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THESIS IN BIOMEDICINE

The Filzig protein affects embryonic cuticle and taenidia organization in *Drosophila*.

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Contents

Introduction	1
Apical extracellular matrix (aECM) deposition by epithelial cells	1
The <i>Drosophila</i> exoskeleton as a model system for studies of aECM formation.....	2
The serine protease Filzig is required for tracheal taenidial organization.....	5
<i>Drosophila</i> as an amenable genetic model organism	7
Aim of the study	8
Methods and Materials	8
Fly lines and Genetics	8
Fly works.....	8
Embryo collection	9
Regular fixation.....	9
Heat fixation.....	9
Antibody staining	9
Cuticle preparation	10
Live imaging	10
Confocal imaging	10
Results and Discussion.....	11
Filzig mutants show disorganized cuticular pattern	11
Filzig is expressed in the trachea, epidermis, head skeleton and hindgut as cuticle deposition begins.	12
Filzig localizes the apical cell surface and to the extracellular cuticle.....	14
Filzig is required for chitin organization, but not for epithelial integrity.....	15
The effects of genes flanking <i>flz</i> on cuticle pattern.....	16
Conclusion.....	17
References	19
Acknowledgements	21

Abstract

The surface of multicellular organisms is covered with epithelial cells that provide a barrier to the external environment. As part of this barrier function, most epithelia produce apical extracellular matrices (aECMs). The generation of such chemical and physical barriers requires specialized deposition of macromolecules and is likely to involve a spatial and temporal coordination of biochemical activities at the apical surface. A challenging task is thus to characterize key proteins that underlie apical cell surface organization and correct aECM assembly. The *Drosophila* trachea provides an excellent system to study aECM formation, as they produce an ordered aECM, called the cuticle. The tracheal cuticle is unique by its presence of cuticular ridges, called taenidial folds, which prevent collapse of tracheal tubes while allowing them to expand and contract along their length. A gene called *filzig* encodes a transmembrane serine protease and is required for taenidial organization. The aim of this research was to further understand Filzig function through characterization of *filzig* mutants and Filzig protein expression. The results showed that Filzig is expressed in cuticle-producing epithelia as cuticle deposition begins. Moreover, Flz localized to the apical epithelial surface, as well as to the aECM. The apical Flz localization does not reflect the pattern of cuticle ridges, indicating that Flz-localization is not a determinant for taenidial patterning. Instead, Flz might act on extracellular targets that localize to the future taenidial folds. Alternatively, Filzig is involved in a cascade of self-organizing activity of cuticular components to form the regular taenidial folds.

Abbreviations

aECM-apical extracellular matrix

AJ-adherens junction

CBP-chitin binding proteins

Crb-crumbs

DECad-drosophila epithelial cadherin

DAAM-dishevelled associated activator of morphogenesis

GFP- green florescent protein

Flz-filzig

RISC- RNA-inducing silencing complex

SAR-sub apical region

SJ-septate junction

TJ-tight junction

TTSP-type II transmembrane serine protease

ZA-zonular adherens

Introduction

Apical extracellular matrix (aECM) deposition by epithelial cells

The surface of vertebrate and invertebrate organisms is covered with epithelial cells that provide a barrier to the external environment. Such epithelia also line most of our inner organs, such as the kidneys, lungs, liver, and vascular system. As part of their barrier functions, most epithelia produce apical extracellular matrices (aECM, Cambell, *et al.*, 2010). To accommodate this function, epithelial cells often form special apical membrane structures, for example microvilli, and specific micro-domains at the apical membrane, to facilitate and coordinate correct build-up of aECMs.

Most epithelia are single cell-layered, with the cells tightly connected to each other via intercellular junctions. A characteristic of all epithelia is that they exhibit an apical/basal polarity: the apical surface faces the exterior (outside of the epidermis and the lumen surface in inner organs), while the basal surface is in contact with internal tissues via a basal lamina. The functionality of an epithelium is highly dependent on the correct polarization of cellular components along the apicobasal axis, and there is an evolutionary conservation of the core molecular mechanisms underlying epithelial cell polarization among animals. However, studies of invertebrate and vertebrate epithelia have illustrated that these core mechanisms operate within the context of slightly different epithelial architecture (Figure 1). In insects, like the fruit fly *Drosophila melanogaster*, epithelia exhibit an apically localized cell-cell adhesive belt known as the Zonula Adherens (ZA). Basal to the ZA are the septate junctions (SJ), which are known to provide the paracellular diffusion barrier. Just apical to the ZA lies the sub apical region (SAR), which has an organizing role in epithelial polarization but is not known to function as a site of cell-cell junctions (Figure 1a). Contrasting with drosophila, vertebrate epithelial cells lack SJs and instead exhibit tight junctions (TJs). The TJs are cell – cell adhesive structures that lie apical to the vertebrate ZA in a position analogous to the drosophila SAR (Figure 1b). The apical TJ complexes between vertebrate epithelial cells serve an organizing role in epithelial polarization and as a paracellular diffusion barrier that restricts the movement of solutes across the cell layer (Gibson and Perriomon, 2003). The TJs effectively segregate the epithelium and surrounding media into immiscible apical and basolateral compartments. Although drosophila SJs lie basal to ZA, the SJs appear to fulfill a similar paracellular barrier role to the vertebrate TJs, and the two junctions appear to have similarities in protein composition. Despite differences in the distribution of cell-cell junctions, conserved sets of polarity proteins govern apico/basal polarization in both drosophila and vertebrate epithelia.

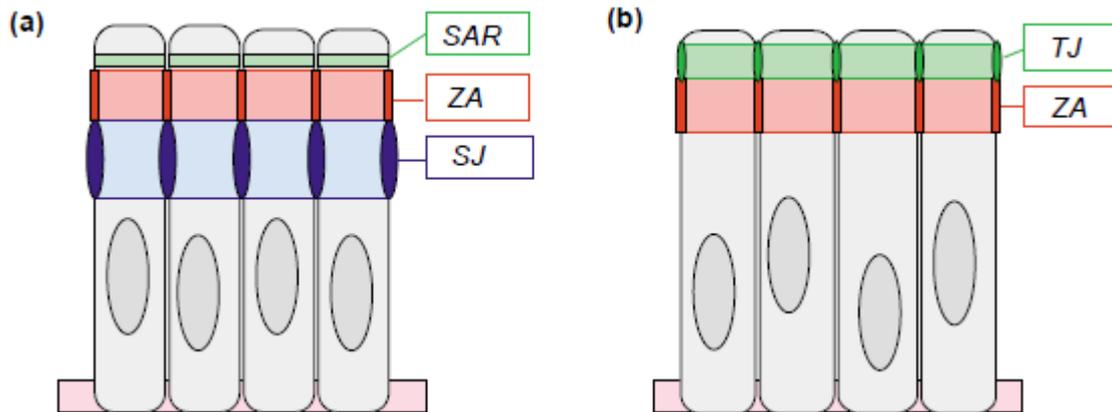


Figure 1. Comparison of vertebrate and insect epithelial junctions. Insect epithelial cells (a) have several junction complexes: The sub apical region (SAR) is essential for establishment of apical/basal polarity, the Zonula Adherens (ZA) contain the cadherin complex important for cell-cell adhesion, and the more lateral septate junctions (SJ) make up the paracellular diffusion barrier. In vertebrate epithelia (b) the tight junctions (TJ) are required to establish apical/basal polarity and also provide the paracellular diffusion barrier, while the ZA is equivalent to that of insect epithelia. Both types of epithelia secrete a basal lamina (pink). *From Gibson and Perrimon,2003.*

While extensive research has been devoted to the composition and function of lateral cell junctions, and to the interaction between the epithelium and the underlying tissues, there has been much less focus on the organization of the apical cell membrane and the correct deposition of aECMs to generate chemical and physical barriers vital to organ function. This activity is likely to require both spatial and temporal coordination of biochemical activities at the apical surface. A challenging task is therefore to identify and characterize key apical proteins that facilitate the organized activity of this cell surface that leads to correct assembly of the aECM.

The *Drosophila* exoskeleton as a model system for studies of aECM formation

Drosophila melanogaster, commonly known as the fruit fly, has been used as a model organism in studying basic mechanisms of inheritance since the time of Thomas Hunt Morgan from the beginning of the twentieth century. The fly's short life cycle, relatively small chromosome number, small genome size, and giant salivary gland chromosomes are some of the advantages that made it a potent model organism in the study of modern genetics (Hartwell *et al.*, 2004). With the emerging understanding in molecular and cell biology, and the realization that most cellular processes are highly conserved between species, the fly became an invaluable model organism also for organ development and function. The *drosophila* genome contains about 13,000 protein-coding genes, compared to the 30,000 genes of the human genome, and generally has less functional redundancy among genes, which is useful in mutant analysis. Moreover, many of the developmental genes and mechanisms operating in the fly have been conserved in a wide variety of animals, and

researches have shown that most of the genes predicted to influence the development of human have already been discovered in drosophila.

Insects also provide an excellent system for the study of aECM formation, as they produce a highly ordered aECM, called the cuticle (Figure 2). The cuticle is the exoskeleton of insects and is a layer of non-cellular material that lines the external surface of the body as well as the respiratory system (trachea), fore- and hind-gut, and part of the genital system. It is produced by the underlying epithelium at the end of embryogenesis before hatching, at each larval molt and during metamorphosis (Devine *et al.*, 2005). Thus, the epithelia are primarily a secretory tissue responsible for producing the cuticle and at least part of the basement membrane (Neville, 1975; Locke, 2001). While the basement membrane is a supportive bilayer of amorphous mucopolysaccharides (basal lamina) and collagen fibers (reticular layer) that serves as a backing for the epidermal cells and effectively separates the homocoel (insect's main body cavity) from the integument, the cuticle has three distinctive layers: the outer most water proof envelope, the middle protein-rich epicuticle and the inner chitinous procuticle that contacts the apical plasma membrane of the epithelial cells and provides physical support and barrier functions to the animal (Figure 2). The most abundant component of the insects cuticle is fibrils of chitin, a β 1-4 linked polymer of N-acetyl-D-glucose amine (Cohen, 1987). Chitin is the structural component of the procuticle and assumes its role through the association with different types of cuticular proteins, there by defining a variety of cuticles with different physical properties. Its synthesis is catalyzed by chitin synthases that are transmembrane proteins situated in the apical membrane. Analysis of the ship blowfly (*Lucila caprina*) chitin synthase suggests that the integral membrane protein has its catalytic domain located on the cytosolic face of the plasma membrane (Tellam *et.al.*, 2000). Thus, the polymers must be extruded across the membrane, perhaps through a pore formed by the transmembrane domains of chitin synthases itself. The deposition and layered formation of cuticular components by the apical cell membrane of epithelial cells results in the formation of a highly organized apical extra cellular matrix.

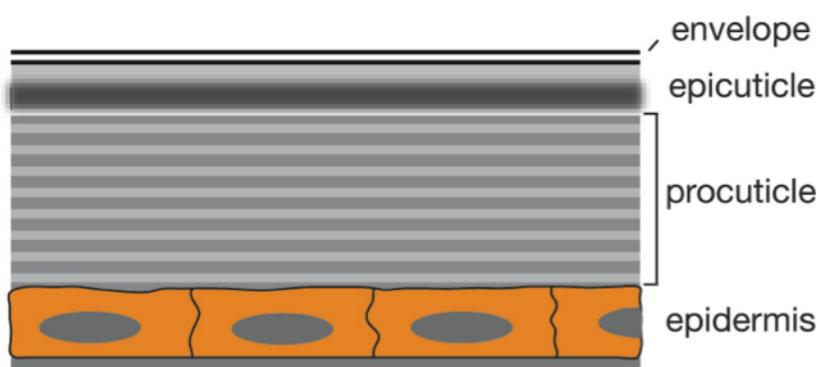


Figure 2. Structure of the *Drosophila* cuticle. The epithelia (orange) deposit the outer-most envelope, the protein-rich epicuticle and the chitinous and lamellar procuticle. Adapted from Moussian and Uv, 2005.

The property of the cuticle varies between developmental stages and among the type of organs where it is found. For instance, the tracheal cuticle is unique from the epidermal cuticle by the presence of cuticular ridges, often called taenidia folds, that are thought to prevent the collapse of tracheal tubes while allowing them to expand and contract along their length (Figure 3) (Matusek *et al.*, 2005). The taenidia contain at least the envelope and

procuticular material, and they lie perpendicular to tube length and form annular rings or run as a helical course around the lumen. Extraordinarily, the taenidia run uniformly across cell boundaries, raising intriguing questions on how they become organized. Deposition of the cuticular constituents of the taenidial folds is preceded by the appearance of membrane folds that reflect the position of the future taenidia. These membrane protrusions disappear as the taenidial material is deposited, so that the apical plasma membrane becomes flattened beneath the growing ridges (Uv *et al.*, 2010).

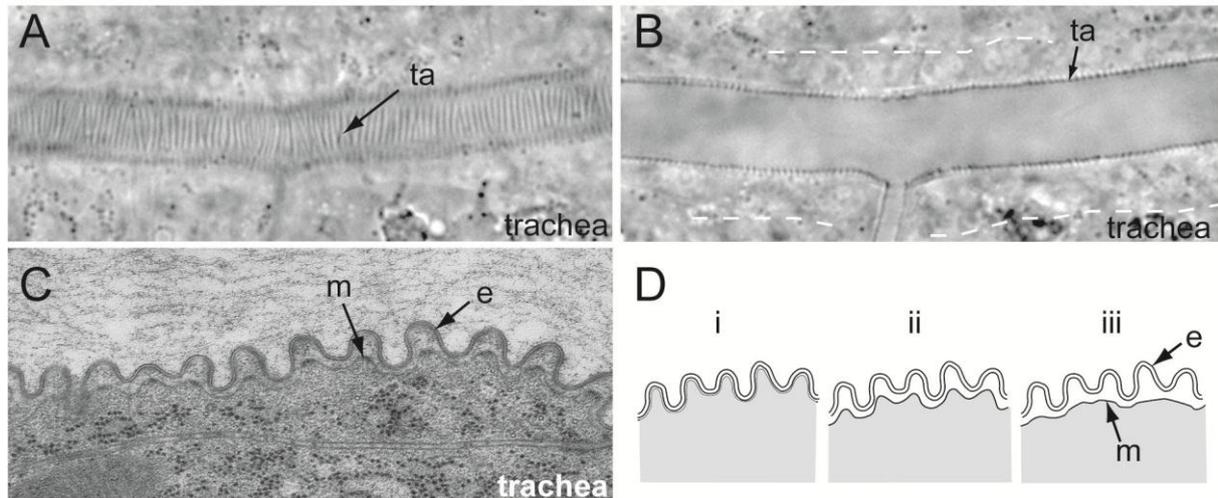


Figure 3. Tracheal taenidial formation. (A-B) The taenidia (ta) of the tracheal cuticle run a helical course around the lumen and can be seen by light microscopy during late embryogenesis. With focus on the lumen surface (B) they appear as dark lines perpendicular to tube length (A, arrow), and when viewing longitudinal sections of the lumen they are seen as dark punctae along the apical surface (B, arrow). Stippled lines mark the basal epithelial surface. (C) TEM analysis of a longitudinal section of a tracheal tube reveals apical membrane (m) bulging during formation of the taenidial folds (e points to the envelope). Note the electron-dense areas at the tip of each membrane fold (m). (D) Apical membrane dynamics during taenidial formation: Initially, the membrane follows the outer lining of the cuticle (i), it then withdraws as taenidial ECM is deposited (ii) and later becomes flattened under the taenidial buckles (iii). From Uv and Moussian, 2010.

There have been different hypotheses regarding the origin of taenidial folds. Thompson (1929) suggested that some simple physical force is responsible, while Locke (1958) proposed that the cuticle is deposited uniformly and that an axially restrain, possibly provided by the epithelium, causes the cuticle to buckle and form taenidial folds. The spiral course of the taenidia could then be caused by some small randomly occurring torsional stress in the tissue. Now a day, it has been noted that there is an active role of the apical cell domain that includes the apical plasma membrane and the cortical cytoskeleton in taenidia formation. A study performed by Matusek and colleagues (Matusek *et al.*, 2005) has shown that mutant embryos lacking the gene *DAAM* exhibit a highly disorganized taenidia pattern. *DAAM* is a drosophila formin and has been shown to affect actin polymerization. Moreover, they found that just before cuticle deposition begins, the tracheal cells from a sub apical pattern of actin filaments that appears to serve as a blue print for the future taenidia pattern. However, there are still puzzles about the establishment of the actin rings, the connection between the actin

ring structures and cuticle deposition, and how the orientation, spacing and uniformity of the taenidial folds are achieved.

The serine protease Filzig is required for tracheal taenidial organization

Most of the major findings that have emerged from research on drosophila were driven by the identification of mutations producing a chosen phenotype via unbiased forward genetic screens. In this case, random mutations are induced by certain mutagens, such as irradiation or chemicals, the mutants are screened for phenotypic defects and the molecular identification of the mutated gene is performed. Genetic screens by Nusslein-Vollhard and Wieschaus examined cuticle patterns of unhatched embryos produced by lethal mutations induced by the chemical mutagen ethyl Methalosulfonate (EMS). The characterization of the genes found in this screen defined many of the fundamental mechanisms that control cuticle development. Further screening of these mutants have been done for defects in taenidial formation (Moussian and Uv, unpublished) and revealed that a mutant called *filzig* (*flz*) developed an irregular taenidial pattern. Genomic mapping and sequencing showed that this phenotype in taenidial formation was due to a mutation in the gene *CG8213* (Uv, unpublished). The *CG8213/filzig* gene is located on the right arm of the second chromosome and is predicted to encode a transmembrane protein with an active trypsin like serine endopeptidase domain (<http://smart.embl-heidelberg.de/>).

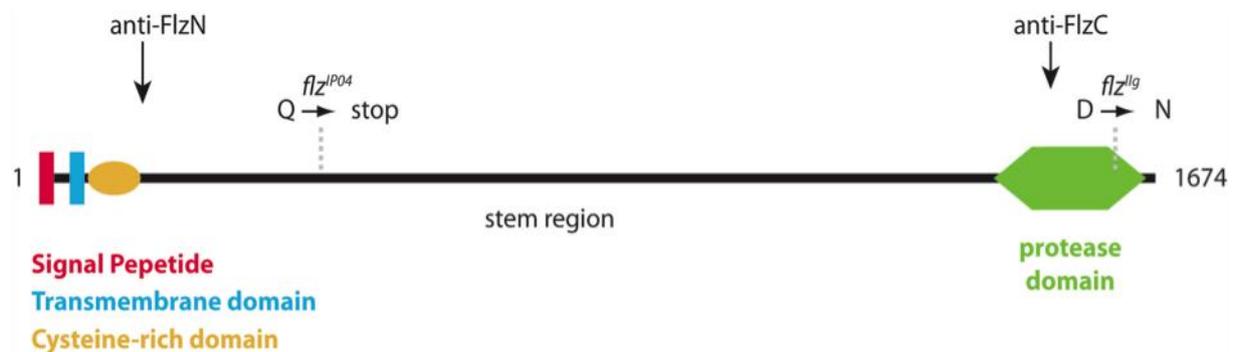


Figure 4. The Filzig protein. Schematic image of the Flz protein with its domains. Starting from the N terminal is a signal peptide, followed by a transmembrane domain, a cysteine-rich domain, a long stem region and, at the C-terminus, the serine endopeptidase domain. Also indicated are the binding epitopes for the antibodies; anti-FlzN (C-terminal to the cysteine-rich domain) and anti-flzC (on the serine endopeptidase domain). The position of the two *flz* mutations identified in the genetic screen are indicated (*flz*^{P04} and *flz*^{lg}).

The *flz* gene spans approximately 16 kb and encodes two predicted proteins of 1674 and 1693 amino acids, respectively (FlyBase, flybase.org). Both proteins are predicted to have a signal peptide, a transmembrane domain and the protease domain, and thus do not have significant physiological differences. In this respect, the Flz protein belongs to the type II transmembrane serine proteases (TTSPs), which are characterized by an N-terminal transmembrane domain that anchors TTSPs to the plasma membrane, a C-terminal protease domain, and a stem region of variable length (Hooper, 2000). Localization to the cell surface gives these enzymes an excellent opportunity to mediate signal transduction between the cell and its extracellular environment and to regulate various cellular responses. These serine proteases are named after

the reactive serine residue located in the active site that is essential for the function of the enzyme. The active site of serine proteases contains three critical amino acids: serine, histidine, and aspartate. These residues are often referred to as the "catalytic triad". When the linear sequence of amino acids folds into its tertiary structure, these three residues are arranged in such a fashion that enables the side chains of the serine residue to become negatively charged through the loss of the hydrogen from the hydroxyl R group to the histidine. This nucleophile can then make an attack on the carbonyl group of the peptide that is to be cleaved. Serine proteases have well characterized roles in diverse cellular activities, including blood coagulation, wound healing, digestion, and immune responses, as well as tumor invasion and metastasis (Hooper *et al.*, 2000). In mammals, the TTPSs currently consists of 17 members, of which 7 are found in man. The TTPSs share a number of common structural features, including (i) the proteolytic domain, (ii) a transmembrane domain, (iii) a short cytoplasmic domain, and (iv) a variable length stem region containing modular structural domains, which links the transmembrane and catalytic domains (Figure 4). It is this unique combination of domains that suggests novel roles for the TTPSs at the cell surface (Bugge *et al.*, 2009).

The proteolytic domains of the TTPSs share a high degree of amino acid sequence identity. In particular, the histidine, aspartate, and serine residues necessary for catalytic activity are present in highly conserved motifs. TTPSs are synthesized as single chain zymogens and are likely activated by cleavage following an arginine or lysine present in a highly conserved motif in the protease domain. Based on the predicted presence of a conserved disulfide bond linking the pro-and catalytic domains, the TTPSs are likely to remain membrane-bound following activation.

The transmembrane region of Filzig contains a hydrophobic domain near the N-terminus. This domain is predicted to span the plasma membrane in such a way that the proteolytic domain lies extracellularly, presumably to localize the proteolytic activity in close proximity to target substrates and /or to permit regulated release of the protein from the cell surface. The Filzig protein has a short cytoplasmic domain, and whether this domain has the potential to support interactions with cytoskeletal components and signaling molecules is not yet known. However, a number of the TTSPs, including corin, stubble, stubbloid, and TMPRSS2 contain consensus phosphorylation sites for either or both of protein kinase C and casein kinase II. In addition, based on the cellular sorting of other integral membrane proteins, it is likely that the cytoplasmic and transmembrane domains also contribute to the targeting of the TTSPs to a particular cell surface in polarized cells (Hooper, 2000).

The stem regions of the TTSPs contain as many as 11 structural domains that may serve as regulatory and/or binding domains. The most common stem region structural domain is the LDL receptor class A domain. Although the function of these domains in the TTSPs has not been demonstrated, in other proteins they bind Ca^+ ions and mediate the internalization of macromolecules including serine protease inhibitor complexes and lipoproteins. However, the Filzig protein is not predicted to contain conserved domains in the stem region.

Drosophila as an amenable genetic model organism

Genome. *Drosophila melanogaster* has four chromosomes, designated numerically as 1-4. Chromosome 1 is the X/Y chromosome; chromosomes 2-4 are autosomes. Each autosome has two arms that are denoted as left (L) and right (R). In addition, each chromosome is divided into cytological regions: X (1-20), 2L (21-40), 2R (41-60), 3L (61-80), 3R (81-100), chromosome 4 (101-102). This chromosome map is used to specify the position of a gene or a breakpoint in a chromosome rearrangement. Sex determination is of the XY type, with females being XX and males XY, and it is the number of X chromosomes to autosomes that determines the sex.

Life cycle. An adult female drosophila has a very high rate of reproduction and can lay about 3,000 eggs in her lifetime. A single male can have the ability to fertilize over 10,000 eggs. The female has spherical sperm storage organs that allow her to produce several hundred progeny after a single mating. Each drosophila egg is about half a millimeter long and is well supplied with yolk that supports embryonic development. Fertilization normally occurs as the egg is being laid. The embryo development follows the 24 hours pattern for completion, and some of the early events happen so rapidly and can be watched in real time. In general, their complete life cycle from fertilization to emergence of the adult fly is about 10 days.

Balancer chromosomes. Chromosomes that carry lethal recessive mutations are difficult to maintain in a population as they are lost in the nonviable homozygous animals and possibly confers a disadvantage on the progeny of heterozygotes. *Drosophila* geneticists created special types of chromosomes that carry multiple, overlapping inversions called balancer chromosomes. They form a very important role in mutagenesis screening, stock maintenance and tracking quantitative traits. The inversions suppress the recovery of viable recombination products over the length of the chromosome, a dominant phenotype that enables the inheritance of the chromosome to be tracked easily in subsequent crosses. Moreover, a recessive lethal mutation carried on eth balancer chromosome eliminates the animals homozygous for the balancer from the population of breeding flies (Hentges *et al.*, 2004).

UAS/GAL4 system. The UAS/GAL4 system is a very important tool for targeted gene expression in drosophila. Targeted gene expression in a temporal and spatial fashion has proven to be one of the most powerful methods for pin pointing gene function *in vivo*. In this system, expression of the gene of interest, the responder, is under the control of five tandemly arrayed and optimized binding sites for the yeast transcription factor GAL4, called the upsetram activating sequence (UAS). Since transcription of the responder requires the presence of GAL4, the absence of GAL4 in the responder lines make them in a transcriptionally silent state. To activate their transcription, responder lines are mated to flies expressing GAL4 in a particular pattern, termed the driver. The resulting progeny then express the responder in a transcriptional pattern that reflects the GAL4 pattern of the respective driver (Duffy, 2002).

Aim of the study

The aim of this research is to further understand Filzig function through characterization of *filzig* mutants and Filzig protein expression.

Methods and Materials

Fly lines and Genetics

The two *flz* mutant alleles, *flz*^{IPO4} and *flz*^{Ilg}, were obtained from Tübingen drosophila stock center and were balanced over *CyO,GFP* (*flz*^{IPO4}/*CyO,GFP* and *flz*^{Ilg}/*CyO,GFP*). The *flz*^{Ilg} allele is a missense mutation of a conserved aspartic acid in the catalytic domain near the active site, while *flz*^{IPO4} is a nonsense mutation at the amino acid residue 391. The two deficiency lines used were: *DF(2R)BSC268* that has a chromosomal deletion causing deficiency for *flz*, the neighboring *CG34350* and 5 additional, and *DF(2R)BSC271* with a chromosomal deletion causing deficiency for *flz*, *CG34350*, *CG8172*, *CG13744*, and another five additional genes. These deficiency lines were obtained from Bloomington fly stock center, Indiana. The deficiency chromosomes were also balanced over *CyO,GFP*. The *UAS-Dicer; Btl-GAL4* flies were crossed with *RNAi* lines (*RNAi-CG8213*, *RNAi-CG34350*, *RNAi-CG8172*, *RNAi-CG13744* and *RNAi-CG8170*) to induce a posttranscriptional targeted gene silencing in tracheal cells. All *RNAi* lines were obtained from Vienna fly stock center, Austria.

Fly works

Fly stocks were kept at 18 °C in 50 ml vials with about 10 ml of corn meal agar media. For analysis of embryonic stages, eggs were collected on apple juice agar plates and were aged at 25°C and 29°C depending on the objective of the experiment. Flies for expansion were cultured in larger 400 ml plastic bottles kept at 25°C.

Table 1. Preparation of cornmeal agar media

Ingredients	Quantity
Water	2.6L
Yeast	24g
Agar	33g
Molasses	200g
Corn meal	163g
Nipagen	38ml

Table 2. Apple juice agar plate recipe

Ingredients	Quantity
Water	600ml
Bacto agar	18g
Sugar	20g
Apple juice	200ml
Nipagen	12ml

Embryo collection

Embryos from wild type (*OrR*), *flz^{IPO4}/Cyo*, *flz^{Ilg}/Cyo*, *DF(2R)BSC268*, and *DF(2R)271* were collected and staged on apple juice agar-plates at 25°C. An inverted fluorescence microscope was used to make sure that they were at the appropriate stage; if not, further aging was done at 29°C. Homozygous mutant embryos were identified by their lack of GFP expression, and staging was done by gut morphology. The embryos were washed off the apple plate in water and were collected in a cut off 50 ml falcon tube covered with mesh at the underside. Then, embryos were dechorionated by immersing them in bleach (NaClO, 2-3 % active chlorine) for 2 minutes followed by immediate washing with embryo washing buffer (0.003 % Triton X100, 0.4% NaCl). Finally, the embryos were subjected to different types of fixation procedures been outlined below.

Regular fixation

The dechorinated embryos were transferred into glass vials that were filled with 5 ml heptane. Properly dried embryos can easily slide down from the mesh into the heptane. A 5 ml fixation solution (0.5 ml 37% formaldehyde in 4.5 ml PEM) was added immediately into the heptane, and the tubes were closed and placed on a rocker for 30 min at 300 rev. After removing the fixation solution (lower phase), 5 ml of 100% methanol was added, followed by hand-shake for 30 seconds to remove the vitelline membrane. It was clear that the vitelline membrane was suspended at the interphase between the heptane and methanol, whereas devitellinized embryos sank down into the methanol and to the bottom of the vial. The heptane along with the vitelline membrane was removed. Then, embryos were transferred along with the methanol into an eppendorff tube and were washed with fresh 99% methanol 3×5 min on a rotator, rehydrated in 50% methanol for 5min, washed with PBT (PBS, 0.1% Triton X100) 3×5 min, and finally they were blocked with PBSBT (PBT, 0.5% BSA) for 2×20 min.

Heat fixation

Previously dechorinated embryos were subjected to heat shock in a cup filled with hot embryo wash buffer followed by an immediate cool down in an ice cold embryo wash buffer. The vitelline membrane was removed after transferring the embryos to fixation tubes filled with 5 ml of heptane and 5 ml of 99% methanol, and shaking the tubes for 30 seconds. The rest of the procedure was as described for regular fixation.

Antibody staining

Primary anti-sera used were: the two Flz anti-sera (obtained from GenScript, USA), anti-FlzN (1:500) and anti-FlzC (1:500), both of which are complete affinity-purified peptide polyclonal antibodies raised in rabbit, rabbit anti-GFP (1:500, Molecular probes, Invitrogen, Sweden), mouse anti-Crb (1:10, DHSB, Iowa) and rat anti-DECad (1:20, DHSB, Iowa). A FITC-conjugated chitin-binding probe (CBP, New England Biolabs) was used at 1:500. Secondary antibodies were conjugated to Alexa488 or Alexa548 (Molecular probe, Invitrogen Sweden), and were used at 1:500. Primary antibodies were mixed in 500 µl PBSBT and incubated together with the embryos overnight at 4°C on a rotator. Washes continued on day 2, first

3×5 min, then 3×20 min with PBSBT on a rotator. Secondary antibodies were mixed with 500 µl PBSBT and incubated together with the embryos in a dark chamber for 2 hours on a rotator. After that, the embryos were again washed, first 3×5 min, then 4×20 min in PBSBT. When CBP was used, the protocol continued with adding 50% glycerol in PBS to the embryos that were allowed to sink before the glycerol was removed. Then, Prolong[®]Gold antifade reagent (Invitrogen[™], Molecular probes[®], USA) was added, and the embryos were mounted (60 µl) and examined. In the absence of CBP, the protocol proceeded with dehydration in ethanol of increasing concentrations: 25%, 50%, 75%, and 99% (5 min on rocker for each dilution). Finally, 500 µl of methyl salicylate (Sigma Aldrich) was added carefully, and the embryos were allowed to sink and settle for at least 30 min before mounting on a glass slide.

Cuticle preparation

Wild type and *flz* mutant flies were caged with apple juice agar plates and incubated at 25 °C. Unhatched embryos from a 24-36 hours old plate were collected. Embryos were dechorinated in 8% sodium hypochlorite solution were rolled on a drop of water to remove the vitelline membrane and transferred onto a glass microscope slide which has a drop of 1:1 Lactic acid:Hoyer's medium (Hoyer's-based mountant) and were thereafter covered with a cover slip. Finally, the slide was incubated overnight in a 65°C oven to allow digestion of internal tissues and clearing of the larvae.

Live imaging

RNAi lines for *CG8213*, *CG35340*, *CG8172*, *CG8170* and *CG13744* were crossed with fly lines of *UAS-dicer*; *Btl-GAL4* in order to induce targeted gene silencing in tracheal cells. Embryo offspring were collected and dechorinated, and were then mounted in halocarbon oil 700 (Sigma Aldrich, USA) and were observed under upright fluorescence and light microscope (Leica DM5500B, Germany). Homozygous mutant embryos for *flz* were identified by their lack of GFP expression.

Confocal imaging

Images were obtained using an upright Biorad Radiance 2000 confocal microscope. Comparable images of wild type and mutant embryos were obtained with similar confocal settings.

Results and Discussion

Filzig mutants show disorganized cuticular pattern

The embryonic tracheal system of *Drosophila* is lined with cuticle that exhibits unique organization. To investigate how such organization was maintained and formed, we took the advantage of the distinctive structures called taenidia, visible ridges of cuticle that run in helical or annular rings perpendicular to the axis of tracheal tubes. The taenidia forms such a nice and highly organized annular pattern in wild type embryos (Figure 5A). But results from *flz* mutant embryos revealed very irregular or disorganized taenidial pattern that appeared to be crisscrossed instead of following the parallel pattern as it has been in the wild type (Figure 5B). A closer look at the taenidia showed that not only the shape of the taenidia, but also the intertaenidial distance has been disrupted. The two *flz* deficiency lines (*Df(2R)BSC268* and *Df(2R)BSC271*) also showed a disorganized tracheal taenidial pattern (data not shown), which proves that *flz* is required for regular taenidia formation.

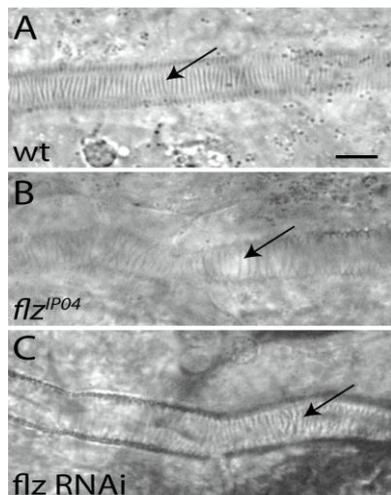


Figure 5. Taenidial cuticle disruption in *flz* mutant embryos. (A-C) Light microscopy images of living larvae before they hatch. (A) The taenidia of the wild type larvae have a regular organization and follow annular rings perpendicular to the tracheal tube (arrow). (B) In *filzig* mutant embryos (*flz*^{IP04} is shown) the pattern of the taenidia is irregular: they appear further apart and often cross each other (arrow). (C) The same taenidial defects seen in (B) are also found in embryos that express a double hairpin of the *filzig* transcript in tracheal cells to cause RNA interference (*flz* RNAi) (arrow). Scale bar: 5µm.

Further characterization of *flz* mutants showed that the denticle belts were also affected (Figure 6). Normally, at the end of embryonic development, the ventral epidermis is decorated by a segmentally repeated denticle pattern. One segmental unit is about 11 cells wide and it comprises 6 rows of cells that secrete denticles while the remaining cells are covered by a naked cuticle. Individual denticle rows (numbered by 1 to 6) are distinguishable by their shape, size, color and polarity. Instead of forming the typical pigmented triangular shaped denticles as in the wild type (Figure 6A), the shape of the denticles in the *flz* mutants appear as small colorless rounded protrusions (Figure 6B).

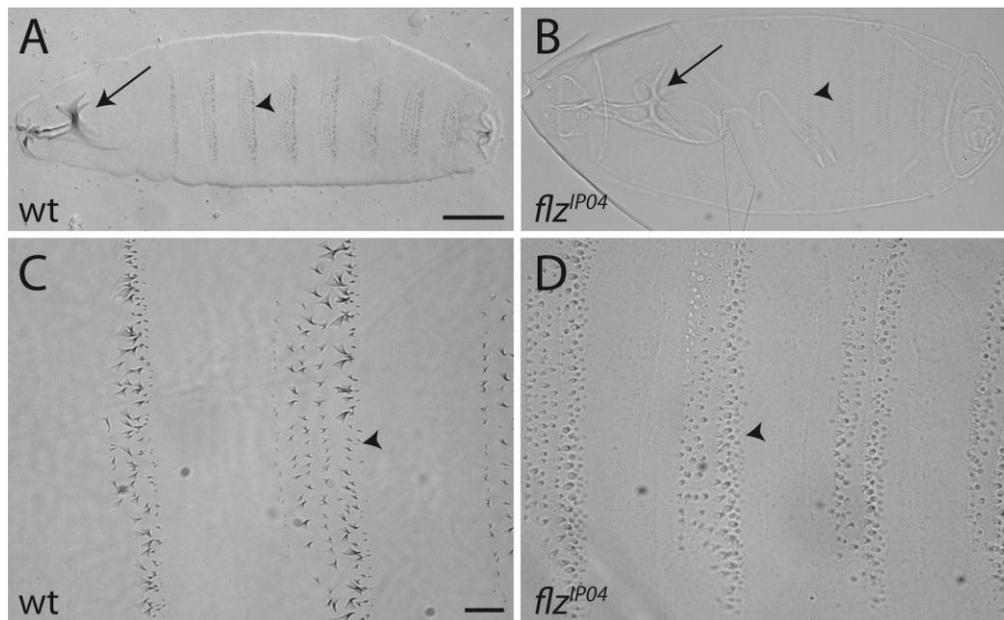


Figure 6. Mutation in the *flz* gene affects epidermal cuticle shape and pigmentation. (A-D) Bright field microscopy of cuticle preparations of mature wild type (A and C) and homozygous *flz*^{IP04} mutant embryos (B and D). The body morphology of *flz* mutant embryos is aberrant, as they have lost the characteristic body shape and appear bigger (compare A and B). Note also the unpigmented head skeleton (arrows) and denticle belts (arrowheads) in the mutants compared to the wild type. The denticle belts (arrowhead) of wild type embryo (C) are arranged in six rows and have a triangular shape. In the *flz* mutant embryos (D), the denticle belts (arrowhead) have lost their characteristic triangular shape and their arrangement is a bit sloppy. In all images, anterior is to the left and ventral is up. Scale bars: 100µm (in A for A and B), 10µm (in C for C and D).

The size of *flz* mutant embryo looks a bit thick dorso-ventrally compared to the wild type probably due to pressure exerted on vetilline membrane before devetillization. Previous works (Alexander, 1999) revealed that the proteins Hedgehog and Wingless regulate the spatial expression of Serrate and Veinlet and embryos lacking either of these proteins results develop denticle defects. However, these denticle defects regard the patterning of the denticle belts in terms of their number, position and type of denticle. Filzig on the other hand appears to form denticles in the right position but the denticles themselves are misshapen. Thus, Filzig does not appear to be involved in the patterning of eth denticles, but in the subsequent step of denticle formation. This in turn suggests that the Flz protein works in a cascade downstream of the patterning genes to execute denticle formation.

Filzig is expressed in the trachea, epidermis, head skeleton and hindgut as cuticle deposition begins.

Previous analyses (Uv, unpublished) have shown the presence of *flz* mRNA in ectodermally derived tissues from stage 15, just before cuticle deposition begins (Figure 7A). To further address the expression and localization of Filzig, antisera were raised against two parts of the Filzig protein: an N-terminal peptide (anti-FlzN) and a C-terminal peptide (anti-FlzC) (see Figure 4).

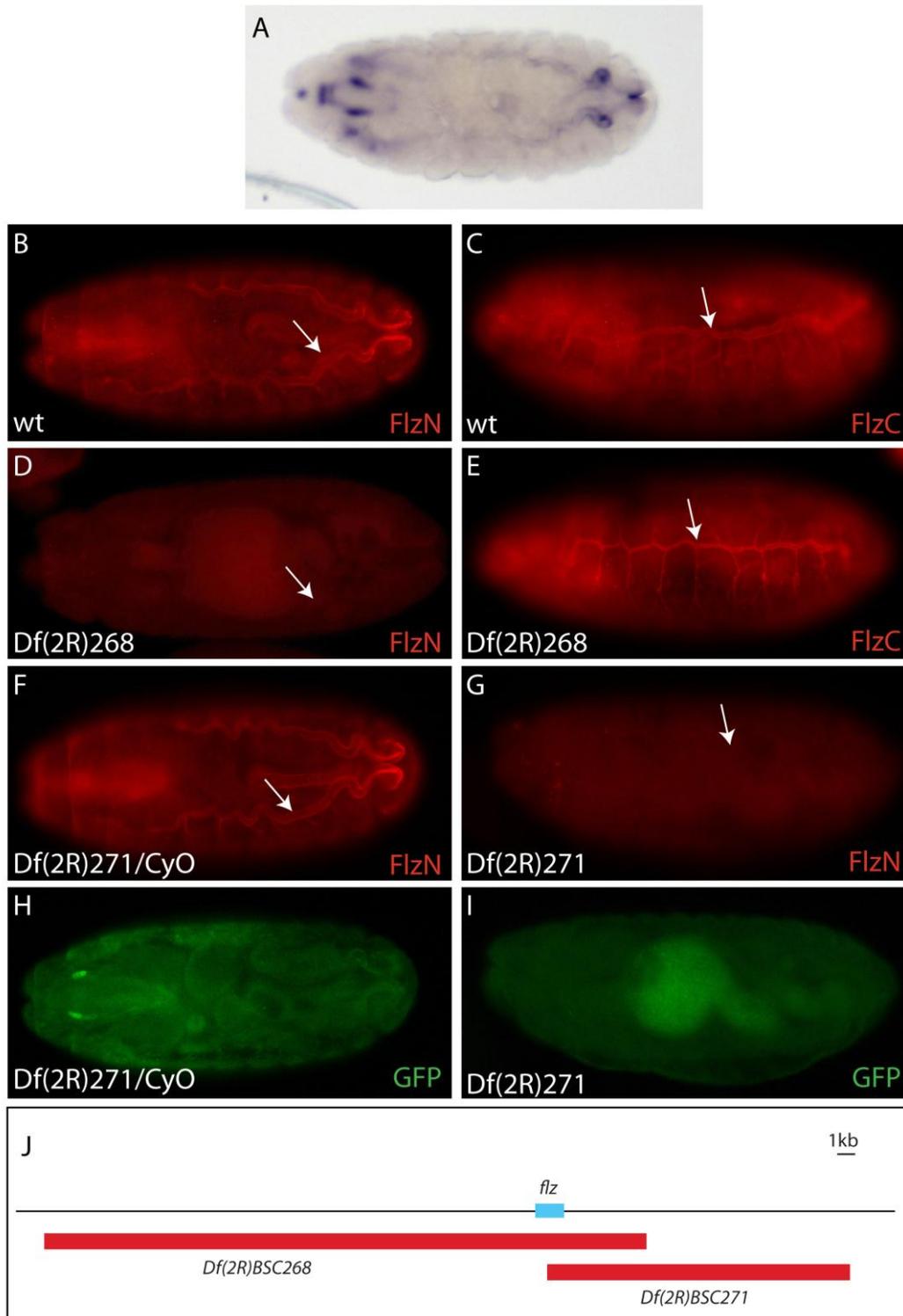


Figure 7. Anti-FlzN specifically recognizes Flz and shows Flz expression in cuticle-producing tissues. A) An image of in situ hybridization to show *flz* transcripts in the head, trachea and hindgut at stage 16.)FlzN stains B) and flzC stains the trachea of wild type and heterozygotes(F) .The *flz* mutants do not show staining for flz(D) and (G).However ,the anti-FlzC was unspecific since it stained the mutant embryos trachea. HomozygousFlz mutant lack GFP expression(I) compared to heterozygotes(H) .chromosomal deletion of genes close to the *flz* gene(J)

To test whether either of the antisera specifically recognizes the Filzig protein, wild type embryos and embryos deficient for *filzig* were stained with anti-FlzN or anti-FlzC. The deficiency lines carry the deficiency chromosome over a balancer chromosome that contains a gene that encodes GFP. Thus, the embryos were double labeled for GFP and Flz, so that homozygous mutant embryos could be identified based on their lack of GFP expression. Stage 16 to 17 embryos were analyzed using fluorescence microscopy (Figure 7B-I). Both anti-FlzN and anti-FlzC stained the trachea of wild type embryos (Figure 7B and C). However, while anti-FlzN failed to produce any staining in embryos deficient for Flz (*DF(2R)BSC268*) (Figure 7D), the anti-FlzC stained these embryos in a similar manner as the wild type (Figure 7F). Thus, the anti-FlzC antiserum was not specific to the Filzig protein. The anti-FlzN stained all wild type embryos from stage 16 in tissues where the *flz* RNA had been detected (trachea, hindgut, epidermis and head skeleton), but not the mutant embryos, consistent with it recognizing the Filzig protein. To confirm the specificity of anti-FlzN, we also used this antiserum to label embryos carrying the deficiency *DF(2R)BSC271*. This deficiency also deletes *filzig*, but the deficiency extends in the opposite direction. Indeed, anti-FlzN stained heterozygous *DF(2R)BSC271/CyO,GFP* embryos, but not embryos homozygous for *DF(2R)BSC271* (Figure 7F-I). Thus, anti-FlzN recognizes the Filzig protein and can be used to study its expression and subcellular localization.

Filzig localizes the apical cell surface and to the extracellular cuticle.

In order to determine the subcellular localization of Flz, double labeling was performed with anti-FlzN and anti-Crumb (Crb). Crumb is an apical transmembrane protein that is required for organizing apical-basal polarity in gastrulating drosophila embryo (Ling *et al.*, 2010). In *Drosophila*, Crb misexpression causes apicalization of cells and consequently gross defects in epithelia organization (Gibson and Perrimon, 2003). Confocal analysis of wild type embryos showed that Flz localizes to the apical cell surface of tracheal cells along the apical Crb protein (Figure 8D-F). This localization agrees with the presence of a transmembrane domain in Flz. However, the Flz protein appears to distribute further into the lumen than Crb.

The pattern of Flz localization at the apical surface of tracheal tubes was visualized by obtaining and merging a stack of images that span half of the tracheal tube (Figure 8A). The Flz-staining appears as dots that cover the entire lumen surface and could not be found to exhibit a pattern that would be reminiscent of the taenidial pattern. Moreover, when the Filzig staining was analysed in the epidermis (Figure 8B) and the head (Figure 8C), it appeared that Flz is not restricted to the apical surface, but is present also in the extracellular cuticle. This was evident by the finding that Flz is present in cuticular hairs (arrow in Figure 8B) and the head skeleton (arrow in Figure 8C) that are cell free structures and too far away from the apical surface to allow transmembrane Flz protein to stain these tissues. In support of this, we observed that a homozygous viable GFP gene-trap of *filzig* (GFP has inserted just C-terminal to the transmembrane domain) expresses Filzig as a secreted protein that fills the gap between the epidermis and the vitelline membrane (data not shown). Thus, it seems that Flz might be cleaved from the apical surface and can perform its function in the extracellular space. To test

this, one could express the Flz protein without the transmembrane domain to test if it can rescue the *flz* mutant phenotype.

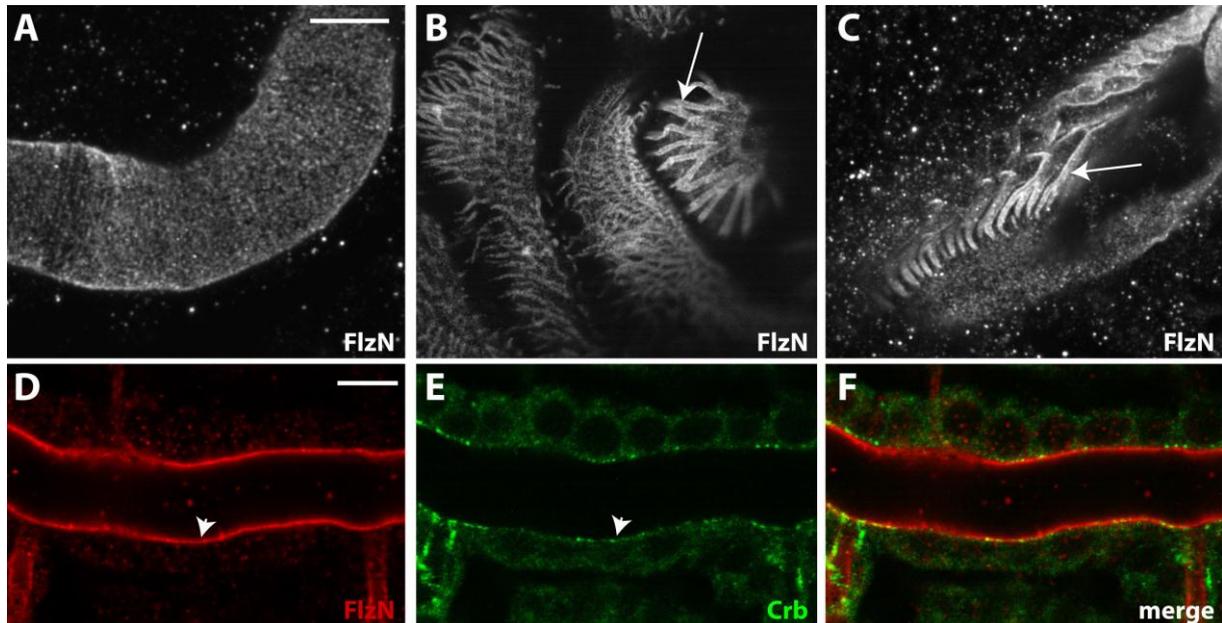


Figure 8. Filzig localizes the apical cell surface and to the extracellular cuticle.(A)the *flz*-staining appears as dots that cover the entire lumen surface and could not be found to exhibit a pattern that would be reminiscent of the taenidial pattern. Flz is present in cuticular hairs(B) and head skeleton(C) that are cell free structures and too far away from the apical surface to allow transmembrane Flz protein to stain these tissues. Flz localizes(D) to the apical surface of tracheal cells along the apical *crb* protein(E) but the merge reveals that Flz protein appears to distribute further into the lumen than Crb..

Filzig is required for chitin organization, but not for epithelial integrity

A further investigation into the cuticle defects in *flz* mutant embryos was performed by staining for the main cuticle component, chitin, using a chitin-binding protein (CBP). *Drosophila* embryos can normally not be labeled by antisera beyond early stage 17 (around 5 hours before hatching) since the cuticle blocks access of antibodies. However, the chitin-binding protein is only 5 kDa and can penetrate the cuticle and label the chitin at late stage 17 (just before hatching) when the embryos are heat-fixed (regular fix does not work). As we found that anti-FlzN does not label heat-fixed embryos, we could not perform a co-staining of such embryos. Embryos stained with CBP show that the chitin fibers were highly disorganized in *flz* mutants (*flz^{IPO4}*) compared to the wild type (Figure 9). Instead of forming uniform bundles that run around the lumen perpendicular to tube length, as in the wild type (Figure 9A), the chitin bundles of *flz* mutant embryos run in different orientations and cross each other, and also appear to have uneven thickness. This suggests that Flz takes part in organizing the regular architecture of chitin bundles, and that in its absence, the resulting chitin disorganization might lead to a subsequent defect in taenidial organization.

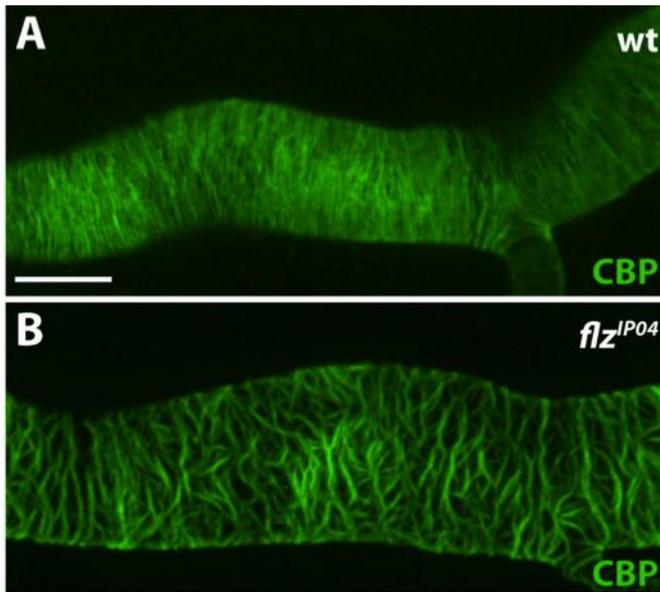


Figure 9. Embryonic trachea labeled with chitin binding protein(CBP) showing the regular pattern of chitin fibers in the wild type and a highly disorganized chitin fibers in *flz* mutant(*flz^{IP04}*) embryos. Scale bars:5μm(A and B).

The grossly disorganized chitin bundles are not associated with detectable anomalies in the underlying epithelium, as seen from staining for drosophila epithelial cadherin (DECad). DECad is a homolog of classic vertebrate cadherins, and is important in setting up boundaries between epithelial compartments, preventing cell populations with different fates from mixing with one another. By merging image stacks that span half of the tracheal tubes, the apical cell outline can be visualized. Both the level of DECad and tracheal cell shape appear to be normal in *flz* mutant embryos (Figure 10). Flz therefore appears to specifically affect the aECM.

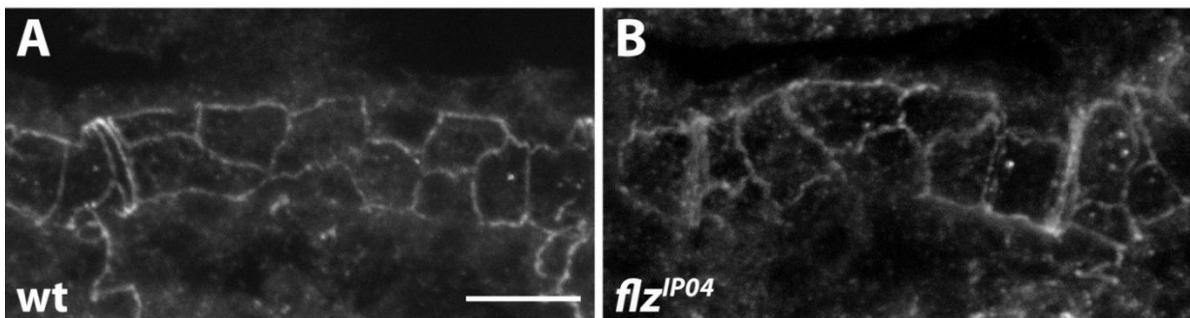


Figure 10. Embryos stained with DE-cadherin which is a junction protein involved in the adhesion between boarder cells looks unaffected by the absence of *flz*. Scale bar: 5μm.

The effects of genes flanking *flz* on cuticle pattern

Genes annotated as *CG34350*, *CG8172*, *CG13744* and *CG8170* are found to be flanking the *flz* gene and are also predicted to secreted or transmembrane serine proteases with a catalytic domain at the very C-terminus. The structural and locus similarity of these serine proteases

with the Flz protein posed an interesting question if these genes are also involved in the development of cuticle. The RNAi gene knock down technique was used to study the effect of these serine proteases on tracheal taenidia formation. RNA interference (RNAi) is a highly evolutionary conserved mechanism of gene regulation. The classic RNAi occurs at the post-transcriptional level and is triggered by short double-stranded RNA also known as short interfering RNA (siRNA), which is processed from long dsRNA by the RNase III enzyme dicer or introduced into a cell exogenously. After being loaded into the so-called RNA-inducing silencing complex (RISC) in the cytoplasm, the siRNA would cause sequence-specific degradation of its endogenous homologous mRNA sequences (Takashi *et al.*, 2003). Fly lines carrying insertions coding for inverted repeats that correspond to the mRNA of different target genes are publicly available from the Vienna *Drosophila* RNAi center. The expression of these inverted repeats is under the control of UAS, and the Btl-GAL4 driver was used to target such gene silencing to the developing tracheal cells. Thus, flies that express GAL4 in tracheal cells (*Btl-GAL4*) were crossed with transgenic flies that carry the inverted repeat under. The offspring will thus have an activated transcription of the UAS-construct driven by GAL4 leading to the formation of dsRNA for a specific transcript in the tracheal cells.

The cross between *RNAi-CG8213* and *UAS-dicer; btl-GAL4* fly lines yielded embryos that had similar tracheal phenotype as that seen in *flz* mutant embryos (Figure 5C). Recent reports (personal contact) have also shown that expression of *RNAi* for *CG34350* produced embryos with irregular taenidia formation. However, the use of RNAi to silence the remaining genes did not produce any visible tracheal phenotype (data not shown).

Conclusion

The current results showed that the Filzig protein localizes to the apical surface of epithelial cells and to the apical extracellular matrix. Moreover, the results indicated that Filzig is involved in cuticle organization, rather than taking an active role in cuticle patterning or in organizing the underlying epithelium. Since there is an intimate relationship between the aECM, the apical plasma membrane and the cytosolic sub apical cytoskeleton at the apical domain of epithelial cells, the Flz protein could exploit its structure to function at the apical extracellular matrix to bridge these domains. Flz appears to be related to another serine protease, Stubble, which has previously been shown to be required for morphogenesis and for cuticle organization during metamorphosis, possibly by loosening the extracellular matrix (Hammonds and Fristrom, 2006). Thus, it was suggested that the unique topology of the membrane and distribution of membrane-bound and -associated effectors depend on the underlain cytoskeleton, in turn influencing the organization of the aECM. However, several observations (Uv and Mousani, 2010) suggest the apical plasma membrane does not simply adopt instructions of the cytoskeleton, instead, it may have the potential to reorganize the cytoskeleton according to its own needs. Even though the molecular determinants of the apical plasma membrane interacting either with the aECM or the cytoskeleton have only been poorly investigated, our results shown above indicate that the correct organization of the

aECM in the *Drosophila* trachea depend on the activity of at least two serine proteases (*CG8213* and *CG34350*) that might function in a cascade to regulate spatial and temporal modifications in extracellular components to shape the extracellular matrix. We suggest further investigation into the cell-autonomous role of Filzig and its possible effect on the subapical cytoskeleton to address interdependence between cuticle organization, apical membrane topology and subapical cytoskeleton.

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