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Review

Recent advances in understanding mechanisms of insect cuticle differentiation

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ABSTRACT

Insects possess a cuticle that covers all tissues exposed to the outside world including the body, the fore- and hindgut and the luminal side of the tracheae. The cuticle is a multifunctional device that protects its carriers against dehydration, arms them against predators, constitutes a physical barrier to prevent pathogen entry and serves as an exoskeleton allowing locomotion. Depending the developmental stage and the body part, the composition and function of the cuticle changes. The body cuticle of larvae of holometabolous insects for example is soft while their cuticular head skeletons used to chew food is hard. In spite of these differences, the basic architecture of the insect cuticle is evolutionarily well conserved between developmental stages and between species.

The insect larval cuticle is formed at the apical site of a monolayer of polarised epithelial cells that differentiate concomitantly during embryogenesis. The stratified structure of the cuticle results from the concerted unfolding of basic cellular functions including timed transcription, biosynthetic enzymatic cascades, secretion and membrane trafficking as well as elaborate extracellular self-organization of the components. The aim of this review is to summarize recent advances in understanding these processes.
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1. Introduction

The insect cuticle is an apical extracellular matrix produced by the epidermis, the tracheal, hind- and foregut epithelia during embryogenesis and renewed during moulting and metamorphosis. Among others a fundamental objective in animal biology has been and is to understand the physiological, cellular, molecular and biochemical aspects of cuticle differentiation and structure. Our knowledge on the histology and physiology of cuticle differentiation derives especially from extensive studies of cuticle renewal during moulting in various insect species that have been excellently summarized by Michael Locke in 2001. A prominent model insect to investigate the molecular mechanisms and the biochemistry of cuticle differentiation is the fruit fly *Drosophila melanogaster*. Particularly the focus on cuticle differentiation in the embryo of *D. melanogaster* has started to revitalize the cuticle research field with some advances that François Payre reviewed in (2004). Since then, the molecular biology of cuticle differentiation has considerably progressed. In fact, *D. melanogaster* offers the opportunity of a genetic approach to study cuticle factors that are essential for survival. Moreover, the genetic accessibility of *D. melanogaster* allows the identification of crucial cuticle factors that would not have been identified biochemically, e.g. Knickkopf (Knk) and Retroactive (Rtv) (Moussian et al., 2005b, 2006b). Thank to the relatively conserved cuticle structure between species, findings in *D. melanogaster* can readily be related to the enormous amount of data collected in other insect species. In the near future, *D. melanogaster* may be also utilized to genetically characterize cuticle proteins that have been systematically identified based on insect genomic sequences (Cornman and Willis, 2009; Magkrioti et al., 2004). Reverse genetics is a powerful tool also in the red flour beetle *Tribolium castaneum* that has already served to analyze some cuticle factors (Arakane et al., 2004, 2005a, 2005b, 2008, 2009a; Zhu et al., 2008). The use of *D. melanogaster* as a model insect to investigate cuticle differentiation would not have been successful without the application of the relatively newly developed technique of high-pressure freezing followed by freeze substitution that enables the adequate fixation and handling of small embryos and larvae destined

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for electron microscopic studies of cuticle ultrastructure (McDonald and Morphew, 1993).

This review deals first with the structure and composition of mainly the *D. melanogaster* larval cuticle. It follows a discussion of problems regarding spatial, temporal and quantitative coordination of cuticle differentiating mechanisms as well as their variations that are pivotal for a guaranteed construction of a structure that coats a very prosperous animal taxon, the insects.

2. Structure and composition of the insect cuticle

2.1. The insect larval cuticle

2.1.1. Nomenclature

As the last step in development, the insect embryo produces the larval cuticle that enables the larva to hatch and qualifies it to live in a hostile environment. The insect cuticle is composed of the polysaccharide chitin, proteins and lipids that are distributed in distinct horizontal layers, and classically, two main layers have been distinguished according to their physiological and biochemical composition: the inner chitinous *procuticle* and the outer chitin-free *epicuticle* (Neville, 1975). Based on the ultrastructural texture, the procuticle may be subdivided into a lower endocuticle and an upper exocuticle, and the epicuticle is made up of two sublayers, an inner and an outer epicuticle, the latter also called the cuticulin layer. A wax and a cement layer may be added to the surface of the larval cuticle after hatching. According to the mode of establishment, which is described below, the sublayers of the epicuticle may be considered as separate entities (Locke, 2001; Payre, 2004), a concept that consequently favors a nomenclature of a three-layered cuticle: the *envelope* (outer epicuticle or cuticulin layer), the *epicuticle* (the inner epicuticle) and the *procuticle* (Fig. 1). Regardless of the nomenclature, the assembly of the cuticle requires controlled deposition and transport of the components to the correct position in the extracellular space where they can unfold their function and interact with each other to structure the cuticle.

2.1.2. Assembly of the non-chitinous regions

The cuticle faces the environment with a composite non-chitinous region that consists of different quinones, lipids and a number of proteins. The hydrophobic nature of this region represents the first barrier of the insect surface that protects the animal against dehydration and swelling (Gibbs, 1998, 2002). The ultrastructural localization and arrangement of these components within this region is not determined, but their distribution is certainly not random and results in a species-specific and stereotypic stratified architecture of the region that upon the mode of construction can be subdivided in two layers: the inner epicuticle and the outer envelope or cuticulin (Fig. 1) that may carry a deposit of waxes and cement (Locke, 1961, 1966, 1969). In the *D. melanogaster* embryo, the envelope is established in two steps (Fig. 2) (Moussian et al., 2006a). The precursory envelope consisting only of an outer electron-dense and a single electron-lucid films is deposited as fragments at the tips of randomly forming plasma membrane protrusions. The fragments eventually fuse together forming a single cover. Later, during cuticle differentiation, a third electron-dense film intercalates.

In contrast to the step-wise assembly of the envelope, the epicuticle thickens gradually during cuticle differentiation in the *D. melanogaster* embryo (Fig. 2) (Moussian et al., 2006a). Epicuticle material is secreted at the valley between newly formed regular corrugations of the plasma membrane (see Section 2.1.3.). Subsequently, the epicuticle is assembled underneath the envelope. The stepless thickening of the epicuticle implies constant synthesis and traffic of organic molecules of the melanization and sclerotization as well as steady supply of cuticular proteins through the secretory pathway. As observed during the construction of other extracellular matrices like the oocytes zona pellucida (Wassarman, 2008), epicuticle establishment may partially involve pre-assembly of the

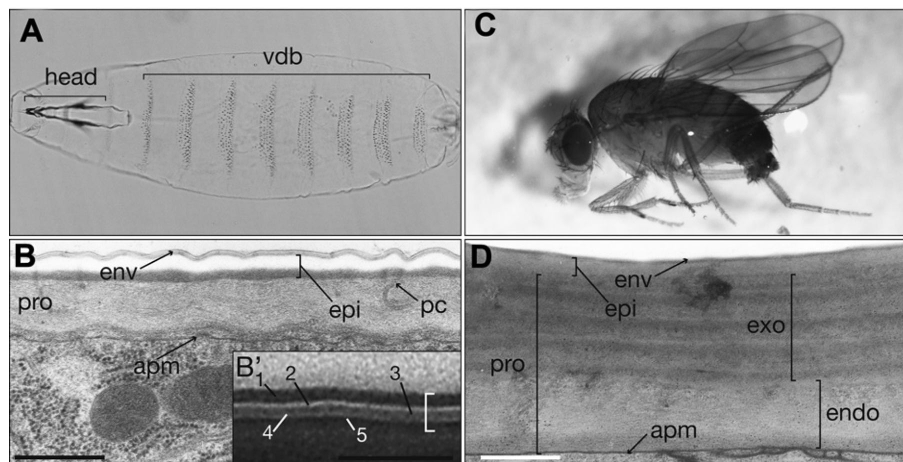

Fig. 1. Ultrastructural architecture of the insect cuticle. (A) The *D. melanogaster* larva has a soft body cuticle. Only the head skeleton (head) and the ventral denticle belts (vdb, bracket delimits the abdominal vdbs that are separated by naked cuticle) are hard and tanned. (B) The *D. melanogaster* larval cuticle produced during embryogenesis is a typical arthropod cuticle with three horizontal layers that are defined by their mode of formation. The procuticle (pro) is the inner chitin-protein matrix that is attached to the apical surface (apical plasma membrane, apm) of the epithelial cell. It is overlain by the protein-network of the epicuticle (epi). The outermost envelope (env) with five alternating electron-dense and electron-lucid sheets shown in the inset (bracket, B′) at a higher magnification faces the environment. Pore canals (pc) running through the cuticle connect the cell with the envelope. (C) The *D. melanogaster* imago is a patchwork of different types of cuticles. The cuticle of the head, the thorax and the leg, for example, is relatively hard, whereas the ventral abdominal cuticle is rather soft. The wing cuticle is flexible and stiff withstanding shearing forces during flight. (D) The three layers of the *D. melanogaster* adult cuticle are built during metamorphosis. The procuticle is thicker than the larval procuticle, and it is subdivided into an upper and a lower zone called the exo- (exo) and the endocuticle (endo), respectively. The exocuticle is electron-denser that the endocuticle due to a higher degree of sclerotization. The epicuticle is a uniform layer. The envelope often collapses due to the fixation procedure. Although the adult cuticle is somewhat more elaborate, the *D. melanogaster* larval and adult cuticles have a comparable ultrastructure. Thus, the study of the simpler larval cuticle may yield results that are largely transferable. (A and C) Light microscopy of a *D. melanogaster* larva (ventral view) and a *D. melanogaster* imago (lateral view). In both images, anterior is to the left. (B and D) Electron micrograph of a cross-section of the *D. melanogaster* larval (B) and adult leg (D) cuticle. Specimens were fixed and treated as described previously by Moussian et al. (2006a). Scale bars in (B) and (D) 500 nm and in (B′) 100 nm.

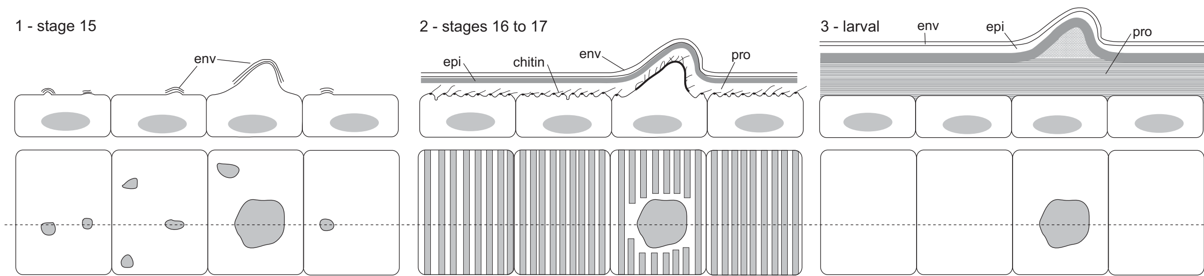

Fig. 2. The course of cuticle differentiation in the *D. melanogaster* embryo. Formally, cuticle differentiation in the *D. melanogaster* embryo runs through three phases underscored by the dynamics of the apical plasma membrane (Moussian et al., 2006a). The upper panel illustrates sections of the *D. melanogaster* embryonic epidermis during cuticle differentiation. The lower panel provides a top view on the scheme shown in the upper panel. First, the apical plasma membrane forms protrusions (grey patches) at random positions at the tip of which fragments of the envelope are deposited (1). Next, regular longitudinal corrugations, the apical undulae, emerge (grey stripes). They subdivide the apical surface in chitin synthesizing plaques at the crest of the corrugations, and sites where vesicle fuse to release their cargo into the extracellular space (2). The molecular mechanisms proceeding during this phase are shown in Fig. 4. The denticle extension of the apical plasma membrane (large grey patch) disrupts the apical undulae and denticular plaques are continuous. The cellular and molecular mechanisms governing the switch from random to ordered subdivision of the apical plasma membrane are obscure. The epi- and procuticle thicken simultaneously. Finally, when cuticle differentiation ceases the apical plasma membrane flattens (3). Chitin microfibrils in the denticles seem to be unorganized (see also Fig. 5A).

components already in the secretory vesicles, and partially rely on in-place maturation. Some evidence supports the notion of the apical plasma membrane being a central control site of a robust epicuticle differentiation. Epicuticle differentiation strongly depends on the apical plasma membrane t-SNARE Syntaxin 1A (Syx1A). A striking phenotype of *syx1A* mutant larvae is the almost complete loss of the epicuticle and the unmelanized cuticle (Moussian et al., 2007). Instead, electron-dense vesicles accumulate at the apical tier of the epidermal cells. These vesicles can be subdivided into at least two groups depending on their recognition by the *Maclura pomifera* agglutinin (MPA) that binds to N-acetylgalactose and galactose residues present e.g. on O-glycans. Although direct evidence is missing, the cargo of these vesicles presumably consists also of epicuticular proteins and components of the melanization pathway. The normal localization of chitin synthesis and organizing factors (see below), by contrast, indicates that cuticle producing and organizing membrane proteins may employ another t-SNARE for sorting of a third type of vesicles. Hence, these observations argue that cuticle differentiation is managed by at least three distinct post-Golgi routes to the apical plasma membrane.

Comparably, we are very much ignorant of the mechanisms of cuticle lipid biology. In a generalized picture, precursors of epicuticular and envelope lipids like hydrocarbons and free fatty acids may directly be produced at the plasma membrane and flipped into the extracellular space (Locke, 1966), for the exertion of their role in protecting the animal against desiccation they need to be further modified by localized proteins. At the same time, these lipid-interacting proteins including enzymes are delivered to the cuticle likely employing again the canonical secretory pathway. One group of extracellular proteins that deserves more future attention comprises lipid-processing enzymes like esterases. Indeed, esterase activity has been localized to the epicuticle and to the surface of several insect cuticles suggesting that the last step of wax ester synthesis may take place in the cuticle (Locke, 1961). Conceivably, these two processes – lipid deposition and protein secretion – that run across the apical plasma membrane have to be coordinated to result in the stereotypic arrangement of especially the envelope.

Epicuticle but not envelope stability or assembly depends also on the integrity of the underlying chitin matrix of the procuticle. In *D. melanogaster* larvae mutant for factors that are required to produce or organize chitin microfibrils epicuticle uniformity is lost (Moussian et al., 2005a, 2005b, 2006b). Taken together, the epicuticle is a complex extracellular matrix that is constructed by the parallel and cooperative activity of cytoplasmic, membrane-associated and extracellular processes.

2.1.3. Architecture of the procuticle

The innermost layer, the procuticle (Fig. 1), harbors a chitin-protein lattice and contacts the apical plasma membrane of the epidermal cell. In the *D. melanogaster* embryo, procuticle formation starts as soon as the envelope is a continuous layer covering the animal (Fig. 2). The defining constituent of the procuticle is the polysaccharide chitin that is assembled to bundles, the microfibrils. Chitin microfibrils adopt a stereotypic organization that accounts for cuticle elasticity and stiffness (Fig. 3) (Raabe et al., 2005;

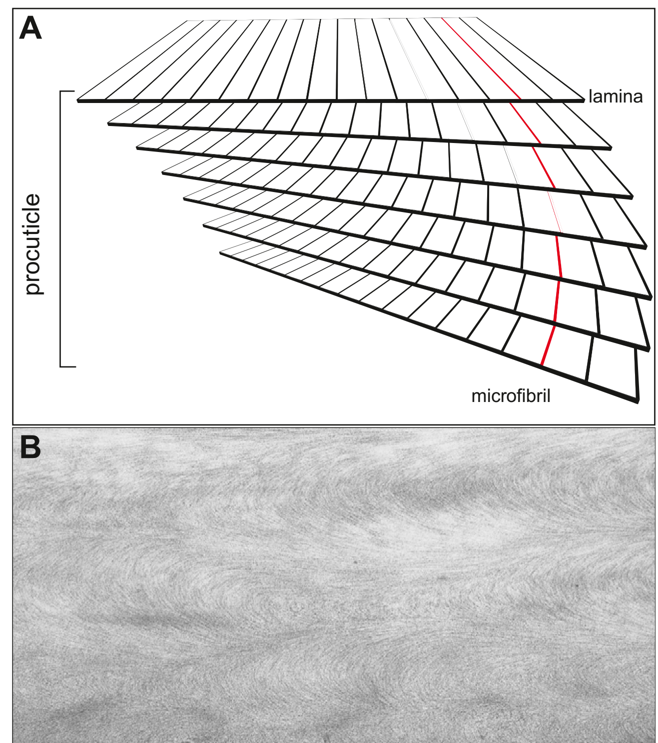

Fig. 3. Model for the organization of chitin in the arthropod cuticle described by Yves Bouligand (1965). (A) Chitin microfibrils that contain around 18 anti-parallel running chitin fibers are ordered in parallel to form horizontal sheets, the laminae (Neville et al., 1976). These sheets are twisted along the apical–basal axis of the procuticle. In the giant water bug *Hydroeyrius colombiae* the laminae rotate by 7–8° (Neville and Luke, 1969a). In oblique sections, this arrangement gives the illusion that chitin microfibrils run as arches through the procuticle. One microfibril in each lamina is highlighted in red to visualise this illusion. (B) An oblique section of the *D. melanogaster* larval procuticle visualizes Bouligand's organization.

Sachs et al., 2006; Vincent and Wegst, 2004). This elaborate organization of chitin microfibrils has been modelled for crustacean cuticles almost half a century ago by the French biophysicist Yves Bouligand (1965) and later confirmed for the insect cuticle by Neville and Luke (1969a). In this widely accepted model, sheets (lamina) of chitin microfibrils that are arranged parallel to each other are stacked helicoidally along the apical–basal axis of the procuticle. The chemical properties of the naked chitin fibre probably are insufficient to guide and stabilize the complex construction of the procuticle. To adopt the Bouligand architecture, chitin conceivably interacts with chitin-binding proteins. One class of evolutionary conserved cuticle proteins features the so-called Rebers and Riddiford (R&R) domain (Rebers and Riddiford, 1988) that in several cases has been indeed reported to bind chitin *in vitro* (Qin et al., 2009; Rebers and Willis, 2001; Togawa et al., 2004). An insect genome harbors multiple copies of R&R protein coding genes that are arranged in clusters (Cornman et al., 2008; Cornman and Willis, 2008; Karouzou et al., 2007; Togawa et al., 2008). Genes within a cluster usually have a similar expression pattern, their expression often peaking around moulting at different stages (Togawa et al., 2008). One may speculate that high copy number of R&R protein coding genes is a means to ensure sufficient supply of the proteins during chitin organization and to counteract spontaneous genetic defects. A distinguished member of the R&R protein family is resilin that is found in the cuticle of body parts with a need for high elasticity and resilience such as the flight apparatus (Bennet-Clark, 2007; Weis-Fogh, 1960). The physical properties of *D. melanogaster* resilin have now been studied in detail confirming the rubber-like behavior of resilin *in vitro* (Elvin et al., 2005; Qin et al., 2009). Another class of potential chitin-organizing proteins characterized in *D. melanogaster* is constituted by the Obstructor proteins that possess *Tachycitin-like* chitin-binding domains, most of which are expressed in cuticle depositing cells (Behr and Hoch, 2005). The *in vivo* function of none of the chitin-binding protein classes has been studied yet.

During procuticle differentiation in the *D. melanogaster* embryo, the apical plasma membrane forms longitudinal protrusions that run perpendicular to the anterior–posterior axis of the developing embryo (Moussian et al., 2006a). These protrusions that are named *apical undulae* are identical to the microvilli mentioned by François Payre (2004) and related to the microvilli described in many different arthropods (Locke, 2003). The apical undulae like the microvilli subdivide the apical plasma membrane into two domains (Figs. 2 and 4). The crests harbor the chitin synthesis complex (plaque), while secretory vesicle fuse with the grooves separating the crests. As the plaques are likely assembled via a yet undiscovered membrane trafficking path, the apical plasma membrane has to have acquired the competence to distinguish between different types of secretory vesicles prior to cuticle differentiation. Newly synthesized chitin microfibrils cross the apical undulae at an angle of 90° suggesting that the topology of the apical plasma membrane and its regionalization are essential for chitin microfibril orientation. This interpretation is in agreement with the analysis of the *syx1A* mutant phenotype (Moussian et al., 2007). In embryos suffering lack of Syx1A function, the apical plasma membrane is flat and chitin is disorganized, while the localization of the membrane-associated chitin synthesis and organizing complex is independent of Syx1A function. Of course, it cannot be ruled out that secretion of extracellular chitin-binding and organizing proteins may be abrogated in *syx1A* embryo, as well. Both failures in *syx1A* mutant embryos may culminate in defects in the assembly or organization of chitin microfibrils in the procuticle.

The crosswise arrangement of the apical undulae and chitin microfibrils does not explain rotation of the laminae at upper positions. This layout may be argued to be a static view of a mechanism of chitin orientation adjacent to the apical site of the epithelial cell. A more dynamic view could implicate movements of the chitin synthesis complex along the apical undulae as has been described for the cellulose synthase producing the cell wall in the plant *Arabidopsis thaliana* (Paredez et al., 2006). In other insects than *D. melanogaster*, as proposed by Michael Locke (1991), the chitin synthesis complex at the tips of swinging membrane protrusions like microvilli could passively be moved back and forth in a defined extracellular area while synthesizing chitin. As opposed to the weaving mechanisms, one could speculate that the rotation of laminae in the procuticle is a self-organizing mechanism triggered by a yet unknown biological, chemical or physical signal.

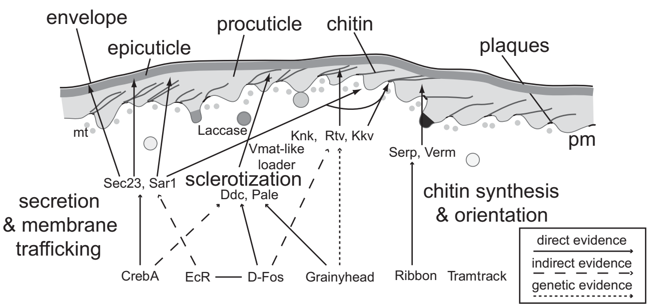

Fig. 4. Summary of molecular interactions implementing the *D. melanogaster* larval cuticle. Differentiation of the *D. melanogaster* larval cuticle involves a network of transcriptional regulation. Several transcription factors control the secretory pathway, sclerotization and chitin synthesis that have to be coordinated to produce a functional cuticle. Secretory vesicles deliver components of all cuticle layers. Membrane trafficking serves to decorate the apical plasma membrane both at the crest to construct the plaques and at the valley between two plaques to convey competence for vesicle docking. The mechanism of plaque assembly is unknown. Likewise, the flux of the sclerotization pathway from the cytoplasm (Ddc, Pale), via a Vmat-like vesicle loader to the extracellular matrix (Laccase) is not understood. Overall, decoration of the apical plasma membrane comprises yet undiscovered factors mediating membrane bending. Margined circles with three grey levels reflect the minimum of three types of cargos constructing the cuticle: extracellular proteins, components of sclerotisation/melanization and subunits of plaques (see also text in 2.1.2.). Microtubules (mt) are depicted to remind the importance of the cytoskeleton for cuticle differentiation. The data summarized in the text argue that the apical plasma membrane of cuticle producing cells plays a central role in coordinating the molecular mechanisms of cuticle differentiation. It is not merely a passive interface that has to be crossed to deliver cuticle material to the extracellular space. The engagement of the apical plasma membrane in coordination is seemingly reflected by its organization in repetitive units. This topology implies that the pre-cuticle producing plasma membrane has to be patterned in order to separately position the chitin synthesis complex and the vesicle docking machinery.

2.2. Sclerotization and melanization

2.2.1. The pathway from the cytoplasm to the extracellular space

Construction and stabilization of all cuticle layers involve sclerotization, which is the covalent cross-linking of cuticle components via tyrosine-derived phenolic compounds that are also used for cuticle tanning (melanization). The underlying biochemical reactions of sclerotization and melanization have been excellently reviewed in various articles published frequently (Andersen, 2010; Hopkins and Kramer, 1992; Riley, 1997; Sugumaran, 2002, 2009; Wright, 1987). Therefore, in order to provide a background for some notions and speculations on cuticle differentiation presented in this review, I am giving only a short summary on the sclerotization and melanization pathways. They start within the cytoplasm, where first tyrosine is hydroxylated to 3,4-dihydroxyphenylalanine (DOPA) by the tyrosine hydroxylase encoded by the *pale* locus in *D. melanogaster* (Neckameyer and White, 1993). Next, DOPA is decarboxylated to dopamine by the DOPA decarboxylase (Ddc) (Hirsh and Davidson, 1981). Mutations in the *pale* and *Ddc* loci are larval lethal underscoring the importance of these events for viability. Still in the

cytoplasm, dopamine also serves to produce sclerogenic and melanogenic precursors such as N-acetyldopamine (NADA) by the Dopamine N-acetyltransferase (Dat) and N-β-alanyldopamine (NBAD) by the NBAD synthase encoded by the *ebony* (*e*) gene (Brodbeck et al., 1998; Hovemann et al., 1998; Richardt et al., 2003). DOPA, dopamine, NADA, NBAD and other DOPA- or dopamine-derivatives are released into the developing cuticle by a yet unknown mechanism. In *D. melanogaster* neuronal cells, dopamine-related neurotransmitters produced in the cytoplasm are loaded into secretory vesicles through the function of the vesicular monoamine transporter (Vmat) that however is not expressed in the epidermis (Greer et al., 2005; Simon et al., 2009). Either supply of these substances is independent from Vmat in cuticle forming tissues, or another Vmat-like protein acts to suffice this demand. The *D. melanogaster* genome harbors a gene coding for a Vmat paralog, CG10251 (Fig. S1). Whether this protein may be responsible for loading of the precursors of melanization and sclerotization reactions into secretory vesicles remains to be tested. Once these molecules are outside the cell, the following reactions are continued by phenol oxidases, one of which, Laccase2, has been intensively studied in *T. castaneum* (Arakane et al., 2005a, 2009b). Laccase2 uses all four secreted molecules as substrate to catalyze the respective quinones, and by consequence, down-regulation of Laccase2 activity causes diminishment of tanning as well as cuticle stiffness. This finding argues that the degree of cross-linking of cuticle components including chitin (see below) defines the stiffness and thereby the function of the cuticle. Covalent binding of the NADA- and NBAD-quinones to cuticle proteins stems directly in cuticle sclerotization, whereas for melanization first DOPA- and dopamine-quinones have to convert to DOPA- and dopaminechrome that next serve to produce the melanogenic substrates 1-carboxyl 5,6-dihydroxyindole (DHICA) and 5,6-dihydroxyindole (DHI) (Sugumaran, 2002, 2009). The latter steps of melanization engage the enzymatic activity of members of the extracellular Yellow protein family that were first described in *D. melanogaster* and later in a variety of other insect (Albert and Klaudiny, 2004; Arakane et al., 2010; Drapeau, 2001). For instance, the *Drosophila* Yellow proteins Yellow-f and -f2 catalyze in the conversion of dopachrome into DHI (Han et al., 2002) that is directly utilized for melanin synthesis in the cuticle. Alternatively, it has been adventurously hypothesized that Yellow proteins may act as hormone-like molecules triggering melanization through the binding to receptors (Drapeau, 2003). There is no experimental evidence supporting this hypothesis. The biological function of Yellow itself in melanization is well examined (Wittkopp et al., 2002). The antagonistic effects of Yellow and Ebony determine the pattern of melanization in the *D. melanogaster* imago. Of note, flies having loss-of-function mutations in *y* or *e* only display a color phenotype and do not die. Consistently, elimination of the function of the transcription factor Broad and the peptide hormone Bursicon, both shown to regulate tanning in the adult insect, does not cause embryonic lethality (Baker and Truman, 2002; Chen et al., 2002). Assuming that inhibition of sclerotization would by contrast be lethal, these observations underline that extracellular melanization and sclerotization reactions are indeed largely independent biochemical processes.

2.2.2. Linking components

There have been numerous endeavors to identify the protein targets of sclerotization and melanization. Systematic biochemical experiments have yielded several protein motifs that are proposed to be potentially important for epicuticle architecture (Andersen et al., 1995). Especially the imidazole ring of histidine residues, the free amino group of lysines and the amino group of the N-terminal amino acid are bound to quinones (Andersen, 2007; Andersen and Roepstorff, 2007). Despite these efforts our knowledge about structural proteins of the epicuticle is limited, since, because of the stability of the epicuticular protein-quinone network, these proteins are difficult to isolate and to investigate. Recent genomic approaches have led to the identification of complete sequences of several classes of related cuticle proteins that potentially structure the epicuticle (Cornman and Willis, 2009; He et al., 2007; Togawa et al., 2007). The expression patterns of the underlying genes that are organized in clusters (Cornman and Willis, 2009) are not strictly stage-specific (Togawa et al., 2007), but presumably reflect tissue-specific requirements. The high number of related proteins also suggests functional redundancy. The multiplication of cuticle genes could accommodate the extensive need for these proteins during cuticle formation by an enhanced transcription rate, at the same time provide a buffer against genetic changes (see also above). A genetically defined class of cuticle proteins is represented by the *D. melanogaster* TweedleD protein (Guan et al., 2006). Mutations in *tweedleD* are dominant and alter the body shape of the larvae, the pupae and the imago. The *D. melanogaster* genome harbors 27 *tweedle-like* partially clustered genes that are also found in other insects (Cornman and Willis, 2009; Zhong et al., 2006). The molecular mechanisms of Tweedle function are not known. Since, as mentioned above, the exact localization of all these proteins is not determined, they naturally could also be elements of the procuticle that occasionally also sclerotizes (see Section 2.1.1.).

Indeed, besides non-covalent interaction with chitin-binding proteins chitin also covalently binds to proteins via quinones during procuticle sclerotization following those biochemical reactions thought to occur during epicuticle and envelope sclerotization (Schaefer et al., 1987; Wright, 1987). Hence, the construction of the procuticle and its stabilization including communication with the above epicuticle is an enormous task that the epidermal cell has to accomplish during cuticle differentiation. The concerted underlying molecular mechanisms are awaiting characterization. Genetic approaches undertaken using *T. castaneum* and *D. melanogaster* as model insects will advance our understanding of these processes.

2.3. Chitin synthesis and orientation

The assessment of the elements i.e. structural proteins, enzymes and organic molecules that build up the cuticle is a prerequisite to understand the mechanisms of cuticle differentiation. However, we are at the beginning of our efforts to dissect the genetics of these mechanisms.

A central enzyme producing an important portion of the cuticle is the membrane-inserted chitin synthase (Merzendorfer, 2006). It synthesizes chitin fibers by catalyzing the b1–4 linkage between N-acetyl-glucosamines (GlcNAc) that are supplied in the cytoplasm, and extrudes them across the plasma membrane. The role of the epidermal chitin synthase in cuticle differentiation has been mainly studied in the red flour beetle *T. castaneum* (Arakane et al., 2004, 2005b, 2008) and *D. melanogaster* where it is called Krotzkopf verkehrt (Kkv) (Gagou et al., 2002; Moussian et al., 2005a). In *T. castaneum*, injections of double stranded RNA (dsRNA) directed against the epidermal chitin synthase transcript *TcCHS-A* into females provoke a twisted, enraged and lethal embryonic phenotype. Lack of chitin in the cuticle of the *D. melanogaster kkv* mutant larva eventually causes detachment of the cuticle from the epidermis and disintegration of the epicuticle.

In the arthropod cuticle, chitin adopts a stereotypic arrangement that probably contributes to the elasticity of the exoskeleton (see above). An essential chitin-organizing factor is Knickkopf (Knk) that is inserted via a GPI-anchor in the plasma membrane harboring three types of domains (Fig. S2) (Moussian et al., 2006b). The two N-terminal DM13 domains are found in organisms that form an external coat including bacteria, ecdysozoans and plants and do not display any homology to known domains and their function is unknown (Iyer et al., 2007; Ponting et al., 2001). The middle

dopamine monooxygenase-like (Domon) domain (Aravind, 2001; Iyer et al., 2007) folds similar to the cytochrome domain of cellobiose dehydrogenase from *Phanerochaete chrysosporium* (Fig. S2) (Rotsaert et al., 2003) that is catalyzing the depolymerization of the polysaccharide cellulose (Mansfield et al., 1997). This domain may bind to heme. The C-terminal domain shows high similarity to plastocyanin and plastocyanin-like domains. This domain may bind a metal ion. Hence both domains may be engaged in electron transfer. We can only speculate on the acceptor of the transferred electron. The best candidate as a substrate of Knk is chitin, as chitin microfibrils are disorganized in *D. melanogaster knk* mutant embryos. However, it is not clear why chitin organization would require a redox reaction. Is in agreement with the function of cellobiose dehydrogenase, Knk activity needed to release chitin fibers from the chitin synthase complex thereby facilitating chitin synthesis? The potential dopamine monooxygenase activity of Knk opens a second possibility: using dopamine as a substrate Knk could produce norepinephrine that subsequently is converted to the sclerogenic agents N-acetyl-norepinephrine (NANE) and N-β-alanyl-norepinephrine (NBANE), which may be used to cross-link chitin with proteins in order to organize the chitin-protein matrix (Andersen, 2010; Hopkins and Kramer, 1992). If this holds true, it would be a spectacular notion of chitin organization. Biochemical and genetic analyses of the Knk function are necessary to solve this problem.

Another membrane-inserted protein needed for correct chitin organization is Retroactive (Rtv) (Moussian et al., 2005b, 2006b). Rtv is a member of the small cysteine-rich snake-toxin like protein family characterized by five β-sheets positioning three flexible loops that present putative partner binding sites. In the case of Rtv and its orthologs, these loops expose each two conserved aromatic amino acids that we have hypothesized to mediate chitin binding as it has been shown in some cases that carbohydrate-binding proteins contact their substrate via aromatic amino acids (Colombo et al., 2005; Hashimoto, 2006). In a simple model, Rtv may bind chitin and assist orienting chitin microfibrils at the surface of cuticle producing cells by its flexible loops. Indeed, chitin organization is lost in *rtv* mutant larvae.

Organization of chitin is not occurring only at the apical plasma membrane. Epidermal cuticle integrity in *D. melanogaster* larvae has been reported to also depend on the chitin deacetylases Serpentine (Serp) and Vermiform (Verm) (Luschnig et al., 2006; Wang et al., 2006). The role of predicted chitin deacetylases (TcCDA) has also been investigated in *T. castaneum* (Arakane et al., 2009a). Upon injection of dsRNA against either of two out of nine TcCDAs (TcCDA1 and 2), moulting was compromised at different developmental stages and animals were unable to shed their old cuticle and died. Of note, only the parental injection of dsRNA against a splice-variant of TcCDA2 affected embryogenesis, suggesting that compared to the situation in *D. melanogaster* only one chitin deacetylase is essential in the embryo of *T. castaneum*. Presumably, modification of chitin including deacetylation is, as proposed by Neville (1975), a prerequisite for its ability to interact with proteins. As the phenotypes of *serp* and *verm* double mutant and *knk* or *rtv* mutant larvae are similar, the underlying factors supposedly act within a single genetic and biochemical pathway of chitin modification and orientation.

2.4. Some aspects about evolution of procuticle differentiation

The factors that are required for chitin synthesis and organization are all also used earlier in development to produce and organize an non-cuticular luminal chitin rod and thereby to regulate the diameter and length of tracheal tubes (Devine et al., 2005; Luschnig et al., 2006; Moussian et al., 2006b; Tonning et al., 2005). While a discussion of this issue is beyond the scope of this review, I would like to hypothesize that the tracheal luminal chitin is an evolutionary relict from embryonic cuticles that are moulted within the egg case and that are still present in many holo- and hemimetabolous insects and crustaceans (Havemann et al., 2008; Konopova and Zrzavy, 2005; Lagueux et al., 1979; Truman and Riddiford, 1999). The analysis of tracheal morphogenesis in embryos of these insects would help to test this hypothesis.

Insects had a common ancestor with fresh water crustaceans around 410 million years ago (Glenner et al., 2006). Crustaceans themselves as the first Arthropods arose during the Cambrian explosion around 550 million years ago. Consistent with the close relationship between insects and crustaceans in particular chitin organization is identical in both taxa (Bouligand, 1965; Havemann et al., 2008; Moussian et al., 2006a; Neville and Luke, 1969b). How is chitin organized in sister groups of Arthropods? Reading the literature, we learn that chitin organization in Onychophorans is according to Bouligand's model, whereas in Tardigrades it is not (Harrison and Rice, 1993). Hence, factors governing chitin orientation such as Knk and Rtv were evolved before the divergence of Arthropods and Onychophorans, but after the separation of these two groups from the Tardigrades. Alternatively, Tardigrades may have lost factors of the respective pathway during evolution. The invention of chitin organization implies that regulation of cuticle differentiation probably also at the transcriptional level is conserved. One candidate that is essential for directing cuticle differentiation in *D. melanogaster* is Grainy head (Grh), an ETS-type transcription factor (Bray and Kafatos, 1991; Dynlacht et al., 1989). Consistently, mutations in *grh* cause a larval *knk*- and *rtv*-mutant-like phenotype (Ostrowski et al., 2002) allowing the assumption that the *grh* larval phenotype reflects loss of chitin organization as observed in *knk* and *rtv* mutant larvae (Moussian et al., 2005b, 2006b). An obvious conclusion would be that Grh regulates the implementation of chitin organization according to Yves Bouligand. A correlation between Grh and chitin organization may also exist during the production of the serosal cuticle, which protects the developing embryo in various insects (but not *D. melanogaster*) against desiccation (Rezende et al., 2008). In mosquitoes, *grh* mRNA is detected in those cells producing the serosal cuticle (Goltsev et al., 2009), in which at least in the locust *Locusta migratoria* chitin is organized as described by Yves Bouligand (Rinterknecht, 1993). The recruitment of Grh as a key regulator of procuticle assembly has been a straightforward step in evolution as an ancestral Grh likely regulated differentiation of the skin already in pre-cambrial animals. This argument is deduced from findings that Grh orthologs are elementary regulators of animal skin differentiation not only in the protostomal arthropods but also in the deutorostomal vertebrates where they control skin ECM organization (Ting et al., 2005; Yu et al., 2006).

Another essential molecule driving insect late embryonic development including cuticle differentiation is ecdysone (see below). The ecdysone-producing enzyme are also found in the crustacean *Daphnia* (Rewitz and Gilbert, 2008). Thus, Grh and ecdysone are ancient factors already controlling cuticle differentiation during the birth of the modern taxa between the Cambrium and the Ordovicium.

3. Regulation and coordination of cuticle differentiation

3.1. Ecdysone drives multiple pathways of cuticle differentiation

Arthropods grow continuously until their cuticle ceases to expand. Further growth of the animal requires peeling of the old cuticle and the differentiation of a new one. These moulting events are regulated by the steroid hormone ecdysone. Ecdysone binds to the nuclear receptor EcR that in the absence of the signal shuffles between the cytoplasm and the nucleus; upon hormone docking EcR accumulates predominantly in the nucleus as it is now able to stably bind to its nuclear partner Ultraspiracle (Usp) (Cronauer et al., 2007;

Hall and Thummel, 1998; Yao et al., 1993). The EcR/Usp heterodimer activates or represses a manifold of target genes changing the developmental and physiological status of the animal. The interaction of EcR/Usp with the chromatin leads to loosening of the DNA forming loops called puffs where transcription of ecdysone response genes is initiated (Ashburner, 1971; Thummel, 1990). The first set of genes activated upon ecdysone signalling code for transcription regulators like the heme binding nuclear receptor E75 (Reinking et al., 2005; Segraves and Hogness, 1990; Segraves and Woldin, 1993) that in turn activate or repress the second set of genes coding for regulatory factors like β-FtzF1 (Broadus et al., 1999). During *D. melanogaster* embryogenesis, ecdysone peaks just at the onset of morphogenesis (Kozlova and Thummel, 2003). Most enzymes acting on the ecdysone biosynthesis pathway have been identified and characterized (Gilbert, 2004; Rewitz et al., 2007). The ecdysone pulse is sufficient to trigger completion of embryogenesis including epidermal and cuticle differentiation. Indeed, mutations in the genes coding for the enzymes that convert cholesterol into 20-hydroxyecdysone, the active form of ecdysone, cause developmental arrest at mid-embryogenesis and formation of a very reduced cuticle (Chavez et al., 2000; Ono et al., 2006; Petryk et al., 2003). The phenotypes of ecdysone-deficient embryos are strong compared to those resulting from defects in ecdysone transduction mediated for example by E75 or β-FtzF1 (Reinking et al., 2005; Ruaud et al., 2010) arguing that multiple transcription factors cooperate to transduce the ecdysone signal during cuticle differentiation. It will be a great and fruitful challenge to dissect these pathways downstream of ecdysone in the *D. melanogaster* embryo.

This scenario drawn for embryonic ecdysone function does not include the enigmatic role of the complex locus *shroud*/*kayak* (*sro*/*kay*) in ecdysone function. This gene encodes the *D. melanogaster* Fos transcription factor (D-Fos) that has four isoforms (Giesen et al., 2003; Hudson and Goldstein, 2008). Mutations in the exon that codes for the N-terminus of two isoforms cause a phenotype that resembles those provoked by ecdysone deficiency although ecdysone levels are normal in the mutant animals (Chavez et al., 2000). By contrast, ecdysone response is diminished in *sro* mutant embryos. Consequently, D-Fos could assist EcR in mediating ecdysone function. Since one of these D-Fos isoforms lacks the DNA-binding domain, the molecular function of D-Fos in mediating the ecdysone response is unclear.

Some ecdysone-responsive transcription factors such as the BTB-type Broad and the ETS-type E74A reported to be important during pupal or adult cuticle differentiation are not essential during embryogenesis (Karim and Thummel, 1991; Karim et al., 1993; Zhou and Riddiford, 2002). Thus, there seems to be a fundamental difference in the ecdysone-dependent regulation of cuticle differentiation at different stages of an insect's life.

What is the impact of ecdysone on cuticle differentiation beyond its requirement to trigger a cascade of transcription regulators? As suggested by the reduced cuticle phenotype of embryos that fail to produce ecdysone, secretion and membrane trafficking may be a cardinal response to the hormone pulse. In agreement with this notion, ecdysone has been reported to induce Golgi formation by driving the transcription of a number of genes coding for factors of the ER and Golgi including the COPII component Sec23 in *D. melanogaster* imaginal discs (Dunne et al., 2002; Kondylis et al., 2001). The transferability of the findings to the embryo has yet to be tested.

The impact of ecdysone on chitin synthesis is illustrated by the ecdysone-dependent expression of the enzyme supplying the chitin monomer GlcNAc, UDP-GlcNAc pyrophosphorylase called Mummy (Mmy) in *D. melanogaster* (Araujo et al., 2005; Schimmelpfeng et al., 2006; Tonning et al., 2006). In ecdysone-deficient *D. melanogaster* embryo, epidermal expression of *mmy* is prematurely induced, while in the tracheae its expression is suppressed. This observation indicates that ecdysone is a modulator rather than an absolute regulator of chitin synthesis.

3.2. Timing of cuticle differentiation

Cuticle differentiation in the embryos of the insects *D. melanogaster* and *Manduca sexta* and in the crustacean *Parhyale hawaiensis* begins around mid-embryogenesis regardless how long the entire development takes (Havemann et al., 2008; Moussian et al., 2006a; Ziese and Dorn, 2003). This observation incites the hypothesis that the period of cuticle differentiation is adapted to the total length of embryogenesis. For instance, the *D. melanogaster* and the *P. hawaiensis* embryos that develop at 25 °C initiate cuticle production 12 and 130 h after egg laying, respectively, and complete it just before hatching, the former requiring 10 h and the latter 120 h. Moreover, the duration of cuticle differentiation does not correlate with the thickness of the cuticle that in the end-products of embryogenesis, the *D. melanogaster* larva and the *P. hawaiensis* juvenile, is actually within the same range. In this perhaps special case, thus, longer cuticle differentiation does not result in a thicker cuticle suggesting that the efficiency of cuticle differentiation in *P. hawaiensis* is lower than in *D. melanogaster*. Postulating that insects and fresh water crustaceans share the vast majority of cuticle factors, this notion allows to ask for the factor or factors that define the length and performance of cuticle differentiation in arthropods. Alterations of the activity of a single factor are unlikely to be responsible for interspecies differences in duration of cuticle differentiation, as the corresponding biochemical mechanisms seem not to be flexible enough to compensate bottlenecks. For instance, reduction or up-regulation of the activity of the chitin synthase would require calibrating the supply of UDP-GlcNAc as well as protein secretion (see also chapter 3.5.). Indeed, down-regulation of chitin synthesis in *D. melanogaster* embryos induced by the chitin synthase specific inhibitor Nikkomycine Z does not result in prolonged cuticle differentiation and embryogenesis but kills the larva suffering *kkv*- and *knk*-like cuticle defects (Gangishetti et al., 2009). In other words, cuticle-producing cells are unable to choke cuticle differentiation in response to the partial suppression of chitin synthase activity. Hence, cuticle differentiation possibly requires rather collective adjustment of effectiveness of the underlying mechanisms than changes in the performance of single factors. Developmental variability may certainly be explained by general physiological differences between, in this case, a water- (*P. hawaiensis*) and an air-based (*D. melanogaster*) mode of life. In a more attractive view, adaptation may rely on sequence variations in the promoter and/or in the coding regions of a battery of cuticle genes slowly introduced during evolution. Spotting these base-pair changes by e.g. QTL analysis will be an important task in the future to assess the control mechanisms of cuticle differentiation deployed across species.

3.3. Transcriptional control of cuticle differentiation

The arthropod cuticle consists of various proteins, lipids and carbohydrates that are not distributed randomly within the extracellular space. In addition, the stratification of the cuticle necessitates scheduled deposition of layer-specific material. Hence, the supply of the components has to be controlled in time in order to ensure a stereotypic arrangement of the cuticle. Control of cuticle differentiation relies conceivably in part on transcriptional regulation. In *D. melanogaster*, several evolutionary conserved transcription factors are involved in cuticle production or organization. An important regulator of cuticle differentiation is the ETS-type transcription factor Grainy head (Grh) that I have already introduced in chapter 2.3. (Bray and Kafatos, 1991). The role of Grh in cuticle differentiation has also been studied during wound healing in the

embryo when the cuticle is reconstituted (Mace et al., 2005). In both contexts, the major task of Grh is to trigger the expression of genes coding for enzymes of the sclerotization and melanization pathway like Pale and Ddc. This indicates that Grh activity is sensitive to different types of signals exciting either cuticle reconstitution or cuticle differentiation. Apparently, the original and immanent function of Grh transcription factors is to control the cross-linking of epidermal extracellular matrices, since, as mentioned above, Grh orthologs not only in invertebrates but also in vertebrates regulate the expression of genes coding for cross-linking enzymes (Jane et al., 2005; Moussian and Uv, 2005).

The relevance of Grh for larval cuticle differentiation is debatable. Ostrowski et al. (2002) observe that mutations in *grh* strongly reduce sclerotization and melanization of the larval head and ventral denticles. By contrast, the *grh* mutant phenotype described by Bray and Kafatos (1991) is definitively weaker than that of *kkv* mutant larvae and resembles the phenotype of *knk* mutant larvae that are characterized by a highly melanized head skeleton (Moussian et al., 2005a, 2006b). The latter observation argues that Grh is a modulator rather than an activator of gene expression. This interpretation is in agreement with recent findings that Grh modulates but is not essential for the expression of cell junction genes in the developing epidermis preparing to produce the cuticle (Narasimha et al., 2008).

The relatively mild macroscopic cuticle phenotype of *grh* mutant larvae underlines that other transcriptional regulators act in concert or in parallel to Grh to drive cuticle construction. The zinc finger protein Tramtrack (Ttk) is one such factor (Araujo et al., 2007). The epi- and procuticle of *ttk* mutant embryos is disorganized arguing that the yet unknown targets of Ttk are essential for chitin organization. Intriguingly, like *grh* transcripts *ttk* transcripts are detected in the serosal cells in mosquito embryos (Goltsev et al., 2007) suggesting that Ttk and Grh may collaborate in driving cuticle production in various tissues. The expression of two genes coding for chitin deacetylases that are predicted to modify procuticular chitin fibers, *serpentine* (*serp*) and *vermiform* (*verm*) depends on Ribbon (Rib), which has a BTB/POZ domain and Pipsqueak DNA-binding motif (Bradley and Andrew, 2001; Luschnig et al., 2006). The cuticular phenotype of *rib* mutant larvae has not been analyzed. However, as Rib has been shown to manipulate apical plasma membrane growth in the salivary glands (Kerman et al., 2008), one can imagine that in the epidermis *rib* mutations interfere with apical undulae formation thereby affecting chitin organization. As Ttk and Rib control morphogenesis during early and mid-embryogenesis before cuticle differentiation starts, we have to consider the possibility that the effect of these transcription factors on cuticle differentiation may be indirect.

We have a more detailed view on the function of CrebA (cAMP responsive element binding factor A). Recently, CrebA was demonstrated to regulate the expression of a battery of genes coding for factors of the canonical secretory pathway such as the COPII elements Sec23 and Sar1 (Abrams and Andrew, 2005). Reduction of CrebA activity results in a pale and thin cuticle suggesting lack of extracellular enzymes of the melanization and sclerotization pathways and reduced deposition of cuticle material. It has not been investigated whether mutations in *CrebA* also affect chitin synthesis. Likewise, mutations in CrebA target genes cause a *CrebA*-similar cuticle phenotype. Interestingly, the expression of some genes coding for secretory factors seems to be independent of CrebA arguing for the requirement of at least one another transcription factor for coordinated expression of all genes encoding factors of the secretory pathway.

In summary, several transcription factors regulate in concert the expression of genes needed for cuticle differentiation. Hence, none of the transcription factors discussed above seems to be the master regulator of cuticle differentiation. This observation raises the question whether cuticle differentiation may at all be under the control of a master regulator. In the scenario of multiple transcription factors regulating cuticle differentiation the different signals have to be somehow integrated for correct cuticle architecture. Conceivably, integration of these signals occurs at the promoter regions of cuticle genes. The identification of such a cuticle-specific *cis*-regulatory module is an important step towards the understanding of coordinated cuticle differentiation.

3.4. Towards a cis-regulatory module of cuticle genes

The DNA binding sites for a handful of transcription factors regulating cuticle differentiation are available. Repeatedly, it was found that Grh-binding sites do not tend to be abundant in the regulatory regions of its target genes (Almeida and Bray, 2005; Narasimha et al., 2008). For example, two binding sites are detected in the septate junction genes *coracle* (*cora*) and *fasciclin III* (*fasIII*), as well as in *pale* (*ple*) and *Ddc* (Mace et al., 2005; Narasimha et al., 2008). The low occurrence of Grh binding sites in the regulatory region of its targets is not necessarily constituting a rule as clustering of six Grh-binding sites has been reported within the fist intron of *kkv* (Pearson et al., 2009). The attempt to characterize the regulatory role of this sequence in the *kkv* gene addressing the function of Grh during wound healing failed, as the observed up-regulation of *kkv* transcription at wound sites does not depend on Grh. The impact of these Grh-binding sites on normal cuticle differentiation remains to be investigated. The first intron of *kkv* also harbors two canonical binding sites for D-Fos that is implicated in wound healing (Pearson et al., 2009). Hypothetically, Grh could cooperate with D-Fos to ensure robust *kkv* transcription in the wound-healing assay and possibly during cuticle differentiation. Consistently, some mutations in the *D-fos* gene, called *shroud* (*sro*, see above) cause a severe cuticle phenotype (Giesen et al., 2003; Jürgens et al., 1984). Interestingly, Grh binding sites are also found in the *ple* and *Ddc* promoter regions close to D-Fos binding sites (Mace et al., 2005). Thus, Grh and D-Fos could act cooperatively to induce the expression of cuticle genes.

Cooperation between Grh and D-Fos may involve a third transcription factor i.e. CrebA (Fig. 4). Besides two Grh- and D-Fos-binding sites, the promoter region of *Ddc* has one CrebA-binding site (Mace et al., 2005). As mentioned above, CrebA is essential for full expression of many genes coding for factors of the secretory pathway, and mutations in CrebA result in a thin and pale larval cuticle (Abrams and Andrew, 2005). At the moment, these observations may all be coincidental. Genetic experiments as well as genome-wide analyses of binding sites for Grh, D-Fos and CrebA should help to elucidate the relevance of interaction between these transcription factors. These approaches will also contribute to formulate cuticle-specific *cis*-regulatory modules, the further dissection of which will enhance our understanding of coordination of processes governing cuticle differentiation.

3.5. Coordination during cuticle differentiation

The stereotypic architecture of the insect cuticle implies that the various mechanisms driving its differentiation have to be coordinated temporarily, spatially and quantitatively (Fig. 4).

Temporal coordination obviously is necessary to schedule the successive events during cuticle differentiation. For example, first the cell has to produce the envelope before chitin synthesis is initiated (Fig. 2). Presumably, scheduling of cuticle differentiation is dictated by activation and repression of gene expression by transcription factors.

Spatial coordination is seemingly occurring at the apical plasma membrane during procuticle establishment (Fig. 2). Epidermal and

hindgut epithelial cells in *D. melanogaster* form apical undulae that subdivide the apical plasma membrane in alternating regions of chitin synthesis and sites of vesicle docking. Thus, chitin fibers and chitin-binding proteins such as R&R and Obstructor proteins enter the extracellular space at different positions and must be brought together to interact, the histological visible extracellular space of interaction being called the assembly zone (Locke and Huie, 1979; Locke, 2003). By this separation the cuticle-producing cell controls the organization of the extracellular matrix (Moussian et al., 2006a, 2007). The dynamics of this process, however, are scarcely explored. Based on work on Syx1A function in *D. melanogaster* (Moussian et al., 2007), we can speculate that different t-SNAREs and associated proteins line up distinct regions of the apical plasma membrane thereby defining the position where specific components reach the extracellular matrix.

Quantitative coordination is required for example during sclerotization. The activity of enzymes and the amount of the organic molecules NADA and NBAD available have to be adjusted to obtain the designated stiffness and elasticity of the cuticle. Hence, how are the functions of Ddc (cytoplasmic), a putative epidermal Vmat-like monamine transporter (membrane-associated) and Yellow (extracellular) balanced? In the locust *Schistocerca gregaria*, Andersen (1974) has demonstrated that the release of organic molecules and not their synthesis in the cytoplasm is the limiting process for sclerotization. During melanization in *D. melanogaster*, by contrast, the amount of available extracellular dopamine for melanization is defined by the activity of the cytoplasmic NBAD synthase Ebony (Wittkopp et al., 2002). Flies mutant for *ebony* are darker than wild-type flies indicating a higher Yellow activity. Thus, the secretion of dopamine is not the limiting process in melanization. The underlying regulatory mechanisms of sclerotization and melanization have not been investigated to date.

One mode to fulfill quantitative coordination is to implement a feedback-circuit to regulate the expression of the respective factors at the transcriptional level. Such a communication may exist during procuticle formation between chitin synthesis and protein secretion. Stereotypic chitin organization implies that the processivity of the chitin synthase complex situated at the plaques and the supply of proteins between two plaques are in tune. The plasma membrane-inserted chitin synthase itself may be responsible for implementing this communication (Moussian et al., 2005a). In *D. melanogaster* embryos mutant for the chitin synthase encoding gene *kkv* excessive amounts of proteins seem to accumulate in the extracellular space suggesting that in the wild-type embryo the chitin synthesis complex formally controls the expression of secreted proteins. This information may directly come from the chitin synthase complex or indirectly emanate from fluctuations in the mechanical properties of the extracellular space that are sensed by the apical plasma membrane that generates a signal, which is subsequently transmitted to the nucleus. In any case, it will be a great challenge to identify and characterize the mechanisms and the transcription factor responsible for this regulatory circuit.

4. Variations

The composition and architecture of cuticles vary depending on their function. To accommodate functional differences the basic mechanisms of cuticle differentiation have to be modified. The ventral epidermal denticles in *D. melanogaster* larvae used for crawling, exemplify a cell-specific cuticle architecture (Figs. 1, 2 and 5A). Especially, chitin microfibrils in the denticular cuticle are disorganized. Unordered chitin correlates with the lack of apical undulae at the apical plasma membrane that serves as a template for the denticle shape. Instead, the denticular plaque is continuous. Supporting the notion that the activity of the apical plasma membrane accounts for the difference in cuticle architecture, several factors including zona pellucida (ZP) proteins specifically localize to the apical plasma membrane of the protruding denticle and are needed for this process (Chanut-Delalande et al., 2006). Initiation of denticle formation *per se* does neither depend on chitin synthesis nor on epicuticle establishment as *kkv* and *syx1A* mutant larvae do possess albeit malformed or small denticles (Moussian et al., 2005a, 2007). Interestingly, these localized modifications of cell morphology and cuticle architecture rely on the function of a single transcription factor, Shavenbaby (Svb) that induces the expression of actin organizing factors, sclerotization enzymes and ZP proteins (Chanut-Delalande et al., 2006; Delon et al., 2003; Payre et al., 1999). The major task of Svb is hence to govern the disruption of the regular apical plasma membrane and to install instead a uniform membrane platform to produce and organize denticles.

Cell-specific distinct cuticle architecture is also realized in the apodemes, the epidermal muscle-attachment sites (Fig. 5B). In these cells the difference of the cuticle architecture to the neighbouring non-apodeme cells is minor (Tepass and Hartenstein, 1994). The egr-like zinc finger transcription factor Stripe is sufficient to dictate the fate of apodemes in *D. melanogaster* (Becker et al., 1997; Frommer et al., 1996; Vorbruggen and Jackle, 1997). While the molecular mechanisms guiding and linking muscles to the basal site of the apodemes are very well investigated (Schnorrer and Dickson, 2004), we do not know much about the mechanisms fixing and organizing the cuticle at the apical site of these cells. A starting point for a detailed analysis could be the ZP proteins Piopio

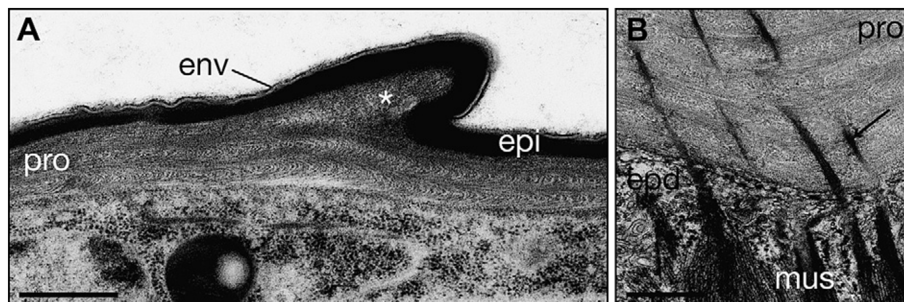

Fig. 5. Variation of cuticle architecture: the ventral denticle and apodemes. (A) Ventral denticles are important devices that allow the larva to move forward (see Fig. 1A). They are extensively melanized and sclerotized. Additionally, the chitin-protein matrix is rather amorphous (*) compared to the organized procuticle (pro) in neighbouring regions. (B) Apodemes are epidermal (epd) muscle (mus) attachment sites and have to withstand forces created during movement. In order to fulfill their role, the cuticle of apodemes is reinforced with tonofilaments (arrow), which are electron-dense stripes of proteins that are connected with the underlying cell (Tepass and Hartenstein, 1994). The tonofilaments do not interrupt chitin organization. Electron micrographs of oblique (A) or cross-sections (B) of the *D. melanogaster* larval cuticle. Specimens were fixed and treated as described previously by Moussian et al. (2006a). Scale bars 500 nm.

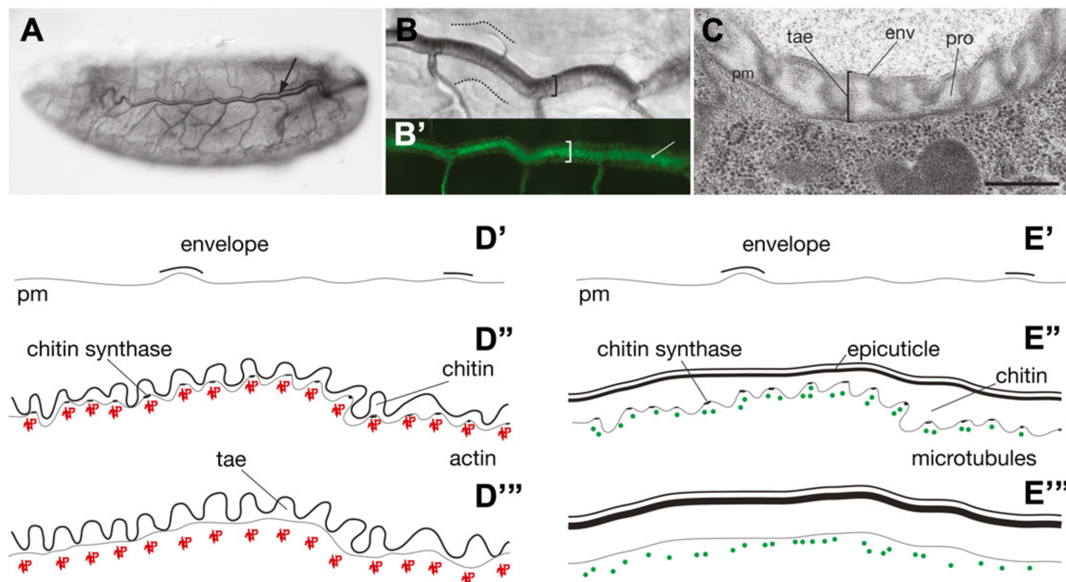

Fig. 6. Variation of cuticle architecture: the taenidial folds. (A) The tracheal tubes provide the animal with air. The arrow points to the dorsal trunk of the tracheal system of the *D. melanogaster* larva, which looks dark when air-filled. (B) A spiral of tracheal cuticle that appears as stripes (taenidiae) by light microscopy is deposited at the apical site of tracheal cells. The bracket marks the tube, and the dotted lines trace the basal sites of tracheal cells. (B′) The rings of taenidial chitin in *D. melanogaster* stage 17 embryos have been highlighted with the dye-conjugated chitin-binding probe (CBP). At this stage, the luminal chitin filament (arrow) is also visible (Tonning et al., 2005). (C) In cross-sections, the taenidiae appear as extracellular bulges (tae) consisting of a procuticle (pro) and an envelope/outer epicuticle (env). Interestingly, the inner epicuticle, a prominent layer in the even epidermal cuticle (Fig. 1), is either comparably thin or seems to be missing in the tracheal cuticle. Is this the reason for the difference between the tracheal and epidermal cuticle? Tracheal (D′) and epidermal (E′) cuticle differentiation start both with the random deposition of the envelope at the apical plasma membrane. In both tissues, after completion of envelope formation, the apical plasma membrane corrugates regularly (D″ and E″). In tracheal cells, these corrugations are underlain by actin cables (Matusek et al., 2006), while in the epidermal cells microtubules run underneath them (Moussian et al., 2006a). When cuticle differentiation is completed, the apical plasma membrane is withdrawn. In the tracheae, the cuticle (tae) follows the former corrugations of the apical plasma membrane (D‴). In the epidermis, the cuticle is flat (E‴). (A and B) Nomarksi microscopy of a living *D. melanogaster* larva ready to hatch. (B) Magnification of the dorsal trunk. (B′) Fluorescence microscopy of the tracheae after incubation of heat-fixed *D. melanogaster* wild-type embryos with Fluorescein-CBP (NEB Biolabs). (C) For electron microscopy, the specimen was fixed and treated as described previously by Moussian et al. (2006a). Scale bar 500 nm.

(Pio) Papillote (Pot) and Dumpy (Dp) that in the epidermis are predominantly expressed in apodemal cells and that have been mentioned to attach the cuticle to the surface of the epidermis in the *D. melanogaster* larva (Bokel et al., 2005; Jazwinska et al., 2003; Wilkin et al., 2000).

A variation that extends over an entire tissue is the spiral of taenidial folds lining the lumen of tracheae (Fig. 6). They run perpendicular to the length of the tracheal tube, thereby stabilising the tissue. Principally, the cellular and molecular mechanisms of taenidial differentiation are similar to those described for the smooth epidermal cells (Fig. 6C and D). Longitudinal protrusions of the apical plasma membrane mark the sites of chitin synthesis that takes place at the crests of the protrusions carrying canonical plaques (Moussian et al., 2006a), and mutations in *kkv* cause occasional flattening of the taenidial folds (Moussian et al., 2005a). However, despite these similarities the taenidial cuticle is bulged, whereas the epidermal cuticle is largely smooth. Moreover, in the taenidia chitin microfibrils are not arranged in laminae, suggesting that different chitin organizing proteins act in the tracheae and the epidermis. The comparative analysis of cuticle differentiation in these two tissues will boost our understanding of the cellular and molecular conditions of cuticle differentiation in general.

5. Outlook

Recent genetic and genomic data have forwarded our knowledge on insect cuticle differentiation and structure. *D. melanogaster* and *T. castaneum* have been proved to be excellent model insects to study the genetics and molecular biology of cuticle differentiation during development. Both species also allow investigating the genetics of cuticle moulting by RNA interference. What comes next? The small size of *D. melanogaster* and *T. castaneum* embryos, larvae and adult animals will facilitate microscopy of cuticle differentiation in living animals using cuticle markers identified today. Despite the difference in appearance, it has become clear that findings are transferable between developmental stages and between insect, and even between arthropod species. Relying on invaluable classical work on insect cuticle, our conjoint efforts should originate a new golden era of cuticle research.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ibmb.2010.03.003.

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