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Abstract

Lipids are precisely distributed in the eukaryotic cell where they help to define organelle identity and function, in addition to their structural role. Once synthesized, many lipids must be delivered to other compartments by non-vesicular routes, a process that is undertaken by proteins called Lipid Transfer Proteins (LTPs). OSBP and the closely-related ORP and Osh proteins constitute a major, evolutionarily conserved family of LTPs in eukaryotes. Most of these target one or more subcellular regions, and membrane contact sites in particular, where two organelle membranes are in close proximity. It was initially thought that such proteins were strictly dedicated to sterol sensing or transport. However, over the last decade, numerous studies have revealed that these proteins have many more functions, and we have expanded our understanding of their mechanisms. In particular, many of them are lipid exchangers that exploit PI(4)P or possibly other phosphoinositide gradients to directionally transfer sterol or PS between two compartments. Importantly, these transfer activities are tightly coupled to processes such as lipid metabolism, cellular signalling and vesicular trafficking. This review describes the molecular architecture of OSBP/ORP/Osh proteins, showing how their specific structural features and internal configurations impart unique cellular functions.

Keywords

Lipid Transfer Protein (LTP), membrane contact sites, Oxysterol Binding Protein (OSBP), sterol, phosphoinositide, structural biology

Introduction

Lipids are precisely distributed in the cell: each organelle membrane has its own lipid composition and thus unique molecular features and identity that are critical for proteins to support many functions including endocytosis and exocytosis, signalling pathways, ionic exchange, cellular movement, and respiratory function. The endoplasmic reticulum (ER), nuclear envelope and the *cis*-Golgi are limited by a membrane that is mostly constituted by neutrally-charged glycerophospholipids with unsaturated acyl chains. The *trans*-Golgi, endosomes and the plasma membrane (PM) contain glycerophospholipids that are more saturated and/or anionic such as phosphatidylserine (PS). Moreover, they are highly enriched in sphingolipids and sterols (Drin, 2014). These organelles also harbour small amounts of phosphoinositides (PIPs) that serve as molecular signposts, for example PI(4)P, which marks the cytosolic leaflet of the *trans*-Golgi and the PM, and PI(4,5)P₂ which is restricted to the PM (Di Paolo and De Camilli, 2006).

At any given time, specific mechanisms are in place to create and maintain the lipid composition of organelle membranes in the face of continuous cellular processes, such as vesicular trafficking that mixes membranes, or signalling cascades that consume lipids. Metabolic pathways ensure the synthesis, interconversion and degradation of lipids, while other processes guarantee the transport of lipids between and within the cellular membranes. Because most of the lipids or lipid precursors are made in the ER, it had early been suggested that lipids

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must have been intensively exported to other organelles as well as to the PM. However, due to their hydrophobic nature, it is impossible for lipids to spontaneously cross the cytosol at a speed that is compatible with cellular functions. Moreover, this process would lead to a random distribution of lipids between cellular compartments. Today it is largely assumed that lipid transport mostly relies on cytosolic proteins called Lipid Transfer Proteins (LTPs), in addition to vesicular trafficking. These proteins contain a structural domain with a cavity to shield one or more specific lipid(s) from the aqueous medium and are able to accurately promote their transfer between two organelles, often in a directional manner (Lev, 2010; Wong et al., 2017). They belong to diverse families and show great diversity both in terms of structural features and domain organization (Chiapparino et al., 2016). Consequently, a broad range of lipids can be carried between different organelles by diverse mechanisms and under specific regulatory controls. This review focuses on a major and evolutionarily conserved family of LTPs whose founding member is OSBP and that comprises the OxySterol-Binding Protein (OSBP)-Related Proteins (ORPs) in higher eukaryotes and the OxySterol-binding Homology (Osh) proteins in yeast. A number of these were found to extract and transfer sterol or PS in exchange for PIPs, whereas others have alternative biochemical functions and possibly act as lipid sensors or partners of lipid-modifying enzymes. A fundamental aspect is that the ability of ORP/Osh proteins to recognize sterol, PS and PIPs as a lipid ligand, assigns these proteins with a pivotal role in the cell at the interface between lipid metabolism, cellular signalling and vesicular trafficking. This review article mostly focuses on the biochemical and structural features of ORP/Osh proteins, showing how diverse they are and how such features impart particular cellular functions to these proteins.

OSBP, the Prototype

In eukaryotes, sterol represents ~20% of total cellular lipids and is critical for the structural integrity of cellular membranes and for cell physiology (Mesmin and Maxfield, 2009; Vance, 2015). In mammals, cellular cholesterol levels are maintained by regulated uptake through receptor-mediated endocytosis of low-density lipoproteins (LDLs) (Goldstein and Brown, 2015) and are controlled by *de novo* synthesis in the endoplasmic reticulum (ER) (Holthuis et al., 2001; Espenshade and Hughes, 2007; Breslow and Weissman, 2010; Henry et al., 2012; Vance and Tasseva, 2013). Despite being made in this compartment, sterol accounts for less than 5 mol% of ER lipids, whereas it represents up to 40 mol% of lipids in the *trans*-Golgi and the PM

(Mesmin and Maxfield, 2009). The accumulation of sterol in the PM is crucial as sterol reduces the flexibility of neighbouring lipids due to its rigid structure and thus guarantees the impermeability of the cell (Mesmin and Maxfield, 2009).

Oxysterols constitute the numerous oxygenated derivatives of cholesterol that have diverse cellular functions (Luu et al., 2016; Griffiths and Wang, 2019). They arise from enzymatic and/or non-enzymatic processes that introduce modifications, hydroxyl and keto groups in particular, to the ring and/or side chain of the sterol molecule. The oxysterol family is primarily known for molecules that control intracellular cholesterol levels via both transcriptional and post-transcriptional mechanisms. Notably, 25-hydroxycholesterol (25-HC) has been known for some time to be able to efficiently inhibit the *de novo* synthesis of cholesterol by downregulating the mevalonate pathway, and also to inhibit sterol uptake via the LDL receptor and by stimulating cholesterol esterification. Of note, certain oxysterols can act as potent endogenous activators of the Hedgehog pathway that has an important role in embryogenesis and dysfunction of which is linked to various types of cancer (Griffiths and Wang, 2019). In addition, some oxysterols, and notably 25-HC, play a role in the innate and/or the adaptive immune system. Lastly, oxysterols have also been implicated in neurodegenerative diseases and atherosclerosis (Luu et al., 2016; Griffiths and Wang, 2019).

In the '70s, very little was known about how oxysterols regulated cellular cholesterol metabolism. This motivated a search for a cellular component that was able to perform this regulatory function upon recognizing oxysterol, and led to the identification of a cytosolic protein that binds 25-HC with high affinity ($K_D \sim 10$ nM) but does not bind cholesterol (Kandutsch et al., 1977; Kandutsch and Thompson, 1980). A good correlation was found between the binding affinities of diverse oxysterols for this protein, partially purified, and their ability to downregulate the mevalonate pathway in the cell (Taylor et al., 1984). Consequently, this protein, designated OSBP, was assumed to be an oxysterol receptor that controls sterol metabolism. Purification of OSBP to homogeneity revealed that it exists in two forms, 96 and 101 kDa (due to phosphorylation), and forms homodimers (Dawson et al., 1989b). Eventually, the cDNAs encoding the rabbit and human OBSPs were cloned by Brown and Goldstein (Dawson et al., 1989a; Levanon et al., 1990); the corresponding proteins have a length of 807 and 809 amino-acids, respectively, and share 98% identity, suggesting that OSBP is essential for cellular sterol metabolism. Analysis of the primary sequence indicated two features: a glycine/alanine rich-region at the N-terminus (segment 1–80) and a potential leucine-

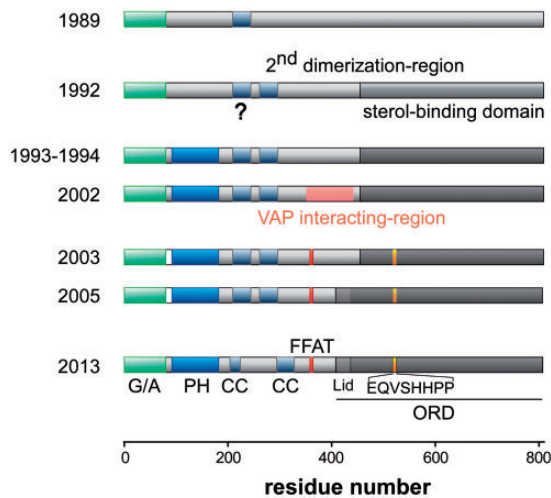


Figure 1. An OSBP Story (See Details in Text).

zipper motif (segment 209–244) responsible for OSBP dimerization (Dawson et al., 1989a) (Figure 1).

In 1992, Ridgway and co-workers found that the C-terminal half (455-809 segment) of OSBP contains the oxysterol-binding domain (Figure 1) (Ridgway et al., 1992). They also reported curious observations: OSBP relocates from the cytosol to the Golgi apparatus once 25-HC is added to the cell. Moreover, when the oxysterol-binding domain is deleted, OSBP permanently associates with the Golgi apparatus, *i.e.* even in the absence of 25-HC. A next step was the identification, just downstream of the G/A-rich region, of a pleckstrin homology domain (PH) (Haslam et al., 1993; Gibson et al., 1994) of ~90 aa (Figure 1) that mediates the translocation of OSBP onto the Golgi surface (Lagace et al., 1997). Levine and Munro identified phosphatidylinositol 4,5-bis-phosphate (PI(4,5)P₂), and phosphatidylinositol 4-phosphate (PI(4)P) as lipids that are able to recruit the PH domain to this compartment. Because these lipids are not uniquely restricted to this organelle, they further examined how the PH^{OSBP} targets the Golgi apparatus in yeast. They were able to silence both Pik1p and Stt4p, two PI 4-kinases, which convert phosphatidylinositol (PI) into PI(4)P at the Golgi and the PM, respectively, and Mss4p, which synthesizes PI(4,5)P₂ at the PM (Walch-Solimena and Novick, 1999; Audhya et al., 2000; Foti et al., 2001). Only the silencing of Pik1p prevents PH^{OSBP} from localizing to the Golgi surface, meaning that it recognizes PI(4)P, and similar results were obtained in mammalian cells (Levine and Munro, 2002). However, when PI(4)P is absent, PH^{OSBP} still binds, albeit weakly, to the Golgi surface as it recognizes a second determinant, found to correspond to the small G protein, Arf1, in its GTP-activated state (Levine and Munro, 2002; Godi et al., 2004).

Finally, it was reported that OSBP also locates to the ER *via* its 351–442 segment that associates with VAP-A (Wyles et al., 2002), one of the three isoforms of the ER-resident integral membrane VAP protein (Nishimura et al., 1999). It turned out that this region contains a short sequence, ₃₅₈EFFDAPE₃₆₄, that corresponds to an FFAT motif (two phenylalanine residues in an acidic tract, EFFDAxE) that is specifically recognized by VAP proteins and their homologue Scs2p in yeast (Loewen et al., 2003) (Figure 1). At that time, it became increasingly appreciated that zones of close apposition between the ER and other compartments, including the PM, *trans*-Golgi, mitochondria and endosomes are crucial for inter-organelle communication and lipid transfer, as well as for the regulation of second messenger molecules, including Ca²⁺ (Levine, 2004). In these regions, called membrane contact sites, the two organelle membranes are 15–30 nm away. The dual ability of OSBP to bind the ER and *trans*-Golgi/*trans*-Golgi network (TGN) membrane suggested that it might populate ER-Golgi contact sites, whose architecture had recently been described (Ladinsky et al., 1999; Marsh et al., 2004).

Despite these advances, the exact function of OSBP remained obscure. Any role in the control of the mevalonate pathway was ruled out by the discovery that the SRE binding protein (SREBP) and the SREBP cleavage-activating protein (SCAP) mediate such a role by detecting the sterol level in the ER (Brown and Goldstein, 1997). Moreover, it was found that the nuclear receptors LXR α and LXR β (liver X receptors α and β), are in fact the *bona fide* mediators of oxysterol-induced transactivation (Janowski et al., 1996; Lehmann et al., 1997). LXRs form heterodimers with the retinoid X receptors (RXRs) and bind to a specific DNA recognition sequence known as the LXR response element (LXRE). Upon binding of oxysterols, LXRs regulate the expression of gene networks involved in sterol absorption, transport and efflux processes, bile acid synthesis and excretion, hepatic lipogenesis, and synthesis of nascent high-density lipoproteins. As such, LXRs operate as sensors which protect the cell from cholesterol overload (for a review see Wang and Tontonoz, 2018).

Nevertheless, tenuous links were found between OSBP and the metabolism of sphingomyelin (SM), a lipid that segregates with sterol and whose abundance in cellular membranes correlates with sterol levels. Indeed, adding 25-HC stimulates SM synthesis (Ridgway, 1995; Ridgway et al., 1998; Storey et al., 1998) and this relies on the shift of OSBP to the Golgi apparatus. Afterward, it was found that OSBP and VAP are essential for the protein CERT to associate with this organelle (Perry and Ridgway, 2006). CERT shuttles ceramide from the ER to the *trans*-Golgi (Hanada et al., 2003), where ceramide is converted into SM.

Interestingly, CERT has a molecular configuration similar to that of OSBP, and can bridge the ER and the Golgi membrane but displays a START domain instead of an sterol-binding domain to transfer ceramide (Hanada et al., 2003; Kawano et al., 2006). Considering that 25-HC causes a depletion of cholesterol at the PM and a consequent increase at the ER (Tabas et al., 1988), and, conversely, that overexpressing OSBP presumably lowers sterol levels in the ER membrane (Lagace et al., 1997), one could surmise that OSBP and oxysterol contribute to regulating cellular sterol and SM distribution.

Intriguingly, OSBP constitutively associates with the Golgi apparatus, independently of 25-HC, if cellular sterol is low or if sterol is improperly distributed between the ER and PM (Ridgway et al., 1998; Storey et al., 1998). If OSBP is already bound to the Golgi apparatus, 25-HC does not activate SM synthesis. Thus, OSBP relocation seems to be a general response to various mechanisms, able to alter intracellular cholesterol levels or distribution and to, in turn, adjust SM metabolism. But it seemed paradoxical that a depletion of cellular cholesterol affects OSBP as 25-HC does, since 25-HC appears when cholesterol levels rise. This suggested that OSBP was not only, or not at all, a signalling protein that tunes sterol metabolism in response to oxysterol. In 2005, it was eventually reported that OSBP is dispensable for the control of cholesterol synthesis by 25-HC (Nishimura et al., 2005). In parallel, it was established that 25-HC can inhibit cholesterol production but does so by preventing SREBP activation (Adams et al., 2004; Radhakrishnan et al., 2007).

Early studies suggested that, in mammals, sterol is quickly transferred (half-time of ~10 min) from the ER to PM, mostly along non-vesicular routes (DeGrella and Simoni, 1982; Urbani and Simoni, 1990). In addition, the ER-to-PM transfer of ergosterol in yeast essentially takes place along such routes to preserve sterol gradient at the ER/PM interface (Baumann et al., 2005). Because sterols are insoluble in water, it was thought that specialized LTPs might exist to transfer these lipids throughout eukaryotic cells and that OSBP was a potential candidate in humans (Raychaudhuri and Prinz, 2010).

OSBP/ORP/Osh Proteins: An Evolutionarily Conserved Family in Eukaryotes

The advent of genomics revealed the existence of many OSBP homologues in eukaryotes. In mid '90s and early 2000s, seven genes were identified in *S. cerevisiae*, on the basis of sequence similarity to the ligand binding domain of OSBP (OSBP-Related Domain or ORD), (Jiang et al.,

1994; Beh et al., 2001). Based on their overall sequence homology, these proteins were referred to as Osh (Oxysterol-binding protein homologs) and classified into four subfamilies: Osh1p (a.k.a Shw1)/Osh2p, Osh3p, Osh4p/Osh5p (*i.e.*, Kes1p and Hes1p), and Osh6p/Osh7p. The first three have complex structures that include a PH domain and an ORD, whereas the others consist only of the ORD. Osh1p and Osh2p contain an ankyrin repeats domain (ARD) near their N-terminus. Note that the classification of Osh proteins into subfamilies primarily arose from divergences in the ORD sequences, which, as seen later, reflects distinct lipid specificity (Figure 2).

Concurrently, Ikonen's, Olkkonen's and Rodriguez's teams identified eleven OSBP-related proteins (ORPs) that, together with OSBP, define the ORP family in humans (Laitinen et al., 1999; Jaworski et al., 2001; Lehto et al., 2001). They were ordered into six subfamilies on the basis of sequence similarity and gene structure: OSBP and ORP4 (subfamily I), ORP1 and ORP2 (II), ORP3, ORP6 and ORP7 (III), ORP5 and ORP8 (IV), ORP9 (V), ORP10 and ORP11 (VI). Within each subfamily, the ORD share 70% identity. A PH domain was identified in all ORPs except ORP2. ORP1 has an ARD at its N-terminus. Potential membrane-spanning segments were identified at the C-terminus of ORP5 and ORP8. All ORPs contain short segments predicted to form coiled-coil structures that could play a role in protein-protein interactions. As described in more detail below, some of these (ORP1, ORP3, ORP4, ORP8, ORP9) are expressed either in a short or a long form. Importantly, sequence analyses, now based on a substantial set of genes (yeast, human and other species), pointed to a conserved EQVSHHPP sequence in the ORD, which became the hallmark signature of the ORP/Osh family (Figure 2).

Due to their homology with OSBP, ORP/Osh proteins were assumed to contribute to sterol homeostasis. Individual Osh genes seemed somewhat redundant and not necessary for yeast viability, but the elimination of the entire gene family was lethal, suggesting that Osh proteins share an essential function (Beh et al., 2001). When each of the OSH genes are deleted individually, a specific range of phenotypes is observed, many of which are consistent with a mild perturbation of ergosterol synthesis or trafficking, indicating that each protein also performs distinct functions. In-depth phenotype analyses of a yeast strain lacking all the Osh proteins suggested they play a role in endocytosis and intracellular sterol distribution (Beh and Rine, 2004). In parallel, it was found that Osh1p has the ability to bind to both the Golgi and to contact sites called nuclear-vacuolar junction (NVJs) and that Osh2p and Osh3p are at ER-PM contact sites. The fact that these proteins are in these regions suggested that ORP/Osh proteins play a role in

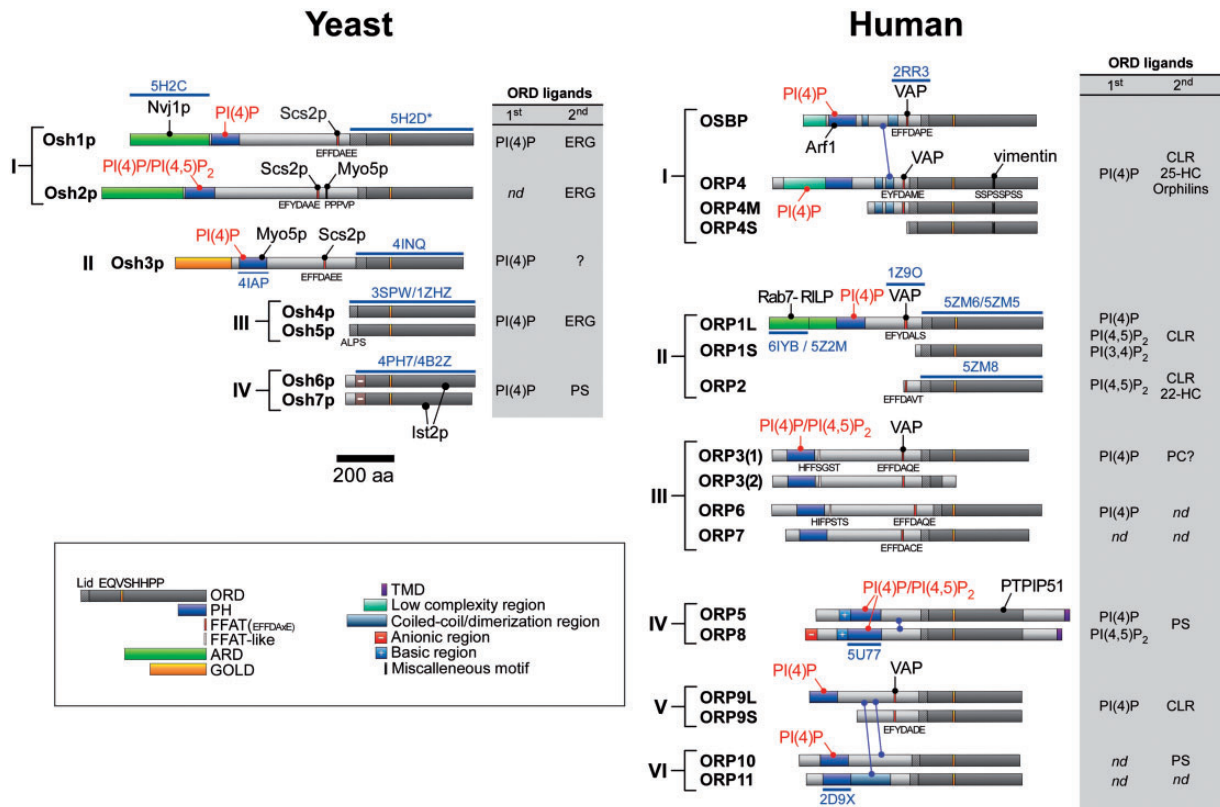


Figure 2. Domain Organization of Osh Proteins in *S. cerevisiae* Yeast and ORPs in Humans. Osh and ORP families include four (I-IV) and six (I-VI) subfamilies, respectively. The domains and motifs that compose these proteins are listed in the legend box. The amino-acid sequence of FFAT, FFAT-like and miscellaneous motifs, mentioned in the main text, is indicated. The interaction between regions of ORP/Osh proteins with PIPs or other proteins are shown by red and black lines, respectively. Interactions between ORPs are indicated by blue lines. The main lipids found to associate with the corresponding ORDs are listed in grey boxes. The first ligands correspond to PI(4)P or another PIP proposed to be used as a “fuel” to transfer a second lipid. Molecules that target the ORD without being necessarily transported are classified as a second ligand. The symbol “?” means that the corresponding ORD neither traps PS nor sterol, suggesting it recognizes a second lipid ligand whose nature remains to be defined. *nd.*: ligand not determined. Osh and ORP segments crystallized in complex with a ligand or a protein partner are indicated by a blue line and labelled with the corresponding PDB ID. Only the structures of ORD in complex with the most biologically relevant ligand are indicated: ORD^{Osh1p}-ERG (5H2D), ORD^{Osh3p}-PI(4)P (4INQ), Osh4p-PI(4)P (3SPW), Osh4p-ERG (1ZHZ), Osh6p-PI(4)P (4PH7), Osh6p-PS (4B2Z), ORD^{ORP1}-PI(4,5)P₂ (5ZM6), ORD^{ORP1}-CLR (5ZM5), and ORD^{ORP2}-PI(4,5)P₂ (5ZM8); (*) 5H2D structure corresponds to the ORD^{Osh1p} of *K. lactis* (77% homology with the ORD^{Osh1p} of *S. cerevisiae*). The lid of Osh4p/Osh5p corresponds to an ALPS (Amphipathic Lipid Packing Sensor) motif, whereas the lid of Osh6p/Osh7p is highly anionic (marked with the symbol “-”). ERG = ergosterol; CLR = cholesterol.

either the local sensing or the transport of lipids, rather than in lipid synthesis as initially assumed for OSBP (Olkkonen and Levine, 2004).

Osh4p Is a Sterol/PI(4)P Exchanger

Osh4p is one of the simplest ORP/Osh proteins, as it consists solely of an ORD. In spite of this simplicity, studies of this protein were decisive to bring light to the cellular role of ORP/Osh proteins. A turning point was in 2005 when Im and co-workers reported that Osh4p corresponds to a 15 stranded β -barrel with a hydrophobic cavity that is able to accommodate one ergosterol molecule (Im et al., 2005), (Figure 3A). The

cavity is sealed by an N-terminal molecular lid of ~ 30 residues. The ligand is in a head-down orientation with its 3-hydroxyl group making contacts with polar residues clustered at the bottom of the cavity (Figure 4). The rest of the lipid makes contacts with the pocket wall and inner face of the lid, stabilizing its closed conformation. Thus, it was revealed that Osh4p can shield a lipid from the water medium, a feature reminiscent of various LTPs.

The crystal structure of Osh4p was also solved in complex with cholesterol, 25-HC and other oxysterols, 7-HC and 20-HC, which are known to be good ligands of OSBP (Dawson et al., 1989a, 1989b). Interestingly, the volume of the cavity is large enough to host a

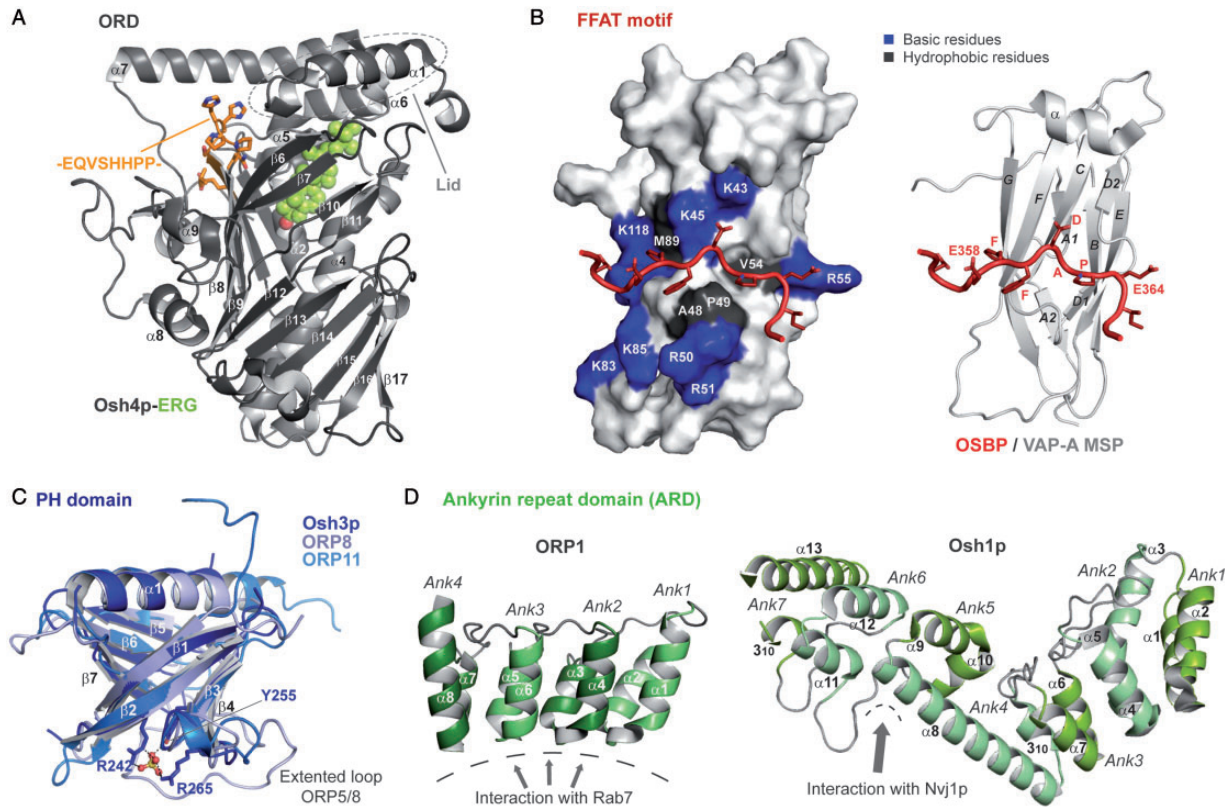


Figure 3. Structural Insights Into the Different Functional Domains of ORP/Osh Proteins. **A:** The OSBP-related domain (ORD) is the common domain of all ORP/Osh proteins. Today, 20 ORD structures with/without ligand, from yeast and humans, have been solved. The architecture of ORDs of all sub-families (described here with the Osh4p-ERG complex, 1ZHZ) is organized around a near-complete β -barrel forming a hydrophobic tunnel that can host one lipid. The pocket entrance is bordered on one side by the EQVSHHPP signature (in orange), and covered on the other side by a lid containing the helix $\alpha 1$. This helix is connected by a long loop to the N-terminal sub-domain that closes the barrel and consists of a two-stranded β -sheet and three α -helices ($\alpha 2$ - $\alpha 4$). The C-terminal region is composed of the conserved helices $\alpha 5$ - $\alpha 7$ followed by a variable sub-domain of ~ 80 amino-acids (see Figure 7 for details). **B:** The FFAT motif (in red, consensus sequence = EFFDAxE), mediates the association of ORP/Osh proteins with the N-terminal MSP domain of VAP proteins. Only two structures of complexes between an FFAT-containing peptide derived from OSBP or ORP1, and the MSP domain of partner VAP-A, give atomic details on this interaction. The motif adopts an extended β -strand like conformation, and binds across MSP^{VAP-A} near the β -strands C, D1, E and F. The FFAT motif is docked into a hydrophobic (in grey) and basic (in blue) cleft. **C:** Despite low sequence similarity, PH domains of ORP/Osh proteins share a common architecture composed of seven β -strands forming two anti-parallel β -sheets that are surrounded by a C-terminal α -helix. It is likely that the presence of a sulphate ion in the crystal structure of PH^{Osh3p}, in interaction with the conserved residues R242, Y255, and R265, corresponds to the 4-phosphate in IP₃, thus defining the binding site (see (Tong et al., 2013)). In comparison, the structure of PH^{ORP8} displays a 25 amino-acid extended loop between $\beta 6$ and $\beta 7$ strands, which covers the PIPs binding site. Together with the presence of a distinct basic cavity at the surface of PH^{ORP8} (Ghai et al., 2017), these features suggest that the mode of PIP-binding is different for PH^{ORP8}, and by extension PH^{ORP5}, which also contains this insertion. **D:** The structures of two ORP/Osh ARDs have been elucidated and reveal different organizations. ARD^{ORP1} adopts the classic architecture of ankyrin repeats, with four repeats of the two antiparallel α -helices ankyrin motifs stacked together to form a linear structure with a slight curvature. Structural analysis showed that Rab7 interacts with the concave side of the ARD^{ORP1}. In contrast, ARD^{Osh1p} forms a bi-lobed structure with seven tandem repeats distributed around the central and long α -helix $\alpha 8$. This organization results in the formation of two clefts on either side of $\alpha 8$, which could accommodate protein partners, as demonstrated for Nvj1p.

single sterol molecule and multiple water molecules (Figure 4), explaining why Osh4p, and by homology, OSBP, bind various sterols. Given that cholesterol is far more abundant than oxysterols in mammalian cells, and that the affinity of OSBP for the two ligands was finally found to be similar, cholesterol appeared to be the physiological ligand for OSBP. The fact that the lid

opens and closes depending on the status of Osh4p (empty or sterol-loaded) led to models describing how an ORD docks onto organelle surface to extract or deliver sterol, and performs transfer cycles. Moreover, *in vitro* assays showed that Osh4p but also OSBP, transferred cholesterol between artificial lipid vesicles (Raychaudhuri et al., 2006; Ngo and Ridgway, 2009).

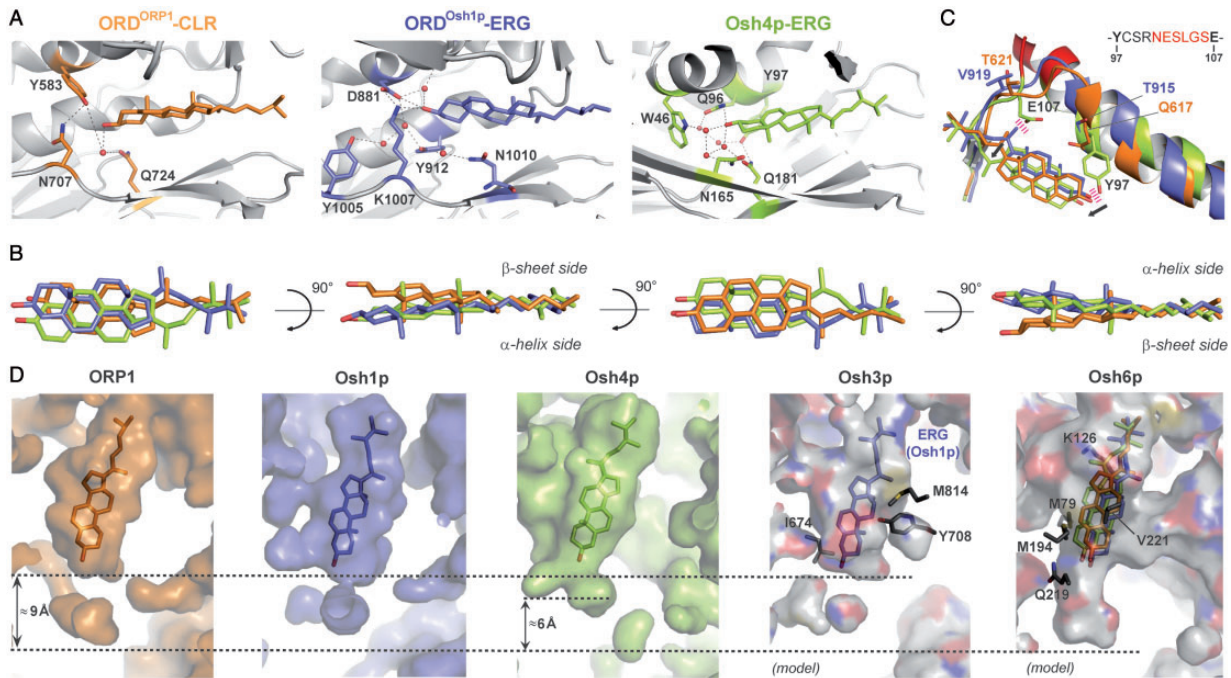


Figure 4. Shared and Specific Features in the Binding Modes of Sterols to ORD Domains. **A:** Close-up view of the pocket of ORD^{ORP1} harbouring a cholesterol molecule (5ZM5, in orange), and that of ORD^{Osh1p} (5H2D, in blue) and ORD^{Osh4p} hosting an ergosterol (1ZHZ, in lime). These structures are the only three available structures for ORP/Osh-sterol complexes. Sterols bind at the same position in the deep pocket of the ORDs thanks to a complex-specific network of hydrogen bonds involving the hydroxyl moiety of sterols, a cluster of non-conserved polar residues, and water molecules. In contrast, the sterol core interacts with hydrophobic residues through unspecific van der Waals contacts. **B:** While all the various sterols co-crystallized with ORD^{Osh4p} are perfectly stackable, the sterols in ORD^{Osh1p} and ORD^{ORP1} are tilted by $\sim 180^\circ$ around their long axes compared to the binding mode in ORD^{Osh4p}. **C:** This difference in orientation could be explained by the insertion of six residues (in red) specific to ORD^{Osh4p/5p} at the end of helix 4, resulting in the formation of a π -bulge that orientates the long side chains of Y97 and E107 toward the pocket, thus inducing steric clashes that are not compatible with the orientation of sterols observed in ORD^{Osh1p} or ORD^{ORP1}. **D:** A comparison of the pocket volume of representative ORDs might shed light on whether or not they trap sterol. Notably, Im and coworkers (Tong et al., 2013) proposed a model for the ergosterol-bound ORD^{Osh3p} with ergosterol in the “ORD^{Osh4p} orientation”, which brings out clashes between the ligand and residues L643, Y708, L778, N780, Q799, and R812. Performing the same analysis with ergosterol in the “ORD^{Osh1p} orientation” (Osh3p pocket), we found that the sterol would clash with some residues (I674, Y708, M814). Thus, the narrower pocket of ORD^{Osh3p} could account for its inability to bind sterol. The fact that the pocket of ORD^{Osh6p} is both long and narrow due to certain residues (indicated in the panel) might also explain why it does not enclose the wrap sterol but this needs to be further examined, for example, by molecular dynamics simulations. Oxygen and nitrogen atoms are in red and blue, respectively, hydrogen bonds are indicated by dashed lines, and water molecules by red spheres. In panel D, the internal surfaces are represented using PyMol (<http://pymol.org/>).

Altogether, these data suggested that ORP/Osh proteins were authentic cellular sterol transporters (Levine, 2005).

Yet this assumption was immediately questioned. Osh4p moves sterol between synthetic membranes, but at low speed (Raychaudhuri et al., 2006) and, except Osh2p and Osh5p, the other Osh proteins have no transfer activity (Schulz et al., 2009). Furthermore, it is unclear whether Osh proteins ensure sterol fluxes in yeast, at least at the ER/PM interface (Schulz et al., 2009; Georgiev et al., 2011). In addition previous findings provided conflicting evidence about the role of Osh proteins, or at least Osh4p, in sterol transport. Indeed, in 1996, the Bankaitis group reported that silencing Osh4p in yeast bypassed the requirement for SEC14, an

essential gene encoding a PI/phosphatidylcholine transfer protein (Fang et al., 1996). Thus, yeasts devoid of Sec14p die but are able to survive if Osh4p is also missing. Sec14p maintains proper PI(4)P levels and thereby the recruitment of key effectors at the Golgi surface, by either delivering PI in the Golgi membrane or presenting PI to Pik1p (Schaaf et al., 2008). In fact, Osh4p reduces the availability of the PI(4)P pool at the Golgi (Fairn et al., 2007), explaining how it counteracts Sec14p. Moreover, Osh4p regulates exocytosis (Fairn et al., 2007; Alfaro et al., 2011), a process that implies the PI(4)P-dependant generation of post-Golgi transport vesicles. In addition, Osh3p was proposed to downregulate PI(4)P to ensure signalling functions at ER-PM contact sites (Stefan et al., 2011). It seemed that Osh

proteins have other roles, linked to PIPs and unrelated to sterol transfer, or a sterol transfer activity somehow relying on PIPs.

Functional links with PIPs were conceivable for longer ORP/Osh proteins that exhibit a PH domain, but less so for simple proteins like Osh4p. Intriguingly, in the empty Osh4p structure, two sulphate ions are bound to a basic surface at the entrance of the binding-pocket: one between Lys109 and Lys336, two highly conserved lysines in ORP/Osh proteins, and the other one near the His143/His144 pair of the EQVSHHPP signature (Im et al., 2005). Because sulphate ions resemble phosphate groups, this might reflect the ability of an ORD to bind the headgroup of glycerophospholipids, *e.g.* PIPs, to better adsorb on membrane surfaces and be more efficient. For example, Osh4p transfers sterol between membranes a little

more quickly when they are doped with anionic lipids like PS or PI(4,5)P₂ (Raychaudhuri et al., 2006). Other ORDs (ORP1 and ORP2) detect the polar head of anionic phospholipids including PIPs (Xu et al., 2001). However, this was insufficient to explain the special relationship between Osh4p and PI(4)P.

We found that Osh4p can extract PI(4)P from membrane, meaning that it does not solely recognise the PI(4)P headgroup on membrane surface like a PH domain but traps the entire PI(4)P molecule. We solved the 1:1 Osh4p-PI(4)P complex structure and found that the sterol-binding pocket hosts the PI(4)P acyl chains, whereas charged residues that define an adjacent and shallow pocket under the lid recognize the PI(4)P headgroup (Figure 5A and B). The PI(4)P acyl chains loosely interact with the sterol-binding site in a rather nonspecific manner. In contrast, the polar head group is

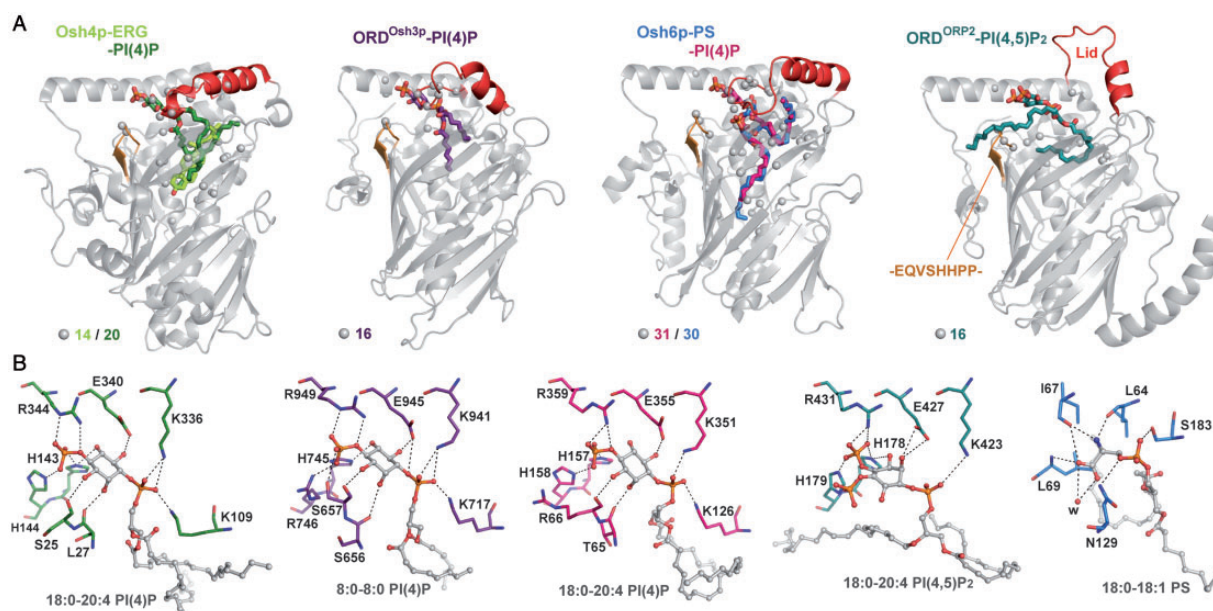


Figure 5. The Conserved Binding Mode of PIPs to ORDs. A: Structures of ORD-PIP complex representatives of various ORP/Osh sub-families. For comparison purposes, the structure of Osh4p and Osh6p, bound to ergosterol (1ZHZ) and PS (4B2Z), respectively, are superimposed to their PI(4)P-bound structures. PIP acyl chains are inserted into the central tunnel of the β -barrel while the inositol rings bind near the protein surface *via* conserved interactions (B). In Osh4p, the acyl chains occupy the same position as ergosterol in the pocket (more details in Figure 4). Because Osh3p (4INQ) has been crystallized with a synthetic PI(4)P bearing short acyl chains (8:0-8:0), it is impossible to define the real occupancy of the pocket by a natural PI(4)P, whose acyl chains are longer. Osh6p structures, either bound to PI(4)P (4PH7) or PS (4B2Z), highlight the similarities in the acyl chains insertion in the pocket. However, the binding mode of the polar head of PS and PI(4)P differ (B). In both ORP1 (5ZM6) and ORP2 (5ZM8) structures, PI(4,5)P₂ adopts the same binding mode, but differs from that of PI(4)P in other ORDs: (i) one acyl chain is located out of the pocket, whereas the second is curled up at the top of the pocket, in contrast with PI(4)P in Osh proteins that plunges into the cavity; (ii) the orientation of the polar head of PI(4,5)P₂ is also different (B). B: The close-up views of the residues that make key contacts with the polar head of PIPs, and partly located in the ORD signature (-HH-; in orange in A), show the high degree of similarity of these residues. The major difference is observed for PI(4,5)P₂ in ORP2 (and ORP1), whose inositol ring is rotated by 180° compared with that of PI(4)P in Osh proteins. This is probably due to the steric clash that would occur between the 5-phosphate group and helix α 7 of proteins in the PI(4)P orientation. Yet this does not prevent the interaction with the conserved patch of residues located at the pocket entrance. In Osh6p, the PS polar-head interacts with the main chain residues L64, I67 and L69, and with the side chain residues N129 and S183, all of which are conserved. Oxygen and nitrogen atoms are coloured in red and blue, respectively. Hydrogen bonds are indicated by dashed lines. In A, the grey spheres indicate the number of contacts between ligands and ORDs.

involved in direct and water-mediated contacts with residues such as Lys336 and the His143/His144 pair, accounting for the specific recognition of PI(4)P by Osh4p (Figure 5B). The lid secures the lipid molecule by covering its glycerol moiety. Of note, the localization of phosphate groups in position 1 and 4 on the inositol ring overlap with that of sulphate ions in the apo Osh4p. Because the residues that recognize PI(4)P are highly, if not strictly, conserved in Osh4p homologues, with some of them belonging to the EQVSHHPP signature, this suggested that all ORP/Osh proteins can extract PI(4)P.

A key element is that Osh4p binds sterol and PI(4)P in a mutually exclusive manner, notably as the acyl chains of PI(4)P occupy the sterol-binding pocket, and exchanges these two lipids between membranes (de Saint-Jean et al., 2011). In eukaryotic cells, PI(4)P is prominent in the Golgi and the PM (Di Paolo and De Camilli, 2006; Strahl and Thorner, 2007) but is absent from the ER due to Sac1, which hydrolyzes PI(4)P into PI (Foti et al., 2001; Faulhammer et al., 2007). Consequently, a steep PI(4)P concentration gradient exists at the ER/Golgi and ER/PM interface. We assumed that Osh4p exploits a PI(4)P gradient at the ER/Golgi interface to vectorially transfer sterol by sterol/PI(4)P exchange cycles. During each cycle, Osh4p would extract a sterol molecule from the ER, exchange it for PI(4)P at the PM, and then deliver PI(4)P to the ER where it is hydrolyzed. The maintenance of a PI(4)P gradient enables non-stop cycles and the build-up of sterol in the *trans*-Golgi (Figure 6A). This explained the existence of genetic interaction between OSH4, SEC14, SAC1 and PIK1 genes (Fang et al., 1996; Li et al., 2002; Fairn et al., 2007), why Osh4p downregulates cellular PI(4)P level (Fairn et al., 2007), and how Sac1p gets access to its substrate. Supporting this model, *in vitro* measurements indicated that Osh4p is 10-fold more efficient as a lipid exchanger than as a mere lipid transporter. Even more, it can create a sterol gradient between membranes by dissipating a pre-existing PI(4)P gradient (Moser von Filseck et al., 2015b). This suggested that ORP/Osh proteins can exploit the energy provided by the ATP-dependent generation of PI(4)P gradients to in turn build the sterol gradients observed in the cell.

OSBP Revisited: Lipid Counter-Exchange and Negative Feedback Loop

In addition to these investigations of Osh4p, determining the structure of complexes between VAP and the FFAT motifs of OSBP and ORP1 (Kaiser et al., 2005; Furuuta et al., 2010) (Figure 3B), Osh3p (Tong et al., 2013) and ORP11 PH domains (Figure 3C) provided key atomistic insights into OSBP and other multi-domain ORP/Osh

proteins. Furthermore, studies on the sterol/PI(4)P exchange activity of Osh4p helped explain OSBP function and cellular behaviour (Mesmin et al., 2013). A model was established: First, OSBP bridges the ER with *trans*-Golgi via its PH domain and FFAT motif. Refined prediction tools suggest that OSBP dimerizes via two short coiled-coil regions, one corresponding to the leucine-zipper found by Dawson et al. (1989a) and the other one overlapping the dimerization segment identified by Ridgway et al. (Ridgway et al., 1992) (Figure 1). Then, OSBP transfers sterol with its ORD from the ER to the Golgi membrane. This is coupled with a backward transfer of PI(4)P from the *trans*-Golgi to ER, where PI(4)P is hydrolysed into PI by Sac1 (Figure 6E). OSBP contributes massively to the ER to TGN transfer of sterol by consuming about half of the cellular PI(4)P pool (Mesmin et al., 2017).

Moreover, as OSBP transfers PI(4)P to Sac1 for hydrolysis, it lowers the Golgi PI(4)P level, thus the number of anchor points for its PH domain and thereby the time it resides at ER-Golgi contact sites. Such a negative feedback loop explains why OSBP, without its ORD, constitutively associates with the Golgi apparatus (Ridgway et al., 1992): because no PI(4)P transfer can occur, PI(4)P levels remain high. The finding that 25-HC slows down sterol/PI(4)P exchange further explained why 25-HC elicits the shift of OSBP to the Golgi. Indeed, when 25-HC is added, PI(4)P level rises at the Golgi, recruiting OSBP in ER-Golgi contacts. CERT also relocates there as it docks onto the Golgi surface in a PI(4)P-dependent manner.

These results, among other findings, helped to better envision how OSBP and CERT cooperate to tune the lipid composition of the TGN (Drin, 2014). The following model could be proposed: Arf1-GTP recruits the PI 4-kinase PI4KIII β (Godi et al., 1999), in addition to OSBP, ensuring the spatial proximity between OSBP and a PI(4)P source (Mesmin et al., 2017). OSBP locates to ER-Golgi contacts and exchanges sterol with PI(4)P. A second, more distant source of PI(4)P can be positively tuned (Mesmin et al., 2017). Indeed, sterol delivery can activate a palmitoyltransferase that grafts a lipid tail to the PI 4-kinase PI4KII α , and promotes its association to the Golgi (Lu et al., 2012). PI(4)P production also brings CERT to the ER-Golgi contact sites (Banerji et al., 2010).

Conversely, the delivery of ceramide by CERT and its conversion into SM produce diacylglycerol (DAG). This lipid recruits the protein kinase D (PKD), which can therefore phosphorylate CERT to restrain its association with PI(4)P (Prashek et al., 2017; Sugiki et al., 2018). PKD enhances PI4KIII β activity (Hausser et al., 2005) but more permanently increases that of OSBP. Overall, this results in a net depletion of Golgi PI(4)P. Consequently, OSBP and CERT disengage from contact

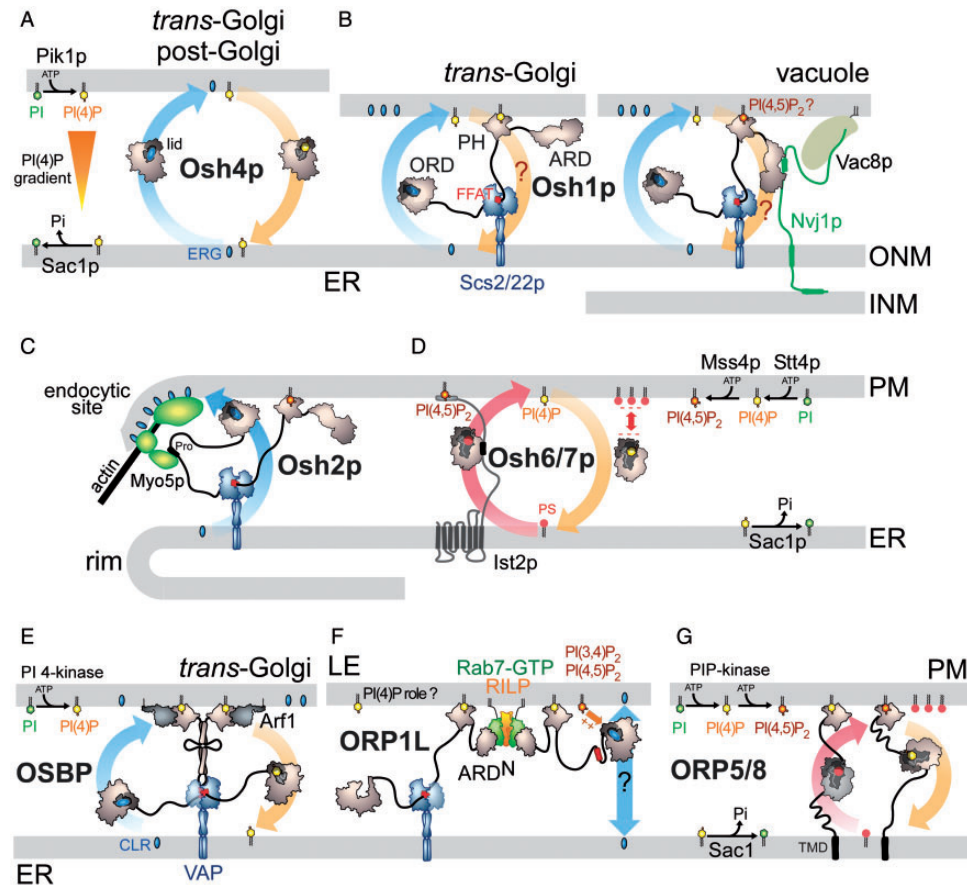


Figure 6. Mode of Action of ORP/Osh Proteins. A: In yeast, Osh4p would transport ergosterol from the ER, where this lipid is made, to the *trans*-Golgi and post-Golgi vesicles, and PI(4)P in the backward direction. At the Golgi, Pik1p phosphorylates PI into PI(4)P in an ATP-dependent manner whereas, at the ER, Sac1p hydrolyses PI(4)P into PI. This creates a PI(4)P gradient that would allow the vectorial ER-to-Golgi transport of ergosterol. B: Osh1p might occupy ER-Golgi contacts by simultaneously bridging the ER and the Golgi membrane via its PI(4)P-recognizing PH domain and by interacting with Scs2p/Scs22p via its FFAT motif and it would exchange sterol for PI(4)P with its ORD. By alternatively binding to Nvj1p via its ARD, Osh1p occupies NVJs where it might also function as an exchanger. Whether its PH domain contributes to recruiting Osh1p to the vacuolar membrane is unclear. C: Osh2p locates to ER-PM contact sites and binds Myo5p to deliver ergosterol at endocytic sites, possibly by sterol/PI(4)P exchange. The function of its ARD is unknown. D: Like sterol, PS is made at the ER. It is conveyed by Osh6p/7p to the PM by PS/PI(4)P exchange. Osh6p interacts with the cytosolic tail of Ist2p to occupy ER-PM contacts and transfer PS. The closing of the lid upon lipid extraction, as it is anionic, limits the length of time that Osh6p stays at the membrane, and thus maintains its rapid activity. E: OSBP interacts via its FFAT motif with the VAP receptors and, via its PH domain, with Arf1 and PI(4)P to perform cholesterol/PI(4)P exchange. F: ORP1L localizes to ER-LE contact sites by interacting with VAP and with Rab7-RILP complex via the N-terminal half of its ARD. ORP1L possibly transfers cholesterol between the ER and late endosomes but the direction is unclear. The role of PI(4)P is unknown and other PIPs might help ORP1L to transfer sterol towards the ER. Moreover, the capture of cholesterol induces a conformational change that masks the FFAT motif and prevents ORP1L from associating with VAP. The exact combinations of these functional traits remain elusive. G: ORP5/8 are anchored to the ER by TMDs, bind to the PM PI(4)P and PI(4,5)P₂ with PH domains and operate PS/PI(4)P exchange at ER-PM contacts. ERG: ergosterol; CLR: cholesterol; INM: inner nuclear membrane; ONM: outer nuclear membrane.

sites and stop their activity (Capasso et al., 2017). These regulatory loops, in addition to other regulatory processes (for more details see Mesmin et al., 2019), could synchronize sterol and ceramide fluxes, allowing the co-enrichment of these two lipids at the Golgi apparatus, while regulating DAG and PI(4)P levels. This would precisely control the lipid composition of the Golgi membrane, and thereby its secretory function (Duran et al., 2012). Recent data confirmed that OSBP

activity is required for vesicular trafficking at the *trans*-Golgi (Peresse et al., 2020). Moreover, this activity also appears to be critical for insulin granule formation at the TGN of pancreatic β -cells, a process that also involves cholesterol (Hussain et al., 2018). Similarly, OSBP locates to ER-endosome contact sites, thus controlling the PI(4)P level in the limiting membrane of endosome and protein recycling by the retromer-dependent trafficking pathway (Dong et al., 2016). OSBP is also

engaged in ER-lysosome contact sites to convey sterol to the limiting membrane of lysosomes. This enables mTORC1, a master regulator of cell growth, to be activated on the lysosome surface (Lim et al., 2019).

Recently, a role has been assigned to the G/A-rich sequence of OSBP. This region is intrinsically disordered and, by occupying a large volume, helps OSBP to maintain its orientation in contact sites while limiting its density and facilitating its dynamics on the membrane surface (Jamecna et al., 2019).

Functional Role and Structural Features of Osh Proteins

Subfamily I: Osh1p and Osh2p

Osh1p and Osh2p (Beh et al., 2001) share the ability to transfer sterol (Schulz et al., 2009; Manik et al., 2017), and likely PI(4)P, but have separate cellular roles, which seem to be dictated by their interactions with distinct partners at specific subcellular localization. Like PH^{OSBP}, PH^{Osh1p} targets the Golgi apparatus (Levine and Munro, 2001, 2002). Osh1p and Osh2p associate with Scs2p, the VAP homologue in yeast (Kagiwada et al., 1998) via an FFAT motif (Loewen et al., 2003). Both proteins differ from other ORP/Osh proteins in that they possess an N-terminal ankyrin repeat domain (ARD) (Jiang et al., 1994; Beh et al., 2001) (Figure 2).

Osh1p localizes to the Golgi apparatus, but also to nuclear-vacuolar junctions (NVJs), which correspond to the close apposition of the nuclear outer membrane with the vacuole membrane (Levine and Munro, 2001). Nvj1p, a protein anchored to the inner and outer nuclear membrane, has a disordered cytosolic tail that contains a binding site recognized by the integral vacuolar protein Vac8p. Together, Nvj1p and Vac8p form Velcro-like patches through which teardrop-shaped portions of the nucleus are pinched off into the vacuolar lumen and degraded by piecemeal microautophagy of the nucleus (PMN). Osh1p can interact via its ARD (Kvam and Goldfarb, 2006; Shin et al., 2020) with the cytosolic part of Nvj1p (Kvam and Goldfarb, 2004), more precisely a conserved sequence in a region (120-177) adjacent to the segment of Nvj1 spanning the outer nuclear membrane (Kvam and Goldfarb, 2006). Structural investigations showed that a short part (139-153) of this region is folded into a helix and is accommodated by a cleft defined by the bi-lobed structure of ARD^{Osh1} with a K_D of 10 μ M (Figure 3D). The ability of Osh1p to simultaneously bind to the ER-resident Scs2p (Loewen et al., 2003) and Nvj1p, itself connected to Vac8p, ensures its localization to NVJs (Figure 6B). One would speculate that the PH domain of Osh1p, given that it recognizes PI(4)P and PI(4,5)P₂, and because the vacuole contains PI(4,5)P₂, helps the protein to

occupy NVJs (Levine and Munro, 2001). However, this mechanism was recently ruled out (Shin et al., 2020) (Figure 6B).

Initial *in vitro* assays suggested that ORD^{Osh1p} encoded by *S. cerevisiae* does not convey sterol (Schulz et al., 2009). In fact, the recombinant version of this domain, purified from *E. coli*, was misfolded. Im and co-workers found that the closely-related ORD^{Osh1p} of *K. lactis* can trap ergosterol or PI(4)P (Manik et al., 2017). Structural analyses showed that, as for Osh4p, ergosterol adopts a head-down orientation in the pocket, and the features of the PI(4)P-binding site are conserved. However, ergosterol is hosted in the pocket of the ORD^{Osh1p} with a 180° rotation along its long axis compared to the binding mode of Osh4p (Figure 4).

Therefore, it was suggested that Osh1p ensures sterol/PI(4)P exchange at NVJs to move sterol from the outer nuclear envelope (in continuity with the ER) to the vacuole (Figure 6B). It is unclear why and how, but one might suspect that Osh1p plays a regulatory role. Indeed, ergosterol, despite being scarce in the vacuolar membranes (Zinser et al., 1993), seems essential for vacuole fusion and, if ergosterol is missing, vacuole fragmentation occurs (Kato and Wickner, 2001; Hongay et al., 2002). Vacuole fusion requires ergosterol, DAG and some PIPs as regulatory lipids, which are needed for the enrichment of SNAREs, small G proteins and tethering factors at the vacuole surface (Kato and Wickner, 2001; Fratti et al., 2004; Li and Kane, 2009).

At the Golgi, Osh1p also likely acts as an ergosterol/PI(4)P exchanger, but with a role in regulating post-Golgi vesicular trafficking (Figure 6B). This would explain why the trafficking of the tryptophan-permease Tat2p to the PM, which relies on TGN ergosterol (Umehayashi and Nakano, 2003) is impaired in Δ *osh1* strains (Shin et al., 2020). Likewise, this is why deleting OSH1 in tryptophan auxotrophs (*trp1*) produces a temperature-sensitive cell growth defect on medium that is tryptophan limited (Jiang et al., 1994; Levine and Munro, 2001; Loewen et al., 2003). A mutation in the FFAT motif of Osh1p blocks Trp uptake by yeast (Loewen et al., 2003), suggesting that Osh1p locates to ER-Golgi contact sites to exchange sterol and PI(4)P.

A fascinating aspect of this system is that the cellular activity of Osh1p is tuned by external signals that modify its repartition between the Golgi apparatus and NVJs (Kvam and Goldfarb, 2006; Shin et al., 2020). During the log phase, Osh1p is equally distributed between these two regions but then exclusively associates with NVJs as cells enter stationary phase (Levine and Munro, 2001) due to the higher expression of Nvj1p, and the concomitant increase in both the size and frequency of NVJ and PMN structures (Roberts et al., 2003). Therefore, Nvj1p negatively regulates tryptophan uptake during nutrient depletion (Kvam and Goldfarb, 2006). Recently, it has

been shown that Osh1p dissociates from the Golgi membrane when the cytosolic pH becomes low in response to glucose deprivation (Shin et al., 2020). This arises from the protonation of the PI(4)P headgroup, to which PH^{Osh1p} is less prone to bind. It is possible that Nvj1p regulation and cytosolic pH serve to boost and shut-down the functioning of NJVs and late Golgi, respectively, in a coordinated manner *via* Osh1p in order for the yeast cell to adapt to its environment.

Osh2p localizes at ER-PM contact sites (Loewen et al., 2003; Roy and Levine, 2004; Schulz et al., 2009; Stefan et al., 2011; Maeda et al., 2013) owing to its PH domain, which targets PI(4)P and/or PI(4,5)P₂ and its FFAT motif (Figure 6C). Ultrastructural investigations revealed that Osh2p is found at the rim of the cER and is physically linked to endocytic invaginations (Encinar Del Dedo et al., 2017). Osh2p associates with Myo5p, a type-I myosin that is required for actin assembly and scission of endocytic vesicles from the PM, *via* a polyproline motif ⁷⁷⁶PPPVP₇₈₀ (between the FFAT motif and the ORD) that is recognized by the Src Homology 3 Domain of Myo5p. ORD^{Osh2p} acts as a sterol transporter *in vitro* (Schulz et al., 2009) and uses this capacity to create sterol-enriched domains at endocytosis sites that facilitate actin polymerization. Thus, Osh2p plays a remarkable role at the PM, at the crossroads between dynamic (trafficking vesicle) and more stable (MCSs) subcellular structures. Osh2p lowers cellular PI(4)P levels (Stefan et al., 2011), suggesting it might act as a sterol/PI(4)P exchanger. It remains unclear how PI(4)P and PI(4,5)P₂ pools are regulated in space and time at endocytotic sites to coordinate the assembly/disassembly of the clathrin coat, the supply of sterol and the polymerization of actin. Interestingly, replacing ORD^{Osh2p} with ORD^{Osh4p} maintains the cellular activity of Osh2p (Encinar Del Dedo et al., 2017). This strengthens the idea that the transfer activity of an Osh protein can support a specific cellular function solely because of its localization. Some other structural traits might explain why the localizations of Osh1p and Osh2p differ. First, when PH^{Osh2p} is expressed alone, it is diffuse in the cytosol, contrary to PH^{Osh1p}, which is docked on the Golgi surface. A region centred on a histidine at position 79 in PH^{Osh1p} (replaced by an arginine in PH^{Osh2p}) could be critical for Golgi targeting (Roy and Levine, 2004). That said, the origin of this targeting is unclear since PH^{Osh1p} was recently shown to be unable to bind Arf1, contrary to PH^{OSBP} (Shin et al., 2020). Secondly, sequence comparison, corroborating early data (Levine and Munro, 2001), suggest that the ARD of Osh2p cannot bind Nvj1p due to a steric clash caused by a single bulky residue in the binding cleft of this domain (Manik et al., 2017). So far, the role of the ARD^{Osh2p} is unknown.

Subfamily II: Osh3p

Osh3p localizes to the ER-PM contact sites where it regulates lipid metabolism or vesicular trafficking (Loewen et al., 2003; Roy and Levine, 2004; Schulz et al., 2009; Stefan et al., 2011). In addition to an ORD at the C-terminus, Osh3p contains a GOLD domain at its N-terminus and an FFAT motif (Loewen et al., 2003) (Figure 2). Genome-wide analysis of yeast PH domains indicated that PH^{Osh3p} displays promiscuous binding to PI(3)P, PI(4)P, PI(3,4)P₂, and PI(4,5)P₂ (Yu et al., 2004). However, in yeast, it essentially recognizes PI(4)P in the PM (Stefan et al., 2011). Osh3p interacts with Scs2p and its homologue Scs22p (Loewen and Levine, 2005) to occupy ER-PM junctions where it downregulates the level of PI(4)P (Stefan et al., 2011) but also its spatial distribution in the PM (Omnus et al., 2020). Indeed, in the budding yeast, Osh3p downregulates PI(4)P in the mother cell, in which ER-PM contact sites are present, but not in the daughter cell, where polarized exocytosis occurs. Interestingly, upon heat shock, Osh3p aggregates due to its GOLD domain and disengages from contact sites. Therefore, PI(4)P is evenly spread in the PM of budding yeast, which diverts polarized exocytosis (Omnus et al., 2020).

Osh3p can trap PI(4)P but not ergosterol since the binding pocket of its ORD is too narrow to host a sterol molecule (Tong et al., 2013) (Figures 4 and 5). Moreover, Osh3p does not extract PS as had been demonstrated for Osh6p/Osh7p (see below). It is possible that at contact sites Osh3p either exchanges PI(4)P with a ligand whose identity remains to be determined, or transfers PI(4)P to the ER without counterexchange. Osh3p might also directly present PI(4)P to the catalytic domain of Sac1p (Stefan et al., 2011). Structural analyses suggested that the N-terminal catalytic domain of Sac1p was separated by a disordered linker of 70 residues from its C-terminal transmembrane domain, which is anchored to the ER. This linker might allow the catalytic domain to cross the gap (15-20 nm) between the ER and the PM and provide access to the PI(4)P pool of the PM (Manford et al., 2010). ORD^{Osh3p} might interact with and help Sac1p to hydrolyse *in trans* the PM PI(4)P pool, but not at the ER, *in cis*, as is expected in the counterexchange model. (Stefan et al., 2011). Yet, this hypothesis was questioned by the observation that a large section of this linker is a functional element of the catalytic domain and that its flexible part is too short to offer adequate freedom for Sac1p to work *in trans* (Cai et al., 2014).

Curiously, it has been reported that Osh3p presents phosphatidylethanolamine (PE) or phosphatidylmethyl-ethanolamine (PME) in the PM to help Opi3p at ER-PM contacts to produce phosphatidylcholine (Tavassoli et al., 2013). Yeast cells lacking Osh3p are

resistant to myriocin, a drug that inhibits the synthesis of long chain bases (LCB), suggesting a functional connection between Osh3p and sphingolipids metabolism (Yano et al., 2004). Intriguingly the regulation of LCB synthesis is linked to Sac1p (Breslow et al., 2010). Osh3p is localized to the cER rim where it interacts with Myo5p *via* its PH domain and contributes to endocytosis vesicles scission in a similar manner to Osh2p, albeit at a lesser extent (Encinar Del Dedo et al., 2017). How this relies on the transfer activity of its ORD is unknown, and overall, many facets of Osh3p remain enigmatic.

Subfamily III: Osh4p and Osh5p

Osh4p served as a great prototypical model to provide insight into the cellular function of ORP/Osh proteins. Paradoxically, it is unclear precisely how its own function relates to its exchange activity. Many clues suggest that Osh4p plays a major role in polarized exocytosis by regulating the life cycle of post-Golgi vesicles. These vesicles bud from the Golgi compartment, move along actin cables, dock to and fuse with the PM for delivering their content. The first stages depend on PI(4)P that recruits proteins on the surface of nascent vesicles whereas at later stages, PI(4)P must be removed to initiate the docking process (Mizuno-Yamasaki et al., 2010). It is unclear how these vesicles grow but, presumably, this process relies on the co-segregation of sterol with sphingolipids. These vesicles contain twice as much sterol as the *trans*-Golgi membrane (Klemm et al., 2009), and might contribute to provisioning the PM with this lipid. Osh4p could consume PI(4)P to supply the *trans*-Golgi with sterol, allowing its proper association with sphingolipids and vesicle genesis. This would explain why Osh4p reduces the availability of PI(4)P in the *trans*-Golgi (Fairn et al., 2007). Osh4p works in concert with Drs2p, a flippase that moves PS within the *trans*-Golgi membrane, generating an asymmetry in the membrane that is essential for the budding of post-Golgi vesicles (Sebastian et al., 2012). Drs2p activity is inhibited by Osh4p, probably because PI(4)P, which is critical for its flippase activity (Natarajan et al., 2009; Azouaoui et al., 2017; Timcenko et al., 2019), is removed from the Golgi by Osh4p. In return, the exposition of PS by Drs2p inhibits sterol delivery by Osh4p (Hankins et al., 2015).

At the post-Golgi level, Osh4p would remove PI(4)P from exocytic vesicles *en route* to the PM, making them competent for docking, and provide PI(4)P to Sac1p (Ling et al., 2014; Smindak et al., 2017). At the docking step, Osh4p could transfer the last remaining PI(4)P molecules from vesicles to the PM in exchange for sterol (Smindak et al., 2017). This would complete the enrichment of vesicles with sterol and possibly initiate the fusion process. Yet it is more likely that the sterol

originates from the ER, according to our sterol/PI(4)P exchange model. Indeed, Osh4p is more efficient in extracting sterol from fluid ER-like than rigid PM-like membranes (Moser von Filseck et al., 2015b). We proposed that, given its abundance (34,000/cell) and lipid exchange activity, Osh4p could transfer sterol in vast quantities from the ER to the *trans*-Golgi to promote the formation of exocytic vesicles and efficiently enrich these vesicles with sterol before they fuse with the PM. It could provide up to 60% of the sterol required for the expansion of the PM surface during the asymmetric division of yeast (Moser von Filseck et al., 2015b). Thus, by exchanging lipids, Osh4p would play a key role during the remodelling of *trans*-Golgi membrane and post-Golgi trafficking and this would explain why it impacts the level and transversal distribution of sterol in the *trans*-Golgi membrane and PM (Beh and Rine, 2004; Proszynski et al., 2005; Georgiev et al., 2011). That Osh4p is cytosolic (Li et al., 2002) and not confined in contact sites fits well with the idea that it works on moving objects such as trafficking vesicles.

In a relative sense, the role of Osh5p remains elusive. Although it shares 70% homology with Osh4p (Beh et al., 2001), Osh5p does not display Sec14p-bypass activity (Fang et al., 1996). Osh5p can transfer sterol (Schulz et al., 2009) and extract PI(4)P *in vitro* (Moser von Filseck, unpublished data) suggesting that it might exchange sterol/PI(4)P in yeast.

Subfamily IV: Osh6p and Osh7p

PS is an anionic glycerophospholipid that represents 2–10% of total membrane lipids (Daum et al., 1999; Leidl et al., 2008; Ejsing et al., 2009; Sampaio et al., 2011). Like sterol, it is allocated along a gradient between the ER, where it accounts for 5–7% of glycerophospholipids, and the PM where its proportion rises up to 30% in the cytosolic leaflet of this membrane (Zinser et al., 1991; Vance and Steenbergen, 2005; Leventis and Grinstein, 2010). This accumulation and asymmetric distribution of PS in the PM are critical for signalling pathways, mediated by cytosolic proteins that are mobilized by this lipid (Leventis and Grinstein, 2010). PS is synthesized in the ER (Vance and Tasseva, 2013), meaning that, like sterol, it must be exported to the PM, but little was known about how this was accomplished (Leventis and Grinstein, 2010).

In 2013, Maeda and co-workers reported that Osh6p and Osh7p can trap PS but not ergosterol (Maeda et al., 2013), explaining why these proteins showed no sterol transfer activity *in vitro* (Schulz et al., 2009). Furthermore, they established that Osh6p/7p transport PS from the ER to the PM, uncovering the existence of LTPs specific for PS and explaining how PS can reach the PM. Solving the structure of the 1:1 Osh6p-PS

complex revealed which residues in the cavity and inner surface of the lid recognize PS (Figure 5).

Yet, it was unclear how PS is transferred in a one-way direction from the ER to the PM. We established that Osh6p/7p can trap PI(4)P, and solved the 1:1 Osh6p-PI(4)P complex structure (Figure 5), confirming the conservation of the PI(4)P binding-pocket in ORP/Osh proteins. Osh6p efficiently exchanges PS for PI(4)P between two artificial membranes. If defective in capturing PI(4)P, Osh6p does not transfer PS in yeast. Likewise, silencing Sac1p to abolish the PI(4)P gradient at the ER/PM interface blocks Osh6p activity. Thus, Osh6p vectorially transports PS *via* PS/PI(4)P exchange cycles (Moser von Filseck et al., 2015a) (Figure 6D).

Osh6/7p are cytosolic but also localize at ER-PM contact sites (Schulz et al., 2009; Maeda et al., 2013), although they are devoid of known membrane-targeting motifs like those of the more complex Osh proteins. Recently, we showed that Osh6p/7p reside in these contact sites by interacting with Ist2p (D'Ambrosio et al., 2020) (Figure 6D). This protein is a homologue of TMEM16 proteins, a family of Ca²⁺-activated lipid scramblases (Wolf et al., 2012; Brunner et al., 2014; Wolf et al., 2014) and one of the few proteins that stabilize ER-PM contacts in yeast (Manford et al., 2012; Collado et al., 2019; Hoffmann et al., 2019). The tethering capacity of Ist2p relies on its long and disordered cytosolic tail whose highly cationic C-terminal end is anchored to PI(4,5)P₂ (Figure 6D). Osh6p recognizes a conserved motif in this tail and disruption of this interaction dramatically impairs PS import into the PM. This unveils a new partnership between an LTP and a tethering factor. The transbilayer movements of PS at the ER are poorly understood and there are conflicting data on the distribution of PS between the luminal and cytosolic leaflet of the ER membrane (Fairn et al., 2011; Tsuji et al., 2019). It is unclear whether Ist2p functions as a scramblase, in addition to its membrane tethering function (Lee et al., 2018). Thus, an open and exciting question is whether Ist2p can support Osh6p activity by regulating the cytosolic pool of PS at the ER.

The cytosolic side of the PM is highly negatively-charged compared to that of other organelles, due to the abundance of PS (Yeung et al., 2008; Leventis and Grinstein, 2010; Bigay and Antonny, 2012). Considering the notion that LTPs must transiently bind to organelle membranes to be efficient (Lev, 2010; Dittman and Menon, 2017; Wong et al., 2017; Wong et al., 2018), we explored how Osh6p and Osh7p can interact with the PM and then easily escape from its electrostatic field to return to the ER, whose surface is less anionic, within one exchange cycle (Zinser et al., 1991; Leventis and Grinstein, 2010). We found that the avidity of Osh6p for anionic membranes is strongly reduced, once it extracts PS or PI(4)P, due to the closing of its

N-terminal lid (Lipp et al., 2019). *In silico* analyses suggest that the lid, when it closes, modifies the membrane-binding features of Osh6p, as it contains an anionic D/E-rich motif and covers a basic area at the entrance of lipid-binding pocket. This electrostatic switch helps Osh6p to self-limit its residency time on anionic membranes and thereby, to efficiently transfer lipids between them. This mechanism, combined with the association to Ist2p, might allow Osh6p/7p to rapidly exchange lipids at ER-PM junctions (Figure 6D).

It has been recently shown that PS transfer by Osh6p/Osh7p, along with the activities of other Osh proteins (likely Osh4p), creates a unique environment in the PM, with unsaturated PS and sterols, that helps Mss4p to make PI(4,5)P₂ from PI(4)P (Nishimura et al., 2019). This helps to envision new links between sterol, PS and PI(4)P fluxes, PIP metabolism and possibly the activity of Ist2p and other PI(4,5)P₂-dependent tethering factors (Manford et al., 2012; Collado et al., 2019; Hoffmann et al., 2019).

Functional Role and Structural Features of ORPs

Subfamily I: OSBP and ORP4

ORP4 has the highest similarity with OSBP and logically shares some features, *i.e.* a capacity to harbour sterols or PI(4)P *via* its ORD, to interact with VAP, and to target PI(4)P *via* its PH domain (Wyles et al., 2007; Goto et al., 2012; Charman et al., 2014). However, ORP4 has distinct cellular functions, and unlike other ORPs, is critical for the survival and proliferation of immortalized and transformed cells (Charman et al., 2014; Zhong et al., 2016a, 2016b). Another peculiarity is that the ORP4 gene encodes a full-length transcript (ORP4L) but also spliced variants without a functional PH domain (ORP4M) (Charman et al., 2014), or lacking the FFAT motif and a leucine repeat that mediates ORP4-OSBP heterodimerization (ORP4S) (Wyles et al., 2007) (Figure 2). ORP4S tightly interacts with vimentin intermediate filaments, and extensively remodels the vimentin network near the nucleus (Wang et al., 2002). This relies on the ORD^{ORP4} (Wyles et al., 2007), *via* a serine-rich loop that can be phosphorylated, (S₇₆₂SPSSPSS₇₆₉), and which is absent from ORD^{OSBP} (Pietrangolo and Ridgway, 2019). In contrast, the full-length ORP4L weakly colocalizes with vimentin, presumably as a part of an ORP4L pool localized at ER-Golgi contact sites, *via* its PH domain that targets the Golgi PI(4)P pool, and through heterodimerization with OSBP (Pietrangolo and Ridgway, 2018) to regulate Golgi PI(4)P homeostasis (Pietrangolo and Ridgway, 2018).

ORP4L is also partially localized at the PM where it serves as scaffolding protein for G-protein coupled

receptors and phospholipase C β 3 (PLC- β 3). This elicits the production of inositol 1,4,5-trisphosphate (IP3) and release of Ca²⁺ from ER stores, a signalling cascade that ensures the proliferation of macrophages and transformed T-cells (Zhong et al., 2016a, 2016b). ORP4L would promote PLC- β 3 translocation from the nucleus to the PM (Pan et al., 2018), and extract PI(4,5)P₂ from the PM to present this lipid to PLC- β 3 and boost its hydrolysis activity (Zhong et al., 2019). ORP4L binds vimentin and VAP simultaneously, yet it is unclear whether ORP4L populates ER-PM contacts in a VAP-dependent manner (Weber-Boyyat et al., 2015b; Pietrangelo and Ridgway, 2018). This would possibly depend on whether the ORD is loaded or empty (Pietrangelo and Ridgway, 2018). Interestingly, 25-HC triggers the translocation of ORP4L to the juxtannuclear region, at ER-Golgi contacts, *via* the enrichment of OSBP and PI(4)P in those areas (Weber-Boyyat et al., 2015b; Pietrangelo and Ridgway, 2018). However, it remains unclear why ORP4L has a different pattern of localization, how its ORD handles sterol *vs* PI(4)P or PI(4,5)P₂, and the role of its interaction with vimentin.

Subfamily II: ORP1 and ORP2

ORP1 exists in a long version, (ORP1L), resembling Osh1p and Osh2p, with an ARD, a PH domain, an FFAT motif and an ORD (Lehto et al., 2001; Loewen et al., 2003) and in a short, ORD-only variant (ORP1S, Figure 2). Concerning ORP1L, it is unclear whether it is a sterol sensor, a sterol transporter, or both. Its PH domain binds PIPs with low specificity and affinity (Johansson et al., 2005), and its ORD can trap either cholesterol or PI(4)P (Vihervaara et al., 2011; Zhao and Ridgway, 2017; Dong et al., 2019) and displays some affinity for oxysterols (Suchanek et al., 2007; Vihervaara et al., 2011). The sterol molecule is in a head-down orientation and in a configuration that is similar to that described with Osh1p (Dong et al., 2019) (Figure 4). What makes ORP1L unique is that it attaches onto late endosomes (LE) and lysosomes (Johansson et al., 2003), by interacting, *via* its ARD, with Rab7, a small G-protein that specifically decorates these membrane-compartments (Johansson et al., 2005) (Figure 6G). The PH domain alone does not target LE/lysosome surfaces, but helps ORP1L to do so (Johansson et al., 2003). Rab7 recruits, once activated in a GTP-bound state, the Rab7-interacting lysosomal protein (RILP). This latter protein recruits p150^{Glued}, a component of dynactin/dyneine motor complex. Altogether, this set of proteins allows LEs/lysosomes to move along microtubules. It is noteworthy that the structural bases of the ARD^{ORP1L}-Rab7-RILP complex have been described (Ma et al., 2018; Tong et al., 2019) (see also Figure 3D).

A central model states that when cholesterol is abundant in the limiting membrane of LEs/lysosomes, ORP1L encapsulates sterol and adopts a conformation that prevents the FFAT motif from interacting with VAP (Rocha et al., 2009; Vihervaara et al., 2011). Consequently, LEs/lysosomes are clustered in a perinuclear area due to their shift towards the minus-end of the microtubules, near the microtubule-organizing centre. In contrast, under low-cholesterol cellular conditions, ORP1L undergoes a conformational change allowing it to interact with VAP and the release of the dynein/dynactin complex. LEs/lysosomes are scattered at the cell periphery where they make contacts with the ER (Rocha et al., 2009; Vihervaara et al., 2011). Besides regulating LEs/lysosomes position, ORP1L might act as an LTP but following distinct modalities that remain enigmatic and hard to dissect due to the presence of other sterol transporters at the endosomal/lysosomal level (OSBP (Dong et al., 2016; Lim et al., 2019), STARD3 (Wilhelm et al., 2017), GramD1 (Hoglinger et al., 2019)). ORP1L would transfer cholesterol from the ER to the limiting membrane of LEs (Eden et al., 2016), where sterol is scarce, possibly by using a local endosomal PI(4)P pool (Hammond et al., 2014) as OSBP does (Dong et al., 2016; Lim et al., 2019). Sterol supply participates in endosome maturation by promoting the formation of intraluminal vesicles into which proteins are trapped for degradation. Contrasting studies suggest that ORP1L conveys LDL-derived cholesterol, which is expelled from the LEs/lysosomes by NPC1, to the ER (Zhao and Ridgway, 2017). ORP1L would transport sterol along its concentration gradient instead of operating a sterol/PI(4)P exchange but, curiously, in a PI(4)P dependent-manner. Furthermore, it has been suggested that ORP1L does not transport PI(4)P and that its sterol transport activity is enhanced by a LE/lysosome PI(3,4)P₂ pool (Dong et al., 2019).

Puzzlingly, these diverse transfer modes, which supposedly occur at ER-LE/lysosome contacts, take place in cellular contexts where such contacts should be rare. Indeed, cholesterol continues to be synthesized (Eden et al., 2016) or imported (Zhao and Ridgway, 2017), and if ORP1L picks up sterol for transfer, it should not associate with VAP (Figure 6F). It is likely that ORP1L proteins do not work in a synchronized manner, meaning that some of them are empty and tether membranes while others transfer sterol. This would ensure that ER-LE/lysosome contacts are quite transient to couple endosome maturation and sterol exchange in a dynamic way.

ORP1S, the shorter version, has a cytoplasmic/nuclear distribution. It moves cholesterol from the PM to the ER and LDs (Jansen et al., 2011), but also from LEs/lysosomes to the PM, counteracting ORP1L action (Zhao et al., 2020). It is unclear how the capacity of

ORP1S to shuttle sterol relates to its ability to capture PI(4)P (Zhao et al., 2020). Intriguingly, the release of sterol to the PM seems to be PI(4)P-independent.

ORP2 is unique amongst ORPs as it only exists as a short variant (Lehto et al., 2001), consisting of an ORD preceded by an FFAT motif (Loewen et al., 2003) (Figure 2). ORP2 holds cholesterol, oxysterols (with a noticeable affinity for 22-HC ($K_D \sim 10^{-8}$ M) or PIPs, including PI(4)P and PI(4,5)P₂ (Wang et al., 2019). ORP2 is ubiquitous in mammalian tissues and is thought to have diverse functions. Early studies showed that overexpressing ORP2 reduces sterol esterification at the ER and increases sterol efflux out the cell, suggesting that it mediates ER-to-PM sterol transfer (Laitinen et al., 2002; Hynynen et al., 2005). Accordingly, ORP2 is able to supply the PM with sterol more efficiently than ORP1S and, intriguingly, by exchanging for PI(4,5)P₂ rather than PI(4)P (Wang et al., 2019). The structure of a 1:1 ORP2-PI(4,5)P₂ complex was solved (Figure 5), revealing how PI(4,5)P₂ is accommodated and how the lid, which is partially closed, mediates the tetramerization of ORP2-PI(4,5)P₂ complex observed in solution (Wang et al., 2019). A current model indicates that ORP2 picks up sterol from endosomes, not the ER, and exploits a PI(4,5)P₂ gradient at the endosome/PM interface to supply the PM with sterol.

In contrast, other studies have suggested that ORP2 localizes near lipid droplets (LD) (Hynynen et al., 2009), *i.e.* storage structures full of triacylglycerol (TAG) and cholesteryl-ester, originating from the ER. It is likely that ORP2 plays a role in TAG metabolism at the ER-LD contact sites (Weber-Boyyvat et al., 2015b). This localization depends on the interaction of ORP2 with VAP *via* its FFAT motif, but it is unclear how ORP2 targets the LD surface. In the presence of 22-HC, ORP2 dissociates from VAP and ER-LD contacts to diffuse in the cytosol and relocate to the PM (Kentala et al., 2015; Weber-Boyyvat et al., 2015b; Wang et al., 2019). Sterol release in the PM is stopped (Wang et al., 2019), suggesting that ORP2 is blocked in a sterol-bound closed state, interacts less with VAP and docks more with the negatively-charged surface of the PM *via* basic patches at the protein surface.

Subfamily III: ORP3, ORP6 and ORP7

Members of subfamily III have different tissue distributions: ORP3 mRNA is abundant in kidney, lymph node, and thymus, ORP6 mRNA is found at high levels in brain and muscle whereas ORP7 mRNA is mostly expressed in the gastrointestinal tract (Lehto et al., 2004). All these proteins contain a FFAT motif (Loewen et al., 2003) and a PH domain with a marked PM specificity (Lehto et al., 2004) (Figure 2). ORP3 and ORP6 contain a secondary, non-canonical FFAT motif

(Weber-Boyyvat et al., 2015a). *In vitro* assays suggest that PH^{ORP3} detects PI(3,4)P₂ and PI(3,4,5)P₃ (Weber-Boyyvat et al., 2015a), whereas PH^{ORP6} detects PI(4)P, PI(4,5)P₂, PI(3,4,5)P₃ and phosphatidic acid (PA) (Mochizuki et al., 2018). Nevertheless, a recent report showed that PH^{ORP3} recognizes PI(4)P and PI(4,5)P₂ (Gulyas et al., 2020) to associate with the PM.

ORP3 is the best characterized member of subfamily III. It localizes to the ER-PM contacts once it is phosphorylated by the protein kinase C upon PMA treatment or agonist stimulation (Lehto et al., 2008; Weber-Boyyvat et al., 2015a; Gulyas et al., 2020), a process that is reinforced by an elevation of intracellular Ca²⁺, mostly via the store-operated Ca²⁺ entry pathway. This phosphorylation step might elicit a conformational change that exposes the PH domain and FFAT motif. ORD^{ORP3} was found to recognize sterol (Suchanek et al., 2007) but sequence analyses suggest it is akin to ORD^{Osh3p} (Zhou et al., 2014), *i.e.*, able to selectively trap PI(4)P but not sterol. This is verified by cellular observations: ORP3 lowers PI(4)P but not PI(4,5)P₂ or PI(3,4,5)P₃ levels, and does not impact pools of cholesterol or PS (Gulyas et al., 2020). ORP3 recruits R-Ras, a small GTPase that controls cell adhesion and migration, thereby contributing to these processes (Lehto et al., 2008; Weber-Boyyvat et al., 2015a). Complementing this work, a recent study links ORP3 activation by PKC and Ca²⁺ entry with a mechanism implicated in focal adhesions dynamics. Once localized at ER-PM contact sites, ORP3 interacts with IQSec1, a guanine nucleotide exchange factors of Arf5 to trigger focal adhesion disassembly (D'Souza et al., 2020). How this relates to the ability of ORP3 to downregulate PI(4)P or possibly to exchange PI(4)P with PC (D'Souza et al., 2020), remains unclear. Of note, there are short ORP3 isoforms in which the ORD is truncated, upstream of the EQVSHHPP motif, and followed by a short sequence of ~60 residues (Collier et al., 2003). The function of these isoforms is unknown.

ORP6 shows functional similarities with ORP3. In HeLa cells, ORP6 localizes to the ER and ER-PM contacts and in primary cerebellar granular neurons it coexists in ER-PM contacts with ORP3. ORP6 can associate with ORP3 or itself *via* a middle segment downstream of the PH domain, and this association guides its presence at junctions. ORP6 downregulates PM PI(4)P levels like ORP3, and seems to be involved in PI(4)P turnover in neurons (Mochizuki et al., 2018).

Subfamily IV: ORP5 and ORP8

ORP5 and ORP8 contain a PH domain as well as an ORD, and have the unique feature to be constitutively anchored to the ER by a C-terminal transmembrane segment, instead of interacting with VAP *via* an FFAT

motif (Yan et al., 2008; Du et al., 2011) (Figure 2). Their ORDs most closely resemble the ORDs^{Osh6p/7p}, and ORD^{ORP5} was found to harbour PS but not sterol (Maeda et al., 2013). Subsequently, De Camilli's group established that ORP5 and ORP8 mediate PS/PI(4)P countertransport at ER-PM contact sites, delivering PS to the PM *via* PI(4)P consumption (Chung et al., 2015) (Figure 6G). The PH domain of ORP5/8 was proposed to selectively target PI(4)P at the PM but other reports suggested that it recognizes PI(4,5)P₂ (Ghai et al., 2017; Lee and Fairn, 2018); both PIPs are most likely recognized (Sohn et al., 2018). A short basic region preceding each PH domain greatly helps ORP5 and ORP8 to associate with the PM (Lee and Fairn, 2018; Sohn et al., 2018). However, the recruitment of ORP8 to the PM is weak, in comparison to ORP5, and depends more on PI(4,5)P₂ due to the intrinsic features of its PH domain and N-terminal end (1-42 segment), which is anionic and restrains its avidity for the PM surface. A natural variant lacking this region, ORP8S, behaves more like ORP5 (Chung et al., 2015).

Interestingly, the ORD^{ORP5/8} might use PI(4,5)P₂ as a counterligand, instead of PI(4)P, for supplying the PM with PS (Ghai et al., 2017). This would explain why ORP5/8 downregulate PI(4,5)P₂ levels at the PM. Yet, this conclusion is disputed (Sohn et al., 2018): PI(4)P would be the true counterligand, and PI(4,5)P₂ levels decrease because PI(4)P, which is consumed in the exchange cycles, is its precursor. Presumably, a rheostat mechanism based on the sensory aptitude of the PH domain of ORP5 and ORP8 regulates PI(4)P, PI(4,5)P₂ and PS levels at the PM. A decrease in the PI(4)P and PI(4,5)P₂ levels would predominantly reduce ORP5 activity, and thus restore proper PIP levels. In contrast, an elevation in PI(4,5)P₂ levels would engage ORP8 at the PM to transfer more PI(4)P to the ER, thereby limiting extra PI(4,5)P₂ production. This mechanism constitutes a variation of the PI(4)P-dependent negative feedback loop that controls the presence of OSBP at ER-Golgi contact sites (Mesmin et al., 2013). Alterations in PS synthesis lead to the Lenz-Majewski syndrome, and impact PI(4)P metabolism *via* ORP5/8 activity (Sohn et al., 2016). Blocking of ORP5/8 activity can lower PS abundance at the PM, reducing oncogenicity of signalling proteins (Kattan et al., 2019). In contrast, overexpression of ORP5 seems linked to increased cancer cell invasion and metastasis (Du et al., 2018). ORP5 positively regulates the mTORC1 complex that plays a key role in activating cell proliferation and survival. A possible reason is that the activity of Akt, which is a major upstream effector of mTORC1, depends on its recruitment to the PM by PS (Huang et al., 2011). Thus, altering ORP5/8 function can profoundly affect the lipid homeostasis of the PM and cellular behaviour.

Additionally, ORP5 and ORP8 occupy ER-mitochondria contact sites and seem critical to preserve the morphology and respiratory function of mitochondria, likely by releasing PS in this organelle (Galmes et al., 2016). ORP5 interacts *via* its ORD with PTPIP51 (protein tyrosine phosphatase interacting protein-51). This outer mitochondrial membrane protein associates with VAP-B and contributes to anchoring mitochondria to the ER for facilitating IP3 receptor-mediated delivery of Ca²⁺ from ER stores to mitochondria and its metabolism (Stoica et al., 2014; Gomez-Suaga et al., 2019). Finally, a recent study suggests that ORP5 localizes to ER-LD contacts and might use PI(4)P on LDs to deliver PS (Du et al., 2020).

Subfamily V: ORP9

ORP9 exists in a long form, ORP9L, with a domain organization and subcellular localization that resemble those of OSBP (Figure 2). It binds to VAP proteins and associates with the *trans*-Golgi/TGN *via* a PH domain that is likely to be quite selective for PI(4)P (Wyles and Ridgway, 2004; Ngo and Ridgway, 2009); however, extra molecular elements seem necessary to reinforce Golgi targeting (Wyles and Ridgway, 2004). *In vitro*, ORP9L can sequester sterol or PI(4)P (Liu and Ridgway, 2014), and transfer sterol between synthetic membranes (Ngo and Ridgway, 2009; Liu and Ridgway, 2014). Despite similarity with Osh6p/7p (Maeda et al., 2013), the ORD^{ORP9} is unable to capture PS (Liu and Ridgway, 2014). ORP9L appears to be important for maintaining ER-to-Golgi vesicular transport and Golgi organization, as well as sterol levels in the post-Golgi and endosomal compartment (Ngo and Ridgway, 2009). ORP9L impacts Golgi PI(4)P levels and cooperates with OSBP, besides ORP10, for building ER-Golgi contacts (Venditti et al., 2019). However, ORP9L seems functionally quite different from OSBP. For reasons yet to be defined, ORP9L activity is insensitive to 25-HC and decoupled from CERT activity (Ngo and Ridgway, 2009). In fact, it is quite probable that, unlike OSBP, ORP9L does not convey sterol to the Golgi by sterol/PI(4)P exchange (Ngo and Ridgway, 2009).

ORP9S, a shorter ORP9 variant, lacks the N-terminal PH domain and is absent from the Golgi surface. Despite this difference, ORP9S can downregulate Golgi PI(4)P levels and, surprisingly, this occurs in a VAP-dependant manner (Liu and Ridgway, 2014), possibly by occupying preformed ER-Golgi contacts. ORP9S can strongly disorganize Golgi structure and ER-to-Golgi trafficking (Ngo and Ridgway, 2009). These observations might explain why ORP9S profoundly inhibits cell growth (Ngo and Ridgway, 2009),

but overall, the precise cellular roles of the two ORP9 variants remain enigmatic.

Subfamily VI: ORP10 and ORP11

ORP10 has a PI(4)P-selective PH domain (Nissila et al., 2012) and an ORD that shares similarity with ORD^{Osh6p} and traps PS (Maeda et al., 2013) (Figure 2). ORP10 associates with the Golgi complex *via* its PH domain and likely further determinants within its N-terminal half (Nissila et al., 2012). Intriguingly, the C-terminal end of its ORD mediates the localization of ORP10 on microtubules. Early studies suggested that ORP10 regulates, by controlling ER-Golgi trafficking, the secretion of apolipoprotein B-100 from hepatocytes (Nissila et al., 2012). Recent data indicated that ORP10 occupies ER-Golgi contacts to deliver PS in the Golgi membrane, presumably by PS/PI(4)P exchange, and stabilizes these contacts (Venditti et al., 2019). Thus, ORP10, possibly by heterodimerizing with ORP9 to supersede its inability to interact with VAP, would colocalize with ORP9 and OSBP in these regions (Nissila et al., 2012) to coordinate PS transfer with ER-to-Golgi sterol flux.

ORP11 is expressed in ovary, testis, kidney, liver, stomach, brain, and adipose tissue. In cells, ORP11 resides on the surface of the Golgi and on Rab7- and Rab9-positive LEs *via* its N-terminal region encompassing the PH domain (1-292 region). A middle region of ORP11 interacts with a similar region of ORP9L whose intracellular levels dictate the recruitment of ORP11 at the ER-Golgi interface. Thus, like ORP10, ORP11 positioning seems to be controlled by the interaction with ORP9 and not VAP. The specificity of ORD^{ORP11} remains undefined: it recognizes sterol (Suchanek et al., 2007), but might also host PS (Maeda et al., 2013) and certainly PI(4)P. In macrophages, ORP11 mediates the protective effect of bis(monoacylglycero)phosphate (BMP), a lipid specific to the LEs, against the proapoptotic effect of 7-HC excess following the uptake of oxidized LDL. It is possible that ORP11 favours the egress of sterol out of the cell (Arnal-Levron et al., 2019). It is not known whether BMP is a ligand of ORP11.

Discussion and Perspectives

Today, the ORD structure of one representative member of each Osh subfamily is known: Osh1p, Osh3p, Osh4p and Osh6p. Similarly, the ORD structural determination for the ORPs has been initiated. One can now see how the specific conservations and variations in the ORD sequences, revealed in late '90s, translate, respectively into a shared aptitude to recognize PI(4)P and different specificities for a second lipid, either sterol or PS (Figures 4 and 5). These outcomes provided evidence that ORP/Osh proteins exploit PI(4)P or possibly other

PIP gradients to transfer sterol or PS between organelles. Evidence suggests that some ORP/Osh proteins use their ability to transfer lipids to accomplish distinct cellular functions. In yeast, Osh1p likely controls both vesicular trafficking at the TGN and vacuole fusion (Kvam and Goldfarb, 2006; Manik et al., 2017; Shin et al., 2020), Osh2p assists endocytosis at the PM (Encinar Del Dedo et al., 2017), and Osh4p regulates polarized exocytosis at the *trans*/post-Golgi level (Ling et al., 2014; Smindak et al., 2017). In human cells, OSBP regulates vesicular trafficking at the *trans*-Golgi (Hussain et al., 2018; Peresse et al., 2020), whereas ORP1L facilitates cholesterol transfer from the ER to LEs to support intraluminal vesicle formation (Eden et al., 2016). Collectively, these data suggest that the shared cellular function of many ORP/Osh proteins is to regulate diverse membrane remodelling events, by handling the same lipid, *i.e.* sterol. The physico-chemical and mechanical properties of membranes where sterol is delivered are locally modified due to the singular features of this lipid. The specific configuration and structural features of each ORP/Osh protein allows them to accurately target unique subcellular regions to transfer lipids, as shown by studies of the PH domain, the FFAT-VAP interaction and ARD/protein interactions. Because sterol is exchanged for PI(4)P, the change in the membrane properties might be synchronized with the recruitment of effectors involved in vesicular trafficking by PI(4)P at the Golgi/endosomal levels, or its derivative, PI(4,5)P₂, at the PM. However, this general assumption needs to be substantiated. So far, the sterol/PI(4)P exchange activity of most of these proteins, including Osh4p, has not been quantified in the cell. Conversely, while there is, for instance, convincing evidence that ORP2 transfers lipids at the endosome/PM interface (Wang et al., 2019), there are no data showing a direct link between this activity and upstream functions at the PM. Many mysteries remain regarding the cellular role(s) of ORP1L, which regulates the positioning of LEs but also exports sterol out of the endosomal compartment following sterol uptake, or imports sterol to support protein degradation. It must be clarified whether ORP1 is a sterol transporter, a lipid exchanger or a lipid sensor, or if it exerts each of these functions in response to distinct cellular contexts. Interestingly, OSBP supports the lysosomal activation of mTORC1, *i.e.* cell growth signalling (Lim et al., 2019), meaning that the delivery of sterol might serve functions that differ from remodelling events.

It has been relatively easy to establish that Osh6/7p and ORP5/8 act as PS/PI(4)P exchangers in cells (Maeda et al., 2013; Chung et al., 2015; Moser von Filseck et al., 2015a; Sohn et al., 2018), probably because these proteins are at the ER/PM interface and as endogenous PIPs or PS levels can be measured in real time in

membranes by fluorescent probes. Our current vision is that these proteins enrich the PM with PS while tightly controlling PI(4)P/PI(4,5)P₂ levels in that membrane. However, it is unknown whether these proteins, notably ORP5/8, influence precise PIPs-dependent signalling cascades at the PM in response to external signals in a physiological context. Nevertheless, these proteins seem able to influence the function of signalling proteins by adjusting the electrostatic properties of membranes through PS delivery (Du et al., 2018; Kattan et al., 2019). ORP5/8 also assist mitochondria and LDs function but how this relates to their ability to capture PS or PI(4)P remains to be elucidated. ORP10 delivers PS in the TGN, and this partially answers the question of how PS accumulates in this compartment (Leventis and Grinstein, 2010). It is unknown whether ORP10 consumes PI(4)P to transfer PS and whether the delivery of PS at the TGN fulfils other functions in addition to stabilizing ER-Golgi contacts (Venditti et al., 2019).

Thus, the main role for ORP/Osh proteins is likely to adjust the abundance of major lipids, sterols or PS, and therefore bulk membrane features (stiffness and electrostatic properties), alongside PIPs, in many subregions of eukaryotic cells to control membrane remodelling and signalling events. Some ORP/Osh proteins might exert a similar role, but not necessarily *via* lipid exchange. ORP4L controls the signalling pathway by directly presenting PI(4,5)P₂ to PLC-β3 at the PM (Zhong et al., 2019). Osh3p, ORP3 and ORP6 regulate the PI(4)P level at the PM but it is unclear whether this implies the exchange of PI(4)P for a second ligand. The precise cellular roles and modes of action of ORP7, ORP9L and ORP11, and splice variants of certain ORPs are yet to be better defined.

From a structural point of view, it is surprising that ORPs can use PIPs other than PI(4)P as ligand. Indeed, we initially reported that Osh4p does not extract PI(3)P, PI(5)P or PI(4,5)P₂ as the phosphate group at the positions 3 or 5 on the inositol ring should sterically clash with the protein (de Saint-Jean et al., 2011). Im and co-workers came to the same conclusion when analysing the ORD^{Osh1p} structure. In ORD^{ORP1}-PI(4,5)P₂ and ORD^{ORP2}-PI(4,5)P₂ complexes, the inositol ring of PI(4,5)P₂ is accommodated with a ~180° rotation compared with that of the PI(4)P headgroup in Osh proteins to avoid this clash (Figure 5A and B). The lid is partially open and mediates protein oligomerization. In the ORP2 tetrameric structure, one of the two acyl chains of PI(4,5)P₂ is squeezed toward its lid or stretched out along a surface hydrophobic groove (Figure 5B and not shown). These structures contrast strongly with Osh structures solved with PI(4)P, sterol or PS. One could wonder whether these oligomeric complexes are predominant in cells, and indeed, they have been obtained *in vitro* by mixing ORD with PI(4,5)P₂, which

is not systematically included in a membrane (Dong et al., 2019; Wang et al., 2019), *i.e.*, under conditions that might force its capture by the hydrophobic cavity of ORDs. Also, while there is clear evidence that ORP1 and ORP2 proteins extract PI(4)P (Zhao and Ridgway, 2017; Dong et al., 2019; Wang et al., 2019), this has not been fully explored from a structural and functional standpoint. *In vitro* assays suggested that ORP1 and ORP2 either weakly or not at all transfer PI(4)P between membranes (Dong et al., 2019; Wang et al., 2019), but these results must be interpreted with caution. The precise nature of sterol (Liu and Ridgway, 2014) or the acyl chains of PI(4)P or PS strongly influence the activity of ORP/Osh proteins ((Moser von Filseck et al., 2015a) and unpublished data). Note also that OSBP poorly exchanges sterol and PI(4)P *in vitro* except in a more sophisticated assay where PI(4)P is hydrolysed by Sac1 (Mesmin et al., 2013). The ORP/Osh protein story is littered with ligand identifications that were eventually found to be false positive (*e.g.*, sterol for ORP5, ORP8 and ORP10 (Suchanek et al., 2007; Du et al., 2011), or PS for Osh4p (Raychaudhuri et al., 2006)). Our opinion is that LTPs can transfer, in a fortuitous manner, lipids between synthetic membranes of low complexity and devoid of the correct lipid ligands, and/or that some auxiliary metabolic processes must be reconstituted to properly measure the activity of these LTPs.

There are additional questions related to ORDs. For instance, why do two distinct clades of Osh proteins use a specific ORD but distinct modalities to recognize the same second lipid, *i.e.*, sterol (Figure 4, Osh1p and Osh4p)? We also note that, intriguingly, an ORD is quite big (>400 aa) relative to other intracellular lipid transfer modules (<350 aa, for a review see (Chiapparino et al., 2016)). Figure 7 shows that the ORD core, *i.e.* the β-barrel, is highly conserved but decorated by external elements that are poorly conserved and mainly correspond to insertions that are specific to each ORP/Osh subfamily. Furthermore, functional data suggest that the ORD of some ORP/Osh proteins interact with partners (Nissila et al., 2012; Pietrangelo and Ridgway, 2019; D'Ambrosio et al., 2020). Thus, variation in the ORD, in addition to conferring distinct ligand specificity, might offer specialized binding zones for specific partners but also enable the protein to associate with organelles in different ways.

In that respect, it is unknown how ORD associates with the lipid bilayer and how its ligands move in and out the binding pocket. Molecular dynamics simulations provided ideas on how Osh4p or Osh6p dock onto membrane (Rogaski and Klauda, 2012; Lipp et al., 2019) and deliver lipids (Canagarajah et al., 2008; Singh et al., 2009), but no experimental answers exist for such questions. That said, it is likely that the lid, as foreseen by Im and co-workers (Im et al., 2005), regulates the

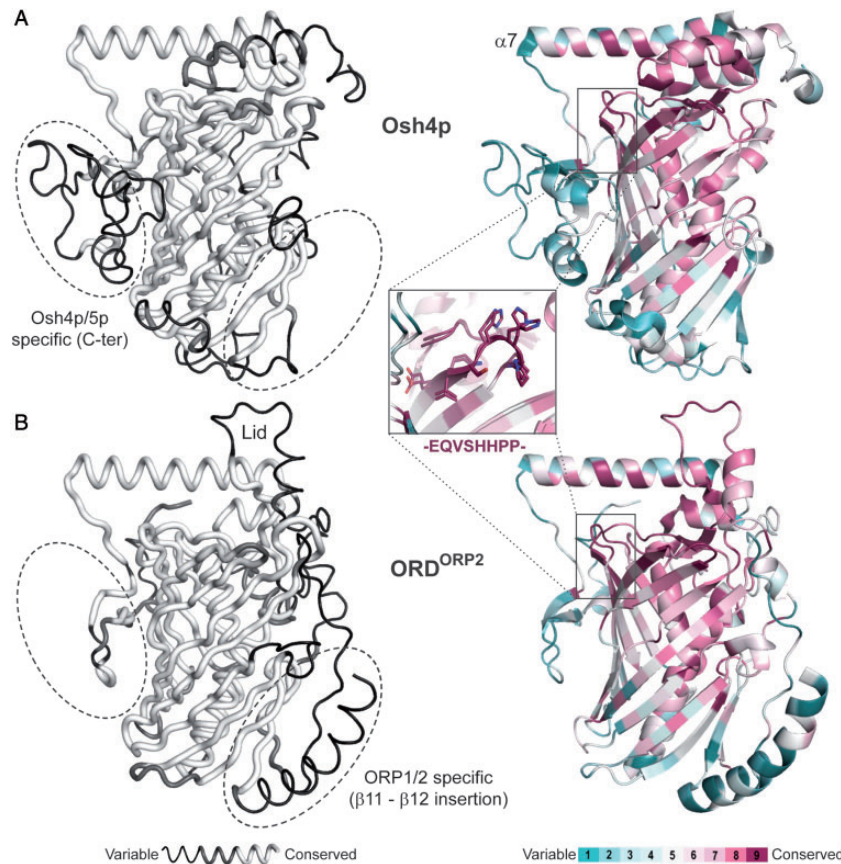


Figure 7. Sequence and Structure Similarity Profiles of Osh/ORP Proteins. Each profile is mapped on the structures of Osh4p (A) and ORP2 (B). The structural profile (left) is based on a multiple structure alignment computed with mTM-align (Dong et al., 2018) using the following structures: ORD^{ORP1}-CLR, ORD^{ORP2}-PI(4,5)P₂, ORD^{Osh1p}-ERG, ORD^{Osh3p}, ORD^{Osh3p}-PI(4)P, Osh4p-ERG, Osh4p-PI(4)P and Osh6p-PI(4)P. The size and colour of the backbone indicate structural similarity in crystals, and were manually assigned for each residue. This profile shows the highly conserved core of ORDs that is mainly composed of a central, near-complete β -barrel (in white). Some elements, i.e. loops that connect β -sheets, are dynamic by nature and are moderately conserved. The less conserved parts (in black) are elements which mainly correspond to insertions that are specific to each sub-family (surrounded by dashed lines), outside the core architecture. Of note, the profile of the N-terminal region more closely reflects the conformational flexibility of the lid in the crystals, linked to the nature of the bound ligands, than variabilities in the secondary structure. Nevertheless, the lid is composed of a conserved helix, which can fold down on the top of the barrel, whereas the N-terminus is quite variable both in length and structure. This profile is based on a few non-redundant available structures, and will be more weighted in future once new structures will be determined. The sequence profile (right) was calculated with the ConSurf Server (Ashkenazy et al., 2016) from a non-redundant multiple alignment of 255 sequence representatives of the Eukaryota kingdom, collected from the OrthoInspector database (Nevers et al., 2019), and aligned with PipeAlign2 (Plewniak et al., 2003). The relative conservation grades (1-9) of each residue, mapped on the structure, are assigned based on the evolutionary rates. This profile does not completely match the structural one. While the least conserved sequence elements are associated with low secondary structure similarity, the sequence of the β -barrel core enclosing the ligand binding pocket is moderately conserved, with just a few extremely well-conserved residues involved both in the interaction with ligands, or in maintaining the architecture. The EQVSHHPP signature is located in a structurally conserved loop at the top of the barrel, and belongs to the set of conserved interacting residues. Furthermore, one side of the top $\alpha 7$ helix (Osh4p numbering), which features key residues for interactions with PIP ligands, also displays a high degree of similarity. The lid includes some conserved residues that interact with ligands or the $\alpha 7$ helix as well as the pocket entrance.

association of the ORD with membrane. In an open state, the lid of Osh4p can fold into an amphipathic helix that detects lipid packing defects in membranes (Drin et al., 2007) and might help Osh4p to target specific organelles like the ER, displaying a high curvature and unsaturated lipids or alternatively the curved surface of secretory vesicles (Ling et al., 2014). Due to its anionic

nature, the lid of Osh6p, once closed, limits the association of the protein to an anionic membrane, and thereby maintains its transfer efficiency (Lipp et al., 2019). Part of this switch mechanism implies the occlusion by the lid of a basic surface well conserved amongst ORDs. Thus, the modification of membrane-binding properties of the ORDs by the opening/closing of the lid is maybe a

general mechanism that helps their transfer activity. For instance, we noticed that ORP1S, which is cytosolic and supposedly associates transiently with cellular membranes (Jansen et al., 2011), disengages from anionic membranes once its lipid ligands are present (Dong et al., 2019), as observed for Osh6p.

Some odd clues suggest that the conformational status of the ORD modifies the FFAT-VAP interaction and *vice-versa*. A prototypical case is ORP1L whose ability to associate with VAP seems to be abolished once its ORD is sterol-bound (Rocha et al., 2009; Vihervaara et al., 2011). Several ORPs, interact more with VAP when they are deficient in loading ligands (Kentala et al., 2015; Weber-Boyvat et al., 2015b). Conversely, VAP lifts an auto-inhibitory mechanism that prevents OSBP from transferring sterol (Mesmin et al., 2013). The structural bases of such observations are unknown.

PH domains of ORP/Osh proteins detect different PIPs and negatively-charged lipids *in vitro*. However, many observations suggest that they primarily recognize PI(4)P and additionally PI(4,5)P₂ to target the Golgi apparatus, the PM or endosomal compartment. Overall, the avidity of the PH domain for PIPs seems weak and extra determinants appear to be required, like Arf1 in the case of OSBP, to recruit ORP/Osh proteins to the Golgi apparatus or the PM. In ORP5/8, other basic or anionic motifs adjacent to the PH domain modulate its targeting properties (Lee and Fairn, 2018; Sohn et al., 2018). For Osh2p and Osh3p, the interaction with Myo5p (Encinar Del Dedo et al., 2017) likely synergizes with the PH domain. In contrast, the PH domain does not seem to strongly contribute to the association of Osh1p and ORP1 with the vacuolar/endosomal membrane, which seems mostly mediated by the ARD (Johansson et al., 2003; Shin et al., 2020). For many ORPs, the binding ability of the PH domain seems reinforced by adjacent elements whose nature remains to be defined. Of great importance, the mutual ability of the PH domain and ORD to recognize PI(4)P and possibly PI(4,5)P₂ constitute the structural basis of negative feedback loops that control how long OSBP and ORP5/8 stay at contact sites (Mesmin et al., 2013; Sohn et al., 2018). Some evidence suggests that this might be valid for other ORP/Osh proteins (*e.g.*, ORP3 (Gulyas et al., 2020)). In summary, the PH domain appears to be a structural module whose association with PIPs is rather weak and can be easily modulated by external factors (small G proteins and pH (Shin et al., 2020)), or the lipid transfer activity of ORP/Osh proteins.

Finally, we know little about the atomistic details and functional roles of the homo/heterodimerization processes that have been reported for many ORPs. Presumably, they modulate how strongly the ORPs associate with organelles *via* their PH domain and FFAT

motif while also synchronizing lipid flux. The role of disordered low-complexity regions in these proteins appears to be important (Jamecna et al., 2019) but remains poorly documented,

Beyond addressing cellular biology question, structural analyses of ORP/Osh proteins might serve therapeutic purposes. Human viruses like rhinovirus, poliovirus or hepatitis C virus, induce an overproduction of PI(4)P to remodel compartments into replication organelles that host molecular supracomplexes, combining viral and host proteins, which replicate the viral genetic material (Romero-Brey and Bartenschlager, 2014). OSBP was found to be pivotal for viral replication as it contributes to the building of these replication organelles by supplying sterol (Roulin et al., 2014; Wang et al., 2014; Strating et al., 2015). Remarkably, molecules collectively named ORPphilins, exert an antiviral action by blocking the sterol/PI(4)P exchange activity of OSBP (Burgett et al., 2011; Wang et al., 2014; Albulescu et al., 2015; Strating et al., 2015). Solving the structure of ORD^{OSBP} bound to ORPphilins might indicate why such compounds are so selective against this protein (and ORP4), and possibly boost the design of new, potent antiviral compounds.

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