Membrane Phospholipid Asymmetry Counters the Adverse Effects of Sterol Overloading in the Golgi Membrane of *Drosophila*

Zhiguo Ma,*,†,1,2 Zhonghua Liu,*,1 and Xun Huang*,2

*State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China, and †Graduate School of the Chinese Academy of Sciences, Beijing 100049, China

ABSTRACT Cholesterol and phospholipids serve as structural and functional components of cellular membranes in all eukaryotes. Heterogeneity in cholesterol and phospholipid content both within and between different organelles is an important characteristic of eukaryotic membranes. How this heterogeneity is achieved and orchestrated to maintain proper cellular physiology remains poorly understood. We previously found that overexpression of the *Drosophila* **o**xy**s**terol-**b**inding **p**rotein (OSBP) leads to sterol accumulation in the Golgi apparatus. Here, we show that *Osbp* overexpression in a set of neuroendocrine neurons compromises the function of the Golgi apparatus. It impairs trafficking of the neuropeptide bursicon and results in post-eclosion behavior defects characterized by unexpanded wings. We performed a genetic screen to identify modifiers that suppress the unexpanded wing phenotype. A putative phospholipid flippase-encoding gene, *CG33298*, was validated, suggesting that a membrane-asymmetry-directed mechanism balances cholesterol chaos within the Golgi membranes. Since the functional connection between cholesterol metabolism and the activity of phospholipid flippase has been implicated in studies in yeast and worms, our findings here support an evolutionarily conserved causal link between cholesterol homeostasis and phospholipid asymmetry that maintains normal cellular physiology.

THE lipid bilayers of eukaryotic cellular membranes are composed of phospholipids within which cholesterol molecules are embedded. The distribution of both phospholipids and cholesterol in cellular membranes is distinct and highly dynamic. Phospholipids are asymmetrically distributed in the leaflets of many bilayer membranes, including the plasma membrane, the endosomal membrane, and likely the Golgi membrane (van Meer *et al.* 2008). For example, in the Golgi apparatus, phosphatidylcholine and sphingomyelin are present only in the inner leaflet, while phosphatidylserine (PS) and phosphatidylethanolamine (PE) are likely concentrated in the outer leaflet. In addition to the heterogeneous distribution of phospholipids within the inner and outer leaflets of lipid bilayer membranes, cholesterol is also unevenly distributed in the membranes of different organelles. From the endoplasmic reticulum (ER) and the Golgi apparatus, to the plasma membrane, an increasing gradient of cholesterol forms via intracellular cholesterol transport by vesicle-mediated and non-vesicle-mediated pathways (Soccio and Breslow 2004; Maxfield and Menon 2006).

The unique distribution of phospholipids and cholesterol in bilayer membranes has been shown to be important for cells to function normally (Mayinger 2009). For example, losing the asymmetric distribution of PS in the plasma membrane is an "eat me" signal to trigger apoptosis (Fadok and Henson 2003). In the Golgi apparatus, the asymmetry of phospholipids is essential for the formation of exocytic vesicles. Mutations in the yeast phospholipid flippase Drs2p lead to defects in the formation of clathrin-coated vesicles but not of other protein transport vesicles (Gall *et al.* 2002). In addition, the relatively high level of cholesterol in the Golgi apparatus was found to be important for the budding of secretory vesicles, probably by generating high membrane curvature (Subtil *et al.* 1999; Wang *et al.* 2000). Although both cholesterol and phospholipids are crucial for proper membrane function, how the proper balance of cholesterol levels and phospholipid asymmetry is achieved and orchestrated to meet the requirements of normal cellular physiology is unclear.

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[1]These authors contributed equally to this article.
[2]Corresponding authors: State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China. E-mail: zgma@genetics.ac.cn and xhuang@genetics.ac.cn

It is thought that the **o**xy**s**terol-**b**inding **p**rotein (OSBP), also called the **O**SBP-**r**elated **p**rotein (ORP) family of oxysterol-binding proteins, may link cholesterol homeostasis, sphingomyelin synthesis, and phospholipid asymmetry (Olkkonen 2004; Raychaudhuri and Prinz 2010). ORPs are implicated in sterol homeostasis by directly binding and sensing intracellular cholesterol or transporting sterol molecules among different cellular organelles (Im *et al.* 2005). Genetic manipulation of ORP levels alters cholesterol distribution and homeostasis in cells. For example, RNA interference (RNAi) depletion of ORP9L leads to profound cholesterol accumulation in lysosomal membranes (Ngo and Ridgway 2009). Similarly, knocking down ORP5 causes cholesterol accumulation in late endosomes and lysosomes in culture cells (Du *et al.* 2011). In cellular membranes, cholesterol and sphingomyelin content is coregulated to accommodate the biophysical properties of these two lipids. In the Golgi apparatus, OSBP mediates the sterol-dependent activation of ceramide transport protein and subsequently increases sphingomyelin synthesis (Storey *et al.* 1998; Banerji *et al.* 2010).

The asymmetry of PS and PE in the Golgi membrane is maintained by phospholipid flippase, also known as type IV P-type ATPase (Pomorski and Menon 2006; Daleke 2007). Residing predominantly in the Golgi apparatus, Drs2p, one of the five phospholipid flippases in yeast, regulates protein sorting and vesicle budding (Graham 2004; Natarajan *et al.* 2004; Liu *et al.* 2008). Interestingly, loss of Kes1p/Osh4p, a yeast ORP, suppresses the growth defect of a partial loss-of-function mutant of Drs2p. On the other hand, Drs2p also antagonizes the activity of Kes1p in intracellular cholesterol trafficking (Muthusamy *et al.* 2009). The exact mechanism behind this mutual antagonistic activity between Drs2p and Kes1p is unknown.

Most of the above ORP studies were performed in yeast or mammalian culture cells. The *in vivo* function of ORP at the whole-organism level has been investigated only in *Caenorhabditis elegans* and *Drosophila*. In *C. elegans*, the ORP family proteins are important for multi-vesicular body formation and exhibit redundant functions (Kobuna *et al.* 2010). There are four ORP family proteins (OSBP/CG6708, CG1513, CG3860, and CG5077) in *Drosophila*. *Osbp/CG6708*-knockout animals exhibit a male sterility phenotype and lose the sterol-rich puncta at the leading edge of the individualization complex in differentiating spermatids (Ma *et al.* 2010). On the other hand, *Osbp* overexpression leads to sterol accumulation in the Golgi apparatus in salivary gland cells (Ma *et al.* 2010). It remains to be determined what the consequences of sterol accumulation in the Golgi apparatus are.

Flies with ubiquitous overexpression of *Osbp* are viable but fail to accomplish post-eclosion behaviors including wing expansion (Ma *et al.* 2010). In this study, we investigated the underlying causes of post-eclosion behavioral failure. We found that *Osbp* overexpression in **c**rustacean **c**ardio**a**ctive **p**eptide (CCAP) neurons impairs the function of the Golgi apparatus. CCAP neurons release the neuropeptide bursicon, which regulates post-eclosion behaviors (Luo *et al.* 2005). The formation of bursicon-containing granules is severely compromised in *Osbp*-overexpressing CCAP neurons. Using an unbiased genetic screen, we identified a putative phospholipid flippase-encoding gene, *CG33298*, as a suppressor of *Osbp* overexpression. These results imply that reducing membrane phospholipid asymmetry may counterbalance the adverse effects of sterol overloading in the Golgi apparatus. Therefore, our results support a causal link between cholesterol homeostasis and the asymmetry of phospholipids in the Golgi apparatus.

Materials and Methods

Drosophila stocks and genetics

Flies were cultured on standard cornmeal medium at 25°. The following transgenic stocks were generated with standard method in a w^{1118} background: *UAS-CG33298*, *UAS-CG30456*, and *UAS-CG15611*. Other transgenic fly strains used were as follows: *CCAP-Gal4* (Park *et al.* 2003), *UAS-ManII-eGFP* (Ye *et al.* 2007), *UAS-GFP*, and *UAS-ANF-GFP* (Rao *et al.* 2001). The Exelixis deficiency stocks and the *UAS-Rab* collection were obtained from the Bloomington Stock Center. To screen for suppressors, virgin *w*; *CCAP-Gal4/CyO*; *UAS-Osbp/TM6B* animals were crossed to deficiency or *UAS-transgene* males. The wing phenotype was scored in the F_1 generation. Since the wing unexpansion phenotype of OSBP-overexpression flies is 100%, the classification of suppression (weak, strong, or full) is arbitrary and is based mainly on the size of the wing. Strong and full suppressions were repeated to confirm the genetic interactions. The $CG33298^{44-5}$ mutant was obtained by imprecise excision with *GS14514* (from the Kyoto Stock Center) as the starting stock. Genomic DNA from $CG33298^{44-5}$ mutants was amplified with primers flanking the deletion region, and molecular lesions were determined by sequencing.

Molecular biology

Full-length cDNAs of *CG30456* and *CG15611* were amplified by RT-PCR and inserted into the *Kpn*I and *Xba*I sites of the *pUAST* vector. *pUAST-CG33298* was generated similarly using the *Spe*I and *Xba*I sites.

Staining and microscopy

Pharate adults that twitched their legs were pulled out of their pupal cases. The heads were cut off with forceps, and the mouth hooks were removed to allow access of the fixative to the cuticle-enwrapped brain. The heads were fixed in 4% paraformaldehyde for 30 min and then washed with 1× PBS. After fixing, we removed the cuticle from the posterior side that abuts the thorax and then separated the brain from the cuticle of the eyes for GFP imaging or immunostaining. For other staining procedures, tissues were dissected and fixed with 4% paraformaldehyde for 20 min followed by 0.3% PBT (PBS + 0.3% Triton X-100) treatment for 30 min. For antibody staining, samples were incubated with primary antibody at 4° overnight. Secondary antibody

(Alexa Fluor 543) incubation lasted for 2 hr at room temperature. The following primary antibodies were diluted in 0.1% PBT before use: rabbit anti-bursicon α (1:2500) (Luan *et al.* 2006) and rabbit/mouse anti-OSBP (1:20) (Ma *et al.* 2010). Filipin (50 μg/ml) (Sigma) was used in sterol staining. Images were taken using equivalent exposure conditions for controls and genetically manipulated samples.

Results

Osbp overexpression impairs neuropeptide trafficking in CCAP neurons

We previously reported that adult flies with *Osbp* overexpression driven by *CCAP-Gal4* showed complete penetrance of post-eclosion behavioral defects, including unexpanded wings and thorax (Ma *et al.* 2010). *CCAP-Gal4* is activated in a set of neuroendocrine CCAP neurons (Zhao *et al.* 2008). The main role of CCAP neurons is to secrete the neuropeptide bursicon, which regulates post-eclosion behaviors in peripheral tissues (Luo *et al.* 2005). Therefore, *Osbp* overexpression might lead to defects in the synthesis or trafficking of bursicon in CCAP neurons. We examined the expression of bursicon with anti-bursicon (α-subunit) antibody staining in P15 pharate adults, the stage with the highest level of bursicon. In control flies, intensive anti-bursicon staining signals were observed in the soma of CCAP neurons, including bursicon-producing neurons in subesophageal (B_{SEG}) and abdominal ganglions (B_{AG}) (Figure 1). In addition, axons with bursicon-positive puncta were found both between the B_{SEG} neurons and along the brain midline reaching to the anterior part of the brain where they spread out laterally and from the B_{AG} neurons to the thoracic neuromeres anterior to the abdominal ganglion (Figure 1). In *Osbp*-overexpressing flies, although the somas of CCAP neurons stained strongly with the anti-bursicon antibody, bursicon-positive axons were either greatly diminished or missing (100%, $n = 10$) (Figure 1). The lack of bursicon-positive axons may be due to impaired neuropeptide transport from the soma to the axon or, alternatively, to a lack of axons. To explore the latter possibility, we labeled CCAP neurons with cytosolic GFP. We found no difference in CCAP axons between control and *Osbp*-overexpressing flies (Figure 2), indicating that the CCAP axons were normal. These results suggest that in *Osbp*-overexpressing CCAP neurons, the synthesis of bursicon and CCAP axon outgrowth are likely normal, but the trafficking of bursicon is impaired.

To demonstrate that *Osbp* overexpression affects neuropeptide trafficking, we analyzed the expression of a previously verified neuropeptide reporter, the rat atrial natriuretic factor (ANF), fused with GFP (Rao *et al.* 2001). Expressing ANF-GFP in CCAP neurons resulted in a pattern similar to bursicon staining (Figure 2). In *Osbp*-overexpressing animals, ANF-GFP-positive axons were diminished or missing, in particular in the anterior part of the brain (Figure 2). Taken together, these results show that *Osbp* overexpression impairs neuropeptide trafficking in CCAP neurons.

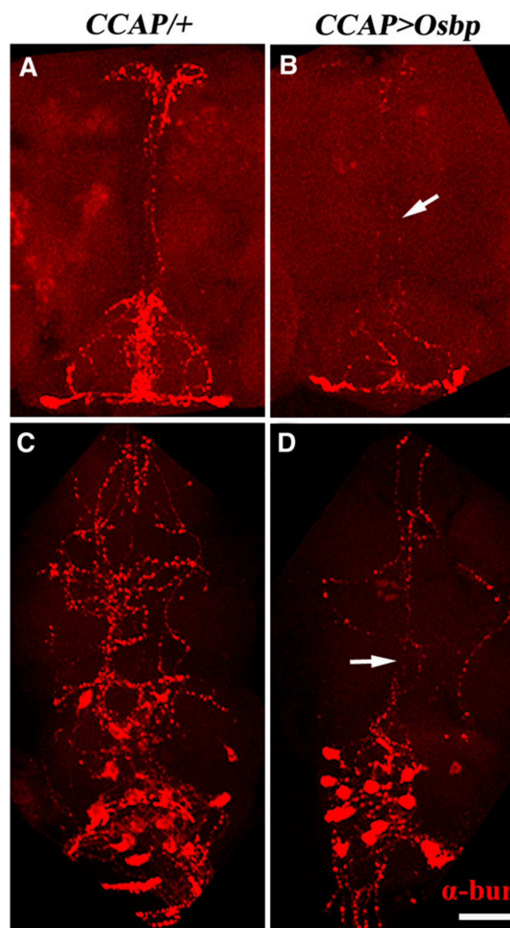

Figure 1 *Osbp* overexpression impairs neuropeptide bursicon trafficking in CCAP neurons. (A and B) Anti-bursicon staining shows bursicon-positive axons in CCAP neurons in the brain and subesophageal ganglion in control (A) and *Osbp*-overexpressing (B) flies. Bursicon-positive axons are greatly diminished in the midline (arrow) of *Osbp*-overexpressing flies. (C and D) Anti-bursicon staining in CCAP neurons in the abdominal ganglion of control (C) and *Osbp*-overexpressing (D) flies. Bursicon-positive axons are greatly diminished in the axons (arrow) of *Osbp*-overexpressing flies. Bar: 50 μm.

Osbp overexpression alters the morphology of the Golgi apparatus and affects the formation of bursicon-positive secretory vesicles

We next explored the underlying causes of impaired neuropeptide trafficking. Neuropeptides are first synthesized in neuroendocrine cells in the rough ER. They are then transported to the Golgi apparatus, where they are sorted and packed at high concentrations into a structure called the secretory granule/vesicle. Secretory granules bud from the Golgi apparatus and are transported along the axons to the terminal, where they are secreted in response to stimulation. It has been reported that the yeast OSBP family protein Kes1p/Osh4p negatively regulates vesicle budding from the Golgi apparatus (Li *et al.* 2002). Likewise, *Drosophila Osbp* overexpression may impair secretory vesicle formation in the Golgi apparatus, resulting in a lack of bursicon-positive granules in the axons. Hence, we focused on the Golgi apparatus in the following studies.

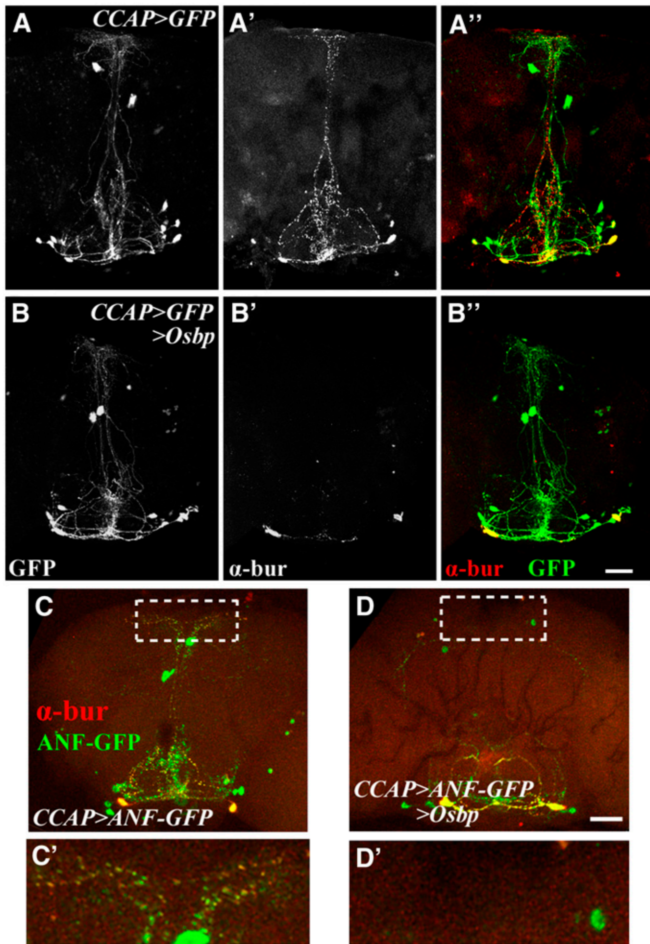

Figure 2 *Osbp* overexpression affects neuropeptide trafficking but not the morphology of CCAP neurons. (A–B′′) Cytosolic GFP labels CCAP neurons in the brain and subesophageal ganglion. Similar to the control (A), CCAP neurons and axons are present in *Osbp*-overexpressing flies (B). Anti-bursicon staining shows a great reduction in bursicon-positive axons in *Osbp*-overexpressing flies (B′) compared to the control (A′). (A′′ and B′′) Merged images (bursicon: red; GFP: green) of A and A′ and B and B′, respectively. Bar: 50 μm. (C–D′) The level of ANF-GFP (green) is greatly reduced in CCAP neuronal axons in *Osbp*-overexpressing flies (D) compared to the control (C). (C′ and D′) Enlarged view of the anterior part of the brain in C and D, respectively. Bar: 50 μm.

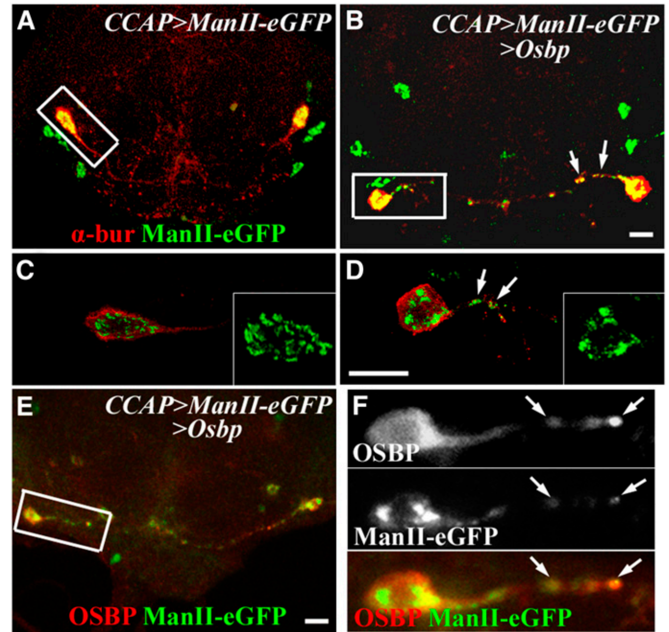

Figure 3 *Osbp* overexpression affects the morphology and function of the Golgi apparatus. (A–D) A pair of CCAP neurons labeled with ManII-eGFP (green) is costained with anti-bursicon antibody (red) in control (A and C) and *Osbp*-overexpressing brains (B and D). (C and D) Enlarged views of the boxed areas in (A and B), respectively. Arrows indicate mislocalization of ManII-eGFP to bursicon-positive puncta in the axon. Insets in C and D represent single-channel images of the Golgi apparatus marked with ManII-eGFP. ManII-eGFP is seen as punctated structures scattered throughout the soma in control, while the punctated structures are lost in *Osbp*-overexpressing neurons. Bar: 50 μm. (E) ManII-eGFP and OSBP (red) are colocalized as puncta in the axons of *Osbp*-overexpressing CCAP neurons. Bar: 20 μm. (F) Magnified single-layer confocal images in the boxed region of E. Arrows indicate colocalization of OSBP and ManII-eGFP in an axon.

Mannosidase II (ManII) is a Golgi-localized enzyme involved in glycoprotein processing and has often been used as a Golgi marker. In control neurons, the Golgi apparatus labeled by ManII-eGFP is seen as punctated structures scattered throughout the soma (Figure 3), a morphology that is typical for the Golgi apparatus in *Drosophila* cells. In *Osbp*-overexpressing neurons, however, ManII-eGFP-punctated structures are often lost and replaced with few large clusters (Figure 3). Therefore, the Golgi apparatus appears to be abnormally distributed in *Osbp*-overexpressing neurons. Interestingly, although the bursicon-staining signal is largely absent from the axons of *Osbp*-overexpressing CCAP neurons, few bursicon-positive puncta are present in axons and are very close to the soma (Figure 3). Normally, as a result of sorting, ManII, as a Golgi protein, is retained in the Golgi apparatus and is therefore not found in axons. In *Osbp*-overexpressing CCAP neurons, ManII-eGFP and OSBP-positive aggregates can be found in the axon (Figure 3). These findings suggest that *Osbp* overexpression may lead to defects in secretory vesicle formation in the Golgi apparatus. *Osbp* overexpression, therefore, not only alters the morphology of the Golgi apparatus but also may impair its function.

Suppressor screen for the post-eclosion behavioral defects caused by Osbp overexpression

OSBP has been shown to be involved in sterol trafficking between the ER and the Golgi apparatus (Ma *et al.* 2010; Raychaudhuri and Prinz 2010). To identify potential regulators of cholesterol homeostasis, we screened for modifiers that, when mutated or overexpressed, can suppress the post-eclosion unexpanded wing phenotype caused by *Osbp* overexpression. Chromosome deficiencies (Exelixis collection), candidate *Osbp*-interacting genes, and a collection of all *Drosophila* Rabs (both dominant negative and constitute active versions) were included in the screen (Figure 4) (Zhang *et al.* 2007). On the basis of the extent of wing expansion, we classified the suppression as weak, strong, or full (Figure

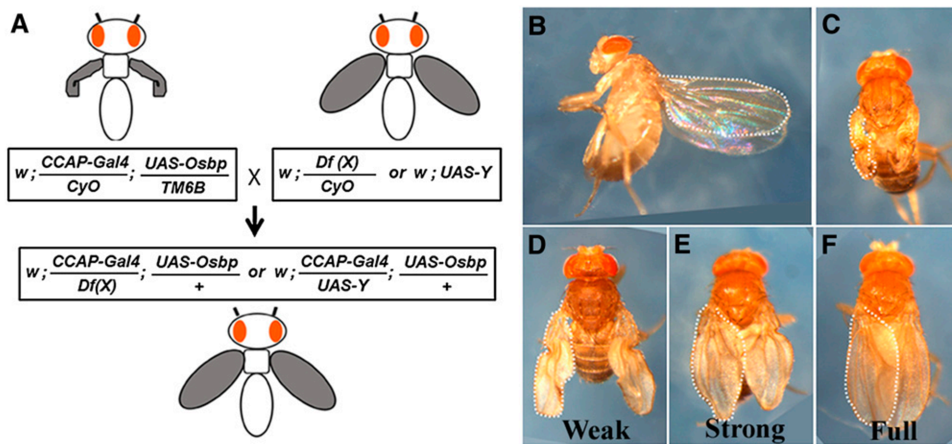

Figure 4 Genetic screen for suppressors of the unexpanded-wing phenotype caused by *Osbp* overexpression. (A) Flow chart of the suppressor screen. (B–F) Different levels of wing expansion. Wings are highlighted by white dotted lines. (B) Normal wing expansion in the wild type. C, D, E, and F represent no, weak, strong, and full suppression of unexpanded wings, respectively.

4). We have shown previously that overexpression of VAP33a and FAN, two ER-associated OSBP-binding proteins, can fully suppress the *Osbp* overexpression phenotype (Ma *et al.* 2010). Of ~400 deficiencies (chromosome II and III), 24 were found to suppress the unexpanded wing phenotype (Table 1). Since single-chromosome deficiency usually covers many genes, we further narrowed down the modifier region of strong or full suppressors by other deficiencies that partially overlap with the Exelixis deficiencies. Modifiers for many of the deficiencies were narrowed down to a few genes, including three genes (*CG12091*, *CG7852*, *CG32313*) in *Df(3L)Exel6087*; three genes (*Sox21b*, *D*, *nan*) in *Df(3L)Exel6120*; four genes (*CG2162*, *Jafrac2*, *scramb2*, *Sk2*) in *Df(3L)Exel6092*; and five genes (*MED27*, *CG2182*, *CG1109*, *Xe7*, *CG1347*) in *Df(3R)Exel7283* (Table 1). For two of the deficiencies, *Df(2L)Exel6022* and *Df(2R)Exel6066*, modifiers were narrowed down to two genes (see below).

Rab proteins are involved in intracellular membrane trafficking. Overexpression of constitutively active Rab18 resulted in strong suppression of the *Osbp*-overexpressing unexpanded wing phenotype (Table 1). Because it has been postulated that Rab18 is involved in ER-Golgi trafficking, and disturbing Rab18 activity affects the secretion of the secretory marker VSVG-GFP from the Golgi apparatus (Dejgaard *et al.* 2008), our results indicate that constitute active Rab18 may suppress the Golgi bursicon secretion defect in *Osbp* overexpression by modulating trafficking in the Golgi apparatus. In addition, expressing constitutively active versions of six other Rabs (Rab10, Rab11, Rab26, Rab35, Rab40, and RabX2) resulted in weak suppression (Table 1). In contrast, mild suppression was observed in only two dominant negative Rabs (Rab7 and Rab19) (Table 1). Interestingly, *Df(3R)Exel6196*, which deletes *Rab7*, is a weak suppressor, while *Df(3L)Exel6112*, which covers *Rab19*, is a strong suppressor (Table 1).

Suppression of RhoGEF genes

The suppressor in *Df(2R)Exel6066* was narrowed down to a two-gene region (*CG30456* and *CG15611*) based on the overlapping deficiencies *Df(2R)Exel6066* and *Df(2R)ED3181*. Interestingly, BLAST searches showed that both CG30456 and CG15611 are homologous to mammalian Rho guanine nucleotide-exchange factors (RhoGEF). The RhoGEF domains of CG30456 and CG15611 share 44%

Table 1 Results of the deficiency screen

Suppressing effects	Deficiency[a]
Full	*Df(2R)Exel6066* (*CG30456*, *CG15611*)
	Df(3R)Exel7283 (*MED27*, *CG2182*, *CG1109*, *Xe7*, *CG1347*)
Strong	*Df(2L)Exel6022* (*CG33298*)
	Df(3L)Exel6087 (*CG12091*, *CG7852*, *CG32313*)
	Df(3L)Exel6092 (*CG2162*, *Jafrac2*, *scramb2*, *Sk2*)
	Df(3L)Exel6102 (*CG7509*, *CG18808*, *Dhc64C*, *Aats-leu*, *CG13708*, *CG13707*, *CG32237*, *CG32235*)
	Df(3L)Exel6112 (66B5-66C8)
	Df(3L)Exel6120 (*Sox21b*, *D*, *nan*)
	Df(3R)Exel6136 (77B2-77C6)
	Df(3R)Exel6167 (*CG8476*, *CG8483*, *CG14380*, *CG8508*, *CG8141*, *CG8138*, *CG42573*, *CG34308*, *timeout*, *pic*, *sim*)
	Df(3R)Exel6211 (*Pkc98E*, *Slu7*, *Cpsf100*, *beta4GalNAcTB*, *Ssl2*, *CG1951*, *eIF4E-6*, *CG14518*, *CG1443*)
	Df(3R)Exel6212 (99A1-99A5)
	Df(3R)Exel6215 (*CDase*, *PH4alphaEFB*, *spdo*, *Jon99Fii*, *Jon99Fi*, *PH4alphaSG2*, *PH4alphaMP*)
Weak	*Df(3R)Exel6143* (82E3-82E7)
	Df(3R)Exel6170 (87F10-87F14)
	Df(3R)Exel6184 (92A5-92A11)
	Df(3R)Exel6193 (94D3-94E4)
	Df(3R)Exel9013 (95B1-95B5)
	Df(3R)Exel6196 (95C12-95D8)
	Df(3R)Exel6200 (96A20-96B4)
	Df(3R)Exel6208 (97E5-97E11)
	Df(3R)Exel6209 (98D6-98E1)
	Df(3R)Exel6213 (99C5-99D1)
	Df(3R)Exel6216 (99F6-99F7)

[a] The chromosome regions covered by the deficiencies are shown. Suppressor regions of some deficiencies were narrowed down to a few genes by overlapping deficiencies and/or mutants.

identity. The RhoGEF domain of CG30456 shares 29% identity and 52% similarity to Kalirin, a RhoGEF in mice. Kalirin has been shown to regulate the maturation of secretory vesicles (Ferraro *et al.* 2007). Overexpression of the Kalirin GEF domain increases secretion from immature vesicles, while inhibiting its GEF activity results in the accumulation of products in mature secretory vesicles. Similarly, CG30456 and CG15611 may modulate the secretory vesicle maturation process in the Golgi apparatus, and reducing the dosage of CG30456 and CG15611 may suppress the defects caused by *Osbp* overexpression.

Potential gene redundancy and lack of mutations of *CG30456* and *CG15611* preclude us from directly testing their suppressive effects on *Osbp* overexpression. In addition, overexpression of these two genes in CCAP neurons driven by *CCAP-Gal4* did not lead to obvious behavioral phenotypes. Therefore, it remains to be determined whether these two *RhoGEF* genes are involved in secretory vesicle processing in *Drosophila*.

CG33298, a putative phospholipid flippase gene, is a suppressor of Osbp overexpression

The suppression locus within *Df(2L)Exel6022* was narrowed down by *Df(2L)Exel8022* and *Df(2L)Exel7042* to a small region including only two genes, *Gdi* and *CG33298* (Figure 5). *Gdi* encodes a Rab GDP dissociation inhibitor and is involved in vesicle-mediated transport (Lloyd *et al.* 2000). *CG33298* is predicted to encode a putative phospholipid flippase that translocates phospholipids PS and PE from the inner leaflet to the outer leaflet across the bilayer membrane. Since the strong loss-of-function, if not null, mutant of *Gdi* ($Gdi^{N7\text{-}3}$) cannot suppress the unexpanded wing phenotype of *Osbp* overexpression, we concluded that *CG33298* is likely the suppressor.

To unambiguously prove that *CG33298* is the suppressor gene, we generated *CG33298* mutations by imprecise *P*-element excision. *P*-element insertion line *GS14514* has a transposon insertion intragenic to the *CG33298* genomic region, close to the start codon (Figure 5). By mobilizing this transposon, we obtained a *CG33298* mutation, $CG33298^{44\text{-}5}$, which deletes the second exon and part of the third exon, including the start codon (Figure 5). This deletion includes the coding region (nt 1–382) for the first 128 amino acids of CG33298. The next available in-frame start codon is 524 nt away from the deletion and starts at amino acid 303. Therefore, $CG33298^{44\text{-}5}$ might produce a truncated protein lacking the N-terminal 302 amino acids. We next introduced one copy of $CG33298^{44\text{-}5}$ into *Osbp*-overexpressing flies and found that the unexpanded wing phenotype was suppressed (Figure 5), demonstrating that *CG33298* is the suppressor gene.

Phospholipid flippase is also known as P4-type ATPase. It uses ATP hydrolysis to provide energy for transporting phospholipids across the membrane bilayer (Pomorski and Menon 2006). Two protein isoforms of CG33298 that differ only at the C-terminal end have been predicted. The predicted P-type ATPase domain of CG33298 is located at the N-terminal part of the protein between amino acids 305 and 576. Similar to other P4-type ATPases (Tanaka *et al.* 2010), 10-transmembrane domains were predicted in CG33298 (using PredictProtein, http://www.predictprotein.org/). The putative truncation (amino acid 1–302) in $CG33298^{44\text{-}5}$ likely includes the first transmembrane domain (amino acid 275–292) but does not extend to the ATPase domain. To understand the nature of $CG33298^{44\text{-}5}$ mutants, we then compared the suppression efficacy of the $CG33298^{44\text{-}5}$ mutant to that of the *Df(2L)Exel6022* deficiency. We found that the suppression efficacy of $CG33298^{44\text{-}5}$ was comparable to that of the deficiency (Figure 5), suggesting that $CG33298^{44\text{-}5}$ is likely a strong loss-of-function allele. Flies that were homozygous for $CG33298^{44\text{-}5}$ or trans-heterozygous to *Df(2L)Exel6022* were viable and showed no discernible phenotypes, indicating that *CG33298* is not an essential gene. In addition, overexpression of *CG33298* also did not lead to an obvious phenotype.

The phospholipid flippase family of proteins is a highly conserved, eukaryotic-specific family with five members in budding yeast, six in *C. elegans*, six in *Drosophila*, and 14 in mice and humans (Tanaka *et al.* 2010). Phospholipid flippases in various organisms have been linked to the function of the Golgi apparatus and sterol metabolism. For example, yeast Drs2p resides predominantly in the Golgi apparatus and regulates protein sorting and vesicle budding (Natarajan *et al.* 2004; Liu *et al.* 2008). Mutants of *C. elegans* phospholipid flippase genes *tat-2*, *tat-3*, and *tat-4* are sensitive to sterol deprivation in a partially redundant fashion (Lyssenko *et al.* 2008). Phylogenetic analysis showed that CG33298 is closely related to Dnf1p in budding yeast, TAT-3 in *C. elegans*, and ATP10D in humans (Tanaka *et al.* 2010). Six phospholipid flippase genes (*CG14741*, *CG31729*, *CG33298*, *CG42321*, *CG4301*, and *CG9981*) exist in the *Drosophila* genome. Since *CG33298* mutants are viable and fertile, redundant substitutes may exist for *CG33298*. To explore the specificity of phospholipid flippases, we examined the suppression effect of other phospholipid flippase genes. Neither *CG42321* nor *CG31729* RNAi, nor the deficiency of *CG14741* or *CG31729*, was able to suppress the effect of *Osbp* overexpression, indicating that mutation of *CG33298* suppresses *Osbp* overexpression specifically.

Mutation of CG33298 suppresses bursicon trafficking and Golgi morphology defects caused by Osbp overexpression

We next explored the possible mechanism(s) of the *CG33298* suppression. Suppression could be achieved by restoring budding of bursicon granules from the Golgi apparatus or, alternatively, by increasing the peripheral response to bursicon. To distinguish between these two possibilities, we stained bursicon in *CCAP-Gal4 > UAS-Osbp* control flies and $CG33298^{44\text{-}5}/+$; *CCAP-Gal4 > UAS-Osbp* flies. We found that in some $CG33298^{44\text{-}5}/+$; *CCAP-Gal4 > UAS-Osbp* animals (45%, $n = 11$) the diminished bursicon staining in

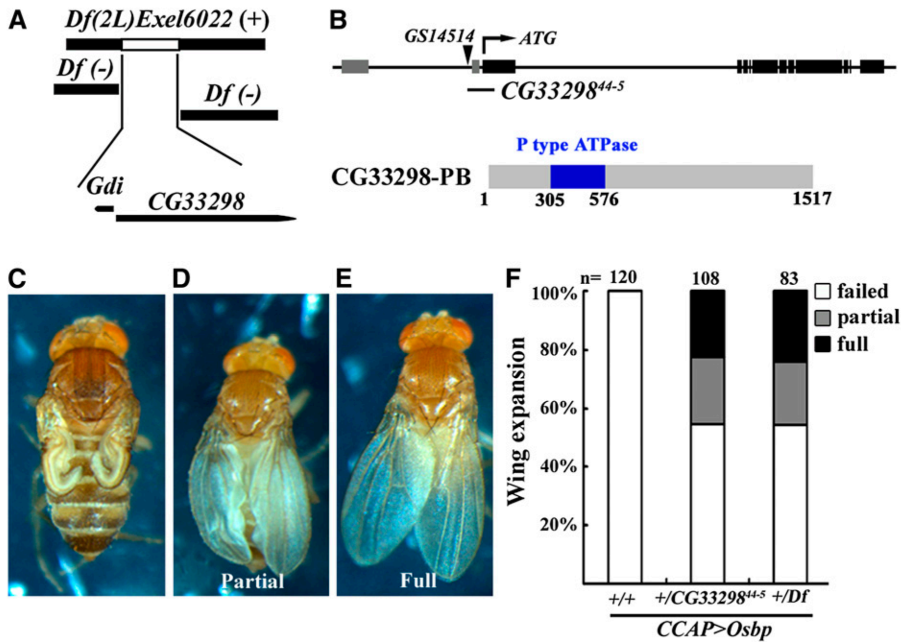

Figure 5 *CG33298* is a suppressor of *Osbp* overexpression. (A) Overlapping deficiencies refine the suppressor region of *Df(2L)Exel6022* to a genomic locus harboring only two genes, *Gdi* and *CG33298*. +, suppression; −, no suppression. (B) The genomic structure of *CG33298* and the protein domain structure of CG33298. Black boxes represent coding regions, and gray boxes represent untranslated regions. The transposon insertion point of the line *GS14514* and the deletion region of *CG33298*$^{44\text{-}5}$ are indicated. The P type ATPase domain is located at aa 305–576 of CG33298-PB. (C–E) Unexpanded wings resulting from *Osbp* overexpression are reverted by reducing one copy of *CG33298*. (C) Unexpanded wings are caused by *Osbp* overexpression. (D) Strong suppression by *CG33298*$^{44\text{-}5}$/+. (E) Full suppression by *CG33298*$^{44\text{-}5}$/+. (F) Quantification of the suppression exerted by *CG33298*$^{44\text{-}5}$ and the deficiency *Df(2L)Exel6022*.

Osbp-overexpressing axons was partially restored (Figure 6). These results indicate that the *CG33298*$^{44\text{-}5}$ mutation could suppress bursicon-trafficking defects.

Since *Osbp* overexpression alters the morphology of the Golgi apparatus (Figure 3), we further examined whether reducing the level of *CG33298* could rescue the Golgi morphology defect. When the copy number of *CG33298* in *Osbp*-overexpressing CCAP neurons was reduced by one, the ManII-eGFP-punctated Golgi structures were partially restored (Figure 6). This result indicates that the suppression exerted by *CG33298* is likely via the restoration of the function of the Golgi.

High levels of cholesterol at the trans-Golgi network have been suggested to be very important for neuropeptide sorting and secretory vesicle formation (Wang *et al.* 2000). Consistent with this, cholesterol synthesis-deficient neuroendocrine/endocrine cells show a significant reduction in secretory vesicle production (Subtil *et al.* 1999), suggesting that secretory vesicle formation is sensitive to membrane cholesterol content. Previously, we have shown that *Osbp* overexpression leads to sterol accumulation in the Golgi apparatus (Ma *et al.* 2010). Therefore, it is possible that excess sterols in the Golgi membrane could compromise the secretory function of the Golgi apparatus. In agreement with this, overexpression of a mutant OSBP protein with point mutations in its sterol-binding domain showed no post-eclosion defects (Ma *et al.* 2010). Moreover, the overexpression of sterol-binding-deficient OSBP caused neither a sterol-overloading phenotype in the Golgi apparatus nor Golgi morphology defects (Figure 6). These data raise the possibility that *CG33298* exerts suppression by reducing the sterol level in the Golgi apparatus. We stained *Osbp*-overexpressing salivary glands with filipin, a sterol dye, and found that the accumulation of sterol was not suppressed by loss of one copy of *CG33298* (Figure 6). Therefore, we conclude that mutation of *CG33298* does not suppress the *Osbp*-overexpressing phenotype by altering the level of sterol in the Golgi apparatus *per se*. It is possible that loss of phospholipid flippase might affect the asymmetry of the Golgi membrane bilayers to counterbalance the effect caused by excess sterol loading.

Discussion

Non-vesicular cholesterol transport by cholesterol-binding proteins plays critical roles in maintaining cellular cholesterol homeostasis. Here we have shown that *Osbp* overexpression leads to elevated levels of cholesterol in the Golgi apparatus and impairs the morphology and function of the Golgi apparatus. In addition, the phospholipid flippase gene *CG33298* was identified in a genetic screen as a suppressor of *Osbp* overexpression.

Several lines of evidence prompt us to link post-eclosion behavioral defects derived from *Osbp* overexpression to abnormal sterol levels in the Golgi apparatus. First, overexpressing OSBP with a mutated sterol-binding domain does not lead to sterol accumulation in the Golgi apparatus or to post-eclosion behavioral defects. Second, expressing the ER-resident protein VAP abolishes sterol accumulation in the Golgi apparatus by recruiting OSBP to the ER. Accordingly, the unexpanded-wing phenotype resulting from *Osbp* overexpression is suppressed (Ma *et al.* 2010). Third, the level of cholesterol has been shown to be critical for the proper budding of secretory vesicles (Subtil *et al.* 1999). Disturbing the level of cholesterol or the balance between cholesterol and other lipids may lead to defects in secretory vesicle formation. In this study, therefore, we utilized this overexpression system to investigate how the Golgi apparatus adapts to cholesterol chaos to maintain physiological function.

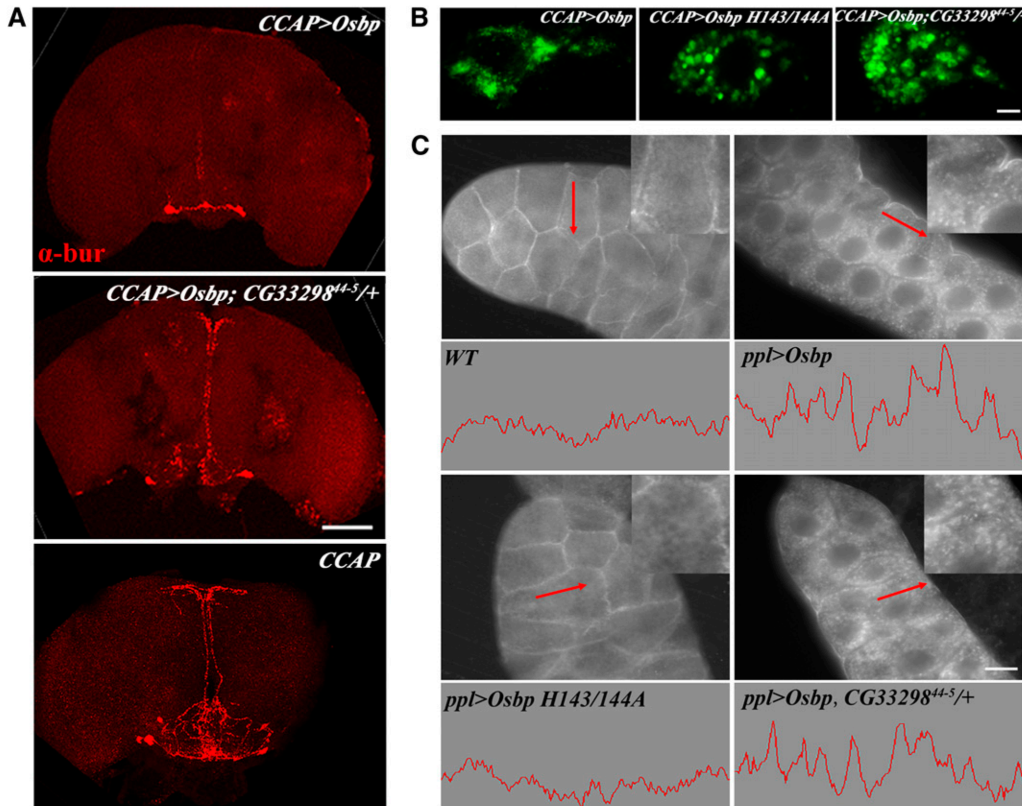

Figure 6 The *CG33298* mutation suppresses the bursicon-trafficking defects and Golgi morphology defects caused by *Osbp* overexpression. (A) Anti-bursicon staining shows bursicon-positive axons in CCAP neurons in the brain and subesophageal ganglion in CCAP control, *CCAP* > *Osbp*, and *CCAP* > *Osbp;CG33298$^{44-5}$*/+ flies. In 5 of 11 *CCAP* > *Osbp;CG33298$^{44-5}$*/+ flies, the diminished midline bursicon staining in *Osbp*-overexpressing flies is partially restored. Note that the CCAP control has a more elaborated bursicon-staining pattern compared to *CCAP* > *Osbp;CG33298$^{44-5}$*/+. Bar: 100 μm. (B) The morphology of Golgi apparatus labeled with ManII-eGFP in CCAP neurons. The punctated Golgi structures are present in *CCAP* > *Osbp* H143/144A and *CCAP* > *Osbp;CG33298$^{44-5}$*/+ backgrounds but not in the *CCAP* > *Osbp* background. Bar: 5 μm. (C) Filipin staining of sterols in salivary gland cells. *Osbp* overexpression with *ppl-Gal4* results in punctated sterol accumulation, which cannot be suppressed in the presence of *CG33298$^{44-5}$* mutation. Expressing a sterol-binding-deficient OSBP (OSBP H143/144A) did not lead to sterol accumulation. Bar: 50 μm. Graphs are line intensity scans drawn from the red arrows. There are multiple peaks in *CCAP* > *Osbp* and *CCAP* > *Osbp;CG33298$^{44-5}$*/+ backgrounds, indicating punctated sterol accumulation. Insets are enlarged views of the line-scanned areas.

On the basis of the screen design used here, several classes of suppressors could be predicted. The first class of suppressors is transcriptional regulators, which regulate the expression level of the *Gal4-UAS* system. This type of suppression is nonspecific for *Osbp* overexpression. Second, the suppression could be achieved by reducing the level of OSBP in the Golgi apparatus, for example, by VAP's recruitment of OSBP to the ER (Ma *et al.* 2010). Third, suppressor genes might be regulators for secretory vesicle formation in the Golgi apparatus, and when overexpressed or mutated, they can suppress defects caused by *Osbp* overexpression. *Rab18* and the putative *RhoGEFs*, *CG30456* and *CG15611*, likely belong to this category. Finally, suppressors could be regulators for lipid homeostasis, mutation of which can offset the cholesterol-overloading effect without lowering cholesterol levels through as-yet-unknown compensatory mechanisms. The identification of phospholipid flippase CG33298 in our screen suggests that an appropriate balance of cholesterol and phospholipids in membrane bilayers is crucial for normal Golgi function.

How can reducing phospholipid asymmetry offset cholesterol overloading in secretory vesicle formation in the Golgi apparatus? Cholesterol increases the membrane curvature and affects the sorting and secretory vesicle-budding processes (Wang *et al.* 2000; Holthuis 2004; Antonny 2011). More cholesterol in the outer leaflet reduces membrane fluidity (Flamm and Schachter 1982). It is possible that reducing phospholipid asymmetry may balance membranes in cholesterol-overloaded Golgi apparatus by influencing membrane curvature or fluidity. Similarly, it has been postulated that the Golgi structural and secretory vesicle formation defects in yeast *drs2* mutants are caused by altered membrane curvature or fluidity (Chen *et al.* 1999).

Phospholipids are important for sorting and exocytic secretion processes in the Golgi apparatus (Mayinger 2009). The Golgi apparatus lies at the center of the cellular endomembrane system, bridging the membrane flow from the ER and the plasma membrane. The lipid composition of the plasma membrane and the ER is significantly different. Cholesterol and sphingolipid levels are high in the plasma membrane but low in the ER (van Meer *et al.* 2008). The Golgi apparatus has a unique lipid composition with intermediate levels of cholesterol and sphingolipids. Phosphatidylinositol 4-phosphate (PI4P) is enriched in the Golgi apparatus (Mayinger 2009). Many PI4P-binding effectors that coordinate vesicle budding and the lipid composition of the Golgi apparatus via binding PI4P have been identified (Dippold *et al.* 2009).

Previous studies have also linked cholesterol-trafficking regulators to phospholipids in the Golgi apparatus. OSBP is an effector of PI4P and may transfer cholesterol between the Golgi apparatus and the ER by binding to PI4P in the Golgi

apparatus with its pleckstrin homolog domain and to VAP in the ER with its FFAT motif. Disturbing the level or the activity of several OSBPs leads to Golgi fragmentation and inefficient protein transport from the Golgi apparatus to the plasma membrane (Ngo and Ridgway 2009; Nhek *et al.* 2010). Interestingly, although PKD inhibits OSBP, it enhances the activity of PI4KIII by phosphorylation and stimulates PI4P production, which might result in the recruitment of more OSBP to the Golgi apparatus (Hausser *et al.* 2005). On the other hand, it is reported that yeast Osh proteins control PI4P metabolism through PI phosphatase Sac1 (Stefan *et al.* 2011). In our study, reducing one copy of wild-type *sac1* cannot suppress OSBP overexpression wing phenotypes.

In addition to the unique lipid composition, the asymmetrical localization of phospholipids also contributes to secretory vesicle formation in the Golgi apparatus. The phospholipid flippase Drs2p is important for vesicle formation in the Golgi apparatus and is another effector of PI4P (Natarajan *et al.* 2004). Drs2p does not depend on PI4P for its Golgi localization, and the level of PI4P is not influenced by Drs2p. PI4P binds to the C-terminal tail of Drs2p and stimulates the activity of Drs2p (Natarajan *et al.* 2009).

The asymmetrical localization of phospholipids has previously been linked to cholesterol homeostasis. Studies in yeast have established a strong link between the phospholipid flippase Drs2p and Kes1p/Osh4p. *kes1* mutants suppress the growth defects of *drs2-ts* alleles. Flippase activity is increased in *kes1* mutants. In addition, both *kes1* and *drs2* mutants exhibit defects in sterol distribution, and the defects are largely diminished in *kes1;drs2* double mutants. These results suggest antagonistic activities or negative correlations between Drs2P and Kes1p (Muthusamy *et al.* 2009). A recent study of *C. elegans tat* mutants also supports a causal link between phospholipid flippase and cholesterol metabolism (Lyssenko *et al.* 2008). *tat-1–6* are six *C. elegans* phospholipid flippase genes. The growth of *tat-2*, *tat-3*, and *tat-4* mutants is hypersensitive to sterol depletion, indicating that phospholipid flippase might be involved in the fine-tuning of sterol metabolism.

In our screen, mutation of *CG33298* can suppress *Osbp* overexpression, indicating a positive correlation between flippase CG33298 and OSBP. How can the different findings in fly and yeast be reconciled? There are several possibilities. First, the phospholipid flippase and OSBP may have different or even opposite relationships in different biological processes. Indeed, although Drs2P and Kes1p have antagonistic effects in many aspects, *kes1* does not suppress the *drs2* defect in secretory vesicle budding in the Golgi apparatus (Muthusamy *et al.* 2009). Alternatively, it is possible that species-specific differences between the fly and yeast lead to different outcomes. For example, yeast does not have PKD, which is a key regulator for OSBP and PI4P. Nevertheless, the genetic interactions between phospholipid flippase and OSBP in both the fly and yeast suggest that an appropriate balance of OSBP and phospholipid flippase is important for normal physiological function.

Taken together, studies in yeast, worms, and flies support an evolutionarily conserved causal link between cholesterol homeostasis and phospholipid asymmetry in maintaining normal cellular physiology. It will be interesting to examine whether this functional connection is also conserved in mammals. Nevertheless, our study pinpoints the importance of orchestrating the homeostasis of various lipids, including cholesterol, sphingolipid, and phospholipid, for maintaining proper functions of the Golgi apparatus.

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