Genetic and molecular regulation of epithelial tube morphogenesis

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Cover picture: An expanding tracheal tube in the *Drosophila* embryo. A luminal chitin filament (green) surrounded by the tracheal epithelium (red).

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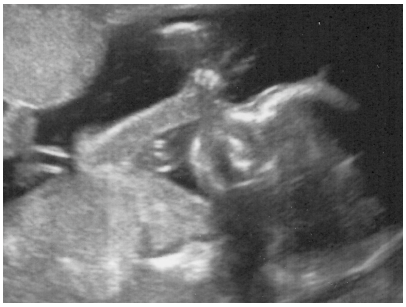

Till min älskade familj

ABSTRACT

Networks of epithelial tubes, such as the vertebrate lung, kidney and vascular system, enable transport of gases and nutrients to all tissues in the body. These tubes are built up by a single layer of polarized epithelial cells, with the apical membrane facing the lumen. For optimal organ function it is critical that each tube in the network attains correct size and shape, as constricted or dilated tubes will affect the flow rate and impede organ function. When tubes form during organ development, they often have a narrow lumen that must expand to attain the typical mature length and diameter. Both apical (luminal) membrane growth and rearrangements of the subapical cytoskeleton are central to tube growth, but the mechanisms that coordinate these events across the tubular epithelium to ensure uniform tubes with functional dimensions have remained unknown.

We have used the respiratory organ (trachea) of the fruit fly *Drosophila melanogaster* as a model system to gain insights into the molecular mechanisms that control tube size and shape. This thesis includes the analyses of five new tracheal tube size genes, which mutational analyses have revealed a new biological principle to ensure uniform functional tubes: newly formed tracheal tubes deposit a broad luminal chitin filament around which the tubular epithelium can rearrange. After tube expansion and before the tubes become functional in transport, the luminal filament is cleared. Four of the genes (*krotzkopf verkehrt*, *knickkopf*, *retroactive* and *mummy*) are required to build the luminal chitin filament, and their loss of function result in severe diameter constrictions and dilations in the expanding tubes. A fifth gene, which we have called *humongous*, encodes a novel protein required for formation of the septate junctions (SJs), which are analogous to vertebrate tight junctions. Many previously identified tracheal tube size genes encode SJ components, but their role in tube expansion has been unclear. We find that intact SJs are essential for correct assembly of the chitin filament, thus reinforcing the central role of the luminal chitin filament in tube size regulation.

Keywords: epithelial tube morphogenesis, trachea, *Drosophila*, chitin, chitin synthase, apical extra cellular matrix, paracellular diffusion barrier, tube expansion, septate junctions

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PAPERS AND MANUSCRIPT

This thesis is based on the following publications, which will be referred to by their roman numbers:

I. **Tonning, A., Hemphälä, J., Tång, E., Nannmark, U., Samakovlis, C. and Uv, A.**
A transient luminal chitinous matrix is required to model epithelial tube diameter in the *Drosophila* trachea.
Developmental Cell 9: 423-430, 2005

II. **Moussian, B., Tång, E., Tonning, A., Helms, S., Schwarz, H., Nüsslein-Volhard, C. and Uv, A.**
Drosophila Knickkopf and Retroactive are needed for epithelial tube growth and cuticle differentiation through their specific requirement for chitin filament organization.
Development 133(1): 163-171, 2006

III. **Tonning, A., Helms, S., Schwarz, H., Uv, A. and Moussian, B.**
Hormonal regulation of *mummy* is needed for apical extracellular matrix formation and epithelial morphogenesis in *Drosophila.*
Development 133(2): 331-341, 2006

IV. **Tonning, A., Wikström, J., Tång, E., Nannmark U. and Uv, A.**
The novel protein Humongous, is required for tubular epithelial barrier function in *Drosophila.*
Manuscript

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ABBREVIATIONS

ADPKD	autosomal dominant polycystic kidney disease
aECM	apical extracellular matrix
AJ	adherens junctions
Arm	Armadillo
Atp□	a-subunit in Na+/K+ ATPase
Baz	Bazooka
Bnl	Branchless
Btl	Breathless
CASPR	Contactin associated protein
CBD	chitin binding domain
CBP	chitin binding probe
Cora	Coracle
Crb	Crumbs
CS	chitin synthase
DaPKC	*Drosophila* atypical protein kinase
DB	dorsal branch
Dlg	Discs large
DPAR6	*Drosophila* Partitioning-defective protein-6
DPatj	*Drosophila* PALS-1 associated tight junction protein
DT	dorsal trunk
ERM	ezrin-radixin-moesin
Fas3	Fasciclin 3
FGF	Fibroblast growth factor
GB	ganglionic branch
GEF	guanine nucleotide exchange factor
GlcNAc	N-acetyl-D-glucosamine
GPI	glycosylphosphatidyl-inositol
Grh	Grainy head
HGF	hepatocyte growth factor
hmn	*humongous*
Ig	Immunoglobulin
kkv	*krotzkopf verkhert*
knk	*knickkopf*
Lgl	Lethal giant larvae
LT	lateral trunk
MDCK	Madin-Darby canine kidney
mmy	*mummy*
MZ	marginal zone
Nrx	Neurexin
PC	polycystin

PDZ	Post synaptic density protein/Discs large/ZO-1 domain
Rib	Ribbon
RTK	receptor tyrosine kinase
rtv	*retroactive*
sad	*shadow*
SB	spiracular branch
Scrib	Scibble
shd	*shade*
Sinu	Sinuous
SJ	septate junctions
SNARE	soluble NSF attachment receptor
Std	Stardust
TC	transverse connectives
TEM	transmission electron microscopy
TJ	tight junctions
VB	visceral branch
WGA	wheat germ agglutinin
wt	wild type

INTRODUCTION

From one cell to many – a need for transport systems develop

Life began with only one cell, with the function to reproduce, respire and obtain energy from metabolites to drive cellular processes. Evolution has resulted in more complex, multicellular organisms where cells come to collaborate to form the different body structures and to perform life-essential processes. A vital step in this evolution has been to develop mechanisms to transport gases and nutrients to all cells in the body. Nature has solved this problem by creating intricate tubular networks, as in the pulmonary system, cardiovascular system, the kidney and the many glands.

Most tubes are built up by a single layer of polarized epithelial cells, with their apical membrane facing the inside of the tube, the lumen, and their basal surface facing the surrounding body tissues. Formation of tubular networks during embryogenesis begins with cell specification and branching morphogenesis to pattern the network, after which each tube matures to form correctly dimensioned and functional tubes (Affolter and Shilo, 2000; Ghabrial et al., 2003). The mechanisms underlying both cell specification and branching morphogenesis appear shared between species and organs (Metzger and Krasnow, 1999). They generally involve transcription factors to specify cell fates within the epithelium, followed by the use of ligands to receptor tyrosine kinases (RTK) to induce and guide branch outgrowth together with tissue-specific guidance cues for certain branches (Nelson, 2003). Unfortunately, little is known about the process by which epithelial cells form functional tubes.

Mature, epithelial tubes are not simply a passive plumbing system, but function as a selective barrier that separate body compartments, and may even modify the transported medium. For these functions, intricate cell-to-cell junctions are established as well as polarized distribution and secretion of molecules (Furuse and Tsukita, 2006; Gibson and Perrimon, 2003; Powell, 1981).

Correct tube size and shape are essential for organ function and tubes are often ordered in a hierarchy with large tubes for fast bulk transport and narrow branches for molecular exchange with target tissues. Tubular constrictions or dilations will cause an uneven flow of the transporting media, and such malformations can give rise to serious pathological conditions, such as polycystic kidney disease, which is one of the most common human genetic diseases, affecting 1 in 1000 individuals (Boletta and Germino, 2003; Horie, 2005). When epithelial tubes form, they often have a narrow lumen, which will expand to attain mature dimensions (Lubarsky and Krasnow, 2003). It has been suggested that tube expansion depends on events occurring within the apical cell domain, such as polarized delivery of apical membrane and luminal components, and the subapical actin cytoskeleton to shape the lumen (Beitel and Krasnow, 2000; Buechner, 2002; Hemphala et al., 2003; Lubarsky and Krasnow, 2003). However, how these events are coordinated across the tubular epithelium to ensure correct size and shape has been unclear.

Aim of this thesis

The aim of the work presented in this thesis is to gain further understanding of the genetic and molecular mechanisms that control epithelial tube morphogenesis.

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

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*.

Drosophila has become an important model system for dissecting molecular mechanisms of development and tissue morphogenesis due to the possibility to perform large-scale genetic screens where randomly mutagenised flies are screened for a certain phenotype, giving an unbiased approach to find genes involved in a specific process. Today there are advanced methods for genetic manipulation in *Drosophila* and numerous molecular and cellular tools are available (St Johnston, 2002). Moreover, analysis of embryonic development is facilitated by a large access of embryos, rapid embryogenesis and the ability to perform whole mount immunohistochemistry.

The respiratory organ of the fruit fly is called the trachea. It is a network of single cell-layered epithelial tubes that extends through the whole organism and distributes air from the exterior through gradually finer tubes for efficient gas exchange with target tissues (Ghabrial et al., 2003). The tracheal network is highly stereotyped, both in branch pattern and tube size, making it easy to recognize deviations from normal development upon genetic manipulation. In contrast to more complex vertebrate systems, development of tracheal tubes does not involve cell proliferation or cell death, but solely rely on cell migration and changes in cell shape and cellular junctions (Beitel and Krasnow, 2000; Samakovlis et al., 1996a). Thus, the *Drosophila* trachea is well suited for elucidating the molecular and genetic mechanisms that underlie epithelial tube morphogenesis.

Tracheal morphology and development

The fully developed embryonic tracheal system contains approximately 1600 epithelial cells with the apical cell surface facing the lumen. The network is built from 20 metameric units, 10 on each side of the embryo, which branch independently in a stereotyped manner and fuse with their neighbours to create a continuous network (one metamere is highlighted in Fig 1). Each branch of the tracheal system has a characteristic number of cells, cellular architecture and tube dimensions, that is invariable from embryo to embryo (Uv et al., 2003).

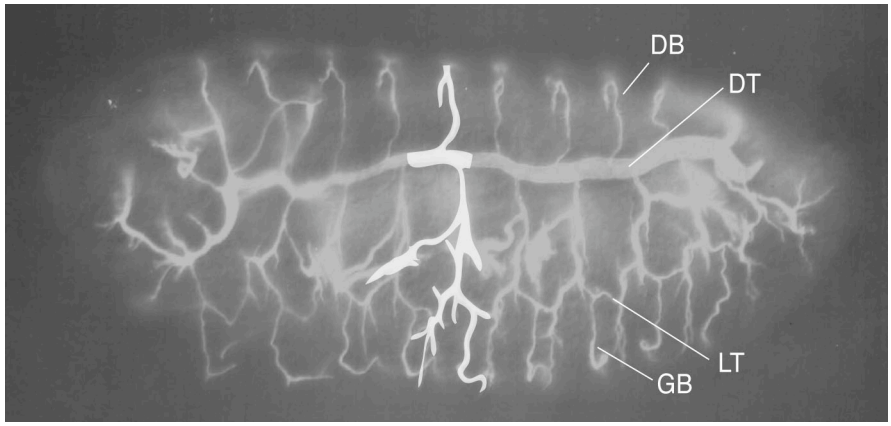

Fig 1. The embryonic tracheal system. Lateral view of a *Drosophila* embryo at stage 16 with the anterior part to the left. The tracheal lumen is visualised by the luminal marker 2A12. One tracheal metamere is highlighted and the main branches are indicated. DB: dorsal branch, DT: dorsal trunk, LT: lateral trunk and GB: ganglionic branch. (Adapted from Uv et al., 2003)

Tracheal development begins at mid-embryogenesis, at stage 10, with the genetic specification of tracheal precursor cells in the ectoderm, the outermost layer of the embryo (Affolter and Shilo, 2000; Zelzer and Shilo, 2000a). This occurs at twenty positions along the anterior/posterior axis, ten on each lateral side of the embryo, and is mediated by the local expression of transcription factors

Trachealess/Tango and Ventral veinless (Anderson et al., 1995; de Celis et al., 1995; Isaac and Andrew, 1996; Wilk et al., 1996; Zelzer and Shilo, 2000b). The 20 clusters of tracheal precursor cells then start to invaginate, creating pouches. During invagination the tracheal cells undergo their final cell division generating approximately 80 cells per pouch. The cell number of approximately 1600 will be invariable throughout tracheal development (Samakovlis et al., 1996a).

The subsequent ramification is highly regulated and depends totally on cell shape changes and migration (Samakovlis et al., 1996a). Within each pouch the cells are predetermined to form either of six primary branches; dorsal trunk anterior (DTa), dorsal trunk posterior (DTp), dorsal branch (DB), transverse connectives (TC), lateral trunk anterior (LTa) and lateral trunk posterior/ganglionic branch (LTp/GB) (Manning and Krasnow, 1993). This commitment of cells to different branches is determined by the spatial expression of Decapentaplegic, Rhomboid, Wingless and Hedgehog in the early embryo (Chihara and Hayashi, 2000; Glazer and Shilo, 2001; Llimargas, 2000; Vincent et al., 1997; Wappner et al., 1997). The actual branch migration is induced and guided by Branchless (FGF, Fibroblast growth factor) and its receptor of RTK type, Breathless (FGF receptor) (Klambt et al., 1992; Sutherland et al., 1996). Trachealess/Tango and Ventral veinless induce expression of the FGF receptor Breathless in tracheal cells at stage 10, whereas the FGF ligand Branchless is expressed in six clusters of epidermal and mesodermal cells surrounding each metamere. Tracheal cells will migrate towards the source of the chemoattractant Branchless creating primary buds. Branch migration continues until the ligand is downregulated when a branch reaches the ligand source. For some branches subsequent upregulation of the ligand in cells further away advances the branch (Anderson et al., 1996; Ribeiro et al., 2002; Sutherland et al., 1996).

Secondary branching commences at stage 14, several hours after initiation of primary budding. In each metamere, 25 secondary branches are formed, of which 20 will become terminal branches and extend to reach their target tissue and 5 will create fusion anastomoses

(Fig 3). During secondary branching, Branchless/Breathless signalling is again used, but in a different manner. It causes cell fate changes in the cells at the tip of each primary branch by inducing the expression of pantip markers such as the transcription factor *pointed* (Hacohen et al., 1998; Ohshiro et al., 2002; Samakovlis et al., 1996b; Scholz et al., 1993). Pointed promotes the next level of branching by inducing expression of *blistered* (or *pruned*) the gene encoding *Drosophila* serum response factor (DSRF) and inhibiting the expression of the fusion gene *escargot*, in terminal cells (Affolter et al., 1994; Guillemin et al., 1996; Samakovlis et al., 1996b) Terminal branches undergo extreme morphogenetic events, forming extensive hollow, single cell projections with a diameter of less than 1 μm that make contact with cells in the target tissue (Uv et al., 2003). Ramification of the terminal branches during larval life is highly variable and regulated by the oxygen-needs of the tissue. Oxygen-deprived cells upregulate expression of *branchless* and thereby induce branch migration, similar to VEGF-induced (Vascular endothelial growth factor) chemotaxis during angiogenesis in vertebrates (Shweiki et al., 1992; Jarecki et al., 1999).

During tracheal development, certain branches (DT, LT and DB) will fuse to connect the network (Fig 3). Dorsal trunk fusion occurs during stage 14, lateral trunk fusion takes place from stage 14 to 16 and dorsal branch fusion occurs during stage 16. At the tip of these branches, there is a specialized fusion cell sending cytoplasmic projections toward the neighbouring fusion cell. After recognition, *Drosophila* E-cadherin (DE-cad) is deposited in a ring at this contact site. The earliest known fusion gene is the one coding for the transcription factor Escargot, which induces expression of later fusion genes and suppresses terminal branching in the fusion cells (Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996).

During tracheal development, the tubes are filled with liquid, which will be cleared just before the larva hatches (Manning and Krasnow, 1993). This is analogous to the first breath of the vertebrate foetus, clearing the lung from amniotic fluid. To prevent the tubes

from collapsing a rigid cuticle is laid down before liquid clearance (Manning and Krasnow, 1993). The cuticle is an apical extracellular matrix that is secreted by the epidermis and the ectodermally derived epithelia in the trachea, hindgut and foregut (Fig 2). This matrix is a specialised structure of proteins and polysaccharides that give structural support and protection against pathogens and dehydration (Locke, 2001).

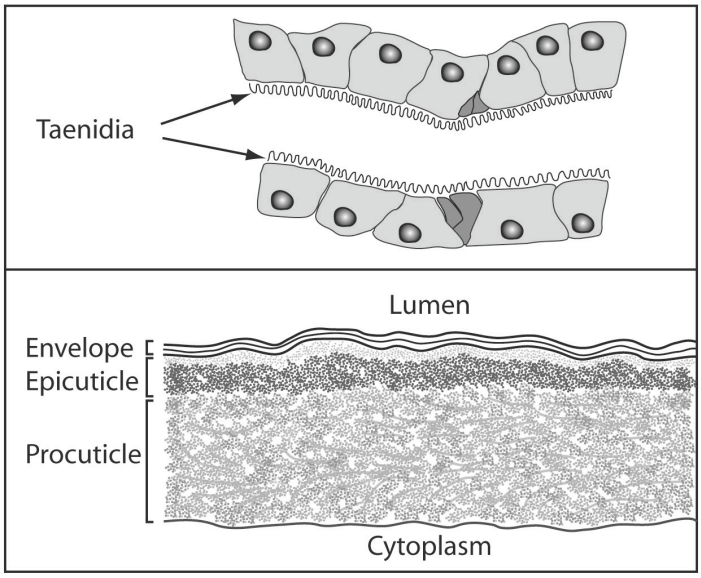

Fig 2: **Tracheal cuticle.** A cuticle with prominent circumferential ridges, called taenidia, is secreted by the tracheal cells late during embryogenesis to provide structural support after liquid clearance. The cuticle is an organized apical extracellular matrix. Its formation begins with secretion of the outer envelope, which is impermeable due to its composition of lipids and waxes. Thereafter, the protein-rich epicuticle and the innermost procuticle are produced. The epicuticle lies beneath the envelope and provides rigidity to the cuticle, whereas the protcuticle contains lamellae built of sheets of chitin microfibrils to provide mechanical support and elasticity (Moussian et al., 2005a).

Tracheal tube morphology

Tracheal cells are squamous, polarized epithelial cells that are connected to each other via junctional complexes along their entire lateral surfaces (Tepass and Hartenstein, 1994). Their specialised apical and basolateral plasma membrane domains are required to create correct tubular structures and to maintain tube wall barrier and integrity (Carlson et al., 2000; Lubarsky and Krasnow, 2003; Tepass et al., 2001).

Tube architecture

The tracheal cells can be grouped into three genetically and morphologically distinct cell types, which express different sets of tracheal genes. Stalk cells constitute the main branches, fusion cells connect the metameres to each other to generate a continuous network, and terminal cells mediate gas exchange with target tissues (Uv et al., 2003).

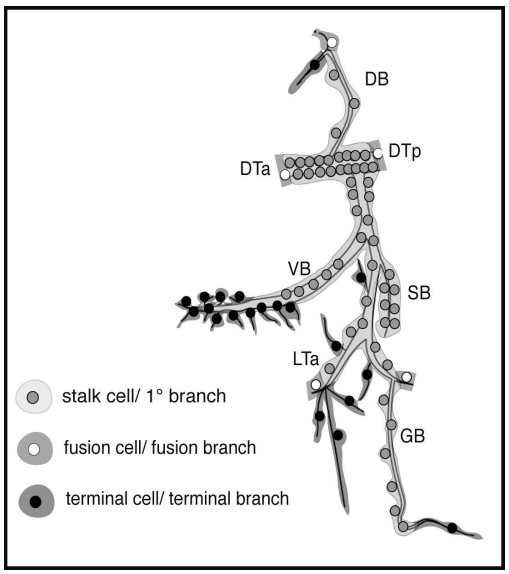

Fig 3. Tracheal cell types. Each tracheal branch is made up by a characteristic number of cells (each cell nucleus is indicated with a circle). The cells that make up the primary branches i.e. the dorsal trunk (DT), dorsal branch (DB), visceral branch (VB), spiracular branch (SB), lateral trunk (LT) and ganglionic branch (GB) are called stalk cells. A single fusion cell is located at the tip of each DB, DT and LT branch and connects with the analogous fusion cell in neighbouring metameres to create the continuous network. Approximately 20 terminal cells form at the tips of primary branches and extend towards target tissues to mediate gas exchange with target tissues. (Adapted from Uv et al., 2003)

Stalk cells assemble into either type I or type II branches (Fig 4). In type I branches, such as the DT, several wedge-shaped cells surround the lumen and are connected with intercellular junctions. In the narrower type II branches, as the DB, single cells are wrapped around themselves and sealed with autocellular junctions (Uv et al., 2003). The fusion cells form type III branches, which consist of two doughnut-shaped cells (one from each neighbouring metamere), connected with each other head to head. Finally, type IV tubes are found in the terminal branches, and are made up by single cells with an intracellular lumen surrounded by a thin wall that facilitates gas exchange with target tissue.

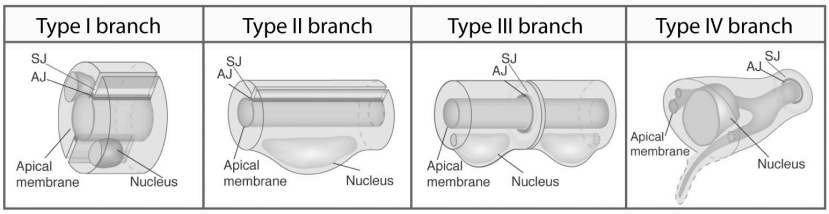

Fig 4. Tracheal branch types. In type I branches several wedge-shaped cells surround the lumen circumference. Type II branches are formed by cells wrapped around the lumen and sealed with autocellular junctions. Type III branches consists of two doughnut-shaped cells. Type IV branches are single cell tubes with intracellular lumen. (Adapted from Uv et al., 2003)

Cell junction organisation

Epithelial development involves the establishment of apical/basal polarity followed by maturation of specialized cell domains that are comprised of transmembrane and cytoplasmic proteins associated with local actin/spectrin cytoskeleton (Tepass et al., 2001).

Tracheal cells are primary epithelia, which are derived from the blastoderm epithelium, and acquire their initial polarization during cellularization in the early embryo (Tepass et al., 2001). Like other epithelia, the tracheal epithelium is connected by junctional complexes, which contain transmembrane proteins that interact with cytoplasmic components to mediate anchoring to the cytoskeleton and intracellular signal transduction (Fig 5, Tepass et al., 2001). These

include the Crumbs- and Bazooka complexes at the apical marginal zone (MZ), the Cadherin-catenin complex at the adherens junctions (AJ), and the baso-lateral septate junctions (SJ, Fig 5).

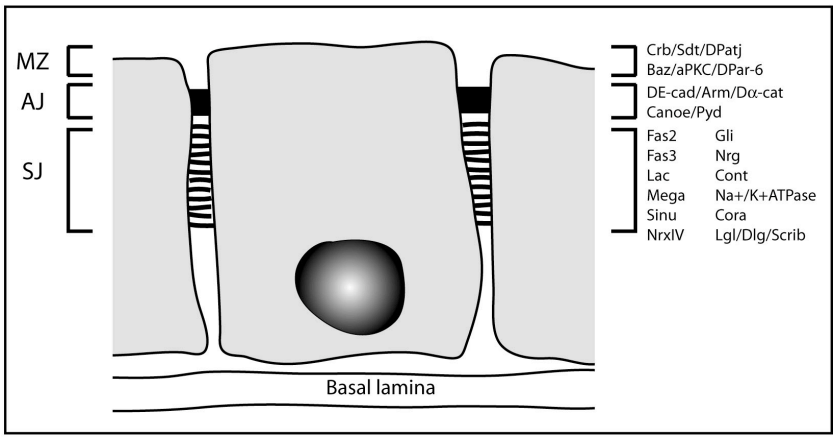

Fig 5. Epithelial cell junctions. A schematic drawing of an epithelial cell with its intercellular junctions to neighbouring cells. Left brackets indicate the positions of MZ (marginal zone), AJ (adherens junctions) and SJ (septate junctions). The localizations of known junction proteins are shown to the right: Crb (Crumbs), Sdt (Stardust), DPatj (*Drosophila* PALS-1 associated tight junction protein), Baz (Bazooka), aPKC (atypical Protein Kinase C), DPar-6 (*Drosophila* Partitioning defective-6), DE-cad (*Drosophila* E-cadherin), Arm (Armadillo), D□-cat (*Drosophila* □-catenin), Canoe, Pyd (Polychaetoid/ZO-1), Fas2 (Fasciclin 2), Fas3 (Fasciclin 3), Lac (Lachesin), Mega (Megatrachea), Sinu (Sinuous), NrxIV (Neurexin IV), Gli (Gliotactin), Nrg (Neuroglian), Cont (Contactin), Cora (Coracle), Lgl (Lethal giant larvae), Dlg (Discs large) and Scrib (Scribble).

The Crumbs complex is composed of Crumbs (Crb), Stardust (Sdt) and PALS-1 associated tight junction protein (DPatj), and is enriched at the marginal zone, although low levels are found along the entire apical membrane in ectodermally derived epithelia (Fig 5, Tepass et al., 2001). The large transmembrane protein Crb is essential for establishment of apical/basal polarity, the biogenesis of the adherens junctions (AJs) and promotes apical membrane differentiation (Grawe et al., 1996; Klebes and Knust, 2000; Tepass, 1996; Wodarz et al., 1995). Crb interacts with the multi-PDZ domain protein DPatj (earlier called Discs lost) and the MAGUK protein Sdt (Bachmann et al.,

2001; Hong et al., 2001; Klebes and Knust, 2000). These two scaffolding proteins are required for correct AJ assembly (Bachmann et al., 2001; Bhat et al., 1999). Crb also interacts with the apical Spectrin membrane cytoskeleton (Medina et al., 2002) via the 4.1 protein DMoesin (Bretscher et al., 2002).

The Bazooka complex contains three intracellular PDZ proteins, the *Drosophila* Par-3 homolog Bazooka (baz), the PDZ protein *Drosophila* Partitioning-defective protein-6 (DPar6) and the atypical protein kinase (DaPKC) (Petronczki and Knoblich, 2001; Wodarz et al., 2000). Similar to the Crb complex, the Baz complex associates with the entire apical membrane, but is concentrated at the marginal zone (Wodarz et al., 2000), and is required for establishment of apical-basal polarity and formation of AJs (Kuchinke et al., 1998; Muller and Wieschaus, 1996). The Baz complex is also required for apical localisation of Crb (Bilder et al., 2003), and DaPKC is shown to activate Crb through phosphorylation (Sotillos et al., 2004).

The Cadherin-catenin complex is the main component of the AJs multiprotein complexes that function as an adhesive belt encircling the apical domain and are required for maintaining tissue architecture (Oda et al., 1994; Tepass et al., 1996). E-Cadherin (DE-Cad) is a calcium-dependent transmembrane homophilic adhesion molecule that interacts with the β-catenin Armadillo (Arm) and α-catenin, which in turn interacts with the cytoskeleton (Oda et al., 1994; Oda et al., 1993; Oda et al., 1997; Pai et al., 1996; Peifer et al., 1993; Tepass et al., 1996).

Cell signalling at AJs A second complex associated with the AJs contains Canoe, the *Drosophila* homologue of Afadin. Canoe appears to be an upstream regulator of Jun N-terminal kinase (JNK) signalling in a process where it interacts with *Drosophila* ZO-1, a MAGUK protein encoded by *polychaetoid* (Noselli and Agnes, 1999; Takahashi et al., 1998; Takahisa et al., 1996). Canoe is also reported to interact

with Ras1 and Notch signalling, and thus represents evidence for cell signalling at the AJs (Matsuo et al., 1999; Miyamoto et al., 1995).

Septate junctions (SJs) form circumferential spirals around the lateral cell domain, basal to the AJs (Fig 5). SJs separate the membranes of the adjacent cells by approximately 15 nm, a space occupied by regularly spaced electron-dense septa, forming a ladder-like structure (Tepass and Hartenstein, 1994; Tepass et al., 2001). SJs develop at midembryogenesis, when epithelial polarity and AJs are already established (Tepass and Hartenstein, 1994). The SJs form a paracellular diffusion barrier (Carlson et al., 2000), as shown by dye exclusion assays and mutational analysis (Baumgartner et al., 1996; Behr et al., 2003; Carlson et al., 2000; Lamb et al., 1998; Paul et al., 2003; Wu et al., 2004), analogous to the barrier function of vertebrate tight junctions (TJs). Despite morphological and molecular differences between SJs and TJs (Anderson, 2001; Tepass et al., 2001), the recent identifications of two claudin-like *Drosophila* SJ proteins, Sinuous (Sinu) and Megatrachea (Mega), suggest that the epithelial barrier function relies on common molecular components (Behr et al., 2003; Furuse and Tsukita, 2006; Wu et al., 2004). SJ components are important for junctional structure and show interdependent localisation (Behr et al., 2003; Lamb et al., 1998; Llimargas et al., 2004; Paul et al., 2003; Wu et al., 2004).
Identified SJ components are summarized in Table 1. Among these is the Lethal giant larvae/Discs large/Scribble (Lgl/Dlg/Scrib) complex (Fehon et al., 1994; Tepass et al., 2001), which has an early role in establishing apical/basal polarity by repressing apical identity along the basolateral surface (Bilder et al., 2003; Tanentzapf and Tepass, 2003), and a later function in SJ formation. Lgl is a myosin II binding protein possibly involved in exocytosis by interacting with SNARE proteins, whereas Dlg and Scrib are multi PDZ domain proteins (Bilder and Perrimon, 2000; Mechler et al., 1985; Strand et al., 1994; Woods and Bryant, 1991; Zhang et al., 2005a). Another SJ component is Neurexin IV (NrxIV), a member of the Caspr (Contactin associated

protein) family, which is present also in vertebrate paranodal SJ between myelin loops and axons at the mammalian blood brain barrier (Baumgartner et al., 1996). The transmembrane Nrx IV interacts with Coracle (Cora), a band4.1 homologue with ERM domains that links the complex to the cytoskeletal proteins Spectrin (Fehon et al., 1994).

Table I. Septate junction components

Protein	Function	Description	Reference
Fasciclin II	Homophilic adhesion	Immunoglobulin (Ig), Fibronectin III (FnIII) domains	(Grenningloh et al., 1991; Lin et al., 1994)
Fasciclin III	Homophilic adhesion	Ig domains	(Snow et al., 1989)
Lachesin	Homophilic adhesion	Ig domains	(Llimargas et al., 2004)
Megatrachea	Homophilic adhesion	Claudin-like	(Behr et al., 2003)
Sinuous	Homophilic adhesion	Claudin-like	(Wu et al., 2004)
Neurexin IV	Heterophilic adhesion	Caspr family	(Baumgartner et al., 1996)
Gliotactin	Heterophilic adhesion	Cholinesterase-like adhesion molecule	(Auld et al., 1995; Schulte et al., 2003)
Neuroglian	Heterophilic adhesion	Ig, FnIII domains	(Bieber et al., 1989)
Contactin	Heterophilic adhesion	Ig, FnIII domains	(Faivre-Sarrailh et al., 2004)
Na^+/K^+ ATPase	Ion pump	P-type ATPases	(Lebovitz et al., 1989)
Coracle	Linker	Band4.1 family	(Fehon et al., 1994)
Lethal giant larvae	Linker	Myosin II binding protein	(Strand et al., 1994)
Discs large	Linker	MAGUK protein	(Woods and Bryant, 1991)
Scribble	Linker	PDZ protein	(Bilder and Perrimon, 2000)

Tube size regulation

Epithelial tubes vary greatly in size, shape and cell number between organs and species. Many tubes are narrow when first formed and later expand to achieve mature dimensions, but the cellular mechanisms that shape tubes are not yet elucidated (Lubarsky and Krasnow, 2003). Combined results from studies in several different systems; cell culture systems, genetic model organisms and human pathological conditions, indicate a common requirement for apical membrane growth and secretion to increase the tubular lumen size (Lubarsky and Krasnow, 2003). Many known tube size mutations are defective in genes encoding apical membrane components or components associated with the subapical cytoskeleton, further emphasising the importance of the apical cell domain in tube expansion (Beitel and Krasnow, 2000; Buechner, 2002; Hemphala et al., 2003; Lubarsky and Krasnow, 2003).

Apical membrane growth and secretion

Tube formation requires the establishment of a luminal (apical) surface. In many tubular organs, this appears accommodated by *de novo* apical membrane biogenesis. For example, when Madin-Darby canine kidney (MDCK) cells, cultured in 3D-collagen to promote cyst formation, are activated with hepatocyte growth factor (HGF) a cellular chord is formed from the cyst. These cells re-establish their polarity and form apical membrane through coalescence of apical vesicles (O'Brien et al., 2002; Pollack et al., 1998). Similarly, lumen formation during vasculogenesis in zebrafish appears mediated by coalescence of intracellular vacuoles (Kamei et al., 2006). The mechanism of lumen formation in the nematode *Caenorhabditis elegans* excretory cell is not fully understood, but is also believed to involve fusion of apical membrane vesicles (Buechner, 2002). In other organs the initial tubular lumen surface is provided by the apical membrane of already polarized cells, but the subsequent lumen

expansion requires apical membrane growth as in the *Drosophila* salivary gland (Myat and Andrew, 2002). Thus, apical membrane growth is central for tube morphogenesis.

Once the lumen is formed a mechanism to separate the apical surfaces and keep the lumen open is required. Secretion of liquid into the lumen has been proposed to fill this need. In both the mammalian lung and *Drosophila* trachea the immature tubes are filled with liquid, which is cleared before the tubes become functional, and the fetal lung liquid is required to keep the lung distended and for correct growth and development (Hooper and Harding, 1995; Manning and Krasnow, 1993). In addition, temporary glycosylated material is observed in the lumens of developing vertebrate lung and kidney, and unidentified luminal content is seen in the excretory cell of *C. elegans* (Buechner, 2002; Gheri et al., 2000; Minuth and Rudolph, 1990). This indicates that the growing lumen is not simply filled with liquid drawn into the lumen, but that apical deposition of luminal components occurs during tube morphogenesis.

Subapical cytoskeleton and apical components

Mutants with defective tube size regulation have been identified in several different model systems. In *C. elegans* two tube size mutants with excessively expanded excretory cell tubes disrupt the genes *sma-1* and *exc-5* (Buechner, 2002). *sma-1* encodes β_H-spectrin, which links the apical cytoskeleton to the plasma membrane, whereas *exc-5* encodes an apically localised homolog of guanine nucleotide exchange factor (GEF) that regulates the actin cytoskeleton through the Rho family of GTPases (Buechner, 2002; Buechner et al., 1999). In addition, the *C. elegans* protein Erm-1, an ortholog of the ERM-family (ezrin-radixin-moesin) of cytoskeleton-membrane linkers, localises to the apical membrane and is required to prevent formation of cystic intestinal tubes (Gobel et al., 2004).

Cystic tubes are also associated with human kidney diseases, as in autosomal dominant polycystic kidney disease (ADPKD). ADPKD is one of the most common human genetic disorders, affecting 1 of

500-1000 individuals (Boletta and Germino, 2003), and is caused by mutations in either of the two genes polycystin-2 (PC2, encoding a cation channel) or polycystin-1 (PC1, stabilizes the PC2 channel). These malformations can lead to complete renal failure with a dependence on dialysis as a consequence for the patient. Both polycystins are located in the primary cilia positioned at the apical surface of renal epithelial cells (Tahvanainen et al., 2005). Together these results imply an important role for the subapical cytoskeleton and apical components in tube size regulation.

Tube size regulation of the Drosophila trachea

Tracheal tube dimensions are tightly regulated, as shown by the invariable branch size between embryos and the narrow time window by which each branch expands to attain its mature diameter. The lumen diameter of the main vessel, the DT, increases 3-5 folds between embryonic stage 14 and 16, a period of 3 hours (Fig 6; Beitel and Krasnow, 2000). During tube expansion, the apical surface increases and remains smooth, whereas the basal diameter remains largely unaltered and irregular in shape (Fig 6). This indicates that the apical surface size and shape is under strict developmental control.

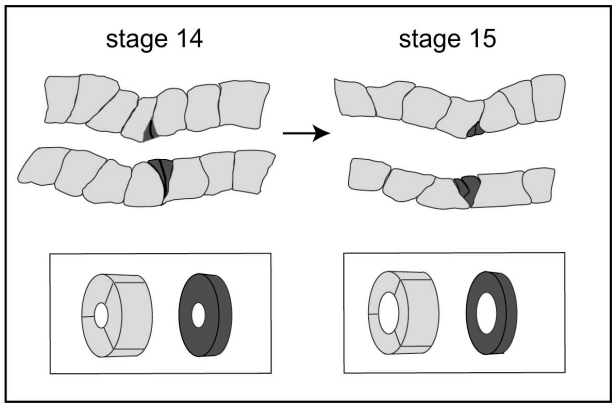

Fig 6. Cellular changes during diameter expansion of the DT. Expansion of the dorsal trunk commences at embryonic stage 14 and results in an increased lumen diameter and flattened tracheal cells. Expansion of the architectural distinct stalk cells (light grey) and fusion cells (dark grey) are coordinated so that an even diameter is maintained throughout expansion.

Tracheal tube expansion occurs without cell divisions, and manipulation of tracheal cell number has little effect on tube size: Tracheal tube diameters are not altered in *cyclinA* mutants, where the last tracheal cell division is prevented, yielding half the normal number of tracheal cells. Conversely, an additional round of tracheal cell division in *dacapo* mutants does not cause an increase in tracheal tube diameter (Beitel and Krasnow, 2000). Lumen expansion is however associated with apical secretion, since secretion of the unknown luminal antigen 2A12 specifically correlates with lumen diameter growth of the different branches. The narrow time windows for lumen diameter expansion also imply regulatory mechanisms that define both the onset and end of lumen growth.

Tracheal tube size mutants

Analysis of *Drosophila* mutants with correct tracheal patterning, but with altered tube dimensions, has begun to elucidate genetic programs for tracheal tube size control. One group of tracheal tube size mutants relate to apical membrane growth and include *ribbon (rib)* and *grainy head (grh)*. Rib is a transcription factor, and *rib* mutants fail to extend the tracheal lumens, as if apical membrane growth is inhibited (Shim et al., 2001). Grh is also a transcription factor, and appears to restrict apical membrane growth. In *grh* mutants, excessive apical membrane forms, resulting in elongated and convoluted tubes, whereas tracheal over expression of Grh prevents lumen extension as in *rib* mutants (Hemphala et al., 2003).

Another group of tube size mutants disrupt genes encoding septate junction (SJ) components ("SJ mutants"). These mutants develop dilated tubes at stage 15 and later, during stage 16, the tubes become too elongated and convoluted with irregular cell shapes. The first "SJ mutant" discovered to have a tracheal tube size phenotype was *atp□,* which lacks a functional □-subunit of the Na^+/K^+ ATPase (Hemphala et al., 2003). Subsequently, mutations in *cora, nrx, neuroglian (nrg), gliotactin* (*gli*) as well as in *nervana2* (*nrv2*), the □-subunit of the Na^+/K^+ ATPase, were shown to display a similar

tracheal tube size phenotype (Paul et al., 2003). Furthermore, loss of Lachesin (Bulbous) or either of the two claudins, Mega and Sinu, causes tracheal phenotypes characteristic for the "SJ mutants" (Behr et al., 2003; Llimargas et al., 2004; Wu et al., 2004). Although these results imply a central role for SJs in tracheal tube size control, the question how they perform this function remains unanswered. One explanation is that the impaired paracellular barrier in "SJ mutants" causes abnormal influx of substances, ions and water across the tube wall, thereby affecting the lumen environment. However, since "SJ mutants" with similar barrier defects cause different tube size defects, and their ultrastructural SJ composition does not correlate with their tube size defects, this explanation has not been favoured (Wu and Beitel, 2004).

Another possibility is that SJs control the extent of apical cell surface (Wu and Beitel, 2004). The SJ complex containing Scrib, Dlg and Lgl have an early function in restricting apical domain by antagonizing the Crb and aPKC complexes (Tanentzapf and Tepass, 2003). If Scrib/Dlg/Lgl continues to restrict apical domain later during embryogenesis, disrupted SJs may lead to an increased apical surface. In support of this, Dlg is mislocalized in several "SJ mutants" (*mega, sinu, nrv2, atp□, lac*) (Behr et al., 2003; Llimargas et al., 2004; Paul et al., 2003; Wu et al., 2004). Moreover, *scrib* mutants also develop the convoluted tracheal tubes seen in "SJ mutants" (Wu et al., 2004).

A third suggested mechanism for SJ function in tube growth is that they affect an unknown aECM, which the cells could sense and adjust their size and shape according to (Wu and Beitel, 2004). This is based on findings that several "SJ mutants" develop an abnormal cuticle with malformed tracheal taenidia and epidermal cuticle (Baumgartner et al., 1996; Wu et al., 2004), and that many "SJ mutants" have reduced luminal levels of the 2A12 antigen (Beitel and Krasnow, 2000; Llimargas et al., 2004).

Although the identified tracheal tube size mutants demonstrate important roles for apical cell surface growth and correctly formed

junctions in tube size regulation, the developmental mechanisms coordinating these events to control tube size and shape are not understood.

RESULTS AND DISCUSSION

The major finding in this thesis is that tracheal tube expansion relies on the synthesis of a luminal chitin filament to ensure uniform lumen diameter. We have dissected four genes required for formation of this filament (Paper I-III).

To gain further insight in the regulation of tracheal tube expansion, we screened additional genes expressed in trachea at the time of tube expansion, and found a new tube size mutant required for epithelial barrier function (Paper IV).

Paper I: A transient luminal chitinous matrix is required to model epithelial tube diameter in the *Drosophila* trachea.

krotzkopf verkehrt (kkv), which encodes Chitin synthase-1, is required for uniform tracheal tube expansion

The tracheal tube size mutant *grh* was first discovered in a screen for cuticle phenotypes in the 1980's (Jurgens et al., 1984; Nusslein-Volhard et al., 1984; Wieschaus et al., 1984). Its cuticle phenotype was similar to that of three other mutants, called *krotzkopf verkehrt* (*kkv*), *knickkopf* (*knk*) and *retroactive* (*rtv*). We therefore analysed the tracheal phenotypes of embryos mutant for *kkv* (Paper I), *knk* and *rtv* (Paper II).

The *kkv* mutation gave rise to tubular constrictions and dilations due to failure to expand the fusion branches and to restrict excessive dilations of other parts of the tube. At later stages these embryos also developed excessively elongated tubes. While we analysed this mutant, Ostrowski and colleagues found that the gene disrupted in *kkv* mutants encodes Chitin synthase-1 (CS-1, Ostrowski et al., 2002). Chitin synthases are large (approximately 180 kDa) proteins with 15-18 transmembrane regions that produce chitin, the second-most

abundant polysaccharide in nature, found in fungi, arthropods and nematodes. Chitin consists of N-acetyl-D-glucosamine (GlcNAc) residues linked to each other by β-(1-4) glycosidic bonds (Cohen, 2001; Merzendorfer, 2006). It is suggested that CS units are transported in an inactive form to the plasma membrane, where they become activated by proteases (Cabib and Farkas, 1971; Merzendorfer, 2006). Chitin chains are then synthesized from cytosolic UDP-GlcNAc residues, linked together at the intracellular catalytic site. The transmembrane domains of the CS are suggested to form a pore through which the polysaccharide chains can be extruded into the extracellular space (Merzendorfer, 2006). Alternatively, chitin synthesis may start in vesicles, so called chitosomes, which fuse with the plasma membrane when chitin-release is required (Merzendorfer and Zimoch, 2003).

Inhibition of chitin synthase activity reproduces the kkv phenotype

Insect chitin has been known for its function in the cuticle, an apical extra cellular matrix that is secreted by epidermal and tracheal cells at the end of embryogenesis after tube expansion. Thus, it was surprising to find a requirement for CS-1 during mid-embryogenesis. We first analysed if the *kkv* tube expansion phenotype actually depended on the chitin synthase function of the CS-1 protein or if this large protein could have another function. This was done by inhibiting the synthase activity of CS-1 with the substrate analogue Nikkomycin Z, which acts as a competitive inhibitor (Cabib, 1991). Indeed, all aspects of both the cuticle and tracheal phenotypes of *kkv* mutants were reproduced, suggesting that chitin synthesis is a prerequisite for correct tube expansion.

Ectopic tracheal expression of a Chitinase reproduces the kkv phenotype

In order to confirm that extra cellular chitin is required for tracheal tube expansion, rather than some other components that could be co-

secreted with chitin, we over-expressed a chitinase in the trachea. Chitinases are secreted enzymes that degrade chitin chains by hydrolysing the β-1,4-glycosidic bond between the GlcNAc residues in chitin (Jollès P., 1999). Chitinases and proteases are found in molting fluid secreted by epidermal cells before molting, breaking down the components of the old cuticle (Jollès P., 1999). Upon tracheal expression of Chitinase-2 (Cht2) the tracheal phenotype of *kkv* mutants was reproduced, showing that luminal chitin is necessary for uniform tube expansion.

A non-cuticle related function of chitin in tube expansion

To test whether the tube expansion defect in chitin-deficient embryos depended on an unexpected early function of the cuticle, we analysed cuticle deposition in both wild type and *kkv* mutant embryos by transmission electron microscopy (TEM) after tube expansion, at stage 16.1. At this stage the envelope is being deposited, appearing as discontinuous flakes, which implies that production of the innermost chitin-containing procuticle has not yet commenced (Locke, 2001; Moussian et al., 2005a). Thus, a non-cuticle related chitin-structure functions in tracheal tube expansion.

A transient luminal chitin filament is required for uniform tube expansion

Our results indicated a new developmental function for chitin in tracheal tube expansion. In order to visualize chitin in the developing trachea, we used Congo Red, a fluorescent dye that intercalates between chitin chains, Wheat germ agglutinin (WGA) that recognizes GlcNAc residues and a fluorescent conjugated chitin binding probe (CBP) (Cohen, 1993; Peters and Latka, 1986; Zhang et al., 2005b). All three reagents detected a filament residing in the tracheal lumen from stage 14, when tube expansion begins. During tube expansion the filament also increases in size, but do not fill the lumen completely. In *kkv* mutants the tracheal chitin filament, as well as the cuticular chitin,

is absent. Following tube expansion, the chitin filament disappears whereas cuticular chitin is detected. Luminal chitin degradation is likely to be mediated by chitinases, and interestingly, one of the *Drosophila* chitinases is endogenously expressed in the developing trachea (BDGP).

The luminal chitin filament has a fibrous appearance, which is likely to reflect chitin microfibrils. It is believed that CS units are clustered in the membrane to facilitate the formation of microfibrils of around 20 nascent chitin polymers. These microfibrils are approximately 3 nm in diameter and are held together by hydrogen bonds between the amine and carbonyl groups in the chitin chains (Cohen, 2001; Merzendorfer and Zimoch, 2003). Chitin microfibrils can contain either anti-parallel chitin-chains as in the α-form, parallel chitin-chains as in the β-form or a combination of two parallel and one anti-parallel strand, as in the γ-form. The different arrangements result in different physical properties of the microfibrils. The α-form has closely packed fibrils, which are stabilized by many hydrogen bonds to give a strong and stabile structure ideal for cuticles. In contrast, the β- and γ-forms contain fewer hydrogen bonds between fibrils and, instead, increased number of hydrogen bonds with water to yield a soft and elastic structure, which is found in the peritrophic matrix (Merzendorfer and Zimoch, 2003). We do not yet know which form the tracheal chitin filament adopts. A soft and elastic chitin-matrix with the ability to bind water and swell upon increase in liquid volume could push the lumen diameter in a uniform manner, whereas a more rigid chitin-form could act to stabilise a luminal matrix that is responsible for uniform lumen diameter expansion.

Chitin polymers can be chemically modified by enzymes and thereby alter its chemical properties. Chitin is characterized by low solubility and high density, properties that can be altered by Chitin deacetylases which convert chitin into Chitosan, a more soluble molecule with lower density than chitin (Cho et al., 2000; Wenling et al., 2005). Interestingly, recent studies have shown that the two tracheal genes *serpentine (serp)* and *vermiform (verm)*, encoding

proteins with chitin binding and deacetylation domains, are required to limit tube elongation (Luschnig et al., 2006; Wang et al., 2006). Serp and Verm are also necessary for correct chitin filament structure and are suggested to bind and modify chitin into a more rigid matrix restricting tube elongation (Luschnig et al., 2006; Wang et al., 2006).

The chitin filament affects the subapical cytoskeleton

At the cellular level, we showed that loss of chitin does not affect epithelial polarity or microtubule orientation. However, the organisation of the subapical cytoskeleton is impaired upon loss of chitin, as shown by irregular and weakened apical staining with an antibody against β_H-spectrin in *kkv* mutants. Thus, the chitin filament may regulate tracheal tube size and shape during tube expansion by organizing the subapical cytoskeleton. However, the mechanism by which the chitin filament is sensed by the surrounding cells is not known. Interestingly, the apical vertebrate Polycystin-1 contains a C-type lectin domain and a G-protein-coupled receptor proteolytic site, suggesting a possible carbohydrate interaction and function as an atypical GPCR (Boletta and Germino, 2003). Similar *Drosophila* proteins, with carbohydrate interaction motifs as well as signalling capacity, would be candidates for mediating an interaction between the chitin filament and tracheal cells. Alternatively, chitin may indirectly influence the tubular epithelium.

Septate junction components are required for correct chitin filament assembly

The organisation and function of SJs seemed unaffected in *kkv* mutant embryos. However, we found that the luminal chitin filament is disorganised in “SJ mutants”, generating a wide and amorphous chitin filament. Genetic interaction experiments showed an additive loss of the luminal antigen 2A12 in double mutants for *kkv* and SJ components, suggesting that SJs may be necessary for deposition of additional luminal components apart from chitin.

Do other tubular systems rely on intraluminal matrices to coordinate tube growth?

The use of an intraluminal chitin filament to shape tracheal tubes is a completely new concept in epithelial tube morphogenesis. It is therefore intriguing to speculate if a similar mechanism for tube size control applies to development of other tubular organs.

The vertebrate counterpart to chitin is hyaluronan, an unbranched polysaccharide made up by repeating disaccharide units of glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) (Toole, 2004). Hyaluronan is synthesized in a similar fashion to chitin, by large transmembrane enzymes (Hyaluronan synthases) that form pores in the membrane through which the polysaccharide can be transported to the extra cellular space (Weigel et al., 1997). Hyaluronan can influence cell behaviour, such as migration and proliferation, during morphogenesis by binding to cell-surface receptors to induce signal transduction or by immobilizing signalling proteins in the hyaluronan matrix (Toole, 2004; Turley et al., 2002). Small fragments or oligosaccharides of hylauronan stimulate proliferation, motility and tube formation of endothelial cells and promote angiogenesis in several different experimental models (Lees et al., 1995; Montesano et al., 1996; Rahmanian and Heldin, 2002; Rahmanian et al., 1997; Slevin et al., 2002). Recent data suggest that the cell surface hyaluronan receptor CD44 is involved in *in vivo* angiogenesis (Cao et al., 2006; Lesley et al., 2000). Vascularization of implants and tumours as well as angiogenesis during wound healing was inhibited in CD44-/- mice. Moreover, the newly formed vessels in CD44-/- mice had an irregular luminal surface similar to that seen in chitin-deficient trachea (Cao et al., 2006). However, the question whether a counterpart to the chitin filament is used in vertebrate epithelial tube morphogenesis has yet to be addressed.

Paper II: Drosophila Knickkopf and Retroactive are needed for epithelial tube growth and cuticle differentiation through their specific requirement for chitin filament organization.

Knickkopf and Retroactive are required for tracheal tube expansion and act in the chitin-dependent pathway

Mutants for *knickkopf* (*knk*) and *retroactive* (*rtv*), which were identified in a screen for cuticle phenotype together with *kkv*, display tracheal phenotypes reminiscent to that of *kkv* mutants. By inhibiting chitin synthesis in these mutants, we could place both *knk* and *rtv* in the chitin-dependent pathway of tube size control. Furthermore, the chitin filament in *knk* and *rtv* mutants lacks a fibrous appearance and fills the entire tracheal lumen. Thus, Knk and Rtv are required for correct organisation of the chitin filament, and thereby tracheal tube expansion.

Furthermore, partial inhibition of chitin synthesis by feeding flies with a low dose of Nikkomycin gives rise to a similar disorganized filament structure as in *knk* and *rtv* mutants. This indicates that a certain amount of chitin is required to produce a correctly organized filament. This may reflect the importance of clustering Chitin synthase entities to acquire chitin microfibril association.

Rtv and Knk are necessary for chitin fibril organization in the cuticle

Rtv has been shown to be necessary for organisation of chitinous lamellae in the procuticle (Moussian et al., 2005b), and we found that Knk has a similar function. In addition, we could show that chitin is present in the *knk* and *rtv* mutant procuticle, indicating a specific role in fibril organisation for these proteins. Rtv is a transmembrane protein with chitin-binding properties, whereas Knk is a novel protein with unknown function. Knk is predicted to be linked to the plasma membrane through a GPI-anchor (glycosylphosphatidyl-inositol-anchor), and by using Phospholipase C, which cleaves GPI-linked

proteins, we could show that Knk indeed is a GPI-linked protein. However, endogenous cleavage of Knk is not necessary for function since a Knk protein in which a transmembrane region replaces the GPI-linkage domain could rescue the *knk* mutant phenotype. Moreover, antisera against Knk revealed its localisation to the apical and apico-lateral domain in tracheal and epidermal cells. Taken together, these results suggest that Knk acts at the apical membrane, and is not part of the chitin matrix itself.

Septate junctions are required for correct localisation of Knk

Genetic interaction experiments with "SJ mutants" and *knk* showed an additive loss of luminal 2A12 antigen levels, similar to that seen in double mutants for *kkv* and SJ components. Furthermore, we found that Knk was mislocalised along the lateral and basal surfaces in "SJ mutants" tracheal cells, suggesting that SJs affect chitin filament formation by promoting polarized protein localisation. However, since over-expression of Knk in "SJ mutants" did not rescue their tracheal phenotype or the chitin filament structure, SJs must have additional roles in tube expansion. Indeed, SJs were recently found to be required for apical secretion of Vermiform, which is required for luminal chitin filament formation.

Composition of the tracheal luminal matrix

In the exoskeletal cuticle, chitin is always associated with proteins, which determines the mechanical properties of the cuticle, such as its elasticity (Merzendorfer and Zimoch). The chitin fibrils are embedded into the protein matrix similar to the way steel bars are used to reinforce concrete.

There are two classes of chitin-binding proteins in *Drosophila*, one class with the chitin-binding domains (CBD) found in lectins, chitinases and peritrophic membranes (cystein-containing CBD), and another class with the type of chitin-binding domains found in many cuticular proteins (R&R consensus, non-cystein containing CBD)

(Rebers and Willis, 2001). There are 15 *Drosophila* proteins registered in SwissProt carrying the R&R consensus sequence, all of them suggested to be structural constituents of the cuticle (Flybase). There are 74 genes coding for proteins with cystein-containing chitin binding domains (SMART database) and 12 of these are analysed by in situ hybridisation (BDGP, Luschnig et al., 2006; Wang et al., 2006). Six of these are expressed in the developing trachea, three in the gut, two in the late epidermis and trachea (probably cuticle specific) and one in tracheal imaginal precursor cells (BDGP, S. Pinnock, unpublished). Future studies will show if some of these chitin-binding proteins are part of the luminal chitin matrix.

Paper III: Hormonal regulation of *mummy* is needed for apical extracellular matrix formation and epithelial morphogenesis in *Drosophila*.

Mummy is a new tube size mutant

In Paper III we identify a new tube size mutant, *mummy* (*mmy*), which develops severe tracheal constrictions and dilations during tube expansion. By using CBP and WGA to visualize chitin and glycosylated residues we noted a complete lack of chitin in the *mmy* mutant tracheal lumen, as well as loss of apical WGA-staining, which probably represents glycosylated residues. Staining with WGA also revealed a decrease in glycosylated residues in non-chitin-producing tissues, such as the Malphigian tubules, early epidermis and salivary glands. However, the protein filament Piopio required for intercalation of type II branches is present in *mmy* mutants, indicating that Mmy is not required for general apical secretion, but instead is required for generation of GlcNAc residues.

We also found that organization of the *mmy* mutant exoskeletal cuticle was severely affected, as all sublayers were thinner than normal, the integrity of the epicuticle was perturbed and the procuticle lacked chitin. Moreover, the SJs in *mmy* mutants were perturbed, since the SJ component Fas3 was mislocalised along the lateral membrane, and the ladder-like SJ structure was diminished, as analyzed by TEM.

Mummy encodes a UDP-GlcNAc-pyrophosphorylase

By genomically mapping the *mmy* mutation, we found that *mmy* encodes the *Drosophila* UDP-GlcNAc-pyrophosphorylase. Mmy therefore catalyses the conversion of UTP and GlcNAc-1-phosphate to UDP-GlcNAc, which is required for chitin synthesis. Since UDP-GlcNAc also is required for protein glycosylation and production of GPI-anchors, we analysed the glycosylated and GPI-linked protein Knk in *mmy* mutants. Mmy was shown to be required for correct

glycosylation of Knk as well as of the plasma-membrane protein, Tout-velu, but not the intracellular component Syntaxin1A. Furthermore, the Mmy-dependent post-translational modifications of Knk are essential for proper localisation of Knk at the plasma membrane. The relatively late onset of the *mmy* mutant phenotype at stage 13/14 is probably due to maternally contributed Mmy product.

Mmy expression is regulated by the molting hormone ecdysone

Although Mmy is a metabolic enzyme required for general protein modification its expression pattern was surprisingly tissue-specific, as it was detected at high levels in epithelial organs such as the trachea, epidermis and salivary gland. For example, *mmy* is specifically upregulated in the trachea from the placode stage until tube expansion is completed, which correlates with luminal chitin filament production.

mmy has previously been characterised as a Halloween mutant, which develop reduced and hardly visible cuticles (Jurgens et al., 1984; Nusslein-Volhard et al., 1984; Wieschaus et al., 1984). Many Halloween-mutants encode enzymes needed for production of the steroid hormone ecdysone. Ecdysone initiates molting, the process by which epidermal cells produce a new and larger exoskeleton and the old cuticle is broken down (ecdysis). This larval ecdysone is biosynthesized in the prothoracic gland from dietary sterols, since insects are unable to synthesize the steroid nucleus, and peaks just before each molt. During molting ecdysone is suggested to have a role in regulating the cyclic turnover of CS units, which are clustered at so called plaques at the tip of microvilli of epidermal cells during cuticle formation (Locke and Huie, 1979). Analysis of CS expression during *Drosophila* metamorphosis shows that downregulation of CS expression correlates with high ecdysone levels (Gagou et al., 2002) and ecdysteroid responsive elements (EcREs) have been identfied in the upstream regions of the CS genes (Tellam et al., 2000).

There is also an ecdysone peak at mid-embryogenesis (Richards, 1981), with unknown function. This ecdysone is produced

in the amnioserosa, since the prothoracic gland is not yet developed, and is required for germ-band retraction, dorsal closure and head involution, as well as cuticle production (Kozlova and Thummel, 2003). Since *mmy* is not required for ecdysone synthesis we investigated if *mmy* expression could be a down-stream effector of the embryonic ecdysone peak (Chavez et al., 2000).

Interestingly, in mutants for two enzymes needed for ecdysone production, *shade (shd)* and *shadow (sad)*, we observed a lack of upregulated *mmy* expression in trachea and a premature upregulation in epidermis, salivary gland and proventriculus. Thus, the mid-embryonic ecdysone-peak is necessary for regulating *mmy* expression in epithelial tissues. Ecdysone may therefore be of importance in tube size regulation in terms of regulating chitin-production and protein modifications through regulation of *mmy*.

Ecdysone is exerting its actions mainly through binding to a heterodimer of the Ecdysone receptor (EcR) and Ultraspiracle (USP), both members of the nuclear receptor superfamily that are sequence-specific transcription factors (Hall and Thummel, 1998; Thomas et al., 1993; Yao et al., 1993). Since this complex is regulating the expression levels of numerous target genes, ecdysone might be a possible coordinator of several processes involved in tracheal tube size regulation.

Paper IV: The novel protein Humongous is required for tubular epithelial barrier function in *Drosophila*.

The novel tube size mutant hmn displays a tracheal phenotype reminiscent to that of septate junction mutants

In Paper IV we identified another new tube size mutant, *humongous* (*hmn*), which develops overgrown and convoluted tracheal tubes, and investigated its functional relationship with that of previously identified tube expansion mutants. Upon inhibition of chitin synthesis in *hmn* mutants, we observe an additive loss of luminal 2A12 as seen in chitin deficient "SJ mutants". Furthermore, the fibrous structure of the luminal tracheal chitin filament had an amorphous appearance in *hmn* mutants similar to that seen in "SJ mutants", as well as in mutants for *knk* and *rtv*. In addition, we observed mislocalisation of the GPI-linked protein Knk along the lateral and basal surfaces and that the chitin-binding proteins Serpentine and Vermiform failed to accumulate in the lumen of *hmn* mutants, analogous to what occurs in "SJ mutants". Thus, *hmn* mutants display a tracheal phenotype reminiscent to "SJ mutants", rather than to *kkv*, *knk* and *rtv*.

Hmn is required for a functional paracellular diffusion barrier and correct septate junction organization

The phenotypic similarities between *hmn* and "SJ mutants" prompted us to investigate the functionality of the paracellular barrier in *hmn* mutants. Indeed, a 10 kDa dextran dye injected into the body cavity of *hmn* mutant embryos could rapidly pass between the epithelial cells in both trachea and salivary gland, demonstrating that Hmn is required to prevent transepithelial diffusion. Ultrastructural analysis of SJs in *hmn* mutants by TEM, further revealed that the ladder-like septa are less electron-dense and sparser than in the wild type. Moreover, several SJ components were reduced in levels and mislocalized along the lateral membranes in the *hmn* mutant tracheal and hindgut epithelia. Thus, Hmn is required for the organisation of SJs, as well as their function

as a paracellular barrier. The observation that embryos mutant for both *hmn* and the SJ component *atp□* show no additive phenotype, further suggests that the tracheal tube size phenotype in *hmn* mutants is related to its dysfunctional SJs.

Hmn is a predicted GPI-linked protein with structure homology to cell surface receptors

We confirmed that the *hmn* phenotype arises form disruption of the gene CG17218. This gene encodes a small protein of 151 amino acids with a predicted N-terminal signal sequence and a short C-terminal motif for GPI-anchoring to the plasma membrane. Interestingly, Hmn has ten conserved cystein residues in the 100 amino acid processed protein, and is predicted to form a 3D-structure similar to that seen in cell surface receptors, such as uPAR, CD59 and BMP receptor type IA. By sequence similarity searches we also identified nine additional small, secreted *Drosophila* proteins with similar pattern of ten conserved cystein residues, six of which are predicted to be GPI-linked. Thus, Hmn belongs to a new family of small, secreted proteins with a 3D structure found in several cell surface receptors.

GPI-linked proteins often localize to the apical cell surface in polarized cells (Schuck and Simons 2006). To investigate the subcellular localisation of Hmn we generated both an anti-Hmn peptide antibody, and transgenic flies that carry a GFP-Hmn fusion construct. However, Hmn localization (detected with anti-Hmn antisera) and GFP-Hmn localization (detected with both anti-Hmn and anti-GFP antisera) was predominantly around the cell nuclei and at the apical cell domain, but not at the SJs. These results can be interpreted in two ways. Hmn may be localized to the apical cell domain and since GPI-linked proteins can be actively recycled from the plasma membrane through receptor-mediated endocytosis, these results could reflect that Hmn is an apical protein that is actively involved in receptor-mediated endocytosis in a novel process that regulates SJs (Shin and Abraham, 2001). Alternatively, the results simply reflect

that the Hmn-antisera only recognized an unfolded state of Hmn in the ER and that the GFP-Hmn fusion protein is unable to fold correctly and therefore also is detected in the ER. Since these issues are not resolved yet, these data are omitted in Paper 4.

Speculations around the role of hmn and septate junctions in tube size control

How may the laterally located SJ complex play a role in the regulation of tracheal tube size? Our findings that intact SJs are required for proper chitin filament organisation and for correct localisation of Knk indicate that one role for SJs in tube expansion is to ensure formation of a functional chitin filament.

The observed mislocalisation of Knk in "SJ mutants" could be due to defects in polarized Knk delivery to the apical membrane. Although this hypothesis was strengthened by studies in vertebrate cell cultures, which showed that GPI-linked proteins are delivered to the apical membrane through an indirect route via the lateral membrane, where the TJs and exocyst complexes are located (Polishchuk et al., 2004), more recent studies show that in fully polarized cells GPI-linked proteins follow a direct route to the apical surface (Schuck and Simons, 2006). In relation to these studies, one might thus question whether the developing trachea is a fully polarised epithelium, since for example maturation of the paracellular barrier is not completed until stage 16. In that case one could envision an analogous indirect route for GPI-linked proteins also in the tracheal epithelium, and that SJs are needed to organize the lateral membrane domain where such protein-sorting could take place.

Another indication that SJ might be involved in apical cell secretion comes from recent studies of the luminal chitin deacetylase Verm. Verm, which normally is a secreted protein, is predominantly found inside the tracheal cells in "SJ mutants" (Wang et al., 2006), suggesting that SJ are required for secretion of a subset of luminal components. The Knk mislocalization in "SJ mutants" could however

be explained also by a fence function of SJs, where SJs normally prevent apical Knk from diffusing to basolateral membrane domains.

SUMMARY AND SIGNIFICANCE

The strength of *Drosophila* genetics has enabled us to identify mutations that cause defects in tube shape and integrity, and to subsequently characterize the targeted gene function. By this unprejudiced approach we have discovered an unexpected concept to ensure uniform tubes of correct size: Newly formed tracheal tubes deposit a temporary intraluminal chitin-based matrix that models the lumen during tube growth. We hypothesize that the chitin filament provides the core of a luminal scaffold that attaches to the apical epithelial surface, thereby promoting equal structuring of the apical cell domains and uniform diameter.

Paper I

- A transient luminal chitin filament is required for uniform tracheal tube expansion and affects the tracheal subapical cytoskeleton.
- Previously identified tube expansion mutants encoding septate junction components are required for correct organisation of the chitin filament.

Paper II

- The apical, GPI-linked protein Knickkopf and the transmembrane protein Retroactive are required for correct organisation of the chitin filament.
- Polarized apical localisation of Knickkopf requires intact septate junctions.

Paper III

- A new tube size mutant, *mummy,* develop severe diameter constrictions and dilations and lack luminal chitin.

- Mummy is a *Drosophila* GlcNAc-Pyrophosphorylase that catalyses the formation of GlcNAc residues required for chitin synthesis, protein glycosylation and GPI-anchor synthesis.
- Expression of *mummy* is regulated by the molting hormone ecdysone, which is present in high levels at the time of tube expansion, suggesting that ecdysone may be a coordinator of chitin synthesis during embryogenesis.

Paper IV

- A new tube size mutant, *humongous*, develops overgrown tracheal tubes.
- Humongous is a novel protein that is predicted to be GPI-linked.
- Humongous is required for epithelial paracellular barrier function and organisation of septate junctions.

Previous studies of epithelial morphogenesis have mainly focused on signals deriving from the basal environment or from neighboring cells by intercellular communications. Our studies on a role for apical extracellular components in providing cues necessary to organize tube morphogenesis thus represent a new biological field. As coordinated changes in cell shape and movements are fundamental to epithelial organ formation, and morphogenesis of tubular networks sets exceptionally high demands on the regulation of these processes, the proposed studies are likely to also reveal apical molecular functions that shape epithelia in diverse organs. Identification of such components in *Drosophila*, which shares two thirds of its genes with humans, should enable the recognition of analogous mechanisms in vertebrates (St Johnston, 2002).

Medical relevance

Pathological conditions like polycystic kidney disease and the abnormal blood vessels caused by ischemic, tumour and chronic

inflammatory diseases have been closely linked to aberrant tube size and barrier function (Nagy and Senger, 2006). Strategies for regeneration or interference with such dysfunctional tubes are wanted, but the cellular and molecular mechanisms that control tube size and integrity are still poorly understood. Our studies of tubulogenesis may thus have impact on the development of treatments for human damaged tubular organs.

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