ABSTRACT

In nature, epithelial tubes are vital structures in organ design and are required for transport of gases and liquids in organs, such as the vascular system, the vertebrate lung and the kidneys. The tubular epithelium is single layered, but is often reinforced by layers of muscular support. It constitutes an apical side facing the lumen and a basal side that contacts surrounding tissues. To ensure optimal flow, it is critical that the tubes are correctly sized and shaped. Epithelial tube growth depends on apical membrane enlargements, as well as sub-apical rearrangements, but the mechanisms involved in the regulation of size and shape of epithelial tubes are yet to be revealed.

In this thesis the *Drosophila* respiratory (tracheal) system has been used as a model organ to identify essential genes and clarify the mechanisms involved in the making and shaping of tubes. Through genetic and molecular analyses, new biological concepts have been uncovered. The main tracheal tube, the dorsal trunk (DT), expands three-fold in diameter during a short interval followed by tube elongation. In this thesis we have dissected the roles of five genes in tube regulation, called *kkv*, *knk rtv*, *dBest2* and *DAAM*. Analysis of *kkv*, *knk* and *rtv* led us to identify an unprecedented need for luminal matrix components in modeling tube shape. A chitinous luminal matrix is deposited in newly formed tubes and constitutes an expanding cord inside the tube that is required for uniform tube diameter growth. *kkv* is required for chitin synthesis while *knk* and *rtv* are needed for chitin filament assembly. If chitin is missing or fail to form an organized matrix, the expanding tubes develop severe local dilations and constrictions.

The subsequent tube elongation requires dBest2 and DAAM. *dBest2* encodes an apical chloride channel and is essential for lumen growth during elongation, suggesting that elongation is driven by an increased luminal osmotic pressure. DAAM has a function in actin organization. In the wild type trachea, actin filaments arrange as sub-apical rings perpendicular to tube length, thus allowing for lumen elongation, but not diametrical expansion, upon the increase in lumen pressure. In *DAAM* mutants, the actin rings are disorganized, thus lumen elongation is inhibited. The luminal chitin matrix has a second role at this stage by preventing excess tube elongation. A balance between combinatorial physical forces exerted by the lumen and sub-apical actin cytoskeleton determines final tube size.

Key words: *Drosophila*, trachea, tubulogenesis, chitin, luminal matrix, tube shape, chloride channel, and sub-apical actin.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Biologiska rör är nödvändiga för att vi ska kunna leva. Man hittar dem i olika organ som t ex i vårt blodkärlsystem för transport av syre och andra blodgaser, eller i njurar för avlägsnande av kroppens slaggprodukter. För ett optimalt flöde måste rören ha korrekt storlek och form, samt förgrena sig för att bilda ett nätverk. De största rören, som aortan, transporterar stora volymer ut till kroppens alla organ, medan de allra minsta rören utbyter syre och koldioxid på molekylär nivå med enskilda celler. De grundläggande molekylära mekanismer som styr nätverkens förgreningsmönster blir nu alltmer kända och verkar kunna appliceras på skilda organ och djurarter. Däremot vet man fortfarande väldigt lite om hur ett rörs storlek och form regleras. En av de vanligaste genetiskt nedärvda sjukdomar som finns är Polycystisk njursjukdom. Små cystor finns redan i det lilla barnets njurar och förstoras längre fram i livet. Livslång dialys eller njurtransplantation är de behandlingar som idag finns att tillgå.

Jag har använt mig av bananflugans andningsorgan, trakéerna, för en ökad förståelse av hur ett rörs storlek och form regleras på molekylär och cellulär nivå. Trakéerna kan jämföras med människans vaskulära system, då de bildar ett förgrenat nätverk som har till uppgift att transportera syre till flugans alla inre organ. Jag har med hjälp av genetiska och molekylära metoder analyserat fem nya gener som är nödvändiga för att bilda rör med rätt diameter och längd i bananflugans embryo. Tre av dessa rör-gener behövs för att producera ett repliknande kitin-matrix som fyller rörens hålutrymme (lumen) under dess bildning. Kitin-matrixet fungerar som en mall för rörets form och om det saknas bildas rör med ojämn och cystliknade diameter, och som mot slutet av embryoutvecklingen blir alldeles för långa. Vidare har vi studerat två gener som visat sig vara essentiella mot slutet av embryogenesen, då röret tillväxer i längd. Den ena genen kodar för en kloridkanal som är lokaliserad till rörets insida och om den saknas blir rören för korta. Vi tror att kloridkanalen behövs för att skapa ett tryck som pressar ut rören. Den andra genen behövs också för att rören skall växa på längden. Den ser nämligen till att arrangera stabila ringar av aktin runt rörets lumen, och vid ett ökat tryck tillåter aktin-ringarna en längdtillväxt. Det intraluminala kitin-matrixet fungerar nu genom att hålla tillbaka och begränsa rörets längdtillväxt.

Det är en ny biologisk princip att det behövs ett formgivande matrix inne i lumen vid rörbildning samt ett nytt fynd att balanserande krafter reglerar rörets slutgiltiga storlek. Då människans gener till två tredjedelar överensstämmer med bananflugans gener, är förhoppningen att dessa upptäckter även får en relevant medicinsk betydelse i framtiden.

PAPERS AND MAUSCRIPT

This thesis is based on the following papers, which will be referred to in the text by their roman numbers (I-III):

I: **Tonning A*, Hemphälä J*, Tång E, Nanmark 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
* These authors contributed equally to this work

II: **Tång E*, Moussian B*, Tonning A, Helms S, Schwartz 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 organisation.
Development 133(1): 163-171, 2006
* These authors contributed equally to this work

III: **Tång E, Chavoshi TM, Uv A**
Balancing physical forces regulate tube size and shape in the *Drosophila* trachea.
Manuscript

TABLE OF CONTENTS

ABBREVIATIONS

AEL	after egg lay
AP	Anterior-Posterior
Arm	Armadillo
AJ	adherens junctions
Baz	Bazooka
BDGP	Berkley Drosophila genome project
Bnl	Branchless
Btl	Breathless
Caspr	Contactin associated protein
CBP	Chitin binding protein
Cor	Coracle
Crb	Crumbs
CR	Congo red
CS-1	Chitin synthase 1
DaPKC	*Drosophila* atypical Protein Kinase C
dBest2	*drosophila* Bestrophin 2
Dfr	Drifter
Dlg	Discs large
DOF/Stumps	Downstream-of-FGF-receptor
DmPAR-6	*Drosophila* Partitioning-defective protein-6
Dpp	decapentaplegic
DT	dorsal trunk
DV	Dorsal-Ventral
Ed	Echinoid
EGF	epidermal growth factor
ERM	Ezrin, Radixin and Moesin
Fas II	Fasciclin II
FasIII	Fasciclin III
FGF	Fibroblast growth factor
GB	Ganglionic branch
GlcNac	N-acetylglucoseamine
Grh	Grainy head
Hkb	Huckbein
Ig	immunoglobulin
IOP	intra ocular pressure
JNK	Jun N-terminal kinase
kkv	*krotzkopf verkert*
Klar	Klarsicht
Knk	Knickkopf
Lac	Lachesin
Lgl	Leathal giant larvae
LT	Lateral trunk
MAGUK	membrane-associated guanylate kinase

Mega	Megatrachea
MZ	Marginal zone
Nrv2	Nervana 2
Nrx-IV	Neurexin-IV
PKD	Polycystic kidney disease
PC-1	Polycystin-1
PC-2	Polycystin-2
Pyd	Polychaetoid
rho	*rhomboid*
Rtv	Retroactive
Scrib	Scribble
Serp	Serpentine
shg	*shotgun*
Sinu	Sinous
SJ	Septate junctions
SRF	serum response factor
Std	Stardust
stra	*straight*
TC	Transverse connective
TGF-β	transforming growth factor-β
TJ	Tight junctions
Tnc	Tencectin
Trh	Trachealess
VB	Visceral branch
Verm	Vermiform
Vvl	Ventral veins lacking
wt	wild-type
ZA	Zonula adherens

INTRODUCTION

The organization of cells into an epithelium brings about the most basic structures in metazoans. The cells arrange themselves side by side and contact each other by intercellular junctions to form epithelial sheets, which divide the organism into separate functional compartments. Some sheets cover the outside of the animal, while others line internal organs. The latter often shape themselves into tubes, a fundamental unit in organ design that is crucial to the life of metazoan organisms. Such tubes need to be of correct diameter and shape to ensure a dynamic flow of gases and liquids and fulfil organ functions. Human conditions associated with malformed tubes can result from expansions of epithelial tubes, such as in polycystic kidney disease (PKD) or blood vessel aneurysms, or stenotic tubes that cause obstructions of blood vessels and other organs (Boletta and Germino, 2003; Lubarsky and Krasnow, 2003). In nature there is a great diversity in epithelial tube diameter, both between different species and within the same organism. Tubes with a large lumen diameter contain several cells in a circumferential cross-section. Such tubes are used for bulk transport of e.g. gases and liquids. Small epithelial tubes are usually made by only one cell, wrapped up around its own axis and sealed by an auto-cellular junction, while tubes with the smallest diameter are formed by junctionless hollowed cells with finger like branch protrusions to provide exchange on a molecular level with surrounding target tissues (Lubarsky and Krasnow, 2003). Although biological tubes are made up by a monolayer of epithelial cells, many tubes need structural support such as layers of muscular cells.

Tubular epithelia, like epithelial sheets, exhibit an apicobasal polarity to achieve correct structure and to form transepithelial barriers. Polarization is accomplished by linking asymmetrically distributed intercellular junctions to the cytoskeleton of individual cells (Gibson and Perrimon, 2003). In tubular epithelia the apical cell surface faces the lumen and the basal surface is exposed to surrounding tissues.

How are tubes formed? Tubes are formed either from an already polarized epithelium (by wrapping or budding) or from clusters of cells that polarize during the process of tube formation (by hollowing or cavitation) (Lubarsky and Krasnow, 2003). When a new branch buds, the apicobasal polarity is maintained during tube formation. Irrespective of the mechanisms that give rise to a tube, this newly formed tube is often small and must expand in length and diameter before becoming functional. For example, the human aorta expands 100-fold from embryo to adult in order to fulfil the

increasing demands of circulation. Tube enlargement involves lumen growth and may also be associated with growth of the basal surface. Lumen size is limited to the size of the apical epithelial surface. Thus, a critical determinant for tube size resides in the growth of the apical cell membrane, and lumen diameter growth is associated with an abundance of secretory vesicles near the apical surface. In addition, it has been noted that the inner (apical) surface of tubes are generally smooth and regular compared to the basal (outer surface), leading to assumptions that the sub-apical cytoskeleton has an important role in defining tube size and shape (Figure 1) (Lubarsky and Krasnow, 2003).

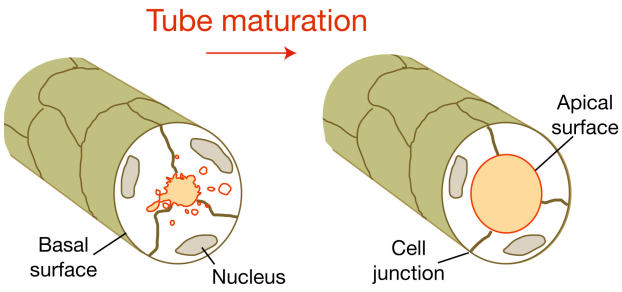

Figure 1. Tubes are composed of living cells that attach to one-another to form an epithelium. The apical surface is lining the lumen and is in contact with luminal media, while the basal surface is facing the surrounding tissues. A newly formed tube (left) has a narrow lumen that expands to acquire functional dimensions (right), in a process that relies on apical cell secretion and rearrangements of the sub-apical cytoskeleton.

How to identify mechanisms that control epithelial tube size and shape

Although much is known about basic molecular mechanisms for patterning of tubular networks, the cell biology underlying the formation of tubes with correct size and shape is not well understood. Investigation of lumen formation in different model systems has revealed many tube size genes that affect the apical cell surface and sub-apical cytoskeleton (Myat and Andrew, 2002). In humans, studies of genetically linked diseases have identified molecular components required to control tube shape. For example, mutations in *PKD1* and *PKD2*, encoding Polycystin-1 (PC-1) and polycystin-2 (PC-2), cause uneven renal tube diameters, and finally a transition from tubes to cysts, causing polycystic kidney disease (Boletta and Germino, 2003). PC-1 and PC-2 are transmembrane proteins localizing to the apical primary cilium of renal epithelial cells, thereby implying a function for the apical surface in tube size control. Zebrafish embryos that lack functional Polycystin-2 also develop renal cysts in

correlation with expansion of the apical cell surface in pronephric duct epithelial cells leading to changes of nephron fluid flow (Obara et al., 2006).

Given that epithelial tubes are necessary for the existence of all higher eukaryotes, the use of simple and genetically amenable model organisms for studies of mechanisms that control tube formation are helpful. Indeed, analyses of tube formation in model organisms such as C. elegans and Drosophila begin to provide cues regarding the molecular and cellular pathways that control tube size. In *C. elegans*, two tubular organs are primarily studied. These are the single-celled tubes formed by the excretory cells (equivalent to the worm's kidney) and the hindgut. A series of mutations affecting the apical cell surface of the excretory cell canals has been identified and named "the Exc mutants" (Buechner et al., 1999). In the Exc mutants the apical membrane swells to produce cyst-like lumens. Two of the Exc mutants display severe phenotypes with excessively dilated canal lumens. The gene disrupted in one of these mutants is identified and encodes a secreted mucin called *let-653* (Jones and Baillie, 1995). Mucins are large glycoproteins found on the apical surface of many epithelia, where they are believed to protect exposed surfaces as in the respiratory system and in the digestive tract. The finding that an apical secreted protein could affect lumen size was unprecedented, and it was suggested that the mucin might help regulate the fusion of vacuoles to the apical membrane or form or retain lumen material needed to control lumen size. The ten other *exc* mutants develop various diameter phenotypes. One of these is disrupted in *sma-1*, a *C. elegans* homologue of β-heavy spectrin, affecting the apical cell membrane (Buechner et al., 1999). In addition, the *C. elegans* ERM orthologue *erm-1* (encoding a cytoskeletal membrane linker) has a crucial role in luminal membrane morphogenesis. Both reduction and over-expression of *erm-1* function cause luminal diameter defects of the *C. elegans* hindgut, excretory canal and gonadal tubular epithelia. ERM-1 is localized to the apical membrane domain, and in *Drosophila*, the ERM orthologue *Dmoesin* interacts with Crumbs and β-heavy spectrin in the apical membrane skeleton (Gobel et al., 2004). Also during formation of the *Drosophila* salivary glands, the apical membrane appears to be important for regulation of tube size. The salivary gland is a single blind-ended tube that arises by invagination from the ectoderm. Mutants for the transcriptional regulator Hairy do however develop an enlarged apical (luminal) surface with protrusions of the normally unbranched tubes. Part of this effect appears mediated by the prolonged expression of another gene regulator (Huckebein) that regulates the expression of Crumbs (Crb), an apical membrane determinant, and Klarsicht (Klar), which mediates microtubuli-

dependent organelle transport (Myat and Andrew 2002). Thus, apical membrane growth may control lumen size.

The respiratory organ of the fly, called the trachea, is another model organ for tube size regulation. The trachea is a branched epithelial network and is a well-established model for branching morphogenesis (Affolter et al., 2003; Ghabrial et al., 2003; Uv et al., 2003). However, during the course of use of the tracheal studies, researchers have also identified a number of mutants that affect tube size. Hence, a new focus in tracheal development is epithelial tube size regulation (Beitel and Krasnow, 2000; Hemphala et al., 2003; Paul et al., 2003; Samakovlis et al., 1996a).

Drosophila as a model organism

A favoured model organism for tubulogenesis is *Drosophila*. *Drosophila* has been an important model organism in genetic studies since 1910, when Thomas Hunt Morgan discovered the sex-linked *white*-eye mutation. The fruit fly is common in nature, is easy to breed, has a short generation time and is cheap to maintain (St Johnston, 2002). Over the last 30 years, the fruit fly has also become an important model organism for developmental biologists.

Drosophila has only three pairs of autosomal chromosomes in addition to the sex chromosomes, X and Y. The X chromosome is often referred to as the first chromosome, and sex determination is based on the X-chromosome ratio. The second and third chromosomes are the two largest autosomes, while chromosome four and the Y chromosome are small and contain very little genetic information. An advantageous genetic characteristic of flies is the lack of genetic recombination in male flies. In addition, two useful genetic tools are identified for flies, the balancer chromosomes and phenotypic markers. The balancer chromosomes contain multiple inversions that prevent segregation of daughter cells with recombined genetic material, one or more dominant markers as well as recessive markers, and they are often lethal as homozygotes. As there are balancer chromosomes for each of the *Drosophila* chromosomes, lethal mutations can be maintained in a population over several generations, and in combination with viable phenotypic markers that affect larval and adult structures, they can be used to follow the segregation of chromosomes and mutations during crossing schemes.

The short *Drosophila* life cycle begins with a 24-hour embryonic development into first instar larvae. After another 24-hour period the larvae molt to second instar

larvae, and after yet a 24-hour period they become third instar larvae. After two days as third instar larvae, they form pupae, and five days later metamorphosis is completed and the adult flies eclose. The speed of this life cycle is dependent on temperature, and if the temperature is shifted from 25°C to 18°C, it takes 19 days to complete instead of only 10 days. Since the female fly can mate with several male flies and store their sperm, it is important to use virgin female flies in crossings. Again, temperature affects the maturation time of the flies, so that flies are ready to mate after 8 hours at 25°C or 18 hours at 18°C. All these features of the fly have made it one of the most unique and strong genetic model organisms existing (St Johnston, 2002). *Drosophila* is now used to study basic principles for embryonic patterning, organ formation, cellular signalling mechanisms, immunity and recently also behaviour studies.

Drosophila Tracheal development

The trachea is built by single layered epithelial tubes that lack muscular support, and has now become the leading model organ for studies of tubular morphogenesis due of its relatively plain architecture and the availability of advanced genetic and molecular tools (St Johnston, 2002). The larval tracheal system consists of approximately 1600 cell and arises from 20 independently branched metameres, 10 on each side of the embryo. Thus, one metameric unit consists of about 80 cells and gives rise to the different tubes of the trachea (Figure 2B). Tracheal branching morphogenesis is a highly stereotyped process and is invariable from embryo to embryo (Samakovlis et al., 1996a; Uv et al., 2003). During branching morphogenesis, each metamere connects with its neighbouring metamers, both on the same side and on the contralateral side of the embryo, to form a continuous tracheal network (Figure 2A). Such branch fusions are mediated by two cells, one at the tip of each branch that will fuse and the process is similar to the formation of vertebrate capillary anastomoses (Gerhardt et al., 2003; Samakovlis et al., 1996b).

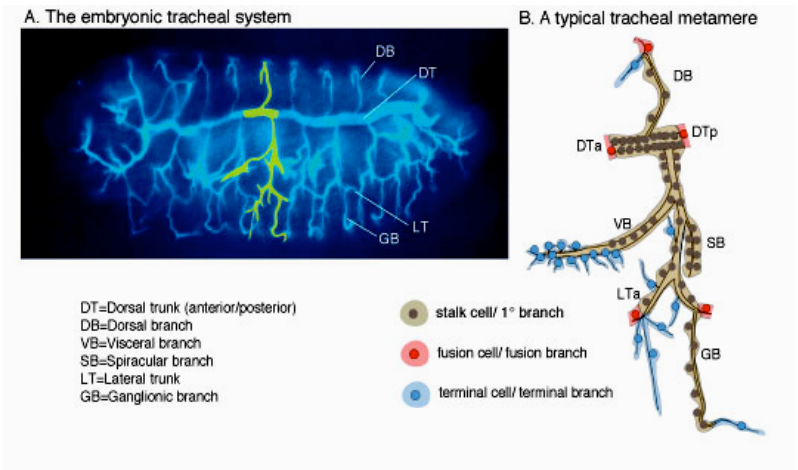

Figure 2. (A) Lateral view of the late embryonic tracheal system. The main airway, the dorsal trunk (DT), runs along anterior-posterior axis of the body and is formed by the linking of metameric units (one unit is drawn in yellow).
(B) A typical metameric unit with the 80 cell nuclei drawn in color as dots: Stalk cells (brown), fusion cells (red) and terminal cells (blue). The different branches that constitute a metamere are marked by their names. *(Adapted from Uv et al. 2003)*

Tracheal cell specification and patterning

Tracheal formation begins at stage 10 (about 5 h hours after egg lay (AEL) at 25°C) by the specification of clusters of ectodermal cells to form epithelial placodes. Each placode contains initially approximately 20 cells that subsequently invaginate to form small pouches of polarized tracheal cells, called the tracheal pits. Tracheal cell identity is specified early in the future placodes by a gene called *trachealess* (*trh*), expressed in the nuclei of tracheal cells throughout tracheal morphogenesis (Affolter and Shilo, 2000). *Trachealess* encodes the bHLH-PAS transcription factor that functions as a heterodimer together with Tango. In *trachealess* mutants, the placodes do not invaginate into tracheal pits, or placodes fail to form at all (Wilk et al., 1996). Although *trachealess* is seen as a master regulatory gene in tracheal morphogenesis, the transcription factor Cf1a, encoded by *drifter* (*dfr*) */ventral veins lacking (vvl),* is also required for specification of tracheal cell fates. In fact, the activation of tracheal-target genes is dependent on the interaction between the Trh PAS domain with the POU-domain of Cf1a (Anderson et al., 1995; de Celis et al., 1995; Zelzer and Shilo, 2000). Both *trh* and *dfr*/*vvl* are expressed under the control of the early patterning genes (de Celis et al., 1995; Wilk et al., 1996).

During the invagination process, the tracheal cells undergo their two final rounds of cell divisions, generating the approximate 80 cells per tracheal pit. After this cell division, there is no change in cell number, as no proliferation or cell death is seen in the embryonic tracheal system (Samakovlis et al., 1996a). Thus, the entire process of tracheal morphogenesis depends solely on cell rearrangements, movements and changes in cell shape. In the tracheal placodes and pits the cells will also orient

themselves in six different domains, corresponding with their future branch-specific localization. Such " branch-specific identities" are also promoted and regulated by the early patterning genes. Expression of *decapentaplegic* (*Dpp*; transforming growth factor-β, TGF-β) allows the tracheal cells to migrate in the dorsal-ventral (DV) direction, while *rhomboid* (*rho*), encodes a transmembrane protease that generates active epidermal growth factor (EGF), and *wingless* (*wg*) direct the cells into the anterior-posterior (AP) pathway (Chihara and Hayashi, 2000; Llimargas, 2000; Vincent et al., 1997; Wappner et al., 1997). Each pit then starts to branch by forming several finger-like protrusions.

Branching morphogenesis

As the tracheal cells starts to bud from the invaginated tracheal pockets, they organize themselves into tubes to form six primary branches. These are called the dorsal trunk anterior (DTa), dorsal trunk posterior (DTp), lateral trunk anterior (LTa), lateral trunk posterior (LTp)/ganglionic branch (GB) and visceral branch (VB). The tracheal cells that remain in the middle of the metameric structure and do not migrate out to form a primary branch will eventually form the transverse connective (TC) (Figure 2B). The main inducer of directed branch migration is Branchless (Bnl), a member of the secreted fibroblast growth factor (FGF) family. Bnl is expressed in cell clusters surrounding the tracheal pits, and functions as a chemo attractant by guiding each primary branch toward its source. Bnl activates the Breathless receptor (Btl; a *drosophila* FGF receptor) in tracheal cells that is under direct transcriptional control of Trh. When the migrating primary branches reach the Bnl source, Bnl expression is shut off, and in some places ligand expression is reactivated in cells further away to advance branch migration (Glazer and Shilo, 1991; Glazer and Shilo, 2001; Klambt et al., 1992; Sutherland et al., 1996). Downstream-of-FGF-receptor (DOF/Stumps) is an intracellular protein required for FGF signal transduction in *Drosophila* (Imam et al., 1999; Vincent et al., 1998), and in *Bnl*, *Btl* or *Dof*/*Stumps* mutants, the six primary branches fail to form.

At the end of stage 14 (11 h AEL), 25 secondary unicellular branches sprout from the tips of primary branches. Most of the secondary branches will form terminal branches, but at five positions fusion cells will form to connect neighboring and contra lateral metameres by fusion anastomoses (Samakovlis et al., 1996a). Secondary branching, as well as primary branching, occurs according to a stereotyped pattern and is also regulated by *bnl*/*btl* signalling (Samakovlis et al., 1996a; Sutherland et al.,

1996). However, at this time Btl-activation leads to differentiation of pantip cells in the branch tips. The pantip gene *pointed* (*pnt*) encodes an ETS-domain transcription factor activated by Bnl signaling via activation of the MAPK pathway. Pnt is required for secondary branch sprouting, and also helps to prevent the trailing stalk cells from adopting a tipcell fate. Pointed maintains the expression of Btl in tipcells, and induces expression of the intracellular Sprouty protein (Hacohen et al., 1998; Mason et al., 2006; Samakovlis et al., 1996a; Scholz et al., 1993). Sprouty inhibits the Btl-signalling pathway, but is antagonized by Corkscrew, which is activated upon Btl-signaling (Jarvis et al., 2006). It is thus possible that the balance within this signaling network singles out tipcells from stalk cells depending on the concentration of Bnl. In addition, Btl-signalling leads to Delta expression, which activates Notch on adjacent cells, to suppress Btl signaling in stalk cells through inhibition of MAPK (Ikeya and Hayashi, 1999).

Five of the 25 secondary branches (Figure 2B) will become fusion cells and arise in the DTa, DTp, LTa, LTp and DB. In the DB, two secondary branches form, fusion branch and one terminal branch. Selection of the fusion cell fate results from cross talk between the two cells. Bnl induces the expression of Delta in tip cells, and increasing levels of Delta in the same cell will inhibit the adjacent cells, through the activation of Notch, from also becoming fusions cells (Llimargas, 1999; Steneberg et al., 1999). The fusion cell is manifested by expression of fusion cell marker, one of which is *escargot*, encoding a zink-finger transcription factor (Samakovlis et al., 1996b). The terminal cells will instead express *pruned*, encoding the *Drosophila* serum response factor (SRF) required for terminal branching. Expression of SRF is Pnt-dependent (Guillemin et al., 1996; Samakovlis et al., 1996a).

The single-celled terminal branches extend far away from the main branches to supply all tissues of the animal with oxygen. During larval life, finger-like protrusions sprout from the single celled tube in a non-stereotyped way, creating ramified networks of tracheal branches that provide almost all cells with oxygen as the animal grows. Cells experiencing hypoxia reactivate Bnl (FGF) secretion to induce and attract additional branch sprouts from nearby terminal cells to provide oxygen to deprived cells (Jarecki et al., 1999). This situation can be compared to capillary branching induced by the angiogenic factors VEGF and FGF secreted by hypoxic cells in vertebrate tissues (Walgenbach et al., 1995 and (Jarecki et al., 1999; Shweiki et al., 1992).

Tracheal tube structure

During embryogenesis the tracheal network is filled with liquid, and right before the larva hatches, the tubes become filled with gas. To prevent the mature tubes from collapsing, the tracheal cells secrete a luminal exoskeleton. This cuticular lining is composed of three layers; [1)] the envelope, the outermost layer, which is rich in waxes and cuticulin, [2)] the epicuticle that is protein-rich and [3)] the procuticle, situated between the epithelial apical membrane and the epicuticle and is built up by sheets of chitin laminae that stack on top of each other in a helicoidal way together with sclerotized proteins (Figure 3A) (Locke, 2001; Moussian et al., 2005a). The apical cuticle of the trachea, contrast to the exoskeleton, by its presence of characteristic cuticular ridges, a spiral-shaped structure called the taenidial folds (Figure 3B) (Wigglesworth, 1990).

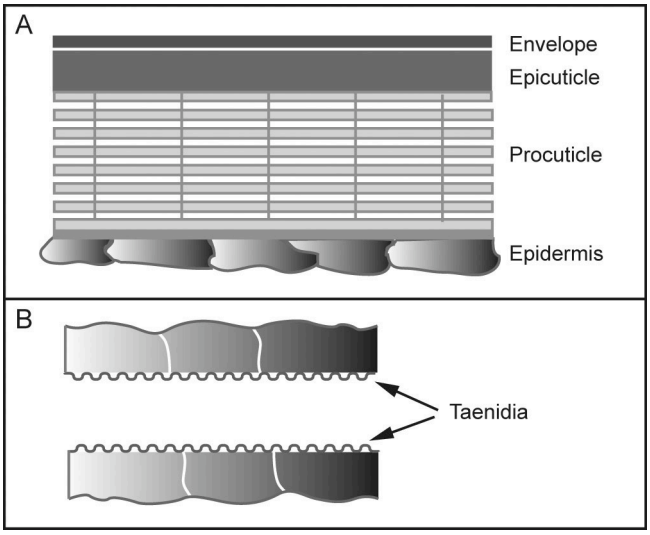

Figure 3. (A) The insect cuticle is a multi-layered protective structure that lines the outer surface of the animal, as well as the inside of tracheal tubes. The cuticle consists of the envelope, epicuticle and procuticle, and is attached to the apical cell surface. *(Adapted from moussian et al. 2005)* **(B)** The cuticular lining is secreted by the tracheal cells during late embryogenesis and prevents collapse of the tubes upon air filling. In the tracheal cuticle, spiral thickenings are formed, called the taenidia.

In the mature tracheal system all branches will fall into either of four types of tubes (Figure 4). Type-I tubes are built from wedge-shaped cells surrounding the lumen. In the tracheal system Type-I branches are represented by the dorsal trunks and transverse connectives, while in vertebrates, type-I tubes can be found in the lungs, vascular system and in glandular organs. In the type-II tubes the lumen circumference is surrounded by a single cell, which folds over it's own axis and is sealed by autocellular junctions, thereby creating a small lumen. Several such cells in a row make up the tube. Narrower primary branches, such as the dorsal branches, ganglionic branches and the visceral branches are all type-II tubes. Type-3 tubes are unicellular

doughnut shaped cells, and two such single seamless cells interconnect to form fusion anastomoses, thereby creating a continuous tracheal network. Finally, the type-IV tubes derive from single terminal cells and create intracellular seamless capillaries to provide the surrounding tissues with oxygen (Uv et al., 2003).

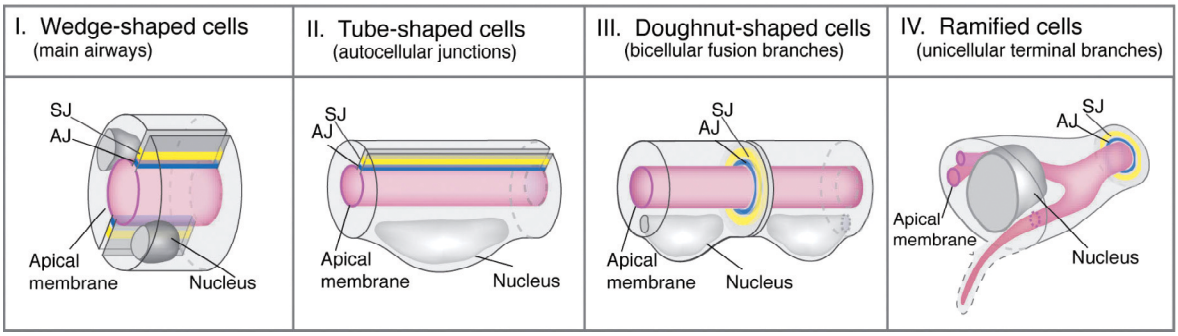

Figure 4. Four different cell types are found in the tracheal system: (1) The wedge-shaped cells that form the main airways, (2) The tube-shaped cells, sealed by autocellular junctions that make up the smaller tubes, (3) The seamless doughnut-shaped cells, represented by the tracheal fusion branches and (4) The blind-ended unicellular terminal tubes. (*From Uv et al. 2003*)

Tubular epithelia, like epithelial sheets, need an apicobasal polarisation to achieve correct structure and transepithelial barriers (Gibson and Perrimon, 2003). Constituent cells of the epithelial tubes are arranged so that the apical surface faces the lumen and the basal surface is exposed to other tissues of the organism. The tracheal epithelium in *Drosophila* is, together with the foregut, hindgut, malphigian tubules and salivary gland, derived from ectodermal primary epithelia. Important cellular juctional complexes in *Drosophila* primary epithelium include the marginal zone (MZ) proteins, the zonula adherens (ZA) junctions and the septate junctions (SJ) (Tepass and Hartenstein, 1994).

The marginal zone

The marginal zone represents a narrow region in cell-cell contact on the most apical side of the cell (Figure 5). Two protein complexes are found at the marginal zone (MZ), which are required for MZ formation and cell polarization in primary epithelia (Roh and Margolis, 2003). The first complex contains Crumbs (Crb), Stardust (Std) and DPatj (earlier called Discs lost, Dlt) and is simply called the Crb complex. Crb is a large transmembrane protein (Tepass et al., 1990) that interacts with the MAGUK (membrane-associated guanylate kinase) protein Std (Bachmann et al., 2001; Hong et al., 2001). Std in turn, binds dPatj to through its cytoplasmic PDZ domain (Bhat et al., 1999; Roh et al., 2002). In embryos mutant for Crb or Std the apicobasal polarity is

lost, but mutants for dPatj are vital, which suggests a dispensable or redundant role for dPatj in the Crbs complex (Pielage et al., 2003). The complex is mainly found at the marginal zone, although low concentrations are also detected at the apical membrane of the epithelial cell (Tepass et al., 2001).

The second complex in the MZ is composed of Bazooka (Baz), the *Drosophila* homologue of *C. elegans* Par-3 and vertebrate ASIP, the *Drosophila* homologue of atypical Protein Kinase C (DaPKC) and *Drosophila* Par-6 (DmPAR-6) (Hutterer et al., 2004; Petronczki and Knoblich, 2001; Wodarz, 2002). The two PDZ domain proteins Baz and Par-6 bind to aPKC in vertebrates and Drosophila (Joberty et al., 2000; Lin et al., 2000; Wodarz et al., 2000). This complex is all critical for the apicobasal polarity of epithelial cells (Wodarz, 2002). Like the Crumbs complex, the Bazooka complex is concentrated to the MZ, but is also found along the apical membrane of the epithelial cell (Tepass et al., 2001).

The zonula adherens junction

The zonula adherens junction consist of two major complexes, the cadherin-catenin complex and the Ed-Canoe complex (Figure 5) (Tepass and Harris, 2007). Together with the other junction proteins the cadherin-catenin complex forms a circumferential adhesion belt at the apical part of the epithelial cell by linking to actin filaments in the cytoskeleton (Gumbiner, 2005; Perez-Moreno et al., 2003; Tepass et al., 2000; Yagi and Takeichi, 2000). Members of the cadherin-catenin complex are DE-cadherin, the *Drosophila* counterpart to E-cadherin that mediates Ca^{2+} dependent homophilic adhesion, encoded by *shotgun* (*shg*), as well as the two cytoplasmic proteins Armadillo (Arm) the *Drosophila* homologue of vertebrate β-catenin and Dα-catenin that binds to the cytoplasmic part of DE-cadherin

The second complex of adherens junction proteins is involved in morphogenesis. Canoe is the *Drosophila* orthologue of the mammalian PDZ-domain protein Afadin and appears to be involved in the Jun N-terminal kinase (JNK) pathway during dorsal closure (Takahashi et al., 1998). Afadin is an actin filament-binding protein that serves as an adaptor by binding several scaffold proteins, including α-catenin (Kobielak and Fuchs, 2004; Takai and Nakanishi, 2003). Canoe binds directly to the MAGUK protein ZO-1 encoded by *polychaetoid* (*pyd*) (Takahashi et al., 1998) and also links the homophilic Ig-type adhesion protein Echinoid (Ed) to the actin-filament at AJs (Wei et al., 2005). Canoe is also proposed to couple Ras signaling with cytoskeleton (Matsuo et al., 1997).

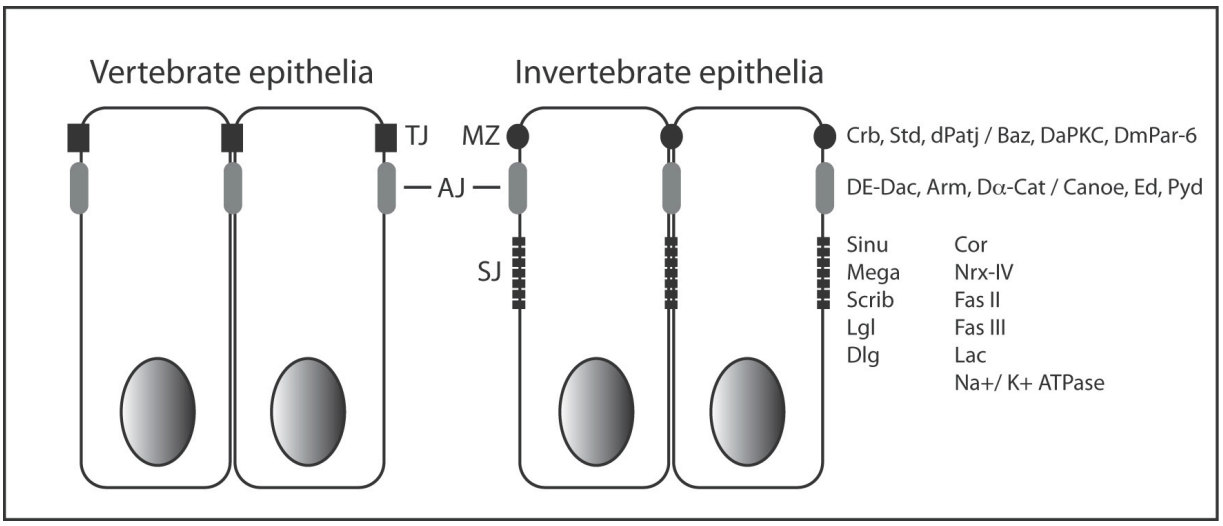

Figure 5. Schematic illustration of cellular junctions in vertebrate and invertebrate epithelia. In vertebrate epithelia, tight junction (TJ), the functional counterpart to invertebrate septate junction (SJ), are located apical to the adherens junction (AJ). The invertebrate marginal zone proteins (MZ) are located on the apical border of the epithelium and consist of Crb, Std and dPatj (the crumbs complex) and of Baz, DaPKC, Par-6 (the Bazooka complex). The AJ also contain two complexes: DE-Cad, Arm and Dα-cat as well as Canoe and Pyd. The SJ are located on the basolateral side of the cell and include Sinu, Mega, Scrib, Lgl, Dlg, Cor, Nrx-IV, FasII, FasIII, Lac and Na+/ K+ ATPase.

The septate junction

Proteins localized to the septate junctions (SJ) appear in ectodermal epithelia first after the apicobasal cell polarity is set up and the ZA has been established. At stage 15 a ladder-like structure is detected basally to the ZA (Figure 5) (Tepass and Hartenstein, 1994). The septae span the intermembrane space and form spirals around the epithelial cells. The SJ proteins create a diffusion barrier for water and solutes across the epithelium and thus SJs show an analogous function to vertebrate tight junctions (TJ) (Anderson, 2001; Carlson et al., 2000). This was seen by injection of dyes into the body cavity of embryos with dysfunctional SJ proteins and by mutational analysis of genes encoding SJ components (Baumgartner et al., 1996; Lamb et al., 1998). Although the SJs are quite different from TJs, both morphologically and molecularly, the discovery of the two *Drosophila* transmembrane caludins, Sinous (Sinu) and Megatrachea (Mega), propose a common biochemical basis and evolutionary origin in the regulation of barrier functions (Behr et al., 2003; Wu et al., 2004).

Despite the fact that cell polarity is already established when the SJs are formed, a complex of proteins found in the SJs are involved also in the control of cell polarization (Bilder et al., 2000; Tepass et al., 2001). This complex is called the Scribble complex and consists of Scribble (Scrib), Discs large (Dlg) and Lethal giant larvae (Lgl) (Bilder et al., 2000). Mutations in either of these components result in

epithelial disorganization with expansion of the apical cell surface (Bilder et al., 2003; Tanentzapf and Tepass, 2003). Dlg is also required for maintenance of the SJ complex. Loss of the Dlg protein leads to a redistribution of the cytoplasmic SJ protein Coracle (Cor) that ends up throughout the cell. Also, the homophilic cell adhesion molecule FasIII that localizes to SJ, and is used as a marker for SJ integrity, is faintly expressed in Dlg mutants (Woods et al., 1996). Thus, there is an interdependence of the components of SJs for their proper localization, which has been confirmed by several studies (Behr et al., 2003; Genova and Fehon, 2003; Hemphala et al., 2003; Lamb et al., 1998; Llimargas et al., 2004; Paul et al., 2003; Wu et al., 2004). A number of additional proteins localize to SJ and are required for SJ formation. These include Neurexin-IV (Nrx-IV), Lachesin (Lac) a protein belonging the immunoglobulin (Ig) superfamily, Fasciclin II (Fas II) a homophilic adhesion protein and Na+/K+ ATPase (Grenningloh et al., 1991; Hemphala et al., 2003; Lebovitz et al., 1989; Llimargas et al., 2004; Paul et al., 2003; Wu and Beitel, 2004).

Regulation of tube size

Creating a lumen of correct size and shape is a critical step in tube morphogenesis. Initially, when a tube is formed the lumen is quite small, and as the animal develops the tube expands to be able to fulfil the needs of functionality. The expansion process can begin as soon as the lumen is formed, and in the developing trachea it occurs at specific time points in development. For example, the DT lumen diameter triples over a two- to three-hour period between embryonic stages 14 and 16 with little growth of the basal DT diameter (Beitel and Krasnow, 2000). At the beginning of tube expansion, we have observed multiple vesicle-like structures in the apical domain of the tracheal cells, which appear to fuse with the apical membrane to make the lumen surface expand (Figure 6). At the same time the apical cytoskeleton has to rearrange to accommodate alterations in cell shape. Tracheal cell division cease after developmental stage 11 (Samakovlis et al., 1996a), and experiments have shown that when tracheal cell number is either halved or doubled in *cyclinA* and *dacapo* mutants, respectively, tube size is not affected (Beitel and Krasnow, 2000). This indicated that the number of cells that makes up the tracheal tubes does not determine tube size. Instead the cells somehow adjust their shape to accommodate the predetermined tube dimensions. Additionally, diameter expansion and elongation of a growing tube seem to be independently regulated. Beitel and Krasnow measured tube length and diameter

growth of two primary branches, the DT and the TC in both embryos and larvae. They found that while diameter growth occurs during discrete periods that are distinct for each branch, tube elongation is a continuous process (Beitel and Krasnow, 2000). Moreover, they identified tube size mutants that selectivity affected tube length.

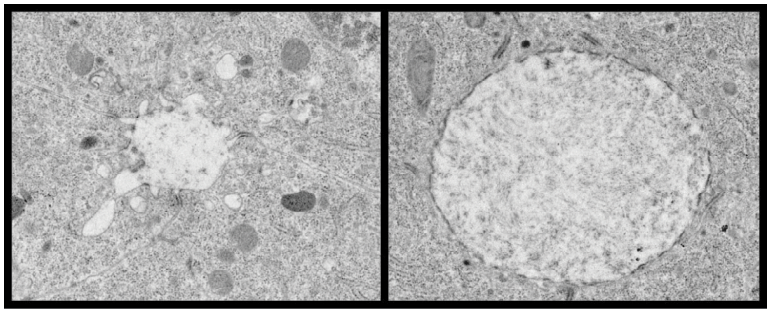

Figure 6. Early in the tracheal tube expansion process (left), vesicles are detected around the small apical lumen. These vesicles are presumed to add apical surface material to expand the tracheal lumen (right). The sub-apical cytoskeleton rearranges to accommodate a smooth growing tube diameter.

Genes affecting tracheal tube size

Studies of the *Drosophila* trachea by various labs have identified a number of tube-size mutants. One such mutant is *shotgun* (*shg*). In *shg* mutants many DT segments fail to fuse, but those that do fuse, do not expand the fusion lumen to proper size, forming a tube with local constrictions (Tanaka-Matakatsu et al., 1996). The part of the tube in-between the fusion junctions are often too wide and too short. As *shg* encodes DE-cad, these findings suggested that reorganization of the AJs is important for lumen growth.

Another group of tube-size mutants are loss-of-function mutations in the genes encoding septate junction (SJ) proteins, the so-called "SJ-mutations". Homozygous SJ mutant embryos develop a normal trachea until tube expansion begins. Towards the end of tube expansion (late stage 15), their DT lumen diameter appears slightly broader than that of the wild type, and from early stage 16 their DT elongates excessively to become extremely elongated and convoluted. These phenotypes have been noted in embryos with loss of function of Mega, Sinu Lac, FasII and the two subunits of the Na+/K+ ATPase ion transporter; ATPα and its β subunit Nervana2 (Nrv2) (Behr et al., 2003; Hemphala et al., 2003; Llimargas et al., 2004; Paul et al., 2003; Wu and Beitel, 2004; Wu et al., 2004). None of the SJ mutants have altered tracheal cell number, but develop defected cell shapes with an increased apical cell circumference. Also, the SJ-mutants fail to display a defective tracheal epithelial barrier. A 10-kDa dextran dye injected into the body cavity of wild-type after stage

15 embryos is excluded from entering the closed tracheal system is able to dye from stage late 15, indicating that the barrier has become functional at this stage (Paul et al., 2003). When the 10-kDa dextran dye is injected into the body cavity of SJ mutants, the dye enters the tracheal lumen within 5 minutes, demonstrating a broken tracheal transepithelial barrier (Behr et al., 2003; Llimargas et al., 2004; Paul et al., 2003; Wu et al., 2004).

A third component required for normal tracheal tube size is the transcription factor Grainy head (Grh). In *grh* mutants, the apical cell surface becomes overgrown, resulting in tracheal branches that are too long and convoluted. In addition the AJs appear atypical, often lying in parallel to the lumen instead of perpendicular, and the overgrown apical membrane often folds over the neighboring cells producing multiple layers of cuticle. The over elongated tube phenotype first appears at stage 16, indicating that Grh restricts tracheal tube elongation. Over-expressing Grh, in contrast, limits luminal growth and generates too short and narrow tubes without affecting tracheal cell fates (Hemphala et al., 2003). Also, the phenotype in *grh* mutants is not due to altered cell number or cell polarity. However, Grh appears to regulate tracheal tube growth through a different genetic program than the SJ genes, as removal of *grh* in a SJ mutant causes a more severe phenotype where the tracheal lumen appears fragmented (Hemphala et al., 2003). It was also shown that tracheal Grh activity is influenced by Bnl/Btl signaling, thus providing a potential link between a signals that induces branch formation with a program that restrict tube growth.

Altogether these results show that tracheal tube growth is highly dependent on intact cell-to-cell junctions, correct apical membrane growth and sub-apical rearrangements, but the mechanism that coordinate cell behavior to produce uniform tubes of correct dimensions is yet to be revealed.

AIM

The aim of this thesis is to reach a further understanding of the mechanisms involved in controlling epithelial tube size, using the developing *Drosophila* trachea as a model system.

RESULTS AND DISCUSSION

During tracheal tube size maturation the dorsal trunks expand at least 3-fold in diameter and elongates by more than 20%. How are these processes regulated to achieve uniform tubes of precise dimensions? This thesis describes a fundamental impact of luminal components on tube shape, which may represent biological principles used to shape epithelial organ lumens in general.

Paper I and paper II describe a novel finding, that an intraluminal matrix is required to model uniform tube diameter during tube dilation. They also provide a possible explanation for the effect of previously identified tube size genes on tube diameter. Paper III focuses on the subsequent tube elongation and reveals that final tube shape is determined by balancing forces exerted by the lumen environment and sub-apical actin cytoskeleton.

Paper I: A Transient Luminal chitinous matrix is required to model epithelial tube diameter in the Drosophila trachea

The transcription factor Grh is required to restrict tracheal tube length, and in *grh* mutants the long and convoluted tracheal tubes are associated with enlarged apical tracheal cell membranes (Bray and Kafatos, 1991; Hemphala et al., 2003). The target genes of Grh are however unknown. In *Drosophila*, genes that act in the same genetic pathway are often identified by similarities in mutant phenotypes. *grh* mutants were first identified in a comprehensive genetic screen and the *grh* phenotype discovered at that time included a disorganized head skeleton and cuticle (Nusslein-Volhard et al., 1984). In the same screen three other mutants were identified and grouped together with *grh*, based on similarities in phenotypes. These were called *Krotzkopf verkehrt* (*kkv*), *Knickkopf* (*knk*), and *retroactive* (*rtv*) (Jurgens et al., 1984; Wieschaus et al., 1984). With the assumption that these mutants might carry disruptions in genes regulated by Grh, their mutant tracheal phenotypes were investigated. Indeed, *kkv*, *knk* and *rtv* developed tracheal tubes that were too long. However, the mutant phenotypes of *kkv*, *knk* and *rtv* were more severe than those of *grh* mutants, since they also include irregular tube diameter with local tube dilations and constrictions. Thus, although the expression of *kkv*, *knk* and *rtv* are unlikely to solely depend on Grh, they represented three new exiting tube size genes.

Analysis of kkv mutants

In paper I we focused on the role of one of these genes, *kkv*, in tracheal tube size growth. The tracheal lumen of *kkv* mutant embryos was examined at different embryonic stages by the use of the lumen-specific antibody 2A12. We found that they become irregular in diameter during stage 15, when tube expansion occurs, as they developed local constrictions at the branch fusion junctions and excess dilations of the tube in-between the fusion junctions. During stage 16, the mutant tracheal tubes also become excessively elongated and convoluted. Despite such severe defects in tube shape, the mutant tracheal epithelium is intact with an apparent normal apical-basal polarity. The only anomaly detected in the mutant epithelium was a slight reduction in the levels of ßHeavy-spectrin, indicating that the organization of the sub-apical cytoskeleton is affected in the mutants.

kkv encodes CS-1

When these analyses were initiated, the gene disrupted by *kkv* was not identified. In 2002 it was reported that *kkv* encodes chitin synthase 1 (CS-1), which is one of two chitin synthases in *Drosophila* (Ostrowski et al., 2002). Chitin is a long extracellular polymer of β-(1-4) linked N-acetylglucosamines (GlcNAc), and one of the most common polymers in nature after cellulose. It functions as a strong scaffold material in the exoskeleton of insects, arthropods and nematodes, as well as in the cell walls of yeast and fungi (Merzendorfer and Zimoch, 2003). In insects, chitin is also important in the peritrophic matrix, which lines the gut and forms a permeability barrier to protect the insect from invasion by microorganisms and parasites (Lehane, 1997).

During *Drosophila* development, chitin is incorporated in the cuticle sometime towards the end of embryogenesis. Tracheal tubes also deposit an apical (luminal) cuticular lining that provides structural support to the tubes. We therefore asked whether the tube size defects seen in *kkv* mutant embryos simply could be due to a defective cuticle. *kkv* mutants develop tube size defects already at the beginning of stage 15, and to address the time of cuticle deposition, we performed TEM analysis. At late stage 15, deposition of the outermost part of the cuticle, the envelope, can be seen as “flakes” that eventually will fuse to a continuous layer. This means that production of the chitin containing procuticle has not yet started (Locke, 2001; Moussian et al., 2005a). Also, we couldn’t detect any procuticle at this stage and the mutant and wild type cuticle structures looked similar. As chitin is a component of the procuticle, we concluded that cuticular chitin is not deposited prior to early stage 16. Consequently,

the tracheal diameter defects seen in *kkv* mutant embryos were not likely to relate to a function of cuticular chitin.

Luminal chitin is required for uniform tube diameter growth

Chitins synthases are large membrane-spanning glycosyltransferases, which catalyze the linkage of cytoplasmic UDP-N-acelylglucosamine into long extracellular polysaccharide chains. Based on sequence homologies, chitin synthases have been grouped together with other glycosyltransferases such as cellulose synthases in plants and hyaluronan synthases in animals (Cohen, 2001; Merzendorfer, 2006). Insect epidermal cells from Brazilian skipper butterfly *Calpodes ethlius* and Australian sheep blowfly *Lucila caprina* produce chitin fibrils directly at the apical plasma membrane (Merzendorfer and Zimoch, 2003). The *Lucilia cuprina* chitin synthase is proposed to be an integral membrane protein with its catalytic domain situated on the cytosolic side of the cellular plasma membrane (Tellam et al., 2000). In *Drosophila*, cuticular chitin is believed to be synthesized in plasma membrane plaques, which are recognized with electron microscopy as densely stained macromolecular structures, consisting of chitin synthases presumably together with different co-factors (Locke, 2001). Chitin would then be made by linking cytosolic UDP-GlcNAc residues together at the CS catalytic site and exported across the plasma membrane through a transmembrane pore formed by the chitin synthase.

The actual chitin synthase domain of CS-1 does however occupy a limited part of the protein. The total CS-1 protein is between 1600 and 1700 amino acids, but the chitin synthase domain consists of 527 residues. Accordingly, the role of CS-1 in tracheal tube expansion could possibly be attributed to protein functions outside the synthase activity. Notably, in fungi, such as *Mucor rouxii* and in *Saccharomyces cerevisiae*, chitin synthases are seen to assemble in so-called chitosomes, which are small chitin synthesizing micro-vesicles (Bartnicki-Garcia, 2006; Siemieniewicz et al., 2007). If CS-1 is transported to the membrane via analogous insect chitosomes, it could in principal affect the assembly or trafficking of such vesicles to the plasma membrane, and thereby the co-transport of other components needed for tube expansion.

To test whether chitin synthesis is required during tracheal tube expansion, we used a chemical inhibitor of chitin synthesis, called Nikkomycin Z. Nikkomycin is a naturally occurring metabolite of *Streptomyces.* It is a competitive structural analogue of UDP-GlcNac that inhibits the enzymatic function of chitin synthase by binding to

its catalytic site (Cohen, 2001). By feeding adult flies with Nikkomycin Z the *kkv* tracheal phenotype was indeed reproduced in the offspring, demonstrating that chitin synthesis is a prerequisite for uniform tube expansion. We next asked whether luminal chitin is required for tube expansion. For this, we generated transgenic flies that ectopically express a chitinase in the trachea. Chitinase secretion normally occurs during ecdysis and pupation for degradation of the cuticle to prepare space for the new cuticle. The transgenic embryos that express ectopic chitinase in the developing trachea displayed tracheal tube size defects similar to those of *kkv* mutants. Together the results demonstrate that luminal chitin is essential to produce tracheal tubes with uniform diameter.

Chitin assembles as a luminal filament

By studying the literature, we found three ways to visualize chitin. One is to use FITC-conjugated Wheat Germ Agglutinin, which selectively binds to N-Acetyl glucosamine (GlcNAc) groups and to sialic acid, thus recognizing glycoproteins in addition to chitin. Another is to use a diazo dye, called Congo red (CR), which has high affinity to structural polysaccharides (Herth, 1980). The third way is to use a specific chitin-binding protein (CBP) conjugated to FITC, provided by New England biolabs. By all three means, we found that chitin is present within the tracheal lumen from stage 14. However, instead of localizing along the luminal surface, where the cuticle is deposited, the early tracheal chitin forms a filamentous cable within the tracheal lumen. The chitin cable appears just prior to diametrical DT expansion and runs like a defined cable throughout the DT. During diameter expansion the filament expands in unison with tube diameter. In *kkv* mutant embryos the filament is undetectable. In conclusion, a luminal chitin filament is required for uniform tube expansion.

Another family of tube size mutants disrupts components of the *Drosophila* SJ ("SJ mutants"), but the involvement of SJ components in tube size regulation was unclear. In "SJ mutants", the tracheal tubes show slight dilations during stage 15 and excess tube elongation during stage 16. We found that in these mutants, the chitin filament has a diffuse texture without the filamentous traits seen in the wt embryos. Thus, intact SJ are required for the proper organization of the luminal chitin filament, possibly explaining the requirement for SJ components in tube size regulation.

A luminal chitin filament models tube diameter

We propose that newly formed tracheal tubes synthesize chitin polysaccharides, which assemble into an organized intraluminal cable. The chitin cable expands in diameter as lumen volume increases, and serves to model the tube wall. The increase in diameter of the chitin-filament may be caused by swelling upon increased lumen volume, require continued chitin synthesis or depend on both of these parameters. A simple mechanical pressure could mediate the effect of the chitin filament on tracheal cell shape and sub-apical cytoskeleton: As the chitin cable expands during tube dilation, it could equalize the pressure on the tube wall, forcing the tube to dilate in a uniform manner. It is also possible that the cable is anchored to the tube wall to actively hold back excess dilation of the lumen in between fusion junctions. In the absence of the chitin cable, the tubes would simply expand where the tube wall is weakest.

A general requirement for intraluminal matrices in tubular organogenesis?

Chitin is not known to be synthesized in mammals, and chitin appears absent in other developing fly tubular organs. Still, our finding may represent a general requirement for intraluminal matrices to shape epithelial organ lumens. During *Drosophila* embryo development, many epithelial organ lumens are shown to contain dense sugar-rich components. These were detected by the use of lectins and an antibody against the mucin-type O-glycosylation (polysaccharides in which single N-acetylgalactosamine molecules are O-linked to serine or threonine (Tian and Ten Hagen, 2007). In addition, TEM analysis of the developing drosophila salivary glands reveals dense material filling the lumen (Abrams et al., 2006). Lectin-stainings in vertebrate embryos have also revealed the presence of temporary sugar-rich components in developing tubular organs, such as the rabbit kidney and chicken lung (Gheri et al., 2000; Schumacher et al., 2002). Do such components have role in shaping the organs lumens? Studies of the *Drosophila* embryonic salivary glands reveal malformed lumens with cyst formations and constrictions in embryos mutant for the *Drosophila* homologue of prolyl hydroxylases, enzymes that hydroxylate proline residues on collagen (Abrams et al., 2006). Collagen is not present in salivary glands and the authors believe that these enzymes hydroxylate proline residues in other secreted or transmembrane proteins. They also noted that apical salivary gland secretions were altered in embryos lacking SG1 and SG2, and suggested that the tube size defects in SG1 and SG2 mutants are due to a defective luminal matrix texture. However, since the identities of the salivary

gland luminal components are unknown, these experiment are only indicative of a function for luminal components in shaping the salivary gland lumen.

Interestingly, a screen for *Drosophila* mucins (proteoglycans rich in substrates for O-glycosylation) revealed that several genes encoding mucin-like proteins are expressed in epithelial organs during embryogenesis (Syed et al., 2008). One of these is called Tenectin (Tnc), and was previously shown to be apical in the hindgut and tracheal lumen (Fraichard et al., 2006). We have continued the studies of *Tnc* and find that it fills the lumen of the hindgut, foregut, trachea, proventriculus and heart. Moreover, mutant analyses show that *Tnc* is required for correct size and shape of the hindgut lumen. Consistent with this finding is the requirement of an intact *let-653* that encodes a mucin-like protein in the *C. elegans* excretory cells. Mutations in this gene cause extremely enlarged canal lumina (Jones and Baillie, 1995). Thus, diverse epithelial tubular organs may commonly depend on luminal components to attain their functional form.

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

In paper II, the functions of *knk* and *rtv* were analyzed. As already mentioned the tracheal phenotype of *knk* and *rtv* mutants is similar to that of *kkv* mutants. All three genes are required for formation of uniform tubes during the expansion period and to restrict tube elongation. While the tracheal phenotype of *knk* mutants is almost undistinguishable from that of *kkv* mutants, a less severe phenotype is detected in *rtv* mutant embryos.

Knk and Rtv are required for chitin matrix organization

Like *kkv* mutants, the tracheal epithelium of *knk* and *rtv* mutants exhibits normal polarity and junctional complexes. To test if they have selective effects on the tracheal luminal chitin cable, we addressed the effects of removing *knk* and *rtv* functions in chitin-deficient embryos. Embryos double mutant for "SJ mutants" and *kkv* were previously shown to display reduced luminal 2A12-staining, but chitin synthesis inhibition in *knk* and *rtv* mutant embryos did not produce such additive loss in 2A12-staining. Also, their tracheal phenotypes looked identical to those of *kkv* mutants.

Moreover, double mutants for *knk* and "SJ mutants" display reduced 2A12-staining. Finally, embryos laid by wild type flies fed with a low dose of Nikkomycin Z, developed tracheal phenotypes similar to those of *rtv* mutants. These results indicated that Knk and Rtv act in a strict chitin-dependent pathway to control tube size.

To analyze the intraluminal chitin cable in *knk* and *rtv* mutants, we used the FITC- conjugated chitin-binding probe (CBP). CBP reveals that the *knk* and *rtv* lumens do contain chitin, but that the chitin has lost its filamentous appearance and fills the entire lumen, rather than forming a defined cable. Thus, Knk and Rtv are required for correct chitin filament assembly. Furthermore chitin synthesis inhibition by the use of Nikkomycin Z, irrespectively of dosage, develops intra luminal chitin of the DT with a weaker appearance compared to both *knk* and *rtv* mutants. This indicates that a critical amount of chitin seems required for the formation of a chitin filament with a defined and fibrous appearance.

In a parallel study, our collaborator Bernard Moussian studied the cuticle of *knk* and *rtv* mutants. TEM analysis revealed disorganized chitin lamellae in the cuticle of *rtv* (Moussian et al., 2005b) and *knk* (this paper) mutants. Moreover, TEM analyses combined with the use of gold-labelled Wheat germ agglutinin (WGA) of *rtv*, *knk* and *kkv* mutant embryos, revealed cuticular chitin in the procuticle of both *knk* and *rtv* mutant embryos, whereas in *kkv* mutant embryos, cuticular chitin was absent. Thus Knk and Rtv seem to have a common role in chitin organization in the tracheal luminal matrix and the cuticle.

rtv encodes a small protein with a predicted N-terminal signal peptide and a C-terminal transmembrane domain. Although Rtv does not contain any known chitin binding domains, it has been demonstrated to be important for laminae organization of the chitin rich procuticle. We found Knk to have a similar role in cuticle organization.

Knk is required at the apical surface

Rtv and Knk are predicted secreted proteins. Rtv has a potential transmembrane region, while Knk is GPI-anchored. Labelling with anti-serum against Knk, reveals that it localizes to the apical surface of the epidermal and tracheal cells. To test if Knk functions at the apical membrane, rather than being released from the membrane to constitute a component of the luminal matrix, we asked whether transmembrane Knk (Knk^{TM}) could rescue the *knk* mutant phenotypes. Ubiquitous expression of a wild type Knk is able to rescue both the tracheal phenotype and lethality. A constructed form of Knk, where the transmembrane domain of transferrin 2 replaced the GPI-anchor, was

similarly able to rescue the *knk* mutant phenotypes. Thus, Knk and possibly Rtv, appear to act at the apical membrane to aid organization of newly formed chitin chains.

Intact proteins of the SJ are crucial for the correct localization of Knk

Returning to SJ mutants, we analyzed the distribution of Knk in these tube size mutants. We found that intact SJ components are necessary for the correct localization of Knk to the apical membrane. In "SJ mutants" Knk is not restricted to the apical surface, but is found also along the lateral and basal surfaces. Thus, intact SJ appear required either to direct Knk to the apical cell surface or to prevent diffusion of Knk to the lateral and basal surfaces of the epithelial cells. As we could not rescue the SJ phenotype by ectopic expression of Knk^{TM}, it is likely that SJ components are required for the correct localization of additional components required for chitin matrix organization. Indeed, it was reported that secretion of the extracellular predicted chitin deacetylase Vermiform (Verm) is impeded in "SJ mutants" (Wang 2006).

Possible roles for Knk and Rtv in chitin organization

The function and structure of the luminal chitin matrix depends on Knk and Rtv. How may these proteins affect chitin organization? Chitin-containing matrices in nature often occur in specific arrangements. The first level of organization is the formation of chitin microfibrils (Merzendorfer, 2006). Newly synthesized chitin chains extrude into the extracellular space, where they come together and hydrogen bond to form such microfibrils. X-ray analyses have revealed three different crystalline forms of chitin microfibrils, called α-, β-and γ-chitin. The α-form is hard and inflexible due to an anti-parallel organization of the chitin polymers, which are stabilized by a high number of hydrogen bonds, leading to a tight packaging of the fibrils. α-chitin is abundant in the insect cuticle, and also in the cell walls of fungi and yeast (Siemieniewicz et al., 2007). In the β-form the chitin chains are arranged in a parallel fashion, and in γ-chitin, two parallel strands alternate with one anti-parallel strand in between. The latter two chitin forms have less inter-fibrillar hydrogen bonds, and are softer since the chains are not as tightly packed as in α-chitin. β-chains and γ-chains allow increased hydrogen bonding with water, and the β-form of chitin is predominant in insect cocoons and present in the peritrophic matrix ((Merzendorfer, 2006)).

A next level of chitin organization appears to involve the bundling of microfibrils. In yeast, for example, nascent chitin microfibrils are thought gradually mature to a more robust form by covalent attachment of chitin to (1,3)- and (1,6)-

glucans, which, in turn, is attached to various cell wall proteins (Hartland et al., 1994; Kollar et al., 1995; Kollar et al., 1997). The insect cuticle and peritrophic matrix are also rich in proteins (Fristrom et al., 1978; Hegedus et al., 2009), some of which contain chitin-binding domains that may help to bundle chitin microfibrils into higher order structures.

The mechanisms by which newly synthesized chitin chains assemble into the right form of microfibrils and higher ordered arrangements are unclear. In yeast, the microfibrils can form different structures, which appear to depend on the specific chitin synthase (Lenardon et al., 2007). It is therefore likely that the chitin synthase together with co-factors have a role in determining the chitin structure already when the chains extrude from the plasma membrane into the extracellular space. Since Knk, and possibly also Rtv, are required at the plasma, they are unlikely to be component of the chitin matrix. Instead, they may localize together with CS-1 at the apical membrane, allowing correct organization of newly formed chitin chains. The dense chitin synthesizing plaques in the cuticle, and the observed disorganization of the chitin matrix upon reduced chitin synthesis (by Niccomycin Z) also suggest that CS-1 molecules are clustered to enable a critical amount of newly synthesized chitin chains for correct assembly. It is thus possible that Knk and Rtv function to cluster CS-1 molecules.

Paper III: Balancing luminal and sub-apical forces regulate tube size and shape in the Drosophila trachea

In this paper, we have studied regulation of tube elongation by the involvement of luminal as well as sub-apical components in the development of the tracheal dorsal trunks.

CONCLUSIONS

I.

- An intraluminal chitinous filament, synthesized by CS-1, is required for uniform tracheal tube growth. In *kkv* mutants, which lack functional CS-1, the tubes become overgrown and develop local constrictions and dilations.

- A group of tube size mutants affecting septate junction components are essential for correct chitin filament organization.

II.

- The two new tube size genes *knk* and *rtv* encode predicted membrane-associated proteins needed for assembly of the intraluminal chitin filament. Embryos deficient for *knk* and *rtv* develop a phenotype resembling that of *kkv* mutant embryos.
- Intact septate junctions are required for correct localization of Knk to the apical cell surface.

The results obtained from these studies point to an important role of the lumen environment and subapical cytoskeleton in the regulation of epithelial lumen size and shape. The principles that a lumen matrix models tube shape, an intraluminal pressure drives lumen volume expansion and specific organizations of the subapical structures of the tube wall influence the direction of lumen expansion, may well hold for epithelial lumen morphogenesis in general.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all people who have contributed to this thesis in one way or another. Special thanks to:

Min handledare **Anne Uv** för att du tog mig under dina vingar och lät mig få vara doktorand i din grupp. Tack också för ditt aldrig sinande engagemang, din otroliga kompetens, proffesionalism och din ständiga närvaro. Handledare som du växer inte på träd!

Anna Nilton, för att du var en så härlig doktorand-medsyster under min första ”halvlek”, jag har verkligen saknat dig! Vi har delat skratt och tårar, deltagit på roliga konferenser samt varvat förlovningar, barn och bröllop. Vi ses snart igen!

Alla”gruppmedlemmar” för många härliga skratt. **Iris**, Jag vet att du har en egen grupp, men det känns ändå som om vi tillhör samma storgrupp! Tack för all vetenskaplig feedback och all peppning, det betyder mycket! **Tina**, för att du är så väldigt omtänksam och energisk samt att du håller så bra ordning och reda på oss alla! Tack också för alla milslånga sms. Boosisar! **Hamid**, för din softa stil och för att du alltid är så hjälpsam! Jag gillar också den lillebrorsaktiga retstickan med glimten i ögat som bor i dig! Thanks also to **Zulfi**, the other half of the ”Akbar brothers”, a true McGyver that has turned out to be indispensible regarding all the technical issues in our group. You are also a truely nice guy! **Fariba**, för att du är så lugn och snäll och alltid omtänksamt frågar hur det går! **Simon**, en bioinformatiker med ett stort hjärta! **Erik**, du är en skön snubbe och utan dig hade jag ju aldrig kunnat lista ut att det funkar att äta tofubiffen med makrill i tomatsås + blue cheese tub-ost! **Jonas** ”Piglet”, det var så roligt att ha dig hos oss! Tack också för Kvack! **Simin** det är jätteroligt att ha dig tillbaka och det är mysigt att fika och luncha med dig. Du har alltid varit en sådan varm och empatisk person. Lycka till med din avhandling!!

Till **Stina Simonsson** och hennes tjejer för att ni har stått ut med mig i ert skrivrum! Tack **Helena** för att du alltid har en så härligt positiv attityd, och **Dzeneta** för din varma personlighet och för att du orkar lyssna när jag behöver gnälla av mig. Det kommer att gå bra för er!!

Yalda, du är en klippa! Du har varit en tillgång för mig i form av rådgivning och stöd kring doktorandandet och annat, i stort och smått, på sista tiden. Tack också för ditt glada leende. Jag önskar dig all lycka!!

Bernard Moussian, for excellent collaborations during the past years. Tanks also for your cheerful spirit. I will never forget your dance on the red carpet! Welcome to Sweden!

All **co-authors** of the papers in this thesis, thank you!

Tack till kollegor och vänner, (nuvarande och före detta):
Tack **Louise** för alla roliga och mysiga stunder med dig och din familj, för alla ovärdeliga ”mamma-tips” och för fikastunder på lab. Jag tycker så mycket om dig, synd bara att du aldrig är här när jag är där, om du förstår vad jag menar…Tack för att du är min vän!!! Tack **Alex**, för en fantastisk vistelse i Amerika. Skönt att ni äntligen har kommit hem nu! Tack också för din positiva energi, den smittar av sig! Men mest av allt skall jag väl kanske tacka dig och **Cicci** för att ni styrde mig på rätt vägar i kärlekens tecken. Tack också till Cicci för

skidsemestrar och allt annat roligt. Lycka till med studierna, du kommer att bli en bra doktor! Tack **Fina** för allt du gjorde för mig första gången jag försökte mig på att doktorera. Det betydde så mycket mer för mig än vad du kan ana!! **Carina** och **Elisabet** för alla glada skratt och snack om vitt och brett. **Åsa**, och **Lizhen** för alla roliga diskussioner. **Jessica**, för ditt otroliga norrländska lugn för pratstunder i korridoren! **Paula**, för att du som jag vet att frukt inte är godis! **Emil** och **Jenny**, för att ni är så goa och för att ni förstår vikten av en riktigt bra vagn ;-). Vem vet, jag kanske hänger med till våren?! Jenny, lycka till med doktorerandet! Tack också **Kornelia** för allt roligt, jag har saknat dig!

Gunnar Hansson med grupp samt **MPE**. Det var bra att ni flyttade ner till oss i källaren så det blev lite liv och rörelse igen! Speciellt tack också till **Frida** för intressanta dagisdiskussioner och för att du är så go!

Susann Teneberg med grupp, **Lena** och **Marianne**, tack för lån av material samt underhållande ”fika-betraktelser”! Tack ocksk **Halina** för trevligt fika sällskap.

Sven Enerbäck med grupp. Tack för lån av spektrofotometer och annan apparatur. Tack också till **Rickard** för all skönsång, **Zahra**, för att du är så gullig och omtänksam. Tack också till **Joakim** och **Gunilla** för all omtanke ända sedan ”Semb-tiden”.

Tack till **Henrik Semb** för att jag, en gång i tiden, fick börja hos dig som doktorand!

Varmt tack till kansliet för all hjälp!

Varmt tack till alla er som håller det fint och rent nere hos oss!

Julia and **Maria** at CCI, thank you for all you help at the confocal!

Till elektonmikroskopifaciliteten med **Yvonne**, **Gunnel**, **Ulf** och **Bengt.**. Tack för en fantastiskt bra kurs och för alla fina bilder som ni hjälpte mig med!!

Tack så mycket till min granne, den nybakade doktorn **Cecilia,** för korrekturläsning. Jag uppskattar det verkligen!! Lycka till nu med bulle nummer två!

Tack alla ni vänner och bekanta som förgyllt livet utanför forskningsvärlden.

Speciellt tack till: **Katarina**, min ”bästis” sedan barndomsåren. Nu firar vi snart 30-års jubiléum, och den här gången måste vi verkligen fira!! **Anne**, tack för alla delade asgarv och tårar, jag gillar dig så fantastiskt mycket och du kommer alltid att ha en speciell plats i mitt hjärta! Nästa gång ni kommer till Göteborg så skall jag och Peter på en ”revival trip” till Topasgatan. **Liselott**, **Catharina** och **Emma**, tack för all omtanke och för alla mysiga luncher, fikor, sommarutflykter, pyssel o.d. Jag ser fram emot att hinna träffas mer! **Anna W** för alla roliga fester och mysiga fikastunder och för att du är en så väldigt go tjej! Lycka till med allt!! **Lovisa**, **Maria** och **Elin** mina pluggkompisar från Umeå-tiden, jag saknar er! **Börje** och **Margareta** de coolaste f.d. bönderna och ”globetrotters” som jag vet!

Eva du är så snäll och gullig emot oss. Jag uppskattar också alla berättelser från när Jonas var liten. Tack också för att du är så snäll mot Viggo!

Janne, tack för att du tar så bra hand om oss och för att du alltid raskt kastar dig i bilen för att hjälpa oss med alla våra projekt. Tack också för att vi får ha ett rum hos dig!

Tack **Svante**, **Helene**, **Moa** och **Isak**, för alla Borrås-vistelser (nej, det heter inte Borås), för all hjälp med renoveringar och allt bus! Tack Moa och Isak för att ni är så goa, jag tycker så mycket om er!

Tack farmor **Ella** för alla varma och salta sommrar på Orust och för att du alltid har varit så intresserad av vad jag har gjort! Tack farfar **Allan** för alla sångstunder och för allt du snickrade åt mig. Tack för alla goa kramar, jag saknar dig! Tack mormor **Vera** för att du har varit och är den bästa mormorn som en liten ”Sippa” kan önska sig! Tack morfar **Lennart** för många ljusa barndomsminnen och för allt godis!

Tobias, tack för att du är du!! Tack också för att du tar så bra hand om Viggo och leker honom trött, det är inte många som klarar det skall jag säga dig! Jag älskar dig!

Mamma **Joyce** och pappa **Pelle**, tack för att ni alltid har funnits där för mig på alla sätt och vis! Ni har kanske prövats lite mer än andra föräldrar men ni har gjort det bra och jag är väldigt stolt över er! Tack också för att ni är världen bästa mormor och morfar och all hjälp jag har fått med hämtningar från dagis och alla barnpassningar! Jag älskar er!

Viggo, min älskade lilla skitunge. Tack för att du förankrar mig i verkligheten och för att du är så himla go och busig! Tack för att du finns!! Mamma älskar dig hela vägen upp till månen!

Jonas, Jag vet att jag att jag inte har varit så lätt att leva med den sista tiden och du förtjänar verkligen ett diplom! Tack för allt stöd, peppning och kärlek, och tack för all mat. Du börjar att bli en j-kel i köket! Tack också för att du är världens bästa pappa! Jag älskar dig❤ och jag ser fram emot allt vi skall göra tillsammans. Du och jag, vi är av samma slag…!

EB

REFERENCES

Abrams, E. W., Mihoulides, W. K. and Andrew, D. J. (2006). Fork head and Sage maintain a uniform and patent salivary gland lumen through regulation of two downstream target genes, PH4alphaSG1 and PH4alphaSG2. *Development* **133**, 3517-27.

Affolter, M., Bellusci, S., Itoh, N., Shilo, B., Thiery, J. P. and Werb, Z. (2003). Tube or not tube: remodeling epithelial tissues by branching morphogenesis. *Dev Cell* **4**, 11-8.

Affolter, M. and Shilo, B. Z. (2000). Genetic control of branching morphogenesis during Drosophila tracheal development. *Curr Opin Cell Biol* **12**, 731-5.

Anderson, J. M. (2001). Molecular structure of tight junctions and their role in epithelial transport. *News Physiol Sci* **16**, 126-30.

Anderson, M. G., Perkins, G. L., Chittick, P., Shrigley, R. J. and Johnson, W. A. (1995). drifter, a Drosophila POU-domain transcription factor, is required for correct differentiation and migration of tracheal cells and midline glia. *Genes Dev* **9**, 123-37.

Bachmann, A., Schneider, M., Theilenberg, E., Grawe, F. and Knust, E. (2001). Drosophila Stardust is a partner of Crumbs in the control of epithelial cell polarity. *Nature* **414**, 638-43.

Bakall, B., McLaughlin, P., Stanton, J. B., Zhang, Y., Hartzell, H. C., Marmorstein, L. Y. and Marmorstein, A. D. (2008). Bestrophin-2 is involved in the generation of intraocular pressure. *Invest Ophthalmol Vis Sci* **49**, 1563-70.

Barro Soria, R., Spitzner, M., Schreiber, R. and Kunzelmann, K. (2006). Bestrophin 1 enables Ca2+ activated Cl- conductance in epithelia. *J Biol Chem*.

Barro-Soria, R., Schreiber, R. and Kunzelmann, K. (2008). Bestrophin 1 and 2 are components of the Ca(2+) activated Cl(-) conductance in mouse airways. *Biochim Biophys Acta*.

Bartnicki-Garcia, S. (2006). Chitosomes: past, present and future. *FEMS Yeast Res* **6**, 957-65.

Baumgartner, S., Littleton, J. T., Broadie, K., Bhat, M. A., Harbecke, R., Lengyel, J. A., Chiquet-Ehrismann, R., Prokop, A. and Bellen, H. J. (1996). A Drosophila neurexin is required for septate junction and blood-nerve barrier formation and function. *Cell* **87**, 1059-68.

Behr, M., Riedel, D. and Schuh, R. (2003). The claudin-like megatrachea is essential in septate junctions for the epithelial barrier function in Drosophila. *Dev Cell* **5**, 611-20.

Beitel, G. J. and Krasnow, M. A. (2000). Genetic control of epithelial tube size in the Drosophila tracheal system. *Development* **127**, 3271-82.

Bhat, M. A., Izaddoost, S., Lu, Y., Cho, K. O., Choi, K. W. and Bellen, H. J. (1999). Discs Lost, a novel multi-PDZ domain protein, establishes and maintains epithelial polarity. *Cell* **96**, 833-45.

Bilder, D., Li, M. and Perrimon, N. (2000). Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. *Science* **289**, 113-6.

Bilder, D., Schober, M. and Perrimon, N. (2003). Integrated activity of PDZ protein complexes regulates epithelial polarity. *Nat Cell Biol* **5**, 53-8.

Boletta, A. and Germino, G. G. (2003). Role of polycystins in renal tubulogenesis. *Trends Cell Biol* **13**, 484-92.
Bray, S. J. and Kafatos, F. C. (1991). Developmental function of Elf-1: an essential transcription factor during embryogenesis in Drosophila. *Genes Dev* **5**, 1672-83.
Buechner, M., Hall, D. H., Bhatt, H. and Hedgecock, E. M. (1999). Cystic canal mutants in Caenorhabditis elegans are defective in the apical membrane domain of the renal (excretory) cell. *Dev Biol* **214**, 227-41.
Carlson, S. D., Juang, J. L., Hilgers, S. L. and Garment, M. B. (2000). Blood barriers of the insect. *Annu Rev Entomol* **45**, 151-74.
Chien, L. T. and Hartzell, H. C. (2007). Drosophila bestrophin-1 chloride current is dually regulated by calcium and cell volume. *J Gen Physiol* **130**, 513-24.
Chien, L. T., Zhang, Z. R. and Hartzell, H. C. (2006). Single Cl- channels activated by Ca2+ in Drosophila S2 cells are mediated by bestrophins. *J Gen Physiol* **128**, 247-59.
Chihara, T. and Hayashi, S. (2000). Control of tracheal tubulogenesis by Wingless signaling. *Development* **127**, 4433-42.
Cohen, E. (2001). Chitin synthesis and inhibition: a revisit. *Pest Manag Sci* **57**, 946-50.
de Celis, J. F., Llimargas, M. and Casanova, J. (1995). Ventral veinless, the gene encoding the Cf1a transcription factor, links positional information and cell differentiation during embryonic and imaginal development in Drosophila melanogaster. *Development* **121**, 3405-16.
Fischmeister, R. and Hartzell, H. C. (2005). Volume sensitivity of the bestrophin family of chloride channels. *J Physiol* **562**, 477-91.
Fraichard, S., Bouge, A. L., Chauvel, I. and Bouhin, H. (2006). Tenectin, a novel extracellular matrix protein expressed during Drosophila melanogaster embryonic development. *Gene Expr Patterns* **6**, 772-6.
Fristrom, J. W., Hill, R. J. and Watt, F. (1978). The procuticle of Drosophila: heterogeneity of urea-soluble proteins. *Biochemistry* **17**, 3917-30.
Genova, J. L. and Fehon, R. G. (2003). Neuroglian, Gliotactin, and the Na+/K+ ATPase are essential for septate junction function in Drosophila. *J Cell Biol* **161**, 979-89.
Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D. et al. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* **161**, 1163-77.
Ghabrial, A., Luschnig, S., Metzstein, M. M. and Krasnow, M. A. (2003). Branching morphogenesis of the Drosophila tracheal system. *Annu Rev Cell Dev Biol* **19**, 623-47.
Gheri, G., Sgambati, E. and Bryk, S. G. (2000). Glycoconjugate sugar residues in the chick embryo developing lung: a lectin histochemical study. *J Morphol* **243**, 257-64.
Gibson, M. C. and Perrimon, N. (2003). Apicobasal polarization: epithelial form and function. *Curr Opin Cell Biol* **15**, 747-52.

Glazer, L. and Shilo, B. Z. (1991). The Drosophila FGF-R homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension. *Genes Dev* **5**, 697-705.
Glazer, L. and Shilo, B. Z. (2001). Hedgehog signaling patterns the tracheal branches. *Development* **128**, 1599-606.
Gobel, V., Barrett, P. L., Hall, D. H. and Fleming, J. T. (2004). Lumen morphogenesis in C. elegans requires the membrane-cytoskeleton linker erm-1. *Dev Cell* **6**, 865-73.
Grenningloh, G., Rehm, E. J. and Goodman, C. S. (1991). Genetic analysis of growth cone guidance in Drosophila: fasciclin II functions as a neuronal recognition molecule. *Cell* **67**, 45-57.
Guillemin, K., Groppe, J., Ducker, K., Treisman, R., Hafen, E., Affolter, M. and Krasnow, M. A. (1996). The pruned gene encodes the Drosophila serum response factor and regulates cytoplasmic outgrowth during terminal branching of the tracheal system. *Development* **122**, 1353-62.
Gumbiner, B. M. (2005). Regulation of cadherin-mediated adhesion in morphogenesis. *Nat Rev Mol Cell Biol* **6**, 622-34.
Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y. and Krasnow, M. A. (1998). sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. *Cell* **92**, 253-63.
Hartland, R. P., Vermeulen, C. A., Klis, F. M., Sietsma, J. H. and Wessels, J. G. (1994). The linkage of (1-3)-beta-glucan to chitin during cell wall assembly in Saccharomyces cerevisiae. *Yeast* **10**, 1591-9.
Hartzell, H. C., Qu, Z., Yu, K., Xiao, Q. and Chien, L. T. (2008). Molecular physiology of bestrophins: multifunctional membrane proteins linked to best disease and other retinopathies. *Physiol Rev* **88**, 639-72.
Hegedus, D., Erlandson, M., Gillott, C. and Toprak, U. (2009). New Insights into Peritrophic Matrix Synthesis, Architecture, and Function. *Annu Rev Entomol* **54**, 285-302.
Hemphala, J., Uv, A., Cantera, R., Bray, S. and Samakovlis, C. (2003). Grainy head controls apical membrane growth and tube elongation in response to Branchless/FGF signalling. *Development* **130**, 249-58.
Herth, W. (1980). Calcofluor white and Congo red inhibit chitin microfibril assembly of Poterioochromonas: evidence for a gap between polymerization and microfibril formation. *J Cell Biol* **87**, 442-50.
Hong, Y., Stronach, B., Perrimon, N., Jan, L. Y. and Jan, Y. N. (2001). Drosophila Stardust interacts with Crumbs to control polarity of epithelia but not neuroblasts. *Nature* **414**, 634-8.
Hutterer, A., Betschinger, J., Petronczki, M. and Knoblich, J. A. (2004). Sequential roles of Cdc42, Par-6, aPKC, and Lgl in the establishment of epithelial polarity during Drosophila embryogenesis. *Dev Cell* **6**, 845-54.
Ikeya, T. and Hayashi, S. (1999). Interplay of Notch and FGF signaling restricts cell fate and MAPK activation in the Drosophila trachea. *Development* **126**, 4455-63.
Imam, F., Sutherland, D., Huang, W. and Krasnow, M. A. (1999). stumps, a Drosophila gene required for fibroblast growth factor (FGF)-directed migrations of tracheal and mesodermal cells. *Genetics* **152**, 307-18.

Jarecki, J., Johnson, E. and Krasnow, M. A. (1999). Oxygen regulation of airway branching in Drosophila is mediated by branchless FGF. *Cell* **99**, 211-20.
Jarvis, L. A., Toering, S. J., Simon, M. A., Krasnow, M. A. and Smith-Bolton, R. K. (2006). Sprouty proteins are in vivo targets of Corkscrew/SHP-2 tyrosine phosphatases. *Development* **133**, 1133-42.
Joberty, G., Petersen, C., Gao, L. and Macara, I. G. (2000). The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. *Nat Cell Biol* **2**, 531-9.
Jones, S. J. and Baillie, D. L. (1995). Characterization of the let-653 gene in Caenorhabditis elegans. *Mol Gen Genet* **248**, 719-26.
Jurgens, G., Wieschaus, E., Nusslein-Volhard, C. and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster. *Roux Arch. dev. Biol.* **193**, 283-295.
Klambt, C., Glazer, L. and Shilo, B. Z. (1992). breathless, a Drosophila FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. *Genes Dev* **6**, 1668-78.
Kobielak, A. and Fuchs, E. (2004). Alpha-catenin: at the junction of intercellular adhesion and actin dynamics. *Nat Rev Mol Cell Biol* **5**, 614-25.
Kollar, R., Petrakova, E., Ashwell, G., Robbins, P. W. and Cabib, E. (1995). Architecture of the yeast cell wall. The linkage between chitin and beta(1-->3)-glucan. *J Biol Chem* **270**, 1170-8.
Kollar, R., Reinhold, B. B., Petrakova, E., Yeh, H. J., Ashwell, G., Drgonova, J., Kapteyn, J. C., Klis, F. M. and Cabib, E. (1997). Architecture of the yeast cell wall. Beta(1-->6)-glucan interconnects mannoprotein, beta(1-->)3-glucan, and chitin. *J Biol Chem* **272**, 17762-75.
Lamb, R. S., Ward, R. E., Schweizer, L. and Fehon, R. G. (1998). Drosophila coracle, a member of the protein 4.1 superfamily, has essential structural functions in the septate junctions and developmental functions in embryonic and adult epithelial cells. *Mol Biol Cell* **9**, 3505-19.
Lebovitz, R. M., Takeyasu, K. and Fambrough, D. M. (1989). Molecular characterization and expression of the (Na+ + K+)-ATPase alpha-subunit in Drosophila melanogaster. *Embo J* **8**, 193-202.
Lehane, M. J. (1997). Peritrophic matrix structure and function. *Annu Rev Entomol* **42**, 525-50.
Lenardon, M. D., Whitton, R. K., Munro, C. A., Marshall, D. and Gow, N. A. (2007). Individual chitin synthase enzymes synthesize microfibrils of differing structure at specific locations in the Candida albicans cell wall. *Mol Microbiol* **66**, 1164-73.
Lin, D., Edwards, A. S., Fawcett, J. P., Mbamalu, G., Scott, J. D. and Pawson, T. (2000). A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. *Nat Cell Biol* **2**, 540-7.
Llimargas, M. (1999). The Notch pathway helps to pattern the tips of the Drosophila tracheal branches by selecting cell fates. *Development* **126**, 2355-64.
Llimargas, M. (2000). Wingless and its signalling pathway have common and separable functions during tracheal development. *Development* **127**, 4407-17.

Llimargas, M., Strigini, M., Katidou, M., Karagogeos, D. and Casanova, J. (2004). Lachesin is a component of a septate junction-based mechanism that controls tube size and epithelial integrity in the Drosophila tracheal system. *Development* **131**, 181-90.
Locke, M. (2001). The Wigglesworth Lecture: Insects for studying fundamental problems in biology. *J Insect Physiol* **47**, 495-507.
Lubarsky, B. and Krasnow, M. A. (2003). Tube morphogenesis: making and shaping biological tubes. *Cell* **112**, 19-28.
Luschnig, S., Batz, T., Armbruster, K. and Krasnow, M. A. (2006). serpentine and vermiform encode matrix proteins with chitin binding and deacetylation domains that limit tracheal tube length in Drosophila. *Curr Biol* **16**, 186-94.
Mason, J. M., Morrison, D. J., Basson, M. A. and Licht, J. D. (2006). Sprouty proteins: multifaceted negative-feedback regulators of receptor tyrosine kinase signaling. *Trends Cell Biol* **16**, 45-54.
Matchkov, V. V., Larsen, P., Bouzinova, E. V., Rojek, A., Boedtkjer, D. M., Golubinskaya, V., Pedersen, F. S., Aalkjaer, C. and Nilsson, H. (2008). Bestrophin-3 (vitelliform macular dystrophy 2-like 3 protein) is essential for the cGMP-dependent calcium-activated chloride conductance in vascular smooth muscle cells. *Circ Res* **103**, 864-72.
Matsuo, T., Takahashi, K., Kondo, S., Kaibuchi, K. and Yamamoto, D. (1997). Regulation of cone cell formation by Canoe and Ras in the developing Drosophila eye. *Development* **124**, 2671-80.
Matusek, T., Djiane, A., Jankovics, F., Brunner, D., Mlodzik, M. and Mihaly, J. (2006). The Drosophila formin DAAM regulates the tracheal cuticle pattern through organizing the actin cytoskeleton. *Development* **133**, 957-66.
Merzendorfer, H. (2006). Insect chitin synthases: a review. *J Comp Physiol [B]* **176**, 1-15.
Merzendorfer, H. and Zimoch, L. (2003). Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. *J Exp Biol* **206**, 4393-412.
Milenkovic, V. M., Langmann, T., Schreiber, R., Kunzelmann, K. and Weber, B. H. (2008). Molecular evolution and functional divergence of the bestrophin protein family. *BMC Evol Biol* **8**, 72.
Milenkovic, V. M., Soria, R. B., Aldehni, F., Schreiber, R. and Kunzelmann, K. (2009). Functional assembly and purinergic activation of bestrophins. *Pflugers Arch*.
Moussian, B., Schwarz, H., Bartoszewski, S. and Nusslein-Volhard, C. (2005a). Involvement of chitin in exoskeleton morphogenesis in Drosophila melanogaster. *J Morphol* **264**, 117-30.
Moussian, B., Soding, J., Schwarz, H. and Nusslein-Volhard, C. (2005b). Retroactive, a membrane-anchored extracellular protein related to vertebrate snake neurotoxin-like proteins, is required for cuticle organization in the larva of Drosophila melanogaster. *Dev Dyn* **233**, 1056-63.
Myat, M. M. and Andrew, D. J. (2002). Epithelial tube morphology is determined by the polarized growth and delivery of apical membrane. *Cell* **111**, 879-91.
Nusslein-Volhard, C., Wieschaus, E. and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle of Drodophila Melanogaster. *Roux Arch. dev. Biol.* **193**, 267-282.

O'Driscoll, K. E., Hatton, W. J., Burkin, H. R., Leblanc, N. and Britton, F. C. (2008). Expression, localization, and functional properties of Bestrophin 3 channel isolated from mouse heart. *Am J Physiol Cell Physiol* **295**, C1610-24.

Obara, T., Mangos, S., Liu, Y., Zhao, J., Wiessner, S., Kramer-Zucker, A. G., Olale, F., Schier, A. F. and Drummond, I. A. (2006). Polycystin-2 immunolocalization and function in zebrafish. *J Am Soc Nephrol* **17**, 2706-18.

Ostrowski, S., Dierick, H. A. and Bejsovec, A. (2002). Genetic control of cuticle formation during embryonic development of Drosophila melanogaster. *Genetics* **161**, 171-82.

Paul, S. M., Ternet, M., Salvaterra, P. M. and Beitel, G. J. (2003). The Na+/K+ ATPase is required for septate junction function and epithelial tube-size control in the Drosophila tracheal system. *Development* **130**, 4963-74.

Perez-Moreno, M., Jamora, C. and Fuchs, E. (2003). Sticky business: orchestrating cellular signals at adherens junctions. *Cell* **112**, 535-48.

Petronczki, M. and Knoblich, J. A. (2001). DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in Drosophila. *Nat Cell Biol* **3**, 43-9.

Pielage, J., Stork, T., Bunse, I. and Klambt, C. (2003). The Drosophila cell survival gene discs lost encodes a cytoplasmic Codanin-1-like protein, not a homolog of tight junction PDZ protein Patj. *Dev Cell* **5**, 841-51.

Pifferi, S., Pascarella, G., Boccaccio, A., Mazzatenta, A., Gustincich, S., Menini, A. and Zucchelli, S. (2006). Bestrophin-2 is a candidate calcium-activated chloride channel involved in olfactory transduction. *Proc Natl Acad Sci U S A* **103**, 12929-34.

Qu, Z., Fischmeister, R. and Hartzell, C. (2004). Mouse bestrophin-2 is a bona fide Cl(-) channel: identification of a residue important in anion binding and conduction. *J Gen Physiol* **123**, 327-40.

Qu, Z., Wei, R. W., Mann, W. and Hartzell, H. C. (2003). Two bestrophins cloned from Xenopus laevis oocytes express Ca(2+)-activated Cl(-) currents. *J Biol Chem* **278**, 49563-72.

Roh, M. H., Makarova, O., Liu, C. J., Shin, K., Lee, S., Laurinec, S., Goyal, M., Wiggins, R. and Margolis, B. (2002). The Maguk protein, Pals1, functions as an adapter, linking mammalian homologues of Crumbs and Discs Lost. *J Cell Biol* **157**, 161-72.

Roh, M. H. and Margolis, B. (2003). Composition and function of PDZ protein complexes during cell polarization. *Am J Physiol Renal Physiol* **285**, F377-87.

Samakovlis, C., Hacohen, N., Manning, G., Sutherland, D. C., Guillemin, K. and Krasnow, M. A. (1996a). Development of the Drosophila tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. *Development* **122**, 1395-407.

Samakovlis, C., Manning, G., Steneberg, P., Hacohen, N., Cantera, R. and Krasnow, M. A. (1996b). Genetic control of epithelial tube fusion during Drosophila tracheal development. *Development* **122**, 3531-6.

Scholz, H., Deatrick, J., Klaes, A. and Klambt, C. (1993). Genetic dissection of pointed, a Drosophila gene encoding two ETS-related proteins. *Genetics* **135**, 455-68.

Schumacher, K., Strehl, R. and Minuth, W. W. (2002). Detection of glycosylated sites in embryonic rabbit kidney by lectin chemistry. *Histochem Cell Biol* **118**, 79-87.

Shweiki, D., Itin, A., Soffer, D. and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* **359**, 843-5.
Siemieniewicz, K. W., Kajla, M. K. and Schrempf, H. (2007). Elucidating the biosynthesis of chitin filaments and their configuration with specific proteins and electron microscopy. *Macromol Biosci* **7**, 40-7.
St Johnston, D. (2002). The art and design of genetic screens: Drosophila melanogaster. *Nat Rev Genet* **3**, 176-88.
Steneberg, P., Hemphala, J. and Samakovlis, C. (1999). Dpp and Notch specify the fusion cell fate in the dorsal branches of the Drosophila trachea. *Mech Dev* **87**, 153-63.
Sun, H., Tsunenari, T., Yau, K. W. and Nathans, J. (2002). The vitelliform macular dystrophy protein defines a new family of chloride channels. *Proc Natl Acad Sci U S A* **99**, 4008-13.
Sutherland, D., Samakovlis, C. and Krasnow, M. A. (1996). branchless encodes a Drosophila FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell* **87**, 1091-101.
Syed, Z. A., Hard, T., Uv, A. and van Dijk-Hard, I. F. (2008). A potential role for Drosophila mucins in development and physiology. *PLoS ONE* **3**, e3041.
Takahashi, K., Matsuo, T., Katsube, T., Ueda, R. and Yamamoto, D. (1998). Direct binding between two PDZ domain proteins Canoe and ZO-1 and their roles in regulation of the jun N-terminal kinase pathway in Drosophila morphogenesis. *Mech Dev* **78**, 97-111.
Takai, Y. and Nakanishi, H. (2003). Nectin and afadin: novel organizers of intercellular junctions. *J Cell Sci* **116**, 17-27.
Tanaka-Matakatsu, M., Uemura, T., Oda, H., Takeichi, M. and Hayashi, S. (1996). Cadherin-mediated cell adhesion and cell motility in Drosophila trachea regulated by the transcription factor Escargot. *Development* **122**, 3697-705.
Tanentzapf, G. and Tepass, U. (2003). Interactions between the crumbs, lethal giant larvae and bazooka pathways in epithelial polarization. *Nat Cell Biol* **5**, 46-52.
Tellam, R. L., Vuocolo, T., Johnson, S. E., Jarmey, J. and Pearson, R. D. (2000). Insect chitin synthase cDNA sequence, gene organization and expression. *Eur J Biochem* **267**, 6025-43.
Tepass, U. and Harris, K. P. (2007). Adherens junctions in Drosophila retinal morphogenesis. *Trends Cell Biol* **17**, 26-35.
Tepass, U. and Hartenstein, V. (1994). The development of cellular junctions in the Drosophila embryo. *Dev Biol* **161**, 563-96.
Tepass, U., Tanentzapf, G., Ward, R. and Fehon, R. (2001). Epithelial cell polarity and cell junctions in Drosophila. *Annu Rev Genet* **35**, 747-84.
Tepass, U., Theres, C. and Knust, E. (1990). crumbs encodes an EGF-like protein expressed on apical membranes of Drosophila epithelial cells and required for organization of epithelia. *Cell* **61**, 787-99.
Tepass, U., Truong, K., Godt, D., Ikura, M. and Peifer, M. (2000). Cadherins in embryonic and neural morphogenesis. *Nat Rev Mol Cell Biol* **1**, 91-100.
Tian, E. and Ten Hagen, K. G. (2007). O-linked glycan expression during Drosophila development. *Glycobiology* **17**, 820-7.

Uv, A., Cantera, R. and Samakovlis, C. (2003). Drosophila tracheal morphogenesis: intricate cellular solutions to basic plumbing problems. *Trends Cell Biol* **13**, 301-9.
Vincent, S., Ruberte, E., Grieder, N. C., Chen, C. K., Haerry, T., Schuh, R. and Affolter, M. (1997). DPP controls tracheal cell migration along the dorsoventral body axis of the Drosophila embryo. *Development* **124**, 2741-50.
Vincent, S., Wilson, R., Coelho, C., Affolter, M. and Leptin, M. (1998). The Drosophila protein Dof is specifically required for FGF signaling. *Mol Cell* **2**, 515-25.
Wang, S., Jayaram, S. A., Hemphala, J., Senti, K. A., Tsarouhas, V., Jin, H. and Samakovlis, C. (2006). Septate-junction-dependent luminal deposition of chitin deacetylases restricts tube elongation in the Drosophila trachea. *Curr Biol* **16**, 180-5.
Wappner, P., Gabay, L. and Shilo, B. Z. (1997). Interactions between the EGF receptor and DPP pathways establish distinct cell fates in the tracheal placodes. *Development* **124**, 4707-16.
Wei, S. Y., Escudero, L. M., Yu, F., Chang, L. H., Chen, L. Y., Ho, Y. H., Lin, C. M., Chou, C. S., Chia, W., Modolell, J. et al. (2005). Echinoid is a component of adherens junctions that cooperates with DE-Cadherin to mediate cell adhesion. *Dev Cell* **8**, 493-504.
Wieschaus, E., Nusslein-Volhard, C. and Jurgens, G. (1984). Mutations affecting the pattern of the larval cuticle of Drodophila Melanogaster. *Roux Arch. dev. Biol.* **193**, 196-307.
Wigglesworth, V. B. (1990). The properties of the lining membrane of the insect tracheal system. *Tissue Cell* **22**, 231-8.
Wilk, R., Weizman, I. and Shilo, B. Z. (1996). trachealess encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in Drosophila. *Genes Dev* **10**, 93-102.
Wodarz, A. (2002). Establishing cell polarity in development. *Nat Cell Biol* **4**, E39-44.
Wodarz, A., Ramrath, A., Grimm, A. and Knust, E. (2000). Drosophila atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. *J Cell Biol* **150**, 1361-74.
Woods, D. F., Hough, C., Peel, D., Callaini, G. and Bryant, P. J. (1996). Dlg protein is required for junction structure, cell polarity, and proliferation control in Drosophila epithelia. *J Cell Biol* **134**, 1469-82.
Wu, V. M. and Beitel, G. J. (2004). A junctional problem of apical proportions: epithelial tube-size control by septate junctions in the Drosophila tracheal system. *Curr Opin Cell Biol* **16**, 493-9.
Wu, V. M., Schulte, J., Hirschi, A., Tepass, U. and Beitel, G. J. (2004). Sinuous is a Drosophila claudin required for septate junction organization and epithelial tube size control. *J Cell Biol* **164**, 313-23.
Xiao, Q., Prussia, A., Yu, K., Cui, Y. Y. and Hartzell, H. C. (2008). Regulation of bestrophin Cl channels by calcium: role of the C terminus. *J Gen Physiol* **132**, 681-92.
Yagi, T. and Takeichi, M. (2000). Cadherin superfamily genes: functions, genomic organization, and neurologic diversity. *Genes Dev* **14**, 1169-80.
Zelzer, E. and Shilo, B. Z. (2000). Interaction between the bHLH-PAS protein Trachealess and the POU-domain protein Drifter, specifies tracheal cell fates. *Mech Dev* **91**, 163-73.