Mechanisms of production, molecular stratification and function of the non-chitinous cuticle part of *Drosophila melanogaster*

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I dedicate this thesis to my mother Barbara,

and to my friend Mustafa.

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Abbreviations

a-Slf	anti-Schlaff specific antibody
a-DT	anti-Dityrosine specific antibody
Alas	Delta-aminolevulinate synthase (CG3017)
CD8	mammalian transmembrane glycoprotein Cluster of Differentiation 8
CPR67b	Cuticle Protein 67b (CG3672)
CySu	Curly Suppressor (CG5873)
DsRed	Discosoma Red Fluorescent Protein
DT	Dityrosine
DUF243	Domain of Unknown Function 243 (Tweedle protein family)
Duox	Dual Oxidase (CG3131)
GFP	Green Fluorescent Protein
NLS	Nuclear Localisation Signal
ObstE	Obstructor E (CG11142)
Phm	Phantom, cytochrome P450
Res	Resilin (CG15920)
RFP	Red Fluorescent Protein
RNAi	RNA Interference Technique
SJ	Septate Junctions
Snsl	Pore canal component Snustorr Snarlik (CG2837)
Snu	ABC transporter Snustorr (CG9990)
Slf	C-Type lctin Schlaff (CG3244)
Tb	Tubby (Tweedle A, CG5480)
Tb1	mutated Tubby protein
Tb93	mutated Twdl L protein
TEM	Transmission electron microscopy
Twdl	Tweedle (protein family)
TwdlD	Tweedle D (CG14243)
TwdlF	Tweedle F (CG14639)
TwdlL	Tweedle L (CG6447)

TwdlM	Tweedle M (CG5468)
TwdlS	Tweedle S (CG14242)
UAS	Upstream Activating Sequence (promoter)
Verm	chitin deacetylase Vermiform (CG8756)

Abstract

Insects constitute the largest group of the animal kingdom that was able to colonize all the continents with the whole game of climates. A key weapon in fight against various environmental difficulties is the hard external scaffold named cuticle. It is a highly organized layered structure, which requires a complex machinery of factors and events to be correctly constructed and fully functional. Until now the main interest of the research was focusing on the thickest innermost chitinous layer called procuticle, whilst the other two – the proteinous epicuticle and lipidic external envelope remained elusive. In this work I present strong evidences that the envelope and the epicuticle constitute significant inward-outward and outward barriers, respectively. Their mis-functioning impairs insect development and often leads to animal death at early developmental stages. Moreover, the epicuticle composed of two-dimensional sheets is also substantial in determining correct larval body shape.

The first main element of the protective cuticular barrier is the mechanical barrier, provided by procuticular hardness and compactness. The second important element presented in my work is the inward-outward barrier, highly depending on the structure of the envelope and pore canals, thin tubes running across the cuticle. A key component in this barrier is a substance, which I named “405 material” because it exhibits auto-fluorescing properties when exposed to violet light (405 nm), visualising the envelope and pore canal tips of *Drosophila melanogaster*. Conducted analyses suggest that the 405 material may be the catecholamine-protein-lipid complex cuticulin, known to confer cuticle waterproofness. I described two factors co-operating during envelope formation and cuticulin delivery to the envelope: the pore canal

constituent Snustorr Snarlik (Snsl) and the ABC-transporter Snustorr (Snu). Snsl is involved in the transport of envelope components through the cuticular pore canals, whilst Snu in transporting Snsl to the cuticular pore canals. Down-regulation of any of them leads to the decreased envelope lipid/cuticulin content and as a consequence animal desiccation, high cuticle permeability to the surrounding environment and larval death.

The envelope inward-outward barrier is not the only barrier existing in the cuticle. I characterised another outward barrier layer at the boarder of the epi- and procuticle, consisting of dityrosinylated proteins, that I called the “intermediate zone”. I described a component Schlaff (Slf) localizing to this zone and being an organizer of the dityrosinylated proteins like Resilin. Animals with down-regulated Schlaff activity lose water and contract, whilst their dityrosine layer is disorganized. Similar phenotypes present mutants of heme synthesis pathway enzymes, what suggests lower heme-dependent peroxidase activity, known to be involved in dityrosine bounds formation. A second role of the dityrosinylated layer seems to be keeping the epicuticle attached to the procuticle, as these two layers strongly detach from each other in animals with down-regulated Slf activity.

The cuticle compactness and correct construction are important factors for animal protection, but also for the body shape. The hard chitinous procuticle has been shown to play an important role in body shaping. Loss of function of many procuticular factors leads to procuticle structure disorders and as a consequence cuticle dilation and animal misshaping. In my research I have revealed significance of the epicuticle for the larval body shaping. I have shown that the cuticular proteins belonging to the Tweedle family do not localize to the chitinous procuticle as previously suspected, but to the upper epicuticle, forming 2-dimentional sheets. Loss of function of the Tweedle

Domain of Unknown Function 243 (DUF243) causes mislocalisation of the aggregated Twdl protein in the procuticle, attracting other protein family members and thereby causing loss of epicuticular and procuticular integrity. The larvae are shorter and thicker compared to the *wild type* ones but their cuticle stretchability and movement capabilities do not change.

Taken together, my research reveals significant role played by two as yet poorly investigated cuticular layers in *Drosophila melanogaster*: the envelope and the epicuticle. This is a breakthrough in the cuticle research field and opens new horizons in understanding cuticle construction and function.

Zusammenfassung

Insekten bilden die größte Gruppe des Tierreichs, die alle Kontinente mit dem ganzen klimatischen Unterschieden besiedeln konnte. Eine Schlüsselstruktur im Kampf gegen verschiedene Umweltfaktoren ist der harte äußere Panzer namens Kutikula. Es ist eine hoch organisierte Schichtstruktur, die erfordert, dass ein komplexes Faktorennetzwerk korrekt aufgebaut und voll funktionsfähig ist. Bisher konzentrierte sich die Forschung auf die dickste innerste Chitinschicht, die als Prokutikula bezeichnet wurde, während die anderen beiden nämlich Epikutikula und die lipidische äußere Envelope schwer fassbar waren. In dieser Arbeit zeige ich starke Hinweise dafür, dass Envelope und Epikutikula signifikante innen- und außen Barrieren darstellen. Ihre Fehlfunktion beeinträchtigt die Insektenentwicklung und führt in frühen Entwicklungsstadien häufig zum Tod der Tiere. Außerdem ist die aus zweidimensionalen Schichten bestehende Epikutikula auch für die Bestimmung der korrekten Körperform der Larven erforderlich.

Das erste Hauptelement der kutikulären Schutzbarriere ist die mechanische Barriere, die durch proktikuläre Härte und Kompaktheit bereitgestellt ist. Die zweite wichtige Barriere die in meiner Arbeit präsentiert wurde, ist die innen- und außen Barriere, stark abhängig von der Struktur der Envelope und den Porenkanälen - dünnen Röhrchen, die durch die Kutikula verlaufen. Eine Schlüsselkomponente dieser Barriere ist eine Substanz, die ich als „405-Material“ bezeichnete, weil sie bei violettem Licht (405 nm) autofluoreszierende Eigenschaften zeigt und die Envelope und die Porenkanälen von *Drosophila melanogaster* visualisiert. Die durchgeführte Analyse legt Nahe, dass das 405-Material ein Katecholamin-Protein-Lipid-Komplex Cuticulin

ist, von dem bekannt ist, dass es die Kutikula wasserdicht macht. Ich habe zwei Faktoren beschrieben, die bei der Bildung von der Envelope und dem Transport der Cuticulin zur Envelope kooperieren: Porenkanälenkomponent Snustorr Snarlik (Snsl) und der ABC-Transporter Snustorr (Snu). Snsl ist beteiligt für den Transport der Envelopekomponenten durch die Porenkanäle der Kutikula, während Snu für den Transport von Snsl zu den Porenkanälen zuständig ist. Die Herabregulierung von jedem von ihnen führt zu einem verringerten Lipid- / Cuticulingehalt der Envelope und als Folge zu einer Austrocknung der Tiere, einer hohen Permeabilität der Kutikula für die Umgebungsfaktoren und einem Embryonal- bzw. Larvaltod.

Die innen und außen Envelope-Barriere ist nicht die einzige die in der Kutikula existiert. An der Grenze der Epi- und Prokutikula habe ich eine andere äußere Barriereschicht charakterisiert, die aus dityrosinylierten Proteinen besteht und die ich als Intermediate Zone bezeichnete. Ich beschrieb das Schlaff (Slf) Protein, die sich in dieser Zone lokalisierte und ein Organisator der dityrosinylierten Proteinen - wie Resilin - war. Tiere mit herunterregulierter Schlaff-Aktivität verlieren Wasser und schrumpfen, während ihre Dityrosinschicht unorganisiert ist. Ein ähnlicher Phänotyp präsentieren die Mutanten mit Häm-Syntheseweg-Störungen, was auf eine geringere Häm-abhängige Peroxidaseaktivität hinweist, von der bekannt ist, dass sie an der Bildung von Dityrosin-Bindungen beteiligt ist. Die zweite Rolle der dityrosinylierten Schicht scheint die Adhesion zwischen der Epikutikula und der Prokutikula zu sein, da sich diese beiden Schichten bei Tieren mit herunterregulierter Slf-Aktivität voneinander lösen.

Die Kompaktheit und die korrekte Konstruktion der Kutikula sind wichtige Faktoren für den Schutz des Tieres gegen Umwelteinflüssen, aber auch für die Körperform. Es wurde wiederholt gezeigt, dass die harte chitinöse Prokutikula eine wichtige Rolle bei

der Körperformung spielt. Funktionsverlust vieler prokutikulärer Faktoren führt zu Störungen der Prokutikulastruktur und als Folge zur Kutikulablösung und Körperformveränderung (Nüsslein-Volhard et al., 1984). In meiner Forschung habe ich die Rolle der Epikutikula in der Körperformung der Larven aufgedeckt. Ich habe gezeigt, dass Kutikularproteine der Tweedle-Familie nicht wie zuvor vermutet in der chitinösen Prokutikula lokalisiert sind, sondern in der oberen Epikutikula, wo sie 2-dimensionale Schichten bilden. Der Funktionsverlust der Tweedle-Domäne mit der unbekannten Funktion 243 (DUF243) verursacht eine Fehllokalisierung der aggregierten Twdl-Proteine in der Prokutikula, wo sie andere nicht mutierte Mitglieder der Proteinfamilie rekrutieren. Diese Fehllokalisierung verursacht den Verlust der epikutikulären und prokutikulären Integrität. Infolgedessen sind die Larven kürzer und dicker als die Wildtyp-Larven, ihre Dehnbarkeit und Bewegungsfähigkeit ändert sich jedoch nicht.

Zusammengenommen zeigen meine Forschungen eine bedeutende Rolle, die zwei bislang wenig erforschte Kutikularschichten in *Drosophila melanogaster* spielen: die Envelope und die Epikutikula. Dies ist ein Durchbruch in einem wichitgen Bereich der Insektenforschung und eröffnet neue Horizonte für das Verständnis der Konstruktion und Funktion der Kutikula.

1. Publications

1. Zuber, Renata; Norum, Michaela; Wang, Yiwen; Oehl, Kathrin; Gehring, Nicole; Accardi, Davide; Bartoszewski, Slawomir; Berger, Jürgen; Flötenmeyer, Matthias and Moussian, Bernard (2017). **The ABC transporter Snu and the extracellular protein Snsl cooperate in the formation of the lipid-based inward and outward barrier in the skin of Drosophila**.
   In: Eur J Cell Biol. 97(2), S. 90-101.

2. Zuber, Renata; Shaik Saheb, Khaleelulla; Meyer, Frauke; Ho, Hsin-Nin; Speidel, Anna; Gehring, Nicole; Bartoszewski, Slawomir; Schwarz, Heinz and Moussian, Bernard (2019). **The putative C-type lectin Schlaff ensures epidermal barrier compactness in Drosophila.**
   In: Scientific Reports 9, Article Number 5374.

Manuscripts ready for submission:

3. Zuber, Renata; Wang, Y; Gehring, Nicole; Bartoszewski, Slawomir and Moussian, Bernard. **Tweedle proteins form extracellular 2D-sheets defining body and cell shape in Drosophila melanogaster.**

2. Contributions to the Publications

Publication 1: **The ABC transporter Snu and the extracellular protein Snsl cooperate in the formation of the lipid-based inward and outward barrier in the skin of Drosophila** (Eur J Cell Biol.).

I designed and performed the detection of the envelope autofluorescence by confocal microscopy in the embryos and larvae of all stages and phenotypes presented in the publication. I analysed the spectral and lifetime properties of the signal emitted by the envelope by linear unmixing function of the confocal microscopy (Zeiss LSM 880).

I planned and accomplished confocal microscopy work showing the localisation of UAS>GFP-SnuA and TwdlM>Snsl-RFP in the epidermis and cuticle of the *wild-type* larvae and *snsl*- and *snu*-deficient larvae.

I designed and carried out visualisation of the larval cuticular pore canals by UAS>CD8-GFP and UAS>CD8-RFP.

I designed and performed bromophenol blue embryonic permeability experiments and Eosin Y permeability experiments in the wings of adult flies.

Publication 2: **The C-type lectin Schlaff ensures epidermal barrier compactness in Drosophila** (Scientific Reports).

I planned and carried out bromophenol blue permeability tests and visualisation of the envelope by confocal microscopy of wild type, *slf, alas* and *duox* mutant embryos.

I performed fixation in Hoyer's medium and visualisation by the confocal microscope of all the phenotypes shown in the publication.

I designed, made respective fly crosses and visualised the CPR67b-RFP and TwdlD-dsRed in the *wild-type* and Slf-deficient larvae by DMI8 fluorescent microscope (Leica) and confocal microscope (Zeiss).

I generated and analysed the phenotype of double *slf-alas* mutant and late instar Alas and Slf RNAi larvae.

I carried out the crosses of flies and obtained the larvae expressing Tb-GFP with Slf-RFP, ObstE-GFP with Slf-RFP, Slf-RFP with Resilin-Venus. I analysed the localisation of Slf-RFP in the cuticle of the genotypes mentioned above.

I examined the localisation of Resilin-Venus in third instar Slf knockdown larvae with additional TwdlF-dsRed expression.

I purified the specific anti-Schlaff antibody by pre-adsorption and performed the detection of the Schlaff protein (a-Slf) and dityrosine network (a-DT) in all the phenotypes presented in the manuscript.

I down-regulated the activity of Slf in the third instar larval and pupal cuticle, as well as the activity of Alas in the second instar larval cuticle, analysed and photographed given phenotypes.

Manuscript: **Tweedle proteins form extracellular 2D-sheets defining body and cell shape in *Drosophila melanogaster***.

I designed and performed analysis of spatial and temporal localisation of Tb-GFP, TwdlS-GFP, TwdlD-dsRed and TwdlF-dsRed at the first, second and third larval stages.

I planned and accomplished the detailed sub-cuticular confocal microscopic analysis of the localisation of the Tb-GFP, TwdlS-GFP and TwdlF-dsRed in combination with the envelope autofluorescence and the procuticular markers Verm-RFP and CPR67B-RFP in the background of *wild type*, Tb1 and Tb93 living larvae.

I designed and carried out the experiments of the localisation of TwdlF-dsRed in the *wild type* and *phantom* mutant embryos.

I mapped the position of the Tb93 mutation, sequenced the Tweedle family genes in the vicinity of the *tubby* gene and assigned the mutation to the *twdlL* gene.

I planned and accomplished the measurements and statistics of the the cell number, surface area and cell dimensions of the wild type, Tb1 and Tb93 larvae.

I designed and performed the cuticle strechability and crawling efficiency assays.

3. Introduction

Insects are the most numerous class of the whole animal kingdom, which invaded almost all the places in the world. These little, cold-blooded creatures were able to adapt to very different environments – from extremely hot and draughty to very cold and humid ones. Great merit in achieving this goal is their external scaffold called cuticle - hard chitinous shell covering their whole body. It allows locomotion and protects insects - not only mechanically, but also constitutes efficient barrier preventing against dessication and penetration of pathogens and harmful substances into the body. In order to understand how the cuticle plays so many roles, how did it ensure ecological success of the insect class and how can scientific research and industry benefit from it, one needs to learn how it is constructed and how does it function. *Drosophila melanogaster* with all its advantages as a model organism is perfect object for studying cuticle development and architecture.

Cuticle of many insect species has been studied since more than hundred years and shown that it is not a simple uniform layer, but stratified complex structure produced by epidermis below. It needs quite a number of factors and precisely regulated processes to be correctly produced and build up. Even one mistake in the production can result in its construction failure and influence animal survival. The detailed form of the cuticle can differ among the species, developmental stages and body parts, but the general construction is based on the three layers. The outermost thinnest envelope is built mostly of lipids and waxes associated with proteins, that prevent animal dessication and environmental factors penetration (Moussian, 2010, Gibbs, 1998; Gibbs, 2002; Wang et al., 2017; Wang et al., 2016). The middle layer

epicuticle is composed of proteins (Moussian, 2013). The thickest innermost procuticle contains protein-chitin microfibrils running either parallel or helicoidally forming laminae (Neville, 1965, Moussian et al., 2005a).

The main interest of the cuticle research focuses on the procuticle. In *Drosophila melanogaster* number of proteins involved in the procuticle development have been identified. Down-regulated activity of these factors leads very often to the disorders of the procuticular structure, what usually causes characteristic noticeable phenotype and decreases animal survival (Nusslein Volhard et al., 1984). For instance, the embryos lacking Chitin Synthase 1 activity do not produce chitin in the integumental cuticle, what results in its incompactness and dilation. These embryos cannot break open the egg case and die in the egg (Moussian et al., 2005a). In particular, severe cuticular dysfunctions causes down-regulation of the activity of chitin organizing factors in many insect species (Moussian et al., 2005b, Chaudhari et al., 2011, Yu et al., 2016). Their misfunctioning at any developmental stage evokes changes of the newly synthesized cuticle and either animal death or developmental arrest.

Despite quite impressive data gathered over dozens of years about the procuticle, the data about the other two cuticular layers is very faint. In my research I focussed on the envelope and the epicuticle, their visualisation, structure, function and the factors involved in their development. I show that the roles played by these two layers are not less important as the role of the procuticle and their correct proper functioning is critical first of all for the animal protection, but also for the body shape.

4. Objectives and expected outcome of doctoral research:

The objective of my doctoral research was to explore the structure and function of the non-chitinous cuticular part consisting of epicuticle and envelope. I wanted to examine how important are these so far two mysterious layers for cuticular structure and insect survival. For this purpose I have planned to:

- specify factors being organizers/structural components of this part,
- down-regulate/knock-down their activity and to observe the consequences of their dysfunction on the cuticle composition and animal survival, thereby defining their roles.
- observe their exact localisation and affiliation to the respective cuticular layer,
- find eventual interactions with the other cuticular proteins and with the chitinous procuticle.

I concentrated on several factors, which when down-regulated gave decent larval phenotypes (Snustorr and Snustorr-Snarlik, Tweedle: Tubby and Tweedle L and Schlaff) in the envelope, epicuticle and at the epicuticular-procuticular interface, respectively.

I expected to reveal severe cuticular disorders leading to animal developmental disorders, resulting from the incorrect envelope/epicuticular construction and thereby to prove the relevance of these two layers. Also, the analysis of various fluorescent-tagged cuticular proteins was supposed to define the molecular stratification of the larval cuticle of *Drosophila melanogaster*, not performed so far.

By this work, I contribute to the understanding of the cuticle of insects, an ecologically key taxon with the highest species number on our planet.

5. Results

5.1 The ABC transporter Snu and the extracellular protein Snsl cooperate in the formation of the lipid-based inward and outward barrier in the skin of Drosophila.

In order to understand how the cuticle functions, it is essential to identify factors involved in its construction and to specify their role. Frequently used, efficient method in searching for such factors is screening for phenotypes. It is a method based on the complete knocking-down or partial down-regulation of the activity of certain candidate genes and searching for animals presenting cuticle deficient phenotypes. In such a screen based on RNA interference method (RNAi), two independent phenotypes of the non-hatching larvae, suffering rapid dehydration have been found. Based on the properties, they have been named Snustorr (Snu, Swedish for bone-dry) and Snustorr-Snarlik (Snsl, Swedish Snustorr-like). Complete removal (deletion) of the *snu* or *sns*l genes resulted in similar phenotypes, what confirmed efficiency and specificity of the RNAi technique. Snu has been identified as CG9990, an epidermal ABC transporter, whilst Snsl as CG2837, is an extracellular insect-specific protein. Snu- and Snsl-deficient embryos after manual freeing from the eggshell loosed water very quickly compared to the *wild type* embryos, what demonstrated that their outward barrier was not completely functional. Was the inward barrier impaired as well? Recently published cuticle permeability Eosin Y assay (Wang et al., 2016) relying on immersing of the late

stage 17 embryos in the coloured solutions with Eosin Y showed the inflow of the substance into the body. Both *snu* and *snsl* deficient animals revealed rapid uptake of Eosin Y, while the *wild type* embryos remained impermeable. Nevertheless, the *snsl* phenotype seemed to be weaker than the *snu* phenotype as in the *snsl* larvae cuticular permeability for Eosin Y occurred at a higher temperature (40°C), whilst in *snu* larvae already at the room temperature (25°C). Additionally, I tested the cuticle permeability properties of both mutants with another hydrophilic chemical substance of similar molar mass, Bromophenol blue. Both *snu* and *snsl* larvae were permeable already at 25°C what demonstrated that the cuticle exhibited different permeability properties for the two tested dyes. These data confirmed that not only the outward, but also the inward barrier was impaired in *snsl* and *snu* larvae.

Study of the cuticle ultrastructure of the *snu*- and *snsl*-deficient embryos by transmition electron microscopy (TEM) revealed unusual electron-dense material accumulating at the surface of the envelope. Furthermore, the envelope of the *snu*-deficient embryos contained only two out of five alternating electron-dense and electron-lucid layers, whilst the envelope of the *snsl*-deficient embryos did not exhibit any remarkable alterations. The other two cuticular layers, epicuticle and procuticle looked unchanged at the TEM level in both cases. Those data indicated that Snu and Snsl proteins were involved in the formation of the envelope, which seemed to constitute inward-outward barrier.

The envelope stratification in *snsl* larvae, in contrast to the *snu* larvae, on the TEM micrographs looked rather unchanged. On the other hand, the permeability features and the electron dense structures at the envelope surface of *snsl* larvae suggested that their envelope structure contained alterations that could not be fully visualised by the TEM method. This prompted me to search for another method for envelope

visualisation. I discovered that the external cuticular surface - putative envelope - emitted a signal of a broad range of blue and green light when excited by 405nm confocal microscope laser. That signal was missing in the *snu* and *snsl* deficient embryos, what indicated that the envelope structure was incomplete in both phenotypes. After deeper analysis, I found the 405-induced autofluorescent signal in the cuticle of all *Drosophila* developmental stages, what underlined the importance of that unusual material, which I called “405 material”, for cuticle composition. In later larval instars with greater epidermal cells and thicker cuticle the blue signal was detectable predominantly at the cuticular external surface – the putative envelope, as a straight line with distinct dots underneath. Moreover, inside epidermal cells the tiny dots were discernable. I reckoned that those dots were transporting vesicles delivering the blue material from the epidermis - where it was produced - to the cuticle. The dots in the cells were observable through the whole larval instar duration and the envelope linear signal thickened with time, what implied that the blue substance was constantly produced and delivered to the putative envelope.

Which components were missing in the envelopes of *snu* and *snsl* animals and what was the source of the mysterious autofluorescence? The literature data comprising analyses of the envelope composition of some insect species indicated neutral lipids, wax esters and proteins as the main envelope constituents (Gibbs, 1998; Gibbs, 2002; Moussian; 2010). Out of those three components, lipids attracted my attention as the fatty acids have been shown to give the blue-green autofluorescent signal when induced with UV light in the rat liver (Crocce and Bottiroli, 2014). Furthermore, the orthologs of Snu in the red flour beetle *T. castaneum* and migratory locusts *L. migratoria* have been reported to be required for the presence of the surface lipids in the larvae and nymphs (Broehan et al., 2013; Yu et al., 2017). Sudan Black B

lipid staining performed by my colleagues revealed weaker signal in *snu*, but not in *snsl* deficient embryos. This result has shown the dependence of the cuticular lipid localisation on Snu, but not Snsl activity what argued with the hypothesis that 405 material missing in *snu* and *snsl* phenotypes might be lipids. However, Sudan Black B might not detect all the lipids incorporated into the envelope. For instance, the probe detected only the surface lipids and not those ones binding inside the cuticle. Analysis of the cuticle sections would be recommended in the future. Perhaps lipids in a complex with the other molecules or sclerotized ones might be not recognizable by a lipid binding probe. A good candidate for a 405 material in that case seemed to be cuticulin, a legendary catecholamine-protein-lipid complex described by Sir V. Wigglesworth as conferring cuticle waterproofness, tanning and stiffness (Wigglesworth, 1933, Wigglesworth, 1990). Detection of the cuticulin complex with argentaffin staining gave definitely weaker signal in both *snu* and *snsl* deficient embryos, what strongly suggested that the autofluorescent enigmatic substance might be cuticulin. Taken together, the aforementioned results above indicated that the 405 material missing in the *snu* and *snsl* deficient embryos contained lipids and with high probability was that cuticulin complex.

In order to learn more about the spatial composition of the putative cuticulin molecule, I examined its conceivable stratification visualizing the spectral differences of the autofluorescence with the Zeiss confocal linear unmixing function. The signal was not uniform, but could be divided into two zones: the upper and the lower one, what suggested that the autofluorescent material differed in its composition in those two sub-regions. In order to characterize in details the mysterious autofluorescent substance in the future I propose two examinations: Lipid mass spectrometry and Rahman spectroscopy. The first one might help in identification of certain lipids in the

405 material. However, covalent cross-links of lipids with other molecules might be an obstacle in this method. Identification of the lipid molecules and their putative association with other molecules, as well as unravelling the chemical differences between the upper and lower part of the 405 material might be possible by means of Rahman spectroscopy. It would be presumably a big step forward in understanding the nature and stratification of the cuticular envelope.

The evident cuticular phenotypes of *snu* and *snsl* deficient larvae demonstrated that both proteins played significant roles in the envelope construction. To learn more about the exact functions of both proteins we generated fluorescent-tagged forms of Snu isoform A (GFP-SnuA) and Snsl (Snsl-RFP) and induced their expression in the larval integumental epidermis. As the embryonic cuticle was very thin and difficult for microscopic analysis, I studied the localisation of the fluorescent-tagged proteins in the cuticle of the third instar larvae with a thicker cuticle. Snu, an ABC transporter with seven transmembrane helices localized predominantly to the apical plasma membrane of epidermal cells. Fluorescent form of the Snsl protein gave decent signal in the envelope, co-localising with the blue dots underneath the blue straight line. In the dorsal cuticular protrusions called hairs these “dots” were forming longitudinal tubes running from the epidermis until the blue straight external line. This suggested that Snsl could be a part of the cuticular pore canals, thin transport canals running across the cuticle and delivering the constituents (including the putative cuticulin) to the envelope. I searched for a marker to be able to visualize the pore canals and I found the fluorescent-tagged mammalian transmembrane glycoprotein Cluster of Differentiation 8 (CD8) marking the pore canals across the cuticle from the epidermis until the Snsl-blue dots, contacting them directly. This was the first evidence for the

pore canals as membranous structures (probably elongations of the cytoplasm) and the first example of visualisation them by fluorescent microscopy. Localisation of Snsl to the tips of the pore canals and overlapping with the blue signal indicated that Snsl constituted a pore canal part and a gate for the “405” material, transported from the epidermis to the envelope through the pore canals.

The signals of GFP-SnuA and Snsl-RFP to a lesser extent were observable inside the cuticle producing epidermal cells as tiny round-shaped dots, what suggested that both proteins were delivered to their destination in transporting vesicles. Were Snu and Snsl transported in the same vesicles? To reply this question, I analysed the signals of GFP-SnuA and Snsl-RFP in the epidermal cells of the molting second instar larvae during new third instar cuticle production. At that time the fluorescence of both proteins became more intensive. Snu and Snsl dots largely overlapped with each other. Moreover, further analysis revealed that the Snu and Snsl dots co-localised also with the blue autofluorescent dots and CD8 dots. The reciprocal overlapping of Snsl, Snu, blue material and membranous CD8 implied that those round-shaped structures were vesicles transporting Snu, Snsl and blue material to the epidermal surface.

The similarity of the phenotypes of Snu- and Snsl-deficient larvae and the transport of both proteins together with the blue material in the epidermal cells prompted me to investigate the reciprocal relation of Snu and Snsl in the transport of the envelope constituents. Assuming that the transporter Snu might be responsible for the delivery of Snsl and the blue substance to the pore canals, I examined how the down-regulation of Snu activity would influence the localisation of Snsl and the blue material. For this purpose, I generated cell clones with reduced Snu activity in other wise *wild type* clones in the epidermis of late second instar larvae producing the new thick third instar cuticle. As a result, the co-localizing Snsl-RFP and blue dots accumulated not in the

envelope, but predominantly in the lower cuticle parts, presumably the procuticlar pore canals. The blue and red signals in the envelope were significantly lower. The effect turned out to be cell-autonomous as the mislocalisation of Snsl and the blue material occurred only in the cell clone areas with down-regulated Snu activity.

The process of the transport of the envelope elements through the pore canals and deposition in the envelope seemed to be complex phenomenon, involving a network of mutually interacting factors. After I had found the co-operation of Snu and Snsl in the transport of the blue autofluorescent substance to the envelope, I searched for another agents involved in this process. In the past few years a phenotype representing embryonic water loss and larval contraction had been reported (Shaik et al., 2012). It was a consequence of the impaired activity of Delta-aminolevulinate Synthase (Alas), the first enzyme in the heme synthesis pathway. Heme is a cofactor of peroxidases, enzymes required for dityrosine network formation, another cuticular barrier conferring resistance to internal hydrostatic pressure (Shaik et al., 2012). Although the electron micrographs of the cuticle of *alas* embryos showed no visible changes in the envelope structure, I examined if any alterations could have been shown by 405-induced putative cuticulin autofluorescence. The blue layer seemed to be intact in *alas* mutant embryos. Furthermore, I tested the inward barrier of the cuticle of *alas* embryos immersing them in bromophenol blue solution. The dye did not penetrate into the body. Thus, I concluded that the heme synthesis pathway did not seem to contribute to the envelope formation and the envelope inward barrier.

Another candidate pathway that might be required for the envelope integrity was the melanisation pathway as the head skeleton and the cuticular denticles of the Snu-deficient embryos were barely melanised. I studied the surface autofluorescence of the mutants in the *pale* gene, encoding the first enzyme in the melanisation pathway.

The blue signal was not reduced, and the cuticle was not permeable for bromophenol blue solution, what revealed that the melanisation pathway was not involved in the envelope structure and waterproofness.

Summarizing, I developed a fast and simple method of the visualisation of the cuticular envelope of the fruit fly. It allows detection of the alterations in the envelope structure not discernable by electron microscopy. I found an innovative method of the cuticular pore canals visualisation by the confocal microscopy in *Drosophila,* which gives new perspectives of studying of the pore canal structure and function *in vivo*. I contributed to the identification of a new pathway deployed during cuticle formation in general and barrier construction in particular. I discovered that the ABC transporter Snu and cuticular protein Snsl cooperate in transport of the envelope constituents, including a 405nm-induced autofluorescent lipidic complex, which is very likely the legendary cuticulin.

5.2 The putative C-type lectin Schlaff ensures epidermal barrier compactness in Drosophila.

As shown in the upper part, proper functioning of cuticular barriers is highly relevant for the survival of insects. Another conspicuous example of the non-functional barrier, lethal in consequences, is knock down of the *schlaff* gene, encoding an extracellular Calcium-dependent (C-type) lectin with an N-terminal extracellular signal. The *schlaff* phenotype has been found in two screens: widely performed screen for mutants with cuticular disorders (Nuesslein-Volhardt et al., 1984) and RNAi screen performed by Bernard Moussian and Stefan Luschnig (Moussian and Luschnig, 2004). Ready-to-hatch RNAi/mutant larvae with down-regulated/deficient Slf activity at the end of embryonic development contract in the egg and lose water, which fills the egg space. In the head region the cuticle is dilated whilst in the abdominal part it is crumpled. The transmission electron microscopy reveals no visible envelope structural disorders. The structures of epicuticle and procuticle seem to be disorganised. The procuticle contains inclusions of various sizes disrupting the laminar organisation. The epicuticle occasionally detaches from the procuticle and its upper sub-layer is not smooth. Regardless of the visible malformations in the epicuticle and procuticle, the water outflow suggests that the *slf* mutants may contain structural alterations in the envelope as the outward barrier is impaired. In order to rule out that the envelope dysfunction of the *slf* larvae is a cause of the contracted phenotype and water loss, I tested the properties of the envelope. First, I analysed the 405nm-induced autofluorescence by confocal microscopy. The blue signal of the *slf* deficient embryos was not reduced in contrast to the signal of the control *snsl* deficient embryos with a defective envelope. Second, I examined the inward barrier of the Slf-deficient embryos immersing them in

bromophenol blue solution. The cuticle of larvae with down-regulated Slf function was impermeable, while Snsl embryos got coloured very rapidly. This revealed that – in contrast to the embryos with envelope disorders - only the outward cuticular barrier of the *slf* mutant embryos was dysfunctional.

Alternative explanation of the massive water loss were defects of the septate junction (SJ) barrier, composed of epidermal structures linking neighbouring epidermal cells. However, dye injection assays and visualisation of the septate junction structure of the *slf* deficient embryos performed by my colleagues did not show any changes in the septate junction structure and function in contrast to the embryos with mutations in a gene coding for one of the SJ component, Coracle.

The effects of the procuticular disorders on the cuticle compactness can be visualized by immersion in the Hoyer's medium on the slide and heating up to 65°C in order to digest all the proteins and to let the cuticle stretch. Any structural disorders of the procuticle lead to body shape changes. For instance, the cuticle of chitin synthase mutants *krotzkopf verkehrt* or chitin organising factor mutants *knickkopf* is dilated in comparison with the cuticle of the *wild type* embryos (Moussian et al., 2005b, Moussian et al., 2006b). Inclusions in the procuticle of the Slf-deficient embryos and visible malformations in the head and abdominal part indicated that Hoyer's preps might be misshapen. Indeed, the head and the dorsal cuticle parts were extremely dilated and in the other body parts the cuticle formed large blisters. With a closer look, the blisters were formed by the external cuticular layer only, whilst the internal was not dilated, what suggested cuticle delamination. Confocal analysis of the 405nm-induced autofluorescence showed the envelope signal in the dilated parts.

The Hoyer's fixation revealed the detachment of the envelope from the lower cuticular layers in the *slf*-deficient embryos, whilst the electron microscopy additionally local separation of the epicuticle and the procuticle. Both methods indicated cuticle delamination, however, it occurred *in vitro*. To investigate the cuticular compactness *in vivo*, I expressed two fluorescent-tagged proteins representing two different cuticular layers: chitin-binding Cuticle Protein 67b-RFP (CPR67b-RFP) and TweedleD-dsRed (TwdlD-dsRed, Guan et al., 2006) in the background of the *wild type* and *slf* mutations. CPR67b-RFP depicted the chitinous procuticle, while TwdlD-dsRed the layer above, probably the epicuticle. The latter one co-localized with the dilated cuticle and the former one with non-dilated, attaching to the body surface in the *slf* mutant embryos. In the cuticle of the *wild type* embryos both fluorescent proteins were attaching to the body surface. This underlined the delamination *in vivo* in the *slf* embryos, showing that the putative epicuticle and envelope detach from the procuticle and form large blisters.

The detection of Schlaff protein using anti-Schlaff specific antibody in the embryos at the end developmental stage revealed Slf signal in the integumental epidermal cuticle and the cuticle surrounding mouth hooks. The detachment of the epicuticle from the procuticle *in vivo* suggested that Slf protein might localize between these two layers. To examine exact Slf localisation, we generated Red Fluorescent Protein-tagged Schlaff and induced its expression in the third instar larvae containing either the epicuticle marked green by Tubby-GFP (Tb-GFP) or the procuticle marked green by ObstructorE-GFP (ObstE-GFP, Tajiri et al., 2017). Slf-RFP localized in the cuticle predominantly below the Tb-GFP and above the ObstE-GFP, weak signal was observable also in the whole procuticle overlapping with the ObstE-GFP fluorescence. Under the procuticle in the epidermis the signal of Slf-RFP was decent in a form of

dots, which were probably the transport vesicles. Slf localisation data corroborated the hypothesis that Slf layer occurs between the epicuticle and procuticle and thereby is important for the adhesion of these two layers.

The role of Slf played in the soft larval integumental cuticle seemed to be essential. However, despite the a-Slf antibody signal detected in the epidermis surrounding the hard cuticle of the mouth hooks, no structural changes of the mouth hooks of the *slf* mutant embryos were observed. Was Slf activity important for the development and function of the hard cuticle? In order to answer this question my colleagues by means of the Flp/FRT system induced the clones of homozygous *slf* mutant cells in the heads of developing adult flies, consisting of both, hard and soft cuticle parts. As a consequence, the non-eclosing flies showed ruptures in the soft ptillinum only, whilst the hard cuticle looked unchanged. It suggested that Slf was needed for the development of the soft but not hard cuticle. To confirm this interpretation, I down-regulated the activity of Slf in the entire epidermal cuticle of developing pupae. I observed necrosis in all the regions with soft cuticle: leg joints, wing hinges, ptillinum, and ventral abdomen, whilst the regions with hard cuticle looked unaffected. The flies either did not eclose or eclosed and died shortly afterwards. This data confirmed that Schlaff is required especially for the soft cuticle of *Drosophila melanogaster*.

As mentioned in the previous thesis part, few years ago a phenotype presenting loss-of-function of *alas* gene has been described (Shaik et al., 2012). The *alas* gene encodes Delta-aminolevulinate Synthase, the first enzyme in heme synthesis pathway. The phenotype of *alas* resembles *slf* phenotype: non-hatching embryos contracted in the egg with dilated head cuticle and massive water loss. The single difference in both phenotypes are non-air-filled, permeable tracheae in *alas* embryos. However, based on the Slf localisation data, Slf does not seem to be expressed and

play any role in the tracheal system, perhaps another Slf homologue is active in the tracheae. In order to learn how similar *alas* and *slf* phenotypes are, I performed several genetic and histological experiments. First, I examined the cuticular structure and permeability of *alas* mutant embryos. Hoyer's preparations, autofluorescent signal visualisation and bromophenol blue penetration assays showed congruent defects with those observed in *slf* mutant embryos. Second, I combined *alas* and *slf* mutations together and generated double *alas-slf* mutant. An additive effect shown by a double mutant would indicate that Slf and Alas are involved in two different processes of cuticle development. As a result, the phenotype of the *alas-slf* mutant was similar to the phenotypes presented by single *alas* and *slf* mutations. It suggested that both proteins played roles in a common pathway of the cuticle formation. Third, I generated the late larval instar *alas* and *slf* phenotypes by a partial down-regulation of gene expression using RNAi technique. In both cases the larvae were contracted and the cuticle carried many dark wounds. The Hoyer's preps of those larvae showed cuticle delamination, i.e. large blisters were detaching from the body surface. All that data suggested that both Alas and Slf proteins were involved in the same biological cuticle synthesis pathway. To confirm this assumption I tested if Slf may be dependent on the Alas function visualizing the localisation of Slf by means of specific a-Slf antibody in a background of *alas* mutant embryos. The Slf signal was present in the cuticle and no difference in Slf-detection between *alas* and *wild type* embryos was observable, what suggested that Slf localisation was unchanged. However, the embryonic cuticle is very thin and there was no possibility to perform more detailed localisation analysis.

Alas is an enzyme in heme synthesis pathway and heme is a cofactor of many proteins, including peroxidases. Peroxidase activity has been shown to entail protein dityrosinylation. It happens either as a side effect of an oxidation or purposely in order

to confer elasticity of the tissue, such as cuticle (Malencik and Anderson, 2003, Edens et al., 2001). Dityrosine network has been documented to be decreased in *alas* mutant embryos (Shaik et al., 2012). Was Slf involved in dityrosine network formation as well? Using dityrosine specific antibody (a-DT) I examined whether dityrosine network was disordered in the late instar embryos with no Slf activity in comparison with the *wild type* and *alas* deficient embryos. In *alas* deficient embryos the dityrosine network was reduced in the integumental and tracheal cuticle in comparison with the situation in *wild type* embryos. In the *slf* mutant embryos the a-DT signal was significantly weaker in the integumental cuticle, whilst in the tracheae it was unchanged. This implied that dityrosine network depended on the presence of Slf, either directly (Slf as a substrate of dityrosinylation) or indirectly (Slf as dityrosine network organizer/stabiliser). Moreover, it confirmed that the Slf activity is required in the integumental but not tracheal cuticle.

The direct role of Slf in dityrosinylation process (as dityrosinylation substrate) seemed to be very unlikely as the Western Blot band of Slf protein was very distinct. In case of dityrosinylated network (covalently cross-linked and thereby difficult to separate into single proteins) we expected to have peptides of different sizes and as a consequence smudged bands. To test the indirect Slf role in DT-network organisation, I focused on Resilin, a protein well known in the cuticle field to form a network of di- and trityrosines, conferring high cuticle resilience (Andersen and Weis-Fogh, 1964). I examined at which cuticular layer Resilin was deposited in the larval cuticle and how the Slf activity depletion influenced Resilin localisation. First, I co-expressed fluorescent-tagged Resilin protein under the control of the Upstream Activating Sequence (UAS>Resilin-Venus) in the cuticle of third instar larvae with Slf-RFP. Both proteins co-localized, what suggested that both occurred in the same

cuticular layer. Second, I expressed UAS>Resilin-Venus in the background of the *wild type* and Slf knockdown larvae with additional expression of the cuticular TweedleF-dsRed (TwdlF-dsRed) possibly representing the epicuticle. In the cuticle of the larvae with normal Schlaff activity Resilin-Venus localized in a form of a thin line below the TwdlF-dsRed layer. In the Slf knockdown larvae Resilin-Venus signal was weaker and broader, spanning across the procuticle until the epidermal border. It suggested that Resilin was mis-localized in the whole procuticle, whilst the localisation of TwdlF-dsRed was unchanged. Summarizing, Resilin localisation analysis implied that Slf is involved in the Resilin/Dityrosine network organisation. Although the exact mechanism is not known yet, there are two most possible scenarios of this process which need to be examined: either delivery of the substrates of dityrosinylation to the Schlaff layer or stabilisation/organisation of the network in the layer.

Protein dityrosinylation process requires peroxidase activity (Malencik and Anderson, 2003). Which peroxidase co-operates with Slf and Alas in dityrosine network formation in *Drosophila* larvae? A good candidate seems to be the membrane-inserted Dual Oxidase Duox, a protein documented to be involved in dityrosinylation in insects and nematodes (Anh et al., 2011; Edens et al., 2001). I examined the signal of dityrosine network in the embryos with *duox* deficiency using a-DT specific antibody. There was no difference in the respective signal between *duox* deficient and the *wild type* embryos. This data suggests that Duox is not involved in the larval dityrosine network formation in *Drosophila melanogaster*. Nevertheless, the other possible scenarios must be considered such as are long-lasting maternal mRNA activity and compensation by another peroxidase activity. Recently Curly Suppressor (CySu) peroxidase has been shown to act together with Duox in protein cross-linking in *Drosophila* wing (Hurd et al., 2015). Perhaps the down-regulation of both, Duox and

CySu together would reveal impaired larval dityrosine network. In order to find out which peroxidase(s) are involved in this process, I would recommend immunofluorescent analysis of the dityrosine network of various peroxidase mutants/RNAi animals.

Taken together, my work revealed Slf localisation in the larval cuticle between the epi- and procuticle and underlined the importance of the presence of Slf for attachment of these two layers to each other. Furthermore, I showed that Slf plays important role especially in the soft cuticle, including larval cuticle and soft body parts in adult fly. Finally, I specified the role of Slf playing in the epicuticle-procuticle adherens zone by organisation of the dityrosine layer in co-operation with the heme synthesis pathway.

5.3 Tweedle proteins form extracellular 2D-sheets defining body and cell shape in Drosophila melanogaster.

Extracellular matrices are produced by all multicellular animals, protecting them against environmental conditions and to a greater or lesser extent conferring animal shape. In arthropods, the body shape significantly relies on the exoskeleton and any changes may influence the quality of animal life. In insects the extracellular matrix cuticle is shed and rebuilt several times, what significantly increases a risk of an error. In the present publication I am focusing on the epicuticle, the non-chitinous, still unexplored cuticular layer, its construction and impact on the other layers, the body shape and insect life.

More than a decade ago a novel cuticular protein family named Tweedle has been discovered in the fruit fly (Guan et al., 2006). The members (Tweedles, Twdls) are insect specific, extracellular proteins with a DUF 243 domain of unknown function. Mutations in this domain in two Tweedle proteins, Tweedle A (Tubby, Tb) and Tweedle D (TwdlD) elicit a dominant effect of body shape change (Guan et al., 2006). The larvae and consequently pupae are shorter and thicker whilst adults slightly more squat than the *wild type* flies. This mutation is commonly used as a marker for the third chromosome balancer TM6 of *Drosophila melanogaster* (*flybase.org*). The spatial and temporal localisation of various Tweedle members performed by Guan's team revealed that Twdls are expressed at different developmental stages and different cuticle producing body parts. However, the exact localisation regarding the cuticular layers was not accomplished. The secondary structure of the Tweedle proteins and the positive result of *in vitro* chitin binding assays suggest that Tweedles localise to the chitinous procuticle (Guan et al., 2006, Tang et al., 2010). In order to find in which

cuticular layer occur Tweedle proteins *in vivo*, I performed a detailed microscopic analysis of Guan's ds-Red-tagged fluorescent Tweedle lines and the GFP-tagged lines generated in our lab. As first, I carried out an overview of the Twdl expression in the whole larval body, verifying the presence of the respective Tweedle proteins at the three larval instars. TwdlD-dsRed was strongly expressed in the first and second instar larvae in a striped pattern, while TwdlF-dsRed was expressed at all three larval stages in the whole cuticular epidermis evenly. The signal of TwdlS-GFP was quite weak in the whole cuticle of the first and second larval stage (L1 and L2) and more decent at the third larval stage (L3). Tubby-GFP (Tb-GfP) was localised in patches at L1 and evenly in the whole cuticle of L2 and L3. Afterwards, I performed confocal microscopy analysis of sub-cuticular localisation of fluorescent-tagged Tweedle proteins in third instar larvae. I combined fluorescently tagged Tweedles with the 405nm-induced autofluorescent signal given by the envelope (Zuber et al., 2018) and procuticular-specific CPR-67B-dsRed and Vermiform-RFP (Verm-RFP, Forster et al., 2010), both containing chitin-binding domains. TwdlS and Tubby conjugated with GFP (TwdlS-GFP and Tb-GFP) formed a line between the autofluorescent envelope and the procuticle marked in red (CPR-67B-dsRed). When combined together, TwdlF-dsRed overlapped with Tb-GFP, but not with TwdlS-GFP, which located just below the TwdlF-dsRed layer. The localisation of the fluorescent layers looked a bit different in the cuticular protrusions, for instance dorsal hairs. Here the Verm-RFP or CPR67B-dsRed marking the chitinous procuticle filled the center of the hair. Tweedle layer, similarly to the uncovered by hairs "naked" cuticle, occurred between the envelope and the procuticle. However, in that case in both Tweedle combinations, the proteins did not overlap but formed the two-dimensional sheets: TwdlF-dsRed - the upper one and Tb-GFP and TwdlS-GFP - the lower one.

The confocal microscopy pictures suggested that Tweedle proteins localised to the putative epicuticle, where they were stratified, forming two-dimensional sheets. To test this notion I monitored the expression and localisation of the TwdlF-dsRed in the ecdysone deficient i.e. *phantom* (phm) mutant embryos. *Phantom* is a gene active in the ecdysone synthesis pathway. Ecdysone has been shown to be required for the epicuticle, but not the procuticle formation (Gangishetti et al., 2010). In *wild type* embryos the signal of TwdlF-dsRed occurred in the whole integumental cuticle, while in *phm* mutant embryos was very faint, visible mainly as tiny dots in the epidermal cells. This result underlined that Twdl proteins belong to the epicuticle, not like suggested before, the procuticle, and their expression is dependent on ecdysone signalling.

The epicuticular localisation of the Tweedle proteins implied that the alterations of that layer were a cause of the squat larvae of the Twdl dominant mutants. To find a source of the shape change I performed several experiments. I started from the analysis of the localisation of various Twdl proteins in the alleles described as the dominant alleles of the *tweedle A (Tubby)* gene: Tb1 and Tb93 (*flybase.org*). In the naked cuticle of the living Tb1 third instar larvae, Tb-GFP, TwdlS-GFP and TwdlF-dsRed were only partially localised in the epicuticle just below the autofluorescence of the envelope. Here, the TwdlS-GFP and TwdlF-dsRed were not seperated but entirely overlapped. Partially, the Tweedle proteins were aggregated together inside the cuticle, probably the procuticle. In the dorsal hairs, the three Tweedle proteins partially accumulated in the epicuticle with no visible stratification and partially at the hair tip entirely overlapping. In the Tb93 larvae, Tb-GFP and TwdlS-GFP were only weakly aggregated in the putative procuticle and at the tips of hairs, whilst TwdlF-dsRed was not aggregated, but correctly localised to the epicuticle. Taken together, the analysis

revealed that in Tb mutant larvae the Tweedle proteins form aggregates below the Tweedle layer and the composition of the aggregates differs between the *tubby* alleles.

The mutation in the *tubby* gene suggested that the mutated protein was a source of the mislocalisation of other family members. In order to confirm this assumption I expressed Tb-GFP and TwdlS-GFP in a background of the mutated fluorescent-tagged Tubby protein, Tb1-RFP (Pina and Pignoni; 2012). The mutated Tubby protein formed numerous aggregates in the putative procuticle of the third instar living larvae. The non-mutated Tubby protein and GFP-tagged TwdlS protein were attracted to the aggregates. Hence, this data confirmed that the mutated Tubby protein is a cause of the aggregation and it attracts the other normal Tweedle proteins, including the non-mutated Tubby version.

The cross-sections of the cuticular ultrastructure of the Tb1 and Tb93 mutant larvae visualized by transmission electron microscopy (TEM) revealed electron dense aggregates immersed in the procuticle. Those aggregates I considered as the Tweedle aggregates visible on the confocal microscopy pictures. In order to test this hypothesis I co-expressed TwdlS-GFP with Verm-RFP and CPR67B-RFP in a background of Tb1 and Tb93 third instar mutant larvae. In ¨the naked cuticle of both phenotypes the aggregates of TwdlS-GFP were surrounded by red fluorescing procuticle. In the dorsal hairs the localisation was different, as the aggregates were the hair tips, whilst red procuticle at the basis below the aggregates. Based on this data, I corroborated my hypothesis that Tweedle aggregates constitute the ectopic epicuticle accumulating in the lower procuticle of the naked cuticle and at the dorsal hair tips.

The mislocalisation of the TwdI proteins in Tb1 and Tb93 mutant larvae was obvious in the transverse (dorsal-ventral) cross-section. Did it occur in the lateral

cross-section? Tweedle D was expressed in a striped pattern at the first and second larval instars. I tested TwdlD-dsRed distribution in the lateral surface in two *tubby* alleles to see if the striped pattern was disordered. In both cases TwdlD was mislocalised perpendicularly aggregating in the lower procuticle. Longitudinally, the aggregates were spread along the whole cuticle without formation of the striped domains. Moreover, in the *wild type* larvae the areas containing the dorsal hairs were lacking TwdlD and that protein was not present in the hairs. In both *tubby* alleles however TwdlD aggregated at the tips of hairs. Thus, the Tweedle proteins were mislocalised in the cuticle two-dimensionally: along the dorsal-ventral and lateral axis. Was the lateral Twdl mislocalisation a consequence of the expression pattern change or the increased lateral mobility of a protein? To test this notion I examined the expression domains of the nuclear binding Red Fluorescent Protein under the control of the *twdlD* promoter (TwdlD>RFP-NLS) in Tb1, Tb93 and the *wild type* larvae. In all cases the expression had a striped pattern, what strongly indicated that the ectopic expansion was related with the mobility of the aggregates in the procuticle. The stable, organized laminar structure of the procuticle argues with the theory of mobility of the proteins/aggregates occurring inside this layer. However, the Tweedle aggregates are formed at the time of cuticle formation where the procuticle perhaps is unorganized yet. Studying of the Tweedle aggregation/mobility processes in the newly formed larval cuticle might help in understanding the mechanisms of cuticular layers/procuticuar laminae deposition.

Mutated Tubby protein attracted the other unmutated epicuticular proteins from the Tweedle family. As shown before by confocal microscopy, the Tweedle protein layer seemed to be in contact with the lower layer of dityrosinylated proteins. If these two layers bind each other, do the ectopic Tweedle aggregates attract the proteins from

dityrosine layer then? To find the answer I co-expressed TwdlF-dsRed with fluorescent-tagged Resilin protein (Resilin-Venus) in a background of the third instar *wild type*, Tb1 and Tb93 mutant larvae. Resilin protein is well known to be dityrosinylated in the insect cuticle (Andersen and Weis Fogh, 1964; Ardell and Andersen, 2001). Resilin-Venus localized just below the TwdlF layer in the naked cuticle and the dorsal hairs of the *wild type* larvae. In the Tb1 larvae Resilin signal was predominantly surrounding the aggregates marked by TwdlF-dsRed. In the Tb93 larvae TwdlF was not mislocalized and Resilin surrounded the unmarked aggregates. In the dorsal hairs of the Tb1 and Tb93 larvae Resilin-Venus was only partially mislocalized, surrounding the aggregates at the hair tips. What is more, I discovered that Resilin-Venus localisation in the cuticle of the Tb larvae was changing with time. In the cuticle of third instar larvae just after hatching Resilin signal was overlapping with the signal of aggregates. After dozen of hours Resilin signal was moving towards aggregate edges and finally it was accumulating around the aggregates, being clearly separated from the aggregates. In the *wild type* larvae, in contrast to the Tb larvae, Resilin signal was stable at the whole larval stage. Concluding, I have shown that the Tweedle aggregates attract Resilin protein representing the lower dityrosine layer and the localisation of Resilin in the aggregates changes with time, what might also reflect changes undergoing in the cuticle during its formation.

Tb1 and Tb93 were considered as the two alleles of the *tubby* gene. However, they revealed differences in the distribution of the fluorescent-tagged Tweedle proteins. Moreover, the two phenotypes differed between the larval stages: Tb1 squat body shape was remarkable since the second instar, while in Tb93 larvae already at first instar. This prompted me to examine the sequences of the two alleles. The deletion of six amino acids in the DUF243 domain of the Tb1 allele has been reported (Guan et

al., 2006). Nevertheless, there was no accessible data about the sequence changes in the Tb93 allele (*flybase.org*). After sequencing I could not find any change in the sequence of the *tubby* gene, what suggested that Tb93 was not an allele of that gene. Thus, I performed recombination tests between the Tb1 and Tb93 alleles. I assumed that the alleles of the two different genes would be able to be recombined on one chromosome and recombination statistics would give approximate position of the Tb93 mutation. As a result, I was able to recombine these two alleles and the rough distance of these two loci was 0,1 cM. Subsequently, I sequenced other Twdl genes placed in the vicinity of the *tubby* gene and I found a missense mutation in a conserved amino acid in the sequence of the DUF 243 domain of a *twdlL* gene. To confirm that Tb93 was an allele of the *twdlL* gene, I down-regulated the activity of the *twdlL* gene in a background of the Tb93 squat larvae. The RNAi expression abolished the contracted phenotype and restored the normal larval and pupal longevity and thereby proved that Tb93 is an allele of the *twdlL* gene.

The contracted body shape might be related with the alterations such as changes in cell morphology and locomotion, which at the end may influence the quality of animal life and survival. I tested both options in Tb1 and Tb93 larvae. First, I examined the integumental epidermal cell number, surface area and shape. In both mutants the cell number and the surface areas were unchanged, whilst their shape, especially in the middle parts of segments was longer along the dorso-ventral axis and shorter along the anterior-posterior axis. Second, I tested the cuticle strechability along the anterior-posterior axis and crawling efficiency of the mutant larvae. I could not find any difference between the Tb1, Tb93 and *wild type* larvae. Taken together, this data indicates that the epicuticular mislocalisation leading to the barrel shape does affect the cuticular strechability and larval locomotion, but seems to affect the cell shape.

The initial explanation for the barrel body shape was the limited cuticle strechability in the anterior-posterior axis, which would “force” the cells to grow laterally. In case of unchanged cuticle strechability another scenario must be considered. I created a model based on the three zones (internal, in-between and external), which might have an impact on the squat body shape. First zone, called “internal” would be the epidermis and the processes taking place inside the epidermal cells. For instance, influence of the mutated Tweedle proteins on the intracellular processes including cell shape determination or intracellular cuticular subunit organisation. This point remains the open question to be answered in the future. Second zone, called “in-between” means epidermal-cuticular interface (attachment site of the epidermis to the cuticle) and the processes undergoing in this narrow region, e.g. transport of the cuticular constituents through the cell membrane to the external space and their deposition outside/preparation for further transport across the cuticle. One possible explanation of the impaired body shape, consistent with the “in-between” model seemed to be mutation in the cuticular Obstructor E (ObstE) presenting the opposite effect of the shape change to the Tubby mutants (Tajiri et al.; 2017). The ObstE mutant larvae did not contract during pupariation, what was linked with the lack of the procuticular basal longitudinal ridges at the procuticular-epidermal attachment zone. I examined the relation between the ObstE and Tweedle protein functions in larval cuticular body shaping. The structure of ridges shown by atomic force microscopy scanning turned out to be flatter and disorganised in Tb1 and Tb93 mutant larvae compared to the longitudinal parallel convexities in the *wild type* larvae. This revealed that correct deposition of the Tweedle proteins is significant for the correct orientation of the procuticular ridges. Moreover, I tested the localisation of the fluorescent-tagged ObstE protein (ObstE-GFP) in the background of the Tb1 and Tb93 mutants, which seemed

to be unchanged. Nevertheless, the changes in the procuticular ridges structure demonstrated the input of the “in-between” zone in the tubby-like phenotype. The importance of the undulated shape of the in-between zone for the cuticular struture underlines the observation that during the embryonic cuticle production the epidermis forms apical corrugations called undulae (Moussian, 2006). At the crests of the undulae reside chitin-synthase complexes. The localisation of these complexes in the crests (and thereby the epidermal undulation) might be essential to produce and to organize chitin microfibrills in a certain manner. Hence, it seems that certain shape of the in-between zone influenced either by the cell or by the cuticle is necessary for the correct cuticle construction and the cell and overall body shape.

The third, “external” zone that might influence the body shape is cuticle – its organisation and properties. The depletion and disorganisation of the epicuticle and dityrosine layer and their ectopic accumulation disrupting procuticular lammellae might alter the physical properties of the cuticle – for instance, the resistibility to the internal hydrostatic pressure. Following the Barlow law, a cylinder with the weak lateral wall expands rather radially than longitudinally, what is consistent with the growth of *Tubby* mutants.

Summarizing, my data demonstrated unexpected epicuticular localisation and additional stratification of the Tweedle proteins in the cuticle of *Drosophila* larvae. It revealed the importance of the correct structure of the epicuticle for the whole cuticular construction and the larval body shape. The results helped in understanding the aberrant larval and pupal barrel-like shape of the well-known and broadly used *tubby* mutants. Moreover, the data uncovered the real source of the Tb93 mutation, inconsistent with the previous findings. The paper sheds new light on the still unexplored epicuticle and opens the doors for further analysis to understand the

functioning of the insect cuticle and to find new practical applications in the agricultural and medical fields.

6. Discussion and perspectives

6.1 Envelope and epicuticle – still unexplored significant cuticle areas

The cuticle research over the past decades concentrated predominantly on the chitinous procuticle, its structure and function. The answer on the coming question: why not on the other cuticular layers? – seems to be simple. First of all, the procuticle is the thickest and toughest layer and any minor changes in its structure are reflected in the distinct early larval phenotypes. Thereby, it is rather easy to discern the larvae with procuticular disorders in a screen. Furthermore, chitin is a unique material in the fungal and animal world and thus an efficient target in a battle against pests. Recently chitin and its derivatives find also novel applications in modern biomaterial branch. All these advantages of the chitinous procuticle attract the attention of many researchers. In my paper I focus on the two other cuticular layers - the envelope and the epicuticle, and I show evidences that the proper structure and function of these two layers are essential for animal survival as well. Furthermore, the epicuticle plays a role in the larval body shaping. The two upper cuticular layers beside the procuticle can become potential sources for a modern medical and agricultural research.

6.1 a) lipidic envelope and its transport through the cuticular pore canals

The envelope of the fruit fly, although very thin, has a complex stratified structure. It constitutes an effective inward- and outward barrier protecting animal against water loss and inflow of the harmful environmental substances into the body. The envelope is known to be composed mainly of lipids, waxes and proteins. However, the exact chemical feature of the constituents and their assignment to the respective sub-layers needs to be investigated. In my research I discovered in the envelope of *Drosophila*

melanogaster a material, which exposed to the 405nm confocal laser fluoresces in a broad blue and green light spectrum. Depletion of this material is related with highly permeable phenotype, lethal in consequences. Conducted experiments strongly suggest that this yet uncharacterised substance contains lipids and with a high probability it is a catecholamine-protein-lipid complex named cuticulin. Discovered and hardly described several decades ago by Sir Wigglesworth, this enigmatic waterproof material still remains unexplored. Simple and quick cuticulin visualisation with a confocal microscopy besides the complex argentaffin staining seems to be a milestone for further investigation of this material. It allows cuticulin identification without introducing any artificial dyes. It enables studying production and delivery of this material in real time in the living animals. What is more, cuticulin transport might be common with the other envelope components, what gives perspectives of studying processes undergoing in the cell and cuticle during envelope production in a broader scale.

In particular, the transport of the 405 material through the pore canals is a highly interesting process. These thin tubes running across the whole cuticle have been discovered dozens of years ago and revealed in the cuticle of many insect species, usually in the fixed and highly processed samples (Locke, 1961; Neville et al., 1969, Noh et al., 2015). However, their construction and exact role at the molecular level is until now poorly understood. A novel method of pore canal imaging with a confocal microscope gives perspectives of studying these structures *in vivo*, especially in combination with other fluorescing components. Presence of the membrane-bound Cluster of Differentiation 8 protein (CD8) almost in the whole length of the pore canals implies that they constitute membranous elongations of the epidermal cells. Perhaps these tubes contain also a cytoplasm and other cellular constituents, not detectable

on the fixed cuticular preparations. Localisation analysis of fluorescent-tagged forms of the respective candidates in the living animals may help in understanding of pore canals structure. Studying the phenotypes with envelope disorders, I discovered a first example of the pore canal transport pathway. I showed that the cuticulin complex is delivered to the plasma membrane of epidermal cells in the transporting vesicles together with the ABC transporter Snu and the cuticular protein Snsl. Afterwards, cuticulin is transported to the envelope through the pore canals, in which Snsl localizes at the upper, non-membranous part and possibly constitutes a gate for molecules passing them to the envelope. This model is rough and there are still many questions to be answered, for instance the exact role of Snu in this process. Modern genetic and molecular biology techniques, as well as rapidly developing bioinformatics offer many possibilities for the identification of the whole network of factors potentially involved in the pore canal function and targets of this transport in order to deepen our knowledge about the pore canal role in cuticle production.

6.1 b) course of envelope production

Another important aspect uncovered from a new side in my research is the envelope production process. As reported before, in *Drosophila melanogaster* the larval cuticle production starts from the envelope formation. Fragments of the envelope consisting of the two electron dense layers separated by the electron lucid one are deposited on the epidermal cell surface and fuse together (Moussian et al., 2006a). Afterwards, the two other cuticular layers – epicuticle and procuticle – are deposited and electron-dense canals - probably the pore canals – are being formed in these two layers. Subsequently, the third electron-dense layer intercalates into the electron-lucid one and the envelope achieves the mature structure consisting of three electron-dense and two electron-lucid sheets. Thereby, the third electron dense layer is produced after

the epicuticle and procuticle deposition and the pore canal formation, what suggests that it is transported to the envelope through the pore canals. This electron dense layer is missing in the *snu* mutant embryos, what strongly implies that the auto-fluorescent blue material, which is also missing, and known to be transported through the pore canals, might be a constituent of this layer. Studying exact mechanism of the transport of the 405 substance can help in elucidating the last step of the envelope production process, which seems to be interesting as it is separated in time from the production of the first three layers and requires formation of highly specialised canal tubes for the transport through the two lower cuticular layers. The straight shape of the pore canals without any branches, running from the cell surface directly until the envelope suggests that these complex structures are formed in order to complete the structure of the envelope only, without taking part in the two other layers' formation. What stands behind the reason of pore canal generation instead of simple production of the whole envelope at the beginning of the cuticle production? The process of blue material synthesis and delivery seems to occur during the whole larval stage, what might be related with cuticle stretching during growth. The need of the constant envelope production in order to strengthen it during larval growth might be a possible explanation of the pore canal formation, in which the cell invests much costs and involves many factors.

Until now very little is known about the exact mechanism of pore canal formation, function and the envelope delivery and production, although this process is essential for the insect survival. My research has uncovered important aspects of this machinery and opened a gate for further investigations to unravel this mystery in the future.

6.1 c) Envelope and epicuticle-procuticle zone – two different waterproof cuticular systems

The envelope constitutes an efficient impermeable inward- and outward barrier, next to the epidermal barrier built up of the epidermal cells tightly bound to each other with septate junctions. An interesting phenotype suggesting breakdown of the cuticular barrier is presented by embryos deficient in cuticular C-type lectin Schlaff: severe haemolymph leakage and as a consequence contracted larvae inside the egg. However, the *slf* phenotype is different from the one represented by *snu* and *snsl* mutant embryos. The envelope structure shown in transmission electron micrographs and the blue material visualisation do not exhibit any visible changes. Furthermore, bromophenol blue and Eosin Y do not penetrate into the body of the *slf* mutant embryos, what reveals that the inward barrier is unchanged. This data indicates that not the envelope is a reason for the haemolymph leakage. The ultrastructure of the septate junctions looks unchanged in the *slf* mutant embryos, as well as the phenotype of the septate junction (SJ) mutants looks and behaves differently from the *slf* phenotype in the permeability dextran injection tests. This disqualifies *slf* mutants as representing broken SJ barrier. The ascertained reason of the haemolymph leakage is probably a broken layer of dityrosinylated proteins at the epicuticle-procuticle border, which is delivered/organised by the Schlaff lectin and produced in participation with the heme synthesis pathway. The dityrosine bounds have been detected in the chorion of insect eggs, conferring protein insolubility and hardening, contributing to the waterproofing (Dias et al., 2013; J.S. Li and J. Li; 2006). This data suggests that most likely the dityrosine layer directly confers waterproofness. However, another explanation of playing this function would be an indirect way of gluing the epicuticle and the procuticle together. Separation of these two layers (delamination) in case of

the dityrosinylated layer disfunction may also contribute to the increased cuticular permeability.

Taken together, there are three known barriers in *Drosophila* larval surrounding scaffold: the envelope inward-and outward barrier preventing water loss and xenobiotics penetration, the epidermal septate junction waterproof barrier and the epicuticle-procuticle dityrosine network waterproof barrier. Possibly, there are more, still unexplored barriers in the insect cuticle, especially in the desert-living species. This shows how complex the cuticle structure is and how important is correct assembly and compactness of all its layers to play its protective role. It underlines importance of the epicuticle and its attachment to the procuticle for the proper functioning of the impermeable dityrosine layer.

6.1 d) epicuticular Tweedle protein family – and its definition of the *Drosophila* larval shape

The insect cuticle plays a role not only in protection against desiccation and xenobiotics penetration, but also in conferring body shape. The procuticle as the thickest cuticular layer has a crucial meaning in body shaping. However, it turns out that the proteinous epicuticle influences this process as well, as disorders in its structure cause severe larval shape changes. Mutations or deletions in the DUF243 domain of unknown function in the family of epicuticular Tweedle proteins lead to the squat larval phenotype. Despite many screens searching for cuticular disorders no lethal mutant in any of the 27 *twdl* genes in *Drosophila melanogaster* has been found. Down-regulation of the activity of a single *twdl* gene does not evoke lethality or even

any visible developmental disorders. This suggests that a single Tweedle protein does not have a significant impact on the cuticle functioning. On the other side, lack of a given phenotype could be explained by a possible mechanism of substitution by another Tweedle protein out of 26 other family members. This notion can be supported by a fact that most of the insect species, which evolved before the dipterans, have only two or four Tweedle protein variants and here the role of these proteins seems to be essential. For instance, the migratory locust, which has only two *tweedle* genes, gives an obvious lethal phenotype after silencing of the *tweedle1* gene activity. Locust nymphs die in a molting process having thinner epicuticle and disorganized chitin in the procuticle (Tian-Qui et al., 2016). From the evolutionary point of view, the "non-importance" of a non-functioning of the single *tweedle* gene among 26 other members seems to be an advantage giving higher survival chances.

Although the impact of the Tweedle proteins in the larval shaping is clear, the exact mechanism is not known. According to the four theories, either the Tweedle proteins have a direct influence on the body shaping (conferring cuticle resistibility to the internal hydrostatic pressure) or indirect (changes/loss of the protein function evokes changes in the epidermal/cuticular structure and properties, what in turn leads to the larval shape change). It seems that mutations in the DUF243 domain leading to the Tweedle protein aggregation in the procuticle probably represent a neomorphic case, but it could also be a loss-of-function case. Fascinating is that one single mutated protein attracts (however selectively) many other "healthy" members and the lower dityrosinylated layer, which encircle the aggregates. Tweedle proteins are aligned in the epicuticle in the two-dimensional sheets, what suggests that they might bind selectively to each other or in a certain order as a consequence of an ordered production or delivery. In the aggregates, the proteins are mixed up without forming

any layers, what is either the result of the neomorphic mutated protein feature, or a result of random binding of the Tweedle members. Studying the processes of production, delivery, organisation into the layer and binding of the respective Tweedle proteins to each other in the *wild type* and mutant larval background will certainly help to understand how the epicuticle is formed and why is disorganized in such way in the squat larvae.

6.1 e) epicuticle – non-chitinous layer?

The division of the cuticle into the epicuticle and procuticle in the insects is based on the chemical composition: the epicuticle is considered as the proteinous, non-chitinous layer, whilst the procuticle consists of the protein-chitin network. Fluorescent-tagged Tweedle family members localize to the epicuticle, above the fluorescent-marked procuticle. Their affiliation to the epicuticle has been confirmed by the dependence of the expression on the ecdysone pathway. What is more, in the squat mutant larvae Tweedle proteins do not spread within the procuticle but aggregate, being clearly separated from the chitinous procuticle by the dityrosine layer. It suggests that the Tweedle proteins do not bind chitin. However, a motif in the sequnces of the Tweedle proteins has been predicted as potential chitin binding site. Barrel-shaped structure formed by beta-strands gives potential interface for the aromatic residues to be stacked and to bind chitin (Hamodrakas et al., 2002; Iconomidou et al., 1999; Guan et al., 2006). Binding of chitin by the silkworm *Bombyx mori* Tweedle 1 BmorCPT1 has been also shown by *in vitro* assays. Is the epicuticle really non-chitinous layer then or are predictions and *in vitro* binding assays far from the *in vivo* reality? Maybe chitin derivatives are present in the epicuticle or very less, undetectable chitin amounts? Maybe chitin binding feature of the Tweedle proteins has extincted over the insect phylum evolution? These discrepancies put in question

the non-chitinous nature of the epicuticle and encourage for more detailed studying of its composition and chitin-binding properties of the Tweedle proteins across the insect groups.

6.2 Envelope and epicuticle - new horizons for biomedicine and agriculture

Insects are indispensable for the functioning of all the ecosystems and one can point out numerous advantages of various insect species for the human culture. However, in some aspects certain insect species can become a nightmare, especially in the agricultural field causing crops destruction and contributing to the epidemic of hunger and poverty. A successful way to find an efficient insecticide, which would reduce a number of certain insect species without harming the other ones and the other animals is deepening the knowledge about the insect cuticle. It is a structure unique only for the arthropod phylum and targeting it seems to be quite safe for the other animals and plants. Particularly procuticular chitin as the unique component for the protists, arthropods and fungi has been considered as an excellent insecticide target (Merzendorfer, 2013). Nevertheless, it is clear that the envelope and the epicuticle are also important for the proper cuticle functioning and perhaps can serve as potential targets in the agricultural battle against pests. It seems that many proteins belonging to the envelope/pore canals and the epicuticle like Snustorr-Snarlik, Schlaff, Resilin or the Tweedle family are insect-specific. The interspecies differences between the structure, function and properties of certain protein groups (e.g. Tweedle) or specificity of certain proteins for some species only may facilitate to find a perfect compound acting against pests and not the other insect groups.

Another potential application of the epicuticular proteins seems to be biomaterial branch. Nowadays, more and more cuticular compounds find an application in a modern biomedicine. For instance, deacetylated chitin derivative chitosan has found

a broad application in tissue engineering and regenerative medicine as a bone graft substitute, in wound healing bandages and as matrix molecule for drug delivery (Ahsan et al., 2018). Another splendid example of the application of the cuticular compound in biomedicine is the rubber-like protein Resilin. Its unusual elasticity, resilience, long fatigue lifetime and high energy storage properties attracted bioengineers' attention. Biomaterials including Resilin polypeptide in combination with other protein domains found numerous applications such as biosensors parts, nanosprings, bio-rubbers or in tissue engineering field (Li and Kiick, 2013). In my research, Resilin protein expressed in the integument of *Drosophila* larvae has been shown to localize at the lower epicuticular edge, where it forms a network by cross-linking with each other and perhaps with other cuticular proteins via tyrosine residues. The C-type lectin Schlaff seems to be involved in the organisation of the dityrosine layer but not directly being dityrosinylated. Ardell and Andersen in the first paper revealing the identification of the *resilin* gene (Ardell and Andersen; 2001) postulated two potential candidates with a sequence similarity to the locusts Resilin protein in the *Drosophila* genome: CG15920, which is considered to be the *resilin* gene and CG9036, known as Chitin Binding Protein 56F (CPR56F). Sequence analysis of the CG9036 protein does not predict any repetitive motif homologous to the elastic motif of the Resilin protein. On the other hand, such a motif can be found in the CG7709 protein, known as Mucin 91c (Muc91c). Both proteins, CPR56F and Muc91c have an extracellular signal and additionally CPR56F chitin binding domain and could be a potential partner of Resilin in dityrosine network formation. Finding more Resilin-like proteins with elastic motifs might bring new insights into the biomaterial research. Insect cuticle consists of three completely different layers of different mechanical properties, which can serve as sources of potential biomaterials. The epicuticle

consists mostly of proteins, largely unidentified ones. Discovering them and characterisation may bring many adventages for the dynamically developing biomedicine.

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   Eur J Cell Biol. 97(2): 90-101.

2019

1. Zuber R, Shaik Saheb K, Meyer F, Ho HH, Speidel A, Gehring N, Bartoszewski S, Schwarz H and Moussian B (2019). **The putative C-type lectin Schlaff ensures epidermal barrier compactness in Drosophila.**
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Co-author

2015

1. Wang Y, Zuber R, Oehl K, Norum M and Moussian B (2015). **Report on Drosophila melanogaster larvae without functional tracheae.** J Zool. 296: 139–145.

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1. Zuber R, Wang Y, Gehring N, Bartoszewski S and Moussian B (2019). **Tweedle proteins form extracellular 2D-sheets defining body and cell shape in Drosophila melanogaster.**

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Appendix 1: The ABC transporter Snu and the extracellular protein Snsl cooperate in the formation of the lipid-based inward and outward barrier in the skin of Drosophila.

Zuber, Renata; Norum, Michaela; Wang, Yiwen; Oehl, Kathrin; Gehring, Nicole; Accardi, Davide; Bartoszewski, Slawomir; Berger, Jürgen; Flötenmeyer, Matthias and Moussian, Bernard (2017). **The ABC transporter Snu and the extracellular protein Snsl cooperate in the formation of the lipid-based inward and outward barrier in the skin of Drosophila**.
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Appendix 2: The putative C-type lectin Schlaff ensures epidermal barrier compactness in Drosophila.

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Appendix 3: Tweedle proteins form extracellular 2D-structures defining body and cell shape in Drosophila melanogaster.

Zuber, Renata; Wang, Yiwen; Gehring, Nicole; Bartoszewski, Slawomir and Moussian, Bernard. **Tweedle proteins form extracellular 2D-structures defining body and cell shape in Drosophila melanogaster.**