

Smt3 is required for *Drosophila melanogaster* metamorphosis

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Sumoylation, the covalent attachment of the small ubiquitin-related modifier SUMO to target proteins, regulates different cellular processes, although its role in the control of development remains unclear. We studied the role of sumoylation during *Drosophila* development by using RNAi to reduce *smt3* mRNA levels in specific tissues. *smt3* knockdown in the prothoracic gland, which controls key developmental processes through the synthesis and release of ecdysteroids, caused a 4-fold prolongation of larval life and completely blocked the transition from larval to pupal stages. The reduced ecdysteroid titer of *smt3* knockdown compared with wild-type larvae explains this phenotype. In fact, after dietary administration of exogenous 20-hydroxyecdysone, knockdown larvae formed pupal cases. The phenotype is not due to massive cell death or degeneration of the prothoracic glands at the time when puparium formation should occur. Knockdown cells show alterations in expression levels and/or the subcellular localisation of enzymes and transcription factors involved in the regulation of ecdysteroid synthesis. In addition, they present reduced intracellular channels and a reduced content of lipid droplets and cholesterol, which could explain the deficit in steroidogenesis. In summary, our study indicates that Smt3 is required for the ecdysteroid synthesis pathway at the time of puparium formation.

KEY WORDS: *Drosophila*, Ecdysone, Metamorphosis, Ring gland, Smt3, Sumoylation

INTRODUCTION

Post-translational modifications modulate the activity of proteins and therefore have crucial roles in many cellular processes. Sumoylation has been involved in the regulation of protein-protein interactions, nuclear localisation, protein-DNA interactions, enzymatic activity and transcription, and can also antagonise ubiquitylation (Geiss-Friedlander and Melchior, 2007; Gill, 2005; Heun, 2007; Ulrich, 2005; Verger et al., 2003). Consequently, it affects diverse cellular processes, such as cell cycle, DNA repair, nuclear body formation, nucleocytoplasmic transport, protein turnover and maintenance of genomic and nuclear integrity. Although a large number of proteins are known to be substrates for SUMO, for most of them the biological function of sumoylation remains to be elucidated.

In yeast, insects and nematodes there is a single SUMO gene (*smt3* in *Drosophila*), whereas in mammals three members have been identified (Johnson et al., 1997). The conjugation of SUMO to target proteins involves four enzymatic reactions. First, a specific hydrolase processes the SUMO precursor into a mature form. Second, an E1-activating enzyme activates mature SUMO. Third, during the conjugation step, SUMO is transferred to the single E2-conjugating enzyme Ubc9 [Lesswright (Lwr) in *Drosophila*]. Subsequently, the covalent interaction between SUMO and the target protein is achieved. Although Ubc9 is able to recognise the sumoylation consensus motif in the target proteins (Rodríguez et al., 2001), efficient and proper modification in vivo requires E3 ligases (Melchior et al., 2003; Sharrocks, 2006). In addition, SUMO

conjugates are susceptible to cleavage by SUMO-specific proteases (Hay, 2007; Yeh et al., 2000). In *Drosophila*, SUMO components are expressed during all developmental stages (Long and Griffith, 2000), although their role in the control of development remains unclear. Previous studies have shown a role for *lwr* in embryonic patterning (Epps and Tanda, 1998). Hypomorphic mutations in *lwr* result in a prolonged larval life followed by death (Chiu et al., 2005), suggesting a role for sumoylation in development and metamorphosis that has been largely unexplored until now.

Three major hormones regulate most aspects of post-embryonic development in holometabolous insects: the prothoracicotrophic hormone (PTTH), 20-hydroxyecdysone (20E) and the juvenile hormone (JH) (Berger and Dubrovsky, 2005). In Lepidoptera, PTTH, produced by a pair of neurosecretory cells located in the dorsomedial region of the brain, is required to stimulate the synthesis of ecdysone (E). In *Drosophila*, E is synthesized in the prothoracic gland (PG) cells of the ring gland and then secreted to the hemolymph and converted to its active form, 20E, in target tissues (for a scheme of the ring gland in *Drosophila*, see Fig. 1A). Active 20E interacts with specific receptors, activates response genes and triggers genetic programs in target tissues (Ashburner, 1974; Thummel, 2002). During larval stages (or instars) periodic pulses of 20E before each larval molt act in concert with the sesquiterpenoid JH, secreted by the corpus allatum (CA) in the ring gland, to ensure the transition to the next larval instar. In *Manduca sexta*, at the end of the last larval instar, the JH titer drops and the peak of 20E initiates metamorphosis (Nijhout and Williams, 1974). However, the roles of JH and PTTH during metamorphosis are less studied in *Drosophila*, where the drop of JH titer or PTTH requirement for ecdysone production have not been demonstrated (McBrayer et al., 2007).

In arthropods, ecdysteroids are synthesized from cholesterol or phytosteroids. The biosynthetic pathway from cholesterol to 20E is not completely characterised, although several members of the Halloween gene family mediate steroid hormone biosynthesis in *Drosophila* (Gilbert, 2004; Gilbert and Warren, 2005; Rewitz et al., 2006). The genes *phantom* (*phm*), *disembodied* (*dib*), *shadow* (*sad*)

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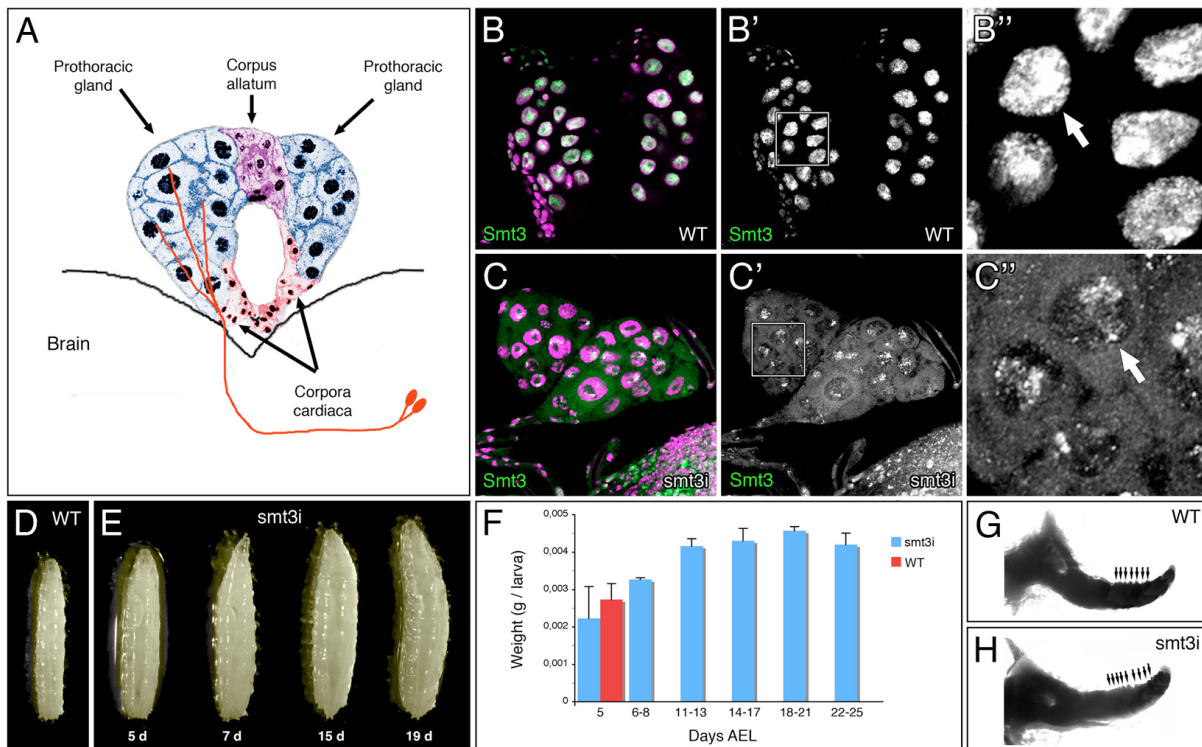


Fig. 1. Smt3 role on development and metamorphosis. (A) Cartoon of the ring gland, a neuroendocrine complex located above the brain, composed of: the PG cells that produce the ecdysone hormone (blue); the corpus allatum cells that produce the juvenile hormone (purple); and the corpora cardiaca (red). The PG is innervated by neurosecretory cells depicted in red (only one hemisphere has been represented). (B,C) Ring gland staining showing nuclei (purple) and Smt3 expression (green). (B',C') Green channels showing Smt3 expression only are shown in black and white. Boxed regions are magnified in B'' and C''. Smt3 levels are strongly reduced in *smt3i* nuclei (C',C'') compared with wild type (B',B''), although some residual protein can be observed in *smt3i* PG cells (arrows). (D-F) *smt3i* larvae do not pupariate but continue growing as larvae, becoming approximately double the weight at 18–21 days AEL (F). (G,H) The morphology and the number of teeth (arrows) in mouth hooks indicate that *smt3i* larvae reached the third instar and stayed at that stage throughout the rest of their prolonged larval life.

and *shade* (*shd*) encode cytochrome P450 enzymes that catalyse the final four sequential hydroxylation steps in the conversion of cholesterol to active 20E. Recently, *spook* and *spookier* (*spok*) have been implicated in ecdysteroid biosynthesis (Ono et al., 2006), although their function is currently unknown. In addition, a Rieske-domain protein, Neverland, has been implicated in the conversion of cholesterol to 7-dehydrocholesterol (7dC), the first enzymatic reaction of the pathway (Yoshiyama et al., 2006). Little is known about the regulation of the ecdysteroid biosynthesis enzymes and only a few transcription factors have been involved in this pathway, including Without children (Woc) (Warren et al., 2001; Wismar et al., 2000), Molting defective (Mld) (Neubueser et al., 2005) and the β isoform of Fushi tarazu-factor1 (β Ftz-f1) (Parvy et al., 2005). Woc controls the conversion from cholesterol to 7dC, Mld is involved in the regulation of *spok*, and β Ftz-f1 is involved in the transcriptional regulation of *dib* and *phm* (Ono et al., 2006; Parvy et al., 2005; Warren et al., 2001). Several other genes in *Drosophila* are implicated in the control of ecdysone titers, such as *ecdysoneless* (*ecd*) (Henrich et al., 1987), *giant ring gland* (*grg*) (Klose et al., 1980), *dare* (Freeman et al., 1999), *giant* (Schwartz et al., 1984), *dre4* (Sliter and Gilbert, 1992) or the *inositol 1,4,5,-tris-phosphate receptor* (Venkatesh and Hasan, 1997), although for most of them their mechanism of participation in steroidogenesis is unclear.

Here, we present our studies on the *in vivo* function of Smt3 during *Drosophila* post-embryonic development. Our results indicate that Smt3 has a role in the regulation of ecdysteroid levels

and is required for the larval to pupal transition. Reduced levels of *smt3* produce low ecdysteroid titers, abnormalities in the subcellular localisation and/or expression levels of factors involved in the regulation of ecdysteroid synthesis, reduced cholesterol content, and alterations in nuclear and plasma membranes in PG cells. Taken together, our results show a specific requirement of Smt3 to complete the developmental transition from larval to pupal stage in *Drosophila*.

MATERIALS AND METHODS

Drosophila strains

Flies were raised on standard *Drosophila* medium at 25°C. Mutant strains *lwr⁴⁻³* and *lwr⁵* were obtained from Bloomington *Drosophila* Stock Centre. The wild-type (WT) control strain was Vallecas. Gal4 strains *Aug21-Gal4/CyO-GFP* (hereinafter *Aug21-Gal4*) and *phm-Gal4,UAS-mCD::GFP/TM6B,Tb* (hereinafter *phm-Gal4*) were obtained from P. Leopold and C. Mirth (Colombani et al., 2005; Mirth et al., 2005). Information about strains not described in the text can be found in FlyBase (<http://flybase.bio.indiana.edu>).

Plasmid construction and generation of transgenic strains

Knockdown experiments were performed using the GAL4/UAS system (Brand and Perrimon, 1993). To generate *UAS-smt3i*, *smt3* cDNA was amplified by PCR using specific primers (Fw 5'-GCTCTAGAGC-ATGCCAGCTTCAACAAGCAACCA-3' and Rev 5'-GCTCTAGAAT-CGATTCTTAGGGCCTGGT-3') containing *Xba*I sites for cloning into pWIZ (Lee and Carthew, 2003). Transgenic lines *UAS-smt3i* were generated following standard transformation procedures (Spradling and Rubin, 1982).

Immunocytochemistry

Adults were allowed to lay eggs during 8 hours. Wandering larvae were collected 5, 6, 11 and 15 days after egg laying (AEL), dissected in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde (PFA) for 20 minutes and washed in PBS-Triton X-100 0.3% (PBT) three times, for 20 minutes each. Tissues were then blocked in PBT-BSA for one hour at room temperature (RT) and incubated with the appropriate antibodies at 4°C overnight. The following polyclonal antibodies were used at the indicated dilution: anti-Woc, 1:500 (Raffa et al., 2005); anti-Smt3, 1:500 (Smith et al., 2004); anti-βFtz-f1, 1:20 (Ohno et al., 1994); anti-Phm, 1:100 (Parvy et al., 2005); anti-Dib, 1:200 (Parvy et al., 2005); anti-Mld, 1:200 (Neubueser et al., 2005); polyclonal goat anti-activated-caspase-9 (Santa Cruz Biotechnology), 1:50; rabbit anti-Sad (Abcam), 1:200; rabbit anti-HRP (Jackson ImmunoResearch), 1:600; and mouse monoclonal anti-lamin Dm0 (Developmental Studies Hybridoma Bank), 1:10. The following day, the tissues were washed with PBT three times, for 20 minutes each, and incubated with secondary antibodies at RT for two hours. Fluorescent Alexa 568- and 633-conjugated secondary antibodies (Molecular Probes) were used at a 1:200 dilution. DAPI (Roche) and Phalloidin-TRITC (Sigma) were used at a 1:2000 dilution. Stained brains and ring glands were mounted in Vectashield mounting medium (Roche). Confocal images were taken with a Leica DM IRE2 microscope and images were processed using the Leica Confocal Software and Adobe Photoshop.

Filipin and Oil Red O stainings

Ring glands were fixed in 4% PFA for 20 minutes, washed twice in PBS and stained with 50 µg/ml of filipin (Sigma) for 1 hour or incubated in an Oil Red O (Sigma) solution at 0.06% for 30 minutes. Samples were washed twice with PBS before mounting in Vectashield (Roche). Pictures were taken with a Leica DM IRE2 confocal microscope.

Quantification of lipid droplets was done on single plane confocal micrographs of Oil Red O or filipin stainings using the 'Analyze particle' tool from ImageJ software. At least 10 independent micrographs were analyzed from WT and *smt3i* PG cells.

Rescue experiments

UAS-*smt3i* flies were crossed with a *phm-Gal4* driver to obtain *smt3i*-RNAi larvae (hereinafter called *smt3i*). *smt3i* larvae (lacking *TM6B,Tb*) and controls were collected at 120 hours AEL and placed in groups of 10 individuals in new tubes supplemented with 20E (Sigma) dissolved in ethanol at 1 mg/ml and mixed with yeast. Control larvae were fed with yeast mixed only with ethanol.

Ecdysteroid titers and weight quantifications

Ecdysteroid levels were quantified by ELISA following the procedure described by Porcheron et al. (Porcheron et al., 1976), and adapted by Romañá et al. (Romañá et al., 1995). 20E (Sigma) and 20E-acetylcholinesterase (Cayman Chemical) were used as the standard and enzymatic tracer, respectively. The antiserum (Cayman Chemical) was used at a dilution of 1:50,000. Absorbance was read at 450 nm using a Multiscan Plus II Spectrophotometer (Labsystems). The ecdysteroid antiserum has the same affinity for ecdysone and 20E (Porcheron et al., 1976), but because the standard curve was obtained with the latter compound, results are expressed as 20E equivalents. For sample preparation, 15 staged larvae were weighed and preserved in 600 µl of methanol. Prior to the assay, samples were homogenized and centrifuged (10 minutes at 18,000 g) twice and the resultant methanol supernatants were combined and dried. Samples were resuspended in 50 µl of enzyme immunoassay (EIA) buffer (0.4 M NaCl, 1 mM EDTA, 0.1% BSA in 0.1 M phosphate buffer).

For weight quantification, *smt3i* and control larvae were collected at 5 days AEL and weighed in groups of fifty larvae. Then, *smt3i* larvae were placed in new tubes and weighed during the next 25 days.

Transmission electron microscopy

smt3i and wild-type wandering third-instar larvae were rinsed in water and opened with forceps in a droplet of 0.1 M PBS, pH 7.3, on a clean microscope slide. The brain with the attached ring gland was removed and immersed directly in ice-cold, freshly prepared fixative containing 2.5% glutaraldehyde and 4% PFA in 0.1 M PBS, pH 7, for six hours. The samples

were then rinsed four times, for 15 minutes each, in PBS, post-fixed for 1 hour in an aqueous 2% solution of osmium tetroxide, rinsed in water, dehydrated in a gradual series of ethanol and acetone, and embedded in EPON (EPON 812 embedding kit 3132, Tousimis) following the manufacturer's instructions. Following the last infiltration step, the samples were moved to pure resin in moulds for polymerization at 60°C for 48 hours. Semi-thin sections (around 2 µm) were cut with a glass knife, mounted on microscope slides, stained with 0.1% boracic Toluidine Blue for histological study and to locate appropriate sites for ultrastructural analysis. Ultra-thin sections (60 to 70 nm) were cut with a diamond knife, contrasted with lead citrate and uranyl acetate, and observed under a JEOL JEM 1010 microscope operated at 80 kV. Images were taken with a digital camera (Hamamatsu C4742-95). Measurements and image processing were done with AMT Advantage CCD and Adobe Photoshop software, respectively. Three to four larvae were analyzed from each sample (genotype and age). *smt3i* samples were fixed at age 120, 144, 168 and 264 hours AEL. Control samples (WT, *phm-GAL4* and *UAS-smt3i*) were all fixed at 144 AEL.

RESULTS

smt3i knockdown produces developmental arrest at third larval instar

smt3i is expressed ubiquitously throughout embryonic and larval stages (see Fig. S1 in the supplementary material) (Lehembre et al., 2000; Shih et al., 2002). To investigate Smt3 function at post-embryonic stages in *Drosophila*, we used the UAS/Gal4 system to drive *smt3i* transgene expression in several tissues, including wing, haltere and eye imaginal discs, salivary gland, ring gland and central nervous system, during development. *UAS-smt3i* with different Gal4 lines produced strong, fully penetrant, phenotypes. Smt3 accumulates at high levels in the nuclei of WT PG cells (Fig. 1B), and its levels were dramatically reduced in *smt3i* larvae when the *phm-Gal4* driver was used (Fig. 1C). As a result of the knockdown, *smt3i* animals arrested their development at the third larval instar (L3) just before pupariation and survived for an additional 3 weeks (Fig. 1D-F). During this time, *smt3i* larvae continued feeding and gaining weight until approximately 21 days AEL (Fig. 1F). These larvae did not present duplicated mouth hooks (Fig. 1G,H), indicating that previous molts were correct, and died as L3 without forming a puparium. Interestingly, *smt3* knockdown in the CA by using *Aug21-Gal4* produced normal progeny with no defects in molting or metamorphosis, and gave rise to normal adult flies. Thus, the role of *smt3* on metamorphosis seems to be due to its role within PG cells. Our results suggest an essential role for Smt3 during post-embryonic development during initiation of the pupariation process.

smt3i larvae show reduced ecdysteroid levels

Insect molting and metamorphosis are controlled by the hormone 20E, which is synthesized from the precursor E produced in the PG. To determine whether the inability of *smt3i* larvae to pupariate was due to reduced levels of ecdysteroids, we measured the ecdysteroid titers in *smt3i* and control larvae. At 120 hours AEL, the levels of ecdysteroids in *smt3i* larvae were only slightly reduced compared with control larvae (Fig. 2A). At 144 hours AEL, these levels increased in the controls, probably corresponding to the level of the 20E peak associated with pupariation, as shown by Warren et al. (Warren et al., 2006). However, the ecdysteroid levels remained unchanged in *smt3i* larvae, suggesting that the 20E peak is absent in the knockdown animals (Fig. 2A). During the abnormally extended larval life of *smt3i* larvae there was a progressive reduction in the ecdysteroid titer, as shown by the levels at 7 and 11 days AEL (Fig. 2A). These results suggest that *smt3i* larvae are not able to produce the ecdysteroid peak required to proceed to pupal stages.

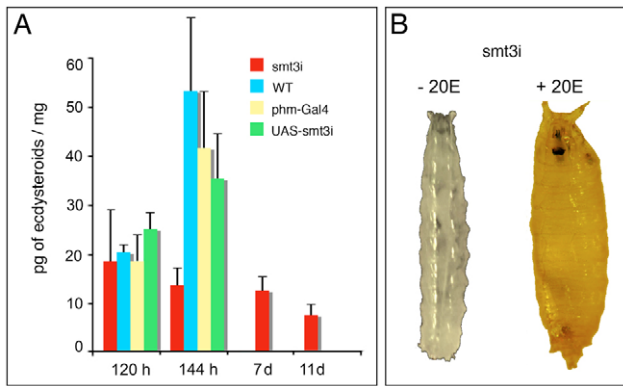


Fig. 2. *smt3i* larvae show low ecdysteroid levels. (A) Graph of the levels of ecdysteroids (E and 20E) in larvae of different genotypes at different times AEL, expressed in pg per mg of larvae. The peak of ecdysteroids that in WT, *phm-Gal4* and *UAS-smt3i* larvae is thought to induce pupariation is not found in *smt3i* larvae. (B) *smt3i* larvae fed with 20E can pupariate. Pupae proceeded to head eversion, but in no instance gave rise to adults.

To further demonstrate that the developmental arrest is due to reduced levels of 20E, we performed ecdysteroid-feeding rescue experiments. L3 *smt3i* fed with medium containing 20E pupariated within 24 hours (100%, $n=30$); however, these pupae were not able to develop further and died, maybe because a higher dose of 20E is required for metamorphosis (Fig. 2B). The control untreated *smt3i* larvae continued as L3 and died several weeks later without signs of molting (Fig. 2B). These results confirm that *smt3i* larvae have reduced levels of ecdysteroids, which could be the reason for their inability to pupariate.

***smt3i* larvae show enlarged PG cells with abnormal nuclei, but not massive degeneration**

The requirement of Smt3 in PG cells for puparium formation prompted us to analyse in detail the ring gland in knockdown larvae. At 120–144 hours AEL, the external morphology of the PGs in *smt3i* larvae (Fig. 3B,F) did not exhibit drastic changes when compared with WT (Fig. 3A). However, some PG cells in *smt3i* larvae had clearly increased size. Nuclei were also enlarged and exhibited an abnormal morphology (compare Fig. 3A with 3B,F). Later, from days 7 to 22 AEL, PG cells and their nuclei continued to grow in size, and, at the same time, the number of PG cells progressively decreased (Fig. 3C,D).

The reduction of ecdysteroids could reflect a massive death of PG cells in knockdown larvae. In fact, Smt3 is necessary for cell survival in some larval structures (Takanaka and Courey, 2005). To analyze this possibility, we used the anti-activated-caspase-9 antibody that recognises the *Drosophila* activated initiator caspase Nc (previously known as Dronc) in apoptotic cells. At 120–144 hours AEL we did not observe features of cell death in most of the *smt3i* PG cells (Fig. 3F), and we only detected active Nc in one or two cells in the PG of some *smt3i* specimens (Fig. 3G). These Nc-positive cells were bigger than the other *smt3i* PG cells and we called them ‘giant cells’ (Fig. 3C,D,G, arrows). Our ultrastructural analysis corroborates these results, showing sporadic apoptotic cells surrounded by non-apoptotic cells (Fig. 3H). There may be a progressive loss of these cells over time, perhaps by detachment from the PG and subsequent loss into the hemolymph.

We focused on the ultrastructural changes described as typical features of PG degeneration during metamorphosis, such as cytoplasmic fragmentation, reduction in the amount of smooth endoplasmic reticulum (SER) and the number of mitochondria, and amplification of autophagic vacuoles and lysosomes (Dai and Gilbert, 1991). We observed no change in SER or mitochondria, and no increment of autophagosomes or lysosomes in *smt3i* PG cells. Therefore, our observations suggest that the impairment of development and the reduced ecdysteroid levels in *smt3i* larvae are not due to massive cell death or premature degeneration of the PG cells at the time of puparium formation. It is likely that the remaining levels of Smt3 in knockdown PG cells are enough to allow cell survival (Fig. 1C’).

Variations in the levels and localisation of steroidogenic factors in *smt3i* larvae

Halloween genes encode for cytochrome P450 enzymes that mediate the conversion of cholesterol to 20E. *phm*, *dib* and *sad*, which encode the C25, C22 and C2 hydroxylases, respectively, are all expressed in PG cells (Chavez et al., 2000; Niwa et al., 2004; Warren et al., 2002; Warren et al., 2004). From early to late third instar larval stages there is an upregulation of P450 enzyme expression that correlates with an increase in the ecdysteroid titers (Warren et al., 2006). We analysed these enzymes in 5- to 6-day-AEL *smt3i* larvae by immunodetection (Fig. 4). We did not observe changes in the expression levels or pattern of Phm, localised in the endoplasmic reticulum (ER) of the PG cells (Fig. 4A,A’,D,D’) (Warren et al., 2004). However, the levels of Dib, which in WT third instar larvae showed a characteristic punctuate-like pattern corresponding to mitochondria (Petryk et al., 2003), were dramatically reduced in *smt3i* larva (Fig. 4B,B’,E,E’). We also analysed the expression pattern of Sad, expressed in WT wandering third instar larvae in the cytoplasm with a pattern similar to Dib, as well as in the nucleus (Fig. 4C,C’). In *smt3i* larvae, the nuclear accumulation was reduced (Fig. 4F,F’). Altogether, these results show that reduced levels of Smt3 in the PG produce changes in the expression levels of enzymes in the ecdysteroid biosynthetic pathway.

We also analysed the transcription factors involved in the regulation of these steroidogenic enzymes. In WT third instar larvae PG cells, Woc is localised in the nucleus (Fig. 4G,G’) and we could not detect remarkable variations in the expression levels or the subcellular localisation of this factor in *smt3i* larvae (Fig. 4J,J’). Mld is also expressed in the nucleus of WT PG cells, in a pattern different than that of Woc (Fig. 4H,H’). We observed a reduction in the expression levels of Mld in the nuclei of *smt3i* PG cells and, interestingly, a change in the localisation of this protein that could now be found in the cytoplasm (Fig. 4K,K’). It has been suggested that β Ftz-f1 regulates both Dib and Phm expression (Parvy et al., 2005), and, as shown for Dib, expression of β Ftz-f1 is drastically reduced in *smt3i* PG cells, in both the nucleus and the cytoplasm (Fig. 4I,I’,L,L’).

If Smt3 is involved in the ecdysteroid biosynthesis pathway, we would expect mutations in other members of the sumoylation pathway to have the same effect on the expression and localisation of ecdysteroidogenic enzymes and factors. We analysed the expression pattern of these in the PGs of *lwr* homozygous mutants that had reached L3 (Chiu et al., 2005; Huang et al., 2005a). Similar to *smt3i*, in *lwr* mutant larvae we detected severe alterations in the expression levels of Dib (not shown) and β Ftz-f1 (Fig. 4O,O’), as well as a mis-localisation of Woc and Mld (Fig. 4M–N’), confirming that *smt3* knockdown alters the sumoylation pathway in PG cells. However, the low levels of these factors might not be sufficient to

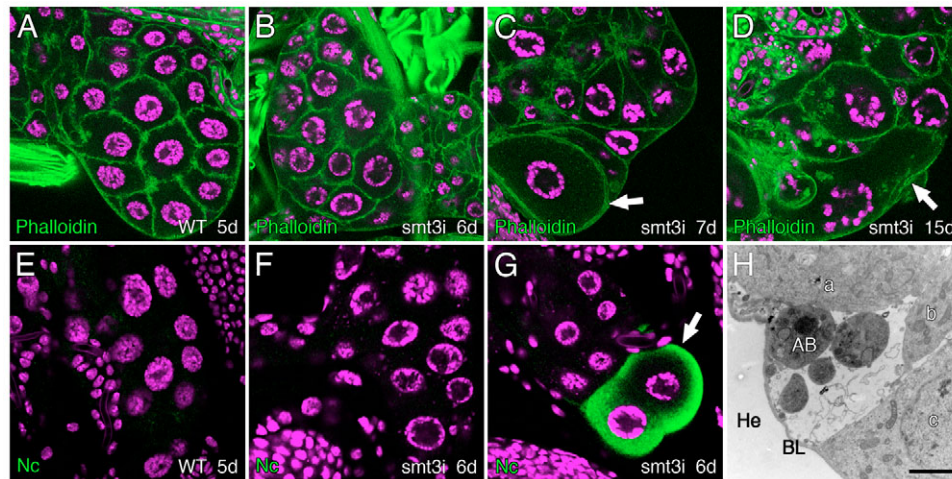


Fig. 3. The PGs of *smt3i* larvae do not show massive degeneration. (A–D) Confocal single plane micrographs showing DAPI staining of nuclei (purple) and Phalloidin (green) to show cell contour. The nuclei of *smt3i* cells (B–D) show a large DAPI-negative space in the centre. Cells are bigger than in WT (A) and some ‘giant cells’ appear in each PG (arrows). With time, *smt3i* PGs show lower number of cells than do WT (C,D). (E–G) Confocal pictures showing nuclei (purple) and the activated initiator caspase Nc (green) that indicates whether the cell has entered into apoptosis. At 5 to 6 days AEL, the PGs do not show massive cell death and the levels of Nc are comparable to WT (E versus F). Only sporadic apoptotic cells are observed in *smt3i* larvae, which could correspond to the ‘giant cells’ (arrow in G). (H) Electron micrograph showing four apoptotic bodies (AB), in a large extracellular space probably representing the remnants of an apoptotic cell, surrounded by non-apoptotic cells (labelled a, b and c) in a 5-day-old *smt3i* ring gland. Scale bar: 2 μ m. He, hemocoel; BL, basal lamina.

explain the reduction in the ecdysteroid titer of *smt3i* larvae, as a decrease in their transcriptional levels does not impair metamorphosis (McBrayer et al., 2007).

The cell membrane is compromised in *smt3i* PG cells

During WT wandering L3 the PG is highly active (Dai and Gilbert, 1991). The conversion of cholesterol into E involves mainly the ER and the mitochondria, as well as the shuttling of intermediate forms between these intracellular compartments. However, as mentioned previously, we did not observe changes in the ER or the mitochondria in *smt3i* larvae (Fig. 5A,B; data not shown). Similar to previous observations (Aggarwal and King, 1969; Dai et al., 1991; King et al., 1966), we observed in WT L3 a very high number of deep invaginations in the membrane of PG cells facing the hemolymph, which form channels reaching deep into the cells (Fig. 5A). These plasma membrane invaginations represent a substantial increase in the cell surface, and are probably relevant for the efficient uptake of lipids and the secretion necessary for the high ecdysteroid titer characteristic of this developmental stage. In addition, we observed elaborated interdigitations, which, overall, produced an extensive extracellular space between the cells (see Fig. S2A in the supplementary material). Interestingly, in *smt3i* PG cells there was a clear reduction in the number and length of the invaginations of the plasma membrane (Fig. 5B), and also a diminution of the interdigitations (see Fig. S2B in the supplementary material). It is difficult to assess whether the balance between the increased size of the cells, and the reduction of the channels and interdigitations in *smt3i* larvae, involves changes in the total cell surface. However, the intracellular channels seem to be of functional relevance for ecdysteroid secretion (Dai and Gilbert, 1991; Dai et al., 1991) and, therefore, their reduction might be important to understand the phenotype of *smt3i* larvae.

Our EM analysis corroborated the abnormal morphology of the nuclei of PG cells in *smt3i* larvae and disclosed the formation of extraordinarily large aggregates of viral-like particles (VLPs).

These particles are frequently detected in low numbers in all tissues in WT strains, although in *smt3i* larvae the quantitative differences were obvious (see Fig. S2B in the supplementary material). In addition to the VLPs, and associated with them, we found a high number of parallel bands of electron-dense material of unknown origin (see Fig. S2B in the supplementary material) that was absent in control larvae. In addition, *smt3i* PG nuclei had thickened nuclear lamina (compare Fig. 5C and 5D), although the nuclear pores seemed to be still present. In agreement with this observation, detection of lamin using Dm0 antibodies showed a thickening of the lamin layer associated with the nuclear envelope in *smt3i* larvae (Fig. S2C,D in the supplementary material). No other ultrastructural changes were observed. Overall, these results show that, at the ultrastructural level, the main organelles affected in *smt3i* larvae are the plasma membrane and the nucleus.

Ecdysone synthesis in the PG is stimulated by the brain neuropeptide PTTH in Lepidoptera. As we used a PG-specific Gal4 to knockdown *smt3*, we assume that PTTH synthesis in the neurosecretory cells of the brain is normal. However, the reception of this signal could be compromised in *smt3i* larvae as a result of the alterations in the plasma membrane. We visualised the nerve terminals reaching the ring gland, by HRP immunostaining, in 5- to 6-day AEL larvae (Fig. 5E–F'). In WT larvae, the axons arborised and extended among the PG cell layers as described (Fig. 5E,E'; see also Fig. S3A–B' in the supplementary material) (Siegmond and Korge, 2001). A similar pattern was found in the PG of *smt3i* larvae (Fig. 5F,F'; see also Fig. S3D–E' in the supplementary material), although some of the nerve endings looked slightly disorganised. Varicose nerve terminals containing the electron-dense vesicles characteristic of neurosecretory endings were detected by EM among the PG cells of WT and knockdown larvae, and no obvious differences were detected among them (see Fig. S3C,F in the supplementary material).

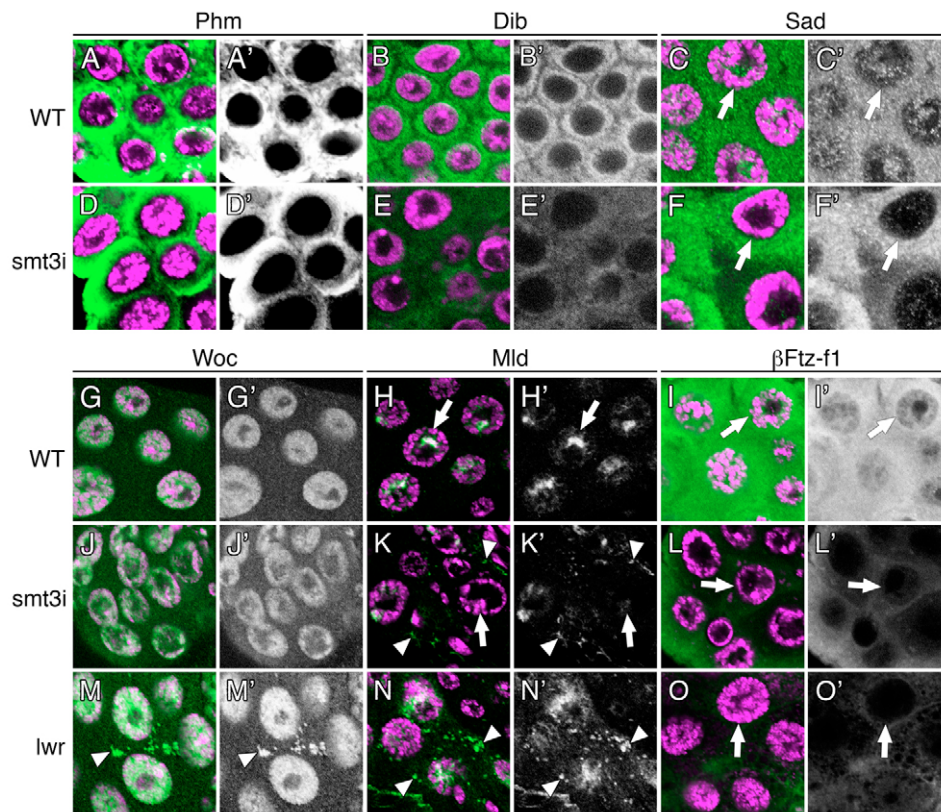


Fig. 4. *smt3i* PG cells show changes in steroidogenic enzymes and transcription factors. (A–O) Single plane confocal micrographs showing expression of the indicated hydroxylase enzymes (Phm, Dib or Sad) or the indicated transcription factors (Woc, Mld or β Ftz-f1) in WT, *smt3i* or *lwr* mutant PG cells. Nuclei are labelled with DAPI and shown in purple; the indicated proteins are shown in green. (A'–O') Single green channels for each panel, showing expression of the indicated proteins, are presented in black and white. White arrows denote differences in the nuclear accumulation of the referred factors between WT and knockdown phenotypes, and white arrowheads denote ectopic cytoplasmic accumulation of certain factors in knockdown or mutant backgrounds. (A,A',D,D') Phm, accumulated in the ER, does not show changes in expression levels or localisation in *smt3i* larvae compared with WT, whereas Dib mitochondrial staining is reduced (B,B',E,E'). Sad nuclear accumulation, but not mitochondrial staining, is reduced in knockdown larvae (C,C',F,F', arrows). (G,J,M) Woc nuclear localisation does not diminish in *smt3i* (J,J') or *lwr* mutant larvae (M,M') compared with WT (G,G'). In *lwr* mutants, Woc accumulates in the cytoplasm (arrowhead). (H,K,N) Mld accumulates in the nucleus in WT cells (H,H', arrow), and is highly reduced in *smt3i* nuclei (K,K', arrow), although cytoplasmic accumulations of the protein can be observed (arrowheads). This is even more noticeable in *lwr* mutant larvae (N,N', arrowheads). (I,L,O) β Ftz-f1 appears to be evenly localised in the nucleus (arrow) and the cytoplasm in WT cells (I,I'), whereas in *smt3i* (L,L') and *lwr* mutant larvae (O,O') it is reduced in the cytoplasm and absent in the nuclei (arrows). All images are shown at the same magnification.

Lipid content is reduced in the PG cells of *smt3i* larvae

The reduction of plasma membrane invaginations observed in *smt3i* larvae has been previously described in PG cells of the ecdysone deficient mutant *l(3)ecd^{lts}* (Dai et al., 1991). However, none of the other characteristic features of *l(3)ecd^{lts}* mutants occur in *smt3i* PG cells, such as accumulation of lipid droplets in the cytoplasm, disappearance of SER or enhancement of electron-dense mitochondria. On the contrary, in 5- to 6-day-old *smt3i* larvae, we found a general diminution of lipid droplets compared with WT. These droplets most likely include sterol precursors required for ecdysteroid production. To better characterise this observation, we used Oil Red O staining to identify the lipid droplets and filipin staining to specifically stain non-esterified sterols. In most *smt3i* PG cells, we observed a clear reduction in the number of lipid droplets (Fig. 6A–B'') and also a diminution of sterols (Fig. 6C,D). Only the PG cells previously described as 'giant cells' had an increased accumulation of lipid droplets (Fig. 6B,B', arrow). We quantified the number of lipid droplets in *smt3i* and WT PG cells, excluding the

'giant cells' as they were apoptotic, as shown by the active Nc-positive staining (Fig. 3G). Whereas each WT PG cell in a single section contained approximately 25 lipid droplets, *smt3i* larvae had only 5 lipid droplets (Fig. 6E). Interestingly, the total lipid content in other tissues of the *smt3i* larvae increased over the course of their expanded life, reflecting the reported body weight increase (Fig. 1F; data not shown). The quantification of filipin-stained drops gave a similar result (see Fig. S4 in the supplementary material).

In summary, our analysis showed that the number of lipid droplets and sterols per cell was significantly reduced in *smt3i* PG cells (Fig. 6E). This could be related to the reduction of intracellular channels and could contribute to the inability of *smt3i* larvae to achieve the ecdysteroid levels required to pupariate.

DISCUSSION

Smt3 is required at the onset of metamorphosis

Steroid hormones have essential physiological and developmental functions in higher organisms. In *Drosophila*, ecdysteroids regulate most of the developmental events required for molting

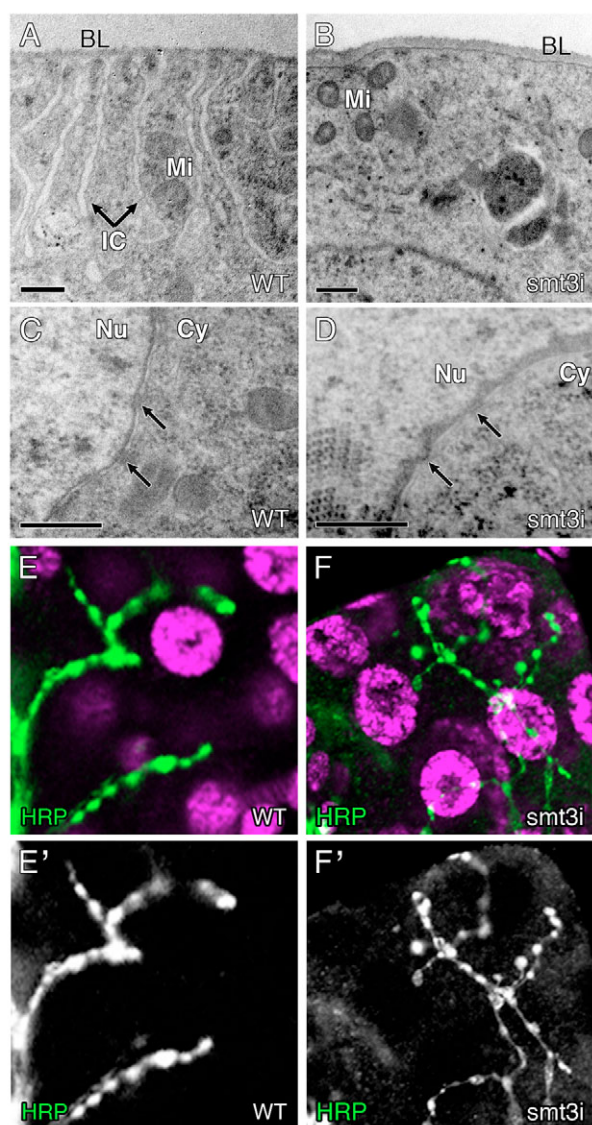


Fig. 5. The structure of the cell membrane is compromised in *smt3i* PG cells. (A-D) Transmission electron microscopy images of PG cells from WT (A,C) and *smt3i* (B,D) larvae. (A,B) Intracellular channels (IC, arrows) are severely reduced in number and size in *smt3i* larvae (B) compared with WT (A), although no differences were observed in number, size or morphology of the mitochondria (Mi). BL, basal lamina. (C,D) The nuclear lamina is thickened in *smt3i* (D) compared with WT (C), but the nuclear pores appear not to be affected (arrows). Nu, nucleus; Cy, cytoplasm. Scale bars: 500 nm. (E,F) Single confocal micrographs showing PG cell nuclei stained with DAPI (purple) and anti-HRP antibodies (green). (E',F') Single green channels for HRP staining shown in black and white.

and metamorphosis, with 20E being the main cholesterol-derived active steroid. *smt3* knockdown in the PG produces diverse defects, such as thickening of the nuclear lamina, severe reduction of the plasma membrane invaginations, changes in the expression levels or localisation of enzymes and transcription factors involved in steroidogenesis, and a reduction of the sterol content of these cells. Our study demonstrates that sumoylation is essential for ecdysteroid biosynthesis, suggesting a specific requirement for Smt3 in the PG during the last larval instar before

pupariation. Therefore, our study implicates for the first time SUMO in the ecdysteroid biosynthetic pathway required for metamorphosis, probably by modification of some of the factors involved in the ecdysteroidogenic pathway.

Loss-of-function studies of *ubc9* and *smt3* show embryonic lethality in *Drosophila* and mice (Epps and Tanda, 1998; Nacerddine et al., 2005; Takana and Courey, 2005). *smt3i* larvae, after surviving the first and second molts, arrest development specifically at the time of pupariation, giving us the possibility to explore the role of sumoylation during metamorphosis.

The reduction in ecdysteroid titers in *smt3i* larvae could not be caused by a premature degeneration of the ring gland, as we did not detect massive cell death or an increase in lysosomes and autophagic vacuoles at the time-point when the larvae should enter pupariation. During their abnormally extended larval life, *smt3i* PGs contain apoptotic cells but never show autophagic features characteristic of WT PG degeneration (Dai and Gilbert, 1991).

***smt3i* changes in the nucleus and cytoplasm**

The hypertrophy of PG cells and their nuclei found in *smt3i* larvae has also been reported in ecdysteroid deficient mutants, such as *mld*, *woc*, *grg* or *dre4* (Klose et al., 1980; Neubueser et al., 2005; Sliter and Gilbert, 1992; Wismar et al., 2000). This could reflect a compensatory mechanism triggered by the abnormally low ecdysteroid levels common for all these genotypes.

The main organelles involved in the ecdysteroid biosynthetic pathway are thought to be the mitochondria and the ER, and changes in these organelles have been reported for some mutants exhibiting reduced ecdysteroids (Dai et al., 1991; Wismar et al., 2000). We did not observe ultrastructural abnormalities in these structures in *smt3i* larvae. However, the nucleus is affected in *smt3i* PGs, showing an abnormal morphology, thickening of the nuclear lamina and hyper-proliferation of VLPs. A similar increase in the amount of VLPs has been found in at least one other ecdysteroid mutant, *grg* (Klose et al., 1980). *smt3i* PG nuclei also showed arrays of parallel electron-dense stripes, a phenotype that increased gradually during the prolonged larval life. These arrays of alternating electron-dense and clear material were always tightly associated with VLPs, but the mechanism by which these bands are formed is unknown.

Reduction of *smt3* results in the thickening of the nuclear lamina beneath the inner nuclear membrane (INM). The INM and its associated layer of lamins have important functions, such as maintenance of the nuclear shape, organization of the nuclear pores, chromatin and transcriptional regulation (Heessen and Fornerod, 2007), and the correct distribution of nuclear pore complexes (Liu et al., 2007). As sumoylation is crucial for the nuclear transport, *smt3i* larvae could abolish the nucleo-cytoplasm transport and, therefore, could contribute to the localisation changes observed in factors necessary for ecdysteroidogenesis. However, this is not a general problem in *smt3i* PG cells, as transport to the nucleus of some of the tested proteins was not affected (for instance Woc). Therefore, despite the ultrastructural aberrations observed, the protein-production machinery and nucleo-cytoplasmic transport are not blocked.

By contrast, the reduced levels of cytoplasmic Dib or β Ftz-f1, or nuclear Mld or Sad, might contribute to the low levels of ecdysteroids in *smt3i* larvae, although this might not be the only cause of impeded pupariation, as the low transcriptional levels of these factors do not stop entry in metamorphosis (McBrayer et al., 2007).

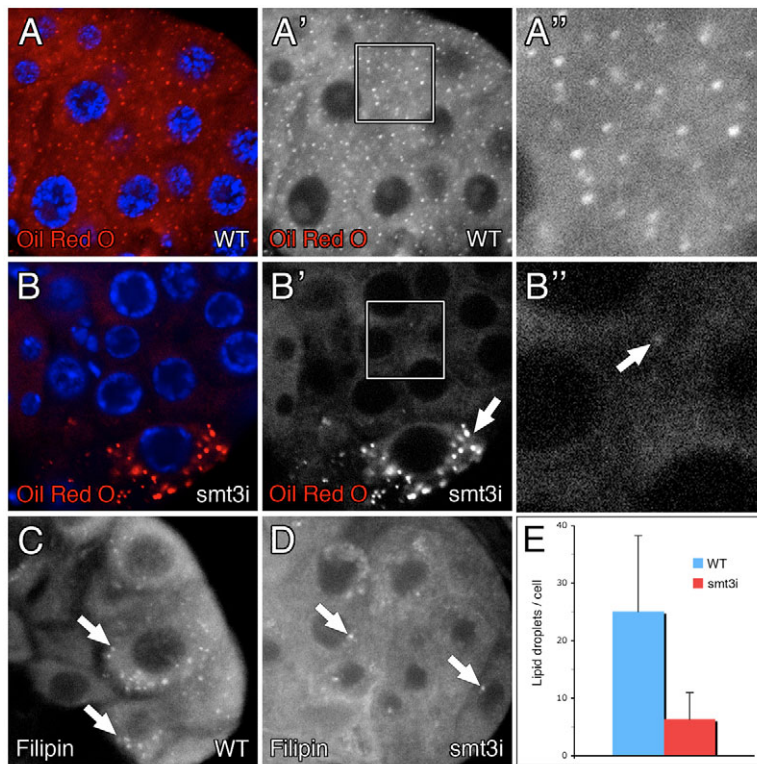


Fig. 6. The lipid content of *smt3i* PG cells is reduced.

(A,B) Confocal micrographs showing nuclei marked with DAPI (blue) and lipid droplets stained with Oil Red O (red) in WT (A) and knockdown (B) larvae. Single red channels are shown in black and white (A',B'), and boxed regions are magnified (A'',B''). Most *smt3i* PG cells contain very few droplets (arrow in B''), except for the 'giant cells', which accumulate large drops (arrow in B'). (C,D) Filipin staining shows a reduction in sterol droplets (white dots) in *smt3i* PG cells (D) compared with the number of droplets in WT (C). Arrows in C indicate cells containing several lipid droplets in WT cells, whereas arrows in D indicate remnant lipid droplets in some cells of the *smt3i* PG. (E) Graph of the average number of Oil Red O droplets per cell present in a single confocal plane in WT versus *smt3i* PG cells, reflecting a one-third reduction of droplets.

Intracellular channel formation is impaired in *smt3i* PG cells

The severe reduction of intracellular channels and interdigitations in *smt3i* PG cells might be essential to the understanding of the L3 arrest phenotype of knockdown larvae. These intracellular channels, typical of an active gland in WT L3 (Dai et al., 1991), could be necessary for the increased rate of ecdysone synthesis required at this stage, perhaps because the amplification of the interface between the PG cells and the hemolymph results in a more efficient uptake of lipids and secretion of ecdysteroids.

How could these defects of plasma membrane explain the impaired metamorphosis phenotype of *smt3i* larvae? We can envisage at least two possibilities: defects in PTTH signalling and/or cholesterol uptake. As PTTH downstream factors have not been identified in *Drosophila*, we cannot further investigate the possibility that signalling is impaired, although no variations were found in the expression of β -tubulin or phosphorylated ribosomal protein S6, two known targets of PTTH in Lepidoptera (data not shown). Thus, we hypothesise that reduced cholesterol uptake contributes to the low ecdysteroid levels described in *smt3i* PG cells.

Smt3 is necessary for cholesterol uptake in PG cells

The reduction of lipid and sterol droplets suggests a problem in cholesterol uptake in *smt3i* PGs, maybe caused by the reduction of the intracellular channels characteristic of *smt3i* PG cells. Interestingly, functionally analogous structures, the microvillar channels, seem to play an important role in lipid uptake in the adrenal gland, the mammalian equivalent of the insect PG (Reaven et al., 1989).

Arthropods are not able to synthesize cholesterol and depend on exogenous cholesterol or related sterols. Receptors involved in cellular cholesterol uptake have been described in various organisms

from nematodes to mammals. Recently, the relevance of lipoproteins and their receptors in embryonic development and steroid hormone signalling has been reported; for example, the delivery of cholesterol to steroidogenic tissues such as the adrenal gland (Willnow et al., 2007). Particularly interesting is the role of scavenger receptor class B type I (SR-BI)-mediated cholesterol uptake, as it has been shown that SR-BI is essential for both microvillar channel formation and HDL localisation (Williams et al., 2002). This receptor has been localised to caveolar rafts, plasma membrane microdomains characterised by their elevated cholesterol content (Martin and Parton, 2005). These specialised regions have been implicated in different cell functions by regulating transduction pathways.

Alternatively, the deficient cholesterol uptake and the reduction of intracellular channels in *smt3i* larvae could be independent processes. The analysis of lipid droplets in *l(3)ecd^{11s}* (Dai et al., 1991) and *woc* mutants (A.T., J.S., R.C., C.P. and R.B.) suggests that diminution of the intracellular channels is not enough to disrupt completely cholesterol uptake. Although both mutants show a clear reduction of plasma membrane folding, they show a high accumulation of lipid droplets (Dai et al., 1991; Wismar et al., 2000). Mutations in other factors involved in cholesterol homeostasis also show an accumulation of cholesterol, caused by intracellular trafficking defects that result in lethality during larval to pupal transition (Huang et al., 2005b; Huang et al., 2007) (for a review, see Huang et al., 2008).

Further studies will be required to understand the mechanism of cholesterol uptake by PG cells, how this is altered in cells that lack interdigitations, and, lastly, how this is related to deficient steroidogenesis. Alterations in the sumoylation pathway could affect steroidogenesis in other cell types and in other organisms. Our research could provide insights into physiological regulation by steroid hormones in higher organisms and into the associated pathologies.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/9/1659/DC1>

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