New roles for apical secretion and extracellular matrix assembly
in *Drosophila* epithelial morphogenesis

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The cover shows an embryonic *Drosophila* tracheal tube. Epithelial cells (in magenta) of the dorsal trunk surround the lumen (in green).

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To my parents and family…
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Abstract

Networks of branched tubular organs, such as the lung and blood circulatory system transport gases and nutrients to different parts of the body. Optimal tissue function relies on the uniform sizes and shapes of the constituting branches in each organ. The *Drosophila* trachea is an epithelial network of branched tubes that transport air to most internal tissues. Thus, the fly airways provide a genetic model system for identification and characterization of tube size regulators. We found that programmed secretion and the assembly of the apical extracellular matrix (ECM) are required for the diametric expansion of the trachea and salivary glands (SG) tubes. The airways of mutants lacking *COPII* and *COPI* components show diminished secretory activity and reduced diameter. The defective lumen enlargement may be due to a shortage in apical delivery of membrane and regulatory proteins or a failure in the assembly of an inflating luminal matrix. The secretion and assembly of the luminal matrix is followed by the endocytic removal of the luminal proteins at the end of embryogenesis. We propose that the precise activation of sequential pulses of apical secretion and endocytosis drive the maturation of embryonic trachea into functional airways. To test the importance of ECM proteins in tube expansion, we characterized Vermiform (Verm) and Serpentine (Serp), two chitin-binding proteins with predicted polysaccharide deacetylase domains (ChLDs). *Verm* and *Serp* mutants show overelongated tubes, suggesting that luminal ECM modification restricts tracheal tube elongation. The luminal deposition of ChLDs, but not other secreted components, depends on the integrity of the paracellular septate junctions (SJs) in the tracheal epithelium. Thus, we identified a new function of SJs in the apical secretion of a subset of the luminal proteins. Deletion of the deacetylase domain (Deac) renders Serp-GFP intracellular, arguing that the deacetylase domain harbors secretion signals. We transferred the deacetylase domain from Serp to Gasp, another tracheal luminal protein, which requires the Emp24 adaptor for ER exit. The GaspDeac-GFP chimera was normally secreted in *emp24* mutants indicating that the deacetylase domain contains potential ER-exit signals. To explore this possibility we identified and characterized conserved sequence motifs in Serp deacetylase domain. We generated amino acid substitution mutants altering the three putative N-glycosylation sites, the predicted enzymatic activity cluster and three phylogenetically conserved motifs. Residue substitutions in the putative catalytic site neither interfered with Serp secretion nor with its ability to rescue the tracheal tube elongation defects of *serp* mutants. Mutations of the N-glycosylation sites gradually reduced the luminal deposition of Serp-GFP constructs suggesting that increased glycosylation enhances apical Serp secretion. By contrast, substitutions in each of the three conserved amino acid stretches completely blocked the ER-exit of Serp-GFP constructs. The mutated proteins were N-glycosylated suggesting that the motifs may be involved in a subsequent protein-folding step or facilitate ER exit through interactions with yet unidentified specific adaptors.
Papers in this thesis


Introduction

Branched tubular organs like the lung, kidney and vascular system facilitate the transport of fluids in many animals. In insects, oxygen supply to internal tissues is largely dependent on the trachea, a tubular network that transports air to most internal tissues. Strikingly many of the cellular and molecular mechanisms involved in the development of mammalian and insect airways appear phylogenetically conserved (Metzger and Krasnow, 1999). The *Drosophila* respiratory system provides an advantageous model system for the genetic study of airway development and tubulogenesis in general.

Biological tubes can develop from unpolarized cell clusters through hollowing or cavitation from a cell mass. For example, hollowing of a cell cluster initiates the formation of the zebrafish intestine and *C. elegans* gut. Tubes can also arise from pre-polarized epithelia sheets through wrapping or budding. A typical example of wrapping is the formation of the neural tube in vertebrates. Where, the neural plate elongates and the lateral ridges rise and extend to fuse in the midline to form a tube. Characteristic examples of budding tube morphogenesis are provided by the formation of the fly and mouse airways. Regardless of the mechanism involved in lumen formation in different organs all tubes need to acquire a distinct size and shape at the end of embryogenesis to fulfill their specific function during animal physiology. A uniform shape and distinct lumen diameter determines the flow rate of the transported fluids through each organ. Accordingly, defects in tube size control can lead to devastating human diseases like polycystic kidney disease (Deane and Ricardo, 2007); (Lubarsky and Krasnow, 2003). An additional critical step in the development of both insect and mammalian airways is the clearance of the embryonic luminal contents.
**Structure and function of epithelial tubes**

The epithelial cells of tubular organs frequently contain specialized cell types capable of performing different functions. For example, most of the intestinal epithelial cells absorb essential nutrients from the ingested food but they are interspersed with goblet cells that secrete the mucosal layer covering the entire gut tube. Both secretion and endocytosis occur at the apical membrane domain of epithelial cells in most organs. These polarized activities of epithelial cells are essential for organ function and become determined during tissue development. The developmental mechanisms that establish the “secretory” or the “absorptive” functions in epithelial cells are not well understood and are only recently becoming addressed in tubular organs (Abrams and Andrew, 2005). The plasma membrane domains of epithelial cells are biochemically distinct. The apical surface, which faces the outside environment, is enriched in glycolipids and cholesterol. The apical membrane has also a distinct composition of proteins, the epithelial cells of collecting tubes in the mammalian nephron carry out their distinct functions due to the presence of characteristic ion cannels on their apical surface (Woudenberg-Vrenken et al., 2009). The basolateral surface of epithelial cells is in contact with adjacent cells and the underlying basement matrix whose composition varies from organ to organ. Cell adhesion to adjacent cell surfaces is mediated through junctional complexes, made of specialized set of proteins that mediate cell-cell contact. These cell junctions block the mixing of membrane proteins in the apical and basolateral surfaces, keeping the membrane domains distinct. In vertebrates, tight junctions (TJs) and adherens junctions (AJs) are found at the apical most portion of the lateral membrane. They function to keep the epithelial cells in contact with each other. Importantly, TJs also form a selective barrier that limits the paracellular diffusion of molecules across epithelium (Knust and Bossinger, 2002; Tepass et al., 2001; Wu and Beitel, 2004).
Thus polarization of the cell membrane in epithelial tissues is an important determinant of tissue function. Epithelial cells become polarized by the asymmetric distribution of regulatory polarity protein complexes. This initial polarization, will then organize and maintain different plasma membrane domains by the asymmetric organization of cytoskeletal and membrane-trafficking systems (Nelson, 2003). A major question in tubular organ morphogenesis is to understand how the polarity complexes organize the distinct cytoskeletal structures and vesicle transport machineries to the apical plasma membrane.

**A short summary of early tracheal development in *Drosophila***

The *Drosophila* tracheal system derives from the epidermis. Six hours (stage 11) after egglaying, ten metameric cell clusters on either side of the embryo invaginate to form a pit and generate a primitive branched tree of 6 branches. Each pit contains in average 80 cells that undergo a remarkable program of stereotypic cell migration and cell rearrangements in each independent metameric unit. Cell proliferation and programmed cell death have not been detected during the branching of embryonic tracheal epithelium. Branch elongation and the formation of new secondary and terminal branches only rely on cell intercalation and cell migration (Samakovlis et al., 1996). The major regulator of these processes is Branchless (bnl), a *Drosophila* homolog of Fibroblast Growth Factors. Bnl is dynamically expressed in surrounding embryonic tissues and attracts tracheal cell migration towards its expression sites (Sutherland et al., 1996). It also induces the activation of gene expression in the leading cells of each branch to specify, which cells will further migrate to generate terminal branches and which cells will form fusing anastomoses to interconnect the network. A parallel program of branch specification is induced in response to Dpp (TGFβ), Wingless and Hedgehog signaling to specify the cell number and identity in each of the six primary branches.
At around 11 hours after egg laying (stage 14), the primitive tracheal network is in place and new unicellular branches form to penetrate the developing embryonic tissues guided by organotypic signals (Englund et al., 1999). During the next 10 hours (from stage 14-17), the branches of the primitive network acquire their final shapes and sizes and transform into a functional respiratory organ. The experiments in this thesis addressed the cellular process of the airway maturation period in the dorsal trunk (DT) airways. These are the major tubes that span almost the entire length of the embryo and connect to the anterior and posterior spiracles (Samakovlis et al., 1996). The tracheal tubes have been classified into four distinct types based on structure, cell junctions and the number of cells involved in the formation of lumen (Figure 1).

Type-I tubes
The dorsal trunk is a multicellular tube composed of wedge-shaped cells lining the lumen. Intercellular junctions hold cells of the dorsal trunk together.

Type-II tubes
The dorsal and ganglionic branches are formed from single row of cells, each cell in the row folding over on itself to form autacellular junctions surrounding a lumen.

Type-III tubes
Fusion cells are seamless, doughnut-shaped cells at the tips of interconnecting branches. These cells attach to each other through intercellular junctions.

Type-IV tubes
Terminal cells harbor hollow finger-like protrusions of lumen through the cell. Junctional complexes are not detected along the lumen of terminal cells and several capillaries can be detected in the cytoplasm of a single cell. These tubes mediate gas exchange with target tissues (Figure 1C) (Baer et al., 2009; Kerman et al., 2006; Samakovlis et al., 1996; Uv et al., 2003).
Figure 1. The embryonic tracheal system and different types of branches and tubes

A) Wild-type stage 16 embryonic *Drosophila* tracheal system stained with the luminal antigen 2A12. B) The main branches in each central tracheal metamere are: the dorsal trunk (DT), dorsal branch (DB), visceral branch (VB), transverse connective (TC), lateral trunk (LT), and ganglionic branch (GB). Anterior is labeled (a); posterior (p). C) The fly respiratory system consists of four different types of tubes. Type I tubes are made up of 3-4 wedge shaped cells with intercellular junctions surrounding the central lumen. Type II tubes are formed by cells wrapped around the lumen and sealed with autocellular junctions. Type III tubes consists of two doughnut shaped (fusion) cells connected with intercellular junctions. Type IV tubes are single (terminal) cells with intracellular lumen and lacks cell junctions.

All branches of the mature *Drosophila* tracheal system are made up of polarized epithelial cells with distinct membrane domains. The apical membrane facing the lumen is in contact with the cuticular lining that strengthens the tracheal tubes, while the lateral membranes of neighboring cells are in close apposition with each other. The lateral membrane can be further sub-divided on the basis of the localization of distinct protein complexes. The apical most part is referred to as the sub-apical zone, containing polarity protein complexes (the Par3/Par6 and Crumbs complexes). These proteins function as regulators of epithelial polarity in *Drosophila*; they are localized to the apical most region.
of the lateral membrane and act as apical determinants in epithelial cell. In addition to its primary role in epithelial cell polarization, Crb may also be essential for the growth of apical membrane (Tanentzapf and Tepass, 2003). In the salivary gland the transcriptional regulation of Crb during development has been implicated in growth of the apical membrane of the cells and gland elongation (Myat and Andrew, 2002).

In contrast to vertebrate epithelial tissues, Drosophila adherens junctions (AJs) are located apically just under the Crb region. AJs are composed of DE-cadherin, α- and β-catenin (the armadillo homolog) and function in cell adhesion, intracellular signaling and the attachment of the actin cytoskeleton to the plasma membrane. Crb mediated polarization is required for AJ formation in Drosophila epithelia (Laprise et al., 2009).

The borders of the apical and basolateral domains are established by the antagonism of the Lgl/Dlg/Scrb complex and the Par3/Par6 complex. The septate junctions (SJs) are found just below the AJs at the basolateral surface of Drosophila epithelial cells. Transmission electron microscopy (TEM) shows SJs as a ladder-like structure between two tightly apposing lateral cell membranes. SJs are composed of a complex of proteins, including Neurexin IV, Sodium potassium ATPase, Coracle, Claudins and several other components (Knust and Bossinger, 2002; Tepass et al., 2001; Wu and Beitel, 2004). The functions of SJs are thought to be analogous to the apical tight junctions found in vertebrate epithelia. Both types of junctions function as selective barriers across epithelial cell surfaces (Figure 2). One important aspect of epithelial cell specification is the establishment and maintenance of membrane domains through polarized secretion. This is achieved by the secretion of sets of proteins to exclusive portions of cell membrane and by preventing them from mixing.
Chitin and apical ECM assembly in tube size control

Chitin is the second most abundant biopolymer in nature, next to cellulose. It is a major Extracellular Matrix (ECM) component, serving as a scaffold for the stiff cuticle that lines and protects the epidermis and tracheal system of insects. During tracheal tube maturation, chitin is deposited into the lumen. The chitin polymer forms an expanding cylindrical structure with uniform arrangement of chitin fibrils. After stage 15 a chitin rich matrix is also laid on the apical surface of the tracheal cells. This matrix will form the characteristic taenidia that reinforce the tube and prevent collapsing. Assembly and growth of the chitin matrices may coordinate with the surrounding epithelial cell for controlled growth. This is based on the observation that tracheal mutants for chitin synthesis showed uncontrolled expansions, with some regions of the epithelium dilating.
poorly while others over-expanding, resulting in wiggly and cystic tracheal tubes. Mechanistically chitin filaments might provide a rigid substrate for apical membrane attachment of tracheal epithelial cells and consequently prevent excessive elongations of the cells. It may also force diametric expansion of the fusion cells. Alternatively, ECM may serve as a uniform structure, which cushions the apical cytoskeleton for consistent diameter expansion (Araujo et al., 2005; Devine et al., 2005; Moussian et al., 2006; Tonning et al., 2005). Secretion of other ECM molecules, was bean shown in the inter-rhabdomeral space of the fly retina. These proteins facilitate the separation of rhabdomeres (Husain et al., 2006; Zelhof et al., 2006).

In amphioxus, a small marine animal closely related to vertebrates, has an ECM structure in its vascular lumen. The lumen is generated between the basal cells surface of the intestinal epithelium and the mesodermal layers. Initially the lumen is filled with a laminin-containing ECM, later it is cleared by phagocytotic blood cells (Kucera et al., 2009). The presence of ECM structures in tubes suggests that, it might function in tube growth in other organisms similar to trachea. Chitin containing structures are absent in vertebrates, but they might contain uncharacterized ECM material like proteoglycans that may play analogous role to coordinate tube growth. An important question is how the epithelial cells generate and sense the cues from the apical ECM (aECM).

Integral membrane proteins mediate coupling of signals outside the plasma membrane to the underlying cytoskeleton or downstream signaling cascade. On the cytosolic side transmembrane receptors might interact with member of the ERM (ezrin, radixin and moesin) proteins, which likely link the apical actin cytoskeleton to the external cues. In vertebrates, integral membrane proteins like layilin, which is a widely expressed hyaluronan receptor, binds to the EMR protein radixin (Bono et al., 2005). L-selectin, a member of the selectin family of cell adhesion molecules, and may also act as a signaling receptor. (Ivetic et al., 2002; Ivetic et al., 2004). In Drosophila several integral
membrane proteins like Piopio, Sas, Cad99c, Stit and RPTPs are apically localized in epithelial cells (Bokel et al., 2005; D'Alterio et al., 2005; Schonbaum et al., 1992; Wang et al., 2009). The ZP-domain containing proteins Piopio and Dumpy link the apical extracellular matrix and the underlying apical epithelial surface to the cytoskeleton. Any of above molecules or yet unidentified ECM sensors may potentially signal from the apical ECM to the sub-apical cytoskeleton or to the unknown intracellular signaling molecules of Drosophila tracheal system.

The deposition and modification of the apical ECM is dependent on the timely secretion of its components. Sensing of the aECM by an apical receptor is a crucial aspect of tracheal tube development.

**Secreted protein transport**

Proteins with specific sorting signals are directed to discrete cell locations. Sorting signals are short sequences of amino acids that are recognized by a sorting machinery, which mediate their proper localization in the cells. Based on the interaction between sorting signals and specific carriers, a protein is either targeted to the endoplasmic reticulum lumen (ER), the nucleus, intracellular vacuoles or the plasma membrane (Bradshaw, 1989). Transport of proteins from the ER is mediated by membrane-bound vesicles that facilitate protein secretion (Sabatini et al., 1982). Schekman and colleagues have isolated temperature-sensitive secretion (sec)-mutants in *S. cerevisiae* and pioneered the genetic identification and analysis of components of the secretory apparatus. Electron microscopy studies of the sec-mutants at non-permissive temperature revealed an accumulation of various types of intracellular membrane-enclosed structures (Barlowe et al., 1994; Barlowe and Schekman, 1993; Bednarek et al., 1995). Depending on the mutant strain, these structures appeared as small vesicles between 60-100nm in size, which presumably correspond to the
transporting vesicles. Mutants also displayed enlarged ER network and abnormal Golgi structures. Each of these structures represented a defective intermediate of the secretory pathway caused by a block in a specific step of protein transport. Subsequent identification of the genes revealed an array of proteins involved in the different stages of transport. Some of the proteins, their importance and functions are discussed in the following sections.

1) Protein synthesis and Endoplasmic reticulum (ER)

The ER plays a central role in protein and phospholipid biosynthesis. The ER extends throughout the cytoplasm, occupying a major volume of the cell. It appears as a tubular structure with flat, sac-like membranous extensions that are held together by the cytoskeleton (Herman, 2008; Terasaki et al., 1986). The endoplasmic reticulum is the site where the majority of the newly synthesized proteins enter the secretory pathway. Initiation of protein synthesis happens on cytoplasmic ribosomes. The shift of newly synthesizing protein from cytosol to the ER membrane only occurs in the presence of an ER signal sequence or signal peptide (SS or SP). The SS is a short stretch of approximately twenty or more hydrophobic residues, either at the amino-terminal or internally in the peptide sequence. It is present on all soluble and membrane secretory proteins. If a protein lacks the ER signal peptide, protein synthesis happens on ribosomes but the protein will stay in the cytoplasm. When an ER signal sequence is produced by the ribosome it becomes recognized by the signal recognition particle (SRP) (Cross et al., 2009; Marrichi et al., 2008). Binding of SRP to the signal sequence emerging from the ribosome directs the polypeptide synthesizing ribosomal complex to the ER membrane (Walter and Blobel, 1981). Ribosomes then engage with a translocon located in the ER membrane. The Sec61 translocon channel is formed by a trimeric complex of α, β, γ integral membrane proteins. The short SS moves across the ER
membrane through the translocon channel into the ER lumen, where it is cleaved by a
signal peptide peptidase (Weihofen and Martoglio, 2003). The nascent polypeptide chain
is then co-translationally injected into the ER lumen, through the further addition of
amino acids (Nakamoto, 2009).

The lipid bilayer of ER encloses a central luminal space, which spans the entire ER (English et al., 2009). While newly synthesized polypeptides are gradually released into the ER lumen, “the protein quality control machinery” or “chaperones” of ER assist the nascent polypeptides for proper folding. The quality control chaperones consists of Hsp70 (also called BiP), PDI, calnexin, calreticulin and other ER resident proteins (Ellgaard and Helenius, 2003). They also perform posttranslational modifications such as disulfide bond formation between cysteine residues [mediated by phospho disulfide isomerase (PDI)] and the addition of preassembled oligosaccharides and lipid anchors (Helenius and Aebi, 2004; Lisanti et al., 1989; Yeaman et al., 1997). Posttranslational modifications of this sort help the nascent polypeptides obtain their biologically active states. Chaperones also decide whether the polypeptide is further transported to downstream compartments like the Golgi apparatus or selected for proteasomal degradation (Hebert and Molinari, 2007). Proteins that fail to fold properly are rerouted to the cytosol for degradation (Meusser et al., 2005). The above processes must be in a balanced state; slight disproportions in these mechanisms might lead to cellular defects and potential disease (Aridor and Balch, 1999; Aridor and Hannan, 2002).

The structure and function of the endoplasmic reticulum varies greatly depends on the cell type. There are two structurally and functionally distinct varieties of ER, rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER). The RER surface is studded with protein-manufacturing units, namely the ribosomes, giving it a "rough" texture in the transmission electron microscopic (TEM) graph, hence the name RER (Kelly, 1985). As ribosomes only bind to the ER once when it begins to synthesize
a protein destined for secretion, one can tell an actively secreting cell by its RER content (Blobel and Potter, 1967). In comparison SER is free of membrane bound ribosomes, and consequently these cells are not considered of highly secretory nature.

For the secretory proteins to exit ER, they must expose the sorting signals for forward transport to Golgi apparatus and finally to their site of activity. The transport of proteins synthesized in ER, to the Golgi and finally to destinations of extracellular space is termed the conventional protein secretory pathway. During “unconventional” protein secretion polypeptides exit the cell independently of the early secretory compartments like the ER and Golgi (Nickel and Rabouille, 2009).

The coat protein (COP) coated (coatomer) vesicles are responsible for the transportation events in the early secretory pathway between the ER and the Golgi apparatus. Anterograde transport of cargo containing vesicles from ER to Golgi is mediated by COPII–coated vesicles. In contrast, vesicles coated by the COPI protein complex function in the retrograde transport of escaped ER-resident proteins from compartments like ERGIC (an intermediate organelle between ER and Golgi) and Golgi apparatus back to ER (Bonifacino and Glick, 2004).

2) COPII coated vesicles and anterograde transport

Components of the COPII coat include the GTPase Sar1 (secretion-associated RAS-related protein 1), Sec23/24 (inner layer) and Sec13/31 (outer layer) dimers (Barlowe, 2003). The membrane-associated Sec12, a guanine nucleotide exchange factor (GEF), initiates COPII coat assembly. Sec12 converts the GDP bound form of Sar1 to the GTP form; as a consequence of GDP to GTP exchange cytoplasmic Sar1 becomes membrane associated and recruits sequentially Sec23/24 and later Sec13/31 forming the pre-budding cargo complex. COPII pre-budding complex formation is
sufficient to deform the ER membrane and generate a coated vesicle (red components in Figure 3). During protein secretion transmembrane cargos directly interact with components of the COPII complex via their cytoplasmic tail. In contrast, soluble cargos within the ER lumen interact with the COPII complex through membrane proteins called adaptor molecules (the p24 family of transmembrane proteins). Interaction of cargo with COPII complex mediates their assembly into the vesicle for the anterograde movement from ER to Golgi (Baines and Zhang, 2007). COPII coatomer disassembly from the vesicle is achieved by hydrolysis of Sar1-GTP. Sec23 an integral member of COPII coatomer acts as GAP protein (GTPase-activating protein), it performs the hydrolysis of Sar1-GTP to Sar1-GDP (Barlowe, 2003; Paccaud et al., 1996; Tang et al., 1997; Tang et al., 2005).

3) COPI coated vesicles and retrograde transport

GTP bound active Arf1 (ADP-ribosylation factor 1, also a small GTPase) is essential to recruit COPI components from the cytosol to Golgi membranes. Additional interactions between the COPI subunits and membrane adaptor proteins facilitate membrane association. The COPI coatomer consists of seven polypeptides arranged in two different layers. The β, δ, γ, ζ subunits form the tetramer subcomplex of the inner layer. The β- and γ- subunits of the subcomplex bind to the active Arf1-GTP. γCOP can also interact with p24 family of membrane adaptor proteins. The outer coatomer subcomplex is a trimer containing the α, β′ and ε subunits. The COPI pre-budding complex can also deform the membrane into a coated vesicle (green components in Figure 3). Unlike the COPII coat, where the Sar1-GAP is an integral member of the coat, stimulation of GTP hydrolysis on Arf1 to promote coat disassembly is not mediated by a subunit of the coat but by a separate Arf-GAP (Bethune et al., 2006; Coutinho et al., 2004; Pimpl et al., 2000).
Figure 3. COPII and COPI vesicular transport
The COPII coatomer protein coated vesicles are involved in the anterograde movement of cargo from the ER to the Golgi (red circles). It comprises of Sar1, Sec13, Sec23, Sec24 and Sec31 subunits. Sar1 and Sec24 interact directly with transmembrane cargo proteins (orange bars) for transport from ER. The p24 transmembrane adaptor proteins (yellow bars) interact with soluble cargo destined to leave the ER.
COPI coatomer coated vesicles are involved in retrograde transport of vesicles from the Golgi to the ER (green circles). Components of COPI coatomer are ARF-1, COP-α, β, β', γ, δ, ε, ζ subunits. COPI protein complex involved in the retrieval of escaped ER resident proteins and p24 membrane adaptor proteins back to the ER. Modification and sorting of cargo molecules happens in Golgi complex, which is a well-organized stack of sack like membranous structure.
4) The Golgi apparatus

Transmission electron microscopy analysis (TEM), reveals the Golgi as stacks of flattened cisternae. These stacks provide for well-ordered compartmentalization of oligosaccharide (covalently attached on proteins) modifying enzymes important for protein maturation. Each layer of the stack contains different sets of enzymes, with a slight overlap, although little is known about how this compartmentalization is established and maintained. Importantly, the Golgi serves as a selective filter to segregate and return escaped ER resident proteins back to ER (Pfeffer, 2007). The Golgi is divided into three primary sub-compartments; closest and the recipient of ER vesicles is the cis-Golgi compartment, at which retrieval of escaped ER resident proteins occurs mediated by COPI coated vesicles. During the secretory process, proteins are transported from the cis-Golgi to the median-Golgi. Finally at the trans-Golgi network (TGN) proteins get sorted, this is the last compartment that faces the plasma membrane of the cell (Figure 3) (Pfeffer, 2007). Two models exist for anterograde movement of cargo through the Golgi. In the cisternal maturation model, stacks of Golgi sacks sequentially mature from cis to trans, while vesicles that carry Golgi resident enzymes move retrogradely. In the vesicular transport model, vesicles shuttle between Golgi cisternae carrying Golgi enzymes and the cargo proteins in both antero- and retrograde directions (Ungar et al., 2006) (Rabouille and Klumperman, 2005). The vesicular transportation events in the intra-Golgi cisternae is controversial, as it is performed by COPI proteins in addition to its functions in retrieval of escaped ER resident proteins (Rabouille and Klumperman, 2005). Once the cargo proteins reach the TGN they are sorted distinctively to cellular compartments like apical or basolateral plasma membranes or even to the cellular exterior, depending on the sorting signal the cargo protein contains. While most sorting occurs within the TGN, studies also emphasize that
sorting also occurs in recycling endosomes which is discussed in the following section (Folsch et al., 2009).

5) Endocytosis

The plasma membrane is a dynamic structure that functions to separate, and act as an interface between the cytoplasm and the extracellular environment. Small essential molecules, such as amino acids, sugars and ions, can pass through the plasma membrane with the aid of channels and pumps. Macromolecules cannot cross via channels or freely diffuse across the membrane. Hence proteins and other molecules must be transported into the cell in an alternative way. Endocytosis is the process in which a portion of cell membrane invaginates into the cell to form a pouch. This invagination then gets pinched off from the membrane, forming a cytoplasmic transporting vesicle enclosing macromolecules, protein and signal transducers. This process occurs by different mechanisms that fall into two categories, phagocytosis and pinocytosis. In many protists like amoeba, phagocytosis is mainly used to engulf the entire particle during feeding. In mammals it is typically observed in specialized immune cells, which includes macrophages, monocytes and neutrophils that function in the removal of large pathogens such as bacteria, debris and the remnants of dead cells. Pinocytosis occurs in almost all the cells, this process is mediated by different cytosolic mechanisms, which includes caveolae and clathrin-mediated endocytosis (CME). Much attention has gone to CME, which mediates the uptake of extracellular material via specific receptor mediated mechanisms. Well studied examples of CME include, low density lipoprotein (LDL) particle internalization after binding to the LDL-receptor and uptake of iron loaded transferrin by transferrin receptor-mediated endocytosis (Conner and Schmid, 2003).
During CME, the cytosolic coat protein clathrin assembles on the plasma membrane, this assembly is sufficient to deform the overlying membrane into intracellular coated pits (Brodsky et al., 2001). The clathrin triskelion complex is composed of three clathrin heavy chain (CHC) and three clathrin light chain (CLC) proteins tightly associated into a polygonal lattice. At normal physiological conditions, clathrin coat assembly and vesicle scission form clathrin-coated vesicles (CCV). This process also requires other components like the cytoplasmic adaptor proteins (APs) and dynamin. Heterotetrameric adaptor complexes AP1-4 are cytosolic proteins, of which only AP2 has been shown to be involved in CCV formation. The GTPase dynamin, is a key regulator of phagocytosis, caveolae and CME (Hinshaw, 2000). It is thought to self-assemble into a scissoring collar at the necks of invaginating coated pits (Conner and Schmid, 2003). Once the vesicle is internalized into the cell the disassembly of the clathrin coat occurs. Upon the formation of cytoplasmic transporting vesicle and coat disassembly, many such internalized vesicles fuse to form the early endosome. Some of the plasma membrane proteins are sorted here and rerouted back to the plasma membrane. Early endosomal structures mature to form recycling endosomal compartment (RE). The cargo vesicles originating from the TGN fuse with RE compartment for further sorting and segregation of cargo into different transporting vesicles for polarized secretion (Maxfield and McGraw, 2004).

6) Vesicle tethering and fusion with target membranes

Once a vesicle is released from its donor compartment, it is targeted to the appropriate acceptor membrane compartment, where the vesicle fuses to releases the contents. Vesicular fusion is mediated by the SNARE (SNAP (Soluble NSF [ N-ethylmaleimide sensitive factor ] Attachment Protein) REceptors) proteins. Each transport vesicle carries a single, specific v-SNARE that binds to three separate relevant
polypeptides t-SNARE on the target membrane. SNAREs are known to perform two major functions. Firstly, the assembly of v- and t-SNAREs (four peptides) supplies the free energy that is necessary to bring apposing membranes close enough to promote membrane fusion. Secondly, SNAREs help to ensure that vesicles fuse only with the specific targeted membrane compartment (Bonifacino and Glick, 2004).

**Membrane trafficking in polarized epithelial cells.**

As mentioned earlier, the asymmetric distribution of polarity complexes results in the differential organization of membrane compartments. Organization of plasma membranes through cell junctions and polarized secretion is essential to maintain polarity in epithelial cells. The initial orientation of cell polarity is determined by the asymmetric localization of intracellular polarity complexes. The Par3/Par6 complex and atypical protein kinase C (aPKC) becomes localized to sub-apical regions. This complex interacts with the Crumbs and Stardust complex and cumulatively they regulate the apical membrane identity and cell junction formation (Bryant and Mostov, 2008; Mostov et al., 2003). The apical and basolateral membrane surfaces of the cell are further polarized by different lipid compositions. The phosphatidyl inositol (4,5)-P$_2$ (PIP2) and (3,4,5)-P$_3$ (PIP3) are crucial determinants of the apical and basolateral membrane surfaces. Normally PIP2 is enriched at the apical surface. Conversely phosphatidyl inositol 3-kinase generating PIP3 is found at the basolateral surface. The lipid phosphatase PTEN, is needed to convert PIP3 to PIP2. PIP2 binds and recruits Annexin 2 to the apical surface, which in turn recruits the GTP-bound form of the Cdc42-GTPase and aPKC to the apical surface. PTEN, PIP2, Annexin 2, Cdc42 and aPKC are all needed to form normal apical surfaces, which line the lumen of cysts formed by MDCK cells in culture (Martin-Belmonte et al., 2007) (Mellman and Nelson, 2008). Annexins serves as apical plasma membrane tethering factors for the apical vesicles.
1) Polarized sorting of apical proteins

A characterized apical sorting signal is the glycosyl phosphoinositol (GPI) lipid anchor. The sugar residue of the glycolipid is covalently attached to the carboxyl terminus of proteins, carrying a consensus sequence for GPI-addition in the ER. The apical sorting is achieved by oligomerization of GPI-anchored proteins into lipid rafts that are formed by sphingolipids and cholesterol (Rodriguez-Boulan et al., 2005; Schuck and Simons, 2006). Additional apical signals include N-linked and O-linked glycosylation. The covalent addition of core oligosaccharides with high mannose content to specific asparagine residues of NXT/S sequence is N-glycosylation (Helenius and Aebi, 2001; Helenius and Aebi, 2004; Wacker et al., 2002). O-glycosylation occurs by linkage of N-acetylgalactosamine to the hydroxyl group of a serine or threonine residues. Both modifications happen during protein synthesis in the ER. Interaction with a sorting receptor that promotes incorporation into an apical transporting vesicle facilitates efficient apical targeting. N-glycans were proposed to sort apically by interacting with a lectin receptor and a sugar interacting protein, galectin (Rodriguez-Boulan et al., 2005; Folsch, 2008).

2) Basolateral sorting of proteins

Compared to apical signals the basolateral sorting signals of transmembrane proteins are better defined. They usually comprise amino acid stretches in the cytoplasmic domain that typically include tyrosine, mono- and dileucine and acidic amino acid motifs. These motifs are recognized by the basolateral sorting machinery. A well-studied basolateral sorting machinery is the exocyst complex. It mediates the tethering of post-Golgi secretory vesicles to the basolateral plasma membrane. The exocyst is an evolutionary conserved octameric protein complex, consisting of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84. In yeast targeting of secretory vesicles to the
plasma membrane involves direct interactions of the Sec3 and Exo70 components with PtdIns (4,5)-P$_2$ (Folsch, 2008; He and Guo, 2009; Lipschutz and Mostov, 2002). Mutations in any member of the exocyst complex block bud growth and cause post-Golgi vesicles to accumulate in the cytosol (Salminen and Novick, 1989).

In polarized MDCK cells Sec8 and Sec6 are recruited to sites of cell-cell contact through protein interactions at sites of E-cadherin localization (Grindstaff et al., 1998). Similarly, in Drosophila epithelial cells Sec5, Sec6 and Sec15 function in the tethering of vesicles originating from the trans-Golgi and recycling endosomes to the lateral membrane, where DE-cadherin is localized. In mutants of exocyst components, delivery of AJ proteins to the lateral membrane was affected (Grindstaff et al., 1998; Langevin et al., 2005). Flies mutant for sec15 showed defects in delivery of specific cell adhesion and signaling molecules that are required for the establishment of synaptic specificity, which caused defects in distinct neuronal targeting steps. This also suggested that components of the exocyst perform different functions (Mehta et al., 2005).

In the TGN and endosomes of MDCK cells the heteromeric cytosolic adaptor protein complex AP1, 3 and AP-4 were shown to mediate the sorting and delivery of cargo to basolateral surfaces (Simmen et al., 2002). The asymmetric distribution of docking and fusion machinery (SNARE) probably regulates polarized secretion in epithelial cells. In MDCK cells, components of the plasma membrane fusion machinery, the t-SNAREs Syntaxin 3 and 4, are localized to the apical and basolateral plasma membranes respectively (Kreitzer et al., 2003).

3) Vesicle transport

Vesicles are transported along cytoskeletal highways. Most polarized cells have specialized organizations of actin and microtubule cytoskeleton. Non-epithelial cells exhibit a radial array of microtubules (MT) with the minus ends emanating from a
centrosome or MT organizing center (MTOC) that is located next to the nucleus and the plus ends extending to the cell cortex, for example this arrangement observed in fibroblasts. When a simple epithelial cell becomes polarized, the MTs acquire vertical arrays of non-centrosomal MTs in which the minus ends mostly face the cell apex. This is typically observed in columnar epithelial cells. Post-Golgi and post-endocytic vesicles associate with the minus end directed motor dynein, which assists in the transport of vesicles towards the minus end facing the apical side. Conversely kinesin is a plus end motor protein (Musch, 2004). Disruption of microtubules in MDCK cells affects the efficient delivery of the Syntaxin 3 (t-SNARE) to the apical cell surface. Instead Syntaxin 3 is also delivered to basolateral membrane. In contrast, MT disruption does not significantly affect the delivery of basolateral membrane protein Syntaxin 4 (Kreitzer et al., 2003). In epithelial cells a subpopulation of MT induce clustering of DE-cadherin protein at the sites of cell contacts. The plus end of MT is highly dynamic, it is seen in contact with cadherin-based cell junctions in fluorescent microscopic studies. Abolishing the dynamic growth of MT without affecting gross morphology results in the disruption of cell junctions (Stehbens et al., 2006).

The delivery of proteins and membrane to specific cell domains is important for the maintenance of polarity and different membrane domains in epithelial cells. Without the coordinated action of these processes, it is difficult to maintain cell polarity and polarized epithelial activities. In the case of tubular organs defects in the polarized vesicle trafficking is expected to affect both lumen formation and its maintenance.
AIMS OF THIS THESIS

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

1) Gain further understanding of the cellular mechanisms that regulate epithelial tube size
2) Characterize previously identified mutations causing tube stenosis or overelongation

RESULTS AND DISCUSSION

Tube overgrowth mutants

How is the diametric expansion and elongation of tubular organs controlled during development? In the *Drosophila* tracheal system, it has been shown that diameter growth and the tube length are two independent events. Tube length increases gradually but diametric expansion occurs abruptly at discrete time points during development (Beitel and Krasnow, 2000). Several mutations have been identified with tracheal tube overgrowth defects. They affect genes encoding septate junction (SJ) components, a transcription factor and genes involved in chitin biosynthesis.

Many mutations affecting genes encoding SJ components show tube overelongation phenotypes in the *Drosophila* tracheal system. For example mutations in the genes encoding the α/β subunits of the Na+/K+ ATPase, which form a complex with SJ components show overelongated tubes (Hemphala et al., 2003; Paul et al., 2003). Similarly the *megatrachea* and *sinuous* mutants affect claudins, are required for SJ assembly and termination of tracheal tube elongation (Behr et al., 2003); (Wu et al., 2004). *bulbous* and *nrx IV* mutants lacking the SJs adhesion molecules, lachesin and Neurexin IV, show SJ integrity and tube over elongation defects (Baumgartner et al., 1996; Llimargas et al., 2004). Finally, *varicose* mutants that perturb the function of
membrane-associated guanylate kinase (MAGUK) super family member. MAGUK forms complex with SJ components, it was identified as a negative regulator of tube growth (Wu et al., 2007). SJ components have been shown to bind to each other and they are mutually required for the assembly of functional junctions at the end of embryogenesis. Thus, SJ assembly and function has a profound role in tube morphogenesis but the mechanism by which SJ mediate termination of tube elongation remained unknown.

The transcription factor Grainy head (Grh) controls apical membrane growth and tracheal tube elongation independent of SJs. *grh* mutants have long and tortuous tracheal tubes (Hemphala et al., 2003). In an over-expression study Grainy head was shown to promote expression of some of the SJ proteins in amnioserosa (Narasimha et al., 2008). The Grh transcriptional targets that regulate uniform tube growth remain to be identified.

Another set of mutants shows defects in both tube elongation and diameter including constrictions and dilations in the trachea. This group encompasses the *krotzkopf verkehrt* mutant affecting chitin synthase-1 (Tonning et al., 2005) and the *cystic/mummy* mutants disrupting the function of UDP-N-acetylglucosamine pyrophosphorylase enzyme that is required for the synthesis of chitin (Araujo et al., 2005; Moussian et al., 2006; Tonning et al., 2006). Similarly, the analysis of *knk* (Knickkopf) mutants affecting a GPI-anchored apical protein (Moussian et al., 2006) further suggested that the assembly of an intraluminal filamentous chitin ECM coordinates for uniform diametric growth of tracheal tubes. In the following sections, I will discuss my work addressing the mechanisms that drive the diametric expansion and control tube elongation in the trachea.
Live Imaging of Airway Maturation (Paper I)

To describe the cellular activities of tracheal epithelial cells during the late phases of tube morphogenesis we examined embryos expressing fluorescent luminal markers. We expressed Gasp-GFP, an endogenous secreted protein with three chitin-binding domains fused to GFP and ANF-GFP (rat Atrial Natriuretic Factor a heterologous secreted protein) in the trachea. We found, precise sequential activation of apical secretion and endocytosis are the characteristic events for embryonic tracheal maturation. The analysis divided tracheal maturation into three steps 1) around 10.5 hrs after egg laying (AEL) epithelial cells of the tube deposit large amounts of proteins into the nascent tracheal lumen. Over the following 30 minutes around 11hrs AEL, the tube diameter expands due to bulk protein secretion into the lumen. 2) Around 18.5 hrs AEL, the same epithelial cells clear proteins and other material from the tracheal lumen through endocytosis. 3) Shortly before the embryo is ready to hatch 20 AEL, the tracheal network clears the remaining liquid and the tubes are filled with gas making the trachea into a functional organ.

Sar1 is required for efficient secretion of luminal proteins

The sar1 mutant allele was identified in a P-element screen for mutants with narrow tracheal tubes (from Hemphälä. J, et al. unpublished data). During embryonic development, zygotic expression of sar1 transcript is initiated in the SG and epidermis (Abrams and Andrew, 2005). To monitor zygotic Sar1 expression in the trachea, we used a Sar1-GFP protein trap line. Strong zygotic expression of GFP in the trachea was evident in the embryos carrying only the paternally supplied Sar1-GFP. As sar1 transcript is expressed in tracheal epithelial cells, we generated and analyzed sar1 null mutants for developmental phenotypes in trachea. At stage 15 of embryonic development, we observed a reduced apical deposition of the luminal marker 2A12 and
its retention in tracheal cells of \textit{sar1} mutant embryos. Conversely at stage 15, 2A12 is exclusively luminal in wild-type embryos. To analyze if the apical secretion of other proteins are affected, we stained for the secreted putative deacetylases Verm and Gasp in \textit{sar1} mutants. Similar to 2A12 we saw reduced apical secretion of these proteins at stage 15. Both Verm and Gasp are retained in tracheal cells of \textit{sar1} mutant embryos. The defects in cellular accumulation of luminal proteins were rescued by tracheal re-expression of \textit{sar1}. Reduced apical secretion might be due to loss of epithelial polarity. Embryos were stained for the apical polarity marker Crumbs, the adherence junction protein E-cadherin and the SJ component Coracle. The localization of these makers was not affected in the trachea of \textit{sar1} mutants. This suggests that the epithelial organization is normal in \textit{sar1} zygotic mutants. This is likely due to maternal contribution of \textit{sar1} transcript (Zhu et al., 2005), which seems sufficient for the basal secretory activity and localization of polarity determinants in \textit{sar1} mutant embryos. We generated a dominant negative (DN) form of Sar1 and expressed it in the trachea. DN-Sar1 intervention completely disrupted secretion and integrity of epithelial. Thus, Sar1 function is necessary throughout embryogenesis. The phenotypes of the zygotic null mutants indicate that the maternal protein is not sufficient to support the increased requirement for efficient bulk apical protein secretion at stage 15 of embryonic tracheal development.

\textbf{Sar1 is required for ER and Golgi Integrity}

Sar1 is well known for its function in COPII vesicle budding from the ER. To determine the Sar1 sub-cellular localization in flies, we stained trachea with an anti-Sar1 antibody. Sar1 predominantly co-localized with the ER marker PDI-GFP. Consistent with its functions in yeast and vertebrates, \textit{Drosophila} Sar1 localizes to the ER.

To test the integrity of the early secretory apparatus, we stained \textit{sar1} mutants with an antibody against KDEL (represents Lys-Asp-Glu-Leu tetra-peptide, which is an
ER retention signal present in many ER residual proteins). The staining intensity for KDEL was strongly reduced in \textit{sar1} mutant trachea. This might suggests a reduction of ER residual proteins, which may in turn affect ER integrity in the mutants. We also addressed Golgi integrity by staining with gp120, a widely used \textit{Drosophila} median-Golgi marker. The gp120 staining displayed numerous punctate structures in wild-type trachea. The abundance of gp120 structures was reduced in the trachea of \textit{sar1} mutants. This suggests that the Golgi units are reduced in \textit{sar1} mutants. Using transmission electron microscopy (TEM) we observed tubular ER decorated with ribosomes in wild-type. By contrast the ER was bloated in the trachea of \textit{sar1} embryos. Thus Sar1 is required for normal structure and function of ER and Golgi.

The TEM analysis also revealed a condensed and distorted electron-dense apical extracellular matrix (aECM) in the tracheal lumen of \textit{sar1} mutant embryos compared to the uniform matrix seen in wild-type embryos. Similar defects were also evident when we stained \textit{sar1} mutants with a chitin binding probe. We noticed a reduced staining intensity of the luminal chitin filament in trachea. This indicates the apical ECM is not properly assembled in the \textit{sar1} mutant trachea. This is likely due to the failure in the apical secretion of chitin binding proteins and matrix modifying enzymes.

**Narrow tubes in \textit{sar1} mutants**

Mutants of \textit{sar1} showed reduction in the apical deposition of proteins at stage 15. \textit{sar1} mutant embryos also displayed narrow lumen diameter in the trachea of stage 16 embryos. This suggests that reduction in diametric expansion might be a consequence of diminished apical secretion. Live imaging of embryos expressing either ANF-GFP or Gasp-GFP, showed retention of fluorescent-tagged proteins in the tracheal cells of \textit{sar1} mutants. Consequently, \textit{sar1} mutants failed to fully expand the lumen diameter of the dorsal trunk (DT). We quantified this defects by measuring diametric growth rate in the
trachea of wild-type and sar1 mutant embryos. While early lumen diameter expansion happens in parallel for both genotypes, later diametric growth in sar1 mutants falls significantly behind in comparison to wild-type embryos. The DT lumen in sar1 mutants reaches only an average of 70% of the wild-type diameter. The sudden initiation of apical secretory burst preceding lumen diametric expansion seems to be developmentally regulated for tracheal maturation. Thus, zygotic Sar1 expression in the trachea is required for efficient diametric expansion of dorsal trunk tubes.

Mutations in COPII components showed secretion and tube expansion defects

Sar1 is required for the budding of COPII vesicles from the ER. We asked if mutations affecting other COPII coatomer proteins show defects in secretion and tube expansion. Components of COPII protein complex like sec13 and sec23 mutants showed reduction in apical secretion, which also correlated with a narrow tube phenotype. Thus, COPII components are a prerequisite, for efficient luminal secretion. Massive luminal dumping of proteins is functionally relevant for diametric growth, as three mutants of COPII complex display narrow tube phenotypes.

Luminal proteins co-localize with endosomal markers during tube clearance

During the initial stage of embryonic development, tracheal cells transiently deposit ECM components to expand their lumen diameter. Protein and matrix filled tubes must be emptied to generate a functional respiratory system. We hypothesized, that clearance of luminal proteins in tracheal tubes might involve a wave of endocytic uptake. To ask if tracheal cells internalize luminal contents, we developed a heterologous endocytosis assay. 10kDa rhodamine conjugated dextran was injected into the hemocoel of wild-type stage 13 embryos. At this stage the tracheal epithelium lacks a functional paracellular diffusion barrier and hence dextran consistently accumulates,
presumably by diffusion into the trachea lumen. Dextran injection at stage 16 does not result in luminal accumulation, indicating that the septate junctions have assembled at this stage and prevent dye leakage. During the phase of protein clearance (at mid 17 stage), we detected numerous rhodamine-dextran puncta inside the tracheal cells of pre-injected embryos. The number of intracellular dextran puncta transiently increased during the period of protein clearance. Interestingly, the dextran puncta co-localized with vesicular structures marked by GFP-Rab7 and GFP-FYVE endosomal markers, suggesting that dextran is endocytosed by tracheal cells. To exclude that intracellular dextran puncta derived from endocytosis happening through the basal membrane surface, we injected the dextran in to the hemocoel of wild-type embryo at stage 16. Dextran puncta were not observed in the tracheal cells of injected embryos that developed functional paracellular barriers. This argues that the apical endocytosis is strongly enhanced in the tracheal system during mid-stage 17. We also detected intracellular puncta staining for the endogenous protein Gasp during the period of protein clearance in tracheal cells. Intracellular Gasp puncta co-localized with Chc, Rab5 and GFP-Rab7 endosomal marker stainings during the phase of protein clearance. This additionally supports that luminal protein clearance occurs through endocytosis performed by the tracheal cells.

**Luminal protein clearance depends on Rab5 mediated endocytosis**

We anticipated that mutations affecting the endocytic pathway would abrogate protein clearance from the tubes. Mutants disrupting endocytic components like *clathrin heavy chain*, *shibire* and *rab5*, were examined for protein and liquid clearance from the lumen. Clathrin heavy chain is a major component of the clathrin coat. Temperature sensitive (ts) *shibire* is a mutation in the dynamin GTPase. At restrictive temperature of 32° C, the protein renders to a non-functional state. Dynamin clips clathrin-coated pits to
form endocytic vesicles from the plasma membrane. An additional key regulator of early endocytosis is the Rab5 GTPase. All the three mutants showed clearance defects, with rab5 mutant showing the most penetrant phenotype in protein and liquid clearance in the trachea.

To address the effect of rab5 mutation on endocytosis, we tested the integrity of different endosomal compartments. We stained rab5 mutants with anti-Chc antibody and expressed different reporters like the early endosomal marker GFP-FYVE, the recycling endosomal marker GFP-Rab11 and late endosomal marker GFP-Rab7. At early stage 17, endocytic markers were unaffected in rab5 mutant embryos. However at mid-stage 17 during the period of luminal protein clearance, the sub-cellular distribution of endosomal markers became abnormal in rab5 mutant embryos. The antibody stainings for anti-Chc showed a discrete apical localization, with a dotty pattern in tracheal cells of wild-type embryos. Apical staining of Chc was lost in the trachea of rab5 mutants. Live imaging of GFP-FYVE showed dynamic movement of large GFP positive apical structures in the dorsal trunk (DT) of wild-type embryo. In rab5 mutants these apical structures were absent. Finally GFP-Rab7 structures were spread along the apicobasal axis in wild-type tracheal cells. These GFP-Rab7 endosomal structures were decreased in rab5 mutant embryos. Thus the localization of Chc, GFP-FYVE and GFP-Rab7 markers were affected in rab5 mutants. This suggests Rab5 is directly or indirectly involved in the formation of endosomes.

We imaged rab5 mutant and wild-type embryos in parallel for protein clearance of ANF-GFP or Gasp-GFP from the tubes. In contrast to wild-type embryos rab5 mutant embryos failed to clear ANF-GFP and Gasp-GFP from their lumen. To rule out persisting presence of over expressed GFP tagged luminal proteins during the phase of clearance, we stained for endogenous luminal antigens like Gasp, Verm and 2A12 in the trachea of rab5 mutants and wild-type embryos. Similar to live imaging data, rab5 mutants at late
stage 17 failed to clear endogenous luminal proteins. The clearance of luminal dextran was also defective in rab5 mutant embryos. Thus we conclude that Rab5 mediated endocytosis is directly involved in clearing proteins from the tracheal lumen.

**Liquid clearance and gas filling during tracheal maturation**

Late stage 17 wild-type embryos were imaged live to visualize the transition of liquid filled trachea, to a gas filled organ. In a standardized chain of events, a bubble of unknown gas appears in one of the DT metamere 4 to 6. This gas bubble quickly expands through the DT first posteriorly and then anteriorly. The bubble then spreads through the posterior anastomosis to the contralateral DT. Within 10 min, the entire tracheal network is filled with gas. The mature larva hatches with a gas filled trachea.

Thus we identified a series of strictly timed sequential events of apical secretion and endocytosis that drive embryonic tracheal maturation. During the transition from nascent, narrow tubes to functional organ, the tracheal cells 1) initiate a Sar1-dependent bulk apical secretion supporting lumen diameter expansion. 2) After the secretory phase the luminal material is cleared by endocytosis. 3) Finally the remaining liquid is exchanged for gas.

**Identification of γCOP (Paper II)**

γCOP like sar1 mutants was found in the same screen for tracheal tube morphogenesis defects. To identify the affected gene, we performed plasmid rescue from the P-element insertion line. We identified that the insertion was located in the 5´ UTR of γCOP locus (from Hemphälä. J, et al. unpublished data). Two deletion alleles were generated by imprecise excision of the transposon. Both deletions remove portions of the open reading frame of γCOP gene.
Expression of γCOP mRNA in tubular organs

We performed in situ hybridization to detect the expression pattern of γCOP transcript in embryos. We observed that γCOP mRNA was maternally contributed. At later stages of embryonic development, zygotic γCOP transcripts preferentially accumulated in epithelial organs like the trachea, salivary gland (SG), hindgut and epidermis. The zygotic γCOP transcripts were undetectable in the γCOP deletion mutants. The transcription factor CrebA was shown to be necessary for the zygotic expression of early secretory pathway genes including γCOP in embryonic SG (Abrams and Andrew, 2005).

γCOP is required for efficient protein secretion

At stage 15 γCOP mutants displayed strong cellular retention of 2A12 in tracheal cells. By this stage, tracheal cells of wild-type embryos have deposited the 2A12 marker into the lumen. γCOP mutants also showed strong cellular retention of the Verm and Gasp proteins. Hence we concluded that γCOP mutants are defective in efficient secretion of luminal proteins. These phenotypes were rescued to wild-type by tracheal expression of γCOP in γCOP mutants. The epithelial organization is not affected in zygotic γCOP mutants presumably due to the abundant maternal contribution of the γCOP transcript.

We observed zygotic expression of γCOP transcript in SGs and hindgut. Thus we were interested to know if γCOP has similar functions in the morphogenesis of other tubular organs. Wild-type embryos show an accumulation of O-glycosylated antigens in the tracheal and SG tubes. We observed a strongly reduced apical secretion of O-glycosylated proteins visualized with an anti-Tn antibody in SG of γCOP mutants. These
defects were rescued by re-expression of γCOP under tubulin α1 promoter in the mutant embryos. Thus, γCOP function is necessary for efficient secretion in trachea and SG at stage 15. From this we conclude that zygotic expression of γCOP is necessary during embryonic tubular organ development.

Lumen diameter expansion defects in the trachea and salivary glands of γCOP mutants

γCOP mutants showed reduced apical proteins secretion and a decrease in lumen diameter expansion of DT at late embryonic stage 16. To visualize the lumen, we stained embryos for Gasp and highlighted tracheal cell membranes by GFP-CAAX expression (a fusion of the membrane targeting CAAX sequence to GFP). At stage 16, the DT tubes of γCOP mutants were thinner compared to wild-type. This failure in tube expansion was also clearly visible in yz-confocal (cross) sections of GFP-CAAX γCOP embryos. To quantify DT tube diameter we measured the diameter of the lumen in wild-type and γCOP mutant embryos expressing GFP-CAAX. The DT diameter at stage 16 for metamere 6 was reduced by 54% in γCOP mutants (3.6 mm) compared to wild-type (6.2 mm). In contrast the length of the DT for metamere 6 was not altered significantly.

We also stained wild-type and γCOP mutant embryos for the apical membrane regulator Crumbs and the cytoskeletal protein αSpectrin to highlight cellular outlines and Piopio to label the SG lumen. Similar to the tracheal tubes, the SG tubes showed a smaller diameter in γCOP mutants compared to wild-type (1.9 µm compared to 5.0 µm). These narrow lumen phenotypes were rescued by re-expression of γCOP in the mutants. In connection with the apical secretion defects in trachea and salivary glands, the luminal ECM was defective in both organs of the γCOP mutants. These phenotypes might be due to inefficient function of COPI vesicles in apical secretion of proteins in the
respective tissues. The similarities in tracheal and SG tube expansion phenotypes in γCOP mutants suggest a common mechanism underlying for diametric growth of tubular organs. Consistent to the epidermal and hindgut expression of γCOP transcript, we observed that γCOP mutants failed to completely perform the dorsal closure of the epidermis. The hindgut tube also showed cellular retention and defective localization of Crumbs proteins in border cells. The dorsal closure defect in the mutants was rescued by ubiquitous expression of γCOP from tubulin promoter.

**Localization of γCOP between ER and Golgi**

To address the subcellular localization of γCOP we used an antiserum raised against mouse γCOP. We first assessed the specificity of the antiserum by RNA intereference (RNAi) against γCOP in S2 cells. The sub-cellular staining intensities were selectively reduced in RNAi against γCOP treated S2 cells. In western blot analysis of extracts from the RNAi treated cells displayed a strong reduction in intensity of a band at around 100kDa, which corresponds to endogenous γCOP protein. RNAi against GFP did not show changes in staining or the band intensity in western blot analysis. Thus we conclude that the antibody against mouse γCOP specifically recognizes *Drosophila* γCOP protein in S2 cells.

To address the sub-cellular localization of γCOP, we co-stained cells with a mouse anti-γCOP antibody together with different endogenous cellular markers. We observed partial co-localization of γCOP with the ER marker KDEL. γCOP strongly co-localized with the widely used cis-Golgi marker GM130 and Lava lamp. The median and trans-Golgi were always seen in close proximity, they did not show complete co-localization with γCOP. In conclusion *Drosophila* γCOP localizes between ER and Golgi, which is consistent with the sub-cellular localization of mouse γCOP.
\( \gamma \text{COP is required for ER and Golgi integrity} \)

As \( \gamma \text{COP} \) protein resides at the early secretory compartments in \textit{Drosophila}, we wanted to assess the integrity of these compartments in the \( \gamma \text{COP} \) mutants. The KDEL marker showed strong reduction in staining intensity in the trachea and SG of mutant embryos compared to wild-type, suggesting that the ER is affected. TEM analysis of wild-type epithelial cells revealed a tubular ER. In \( \gamma \text{COP} \) mutants we found ballooned ER structures both in trachea and SG. In wild-type SGs we observed abundant apically localized secretory granules. These granules were either very few or completely missing in the cells of \( \gamma \text{COP} \) mutants. Accordingly the luminal ECM appeared irregular in the SGs of \( \gamma \text{COP} \) mutants compared to wild-type. These observations further argue for an important role of \( \gamma \text{COP} \) in fueling and maintaining the secretion capacity of the cells.

\( \gamma \text{COP} \) mutants also showed a decrease in the staining intensity and number of Golgi puncta visualized by Lava lamp antibodies in \( \gamma \text{COP} \) mutants, which is not the case in the wild-type embryos. Thus, \( \gamma \text{COP} \) is required directly or indirectly for the integrity and function of both ER and Golgi.

\textbf{Un-folded Protein Response (UPR) in \( \gamma \text{COP} \) mutants}

Over-accumulation of proteins in the ER caused by defects in folding or secretion can activate the ER stress response. ER stress is further transmitted to cytosol by an ER localized transmembrane protein IRE1. It is an endonuclease, which performs splicing of \textit{xbp1} transcript only in the presence of ER stress. \textit{xbp1} mRNA codes for a transcriptional factor. Once spliced it becomes translated and moves into the nucleus to up-regulate downstream genes that help the cell to cope with the ER stress. For example upon UPR activation, the ER undergoes a series of structural changes and autophagy (Bernales et al., 2006; Friedlander et al., 2000; Ryoo and Steller, 2007). Splicing of \textit{xbp1} mRNA
provides a sensitive tool to address ER stress (Plongthongkum et al., 2007). We investigated if \( \gamma \text{COP} \) mutants activate UPR in response to the abnormal intracellular accumulation of secretory cargo. As positive and negative controls we used DTT treated or untreated S2 cells. DTT hinders disulfide bond formation during protein folding and is expected to activate UPR. We observed splicing of \( xbp1 \) in cells treated with DTT. \( xbp1 \) splicing was also readily detected in \( \gamma \text{COP} \) mutants but not in heterozygous embryos. Thus we concluded that ER structural defects might be due to activation of the unfolded protein response, which is supported by the presence of spliced form of \( xbp1 \) transcript in the \( \gamma \text{COP} \) mutants.

**COPI and COPII are interdependent for lumen diameter expansion**

Sar1 and components of COPII complex, function in anterograde movement of protein containing vesicles from ER to Golgi. \( \gamma \text{COP} \) together with other COPI components operates in retrograde movement of vesicles, in recovering ER resident proteins from Golgi back to ER. \( \delta \text{COP} \) mutants also displayed defects in apical secretion leading to reduced lumen diameter expansion in the trachea and SGs. This suggests other components of COPI complex are also required for efficient diameter expansion of tubes.

Thus mutations disabling both COPII and COPI components show similar phenotypes of decreased protein secretion and reduced diameter expansion of the tubes. To test if they function in the same pathway during tube morphogenesis, we generated \( sar1 \ \gamma \text{COP} \) double mutants. We did not detect any additive phenotypes in the trachea of \( sar1 \ \gamma \text{COP} \) mutant embryos. The severity of the defects in lumen diameter was similar to the defect in the single \( sar1 \) and \( \gamma \text{COP} \) mutants, suggesting that they function in a linear pathway. Thus up-regulated bidirectional ER-Golgi vesicular activity
is essential to support the high secretory needs of epithelial tissues like trachea and SG during lumen growth.

During tracheal and SG development, epithelial cells initiate a dynamic secretory burst that deposits a transient filamentous matrix. This process is mediated by COPII and COPI vesicles. The diameter expansion defects in COPI and COPII mutants may be explained by 1) defective secretion leads to the reduced delivery of apical membrane defects in diametric growth. 2) Defects in the delivery of luminal proteins that lead to structural defects in the swelling of the luminal matrixes and the pressure it may exert to the epithelium. 3) The failure in deposition of tube expansion regulators, like ion channels (Berry et al., 2003) on the apical membrane of the tracheal cells (Figure 4). The critical function of channels and osmotic pressure in tubulogenesis has been demonstrated in the formation of the zebrafish gut tube (Bagnat et al., 2007) and they have also been implicated in the expansion of the lungs (Olver et al., 2004).

Zebrafish mutants for coatomer α, β and β’ genes also displayed block in export from ER and also showed structural defects in the Golgi. These mutants fail to expand their notochord and larval tail. These phenotypes could be partly due to defects in laminin secretion and the assembly of extracellular matrix (Coutinho et al., 2004). We speculate that the fly trachea and SGs transiently deliver an expanding ECM into their lumen, which may facilitate efficient tube expansion by exerting a physical pressure to the apical membrane.
Figure 4. Mechanisms of tube expansion in *Drosophila* epithelial organs

Different models for the role of secretion in diametric expansion of the lumen. In the first model assembly the luminal matrix exerts an inflating force that expands the tube. In the second model a burst of apical secretion deposits the necessary membrane for luminal expansion. In the third model the increased membrane deposition of apical regulators (like Crb) may control the composition of the cytoskeleton to stretch the apical surface. The three proposed models are not mutually exclusive.
**Chitin deacetylases (ChLDs) restrict tracheal tube length (Paper III)**

The *Drosophila* genome encodes many putative chitin binding proteins (ChtB). We identified two of them *vermiform* (*verm*) and *serpentine* (*serp*). The names are because of the convoluted tracheal phenotypes in mutants lacking these proteins. Verm and Serp belong to the protein family of ChLDs, as they also contain an additional Low-Density Lipoprotein Receptor (LDLR) domain and a polysaccharide deacetylase (Deac) domain. The *Drosophila* genome contains three ChLD encoding genes and three Chitin deacetylases (CDA) genes (Dixit et al., 2008). The presence of the ChtB and the putative enzymatic Deac domains suggest that ChLD proteins may be involved in the assembly and maturation of luminal chitin ECM. In situ hybridization of *verm* and *serp* transcripts showed tracheal cells expression at stage 12 of wild-type embryos. Epidermal expression of transcripts commences at stage 16. We used P-element insertion allele of *verm*, which lacks Verm protein expression; and a *serp* excision allele with reduced transcript expression to characterize their role in tracheal development. Mutants of *verm* and *serp* have overelongated tracheal tubes, when compared to wild-type. A deficiency strain that lacks both genes shows increased convolution in comparison to the single mutants. This suggests that both Verm and Serp are required to halt tracheal tube elongation.

**Verm and Serp are chitin-binding proteins secreted into the tracheal tubes**

To follow the localization of Verm during tracheal development, we raised antisera against Vermiform. Wild-type embryos stained with anti-Verm antibody, revealed cellular accumulation of Verm protein at stage 13. During tracheal maturation, Verm secretion into the lumen is initiated at stage 14 and finishes by stage 15. By late stage 17, Verm becomes cleared from the lumen and is only detected along the apical
surface of the tracheal epithelium. We made Verm and Serp constructs fused to a carboxy-terminal GFP tag. When they are expressed in the tracheal network they are secreted into the lumen. The overexpression of GFP constructs also caused tube convolution phenotypes resembling the single null mutants.

As Verm and Serp are luminal proteins with a chitin-binding domain, we were interested to test if they function in the assembly of the chitinaceous luminal ECM. Staining of wild-type embryos with a fluorescent chitin binding probe revealed a cylindrical structure of tightly packed luminal chitin polymers. In ChLD mutant embryos, this structure appeared diffused and dilated at stage 15. At later stages, staining intensities of the probe were severely reduced in ChLD mutants. In comparison, the wild-type embryo showed strong and uniform staining in the lumen. Similar degrees of intensity differences were observed in embryos expressing GFP tagged Verm and Serp constructs. TEM analysis of DTs in wild-type embryos revealed a homogenously packed luminal fibrils, which were distorted in verm mutants. Apical chitinous taenidial structures were affected or lost in verm mutants. This indicates that ChLDs are important for the organization of intraluminal chitin assembly.

**Luminal deposition of Chitin deacetylases require functional SJs**

Like verm and serp mutants, embryos lacking components of SJs show tortuous tracheal tubes. We asked if the SJ components and the putative deacetylases function in the same pathway of tube size regulation. We first analyzed for SJ component localization and function in wild-type and ChLD mutants. The localization of Neurexin IV, a well characterized SJ protein was not affected in verm mutants. Also the ladder-like structure of the SJs in verm mutants was very similar to wild-type SJs. Finally, we tested the paracellular barrier function of the SJs in verm mutants by a dye leakage assay. We did not observe dye accumulation into the DT lumen of verm mutant embryos.
contrast the DT tubes of sodium potassium ATPase mutants (Atpα) became filled with the dye 20 minutes after injection. This indicates that ChLD mutants have normal functional SJs. We then asked if ChLD expression and luminal localization is affected in SJs mutants. Wild-type embryos and Atpα mutants were analyzed by Verm and 2A12 stainings. Both markers are secreted in to tracheal lumen by stage 15 in wild-type. However, in Atpα mutants, we observed selective intracellular accumulation of Verm, while the 2A12 antigen was normally deposited into the lumen. By stage 16, Verm staining in the trachea was lost in Atpα mutants. Similar cellular retention defects of Verm were observed in other “SJs mutants” like lachesin and sinuous. 2A12 was again normally deposited and maintained into the lumen. Thus, we identified a new function of SJs in selective apical secretion of ChLD proteins and we proposed that convoluted trachea of SJs mutants might be a consequence of inefficient luminal deposition of ChLDs. In the lumen of wild-type embryos ChLDs are expected to modify the structure of the intraluminal ECM. We speculate that these modifications are sensed by the tracheal cells and instruct them to terminate apical membrane and cell shape changes that lead to tube elongation.

Selective requirement of a deacetylase domain for Emp24 independent luminal secretion in the Drosophila trachea. (Manuscript)

We were intrigued by the selective requirement of SJs for Verm and Serp apical secretion. It suggested a specific apical secretion route for these proteins. To identify the signals for the SJ-dependent apical secretion of ChLDs, we performed a domain deletion analysis of Serp-GFP. We found out that chitin binding and LDLr domains are not required for apical secretion of Serp-GFP. Deletion of the deacetylase domain rendered the protein intracellular in the trachea. This suggested that the C-terminal Deacetylase
domain harbors stretches of amino acids that are necessary for Serp secretion. We took two approaches to uncover the apical secretory pathway of ChLDs. In the first, we identified and mutated phylogenetically conserved residues in the Deac domain to test their impact on luminal secretion. In the second approach, we screened for potential adaptor proteins, which may selectively assist the ER exit of Serp or Gasp.

**Sequence alignments of the Deacytylase domain**

Protein sequence alignment of deacytylase domain identified several conserved motifs. 1) Partially conserved N-glycosylation sites. 2) Residues potentially involved in enzyme activity. 3) Three highly conserved, uncharacterized amino acid motifs. The requirements of these sequences in Serp-GFP secretion were addressed by alanine substitutions and expression of the constructs in the embryonic trachea.

**N-glycosylations are required for efficient secretion of Serp**

To assess if Serp is an N-glycosylated protein, we performed N-Glycosidase F digestions to cleave the N-linked oligosaccharides from the peptide chain. We expressed V5-tagged Serp in tissue culture cells and collected the secreted protein from the culture medium. A fraction of Serp was incubated with N-Glycosidase F enzyme and its electrophoretic mobility was compared to the untreated protein. We observed a clear difference in the mobility of the enzyme treated sample, which was running faster in comparison to untreated sample. This suggests that Serp protein is N-glycosylated. To address the importance of the potential N-linked glycosylations in the Deac domain of Serp, we substituted three of the N-glycosylation sites (asparagine residues of NXT/S sequence) to alanine. The V5-tagged triple N-glycosylation substitution mutant and V5-Serp were expressed in S2 cells. Both proteins became secreted into the cell culture medium. We subjected fractions of the two proteins to N-Glycosidase F treatment to test
if the substitutions affected the sensitivity of the protein to the enzyme. In contrast to the wild-type V5-Serp, we did not observe a mobility shift upon enzyme treatment of triple N-glycosylation substitution mutant. Both enzyme-treated and untreated bands migrated to the same level as the digested wild-type V5-Serp construct. This indicates that Serp is N-glycosylated in vivo and suggests that the triple N-glycosylation substitution mutant lacks the modifications.

To test the significance of these modifications in Serp apical secretion we compared the luminal deposition of Serp-GFP, three single N-residue substituted Serp-GFP proteins and a triple Serp-GFP mutant construct lacking all three N-residues. Wild-type Serp-GFP was secreted into the lumen. In contrast the single substitution constructs was found mostly in the lumen with a minor retention in the tracheal cells. The triple-N mutated Serp-GFP was strongly retained in the trachea cells and a fraction of the mutated protein was also detectable in the lumen. But triple-N Serp-GFP mutant secretion was not sufficient to cause convolutions like the wild-type and single N-residue substitution of Serp-GFP. This suggests that N-linked glycosylations contribute to efficient apical secretion of Serp.

**Verm is normally N-glycosylated in ATPα mutants**

To analyze if the defect in apical secretion of Verm in SJs mutants reflects a defect in N-linked glycosylation of ChLDs, we performed N-Glycosidase F enzyme treatments of wild-type and ATPα mutant embryonic extracts. We detected no difference in the electrophoretic mobility shift of Verm protein deriving from wild-type or the ATPα embryonic extracts. This suggests that the mutants show a normal N-glycosylation pattern of Verm and argues against a requirement of SJ in ChLD N-glycosylation.
Residues involved in the predicted deacetylase activity are not necessary for Serp secretion or tracheal function

Polysaccharide deacetylases catalyze the removal of acetate from chitin. The fungal enzyme is well characterized. It is a Zn$^{2+}$ containing metalloenzyme, the Zn$^{2+}$ ion in the catalytic center is held in place by an aspartic acid and two histidine residues (Blair et al., 2006). The characteristic amino acids for enzymatic activity are found in Serp, but not in Verm. To ask if the predicted enzymatic activity residues may also be required for Serp apical secretion we mutated them to alanine to abrogate Zn2+ binding and the putative chitin deacetylase activity. None of the substitution constructs showed pronounced cellular retention defects. Thus, the amino acids involved in the predicted enzymatic activity were not necessary for Serp secretion. Surprisingly, expression of the mutated constructs in serp mutants, rescued the tube convolution phenotype as efficiently as the transgenic expression of the wild-type Serp protein. This indicates that the deacetylase activity of Serp is redundant for its tracheal function.

Conserved motifs are required for Serp exit from the ER

To understand the function of four short, highly conserved amino acids stretches, we substituted three of them with alanine at positions TYF$^{237-239}$ to AYA, E$^{259-260}$ to AA and RAP$^{308-310}$ to AAA. We expressed the Serp-GFP and GFP-tagged mutants in the tracheal system to address their potential impact on Serp-GFP secretion. We found that all the three mutated proteins were retained in the tracheal cells. To test if the overexpression of the constructs may cause a general secretion block we stained embryos expressing each of the mutated constructs with the 2A12 antibody. The luminal deposition of the 2A12 antigen in these embryos was indistinguishable from the wild-type arguing against the “clogging” hypothesis.
Consecutive substitutions at conserved positions might cause protein missfolding and ER retention of the mutated Serp-constructs. Thus we made a single substitution at position EI$^{259-260}$ to Al. This Serp-GFP mutant was also retained in the tracheal cell. This suggests that the cellular retention of substitution mutant is not due to an indirect effect of the consecutive residue replacements.

GFP is known to potentially polymerize and to form protein aggregates in cells. To rule out that cellular retention of mutant Serp proteins is not caused by GFP, we expressed the RAP$^{308-310}$ to AAA substitution mutant of Serp without the GFP tag in the trachea. This GFP-less, mutant Serp still showed complete intracellular retention indicating that the GFP tag does not mediate its cellular retention. To test where in the secretory apparatus are conserved substitution mutants retained. We stained embryos expressing the mutated constructs with secretory compartment makers. We found complete co-localization of the GFP tagged mutant proteins with KDEL staining. This suggests that the mutated Serp proteins are retained in the ER lumen. We therefore tested if the substitution mutants are N-glycosylated. We expressed V5-tagged, wild-type and Serp mutant versions in S2 cells. Unlike wild-type Serp, which is secreted into the culture medium, the substitution mutants were retained inside the S2 cells. Interestingly, the electrophoretic mobility shift assay after N-Glycosidase F digestion showed that the three mutant versions are normally glycosylated and potentially folded normally in the ER. Thus mutant protein retention occurs despite to their glycosylations in the ER. This suggests, that the three-conserved motifs in the deacetylase domain are necessary for Serp exit from the ER or that they are required for folding after N-glycosylations.

The deacetylase domain can divert Gasp secretion

In eukaryotic cells, all secretory proteins are synthesized and deposited into the ER lumen. They are packed into COPII-coated vesicles for transportation through the
early secretory pathway. Two theories were put forward to explain soluble secretory protein export from ER: 1) COPII vesicles mediate bulk flow of soluble proteins, 2) soluble cargo proteins interact with specific transmembrane adaptor proteins for efficient ER exit (Barlowe, 2003). Data from yeast and other systems favor the second model in which, certain integral membrane adaptor molecules like Erv29, Emp24, and ERGIC-53 are shown to directly bind to cargo protein at the luminal side. The same adaptor proteins bind with the COPII protein complex, through their cytoplasmic tail. Thus adaptor proteins mediate the concentration of soluble cargo into ER derived COPII vesicles (Belden and Barlowe, 2001; Otte and Barlowe, 2004); (Baines and Zhang, 2007; Nyfeler et al., 2008).

Our analysis on SJ mutants had shown that the luminal proteins Gasp and Verm/Serp may follow two different secretory paths into the lumen (unpublished data). We searched for membrane adaptor molecules, which may assist in the selective secretion of these cargos from the ER. We collected Drosophila mutants that potentially inactivate several homologous proteins of the previously characterized adaptor molecules. Emp24, is such a transmembrane adaptor required for efficient soluble protein secretion from the ER. Drosophila mutants for emp24 show a cellular retention of Gasp in the trachea at stage 15, but displayed normal deposition of Verm into the lumen. Interestingly the intracellular retention of endogenous Gasp is transient and seen only at stage 15. As Gasp is also found in the lumen at stage 16, this suggests the presence of alternative secretion routes.

We hypothesized that if the Deacetylase domain of Serp contained specific and different ER exit signals, then it might redirect the secretion of Gasp through a yet unidentified Emp24-independent pathway of secretion. To address this we made a GaspDeac-GFP chimeric construct. The Deacetylase domain of Serp was placed adjacent to the carboxy terminus of Gasp and expressed in the trachea of emp24 mutant
embryos. In parallel, we also expressed Gasp-GFP in the mutants. We found that the addition of the Serp deacetylase domain could circumvent the requirement of Emp24 for Gasp secretion at stage 15. This suggests that the deacetylase domain can redirect Gasp secretion. Thus, we propose that the deacetylase domain contains specific ER exit signals, which can divert soluble protein secretion to an Emp24 independent pathway. It will be interesting to test the diverting ability of the Serp deacetylase domains carrying conserved amino acid substitutions in this assay. The anticipated selective adaptors for this new ChLDs-ER exit pathway remain unknown. This opens for more experiments towards the identifications of the signals and factors involved in this pathway.

Conclusions

My work led to the proposal that the initiation of a transient protein secretion pulse during tracheal development is essential for diametric tube expansion. COPII and COPI mediated vesicular transport plays a key role in tube diameter expansion of the trachea and SGs. Diametric expansion is mediated through protein secretion and constant membrane addition to the lumen. We proposed that the deposition of proteins and polysaccharides facilitates the growth of transient apical ECM, which may also inflate the lumen from inside. The luminal proteins and liquid must be removed to facilitate the function of the trachea in respiration. This is achieved by a wave of Rab5 mediated endocytosis, which is performed by the same tracheal cells. When endocytosis is disrupted, the protein clearance from the lumen is abolished. At the final stages of embryonic tracheal maturation, liquid in the lumen is exchanged for gas.

The work with Verm and Serp identified an important role of chitin modifications in tube size termination. Intact septate junctions (SJs) are selectively required for the apical secretion of ChLD proteins. Thus, we identified a new role of SJs in apical secretion of ChLD proteins and provided a molecular explanation for the roles of SJ
proteins in tube size regulation. The polysaccharide deacetylase domain is necessary for Serp secretion.

The subsequent work with the Deac domain of Serp showed that N-glycosylation is required for efficient apical secretion. We identified three conserved amino acid motifs necessary for Serp exit from the ER. Emp24 is required for Gasp but not ChLD secretion. By adding the Deac domain of Serp to Gasp we diverted the GaspDeac chimera to an Emp24-independent secretion pathway. This suggests that the Deac domain contains an ER exit signals that can circumvent the requirement of emp24 for Gasp luminal secretion.

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References


