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OSBP- and FAN-mediated sterol requirement for spermatogenesis in *Drosophila*

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SUMMARY

Members of the oxysterol binding protein (OSBP) family are involved in diverse biological processes, including non-vesicular sterol transport and vesicle trafficking. The mechanisms by which OSBPs integrate functionally with developmental and physiological processes remain elusive. Here, we report the *in vivo* analysis of OSBP function in the model organism *Drosophila*. *Osbp* mutants are male-sterile and exhibit defects in individualization, the process by which each spermatid is packaged into its own membrane. Overexpression of OSBP leads to post-eclosion behaviour defects that can be suppressed by co-expression of endoplasmic reticulum-specific VAP family proteins. Most notably, FAN, a testis-specific VAP protein, acts together with OSBP genetically and physically to regulate the individualization process. OSBP-positive and sterol-enriched speckles are found at the leading edge of the individualization complex in wild type but not in *Osbp* or *fan* mutants, suggesting that sterol trafficking might play key roles during the membrane-remodelling phase of individualization. In addition, *Osbp* mutants that are fed additional sterols partially recover fertility, implying that male sterility is attributable to sterol shortage. Thus, we have identified an OSBP- and FAN-mediated sterol requirement in *Drosophila* spermatogenesis.

KEY WORDS: OSBP, *Drosophila*, Sterol, Spermatogenesis, Individualization

INTRODUCTION

Sterols are essential components of cell membranes and are precursors of various steroid hormones. Sterol homeostasis is crucial for normal cellular behaviour and physiology. Dysfunction of sterol homeostasis leads to disease conditions in humans, such as hypercholesterolaemia, caused by excess sterols, or Smith-Lemli-Opitz syndrome, caused by lack of sterols (Herman, 2003). Studies of the regulation of sterol biosynthesis and uptake have led to the discovery of the low-density lipoprotein (LDL) endocytotic pathway and the sterol regulatory element-binding protein (SREBP) pathway (Brown and Goldstein, 1997; Goldstein and Brown, 2009). Despite this, many fundamental aspects of intracellular sterol transport and distribution are not well characterized (Maxfield and Menon, 2006).

Within cells, sterols are distributed unevenly with the highest level in the plasma membrane and the lowest level in the endoplasmic reticulum (ER). It is not known how this uneven sterol distribution is achieved. Both vesicular and non-vesicular trafficking systems have been implicated in sterol transport and distribution (Maxfield and Wustner, 2002; Soccio and Breslow, 2004). The OSBP family consists of sterol-binding proteins that have been postulated to be involved in intracellular non-vesicular trafficking of sterols. OSBP proteins are conserved from yeast, worms and flies to mice and humans. OSBP was originally characterized in the 1980s as a cytosolic receptor for oxysterol, hence the name oxysterol-binding protein (OSBP) for the founder member and, subsequently, OSBP-related proteins (ORPs) for the

other family members (Kandutsch and Thompson, 1980; Olkkonen, 2004). Besides the oxysterol-binding protein (oxysterol-BP) motif, which has sterol binding function, some ORPs contain other protein-protein or protein-lipid binding domains. For example, the N-terminal pleckstrin homology (PH) domain can anchor OSBP to the Golgi apparatus by interacting with phosphatidylinositol 4-phosphate (PI4P) and the small GTPase ADP-ribosylation factor 1 (ARF1) (Levine and Munro, 2002). The internal FFAT motif can localize OSBP to the ER by binding to the ER protein VAP (VAMP-associated protein) (Loewen et al., 2003).

OSBP and ORPs were subsequently found to bind to cholesterol and are implicated in sterol transport, vesicle trafficking and cell signalling (Olkkonen et al., 2006). For example, in yeast, Osh4 is essential for rapid transport of cholesterol between the plasma membrane and ER, and specifically facilitates the non-vesicular transfer of sterols between membranes *in vitro* (Raychaudhuri et al., 2006). Upon binding 25-hydroxycholesterol, OSBP translocates from the cytosol to the Golgi, but the consequence of this is unknown (Ridgway et al., 1992). Knockdown of ORP9 in cells results in Golgi fragmentation, accumulation of sterols in endosomes or lysosomes and, subsequently, ER-to-Golgi transport defects, indicating that the partitioning of sterols in the Golgi and other organelles affects intracellular transport (Ngo and Ridgway, 2009). Furthermore, in CHO-K1 cells, OSBP synergizes with ceramide transport protein (CERT) in ER-to-Golgi ceramide transport, and the interaction of OSBP with the ER and Golgi apparatus is required for Golgi translocation of CERT (Perry and Ridgway, 2006). OSBP has also been identified as a scaffold protein for the protein phosphatase PP2A in the regulation of ERK signalling (Wang et al., 2005). The structural analysis of yeast Osh4 suggests a model in which sterol- and membrane-binding of Osh4 promote reciprocal conformational changes that facilitate sterol transfer (Im et al., 2005). Simultaneous knockout of all seven ORPs is lethal in yeast, showing that OSBP family proteins have essential and redundant functions (Levine and Munro, 2001).

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Despite numerous studies of the molecular functions of OSBP at the intracellular level, the functions of OSBP in multicellular animals remain to be determined. What is the biological significance of OSBP-mediated sterol transport in animal development and, in particular, in brain and testis, both of which are abundantly enriched with membranous structures (Cross, 1998; Dietschy and Turley, 2001)? There are at least 12 genes encoding OSBP family proteins in mice, but knockout mutants have not yet been reported (Fairn and McMaster, 2008). Interestingly, *Drosophila* and *Caenorhabditis elegans*, two model organisms that cannot synthesize sterols de novo, both have only four OSBP family proteins, which is even fewer than yeast. Therefore, it is relatively easy to investigate the function of OSBP family proteins in worms and flies, as sterol trafficking, which mainly involves dietary-derived sterols, is less complicated in these organisms.

Here, we describe the use of various genetic tools in *Drosophila* to study the in vivo functions of OSBP proteins. We show that loss of function of OSBP leads to a male-sterile phenotype with spermatogenesis defects, especially in the individualization process. Individualization is a membrane reorganizing process during which an actin-based complex, the individualization complex (IC), composed of a cohort of 64 actin cones, progresses caudally along the spermatid heads and remodels the syncytial membrane to remove excess cytosol and pack each spermatid into its own plasma membrane (Fuller, 1993). Mutants that compromise the caspase pathway, the integrity or the movement of the IC, or the trafficking of intracellular vesicles have been shown to affect the individualization process (Fabrizio et al., 1998; Huh et al., 2004; Noguchi et al., 2006; Noguchi and Miller, 2003; Sevrioukov et al., 2005). We found that OSBP-positive and sterol-rich speckles are present at the leading edge of the IC, suggesting that they might participate in the membrane remodelling process. The *Osbp* male-sterile phenotype can be partially rescued by exogenous cholesterol, indicating that OSBP deficiency is associated with a lack of sterols. Furthermore, we show that FAN, one of the three VAP proteins in *Drosophila*, functions together with OSBP in spermatogenesis. In summary, we report an OSBP- and FAN-mediated sterol transport pathway that functions in *Drosophila* spermatogenesis.

MATERIALS AND METHODS

Drosophila stocks

Flies were cultured on standard corn-meal medium at 25°C. The following transgenic stocks were made: *UAS-Osbp*, *UAS-Osbp Δ PH*, *UAS-Osbp F276A*, *UAS-Osbp HH503/504AA*, *UAS-CG3860*, *UAS-CG5077*, *UAS-CG1513*, *UAS-ha-fan*, *P β 2-tub-fan^{RNAi}*, *P β 2-tub-fan*. Other transgenic fly strains used were: *UAS-dvap33a*, *UAS-ManII-eGFP*, *CCAP-Gal4*, *386Y-Gal4*, *ppl-Gal4*, *Hsp83-Gal4*, *nanos-Gal4::VP16*, *UAS-ER-YFP* and *sqh-EYFP-Golgi*. Ends-out gene targeting methods were used to generate *Osbp* and *fan* knockout flies (Chen et al., 2009; Gong and Golic, 2003). Long-range PCR, RT-PCR and Southern blotting were performed to verify the knockouts.

Molecular biology

Full-length cDNAs of *Osbp*, *CG1315* and *CG5077* (CG42668 – FlyBase) were amplified by RT-PCR. cDNA of *CG3860* was obtained from the cDNA clone *GHI2064*. These cDNAs were subcloned into the *pUAST* vector to create *UAS* transgene constructs. The *Osbp* cDNA lacking the PH-domain coding sequence was created by PCR with an in-frame start codon (ATG) after the PH domain. The OSBP point mutations F276A and HH503/504AA were generated by site-directed mutagenesis using the QuickChange Mutagenesis Kit (Stratagene). Full-length *fan* cDNA was amplified by RT-PCR. The cDNA was inserted in frame into the *pCMV-HA* vector, resulting in HA-FAN fusion. The *ha-fan* fragment was

subcloned into *pUAST* to generate a *pUAST-ha-fan* construct. The construct for *fan* genomic rescue was created by fusing a *ha-fan* fragment with a 5' promoter fragment (~0.55 kb) and a 3' downstream region (~0.56 kb) in genomic array order. This modified genomic sequence was cloned into the *Drosophila* transformation vector *pCaSpeR4* to generate the final construct *P β 2-tub-fan*. To create the construct for *fan* RNAi in the germline, two full-length *fan* cDNA fragments (~0.7 kb each) were cloned in opposite orientations into the *pWIZ* vector then inserted downstream of the β 2-tub promoter in *pCaSpeR3* to create *P β 2-tub-fan^{RNAi}*.

Antibodies and immunostaining

A cDNA fragment encoding amino acids 183-603 of the OSBP protein was cloned in frame into the *pATH11* vector and the TrpE-fused OSBP fragment was expressed in BL21 bacteria. A polyclonal antibody was generated by immunizing rabbits with the resulting purified OSBP antigen. The same antigen was used to immunize mice to produce the monoclonal antibody mAB7.

For immunostaining, tissues were fixed with 4% paraformaldehyde for 20 minutes followed by 0.3% PBT (PBS + 0.3% Triton X-100) treatment for 30 minutes. Filipin (50 μ g/ml; Sigma) was used for sterol staining. 1 μ g/ml DAPI or 1.5 μ M propidium iodide (PI) was used for nuclei staining. For antibody staining, the samples were incubated with primary antibody (diluted in 0.1% PBT) at 4°C overnight then with secondary antibody for 2 hours at room temperature. Primary antibodies used were: rabbit anti-LVA (kindly provided by Drs John Sisson and Ophelia Papoulas, University of Texas at Austin, TX, USA; 1:250), rabbit anti-HA antibody (Santa Cruz; 1:100), mouse anti-OSBP monoclonal antibody (this study; undiluted) and rabbit anti-cleaved caspase 3 (Asp175) antibody (Cell Signaling; 1:200). The following secondary antibodies were used at 1:2000 in this study: anti-rabbit Alexa Fluor 488, 568 and anti-mouse Alexa Fluor 594 (Molecular Probes). Alexa Fluor 488 phalloidin or Alexa Fluor 543 phalloidin (Molecular Probes) was added to the secondary antibody at a final concentration of 2 Units/ml when counterstaining with anti-OSBP antibody or anti-cleaved caspase 3 antibody. Images of controls and genetically manipulated samples were taken with equivalent exposure conditions and scale.

Electron microscopy

Testes were dissected from 2- to 3-day-old males and fixed with 2% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The samples were treated with 1% osmium tetroxide followed by 1% uranyl acetate, then dehydrated in an ethanol series and infiltrated with, and embedded in, embed 812 resin (Electron Microscopy Sciences). Ultrathin sections were cut with a Diatome diamond knife and mounted on formvar-coated grids then stained with 0.08 mM lead citrate trihydrate for 10 minutes. The sections were examined using an FEI Tecnai 20 electron microscope at 120 kV and photographed using a CCD camera.

Western blot and co-immunoprecipitation

To detect OSBP, dissected testes were treated with lysis buffer [50 mM NaCl, 1% NP40, 50 mM Tris (pH 7.4)] containing a complete protease inhibitor pellet (Roche). Lysates were resolved on 10% SDS-PAGE gels, transferred to nitrocellulose and probed with anti-OSBP rabbit serum (1:500). For co-immunoprecipitation (co-IP), adult lysates were applied to pre-adsorbed anti-HA antibody beads, shaking head-to-tail at 4°C overnight. After extensive washing with lysis buffer, the samples were resolved, transferred and probed using standard western blot procedures. For endogenous co-IP, the testis lysates from *P β 2-tub-fan* transgenic animals were used.

Sterol feeding and fertility assays

To examine fertility, individual adult males were mated to three wild-type virgin females in separate vials. Unless otherwise specified, the females were transferred after 9 days at 25°C to fresh vials. Progeny from the original vial and the first transfer vial were counted. For the sterol feeding assay, flies were raised on normal corn-meal food with additional sterols (0.14 mg/g 7-dehydrocholesterol or cholesterol) and subsequently mated. One week after mating, the adults were removed and the fertility rate and progeny number were scored.

RESULTS

Osbp mutants are male sterile

Four OSBP family proteins, OSBP (previously known as CG6708), CG1513, CG3860 and CG5077 were identified by Blast analysis of the *Drosophila* genome database with the human OSBP protein sequence (Fig. 1A). OSBP contains a PH domain, an FFAT domain and an oxysterol-BP domain, which is the signature motif of OSBP family proteins. CG1513 has a PH domain and an oxysterol-BP domain, whereas CG5077 has five predicted splicing variants, including long isoforms containing a PH domain and short isoforms without a PH domain. CG3860 has an oxysterol-BP domain only. We examined the expression patterns of these *Osbp* family genes by semi-quantitative RT-PCR and found that they are broadly expressed throughout development (see Fig. S1 in the supplementary material; data not shown).

Next, we explored the function of OSBP family proteins during development by analyzing mutant phenotypes. We generated knockout mutants of *Osbp*, *CG5077* and *CG3860* by ends-out gene targeting (Chen et al., 2009). We also obtained *CG1513^{c05643}*, a P-element insertion allele that deletes the C-terminal 71 amino acids of the protein, including part of the oxysterol-BP domain. All these mutants are viable and fertile except for *Osbp*. Therefore, we focused our studies on *Osbp*. Flies homozygous for *Osbp* knockout allele *Osbp¹* were viable but male-sterile and were indistinguishable from that of *Osbp¹* over deficiency *Df(3R)ED6220*, suggesting that they are null alleles and that the male-sterile phenotype is owing to *Osbp* mutation (Fig. 1B). The nature of the *Osbp* mutation was also confirmed by western blot using an anti-OSBP antibody. A band (~89 kDa) was detected in protein extracts from wild type and flies with germline-specific OSBP overexpression but not from *Osbp* mutants (Fig. 1C).

Osbp mutants exhibit individualization defects

We next addressed the cause of male infertility in *Osbp* mutants. The testes of *Osbp* mutants appeared the same as that of wild type. During spermatogenesis in *Drosophila*, a pair of cyst cells encloses a primary spermatogonial cell and its progeny. Within this structure, which is called a cyst, proliferating germline cells connected by cytoplasmic bridges differentiate in synchrony from round to elongated spermatids. In *Osbp* mutants, besides spermatocytes, the round and elongated spermatids were comparable with wild type when examined using phase-contrast microscopy (Fig. 2A). However, the seminal vesicles, where the mature motile sperm are stored in wild type, were devoid of sperm in *Osbp* mutants (Fig. 2B). In addition, sperm debris accumulated in the basal region of the testis. These phenotypes were 100% penetrant in *Osbp* mutants. Therefore, *Osbp* mutants have a defect in spermatogenesis that occurs between the production of elongated spermatids and the maturation of motile sperm.

We then examined individual elongated spermatids in squashed testis. Compared with the smooth appearance of individual elongated spermatids in wild type, numerous blebs along spermatids, indicative of unremoved cytoplasm, could be found in *Osbp* mutants, suggesting that the individualization process is impaired (see Fig. S2 in the supplementary material) (Fabrizio et al., 1998). The membrane-remodelling individualization process follows the differentiation of round spermatids into elongated spermatids. Individualization is achieved through coordinated caudal movement of a cohort of F-actin-based triangular cone

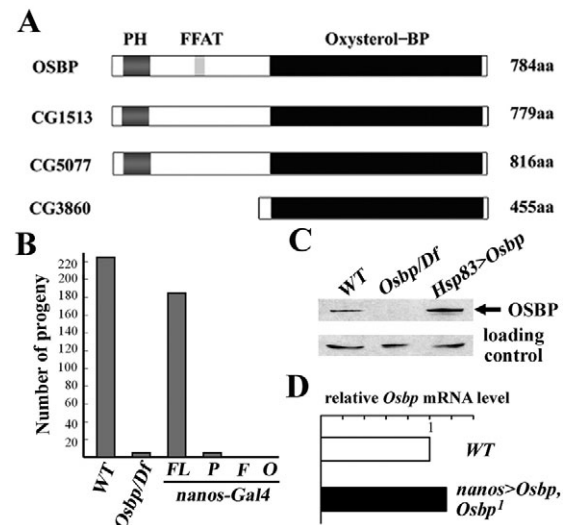


Fig. 1. *Osbp* is required for male fertility in *Drosophila*.

(A) Schematic of the structure of OSBP family proteins. PH, FFAT and oxysterol-BP domains are indicated. (B) Mean number of progeny for flies of different genotypes. Progenies from 10–20 males were counted. WT, wild type; *Osbp/Df*, *Osbp* loss-of-function; FL, full-length *Osbp*; P, *Osbp* with a deletion of the PH domain; F, *Osbp* with a point mutation in the FFAT motif; O, *Osbp* with point mutations in the oxysterol-BP domain. (C) Western blot of OSBP protein extracted from testes. The arrow indicates the predicted OSBP band. A non-specific band below the predicted OSBP band was used as loading control. (D) Mean mRNA levels of *Osbp* measured by quantitative RT-PCR. The *Osbp* mRNA in WT was set at 1. Experiments were repeated three times.

structures, collectively called the individualization complex (IC) (Fig. 2C). As the IC moves through the cyst, the caspase interleukin-1 β -converting enzyme (ICE) remains activated throughout the non-individualized part of the sperm. In the individualized portion of the sperm, the activated caspase signal is absent (Huh et al., 2004). Disrupted IC and leakage of activated caspase/ICE into the individualized part of the sperm are two examples of individualization defects. The wild-type IC, visualized by phalloidin staining, is rectangular- or fan-shaped and contains many individual triangular cone structures. In *Osbp* mutants, the IC cohort was not fragmented but appeared mildly disorganized, displaying irregular shapes with fewer triangular cone structures (~50%; $n=55$) (Fig. 2D,E). Additionally, residual activated caspase/ICE was found in the individualized trailing end of ICs in *Osbp* mutants (~75%; $n=126$), suggesting that *Osbp* mutants are defective in the individualization process (Fig. 2F,G).

To examine the *Osbp* phenotype further, we performed ultrastructural analysis of *Osbp* mutants. In wild type, each individualized spermatid was enclosed within its own membrane (Fig. 2H,H'). However, in *Osbp* mutants, we found that many spermatids were enclosed within one membrane, a phenotype typically present in mutants with individualization defects (Fig. 2I,I'). The individualization phenotype was further confirmed with DJ-GFP, a mitochondrial protein marker. In wild type, DJ-GFP was evenly distributed throughout each individualized spermatid, whereas in *Osbp* mutants, DJ-GFP formed aggregates that were dispersed along the spermatid (see Fig. S2 in the supplementary material). Taken together, our results suggest that *Osbp* is important for individualization during spermatogenesis in *Drosophila*.

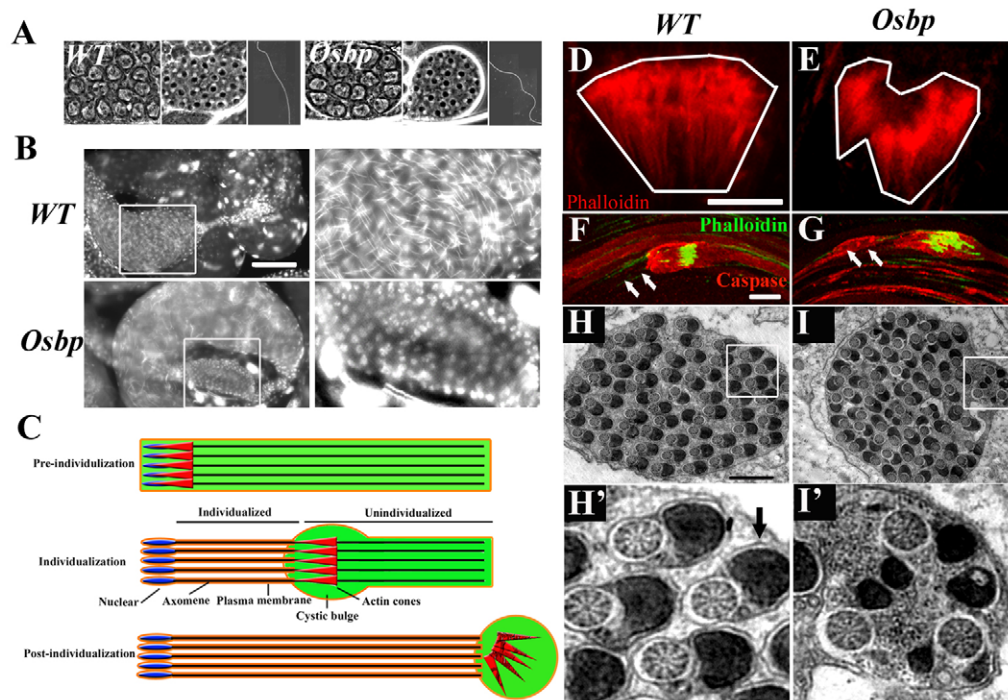


Fig. 2. *Osbp* mutants are defective in individualization. (A) Spermatocytes (left), round spermatids (middle) and elongated cysts (right) in wild type and *Osbp* mutants. (B) Detection of sperm by nuclear staining with DAPI in seminal vesicles. Boxed areas are magnified on the right. The characteristic needle-like nuclei of mature sperm can be seen in the wild type (WT) but not in *Osbp* mutants. Scale bar: 50 μ m. (C) Diagram of the individualization process. Actin cones of the individualization complex (red) initially assemble around the nuclei (blue). During individualization, the actin cones move caudally. Simultaneously, excess cytoplasm (green) is expelled into the cystic bulge and individualized parts (orange) of sperm are packaged into their own plasma membrane. When actin cones reach the end, degraded actin cones and discarded cytoplasmic components are detached from the sperm bundle. (D,E) Phalloidin staining revealing individualization complex (IC) morphology. In *Osbp* mutants (E) the IC has an irregular shape (outlined) in contrast with the fan-shaped IC of the WT (D). Scale bar: 20 μ m. (F,G) Activated caspase/ICE detected with an anti-cleaved caspase 3 antibody. In wild type, activated caspase (red) is absent from the individualized part of the cyst (arrows in F) but in *Osbp* mutants it leaks into the individualized region (arrows in G). Phalloidin staining is in green. Scale bar: 20 μ m. (H-I') Transmission electron microscope images of cysts from wild type (H,H') and *Osbp* mutants (I,I'). Boxed areas in H and I are enlarged in H' and I', respectively. Individualized sperm cells within their own plasma membranes can be seen in wild type (arrow in H') whereas sperm are non-individualized in *Osbp* mutants (I'). Ten testes were examined and, on average, 9% spermatids has individualization defects in each section. Scale bar: 1 μ m.

Cell-autonomous function of OSBP in the germline

The male infertility of *Osbp* mutants could result from defects in germ cells or cyst cells or both. To identify where OSBP is required, we expressed *Osbp* using the *UAS-Gal4* system, in which a target gene linked to *UAS* promoter elements is activated by Gal4 protein expressed under the control of a chosen promoter (Brand and Perrimon, 1993). When driven by a germline-specific *Gal4*, *nanos-Gal4::VP16*, the *UAS-Osbp* transgene significantly restored the fertility of *Osbp* mutants, indicating that *Osbp* is likely to regulate male fertility in a cell-autonomous manner (Fig. 1B,D; see Fig. S2 in the supplementary material). Germ cell-specific expression of the other three *Drosophila* *Osbp* family genes, *CG1513*, *CG3860* and *CG5077*, did not rescue the fertility of *Osbp* mutants, implying that OSBP has a distinct function in the *Drosophila* testis (data not shown).

To determine whether the conserved PH, FFAT and oxysterol-BP domains of OSBP are required for male fertility, we tested the rescuing activity of *UAS-Osbp* transgenes carrying domain-specific mutations. Point mutations in the FFAT domain (F276A) and the oxysterol-BP domain (HH503/504AA) abolished the rescuing activity of OSBP, as did deletion of the PH domain (Fig. 1B). These results suggest that all three domains are essential for the function of OSBP in testis.

VAP family proteins suppress post-eclosion behaviour defects resulting from OSBP overexpression

To investigate how OSBP regulate individualization, we then searched for its genetic partners in testis. As it is difficult to genetically identify potential interactors of OSBP based on the loss-of-function individualization defects, we examined the overexpression phenotype of *Osbp*. During the course of the rescuing experiments, we observed that adult flies with *Osbp* overexpression driven from *UAS-Osbp* by a ubiquitous *Gal4* (*Act5C-Gal4*) did not expand their wings and thorax and had abnormally tanned cuticles (data not shown). Overexpression of the other three OSBP family proteins (*CG1513*, *CG3860* and *CG5077*) did not result in any obvious phenotype (data not shown). As a control, *Act5C-Gal4* flies without a *UAS* transgene completed these morphological transitions (which are known as post-eclosion behaviours) normally (data not shown). A set of neuroendocrine neurons, called crustacean cardioactive peptide (CCAP) neurons, has been shown to initiate these post-eclosion behaviours by secreting the neuropeptide bursicon which activates the DLGR2 (RK – FlyBase) receptor (Luo et al., 2005). Both the pan-neuropeptidergic driver *386Y-Gal4* and the CCAP neuron-specific driver *CCAP-Gal4* resulted in 100% failure of post-eclosion

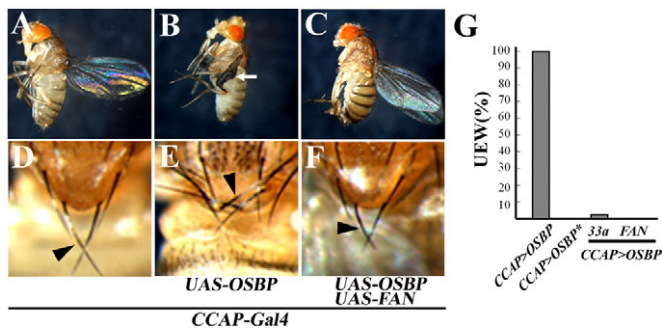


Fig. 3. Co-expression of VAP proteins suppresses the post-eclosion behaviour defects in *Osbp* overexpressing flies.

(A–F) Wing and thorax expansions after eclosion. The white arrow in B indicates an unexpanded wing. Posteriorly oriented bristles (arrowheads in D, F) indicate an expanded thorax, whereas anteriorly oriented bristles (arrowhead in E) are present on an unexpanded thorax.

(G) Quantification of unexpanded wing (UEW) phenotype in flies with *CCAP-Gal4*-driven *UAS-Osbp* or *UAS-Osbp** transgenes. More than 200 animals were scored. Overexpression of wild-type OSBP induces nearly 100% UEW phenotype, whereas mutated OSBP does not lead to this phenotype. Co-expression of VAP family proteins in CCAP neurons fully suppresses the UEW phenotype. OSBP* is OSBP with point mutations in the oxysterol-BP domain. 33a or FAN: co-expression of OSBP with VAP33A or FAN, respectively.

behaviours in *UAS-Osbp* flies (Fig. 3; data not shown). These results demonstrate that *Osbp* overexpression impairs the function of CCAP neurons, resulting in post-eclosion behaviour defects.

To identify potential partners of OSBP, we screened for modifiers that can suppress the post-eclosion behaviour defects induced by overexpression of *Osbp*. Chromosome deficiencies, gain-of-function enhancer-promoters (EPs) (*UAS* insertion lines) and candidate interactors were used in the screen. From the screen, two *Drosophila* VAP genes, *dvap33a* (*Vap-33-1* – FlyBase) and *fan*, were identified as genetic interactors of *Osbp*. VAP33A has been shown to be involved in synaptic bouton formation at neuromuscular junctions and acts as a ligand for the Eph receptor (Pennetta et al., 2002; Tsuda et al., 2008). Post-eclosion behaviour defects resulting from *Osbp* overexpression were almost totally suppressed by co-expression of *dvap33a* (Fig. 3G). Like *dvap33a*, overexpression of *fan* in CCAP neurons completely suppressed the post-eclosion behaviour defects resulting from OSBP overexpression (Fig. 3C,F,G).

***fan* mutants have reduced fertility and exhibit individualization defects**

VAP33A is unlikely to partner OSBP in the control of male fertility because it is mainly expressed in neurons (Pennetta et al., 2002). The other VAP protein, FAN, is reported to be essential for male fertility (U. Renner, M. Hollmann and M. A. Schafer, unpublished) and is expressed specifically in testis (Pennetta et al., 2002). We confirmed the testis-specific expression of *fan* by RT-PCR and by creating a genomic transgene with an HA tag (Fig. 4A). In addition, HA staining showed that FAN is expressed in testis from spermatocytes to elongated spermatids and is colocalized with the ER marker PDI-GFP (Fig. 4B). Therefore, FAN is a possible partner of OSBP in testis. To undertake loss-of-function analysis of *fan*, we generated *fan RNAi* flies by expressing hairpin fragments under control of a strong germline-specific β_2 -*tub* promoter. RT-PCR

analysis showed that the *fan* mRNA level was dramatically reduced in the testis of newly-eclosed flies with the *RNAi* transgene (*fan^{RNAi}*; Fig. 4C). Interestingly, RNAi of *fan* resulted in reduced fertility but not sterility (Fig. 4I). To rule out the possibility that residual levels of *fan* keep the animals fertile, we generated a *fan* knockout mutant and found that *fan* mutant males also exhibited reduced fertility (Fig. 4I). In addition, when we measured fertility at 5-day intervals, *fan* mutants or *fan^{RNAi}* animals exhibited a rapid decline in fertility ~10 days after eclosion, indicating that *fan* is partially essential for male fertility (Fig. 5A; data not shown).

We then examined the individualization process in *fan^{RNAi}* and *fan* mutants. As observed in *Osbp* mutants, leakage of activated caspase/ICE was found at the trailing end of ICs (88%; $n=162$), consistent with defects in individualization (Fig. 4D,E). Moreover, the ICs in *fan* mutants were severely disorganized and the individual actin cone structures were scattered (Fig. 4F,G,H). Therefore, like *Osbp*, *fan* is also required for individualization in *Drosophila* spermatogenesis. Together with the relatively mild IC phenotype in *Osbp* mutants, these results indicate that the integrity of the IC does not fully depend on OSBP and that FAN has OSBP-independent functions.

***Osbp* and *fan* interact genetically and physically**

To further investigate the relationship between *Osbp* and *fan*, we examined their genetic interactions. As *Osbp* mutants are fully sterile and *fan* mutants are only partially sterile, it is not surprising that *Osbp;fan* double mutants were also fully sterile (Fig. 4I). We then tested the dosage interaction between *Osbp* and *fan*. We found that germ cell-specific overexpression of OSBP significantly rescued the reduced fertility in *fan^{RNAi}* animals (Fig. 5A). In addition, mutation of *Osbp* increased the percentage of disrupted ICs with scattered actin cones in animals with one copy of *fan^{RNAi}* (Fig. 4J). Moreover, in *Osbp* and *fan* double mutants, few progressing ICs were found and all those found were disrupted (Fig. 4J; data not shown). These results suggest that *Osbp* interacts genetically with *fan*.

The phenotypic analysis described above raised the possibility that *Drosophila* OSBP, which contains a VAP-binding FFAT domain, might interact physically with FAN, as do their counterparts in mammals (Wyles et al., 2002). To examine the physical interaction between OSBP and FAN *in vivo*, co-immunoprecipitation was performed. We used *Act5C-Gal4* to drive simultaneous expression of *UAS-Osbp* and *UAS-ha-fan* (which produces HA-tagged FAN) and found that when extracted protein samples were applied to anti-HA-coupled beads, OSBP was robustly detected by a polyclonal anti-OSBP antibody on a western blot (Fig. 5B). We next asked whether the FFAT domain is essential for the interaction between OSBP and FAN. Using the same strategy, we found that the interaction was abolished by a point mutation (F276A) in the FFAT domain (Fig. 5B). We also confirmed the physical interaction between endogenous OSBP and endogenous promoter-driven HA-tagged FAN (Fig. 5C). Taken together, these results demonstrate that OSBP interacts with FAN *in vivo*, probably through the FFAT domain.

FAN-dependent OSBP-positive and sterol-rich speckles form at the leading edge of ICs during individualization

Next, we examined the subcellular localization of OSBP in testis to explore further how OSBP functions in late spermatid differentiation. Endogenous OSBP formed many speckles at the

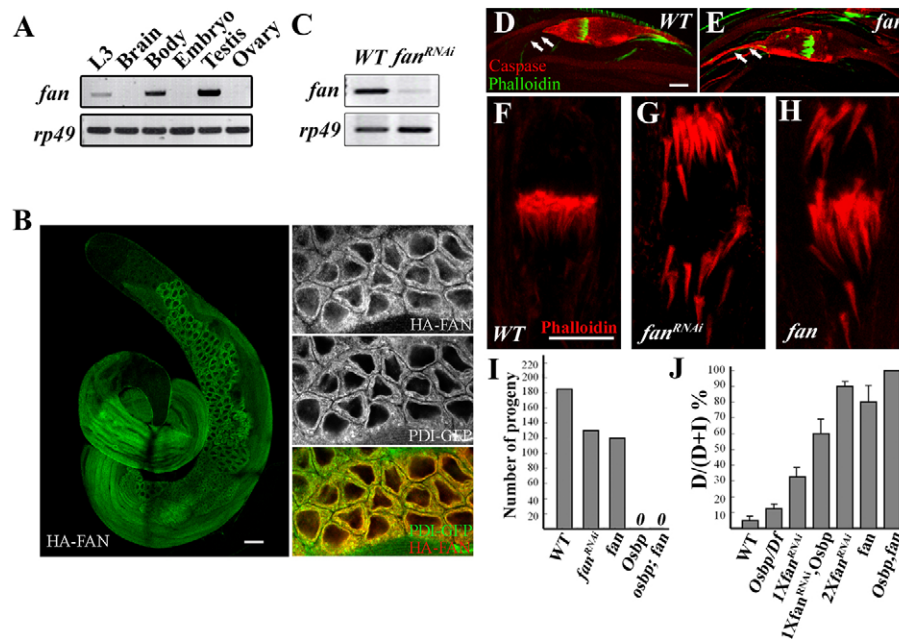


Fig. 4. *fan*, the gene encoding a testis-specific VAP, is required for individualization. (A) RT-PCR shows that *fan* is specifically expressed in testis. L3, third instar larvae. (B) The expression of HA-tagged FAN in testis. Panels on the right are enlarged view showing the co-localization of HA-FAN with the ER marker PDI-GFP. Scale bar: 50 μ m. (C) RT-PCR amplification of *fan* mRNA from testes of wild type (WT) and *fan*^{RNAi} mutants. *rp49* was used as a loading control. (D,E) Activated caspase/ICE (caspase; red) staining. Activated caspase signal is absent in the individualized portion of the cyst in wild type (arrows in D), but is present in *fan* mutants (arrows in E). Phalloidin staining is in red. Scale bar: 20 μ m. (F-H) Progressing ICs stained with phalloidin. ICs are severely disorganized with scattered actin cones in *fan*^{RNAi} (G) and *fan*^{KO} (H) mutants compared with wild type (F). Scale bar: 20 μ m. (I) *fan*^{RNAi} and *fan*^{KO} mutants have reduced fertility. Bars represent mean number of progeny. Progenies from 10-20 males were counted. (J) Disruption of progressing ICs in different genotypes. A ratio was calculated by dividing the percentage of disrupted ICs (D) by the total number of progressing ICs (D+I). Data are expressed as mean \pm s.d.

leading edge of the IC and few at the trailing end, suggesting that OSBP functions predominantly at the leading edge during individualization (Fig. 6A,B). Interestingly, OSBP speckles were still present at the trailing end of the IC in *fan* mutants but those at the leading edge were greatly diminished compared with wild type (Fig. 6C). Genomic rescue of *fan* mutants fully restored the leading edge OSBP speckles, indicating that this phenotype is caused specifically by the absence of *fan* in testis (data not shown). Two possibilities could account for the reduction of OSBP speckles in

fan mutants: either OSBP protein levels are reduced owing to loss of *fan*, or OSBP speckle formation depends on FAN. Western blot analysis excluded the first possibility because the levels of OSBP in the testis of wild type and *fan* mutants were comparable (Fig. 6D). Therefore, FAN might take part in the formation of OSBP speckles.

As OSBP proteins are implicated in the normal functioning of the ER and Golgi, we thus examined the potential connections of OSBP speckles to the ER and Golgi. We used the ER marker PDI-

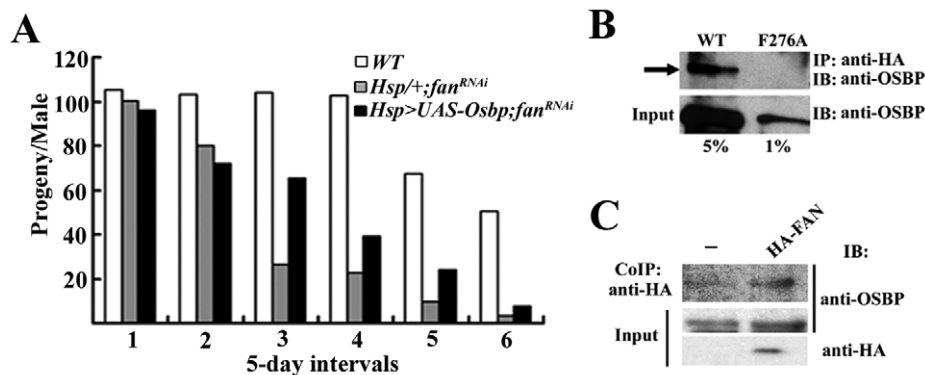


Fig. 5. *Osbp* and *fan* interact genetically and physically. (A) Germline-specific overexpression of *Osbp* (*Hsp>UAS-Osbp; fan*^{RNAi}) partially rescues the semi-sterility of *fan*^{RNAi} (*Hsp+/+; fan*^{RNAi}) as determined by the total number of progeny per male over the course of several 5-day intervals. Progenies from 10-20 males were counted. Bars represent mean number of progeny. (B) OSBP co-immunoprecipitates with FAN in vivo. The F276A mutation in the FFAT domain abolishes the physical interaction. IB, western blot for OSBP; IP, immunoprecipitation. Arrow indicates OSBP band. Input refers to testis lysates before IP. Percentages of input used in the western blot are indicated. (C) The physical interaction between endogenous OSBP and HA-tagged FAN. The negative control (left-hand column) is co-immunoprecipitation (coIP) from testes without expressing HA-FAN.

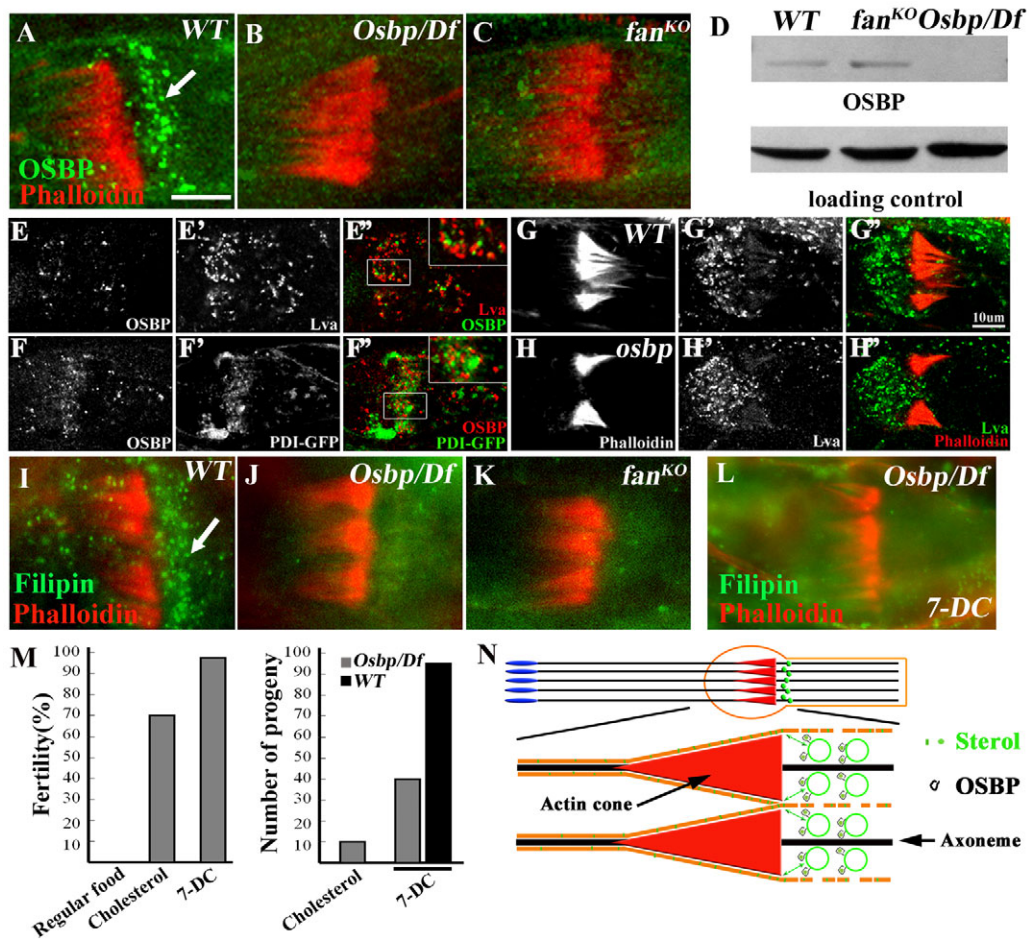


Fig. 6. FAN-dependent OSBP-positive and sterol-rich speckles are present in the IC. (A-C) OSBP-positive speckles labelled by an anti-OSBP monoclonal antibody are present at the leading edge of ICs (labelled by phalloidin) in wild type (arrow in A) but not in *Osbp* (B) or *fan* (C) mutants. Scale bar: 20 μ m. (D) Western blot of OSBP protein in testes of flies with the indicated genotypes. (E-F'') Relative location of OSBP speckles (E,F) to the Golgi apparatus (Lva; E') and ER (PDI-GFP; F'). Merged images are shown in E'' and F''. Boxed areas are magnified in right upper insets. (G-H'') Gross morphology of the Golgi in the cystic bulge in WT (G-G'') and *Osbp* mutants (H-H''). (I-L) Sterol-rich speckles are present at the leading edge of ICs in wild type (arrow in I) but not in *Osbp* (J) or *fan* (K) mutants. The speckles are occasionally found in *Osbp* mutants fed 7-DC (L). (M) Rescuing activity of dietary 7-DC and cholesterol. Progenies from 15-20 males were counted. Left: Fertility rate of *Osbp* mutants fed no additional sterols, additional cholesterol or 7-DC, respectively. Right: Mean number of progeny produced by males of the indicated genotypes raised on additional sterols. (N) Model of OSBP-mediated sterol trafficking during individualization. Enlarged view of two spermatids with progressing actin cones is shown. The individualized portion of spermatids is packaged into its own plasma membrane (continuous) whereas the unindividualized parts possess gaps, indicating connection by cytoplasmic bridges. OSBP mediates sterol trafficking between sterol-rich speckles and the plasma membrane to fulfill the requirements of sterols during membrane remodeling in the process of individualization.

GFP and an anti-LVA antibody to visualize the ER and Golgi, respectively. Interestingly, the distribution patterns of the ER and Golgi were similar to that of OSBP speckles, i.e. concentrated at the leading edge of ICs and scattered around the trailing edge (Fig. 6E,F). Co-staining analysis with the anti-OSBP antibody revealed that OSBP-speckles were located close to the ER and Golgi, often partially overlapping with these two organelles (Fig. 6E,F). Subsequently, we examined the gross morphology of the Golgi in *Osbp* mutants. Immunofluorescent analysis with anti-LVA revealed no apparent differences in the Golgi around the ICs between *Osbp* mutants and wild type (Fig. 6G,H), implying that Golgi apparatus are intact during individualization in *Osbp* mutants.

As OSBP is a sterol-binding protein, we examined the sterol distribution in the IC area using filipin, a fluorescent dye that binds to free sterol, in order to investigate the nature of the OSBP-

positive speckles further. We found that filipin staining highlighted many speckles of similar size to OSBP speckles at the leading edge of the IC (Fig. 6I). Unfortunately, we were unable to co-stain using the anti-OSBP antibody and filipin because of incompatibility of the staining protocols. Nevertheless, the sterol-rich speckles are absent in *Osbp* mutants (Fig. 6J) without apparent alterations in sterol distribution in whole testes, spermatocytes and spermatid bundles (see Fig. S3 in the supplementary material). Moreover, consistent with the finding that FAN is required for the formation of OSBP-positive speckles, the filipin-positive speckles were no longer present in *fan* mutants (Fig. 6K). These results suggest that a FAN- and OSBP-mediated sterol trafficking pathway is required for individualization, and that defective or partially reduced sterol trafficking leads to individualization defects in *Osbp* or *fan* mutants.

The sterility of *Osbp* mutants is rescued by dietary sterols

The lack of sterol-rich speckles in *Osbp* mutants led us to explore the connection between sterols and male sterility in *Osbp* mutants. As overexpression of OSBP results in the accumulation of sterols (see below), and point mutations in the OSBP oxysterol-BP domain abolish its rescuing activity, we investigated whether the sterility of *Osbp* loss-of-function mutants is attributable to sterol shortage. Animals were raised on medium with or without additional cholesterol or its metabolic intermediate 7-dehydrocholesterol (7-DC) to test whether excess sterols could recover the fertility of *Osbp* mutants. Elevated sterol levels, as revealed by filipin staining, were observed in the testes of sterol-fed *Osbp* mutants, indicating the efficiency of the sterol supplement (see Fig. S3 in the supplementary material). All *Osbp* mutants raised on normal food were sterile, whereas 95% of mutant males fed supplementary 7-DC, and 70% of mutant males fed supplementary cholesterol, recovered some fertility (Fig. 6M). As well as being more fertile, 7-DC-fed males produced more progeny than did cholesterol-fed males (Fig. 6M). Consistent with the recovered fertility, the mildly disorganized ICs were also rescued by additional sterols (see Fig. S4 in the supplementary material). However, sterol-rich speckles were found only occasionally at the leading edge of ICs (Fig. 6L). These results suggest that adequate levels of sterols are required for late spermatid differentiation and that they are probably delivered by OSBP (Fig. 6N).

FAN modulates OSBP-mediated sterol trafficking

How can FAN regulate OSBP-mediated sterol trafficking? We examined salivary glands to investigate the interaction of OSBP and FAN because this organ has giant cells and well-developed, easily observed secretory organelles, including the ER and Golgi. Using a salivary gland *Gal4* driver, *ppl-Gal4*, we expressed *Osbp* in salivary glands and examined the sterol level using filipin. We found dramatic elevation of sterols in the Golgi in *Osbp*-overexpressing salivary glands, revealed by colocalization of filipin and a Golgi-specific GFP marker (Fig. 7A,B; see Fig. S5 in the supplementary material). Therefore, when overexpressed, OSBP might deliver more sterols to the Golgi. Consistently, EYFP tagged OSBP partially overlapped with the Golgi (labelled by anti-LVA), indicating that OSBP probably transports sterols to the Golgi apparatus directly (see Fig. S5 in the supplementary material).

As overexpression of FAN can suppress the post-eclosion behaviour defects in *Osbp*-overexpressing flies (Fig. 3C), we also investigated the impact of FAN overexpression on the level of sterols in *Osbp*-overexpressing salivary glands. When expressed individually, FAN is localized to the ER whereas OSBP is only partially localized to the ER (see Fig. S5 in the supplementary material). Interestingly, elevated sterol levels in *Osbp*-overexpressing salivary glands were strongly suppressed by FAN co-expression (Fig. 7C). Moreover, co-expression of OSBP and FAN resulted in the formation of OSBP-FAN vesicles that originated from the ER (Fig. 7D,E). These vesicles can be outlined by filipin, suggesting that relatively high sterol levels were present in the membrane of these ER-derived vesicles (Fig. 7F-F''). These results indicate that FAN might recruit OSBP to specific sites on the ER, thereby inducing a local elevation of sterols that, in turn, causes sterol-rich vesicles to bud from the ER so that fewer sterols subsequently reach the Golgi. Combining these results with the finding that both OSBP-positive and sterol-rich speckles are absent in *fan* mutants, we propose that FAN participates in the formation of OSBP-positive speckles and in the trafficking of sterols in vivo.

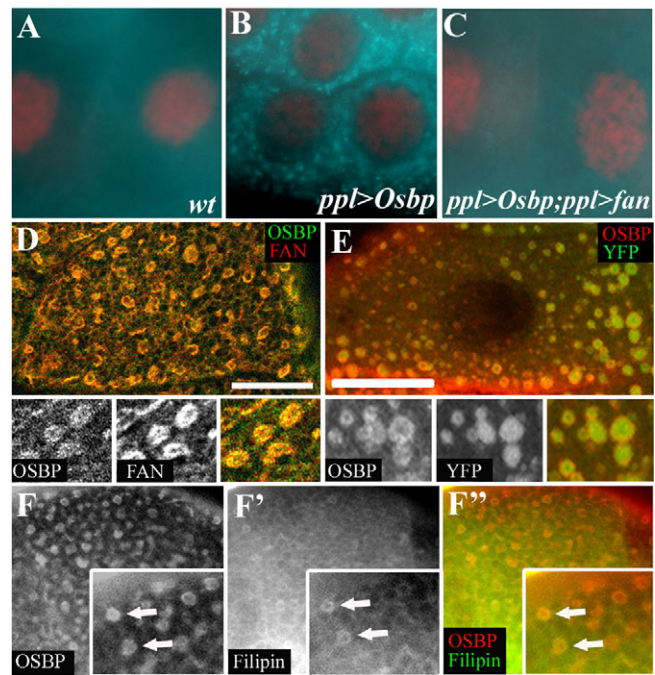


Fig. 7. FAN modulates OSBP-mediated sterol trafficking in salivary glands. (A-C) Filipin staining (blue) of sterols in salivary gland cells of wild type (*wt*; A), *ppl>Osbp* (B) and *ppl>Osbp;ppl>fan* (C) mutants. Nuclei were marked with propidium iodide (red). (D-E) Co-expression of OSBP and FAN in salivary gland cells. OSBP-labelled vesicles are positive for FAN (D) and an EYFP ER marker (YFP; E). Lower panels are enlarged images. Scale bars: 20 μ m. (F-F'') Filipin staining outlines OSBP-circled vesicles (arrows in insets).

DISCUSSION

Despite numerous cellular studies supporting their roles in sterol trafficking, the *in vivo* physiological function of the OSBP protein family members in multicellular organisms remains elusive. In this report, we have demonstrated that spermatogenesis in *Drosophila* requires sterols in an OSBP-dependent manner. In addition, FAN, a testis-specific *Drosophila* VAP protein, interacts genetically and physically with OSBP in the regulation of spermatogenesis.

From *in vivo* loss-of-function analysis, we found that *Drosophila* OSBP is required for spermatogenesis and, in particular, for the individualization process. Although the biological pathways and mechanisms involved in individualization are largely unknown, individualization requires synchrony of plasma membrane remodelling, F-actin-based IC movement and removal of excess cytoplasm. Defects in any one of these three could influence the others to some extent and impair the highly coordinated process of individualization (Huh et al., 2004; Noguchi and Miller, 2003). The membrane reorganization phase must have at least two requirements: one for membrane components and the other for membrane curvature upon remodelling. It has been shown that sterols could fulfil both requirements (Holthuis, 2004). In addition, it has been reported previously that there are many vesicles in the IC area, which might provide the necessary membrane components for the completion of individualization (Tokuyasu et al., 1972). Exchange of sterols between the plasma membrane and these vesicles might be important for plasma membrane reorganization and for maintaining the competence of the plasma membrane during individualization. Consistent with this idea, we observed

OSBP-concentrated sterol-rich speckles in the IC area (Fig. 6). Therefore, we hypothesize that OSBP might participate in the unidirectional or bidirectional transport of sterols between the intracellular vesicles and the plasma membrane during membrane remodelling in individualization (Fig. 6N). In *Osbp* mutants, fewer sterols are transported to the plasma membrane, resulting in membrane defects during individualization that eventually lead to the absence of mature sperm. An excess sterol load could partially compensate for the reduced sterol trafficking, explaining why supplementary cholesterol or 7-DC can partially rescue the male sterility in *Osbp* mutants. We cannot exclude the possibility that OSBP might also have a role in other spermatogenesis steps after individualization. In agreement with the idea that proper sterol trafficking is important for the development of sperm, mutants of *Drosophila Npc1a*, which have low levels of available sterols owing to trapping of exogenous sterols in endosomes and/or lysosomes (Huang et al., 2005), also display defects in individualization and are male sterile (X.H. and Chao Wang, unpublished).

Encapsulation of sperm in individual plasma membranes and removal of excess cytoplasm during spermatogenesis is not peculiar to *Drosophila*. Similar biological processes, which are cooperatively accomplished by the residual body and Sertoli cells, occur during mammalian spermatogenesis. Recent studies on sterols in the mammalian testis have focussed mainly on the role of steroids during early spermatogenesis and sterols in the capacitation of sperm just before they reach the egg (Cross, 1998; O'Donnell et al., 2001). It is not known whether *Drosophila* sperm has a capacitation process and, if they do, whether OSBP has a role in this process. Our data might have implications for research into sterol function in other aspects of spermatogenesis in mammals. In support of this, it has been reported that *Npc1a* mutant mice exhibit profound spermatogenesis defects (Fan et al., 2006).

Our results also reveal the important role of FAN, a testis-specific VAP protein, in individualization. Cells maintain a sterol gradient from the ER (low sterol levels), through the Golgi to the plasma membrane (high sterol levels). OSBP proteins with PH domains can bind to phosphoinositides in the Golgi or plasma membrane. OSBP proteins can also localize to the ER by interacting with ER-specific partners, such as VAP proteins, through the FFAT motif. These specific interactions, mediated by functional domains, could endow OSBP proteins with the capacity to transport sterols between the ER, Golgi and plasma membrane in order to regulate the uneven distribution of sterols. In our study, we found that OSBP overexpression resulted in elevated Golgi sterol levels and probable overall sterol level. OSBP might transport much more sterol to the Golgi from other places, such as the ER, and cells might respond to the sterol shortage in the ER by increasing sterol uptake. Interestingly, co-expression of OSBP and the ER protein FAN causes sterol-enriched vesicles to bud from the ER. FAN might confine OSBP at the ER and prevent OSBP from associating with the Golgi, thereby protecting the Golgi from sterol accumulation. Meanwhile, the reduction of overall intracellular cholesterol levels, revealed by filipin staining, might be a result of conversion of free sterols to sterol esters, which cannot be probed with filipin, at the ER. Alternatively, as a sensor of intracellular sterol levels, the ER might signal the cell to downregulate sterol uptake. These results suggest that OSBP and FAN might participate in sterol-rich vesicle formation, which could be important for membrane reorganization during individualization in spermatogenesis. Loss of sterol-rich speckles in both mutants is consistent with such an explanation.

Although both *Osbp* and *fan* mutants have individualization defects, they are slightly different in several respects. *Osbp* mutants are sterile with a mild effect on the integrity of ICs, whereas *fan* mutants are semi-sterile and have severely disorganized ICs with scattered actin cones. Sterile mutants with individualization defects but intact ICs, reminiscent of *Osbp* mutants, have been reported previously, indicating that IC integrity is not sufficient for accomplishing individualization and producing mature sperm (Fabrizio et al., 1998). Thus, we reasoned that the fundamental function of FAN- and OSBP-mediated sterol transport is probably not directly associated with the maintenance of IC structure during individualization. *fan* mutants are fertile, probably because OSBP is still functional, albeit at a low efficiency because of a reduction of FAN-dependent OSBP speckles. The disorganization of ICs in *fan* mutants suggests that *fan* might have other partners in the maintenance of IC integrity. More research on FAN is required to reveal this.

Studies in yeast have shown that OSBP family proteins have redundant and distinct functions (Beh et al., 2001; Levine and Munro, 2001). This also appears to be the case in *Drosophila*. The specific phenotypes caused by overexpression or loss of function of OSBP, and the failure of other OSBP family proteins to rescue the male sterility of *Osbp* mutants, argue for a distinct role for OSBP. The redundant role of OSBP family proteins could be revealed by double mutants. For example, double mutants of *Osbp* and *CG3860* are larval lethal (Z.M. and X.H., unpublished). Further studies are needed to elucidate other *in vivo* functions of OSBP family proteins and their binding partners. For instance, VAP33A, the possible partner of OSBP in *Drosophila* and the homologue of human VAP (the protein affected in human amyotrophic lateral sclerosis), is reported to affect synaptic growth and neurotransmitter release by interactions between microtubules and the presynaptic membrane (Pennetta et al., 2002).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.049312/-/DC1>

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