrial et al., 2003; Uv et al., 2003), the later events of morphogenesis and tube maturation into functional airways have yet to be elucidated. As the nascent, liquid-filled tracheal network develops, the epithelial cells deposit an apical chitinous matrix into the lumen. The assembly of this intraluminal polysaccharide cable coordinates uniform tube growth (reviewed by Swanson and Beitel, 2006). Two luminal, putative chitin deacetylases, Vermiform (Verm) and Serpentine (Serp), are selectively required for termination of
branch elongation. The analysis of *verm* and *serp* mutants indicates that modifications in the rigidity of the matrix are sensed by the surrounding epithelium to restrict tube length (Luschnig et al., 2006; Wang et al., 2006). What drives the diametric expansion of the emerging narrow branches to their final size? How are the matrix- and liquid-filled tracheal tubes cleared at the end of embryogenesis?

Here, we use live imaging of secreted GFP-tagged proteins to identify the cellular mechanisms transforming the tracheal tubes into a functional respiratory organ. We characterize the precise sequence and cellular dynamics of a secretory and an endocytic pulse that precede the rapid liquid clearance and gas filling of the network. Analysis of mutants with defects in gas filling reveals three distinct but functionally connected steps of airway maturation. Sar1-mediated luminal deposition of secreted proteins is tightly coupled with the expansion of the intraluminal matrix and tube diameter. Subsequently, a Rab5-dependent endocytotic wave frees the lumen of solid material within 30 min. We show that the precise coordination of secretory and endocytotic activities first direct tube diameter growth and then ensure lumen clearance to generate functional airways.

**RESULTS**

**Live Imaging of Airway Maturation**

We established a live-imaging approach to analyze the maturation of the *Drosophila* tracheal network. Using *btl*-GAL4, we coexpressed heterologous or endogenous GFP-tagged secreted molecules along with cytoplasmic RFP reporters to label tracheal cells (Shiga et al., 1996). Live embryos were then imaged by wide-field or confocal microscopy.

ANF-GFP (rat Atrial Natriuretic Factor fused to GFP) serves as a functionally inert, heterologous secretion marker in *Drosophila* (Rao et al., 2001). We recorded the development of the dorsal trunk (DT) of embryos expressing *btl* > ANF-GFP in real time (*n* = 237). Tracheal cells were marked by the (A) *btl*-mRFP1-moe or (B–D) *btl* > myr-mRFP1 transgenes (magenta). SEM denotes the standard error of means. Scale bars are 10 µm in (A)–(D) and 20 µm in (E).

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Figure 1. Identification of Four Distinct Events in Tracheal Tube Maturation

(A and B) Selected images from two time-lapse confocal movies of live wild-type *btl* > ANF-GFP embryos showing the (A) luminal deposition burst of ANF-GFP at 10.5 hr after egg laying (AEL) and the (B) luminal ANF-GFP clearance at 19 hr (green) (Movies S1 and S2).

(C and D) (C) Deposition and (D) clearance of Gasp-GFP in *btl* > Gasp-GFP embryos (green) (Movies S3 and S4).

(E) Two selected images from a wide-field movie illustrating the liquid clearance/gas filling of a wild-type embryo. The upper panel shows initiation of the gas filling (first gas bubble), while the lower panel shows its completion, when all tracheal branches are gas filled (Movie S5).

(F) Summary of recordings showing the maturation events of embryos expressing *btl* > ANF-GFP in real time (*n* = 237).

Tracheal cells were marked by the (A) *btl*-mRFP1-moe or (B–D) *btl* > myr-mRFP1 transgenes (magenta). SEM denotes the standard error of means. Scale bars are 10 µm in (A)–(D) and 20 µm in (E).
Embryos expressing ANF-GFP signal residest mostly in the lumen, and, concomitantly, diametric expansion ended. Thus, ANF-GFP reports a sudden luminal deposition event.

At stage 16, the DT lumen contains multiple proteins and a chitinous cable (Devine et al., 2005; Tonning et al., 2005). To follow the fate of the luminal proteins, we imaged embryos expressing ANF-GFP and the membrane marker myr-RFP in the trachea from stage 16 onward (Figure 1B; Movie S2). Initially, high levels of ANF-GFP filled the lumen. At mid-stage 17, luminal ANF-GFP rapidly declined within 24 min to very low levels. In contrast, the cytoplasmic ANF-GFP levels remained constant and low. Therefore, the dramatic drop in luminal ANF-GFP intensity highlights a wave of luminal protein clearance.

How does the dynamic ANF-GFP deposition and clearance relate to endogenous luminal markers? Live imaging of embryos expressing the luminal protein Gasp tagged to GFP (Barry et al., 1999) shows that Gasp-GFP first accumulates in the lumen and is then removed similarly to ANF-GFP (Figures 1C and 1D; Movies S3 and S4). The dynamic localization of GFP-tagged variants of Verm or Serp was identical (data not shown). Furthermore, analysis of fixed embryos shows that endogenous Verm and Gasp are first deposited and then cleared from the tracheal lumen very much like ANF-GFP (Figure S1). Finally, we fixed btI-GFP embryos at set times before and after the luminal ANF-GFP clearance (18 hr and 20 hr after egg laying [AEL]) for Transmission Electron Microscopy (TEM). Also, this assay showed that the luminal chitinous cable disappeared within the interval of ANF-GFP clearance (Figure S1). Thus, ANF-GFP faithfully reports the rapid and massive deposition of endogenous luminal content at stage 13 and its complete removal at mid-stage 17.

To visualize the transition from liquid- to gas-filled trachea, we recorded late stage-17 embryos (Figure 1E; Movie S5). In a stereotypic sequence, a bubble of a so far unknown gas appears stochastically in one of the DT central metameres (4–6) and quickly expands first posteriorly and then anteriorly. The bubble then spreads through the posterior anastomosis to the contralateral DT, where it again rapidly extends anteriorly. Within 10 min, the entire tracheal tree fills with gas. Approximately 80 min later, the mature larva hatches.

To embed the single steps in a developmental framework, we recorded multiple embryos expressing btI-GFP and timed each of the morphological changes. All transitions occurred robustly within a defined, precise time interval (Figure 1F; Experimental Procedures).

Taken together, our live-imaging approach identifies three dramatic and dynamic changes in luminal morphology that divide tracheal maturation into precisely timed steps. A secretory burst deposits large quantities of luminal proteins into the tracheal lumen and tightly precedes tube diameter expansion. The tracheal epithelium then clears these proteins and liquid by two sequential pulse-like events generating functional, mature airways. What are the underlying cellular and molecular mechanisms executing the rapid transitions? What is the functional relevance of the transitions for airway maturation? We addressed those questions by analyzing mutants with defects in gas filling.

**sar1 Is Required for Efficient Secretion into the Tracheal Lumen**

We identified two strong, hypomorphic sar1 alleles in screens for mutants with tracheal tube defects (see Experimental Procedures and Figure S2). *sar1* encodes a small GTPase that regulates COPII vesicle budding from the endoplasmic reticulum (ER) to the Golgi apparatus (reviewed in Bonifacino and Glick, 2004). In wild-type embryos, the bulk of luminal markers 2A12, Verm, and Gasp has been deposited inside the DT lumen by stage 15 (Figures 2A, 2D, and 2G). However, in zygotic *sar1* mutants (hereafter referred to as *sar1*), luminal secretion of 2A12, Verm, and Gasp was incomplete. The tracheal cells outlined by GFP-CAAX partially retained those markers in the cytoplasm (Figures 2B, 2E, and 2H). *sar1* zygotic mutant embryos normally deposited the early luminal marker Piopio by stage 13 (Jazwinska et al., 2003). Luminal chitin was also detected in *sar1* mutants at stage 15. However, the luminal cable was narrow, more dense (Figure S3), and distorted compared to wild-type (Figures 3E and 3F). To test if the *sar1* secretory phenotype in the trachea is cell autonomous, we reexpressed Sar1 specifically in the trachea of *sar1* mutants by using btl-GAL4. Such embryos showed largely restored secretion of 2A12, Verm, and Gasp (Figures 2C, 2F, and 2I). Thus, we conclude that tracheal *sar1* is required for the
efficient secretion of luminal markers, which are predicted to associate with the growing intraluminal chitin matrix.

Zygotic sar1 Mutants Have Reduced Sar1 Function in the Trachea

sar1 mRNA has been reported to be abundantly maternally contributed (Zhu et al., 2005). At later stages, zygotic expression of sar1 mRNA is initiated in multiple epithelial tissues (Abrams and Andrew, 2005). To monitor Sar1 zygotic expression in the trachea, we used a Sar1-GFP protein trap line (Wilhelm et al., 2005). Embryos carrying only paternally derived Sar1-GFP show a strong zygotic expression of GFP in the trachea (Figure S2). We also used an anti-Sar1 antibody to analyze Sar1 expression in the trachea of wild-type, zygotic sar1P1, and sar1EP3575D2 null mutant embryos (Zhu et al., 2005) (Figure S2). Both zygotic mutants showed a clear reduction, but not complete elimination, of Sar1 expression in the trachea. To test the effects of a more complete inactivation of Sar1, we generated transgenic flies expressing a dominant-negative sar1T38N form in the trachea (Kuge et al., 1994). In btl > sar1T38N-expressing embryos, we observed early defects in tracheal branching and epithelial integrity as well as a complete block in Verm secretion (Figure S2). In contrast to btl > sar1T38N-expressing embryos, zygotic sar1P1 mutant embryos show normal early tracheogenesis with no defects in branching morphogenesis and epithelial integrity (Figure S3).

In summary, tracheal expression of Sar1 is markedly reduced in zygotic sar1 mutant embryos. While maternally supplied Sar1 is sufficient to support early tracheal development, zygotic Sar1 is required for efficient luminal secretion.

Sar1 Localizes to the ER and Is Required for ER and Golgi Integrity

Given the conserved role of Sar1 in vesicle budding from the ER, we determined its subcellular localization in the trachea by using anti-Sar1 antibodies. Sar1 localizes predominantly to the ER (marked by the PDI-GFP trap) (Figure S2). Continuous COPII-mediated transport from the ER is required to maintain the Golgi apparatus and ER structure (Altan-Bonnet et al., 2004; Yamanushi et al., 1996). To test if zygotic loss of Sar1 compromises the integrity of the ER and Golgi in tracheal cells, we stained sar1 mutant embryos with antibodies against KDEL (marking the ER lumen) and gp120 (highlighting Golgi structures). In sar1 mutant embryos, we observed a strongly disrupted ER structure and loss of Golgi staining in DT cells at stage 14 (Figures 3A–3D). Additionally, TEM of stage-16 wild-type and sar1 mutant embryos showed a grossly bloated rough ER structure in DT tracheal cells (Figures 3E and 3F). Consistent with its functions in yeast and vertebrates, Drosophila Sar1 localizes to the ER and is not only required for efficient luminal protein secretion, but also for the integrity of the early secretory apparatus.

Zygotic sar1 Is Selectively Required for Luminal Diameter Expansion

To analyze tracheal maturation defects in sar1 mutant embryos, we generated and imaged sar1 strains that carry either btl > ANF-GFP btl-mRFP-moe or btl > Gasp-GFP...
In sar1 mutants, luminal deposition of both ANF-GFP and Gasp-GFP is reduced (Figures 4A and 4B; Figure S4). Like endogenous Gasp in the mutants, ANF-GFP was also retained in the tracheal cells of sar1 mutants, but to a lesser extent. Strikingly, sar1 mutants failed to fully expand the luminal diameter of the DT outlined by the apical RFP-moe localization (Figure 4B). We quantified this defect by measuring diametrical growth rates in metamere 6 for wild-type (n = 11) and sar1 mutant (n = 7) embryos. While early lumen expansion commences in parallel in both genotypes, the later diametrical growth of sar1 mutants falls significantly behind compared to wild-type embryos. The DT lumen in sar1 mutants reaches only an average of 70% (4.05 μm ± 0.2 SEM) of the wild-type diameter (5.75 μm ± 0.2) at early stage 16 (Figure 4H). We detected identical diametrical growth defects in fixed sar1 mutant embryos expressing btl > ANF-GFP-CAAX by analysis of confocal zy sections or TEM (Figures 4C–4F). Reexpression of sar1 in the trachea of sar1 mutant embryos not only rescued secretion, but also the lumen diameter phenotype at stage 16 (Figure 4G). In contrast to the diametrical growth defects, DT tube elongation in sar1 embryos was indistinguishable from that in wild-type (Figure 4I). This demonstrates distinct genetic requirements for tube diameter and length growth. It also reveals that the sar1 DT luminal volume reaches less than half of the wild-type volume. Prolonged live imaging showed that sar1 mutants are also completely deficient in luminal protein and liquid clearance (Figure 4J; Figure S4). Up to 80% of the rescued embryos also completed luminal liquid clearance, suggesting that efficient tracheal secretion and the integrity of the secretory apparatus are prerequisites for later tube maturation steps (Figure 4J). Taken together, the above-described results show that tracheal Sar1 is selectively required for tube diameter expansion. Additionally, subsequent luminal protein and liquid clearance fail to occur in sar1 mutants.

Mutants in COPII Subunits Phenocopy sar1 Secretion and Diameter Phenotypes

Do the tracheal defects of sar1 reflect a general requirement for the COPII complex in luminal secretion and diameter expansion? To test this, we analyzed lethal P element insertion alleles disrupting two additional COPII coat subunits, sec13 and sec23 (Abrams and Andrew, 2005). We stained mutant sec13 and sec23 embryos for luminal Gasp and for Crb and α-Spectrin to highlight tracheal cells. At stage 15, embryos of both mutants show a clear cellular retention of Gasp (Figure S5). Furthermore, stage-16 sec13 and sec23 embryos show significantly narrower DT branches when compared to wild-type. The average diameter of the DT branches in metamere 6 was 4.8 μm (±0.2 μm) and 4.4 μm (±0.1 μm) in fixed sec13 and sec23 embryos, respectively, compared to 6.3 μm (±0.1 μm) in wild-type (Figure S5). Therefore, sec13 and sec23 mutants phenocopy sar1. The phenotypic analysis of three independent mutations disrupting ER-to-Golgi transport thus provides a strong correlation between deficits in luminal protein secretion and tube diameter expansion (Figure 7A).
Luminal Protein Clearance Depends on Tracheal Rab5 Activity and Endocytosis

Anticipating that the wave of luminal protein clearance may depend on endocytic uptake, we screened several mutations with known defects in endocytosis. To directly assess luminal protein clearance, we generated strains of endocytic mutants carrying btl > ANF-GFP and analyzed them live. We found that clathrin heavy chain\(^1\) (chc\(^1\)), shibire\(^{ts2}\) (shi\(^{ts2}\) at the restrictive temperature of 32°C), and rab5\(^2\) null mutant embryos all display profound defects both in luminal protein and liquid clearance (Figure S6). Chc is the heavy chain of the Clathrin coat (Bazinet et al., 1993), and shi codes for the Dynamin GTPase that clips Clathrin-coated pits off the membrane to form endocytic vesicles (van der Bliek and Meyerowitz, 1991). rab5 encodes for a small GTPase that is a key regulator of early endocytosis (Wucherpfennig et al., 2003). We focused our further analysis on rab5, since rab5\(^2\) showed the most penetrant defects in protein clearance (Figure S6). To follow luminal protein clearance live, we imaged wild-type and rab5\(^2\) mutant embryos expressing ANF-GFP or Gasp–GFP. rab5\(^2\) mutant embryos failed to eliminate both luminal GFP markers (Figures 5A–5D; Movies S8–S10). In contrast to wild-type, rab5\(^2\) mutants also retained substantial quantities of endogenous luminal Gasp, Verm, chitin, and 2A12 antigen inside the tracheal tubes (Figures 5E–5H; Figure S6). When we expressed GFP-rab5 specifically in the trachea of rab5 mutant embryos, luminal clearance was rescued significantly (Figure 5I). Our analysis thus shows that rab5 is required in the trachea for proper clearance of luminal proteins and chitin. The additional analysis of chc\(^1\) and shi\(^{ts2}\) mutant embryos argues that not only Rab5, but endocytosis in general, is required for the clearance of luminal solids.

Normal Secretory Capacity and Epithelial Integrity, but Defective Endosomal Structures, in rab5 Mutants

We examined whether the clearance phenotypes of rab5 mutants may be secondary to disruptions of either secretion (as for sar1) or loss of epithelial integrity. Apart from a minor and partially penetrant tube elongation phenotype, rab5\(^2\) mutants develop an essentially wild-type tracheal network. They show normal secretion of luminal markers at stage 15, as well as intact tracheal epithelial polarity at stage 16. We also analyzed DTS of late-stage rab5\(^2\) mutant embryos by TEM. These embryos display a wild-type cellular ultrastructure and apical luminal lining in the DT tubes (Figure S7). Finally, we performed a dextran injection assay that showed that rab5\(^2\) mutant embryos have intact paracellular junctions (Figure S9). Thus, zygotic rab5 mutant embryos show an overall normal tracheal development and secretion.

We tested the integrity of several distinct endosomal compartments in rab5\(^2\) mutants. We visualized Clathrin-coated vesicles (CCV) by using anti-Chc antibodies in fixed embryos. We also used GFP-tagged reporters expressed in the trachea to analyze early endosomes (EE) and multivesicular bodies (MVB) by using GFP-myc-2xFYVE, recycling endosomes (RE) by using GFP-Rab11, and late endosomes (LE) by using GFP-Rab7 (Emery et al., 2005; Wucherpfennig et al., 2003). For the latter three markers, the analysis of live and fixed embryos yielded identical results.

We found no changes between wild-type and rab5\(^2\) mutant embryos in the predominantly apical localization of GFP-Rab11-positive REs (Figure S8). The remaining endocytic markers were also unaffected during tracheal development in rab5\(^2\) mutant embryos up to early stage...
The Tracheal Epithelium Directly Internalizes and Clears Luminal Material by Endocytosis

Our mutant analysis shows that endocytosis is required for luminal material clearance. Protein trafficking through endocytic compartments may either directly absorb and clear luminal proteins or indirectly facilitate the extinction of luminal material. If the tracheal epithelium directly internalizes luminal material by endocytosis, we expect that: (1) tracheal cells take up luminal markers, and that (2) luminal markers colocalize with endosomal structures inside the tracheal cells during tube clearance.

To test if endogenous proteins are taken up into tracheal cells during luminal solid clearance, we used an amplified staining protocol that allows for the detection of the luminal marker Gasp in mid-stage-17 embryos. gasp mutants were devoid of any Gasp staining throughout tracheal development (Figure S9; K.T. and C.S., in preparation). In early stage-16 embryos marked by the DT (Figure 6A), Live imaging of btl > GFP-FYVE embryos at mid-stage 17 reveals the dynamic movement of large, GFP-FYVE-positive apical structures in the tracheal cells in a background of small endosomal vesicles. Strikingly, GFP-FYVE labeling of those apical structures was absent in rab5 mutants, leaving few, small and randomly distributed vesicles (Figure S8; Movie S11). The lack of the extensive GFP-FYVE-positive structures in the mutants may either reflect the function of Rab5 generating the FYVE ligand phosphatidylinositol-3-phosphate or a direct role of Rab5 in endosomal assembly and growth (Christofidis et al., 1999). In wild-type, GFP-Rab7 highlights late endosomal structures spread along the apico-basal axis of the tracheal cells. In most rab5 mutant embryos, the abundance of GFP-Rab7 endosomes was decreased or completely lost (Figure S8).

Taken together, rab5 mutants show normal epithelial development and secretion in the trachea, but aberrant localization and abundance of Chc-, GFP-FYVE-, and GFP-Rab7-positive structures at mid-stage 17. This suggests that Rab5 either directly mediates or indirectly facilitates the formation and intracellular organization of CCVs and LEs. The manifestation of endocytic phenotypes in the trachea of rab5 mutants precisely correlates with the initiation of solid luminal material clearance in the wild-type.

We analyzed the distribution of intracellular Gasp puncta relative to endosomal markers in the trachea of fixed embryos. We tested the following genotypes of embryos for colocalization: btl > Clathrin light chain-GFP (Clc-GFP) for CCVs, btl > GFP-Rab5 for EEs, btl > GFP-Rab7 for LEs, and btl > GFP-LAMP1 (GFP-Lysosomal Associated Membrane Protein 1) for lysosomes (Chang et al., 2002; Pulipparacharuvil et al., 2005). While intracellular Gasp staining only occasionally colocalized with GFP-Rab5-positive endosomes, Gasp puncta more consistently colocalized with Clc-GFP, GFP-Rab7, and GFP-LAMP1 staining (Figure S9). Thus, considerable portions of intracellular Gasp staining coincide with CCVs, LEs, and lysosomal markers, suggesting that Gasp may traverse those endosomal compartments. The lysosomal localization of intracellular Gasp indicates that Gasp may be degraded inside tracheal cells during clearance of solid luminal material.

While the detection of endogenous Gasp in endosomal structures during stage 17 argues for a direct role of endocytosis in tube clearance, the localization of intracellular Gasp puncta to specific endosomal compartments was relatively infrequent. To visualize the cellular dynamics during luminal protein clearance directly, we established a live endocytosis assay in the trachea by using the fluid-phase marker dextran (Swanson, 1989). We injected 10 kDa dextran into the hemocoel of stage-13 to stage-14 embryos, which lack the paracellular barrier function provided by fully assembled septate junctions (Tepass and Hartenstein, 1994). In these experiments, the fluorescent dextran consistently accumulated inside the tracheal lumens of wild-type embryos (Figure 6D). Taking advantage of this assay, we preloaded the trachea of btl > ANF-GFP embryos with rhodamine-labeled dextran and live imaged ANF-GFP along with the injected marker. Both ANF-GFP and dextran were cleared from the DT lumen simultaneously (Movie S12), suggesting that exogenous luminal dextran is cleared similarly to other luminal constituents.

Is dextran taken up into tracheal cells during luminal protein clearance? We injected 10 kDa dextran into early embryos carrying the tracheal btl > GFP-CAAX marker and analyzed them. We initially detected only few intracellular puncta in such embryos at stage 16 (Figure 6H and data not shown). However, from early to mid-stage 17 and before the drop in luminal fluorescent intensity, the number of rhodamine-dextran puncta distinctly increased inside tracheal cells (Figure 6D, 0 min and 28 min). Upon luminal clearance completion (late stage 17), the count of intracellular dextran puncta was diminished to basal levels (Figure 6D, 50 min). Quantification of intracellular dextran puncta demonstrates a dynamic peak of dextran internalization just before and during luminal protein clearance (Figure 6H).

Since not all of the injected dextran is taken up into the tracheal lumen, the detected intracellular dextran signals may derive either from the lumen or the hemocoel. To test the origin of intracellular dextran, we injected rhodamine-labeled dextran into the hemocoel of late stage-16 embryos. These embryos display fully developed
paracellular barriers that prevent the luminal access of the 10 kDa dextran (Lamb et al., 1998). Importantly, we did not find any fluorescent signal inside the tracheal cells at mid-stage 17 in such embryos (Figure S9). This demonstrates that the transient intracellular dextran puncta in embryos preloaded at stage 13 indeed derive from the lumen. We next asked if dextran internalization requires Rab5-dependent endocytosis. We injected rhodamine-labeled dextran into rab5\(^2\) mutant embryos with tracheal cells labeled by \(btl > \text{CD8-GFP}\). A total of 56\% of rab5\(^2\) mutant embryos (\(n = 11\)) failed to show the characteristic transient peak of intracellular dextran accumulation during the period of luminal protein clearance (Figure 6E). Thus, tracheal cells internalize the preloaded dextran from the lumen in a Rab5-dependent manner exactly before and during the peak of luminal solid clearance.

To test if intracellular dextran dots localize to endosomes, we expressed GFP-Rab5, GFP-2x-FYVE, and GFP-Rab7 in the trachea under the control of \(btl\)-GAL4. Embryos of each genotype were preloaded with 10 kDa rhodamine-labeled dextran and analyzed live during its luminal clearance. A significant increase in the number of internalized dextran occurs before luminal protein clearance (17–19 hr AEL). The green arrow indicates the time of luminal clearance. Values represent mean and standard error from five independent experiments. For all developmental time points \(n = 24\), except for 13 hr AEL, where \(n = 8\). The asterisk denotes \(p < 0.01\) by Student’s t test comparing the data sets between two time points. Bars represent means ± SEM. Scale bars are 10 \(\mu\)m.
to GFP-FYVE-positive EE and MVBs, and to GFP-Rab7-marked LEs, was 8.0% and 31%, respectively (n = 5) (Figure 6F).

In summary, our data show that endogenous Gasp and injected rhodamine-labeled dextran are internalized by tracheal cells before and during the clearance interval. Both markers localize to endosomal structures in tracheal cells (Figure 7B). We conclude that the tracheal epithelium directly removes luminal Gasp protein and the preloaded dextran polymers by a transient, Rab5-dependent, endocytic wave.

DISCUSSION

Our live-imaging approach defines the developmental dynamics of functional tracheal maturation. At the organ level, we identified three sequential and rapid developmental transitions: (1) the secretion burst, followed by massive luminal protein deposition and tube diameter expansion, (2) the clearance of solid luminal material, and (3) the replacement of luminal liquid by gas. Live imaging of each event additionally revealed insights into the startlingly dynamic activities of the tracheal cells. ANF-GFP-containing structures and apical GFP-FYVE-positive endosomes rapidly traffic in tracheal cells during the secretion burst and protein clearance. The direct live comparison between wild-type and mutant embryos further highlights the dynamic nature of epithelial activity during each pulse.

We identified several mutations that selectively disrupt distinct cellular functions and concurrently interrupt the maturation process at specific steps. This clearly demonstrates the significance of phenotypic transitions in epithelial organ maturation and establishes that secretion is required for luminal diameter expansion and endocytosis for solid luminal material clearance.

The Secretory Burst and Tube Diameter Expansion

The sudden initiation of an apical secretory burst tightly precedes diametric tube expansion. The completion of both events depends on components of the COPII complex, further suggesting that the massive luminal secretion is functionally linked to diametric growth. How does apical secretion provide a driving force in tube diameter expansion? In mammalian lung development, the distending internal pressure of the luminal liquid on the epithelium expands the lung volume and stimulates growth. Cl⁻ channels in the epithelium actively transport CI⁻ ions into luminal liquid. The resulting osmotic differential then forces water to enter the lung lumen, driving its expansion (Olver et al., 2004). By analogy, the tracheal apical exocytic burst may insert protein regulators such as ion channels into the apical cell membrane or add additional membrane to the growing luminal surface. Since the ER is a crucial cellular compartment for intracellular traffic and lipogenesis, its disruption in sar1 mutants may disrupt the efficient transport of so far unknown specific regulators or essential apical membrane addition required for diametric expansion. Alternatively, secreted chitin-binding proteins (ChB) may direct an increase of intraluminal pressure and tube dilation. Overexpression of the chitin-binding proteins Serp-GFP or Gasp-GFP was insufficient to alter the diametric growth rate of the tubes, suggesting that luminal diameter expansion is insensitive to increased amounts of any of the known luminal proteins (unpublished data). In sar1 mutants, the secretion of at least two chitin-binding proteins, Gasp and Verm, is reduced. Chitin, however, is deposited in seemingly normal quantities, but assembles into an aberrantly narrow and dense chitinous cable. This phenotype suggests that the correct ratio between chitin and multiple interacting proteins may be required...
for the correct assembly of the luminal cable. Interestingly, sar1, sec13, and sec23 mutant embryos form a severely defective and weak epithelial cuticle (Abrams and Andrew, 2005). The luminal deposition of ChB proteins during the tracheal secretory burst may orchestrate the construction and swelling of a functional matrix, which, in turn, induces lumen diameter dilation. While we favor this later hypothesis, we cannot exclude that other mechanisms, either separately or in combination with the dilating luminal cable, drive luminal expansion (Figure 7A).

**Endocytotic Clearance of Luminal Material**

During tube expansion, massive amounts of luminal material, including the chitinous cable, fill the tracheal tubes. We found that Dynamin, Clathrin, and the tracheal function of Rab5 are required to rapidly remove luminal contents, indicating that endocytosis is required for this process. Several lines of evidence argue that the tracheal epithelium activates Rab5-dependent endocytosis to directly internalize luminal material. First, the tracheal cells of rab5 mutants show defects in multiple endocytic compartments. These phenotypes of rab5 mutants become apparent during the developmental period matching the interval of luminal material clearance in wild-type embryos. Second, tracheal cells internalize two luminal markers, the endogenously encoded Gasp and the dextran reporter, exactly prior and during luminal protein clearance. The number of intracellular dextran puncta reaches its peak during the clearance process and ceases shortly thereafter. Lastly, intracellular puncta of both Gasp and dextran colocalize with defined endocytic markers inside tracheal cells. The colocalization of Gasp and dextran with GFP-Rab7 and of Gasp with GFP-LAMP1 suggests that the luminal material may be degraded inside tracheal cells. Taken together, our data show that the tracheal epithelium activates a massive wave of endocytosis to clear the tubes (Figure 7B).

Endocytic routes are defined by the nature of the internalized cargoes and the engaged endocytic compartments (Conner and Schmid, 2003). What may be the features of the endocytic mechanisms mediating the clearance of luminal material? The phenotype of chc mutants and the presence of intracellular Gasp in CCVs indicate that luminal clearance at least partly relies on Clathrin-mediated endocytosis (CME). In addition to CME, Dynamin and Rab5 have also been implicated in other routes of endocytosis, suggesting that multiple endocytic mechanisms may be operational in tracheal maturation. The nature of the endocytosed luminal material provides an additional perspective. While cognate uptake receptors may exist for specific cargos such as Gasp, Verm, and Serp, the heterologous ANF-GFP, degraded chitin, and the fluid-phase marker dextran may be cleared by either fluid-phase internalization or multifunctional scavenger receptors. Interestingly, Rab5 can regulate fluid-phase internalization in cultured cells by stimulating macro-pinocytosis and the activation of Rabankyrin-5 (Bucci et al., 1992; Schnatwinkel et al., 2004; Stenmark et al., 1994). The defective tracheal internalization of dextran in rab5 mutants provides further loss-of-function evidence for Rab5 function in fluid-phase endocytosis in vivo. The above-described arguments lead us to speculate that additional Rab5-regulated endocytic mechanisms may most likely cooperate with CME in the clearance of solid luminal material.

How is liquid cleared from the lumen? While we still know very little about this fascinating process (Kallis, 1939), some developmental and mechanistic arguments suggest that this last maturation step is mechanistically distinct. First, the interval of luminal liquid clearance is clearly distinct from the period of endocytic clearance of solids. Second, the dynamic internalization of dextran and the abundance of GFP-marked endocytic structures decline before liquid clearance. Finally, our assessment of liquid clearance further suggests that it requires a distinct cellular mechanism (K.-A.S. and V.T., unpublished data).

Viewing the entire process of airway maturation in conjunction, some general conclusions may be drawn. First, the three epithelial pulses are highly defined by their sequence and exact timing, suggesting that they may be triggered by intrinsic or external cues. Second, the analysis of mutants that selectively reduce the amplitude of the secretory or endocytic pulses demonstrates the requirement for each epithelial transition in the completion of the entire maturation process. These pulses are induced in the background of basal secretory and endocytic activities that operate throughout development. Third, specific cellular activities exactly precede each morphological transition. Finally, the separate transitions are interdependent in a sequential manner. Efficient secretion is a prerequisite for the endocytic wave. Similarly, protein endocytosis is a condition for luminal liquid clearance. This suggests a hierarchical coupling of the initiation of each pulse to completion of the previous one in a strict developmental sequence (Figure 7C).

Our study provides a striking example of how pulses of epithelial activity drive distinct developmental events and mold the nascent tracheal lumen into an air delivery tube. Our findings are likely to be relevant beyond the scope of tracheal development. The uniform growth of salivary gland tubes in flies and the excretory canal and amphib channel lumen in worms also require the assembly of a luminal matrix for uniform tube growth (Abrams et al., 2006; Perens and Shaham, 2005). Luminal material is also transiently present during early developmental stages in the distal nephric ducts of lamprey (Youson, 1984). Thus, the coordinated, timely deposition and removal of transient luminal matrices may represent a general mechanism in tubulogenesis.

**EXPERIMENTAL PROCEDURES**

**Genetics**

The P element alleles l(3)s009124 (sar11P in text) and l(3)s05712 fail to complement the nulls sar11EP357528, 205 and the GFP trap sar11CA07674. Insertion sites were determined by plasmid rescue and PCR. Precise excision of sar11P reverted tracheal phenotypes. The COPII subunit...
 mutatations are l(3)Y1031 (sec13) and l(3)Y13C8 (sec23). For more strain information, see the Supplemental Data.

Molecular Biology
PCR subcloning was used to generate pET28a His-Gasp (14–189 aa) for recombinant protein expression and pUAST-Gasp-GFP construction from clones RH12464 and RH10284, respectively. Purified recombinant Gasp was used to immunize guinea pigs. pUAST-sarl and pUAST-sarlTRM were generated by subcloning of RE74312 and PCR mutagenesis.

Immunostaining, TEM, and Dextran Injections
Immunohistochemistry, TEM and 10 kDa dextran (Molecular Probes) injections was performed as described (Lamb et al., 1998; Wang et al., 2006). Additional antibodies used were: guinea pig anti-Coracile (from R.G. Feigon), mouse mAb anti-gp120/Golgi (clone7H6D7C2, Calbiochem), mouse mAb anti-KDEL (clone10C3, Nordic Biosite), goat anti-Clathrin (Sigma), chicken anti-Human Sar1 (tiprosco Inc.), and guinea pig anti-Gasp. DmSar1 protein is 70% identical to HsSar1. Anti-Gasp staining was amplified using a secondary goat anti-guinea pig and tertiary donkey anti-goat antibodies.

Live Imaging
For wide-field live imaging, dechorionated embryos were mounted on a gas-permeable membrane stretched over two silicon bars on the top of a slide. Embryos were imaged on an Axioplan2 microscope by using either a x20/0.5NA Plan-Neofluar or a x63/1.3NA C-Apochromat water-immersion objective and an attached CCD camera controlled by the AxioVision software 4.5. Generally, 12–17 focal sections (0.5–2.5 μm apart) were recorded every 1–3 min. For confocal live imaging, a laser-scanning confocal microscope (LSM 510 META, Zeiss) with an Argon 2/488 nm, a HeNe 543 nm laser, and a 63/1.3NA C-Apochromat water-immersion objective was used. Individual Z stacks with a step size of 0.7–1.0 μm were taken every 2–4 min over a 2–5 hr period. Image-lapse movies were created from the Z stacks by using NIH ImageJ (Abramoff et al., 2004; http://rsb.info.nih.gov/j/). Live imaging was typically conducted for tracheal metameres 6–8.

ANF-GFP Localization Dynamics and DT Morphometric Analysis
btl > ANF-GFP embryos were collected for 10 min, dechorionated, and imaged by wide-field microscopy. ANF-GFP localization was scored every 5 min for a period of at least 3 hr. The analysis covered 1 or 2 maturation events for 237 embryos at 25 °C.

The secretory pulse initiates at 10 hr 30 min (±17 min, n = 45) and is followed by diameter expansion starting at 11 hr 03 min (±16 min, n = 24). ANF-GFP is removed at 18 hr 48 min (±22 min, n = 58), and luminal liquid clearance occurs at 20 hr 8 min (±18 min, n = 56). The embryo hatches at 21 hr 29 min (±29 min, n = 108). We also recorded developing btl > ANF-GFP embryos at 22 °C during the entire maturation process (n = 7). This analysis showed the same order of events with the following time profile: luminal secretion at 12 hr 6 min (±18 min), diameter expansion at 13 hr 18 min (±14 min), protein clearance at 21 hr 50 min (±21 min), and liquid clearance at 24 hr 10 min (±20 min).

The diameter and length of DT6 were measured in live embryos expressing ANF-GFP and btl-mRFP1-moe. The diameter was measured at a right angle in three separate positions within metamere 6 for each sample to average tube tapering. Tube length was measured by tracing the apical mRFP1-moe from fusion cells DTa6 to DTp6. Recordings covered a period of 4.5 hr with 5 min elapsed time. All measurements were done with AxioVision 4.5 (Zeiss).

Phenotypic Analysis of Endocytic Compartments in Live Embryos
The diameter of endosomal compartments labeled by GFP-FYVE was measured from Z stacks of ten tracheal cells per embryo labeled by btl > myr-mRFP1. Endosomal structures with a diameter < 3 μm were not included in the assessment. The number of positive GFP-Rab7 structures was estimated from wide-field sections. Five to six time points, 15 s apart, were estimated for each embryo. The measurements were assembled with AxioVision 4.5. Intracellular puncta of dextran were assessed from Z stacks of confocal sections (DT metamere 7).

Supplemental Data
Supplemental Data include 12 movies, 9 figures, Supplemental Experimental Procedures, and Supplemental References and are available at http://www.developmentalcell.com/cgi/content/full/13/2/214/DC1/.

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Supplemental Data

Sequential Pulses of Apical Epithelial Secretion and Endocytosis Drive Airway Maturation in Drosophila

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Figure S1. Luminal Deposition and Clearance of ANF-GFP Matches Endogenous Verm and Gasp

(A-H) Confocal micrographs of embryos expressing bit>ANF-GFP stained for GFP (green) (A)-(H), Verm (A)-(D) and Gasp (E)-(H) (magenta). ANF-GFP and Verm (A) and Gasp (E) are largely cytoplasmic prior to the secretory burst at stage 13 (A) and (E). Shortly thereafter at late stage 13, all three markers are detected in the lumen (B) and (F). At late stage 16, the markers are found predominantly in the lumen and along the apical
lining of trachea (C) and (G). At late stage 17, ANF-GFP, Verm and Gasp are absent from the luminal cavity, but remain localized to the apical lining of the tubes (D) and (H). Minor-moderate levels of ANF-GFP are present in the cytoplasm of the tracheal cells throughout late stage 16 till mid stage 17 (C), (D), (G), (H).

(I and J) TEM sections of DT of a wild type embryo expressing btl>ANF-GFP. Embryos were fixed before the anticipated ANF-GFP clearance (I) and after disappearance of luminal fluorescence in (J). The electron dense luminal cable structure is removed during the luminal protein clearance. Scale bars 10 \(\mu\)m (A)-(H) and 1 \(\mu\)m (I), (J).

Figure S2. Sar1 Expression and Function in Different sar1 Mutant Conditions
(A) Genomic organisation of sar1 locus and position of P element insertions.
(B-C) Confocal micrographs of wild type (B) and btl>\textit{sar1}^{DN} embryos (C) stained with anti-Verm (green) and anti-GFP stainings. Trachea development is completely arrested in \textit{sar1}^{DN} before the formation of primary branches (white arrowhead) in (D).
(D-D’) Confocal section of an embryo with only paternally contributed \textit{sar}1\textsuperscript{GFP-trap} stained with anti-GFP (white) in (D) and (green) in (D’) and anti-Crb in (D’) (magenta). Tracheal cells show strong zygotic expression of Sar1.

(E-G’) Confocal sections of Stage 15 wild type (E), (E’), \textit{sar}1\textsuperscript{P1} (F), (F’) and zygotic null \textit{sar}1\textsuperscript{EP3575\Delta28} embryos (G), (G’) expressing \textit{btl}>GFP-CAAX stained for Sar1 and GFP. (E-G) show Sar1 staining alone (white) (E’-G’) represent merged images of Sar1 (green) and GFP staining (magenta). (H and H’) Sar1 localizes to the periphery of the ER lumen marked GFP staining of PDI-GFP trap embryos. Sar1 is labelled white in (H) and magenta in (H’). The ER lumen is visualized by anti-GFP staining in (H’) (green). Zygotic Sar1 expression was strongly reduced mutants of both \textit{sar}1 alleles (F) and (G), suggesting that \textit{sar}1\textsuperscript{P1} represents a strong hypomorph (F). Scale bars, 10 \textmu m

\begin{figure}
\centering
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\caption{\textbf{Figure S3.} \textit{sar}1\textsuperscript{P1} Mutant Embryos Show Normal Epithelial Organization}
\end{figure}

(A-L) Confocal micrographs show DTs of wild type (A), (C), (E)-(H) and \textit{sar}1\textsuperscript{P1} mutant embryos (B), (D), (I)-(L). All micrographs are single confocal sections except (G) and (K), which represent projections. Trachea cells were labeled by the transgenic expression of \textit{btl}>GFP-CAAX.

(A-C) Localization of Piopio (green) at stage 13 (A), (B) and of chitin (white) at stage 15 (C), (D) Insets in (C) and (D) represent yz projections of (C) and (D). There are no luminal deposition defects or any excessive cellular accumulation apparent for either of
these luminal markers in $sar^P1$ embryos compared to wild type. Note that the chitinous cable is narrower and more dense in $sar1$ mutants (D) compared to wild type (C).

(E-L) Epithelial organization assessed from embryos at early stage 16 by following staining for the apical marker Crb (E), (I) (green), adherens junction marker E-cad (F), (J) (green), projections of (F) and (J) are shown in (G) and (K) (white) and septate junction marker coracle (H), (L) (green). None of the stainings show discernable phenotypes in the localization or organization of epithelial markers in $sar^P1$ embryos. Further, no defects in the epithelial cell arrangements (like irregular cellular intercalation) were detected (G, K). Scale bars 10 $\mu$m.

**Figure S4.** $sar1$ Is Required for Gasp-GFP Secretion and ANF-GFP Clearance

(A and B) Frames of widefield movies of a wild type (A) and $sar^P1$ mutant (B) embryos expressing $btl>Gasp$-GFP. While wild type embryos secrete Gasp-GFP efficiently and expand the luminal diameter normally, $sar^P1$ mutant embryos partially retain Gasp-GFP intracellularly (0 min and onwards) and fail to expand luminal diameter (90-180 min). Imaging started 10 hours AEL and acquired every 3 min for 5 hours. For simplicity the initiation of luminal secretion is presented as time 0 in the figure.
Frames from widefield movies showing stage 17 wild type (C) and sar1P1 mutant embryos expressing btl>ANF-GFP (D) (white). Wild type embryos clear ANF-GFP from the lumen, while sar1P1 mutant embryos retain it. Scale bars 10 µm.

Figure S5. sec13 and sec23 Mutants Show Defects in Secretion and Luminal Diameter Expansion

(A-F) Confocal sections of the DT of wild type (A), (B), sec13 (C), (E) and sec23 embryos (D), (F). Embryos were stained with anti-Gasp (green) and a mixture of anti-Crb and anti-α-Spectrin to show tracheal cells (magenta). sec13 and sec23 mutant embryos
retained a considerable amount of Gasp inside tracheal cells at stage 15 (C) and (E). At early stage 16, sec13 and sec23 (D), (F) embryos show narrower DT lumina when compared to wild type (B).

(G) Lumen diameter of DT at metamer 6 was measured in wild type, sec13 and sec23 embryos at stage 16 using Crb staining as an apical cell marker. Embryos of both mutants show a significant reduced lumen diameter when compared to wild type embryos. Bars are means ± s.e.m. * denotes statistical significant differences at p< 0.0001 by a Student’s t test. Scale bars 10 μm.

**Figure S6.** shi$^{ts2}$ and chc$^{1}$ Mutant Embryos Fail to Clear ANF-GFP and Luminal Liquid  
**rab5$^{2}$** is Required for Clearance of Endogenous Luminal Proteins  
(A-C) Widefield microscope images of live embryos expressing btl>ANF-GFP depict a wild type (A), a shibire$^{ts2}$ (shi$^{ts2}$) at restrictive temperature 32°C from 13 h to 16 h (B) and
a clathrin heavy chain\(^1\)\(^{(\text{chc}^1)}\) mutant embryo (C). \(\text{shi}^{ts2}\) and \(\text{chc}^1\) show strong defects in the luminal clearance of ANF-GFP at mid stage 17.

(D) Quantification of the luminal protein and liquid clearance defects for wild type, \(\text{rab}^5\), \(\text{shi}^{ts2}\) and \(\text{chc}^1\).

(E-H) Confocal sections of DT in late stage 17 wild type (E), (F) and \(\text{rab}^5\) mutant embryos (G), (H) stained with 2A12 (E), (G) and Chitin binding probe (CBP) (F), (H) (white). \(\text{rab}^5\) mutant embryos retain endogenous luminal antigens at late 17.  

Scale bars 10 µm.

**Figure S7. \(\text{rab}^5\) Mutants Show Normal Luminal Marker Secretion and Epithelial Organization**

(A-O) Confocal sections show the DT of wild type (A)-(D), (I)-(K) and \(\text{rab}^5\) mutant embryos expressing \(\text{btl}>\text{CD8-GFP}\) (E)-(H), (M)-(O). Staining against GFP is shown in magenta in all panels. To analyze the secretion pulse, stage 15 embryos were stained for 2A12 (A), (E), Verm (B), (F), Gasp (C), (G) (green) and Chitin binding probe (white) (D), (H). \(\text{rab}^5\) mutants show no discernable defects in luminal secretion or luminal chitin deposition. To assess epithelial organization, we stained early stage 16 embryos for the apical domain marker Crb (I), (M), the AJ marker E-Cad (J), (N) and SJ marker Coracle (K), (O) all shown in green. None of the analysed epithelial markers showed any
apparent mislocalization in $rab5^2$ mutant embryos. However, a partially penetrant frequency of mild tube elongation phenotypes in $rab5^2$ mutant DT was noticed. (L-P) TEM of DT in late stage 16 wild type (L) and $rab5^2$ mutant (P) embryos shows that both genotypes display indistinguishable apical lining and overall cellular ultrastructure. Scale bars 10 µm (A)-(O) and 1 µm (L), (P).

Figure S8. Rab5 Is Required for the Integrity of Endosomal Compartments in Trachea Cells

(A and B) Confocal sections show the DT of wild type (A) and $rab5^2$ (B) mutant embryos stained with anti-Chc. In wild type embryos, Chc staining is localised apically (A) while in $rab5^2$ mutant embryos, the discrete Chc localisation is lost at mid stage 17. (C and D) Images from a widefield time-lapse movie showing the endosomal structures of the tracheal cells in wild type (C) and $rab5^2$ embryos (D) expressing GFP-FYVE (white) at mid stage 17. Loss of Rab5 results in drastic reduction of the endosomal labeling while diffuse cytoplasmic accumulation of GFP-FYVE is detected (movie S11). (E - H) Confocal sections of the DT of wild type (E), (G) and $rab5^2$ (F), (H) mutant embryos expressing $btl>\text{GFP-Rab7}$ (E), (F) and $btl>\text{GFP-Rab11}$ (G), (H) stained with anti-GFP. $rab5$ mutant embryos showed a reduced number of GFP-Rab7 positive endosomal puncta when compared to the wild type. No changes were detected between
wild type and $rab5^2$ mutant embryos for the predominantly apically localised GFP-Rab11-positive REs irrespective of embryonic stage.

(I) Quantifications of the diameter of the GFP-FYVE positive endosomal puncta at 13 h AEL and 18.30 h AEL for wild type and $rab5^2$ embryos. A significant reduction of the size of the endosomal structures was detected. * indicates statistically significant differences between $rab5^2$ and wild type embryos ($p<0.001$). At least 80 tracheal cells were examined for each developmental time point.

(J) Plot comparing the number of intracellular GFP-Rab7 positive puncta for metamere 7 between live wild type and $rab5^2$ embryos. About 80% of the analyzed wild type embryos exhibited moderate to high number of GFP-Rab7 positive puncta. Only 30% of the $rab5^2$ embryos had moderate number of GFP-Rab7 positive structures while none of the analyzed $rab5^2$ embryos showed high number of puncta. The number of observed intracellular puncta were organized into the following categories: low (0-5 puncta), moderate (6-15 puncta) and high (>15 puncta). Scale bars 10 µm.
Figure S9. A Proportion of Intracellular Gasp Puncta Transiently Colocalize with Specific Endosomal markers

(A-B) Confocal micrographs of early stage 17 wild type (A) and gasp mutant embryos (B) stained for Gasp (green) and a mixture of anti-Crb and anti-α-Spectrin to highlight tracheal cells (magenta). y-z confocal sections correspond to the DT position indicated by white vertical lines in xy sections.

(A’-B’) displays Gasp staining (white) of the images (A) and (B).

(C-E) xy confocal sections, xz, and yz projections of DT of early stage 17 embryos expressing *btl>*Clc-GFP (C), *btl>*GFP-Rab7 (D), and *btl>*GFP-LAMP1 (E) stained for Gasp (green) and GFP (magenta). White vertical and horizontal thin lines indicate the plain of xz and yz confocal projections and each indicate a Gasp dot localizing to a marked endosomal structure. Additional co-localising puncta are marked by orange
arrowheads. A subset of intracellular Gasp puncta co-localize with Clc-GFP (CCV), GFP-Rab7 (LE) and GFP-LAMP1 (lysosomes).

(F) Quantification of the number of Gasp puncta localizing to a specific endosomal marker relative to the total intracellular Gasp puncta per confocal section. For each marker four confocal sections were analysed from a total of four embryos. Error bars denote s.e.m.

(G) Confocal micrograph of the DT of a live, early stage 17 embryo expressing $btl>\text{GFP-CAAX}$ (green) that was pre-injected with dextran (magenta) at stage 16. No intracellular dextran puncta can be seen, indicating that intracellular puncta may not derive from basal internalization.

(H) DT of a live, early stage 17 $rab5^2$ mutant embryo expressing $btl>\text{CD8-GFP}$ (green) that was injected with dextran (magenta) at stage 16. $rab5^2$ mutant embryos show intact paracellular junctions. Scale bars, 10 µm.

**Supplemental Experimental Procedures**

$btl\text{-GAL4}$ (on 2nd) and UAS-ANF-GFP (on X) were mobilised to generate additional autosomal insertions that were meiotically recombined with different GFP transgenes. Fixed and live mutant embryos were identified using appropriate balancers (Casso et al., 2000; Halfon et al., 2002; Le et al., 2006). Additional insertions used were UAS-CD8-GFP (Lee and Luo, 2001), UAS-GFP-$\text{rab5}$, UAS-2xmyc-FYVE-GFP, UAS-GFP-$\text{rab11}$, UAS-$\text{clc-GFP}$, GFP-trap for PDI (on 3rd) (Bobinnec et al., 2003), UAS-GFP-CAAX, UAS-GFP-$\text{rab7}$, and UAS- GFP-LAMP1 (on 2nd), $btl\text{-mRFPl-moe}$ and UAS-myr-mRFPl (on both on 2nd and 3rd).
Supplemental References


Introduction

The acquisition of optimal tube dimensions during development is critical for the function of organs like the lung, renal and vascular systems. This is reflected by the high incidence of human pathologies associated with tube size defects. For example, cystic overgrowth in the collecting ducts is a landmark of polycystic kidney disease, whereas stenotic tubes cause obstructions of blood vessels and other organs [1,2]. We use the Drosophila respiratory network as a genetic model to understand the determinants of tube size expansion. The tracheal system derives from twenty metameric cell clusters that undergo their last cell division as they invaginate from the epidermis. Cell migration and a series of coordinated tracheal cell rearrangements generate a tubular network that sends branches to all developing tissues [3–5]. Branching is followed by three precise morphological events instructive role of the extracellular luminal matrices in tube growth and assembles a massive chitinous extracellular matrix. Mutations affecting chitin biosynthesis (kkv) and matrix assembly (kk) grow disproportional cystic and over elongated tubes [8–10]. In contrast to diametric expansion, tube elongation is continuous during tracheal development. Its termination requires secreted chitin deacetylases that presumably modify the structure of the ECM and thereby provide a stop signal to the epithelium [11,12]. Collectively, tube size mutants in flies and worms suggest an instructive role of the extracellular luminal matrices in tube growth coordination and termination [13].

What triggers and drives tube expansion? Studies of lumen formation in other systems indicate that apical polarization and targeting of membrane and vesicles to the lumen are central processes in the initiation of tube morphogenesis [2,14]. In the luminal liquid is replaced by gas to generate a functional respiratory network [6]. Tube diameter expansion occurs within a defined time interval without changes in the number or shape of the constituent cells [7]. During this interval the tracheal epithelium deposits proteins and polysaccharides into the lumen and assembles a massive chitinous extracellular matrix. Mutations affecting chitin biosynthesis (kkv) and matrix assembly (kk) grow disproportional cystic and over elongated tubes [8–10]. In contrast to diametric expansion, tube elongation is continuous during tracheal development. Its termination requires secreted chitin deacetylases that presumably modify the structure of the ECM and thereby provide a stop signal to the epithelium [11,12]. Collectively, tube size mutants in flies and worms suggest an instructive role of the extracellular luminal matrices in tube growth coordination and termination [13].

What triggers and drives tube expansion? Studies of lumen formation in other systems indicate that apical polarization and targeting of membrane and vesicles to the lumen are central processes in the initiation of tube morphogenesis [2,14]. In the
zebrafish gut, luminal fluid influx generates a distending force that drives coalescence of narrow tubes into a single lumen [15]. The developing trachea tubes and other organs deposit transient solid matrices, which may provide a distending force expanding tube diameter. Mutants affecting COPII vesicle budding and coat assembly show defects in the secretion of luminal proteins and diametric tube growth. The failure in the assembly and expansion of the chitin matrix may be the underlying cause of the narrow tube phenotypes in these mutants. Alternatively, the diametric expansion defects could be due to reduced delivery of apical membrane or transmembrane regulators to the cell surface [6,16,17].

Secrated proteins traffic through the Endoplasmic Reticulum (ER), the Golgi apparatus and the exocytic post-Golgi structures to their final destination. Anterograde and retrograde transport between the ER and the Golgi depends on two distinct types of coated vesicles: COPII vesicles bud from the ER and shuttle nascent secreted proteins to the Golgi apparatus. Conversely, COPII coated vesicles retrieve escaped luminal ER enzymes and recycling cargo adaptor proteins (such as the p23/24 type of proteins) from the Golgi and shuttle them back to the ER. [18]. Within the Golgi apparatus, COPII vesicles have been proposed to mediate anterograde transport of secreted cargo or retrograde traffic of Golgi enzymes between different cisterns of the Golgi [19]. The coatamer complex of COPII vesicles is composed of two different layers. The β, δ, γ, ζ subunits form the trimeric subcomplex of the inner layer. The β- and γ- subunits bind to active Arf1-GTP, γCOP also binds to p23. The outer coat layer subcomplex is trimeric and contains the ζ, β′ and ε subunits [20].

Here we present the functional analysis of COPII function in tube organogenesis. We find that COPII vesicle transport is required to: a) secrete of multiple luminal proteins, b) assemble a luminal matrix and c) expand the diameter of tubular organs. These defects are not confined to the chitin-lined tracheal tubes but also extend to the salivary glands. In tubular epithelia of γCOP mutant embryos, the structure of both the ER and Golgi is compromised indicating that efficient luminal secretion is highly dependent on a functional secretory apparatus. The results highlight the major role of membrane traffic in the expansion of luminal extracellular matrices and apical membrane during tubulogenesis.

**Results**

**γCOP is required for tube diameter expansion**

We identified γCOP mutants by screening a collection of lethal P-element insertions for tracheal tube size defects [21–25]. At early stage 16, the dorsal trunk (DT) tubes of γCOP mutants were narrower compared to wild type. Using imprecise excision, we generated two independent molecular null alleles including the γCOP1114 lethal allele, which we analyzed for tube expansion (hereafter referred to as γCOP mutants) (Figure S1). To visualize the tracheal lumen, we stained embryos for a secreted chitin binding protein, Gasp in embryos expressing GFP-CAAX in the trachea (btl>GFP-CAAX) [6]. At early stage 16, the DT tubes of γCOP mutants were thinner compared to wild type (Figure 1A, B). This failure in tube expansion was also evident by comparison of zy-confocal sections (Figure 1D, E). Re-expression of γCOP in the trachea of mutant embryos rescued the phenotype, indicating that γCOP is required in the tracheal epithelium for diametric tube growth (Figure 1C, F). To quantify tube diameter, we measured the distance between the apical surfaces of cells lining the DT in confocal sections of wild type and γCOP mutant embryos expressing btl>GFP-CAAX. At early stage 16, the DT diameter in metamere 6 was reduced by 54% in γCOP mutants (3.6±0.5 μm, n = 10) compared to wild type (6.2±0.36 μm, n = 9) (Figure 1G). By contrast, DT metamere 6 length was not altered in the mutants (32.7±1.1 μm in wild type n = 4 and 33.1±0.7 μm in γCOP mutant embryos n = 4). These measurements indicate that the DT volume capacity of the γCOP embryos is reduced to the third compared to wild type.

Wild type embryos initiate diametric tube expansion at stage 14 and nearly complete it within 3.5 hours [6]. To determine when the γCOP phenotype is manifested, we imaged live wild type embryos and γCOP mutants expressing btl>GFP-CAAX. We found that γCOP mutant embryos already show a narrower DT diameter at stage 14 and later expand their tubular diameter with a slower rate (0.45 μm/h) compared to wild type (0.71 μm/h) (Figure 1H). Thus, zygotic γCOP function is required before and during the diameter expansion interval.

To investigate where and when γCOP is expressed, we performed mRNA in situ hybridizations. Maternal γCOP transcripts are abundant in early embryos (Figure S1). Zygotic γCOP expression commences in the epidermis and salivary glands (SG) form stage 11, initiates in the trachea at stage 13 and at early stage 16 is also detected in the foregut and hindgut tubes (Figure S1). Consistent with the epidermal expression of γCOP, we found that γCOP mutants fail to complete dorsal closure. This defect can be rescued by ubiquitous γCOP expression from a tubulin1α-promoter in the mutant background demonstrating that γCOP is required for epidermal morphogenesis (Figure S1).

γCOP and other genes encoding components of the secretory apparatus are prominently expressed in developing SGs [26]. This prompted us to analyze SG size growth in γCOP mutants. We used antibodies against the apical regulator Crumbs and cytoplasmic α-Spectrin to highlight SG cellular outlines and Piopio to label the SG lumen in wild type and γCOP embryos [27]. Like in tracheal tubes, the salivary glands of γCOP mutants were thinner compared to wild type (Figure 1I, J, L, M). The SG tube diameter is strikingly reduced in γCOP mutants from 5.0±0.76 μm (n = 7) in the wild type to 1.9±0.36 μm (n = 7) in γCOP (Figure 1O) (errors are SD). This phenotype can be rescued by ubiquitous expression of tub1α-γCOP in mutant embryos (Figure 1K, N, O). Also SG tube elongation is impaired in γCOP null mutant embryos. The distance between the most distal cells of the salivary duct expressing btl>GFP-CAAX to the gland tip visualized by E-Cadherin (E-cad) staining, was reduced from 50.2±5.8 μm (n = 7) in wild type to 36.2±4.3 μm (n = 11) in γCOP mutants at stage 16 (p<0.0001). The similarities of tracheal and salivary gland phenotypes in γCOP mutants suggest a common cellular mechanism expanding tubular organs.

**γCOP is required for luminal secretion and assembly of ECM components**

A COPII-mediated secretory burst of luminal proteins at late stage 13 drives the diametric expansion of tracheal DT tubes [6]. During this interval luminal chitin and chitin binding proteins assemble into an expanding matrix. To test whether luminal protein deposition is affected in γCOP mutants, we stained wild type and mutant embryos expressing btl>GFP-CAAX for the luminal antigen 2A12 and the chitin binding proteins Gasp and Verm. While 2A12, Gasp and Verm predominantly localize to the tracheal lumen in wild type stage 15 embryos, those markers are strongly retained inside tracheal cells in γCOP mutants (Figure 2A, B, D, E, G, H). This retention can be rescued by tracheal specific btl>γCOP expression in γCOP mutants (Figure 2C, F, I). Thus, γCOP is required in the tracheal epithelium for luminal protein secretion and the completion of diametric tube expansion. Does
Figure 1. γCOP is required for efficient lumen diameter expansion. (A–F, I–N) Confocal micrographs of early stage 16 wild type (A, D, I, L), γCOP mutant (B, E, J, M) and btl>γCOP; γCOP,γCOPembryos (C, F). GFP stainings of btl>GFP-CAAX embryos reveal tracheal DT cells (A–C, in magenta and D–F, in white). Anti-Crumbs and anti-α-Spectrin stainings show SG cells (I–K, in magenta and L–N, in white). Embryos were stained for the luminal antigens Gasp (A–C) and Piopio (I–K) (green). All micrographs show confocal sections except for (D–F) and (L–N) which show yz-confocal sections of DT and SG. (G and O) Lumen diameter of DT metamere 6 using btl>GFP-CAAX (G) and diameter of SG measured for Crumbs stainings (O). γCOP mutant embryos show significantly (p < 0.001) narrower trachea and SG tubes. Transgenic expression of γCOP specifically in the trachea (C, F) or under the tubulin1a promoter for SG (K, N) largely rescues the narrow lumen phenotype of γCOP mutant embryos. (H) Plot showing DT diameter expansion of live wild type and γCOP embryos expressing btl>GFP-CAAX. Embryos were recorded from late stage 13 to stage 15. Error bars are means ± SD. Scale bars are 10 μm (A–C, I–K), 5 μm (D–F) and 10 μm (L–N).

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the defective deposition of chitin binding proteins into the tubes affect the assembly and expansion of the luminal matrix? Staining with a fluorescent chitin binding probe and TEM analysis revealed that the tracheal luminal matrix in \( \text{cCOP} \) embryos remained narrow and dense at early stage 16 embryos (Figure 2J, K, L, M).

The developing SGs also form an intraluminal matrix. Embryos lacking \( \text{pasilla} \) or a gene cluster encoding putative prolylhydroxylases show disrupted matrix structure and cystic and malformed glands [28,29]. Do the \( \gamma \text{COP} \) defects in gland expansion coincide with a failure in the secretion and assembly of the intraluminal matrix? The molecular composition of the SG luminal matrix is unknown, but unlike the tracheal one it does not depend on chitin biosynthesis (data not shown). Recently, the SG lumen has been shown to contain glycans with a single N-acetyl-Galactosamine O-linked to Serine or Threonine [30]. To visualize the luminal secretion of those O-glycans in the salivary gland, we stained fixed embryos with the specific Tn antibody. Directly after SG invagination, minor amounts of Tn antigen were detected in the

![Figure 2. \( \gamma \text{COP} \) is required for efficient luminal protein secretion and matrix expansion in the trachea.](image-url)

(A–I) Confocal micrographs of DT of wild type (A, D, G, J), \( \gamma \text{COP} \) mutant (B, E, H, K), \( \text{btl}\gamma \text{COP}; \gamma \text{COP}^{P1} \) rescued embryos (C, F, I). \( \text{btl}\gamma \text{COP} \) embryos were stained for the tracheal luminal antigens 2A12 (A–C), Verm (D–F) and Gasp (G–I) (green), GFP (magenta) and with a chitin binding probe (CBP), white in (J,K). All micrographs are single confocal sections except (J–K), which represent confocal projections. The inserts in J and K show y-z projections. \( \gamma \text{COP} \) mutant embryos show strong intracellular retention of tracheal luminal markers. Transgenic re-expression of \( \gamma \text{COP} \) largely rescues the luminal secretion phenotype of \( \gamma \text{COP} \) mutant embryos. (K and M) The luminal ECM is defective in the trachea of \( \gamma \text{COP} \) mutants. Scale bars are 10 \( \mu \text{m} \) in A–K and 0.5 \( \mu \text{m} \) in L and M.

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lumen. The Tn antigen levels markedly increased and localized in intracellular puncta and in the SG lumen at stage 12 and 13. However from stage 14 onwards, the intracellular and luminal levels decline until only very little remains along the apical lining of the SG a stage 15 (Figure 3A, B, C, D). A similar, dynamic localization of Tn antigen is also evident in the trachea (data not shown). Thus, salivary gland expansion is accompanied by a sharp burst of luminal secretion of O-glycosylated proteins. Is γCOP required for this secretory burst? Importantly at stage 13, γCOP mutant embryos showed strongly reduced intracellular accumulation and luminal deposition of Tn antigens compared to wild type embryos. This phenotype was fully rescued by the tub1-γCOP transgene (Figure 3G). Like γCOP, sar1 mutant embryos also show a reduced secretion in the SG (Figure 3G), suggesting both COPI and COPII are required for efficient luminal deposition in the SG. To determine if the defects in O-glycan secretion are accompanied by defects in luminal extracellular matrix assembly, we analysed the SGs of γCOP mutant embryos by TEM. In striking contrast to wild type embryos that show a uniform and space-filling luminal matrix at early stage 16, γCOP mutant embryos showed an atrophic, deformed and abnormally electron dense luminal matrix in the narrow SG lumen (Figure 3I, J). Additionally, the lack of abundant dark apical granules in γCOP mutants further supports the role of γCOP in luminal material deposition. Thus, γCOP function is required for the secretion of luminal antigens and assembly of an extracellular matrix.

δCOP mutants phenocopy γCOP secretion and diameter phenotypes

As the coatamer complex comprises seven subunits that form the COPI vesicle coat, we asked if mutants in other coatamer components show defects in tube expansion. δCOP mutants retain the Verm and Gasp luminal proteins inside the tracheal cells at stage 15 and fail to fully expand DT tube diameter at early stage 16 (Figure 4A, B, C, D). The diameter of the DT tube of metamere 6 was reduced by 28% in δCOP mutants compared to the wild type (Figure 4E, F, I). A similar phenotype is evident in the SGs (Figure 4G, H). These results confirm that COPI vesicle trafficking mediates luminal secretion and efficient diametric expansion of tracheal and salivary gland tubes.

Figure 3. γCOP is required for deposition of O-glycans and luminal ECM assembly in the SG. (A–H) Confocal projections show developmental stages of salivary gland (A–D) stained for Tn-antigen (green) and Crumbs in (magenta). (A’–D’) represent confocal sections of the (A–D) projections focused inside the lumen. (A, A’) show stage 11, (B, B’) stage 12, (C, C’) stage 13, (D, D’) stage 15 wild-type embryos. The salivary gland epithelium dynamically deposits Tn antigen into the lumen. While at stage 11 luminal levels of Tn antigen are low, they increase dramatically during stages 12 and 13 and localize to intracellular puncta and lumen. Later, luminal Tn antigen levels decline but a minor proportion remains along the apical epithelial membrane. (E–H) Confocal projections show stage 13 wild-type (E), γCOP/γCOPP1 (F), sar1EP28 (G) and tub-γCOP; γCOP/γCOPP1 mutant embryos (H) stained for Tn antigen (white). γCOP and sar1 mutant embryos show reduced intracellular puncta and luminal deposition of Tn antigen. (I and J) TEM of salivary gland cross-sections from stage 16 wild-type and γCOP mutants. The intraluminal matrix and the electron-dense granules are severely reduced in γCOP mutants compared to wild type. Scale bars are 10 μm in A–H. and 2 μm in I and J.

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mutants (Figure 5P–S). Thus, loss of γCOP or sar1 does not affect epithelial integrity (Figure 5E, J, O). In summary, this suggests that apical secretion and luminal matrix assembly are common strategies for tubular organ expansion in Drosophila.

γCOP co-localizes with ER and Golgi markers

We used S2 cells and an antisense against the mouse γCOP [31] to address the subcellular localization of γCOP in Drosophila. The γCOP antibody showed a punctate cytoplasmic signal in S2 cells or cells treated with double stranded (ds) RNA against GFP. γCOP dsRNAi treatment abolished the punctate staining and showed a specific reduction of a 26 kDa protein in γCOP dsRNAi treated cells compared to GFP dsRNAi treated cells (Figure S2). To reveal the identity of the γCOP puncta, we co-stained S2 cells with either ER or Golgi markers. Confocal analysis showed a partial overlap of γCOP with the ER markers KDEL (the ER retention signal found in many ER proteins) and Calreticulin (Figure S2 and not shown). Co-staining for the cis-Golgi markers GM130 and Lava lamp showed a discrete co-localisation of γCOP with these structures. By contrast, the γCOP puncta did not overlap with the median (gp120) and trans Golgi marker (peanut agglutinin) staining (Figure S2). Thus γCOP localizes in the ER and cis-Golgi units in Drosophila cells, consistent with the localization of its mammalian and plant homologs [31,32].

ER and Golgi are disrupted in γCOP mutant embryos

The prominent secretory deficits in the γCOP mutants and the co-localization of γCOP with ER and cis-Golgi markers suggested that the basolateral secretory apparatus may be defective in γCOP embryos. We therefore stained wild type and mutant embryos for KDEL, the transmembrane adaptor p23/Baiser and Calreticulin, an abundant ER chaperone [33–35]. We observed a drastic reduction in the staining intensities of all markers in the trachea and SGs of the mutants compared to wild type embryos (Figures 6A–D, K, L and S3). Thus, the reduced intensity of Calreticulin staining in γCOP mutants was also evident in epidermal cells (Figure S3), which also showed an abnormally high intracellular accumulation of Verm at stage 16 (data not shown). We used TEM to visualize the tracheal and SG ER in wild type and γCOP mutants. In contrast to the uniformly organized tubular ER of the wild type, ER structures were severely bloated in the mutants particularly in the trachea (Figure 6I, J, O, P). These structural defects are accompanied by a mild activation of the unfolded protein response evidenced by the presence of processed xbp1 transcripts in γCOP mutants (Figure S3) [36]. The above data show that COPI vesicle transport is required for maintenance of ER integrity in epithelial tissues. We analyzed the Golgi status by staining embryos for the Lava lamp and gp120 markers [37]. As expected the intensity of Lava lamp and gp120 puncta were reduced in the trachea (Figure 6E–H) and salivary glands of mutant embryos (Figures 6M, N and S3). Thus, the deficits in luminal protein deposition and tube expansion are due to structural defects in the secretory apparatus of γCOP mutants.

COPI and COPII vesicle trafficking drive tube expansion

COPI coatomer subunit mutations cause tube expansion and cellular defects that closely resemble the phenotypes of COPII coat mutants [6]. However, only γCOP mutants show dorsal closure phenotypes suggesting a selective role of COPI vesicles. To determine if mutants in COPI and COPII components show additive phenotypes in the trachea, we analyzed zygotic single sar1 or γCOP null mutant embryos and sar1/γCOP double mutants. Epithelial organization is not affected in zygotic γCOP mutants

Reduced apical secretion in γCOP mutants may be an indirect consequence of defects in epithelial polarization. We addressed this by staining for apical and junctional epithelial polarity markers, such as Crumbs, the adherence junction marker E-Cadherin and the septate junction marker Coracle in the trachea and SGs. Except for a minor decrease in the staining intensity of the markers, their localization and the revealed epithelial cell shapes were indistinguishable between mutants and wild type embryos (Figure 5A–O). Further, we found that both adherens junction structure (Figure 6I, J), as well as the transepithelial barrier function of the septate junctions were intact in γCOP mutants (Figure 4). Epithelial organization is not affected in zygotic γCOP mutants.
Interestingly, sar1 γCOP double mutant embryos showed as narrow DT tubes, as sar1 or γCOP single mutants (Figure 7B, C, D). The lack of an additive phenotype in the double mutants suggests that anterograde COPII and retrograde COPI vesicles transport are equally important to maintain both ER and Golgi structures and that the dynamic bidirectional ER-Golgi traffic is essential for the secretory activity of epithelial cells during tube expansion.

**Discussion**

COPI and COPII mutations strictly affect diameter but not length expansion in the trachea. SG expansion is mainly defective in diameter and to lesser degree also in length. This differential effect may be explained by the dramatic expansion of SG in both diameter and length during the SG secretory burst. The tracheal tubes on the other hand, elongate continuously and the programmed boost of secretory activity precedes the short interval of diametric expansion.

γCOP and sar1 mutants show qualitatively similar phenotypes in tube expansion in the trachea and the SG. γCOP and sar1 gene products are deposited in the oocyte at sufficient levels to support early embryogenesis. Distinct levels in maternal contribution or zygotic expression of γCOP and Sar1 may contribute to the quantitative differences in tube expansion phenotypes. In sharp contrast to the similarities of tubular defects, γCOP mutant embryos fail to complete dorsal closure while sar1 mutants close normally (data not shown). While we cannot exclude that the γCOP maternal product is less stable than Sar1, this phenotypic difference suggests that not all developmental processes require both COPII and COPI function equally. This may be attributed to a specific COPI function in intra Golgi trafficking.

The developing trachea and SG tubes initiate a dynamic secretory burst that deposits transient solid matrices. The diametric expansion defects in mutants for COPI and COPII components may in part be due to reduced delivery of apical membrane or transmembrane regulators to the cell surface. The
Figure 6. Defective ER and Golgi in \(\gamma\text{COP}\) embryos. (A–H, K–N) Confocal sections of stage 16 wild type (A, B, E, F, K, M) and \(\gamma\text{COP}\) mutant embryos (C, D, G, H, L, N) expressing \(btl\)-GFP-CAAX (magenta). Embryos were stained with anti-KDEL for ER in white (A, C, K, L), green in (B, D) and with anti-Lava lamp for Golgi in white (E, G, M, N), green in (F, H). \(\gamma\text{COP}\) mutant embryos show reduced ER staining intensity (C, D, L) and a marked decrease in the number of Golgi puncta both in trachea and SG cells (G, H, N). TEM sections of DT (I–J) and SG (O–P) of stage 16 wild type (I, O) and \(\gamma\text{COP}\) embryos (J, P). In wild type both tracheal and SG cells show a tubular organization of the rough ER, studded with ribosomes (white arrow in I, O). \(\gamma\text{COP}\) mutant cells show disrupted and bloated rough ER structure (white arrow in J, P). The SG intraluminal matrix is indicated by an asterix in (O) and (P). Scale bars are 10 \(\mu\text{m}\) (A–H, K–N), 0.5 \(\mu\text{m}\) in (I–J) and 1 \(\mu\text{m}\) in (O, P).

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and secretion of water absorbing glycosaminoglycans has been proposed to generate the turgor for notochord expansion [42]. Zebrafish mutants in coatomer encoding genes fail to expand their notochord and larval tail. These phenotypes could be partly due to defects in laminin secretion and the assembly of the basal extracellular matrix [43]. The recent visualisation of vacuolar membrane fusion with the luminal cavity in ascidians and our analysis of COPI mutants further implicate luminal secretion in notochord expansion. Thus, the programmed deposition of swelling luminal matrices may be a common strategy of tube expansion.

Materials and Methods

Drosophila Strains

The P element alleles l(3)057302 and KG06383 (γCOPP) and γCOPP respectively; Figure S1) fail to complement each other. Insertion sites were determined by plasmid rescue and PCR. P-element excision of γCOPP generated a precise excision reverting the tracheal phenotype, along with two independent null lethal alleles: A128, a smaller deletion, taking out the 5’UTR and a major part of the first exon and A114 (γCOP in text) which lacks the 5’UTR, first exon and the majority of second exon (Figure S1). All the analysis was performed on A114. Rescue with blt>γCOP or tubulin1z-γCOP was performed in γCOPP;γCOPP transheterozygous mutant background. Other mutants used were KG07426 (δCOPP), l(3)05712 (sar1P1), sar1A28 (sar1 P114 [6], E(4)575A28) [6], kkδOzI [8], Atpx267611 [22], and NPS464 (p23/baiser).

Molecular biology
ds-RNAi, RT-PCR. pUAST-γCOP and tubulin1z-γCOP were generated by sub-cloning the cDNA from RE37840 into pUAST and plb1 alpha-GAL80 [44]. Down regulation of γCOP by RNAi in S2 cells was performed as described in [45]. Primer pairs tagged with T7 RNA polymerase promoter were used to amplify a PCR fragment from the cDNA clone. Primers used in PCR amplification 5’-TTAATACGACTCACTATAGGGAGACGCACAATAGGGCTCTCCAAGATGA-3’ and 5’-TTAATACGACTCATTAGAGGAGACCGAGAGGGTTTGAACAGCGGCA-3’ and 5’-TTAATACGACTCATTAGAGGAGACCGAGAGGGTTTGAACAGCGGCA-3’ and 5’-TTAATACGACTCATTAGAGGAGACCGAGAGGGTTTGAACAGCGGCA-3’ and 5’-TTAATACGACTCATTAGAGGAGACCGAGAGGGTTTGAACAGCGGCA-3’. The 900 bp PCR product was then used as template for dsRNA production with the MEGAscript RNAi kit (Ambion).

For RT-PCR, total mRNA was isolated from stage 16 wild type and γCOP mutant embryos using oligo(dT)-coupled beads (Dynabeads). Unfolded protein response was induced in S2 cells by treatment with 10mM DTT. Reverse transcription was performed with SuperScript-II (Invitrogen). Primers flanking the splice-site of xbp-1 mRNA were used, 5’-CGCCACGCCGCTGAGGAGG-3’ and 5’-CTGCTCCGCCAGCAAGTGACGATGAA-3’. Rescue with blt>γCOP in text) which lacks the 5’UTR, first exon and the majority of second exon (Figure S1). The primer pairs tagged with T7 RNA polymerase promoter were used to amplify a PCR fragment from the cDNA clone. Primers used in PCR amplification 5’-TTAATACGACTCACTATAGGGAGACGCACAATAGGGCTCTCCAAGATGA-3’ and 5’-TTAATACGACTCATTAGAGGAGACCGAGAGGGTTTGAACAGCGGCA-3’ and 5’-TTAATACGACTCATTAGAGGAGACCGAGAGGGTTTGAACAGCGGCA-3’ and 5’-TTAATACGACTCATTAGAGGAGACCGAGAGGGTTTGAACAGCGGCA-3’. The 900 bp PCR product was then used as template for dsRNA production with the MEGAscript RNAi kit (Ambion).

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Immunostaining, TEM and Western blotting

Immunostainings, Western blots and TEM were performed as in [6,45] with the following additional antibodies: Rabbit anti-Mouse γCOPI raised against C-terminal region [31] (The Drosophila γCOP protein is 50% identical to mouse γCOP1), rabbit anti-Lava Lamp [37], rabbit anti-Galleria mellonella calreticulin [35]. Rabbit anti-p23/Baiser raised against cytoplasmic peptide tail [33]. Fluorescin-Peanut agglutinin (PNA) (Sigma), rabbit anti-dGM130 [46] (Abcam). The mAB anti-Tn clone B1.1 (Boimeda) [30] recognizes the unmodified N-acetyl-Galactosamine-group O-linked to serines or threonines on protein substrates (Tn antigen). To examine the Sj barrier function in

Figure 7. COPI and COPII function in tube size control. (A–D) Confocal sections of early stage 16 wild type (A), sar1A28 (B), γCOP (C) and sar1A28, γCOP double mutant embryos (D). Cells of tracheal DT stained for anti-Crumps and anti-α-Spectrin shown in magenta (A–D) and for the tracheal luminal antigen Gasp (A–D) (green). (E) Schematic illustration of a cross-section through an epithelial tube. The ER, Golgi and post-Golgi vesicles carry secreted proteins (green) into the lumen. Both COPI and COPII vesicular transport between the ER and Golgi are required for intraluminal matrix assembly and apical membrane addition (red). Green arrows indicate the proposed pressure exerted on the cells by the expanding matrix. Scale bars are 10 μm.

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strict correlation between the luminal matrix assembly defects and the tube expansion phenotypes strongly suggest that the swelling luminal matrices directly enlarge the tubes from the inside (Figure 7E). This is analogous to the distending pressure from the transient fluid influx into the lumen that expands the lung and coalesces the gut lumen in zebrafish [15,38]. “Inside out molding” by swelling luminal matrices may be a common mechanism in tubulogenesis extending to vertebrates. The notochord of many ascidian species expands by coalescing vacuoles into a single continuous lumen stretching through the entire notochord [39], while other vertebrate species rely on an immense intracellular vacuolarisation [40]. The luminal/vacuolar turgor of the notochord is contained by a thick basement membrane ECM. This renders the expanding notochord into a stiff, rod-shaped organ that elongates the tail and the entire animal [41]. The luminal secretion of water absorbing glycosaminoglycans has been
tracheal cells, embryos were injected at late stage 16 with 10 kDa Rhodamine-Dextran (Molecular Probes).

Supporting Information

**Figure S1** γCOP genomic locus and expression pattern. (A) The γCOP locus and positions of P element insertions and deletions. (B–E) Bright field images of wild type stage 1 (B), early stage 16 (C) and γCOP mutant embryos stained for γCOP (E) with anti-sense RNA probes (grey). (D) shows an embryo stained with a “sense” RNA probe. Expression of γCOP transcripts was strongly reduced in zygotic γCOP mutant embryos. (E, F–K) Confocal sections of late-stage 1 embryos showing zygotic expression of γCOP in the SG (F) and DT (H, I) with γCOP anti-sense probes. No staining was detected with the sense probe in SG (G) and DT (J, K). Tracheal cells are visualized by anti-β-Gal staining (magenta in I, K). Zygotic expression of γCOP transcripts is observed in SG and trachea. (L–N) Confocal projections of wild type (L), γCOP mutant (M) and tub-γCOP (N) embryos (N) stained for Coracle to visualize the dorsal epidermis. γCOP mutant embryos fail to close dorsally. Scale bars are 30 μm in (B–E) and 10 μm in (F–G, H–K, L–N).

**Figure S2** γCOP co-localizes with ER and Golgi markers. (A–J) Confocal sections of stage 2 cells stabilized with anti-γCOP (green) and gpl120 (A–D, I), or KDEL (F), or GM130 (G), or Lava lamp (H), or PNA (J) (red). S2 cells were either mock treated (A) or treated with dsRNA for GFP (B) or for γCOP for 3 days (C) or 6 days (D). Golgi and γCOP staining were reduced in γCOP treated cells. (E) Western blot of S2 cell extracts show a marked reduction of ~97 kDa protein (equivalent to predicted molecular weight) in dsCOP treated cells, but not in untreated cells. Lamin was used as loading control. γCOP shows partial overlap with KDEL and clear co-localization with cis-Golgi markers. Scale bars are 10 μm.

References

**Figure S1**

A diagram illustrating the genomic DNA region with alleles $\gamma\text{COP}^{P2} = P(\text{SUP-orP})\text{ KG06383}$ and $\gamma\text{COP}^{P1} = P(\text{lacW})\text{ I(3)s057302}$. The region $\gamma\text{COP}^{\Delta^{128}}$ and $\gamma\text{COP}^{\Delta^{114}}$ are indicated.

**Panel B**
- wt (wild type) and Anti (antibody) staining compared to sense (control) staining.

**Panel C**
- wt (wild type) and Anti (antibody) staining.

**Panel D**
- wt (wild type) and Sense (control) staining.

**Panel E**
- wt (wild type) and Anti (antibody) staining.

**Panel F**
- wt (wild type) and Anti (antibody) staining.

**Panel G**
- wt (wild type) and Sense (control) staining.

**Panel H**
- wt (wild type) and Anti (antibody) staining.

**Panel I**
- wt (wild type) and Merge (combination of images).

**Panel J**
- wt (wild type) and Sense (control) staining.

**Panel K**
- wt (wild type) and Merge (combination of images).

**Panel L**
- wt (wild type) and Cor (corner) staining.

**Panel M**
- wt (wild type) and $\gamma\text{COP}$ staining.

**Panel N**
- wt (wild type) and $\text{tub}\gamma\text{COP}$; $\gamma\text{COP}$ staining.
Figure S2

(A) Untreated

(B) GFPi

(C) γCOPi d3

(D) γCOPi d6

(E) γCOP

(F) KDEL

(G) GM130

(H) Lava

(I) gp120

(J) PNA

Bar scale: 5 μm
Epithelial septate junction assembly relies on melanotransferrin iron binding and endocytosis in *Drosophila*

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Iron is an essential element in many biological processes. In vertebrates, serum transferrin is the major supplier of iron to tissues, but the function of additional transferrin-like proteins remains poorly understood. Melanotransferrin (MTf) is a phylogenetically conserved, iron-binding epithelial protein. Elevated MTf levels have been implicated in melanoma pathogenesis. Here, we present a functional analysis of MTf in *Drosophila melanogaster*. Similarly to its human homologue, *Drosophila* MTf is a lipid-modified, iron-binding protein attached to epithelial cell membranes, and is a component of the septate junctions that form the paracellular permeability barrier in epithelial tissues. We demonstrate that septate junction assembly during epithelial maturation relies on endocytosis and apicolateral recycling of iron-bound MTf. Mouse *MTf* complements the defects of *Drosophila MTf* mutants. *Drosophila* provides the first genetic model for the functional dissection of MTf in epithelial junction assembly and morphogenesis.

Epithelial sheets provide diffusion barriers that generate distinct chemical compartments within tissues and organisms. Specialized epithelial-cell junctions—tight junctions in vertebrates⁵ and septate junctions in insects⁶—prevent passive diffusion between cells and separate the apical and basal environments. Despite structural differences, septate- and tight-junctions share essential components, such as claudins, and might be derived from a common ancestral junction⁷. Genetic and molecular analysis of *Drosophila* septate junctions has identified 15 components, including adhesion molecules and scaffolding proteins⁸. Among these, Coracle, Neurexin IV and Na⁺, K⁺-ATPase α form a functional sub-group with the FERM-protein, Yurt (Yurt–Cora group)⁹, to maintain epithelial polarity. Subsequently, septate junction protein complexes assemble into junctions to establish the paracellular epithelial barrier⁹ and control apical membrane growth during tubular organ expansion⁷. The maturation mechanism of septate junction complexes into functional junctions remains unknown.

MTf is a GPI (glycosyl phosphatidylinositol)-anchored, iron-binding protein found in several mammalian epithelial tissues⁹. Human MTf is apically localized in polarized cells⁹, and its levels are increased in melanoma patients. Consequently, MTf has been suggested to modulate melanoma-cell migration and metastasis⁸. However, its function in vivo remains elusive. Mice that lack or overexpress MTf have no detectable abnormalities⁸,¹¹,¹², presumably because of compensatory mechanisms involving the other proteins of the transferrin-like family. *Drosophila* MTf (CG10620), similarly to its vertebrate homologues, contains a signal peptide, two putative iron-binding domains and a carboxy-terminal GPI modification sequence (Fig. 1a). In embryos, MTf is expressed in all ectodermal epithelial tissues¹¹. We generated *MTf* null mutants (Supplementary Information, Fig. S1a) and focused our analysis on the trachea. The dorsal trunk airways were tortuous in *MTf*-mutant embryos, compared with wild-type (Fig. 1b, c and Supplementary Information, Fig. S1b). To investigate whether insect and mammalian MTf are phylogenetically conserved, we expressed *Drosophila* MTf cDNA in the trachea of *MTf* mutants. Both the *Drosophila* and mouse MTf rescued the tracheal phenotypes in 100% (n = 9) and 60% (n = 10) of the mutant embryos, respectively (Supplementary Information, Fig. S1c, d). Thus, mouse MTf can largely substitute for *Drosophila* MTf in trachea suggesting that they share essential molecular functions.

The airway phenotype of *MTf*-mutant embryos resembles the defects of mutants lacking septate junction components. Septate junctions are required for luminal accumulation of the putative chitin deacetylases, vermiform and serpentine, and for chitin matrix modification¹¹. These modifications are thought to limit branch elongation. *MTf*-mutant embryos failed to secrete vermiform into the growing tracheal tubes and showed diffuse luminal chitin and tube overelongation (Supplementary Information, Fig. S1e-h). We further assessed septate junction permeability in *MTf* mutants by dye-injection experiments¹¹. In *MTf* mutants, but not in wild-type embryos, 10 K fluorescent dextran leaked rapidly into the airways (Supplementary Information, Fig. S1i, j). These phenotypes indicate that septate junction functions are compromised in *MTf*-mutant embryos.

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To investigate septate junction integrity, wild-type and MTf-mutant stage-16 embryos were stained for the septate junction proteins, Neurexin IV (Nrx)\(^4\); Fig. 1d, e) and Coracle (Cora)\(^7\); Supplementary Information, Fig. S1k, l). In contrast to wild-type, where these markers were localized apically on the lateral membrane, Nrx and Cora were spread basolaterally in the trachea of MTf mutants. Transmission electron microscopy (TEM) analysis of the mutants indicated a strong reduction in paracellular septa, compared with the ladder-like septate junctions of wild-type embryos (Fig. 1f, g). No defects were detected in the adherens junctions of MTf mutants (Supplementary Information, Fig. S1n). Therefore, MTf is selectively required to establish or maintain septate junctions.

We hypothesized that MTf might be a septate junction component. Immunostaining of embryos with antibodies against MTf (Supplementary Information, Fig. S1o, p), Nrx (Fig. 1h, i and Supplementary Information, Fig. S1r, s) and Discs large (Dlg)\(^8\) (Supplementary Information, Fig. S1t, u) demonstrated that MTf co-localizes with these septate junction markers both in the trachea and in the hindgut. To determine whether MTf localization depends on septate junction components, MTf localization was analysed in cora and ATPax mutants\(^16\,19\) (Fig. 1j, k). Although the staining intensity was not affected, a pronounced basolateral spread of MTf was detected in the mutants. Thus, MTf localization requires septate junction integrity, and vice versa, suggesting that MTf is an integral septate junction component. To directly test if MTf associates with septate junction proteins, co-immunoprecipitation was performed from embryonic extracts. The MTf antisera co-immunoprecipitated Nrx, Contactin (Cont)\(^21\) and neuroglian (Nrg)\(^6\). In a complementary experiment Nrx antibodies precipitated MTf and Cont (Fig. 1l), Thus, MTf forms complexes with Nrx, Cont and Nrg during late embryogenesis. Adherens junction components were not detected in the anti-MTf immunoprecipitates, and anti-DE-cadherin antibodies did not co-immunoprecipitate MTf, indicating that MTf interacts specifically with septate junction proteins (Supplementary Information, Fig. S1v). Thus, MTf is a septate junction component required for junction integrity.

Next, we assessed how septate junction proteins assemble into functional junctions. We monitored MTf and Nrg localization in tracheal and hindgut cells of wild-type embryos during early- and late-stages of epithelial morphogenesis. At stage 13, before septate junction formation, MTf and Nrg localized evenly along the lateral and basal epithelial cell membranes. A fraction of the proteins were also detected in intracellular puncta (Fig. 2a, c and Supplementary Information, Fig. S2a).

Interestingly, co-immunoprecipitation experiments using stage 10–13 embryonic extracts demonstrated that a substantial portion of MTf is already forming a complex with Cont and Nrx at early stages of septate junction formation (Supplementary Information, Fig. S2c). The widespread distribution of these complexes along basolateral membranes at stage 13 markedly contrasts their restricted apicolateral localization at stage 16 (Fig. 2b, d and Supplementary Information, Fig. S2b). TEM analysis and epithelial permeability assays from previous studies suggest that mature septate junctions are established during the interval between stage 13 and 16 (ref. 2). We hypothesized that septate junction assembly may involve endocytosis of septate junction components from the basolateral surface. We therefore assessed the potential co-localization of septate junction proteins with endosomal markers at different stages. We observed a substantial overlap of MTf staining with the GFP–2xFYVE and the GFP–Rab5 markers in early endosomes and multivesicular bodies at stage 13 (Fig. 2e, f). To extend these findings and avoid potential reporter-derived artifacts, endogenous Rab5 (ref. 22), Rab11 (ref. 23) and...
MTf were immunostained in the hindgut epithelium. MTf co-localized with Rab5 puncta at stage 13 indicating that MTf is internalized by Rab5-mediated endocytosis (Fig. 2g and Supplementary Information, Fig. S2d–f). Interestingly, partial co-localization of MTf with Rab11 at stage 13 was also detected, suggesting that MTf is recycled to the apicolateral membrane region by Rab11 (Fig. 2h). At stage 16 however, when septate junctions are fully functional, we did not detect MTf puncta or co-localization of MTf with endocytic markers (Supplementary Information, Fig. S2g–j). Next, we investigated whether other septate junction proteins like Cora, gliotactin and sinu are also redistributed during septate junction maturation. All three septate junction proteins were transiently detected in intracellular puncta at stage 13, but not at stage 16 (Supplementary Information, Fig. S3a–f), consistent with the low turnover of septate junction proteins at stage 16 (ref. 24). To corroborate the endosomal MTf localization, we fractionated wild-type membrane extracts by equilibrium density gradient of wild-type membrane extracts. (j) Membrane extracts from wild-type embryos at stage 10–13 and stage 14–17 expressing GFP–Rab5, GFP–Rab11 and GFP–GPI were immunoprecipitated with anti-GFP. Co-immunoprecipitated proteins were resolved and identified by western blotting using the indicated antibodies. Input; 25% of extract that was used for immunoprecipitation.

Figure 2 MTf co-localizes with early and recycling endosomes. (a–d) Confocal microscopy section of trachea (a, b) and hindgut (c, d) tissues immunostained with anti-MTf. MTf is localized along the basolateral membrane and in intracellular puncta at stage 13 in tracheal (a) and hindgut (c) cells. At stage 16, MTf accumulates predominantly at the apicolateral membrane in the trachea (b) and hindgut (d) cells. (e, f) Confocal microscopy section of tracheal cells from a stage 13 embryo, stained with anti-MTf and expressing GFP–ZF (e) or GFP–Rab5 (f). (g, h) Confocal microscopy of hindgut sections from stage 13 embryos, immunostained with anti-MTf and anti-Rab5(g) and anti-MTf and anti-Rab11 (h). Scale bar a–h, 10 μm. Images on the right of e–h show localization of individual proteins (top, middle) and a merge of these images (bottom) from a region of the image on left. (i) Immunoblots of fractions (1; top–9; bottom) from an equilibrium density gradient of wild-type membrane extracts. (j) Membrane extracts from wild-type embryos at stage 10–13 and stage 14–17 expressing GFP–Rab5, GFP–Rab11 and GFP–GPI were immunoprecipitated with anti-GFP. Co-immunoprecipitated proteins were resolved and identified by western blotting using the indicated antibodies. Input; 25% of extract that was used for immunoprecipitation.
To elucidate the molecular role of MTf in septate junction assembly, we analysed its conserved sequence motifs. First, we tested if Drosophila MTf is a GPI-linked membrane protein, similarly to its human homologue. We probed membrane and cytosolic fractions of embryonic lysates for MTf, the transmembrane protein Syntaxin 1A (Syx1A)29, and the GPI-linked protein, Knickkopf (Knk)30. MTf was predominantly detected in the membrane fraction together with Syx1A and Knk (Fig. 4b). Incubation with phosphatidylinositol-phospholipase Cγ (PI-PLCγ) liberated MTf and Knk, but not Syx1A, into the supernatant, indicating that MTf associates with the plasma membrane through a GPI-modification motif (Fig. 4b). To test significance of the GPI-modification motif, we deleted it from MTf (generating a secreted MTfΔGPI protein), or exchanged it for the mouse CD8 transmembrane domain (MTf-TM). We overexpressed MTf, or the MTfΔGPI or MTf-TM constructs in the trachea of MTf mutants. Soluble MTfΔGPI rescued Nrx mislocalization and tube overelongation, presumably by binding to membrane-associated septate junction proteins. (Fig. 4c–e). Notably, MTf-TM did not rescue these MTf phenotypes, although it was expressed at similar levels (Fig. 4f). This suggests that the MTf GPI-modification may be cleaved or have another function.

Next, we investigated if Drosophila MTf binds iron. Iron-binding by serum transferrin is mediated by Tyr 188 of the amino-terminal lobe and the Tyr 426 of the carboxy-terminal lobe. To test the iron-binding capacity of Drosophila MTf, we mutated the corresponding tyrosines, Tyr 231 or Tyr 533, to phenylalanine31, and also deleted the GPI-modification sequence to generate soluble forms of His-tagged wild-type and mutated MTf in S2 cells (Fig. 4a). All constructs were expressed and secreted at similar levels, suggesting that the substitutions did not alter protein secretion or stability. Secreted MTf proteins were affinity purified along with mouse MTf and vermillon as positive and negative controls, respectively. Atomic absorption spectroscopy detected similar levels of bound iron to the wild-type Drosophila and mouse MTf proteins (Supplementary Information, Fig. S6a). The Y231F substitution in the N-terminal lobe of MTf reduced iron binding, whereas the Y533F mutation completely abolished it. To further investigate MTf iron binding, the electron paramagnetic resonance (EPR) spectra of the MTf proteins were compared (Fig. 4i). The Drosophila and mouse MTf spectra were strikingly similar. These EPR profiles are comparable to those of other Tf proteins, and originate from a high-spin Fe(III)-ion34. Vermiform and MTfΔGPI, at comparable protein concentrations, did not have a detectable EPR signal (Fig. 4i), supporting the hypothesis that the MTf C-terminal lobe is the site of Fe(III)-binding specificity. Next, we investigated if Fe(III)-binding is required for MTf function in epithelial junction assembly. Tracheal overexpression of the Y231F MTf protein, which only partly reduces iron-binding, fully rescued the tube overgrowth and the Nrx mislocalization defects in MTf mutants (Fig. 4g). This construct also partially rescued the lethality of MTf mutant larvae when expressed by the ectodermal driver 69B-GAL4 (data not shown).

Conversely, expression of the iron-deficient Y533F construct failed to rescue the MTf mutant phenotypes (Fig. 4h). This indicates that iron-binding is crucial for MTf function and suggests that iron-bound MTf has a direct role in septate junction assembly. Additionally, it may reflect a role for MTf in iron homeostasis. To test this, we monitored ferritin levels, which provide a sensitive indicator of aberrant cellular iron concentration35. Fer1 and Fer2 levels remained unaffected in MTf mutants indicating that MTf does not have a major role in cellular iron uptake.

( Supplementary Information, Fig. S4). The paracellular barrier integrity of chc1 mutants was compromised, as dye leaked into the trachea in 36% of the analysed mutants (n = 11). This genetic analysis indicates that endocytosis is required for functional septate junction assembly. Endocytosis also has a prominent role in adherens junction remodelling and stabilization. Previous studies demonstrated that DE-cadherin accumulates intracellularly in papal tissues of shits mutants at the restrictive temperature25,26. In contrast, the localization and the levels of Crb and DE-cadherin were not affected in our experiments (Supplementary Information, Fig. S4). Additionally, the endocytic binding capacity of Drosophila MTf is crucial for MTf function and suggests that iron-bound MTf has a direct role in septate junction assembly. Additionally, it may reflect a role for MTf in iron homeostasis. To test this, we monitored ferritin levels, which provide a sensitive indicator of aberrant cellular iron concentration. Fer1 and Fer2 levels remained unaffected in MTf mutants indicating that MTf does not have a major role in cellular iron uptake.

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Figure 4 MTf is a GPI-anchored iron-binding protein. (a) Schematic representation of the MTf protein. Arrows indicate the residues at which substitution mutations were made (Y231F and Y533F), and the GPI domain, which was deleted in the MTf∆GPI mutant. (b) Proteins in membrane and supernatant fractions of lysed wild-type embryos treated with PI-PLC, as indicated, were resolved and identified by western blotting. The GPI-anchored protein Knk and the transmembrane protein Syx1A were used as controls. (c–h) Confocal microscopy images of the trachea in an MTf mutant (c), and in MTf mutants expressing MTf(d), MTf∆GPI (e) MTf−TM (f), MTfY231F (g) and MTfY533F (h) under the control of the btl-GAL4 driver. Cells were immunostained with antibodies specific to MTf and Nrx; images on the left indicate localization of Nrx and images on the right indicate co-localization of Nrx and MTf. Scale bar, 10 µm. (i) EPR spectra of MTf, MTfY533F, mouse MTf (mMTf) and vermiform (Verm). A g-factor signal of 4.3 indicates a high-spin Fe(III) atom is bound to the protein. The signal was absent in the preparations containing the mutated MTf or the unrelated protein vermiform.

(Supplementary Information, Fig. S6b–e). This is consistent with the functional analysis of mouse and human MTf
c. In converse experiments, no septate junction abnormalities were found in fer1 and fer2 mutants suggesting that iron homeostasis does not directly affect septate junction assembly (Supplementary Information, Fig. S6f, g). Thus, the genetic analysis suggests that MTf iron binding and endocytosis are specifically required for septate junction maturation.

Next, we investigated if endocytosis of Fe(III)-bound MTf is sufficient to induce functional septate junction assembly. We expressed wild-type MTf, MTf∆GPI and MTfY233F/333ΔGPI in epidermal stripes of MTf/mutants using en–GAL4. The MTf constructs were detected in en–GAL4 expressing cells, which were visualized by parallel expression of UAS–GFP–CAAX (CAAX, membrane-targeting motif, where X is any residue; Fig. 5a, c, e). The wild-type protein was undetectable in surrounding cells lacking transgenic MTf expression. Strikingly, MTf∆GPI (Fig. 5c, d) also accumulated in cells not expressing en–GAL4. Thus, epidermal cells that lack MTf bind and internalize exogenous, secreted MTf∆GPI. The exogenous MTf∆GPI partly localized in Rab11-positive puncta and also along the lateral membranes of the receiving cells, suggesting that it is endocytosed (Fig. 5g, h). MTfY533F/333ΔGPI was not detected in adjacent cells, despite its expression at comparable levels in cells expressing en–GAL4, indicating that MTf internalization requires Fe(III)-binding. Next, we investigated if MTf endocytosis induces septate junction assembly in the recipient cells. We immunostained MTf mutant epidermal cells, expressing GFP and MTf constructs, with antibodies specific to Nrx and MTf. The wild-type protein induced Nrx apicolateral accumulation only in the en–GAL4 expressing cells (Fig. 5a, b). As expected, the iron-binding deficient construct, MTfY533F/333ΔGPI, did not rescue the MTf mutant (Fig. 5e, f). Conversely, MTf∆GPI from the en–GAL4 cells fully rescued Nrx mislocalization in these cells, as well as in the adjacent cells (Fig. 5c, d). In these embryos there was also rescue of the convoluted tracheal phenotype, indicating that exogenous MTf from the epidermis was endocytosed, which restored tracheal septate junction assembly and function. These results demonstrate that endocytosis of Fe(III)-bound MTf is sufficient to induce functional septate junction assembly in MTf-mutant cells.
We have identified MTf as a component of Drosophila septate junctions. We suggest that Fe(III)-binding may enhance its affinity for transmembrane septate junction components and induce endocytosis and clearance of septate junction protein complexes from the basolateral surface of epithelial cells at stage 13. On internalisation, the complexes may dissociate in endosomes, freeing MTf and septate junction proteins for recycling to the apicolateral membrane. The targeting mechanism of the recycling vesicles remains unknown but their localized delivery at the apicolateral region may increase the local concentration of adhesion molecules on neighbouring cell surfaces and promote septate junction formation. Once at the apicolateral membrane region, MTf may bind circulatory iron and also stabilize the junctions (Fig. 5i). Our data suggest that MTf may also function in paracellular junction assembly in vertebrates and provide clues for the elucidation of its postulated role in melanoma tumorigenesis.
METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology/

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AUTHOR CONTRIBUTIONS

C.S., K.S. and K.T. designed the experiments. K.T. performed the experiments. S.W. identified the tracheal overelongation phenotype of the MTF-P element mutant. A.G. analysed the EPR data. C.S., K.T. and K.S. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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METHODS

**Drosophila strains.** The KG-type P element, KG01571, is inserted into the 5′ UTR (untranslated region) of the **MTf** gene. Imprecise and precise excisions of the P element yielded several viable and lethal strains (non-complementing the lethality of Dh(3L)5492). One of the lethal strains, excision #234, contains a 626 bp deletion that removes the **MTf** 5′ UTR and 352 bp coding sequence, and was used for the analysis. The deletion is flanked by two following sequences: 5′-CACAAGAACATCTATTAA-3′ and 5′-GAACCGGCAAACCTAACAGG-3′. w1118 was used as the wild-type strain. Other alleles used include Fer1HCH and Fer2LCH. For co-localization experiments we used the UAS transgenes GFP–2×FYVE, GFP–Rab5 and GFP–Rab11 (ref. 22). Other constructions used were UAS–Rab5K (ref. 37) and UAS–Rab11K (ref. 38). chic and *sh* mutants are described in Flybase (http://flybase.org/). Crosses for ectopic expression using btl–GAL4, en–GAL4 and 69B–GAL4 drivers were performed at 25 °C, except for the *sh* mutant where embryos were raised at 22 °C for 8 h and then shifted to 31 °C for 5 h. In all experiments CyO, TM3 and FM7c balancer strains carrying GFP or lacZ transgenes were used as necessary to identify embryos with the desired genotypes.

**Immunostaining, TEM and dextran injection.** Immunohistochemistry, TEM and 10 K dextran (Molecular Probes) injection was performed as previously described14,15. Images were acquired with a Zeiss Meta confocal microscope and the GPI modification site was predicted by http://mendel.imp.ac.at/gpi/. 626 bp deletion that removes the UTR (untranslated region) of the *Drosophila* strain Df(3L)5492). One of the lethal strains, excision #234, contains a 798. The GPI modification site was predicted by http://mendel.imp.ac.at/gpi/. For co-localization experiments we used the UAS transgenes GFP–2×FYVE, GFP–Rab5 and GFP–Rab11 (ref. 22). The following antibodies were used at the indicated dilutions: guinea pig anti-MTF (1:500 for immunostaining; 1:1,000 for western blotting); mouse anti-trachael luminal 2A12 (1:5; DSHB), guinea pig anti-Cor (1:5,000), rabbit anti-Nrx (1:1,000), mouse anti-Dlg (1:10; DSHB), mouse anti-Nig I/7 (1:500; ref. 41), guinea pig anti-Cont (1:1,000), rabbit anti-sinuous (1:300), mouse anti-gliotactin (1:200), mouse anti-GFAP 12 (1:100; Clontech), rabbit anti-GFP (1:500; Molecular Probes), guinea pig anti-vermiform (1:500), guinea pig anti-gaspar (1:1,000), rabbit anti-Galleria mellonella calreticulin (1:500), mouse anti-synaptopodin (1:100, BC2; DSHB), rabbit anti-synaptopodin 16 (1:500; abcam), rabbit anti-chnk (1:500), rabbit anti-Rab5 (1:500), rabbit anti-Rab11 (1:500), rabbit anti-Fer1HCH (1:400), rabbit anti-Fer2LCH (1:400), rabbit anti-β-gal (1:500; Cappel), fluorescein-conjugated ChtB (1:500; New England Biolabs).

**EPR spectroscopy.** EPR spectra were recorded on an Elexys CW X-band spectrometer (Bruker) with an ESR900 Helium Cryostat (Oxford Instruments) at 12.0 K ± 0.2 K, 0.63 mW microwave power, approximately 9.63 GHz microwave frequency and 0.5 mT modulation amplitude. A dual mode ER 4116DM microwave cavity in perpendicular mode was used. All spectra were recorded under identical conditions. The estimated concentration of the measured proteins was: MTI, 10 ng µl⁻¹, MTI 10 ng µl⁻¹, mMTf 20 ng µl⁻¹ and vermiform, 25 ng µl⁻¹.

**Immunostaining and western-blotting antibodies.** The following antibodies were used at the indicated dilutions: guinea pig anti-MTF (1:500 for immunostaining; 1:1,000 for western blotting); mouse anti-trachael luminal 2A12 (1:5; DSHB), guinea pig anti-Cor (1:5,000), rabbit anti-Nrx (1:1,000), mouse anti-Dlg (1:10; DSHB), mouse anti-Nig I/7 (1:500; ref. 41), guinea pig anti-Cont (1:1,000), rabbit anti-sinuous (1:300), mouse anti-gliotactin (1:200), rat anti-DE-cadherin (1:50; DSHB), mouse anti-arnardillo (1:50; DSHB), mouse anti-GFAP (1:500; Molecular Probes), guinea pig anti-vermiform (1:500), guinea pig anti-gaspar (1:1,000), rabbit anti-Galleria mellonella calreticulin (1:500), mouse anti-synaptopodin (1:100, BC2; DSHB), rabbit anti-synaptopodin 16 (1:500; abcam), rabbit anti-chnk (1:500), rabbit anti-Rab5 (1:500), rabbit anti-Rab11 (1:500), rabbit anti-Fer1HCH (1:400), rabbit anti-Fer2LCH (1:400), rabbit anti-β-gal (1:500; Cappel), fluorescein-conjugated ChtB (1:500; New England Biolabs).

Figure S1. MTf mutant embryos have non-functional SJs (a) The MTf locus including the P element (KG01571) insertion site and the MTf mutant deletion is indicated by the black bar. (b) Histogram showing that dorsal trunk length is significantly different in MTf mutant compared to wild-type embryos (p<0.0001, n=7). Dorsal trunk length is expressed as a ratio of dorsal trunk length (metamer 1-8) / embryo length. (c, d) Expression of UAS-MTf and UAS-mMTf in MTf mutants with the tracheal driver btl-GAL4 rescues the Verm secretion and tube over-elongation phenotypes. (e, f) Wild-type and MTf mutants expressing GFP-CAAX in the trachea stained for Verm and GFP. Verm is secreted to the tracheal lumen in wild-type embryos, but remains intracellular in MTf embryos at stage 15. (g, h) Wild-type and MTf mutant trachea labeled with a FITC-conjugated chitin binding probe (ChtB). Inserts display y-z projections. The filamentous cable is detected in the wild-type embryos (g). Its structure is diffuse and expanded in the mutants (h). Scale bar: 10 μm. (i, j) Injection of rhodamine-labeled dextran into the body cavity of stage 15 wild-type embryos reveals an intact paracellular barrier as the dye fails to penetrate into the lumen of the trachea (i). In MTf mutant embryos the dye penetrates the lumen of the trachea indicating the defects of the paracellular barrier (j). Scale bar: 25 μm. (k, l) A confocal microscopy section of wild-type and MTf mutant stained for the SJ component Cora. In MTf mutant, Cora is mislocalized and spread basolaterally. (m, n) A confocal microscopy projection of wild-type and MTf mutant trachea stained for DE-cad. DE-cad is properly localized in MTf mutant. (o, p) btl-GFP-CAAX; MTf mutant embryos stained with MTf antiserum. GFP immunostaining visualized tracheal cells. (q, r) Immunostaining of embryonic hindgut cells reveals colocalization of MTf and Nrx in confocal sections. (s, t) Immunostaining of embryonic tracheal cells shows colocalization of SJ components Dlg and MTf. Scale bar: 10 μm. (v) MTf and Nrx are coimmunoprecipitated by MTf antibodies from extracts of wild-type embryos, but not DE-cad and Arm. DE-cad and Arm are precipitated by DE-cad antibodies. 5% of extract that was used for IP was loaded in the input lane.
Figure S2 SJ protein complexes transiently colocalize with endosomal markers. (a, b) A confocal section of hindgut shows Nrg localization along the basolateral membrane and in intracellular puncta at stage 13 (a) and apicolateral accumulation at stage 16 (b). (c) Nrx and Cont coimmunoprecipitate from extracts of stage 10-13 wild-type embryos with MTf. Cont precipitates Nrx from stage 10-13 wild-type embryo extracts using Nrx antibody. Pre-immune serum was used as the negative controls. 5% of extract that was used for IP was loaded in the input lane. (d, e, f) Overlap between MTf and Rab5 positive intracellular vesicles at stage 13. (g, h) Minimal colocalization between MTf and expressed GFP-2x-FYVE (g) and MTf and expressed GFP-Rab5 (h) in the tracheal cells at stage 16. (i, j) Minimal colocalization between MTf and Rab5 (i) and MTf and Rab11 (j) in the hindgut cells at stage 16. Scale bar: 10 μm.
Figure S3 SJ components partially colocalize with early and recycling endosomes at stage 13. Confocal microscopy sections of stage 13 hindgut (a-f'). (a-c') 69B-GAL4 embryos expressing GFP-Rab5 stained for GFP (a', b', c') and SJ components: Cora (a), Glio (b), Sinu (c). (d-f') 69B-GAL4 embryos expressing GFP-Rab11 stained for GFP (d', e', f') and SJ components: Cora (d), Glio (e), Sinu (f). Confocal microscopy sections of stage 16 hindgut (g-l'). (g-l') 69B-GAL4 embryos expressing GFP-Rab5 stained for GFP (g', h', i') and SJ components: Cora (g), Glio (h), Sinu (i). (j-l') 69B-GAL4 embryos expressing GFP-Rab11 stained for GFP (j', k', l') and SJ components: Cora (j), Glio (k), Sinu (l). Scale bar: 10 μm. (m) Western blots from stage 10-13 and stage 14-17 wild-type embryos expressing GFP-Rab5, GFP-Rab11 and GFP-GPI with 69B-GAL4 driver showing the expression of the constructs.
Figure S4 The apical marker Crb and the AJ marker DE-cad are not altered in endocytic mutants. Confocal microscopy sections of stage 13 hindgut (a-e). Wild-type embryos (a), embryos expressing dominant-negative rab5 (b) and dominant-negative rab11 (c) in hindgut cells show MTI along the basolateral membrane at stage 13, as visualized by MTI immunostaining. (d, e) chc1 and shp1 mutant embryos display localization of MTI along the basolateral membrane at stage 13. Confocal microscopy sections of stage 16 hindgut (a', a'', b'-b''', c'-c'''', d'-d'''', e'-e''') and trachea (a'''', b'''', c'''', d'''', e'''') and trachea (a'''', b'''', c'''', d'''', e'''). (a'-a''') Wild-type embryos, (b'-b'''') 69B-GAL4 embryos expressing dominant-negative rab5, (c'-c'''') 69B-GAL4 embryos expressing dominant-negative rab11, (d'-d''') chc1 mutant embryos and (e'-e''') shp1 mutant embryos stained for the apical marker Crb (green), AJ's component DE-cad (red) and SJs component MTI (blue). Crb and DE-cad localization is not altered in the mutant embryos, but MTI is mislocalized and the trachea displays a convoluted phenotype. (f, g) chc1 mutant embryos show mislocalization of Cora along the basolateral membrane at stage 16 compare to the wild-type embryos. Scale bar: 10 μm.
Figure S5  Endocytosis is not affected in MTf mutant. (a-d) MTf mutant embryos show similar levels and localization of Rab5 (b) and Rab11 (d) compared to wild-type (a, c) in the hindgut at stage 16. Dashed lines mark the apical and basal side of the cells. Basal is the upper line. (e-h) Confocal sections of the trachea of wild-type and MTf mutant embryos expressing btl >GFP-CAAX at stage 16 and stage 17. Embryos are stained with anti-Gasp and anti-GFP. At stage 16, Gasp is intra-luminal and at stage 17 after the luminal protein clearance, it is detected in the cells of wild-type (e, f) and MTf mutant embryos (g, h). Scale bar: 10 μm.
Figure S6 MTf binds iron but Feritin mutants do not show SJs defects in the trachea. (a) Atomic absorption spectroscopy measurements of iron in purified soluble MTf proteins. Decreased levels of iron are detected in constructs containing mutated iron-binding residues. Mouse MTf was used as positive control and Verm protein as a negative control. (b-e) A confocal section of wild-type and MTf mutant trachea at stage 16 stained for Fer1 (b, c) and Fer2 (d, e). The levels of Fer1 and Fer2 are similar in wild-type and MTf mutant. (f, g) Immunostainings of fer1 and fer2 mutant embryos show MTf localized in SJs at stage 16. Tracheal tube length is not affected. Scale bar: 10 μm.
Figure S7 Full scans and panels of key Western data
Control of tube diameter expansion by secreted chitin-binding proteins

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Abstract

The size and shape of epithelial tubes determine the transporting capacities of tubular organs. Here, we analyze two genes involved in airway tube size regulation in Drosophila. Obst-A and gasp encode secreted proteins with chitin binding domains that are conserved among insect species. mRNA in situ hybridizations show that both genes are strongly expressed during airway tube expansion. Gasp protein is secreted into the airway tubes and colocalizes with a chitin binding probe and other chitin binding proteins. Analysis of obst-A and gasp single mutants and obst-A; gasp double mutant shows that both genes are required for larval elongation and airway tube dilation. The assembly of the apical chitinous matrix of the airway tubes is defective in gasp and Obst-A mutants. The defects become exaggerated in double mutants indicating that the genes have partially redundant functions in chitin structure modification. The phenotypes in luminal chitin assembly in the airway tubes are accompanied with a corresponding reduction of tube diameter in the mutants.

Conversely, overexpression of Obst-A or Gasp in the airways expands the tube circumference. Our results indicate that the level of distinct matrix binding proteins in the tubes determines the extent of diametric growth. We propose that Obst-A and Gasp organize the assembly of the luminal matrix and thereby provide distending forces that stretch the apical cell membranes to expand tube diameter accordingly.
Introduction

An important late step in the morphogenesis of branched tubular organs is the final acquisition of branch sizes. The fixed length and diameter of tubes dictate the flow rates of the transported fluids and are therefore major determinants of optimal organ function. The major tracheal airways, of the Drosophila respiratory network are made by a single epithelial cell layer and provide a relatively simple system for the genetic dissection of tube size control.

Genetic analysis in the Drosophila airways has revealed several of the cellular mechanisms in epithelial tube size regulation (Ghabrial, Luschnig et al. 2003; Uv, Cantera et al. 2003; Wu and Beitel 2004; Swanson and Beitel 2006; Casanova 2007; Andrew and Ewald 2010; Schottenfeld, Song et al. 2010). The length and diameter of the different tracheal branches are controlled independently, branch length increases continuously but tube diameter expansion occurs step-wise. Furthermore, neither cell size or cell number are primary determinants of tube expansion in the embryonic trachea (Beitel and Krasnow 2000). Analysis of mutants with selective size defects indicates that genes encoding proteins with very diverse molecular functions, control airway tube size in Drosophila embryos.

One group of mutants shows predominantly overelongated tubes but some of the members also display local dilations and constrictions. The mutations in this group affect genes encoding proteins involved in chitin biogenesis or polymer assembly. These genes include cystic/mummy (mmy), kkv, knk, retroactive (rtv), vermiform (verm) and serpentine (serp). mmy encodes the Drosophila homolog of UDP-N-acetylglucosamine diphosphorylase. This enzyme is required for the production of UDP-N-acetylglucosamine, a substrate for chitin synthesis (Araujo, Aslam et al. 2005; Devine, Lubarsky et al. 2005; Tonning, Helms et al. 2006). kkv encodes a chitin
synthase that links UDP-N-acetylglucosamine residues into β1,4 glucan chains (Ostrowski, Dierick et al. 2002; Tonning, Hemphala et al. 2005). *knk* and *rtv* encode novel proteins implicated in chitin filament organization (Ostrowski, Dierick et al. 2002; Moussian, Tang et al. 2006). *verm* and *serp* encode two related putative luminal chitin deacetylases with a chitin binding domain and are required for the structural modification of the apical ECM (Luschnig, Batz et al. 2006; Wang, Jayaram et al. 2006). An intraluminal chitin cable is detected at stage 13, just before the expansion of the tracheal tubes. The luminal chitin matrix forms a tightly packed cylindrical structure that grows during tube expansion at stages 14 and 15 and disappears at stage 16 (Devine, Lubarsky et al. 2005; Tonning, Hemphala et al. 2005). The analysis of the mutant phenotypes in the “chitin group” of genes suggests that the apical chitin matrix provides either a physical scaffold that defines the shape of the underlying epithelial cells or it locally signals back to the epithelial cells to adjust their shape in a coordinate manner. Interestingly, *kkv* mutants show an irregular subapical cytoskeleton (Tonning, Hemphala et al. 2005), suggesting that the chitin cable provides cues for the coordinated organization of the apical epithelial cell surfaces along the length of the tubes.

A second group of tube size regulators consists of genes encoding proteins associated with the paracellular septate junctions (SJs). This group of mutants shows overelongated tubes with a defective paracellular barrier. One possible mechanism of SJ-mediated control of tracheal tube size is through the regulation of the activity of the apical polarity proteins such as Crumbs (Crb) and atypical protein kinase C (aPKC) (Laprise, Paul et al. 2010). Another way to explain the functions of the SJ-associated proteins in tube size control derives from their potential involvement in the assembly of the luminal chitin matrix (Wu and Beitel 2004). SJs have been directly
implicated in the luminal deposition of the chitin deacetylases Verm and Serp, which suggests that the function of SJ components is to facilitate the targeting of luminal proteins and to the apical cell surface (Wang, Jayaram et al. 2006). A third group of mutants shows selective defects in tube diameter expansion. They are characterized by narrow airways and they all affect genes encoding COPII and COPI vesicle components (Tsarouhas, Senti et al. 2007; Grieder, Caussinus et al. 2008; Jayaram, Senti et al. 2008; Forster, Armbruster et al. 2010; Norum, Tang et al. 2010).

Here, we describe two new genes, obst-A and gasp required for larval growth and diametric tube expansion. They encode secreted luminal proteins with chitin binding domains. Obst-A and Gasp are required for the assembly of the intraluminal chitin filament and taenidial integrity. Overexpression of either of the proteins in wild type animals can cause tube dilation. Our analysis argues that Obst-A and Gasp modify the luminal matrix to provide an expanding mold that determines airway tube expansion.
Results and Discussion

Obst-A and Gasp are expressed and secreted into the airways during tube expansion

To identify new genes involved in tube morphogenesis, we surveyed the BDGP gene expression database for genes, which are expressed in the trachea (Tomancak, Beaton et al. 2002). Several members of the Obstructor gene family were strongly expressed in the airways during the tube expansion period. This gene family is conserved among insects and can be subdivided into two subgroups, based on protein domain arrangement (Behr and Hoch 2005). We focused our analysis on Obstructor subgroup 1 because it contains genes with a pronounced tracheal expression pattern. The members of this subgroup (Obst-A, B, C, D) are weakly expressed during early embryogenesis but their expression increases dramatically during late embryogenesis. This increase in gene expression coincides with cuticle formation in ectodermal tissues and tube expansion in the airways. Obst-A (Fig. 1a) and Obst-B are expressed in the trachea and epidermis; Obst-C (Gasp) (Fig. 1b) shows expression in the tracheal system, foregut and salivary glands. Obst-D and Obst-E are predominantly detected in the midgut and epidermis. The expression of the genes in subgroup 2 is not elevated during embryogenesis, arguing against a role for them in tube morphogenesis. To examine the phylogenetic relationships among the Obst family members, we constructed a phylogenetic tree based on the protein sequences (Fig. 1c). Obst-A and Gasp are the most closely related genes expressed in the trachea. The predicted proteins contain a signal peptide at the amino terminus, followed by three chitin-binding domains type 2 (CBD2) (Fig. 1d). CBD2 is an extracellular domain that contains six conserved cysteines that probably form three disulphide bridges. These domains are connected with linker regions of similar length in both proteins.
Alignment of the protein sequences revealed 37% identity. The features of the two proteins and the timing of their expression in the trachea indicate that they may function in tube expansion.

To investigate the function of Obst-A and Gasp proteins during tracheal development we characterized strong loss of function mutants for both genes. We first generated a null obst-A mutant by the FLP-FRT technique (Parks, Cook et al. 2004). mRNA in situ hybridizations of mutant and wild type embryos confirmed that obst-A was deleted since we could not detect any obst-A mRNA expression in the mutants (Fig. S1b). For gasp, we used a transposon-induced mutation, which carries a transposon 66bp upstream of the predicted translation initiation site of the gasp gene. We analyzed these mutants using a polyclonal antibody against recombinant Gasp expressed in bacteria (Tsarouhas, Senti et al. 2007). We could not detect any Gasp protein in the mutant embryos suggesting that they can be considered as null (Fig. S1d). During these experiments we also noticed that the Gasp protein and the unknown antigen recognized by the 2A12 antibody show a similar pattern of protein localization in the trachea. Initially during embryonic stages 13 and 14, both Gasp and the 2A12 antigen predominantly localized in the cytoplasm. At stage 15 both of them began to be deposited into the lumen and by stage 16 we only detected a luminal signal for both antigens. Surprisingly, when we stained gasp mutant embryos for both Gasp and the 2A12 antigen we did not detect any signal from either of these antibodies (Fig. 2b, c). Parallel stainings for other luminal markers in the mutants still gave the expected expression patterns. These results suggested that the antigen for 2A12 is Gasp or that the 2A12 antigen is encoded by another gene, whose expression is severely reduced in Gasp mutants. To test this, we expressed Gasp protein using the en-GAL4 driver in the dorsal part of the hindgut. We stained those embryos for Gasp
(Fig. 2d), 2A12 (Fig. 2e) and Verm, a luminal chitin deacetylase (Fig. 2f). Exogenous expressed Gasp was detected both by Gasp and 2A12 antibodies. We could not detect any signal with the Verm antibody. This argues that the 2A12 monoclonal antibody recognizes specifically an epitope on the Gasp protein. Ongoing experiments address whether the 2A12 antibody recognizes recombinant Gasp on Western blots. These results identify the antigen recognized by the most widely used airway marker in *Drosophila*. The discovery of Gasp as the 2A12 antigen opens the possibility to investigate the signals determining the different routes for the apical deposition of Verm and 2A12 (Wang, Jayaram et al. 2006; Massarwa, Schejter et al. 2009).

**Obst-A and Gasp control larval size**

Mutations in several of the genes involved in chitin synthesis and assembly affect larval body size (*tweedleD*) (Guan, Middlebrooks et al. 2006) and cuticle differentiation (*mummy*, *retroactive*, *knickkopf*) (Moussian, Seifarth et al. 2006; Moussian, Tang et al. 2006; Tonning, Helms et al. 2006). Therefore, we analyzed the size and cuticle structure of *obst-A* and *gasp* single and double mutants at the first instar larval stage (Fig. 3).

Both *obst-A* and *gasp* single mutant larvae are shorter compared to heterozygote siblings. The body length of *obst-A* larvae was reduced by 8% and the length of *gasp* mutants was 14% shorter compared to the wild type. *obstA; gasp* double mutants showed a more pronounced reduction of 25% compared to the wild-type larval length (Fig. 3a-e). The width of the mutant larvae did not show any major defects both in single and double mutant combination (Fig. 3f). The increased phenotype severity in the double mutants suggests that Obst-A and Gasp have overlapping additive roles in regulating larval length. We compared cuticle preparations of first instar larvae of the
three mutant genotypes to wild-type preparations to assess the potential roles of role of *Obst-A* and *gasp* in epidermal differentiation (Fig. 3g-j). Both *obst-A* and *gasp* single mutants showed normal cuticular structures. We did not detect any obvious patterning defects or characteristic defects for dorsal closure or head involution phenotypes. In contrast the *obst-A; gasp* double mutant showed a bloated cuticle. The cuticle defect in the double mutants suggests a function for Obst-A and Gasp in chitin fibril assembly and cuticle integrity.

**Obst-A and Gasp are required for apical matrix formation in the airways**

To examine the potential roles of Obst-A and Gasp in tube morphogenesis we analyzed the trachea of the mutants. The airways of single *obst-A* and *gasp* mutants were indistinguishable from wild type and became gas filled at the end of embryogenesis (Fig. 3a-c). However, the double mutant showed a fully penetrant gas-filling defect (Fig. 3d). We also analyzed the epithelial apical cell membrane and adherens junction integrity by staining of single and double mutants for apical markers, like Crumbs and AJs proteins, like DE-cadherin (Fig. S2). Both single and double mutants did not show defects in cell epithelial organization.

To assess the function of ObstA and Gasp in the assembly of the transient luminal chitin cable, we visualized its deposition using a fluorescent chitin-binding probe (ChtB) (Fig. 4a-d). Chitin accumulates inside the dorsal trunk tubes in wild-type embryos at stage 14. The size of the cable increases as the tracheal lumen expands in diameter and reaches its final size at stage 16 (Tonning, Hemphala et al. 2005). Both *obst-A* and *gasp* single mutants showed a decreased and irregular staining intensity of the chitin cable compared to wild type embryos (Fig. 4b, c). In *obst-A; gasp* double mutants the staining of the intraluminal matrix was even more diffuse
compared to the single mutants or to wild type embryos (Fig. 4d). To further investigate if other chitinous matrix modifying proteins are affected, we used antisera against Verm (Wang, Jayaram et al. 2006). Verm staining was reduced both in single and double mutants (Fig. 4e-h). The reduction of ChtB and Verm point to function of Obst-A and Gasp in luminal chitin assembly. It will be interesting to determine whether the reduction in the staining intensities of chitin polymers and Verm in the mutants are due to decrease in the expression of genes involved in polymer synthesis and assembly or due to a direct reduction of matrix stability and integrity. The presence of three chitin binding domains in Gasp and Obst-A suggest that the proteins bind to the luminal chin polymers and aid their assembly into a homogeneous and stable chitin filament.

To further test the potential structural role of Obst-A and Gasp in chitin assembly, we analyzed the chitin in the taenidia, the tracheal cuticular ridges that line the airway tubes (Fig. 5). Transmission electron microscopy (TEM) reveals three layers of cuticle in wild-type embryos: the envelope layer, the proteinaceous epicuticle and the chitin-rich procuticle (Fig. 5a). TEM analysis of obst-A; gasp double mutants indicated an irregular shape and size of the taenidial folds (Fig. 5b). The outermost layers of the stratified cuticle, the envelope, and the dark epicuticle appeared normal in the mutants. The chitin-rich space between the epicuticle and the epidermal surface, was however, disorganized and granular compared to the uniform procuticle layer of the wild-type embryos.

Thus, the reduced staining intensity of the luminal chitin cable and the defects in the procuticle layer and the irregular taenidial folds in obst-A; gasp mutant embryos suggest a direct function of Obst-A and Gasp in chitin fibril organization and maintenance.
Obst-A and Gasp selectively regulate tracheal tube diameter

To assess the impact of Obst-A and Gasp function in tube size control we measured tracheal tube diameter of single and double mutant embryos. We stained \textit{obst-A} and \textit{gasp} single mutants for Crumbs and α-Spectrin. Crumbs is localized in the apical membrane (Tepass, Theres et al. 1990) and α-Spectrin (Pesacreta, Byers et al. 1989) is distributed along the lateral domains of epithelial cells. The combination of these two markers visualizes the apical and lateral cell surfaces and facilitates the measurement of tube diameter. Because the airway tubes are tapered we measured dorsal trunk (DT) diameter in three consecutive tracheal metameres to obtain a representative view (n=6) (Fig. 6a). Both mutants showed a small reduction in airway diameter, suggesting a role for Gasp and Obst-A in tube expansion. \textit{obst-A} mutants showed an average 5% reduction in tube diameter whereas in \textit{gasp} mutants diameter was reduced by 10% compared to wild type embryos. In \textit{obst-A; gasp} double mutants the DT diameter reduction was by 15% in comparison to wild type. This suggests that Obst-A and Gasp secretion into the lumen aids the assembly of the chitin cable, which in turn provides a distending force that stretches the apical surfaces of the epithelial cells. To address if overexpression of Obst-A or Gasp in the trachea may be sufficient to induce an increase in tube diameter we expressed Obst-A or Gasp or an unrelated secreted protein (ANF-GFP) with the \textit{btl}-GAL4 driver in wild type embryos. We labeled the trachea with antibodies against Crumbs and α-Spectrin and performed the diameter measurements of the DT tubes as described above. Obst-A overexpression caused a ~7% increase and Gasp overexpression generated a ~13% enlargement in the diameter of the DT tubes. ANF-GFP expression did not have any effect on tube size
(Fig. 6b). These results suggest that the levels of the chitin binding proteins in the tracheal tubes can directly influence tube diameter expansion.

What is the role of Obst and Gasp in tube diameter expansion? There are few possibilities. One is that the luminal chitin has to be organized to a certain structure, which swells and generates and intra-luminal force that expands the apical surface of the underlying epithelial cells. Expanded chitin maybe sensed by unknown receptors on the apical membrane, which cause changes in cell shape leading to tube expansion. In another model, luminal chitin-binding proteins or chitin modifying enzymes may generate and send chemical signals to the cells to modify their apical cytoskeleton and allow tracheal expansion.
Figure legends

Figure 1

Obstructor family

(a, b) Whole-mount in situ hybridization of wild-type embryos with obst-A (a) and
gasp (b) probes. obst-A and gasp mRNA are detected in tracheal cells from stage 13
onward. Note the gaps in obst-A expression correspond to the position of the fusion
cells. Scale bar: 25 μm (c) A phylogenetic tree of Obstructor proteins based on
ClustalW method. (d) Schematic representation of the Obst-A and Gasp protein
domains. N-terminal signal sequence is followed by three chitin-binding domains type
2 (CBD2).

Figure 2

An unknown antigen recognized by 2A12 antibody is Gasp protein

(a) Wild-type embryo stained with antibodies against the luminal antigen 2A12. (b, c)
gasp mutant embryo stained with 2A12 (b) and Gasp (c) antibodies. 2A12 and Gasp
staining is not detected in gasp mutant. Scale bar: 25 μm (d-f) Confocal sections of
embryonic hindgut of en>gasp embryos labeled with Gasp (d), 2A12 (e) and Verm (f)
antibodies. The luminal staining is more pronounced with the Gasp antiserum. Gasp
protein expressed in the dorsal part of the hingut is recognized by Gasp and 2A12
antibodies, but not by Verm antibody. Scale bar: 10 μm

Figure 3

Gas filling and cuticle defects in obst-A; gasp mutant

(a-d) Bright field photomicrographs of wild type (a), obst-A (b), gasp (c) and obst-A;
gasp (d) first instar larvae. Refracted light makes the gas filled trachea clearly visible
in the wild type and single mutant larvae and shows that the trachea is not gas filled in
the double mutant. Scale bar: 100 µm (e, f) Body size measurement of wild type,
*obst-A, gasp* and *obst-A; gasp* first instar larvae. Body length (e) and body width (f)
(n=10). P value is p<0.0001 in all cases comparing wild type animals with mutants.
(g-j) Dark field of cuticle preparations of wild type (g), *obst-A* (h), *gasp* (i) and *obst-
*A; gasp* (j) mutant embryos. *obst-A; gasp* double mutant shows dilated cuticle. Scale
bar: 25 µm

**Figure 4**

*obst-A and gasp* mutants have defects in the luminal chitin

(a-d) Confocal microscopy projections of the trachea labeled with a FITC-conjugated
chitin-binding probe (ChtB). The filamentous chitin is detected in the wild type
embryos at stage 16 (a). The chitin intensity is weaker in the *obst-A* (b), *gasp* (c) and
*obst-A; gasp* (d) mutant embryos. (e-h) The tracheal sections stained for Verm. Verm
is secreted to the tracheal lumen in wild type embryos at stage 16 (e). The amount of
secreted Verm is reduced in both *obst-A* (f) and *gasp* (g) single mutant embryos and
*obst-A; gasp* (h) double mutant embryos. Scale bar: 10 µm

**Figure 5**

Ultrastructure of the tracheal lumen of *obst-A; gasp* mutant

(a-c) Transmission electron micrographs of wild type and *obst-A; gasp* dorsal trunk at
the end of embryogenesis. The wild type embryos (a) show uniformly distributed
chitin rich procuticle. In *obst-A; gasp* double mutant (b, c) an irregular shape and size
of the taenidial folds and the procuticle with amorphous composition can be seen. pro
= procuticle. Scale bar: 0.5 µm
Figure 6

Lumen diameter expansion requires Obst-A and Gasp

(a, b) Tracheal diameter measurements of wild type, obst-A, gasp and obst-A; gasp embryos (a) and embryos overexpressing Obst-A and Gasp with tracheal specific driver (b) at stage 16.1. The diameter measurements are in three metamers: 4, 5 and 6. Number of embryos used for measurement of each genotype n=6. y axis represents the diameter in micrometers. P value is p<0.05 in all cases comparing wild type embryos with mutant or overexpressed embryos.

Supplementary figure legends

Figure S1

obst-A and gasp mutants are the null mutants

(a, b) Whole-mount in situ hybridization of obst-A heterozygous (a) and obst-A mutant (b) embryos with obst-A probe and GFP antibody. GFP stained the balanced chromosome to separate the mutant and heterozygote embryos. The obst-A mRNA is not detected in obst-A mutant embryos. (c, d) Wild type (c) and gasp mutant (d) embryos stained with antibodies against the protein Gasp. Gasp staining is not detected in gasp mutant embryos. Scale bar: 25 µm

Figure S2

Apical membrane and AJs are not affected in the obst-A and gasp mutants

Confocal microscopy sections of the tracheal dorsal trunk labeled with an apical marker Crumbs (Crb) (a-d) and adherent junctions marker DE-cad (e-h). Crb is localized apically both in wild type (a) and obst-A (b), gasp (c), obst-A; gasp (d)
mutant embryos. DE-cad localization is the same in wild type (e) and obst-A (f), gasp (g), obst-A; gasp (h) mutant embryos. Scale bar: 10 µm

**Materials and Methods**

**Drosophila strains**

The obst-A null mutant was generated by flip FRT technique using two piggyBac elements (f02348, f03687). w1118 was used as the wild type strain. gasp (d02290) mutant is described in Flybase. Crosses for ectopic expression using btl-GAL4, en-GAL4 and 69B-GAL4 drivers were performed at 25 °C. In all experiments CyO, TM3 and FM7c balancer strains carrying GFP or lacZ transgenes were used as necessary to identify embryos with the desired genotypes.

**Immunostaining, TEM**

Immunohistochemistry and TEM were performed as described (Lamb, Ward et al. 1998; Wang, Jayaram et al. 2006). The following antibodies were used: mouse anti-tracheal luminal 2A12 (DSHB), rat anti-DE-Cad (DSHB), mouse anti-Crb (DSHB), mouse anti-αSpectrin (DSHB), rabbit anti-GFP (Molecular Probes), gp anti-Verm (Wang, Jayaram et al. 2006), gp anti-Gasp (Tsarouhas, Senti et al. 2007), rabbit anti-β-gal (Cappel), fluorescein-conjugated ChtB (New England BioLabs). Images were acquired with a Zeiss Meta confocal and Tecnai G2 Spirit BioTWIN electron microscope. Images were post-processed with Adobe Photoshop.

**Molecular Biology**

UAS-Obst-A transgene was generated by subcloning the insert of LD43683 into pUAST.
References


Sup. Figure 1

a) obst-A / FM7 twist-GAL4 UAS-GFP
b) obst-A

GFP, obst-A mRNA

(c) WT
(d) gasp

Gasp

Gasp