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Hormonal regulation of *mummy* is needed for apical extracellular matrix formation and epithelial morphogenesis in *Drosophila*

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Many epithelia produce apical extracellular matrices (aECM) that are crucial for organ morphogenesis or physiology. Apical ECM formation relies on coordinated synthesis and modification of constituting components, to enable their subcellular targeting and extracellular assembly into functional matrices. The exoskeleton of *Drosophila*, the cuticle, is a stratified aECM containing ordered chitin polysaccharide lamellae and proteinaceous layers, and is suited for studies of molecular functions needed for aECM assembly. Here, we show that *Drosophila mummy* (*mmy*) mutants display defects in epithelial organisation in conjunction with aberrant deposition of the cuticle and an apical matrix needed for tracheal tubulogenesis. We find that *mmy* encodes the UDP-N-acetylglucosamine pyrophosphorylase, which catalyses the production of UDP-N-acetylglucosamine, an obligate substrate for chitin synthases as well as for protein glycosylation and GPI-anchor formation. Consequently, in *mmy* mutants GlcNAc-groups including chitin are severely reduced and modification and subcellular localisation of proteins designated for extracellular space is defective. Moreover, *mmy* expression is selectively upregulated in epithelia at the time they actively deposit aECM, and is altered by the moulting hormone 20-Hydroxyecdysone, suggesting that *mmy* is part of a developmental genetic programme to promote aECM formation.

KEY WORDS: Chitin, Knickkopf, Apical ECM, Cuticle, Epidermis, Trachea, Mummy, Pyrophosphorylase, Udp-Glcnac, Ecdysone, *Drosophila*

INTRODUCTION

Epithelia that line the animal body and internal organs synthesise and secrete proteins and polysaccharides at their apical surface to build specialised apical extracellular matrices (aECMs). Such aECMs may serve as protective barriers against environmental influences, provide apical attachment sites for the underlying epithelium, or temporarily assist in epithelial remodelling (Sebastiano et al., 1991; Jovine et al., 2002; Roch et al., 2003; Bökel et al., 2005; Moussian and Uv, 2005). Apical ECMs are rich in carbohydrates such as glucosaminoglycans and glycosyl-groups on proteins. These sugars are important both for the polarised deposition of aECM components and their assembly into functional aECMs. The glycosyl-groups bound up in aECMs originate from cytosolic residues that are imported into the secretory compartments or used directly by transmembrane enzymes to generate extracellular polysaccharides (Wilson, 2002; Huet et al., 2003; Moore, 2003; Mayor and Riezman, 2004). Many enzymes acting in protein maturation are identified, but their contribution to and requirements for aECM differentiation and organ morphogenesis in complex multicellular organisms has been little investigated.

Differentiation of the *Drosophila* exoskeleton (cuticle), a stratified highly ordered aECM, relies on a carefully orchestrated apparatus for biosynthesis and modifications of proteins and the polysaccharide chitin. The *Drosophila* cuticle is deposited during late embryogenesis to cover the apical surface of the epidermis, as well as of the respiratory organ (tracheae) and the fore- and hindgut (Neville, 1975). The first step in cuticle deposition is the formation of an outer impermeable envelope. Subsequently, the middle protein-rich epicuticle responsible for cuticle stiffness is formed and, finally, an innermost procuticle loaded with lamellar linear chitin is assembled to confer stability and elasticity to the cuticle (Locke, 2001).

Several *Drosophila* mutants have been identified that show defects in cuticle formation without affecting the overall embryonic patterning (Jürgens et al., 1984; Nüsslein-Volhard et al., 1984; Wieschaus et al., 1984; Ostrowski et al., 2002). One of these is *krotzkopf verkehrt* (*kkv*), which encodes the epidermal chitin synthase CS-1. CS-1 is a transmembrane enzyme that links cytosolic UDP-N-acetylglucosamine (UDP-GlcNAc) into long GlcNAc polymers (chitin) that extrude from the apical surface. Loss of chitin destabilises not only the procuticle, but also affects the integrity of the epicuticle during cuticle differentiation suggesting that a proper procuticle is required for epicuticle formation (Moussian et al., 2005a). Loss of function of two other factors, the predicted extracellular molecules encoded by *retroactive* (*rtv*) and *knickkopf* (*knk*), causes similar cuticle defects as mutations in *kkv* and is required for chitin filament assembly (Moussian et al., 2005b; Moussian et al., 2006). Another group of mutations that affects cuticle differentiation disrupt genes that encode components of the septate junctions (SJs). SJs share functions with vertebrate tight junctions, confer a paracellular diffusion barrier in *Drosophila* epithelia (Lamb et al., 1998; Wu and Beitel, 2004) and are necessary for polarised deposition of components needed for cuticle assembly such as Knk (Moussian et al., 2006). Both groups of mutants additionally have impaired tracheal tube size regulation, which is associated with a defective temporary chitin-containing luminal matrix that is essential for uniform diameter growth (Tonning et al., 2005).

We report on the phenotypic and molecular characterisation of *mummy* (*mmy*), a mutation that is described to cause severe cuticle defects (Nüsslein-Volhard et al., 1984). The *mmy* mutant

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phenotype is similar to that of the so-called 'Halloween' mutants, which fail to produce the differentiation hormone 20-Hydroxyecdysone (Gilbert, 2004), and whose role is still an enigma during insect embryogenesis. We find that *mmy* codes for the *Drosophila* UDP-GlcNAc pyrophosphorylase that functions in aECM formation by producing GlcNAc residues needed for chitin synthesis and protein glycosylation, and that dynamic *mmy* expression is hormonally regulated in aECM differentiating tissues.

MATERIALS AND METHODS

Fly strains and chemical treatments

Unless otherwise noted, fly strains were obtained form Bloomington Stock Centre (Indiana, USA) and Tübingen Stock Centre (Germany). The mutant alleles of *mmy* used in this study were mmy^{IK63}, mmy^{IL07}, $mmy^{KG04349}$ and $mmy^{KG08617}$ (Nüsslein-Volhard et al., 1984; Bellen et al., 2004). The deficiency uncovering the *mmy* locus and used in this work is Df(2L)BSC6. Other mutants used were $shade^{7C}$ (*shd*), $shadow^{10D}$ (*sad*), kkv^{14C73} and kkv^{DZ8} (Jürgens et al., 1984). Germline clones were generated using the Flp/FRT technique (Chou et al., 1993). Tracheal expression of *UAS-Nod-lacZ* (a chimeric construct of *lacZ* and the microtubule minus-end motor Nod; provided by Yuh-Nung Jan) was driven by *Btl-Gal4*, which expresses GAL4 in all tracheal cells from stage 12-13. The enhancer-trap marker *1-eve-1* (Tracheal-1) expresses *lacZ* in all tracheal cells from tracheal cell invagination at stage 11 and has been described previously (Samakovlis et al., 1996).

Transmission electron microscopy

For transmission electron microscopy (TEM), embryos were cryoimmobilised, sectioned and contrasted as previously described (Moussian et al., 2005a). For gold-labelling of chitin, thin-sections of embryos were incubated with biotinylated Wheat Germ Agglutinin (WGA; 1:500, Vector Laboratories) that was recognised by an anti-biotin antibody (1:300, Enzo Diagnostics) that in turn was detected by protein A conjugated to 10 nm gold particles (1:100, York Stierhof). Gold-labelled specimens were contrasted for only 3 minutes instead of 10 minutes.

Immunohistochemistry

Embryos were fixed and stained according to standard procedures. The following primary antibodies were used: tracheal lumen-specific antibody, mouse monoclonal IgM 2A12 (1:10; Developmental Studies Hybridoma Bank, DSHB), rabbit anti-β-gal (1:500; Jackson), rabbit anti-GFP (1:500; Molecular Probes, MP), mouse IgG1 monoclonal anti-Crumbs (1:10; DSHB), mouse IgG monoclonal anti-Discs large 1 (1:10; DSHB), mouse monoclonal IgG2a anti-Fasciclin 3 (1:10; DSHB) and rabbit IgG polyclonal anti-Piopio (1:20; M. Affolter). For fluorescent visualisation, the following secondary antibodies were used: Alexa488 goat anti-rabbit IgG (1:500, MP), Alexa488 goat anti-mouse IgM (1:500, MP), Alexa594 goat anti-mouse IgM (1:500, MP) and Alexa546 goat anti-mouse IgG (1:500, MP). Labelling with fluorescein-conjugated Chitin-binding probe (1:500, New England Biolabs, NEB) was performed according to manufacturers recommendations. A BioRad Radiance 2000 was used to obtain confocal images, and Nikon eclipse E1000 was used for obtaining fluorescent images. Images were processed using Adobe Photoshop 7.0.

Immunoblot analysis

Protein extracts were prepared form manually selected larvae in PLC-buffer or in the EndoH-buffer provided by the manufacturer (NEB), and correct genotypes were identified based on their absence of a GFP-marked balancer chromosome. For protein extraction, around 500 embryos were homogenised in PLC buffer on ice, the extract was centrifuged to remove cellular debris, and 15 μg protein was loaded in each lane on SDS-gel electrophoresis. Separated proteins were blotted onto a nitrocellulose membrane, and probed with the primary antibodies against Knk (1:2000), Ttv (1:1000; I. The), Syx1A (1:5, DSHB) and α-tubulin (1:2000, Sigma). The primary antibodies were detected with HRP-conjugated secondary antibodies. EndoH treatment was performed according to the manufacturer (NEB).

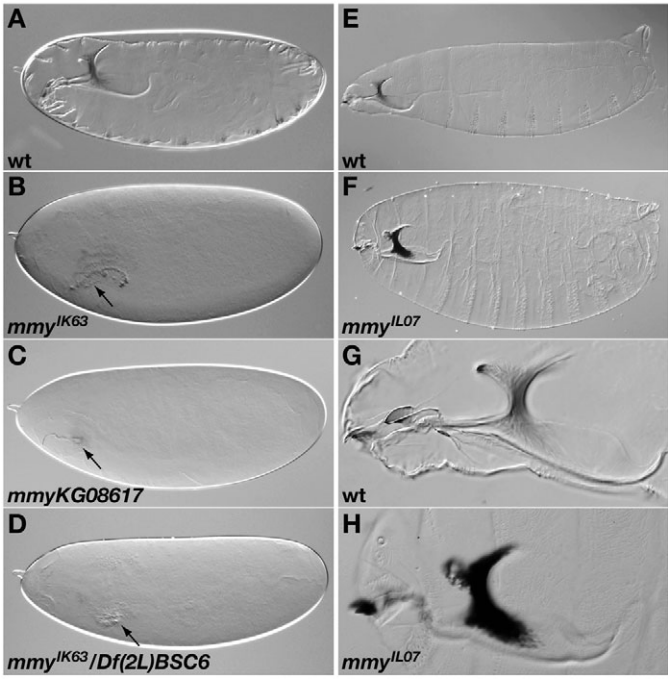

Fig. 1. Defective differentiation of *mmy* mutant cuticles. (A-G) Light microscopy of wild-type and *mmy* mutant larvae. (**A**) The wild-type larva has a colourless cuticle with tanned ventral denticles and head skeleton that is visible through the vitelline membrane. In larvae homozygous for mmy^{IK63} (**B**) or for the P-element insertion KG08617 (**C**), no ventral denticles, head skeleton or cuticle are visible, and transheterozygous mmy^{IK63}/Df(2L)BSC6 larvae (**D**) display similar cuticle defects to homozygous mmy^{IK63} mutant larvae. In all these mutant genotypes, the salivary glands accumulate abnormal debris (arrows in B-D). (**E**-**H**) Removal of the vitelline membrane reveals that larvae mutant for mmy^{IL07} (F) have a distended cuticle compared with the wild type (E). Moreover, the mmy^{IL07} mutant head skeleton (H) is darker and appears discontinuous compared with the wild type (G).

In situ hybridisation

Whole-mount in situ hybridisation was performed with digoxigenin-labelled RNA sense and antisense probes as described previously (Tonning et al., 2005). RNA probes for *mmy* were generated from the *mmy* cDNA LD24639 with SP6 and T7 polymerases.

RESULTS

Cuticle composition and epidermal integrity are disrupted in *mmy* mutant larvae

Two alleles of *mmy* were isolated in a screen for EMS-induced lethal zygotic mutations due to their effects on cuticle differentiation (Nüsslein-Volhard et al., 1984). Compared with the wild-type larval cuticle (Fig. 1A), the cuticle of larvae harbouring the strong mmy^{IK63} allele is hardly visible (Fig. 1B), whereas larvae mutant for the weak mmy^{IL07} allele develop a bloated cuticle and a deformed and strongly melanised head skeleton (Fig. 1F,H).

In order to better understand the role of *mmy* in cuticle differentiation, we compared the *mmy* mutant and wild-type larval epidermis by transmission electron microscopy (TEM). Wild-type cuticle is composed of three layers (Locke, 2001): the outermost envelope characterised by five alternating electron-dense and electron-lucid sheets, the underlying epicuticle built up by an upper electron-lucid and a lower electron-dense sublayer, and the innermost procuticle structured by lamellar chitin microfibrils and contacting the apical plasma membrane of the epidermal cells (Fig.

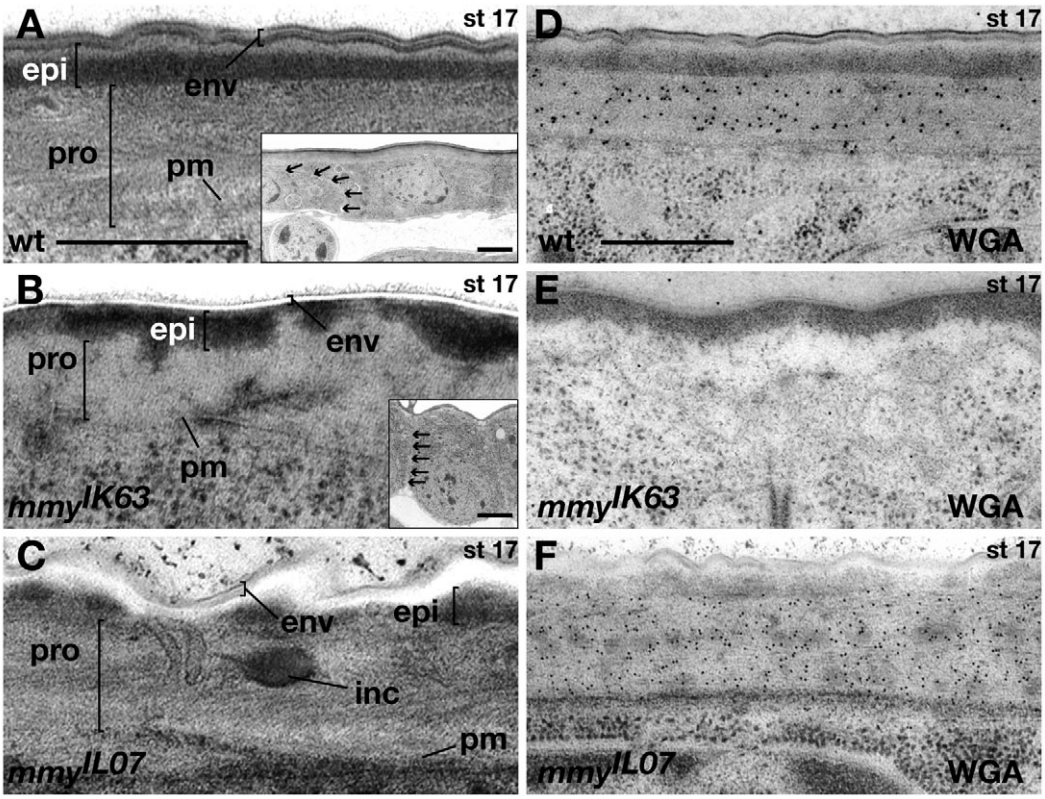

Fig. 2. Chitin is absent in the *mmy* mutant cuticle. (**A**-**C**) Comparison of wild-type and *mmy* mutant larval (end of stage 17) epidermis by transmission electron microscopy (TEM). The wild-type epidermis (A) consists of an epithelial monolayer of flattened cells (inset) beneath a stratified cuticle. The cuticle has three distinct layers: the outermost envelope (env), the middle protein-rich epicuticle (epi) and the underlying chitin-rich lamellar procuticle (pro) attached to the apical plasma membrane (pm). The epidermis of mmy^{IK63} mutant larvae (B) is an epithelium with cuboidal cells (inset) covered by a disorganised cuticle. Arrows in the insets of A and B indicate the lateral membrane that is undulated in the wild-type but not in the mmy^{IK63} mutant epidermal cell. The mmy^{IK63} procuticle varies in thickness and is devoid of lamellar chitin microfibrils, the overlying epicuticle is irregular, and the outermost envelope is reduced to three, instead of five, alternating electron-dense sheets. The envelope and the epidermis of larvae homozygous for the weaker mmy^{IL07} allele (C) appear to be normal, the epicuticle is, however, discontinuous and the procuticle harbours electron dense inclusions, probably orphan proteins. (**D**-**F**) Detection of chitin in wild-type, mmy^{IK63} and mmy^{IL07} mutant cuticles with gold labelled WGA. Chitin is detected in the wild-type and mmy^{IL07} mutant procuticle (D,F; black dots), but is absent in the mmy^{IK63} cuticle (E). Scale bars: 0.5 μm (bar in A applies to A-C; bar in D applies to D-F); 1 μm for insets.

2A). All three cuticle layers are affected in mmy^{IK63} larvae (Fig. 2B). The outer envelope is thinner than in the wild type with only three sheets, and the electron-dense sub-layer of the epicuticle disintegrates and spreads into the upper electron-lucid sub-layer and the procuticle. The procuticle is also reduced in thickness and seems to be devoid of chitin microfibrils; occasionally, the cuticle detaches from the epidermal surface. The cuticle of larvae mutant for the weak mmy^{IL07} allele is stratified as in the wild type, and the procuticular chitin microfibrils appear correctly oriented (Fig. 2C). However, the mmy^{IL07} mutant procuticle contains abnormal inclusions of electron-dense material that are scattered below the epicuticle, presumably orphan proteins, suggesting that the coordinated assembly of the epi- and procuticle is impaired. Taken together, cuticle assembly requires *mmy* activity.

As the chitin containing procuticle is affected in both *mmy* mutant larvae, we tested for the presence of chitin using gold labelling with the lectin wheat germ agglutinin (WGA). WGA binds GlcNAc-groups on glycosylated proteins, glycosaminoglycans as well as chitin (D'Amico and Jacobs, 1995), but on a specimen embedded in Epon and sectioned for TEM, WGA recognises only chitin (Peters and Latka, 1986). Although chitin is present in wild-type and in the mmy^{IL07} mutant procuticle (Fig. 2D,F), no chitin is detected in mutant larvae homozygous for the strong mmy^{IK63} allele (Fig. 2E), arguing that *mmy* is essential for chitin synthesis. However, *mmy* appears to have additional roles in cuticle differentiation, as loss of chitin causes a compensatory increase in cuticular protein deposition (Moussian et al., 2005a), which is not detected in mmy^{IK63} mutants.

mmy is required for epithelial organisation

Our TEM analysis of the mmy^{IK63} cuticle also revealed irregular cell shapes in the underlying epidermis. These cells are cuboidal, rather than flattened like the wild-type epidermal cells (Fig. 2A,B insets), and their lateral membranes are not undulated. Epithelial cell shape is determined by the cytoskeleton and cell-cell contacts. Using high resolution TEM analysis, we find that the orientation of microtubules in wild-type, mmy^{IL07} and mmy^{IK63} mutant larval epidermal cells are indistinguishable (not shown), and that the adherens and septate junctions (AJ and SJ) are positioned normally along the lateral membrane (Tepass and Hartenstein, 1994). However, in the mmy^{IK63}, but not mmy^{IL07} mutant embryos, spacing between the epidermal cells at the AJ appears wider than in the wild type (not shown), and the characteristic ladder-like structure of the SJs is missing (Fig. 3A-C). We also analysed SJ integrity in the two *mmy* mutant epithelia, by labelling for the two SJ components

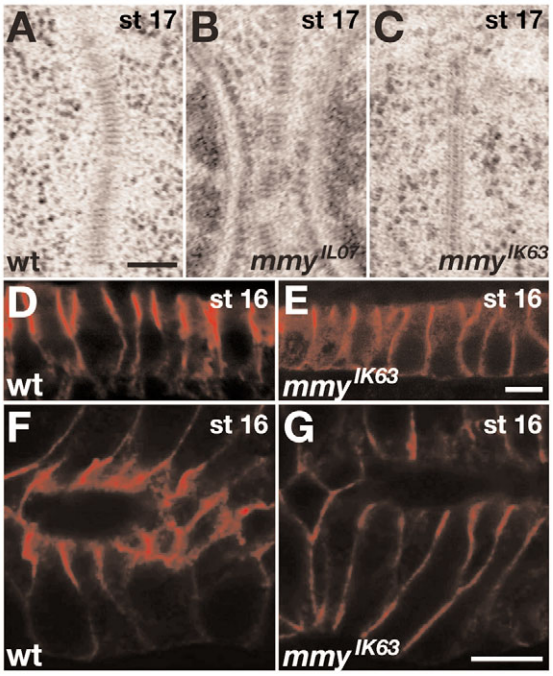

Fig. 3. Septate junctions (SJs) are defective in *mmy* mutant epithelia. (**A**-**C**) TEM-analysis of septate junctions (SJs) of wild-type, mmy^{IL07} and mmy^{IK63} mutant larval (end of stage 17) epidermal cells. The SJs of the wild-type (A) and mmy^{IL07} mutant (C) epidermal cells are seen as ladder-like structures. In mmy^{IK63} mutant epidermal cells (B), this ladder-like assembly is absent, but electron-dense intercellular material at the position usually occupied by the SJ is present. (**D**-**G**) Fas3 (red) is occasionally mislocalised in mmy^{IK63} mutant epithelia. In wild-type (D) hindgut epithelia, Fas3 concentrates within the apical-most region of the lateral membrane, whereas in mmy^{IK63} mutants (E) Fas3 is found along the entire lateral cell surface. Fas3 localisation is similarly affected in the mmy^{IK63} mutant salivary gland (G) when compared with the wild type (F). Scale bars: 0.125 μm in A-C; 5 μm in D-G.

Fasciclin 3 (Fas3) and Discs large 1 (Dlg1). Both SJ proteins are normally distributed in the weak mmy^{IL07} epithelia, and the intracellular Dlg1 protein is also normally localised in the more severe mmy^{IK63} mutants (not shown). By contrast, the transmembrane Fas3 is mislocalised along the lateral membrane in the columnar epithelia of the hindgut and salivary gland in some mmy^{IK63} embryos (Fig. 3D-G). Thus, the disrupted SJ ladder in mmy^{IK63} mutants may reflect a requirement for Mmy in the correct localisation of membrane-bound SJ components. As chitin-deficient embryos have no detectable defects in SJ assembly or function (Moussian et al., 2005a), and chitin-deposition appears unaffected in embryos with disrupted SJ components (Tonning et al., 2005), these results point to two parallel requirements for *mmy* in chitin synthesis and maturation of cell-cell contacts (SJs).

Mmy is required for generation of GlcNAc-groups, including tracheal luminal chitin

The tracheal (respiratory) tubular network is an epithelium that undergoes extensive cell rearrangements during embryonic development, and relies on a luminal (apical) matrix for uniform tube growth (Beitel and Krasnow, 2000; Ghabrial et al., 2003; Uv et al., 2003; Tonning et al., 2005). As chitin is an essential component of this luminal matrix, we initially tested whether *mmy* mutant embryos, which lack cuticular chitin, also display tracheal tube size defects. The wild-type developing tracheal system can be visualised with the lumen specific antibody 2A12 (Fig. 4A). The same staining reveals a perfectly patterned tracheal system in embryos homozygous for the weak mmy^{IL07} allele, although the dorsal trunk (DT) lumen diameter is slightly irregular at stage 15 (Fig. 4B) and becomes excessively elongated at stage 16 (not shown). The tracheal mmy^{IK63} mutant lumen, however, fails to label with the 2A12-antibody, although the typical intracellular vesicular 2A12-staining is present (Fig. 4C). Tracheal tube shapes in mmy^{IK63} mutants were therefore visualised with the pan-tracheal *LacZ*-enhancer-trap *1-eve-1*. Detection of the *1-eve-1* product shows that also mmy^{IK63} mutants display normal tracheal branch patterning, but tube diameter is severely defective (Fig. 4D). The mmy^{IK63} DT fusion branch lumens fail to expand and remain constricted, whereas other parts of the DT trunk become excessively dilated (Fig. 4E,F); during stage 16, these DTs develop huge cyst-like structures (Fig. 4G). Thus, *mmy* is indeed required for uniform tube expansion.

The luminal tracheal chitin matrix can be detected both with a FITC-conjugated chitin-binding probe (CBP) and WGA (Tonning et al., 2005). CBP appears specific for chitin and labels a broad filamentous chitin cable within the wild-type tracheal lumen (Fig. 5A) that is absent in *kkv* mutant embryos (Tonning et al., 2005). In mutants for the weak mmy^{IL07} allele, CBP labels a luminal cable with similar intensity to the wild type (Fig. 5B), but its filamentous appearance is slightly perturbed, implying that the chitin matrix is not properly assembled. By contrast, embryos homozygous for the strong mmy^{IK63} allele completely fail to label with CBP (Fig. 5C), indicating a need for *mmy* also in tracheal chitin synthesis. We also compared WGA labelling of wild-type and mmy^{IK63} mutant embryos. In the wild-type tracheae, WGA labels not only the broad luminal chitin cable, but also the luminal surface that probably represents glycosylated proteins (Fig. 5D). In the mmy^{IK63} mutant tracheae, both the luminal and apical WGA-staining is severely reduced (Fig. 5E). This is different from chitin-deficient embryos, where WGA detects GlcNAc-groups along the apical cell surface and within the lumen, although the broad luminal cable is absent (Fig. 5F), arguing that *mmy* function is required not only for chitin synthesis, but also for other GlcNAc-containing components.

Despite the severe tracheal tube defects in mmy^{IK63} mutants, we find that apicobasal polarity appears normal. The transmembrane protein Crumbs (Crb), which is required for cell polarisation and assembly of the adherens junctions (AJs) (Tepass et al., 1990), localises to the apical tracheal membrane in both wild-type and mmy^{IK63} mutant embryos (Fig. 5H,K), and the microtubule minus end reporter, Nod:β-gal (Clark et al., 1997), appears to accumulate correctly in the apical tracheal cell domain in the mutant (Fig. 5I,L). Furthermore, no significant defects in Fas3 localisation was observed in the mmy^{IK63} mutant tracheal epithelium (not shown) and apical secretion of the luminal protein Piopio (Pio), which is required to form narrow tubes with autocellular junctions (Jazwinska et al., 2003), was normal in mmy^{IK63} mutants (Fig. 5G,J). This indicates that zygotic *mmy* is not generally required for apical protein secretion.

We also observed that mmy^{IK63} mutants display severely reduced WGA-labelling in embryonic tissues not known to produce chitin, including the early epidermis and the salivary glands (Fig. 5M-T). Moreover, labelling for Crb revealed local tube dilations in the single-cell-layered Malphigian tubules (both in the common ureter and in the individual tubules), a failure of the salivary gland lumens to enlarge in late embryogenesis, and a dorsal open phenotype in ~10 % of the *mmy* mutant embryos (not shown), which may reflect a need for high levels of GlcNAc-containing substances for these processes.

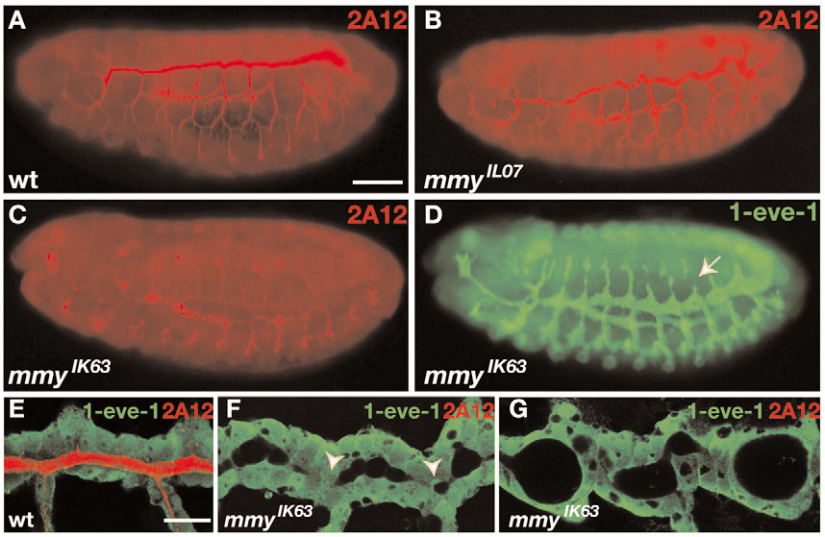

Fig. 4. Mmy is required for uniform tracheal tube growth. (**A-C**) Lateral view of wild-type and *mmy* mutant embryos labelled with the tracheal lumen-specific antibody 2A12. The 2A12 antibody marks the lumen of wild-type (A) and mmy^{IL07} mutant (B) tracheae. The dorsal trunks (DTs) of mmy^{IL07} embryos are slightly convoluted compared with wild type. The tracheal lumen of mmy^{IK63} mutant embryos fails to label with 2A12 (C). (**D**) Expression of the pan-tracheal *lacZ* marker *1-eve-1* in mmy^{IK63} mutants and labelling with anti-β-gal reveal a correctly patterned tracheal network in these mutants, but some of the narrower ganglionic and dorsal branches are discontinuous (arrow indicates a dorsal branch gap). (**E-G**) Confocal analysis of two or three DT segments in embryos that carry the pan-tracheal lacZ marker *1-eve-1*, doubly labelled with 2A12 (red) and β-gal (green). In stage 15 mmy^{IK63} mutants (F), the DT fusion branches fail to expand (arrowheads); at stage 16 (G), the DTs develop severe constrictions and dilations, when compared with the wild type (E). Scale bars: 50 μm in A-D; 10 μm in E-G.

mmy encodes the *Drosophila* UDP-N-acetylglucosamine-pyrophosphorylase

In order to identify the gene disrupted in *mmy* mutants, we first mapped the mutation to deficiency Df(2L)BSC6 on chromosome 2 (Fig. 1D). P-element induced mutant fly strains known to segregate lethal mutations in this region were subsequently tested in complementation crosses to the EMS-induced mmy^{IK63} and mmy^{IL07} heterozygous flies. Two P-element insertions isolated in the BDGP Gene Disruption project (Bellen et al., 2004), KG08617 and KG04349, failed to complement the *mmy* EMS-induced mutations. KG08617 fails to complement the lethality of both *mmy* EMS-alleles, and embryos homozygous for this P-element insertion develop a cuticle phenotype similar to homozygotes for mmy^{IK63} (Fig. 1C and not shown), whereas KG04349 complements lethality, but the transhetereozygous flies are sterile.

KG08617 is located within the first intron of the predicted gene CG9535, and KG04349 resides in the 5′ UTR of CG9535 (Fig. 6A). To verify that the P-element insertion in KG08617 was indeed responsible for the lethality and the *mmy* phenotype, we excised this P-element and established 13 independent excision lines. Three of these are homozygous viable and naturally complement mmy^{IK63}, whereas 10 new lethal alleles were generated that fail to complement mmy^{IK63}. To further prove that *mmy* is equivalent to CG9535, we sequenced the CG9535 open reading frame in both mmy^{IL07} and mmy^{IK63} EMS-induced mutant alleles (Fig. 6B). The mmy^{IK63} allele harbours a base-pair change that causes the exchange of an invariant $glycine^{261}$ to valine, and in the mmy^{IL07} allele a singe base-pair changes alters the $isoleucine^{197}$ codon to that of asparagine. Taken together, these experiments prove that CG9535 is indeed *mmy*.

CG9535 is predicted to code for the *Drosophila* UDP-N-acetylglucosamine-pyrophosphorylase (UDP-GlcNAc-Pyp), a cytosolic enzyme catalyzing the formation of UDP-N-acetylglucosamine (UDP-GlcNAc) from UTP and GlcNAc-1-Phosphate (Fig. 6C) (Merzendorfer and Zimoch, 2003). CG9535 is the only predicted UDP-GlcNAc-Pyp in *Drosophila*, and displays high amino acid homology to the UDP-GlcNAc-Pyp in both yeast and humans (39% and 51% identity, respectively) (Fig. 6B). As UDP-GlcNAc is the substrate for chitin synthases (Merzendorfer and Zimoch, 2003), a reduced level of UDP-GlcNAc in zygotic mmy^{IK63} mutants would explain their chitin-deficiency in the luminal tracheal matrix and in the cuticle, as well as a general loss of GlcNAc-group in aECMs.

Protein modification is impaired in mmy^{IK63} embryos

Yeast and human UDP-GlcNAc-Pyp have been shown to exhibit also a UDP-N-acetylgalactosamine (UDP-GalNAc)-Pyp activity (Wang-Gillam et al., 2000; Peneff et al., 2001). Moreover, UDP-GlcNAc can be converted to UDP-GalNAc by UDP-N-acetylglucosamine 4-epimerase (Winans and Bertozzi, 2002; Mok et al., 2005). Both UDP-GlcNAc and UDP-GalNAc are essential components of the polysaccharide moieties that are added to secreted proteins upon glycosylation and GPI-anchor synthesis in the ER and the Golgi apparatus.

In order to test whether *mmy* mutants display dysfunctional protein maturation along the secretory pathway, we compared the size of the extracellular cuticle protein Knickkopf (Knk) (Jürgens et al., 1984; Ostrowski et al., 2002) in wild-type and *mmy* mutant larvae on Western blots (Fig. 7A). The 689 amino acids Knk protein is predicted to be N-glycosylated at three positions (N^{170}, N^{354} and N^{636}), O-glycosylated at S^{209} and T^{468}, and to possess a GPI-anchor (Mayor and Riezman, 2004; Moussian et al., 2006). In extracts of mmy^{IK63} and $mmy^{KG08617}$ larvae, no wild-type sized Knk can be detected. Instead several proteins of smaller molecular weight are recognised by the Knk antiserum, indicating that post-translational modifications of Knk indeed are affected in the mutant larvae. In addition, smaller molecular weight species of the plasma-membrane protein Tout-velu (Ttv) (Bellaiche et al., 1998), which is predicted to be N-glycosylated at N^{71}, N^{327} and N^{476}, are present in *mmy* mutant larvae when compared with the wild type, whereas no size changes are observed for the intracellular membrane-bound Syntaxin1A (Schulze and Bellen, 1996) (Fig. 7B). Treatment of protein extracts from wild-type larvae with EndoH, an enzyme that removes N-glycosylated modification from proteins, produce Knk and Ttv proteins of even higher mobility than those seen in *mmy* mutant larval extracts (Fig. 7A,B), indicating that protein glycosylation is not completely abolished in *mmy* mutant larvae. By contrast, in protein extracts from mmy^{IL07} mutant larvae, which display strikingly similar cuticle phenotypes as *knk* and *rtv* mutants (Ostrowski et al., 2002; Moussian et al., 2005b), and $mmy^{KG04349}$ mutant larvae, Knk and Ttv migration is not detectably altered, indicating that protein modification in these mutant backgrounds is not dramatically impaired.

To test whether impaired modification of Knk may affect its subcellular localisation, we analysed the distribution of Knk in the epidermis of stage 16 wild-type and mmy^{IK63} embryos labelled with

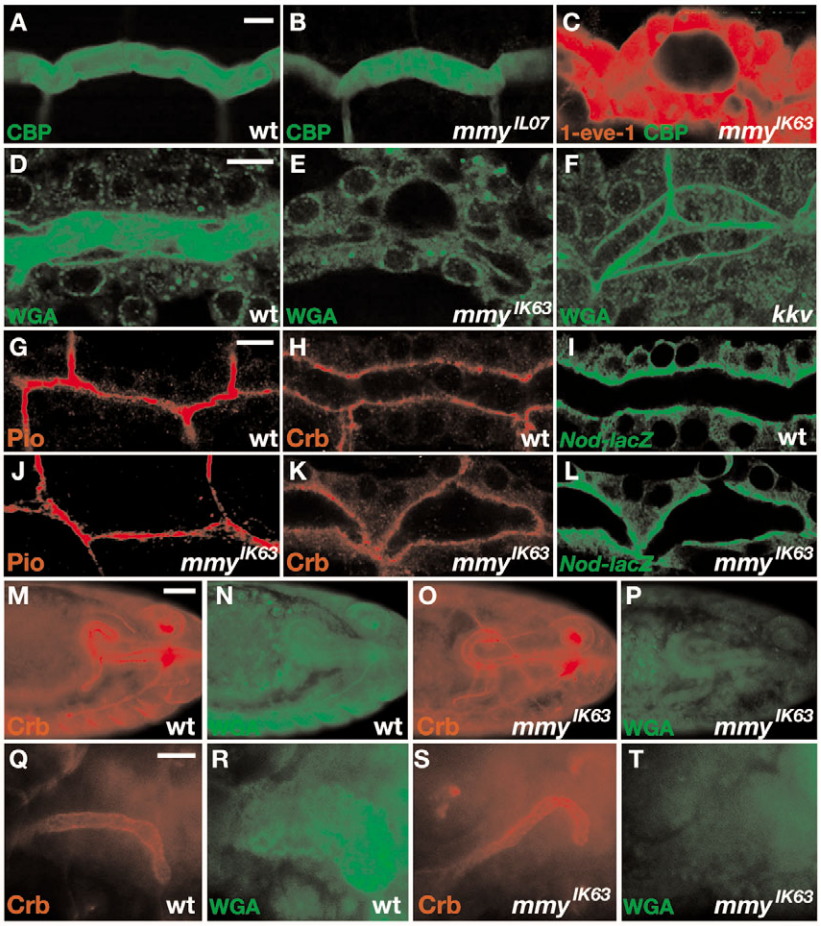

Fig. 5. GlcNAc-groups, including tracheal chitin, are severely reduced in *mmy* mutants. (**A-C**) Detection of chitin with a FITC-conjugated chitin-binding probe (CBP) in wild-type and *mmy* mutant embryos. CBP labelling reveals a broad intraluminal chitin cable with filamentous appearance in stage 15 wild-type tracheae (A). The chitin cable in mmy^{IL07} mutants has a slightly grainy appearance (B), and is absent in mmy^{IK63} mutants (C). The mmy^{IK63} embryo in C is also labelled for the *1-eve-1* marker with β-gal (red) to visualise the tracheal epithelium. (**D-F**) Detection of GlcNAc-group in tracheae with FITC-conjugated WGA. In wild-type tracheae, WGA labels the lumen and the apical surface of tracheal cells (D). In mmy^{IK63} mutant tracheae, neither the lumen nor the apical plasma membrane is labelled with WGA (E). By comparison, in chitin-deficient kkv^{1} embryos, the luminal detection is reduced to a thread of non-chitinous filament, whereas labelling of the apical plasma membrane appears to be normal (F). (**G-L**) mmy^{IK63} mutants display normal apicobasal polarity and can secrete the luminal Pio protein. Stage 15 wild-type (H) and mmy^{IK63} (K) embryos stained with Crb (red) reveal a correct localisation of this apical determinant. The microtubuli minus-end reporter Nod-β-gal localises to the apical cortex in wild-type tracheal cells (I) as well as in mmy^{IK63} mutant tracheae (L). Wild-type (G) and mmy^{IK63} mutant embryos (J) stained with the Pio antibody (red) reveals that the Pio filament is present in mmy^{IK63} mutant tracheae. One segment of the DT is shown in G-L. (**M-T**) Double labelling with WGA and Crumbs in wild-type and mmy^{IK63} mutant embryos. In wild-type stage 14 embryos (M,N), WGA labels the tracheal lumen as well as the developing epidermis (M), whereas mmy^{IK63} mutants at the same stage (O,P) show severely reduced WGA-labelling in both tissues (P). WGA also detects intracellular spots in wild-type stage 15 salivary glands (Q,R), presumably secretory vesicles, but not in mmy^{IK63} mutants at the same stage (S,T). Scale bars: 3 μm in A-C; 4 μm in D-L; 30 μm in M-P; 10 μm in Q-T). Scale bars: 3 μm in A-C; 4 μm in D-F; 30 μm in G-J; 10 μm in K-N; 4 μm in O-T.

the Knk antiserum (Fig. 7C,D). In the wild-type embryo, Knk is detected along the apical surface of the epidermal cell (Fig. 7C), whereas in mmy^{IK63} mutant embryos, only low levels of Knk signal are detected at the apical epidermal surface in addition to a faint cytoplasmic signal (Fig. 7D). This indicates that modification of Knk is required for its stability and localisation to the apical plasma membrane.

mmy expression is temporally up-regulated in developing epithelia

Although Mmy is a metabolic enzyme presumably required for housekeeping functions in most cells, the specific requirements for zygotic *mmy* in epithelial organisation and aECM formation lead us to investigate its developmental expression pattern. In situ hybridisations to detect *mmy* mRNA produced a strong signal in early embryos before zygotic mid-blastula transcription starts (Fig. 8A), indicating a maternal supply of *mmy* RNA. Indeed, embryos derived from *mmy* mutant germline clones arrest development before cellularisation (not shown), suggesting that maternal *mmy* provides UDP-GlcNAc for basic cellular needs during early embryogenesis. The zygotic *mmy* RNA is detected from stage 11, displaying a strong and temporary upregulation in different epithelial tissues. The *mmy* gene is first transcribed in tracheal cells just after they have invaginated as epithelial sacks from the ectoderm (Fig. 8C) (Manning and Krasnow, 1993), and continues to be expressed at high levels in the tracheae until stage 15 (Fig. 8D,E,G). This mid-embryonic *mmy* expression correlates with luminal chitin deposition during tube expansion (Tonning et al., 2005). At stage 16, *mmy* is strongly expressed in the epithelium of the developing salivary glands (Fig. 8F,H), which are not known to synthesise chitin, but may require high levels of *mmy* to accommodate extensive protein secretion. First, at late stage 16, and concurrent with chitin deposition, the *mmy* transcript is detected in the epidermis (Fig. 8F,I).

mmy expression is altered in mutants that disrupt 20-Hydroxyecdysone biosynthesis

The dynamic and tissue-specific zygotic expression of *mmy* may be subjected to feedback regulation triggered by UDP-GlcNAc consumption during aECM formation. However, a hypothetical accumulation or lack of UDP-GlcNAc in *kkv* or mmy^{IK63} mutant embryos, respectively, did not detectably alter the levels of *mmy* transcript, as *mmy* was normally expressed in these mutants (not shown).

As *mmy* belongs to the group of Halloween mutants, including *shadow* (*sad*) (Warren et al., 2002) and *shade* (*shd*) (Petryk et al., 2003), which disrupt enzymes needed for biosynthesis of the insect hormone 20-hydroxyecdysone (Gilbert, 2004), we asked whether temporal expression of *mmy* in different epithelia may depend on 20-hydroxyecdysone. The *sad* gene encodes a mitochondrial P450 enzyme (CYP315A1), a C2-hydroxylase that catalyses the

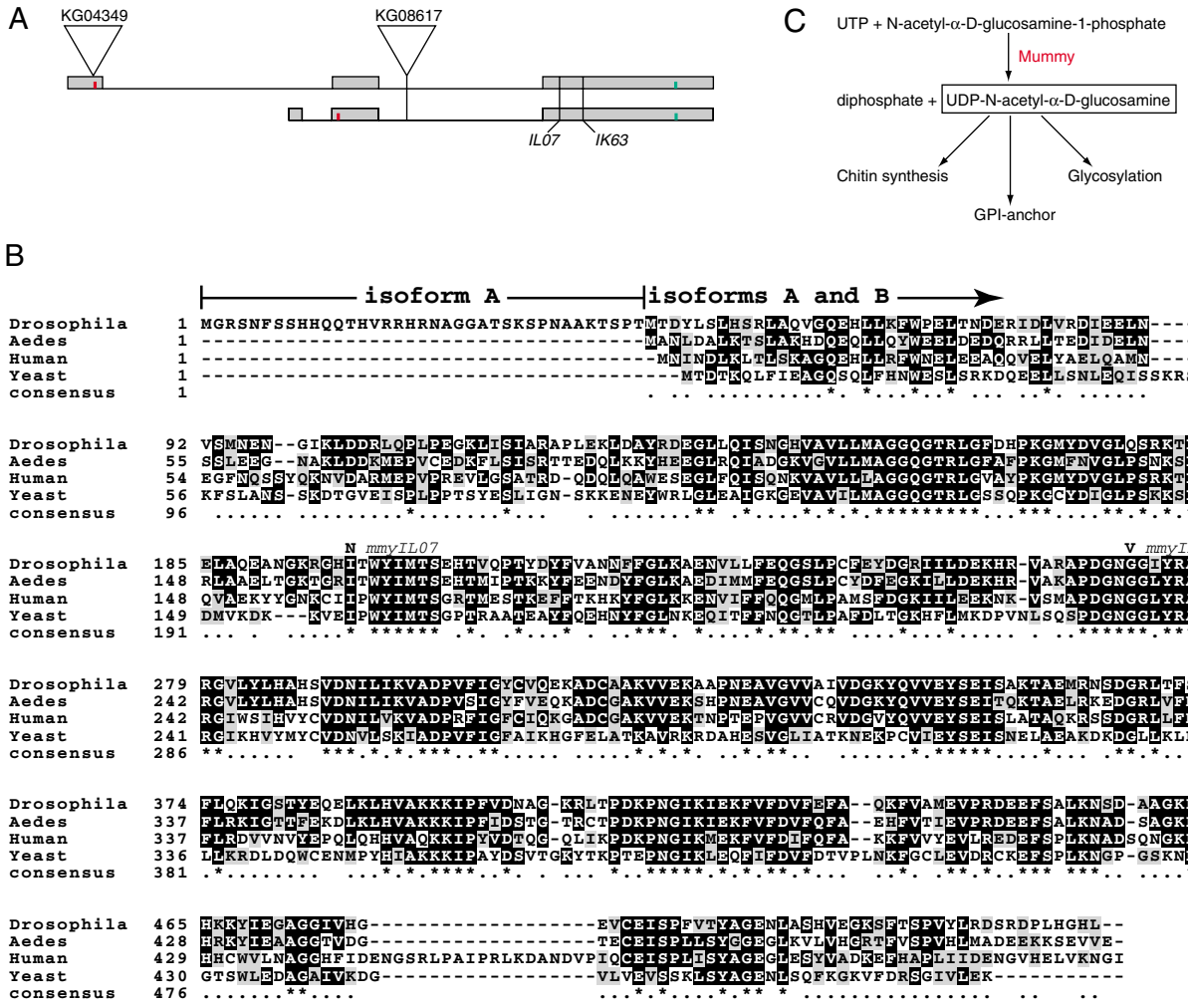

Fig. 6. Mmy encodes the *Drosophila* UDP-N-acetylglucosamine pyrophosphorylase. (**A**) Genome organisation of *mmy*, showing the exons (grey boxes) used in each of the two *mmy* transcripts RA and RB. Start and stop codons are marked in red and green, respectively. The two EMS-induced *mmy* mutations mmy^{IK63} and mmy^{IL07} introduce single mis-sense mutations in the third common exon, whereas P-element KG04349 is inserted 5′ to the ATG of RA, and P-element KG08617 disrupts the second intron of both RA and RB. (**B**) Sequence alignment of the Mmy protein with its orthologues in yeast, human and *Aedes*. The mis-sense mutations in mmy^{IK63} and mmy^{IL07} cause substitution of conserved amino acids as indicated above the sequence. The extra 37 amino acids in isoform A are underlined and do not show homology to Mmy orthologues. (**C**) Illustration of the biochemical reaction catalysed by Mmy and the main biological processes that require the Mmy product.

formation of ecdysone from 2-deoxyecdysone; *shd* encodes the enzyme CYP314A1, which is responsible mono-oxygenation of ecdysone to generate the active 20-hydroxyecdysone. Indeed, two aspects of *mmy* expression are similarly altered in embryos mutant for *sad* and *shd*. First, we find that *shd* and *sad* mutants lack the mid-embryonic up-regulation of tracheal *mmy* expression, and second, *mmy* expression is prematurely upregulated in the epidermis, salivary gland and proventriculus (Fig. 8J-O). Thus, a mid-embryonic ‘ecdysone pulse’ appears essential to control the temporal expression of *mmy* in different embryonic epithelia.

DISCUSSION

We have reported a requirement for Mmy in apical extracellular matrix (aECM) deposition and epithelial morphogenesis, and identified Mmy as the *Drosophila* UDP-N-acetylglucosamine-pyrophosphorylase (UDP-GlcNAc-Pyp), a cytosolic enzyme that provides UDP-GlcNAc for protein maturation and polysaccharide formation.

mmy encodes the *Drosophila* UDP-N-acetylglucosamine-pyrophosphorylase

Formation of UDP-GlcNAc from GlcNAc-6-phosphate and UTP has been demonstrated in vitro for the yeast protein UDP-GlcNAc-Pyp (UAP1) (Mio et al., 1998). Yeast cells lacking UAP1 activity have an abnormal bloated cell shape indicative of a weakened cell wall. The human orthologue of UAP1 (AGX) was identified in the same study and was able to rescue the cell lethal phenotype of *UAP1* mutants, demonstrating a conserved function for UDP-GlcNAc-Pyp among species. Mmy (CG9535) is the predicted *Drosophila* orthologue of yeast and human UDP-GlcNAc-Pyp, and is thus expected to be responsible for UDP-GlcNAc synthesis in *Drosophila*. UDP-GlcNAc-Pyp from different species have also been shown to have UDP-GalNAc-Pyp activity (Szumilo et al., 1996; Peneff et al., 2001). In addition, UDP-GlcNAc is converted to UDP-GalNAc by the UDP-*N*-acetylglucosamine 4-epimerase (Winans and Bertozzi, 2002). Hence, *Drosophila* Mmy may also be responsible for UDP-GalNAc production.

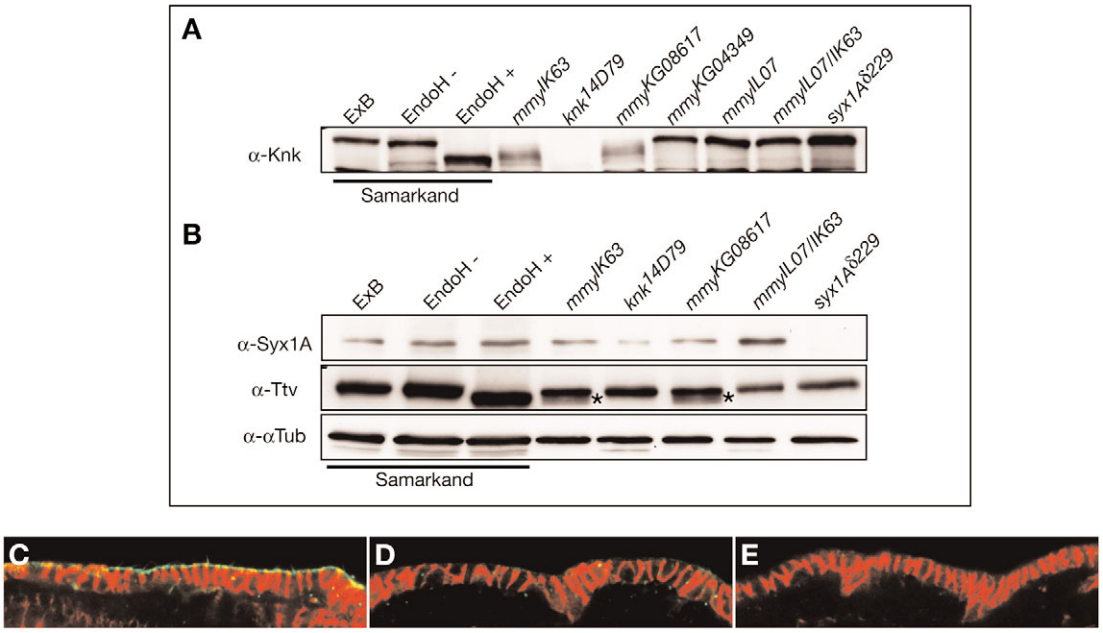

Fig. 7. Post-translational modification of Knk depends on Mmy activity. (**A**) Western blot with protein extracts from wild-type and different mutant larvae labelled with a specific antibody against the extracellular cuticle protein Knk. In the wild type, Knk migrates as a single band (ExB and EndoH-), and EndoH treatment to cleave N-glycosylated sugar-residues generates smaller co-migrating Knk species (EndoH+). In *mmy*^{IK63} and *mmy*^{KG08617}, several Knk proteins with different sizes are present. No Knk protein is detected in *knk*^{14D79} mutants, and Knk protein migration is not affected in the remaining mutants tested. (**B**) Western blot with the same extracts as in A labelled with antisera against the membrane-attached Syntaxin1A (Syx1A), the transmembrane protein Tout-velu (Ttv) and cytosolic α-Tubulin (αTub) as loading control. Ttv also migrates as a smaller protein after EndoH-treatment, and in *mmy*^{IK63} and *mmy*^{KG08617} mutant larvae differently sized Ttv proteins are present (stars). (ExB, extraction buffer PLC). (**C**-**E**) Co-labelling of stage 16 wild-type, *knk* mutant and *mmy*^{IK63} mutant epidermis with anti-Knk (green) and anti-Fas3 (red). Knk localises to the apical plasma membrane in the wild type (C) and is absent in *knk* mutants (E). In the *mmy*^{IK63} mutant epidermis, the amount of Knk is severely reduced in the apical membrane, and some signal is detected within the cell (D).

The EMS induced mutations *mmy*^{IK63} and *mmy*^{IL07} identify important amino acids for Mmy activity. Gly^{261} that is exchanged to valine in the Mmy^{IK63} protein corresponds to Gly^{224} in the human AGX that has been shown to be essential for enzyme activity by site-directed mutagenesis and lies in a loop contacting uridine, as demonstrated by crystallographic experiments (Wang-Gillam et al., 2000; Peneff et al., 2001). As the phenotypes caused by the exchange of Gly^{261} and by the deletion of *mmy* in our P-element excision-lines are identical, it is conceivable that this mutation causes a loss of Mmy function. The highly conserved Ile^{197} that is mutated in the *mmy*^{IL07} allele has not yet been described to be essential for UDP-GlcNAc-Pyp activity. This amino acid exchange permits the production of some UDP-GlcNAc, as many processes that require zygotic Mmy activity appear normal in *mmy*^{IL07} embryos.

Mmy has distinct functions during aECM formation

Embryos homozygous mutant for *mmy* have severe defects in their epidermal and tracheal organisation. In particular, consistent with the molecular function of Mmy, the most striking deficiency in these tissues is the absence of chitin both in the procuticle and the developing tracheal lumen. However, the *mmy* mutant epidermis and tracheae display additional defects compared with embryos lacking chitin. For example, the *mmy*^{IK63} mutant tracheal lumen fails to label with the 2A12 antibody, and the *mmy*^{IK63} mutant procuticle appears collapsed, neither of which is seen in chitin-deficient embryos. Another component essential for cuticle differentiation and luminal tracheal matrix formation is the glycosylated and GPI-anchored extracellular protein Knk (Ostrowski et al., 2002; Moussian et al., 2006). We find that Knk is a target of Mmy activity, as embryos homozygous for the amorphic alleles *mmy*^{IK63} and *mmy*^{KG08617} produce Knk proteins with reduced molecular weight, compared with wild-type Knk, indicative of impaired glycosylation, obviously affecting its localisation and function. However, paired Knk function in combination with lack of chitin is not sufficient to explain fully the aECM defects in *mmy* mutants, as loss of Knk and chitin cause similar phenotypes to loss of chitin only (Ostrowski et al., 2002; Moussian et al., 2006). The observed requirement of zygotic Mmy for Knk modification instead suggests that additional proteins needed for cuticle differentiation and tracheal luminal matrix formation, such as Retroactive (Rtv) (Moussian et al., 2005b), are affected in *mmy* mutants.

The disrupted aECMs in *mmy* mutants may also be contributed to by disorganised aECM-producing epithelia. Although zygotic Mmy is not required for epithelial apicobasal polarity, we find that SJ in *mmy* mutant epithelia fail to form the typical ladder-like structure, which occasionally is accompanied by slight defects in Fas3 localisation at these junctions. Mutants lacking single SJ components display defects both in tracheal luminal matrix formation and cuticle deposition (Llimargas, 2000; Behr et al., 2003; Wu et al., 2004). This role of SJ components may be, at least partially, mediated through correct localisation of the cuticle organising Knk protein (Moussian et al., 2006). Moreover, the tracheal lumen of embryos deficient for both chitin and single SJ components fails to label with the 2A12 antibody, as seen in *mmy*

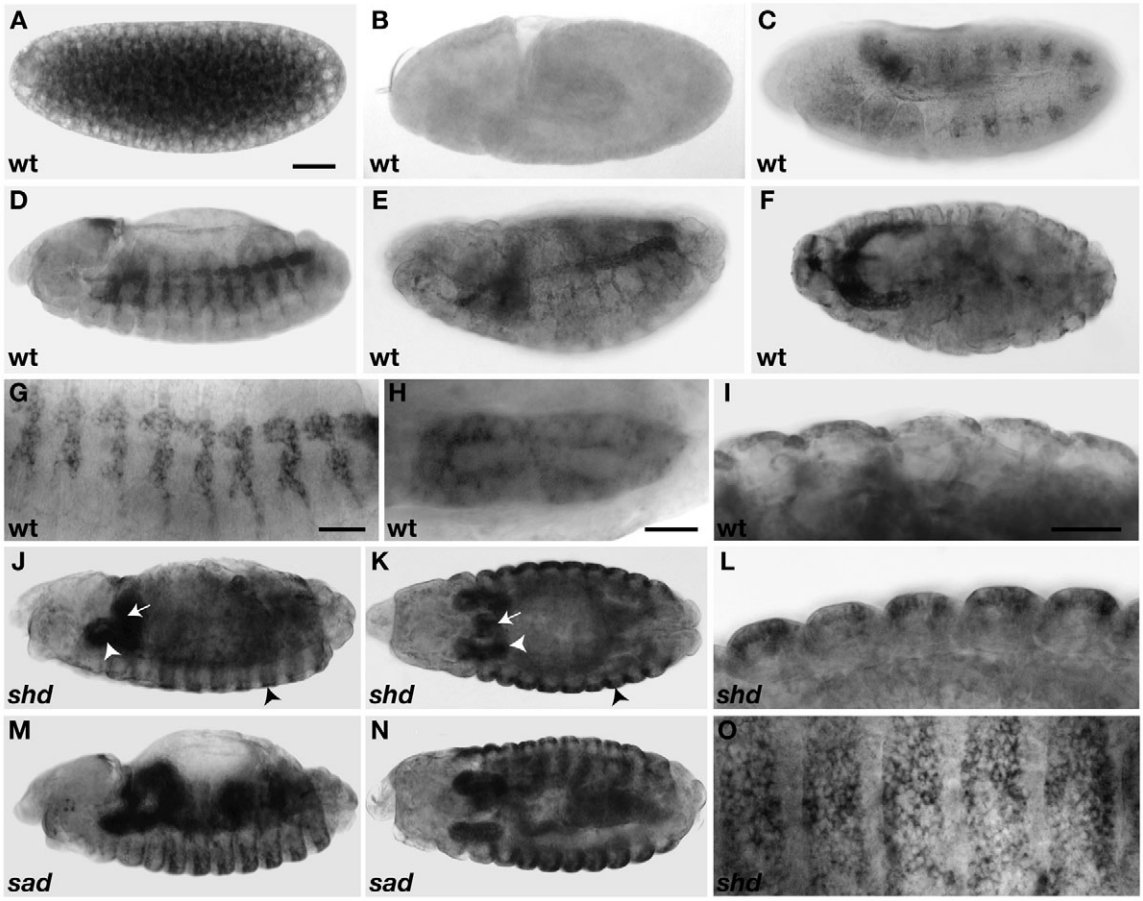

Fig. 8. Mmy expression is temporally upregulated in developing epithelia and altered in mutants required for 20-hydroxyecdysone biosynthesis. (**A-I**) In situ hybridisation of wild-type embryos with *mmy* antisense probe. Embryos at early blastoderm stage display high levels of maternal *mmy* RNA (A), but no *mmy* transcript is detected in embryos between stages 5-10 (B; stage 8). Zygotic *mmy* expression is observed from stage 11 in tracheal cells as they invaginate from the ectoderm (C) and persists in the developing tracheal system until stage 15 (D,E,G) after which it decreases. At stage 16 *mmy* is strongly expressed in the salivary glands (F,H) and weakly detected in the epidermis (F,I). (**J-O**) In *shade* (*shd*) mutant embryos labelled with the *mmy* antisense probe, tracheal expression of *mmy* is not detected (J), instead *mmy* is prematurely expressed in the epidermis (J, black arrowhead; L,O), salivary gland (J,K; white arrowhead) and proventriculus (J,K; white arrow) during stage 15. A similar pattern of *mmy* expression is also found in mutants for *shadow* (*sad*; M and N). Scale bars: 50 μm in A-F,J,K,M,N; 25 μm in G; 10 μm in H; 25 μm in I,L,O.

mutants (Tonning et al., 2005). Thus, *mmy* may also affect the aECM through its need for SJ assembly. Given the requirements for *mmy* in protein modification along the secretory pathway, Mmy is likely to affect primarily the localised deposition and function of extracellular SJ proteins.

Regulation of *mmy* expression during development

Both UDP-GlcNAc and UDP-GalNAc are imported from the cytosol into the endoplasmic reticulum, where they are used by specific transferases for glycosylation and GPI-anchor synthesis. Mmy is therefore expected to be essential for cellular housekeeping functions, and its activity regulated to provide sufficient, but not excess accumulation of its end product. Indeed, the activity of UDP-GlcNAc-Pyp in the human parasite *Giardia lamblia* is under allosteric control of the upstream metabolite glucosamine-6-phosphate (Bulik et al., 2000). In addition, transcription control of chitin synthase and components of the hexosamine pathway occurs in the mosquito midgut epithelium when the apical chitin-containing peritrophic matrix is altered upon contact with a blood meal (Lemos et al., 1996; Smartt et al., 1998; Ibrahim et al., 2000). We find that *mmy* is regulated also at the transcriptional level in that it is strikingly upregulated in different epithelia at the time they produce aECMs. High levels of *mmy* transcript, for example, are limited first to the period of luminal chitin accumulation during tube expansion and later turns on when cuticle deposition begins.

Our data indicate that *mmy* expression is under the control of the insect steroid hormone 20-hydroxyecdysone. There are several peaks of 20-hydroxyecdysone levels during insect development, and the role of these peaks during larval moults and metamorphosis are well studied (Thummel, 2001). In addition, 20-hydroxyecdysone levels peak at mid-embryogenesis, but the cellular mechanisms activated by this rise of 20-hydroxyecdysone have been unclear. Phenotypically, *mmy* belongs to the so-called Halloween mutants (Chavez et al., 2000), which develop a faint cuticle including *shadow* (*sad*) and *shade* (*shd*) (Jürgens et al., 1984; Nüsslein-Volhard et al., 1984) coding for 20-hydroxyecdysone-producing enzymes (Gilbert, 2004). Unlike *sad* and *shd*, *mmy* mutant embryos can activate the 20-hydroxyecdysone response element (Chavez et al., 2000), suggesting that zygotic *mmy* itself is not a component of ecdysone signalling pathway. Here, we show that loss of 20-hydroxyecdysone in *sad* and *shd* mutant embryos prevents the strong tracheal *mmy*

expression normally seen from stage 12 to 15. These results indicate that a function of the mid-embryogenic pulse of 20-hydroxyecdysone is to activate a programme for aECM differentiation in the developing tracheae. To date, we cannot explain the implications of the premature upregulation of *mmy* in the epidermis and the salivary glands of 20-hydroxyecdysone deficient embryos; however, 20-hydroxyecdysone levels appear important for early epidermal morphogenesis as *sad* and *shd* mutant embryos fail to undergo head involution and dorsal closure. The temporal onset of *mmy* expression to accommodate sufficient UDP-GlcNAc/GalNAc for aECM formation thus appears ensured by a composite *mmy* promoter that can positively and negatively respond to 20-hydroxyecdysone, depending on tissue type and developmental time frame.

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