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 (Rodriguez 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 prothoracicotropic 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 dorso medial 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; Hummel, 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 (Nanhout 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)
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 (βFtz-f1) (Parvy et al., 2005). Woc controls the conversion from cholesterol to 7dC, Mld is involved in the regulation of spok, and βFtz-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 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’-GCTCTAGACGATGCCAGCTTCAACAAGCAACCA-3’ and Rev 5’-GCTCTAGACTAGTACTATCGATTCTTAGGGGCTTTGGT-3’) containing XhoI 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-Trition 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-GRP (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 (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 smt3-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-acyetylcholinesterase (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 ecysone 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, Toussimis) 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 3 μm) were cut with a glass knife, mounted on microscope slides, stained with 0.1% boric 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**

smt3 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, smt3i 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.
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 titer (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 larva 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 larva 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 βFtz-f1 regulates both Dib and Phm expression (Parvy et al., 2005), and, as shown for Dib, expression of βFtz-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 βFtz-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...
channels and interdigitations in between the increased size of the cells, and the reduction of the supplementary material. It is difficult to assess whether the balance (5B), and also a diminution of the interdigitations (see Fig. S2B in the supplementary material). Interestingly, in smt3i larvae, involves changes in the intracellular compartments. However, as mentioned previously, we did not observe changes in the ER or the mitochondria in smt3i larvae (Fig. S2A, 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. 5E,F 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) (Sieg mund 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. 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 μm. He, hemocoel; BL, basal lamina.

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.
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)ecd1ts (Dai et al., 1991). However, none of the other characteristic features of l(3)ecd1ts 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 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',E',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) βFtz-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.

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.
and metamorphosis, with 20E being the main cholesterol-derived active steroid. \textit{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 ecdysteroidogenenic pathway.

**Loss-of-function studies of ubc9 and smt3 show embryonic lethality in \textit{Drosophila} and mice (Epps and Tanda, 1998; Nacerdine et al., 2005; Takanaka and Courey, 2005).** \textit{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 \textit{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, \textit{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 \textit{smt3i} larvae has also been reported in ecdysteroid deficient mutants, such as \textit{mld}, \textit{woc}, \textit{grg} or \textit{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 \textit{smt3i} larvae. However, the nucleus is affected in \textit{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, \textit{grg} (Klose et al., 1980). \textit{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 \textit{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, \textit{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 \textit{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 \textit{βFtz-f1}, or nuclear Mld or Sad, might contribute to the low levels of ecdysteroids in \textit{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).
Intracellular channel formation is impaired in smt3Δ PG cells

The severe reduction of intracellular channels and interdigitations in smt3Δ 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 smt3Δ 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 smt3Δ 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 smt3Δ PGs, maybe caused by the reduction of the intracellular channels characteristic of smt3Δ 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 smt3Δ larvae could be independent processes. The analysis of lipid droplets in (l3)ecd1ts (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 lipoprotein 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).

Fig. 6. The lipid content of smt3Δ 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 smt3Δ 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 smt3Δ 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 smt3Δ PG. (E) Graph of the average number of Oil Red O droplets per cell present in a single confocal plane in WT versus smt3Δ PG cells, reflecting a one-third reduction of droplets.
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Supplementary material
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