Role of Wol and Alas in *Drosophila* Skin Differentiation

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

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Tübingen

2012

Tag der mündlichen Prüfung :	20. Februar 2012
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There is one thing one has to have:
either a soul that is cheerful by nature,
or a soul made cheerful by work, love,
art, and knowledge.

(Friedrich Nietzsche)

Dedicated to My Parents

ACKNOWLEDGEMENT

I am very thankful to Dr. Bernard Moussian, for his constant motivation, guidance and indefinite support during my PhD studies. His scientific advices and ways of experimental designing improved my scientific thinking process. I would also like to thank Dr. Andrew renault, Dr. Uve Irion and Dr. Rolf Reuter for their advises and fruitful discussions during lab meetings. I would like to thank my lab mates Umesh Gangishetti, Michaela Norum for creating friendly working environment. I am also thankful to Ines Pramme for her assistance in my work and Koteswara rao Thota for his friendly company. I would like to remember my friends Gandhi, Vinod, Diwakar and other friends for their friendship who filled memorable moments during my stay in Tuebingen. Last but not least, my hearty gratitude for my beloved wife Dr. Shaik Nazneen for her endless care, help and love during my PhD studies.

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Zusammenfassung

Während der Differenzierung der Insektenhaut wird die extrazelluläre Kutikula durch die geordnete Sekretion ihrer Komponenten gebildet. Zur Aufklärung der Bedeutung der frühen N-Glykosylierung bei der Sekretion von Kutikulamaterial untersuchte ich in meinem ersten Projekt die Funktion des *wollknäuel* (*wol*)-Gens in der Taufliege *Drosophila melanogaster*. Das *wol*-Gen kodiert für das *Drosophila* Alg5-Enzym, das die Glukosylierung des Dolichol-gebundenen Oligosaccharids initiiert, das während der N-Glykosylierung an ein extrazelluläres oder Membran-gebundenes Protein angehängt wird. Die Stufenweise Reduktion von Wol-Aktivität führt zur Schrittweisen Verschlechterung des Kutikula-Phänotyps. Die Aufhebung der Wol-Funktion führt zu verringertem Glukosylierungsgrad und folglich reduzierten Mengen an N-glykosylierten Proteinen. Zum Beispiel sind die Pegel des Kutikula-organisierenden Faktors Knickkopf (Knk) reduziert, während es richtig zur Plasmamembran lokalisiert wird. Interessanterweise akkumulieren gleichzeitig die Zellpolaritätsdeterminanten Crumbs (Crb, membranär) und atypische Proteinkinase (aPKC, cytosolisch) an der apikalen Plasmamembran in Wol defizienten Larven. Ich folgere, dass die Hypoglukosylierung das Gleichgewicht der epidermalen Differenzierungsfaktoren stört. Die beobachteten Defekte können indirekt ausgelöst sein durch die so genannte „*unfolded protein response*" (UPR), die üblicherweise durch ER-Stress verursacht wird und zur Reduktion der Transkription und Translation im Allgemeinen führt. In diesem Szenario gibt es keine einfache Erklärung für das Verhalten für Crb und aPKC. Alternativ nehmen wir an, dass die Glukosylierung ein wichtiger direkter Kontrollpunkt für effiziente und ausgewogene Versorgung der epidermalen Differenzierung mit sekretierten und membranassoziierten Proteinen sein könnte.

Eine wesentliche Aufgabe der Arthropoden Kutikula ist, das Tier vor dem Austrocknen zu schützen. Zum besseren Verständnis der molekularen Mechanismen des Wasserrückhaltevermögens der Insektenkutikula studierte ich in meinem zweiten Projekt die Folgen einer P-Element Insertion in das Gen *CG3017*, die Barriere-Defekte der Kutikula verursacht. *CG3017* kodiert für das Enzym δ-Aminolevulinat-Synthase (Alas), das den ersten Schritt der Häm-Biosynthese katalysiert. Mutationen in *alas* führen zu einer Verringerung des Häm-Gehalts, was unter anderem erhöhte Phosphorylierung des Signaltransduktionsfaktors ERK und des Translationsinitiationsfaktors eIF2α zur Folge hat. Überphosphorylierung von eIF2α führt zur Verlangsamung der Translation, was die Reduktion an Knk und Cytochrom c erklären könnte. Auf der ultrastrukturellen Ebene zeigen *alas* Mutanten eine Zerfransung der Prokutikula besonders an ihrer basalen Seite, was zur Ablösung der Kutikula führt, ein

Phänotyp, der dem ähnelt, der durch Reduktion der Dualoxidase (Duox)-Aktivität in dem Fadenwurm *C. elegans* hervorgerufen wird. Duox ist ein Häm-bindendes Enzym, das zur Stabilisierung der extrazellulären Matrix die Vernetzung von Tyrosinresten von kutikulären Proteinen katalysiert. Tatsächlich ist die Dityrosin-Vernetzung in *alas*-mutanten Larven reduziert. Im Gegensatz dazu sind die parazellulären Zellkontakte, die als eine physikalische Barriere in Epithelien wirken und die Transglutaminase-kontrollierte Vernetzung von extrazellulären Proteinen in der Haut von *alas* Mutanten normal. Zusammengenommen konnte ich eine bisher nicht identifizierte Funktion für die Dityrosin-Protein-Vernetzung in der Insektenkutikula zeigen, die als eine physikalische transzelluläre Barriere gegen Austrocknung erforderlich ist.

ABSTRACT

Insect skin differentiation involves the construction of the extracellular cuticle that is formed by the systematic secretion of its components. To elucidate the importance of initial facets of N-glycosylation during secretion of cuticle material, I investigated the function of the *wollknäuel* (*wol*) gene in *Drosophila. Wol* codes for the *Drosophila* Alg5 that initiates the glucosylation of the dolichol-linked oligosaccharide destined to be linked to an extracellular or membrane-bound protein. Stepwise reduction of Wol activity leads to gradual worsening of cuticle phenotype. Abrogation of Wol function results in decrease of glucosylation and by consequence lowered amounts of N-glycosylated proteins. For instance, the levels of the cuticle organizing factor Knickkopf (Knk) are reduced, while it continues to be correctly localized to the plasma membrane. Interestingly, at the same time, the polarity determinants Crumbs (Crb, membrane-inserted) and the kinase aPKC (cytosolic) accumulate at the apical plasma membrane in *wol* deficient embryos. Hence, hypoglucosylation perturbs the balance of epidermal differentiation factors. The observed defects may indirectly be triggered by the unfolded protein response (upr), which is commonly caused by ER stress and reduces transcription and translation in general. In this scenario, there is no simple explanation for the behaviour for Crb and aPKC. Alternatively, glucosylation may be an important checkpoint directly controlling efficient and balanced supply of secreted and membrane-associated proteins during epidermal differentiation.

An essential function of the arthropod cuticle is to guard the animal against dehydration. To understand the molecular mechanism of water retention capacity of the insect cuticle, I studied the consequences of a P-element insertion in the gene *CG3017* that causes liquid barrier defects. *CG3017* codes for the rate limiting enzyme δ-aminolevulenic acid synthase (Alas) catalyzing the initial step in heme biosynthesis. Consistently, mutations in *alas* reduce the heme content, which has several cellular consequences including higher double phosphorylated ERK levels and increased phosphorylation of eIF2α that results in attenuated translation, which could explain the reduced levels of Knk and cytochrome C. At the ultrastructural level, *alas* mutants show rupturing of procuticle especially at its basal side, a phenotype that is similar to the Dualoxidase (Duox) mutant cuticle phenotype in *C. elegans*. Duox is a heme binding enzyme that catalyzes the cross-linking of tyrosine

residues involved in the stabilization of cuticular extracellular matrix. Indeed, dityrosine cross-links are reduced in the *alas* mutant cuticle. By contrast, the septate junctions that are known as physical barriers in epithelia and transglutaminase function that is known for water barrier function in vertebrates are normal in *alas* mutant. Taken together, I show a previously not identified function for the dityrosine protein cross-link in the insect cuticle that is required as a physical barrier for water retention.

2. INTRODUCTION

2.1 Background:

The cuticle is a chitin containing exoskeleton covering the insect body. The impressive adaptation of insects to diverse environmental conditions can be attributed to the wonderful mixture of flexibility and strength of the insect integument. It functions as a physical barrier protecting the body from physical injury, pathogens and water loss. It is also important for the body architecture and locomotion through functioning as the base for muscle attachment. As a structure specific to arthropods, the processes of cuticle differentiation are excellent targets in pest control both in agriculture and disease management (e.g. Malaria). The cuticle is secreted by the epidermal cells during late embryogenesis and before each moult when the animal grows. It consists of the outer most protective *envelope*, upper protein-rich *epicuticle* and inner chitinous *procuticle* (fig.1) [1]. The envelope is the first layer to be formed at the plasma membrane surface at the tip of microvilli-like structures. The epicuticle is deposited on the inner surface of the envelope. Usually, the procuticle is much wider than the other two layers and contains a protein-chitin matrix mainly providing elasticity to the cuticle.

Organisation of the cuticle

After cellulose, chitin is the second most abundant polysaccharide in nature. In 1823, chitin was described for the first time as an essential component of the arthropod exoskeleton by the French naturalist Auguste Odier [2]. The chemical structure of chitin was resolved in 1901 by S. Fränkel and A. Kelly [3]. It is a polymer of β1,4-linked N-acetyl-glucosamine (GlcNAc), some of which are deacetylated, and deacylation of chitin has been proposed to allow the interaction of chitin with certain, yet unidentified proteins that may be responsible for correct organisation of chitin [4]. In 1965, the French biophysicist Yves Boulingand described the architecture of chitin microfibrils in the cuticle of the crustaceans *Acanthocyclops viridis, Carcinus moenas, Macropipus puber* and *Cancer pagurus* [5]. He described that the arrays of chitin microfibrils arranged in parallel to the apical plasma membrane of the epidermal cell are piled up helicoidally in respect to microfibril orientation (Fig.1c). Subsequently, it was found to be also true for insects [6].

Chitin is produced at the apical side of epidermal, tracheal and gut epithelial cells by probably dimmers of chitin synthases that are integral membrane proteins [7]. In insects, chitin synthases are grouped into two classes. Chitin in the epidermis and the trachea is synthesized by class A enzymes, whereas in the midgut it is produced by class B enzymes. Jürgens and colleagues, in 1984 identified mutations in the locus *krotzkopf verkehrt* (*kkv*) that affect the larval cuticle and turned out to code for the epidermal and tracheal Chitin Synthase-1 (CS-1), a class A chitin synthase. Chitin Synthase-2 gene codes for the class B enzymes [8,9].

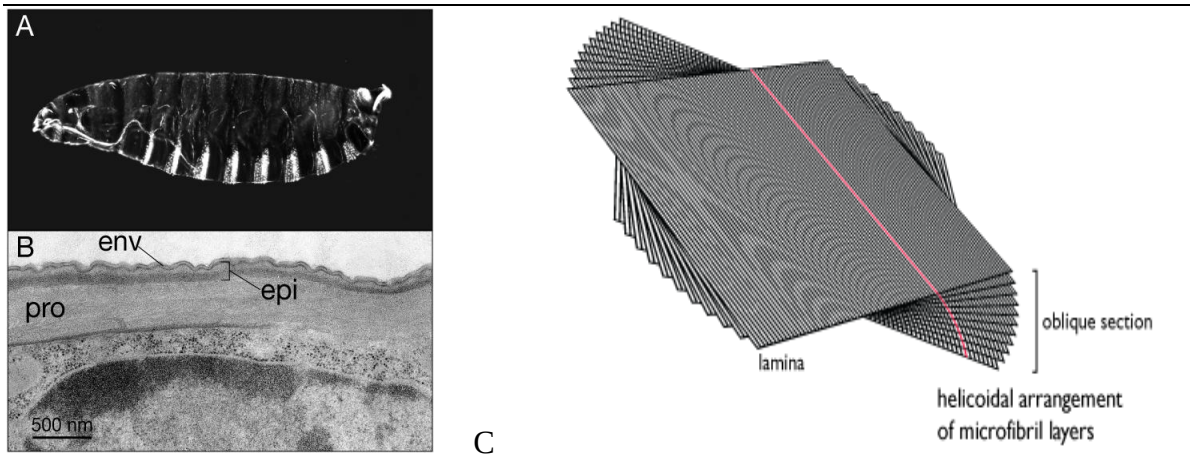

Figure 1: A) Cuticle preparation of a wild-type *Drosophila melanogaster* larvae B) Transmission electron microscopy showing the cuticle in wild type larvae consists of three layers: the outermost envelope (env), the middle electron dense epicuticle (epi), and the inner procuticle containing chitin laminae (pro). C) Chitin organization in procuticle shows parallel chitin micro fibrils (black lines) arranged in sheets called laminae and these laminae are arranged in helical stacks. (Adopted from Moussian *et.al.*2006).

According to the classical model described by Michael Locke, microvilli-like structures carry at their tips the chitin synthesis machinery and through oscillation direct the orientation of the nascent chitin microfibril. In the *Drosophila* embryo, Moussian and colleagues discovered that the apical plasma membrane forms longitudinal microtubule stabilized corrugations called *apical undulae* instead of microvilli-like structures [10]. The highly ordered arrangement of the plasma membrane indicates a fundamental function of the apical plasma membrane in chitin orientation.

Some factors that may be involved in chitin organisation have been identified and characterised genetically. Retroactive (Rtv) and Knickkopf (Knk), both membrane bound proteins are among genetically identified chitin organizing factors, which are involved in chitin microfibril formation and orientation at the apical side of the epidermal cell.

Mutations in the *rtv* gene result in disarrangements of chitin fibres in the cuticle of *Drosophila* larva [11] suggesting that it is important for chitin organisation. Rtv belongs to a new family of disulfide-rich proteins in insects that are related to vertebrate snake neurotoxin-like and small membrane anchored proteins and does not have canonical chitin binding domains such as a R&R chitin binding motif or a tachycitin-like chitin binding domain [12, 13, 14]. Instead, it harbours six evolutionary conserved aromatic amino acids. Aromatic amino acids of carbohydrate binding proteins have been demonstrated to mediate the interaction between these proteins and their substrates [15, 16, 17, 18]. It was therefore hypothesised that the six conserved aromatic residues present in Rtv i.e. four tyrosine and two phenylalanine may play an important role in chitin binding. Knk is a GPI anchored protein with a tandem of DM13, a DOMON (dopamine β-monooxigenase N-terminal) domain and a plastocyanin domain and acts at the apical plasma membrane. Knk and Rtv are also needed for tracheal luminal chitin filament assembly [19]. The biochemical functions of Rtv and Knk are still unknown. Chitin orientation also requires the activity of two extracellular deacetylases Vermiform (Verm) and Serpentine (Serp), supporting the notion that deacetylated GlcNAc residues are important for chitin architecture probably as binding sites for cuticular proteins [20].

An important process during cuticle differentiation is the interaction of chitin with cuticular proteins [21]. The flexibility and diverse mechanical strength of the cuticle is partly attributed to hardening called sclerotization, a process of crosslinking of proteins and chitin with catecholamines and phenols. Tyrosine derived phenolic compounds are also used in the cuticle tanning process called melanization. The proposed steps in the sclerotization process are hydroxylation of tyrosine to dopa by the tyrosine hydroxylase encoded by the *pale* locus in *Drosophila* and decarboxylation of dopa to dopamine by the DOPA decarboxylase (Ddc). Dopamine is used as a starting material for sclerogenic and melanogenic precursors such as N-acetyldopamine (NADA) by the Dopamine N-acetyltransferase and N-b-alanyldopamine (NBAD) by the NBAD synthase encoded by the *ebony* gene. [22].

Taken together, despite identification of many of the factors essential for chitin synthesis and organization, how these function remains sparse.

2.2 Role of Secretion & Wollknäuel in epidermal differentiation:

Secretion control

The regular stereotypic architecture of apical extra cellular matrix (aECM) requires systematic functioning of the apical plasma membrane. The role of the epidermal apical plasma membrane during cuticle differentiation is well demonstrated in larvae mutant for *syntaxin1A* (*syx1A*) that codes for the apical plasma membrane t-SNARE Syntaxin 1A. The cuticle layers of *syx1A* deficient larvae are less thickened, but nevertheless stratified. The secretion of certain O-glycosylated proteins and components involved in pigmentation, in sclerotization and protein cross-linking is mediated by the Syx1A. However, localization of chitin synthesis and organising proteins to the apical plasma membrane or to the extracellular space does not depend on Syntaxin 1A activity. But, chitin microfibrils are randomly oriented in *syx1A* mutants. The corrugations at the apical plasma membrane of epidermal cells, the apical undulae are absent in these mutants. Therefore, Syx1A is required for the correct apical plasma membrane topology and also mediating secretion of a subset of extracellular proteins required for layer organisation. One can conclude that two apical secretion routes one Syx1A dependant and another unidentified t-SNARE is needed to deliver extracellular material for complete cuticle assembly [23].

These two postulated vesicle routes converge, as expected, at the ER level. Indeed, the two genetically identified factors Haunted and Ghost, which are the *Drosophila* COPII vesicle-coating components Sec23 and Sec24 respectively are absolutely essential for polarity maintenance, membrane topology and for secretion of the tracheal luminal matrix and the cuticle. These two factors are involved in vesicle transport from the ER to the Golgi apparatus. Based on these findings, one can generalize that epithelial differentiation during Drosophila embryogenesis is a cooperative action of ECM formation, plasma membrane remodeling and maintenance of cell polarity that all three depend on the canonical secretory pathway from the ER over the Golgi apparatus to the plasma membrane [24].

N-glycosylation

Considerable secretion regulation occurs during the entry of proteins into the secretory pathway in the ER. Many secreted or membrane proteins are modified during their travel through the secretion compartments. These modifications comprise N-glycosylation and GPI-anchor formation. A major molecule used for protein modification is N-acetylglucosamine (GlcNAc). It is a copious hexose that as a monomer or as part of

macromolecules plays various roles in eukaryotic cells. GlcNAc contributes to the organisation and function of various ECMs. In ECMs, GlcNAc is present in several protein conjugates and polysaccharides. Generally, oligo- and polysaccharides are covalently attached to proteins, comprising N- and O-glycans representing the quality of the protein. GlcNAc is also part of membrane inserted sugar–lipid complexes like glycosphingolipids and glycosylphosphatidylinositol (GPI) anchors that define the properties of the membrane. Lastly, GlcNAc is a subunit of free polysaccharides i.e. that are not covalently bound to other molecules, like hyaluronan (HA) and chitin that are the basis for the physical properties of ECM. The active form of GlcNAc in biochemical reactions is uridine di-phosphate (UDP)-GlcNAc that is the end product of the so-called Leloir pathway [25]. N-glycosylation of proteins involved in extracellular matrix formation is essential for their proper functionality. Membrane-bound and secreted proteins are transported through the secretory pathway where they are modified and trimmed for correct folding and function. A major type of modification is N-glycosylation of certain asparagine residues via an evolutionarily conserved mechanism in the early ER [26, 27]. The N-glycosylation pathway has been extensively studied in yeast. The precursor oligosaccharide is assembled on the polyisoprenol carrier lipid, dolichyl phosphate, through a stepwise addition of monosaccharides in a series of reactions catalyzed by glycosyltransferases. Specific glucosyltransferases transfer Mannose (Man), GlcNAc or Glucose (Glc) from the respective dolichol-sugar substrates to the growing oligosaccharide chain to make the oligosaccharide Glc3Man9GlcNAc2. All ER glycosyltrasferases are transmembrane proteins. Basically, the oligosaccharide transferase (OST) at the ER membrane inserts a dolichol-anchored oligosaccharide Glc3Man9GlcNAc2 to the target asparagine amino acid residue. [26]. Glucosidase I and glucosidase II remove the two Glc residues respectively from the protein-linked glycan making the protein competent to interact with the ER chaperones Calnexin or Calreticulin helping in the folding of the protein that may be stabilized by the introduction of disulfide bonds by ERp57 [28]. The removal of the last Glc by glucosidase II releases the folded protein and enters the Golgi cisternae. Misfolded proteins that are not bound to chaperone are exported from the ER and directed to the cytosolic proteasome system for degradation [29]. The UDP-glucose-glycoprotein glucosyltransferase (UGT) adds a Glc to the Man9GlcNAc2 within the ER lumen allowing the protein to interact with calnexin-calreticulin cycle for correct folding. Mutations in

genes involved in N-glycosylation are linked to human disorders, some of them classified as congenital disorder of glycosylation (CGD) type Ia-l and IIa-d. Type I defects are due to impairment in the synthesis and transfer of the lipid linked oligosaccharide (LLO) and Type II defects are caused by the loss of modification process of protein-bound oligosaccharides [30,31, 32]. The important clinical feature of patients suffering from the different types of CGD is defects in some ECMs although the defects are not understood in detail. Though N-glycosylation mechanisms are extensively studied, their implication on ECM differentiation particularly in multicellular organisms is understudied. A good model system for understanding the ECM defects of N-glycosylation is the *Drosophila* larval cuticle through the help of well characterized development and easy genetic manipulation. Tonning and colleagues have investigated in 2006 the impact of mutations in the *mummy* gene (*mmy*) that codes for the UDP-GlcNAc pyrophosphorylase on the *Drosophila* cuticle. UDP-GlcNAc pyrophosphorylase produces GlcNAc residues that are required for chitin synthesis, N-glycosylation and GPI anchor formation. Reduced GlcNAc levels in the epithelial cells of *mummy* mutants strongly affect the cuticle. Knk, a chitin organizing factor is hypoglycosylated in this mutant partially explaining the cuticle phenotype [33, 34].

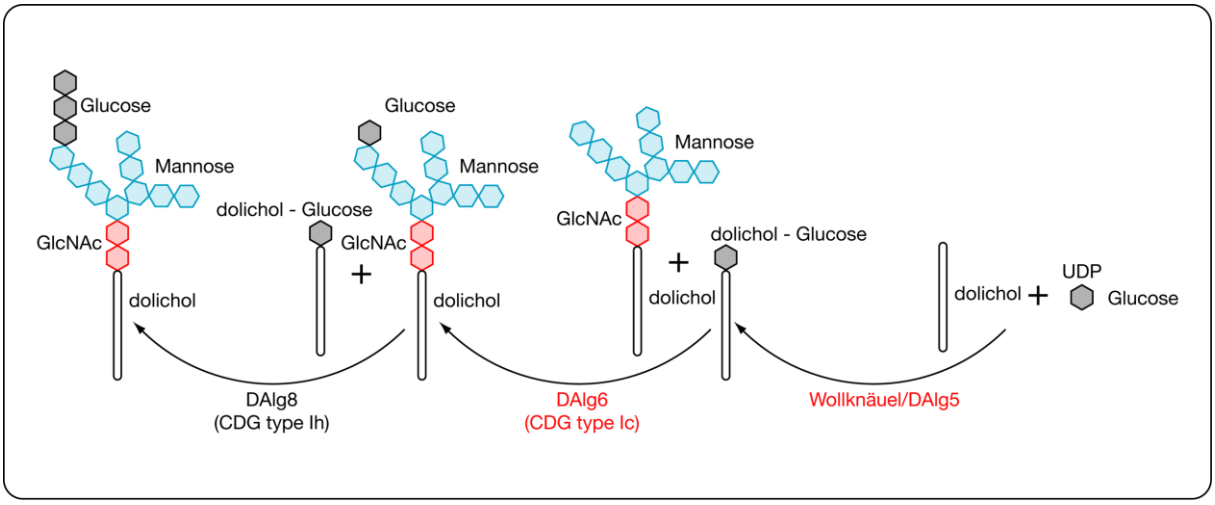

Figure 2: Molecular function of Wol and the requirement of its product dolichol:glucose. The role of the *Drosophila* ALG5 called Wollknäuel (Wol) that is catalysing the formation of dolichol:glucose, a substrate of ALG6 and ALG8 that transfer glucose to the oligosaccharide moiety to be attached to Asn residues of many secreted proteins involved in the cuticle differentiation. CDG stands for congenital disorders of glycosylation. Type I CDGs include disrupted synthesis and transfer of lipid-linked oligosaccharide (LLO).

GlcNAc as part of N-glycans and GPI-anchors and as the monomer of chitin is an abundant substance in the cuticle, thus, the phenotypic information gained from analysing *mmy* mutant embryos is only of partial relevance to understand the regulatory effect of N-glycosylation on the cuticle development. By studying the effects of mutations specifically blocking the early steps of N-glycosylation, it would possible to dissect the early ER functions, which control the folding and secretion of factors required for cuticle differentiation. Hence, I have chosen to analyse the roles of the genes *Dalg5* and *Dalg6* that code for two enzymes catalysing the addition of Glc units to the oligosaccharide, UDP-Glc: dolichol transferase (pfam00535) and dolichol-Glc: Man9-GlcNAc2-dolichol glucosyltransferase (pfam03155), respectively. The ALG5 locus in *Drosophila* is called *wollknäuel* (*wol*) based on its cuticle phenotype, which means ball of wool in German. Phenotypes of *wol* mutants comprise posterior segmentation defects, reduced Dpp signaling, defective mesoderm invagination and germband elongation at gastrulation. Haecker *et al.*, 2008 have proved the biochemical activity of Wol through complementation of the hypoglycosylation phenotype of *alg5* mutant *S. cervisiae* by the *wol*/*ALG5* cDNA. Reduced glycosylation levels in *wol* mutant embryos elicit endoplasmic reticulum stress and the unfolded protein response (UPR). In *wol* mutants phosphorylation of the translation factor eIF2α is increased and results in reduced protein synthesis [35,36]. A P-element insertion in the *Drosophila alg6* locus causes a *wol*-like phenotype, we therefore called this mutation *garnysstan* (*gny*), which means ball of wool in Swedish; it has not been described previously. It transfers the glucose from dolichol-glucose to dolichol oligosaccharyl moiety.

2.3 Role of Alas in liquid barrier function:

Knk is an apical GPI-linked protein that acts at the plasma membrane and is essential for cuticle formation through a specific role in directing chitin filament assembly. The *knk* mutant embryo shows a bloated phenotype due to the detachment of the reduced cuticle from the epidermal cells [37]. The Knk protein contains three kinds of conserved domains, which are two DM13 domains, a DOMON domain and a plastocyanin domain. L.M.Iyer and colleagues (2007) predicted that DOMON domains might be involved in heme or sugar binding. They also presented bioinformatical evidence that the DOMON domain along with the DM13 domain comprises a novel electron-transfer system possibly involved in oxidative modification of extracellular proteins. The DM13 domain and the classical family of the DOMON domain are evolved from bacteria and seem to have expanded to higher

order extracellular proteins such as Human CG-6 protein, Mouse SDR2 [38]. My primary aim was to identify the binding partner of DOMON domain. I was not able to biochemically test for the heme as binding partner for the Knk DOMON domain through heme binding assays[1]. An alternative method is to analyse the phenotypes of heme biosynthesis mutants testing whether they phenocopy the *knk* mutant phenotype. To understand the role of heme and its biosynthesis during *Drosophila* cuticle development, I choose P-element insertions in genes coding for the enzymes involved in Heme biosynthesis pathway that are available at Bloomington *Drosophila* stock center (figure 3). Heme is a tetrapyrrole molecule serving as prosthetic group in many essential enzymes. These enzymes are part of important processes like electron transport, apoptosis, detoxification, nitrogen monoxide synthesis, and oxygen transport. Heme also functions as a signaling molecule for various regulatory processes [39]. It controls the activities of a variety of signal transducers and transcriptional regulators. In reticulocytes when heme deficiency occurs protein translational is inhibited by the Heme Regulated Inhibitor (HRI), later known as Heme-regulated eIF2α kinase, which phosphorylates the α-subunit of the eIF2α. This phosphorylation results in inhibition of GDP-GTP exchange on eIF2 by the guanine nucleotide exchange factor eIF2B, thus inhibiting recycling of the ternary complex containing the initiator methionine Met-tRNAi [40].

The first step in heme biosynthesis is the formation of δ-aminolevulinic acid through condensation of glycine and succinyl-CoA by δ-aminolevulinate synthase (ALAS). The ALAS enzyme functions as a homodimer in the mitochondrial matrix and it is a pyridoxal phosphate-dependent enzyme. In vertebrates, two different genes ALAS1 and ALAS2 are present for executing the first step in heme biosynthesis.

alas2 or *alas-E*, which is exclusively expressed in erythroid cells, is necessary for hemoglobin synthesis. *alas1* or *alas-N* is the nonspecific or housekeeping isoform and is expressed in all cell types (including erythroid) with the maximum expression found in liver, where it is essential for the synthesis of cytochromes P450 [41].

Among the *Drosophila* seven P-element mutants analysed, embryos mutant for the first enzyme Alas die at late stage 17. *alas* mutant embryos showed an interesting phenotype with ruptures in the procuticle and loss of liquid barrier functions leading to liquid leakage from the embryos. However, despite the relatively mild phenotype, mutations in *alas* did

[1] In bacteria, recombinant Knk protein localises to inclusion bodies and is therefore denaturated and not usable for biochemical assays. Likewise, I did not succeed in expressing Knk in mammalian cells.

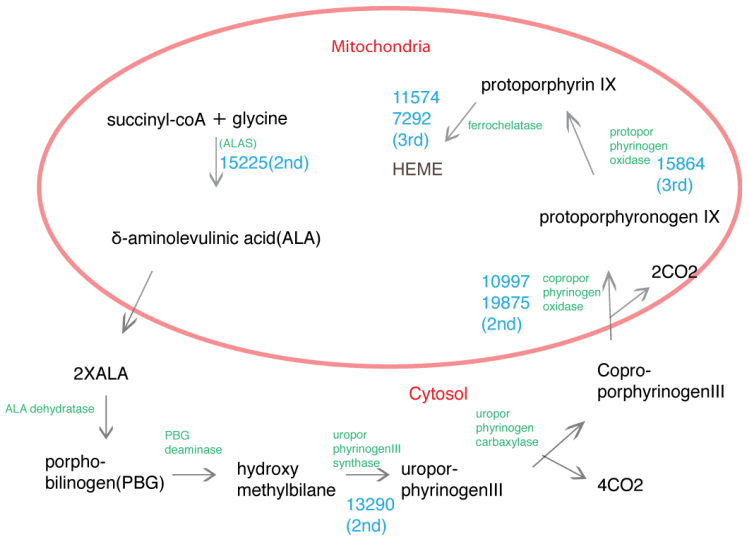

Figure 3: Heme Biosynthesis pathway and *Drosophila* P-element insertional mutants.

Heme Biosythesis takes place in Mitochondria and partly in cytosol. Aminolevulinic acid synthase (Alas) is the rate limiting enzyme in heme biosynthesis which synthesises Aminolevulinic acid from succinyl-CoA and glycine. Numbers indicated in blue colour are flybase P-element stock numbers.2nd, 3rd stands for the location of the genes on the 2nd and 3rd chromosomes respectively.

not phenocopy *knk* mutants. Excitingly, *alas* mutation emerged as a good model for understanding the liquid barrier mechanisms in the *Drosophila* extracellular matrix. The skin in vertebrates and the cuticle in insects serve as barriers against physical, chemical, liquid and pathogen infection. Both are layered structures. The outermost stratum corneum of the vertebrate skin comprises highly cross-linked keratinocytes, proteins, and lipids. The stratum corneum consists of distinctive lipid composition, with long chain ceramides, free fatty acids and cholesterol as main lipid classes. In vertebrates skin with reduced barrier function correlates with altered lipid composition and organization (figure 4) [42]. JA Tunggal and colleagues (2005) described the importance of tight junctions in controlling the water barrier function of epidermis. Permeable tight junctions formed by the absence of E-cadherin in the epidermis resulted in perinatal death of mice due to the inability to retain a functional epidermal water barrier [43]. The claudin-1 deficient mice, which die of massive trans epidermal water loss, revealed the functional evidence for tight junctions in the epidermal barrier function [44]. Another specialized structure in the

skin required for the water-barrier function is the cornified cell envelope that is insoluble proteinaceous layer formed by cornified cells. Transglutaminases (TGs) catalyse the crosslinking of protein-lipid components of the cornified cell envelope. The isopeptide bonds formed by these enzymes are responsible for the chemical and physical resistance of the skin [45]. Another type of protein crosslink present in the extracellular matrix is the dityrosine network that catalysed by the heme enzyme peroxidase/oxidase system also known as Dual oxidase (Duox). In *Anopheles*, the gut permeability for immune elicitors has been shown to decrease upon the formation of dityrosine network. Interruption of this dityrosine network barrier results in the effective pathogen-specific immune responses [46]. In *Caenorabditis elegans* hypodermal cells underlying the cuticle express a protein called Duox (for dual oxidase) because they have both a peroxidase homology domain and gp91phox domain. The cross-linking of tyrosine residues that are involved in the stabilization of cuticular extracellular matrix are catalyzed by Ce-Duox [47].

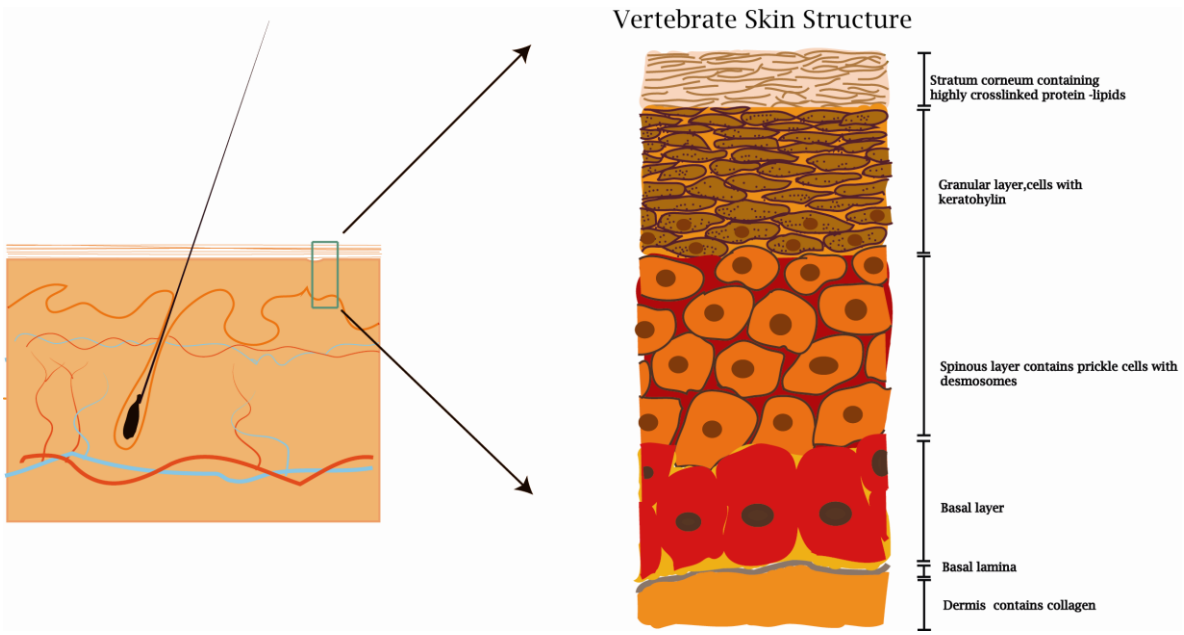

Figure 4: Epidermal differentiation in vertebrate skin: The epidermis is the outermost layer of the skin consisting of Keratinocytes and basal lamina separates it from the underlying dermis. Keratinocytes move forward and differentiate from spinious layer to granular layer and then to stratum corneum. They gradually become anucleated and increasingly compressed.

While huge information is available on the vertebrate skin barrier functions, little information is available on the insect cuticle barrier functions. The insect cuticle is a complex extracellular structure with chitin, protein, chatecholeamines and lipid as components (figure 1). Direct molecular evidence for the functions of each of these cuticular components in liquid barrier functions is missing. However, we can infer the role by the phenotypes of mutants lacking particular components in the extracellular matrix. For instance, chitin synthase-1 (*kkv*) mutants that lack chitin in their ECM do not show liquid barrier defects. This suggests that chitin is not essential for liquid barrier function. Similarly *Drosophila* tyrosine hydroxylase (*pale*) mutants deficient in catecholamine biosynthesis do not show barrier defects indicating that catecholamines might not be essential for liquid barrier function. The role of the protein crosslinking carried out in the extracellular matrix by transglutaminase (TG) and peroxidase/oxidase (Duox) system in the liquid barrier function is unknown. Tight junctions and lipids are other components of the cuticular epidermis that are candidates for the barrier functions. In insects, the role of tight junctions in barrier functions are broadly renowned [48,49]. By the work on *alas*, I contribute the first molecular view on skin barrier function in insects. Regarding the similarity between the insect and mammalian skin in respect to water balance, the *Drosophila* skin may serve as a model tissue to study the underlying and relevant molecular mechanisms.

3. PUBLICATIONS

3.1 Publication Shaik Khaleelulla Saheb et al., 2011(Glycobilogy)

The Alg5 ortholog Wollknäuel is essential for correct epidermal differentiation during *Drosophila* late embryogenesis

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Keywords: ECM, glycosylation, CDG, Drosophila, cuticle

Abstract

The formation of an extracellular matrix (ECM) presupposes an ordered delivery of its components to ensure its stereotypic architecture. The *Drosophila* cuticle is an ECM produced by the epidermis at its apical site and is characterised by a layered organisation. To understand the mechanisms of cuticle assembly during development, we have investigated early aspects of protein N-glycosylation, i.e. the attachment of a dolichol-linked oligosaccharide to distinct Asn sites of a protein known to be essential for sorting in the secretory pathway. Mutations in the *Drosophila alg5* gene *wollknäuel* that codes for an enzyme initiating the glucosylation of the dolichol-linked oligosaccharide decrease, as expected, glucosylation and the amounts of N-glycosylated proteins such as the cuticle organising factor Knk, without affecting their correct localisation. At the same time, the polarity determinants Crb and aPKC accumulate at the apical plasma membrane in *wol* deficient embryos. In part, these perturbations may also be caused by the unfolded protein response, which is commonly triggered by ER stress and downsizes transcription and translation in general. In any case, they are associated with the loss of cuticle layering and aberrant apical plasma membrane organisation suggesting that glucosylation, either directly or indirectly through controlling protein degradation, is important for the efficient and balanced deployment of the biochemical functions of secreted and membrane-associated proteins during epidermal differentiation.

Introduction

Extracellular matrices (ECMs) adopt a stereotypic architecture that is essential for their function as physiological milieus, barriers and cell- and tissue shaping elements. Construction of ECMs implies correct folding, ordered secretion and deposition of their components including structural and organising proteins and free or protein-attached oligo- and polysaccharides like haparan sulfates, hyaluronic acid, cellulose and chitin into the extracellular space. Usually, membrane-bound and secreted proteins run through the secretory pathway where they are modified and trimmed for correct folding and function (Caramelo and Parodi, 2007). A major type of modification is N-glycosylation of certain asparagine residues via an evolutionarily conserved mechanism in the early ER (Vagin *et al.*, 2009). The enzymatic steps of N-glycosylation have been well studied especially in yeast. In principle, a dolichol-anchored oligosaccharide (Glucose $(Glc)_3$ Mannose $(Man)_9$ N-acetylglucosamine $(GlcNAc)_2$) is added to the target asparagine by the oligosaccharide transferase (OST) at the ER membrane. This oligosaccharide is built by glucosyltransferases that transfer Man, GlcNAc or Glc from the respective dolichol-sugar substrates to the growing oligosaccharide chain (Burda and Aebi, 1999). Removal of two Glc residues from the protein-linked glycan by glucosidase I and glucodiase II respectively renders the protein competent to interact with the ER chaperones Calnexin or Calreticulin catalysing the folding of the protein that may be stabilised by the establishment of disulfid bounds assisted by ERp57 (Anelli and Sitia, 2008). The travelling protein is then released and can enter the Golgi cisternae after the last Glc has been removed again by glucosydase II. Cutting off all Glc

residues before chaprone function is completed may result in misfolded proteins that are consequently exported from the ER and are directed to the cytosolic proteasome for degradation (Malhotra and Kaufman, 2007). However, a rescue reaction may occur through the addition of a Glc to the $Man_9GlcNAc_2$ oligosaccharide by the UDP-glucose-glycoprotein glucosyltransferase (UGT) within the ER lumen, thereby allowing the protein to interact with Calnexin again. Several mutations in genes coding for the early enzymes of N-glycosylation are associated with human diseases, some of them subsumed as congenital disorder of glycosylation (CGD) type Ia-l and IIa-d (Freeze, 2006, Jaeken and Matthijs, 2007, Marquardt and Denecke, 2003). Patients suffering from the different types of CGD are reported to have defective ECMs. The defects, however, have not been analysed in detail.

Despite the exhaustive work on the molecular mechanisms of N-glycosylation, its significance on ECM differentiation especially in multicellular organisms is investigated only in few cases. An amenable ECM for studying the relevance of N-glycosylation for ECM formation is the *Drosophila* larval cuticle. It is a typical arthropod cuticle with three biochemically distinct layers (Locke, 2001, Moussian, 2010). The layer adjacent to the apical surface of the epidermal cell is the procuticle that harbours a matrix composed of the polysaccharide chitin and associated proteins. The second layer is the epicuticle that is built up of proteins cross-linked with catecholamines. The pro- and the epicuticle together are responsible for cuticle stiffness and elasticity. Finally, the outermost layer is the envelope that consists of proteins and lipids representing the outer barrier against dehydration of the animal. Some little progress on the role of N-glycosylation in cuticle differentiation has been

published recently (Tonning *et al.*, 2006). For instance, reduction of GlcNAc levels in epithelial cells of the *Drosophila* embryo through mutations in the *mummy* gene (*mmy*) that codes for the UDP-GlcNAc pyrophosphorylase cause a strongly depleted cuticle. This phenotype is accompanied by hypoglycosylation of extracellular or membrane-associated proteins such as the chitin organising factor Knickkopf (Knk) (Moussian *et al.*, 2006). Since GlcNAc as part of N-glycans and GPI-anchors and as the monomer of chitin is a prevalent molecule in the cuticle (Moussian, 2008) , the phenotypic information retrieved from analysing *mmy* mutant embryos is only of limited relevance to understand the impact of N-glycosylation on cuticle morphology. To study the importance of early ER functions in controlling folding and secretion of factors acting during cuticle differentiation, we therefore characterised the effects of mutations specifically impeding early steps of N-glycosylation. We have chosen to analyse the roles of the genes *Dalg5* and *Dalg6* that code for two enzymes catalysing the addition of Glc units to the oligosaccharide, UDP-Glc: dolichol transferase (pfam00535) and dolichol-Glc: Man_9-$GlcNAc_2$-dolichol glucosyltransferase (pfam03155), respectively (Figure 1). The *Drosophila* DAlg5 called Wollknäuel (Wol) has been shown to be essential for patterning of the early embryo (Haecker *et al.*, 2008). Defects in *wol* mutant embryos were mainly attributed to the unfolded protein response, which is induced by ER malfunction and results in protein degradation and translation stop (Malhotra and Kaufman, 2007, Ruddock and Molinari, 2006). Mutations in *Dalg6* that we call *garnysstan* (*gny*) have not been described previously. In the present work, we demonstrate that abrogation of Wol function among others lowers the amounts of N-glycans such as Knk, but increases the amounts of the

membrane-organising factor Crumbs (Crb). We conclude that the stoichiometry of differentiation factors is critical for normal epidermal development.

Results

wol and gny are needed for cuticle differentiation and act in one pathway

The wild-type larva has a spindle-like shape covered by the cuticle that had been produced during embryogenesis (Figure 2A). Larvae homozygous mutant for *wol* or *gny* or *wol* over a deficiency uncovering the respective region hatch and have a wild-type-like cuticle by light microscopy (Figure 2B and C). They eventually die after one moult without displaying any obvious phenotype. Removal of maternal *wol* or *gny* function by inducing mutant clones in the female germ line results in larvae with patterning and morphological defects, the most prominent one being the failure to form a normal head skeleton (Figure 3A-D, (Haecker *et al.*, 2008)). Due to the morphological defects, the cuticle of these larvae is discontinuous and their body contours are irregular. At the edges of ruptures, the cuticle is often melanised. Specialised cuticle structures such as denticles are present. Lack of maternal and zygotic *wol* and *gny* function causes additional reduction of cuticle deposition and a complete failure of denticle formation and melanisation (Figure 3G-I). These larvae resemble those homozygous mutant for *mummy* (*mmy*), which is coding for the last enzyme of the UDP-GlcNAc producing pathway (Figure 3F) that among others supplies N-glycosylation (Moussian, 2008). Of note, the phenotypes of *wol* mutant animals harbouring a transposon insertion (wol^{P}), which is probably a null-mutation (Table1) or an EMS induced point mutation (wol^{1} or wol^{2}) are identical. Overall, the

phenotypic analysis of these mutant larvae underlines that Wol and Gny contribute to cuticle differentiation.

The gene products of *wol* and *gny* are reported to act in a linear pathway to add glucose residues to the oligosaccharide. Indeed, the phenotypes of probably null *wol* and *gny* mutant animals (Table 1) are indistinguishable (Figure 2 and 3). To test whether Wol and Gny may nevertheless have other independent functions as well, we generated embryos double mutant for *wol* and *gny*. These embryos fully develop into larvae that hatch and die at the second instar (Figure 2D). Embryos derived from double mutant germline clones show the same phenotype as embryos produced from either single mutant germline clone (Figure 3E and J). Thus, since the defects in *wol* and *gny* mutant embryos are not additive, we assume that they function in a common pathway and do not exert other functions. Moreover, the failure of the *gny* mutation to deteriorate the phenotype of *wol* mutant embryos indicates that mutations in *wol* indeed completely eliminate Wol activity.

wol is needed to assemble the layered cuticle

For a detailed assessment of the role of *wol* in cuticle differentiation, we have analysed the ultrastructure of the cuticle of *wol* mutant larvae suffering reduction or absence of Wol activity (Figure 4). We focus our following analyses on the ultrastructure of the cuticle of *wol* mutant larvae, as the cuticles of *wol* and *gny* mutant larvae are identical. Compared to the wild-type cuticle, the cuticle of larvae lacking zygotic *wol* function only is less compact, as fissures loosen the procuticle without, however, grossly disrupting chitin organisation (Figure 4A and B). In addition to this defect, the procuticle of larvae lacking maternal *wol* function

harbours electron-dense material, probably coagulated proteins (Figure 4C). Elimination of maternal and zygotic *wol* function results in loss of chitin orientation in the procuticle, and dislocation of proteins of the lower electron-dense portion of the epicuticle into the upper electron-lucid sub-layer (Figure 4D). In the most severe cases, *wol* maternal and zygotic mutant larvae have an expanded and spongy extracellular matrix beneath the envelope. Traces of electron-dense material in the extracellular space suggest that some proteins are being secreted but mislocalise. As shown by chitin detection with gold-conjugated WGA, this rudimentary cuticle contains chitin (data not shown). Taken together, construction of the cuticle, in particular chitin orientation depends on the dosage of Wol.

Wol is required for shaping of the plasma membrane of epithelia

During cuticle differentiation, the apical plasma membrane of epidermal cells actively participates in organising the cuticle (Moussian *et al.*, 2006, Moussian *et al.*, 2007). We therefore explored whether the disorganised cuticle in *wol* mutant embryos may be associated with malfunctions of the apical plasma membrane. Occasionally, in larvae with partially (maternal) reduced Wol function, a normal layered cuticle is separated from the surface of the epidermis by an amorph chitinous matrix. The apical plasma membrane of these cells protrudes ectopic structures into the extracellular space, whereas the wild-type apical plasma membrane is smooth at this stage (Figure 5A-C). We also assessed whether elimination or reduction of Wol activity has an impact on the so-called apical undulae, which are longitudinal corrugations of the apical plasma membrane that occur during chitin deposition (Moussian *et al.*, 2006) (Figure 5D-F). As in the wild-type embryo at early stage 17,

the apical plasma membrane of *wol* maternal mutant embryos elaborate the regular arrangement of apical undulae. By contrast, the apical undulae of *wol* maternal and zygotic mutant embryos appear less regular. Hence, the establishment of repetitive protrusions of the epidermal apical plasma membrane during cuticle differentiation implies full Wol function as does flattening of the apical plasma membrane when cuticle differentiation ceases.

To answer the question whether *wol* is generally needed for correct epithelial apical plasma membrane topology, we inspected the shape of the apical plasma membrane of epithelia of other organs such as the midgut and the hindgut (Figure 5G-L). The apical plasma membrane of the midgut of wild-type larvae mounts a dense array of microvilli (Figure 5G). The arrangement of microvilli seems to be normal in *wol* maternal mutant larvae (Figure 5H). By contrast, in *wol* maternal and zygotic mutant larvae, the midgut microvilli are separated from each other by large gaps (Figure 5I). In the same larvae the apical plasma membrane of the hindgut is highly irregular compared to the smooth one of the wild-type and *wol* maternal mutant hindgut cells (Figure 5J-L). Taken together, dynamics of plasma membrane topology in many epithelia involves Wol function.

Wol is needed for organising the lateral plasma membrane during cuticle differentiation

Concomitant with the formation of the apical cuticle, the lateral plasma membrane of epidermal cells extends, undulates and gets decorated by junctions including the ladder-like septate junctions (SJ) to allow cell communication and to strengthen cell-cell contacts (Figure 6A). By contrast, the *wol* maternal and zygotic mutant epidermal cell fails to extend its lateral surface and the arrangement of the SJs is disordered

(Figure 6B). This phenotype is less severe than displayed by larvae mutant for SJ factors such as *coracle* (*cora*) and *gliotactin* (*gli*) where SJ are missing (Genova and Fehon, 2003).

For the examination of the molecular constitution of junctions in *wol* mutant embryos, we performed immunohistochemical experiments using antibodies directed against the membrane-inserted factors Crumbs (Crb), an apico-lateral determinant of cell polarity, and Fasciclin 3 (Fas3) that is associated with the more baso-lateral SJ (Assemat *et al.*, 2008, Bryant, 1997). Fas3 probably harbours three N-glycosylation sites and is localised to the entire lateral plasma membrane being more abundant at the apical half than at the basal half of it (Figure 6C) (Nilton *et al.*). In *wol* maternal mutant embryos Fas3 localisation is normal (Figure 6D). By contrast, in *wol* maternal and zygotic mutant embryos, it hardly shows a bipartite distribution (Figure 6E). Localisation of Crb that has ten predicted N-glycosylation sites is normal in *wol* maternal and zygotic mutant embryos (Figure 7A-B). In spite of this, Crb amounts are elevated in *wol* mutant embryos. To investigate whether besides Crb other determinants of the apical membrane show a similar *wol*-dependent behaviour, we examined the localisation and amounts of aPKC, a cytosolic kinase (Kim *et al.*, 2009, Wodarz *et al.*, 2000). Like Crb, in *wol* mutant embryos, aPKC accumulation at the apical plasma membrane is more pronounced that in wild-type embryos (Figure 7D-F). In summary, our data suggest that the proper organisation of the lateral and apical plasma membrane involves full Wol function.

Glycosylated proteins reach the plasma membrane despite lack of full Wol activity

Reduction of Fas3 in the lateral plasma membrane argues that glucosylation is controlling the amount of proteins delivered to the plasma membrane or extracellular space. To test to what extend its activity is needed for correct trafficking of N-glycosylated proteins from the ER to the plasma membrane of epidermal cells, we monitored the behaviour of secreted proteins with dye-conjugated lectins or agglutinins binding to sugar residues on glycans in wild-type and *wol* mutant embryos. Rhodamine-conjugated *Pisum sativum* agglutinin (PSA) that recognises α-1,6-core-fucosylated mannose residues (Kornfeld *et al.*, 1981, Luhn *et al.*, 2004) detects some N-glycans mainly at the epidermal apical plasma membrane (Figure 7A'). In *wol* maternal and maternal zygotic mutant embryos localisation of PSA positive signal is gradually reduced (Figure 7B',C'). Likewise, staining with rhodamine-conjugated soybean agglutinin (SBA) that binds to galactose, which is a typical residue of O-glycosylated proteins but a rather minor constituent of N-glycans (D'Amico and Jacobs, 1995, Tian and Ten Hagen, 2007), is diminished in *wol* mutant embryos (Figure 7D'-F'). Despite reduction of PSA and SBA signal intensity in *wol* mutant embryos, the distribution of the recognised epitopes is rather normal. We have also followed the behaviour of GlcNAc containing glycans by staining embryos with rhodamine-conjugated wheat germ agglutinin (WGA, (D'Amico and Jacobs, 1995, Tian and Ten Hagen, 2007)) (Figure 7G-I). WGA staining is almost normal in embryos with abolished maternal Wol contribution, but is clearly weakened in embryos with no Wol function. Since WGA recognises also the GlcNAc polymer chitin (Moussian *et al.*, 2006), it is unclear whether glycan or chitin amounts are lower

in *wol* maternal and zygotic embryos compared to wild-type animals. Using rhodamine-conjugated Concanavalin A (ConA) that recognises glucose and mannose residues (D'Amico and Jacobs, 1995, Tian and Ten Hagen, 2007), we are able to distinguish a minor difference between signal strength in wild-type and *wol* mutant embryos (Figure 7J-L), but it seems that in embryos lacking maternal and zygotic Wol function the predominantly apical distribution of ConA epitopes is uneven. Consistently, localisation of Knk, a GPI-anchored and N-glycosylated protein that is involved in chitin microfibril orientation (Moussian *et al.*, 2006), is independent from Wol function (Figure 7J'-L').

The quantification by immunohistochemistry revealed only a minor effect of *wol* mutations on the amounts of ConA-binding glycans and Knk in the epidermis of stage 16 embryos (Figure 7J-L'). To find out whether quantity changes in ConA-binding glycans and Knk may be more dramatic when epidermal cells are engaged in massive cuticle production, we analysed the behaviour of these factors in stage 17 *wol* mutant embryos by Western blot experiments using unconjugated ConA or a Knk-specific antiserum (Figure 8). Wild-type ConA-positive proteins show a characteristic migration behaviour that is unchanged in *wol* deficient larvae (Figure 8A). The amounts of ConA-positive proteins are however decreased in the later animals. Mobility of Knk from wild-type and *wol* mutant late embryonic protein extracts is identical in Western blot experiments (Figure 8B). Likewise, Knk mobility is unchanged in *wol* zygotic mutant larvae (data not shown). By contrast, the Knk signal from *wol* mutant embryos is weaker. Hence, Knk protein levels depend on Wol activity.

To obtain an alternative to the immunohistochemistry and Western blot results presented in figures 7 and 8, we measured the proportion of glycans in total protein *wol* deficient embryos by mass spectrometry (Table 2). On average, in *wol* mutant embryos glycosylation is reduced by about 25%. In agreement with this finding, we detect significant lower levels for some transcripts coding for factors acting in the early pathway of dolichol-anchored oligosaccharide synthesis (Table 3). Taking all these results together, we conclude that Wol function contributes to normal levels of N-and O-glycans.

The unfolded protein response in wol mutant larvae does not affect transcript levels of membrane factors

Reduction of Knk amounts may result from proteasome-mediated degradation of the protein caused by the unfolded protein response pathway that occurs in *wol* mutant embryos (Supplementary figure 1 and (Haecker *et al.*, 2008)). The unfolded protein response has also been reported to induce decay of mRNA associated with the ER membrane coding for proteins to be loaded into the ER (Hollien and Weissman, 2006). To answer the question whether *wol*-dependent induction of unfolded protein response may cause mRNAs of membrane-associated cuticle factors like *knk* and *CS-1/kkv* coding for the chitin synthase to be degraded, we performed quantitative PCR experiments to monitor their mRNA levels in *wol* maternal and zygotic mutant stage 17 embryos (Table 3). Compared to wild-type, the transcript levels of *knk* and *CS-1/kkv* are not affected considerably.

Mutations in *wol* reduce the expression of *caudal* mRNA that codes for a homeobox containing transcription factor (Haecker *et al.*, 2008). To investigate whether the

expression of transcription factors controlling cuticle differentiation such as *grainyhead* (*grh*) and *Creba* also depends on Wol regulated unfolded protein response, we determined the expression levels of their transcripts in *wol* maternal and zygotic mutant stage 17 embryos (Table 3). The amounts of *grh* and *Creba* transcripts are lowered in these embryos. Taken together, Wol activity has an influence on *grh* and *Creba* transcript levels. In conclusion, through downscaling of mRNA of especially transcription factors, the suppressed activities of a plethora of differentiation factors may contribute to *wol*-dependent defects observed in epidermal cells involved in cuticle production.

Wol acts at the ER and affects ER morphology

Wol is a key enzyme for glycosylation taking place in the ER. Consistently, we find that the V5-tagged Wol protein is associated with ER in *Drosophila* S2 cells, whereas it does not co-localise with the Golgi-marker GM130 (Figure 9A and B). Perturbation of ER function e.g. by mutations in the gene coding for Sec23, Sec24 or Sar1 result in aberrant ER morphology (Norum *et al.*, 2010, Tsarouhas *et al.*, 2007). To inspect whether *wol* mutations have an influence on ER morphology, we examined the ultrastructure of the ER in stage 16 embryos, which have initiated cuticle production. The early stage 16 ER tubules in wild-type epidermal cells are slightly cystic (Figure 9C and D). In the respective embryos suffering loss of maternal and zygotic Wol activity, by contrast, ER tubules are rather smooth. In conclusion, these data argue that Wol acts at the ER and that Wol activity contributes to the ER shape.

Wol is required for ER output

To gain detailed information on the importance of Wol for N-glycosylation, we determined the ratio of the mature oligosaccharide $Glc_3Man_9GlcNAc_2$ and its intermediates in *wol* mutant and wild-type embryos by mass spectrometry (Figure 10). In *wol* mutant embryos, N-glycans contain markedly less glucose units than in wild-type embryos, especially the second and third Glc residues are almost undetectable in *wol* mutant embryos. Concurrently, levels of the intermediates $Man_3GlcNAc_2$ and $Man_5GlcNAc_2$ are slightly increased in these embryos. The significance of this observation is not obvious.

To verify whether N-glycans arrive at the Golgi apparatus and are correctly processed, we tested in our mass spectrometry experiments for the presence of oligosaccharide modifications like e.g. fucosylation that occur in the Golgi apparatus (Table 4). The addition of fucoses to the oligosaccharide is normal in *wol* mutant embryos. This finding suggests that hypoglucosylated proteins are correctly passed through the secretory pathway.

Taken together, Wol activity is needed for full glucosylation of proteins, underlining that it is indeed the *Drosophila* Alg5 ortholog.

Discussion

An early role of N-glycosylation in the ER is to initiate the folding reaction of proteins for correct localisation and function. Recognition of the yet unfolded protein depends on a single glucose residue at the N-glycan. The importance of glucosylation of N-glycans has mainly been investigated in yeasts and cultured cells.

To understand the role of the N-glycan glucose in a multicellular organism, we have studied the role of *Drosophila* Wol (DAlg5) and Gny (DAlg6) that regulate early steps of glucosylation at the ER membrane.

Glucosylation promotes cuticle differentiation

Mutations in *wol* in *Drosophila* disrupt the architecture of the larval cuticle, which is an apical ECM produced by the epidermal cells. Stepwise reduction of Wol activity results in a gradual worsening of cuticle defects ranging from small lesions in the procuticle of zygotic mutant larvae, over mislocalised cuticle proteins in maternally mutant but zygotic wild-type larvae to chitin disorganisation and protein depletion upon additional removal of zygotic enzyme activity. These observations signify that the amount of Wol activity provided by zygotic expression alone is not sufficient but nevertheless necessary to support embryogenesis, whereas maternal supply of Wol only is despite some tolerable errors adequate for development until after first moulting of the larva. Hence, the rate of glucosylation is critical for embryogenesis and development in general and cuticle differentiation in particular.

One may postulate that hypoglucosylation yields fewer biochemically active extracellular and membrane-bound proteins, a situation that may retard especially secretion-related aspects of differentiation. However, perturbed plasma membrane and cuticle organisation cannot be satisfactorily explained by slower development. This is a similar scenario encountered when chitin synthesis is decreased but not abrogated upon application of insecticides (Gangishetti *et al.*, 2009). In brief, the epidermal system is unable to cope with biochemical attenuation of chitin synthesis implying that the activities of the cooperating factors are tightly adjusted and

coordinated. Full interpretation of the *wol* phenotypes, thus, probably also calls for the assumption of problems in coordination between effectors. Our molecular data discussed in the following deal with these genetic arguments.

ER output relies on Wol function

N-glycans are hypoglucosylated in *wol* mutant embryos that concomitantly suffer reduced glycosylation. One can envisage two explanations for these observations. A simple one is that the glucose residues of the oligosaccharide have an influence on the rate of glycosylation. In yeast, indeed, glucose residues on the oligosaccharide have been demonstrated to enhance the activity of the oligosaccharide transferase (Burda *et al.*, 1998, Karaoglu *et al.*, 2001). Problems with glucosylation possibly lower the output of the ER that exhibits a depleted morphology in epidermal cells at the onset of cuticle deposition in *wol* mutant embryos. In addition, elimination of Wol function induces lessening of transcripts coding for enzymes involved in dolichol-anchored oligosaccharide synthesis, an effect that contributes to the attenuated ER flow-through. The activity of the ER luminal UDP-glucose transferase (UGT) adding a glucose to the unglucosylated N-glycan (see below) and the supply with auxiliary chaperones incited by the unfolded protein response apparently do not suffice to normalise ER function. At the contrary, unfolded protein response is probably also responsible for reduced N-glycans and perturbed differentiation. Indeed, the consequences of the unfolded protein response - suppression of transcription and translation (Malhotra and Kaufman, 2007, Ruddock and Molinari, 2006) - constitute the second explanation for the defects (including O-glycan decimation) caused by *wol* mutations.

Despite the initial problems at the gate of secretion, reduced glycosylation and hypoglucosylation seem not to interfere with modifications of proteins in the Golgi apparatus and correct localisation of N-glycosylated proteins to the plasma membrane or the extracellular space. Consequently and in agreement with our genetic argument stated above, the *wol* mutant phenotype could be described as the sum of reduction of membrane and extracellular protein activities through hypoglucosylation and the unfolded protein response. This argument implies that passage through the secretory route *per se* does not depend on glucosylation. Consistently, in *Saccharomyces cerevisiae* cells, deletion of either *alg5* or *alg6* does not result in growth defects indicating that secretion is normal despite hypoglucosylation (Heesen *et al.*, 1994, Reiss *et al.*, 1996).

Wol integrates the activity of differentiation factors

A particular feature of *wol* mutant defects is the lowering of Knk amounts and the accumulation of Crb in the apical plasma membrane. Decrease of Knk may in part be responsible for loss of chitin orientation and procuticle organisation in *wol* mutant larvae (Moussian *et al.*, 2006), and higher Crb (and aPKC) levels may account for the deformation of their epidermal apical plasma membrane (Laprise *et al.*, 2006, Wodarz *et al.*, 1995). Hence, if we generalise our findings, one may argue that Wol activity contributes to equilibrate the activity of factors that direct epidermal differentiation. In other words, glucosylation is critical for balanced and robust differentiation, either directly as a step of glycosylation or indirectly by preventing the unfolded protein response.

Attenuated translation and protein degradation may conceivably explain the consequences of *wol* mutations on Knk, but not on Crb. The behaviour of Crb in *wol* mutant embryos rather exemplifies that some proteins may gain function out of their normal context through the deleterious effects of *wol* deficiency. In the case of Crb, this may indirectly follow from depletion of a factor participating at the complex mechanisms of Crb positioning in the apical plasma membrane. In line with this view, absence of the recycling endosome syntaxin Avalanche causes accumulation of Crb in the apical plasma membrane of various epithelial cells in *Drosophila* (Lu and Bilder, 2005). Alternatively, deviation of normal apical plasma membrane dynamics may start with aPKC that has been shown to support Crb localisation by phosphorylation (Sotillos *et al.*, 2004). In this scenario, the *wol*-induced unfolded protein response may by an unknown mechanism lead to the concentration of aPKC at the apical plasma membrane and thereby stabilise Crb localisation. In any case, the differential response of proteins to glycosylation (including Wol function) may reflect a glycosylation-dependent mechanism of adjusting the stoichiometry of factors that assemble the cuticle in a certain time window during differentiation. Elaborate genetic tools comprising *wol* will be required to test this hypothesis.

Drosophila and CDG

Mutations have been identified in the human *alg6*, but not in the human *alg5* gene (Freeze, 2006, Jaeken and Matthijs, 2001, Jaeken and Matthijs, 2007). Patients carrying *alg6* mutations suffer a plethora of developmental and physiological defects, but survive at least a few years. Hence, these mutations either do not eliminate enzyme activity completely, or like in *Drosophila* the putative maternal product is sufficient to

support development. In cultured fibroblasts of *alg6* mutant patients, the amounts of the non-glucosylated dolichol-linked oligosaccharide $Man_9GlcNAc_2$ are increased at the expense of the fully glucosylated $Glc_3Man_9GlcNAc_2$. By consequence, serum transferrin is hypoglycosylated and therefore probably inactive in these patients. In *Drosophila*, by contrast, as demonstrated for Knk, extracellular and membrane-associated proteins are probably not hypoglycosylated. This difference may point to a species-specific substrate affinity of the OST that transfers the oligosaccharide to the protein in the ER lumen. Even though the presence of glucose has a greater importance for glycosylation in humans, the trace amounts of dolichol-linked oligosaccharide argue that reduced Alg6 function may lead to a dampened and disordered secretion as it occurs in *Drosophila* as well. Thus, the use of *Drosophila* as a model system may help to elucidate the molecular basis of human CDG.

Materials & Methods

Flies and genetics

The mutations used in this work are listed in table 1. The *Drosophila* Alg6 ortholog (CG5091) was identified on the right arm of the second chromosome by a BLAST search using the yeast and human Alg6 protein sequences (Supplementary figure 2). A P-element inserted into the third exon of the gene is available at the Bloomington stock centre. Following *Drosophila* practice to baptise mutations, this mutation was called *garnysstan* (*gny*), Swedish for wool ball, which means Wollknäuel in German. The *gny* P-element was recombined to the FRT site on the cytologic position 42D for generation of germ-line clones (see below).

For collection of homozygous mutant embryos and larvae, fly stocks segregating the respective mutation balanced over *CyO*, *Kr:Gal4*, *UAS:GFP* were kept in cages on apple juice agar plates spotted with fresh yeast paste. To obtain embryos lacking maternal contribution of a respective gene, germline clones were generated using the Flp/FRT technique developed and described by Chou, Noll and Perrimon (1993). For subsequent experiments, embryos were staged after Hartenstein and Campos-Ortega (1985).

Quantitative PCR

For quantitative PCR (qPCR), cDNA was synthesised with the Superscript II RT kit (Invitrogen) from total RNA extracted from 60 embryos with the RNeasy-Microkit (Qiagen). The quantitative PCR was performed on an Opticon Continuous Fluorescence Detector (MJresearch) applying the iQ™ SYBR (Bio-Rad) technique. Two independent RNA extractions for each genotype were assayed by qPCR three (CrebA) or six (*knk*, *kkv* and *grh*) times in parallel. For the determination of the crossing point differences (δCP value) of individual transcripts in mutant (sample) and wild-type (control) embryos the REST® software was used (Pfaffl *et al.*, 2002). The δCP value of histone H4 expression was used as a reference to calculate the efficiency (E) corrected relative expression ratio of a putative target gene:

$$\text{Ratio} = E(\text{target})^{\partial CP \text{ target (control – sample)}} / E(\text{reference})^{\partial CP \text{ reference (control - sample)}}$$

Expression changes up to two-fold were considered as no significant as two-fold change in gene expression may be observed also when different wild-type samples are compared (data not shown).

Cell culture

S2 cells were transfected with pMT-*wol-V5* using the Fugene method (Roche). The day before transfection, $0.5x10^6$ cells/ml were seeded in 6 well plate. On the day of transfection, cells were incubated with serum free medium. For optimizing the transfection efficiency, transfection was done using two different ratios 8:2 and 12:2 of reagent (µl) to plasmid construct (µg). 100 µl of DNA solution was prepared by dissolving plasmid DNA in sterile water. Fugene HD was added to the DNA drop wise without touching the walls of the reaction tube. The Fugene-DNA solution was vigorously mixed and incubated for 15 minutes at room temperature. For transfection, the solution was added drop wise to the cells. After 8 hours, expression was induced in complete medium containing 500 µM $CuSO_4$. 72 hours after transfection, S2 cells were plated on 0.01% poly-lysine coated cover slips for 1 hour and washed with 1x phosphate buffered saline (PBS). Cells were then fixed with 4% formaldehyde for 30 minutes at 37°C followed by washes with PBS. Permeabilisation was done with 0.2% Triton-X100 in PBS for 10 minutes. After washes with PBS, the cells were blocked using 3% normal goat serum (NGS) for 1 hour at room temperature. Mouse anti-V5 tag antibody (Abcam) was diluted to 1:100, Rabbit anti-calnexin (Abcam) was diluted to 1:200 and Rabbit anti-GM130 (Abcam) was diluted to 1µg/ml in PBS containing 1% NGS and 0.01% Triton-X100 (NGS-PBST) and incubated for 2 hours at room temperature. Cells were washed with PBS. Anti-mouse Cy3 and anti-rabbit Alexa 633 secondary antibodies (Molecular Probes) were diluted to 1:500 in 1% NGS-PBST. Cells were incubated in secondary antibody for 45 minutes and washed with PBS. Cover slips containing cells were mounted on slides using

mounting medium (AquaMount®, Polyscience) and left for one hour for solidification at room temperature. The slides were observed by confocal microscopy (see below).

Microscopy

For light microscopy using Nomarski optics, mature embryos or larvae were freed from the egg case by removing first the chorion in 50% Klorix® and second the vitellin membrane by vigorously shaking the eggs in a 1:1 biphasic mixture of methanol and heptane. The mature embryos or larvae were transferred in methanol onto a slide, mounted in Hoyer's medium and incubated over night at 65°C.
For immunohistochemical experiments, dechorionated embryos were fixed at the interface of heptane and 3,7% formaldehyde diluted in Hepes buffer (pH 7.0) for 20 minutes at room temperature. The fixative was removed and replaced by methanol, and embryos were divitellinsed by vigorous shaking. They were washed five times with methanol and stored at 4°C. The following day the embryos were rehydrated gradually with PBS supplemented with 0,2% Triton X-100 (PBST). Before incubation with antibodies, embryos were blocked in 2% bovine serum albumin in PBST (BSA-PBST) for 30 minutes at room temperature. Primary antibodies were applied at respective concentrations over night at 4°C in BSA-PBST. The following day, embryos were washed five times 15 minutes with PBST, blocked with BSA-PBST and incubated with the secondary antibody or a dye-coupled lectin or agglutinin (ConA, SBA, WGA and PSA, VectorLab) diluted in BSA-PBST for three hours at 4°C. Embryos were then washed five times with PBST and deposited on a slide. PBST was almost completely removed, and embryos were mixed with mounting medium

(AquaMount®, Polyscience). Laser scanning confocal microscopy of immunodetected epitopes was performed on an Olympus confocal microscope. Signal quantification was performed with the "measurement log" device of Adobe Photoshop® CS4. For transmission electron microscopy, specimens were prepared and analysed according to the method described previously (Moussian *et al.*, 2006).

Mass spectrometry

Protein enrichment, purification and N-glycan release from glycoproteins was done using a "mini-prep SDS page" protocol (Rendic *et al.*, 2007) described in a recent work (Pabst *et al.*, 2007). Released glycans were borohydride reduced and further analyzed using Porous Graphitized Carbon Chromatography online coupled to electrospray ionization mass spectrometry (PGCC-ESI-MS) (Pabst *et al.*, 2007, Stadlmann *et al.*, 2008). Glycans were detected using an ESI Q-TOF MS from Waters (Micromass, Q-TOF Ultima Global). Data analysis was performed using MassLynx 4.0 SP4 Software, where the total ion counts of the respective glycan structures were used for quantification. Descriptive statistics and statistical significances of LC-MS data were evaluated using SPSS (Statistical Package for the Social Sciences, IBM). One-way ANOVA was performed to evaluate statistical significance. Changes were considered significant, if their corresponding p values were below $p<0.05$ ($n=3$).

Western blot experiments

Dechorionated stage 17 embryos were homogenized in PLC buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 0.1% Triton-X100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM NaP_2O_7, 100 mM NaF, 1 mM Na_3VO_4) containing protease inhibitors for protein extraction. Protein amount was estimated by photometry at

280nm (Nanodrop, Peqlab, Germany). From each extract, about 20µg protein was separated by SDS-PAGE (7.5% polyacrylamide). After gel electrophoresis, proteins were transferred to a nitrocellulose membrane (Whatman) by the semi-dry method. The transferred membrane was blocked with Odyssey blocking solution for 30 minutes. For Concanavalin A (ConA) lectin detection, the blot was incubated with unconjugated ConA lectin (Vector laboratories) in 10µg/ml in Odyssey blocking buffer for 30 minutes. The membrane was washed three times with PBS/0.1% Tween-20 for 5 minutes. Goat anti-ConA antibody (Vector laboratories) was used at 5µg/ml dilution in Odyssey buffer with 0.1% Tween-20 and incubated overnight at 4°C. The membrane was washed three times with PBS/0.1% Tween-20 for 5 minutes. Then the blot was incubated with the secondary anti-Goat 680 antibody (LICOR Biosciences) at a dilution of 1:7500 for 1 hour at room temperature. Again, the membrane was washed three times in PBS/0.1% Tween-20 for 5 minutes. Afterwards the blot was scanned using the Odyssey infrared imaging software (LICOR Biosciences). For tubulin detection, after blocking the membrane in Odyssey buffer for 30 min, incubated in mouse α-Tubulin antibody (Sigma-Aldrich) at a 1:5000 dilution overnight at 4°C. The membrane was washed three times with PBS/0.1% Tween-20 for 5 minutes. Secondary anti-mouse 800CW antibody was used (LICOR Biosciences) at a 1:7500 dilution for 1 hour at room temperature. After three times washing with the PBS/0.1% Tween-20, detection was scanned with Odyssey IR imaging software. Western blots using Knk antiserum were performed as previously described (Moussian *et al.*, 2006). Signals were quantified with the "measurement log" device of Adobe Photoshop® CS4.

Acknowledgments

The authors are grateful to the technical assistance of Ursula Müller, Brigitte Sailer and Anna Speidel. This work was supported by the German Research Foundation (DFG, MO 1714/1-2 and 2-1).

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Legends to figures

Figure 1.

Addition of glucose residues to the dolichol-linked oligosaccharide that will be transferred to secreted or membrane-associated proteins requires the three glycosyltransferases Alg5 (Wol), Alg6 (Gny) and Alg8. Mutations in the human *alg6* and *alg8* gene cause the congenital disorder of glycosylation type-Ic (CDG-Ic) and CDG-Ih, respectively (Freeze, 2006, Jaeken and Matthijs, 2001, Jaeken and Matthijs, 2007).

Figure 2.

Mutations in genes involved in N-glycan glucosylation do not affect the larval body shape.

Light microscopy using Nomarski optics of wild-type and homozygous *wol* and *gny* (*alg6*) zygotic mutant larvae. The wild-type larva is covered by the cuticle that supports its spindle-like body shape (A). The body shape of larvae carrying mutations in *wol* (wol^2, B), *alg6* ($alg6^P$, C) or both genes at the same time (wol^2, $alg6^P$, D) is normal. Likewise, the texture of the head skeleton is normal in *wol*, *alg6* and wol^2, $alg6^P$ mutant larvae (insets in A-D).

Figure 3.

Abrogation of N-glycan glucosylation causes loss of cuticle integrity.

Light microscopy using Nomarski optics of wild-type and *wol*, *gny* (*alg6*) and *mmy* mutant larvae within the egg case (marked by a star in each individual figure). The wild-type larva possesses a cuticle coating the epidermis (arrowhead) and building

up the head skeleton (bracket, A). Larvae lacking maternal *wol* or *gny* (B,B'-D,D') have pronounced morphological defects as for example they fail to close the dorsal hole (black arrow in B') and have melanised (m) deformations instead of a head skeleton, while their cuticle and the cuticular denticles (white arrows) are well discernable (B'-D'). By contrast, larvae maternally and zygotically mutant for *wol* do not produce a proper cuticle (D and F). Larvae maternally double mutant for *wol* and *gny* resemble either respective single mutant larva (E,E'). The cuticle including the head skeleton of strong *mmy* mutant larvae mmy^{IK63} is only poorly formed (F). Larvae maternally and zygotically mutant for *wol* or *gny* or double mutant for *wol* and *gny* (G-J) remind of the mmy^{IK63} mutant phenotype.

Anterior is to the left, dorsal faces up. Wild-type and maternal mutant larvae are shown in two focal planes: left images (A-E) focus on the head and middle images focus on the surface of the larva (A'-E'). The egg case is about half a millimetre long.

Figure 4.

The architecture of the cuticle is sensitive to the dose of Wol.

Ultrastructure of the cuticle of wild-type and *wol* mutant larvae by electron microscopy. The wild-type cuticle is composed of three biochemically distinct layers: the outermost envelope (env), the middle epicuticle (epi) and the innermost procuticle (pro, A). Layering of the cuticle of larvae homozygous mutant for *wol* is normal (B). However, procuticle homogeneity is occasionally disrupted (black arrows). The cuticle of larvae lacking maternal function of *wol* additionally contains electron-dense inclusions within the procuticle (C). Upon removal of maternal as

well as zygotic Wol function, the electron-dense lower sublayer of the epicuticle contacts the envelope (white arrow) at the expense of the electron-lucid sublayer (D). Scale bar in (A) is 500 nm and also applies to (B-D).

Figure ***5****.*

Dynamics of apical plasma membrane topology depends on Wol activity.

The apical plasma membrane (apm) of larval epidermal cells is flat when the cuticle (bracket) is fully differentiated (A). By contrast, the surface of epidermal cells of larvae with reduced or eliminated Wol function protrudes into the extracellular space (bracket, B,C). During cuticle differentiation at stage 16, the apical plasma membrane of wild-type epidermal cells forms regular protrusions (D). These protrusions seem to be normal in embryos lacking maternal Wol function (E). In embryos suffering complete Wol function, they are rather irregular (F). The apical plasma membrane of wild-type larval midgut epithelial cells forms an array of tightly packed microvilli called the brush border (G). Maternal reduction of Wol function has no visible effect on the brush border of midgut epithelial cells (H). In *wol* deficient larvae, by contrast, the microvilli are less tightly packed (I). As in the epidermis, the apical plasma membrane of wild-type hindgut larval epithelial cells is flat (J). Compared to the wild-type hindgut cuticle (bracket), the hindgut cuticle of maternaly mutant *wol* larvae is expanded, while the apical plasma membrane is flat as well (K). Complete loss of Wol function results in dramatic protrusions of the apical hindgut epithelial cells that do not produce any cuticle (L).

Scale bars are 500 nm.

Figure 6.

Integrity of the epidermal lateral membrane depends on Wol activity. Ultrastructure of the lateral membrane of wild-type and *wol* mutant larvae by electron microscopy, and immunohistochemistry with the lateral membrane marker Fas3 in wild-type and *wol* mutant embryos. The lateral membrane of wild-type larval epidermal cells is curvy and composed of the apical adherens junctions (aj) and the more basal ladder-like septate junctions (sj, A and A′, which is a higher magnification of the framed region in A). In larvae lacking maternal and zygotic Wol activity, the adherens junctions appear to be less tight, and the array of septate junction steps at the rather straight lateral membrane is less regular than in wild-type larvae (B and B′, which is a higher magnification of the framed region in B). Fas3 (white signal) marks the lateral membrane of wild-type stage 16 embryos (C). The Fas3 signal at the apical half is stronger than at the basal half of the lateral membrane (bracket). In respective embryos lacking maternal Wol activity the distribution of Fas3 is normal (D). By contrast, in stage 16 embryos with probably complete loss of Wol activity the Fas3 signal is not split into two domains (E).
Scale bar in (A) is 500 nm and also applies to (B). Scale bar in (A′) is 250 nm and also applies to (B′). cut cuticle.

Figure 7.

Amounts of glycosylated proteins are perturbed in the epidermis of *wol* deficient embryos.

In wild-type stage 16 embryos, Crb localises to the apical plasma membrane of epidermal cells (A). Localisation of Crb is normal in *wol* maternal (B) and maternal

zygotic mutant embryos (C). However, compared to wild-type the amounts of Crb in the apical plasma membrane of *wol* mutants is elevated (maternal: 2,7 times, SD 20%, n=6, $p=4e^{-5}$; maternal-zygotic: 1,3 times, SD 5%, n=6, p=0,004). PSA detects mainly N-glycans in the apical plasma membrane of wild-type stage 16 embryos (A'). In respective *wol* maternal and maternal-zygotic mutant embryos the amounts of PSA signal is diminished (B',C') (maternal: 59% of wild-type signal, SD 13%, n=6, $p=2e^{-5}$; maternal-zygotic: 12% of wild-type signal, SD 11%, n=5, $p=2e^{-9}$). The atypical protein kinase C (aPKC) is detected close to or at the apical plasma membrane in the wild-type stage 16 embryo (D). In *wol* maternal and maternal-zygotic mutant embryos, the apical localisation of aPKC is enhanced (E,F) (maternal: 1,3 times, SD 12%, n=5, p=0,006; maternal-zygotic: 1,1 times, SD 6%, n=5, p=0,06). SBA recognises mainly O-glycans at the apical plasma membrane of wild-type stage 16 embryos (D'). In *wol* maternal and maternal-zygotic mutant embryos, SBA signal intensity is markedly weakened (E',F') (maternal: 52% of wild-type signal, SD 9%, n=5, $p=1e^{-7}$; maternal-zygotic: 28% of wild-type signal, SD 10%, n=5, $p=3{,}8e^{-10}$). WGA binds to GlcNAc residues in glycans at the apical plasma membrane of wild-type stage 16 embryos (G). In *wol* maternal mutant embryos, WGA detection seems to be only slightly changed (H) (84% of wild-type signal, SD 13%, n=6, p=0,02). By contrast, the amount of GlcNAc is halved in *wol* maternal-zygotic mutant embryos (I) (46% of wild-type signal, SD 15%, n=5, $p=5e^{-7}$). ConA marks the apical plasma membrane of wild-type stage 16 embryos (J). In *wol* maternal mutant embryos, ConA detection is somewhat reduced (K) (87% of wild-type signal, SD 13%, n=6, p=0,07). In *wol* maternal-zygotic mutant embryos, ConA staining is as in *wol* maternal mutant embryos (L) (87% of

wild-type signal, SD 11%, n=5, p=0,04). The cuticle organiser Knk localises to the apical plasma membrane of stage 16 wild-type embryos (J'). Some trace amounts are also found within the cell, probably representing the protein in the secretory pathway. The light green dots are background signals also present in *knk* mutant embryos (Moussian *et al.*, 2006). Detection of Knk is rather normal in *wol* maternal and maternal-zygotic mutant embryos (K',L') (maternal: 94% of wild-type signal, SD 10%, n=7, p=0,26; maternal-zygotic: 96% of wild-type signal, SD 10%, n=6, p=0,5).

Figure 8.

Western Blot analyses of ConA binding N-glycans and of the cuticle organising factor Knk.

Western blot experiments with Concanavalin A on protein extracts from wild-type, maternally (m) or maternally and zygotically (m&z) mutant *wol* mutant larvae (A). For normalisation, the amount of glycans was related to the amount of αTubulin (Tub). These experiments reveal that the total amount of mannose and glucose containing proteins is reduced in *wol* larvae with reduced or eliminated Wol function (standard deviation of three independent experiments: maternal 4%, maternal and zygotic 3%). Moreover, the pattern of protein migration is unchanged in *wol* mutant as compared to wild-type larvae.

Western blot experiments with a Knk antiserum on protein extracts from wild-type maternally (m) or maternally and zygotically (m+z) *wol* mutant larvae (B). Knk is inserted into the apical plasma membrane via a GPI-anchor and carries three N-glycans (Moussian *et al.*, 2006). Migration of Knk is identical in wild-type and *wol* mutant larvae, while Knk from EndoH treated wild-type protein extracts migrates

faster. In spite of this, Knk amounts are reduced in *wol* mutant larvae (standard deviation of three independent experiments 7% (mat) and 5% (m+z)). The ratios of ConA/Tub and Knk:Tub were calculated using values obtained by the histogram measurement option integrated in the Adobe Photoshop® CS4 software. Three independent Western blots (independent collection of larvae and independent blotting) were analysed, and the ratio was set to 1 for wild-type.

Figure 9.

Wol is associated with the ER and influences its capacity.

The V5-tagged Wol protein co-localises largely with the ER marker KDEL (red, A), but not with the Golgi marker GM130 (blue, B).

Ultrastructure of the epidermal ER of wild-type and *wol* mutant stage 16 embryos by electron microscopy. During cuticle production, the epidermal cell contains cystic ER tubules (C). In the absence of Wol activity, the ER fails to form cysts and consists rather of straight tubules (D). Scale bar in (C) is 500 nm and applies also to (D).

Figure 10.

Wol activity is crucial for glycan composition and amounts.

Mass spectrometry of PNGAse A released N-glycans in wild-type and *wol* deficient larvae (A). In this graph, only the oligomannosidic structures are shown ($Man_{3-9}GlcNAc_2$ glucose residue bearing $Man_9GlcNAc_2$ structures). Terminal Glc residues are almost eliminated in *wol* mutant larvae. Values are in % of total oligosaccharide content.

Tables

Table 1.

Mutations used in this work.

Genotype	Mutation	Reference
wol^{1}*: al dp wol*^{1} *b pr* FRT2A	R^{209}>W	(Haecker *et al.*, 2008)
wol^{2}*: al dp wol*^{2} *b pr* FRT2A	W^{316}>stop	(Haecker *et al.*, 2008)
wol^{P}*: PBac RB*^{e04276} FRT2A	insertion in exon 2	Flybase
gny: PBac WH^{f04215} FRT2A	insertion in exon 3	Flybase
mmy^{IK63} *cn bw*	G^{261}>V	(Tonning *et al.*, 2006)
Df(2L)BSC111	deletion of *wol* region	Flybase
Df(2L)BSC6	deletion of *mmy* region	Flybase

The mutations in *wol*^{1} and *wol*^{2} were induced on the FRT2A chromosome (Luschnig *et al.*, 2004). The piggybac insertions in *wol* and *gny* were recombined on the FRT2A chromosome by standard genetic methods. These insertions possibly cause truncated loss-of-function proteins. In *wol*, the insertion disrupts translation after 80 amino acids that lack almost the entire glycosyltransferase domain of 182 residues starting at position 69. In *gny*, the insertion truncates the protein by 20%, including the C-terminal sequences of the glycosyltransferase domain (residues 12-465).

Table 2.

Glycosylation is reduced in *wol* maternal and zygotic mutant embryos.

	wt	*wol*	*reduced in wol (%)*
protein *(µg per extract)*	1.63	1.50	8 %
glycan *(relative MS signal)*	100	75	25 %

The glycan ratio was determined by mass spectrometry and the protein ratio was evaluated using a micro BCA protein assay kit (Thermo Scientific).

Table 3.

Assessment of transcript levels of some extracellular, glycosylation and transcription factors in wild-type and *wol* mutant embryos by qPCR.

transcript	*fold change*	*p-value*
knk	-1,60 ±0,75%	0,01
CS-1/kkv	-1,54 ±1,27%	0,01
grh	-5,24 ±0,42%	0,01
CrebA	-5,00 ±0,79%	0,001
CG10166[1]	-4,03 ±0,58%	0,001
wol	-1,68 ±0,71%	0,001
gny[2]	-1,69 ±1,28%	0,03
CG4542[3]	-4.92 ±0,0,51%	0,001

A two-fold difference in gene expression is considered as no change as it is also observed when expression levels of different wild-type samples are compared (data not shown). By trend, transcript levels are lower in *wol* maternal and zygotic mutant than in wild-type larvae. Especially transcripts coding for transcription factors such as *grh* and *CrebA* are affected. Likewise, the expression of *CG4542* and *CG10166*, both encoding factors involved in the synthesis of the dolichol-anchored oligosaccharide, is down-regulated in *wol* maternal and zygotic mutant larvae.

[1] dolichol-phosphate mannosyltransferase, [2] *CG5091* (*Dalg6*), [3] *Dalg8* (see supplementary figure 2).

Table 4.

Modification of N-glycans in the Golgi apparatus is not affected in embryos with eliminated *wol* activity.

Composition	Oligosaccharide	*wild-type*	*wol mat&zyg*	
		% ± st.d.	% ± st.d.	*p-value*
H2N2F	MUF	12.03 ± 0.32	10.49 ± 0.13	< 0.01
H3N2F	Man3F	23.15 ± 0.23	22.72 ± 0.43	
H4N2F	Man4F	0.19 ± 0.11	0.11 ± 0.04	
H2N2F2	MUFF	0.38 ± 0.08	0.41 ± 0.12	
H3N2F2	Man3FF	1.01 ± 0.06	1.35 ± 0.26	0.14
H3N3	GnM	1.43 ± 0.0	1.43 ± 0.17	
H3N4	GnGn	0.03 ± 0.01	0.04 ± 0.02	
H5N4	AGn	0.07 ± 0.03	0.17 ± 0.07	0.12
H3N3F	GnMF	2.00 ± 0.02	1.78 ± 0.26	
H3N4F	GnGnF	0.03 ± 0.01	0.03 ± 0.02	

N-glycans in wild-type embryos (n = 3) and embryos with eliminated maternal and zygotic Wol activity (n = 6) were analyzed by PGCC-ESI-MS. Statistical significance for differences is given where p was below 0.2. N = N-acetylglucosamine, H = hexose (mannose or galactose), F = fucose. N-Glycan nomenclature is according to the Proglycan system (http://www.proglycan.com/).

Figure 1

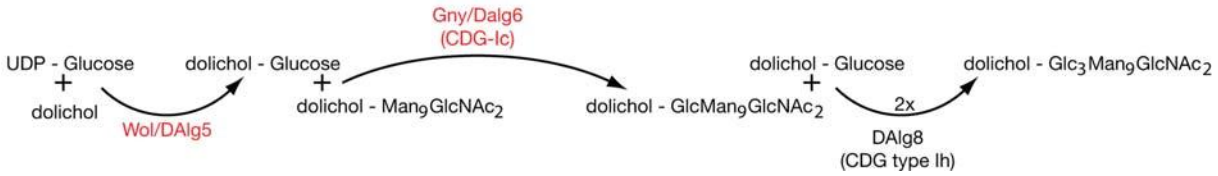


Figure 2

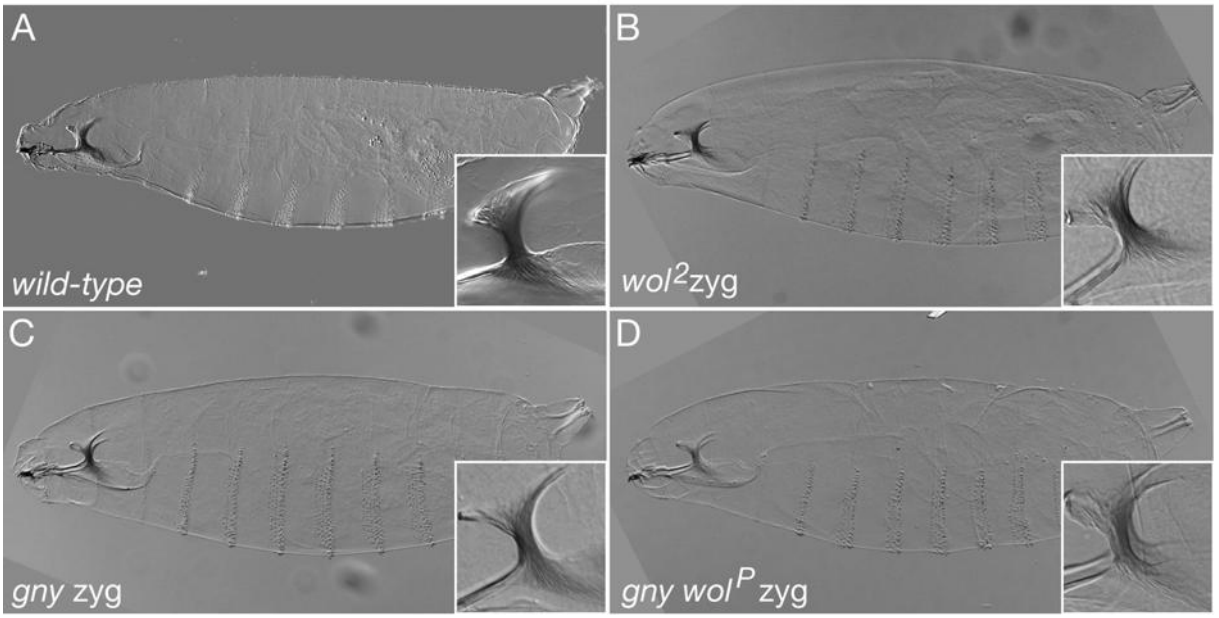


Figure 3

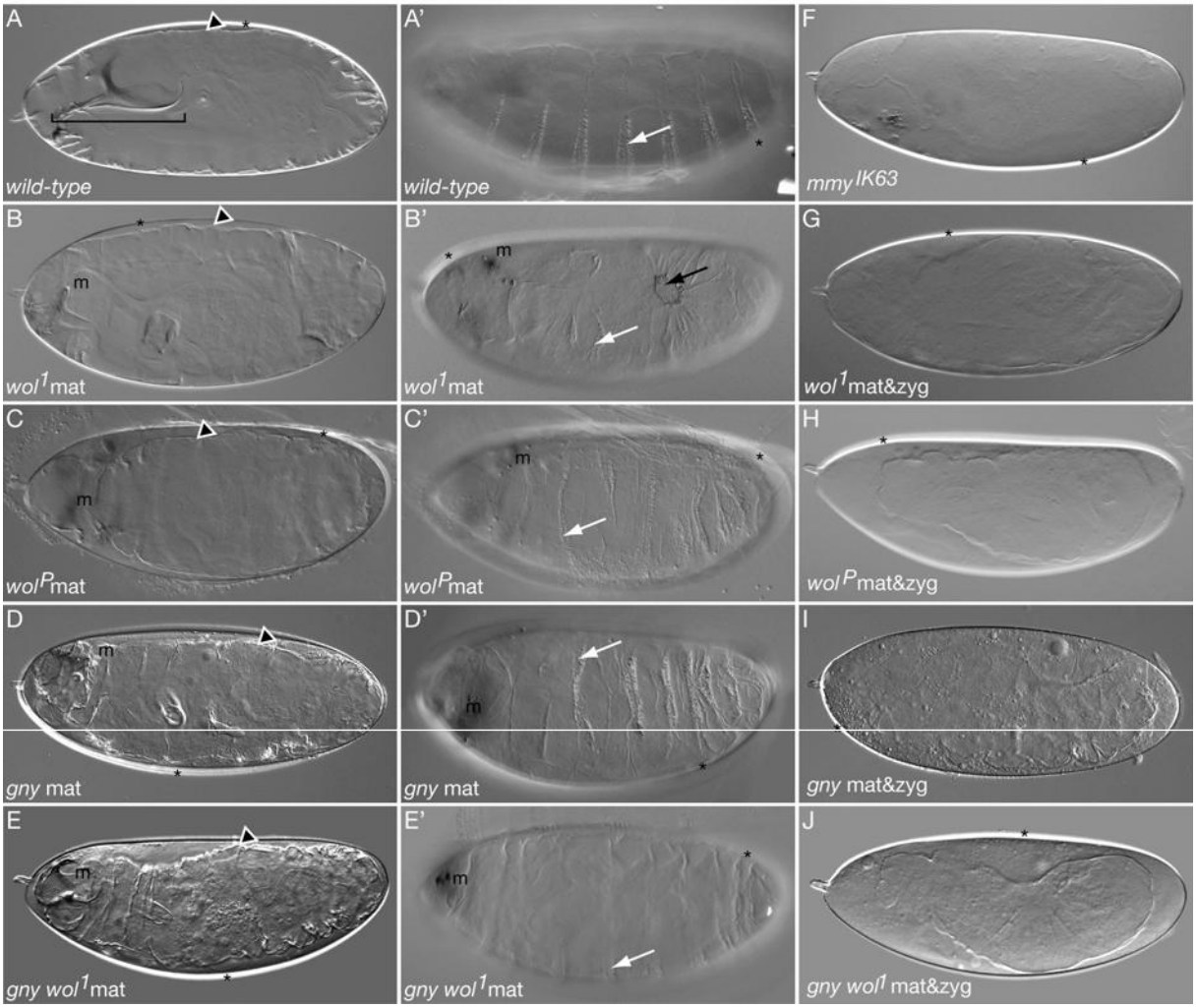


Figure 4

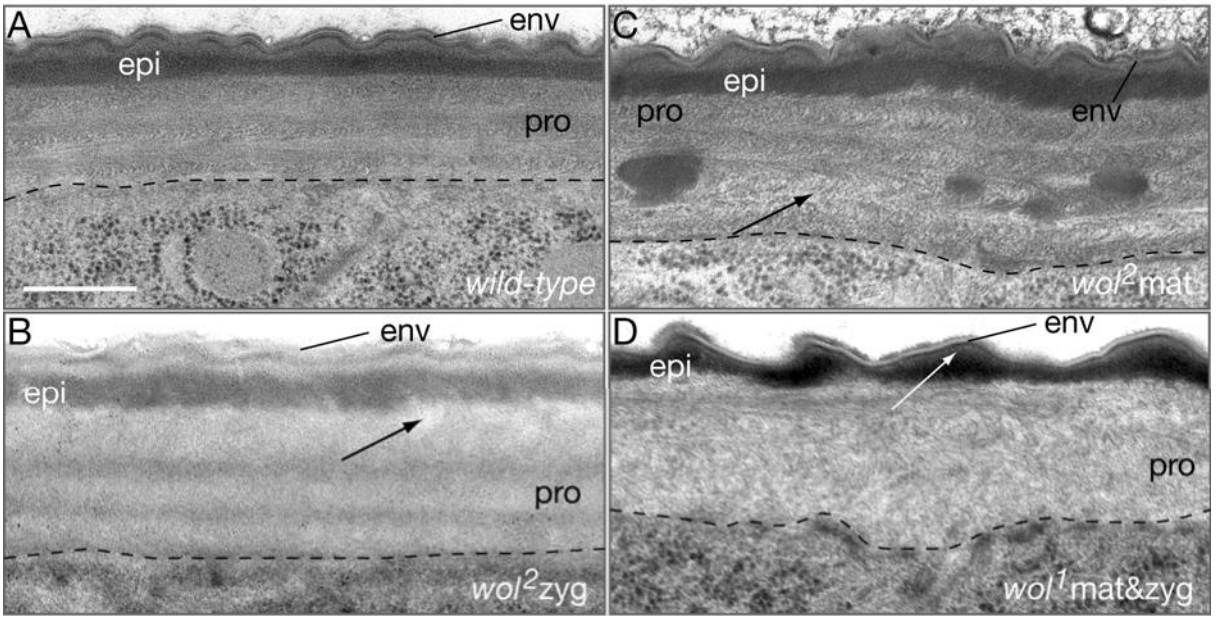


Figure 5

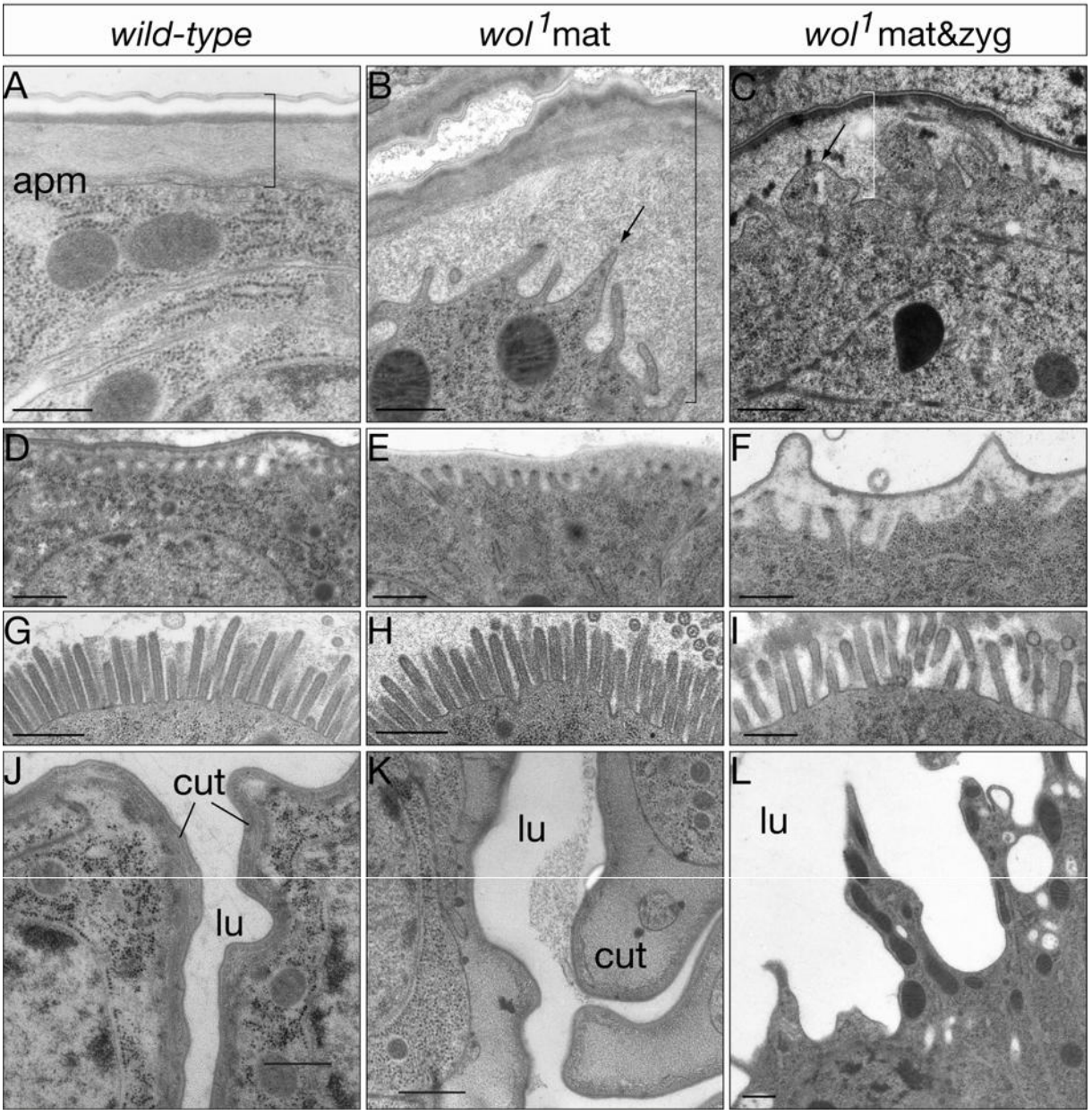


Figure 6

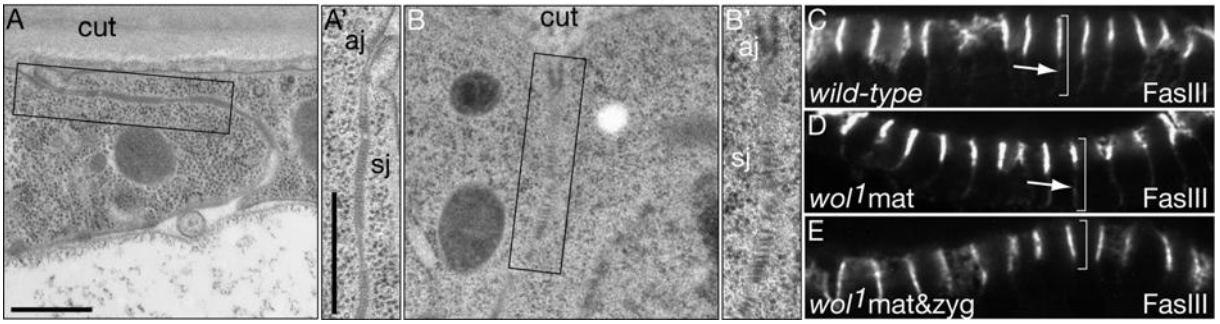


Figure 7

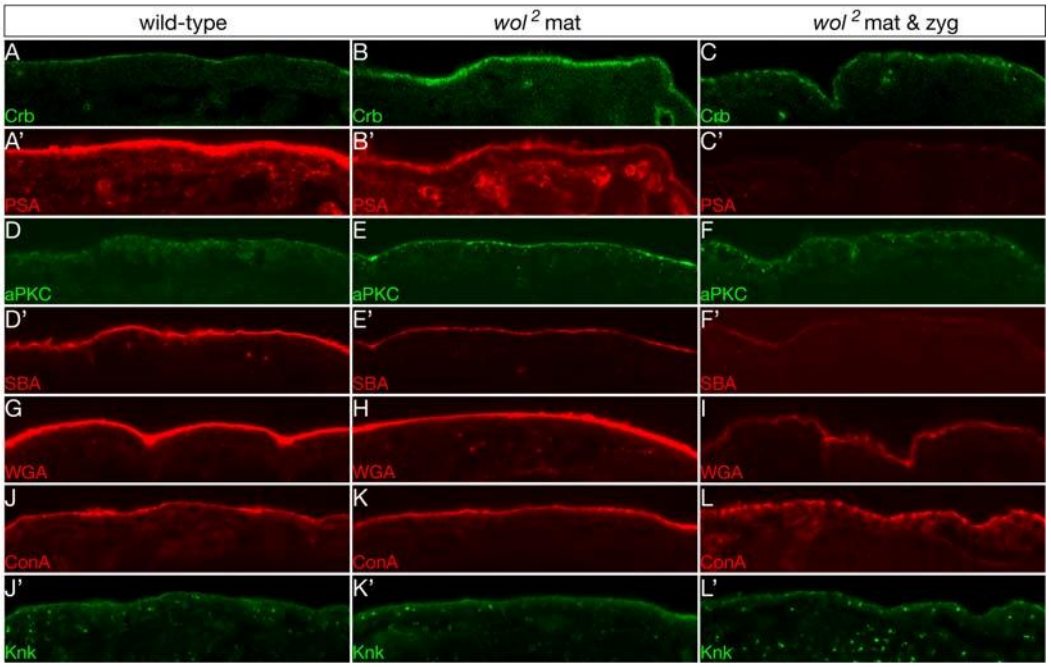


Figure 8

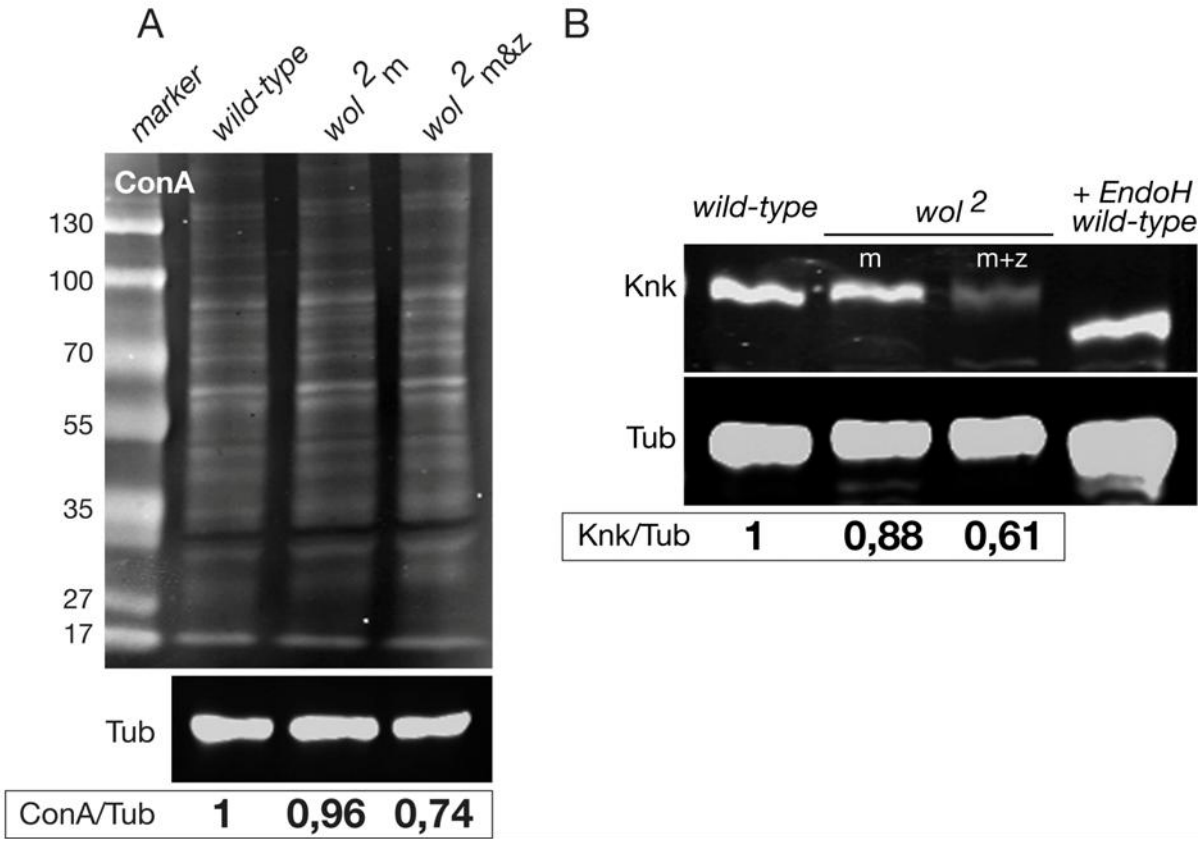


Figure 9

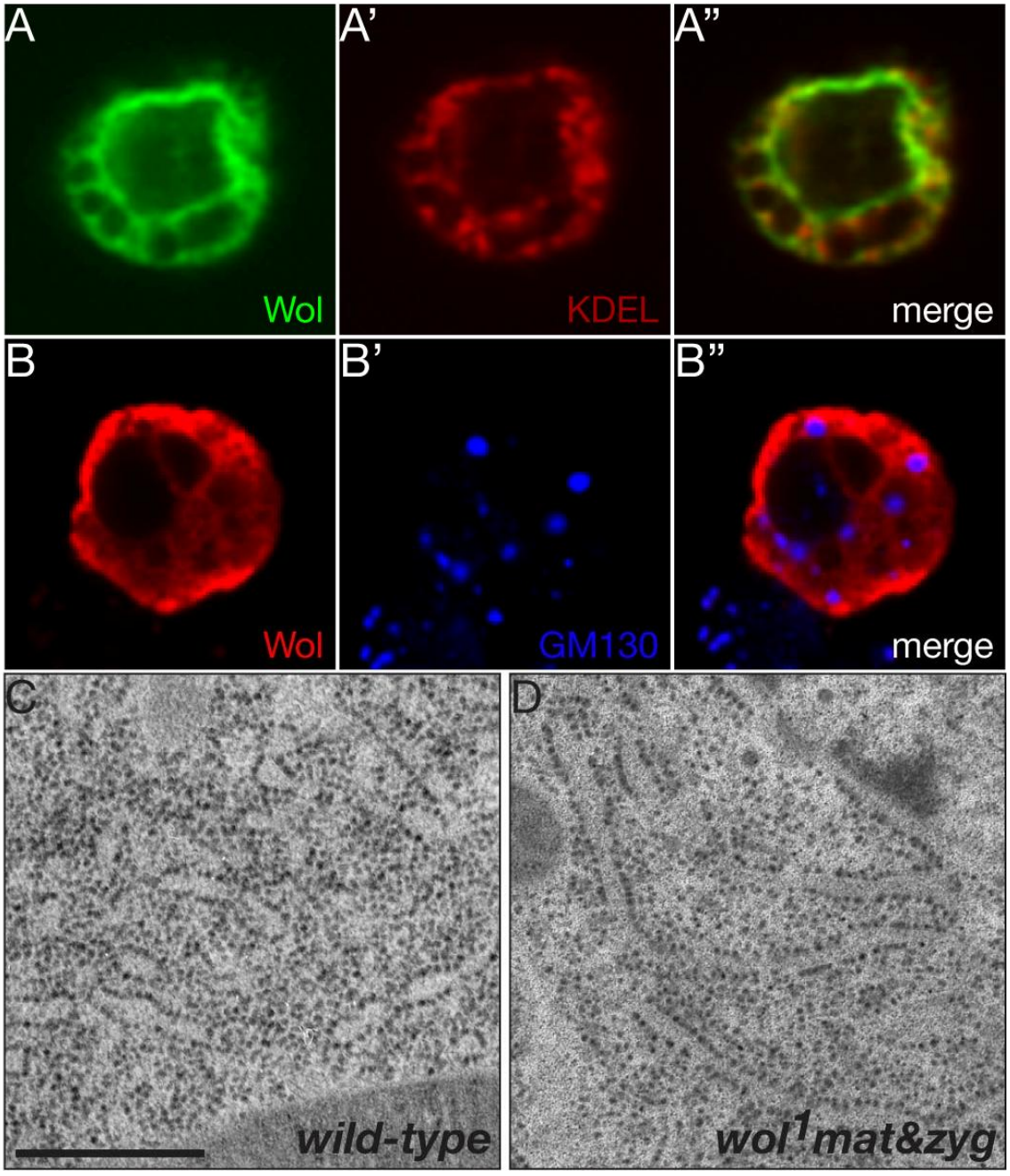
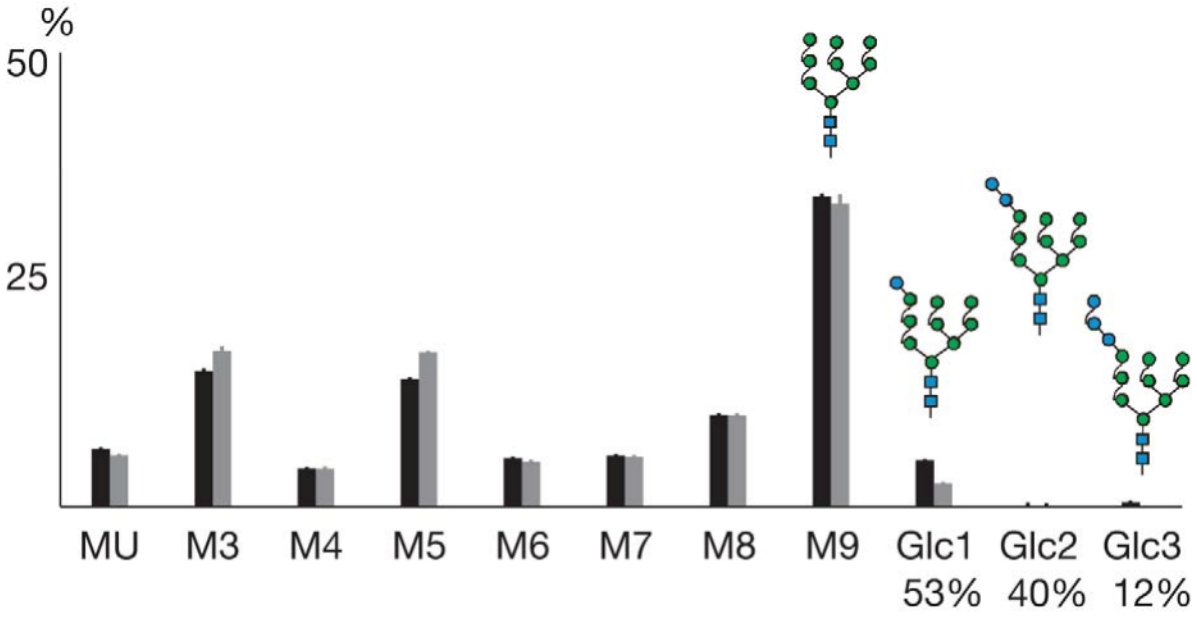


Figure 10



3.2 Publication Shaik Khaleelulla Saheb et al., 2011 (Submitted to JBC)

δ-AMINOLEVULINATE SYNTHASE IS REQUIRED FOR APICAL TRANSCELLULAR BARRIER FORMATION IN THE SKIN OF THE *DROSOPHILA* LARVA*

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Running head: Transcellular barrier formation requires Alas

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Animals construct a layered skin to prevent dehydration and pathogen entrance. The barrier function of the skin relies on the extensive cross-linking of specialised components. In insects, for instance, epidermal cells produce an apical extracellular cuticle that consists of a network of proteins, chitin and lipids. We have identified mutations in the *Drosophila* gene coding for the δ-Aminolevulinate synthase (Alas) that cause massive water loss. Reduction of Alas function results in the breakdown of the extracellular dityrosines network in the cuticle, whereas glutamyl-lysine isopeptide bonds are not affected. The lateral septate junctions of epidermal cells that serve as a paracellular plug are intact, as well. The inflow of liquid into the cuticle, by consequence, heavily destroys chitin organisation in *alas* mutant larvae. Hence, Alas activity, which initiates heme biosynthesis in the mitochondrion, is needed for the formation of a dityrosine-based barrier that protects both the cuticle from transcellular infiltration of body fluid and the animal from dehydration. Taken together, we conclude that at least two modules – an apical protein-chitin lattice and the lateral septate junctions, act in parallel to ensure *Drosophila* skin impermeability.

Organisation of an extracellular waterproof barrier is a major task of the animal epidermis. Sealing of the epidermis involves extensive cross-linking of the extracellular components. In the mammalian skin, a layered lattice of outer lipids mainly ceramides and fatty acids and inner extracellular proteins including, among others, keratin and involucrin constitutes the water-resistant stratum corneum (SC) (1-3). The insect skin, the cuticle, is based on an extracellular chitinous matrix and chitin associated proteins, which are overlaid by lipids and waxes (4). One major type of cross-linking in both cases is the formation of glutamyl-lysine isopeptide bounds between extracellular proteins that is catalysed by extracellular transglutaminases (5-7). Loss of transglutaminase activity in humans results in lamellar ichthyosis, which is characterised by the disorganised SC. In *Drosophila*, reduction of transglutaminase activity does not seem to have dramatic effects on animal viability(7,8). However, adult transglutaminase deficient flies suffer morphological abnormalities of the wing and abdominal cuticle. Together with glutamyl-lysine isopeptide bounds, covalent dityrosine bridges between extracellular proteins seem also to contribute to the cuticular barrier organisation in insects (9-11). The molecular pathway, however, that administrates the establishment of the dityrosine network in the insect epidermis is unknown. Possibly, the same enzymes are employed that act to shield the non-cuticle producing midgut epithelium of the mosquito *Anopheles gambiae* against pathogens (12). Here, oxidation of tyrosine residues of extracellular proteins to dityrosines is mediated bythe membrane-bound dual oxidase (duox) that has a cytosolic heme-binding and an extracellular peroxidase domain.

To learn more about epidermal barrier organisation, we screened a *Drosophila* collection of transposon insertion lines from the Bloomington stock centre, Ohio, for cuticle defective phenotypes. Among others, we identified a P-element insertion in the first exon of the *Drosophila* gene coding for the δ-Aminolevulinate synthase (Alas, EC 2.3.1.37). Alas is a mitochondrial enzyme that initiates the heme biosynthetic pathway catalysing the condensation of succinyl-CoA and glycine into d-aminolevulinic acid. Heme is a co-factor of mitochondrial cytochromes that transfer electrons to different substrates during respiration or the biosynthesis of steroid hormones like ecdysteroids. Moreover, heme is a co-factor of proteins acting in signalling pathways such as nitric oxydase and the ecdysone-dependent transcription factor E75 (13). Despite this broad requirement, larvae homozygous for the insertion *KG10015* have a distinct molecular defect in their cuticle. Whereas distribution and

amounts of glutamyl-lysine isopeptide bounds seem to be normal in these larvae, their dityrosine network is unstable. This leads to a severe failure of epidermal barrier function and water loss. Our data indicate that a heme-dependent mechanism is essential for the construction of a functional skin in *Drosophila*.

EXPERIMENTAL PROCEDURES

Fly genetics. Flies were kept at room temperature (22°C). Mutations and deficiencies listed in table 1 were maintained over balancers that harbour transgenic insertions encoding GFP or YFP expressed under the control of the *Krüppel* or *Deformed* promoter, respectively. For embryo and larva collection, flies were incubated in cages on apple juice agar plates garnished with a spot of yeast. Mutantembryos or larvae were recognised by the lack of GFP or YFP expression. Fly stocks were purchased from the Bloomington Stock Centre. The alas P-element KG10015 (P(SUPor-P)) was mobilised by Hop6 encoded on the X-chromosome. White-eyed F2 males were screened for lethality. The chromosomal deletion for the allele $alas^{3b}$ was characterised in appropriate PCR experiments and sequencing. TweedleF-RFP flies were generated by Fly Facility (Clermont-Ferrand, France) using a construct published in (18). A 2nd chromosomal insertion was recombined to $alas^{KG10015}$.

Microscopy and immunofluorescence. For electron microscopy, specimens were prepared as described in Moussian and Schwarz (31). For data collection by Nomarski optics a Zeiss Axiophot was used. Signal detection by laser scanning microscopy, was performed on an Olympus flowview FV1000. Injections were done with 10 KDa dextran conjugated with FITC (Sigma). For immunodetection, the following antibodies were used: anti-cytochrome c (Abcam) that cross-reacts with the respective *Drosophila* protein; anti-dityrosine (Cell laboratories); anti-Fasciclin3 and anti-Neurotactin, both septate junction proteins, from the Developmental Studies Hybridoma Bank (DSHB, University of Iowa), anti-Knk, which was described in (28). Rhodamine-conjugated wheat germ agglutinin (WGA) and soybean agglutinin (SBA) both from Vector Lab and rhodamine-conjugated chitin-binding probe (CBP) from NEB Biolabs were used as counter stains. Images were prepared with Adobe Photoshop CS4 and Adobe Illustrator CS4 software.

Molecular biology and biochemistry. Western blots were carried out following the protocol described in Norum et al. (32). The antibodies used were: anti-cytochrome c (Abcam), anti-Knk (28) and anti-eIF2a, phosphorylated and non-phosphorylated (Abcam). The extension of the $alas^{3b}$ deletion was determined by standard PCR of the flanking region. Xbp1 RT-PCR was performed as described in (33). Heme content was measured using the heme assay kit from Quantichrom™. In brief, two times 100 larvae of each genotype were homogenised in the detection buffer. The concentration of heme was measured at 405nm in an Elisa reader. The experiment was repeated once equalling four independent embryo collections.

RESULTS

Mutations in alas cause loss of cuticle impermeability. The *Drosophila* protein coded by the *CG3017* locus is 71% similar and 57% identical to the human erythroid-specific 5-aminolevulinate synthase (Supplementary Fig. 1). A P-element insertion in the 5' UTR of the *Drosophila CG3017/alas* gene ($alas^{KG10015}$) is a homozygous recessive allele that causes larval lethality before hatching (Fig. 1A). In these larvae, heme concentration is reduced by 12 % (wild-type larvae 25µM/µg protein, *alas* mutant larvae 22µM/µg). We excised the P-element in order to verify whether the insertion is responsible for the observed phenotypes described below. Several independent excision events restored lethality, and adult animals were healthy and fertile. Several other independent excision events, at the same time, failed to restore lethality. The respective alleles caused an embryonic phenotype similar to the one caused by the P-element insertion. Compared to the ready-to-hatch wild-type larva, homozygous *alas* mutant larvae are smaller, and the space between the egg case and the larva is filled with liquid (Fig. 1B-E). This phenotype becomes apparent during late embryogenesis but before parts of the cuticle such as the head skeleton are fully melanised. In addition, the tracheal tubes of *alas* mutant larvae are not air-filled. Wild-type larvae fixed in Hoyer's medium are cone-shaped (Fig. 1F). By contrast, respective *alas* mutant larvae are rather rectangular and the cuticle partially detaches from the surface of the larva (Fig. 1G). There are no holes or scars in the cuticle of *alas* mutant larvae. Identical microscopic traits are observed in larvae transheterozygous for any *alas* allele and a deficiency that uncovers this locus.Taken together, Alas activity is necessary for normal

body shape, cuticle integrity and tracheal differentiation.

Tracheal barrier breaks down in alas mutant embryos. The liquid in the perivitellin space and the failure to air-fill the tracheae suggest that the barrier function of the epithelia is lost in *alas* mutant larvae. In order to test this assumption, we injected living embryos at different stages of development with dye-conjugated dextran that is retained in the body cavity of wild-type larvae, but should penetrate into the tracheal lumen of mutant larvae. This method has been applied to demonstrate loss of barrier function in various septate junction mutants (14,15). Injection of 10kDa FITC-conjugated dextran into late stage 16 wild-type and $alas^{KG10015}$ embryos reveals no difference in tracheal impermeability between these embryos at this stage (Fig. 2A,B). However, in late stage 17 embryos when some body parts are melanised, FITC-conjugated dextran leaks into the tracheal tubes of $alas^{KG10015}$ embryos (Fig. 2D). We conclude that Alas function is needed to establish or maintain late tracheal impermeability.

The septate junctions are normal in alas mutant animals. Loss of barrier function in *alas* mutant animals may in part be due to defective septate junctions that are indeed involved in establishing epithelial impermeability in several instances (16). We tested whether septate junction components distribution is normal in *alas* mutant embryos in immunofluorescence experiments (Fig. 3A-F). Neurotactin (Nrt) and Fasciclin3 (Fas3) localise to the lateral membrane of cuticle producing cells in wild-type and *alas* mutant embryos. At the ultrastructural level, the septate junctions appear to be unaffected in *alas* mutant larvae (Fig. 3G,H). Together, these results indicate that the lateral paracellular barrier is intact despite mutations in *alas*.

Mutations in alas disrupt cuticle stability. To understand the cellular role of Alas, we compared the ultrastructure of the epidermal cuticle in wild-type, $alas^{KG10015}$ and $alas^{3b}$ mutant embryos and larvae. In late wild-type embryos, the developing cuticle can be subdivided into three histologically distinct composite layers (17): the outermost envelope, the middle epicuticle, and the inner procuticle (Fig. 4A). In late *alas* mutant embryos the cuticle architecture is unchanged (Fig. 4B). Later, in wild-type larvae, the stratified cuticle has thickened and the chitinous procuticle becomes laminar (Fig. 4C). The cuticle of *alas* mutant larvae is, by contrast, severely disrupted (Fig. 4D). Electron-lucid gaps separate the epicuticle from the envelope. The basal side of the procuticle is dishevelled and detaches from the apical surface of the epidermis. There is no cell death in the epidermis or the tracheae. Furthermore, the distribution of TweedleF, an abundant cuticle protein (18) is similar in wild-type and $alas^{KG10015}$ mutant animals (Fig. 4E,F). Taken together, Alas activity is required for cuticle stability at the end of embryogenesis but not for its organisation per se.

Alas activity is needed for dityrosine cross-linking of cuticle proteins. Cross-linking of extracellular proteins in insects comprise dityrosines that are probably introduced by membrane-bound and heme-utilizing dual oxidases (duox) and glutamyl-lysine isopeptide bonds that are catalysed by transglutaminases (7,19,20). To verify whether mutations in *alas* may cause a decrease or loss of isopeptide bonds or dityrosines in late *Drosophila* embryos and larvae, we analysed the presence of these modifications in wild-type, $alas^{KG10015}$ and $alas^{3b}$ mutant animals in immunofluorescnece experiments using specific antibodies against isopeptide bonds or dityrosine (Figure 5). The isopeptide signal lines the apical site of epidermal cells of stage 17 wild-type embryos (Fig. 5A,B). The respective signal in stage 17 *alas* mutant embryos is normal. The dityrosine antibody recognises an epitope at the surface of epidermal cells in stage 17 wild-type embryos (Fig. 5C). In stage 17 *alas* deficient embryos, the distribution of the dityrosine signal in the epidermis is clearly weaker (Fig. 5D). Thus, the heme-dependent cross-linking of tyrosine residues is compromised in *alas* mutant embryos.

Effects of alas mutations on cell functions. To evaluate the severity of the *alas* mutant phenotype, we investigated the impact of *alas* mutations on basal cell functions associated with heme. Mitochondrial function depends largely on the cytochrome co-factor heme, the end product of the biosynthetic pathway initiated by Alas. To quantify Cytochrome c levels, we performed quantitative Western blot experiments using an antibody against Cytochrome c (Fig. 4A). The amount of cytochrome c is decreased by 7 % in $alas^{KG10015}$ compared to wild-type larvae. We also compared the distribution of cytochrome c in wild-type and *alas* mutant embryos by immunofluorescence using the same antibody (Fig. 6B,C). In wild-type stage 17 epidermal cells, dots of cyrochrome c signal mark presumably the mitochondria. In $alas^{KG10015}$ mutant epidermal cells at stage 17 the, cytochrome c signal is unchanged.

Mitochondrial dysfunctions are reflected by loss of mitochondrial morphology (21). To learn about the role of Alas in mitochondrial integrity, we studied the shape of mitochondria in the epidermis of wild-type and *alas*^{KG10015} larvae (Fig. 6D,E). In ultrathin sections, wild-type larval mitochondria appear as oval structures within the cytoplasm. In *alas*^{KG10015} larvae, the mitochondria occasionally are branched, but they do not disintegrate. In summary, cytochrome c activity but not mitochondrial integrity is impaired in *alas* mutant animals.

Heme deficiency increases phosphorylation of the eukaryotic translation elongation factor 2 (eIF2) thereby attenuating translation in mammals (22). Using antibodies against phosphorylated and non-phosphorylated eIF2, we determined the ratio between these two states of eIF2 in quantitative Western blots (Fig. 6F). Indeed, we find that eIF2 phosphorylation is increased by around 30% in *alas* mutant larvae. Consistently, the amounts of Knk, a cuticle organising factor, are decreased by more than 20% in these larvae (Fig. 6G).

Some heme-binding factors such as cytochromes localise to the ER membrane. Heme reduction may therefore lead to ER stress launching a compensatory mechanism that implies the splicing of an inhibitory intron from the mRNA of the transcription factor Xbp1 allowing the correct translation of Xbp1 that in turn activates downstream factors (23). To check whether mutations in *alas* cause ER stress, we measured the presence of spliced Xbp1 transcripts in *alas* mutant larvae. As in wild-type larvae, no spliced Xbp1 transcript is detectable in *alas* mutant larvae (Fig. 6H). This suggests that ER stress is not induced upon heme deficiency.

In summary, reduction or elimination of Alas function incites the phosphorylation of eIF2 that influence the expression and activity of a number of effectors. ER stress, by contrast, is not induced upon Alas malfunction.

DISCUSSION

The animal skin is an elaborate structure that protects the organism against pathogen entry and dehydration. In our search for molecular determinants of skin barrier formation in *Drosophila*, we have analysed the role of Alas, the first enzyme of the heme biosynthesis pathway.

Translation is attenuated in alas mutant larvae. Heme is an essential co-factor of a plethora of factors acting in the mitochondrion, the ER tubules or at the plasma membrane. Mutations in *alas*, however, have a minor effect on embryogenesis as embryos fully develop to larvae that merely suffer skin barrier defects. In brief, chitin laminae especially those close to the apical plasma membrane are dishevelled in the procuticle of *alas* mutant larvae and the cuticle detaches from the epidermis. The disruption of cuticle architecture is accompanied by loss of liquid that accumulates between the larva and the egg case. Apparently, maternally provided *alas* (24) is sufficient to support development and mitochondrial function until late embryonic stages. Indeed, heme concentration is reduced only by 12%, the morphology of mitochondria is normal, and ER stress is not induced in mutant larvae. The relatively and unexpected mild *alas* mutant phenotype can be explained by a long half-life of heme and heme-binding enzymes. This explanation is partly supported by the observation that in *Drosophila* mitochondria may have a half-life longer than the 22 hours of embryogenesis (25). Deleterious effects of *alas* mutations probably derive rather from downscaled protein synthesis as exemplified by 20% reduction of cytochrome c and Knickkopf (Knk) levels triggered by eIF2α phosphorylation.

Alas activity is not required for Knk function. Besides of being a putative victim of elevated eIF2α phosphorylation in *alas* mutant larvae, Knk function might also directly depend on heme. Knk is a GPI-anchored protein with a central Domon domain that has been predicted to bind to heme (26,27). Mutations in the *knk* gene disrupt chitin laminae formation without affecting the barrier function of the cuticle (28). Moreover, the presence of chitin laminae in the cuticle of *alas* mutant larvae argues that Knk is presumably not a heme binding protein. Hence, the *alas* mutant phenotype is probably not due to abrogated Knk function. Beyond this, chitin itself is unlikely to be an important component of the waterproof barrier as in chitin deficient larvae the cuticle is not ruptured and the barrier function is not impaired (29). We conclude that fragmentation of chitin laminae is a secondary consequence of loss of skin barrier function i.e. breakdown of the dityrosine network. The internal liquid flows in and compromises chitin stability.

Extracellular dityrosine formation is compromised in alas mutant larvae. Reduction of translation upon mutations in *alas* may especially affect the synthesis of cuticular proteins that constitute the waterproof barrier. In this case,

barrier disruption would be an indirect consequence of impaired Alas respectively heme function. A direct role of Alas in promoting the construction of the skin barrier could be to provide heme for the membrane-associated and heme-dependent dual oxidase that catalyses protein polymerisation through oxidation of two tyrosines to dityrosine. Loss of duox activity and concomitant reduction of dityrosine moieties in the *Caenorabditis elegans* cuticle correlates with cuticle blistering (30), a phenotype that occurs also in *alas* mutant larvae. In *alas* mutant embryos and larvae, the dityrosine network is reduced whereas the glutamyl-lysine isopeptide network is intact arguing that the dityrosine network is the structure that confers protection against dehydration. We may also assume that duox or a duox-like enzyme is sensitive to moderate heme reduction. The disintegration of the procuticle finally suggests that the dityrosine network is formed close to the apical plasma membrane. In summary, along with the paracellular plug, the *Drosophila* larval epidermis constructs a second, apical transcellular dityrosine-based barrier in order to function as a tight epithelium that impedes water flow towards the relatively dry environment. Whether other epithelia may follow this example remains to be investigated.

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FOOTNOTES

* We would like to thank Brigitte Sailer for technical assistance. This work was supported by the German Research Foundation (DFG, MO1714/2-1 & 3-1).

The abbreviations used are: Alas, δ-Aminolevulinate synthase; CBP, chitin-binding probe; cora, coracle; eIF2, eukaryotic translation elongation factor 2; Fas3, fasciclin3; Knk, Knickkopf; Nrt, neurotactin; SBA, soybean agglutinin; SC, stratum corneum; WGA, wheat germ agglutinin.

FIGURE LEGENDS

Fig. 1. Mutations in the *Drosophila alas* gene cause cuticular defects.
The *alas* gene is located at the tip of the right arm of the second chromosome. It is composed of three exons separated by two introns (A). The P-element *KG10015* is inserted in the 1st exon before the start codon. Two additional genes, *CG34213* (homologous to human "nuclear receptor 2C2-associated protein") and *orange* (a subunit of the clathrin adaptor complex required for eye colour synthesis) are deleted in the excision allele #3 (dashed line). At mid-stage 17, wild-type and *alas* mutant embryos are indistinguishable and develop tightly encased by the eggshell (B and C). Whereas the wild-type larva continues to occupy the entire space of the egg (D), the *alas* mutant larva separates from the eggshell (E), the free space being filled with liquid (white arrows). The tracheae are air-filled in wild-type larvae thereby becoming visible (white arrow in D). The luminal surface of the "empty" tracheae is lined by the chitinous cuticle as detected by the rhodmaine-conjugated chitin-binding probe (CBP, inset in D). The tracheae of *alas* mutant larvae remain invisible as at younger stages, but their lumen is "empty" and the apical surface of tracheal cells is correctly marked by CBP (inset in E). The spindle-like body of the wild-type larva is covered by the cuticle that also constitutes the head skeleton (F). Larvae with mutations in *alas* are smaller and deformed (G). Their cuticle is nevertheless not ruptured and their head skeleton is normal.

Fig. 2. Late tracheal barrier is broken in *alas* mutant embryos.
The tracheal system (black arrow) of wild-type and *alas* early stage 17 embryos is filled with liquid (A,B). Injection of FITC-conjugated 10 kDa dextran into the body cavity is excluded from the tracheal lumen of these embryos (A',B'). By contrast, the tracheal system of *coracle* mutant embryos at the same stage (black arrow in C) fails to exclude the dextran signal from the lumen (white arrow in C'). Likewise, in late stage 17 *alas* mutant embryos, the tracheal system does not air-fill (black arrow, D) and the dextran dye leaks into the tracheal lumen (white-arrow in D').

Fig. 3. The paracellular barrier is intact in *alas* mutant embryos and larvae.
Fasciclin3 (Fas3) is a component of the lateral septate junctions that stabilise the tracheal epithelium (A). At early stage 17, the tracheal tubes contain a luminal chitin rod visualised by rhodamine-

conjugated chitin-binding probe (CBP). Fas3 localisation and the luminal chitin rod are normal in tracheae of *alas* mutant embryos at early stage 17 (B). Fas3 (tangential optical section) as well as Neurotactin (Nrt, longitudinal optical section) are present in the lateral membrane of epidermal cells (C,D). Their localisation is unchanged in *alas* mutant embryos (E,F). In larvae, the epidermal septate junction (sj) are characterised by a ladder-like structure that are arranged basal to the adherens junctions (aj, G). This arrangement is normal in *alas* mutant larvae (H).

Fig. 4. Cuticle architecture is disrupted in *alas* mutant larvae.
At mid-stage 17, the wild-type cuticle consists of the envelope (env), the epicuticle (epi) and the procuticle (pro, A). The cuticle in mid-stage 17 *alas* mutant embryos is indistinguishable from the respective wild-type cuticle (B). The wild-type larval cuticle has thickened considerably and chitin laminae (lam) become apparent (C). The cuticle of *alas* mutant larvae is severely disorganised (D). Chitin laminae occasionally break apart and are separated from the apical surface of the epidermal cells. The epicuticle loses contact to the envelope, which itself appears to be normal. RFP-tagged TweedleF lines the wild-type and *alas* mutant larval cuticle (E,F). Scale bars 500 nm. Scale bar in A applies also to B; scale bar in C applies also to D.

Fig. 5. Dityrosine network breaks down in *alas* mutant larvae.
Isopeptide bonds are detected by a specific antibody (α-KQ) at the surface of epidermal cells in wild-type embryos at late stage16/ early stage 17 that is marked by the soybean agglutinin, SBA, which recognises extracellular O-glycans (A-A''). The signal of the transglutaminase product is unchanged in *alas* mutant embryos (B-B''). An antibody against dityrosine (α-DT) marks the apical side of wild-type early stage 17 epidermal cells lined by SBA (C-C''). In early stage 17 *alas* mutant embryos, the dityrosine signal (arrow) is markedly reduced (D-D''). d ventral denticles

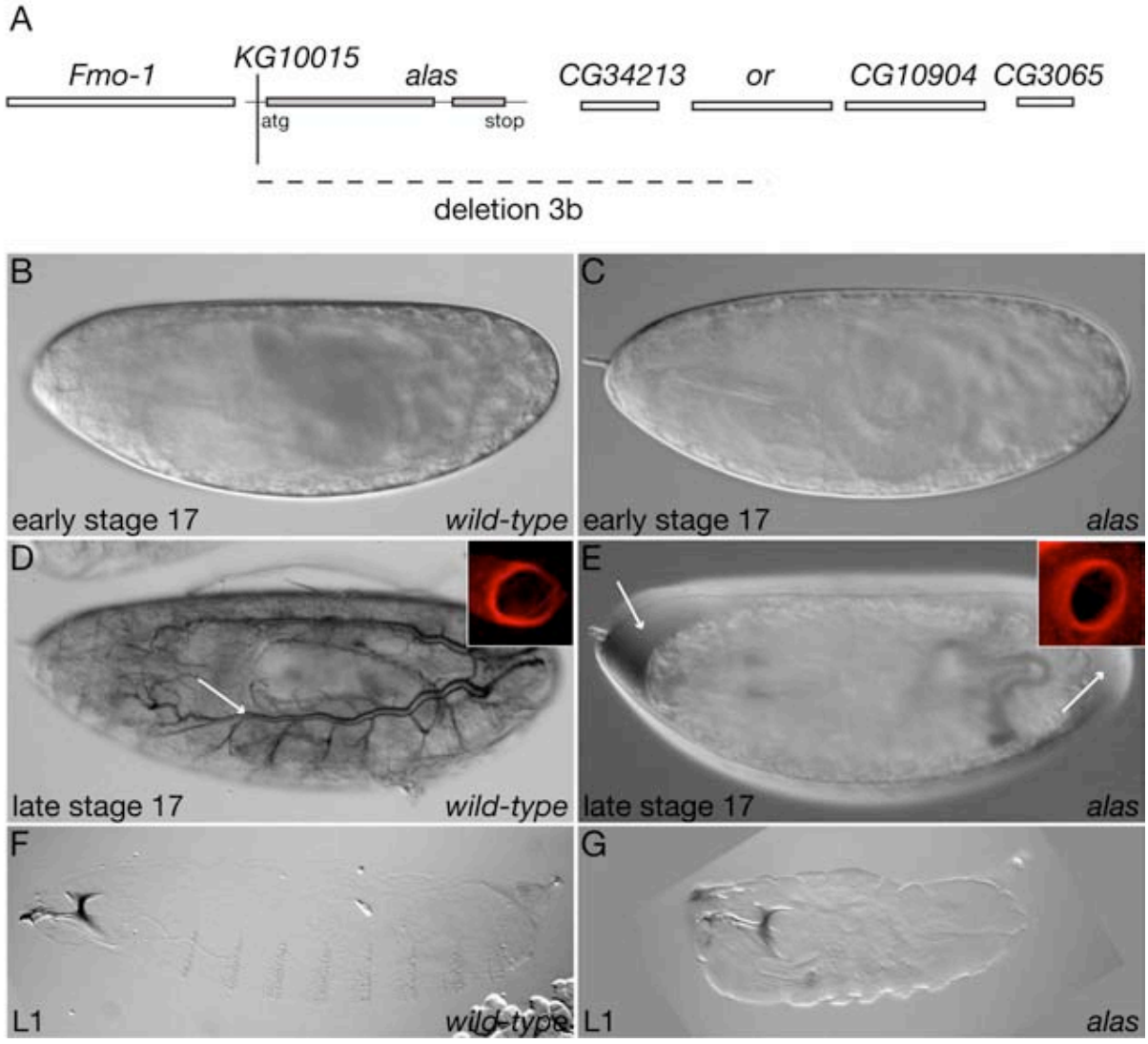
Fig. 6. General translation attenuates upon reduced Alas function
Compared to wild-type, in *alas* mutant larvae, the amounts of cytochrome c, a major enzyme in mitochondria are reduced as revealed by quantitative Western blot experiments (A). Despite this difference, the distribution of the cytochrome c signal (green) within epidermal cells of wild-type and *alas* mutant stage 17 embryos is indistinguishable (B and C). The apical surface of cells and punctae representing WGA-positive ER are marked by rhodamine-labelled WGA (red). To visualise the cytoplasmic distribution, the WGA signal was enhanced. The morphology of mitochondria in wild-type and *alas* mutant larvae is similar (D and E). Phosphorylation of eIF2α at the position 51 is enhanced by more than 30% in *alas* mutant larvae as compared to wild-type on Western blots (F). Concomitantly, in Western blot experiments, the Knk signal is diminished by 23% in *alas* mutant larvae (G).

Tables

Table 1. Fly stocks used in this work.

genotype	***characteristic***
Samarkand	wild-type
$alas^{KG10015}$	P-element insertion in 5' UTR of *alas*
$alas^{3b}$	Imprecise $alas^{KG10015}$ excision
Df(2R)BSC136	Deletion of 5 genes including *alas*
$cora^{14}$	R1607>stop
TweedleF-RFP	Transgenic on 2nd

Figure 1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Fmo-1
KG10015
alas
CG34213
or
CG10904
CG3065
atg
stop
deletion 3b
B
early stage 17
wild-type
C
early stage 17
alas
D
late stage 17
wild-type
E
late stage 17
alas
F
L1
wild-type
G
L1
alas

Figure 2

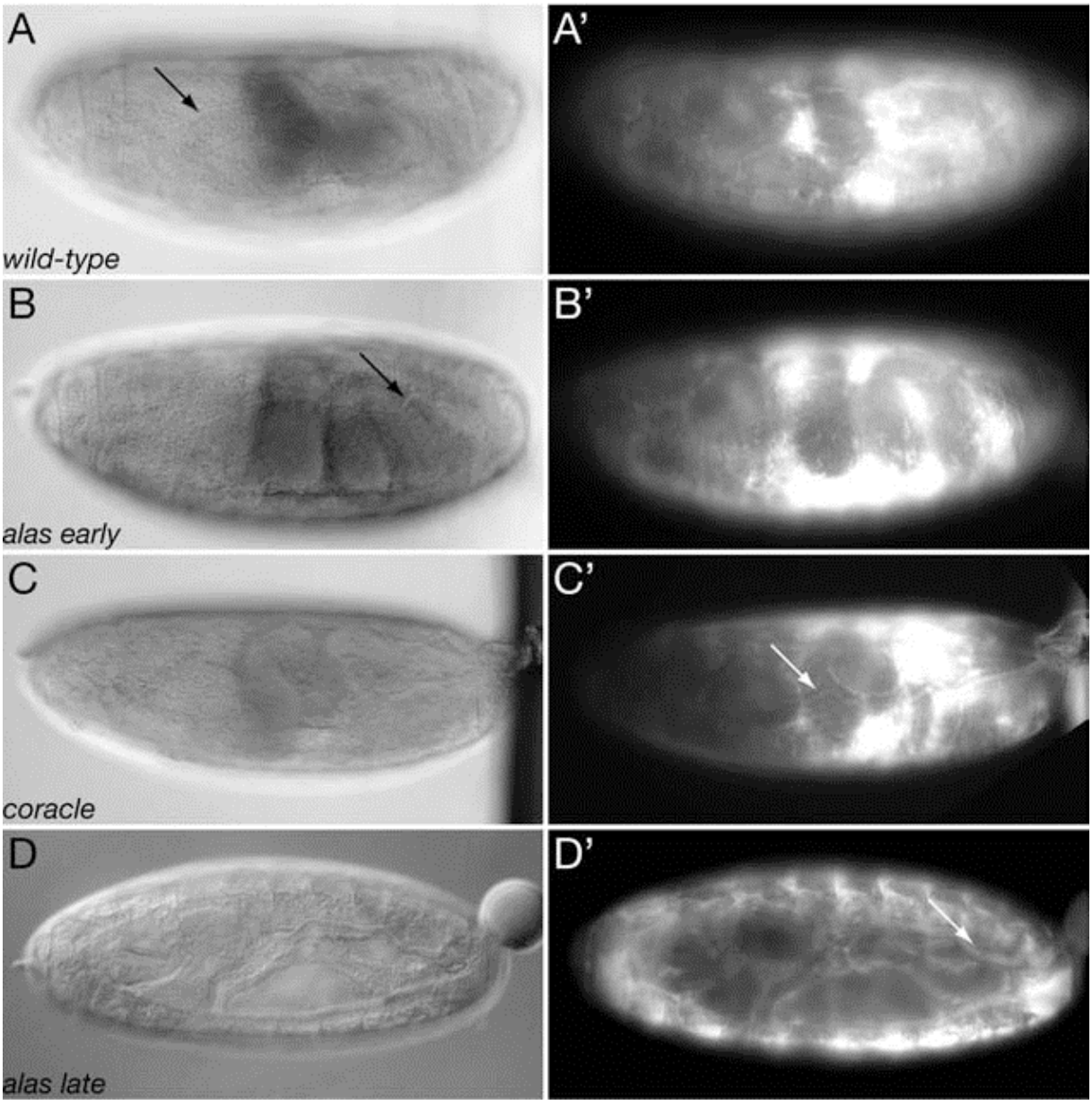


Figure 3

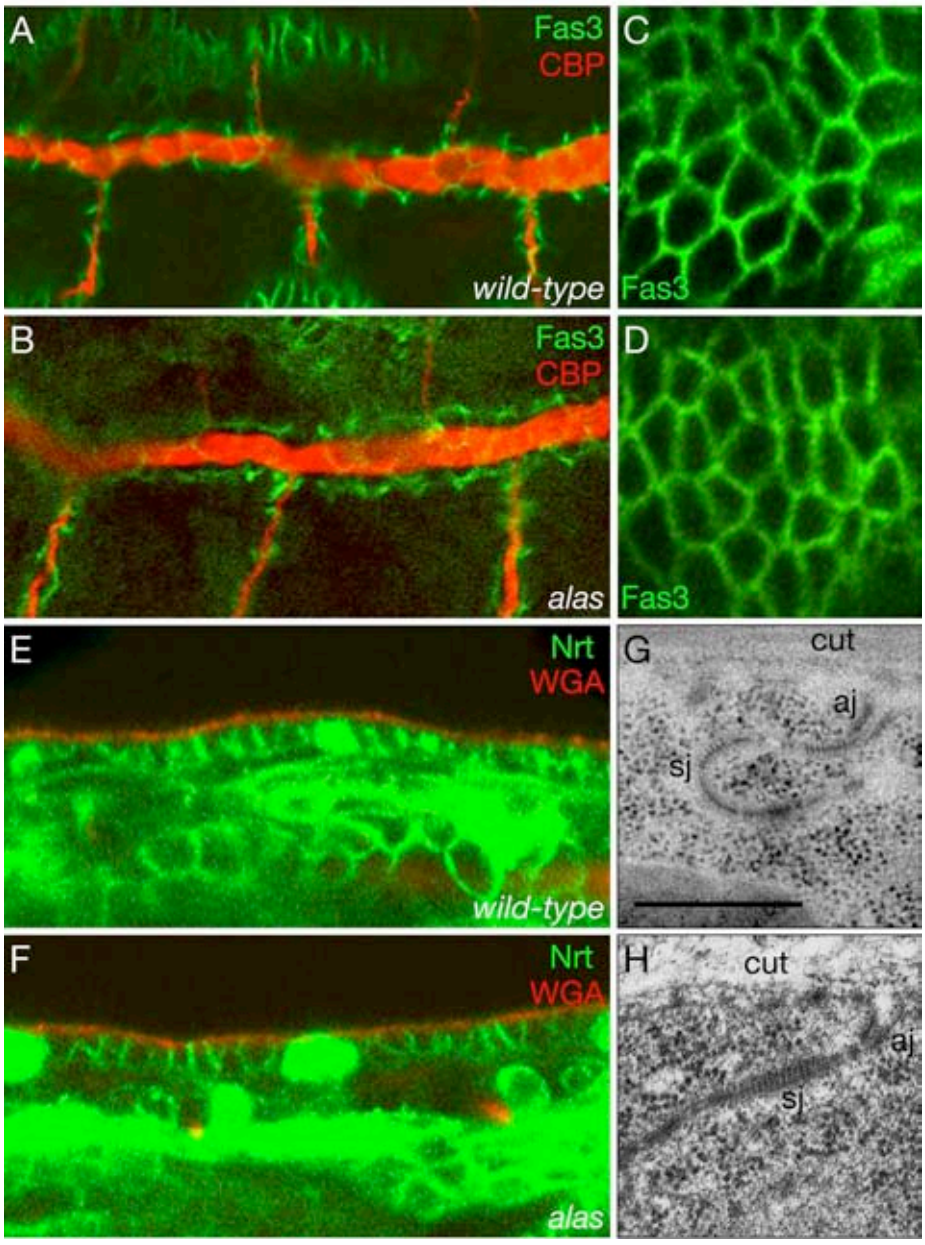

A
Fas3
CBP
wild-type
C
Fas3
B
Fas3
CBP
alas
D
Fas3
E
Nrt
WGA
wild-type
G
cut
aj
sj
F
Nrt
WGA
alas
H
cut
aj
sj

Figure 4

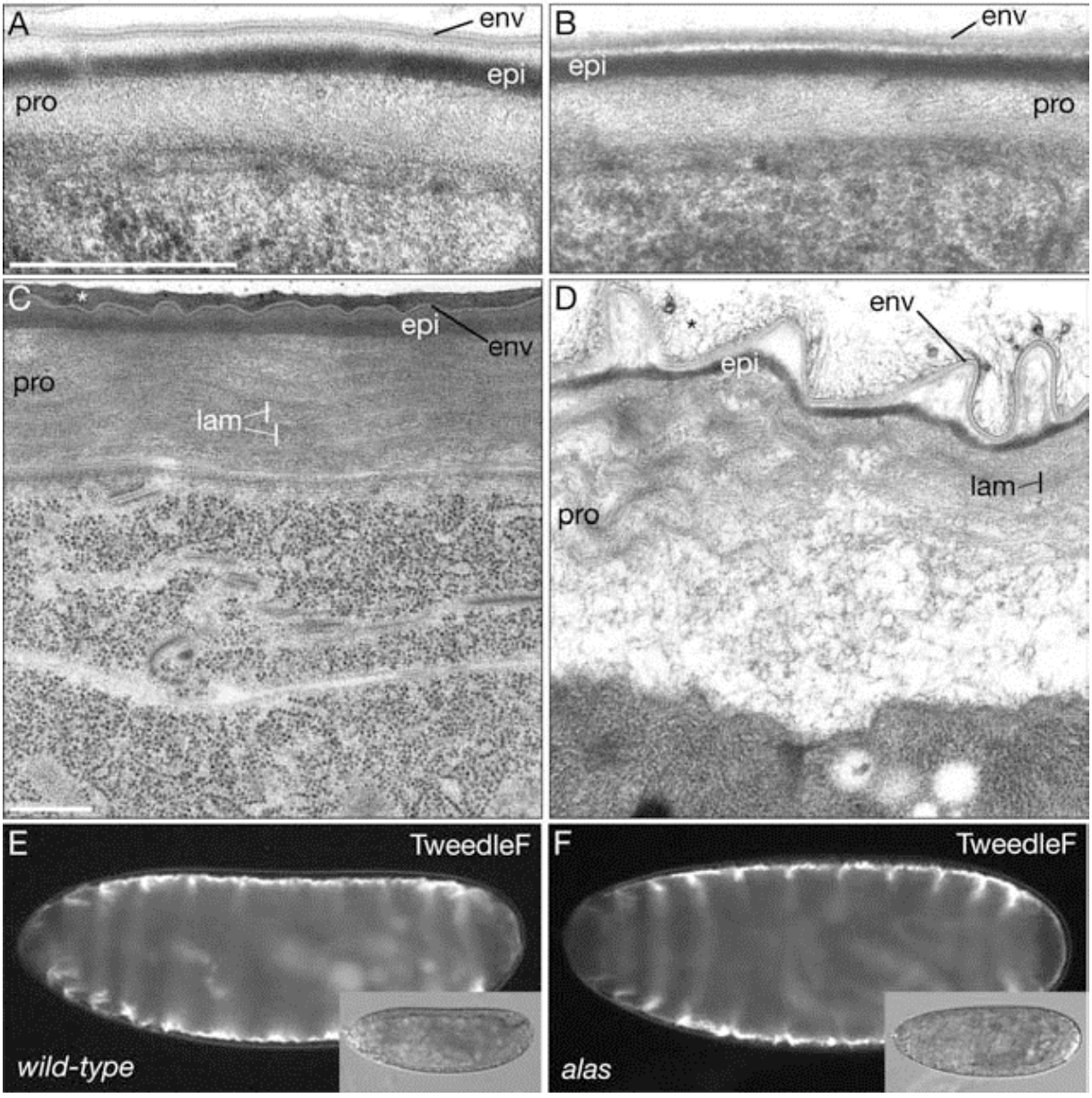

A
env
epi
pro
B
env
epi
pro
C
epi
env
pro
lam
D
env
epi
lam
pro
E
TweedleF
wild-type
F
TweedleF
alas

Figure 5

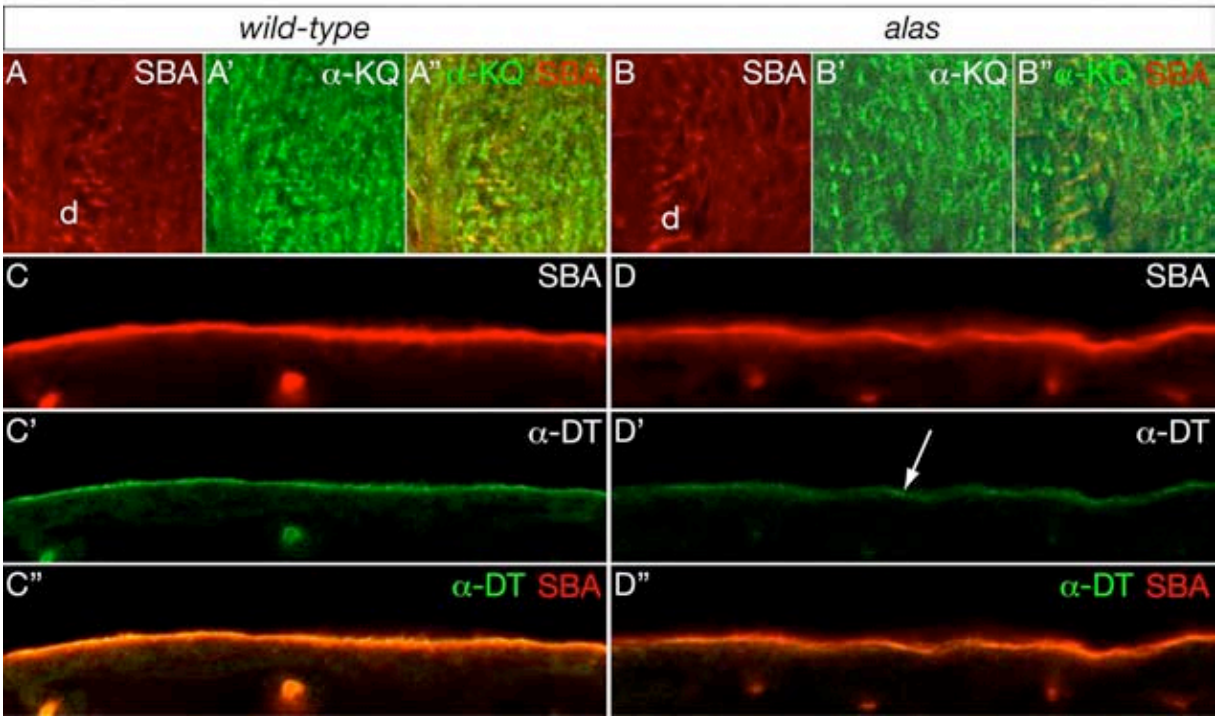
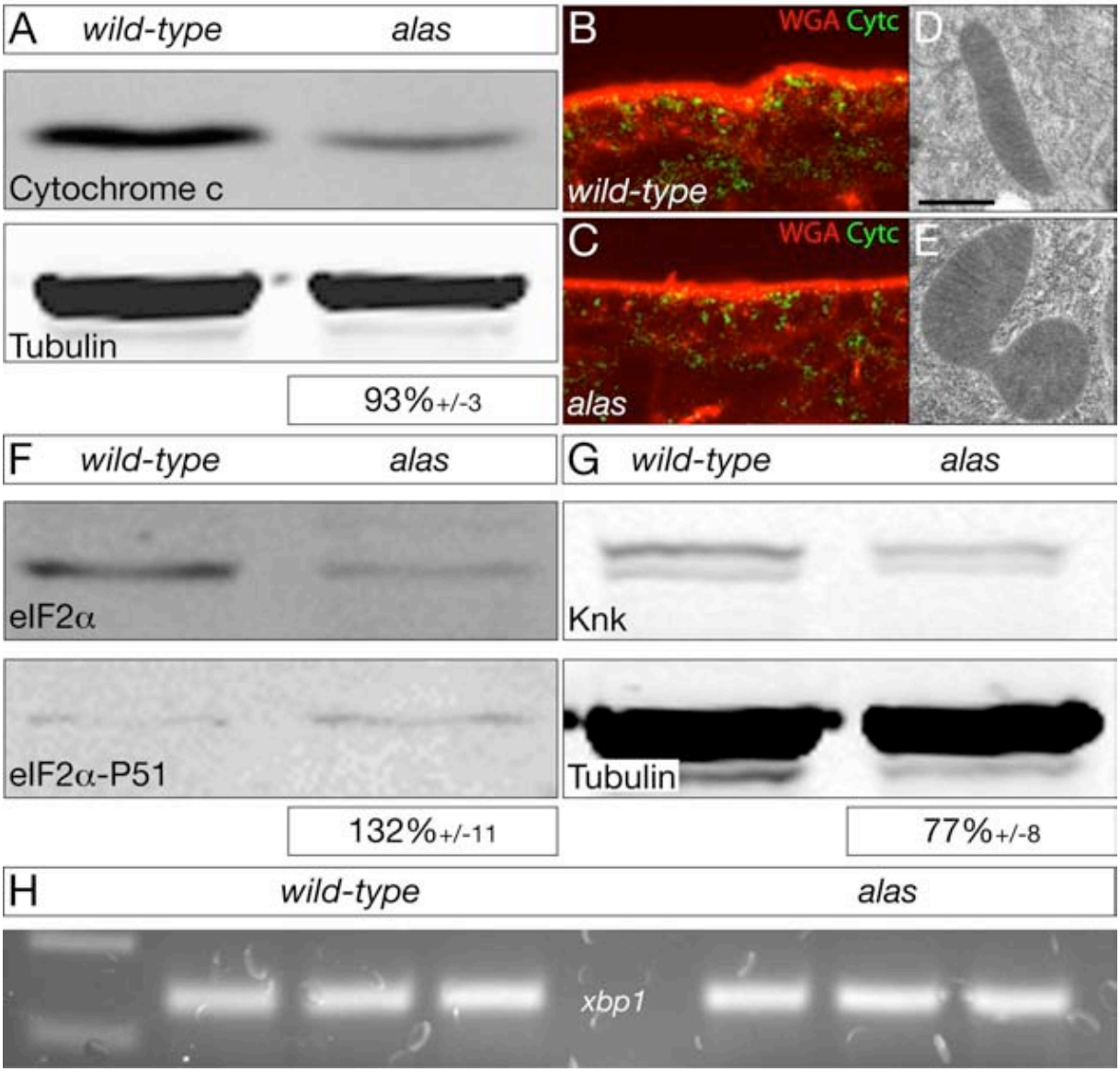

wild-type
alas
A
SBA
A'
α-KQ
A"
α-KQ SBA
B
SBA
B'
α-KQ
B"
α-KQ SBA
d
d
C
SBA
D
SBA
C'
α-DT
D'
α-DT
C"
α-DT SBA
D"
α-DT SBA

Figure 6


A wild-type alas
Cytochrome c
Tubulin
93%+/-3
B WGA Cytc
wild-type
C WGA Cytc
alas
D
E
F wild-type alas
eIF2α
eIF2α-P51
132%+/-11
G wild-type alas
Knk
Tubulin
77%+/-8
H wild-type alas
xbp1

4. DISCUSSION:

4.1 Role of Wol in the cuticle differentiation:

During cuticle differentiation different secretory routes have to be coordinated in order to ensure correct cell shape changes and cuticle deposition. The proteins that are preordained for secretion are co-translationally modified (N-glycosylation, GPI anchor) in the ER, then the protein transported to the plasma membrane through the Golgi apparatus involving vesicular transport. To understand the role of secretion in cuticle differentiation, we previously analysed the *mummy* (*mmy*) mutants that lack UDP-N-acetyl glucosamine pyrophosphorylase, an enzyme catalysing the synthesis of N-acetylglucosamine (GlcNAc). The effects of *mmy* mutations are pleiotropic due to the use of GlcNAc as a general structural component in N-glycosylation, GPI anchor and chitin. To recognize a subtler phenotype than *mmy* I studied animals lacking the function of the *Drosophila alg5 wollknäuel* (*wol*) that is required for early N-glycosylation steps in the ER. Wol/Alg5 is an UDP-glucose: dolichyl-phosphate glycosyltransferase that catalyzes the transfer of glucose from UDP-glucose to dolichyl phosphate. This glucose is added to the oligosaccharide chain that is assembled in the ER prior to the transfer to the nascent polypeptide by the oligosaccharyl transferase (OST). The OST reaction is known as the rate-limiting step in N-glycosylation [50].

In *wol* zygotic mutants the procuticle showed small lesions and in maternal mutants additionally cuticle proteins are mislocalised, whereas in maternal and zygotic mutants the cuticle displayed chitin disorganisation and protein depletion. In brief, the cuticle phenotypes are gradually worsening upon stepwise reduction of Wol activity. In addition, the apical plasma membrane forms ectopic protrusions in maternal and maternal and zygotic *wol* mutant larvae. There are two alternative explanations for the contribution of Wol to cuticle differentiation. First, the glucose residues of the oligosaccharide influence the rate of glycosylation. Consistently, we have shown that in *wol* mutants, N-glycans are hypoglucosylated and reduced suggesting that the rate of glucosylation is critical for glycosylation and ultimately for cuticle differentiation. In yeast cells, glucosylation affects the efficiency of OST function (Burda *et al.*, 1998, Karaoglu *et al.*, 2001). Analogous, hypoglusoylation in *wol* mutant animals may attenuate glycosylation in the ER resulting in a depleted and unorganized cuticle.

The second explanation for the defects caused by *wol* mutations is that the induced unfolded protein response (upr) suppresses transcription and translation thereby generally harming the cell (Malhotra and Kaufman, 2007, Ruddock and Molinari, 2006). Lessening of transcripts coding for enzymes involved in dolichol-anchored oligosaccharide synthesis contribute to the decreased glycosylated protein production in the ER. The supply with auxiliary chaperones prompted by upr and the activity of the ER luminal UDP-glucose transferase (UGT) adding a glucose to the unglucosylated N-glycan in a salvation pathway apparently do not suffice to normalise ER function. It should be noted that early complications at the ER, which is the gateway of the secretory pathway, through reduced glycosylation and hypoglucosylation seem not to restrict protein modifications in the Golgi apparatus and correct localisation of N-glycosylated proteins to the plasma membrane or the extracellular space. This argument suggests that the secretory pathway *per se* is not affected by hypoglucosylation. Heesen *et al.*, 1994 and Reiss *et al.*, 1996 demonstrated that in *Saccharomyces cerevisiae* cells, deletion of either *alg5* or *alg6* does not affect the growth supporting the notion that secretion is normal despite hypoglucosylation. Taken together, in both scenarios (hypoglucosylation versus upr) the *wol* mutant phenotype could be defined as the sum of reduction of membrane and extracellular protein activities.

A central feature of *wol* mutant defects is the lowering of Knk amounts and the accumulation of Crb and aPKC in the apical plasma membrane. The loss of chitin orientation and procuticle organisation in *wol* mutant larvae may in part be explained by the decrease of Knk and the deformation of the epidermal apical plasma membrane by elevated Crb and aPKC levels (Laprise *et al.*, 2006, Wodarz *et al.*, 1995). The consequences of *wol* mutations on Knk can be certainly explained by the diminished translation and protein degradation. These defects can, by contrast, not explain increased Crb amounts. One may speculate that depletion of a factor participating to the complex mechanisms of Crb positioning in the apical plasma membrane may indirectly boost accumulation of Crb. In fact, Lu and Bilder in 2005 showed that absence of recycling endosome syntaxin Avalanche causes accumulation of Crb at the apical plasma membrane of various epithelial cells in *Drosophila*. A second explanation could be that the *wol*-induced unfolded protein response may by an unknown mechanism lead to the concentration of aPKC at the apical plasma membrane that in turn stabilises Crb localisation (Sotillos *et al.*, 2004). In summary, these observations reveal a glycosylation-dependent mechanism of adjusting the homeostasis of

factors that assemble the cuticle in a certain time window during differentiation.

In general, glucosylation is important for balanced and robust epidermal differentiation, either directly as a step of glycosylation or indirectly by preventing the unfolded protein response. Mutations have been known in the human *alg6* that cause congenital disorders of glycosylation (CDG), but not in the human *alg5* gene (Freeze, 2006, Jaeken and Matthijs, 2001, Jaeken and Matthijs, 2007). Patients carrying *alg6* mutations suffer a plethora of developmental and physiological defects, but survive at least a few years. Serum transferrin in these patients is hypoglucosylated due to an increase in the amount of non-glucosylated dolichol-linked oligosaccharide compared to fully glucosylated $Glc_3Man_9GlcNAc_2$. In contrary, *Drosophila* extracellular and membrane-associated proteins in *wol* mutants are probably not hypoglycosylated as demonstrated for Knk. This could be due to species-specific substrate affinity by OST that transfers the oligosaccharide to the protein in the ER lumen. *Drosophila* can be beneficial as a model system to define complex molecular interactions and the pathological mechanisms in CDG through molecular and genetic approach.

4.2 Role of alas in liquid barrier functions:

An important function of the arthropod skin is to protect the animal against dehydration. To investigate the molecular basis of dehydration mechanism of the insect skin, I studied the cuticular components of *alas* (δ-*aminolevulinic acid synthase*) mutant cuticles that showed liquid barrier defects. Alas is the rate-limiting enzyme in the heme biosynthesis, as expected *Drosophila alas* mutant larvae showed 12% reduction of heme content compared to the wild type at late embryogenesis. Normal development of *alas* embryos until the late embryogenesis can be explained by the maternal contribution of Alas that synthesizes the required heme for hemoproteins involved in embryonic development [52]. At the electron microscopic level, *alas* mutant larvae showed a ruptured procuticle, a phenotype that resembles the Dualoxidase (Ce-Duox) mutant cuticle phenotype in *C. elegans* (Edens *et al.*, 2001). Duox is the heme-binding enzyme that catalyzes the cross-linking of tyrosine residues involved in the stabilization of cuticular extracellular matrix. Indeed, dityrosine cross-links are reduced in *alas* mutant at the apical extracellular matrix. By contrast, other structures potentially involved in skin barrier function such as septate junctions and N^{ε}-(γ-glutamyl)-lysine isopeptide bonds catalysed by transglutaminases are normal in *alas* mutant larvae. In summary, the *alas* mutant phenotype in *Drosophila* larvae might be due to

reduced Duox function. Of course, this has to be shown. The role of dityrosine in water barrier function is not been studied neither in vertebrate nor invertebrates. This is the first evidence that showed reduced dityrosine could be involved in liquid barrier function of the insect skin.

Mutations in *alas* in addition to reduced dityrosines in the cuticle influence other cellular processes. For instance, increased phosphorylation of eIF2α in *alas* mutant causes the inhibition of protein translation. These could explain the reduced levels of Knk and cytochrome C in *alas* mutant larvae. Other possible reason could be the heme deficiency observed in *alas* mutants could destabilise the heme requiring protein cytochrome C. The presence of chitin laminae in the cuticle of *alas* mutant larvae argue that Knk is presumably not a heme binding protein. Hence, the *alas* mutant did not phenocopy Knk mutant [38, 35]. In *alas* mutant larvae, we also observed higher double phosphorylated ERK levels. This could be due to a wound healing response being provoked by the damaged cuticle (Wang S *et al.*, 2009).

Despite the clear reduction of extracellular dityrosines, we cannot exclude that the organization of extracellular lipids and waxes, which are classically viewed as constituents of a skin water proof barrier in vertebrates as well as in insects, may be defective in *alas* mutant larvae. In the vertebrate skin ceramide lipids have been proposed to be responsible for water barrier function [49]. In insect, epicuticular lipids have been known to be the primary barrier to cuticular water loss [53]. Heme deficiency might affect the activity ER-associated lipid desaturases and elongases that are heme-binding enzymes. By consequence, cuticular lipids are either not produced or are dysfunctional in *alas* mutant animals. This notion certainly deserves more attention and should be tested in genetic and molecular analyses. Taken together, due to quite some similarities between insect and vertebrate skin architecture, investigations of the skin water-proof barrier in *Drosophila* may have a broad impact on the understanding of this essential skin function in general.

5. CONTRIBUTION TO THE PUBLICATIONS

Publication Shaik Khaleelulla Saheb et al., 2011(Glycobilogy)

The Alg5 ortholog Wollknauel is essential for correct epidermal differentiation during *Drosophila* late embryogenesis.

Contribution: Imunohistochemical analysis of Crumbs (Crb), atypical Protein Kinase C (aPKC), Knickkopf (Knk) and different Lectins (SBA, ConA, WGA, PSA) in *wol* embryo. Westren blot analysis of glycosylation in wild type and *wol* mutants using Concavalin A lectin and Knk antibody. Immunocytological localisation of Wol in ER and Golgi in S_2 cells. Protein sample preparation of wild type and *wol* mutant for mass spectrometry analysis. Quantitative PCR analysis of different cuticle differentiation factors in *wol* mutants.

Publication Shaik Khaleelulla Saheb et al., 2011 (Submitted to Journal of Biological Chemistry)

δ-Aminolevulinate synthase is required for apical transcellular barrier formation in the skin of the *Drosophila* larva".

Contribution: Cuticle preparation analysis of *alas* mutant. Imunohistochemical analysis of dityrosine, lys-glutamyl isopeptide, Cytochrome C (Cyt C), Fas III, Neurotactin , Neuroglian, Knk , WGA, SBA and CBP. Western blot analysis of phosphorylated eIF2α, Double phosphorylated ERK, Cyt C, Knk levels. Heme content measurement by Quantichrom Heme assay kit in *alas* and wild type. PCR analysis of Xbp1 splicing in *alas* mutant. Characterization of $alas^{3b}$ P-element excision by PCR. Quantitative PCR analysis of PEK, GcN2 levels in *alas* mutant.

Publication Gangishetti U, Breitenbach S, Zander M, Shaik Khaleelulla Saheb, Müller U, Schwarz H, Moussian B. 2009 (Europian Journal of Cell Biology)

Effects of benzoylphenylurea on chitin synthesis and orientation in the cuticle of the *Drosophila* larva.

Contribution: Western Blot analysis of Knk levels in wild type and benzoylphenlyurea treated embryos.

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