Scavenger Receptors Mediate the Role of SUMO and Ftz-f1 in Drosophila Steroidogenesis

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Abstract

SUMOylation participates in ecdysteroid biosynthesis at the onset of metamorphosis in Drosophila melanogaster. Silencing the Drosophila SUMO homologue smt3 in the prothoracic gland leads to reduced lipid content, low ecdysone titers, and a block in the larval–pupal transition. Here we show that the SR-BI family of Scavenger Receptors mediates SUMO functions. Reduced levels of Snmp1 compromise lipid uptake in the prothoracic gland. In addition, overexpression of Snmp1 is able to recover lipid droplet levels in the smt3 knockout prothoracic gland cells. Snmp1 expression depends on Ftz-f1 (an NR5A-type orphan nuclear receptor), the expression of which, in turn, depends on SUMO. Furthermore, we show by in vitro and in vivo experiments that Ftz-f1 is SUMOylated. RNAI-mediated knockdown of ftz-f1 phenocopies those of smt3 at the larval to pupal transition, thus Ftz-f1 is an interesting candidate to mediate some of the functions of SUMO at the onset of metamorphosis. Additionally, we demonstrate that the role of SUMOylation, Ftz-f1, and the Scavenger Receptors in lipid capture and mobilization is conserved in other steroidogenic tissues such as the follicle cells of the ovary. smt3 knockdown, as well as ftz-f1 or Scavenger knockdown, depleted the lipid content of the follicle cells, which could be rescued by Snmp1 overexpression. Therefore, our data provide new insights into the regulation of metamorphosis via lipid homeostasis, showing that Drosophila Smt3, Ftz-f1, and SR-BIs are part of a general mechanism for uptake of lipids such as cholesterol, required during development in steroidogenic tissues.

Introduction

Larval molting and metamorphosis in Drosophila melanogaster relies on pulses of ecdysoidic hormones. During the larval stages, the prothoracic gland (PG) is the tissue responsible for the synthesis of the steroid hormone ecdysone that is secreted to the hemolymph and converted to 20-hydroxyecdysone (20E) in target tissues [1]. Other tissues releasing ecdysteroids in the adult are the gonads, ovaries and testes. Cholesterol is the precursor of all steroid hormones. In arthropods, which are unable to synthesize cholesterol, ecdysteroids are synthesized from dietary cholesterol or phytosteroids. Cholesterol is converted to 20E through a series of enzymatic reactions that involve the cytochrome P450 enzymes coded by the Halloween genes spook, spooker, phantom (phm), disembodied (dbi), shadow, shade and the Rieske non-heme iron oxygenase gene neverland [2,3]. A transcriptional cascade triggered by 20E occurs at the onset of metamorphosis that leads to the sequential expression pattern of the transcription factors DHR3, Ftz-f1, E74 and E75 [4]. A similar transcriptional cascade is required during embryogenesis and could also be required for each larval molting [5]. However, many of the aspects involved in the regulation of ecdysteroid biosynthesis remain unknown.

The conjugation of SUMO (Small Ubiquitin-related MOdifier) to target proteins is a reversible post-translational modification highly conserved in all eukaryotic organisms. SUMOylation regulates diverse cellular processes including cell survival and proliferation, nuclear import, intracellular trafficking, transcriptional regulation and maintenance of genomic and nuclear integrity [6]. In addition, Smt3, the only SUMO homologue in Drosophila, has a role in the regulation of ecdysteroid levels during post-embryonic development [7]. Smt3 is required in the PG to produce the ecdysteroid peak necessary for the larval to pupal transition. Interestingly, smt3 knockout PG cells results in reduced intracellular channels and, as a consequence, exhibit low levels of lipid and sterol droplets indicating that impaired cholesterol uptake could contribute to the low ecdysteroid levels observed.

The nuclear hormone receptor superfamily function as transcription factors that regulate several functions such as metabolism, development and homeostasis. Recent studies have implicated the Drosophila nuclear receptors DHR96 and dHNF4 in cholesterol and triacylglycerol homeostasis and in lipid mobilization and fatty acids β-oxidation [8–11]. However, it is unknown whether the nuclear receptors might regulate cholesterol homeo-
Steroid hormones are cholesterol derivatives that control many aspects of animal physiology, including development of the adult organisms, growth, energy storage, and reproduction. In insects, pulses of the steroid hormone ecdysone precede molting and metamorphosis, the regulation of hormonal synthesis being a crucial step that determines animal viability and size. Reduced levels of the small ubiquitin-like modifier SUMO in the prothoracic gland block the synthesis of ecdysone, as SUMO is needed for cholesterol intake. Here we show that SUMO is required for the expression of Scavenger Receptors (Class B, type I). These membrane receptors are necessary for lipid uptake by the gland. Strikingly, their expression is sufficient to recover lipid content when SUMO is removed. The expression of the Scavenger Receptors depends on Ftz-f1, a nuclear transcription factor homologous to mammalian Steroidogenic Factor 1 (SF-1). Interestingly, the expression of Ftz-f1 also depends on SUMO and, in addition, Ftz-f1 is SUMOylated. This modification modulates its capacity to activate the Scavenger Receptor Snmp1. The role of SUMO, Scavenger Receptors, and Ftz-f1 on lipid intake is conserved in other tissues that synthesize steroid hormones, such as the ovaries. These factors are conserved in vertebrates, with mutations underlying human disease, so this mechanism to regulate lipid uptake could have implications for human health.

Author Summary
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Results

**ftz-f1 knockdown in the PG phenocopies smt3i phenotype**

In *Drosophila*, ftz-f1 encodes for two protein isoforms with distinct temporal expression patterns: αFtz-f1 expressed early in embryogenesis and βFtz-f1 expressed later in embryogenesis and during larval, prepupal and early pupal stages [27,28]. βFtz-f1 has previously been implicated in regulating ecdysteroid titers during post-embryonic development, specifically at the prepupa to pupa transition [26,28]. To investigate whether Ftz-f1 contributes to the smt3i knockdown phenotype (herein referred to as smt3i), we silenced both isoforms of ftz-f1 in the PG using UAS-βtaz-f1 lines and the phm-Gal4 driver. The resulting larvae will be referred to as ftz-f1i. Reduced levels of ftz-f1 led to arrested development at larval stages (Figure 1A, 1B). Similar to the smt3i phenotype, ftz-f1i larvae arrested at third instar (L3) before pupariation, as shown by the mouth hook morphology (Figure 1B). These larvae did not pupariate and survived as L3 for several weeks. In contrast to the smt3i phenotype, we also observed larvae that arrested development at the transition from the second instar (L2) to L3, as shown by the double mouth hooks. These larvae die at 120 hours after egg laying (AEL; Figure 1B). The two larval phenotypes could reflect the silencing of the two ftz-f1 isoforms.

The developmental arrest at L3 suggested that ftz-f1i larvae were not able to synthesize normal levels of ecdysteroids at the onset of pupariation. Accordingly, and similarly to other low ecdysteroid mutants including the smt3i larvae, ftz-f1i larvae fed with exogenous 20E pupariated (Figure 1C).

smt3i PG cells show a reduction in lipid droplets and sterol levels, in addition to changes in steroidogenic enzymes and transcription factors [7]. ftz-f1i PGs also showed reduced levels of lipid droplets per cell (Figure 1D, 1E), as well as reduced levels of the steroidogenic enzyme Dbib (data not shown). Taken together, these results show that Ftz-f1 is required in the PG at the end of L3 to acquire appropriate levels of cholesterol and to process it into ecdysone.

**Regulation of Ftz-f1 by SUMOylation**

It was previously reported that Ftz-f1 protein is reduced in smt3i PGs [7], which could explain why ftz-f1 silencing phenocopies SUMO downregulation. SUMO could be necessary for ftz-f1 transcription, for Ftz-f1 protein modification, or both. To clarify this point we analyzed ftz-f1 expression in PGs from 120 hours AEL blue-gut wandering larvae (i.e. 5–12 hours before pupariation), and 120 hours AEL clear-gut larvae (i.e. 1–6 hours before pupariation). ftz-f1 transcription is upregulated in clear-gut larvae compared to blue-gut (Figure 1F, 1G). However, ftz-f1 expression is lower in smt3i PGs and it does not get upregulated in older larvae (Figure 1H, 1I). This indicates that SUMO is involved in ftz-f1 transcriptional regulation.

It has been reported that the mammalian homologues of Ftz-f1 are modified by SUMOylation [14,18–21]. Therefore, we decided to test whether Ftz-f1 can be modified by SUMO. We determined the potential SUMOylation sites in Ftz-f1 using the SUMOplot analysis program and the Phosida Posttranslational Modification Database (Figure 1J). The two isoforms share the C-terminal region, but contain different N-terminal regions (Figure 1J). The in silico analysis showed that αFtz-f1 and βFtz-f1 share four SUMO consensus sequences, all of them conserved among insects, of which three have high prediction scores (Figure 1J and data not shown). The SUMOylation consensus sites are located in the DNA binding domain, in the hinge region and two of them in the ligand binding domain (Figure 1J and Figure S1). Those located in the
Figure 1. Ftz-f1 is required for pupariation and lipid uptake and is modified by SUMOylation in vivo and in vitro. (A) Wild type (WT) L3 larva and mouth hooks. (B) Most of the phm-Gal4>UAS-ftz-f1 RNAi (ftz-f1i) larvae arrested development at L3, while some of them stop at L2–L3 transition, as shown by the mouth hook morphology. (C) L3 ftz-f1i larvae fed with 20E were able to pupariate. (D, E) Single plane confocal micrographs showing nuclei marked with DAPI (purple) and lipid droplets, indicated by yellow arrowheads, stained with Oil Red O (green) in L3 control (D) and ftz-f1i PGs (E). (D’, E’) Single green channels are shown in black and white. L3 ftz-f1i PG cells contain reduced number of lipid droplets. All pictures were taken under the same intensity settings. Scale bars indicate 10 μm. (F–I) Micrographs of ftz-f1 mRNA in situ hybridization in PGs from WT (F, G) or phm-Gal4>UAS-smt3 RNAi (smt3i) larvae (H, I) at the indicated hours AEL. ftz-f1 expression increases in clear-gut respect to blue-gut larvae (G versus F, respectively). In smt3i larvae ftz-f1 expression is downregulated (H, I). All the in situ reactions were stopped at the same time and pictures were taken at the same magnification. (J) Schematic representation of α- and β-Ftz-f1 isoforms indicating the predicted SUMOylation sites (arrows) and the sequences for the high-scoring sites found in Ftz-f1. Pink arrows indicate the SUMOylation sites conserved in insects and orange arrows the SUMOylation sites conserved from insects to vertebrate NR5A2. Pink and orange Ks indicate the lysines where SUMO attachment could take place. Numbering is based on the β-Ftz-f1 isoform. (K) In vitro SUMOylation assay. Incubation of the Ftz-f1 protein in the presence (+) of SUMO1 changes the mobility of the protein (asterisks). Arrowhead indicates the unmodified protein and arrow indicates an unspecific band. Molecular weight markers are shown to the left. (L) In vivo SUMOylation assay. Incubation of the Ftz-f1 protein in the presence (+) of SUMO1 changes the mobility of the protein (asterisks). Arrowhead indicates the unmodified protein and arrow indicates an unspecific band. Molecular weight markers are shown to the left.
DNA binding domain and in the ligand binding domain, are conserved between insects and NR5A2 (LRH-1) but not NR5A1 (SF-1; Figure 1J and Figure S1).

Using an in vitro SUMOylation assay, our results showed that Ftz-f1 protein is modified in the presence of SUMO, appearing as additional slow-migrating bands (Figure 1K). In order to analyze whether Ftz-f1 can also be SUMOylated in vivo, we developed a SUMOylation assay in cultured Drosophila cells. Smt3 was expressed as a fusion with a biotinylatin-target peptide (bio-SUMO, see Materials and Methods), along with Ftz-f1 and BirA enzyme. In this assay, biotinylated SUMO-conjugated proteins were bound to NeutrAvidin beads allowing for the specific isolation of SUMOylated material. Our results show that full-length 2Ftz-f1 is SUMOylated in Drosophila S2R+ cells (Figure 1L), with the estimated molecular weight for the main band (low asterisk) corresponding to the addition of one bioSUMO moiety. An additional but weaker band was observed at a higher molecular length (upper asterisk in Figure 1L), suggesting the possibility that Ftz-f1 could also be modified by more than one bioSUMO moiety in vivo.

Taken together, these results show that SUMOylation regulates Ftz-f1 in two ways. On one hand, it is necessary for ftz-f1 expression and, on the other hand, the protein Ftz-f1 is modified by SUMO in vitro and in vivo, suggesting that the post-translational modification of Ftz-f1 could potentially contribute to Ftz-f1 function in the PG.

SR-Bls expression is regulated by Smt3 and Ftz-f1 in PG cells

The CD36 family of Scavenger Receptors is necessary for lipid uptake in various mammalian cell types [29]. We hypothesized that members of this family could be necessary for sterol uptake in the PG and could mediate some of the functions of Smt3 and/or Ftz-f1. Fourteen members of the CD36 family were identified in Drosophila melanogaster [30]. By in situ hybridization it was recently shown that three of these receptors, peste (pes), croquemort (crq) and Sunt1 were upregulated in the PG at the onset of pupariation [31]. We confirmed this upregulation by quantitative real-time PCR (qPCR) of cDNA samples from precisely staged PGs at 96 hours AEL, 120 hours AEL, 120 hours AEL, and 120 hours AEL clear-gut larvae (Figure 2A).

By immunostaining using specific antibodies we observed that Snmp1 was expressed in Drosophila PG cells at 120 hours AEL (Figure 2B, 2C). Interestingly, Snmp1 expression was upregulated from L3 blue-gut (Figure 2B) to L3 clear-gut larvae (Figure 2C). The same results were observed when antibodies against Crcq were used (Figure 2D, 2E), suggesting a requirement for high levels of the Scavenger Receptors at the end of L3. The Scavengers expression upregulation from blue- to clear-gut larvae coincides with an increase in the content of lipid droplets of the clear-gut PGs (Figure 2F, 2G).

As reported previously, the expression of Snmpl1 is upregulated at the level of mRNA from blue- to clear-gut larvae (Figure 3A, 3B) [31]. Interestingly, we observed that the levels of expression of Smpl1 are reduced, and are not upregulated, in smt3i PGs (Figure 3C, 3D). Similar results were obtained when specific antisense probe for crcq was used (Figure 3A–3D). However, pes expression is still present in smt3i PGs (Figure 3E–3H), indicating different regulatory requirement for pes respect to crq and smpl1.

At the level of proteins, we observed that Snmpl1 expression was reduced in smt3i and in ftz-f1i PG cells (Figure 3E, 3F, 3G). Although basal levels of expression were evident in smt3i 120 hours larvae (Figure 3E), the upregulation of Smnpl1 in smt3i PGs was never observed, even when we analyzed 144 hours AEL larvae (Figure 3F, compare to Figure 2C). The same results were observed when anti-Crcq antibodies were used (Figure 3H, compare to Figure 2E).

These results indicate that SR-BIs are downstream of Smt3 and Ftz-f1 in the PG and suggest that SR-BIs might be required to mediate the role of Smt3 and Ftz-f1 in steroidogenesis during the larval to pupal transition.

SR-Bls silencing in the PG phenocopies smt3i and ftz-f1i L3 developmental arrest

To test the implication of the three Drosophila SR-BI members expressed in the PG in cholesterol uptake and steroidogenesis, we used the plh-Gal4 line to silence crcq, pes or Snpml1 specifically in the PG. Interestingly, at 25°C Snpml1 knockdown in the PG (herein called Snpml1i) led to L3 developmental arrest (Figure 4B). The Snpml1i L3 larvae survived for approximately ten days, darkened in the anterior part but failed to form a cuticle, the pseudo-pupa dying at this stage (Figure 4B). Unlike smt3i or ftz-f1i, the long-lived Snpml1i larvae had normal levels of expression of the cytochrome P450 enzyme Dih, indicating that the receptor does not have a major role in regulating this steriodogenic enzyme (Figure 4D, 4E). However, similarly to smt3i and ftz-f1i, Snpml1i PGs had a reduced content of lipid droplets, reaching between 5 to 16% of droplets per cell in comparison to controls (Figure 4C, 4F, 4G). Intriguingly, these droplets were abnormally big, being about three times bigger in Snpml1i cells compared to controls (Figure 4C, 4F, 4G), When Snpml1i L3 larvae were fed with exogenous 20E, 100% of the animals pupariated, and 66% led to adult flies (n = 30).

In contrast, knockdown of crq or pes in the PG at 25°C did not lead to any obvious phenotype. However, we observed L3 developmental arrest when silencing pes at 29°C (data not shown). Also in this case, the lipid droplets in the PG were less in number and larger in size than in controls (data not shown), which suggest that these receptors are involved in lipid uptake as well as in lipid droplet mobilization and/or the control of lipid droplets size.

Smt3 is involved in lipid uptake in ovarian follicle cells

Besides PG, we wondered whether the molecular mechanism of lipid transport would be conserved in other steriodogenic tissues. The ovary is one of the sources of ecysteroids in female adult insects. In cockroaches and locusts it has been shown that the follicle cells can synthesize and secrete ecysnone [32–34]. In Drosophila, in vitro synthesis and secretion of ecysteroids has also been described in the ovary [35,36]. smt3 and other SUMOylation genes are expressed during oogenesis in Drosophila [37]. We examined Smt3 protein expression, which was localized mainly to the nucleus, and observed the highest levels in the germarium and in the follicle cells at stages 2–3 egg chambers, with weaker expression also evident in nurse cells at these stages (Figure 5A, 5B). At later stages of oogenesis, Smt3 expression in the follicle and nurse cells was maintained (data not shown). To ask whether the Smt3 requirement for sterol uptake is a common feature for steriodogenic tissues, we silenced smt3 in the follicle cells using UAS-sm3i lines and the follicle-specific T155-Gal4 driver (Figure 5C) [38].

The ovary expresses several members of the cytochrome P450 enzyme family involved in ecysteryoid synthesis such as Phm, Dib, Shadow, Shade, Neverland and Dare [39–43]. We have focused our study from stage 8 until stage 10–11 of oogenesis, when the highest expression levels of these enzymes have been observed. In control female ovaries Dib expression started at stage 8 in the follicle cells, with the highest levels at stages 9–10, in correlation
with the peak of ecdysone synthesis in the ovary (Figure 5D and data not shown) [44,45]. Similarly to what happens in the PG [7], the expression levels of Dib in smt3 knockdown follicle cells were drastically reduced (Figure 5E). In addition, Ftz-f1, which was expressed both in nurse and follicle cells, was also reduced in the smt3 silenced follicle cells (Figure 5F, 5G), as shown in the PG [7].

At the stages analyzed, the follicle and nurse cells and the oocyte show high number of lipid droplets (Figure 6A). Interestingly, we observed a strong reduction in the number of lipid droplets in smt3i follicle cells, which correlate with the observations shown in the PG (Figure 6B and data not shown). However, the oocyte was not completely depleted of lipids as it received lipids from the nurse cells [46].

**Figure 2. SR-BIs expression and lipid uptake is upregulated at late L3.** (A) Graphical representation of the qPCR results showing the upregulation of the *Drosophila* SR-Bi members pes, crq and Snmp1 from 96 hours AEL to 120 hours AEL blue-gut and 120 hours AEL clear-gut larvae. Error bars indicate standard deviation. One asterisk (p<0.001) and two asterisks (p<0.0001) indicate significant upregulation respect to 96 hours larvae. (B–G) Single plane confocal micrographs taken under the same intensity settings. Nuclei are marked with DAPI (purple). (B–C) Expression of Snmp1 (green) is upregulated in WT PGs from 120 hours AEL blue-gut larvae (B) to 120 hours AEL clear-gut larvae (C). (D–E) Crq expression (green) is upregulated in WT PGs from 120 hours AEL blue-gut larvae (D) to 120 hours AEL clear-gut larvae (E). (F, G) The number of lipid droplets shows simultaneous increase in the PGs during the same time, as shown by Oil Red O staining (green). (B’–G’) Single green channel are shown in black and white. Scale bars indicate 20 μm.
cells throughout the ring canals (Figure S3A), which might explain why these oocytes are able to give rise to viable embryos. The reduction of Ftz-f1 levels in the follicle cells produced a more severe phenotype with death of many ovarioles. However, those that survived showed also a reduction in the number of lipid droplets in the follicle cells, this reduction being not as strong as in the smt3i phenotype (Figure 6C).

To follow up our observations in the PG, we analyzed whether the SR-BI family could be involved in the lipid uptake in the ovary. Indeed, we observed that Snmp1 knockdown in the follicle cells clearly reduced the lipid droplet content in these cells (Figure 6D). Interestingly, the few droplets observed in some cells were located in the basal part, suggesting a mobilization impairment of droplets from the basal to the apical surface of follicle cells (Figure S3B).

During the stages 8–10 of oogenesis, follicle cells accumulate over the oocyte and become columnar, showing numerous microvilli on their apical surface [46]. These microvilli can be detected by the expression of Cad99C, a cadherin involved in the regulation of the microvilli length [47,48]. We detected Cad99C protein on the apical plasma membrane of the follicle cells surrounding the oocyte (Figure 6E). However, in smt3i follicle cells Cad99C expression was highly reduced, which suggests a microvilli malformation in these cells (Figure 6F). DE-Cad, which marks adherent junctions and is required for centripetal cell migration, was also greatly reduced in smt3i follicle cells (Figure 6G, 6H) [49]. We observed a slight delay in the centripetal migration of follicle cells and very small gaps in the vitelline membrane that might be attributed to the reduction in the expression level of cadherins (data not shown). Even with these changes, egg fertility was only slightly affected and viable embryos were obtained.

These results show that reduced levels of Smt3 in follicle cells affect the levels of Dib and Ftz-f1, alter lipid droplet size and distribution, and affect membrane surface area (in this case, by altering microvilli), suggesting that Smt3 performs parallel functions in the PG and in the ovary. Moreover, Snmp1 could play similar roles in lipid uptake and mobilization in the PG and in the follicle cells.
Snmp1 restores the lipid droplet content of smt3 knockdown in PG and follicle cells

Our results showed that silencing Snmp1 phenocopies the impairment of lipid uptake of smt3 or ftz-f1 knockdowns. To test the role of Snmp1 in the smt3i phenotype in the PG, we over-expressed Snmp1 in an smt3i background. Interestingly, we observed the rescue of the number of lipid droplets per cell in 51% of the larvae (n = 29; Figure 7A, 7B). The lipid droplets were comparable to the controls in size, indicating that the overexpression of Snmp1 was able to restore the uptake of lipids and their mobilization in the PG. Furthermore, Snmp1 was able to rescue the lipid droplets content in smt3i follicle cells (Figure 7C). These results demonstrate the role of Snmp1 in sterol uptake in both the PG and the ovary and underline the relevance of the Drosophila SR-BI family in steroidogenesis.

SUMOylation of Ftz-f1 modulates Snmp1 expression

Our results suggest that SR-BIs are downstream of Ftz-f1. To study whether Snmp1 transcription is mediated, at least in part, by Ftz-f1 we analyzed its promoter region. Interestingly, the Snmp1 locus contains two putative Ftz-f1 binding sites (TCAAGGTCG, position −1410 from the initial methionine; CCAAGGgCA, position +1666) that only differ in one nucleotide from the consensus sequence 5’-PyCAAGGPyCPu-3’. In addition, an
atypical SF-1 binding site (TtGGCC, position −1974) that contains the core of the consensus sequence TCAGGGCCA [50], is present in the promoter and could also be implicated in Snmp1 regulation. We examined whether Ftz-f1 could activate Snmp1 transcription using a reporter gene cloned downstream of a 2 Kb fragment located at 5′ of the Snmp1 transcription initiation point. Interestingly, α and βFtz-f1 activated luciferase activity significantly in S2R+ Drosophila cells (Figure 8A). A mutation in position −1971, which eliminates the atypical SF-1 binding site reduces luciferase activity in presence of αFtz-f1 (Figure 8B), suggesting that activation depends of Ftz-f1 binding to that site.

Fusions of SUMO with the protein of interest can be used as a model of constitutive SUMOylation without the pleiotropic effects of overexpressing SUMO in the cells [51]. In addition, fusions of Ubc9, the E2 SUMO conjugating enzyme, are also used successfully as a model for constitutive SUMOylation [52]. To examine the effect of Ftz-f1 modification on Sump1 activation, we analyzed the transcriptional activity of Smt3-βFtz-f1 and Smt3-αFtz-f1 fusion proteins (Figure 8A). Cotransfection assays showed that while α or βFtz-f1 were able to increase the luciferase activity compared with the control vector, Smt3-αFtz-f1 or Smt3-βFtz-f1 caused a reduction in the level of transcription (Figure 8A). This effect was reproduced using the Ftz-f1 proteins fused to Lesswright, the Drosophila homologue to Ubc9 (data not shown). These results suggest that SUMOylation reduces Ftz-f1 activation capacity on Sump1.

According to our previous data, ftz-f1 expression depends on SUMO. In turn, Sump1 expression depends of Ftz-f1. In fact, α or βFtz-f1 overexpression in the PG rescues Sump1 expression in an smt3i background in 100% of the PGs analyzed (n = 37 and n = 36, respectively; Figure 8C, 8E, 8G, compare with Figure 3E).
then examined in vivo the differences of activity between α or βFtz-f1 and Smt3-α or Smt3-βFtz-f1. Smt3-αFtz-f1 or Smt3-βFtz-f1 overexpression only recovered Snmp1 expression in 8 or 25% of the PGs, respectively (n = 25 and n = 39; Figure 8D, 8F, 8G), suggesting a difference in the activity of Ftz-f1 depending on its SUMOylation status.

Discussion

Here, we have investigated the effect of SUMOylation on SR-BI and Ftz-f1 activities in the context of steroidogenesis. We show that SUMOylation has a dual role on Ftz-f1 function. On one hand, ftz-f1 transcription depends on SUMO. On the other hand, SUMO modifies Ftz-f1, reducing its capacity to activate Snmp1 transcription. In addition, we demonstrated that Drosophila SR-BI family is involved in steroidogenesis by regulating the cholesterol uptake in the PG required for the synthesis of ecdysone. Our results also show that Snmp1 is involved in cholesterol uptake, acting downstream of SUMO and Ftz-f1, and is able to recover the lipid content in smt3i PGs. Furthermore, we showed that SUMO and the Scavenger Receptors are also involved in lipid capture and mobilization in the ovarian follicle cells.

SUMOylation in steroidogenesis

SUMO modification, a highly conserved pathway throughout evolution, is known to impact the activity, interactions, localization and stability of proteins [53]. A number of studies during the last years have clearly established the essential role of SUMOylation during development. In Drosophila melanogaster, the single SUMO protein Smt3 is expressed during development and is highly enriched during embryogenesis and in adult females [54–56]. At later stages it is predominantly expressed in the central nervous system and in the gonads [37,56,57]. Components of the Drosophila SUMO conjugation pathway have also been implicated in diverse processes such as embryogenesis, wing morphogenesis, central nervous system development, neurodegeneration, photoreceptor development and immune response (reviewed in [58,59]). In addition, Smt3 is required in the PG for the correct function of the ecdysteroid biosynthetic pathway at the time of puparium formation, which derives from defects in cholesterol uptake and formation of intracellular channels [7]. We have now shown that Smt3 requirement for lipid uptake is a common feature for steroidogenic tissues by analyzing its function in the ovary, a tissue that also requires cholesterol for the synthesis of ecdysone. In addition, we show that SUMO is required to activate ftz-f1 transcription. During the late

Figure 6. Smt3 and Snmp1 are necessary for lipid uptake in follicle cells. (A–H) Single plane confocal micrographs taken under the same intensity settings. Nuclei are labelled with DAPI (purple). F-actin cytoskeleton is shown in blue. Follicle cells are indicated by yellow dotted lines. (A’–H’) Single green channels are shown in black and white. Close-up of the follicle cells are shown in insets in A’–D’. Scale bars indicate 20 μm. (A–D) Lipid droplets shown by Oil Red O staining (green) in follicle cells of WT (A), T155-Gal4>UAS-smt3i (B), T155-Gal4>UAS-ftz-f1i (C) or T155-Gal4>UAS-Snmp1i (D) adults. (E, F) Cad99C (green; yellow arrowheads) is very much reduced in T155-Gal4>UAS-smt3i (B) respect to WT (E). (G, H) DE-Cad (green; yellow arrowheads) is also reduced in T155-Gal4>UAS-smt3i (H) respect to WT (G).
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larval pulse of ecdysone, the transcription factors E75, DHR3 and the Nitric Oxide Synthase (NOS) are known to regulate Ftz-f1 expression. Ftz-f1 is a direct target of DHR3 [60,61] and E75 suppresses this DHR3 mediated expression of Ftz-f1 [62]. NO, produced by NOS, prevents the E75 function as suppressor of DHR3 [63]. Therefore, these factors could be responsible for the SUMO dependent ftz-f1 transcriptional activation. Noteworthy, NOS is modified by SUMO [64,65] and its downregulation in the PG prevents pupariation [63]. An interesting question to be analyzed in the future will be the study of the biological role of NOS SUMOylation in the regulation of ftz-f1 expression.

Figure 7. Snmp1 restores the lipid droplets content in smt3i cells. (A–B’) Single plane confocal micrographs taken under the same intensity settings showing Oil Red O staining (green) in PGs of phm-Gal4>UAS-GFP;UAS-smt3i (A) or phm-Gal4>UAS-Snmp1;UAS-smt3i (B). Arrowheads in A indicate the brain hemisphere with normal levels of lipid droplets, as smt3 was not silenced there. (C) Confocal micrograph showing Oil Red O staining (green) of follicle cells T155-Gal4>UAS-Snmp1;UAS-smt3i. GFP is shown in blue. Follicle cells are indicated by yellow dotted lines. (A’–C’) Single green channels are shown in black and white. Nuclei are labelled with DAPI (purple). Scale bars indicate 20 μm.

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Although several Drosophila Smt3-modified proteins have been identified, the effect of SUMOylation remains unknown for most of them. A proteomic study in early Drosophila embryos identified 140 SUMOylation targets that confirmed the role of this pathway in Ras signaling, cell cycle and pattern formation [66]. Smt3 also regulates negatively JNK signaling through sequestering HipK in the nucleus [67]. Further identification of SUMO targets at different developmental stages will be particularly important. We are especially interested in PG proteins modified by SUMO during the larval-pupal transition since Smt3 function is required in this crucial developmental window. In that context, the SUMO
Figure 8. SUMOylation of Ftz-f1 modulates Snmp1 expression. (A, B) Graphical representations of the luciferase activity from WT Snmp1-Luc (A) or the indicated mutant vectors (B) cotransfected in S2R+ Drosophila cells with plasmids expressing the indicated proteins. As control, empty pUASAttB vector was used. A single asterisk indicates $p < 0.05$, two asterisks $p < 0.001$ and three asterisks $p < 10^{-6}$. (C–F) Single plane confocal micrographs taken under the same intensity settings showing Snmp1 staining (green) in PGs of phm-Gal4;UAS-smt3i;UAS-aFtz-f1 (C), phm-Gal4;UAS-smt3i;UAS-Smt3-aFtz-f1 (D), phm-Gal4;UAS-smt3i;UAS-bFtz-f1 (E) or phm-Gal4;UAS-smt3i;UAS-Smt3-bFtz-f1 (F). Nuclei are labelled with DAPI (purple). (C–F) Single green channels are shown in black and white. Scale bars indicate 20 μm. (G) Graphical representation of the proportion of PGs that show Snmp1 expression in an smt3i background (grey bars). White bars indicate no Snmp1 expression rescue. The transgenes used for the rescue experiments are indicated. (H) Schematic summary of the results, where S means Smt3. Grey arrows indicate the requirement of SUMOylation for Ftz-f1 and SR-BI expression, and the regulation of SR-BI by Ftz-f1 or Smt3-Ftz-f1. Broken open arrow indicates hypothesized posttranslational modification of Scavengers by Smt3.

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modification of *Drosophila* Ftz-f1 described here, to our knowledge, for the first time is particularly exciting. Ftz-f1 hypomorphic regulatory mutants show defects at the prepupal-pupal transition, such as failure of head eversion and salivary gland cell death, and suggest a function for this transcription factor in muscle contraction events at this transition [28,68,69]. Ftz-f1 expression in late first and second instar larvae and its role in molting has also been described in *Drosophila* and other insects [28,70,71]. However, the precise expression pattern and the role of Ftz-f1 in *Drosophila* PGs from third instar larvae were not completely understood. Our results show that disruption of Ftz-f1 in the PG by RNAi impairs development at late L3, which clearly proves that Ftz-f1 is required at the larval-pupal transition. Interestingly, Ftz-f1 knockdown in the PG results not only in reduction of Dbh expression as expected, but also in diminution of Sump1 expression and a significant decrease of lipid levels. Therefore, our study implicates Ftz-f1 in sterol homeostasis in the PG as well as in the ovary, suggesting that this role could be extended to more steroidogenic tissues. Interestingly, Nhr-25, the only *C. elegans* member, controls the larval to adult transition by regulating an endocrine program of lipid uptake and synthesis [72,73].

A growing number of nuclear receptors are also known to be targets of SUMOylation (reviewed in [74,75]). In mammals, the nuclear receptor coregulator KLF5 (Krüppel-like transcription factor 5) uses SUMOylation as a molecular switch to repress or activate genes involved in lipid catabolism [76]. Other transcription factors modified by SUMO and involved in energy metabolism include PPAR-γ, C/EBPs and SREBPs [77–79]. In addition, a deSUMOylating enzyme, ScNp2 plays a critical role in the control of adipogenesis [80]. SUMOylation of SF-1, as suggested for other transcription factors, attenuates its transcriptional activity [18]. However, only a subset of SUMO sensitive targets seems to be affected [81]. On the other hand, Androgen receptor interacting protein 4, which interacts with SUMOylated SF-1, suppresses SF-1 mediated transcription [82]. Recently, the elimination of SF-1 SUMOylation in mice has been described [21]. UnSUMOylable SF-1 mutants activated Sonic hedgehog signaling and altered other potential SUMO sensitive targets, leading to endocrine abnormalities and changes in cell fate. SUMO modification has also been associated with increased transcriptional activity of nuclear receptors, as reported for retinoid acid receptor related orphan receptor 2 (ROR 2) and estrogen receptor 2 (ER2). Interestingly, SUMOylation at the hinge region of both nuclear receptors has been associated to transcriptional activation [83,84]. SUMOylation of Ftz-f1, as occurs with its orthologue SF-1, could be an important mechanism to control its activity and probably a correct ratio of SUMOylated to unmodified Ftz-f1 must be maintained for proper development. As for SF-1, SUMOylation of Ftz-f1 seems to reduce its capacity of transcriptional activation on Sump1. What could be the biological function of Ftz-f1 SUMOylation? One possibility might be that SUMOylation attenuates Ftz-f1 function after pupariation. As the first peak of ecdysone production subsides, perhaps Ftz-f1 SUMOylation and reduced levels of SR-BI contribute to this downregulation, separating it from the second ecdysone peak that drives pupation itself (10–12 hours after pupariation formation).

**Drosophila** SR-BIs requirement for cholesterol uptake and mobilization in the steroidogenic tissues PG and ovary

Cholesterol, a main component of the cell membranes, is also important for the synthesis of steroid hormones. Steroid hormone biosynthesis requires, in addition to correct cholesterol uptake, appropriate intercellular and intracellular transport. Insects, which are incapable of synthesizing cholesterol, incorporate it from the diet through intestinal absorption and then transport it to different tissues via open circulation in the hemolymph associated with the lipoprotein lipophorin [85]. Several ultrastructural changes have been described in active PG cells, such as increased agranular ER, mitochondria and increased intracellular channels and nuclear folding that correlate with the sterol uptake and/or release of ecdysone [78,79–85]. However, the mechanism used to incorporate cholesterol in the PG and secrete the ecdysone are still largely unknown.

Two main pathways have been described for cellular uptake of lipoprotein-cholesterol esters: the low-density lipoprotein (LDL) receptor mediated endocytic uptake of LDL-cholesterol and the “selective” cholesteryl ester uptake pathway mediated by SR-BI. In insects, proteins related to the mammalian LDLR, lipophorin receptors, have been identified [89]. Recently, the function of *Drosophila* Lpr1 and Lpr2 for neutral lipid uptake in imaginal disc cells and oocytes have been described; however, the phenotype of *lpr1* and *lpr2* mutants does not suggest a role for these receptors in the PG or in the larval-pupal transition [89].

In mammalian steroidogenic tissues the SR-BI “selective pathway”, without endocytic uptake, seems to be the main one used to satisfy the cholesterol requirements for hormone synthesis. [90–92]. Interestingly, SR-BI is also necessary for the formation of the microvillar channels of the adrenal gland, as shown by the reduction of channels in SR-BI null mice and the increased formation of these channels after overexpression of SR-BI [24,93–96]. SR-BI is also expressed in the rat ovary where, similar to adrenocortical cells [95], it is detected on microvilli and membranes of microvillar channels that contained trapped lipoproteins [97]. The expression of SR-BI is regulated by the nuclear receptors SF-1 and LRH-1, supporting the significance of these receptors in lipid capture for steroidogenesis [22,23,98]. Other factors such as the hormones ACTH, estrogen or gonadotropin induce SR-BI expression [99,100].

The *Drosophila* CD36 gene family consists of 14 genes [30,101]. Recently, the expression of Sump1, Crq and Peste in steroidogenic tissues such as PG, ovaries and testes was described [31], pointing to a role for these receptors in these tissues. Significantly, the expression of these receptors in the PG was upregulated at the moment of pupariation when high levels of ecdysteroids are required [31] and this work). Silencing *Sump1* or *pes* in the PG produced the developmental arrest at L3 prior to pupariation. These results indicate that these receptors are not functionally equivalent and the presence of one of them cannot substitute for the other in the PG. Alternatively, reduction of the levels of one of the receptors might be enough to lower the total content of Scavengers and, therefore, the total capacity of the cell for capturing lipids. Interestingly, overexpression of *Sump1* is able to rescue the lipid content of smt3i PG cells. However, this rescue of lipid content is not sufficient to allow the larval-pupal transition, suggesting that the cells are still unable to produce a threshold level of ecdysone. There could be several explanations for this. For instance, overexpression of one of the receptors would be enough for lipid capture but not for lipid mobilization. In this respect, abnormally large lipid droplets were observed in Sump1-lpr1 PG cells, which suggest an additional role in sterol mobilization or a function in the regulation of the lipid droplet size. Several proteins have been shown to affect the size of the lipid droplets such as Rab small GTPases, sterol regulatory element binding protein cleavage activating protein (SCAP) and isoforms of phosphocholine cytidylyltransferase [102,103]. Mutants for other proteins that promote intracellular transport of lipids in the PG, such as *npelA* mutants, have abnormal accumulation of intracellular sterol and are unable to molt due to low levels of ecdysone [104–106].
We showed that the expression of SR-BIs increases at the onset of pupariation, coinciding with an increase in PG's lipid content. Moreover, our results showed that SR-BIs are regulated by Ftz-f1. Interestingly, the Snmp1 locus contains two putative Ftz-f1 binding sites and an atypical SF-1 binding site that could be implicated in Snmp1 regulation. Indeed, experiments in cultured cells and in vivo showed that Ftz-f1 is able to activate Snmp1 promoter. Snmp1 might not be the only Scavenger Receptor regulated by Ftz-f1 in the PG. Furthermore, in addition to SUMOylation influencing the capacity of Ftz-f1 to regulate SR-BIs expression (Figure 8H), we observed that Snmp1 contains two high score putative SUMOylation sites (data not shown). Is Snmp1 modified by SUMO and could this modification affect its function in cholesterol uptake/transport during the larval to pupa transition? Does SUMOylated Ftz-f1 affect the regulation of other SR-BIs in clear-gut larvae? We cannot discard the possibility that other proteins involved in ecdysone synthesis or transport are SUMOylated. The fact that viability is not rescued by Snmp1 overexpression, suggests that this is indeed the case. These questions remain unanswered and will be addressed in the future.

In summary, we demonstrated that Drosophila SR-BI family and Ftz-f1 participate in steriodogenesis downstream of SUMOylation by regulating the lipid uptake in the PG required for the synthesis of ecdysones. The participation of these factors in lipid uptake is conserved in other steriodogenic tissues, suggesting a general role for SUMO, Ftz-f1 and SR-BI in lipid uptake. Our data provide new insight into the lipid homeostasis of the organism.

**Materials and Methods**

**Drosophila strains**

Flies were raised on standard Drosophila medium at 25°C. The wild-type (WT) control strain was Vallecass. Ga4 strains were pnm-Gal4, UAS-smt3::GFP/ TM6B, Tb (called pnm-Gal4, obtained from P. Leopold and C. Mirth) [107,108] and p(Uac6B)T155-Gal4, UAS-mCD8::GFP/TM6B, Tb1 (Bloomington Drosophila Stock Center- BDSC). UAS-RNAi lines were: UAS-smt3i [7]; UAS-ftz-f1i (Vienna Drosophila RNAi Center-VDRC- #2959, which recognizes z and ftz-f1i isoforms); UAS-pes (VDRC- #33153); UAS-ceg (VDRC- #45883); UAS-Snmp1i (VDRC- #04210) and UAS-Snmp1i (NIG-FLY #7000R-3); Overexpression UAS lines used were obtained from BDSC: UAS-Snmp1i (Bloomington Drosophila Stock Center- BDSC). All constructs were fully sequenced.

**In vivo and in vitro SUMOylation assays**

SUMOylation motifs were identified using SUMOplot (http://www.abgent.com/sumoplot) software and Phosida Posttranslational Modification Database (http://www.phosida.com). For the in vitro procedure ftz-f1 cDNA (LD34899, DGRC) was translated using TNT-T7 (wheat germ extract; Promega), to which 35S-methionine was added (Amersham Biosciences and Pierce). This cDNA construct contains three out of the four conserved SUMOylation consensus sites. Translated Ftz-f1 was incubated with an ATP-regenerating system, SUMO-1, Ub9 and E1 activating enzyme (Biomol). Reactions were incubated at 30°C for 2 h, resolved by SDS-PAGE and exposed.

For cellular SUMOylation assays we developed new technology based on Franco et al. [113]. In brief, a plasmid encoding a form of Smt3, capable of being biotinylated, as well as the enzyme necessary for biotinylation, BirA, were introduced into cells. Any proteins that undergo SUMOylation will also be biotinylated facilitating their recovery using streptavidin beads. BirA was amplified from the UAS-(bioUb)-birA vector [113] and cloned into pAc5-STABLE2-Neo [111] by substituting GEP, generating pAc5-FLAGmCherry-BirA. To generate pAc5-bioSUMO-Blc, degenerated Drosophila smt3 sequence (EMBL database accession number FN539078) [110] was fused to a biotin tag according to the strategy described [113]. The fusion was cloned into the pAc5-FLAGmCherry vector by substituting FLAGmCherry.

**Plasmid construction and generation of transgenic strains**

Full length z and ftz-f1 cDNA sequences (EMBL database accession numbers HE169565 and HE169566, respectively; GeneArt) were cloned into the EcoRI-XbaI sites of pUASTattoh vector [109] to generate pUASTattoh-zftz-f1 and pUASTattoh-ftz-f1, respectively. xFlag-Flag respectively were inserted into the EcoRI-BglII sites. To generate constitutively SUMOylated forms, degenerated nucleotide smt3 sequence that encodes for WT Smt3 protein [EMBL database accession number FN39078] [110] was introduced into the Acd and Pcad sites of the previous vectors to generate pUASTattoh-Smt3-zftz-f1 and pUASTattoh-Smt3-ftz-f1, respectively.

Renilla and firefly luciferase (Fluc) were amplified from psiCHECK2 (Promega) by PCR and exchanged for GFP in Ac5-STABLE1 (EcoRI-HindIII) [111] to generate Ac5-FFLuc-STABLE1. For the construction of pSmp1-FLuc genomic DNA was used as template to amplify a PCR fragment containing 2 Kb of the Smp1 upstream region (including 77 bp of 5’UTR) using the oligonucleotides Smp1fl(-2000) (5’-GATCGAGATCTGGAGCATGGCATTATCCAAACATGATTTGGG-3’) and Smp1fl(+1) (5’-GATGGATCTCCTCTGGGCAATGTTTCGATCTCTACTC-3’) (numbering based on initiating methionine). The resulting BgII-EcoRI fragment was used to replace the ActinIC promoter in Ac5-FFLuc-STABLE1. Two potential Ftz-f1 binding sites were identified based on published consensus sites. Fragments were prepared for individual and double mutants in these potential Ftz-f1 binding sites using 2-step overlap extension PCR, with the forward and reverse primers Ftz-f1Mut(-1971) (5’-CCATGGCATTTCTTAAACGTATTTCGACGAAATTTGTCGATTC-3’) and Ftz-f1Mut(-1410) (5’-GAGGCCAGTTAGGCCTCAAATGGCAGCAGTCTAATGTTA-3’) (mutated nucleotides in lowercase and bold). Resulting fragments were used to replace Smp1 WT promoter sequence to generate pSmp1Mut-FLuc plasmids. All constructs were fully sequenced.

To generate pUASAttb-crq and pUASAttb-pec, ESTs RE20270 and RE21078 inserted in the PFLC1 plasmid (Drosophila Genomics Resource Center- DGRC), were digested with EcoRI and BamHI, or Mfi and BgIII, respectively. Fragments were inserted in pUASAttb [109] digested with EcoRI and BgIII. Transgenic lines were generated following standard transformation procedures [112].
DTT. The eluted sample was separated from the beads using a Vivaclear Min 0.5 μm PES microcentrifuge filter unit. The recovered volume for both control and experimental samples was 30 μl.

For Western blots we used mouse monoclonal anti-Flag M2 antibody (1:2000; Sigma), HRP-conjugated secondary antibody (1:5000; Jackson ImmunoResearch) and HRP-linked anti-Biotin antibody (1:200; Cell Signaling Technology).

**RNA probe preparation and in situ hybridization**

ESTs from the DGRc cDNA collections were used as templates for the synthesis of the RNA probes (IP13851 for Snmp1, RE21076 for pes, RE2070 for crq and LD3409 for fzt-f1). RNA labeling was performed using the DIG RNA labeling Mix (Roche) according to the manufacturer instructions, using 1 μg of linearized DNA.

RNA probes were hybridized to larval tissues at 55°C in 50% deionized formamide (Sigma), 5× saline sodium citrate, 50 μg/ml heparin sodium salt (Sigma), 0.1% Tween 20, and 100 μg/ml of phenol extracted sonicated salmon sperm DNA (Amersham Biosciences). Samples were incubated with anti-digoxigenin antibody (1:2000; AP Fab fragments, Roche) and signal was detected using 4-Nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl-phosphate (Roche).

**qPCR analysis**

RNA was extracted from isolated ring glands complexed with brain hemispheres placed in RNAlater (Ambion) and frozen in liquid nitrogen. At least two different pools of 50 to 100 specimens were collected per genotype. Total RNA extracts were obtained using the “mirVana miRNA isolation kit” (Ambion) according to the manufacturer’s instructions and were quantified using the NanoDrop (Thermo Scientific). cDNAs were prepared from 0.2 μg of RNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) at a 10 μl volume per reaction, following manufacturer’s instructions.

Oligonucleotides for pes, crq, Snmp1 and RpL32 were designed using NCBI primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast). RpL32 was used as control. Oligo sequences were:

Oligonucleotide for pes: 5'-TGGGCTGGTGCAATGTGACATA-3';

Oligonucleotide for crq: 5'-CACGTCTTAGCAGCACTGACGCTTGGATAG-3';

Oligonucleotide for Snmp1: 5'-ATGGGTCAGGCCAATCACTCGGC-3';

Oligonucleotide for RpL32: 5'-ATCCACTTCTTTCGTCACAGTCAGC-3'.

**Luciferase assay**

_Drosophila_ S2R+ cells were seeded in 24-well plates and transfected with 150 ng of _pSnmp1-FLuc_ or _pSnmp1Mut-FLuc_, either alone or co-transfected with the same quantities of _pUASTattb-fzt-f1_, _pUASTattb-crq_, _pUASTattb-Snmp1_ or _pUASTattb-Snmp1i_. 150 ng of _pκ-C-Gal4_ [116] and 50 ng of _pκ-Relish_ were added to all the wells. Transcriptional activity was measured 48 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega), following the manufacturer’s instructions. Luminescence was measured in a microplate luminometer (Veritas). Results are given as mean±S.D. Differences between groups were calculated using Student’s t test in Microsoft Excel.

**Immunocytochemistry**

Adults were allowed to lay eggs during 8 hours and wandering larvae were collected 5 days AEL. Larvae and ovaries from adult flies were dissected in PBS, fixed in 4% paraformaldehyde for 20 minutes and washed three times in PBT (PBS, 0.3% triton X-100) for 20 minutes. Samples were blocked in PBT +1% BSA for one hour and incubated with the appropriate antibodies at 4°C overnight. Next day, tissues were washed with PBT three times, for 20 minutes each and incubated with secondary antibodies at room temperature for two hours. The following primary antibodies were used: guinea pig polyclonal anti-Cad99C (1:3,000 [47]; mouse monoclonal DE-Cad (1:25; DCAD2), Developmental Studies Hybridoma Bank; rabbit polyclonal anti-Sm3 (1:500 [117]); goat polyclonal anti-Ftz-f1 (1:25; Santa Cruz, Sc-27221); rabbit polyclonal anti-Crq (1:100 [118]); rabbit polyclonal anti-Snmp1 (1:1000) [119] and rabbit polyclonal anti-Dib (1:200) [26]. Fluorescence Alexa 568 secondary antibody (Molecular Probes) was used at 1:200 dilution. DAPI (Roche) was used at 1:2000 dilution. Phalloidin-TRITC (Sigma) was used 1:1000. Samples were mounted in Vectashield (Roche) mounting medium. Confocal images were taken with a Leica DM IRE2 confocal microscope.

**Oil Red O stainings**

Ring glands were fixed in 4% paraformaldehyde for 20 minutes, washed twice in PBS and stained with Oil Red O (Sigma) solution at 0.06% in isopropanol for 30 minutes. Samples were washed twice in PBS before mounting in Vectashield. Images were taken with a Leica DM IRE2 confocal microscope.

Quantification of lipid droplets was done on single plane confocal micrographs of Oil Red O stainings using the Analyze Particle tool from ImageJ software. At least 10 independent micrographs were analyzed per genotype. Measurements were analyzed and plotted using Microsoft Excel.

**20E feeding experiments**

_phm-Gal4->UAS-ftz-f1_ and _phm-Gal4->UAS-Snmp1i_ larvae were collected at 120 hours AEL and placed in groups of 10 individuals in new tubes. These were supplemented with 20E (Sigma) dissolved in ethanol and mixed with yeast at 1 mg/ml. Control larvae were fed with yeast supplemented with ethanol alone.

**Supporting Information**

_Figure S1_ Conservation of SUMOylation consensus sites in Ftz-f1 related sequences from vertebrates to insects. ChastaW analysis of the Fzt-f1 homologues in the indicated species. SUMOylation consensus sites conserved only among insects are marked in blue, those conserved between insects and NR5A2 homologues are highlighted in orange and those conserved among the vertebrate NR5A2 and NR5A1 (SF-1) are indicated in pink. Orange and...
purple asterisks mark the sites SUMOylated in rat LRH-1/ NR5A2 [14]. Blue and purple asterisks indicate the sites SUMOylated in mouse SF-1 [21]. The blue bar above the alignments indicates the DNA binding domain, while the red bars indicate the ligand binding domains. Below the alignments, asterisks indicate identical amino acids, colons indicate conserved substitutions and periods indicate semi-conserved substitutions. Accession numbers of the sequences used for the analysis: Aedes aegypti, XP_001654601.1; Anopheles gambiae, XP_315680.4; Apis mellifera, XP_001122182.2; Blattella germanica, CAQ57670.1; Bombayx mori, BAK53999.1; Drosophila melanogaster, NP_324143.2; Gallus gallus NR5A2, NP_990409.1 and SF1, BAA76713.1; Homo sapiens NR5A2, NP_003813.1 and SF-1, NP_004950.2; Monodelphis domestica NR5A2, NP_100601 and SF-1, NP_620639; Rattus norvegicus NR5A2, NP_068510 and SF-1, NP_001178028.1; Tribolium castaneum, XP_970369.2; Xenopus laevis NR5A2, NP_001081185.1 and SF-1, NP_001091438.1.

Figure S2

Expression of Scavenger Receptors in PGs of WT or smt3i backgrounds. (A–H) Micrographs of cry (A–D) or pex (E–H) mRNAs in situ hybridization in PGs from WT (A, B, E, F) or phm-Gal4–UAS–smt3i RNAi (smt3i) larvae (C, D, G, H) at the indicated hours AEL. cry mRNA is upregulated in WT clear-gut larvae (B, compare with A). However, no expression is visible in smt3i PGs (C, D). pex mRNA is also moderately upregulated in WT clear-gut larvae (B, compare with A) but, in contrast to cry and Smp1, is still expressed in smt3i larvae (G, H). All the in situ reactions were stopped at the same time and pictures were taken at the same magnification.

(TIF)

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Figure S3

Distribution of lipid droplets when smt3 or Smp1 are silenced. (A–B) Single plane confocal micrographs taken under the same intensity settings showing the lipid droplets marked by Oil Red O staining (green). Nuclei are labelled with DAPI (purple). F-actin cytoskeleton is shown in blue. Follicle cells are indicated by yellow dotted lines. (A’–B’) Single green channels are shown in black and white. (A) In T155-Gal4–UAS–smt3i follicle cells show reduced lipid droplets. However, the oocyte (its nucleus in purple indicated by a yellow arrowhead) gets lipids through the ring canals (white arrowhead) from the nurse cells. Lipids are indicated by yellow arrowheads in A’. (B) In T155-Gal4–UAS–Smp1i, the lipid intake by follicle cells stops. Droplets only occupy the basal side of the cells (b; indicated by a yellow arrowhead), while the apical side remains depleted (a).

(TIF)

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Author Contributions

Conceived and designed the experiments: AT JDS RB. Performed the experiments: AT LH LP CP MG JS FL-O JDS RB. Analyzed the data: AT LH JDS RB. Contributed reagents/materials/analysis tools: UM MSR. Wrote the paper: AT RB.
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