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Review

# Structural insights into nonvesicular lipid transport by the oxysterol binding protein homologue family☆

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ABSTRACT

Sterols such as cholesterol in mammals and ergosterol in fungi are essential membrane components and play a key role in membrane function and in cell signaling. The intracellular distribution and processing of sterols and other phospholipids are in part carried out by oxysterol binding protein-related proteins (ORPs) in eukaryotes. Seven ORPs (Osh1–Osh7 proteins) in yeast have distinct functions in maintaining distribution, metabolism and signaling of intracellular lipids but they share at least one essential function. Significant progress has been made in understanding the ligand specificity and mechanism of non-vesicular lipid transport by ORPs. The unique structural features of Osh proteins explain the diversity and specificity of functions in PI(4)P-coupled lipid transport optimized in membrane contact sites. This review discusses the current advances in structural biology regarding this protein family and its potential functions, introducing them as the key players in the novel pathways of phosphoinositide-coupled directional transport of various lipids. This article is part of a Special Issue entitled: The cellular lipid landscape edited by Tim P. Levine and Anant K. Menon.



## 1. Introduction

Membrane lipids, including cholesterol and phosphoinositides are heterogeneously distributed throughout the cell [1]. The uneven distribution of membrane lipids defines the identities of membrane compartments and maintains proper cell function. For example, cholesterol is synthesized in the endoplasmic reticulum (ER), which is remarkably cholesterol poor with 5% of cellular sterols [2]. By contrast, the plasma membrane (PM) is highly cholesterol-enriched, holding almost two-thirds of cellular cholesterol. Phosphoinositides are phosphorylated forms of phosphatidylinositol (PI) which are enriched in various parts of organellar membranes. Phosphatidylinositol 4-phosphate is the major phosphoinositide on the Golgi, and phosphatidylinositol 4, 5-bisphosphate predominates at the PM. This unique distribution of lipids may serve as a mechanism by which the direction of membrane trafficking, membrane function or protein targeting is controlled [3].

Most membrane lipids are synthesized in the ER and they need to be transported to their final destinations. Given that most cellular lipids are highly hydrophobic, their free diffusion through aqueous cytoplasm is very slow and insufficient to support substantial transport of most lipids [4]. The intracellular lipids are distributed by several different mechanisms. Phospholipids spontaneously flip-flop from one leaflet of a bilayer to the other very slowly or efficiently by the help of flippases [5]. Lipid molecules diffuse along membrane bilayers and between the contacting membrane leaflets [6]. Bulk of lipids is moved between membranes by transport vesicles and tubular carriers, which is common in the secretory and endocytic pathways [7,8]. Lipid monomers are transferred by soluble lipid binding/transfer proteins (LTPs) [6,9]. Vesicular transport requires metabolic energy, intact cytoskeleton, and connection to the vesicular transport machinery [10,11]. Although vesicular lipid transport mediates the bulk transport of many lipids, increasing lines of evidence suggest that nonvesicular lipid transport is the major transport route for certain lipid types including cholesterol and ceramide [12–14]. Non-vesicular lipid transport mediated by soluble carriers plays significant roles in lipid fluxes between organelles that are not connected to membrane trafficking pathways [6,15].

LTPs are intracellular proteins that can carry a lipid molecule in a hydrophobic pocket and transfer it between membranes through an aqueous phase [15]. In general, LTPs have specificity for one or more lipid types, and may contain only a single lipid-transfer domain, or additional domains with various functions. LTPs extract a lipid from a donor membrane. After enclosing the bound lipid in a shielded pocket, LTPs might

*Abbreviations:* CERT, ceramide transfer protein; ER, endoplasmic reticulum; FFAT, two phenylalanines in an acidic tract; LE, late endosome; LTP, lipid transfer protein; MCS, membrane contact site; OSBP, oxysterol-binding protein; ORP, OSBP-related protein; ORD, OSBP-related domain; PC, phosphatidylcholine; PH, pleckstrin homology; PI, phosphatidylinositol; PM, plasma membrane; PI(4)P, phosphatidylinositol 4-phosphate; PS, phosphatidylserine; VAP, vesicle-associated membrane protein (VAMP)-binding protein.

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diffuse through the cytoplasm or transfer the lipids to an acceptor membrane. Membrane contact sites (MCSs) are close appositions of two different membranes typically within the distance of 30 nm. Particularly, ER as a major site of lipid synthesis makes extensive contacts with other organellar membranes such as PM, mitochondria, endosomes, and Golgi [16]. The MCSs facilitate signaling and non-vesicular lipid trafficking by allowing a short distance of transport and close tethering of related proteins. Although nonvesicular lipid transport has been extensively studied for more than four decades, its underlying molecular mechanism and regulation have not been fully explored.

Oxysterol binding protein-related proteins (ORPs), including the yeast proteins encoded by the OSH gene family (*OSH1–OSH7*), were proposed as intracellular LTPs facilitating transport of sterols and other phospholipids between intracellular membranes [17]. Many ORPs accumulate at contact sites between the ER and the PM and promote the exchange of specific lipids, which helps to maintain the distinct membrane identity. In the past few years, researchers have made significant progress toward understanding the routes and modes of lipid transport by ORPs. Biochemical and structural studies using fluorescent lipids and recombinant proteins allowed characterization of molecular mechanism of ORPs in non-vesicular lipid transport. Sterol and other lipid transport by ORPs coupled with phosphatidylinositol 4-phosphate exchange at membrane contact sites has emerged as a new theme of lipid transport against the concentration gradient between organellar membranes [17]. This review will summarize some of the key findings on ORPs and discuss the structural basis for the mechanism of non-vesicular lipid transport.

## 2. Domain architectures of ORPs

The OSBP was initially discovered as a cytosolic receptor for oxysterols during a search for the mechanisms that provide downregulation of sterol biosynthesis [18,19], though OSBP does not appear to be involved in this process [20]. The cloning of OSBP enabled the identification of a large family of OSBP-related proteins (the ORPs) from various eukaryotes. Yeast has seven ORP members and human has 12 ORP genes with four additional protein products by splicing variations [21]. All ORPs contain an OSBP-related domain (ORD) that mediate lipid binding. ORD domain is characterized by the presence of a conserved signature motif EQVSHHPP in the N-terminal region of ORD domain. The majority of ORPs in human and three ORPs in yeast contain other additional domains in the N-terminal region to the ORD domain [22]. The domain organization of human and yeast ORPs is shown in Fig. 1. Many ORPs contain pleckstrin homology (PH) domains and FFAT (two phenylalanines in an acidic tract) motifs in the N-terminal region to the proteins. PH domains localize ORPs by binding to phosphoinosidides in organellar membranes [23]. FFAT motifs present in many cytosolic lipid-binding proteins interact with ER-resident proteins called VAPs (vesicle-associated membrane protein (VAMP)-associated proteins) in humans [24,25]. Some ORPs also contain ankyrin repeats which mediate protein localization by protein–protein interaction [22]. Yeast Osh3 contains GOLD (Golgi dynamics) domain which is predicted to mediate protein–protein interaction. Two of the human ORPs, ORP5 and ORP8 contain single C-terminal transmembrane regions that anchor the proteins to the ER. Yeast Osh proteins are cytosolic proteins that can diffuse through the cytoplasm as most other ORPs except mammalian ORP5 and ORP8, but a variety of N-terminal domains confer membrane targeting to most ORP members. The ORP variants are divided into two groups denoted as long (L) and short (S) forms depending on the presence of an N-terminal PH domain to the conserved ORDs. The human ORPs can be classified into six subfamilies based on gene structure and amino acid homology (Fig. 1) [21]. The ORPs show higher than 70% amino acid identity in the ORD domain within each subfamily while displaying much lower identities between different subfamilies. The expression of some ORP genes is specific to certain tissues by transcriptional regulation [21,26]. The high sequence conservation and accumulating evidences indicate that the structure and the functions within subfamilies are well conserved (Table 1).

## 3. OSBP-related ligand-binding domain (ORD)

The unifying feature of all ORP homologs is a unique C-terminal OSBP-related domain (ORD), which mediates bindings of sterols and phospholipids [27,28]. The first crystal structure of ORD reported was yeast Osh4 (also known as Kes1) [28]. This ORP has 434 amino acids and contains the core ORD domain without the other N-terminal upstream domains such as PH domain and FFAT motif present in many ORPs. The overall structure of Osh4 (Fig. 2) displays an incomplete β barrel containing a central ligand binding tunnel with a flexible lid covering the tunnel entrance. The central β-barrel (17 β-strands) is closed by an N-terminal subdomain (residues 30–117) consisting of a two-stranded β-sheet and a three α-helix bundle that fill the incomplete

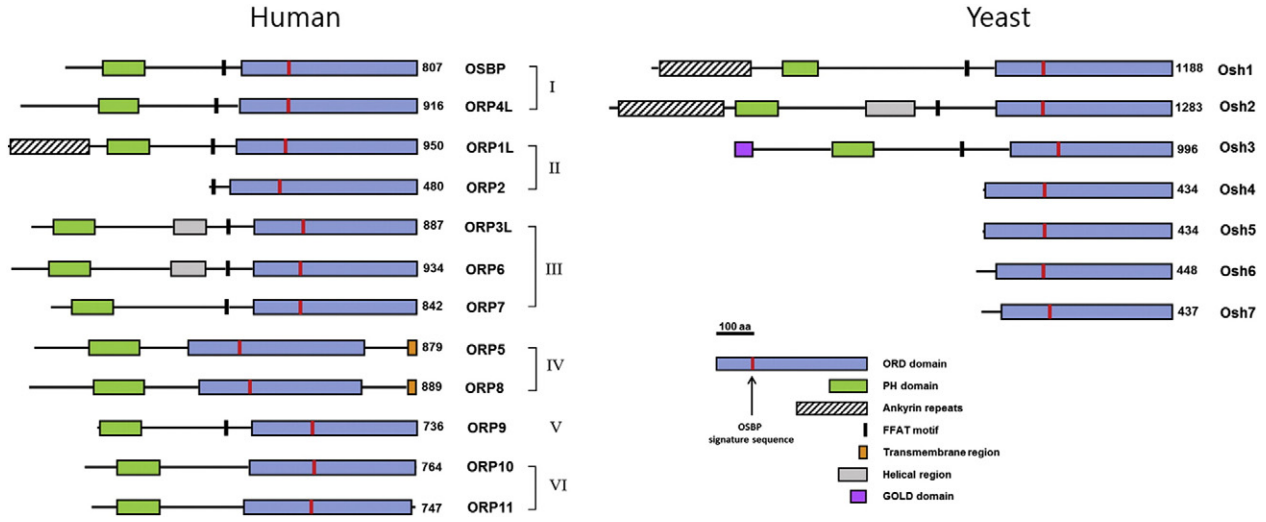


**Fig. 1.** Domain organization of the oxysterol-binding protein (OSBP)-related proteins (ORPs) from human and yeast. Humans have 12 ORP encoding genes which give 16 main ORPs through alternative translation or splicing. Splicing variants of human ORPs are not shown. The human ORPs are divided into six groups (indicated by Roman numerals) based on gene structure and amino acid sequence homology [21]. The *S. cerevisiae* genome encodes seven ORP homologues (Osh1 – Osh7) which are divided into four subgroups (Osh1/Osh2; Osh3; Osh4/Osh5; Osh6/Osh7).

**Table 1**
Structures of ORPs and their domains currently available.

| Protein | Species | Domain | Ligand | PDB entry and Reference |
|---|---|---|---|---|
| Osh3 | *S. cerevisiae* | PH (residue 222–317) | | 4IAP [49] |
| | | ORD (residue 605–996) | Apo form | 4IC4 [49] |
| | | ORD | PI(4)P | 4INQ [49] |
| Osh4/Kes1 | *S. cerevisiae* | ORD without lid (residue 30–434) | Apo form | 1ZI7 [28] |
| | | Full length (ORD) | Ergosterol | 1ZHZ |
| | | | Cholesterol | 1ZHY |
| | | | 25-Hydroxycholesterol | 1ZHX [28] |
| | | | 20-Hydroxycholesterol | 1ZHW |
| | | | 7-Hydroxycholesterol | 1ZHT |
| | | Full length (ORD) | PI(4)P | 3SPW [55] |
| Osh6 | *S. cerevisiae* | Full length (ORD) | PI(4)P | 4PH7 [50] |
| | | Full length (ORD) | Phosphatidylserine | 4B2Z [51] |
| OSBP | *H. sapiens* | FFAT motif (residue 346–379) | In complex with VAP-A MSP (residues 11–135) | 2RR3 (NMR) [87] |
| ORP1 | *R. norvegicus* | FFAT motif (residue 472–481) | In complex with VAP-A MSP (residues 1–125) | 1Z9L, 1Z9O [86] |
| ORP8 | *H. sapiens* | PH (residue 150–265) | | 1V88 (NMR) |
| ORP11 | *H. sapiens* | PH (residue 59–165) | | 2D9X (NMR) |

β-barrel, and a small C-terminal subdomain with around 100 amino acids. Structures of other ORDs from Osh3 and Osh6 show that the β-barrel fold of ORD domains is well conserved with a central hydrophobic tunnel that can accommodate a single lipid molecule (Fig. 2). The tunnel entrance is covered by a "lid" region containing a small amphipathic α-helix connected by a flexible loop.

The fold of ORDs is unique among LTPs, but the structural features are similar to other lipid transfer proteins. The burial of its ligands in a central hydrophobic tunnel and the lid covering the binding pocket are similar structural features to other lipid binding and transport proteins. The ORDs display the highest structural similarity to the steroidogenic acute regulatory protein (StAR) transport (START) domains of MLN64 [29] and StarD4 [30] proteins that accommodate cholesterol in the central hydrophobic pocket of the β-barrel. In these structures, the hydrophobic parts of ligands are entirely sequestered from solvent. The structural features of ORDs are generally consistent with the proposed role of ORPs as LTPs. The ORD structures reported to date are limited to yeast Osh homologs, but the sequence conservation and similarity of properties in biochemical analysis suggest the structural folds of ORPs are well conserved in all eukaryotic species. For example, *Arabidopsis* and rice genome encode 12 ORPs and 6 ORPs respectively. The plant ORP homologs contain the conserved OSBP signature motifs and display similar domain organization to human and yeast ORPs [34,35]. ORPs were also identified in protists such as *Dictyostelium*, *Cryptosporidium*, *Toxoplasma*, and *Plasmodium* [34,36].

## 4. Ligand binding by ORDs

### 4.1. Sterol binding

OSBP was identified as a cytosolic protein that binds oxysterols, which are oxygenated derivatives of cholesterol produced in very low amounts by enzymatic and nonenzymatic reactions [37,38]. OSBP and its close homolog ORP4 were found to bind cholesterol and 25-hydroxycholesterol with nanomolar affinities [39–41]. The Yeast ORP, Osh4 binds these sterols with similar affinities [28]. Osh5, which shares the highest degree of similarity to Osh4, also binds and transports sterol at a comparable level to that of Osh4 [42]. Using [$^3$H]25-hydroxycholesterol, Olkkonen and coworkers found that ORP1 and ORP2 bind sterol [43]. They observed most human ORPs bind photo-crosslinkable derivatives of sterols, but this needs further investigation since crosslinkable sterols do not represent the real nature of free sterol ligands. Other studies also provided evidences of sterol binding to ORP1 [44], ORP2 [45], ORP8 [46] and ORP9 [47,48].

The structure of Osh4 was solved with five different sterol ligands such as cholesterol, ergosterol, and other oxysterols [28]. All these structures were almost identical with bound sterols similarly positioned with in the central tunnel. Osh4 binds a single sterol in an internal cavity closed by a lid. The 3β-hydroxyl group of the sterols is oriented toward the bottom of the hydrophobic tunnel making direct and water-mediated contacts with polar residues at the bottom of the pocket (Fig. 2, 3A). Remarkably, there is no direct interaction between the unique hydroxyl groups of the oxysterols (except the conserved 3-hydroxyl group in all sterols) and the protein, suggesting that Osh4 can accommodate a wide range of sterols with similar structural features. The hydrophobic side chain of sterol makes contact with the inside of the lid, possibly leading to the closed conformation with a completely sealed environment for the sterol. This means the lid should undergo 'open and close' conformational changes to uptake or release the ligand. Consistently, when Osh4 is empty, the N-terminal lid is susceptible to proteolysis suggesting that the lid is flexible or open leaving the pocket accessible for ligand uptake [28] (Fig. 3B).

Sterol binding is not a common theme for ORPs. The lid region and the basic cluster at the tunnel entrance are strictly conserved in all ORPs, whereas the residues recognizing 3-hydroxyl group of sterols and composing the wall of hydrophobic tunnel are not conserved, implying that many ORPs might have different ligand specificities. The residues recognizing sterols in Osh4 are conserved only in the close homolog, Osh5. The structure of Osh3 ORD shows that Osh3 does not bind sterol due to the small hydrophobic pocket [49] (Fig. 3C). Poor

conservation of residues and a different shape of hydrophobic tunnel suggest Osh3 might recognize undiscovered ligands other than sterols. Consistently, in a survey of cholesterol transport by yeast ORPs (Osh1–7), only Osh2 and Osh5 showed significant sterol transfer activity compared to Osh4 suggesting that sterol binding is specific to certain ORP homologs [42,50,51]. ORDs of human ORP5 and ORP10 did not show detectable binding to cholesterol compared to the sterol binding of Osh4 [51].

### 4.2. Conserved PI(4)P binding by all ORDs

PI(4)P is the major phosphoinositide on the trans-Golgi membranes and in the PM and is essential for the recruitment of multiple effectors at the surface of these compartments [52]. PI(4)P pools in the Golgi and the PM are produced from phosphatidylinositol (PI) by the phosphoinositide 4-kinases, Pik1 and Stt4 respectively, in yeast [53]. Osh4 transfers sterols much faster if liposomes contain anionic lipids such as phosphatidylserine, PI(4)P, and PI(4,5)$P_2$ [54]. PI(4,5)$P_2$ was known to bind polybasic patches on the surface of Osh4 and phosphoinositides in an acceptor membrane were found to change the ability of Osh4 to extract sterol from donor liposomes [42]. PI(4,5)$P_2$ stimulated sterol extraction by Osh4 from liposomes, while PI(4)P inhibited sterol extraction [42]. This intriguing relationship of Osh4–phosphoinositide interaction led Drin's group to discover the competitive binding of PI(4)P to the same internal binding site as sterol by monitoring protein–lipid interaction between Osh4 and dehydroergosterol in real time using FRET (fluorescence resonance energy transfer) technique [55] (Fig. 3D). They confirmed this finding by determining the structure of Osh4 complexed with PI(4)P.

Osh4 accommodates the PI(4)P ligand by holding the two acyl chains in the central hydrophobic tunnel where sterol-binding site is located (Fig 3E). The acyl chains of PI(4)P make loose nonspecific interaction with the hydrophobic tunnel. The phosphorylated inositol head group is recognized by a shallow pocket around the tunnel entrance covered by the lid (Fig. 3F). The 4-phosphate group makes direct hydrogen-bonds with the two histidines of the OSBP-signature sequence and the conserved arginine from α7. The 1-phosphate group in the inositol ring is hydrogen bonded to the conserved lysine residues from β1 and α7 (K109 and K336 in Osh4). The mutations (K109A, H143A, and H144A) on the strictly conserved residues contacting with the polar head group of PI(4)P inhibit the PI(4)P binding without affecting sterol binding [28,55]. The structure of the PI(4)P complex is highly similar to that observed in the presence of ergosterol [28] with slight different conformation of lid to accommodate the PI(4)P molecule [55]. Due to the steric clashes with the protein backbone and side chains, phosphate groups at position 3 and 5 in the inositol ring are not allowed, suggesting that ORPs do not accept PI(3)P or PI(4,5)$P_2$ but bind specifically to PI(4)P. This specific recognition of PI(4)P is also seen for other ORD structures of yeast Osh3 and Osh6 [49,50] (Fig. 2). Binding and transfer activity of PI(4)P was also confirmed in many ORP homologs OSBP [56,57], ORP4 [41], ORP5 [58], ORP8 [58], ORP9 [48]. Therefore, the "OSBP fingerprint motif" EQVSHHPP conserved in all ORDs can be defined as a binding motif for the

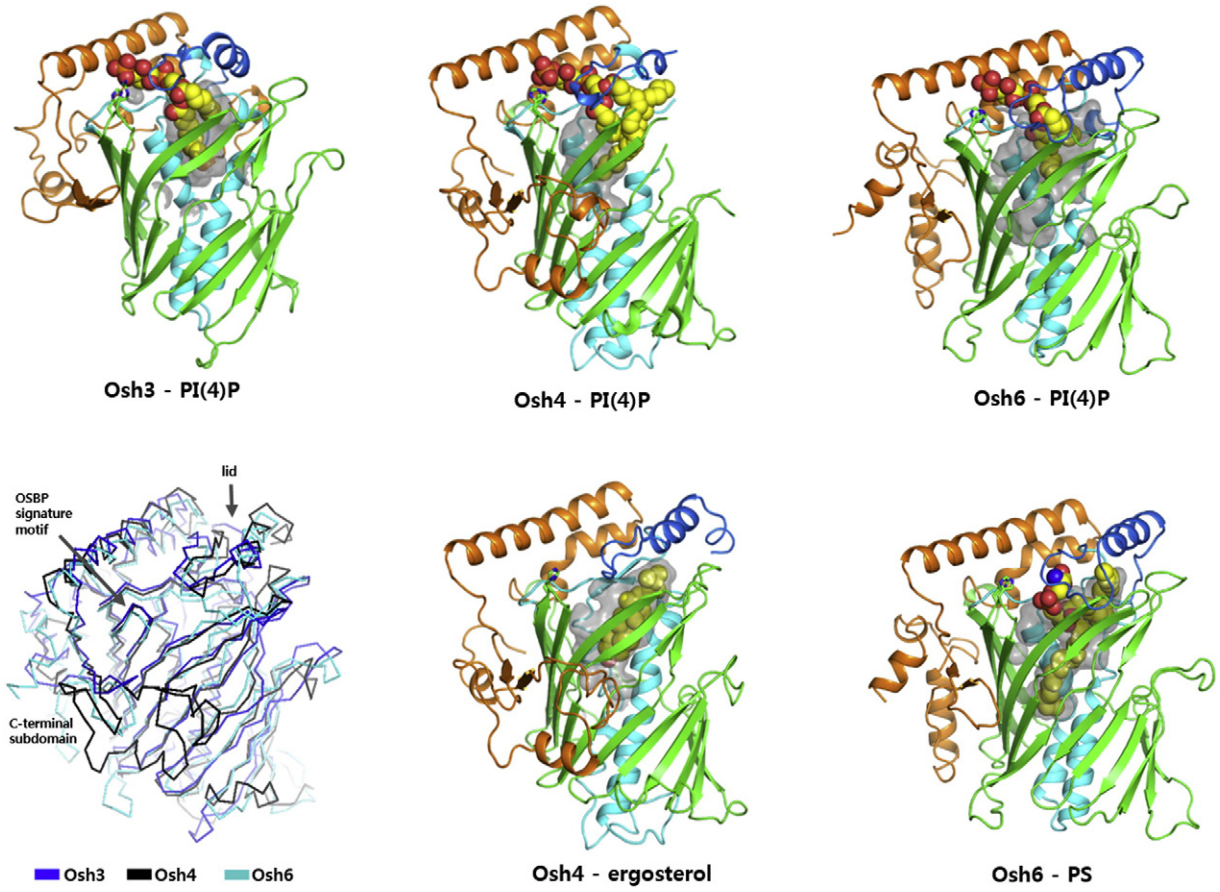


**Fig. 2.** Structures of ORDs from several Osh homologues. The bound ligands are shown in sphere models. The central hydrophobic tunnels are shown in transparent surfaces. The hydrophobic tunnel of Osh4–PI(4)P is drawn partially because disordered residues are missing in the crystal structure. PDB IDs for the illustrated structures of Osh homologs are 4INQ, 3SPW, 4PH7, 1ZHZ, and 4B2Z from left to right, respectively. The ORD domains are colored in blue, cyan, green and orange for the lid, central α-helices, β-barrel and the C-terminal subdomain, respectively. Superposition of backbone structures from Osh3, Osh4, and Osh6 was shown in the left bottom for comparison.

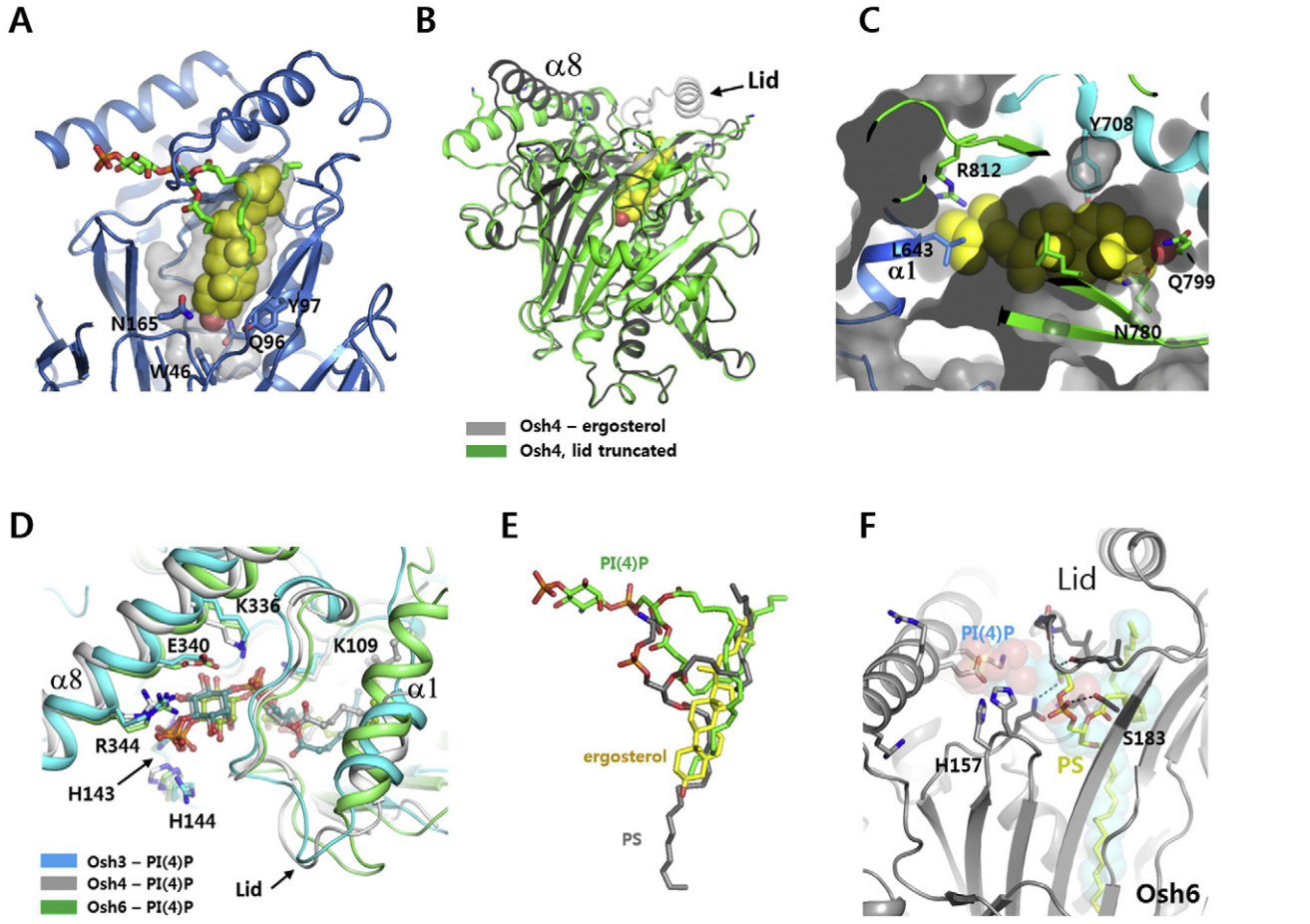


**Fig. 3.** Ligand binding of ORDs. A, Osh4 has an overlapping binding site for PI(4)P and sterol. The bound ergosterol is shown in spheres and the PI(4)P in sticks. The polar residues on the bottom of the tunnel are shown in sticks. B, Structural comparison of ergosterol-bound Osh4 in a closed form and the lid-truncated Osh4 in an open conformation. C, Osh3 ORD does not accept sterols due to the small hydrophobic tunnel. The ligand-binding tunnel is contoured in a transparent surface. The ergosterol was modeled into the tunnel. D, Conserved PI(4)P binding of Osh homologs. Structures of PI(4)P-bound Osh3, Osh4 and Osh6 are superimposed. The conserved residues recognizing PI(4)P head group are shown in sticks. E, Structures of bound lipids extracted from the superposed structures of Osh4–PI(4)P, Osh4–ergosterol and Osh6–PS complexes. F, Overlapping binding of PS and PI(4)P in Osh6. Bound PS is shown in sticks and PI(4)P is shown in a transparent sphere model. The interactions of the unique residues in Osh6 and PS head group are shown in dotted lines.

phosphorylated head group of PI(4)P ligand. The strict sequence conservation of the residues recognizing PI(4)P in ORP/Osh family members certifies the idea that PI(4)P is a ligand common to all ORPs [49,55] (Fig. 3D).

Yeast ORPs have at least one essential function shared by all homologs, which was inferred from the observation that introduction of any one of the OSH alleles makes the seven Osh knockout strain viable [59]. The introduction of human ORP1 gene recovers viability from the lethality of Osh-knockout in yeast cells, suggesting that the key function of ORPs is common for different species [60]. Consistently, we observed expression of ORD domains of either human OSBP1 or ORP3 rescues the OSH-deletion yeast strain (Tong and Im, unpublished observations). Any mutations that abrogate PI(4)P binding in ORDs are lethal [28,49] indicating that the PI(4)P binding of the ORDs is the key feature for the common essential function of all ORPs.

### 4.3. PS binding

Phosphatidylserine (PS) is synthesized in the ER and then transported to the inner leaflet of the PM [1]. PS is more enriched in the PM than in the ER, which recruits and activates various signaling proteins [61]. Non-vesicular route of lipid transport for PS was previously unknown. Gavin and coworkers discovered that PS is transferred from the ER to the PM by Osh6 and Osh7 in yeast [58]. Osh6 and Osh7 display almost no activity to transport or extract sterols from membranes in vitro [42]. They instead bind PS and participate in PS homeostasis by transporting this lipid to the PM in exchange of PI(4)P to the ER in vivo [50,51]. These Osh homologs have subtle structural variations in the ORDs, which confer distinct PS specificity in addition to the conserved PI(4)P binding [50] (Fig. 3E, 3F). Osh6 is similar to Osh4 with 20% sequence identity. The Osh6 structure displays a conserved ORD fold consisting of β-barrel with the central ligand-binding tunnel with a lid and a C-terminal α-helical region (Fig. 2). The structure of PS-bound Osh6 is similar to that of PI(4)P bound form (with root mean square deviation = 0.36 Å for 403 residues). The Osh6 tunnel is mainly hydrophobic and is slightly deeper than that of Osh4. The tunnel accommodates one of the long acyl chains of PS. The head group and the other acyl chain of PS are bound around the tunnel entrance. The α1–β1 loop in the lid and the region near the tunnel entrance are involved in PS recognition by hydrophobic interaction with the acyl chain and by hydrogen-bonding with the carboxylate head group of PS. The binding of PS involves several key residues that are not conserved in other ORP members, which represents unique structural features specific for PS recognition. These key residues are in the α1–β1 loop in the lid and S183 in the wall of β-barrel making a contact with the PS head group. These sequence features adapted for PS recognition are observed in several other metazoan ORPs such as ORP5, ORP8, ORP9, ORP10 and ORP11 representing a separate clade of ORP members with different

ligand specificities. Mammalian ORP homologs, ORP5, ORP8 and ORP10 share the ability to bind PI(4)P and they recognize PS as a second lipid [51,58].

### 4.4. Other non-lipid ligands for ORDs

Several types of non-lipid compounds were discovered as OSBP ligands that regulate OSBP-mediated biological processes. Natural products such as cephalostatin 1, OSW-1, ritterazine B and schweinfurthin A were recently reported to inhibit the growth of cultured human cancer cell lines by directly binding to ORDs of OSBP and ORP4 and therefore they were collectively termed ORPphillins [62]. Recently, Strating et al. found that an antifungal drug itraconazole (ITZ) is a potent inhibitor of enterovirus replication [63]. Its antiviral function depends on the direct binding to OSBP1 and ORP4 and the subsequent inhibition of cholesterol/PI(4)P exchange between membranes. ITZ binds OSBP and inhibits its function, thereby perturbing the virus-induced membrane alterations essential for viral replication organelle formation [63,64]. Consistently, OSW-1 specifically inhibits enterovirus and hepatitis C virus replication through OSBP [63,65,66] and a number of other compounds were suggested to inhibit enterovirus replication by targeting OSBP [64]. A known ligand for OSBP, 25-hydroxycholesterol also weakly inhibits enterovirus replication [64,67], suggesting these ORPphillins might bind to the hydrophobic pocket in the ORDs, competing with their natural lipid ligands. The essential role of OSBP/ORP4-mediated lipid exchange or cell signaling in virus replication and in cancer cell survival suggests that they can serve as a potential target for antiviral and anticancer drug development by providing a drug binding site in the ORD as a good pharmacopore.

## 5. Other domains commonly found in ORPs

### 5.1. N-terminal PH domains

The ORPs are in principal cytosolic proteins, but many of them have been observed to associate with specific membrane compartments by small membrane-targeting domains such as PH domain [22]. PH domains with 100–120 amino acids have a common structure consisting of seven-stranded two anti-parallel β sheets and a C-terminal amphipathic helix [68]. The sequence conservation among different PH domains is low with 7–30% identity due to the variations of the key residues in the PH domain and in the length of loops connecting the β-strands [69,70]. However, most PH domains with known 3D structures show high structural similarity [71]. Structures of PH domains from several ORPs were reported to have similar structures to other PH domains [49,72] (Fig. 4A). PH domains commonly have strong positive charges on the end of β-sheets compared to the other side. The positive surface of PH domain often interacts with the negative head

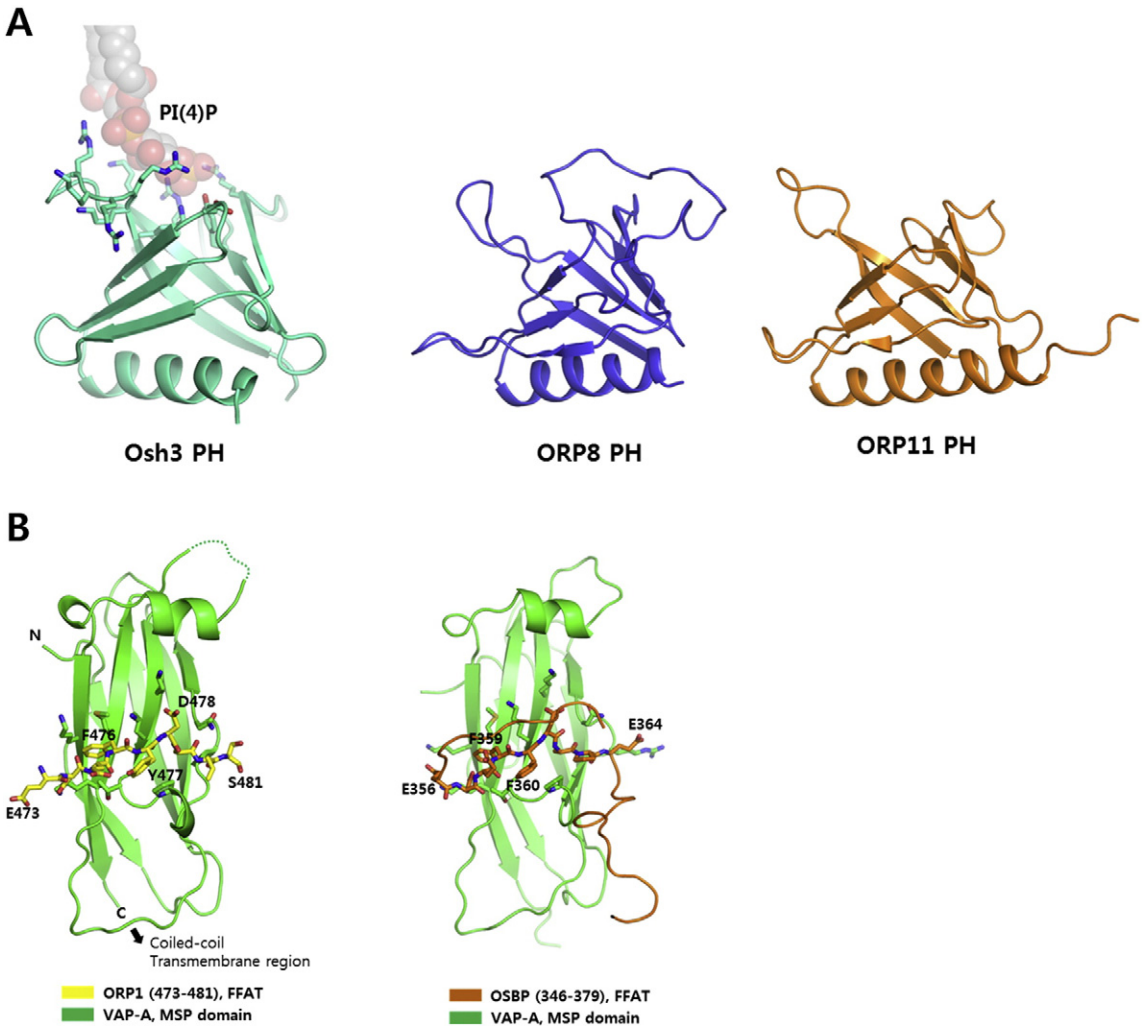


**Fig. 4.** Structures of additional domains in long ORPs. A, Structures of PH domains from Osh3 (PDB id 4IAP), ORP9 (PDB id 1V88) and ORP11 (PDB id 2D9X). The bound PI(4)P in Osh3 PH was modeled as transparent spheres based on the structure of inositol (1,4,5) triphosphate-bound PH domain (PDB id 1U29). B, Structures of MSP domain of VAP-A in complex with FFAT motifs from ORP1 (PDB id 1Z9O) and OSBP (PDB id 2RR3). The FFAT motif sequence of OSBP (ENEFFDAPE) is shown in a stick model and the rest are shown in a cartoon model.

group of phosphoinositides [73]. Distinct PIP species are enriched on different intracellular membranes. Therefore, binding to specific PIPs by PH domains can promote to dock these lipid-transfer proteins to their target membranes, which is common for ORPs [23,74–77], CERT [14,22] and FAPP2 (four-phosphate-adaptor protein 2) [78]. PH domains display a wide variation in terms of ligand affinity and specificity. Most PH domains have weak binding to phosphoinositides with micromolar affinities [70,72] but some show strong interaction with nanomolar affinities especially for PtdIns(3,4,5)$P_3$ [3,79]. Through these interactions, PH domains recruit proteins to different membranes enabling them to function at proper cellular locations or to interact with other components of signaling pathways.

The PH domains of OSBP and ORP9L target the proteins to the trans-Golgi by interacting with PI(4)P abundant in these membranes [47]. Similarly, the PH domain of Osh1 interacts with PI(4)P and displays Golgi targeting [77]. The PH domain of ORP1L shows targeting to late endosomes, and the PH domains of ORP3 [80,81], ORP6, and ORP7 show localization to PM [82]. Overall membrane targeting of ORPs arises from a combination of their PH domains and other targeting domains such as FFAT motif and ANK repeats. ANK region and the PH domain of ORP1L synergize in targeting the protein to late endosome [83]. The coincident detection of PI(4)P and Arf1-GTP by the OSBP PH domain could enable precise localization of OSBP on the trans-Golgi network initiating the MCS scaffolding at the right place [56,84].

### 5.2. FFAT motif

The FFAT motif with the consensus amino acid sequence of EFFDAxE serve as a targeting signal for localizing cytosolic proteins to the ER surface and to the nuclear membrane [24,25]. Many long ORPs (OSBP, ORP1, ORP2, ORP3, ORP4, ORP5, ORP6, ORP7, and ORP9 in human) containing the FFAT motifs are recruited to the cytosolic surface of the ER [81,82]. FFAT motifs bind to the VAMP-binding proteins (VAPs), which are anchored to the ER by a C-terminal transmembrane helix. VAPs are type II integral membrane proteins which are composed of three conserved domains including an N-terminal MSP (major sperm protein) homology domain, central coiled-coil domain and a C-terminal transmembrane region [85]. The N-terminal MSP-like motif folds into the immunoglobulin-like β sandwich structure that binds FFAT sequence [86] (Fig. 4B). The central coiled-coiled domain is involved in multimerization and the C-terminal transmembrane region anchors the VAPs to the ER membrane. The FFAT motif binds VAP on a conserved positive patch and a hydrophobic pocket on the VAP protein surface [86,87]. The complex of MSP domain and FFAT motif forms dimers in the crystals suggesting that VAP-A might interact with FFAT motifs in a 2:2 complex assisted by the association of coiled-coil regions [86]. However, physiological significance of the dimer needs further investigation. Scs2, the yeast VAP homolog, binds several FFAT-containing ORPs including Osh1, Osh2 and Osh3 [24,88], and the yeast lacking Scs2 mislocalizes FFAT-targeted proteins to the cytoplasm [24,25].

The affinity of VAPs to the FFAT motifs of ORPs seems to be not strong with the $K_D$ of a few μM ranges [25,87], which allows FFAT proteins compete for VAP interaction and dispersed distribution of ORPs on the ER membrane and in the cytosol. The primary sequences of FFAT motifs in ORPs have a variation with EF(Y)F(Y)DAxE(S,T) which might result in the difference of relative affinities of various FFAT motifs. All long ORPs in human and yeast contain multiple targeting domains among PH, FFAT motif, ankyrin repeat, transmembrane region, and GOLD domain except ORP10 and ORP11 which have only a PH domain.

### 5.3. Ankyrin repeat domains

Some OPRs including ORP1L, Osh1 and Osh2 contain N-terminal ankyrin repeat motifs, which mediate protein–protein interaction. Ankyrin repeats are tandemly repeated modules of 33 amino acids which fold into a two antiparallel α-helices separated by a β-hairpin loop [89]. The tandem repeats are packed linearly to form helix–turn–helix bundles connected by flexible loops, which provides a protein–protein interaction surface [90]. The ankyrin repeat region contributes to specific membrane targeting of several ORP members via protein–protein interactions. The ankyrin domain in Osh1 interacts with the Nvj1, an integral membrane protein in the outer nuclear envelope, which is required for the localization of Osh1 to the nucleus–vacuole junction [91,92]. The ankyrin repeat in ORP1L synergistically with the PH domain contributes to the localization of this protein to the contact sites between the ER and late endosomes by interacting with the endosomal GTPase Rab7. The ankyrin repeat domain of ORP1L interacts with the GTP-bound active form of Rab7 which is anchored to the endosomal membrane by its C-terminal prenylation [75,93]. ORP1L–VAP complexes bring ER membrane into contact with late endosomes (LE) and lysosomes by the interaction of ORP1 with Rab7 in the LE. ORP1L was known to regulate the mobility and subcellular distribution of LE with Rab7 and its effector protein, RILP (Rab7-interacting lysosomal protein) which is connected to dynein/dynactin motor complexes [44,94]. Whether the protein–protein interaction mediated by ankyrin domains is involved in the regulatory function of ORPs or simply it serves as protein targeting role requires further investigation.

### 5.4. Other domains

At the extreme N-terminus of the yeast long ORP, Osh3, there is a GOLD (Golgi dynamics) domain which might bind other proteins [95]. The domain is a protein module with typically between 90 and 150 amino acids and found in several eukaryotic Golgi and lipid-traffic proteins [95]. The GOLD domain seems to form a compact β-sandwich fold with six to seven strands with a single-large loop insertion when estimated by the secondary structure prediction using the PHD program [96]. So far, no homologous structure was reported for the GOLD domain of Osh3 and the functional role of the GOLD domain in ORPs has not yet established. Two long ORPs, ORP5 and ORP8 lacking the FFAT motif contain a transmembrane helix in the C-terminal end. This single transmembrane region anchors the ORPs in the ER membrane, which might replace the role of FFAT motif [46].

## 6. Conformational changes of ORDs in lipid transport cycles

The lipid transport by LTPs is mediated by the interaction of protein with membrane and transitions between different conformational states [15]. The lipid transfer cycle starts from the association of protein with a donor membrane, which accompanies conformational changes that uncover ligand-binding pocket. The tunnel opening is generally mediated by displacement of the lid covering the binding pocket allowing uptake or release of a lipid molecule. The LTPs extract the lipid from the membrane by providing a hydrophobic environment as well as specific interaction. Dissociation from the donor membrane shifts the conformation to a closed form. Then, the LTPs loaded with a cargo lipid diffuse through the aqueous phase. Thus, LTPs may exist in at least two distinct conformations: a “closed” state in which one lipid molecule is accommodated in the tunnel for transport and an “open” state which is preferred when bound to membrane [15,97].

The structures of several yeast Osh homologs (Osh3, Osh4, and Osh6) in apo form and in complex with various bound-lipids provide substantial understanding on the structural mechanism of lipid transport and signaling by ORPs [28,49–51,55]. The conformational changes in the ORDs are essentially accompanied in each step of the lipid transfer cycles. The N-terminal lid in ORDs covering the hydrophobic tunnel serves as a gate for lipid uptake and release [28]. In the presence of bound ligands such as PI(4)P, sterols and PS, the ligand-binding tunnel is closed by the lid. The closed conformations of ORDs are slightly different depending on the type of bound lipids. The bound ligands within the complex are buried inside the pocket and inaccessible from the outside suggesting a conformational change of lid is accompanied

during uptake and release of the lipids. Molecular dynamics simulations of sterol movement in the binding pocket of Osh4 suggest that opening the lid is slow and energetically unfavorable in aqueous environment [98,99]. Lid opening may be induced by the interaction of a hydrophobic side of the lid with membranes. The lid of Osh4 contains a small amphipathic α-helix named as ALPS (ArfGAP1 lipid packing sensor) motif which binds efficiently to membranes with high positive curvature, suggesting an ability of Osh4 to extract sterols from curved membranes [100,101]. Considering the requirement of membrane association for lipid extraction, membrane binding of the lid domains seems to be a common feature for many ORPs lacking the ALPS motif. The N-terminal lid is not only involved in membrane binding but also seems to be important for the directionality of sterol transport. The lid allows Osh4 to release sterol against its concentration gradient, into the trans-Golgi but slow down the back transport of sterol in the ER [102]. The opening of the lid, when Osh4 is bound to sterol, is fast when the membrane is anionic [55]. However, it prevents the release of sterol into neutral liposomes comparable to ER. The ligand bound structures of Osh4 and Osh6 display very similar conformations of ORDs regardless of lipid types such as PI(4)P, PS and sterols. For example, the structure of PI(4)P bound Osh6 is similar to that of PS-bound form with root mean square deviation of 0.36 Å. There is little conformational change of ORDs between the PI(4)P-bound Osh3 and the un-liganded Osh3 which have a closed conformation of lids in both states. This suggests that ligand-selective signaling might not be a major function in these Osh homologs. In contrast, there is a big conformational change in the ORD domain between apo-form of Osh4 and sterol-bound Osh4 [28] (Fig. 3B). Binding of sterol ligand to Osh4 protects proteolysis of the N-terminal lid suggesting that the lid is disordered in apo-form and closed upon ligand binding. The crystal structure of a truncated form of Osh4 lacking N-terminal lid (residues 1–29) shows Osh4 exposes a relatively flattened basic surface by conformational changes of helix α7, the conserved basic cluster at the tunnel entrance, and several loop regions near the tunnel opening. The structure of truncated Osh4 seems to represent the open conformation of ORD that may interacts with a membrane bilayer, allowing the protein to extract or deliver the lipids to the membranes [28].

## 7. PI(4)P-coupled lipid transport by ORPs

Key insights into the lipid transport function of Osh proteins came from the structural and biochemical analysis of Osh4. Drin and coworkers found that Osh4 binds ergosterol and PI(4)P in a mutually exclusive manner and it transfers sterol and PI(4)P along opposed directions between distinct membranes by counter exchange [55]. This observations led them to propose a model that Osh4 function as PI(4)P/sterol exchanger using PI(4)P gradient as energy source for the transport of secondary lipid against concentration gradient (Fig. 5). This vectorial exchange of lipids driven by PI(4)P hydrolysis catalyzed by the ER-localized Sac1 phosphatase was experimentally confirmed in vitro [56,102].

PI(4)P is most enriched in the cytosolic surface of the trans-Golgi and PM but is absent from the ER, implying that a steep PI(4)P gradient exists between these membrane compartments. In yeast this gradient is maintained by spatially distant phosphoinositide 4-kinases, Pik1 and Stt4, generating PI(4)P by phosphorylation of PI in the trans-Golgi and PM, respectively [53]. In contrast, Sac1, the major phosphatase in the ER degrades PI(4)P into PI. Meanwhile, Osh4 was known to downregulate cellular PI(4)P levels and the availability of this lipid on Golgi surface by extracting PI(4)P [103]. Osh4 counteracts Pik1 and Sec14, a PI/PC LTP that coupled with Pik1 to yield Golgi PI(4)P [103–105]. Osh4 has an ability to transfer sterol supplying the trans-Golgi and PM with sterols taken from the ER [59,106]. Consequently, they proposed an elegant model that Osh4 uses the PI(4)P gradient at the ER/Golgi interface to supply the trans-Golgi with sterol [17]. Osh4 extracts sterol from the ER, then releases it by counter exchange with PI(4)P at the Golgi, and transports PI(4)P to the ER. The consumption of PI(4)P to PI at the ER by Sac1 would sustain sterol/PI(4)P transport cycles. The ATP-dependent production of PI(4)P from PI by Pik1 would energetically drive sterol/PI(4)P cycles by Osh4, thereby promoting the active release of sterol into the trans-Golgi and the creation of a sterol gradient [102].

PI(4)P-coupled transport of lipid by ORPs is not limited to sterols. Recent studies on Osh6, ORP5 and ORP8 revealed that PS transport to PM by these ORPs is fueled by PI(4)P metabolism through PS/PI(4)P exchange cycles [50,51,58]. Osh6 and Osh7 extract PS from the ER, exchange PS with PI(4)P at the PM, and transport PI(4)P to the ER.

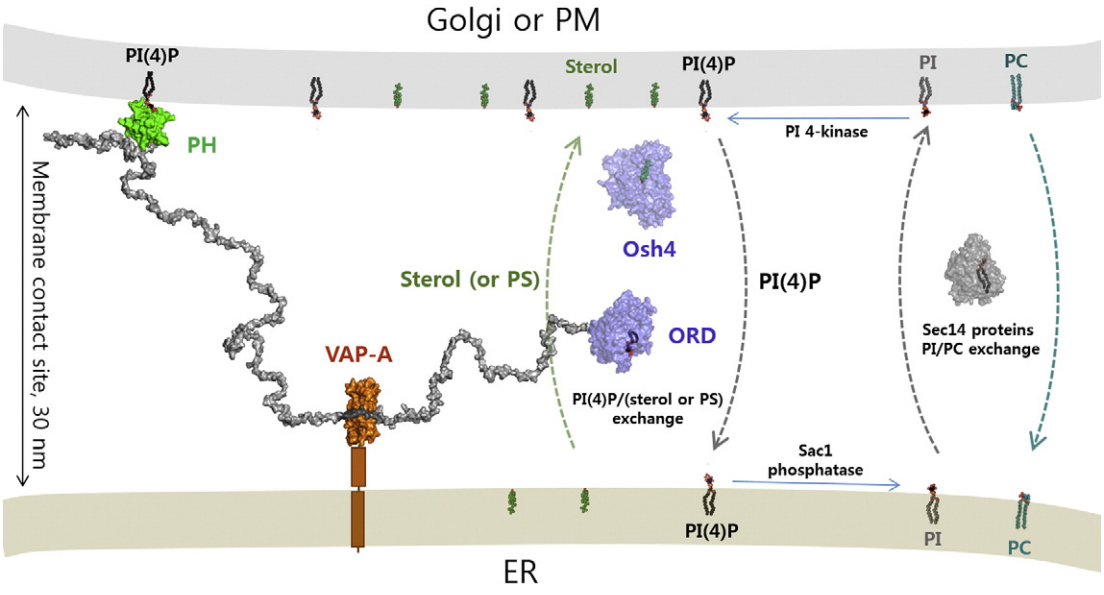


**Fig. 5.** Structural model of lipid transport by ORP homologues in a membrane contacts site. The structure of near-full length Osh3 (residue 176–996) was modeled except the N-terminal GOLD domain based on the structures of PH and ORD domains from Tong et al. [49]. The functional model for PI(4)P/sterol counter exchange by ORPs was based on the proposal by Drin's group [17]. The structures of Osh4 and Sec14 protein (Shf1) bound to ergosterol and PI were taken from the PDBs1ZHZ and 3B7N respectively [104]. The structures of proteins and lipids are drawn in an absolute scale to represent the exact size of each molecules.

Maintenance of a PI(4)P gradient by Sac1 (and Stt4 producing PM PI(4)P) sustains PS/PI(4)P exchange cycles and PS delivery to the PM against its concentration gradient. These results, along with those obtained with sterol/PI(4)P exchangers, Osh4 and OSBP suggest a general mechanism by which ORP/Osh proteins use PI(4)P metabolism to build distinct intracellular lipid gradients [50].

## 8. Dual ligand specificities of ORDs

Many lines of recent evidences strongly suggest several ORPs are bona fide lipid transfer proteins that exchange PI(4)P with a secondary lipid between organellar membranes. Despite of wealth of information of ligand specificities of many ORP homologs, so far the secondary ligands for many ORPs (Osh1, Osh3, ORP3, ORP6, ORP7, ORP9, and ORP11) are not known clearly. Variation in the residues and shapes of the hydrophobic binding pockets in ORPs implies that the secondary ligand is unique to certain ORP members. Therefore, the possibility that which counter ligands are exchanged for PI(4)P by different ORPs should be tested individually. The ability to exchange one lipid for another has been also found for other LTPs such as α-tocopherol binding protein [107] (exclusive binding of α-tocopherol and PI(4,5)$P_2$), Sec14 family PI transfer protein (PI and PC) [104] and the ceramide transporter CERT (ceramide and diacylglycerol) [108], and may be critical for directional lipid exchange at MCSs. In these LTPs including ORPs, the polar head groups of lipids are recognized by the protein surface specific to each ligand types while the aliphatic chains are accommodated by the same binding pockets. This partial overlapping of ligand binding sites allows only one lipid molecule in the binding pocket, suggesting that these LTPs are lipid exchangers rather than unidirectional transporters [16].

So far, structural studies of ORDs are limited to yeast Osh homologs. Considering high degree of structural and functional conservation, key structural features are expected to be conserved between different eukaryotic species. Osh3, Osh4, and Osh6 represent different clades of ORPs with distinct ligand specificities. Osh4 displays structural features specific to sterol/PI(4)P exchangers, while Osh6 has a slight structural variation suited for the recognition of PS as a secondary ligand. Structure of Osh3 shows a conserved PI(4)P binding with a small hydrophobic pocket that excludes sterol binding. Osh3 might have an undiscovered secondary ligand that might bind in the hydrophobic pocket, which is analogous to the concept proposed for PI(4)P/(sterol or PS) exchangers. However, the possibility that Osh3 might transfer PI(4)P as a single unique ligand cannot be excluded. Assuming that the general function of ORP/Osh proteins is to exchange PI(4)P with a secondary ligand between organelles, more investigations are required to explore the second ligand of Osh3 and other ORP members.

## 9. ORPs and nonvesicular lipid transport at the MCSs

Membrane contact sites (MCSs) are closely apposed regions where membranes of two organelles are in a close proximity, typically less than 30 nm [16]. These dynamic structures are highly enriched in lipid biosynthetic enzymes and LTPs, providing hot spots for cell signaling, metabolism, and the transport of metabolites [109–112]. LTPs could transport lipids by either shuttling between two membranes at MCSs or by rotation while simultaneously bound to both membranes. The association of LTPs with specific membranes is mediated by membrane targeting determinants that present on the lipid-transfer domain or on other additional domains. These targeting determinants can interact with either membrane proteins or lipid molecules, and can markedly contribute to the specificity and the direction of the lipid transport event [15].

The ORPs are in principal cytosolic proteins with abilities to associate with specific membrane compartments by membrane-targeting domains, which may reflect diffusible properties of ORPs between cytosolic and membrane localizations. Osh4 ORD has at least two membrane-binding surfaces that bind phosphoinositides or anionic lipids, one near the entrance of the sterol-binding tunnel and another at a distal site that binds PI(4,5)$P_2$ [42]. The combination of FFAT and PH domains provides most long ORPs with dual membrane-targeting activity, which might increase the avidity of membrane binding at MCS. The efficient lipid transport by tethering two distinct membranes was experimentally confirmed for OSBP. Drin's group provided a detailed view on how OSBP mediates sterol/PI(4)P exchange between the ER and Golgi in mammalian cells [56]. OSBP contains a C-terminal ORD homologous to Osh4 and also possesses the PH domain and FFAT motif for membrane targeting at the N-terminal region [22]. The PH and FFAT domains of OSBP provide a tight tethering between the ER and Golgi membranes by binding PI(4)P at the Golgi and VAP-A on the ER, respectively [56]. The bridging of the ER and Golgi membranes by OSBP provides the basis of a model for how OSBP, VAP-A and the PI(4)P phosphatase Sac1 cooperate to promote the specific exchange of lipids. At MCSs between the ER and the Golgi, sterols and PI(4)P are efficiently transferred by the ORD of OSBP. ER-anchored ORP5 by its C-terminal transmembrane helix was suggested to cooperate with NPC1 (Niemann Pick C 1) protein to mediate the exit of cholesterol from endosomes/lysosomes and transfer sterols through a MCS to the ER for esterification [113].

The domain architectures of most long ORPs correlate with their lipid transfer function at MCS. All long ORPs contain at least two membrane targeting determinants (Fig. 1). The structural modeling of intact Osh3 well corresponds to the concept of membrane tethering by ORPs [49]. The lengths of loops connecting PH domains and FFAT motifs in ORPs vary between 141 and 358 residues roughly corresponding to at least 35 nm when calculated with the average Cα distance of 2.5 Å between residues. The flexible loops connecting the FFAT motifs and ORDs are generally around 50 amino acid residues which cover 12.5 nm in distance. Therefore, ORPs can either shuttle across MCSs or transport lipids by bridging an MCS. The absence of a diffusion step through the cytosol would increase the efficiency of lipid transfer.

## 10. Conclusion

Increasing evidence suggests that non-vesicular lipid transport is the primary role of ORPs. In particular, lipid transport coupled with PI(4)P exchange seems to be a major route for sterol and PS transfer by ORPs from the ER to the Golgi and PM against concentration gradient [50,51,55,56,58,102]. The PI(4)P/sterol exchanger model by Osh4 clearly explains the long-established genetic interactions between *OSH4*, *SEC14*, *SAC1*, and *PIK1* genes [55,84,114]. The one essential function shared by all ORP homologs in yeast and perhaps in mammals as well might be the lipid homeostasis by balancing the distribution of PI(4)P in cellular membrane compartments. Although the PI(4)P-coupled lipid transport provides a promising model for ORP function, it does not explain the diverse regulatory roles of many ORP members. For examples, OSBP was identified as a sterol-sensing scaffold that regulates the activity of ERK (extracellular-signal-regulated kinase) in a ligand dependent manner [39,40]. ORP9L has been implicated in the regulation of Akt activity raising a possible link to the proliferative pathway [115]. ORP1L and ORP8 affect expression of *ABCA1* and other *LXR* target genes perhaps by a signaling-related mechanism [46,75,116,117]. So far no direct structural evidence was obtained to delineate the regulatory mechanism of these ORPs.

Cells lacking all seven Osh proteins showed a dramatic reduction in sterol transport between PM and the ER [51]. The finding that Osh4 moves sterol and PI(4)P in opposite directions supports a direct role for the Osh proteins in sterol transport from the ER to PM [79]. However, although at a much slower rate than in wild-type cells, sterols are still transferred between the ER and PM in Osh deficient cells [110]. This movement might imply the action of other undiscovered sterol transfer proteins in yeast. Recently, a new family of StART domain proteins (Ysp1, Ysp2, Sip3, Lam4, Lam5 and Lam6/Ltc1) were discovered to have a role in sterol transport in yeast [118–120]. These proteins contain

at least two membrane targeting domains such as PH and the C-terminal transmembrane region. For example, Ysp2 and its paralog Lam4 specifically bind sterol and act in trans to mediate sterol exchange at the MCS between the PM and ER. These findings require further investigation to establish a mechanism of sterol transport and how these proteins participate intracellular sterol distribution along with ORPs.

The collected evidence suggests a model that each ORP contains a specific function, but all homologs seem to share an essential function on maintaining a cellular lipid distribution, which may influence diverse membrane trafficking and signaling, as well as local lipid metabolism. Given the molecular and functional diversity within the ORP family, one of the major challenges for the future will be to unravel the structural mechanism of the relationship between the conserved PI(4)P-coupled lipid trafficking and the diverse roles of ORPs in cell signaling.

## Transparency document

The Transparency document associated with this article can be found, in the online version.

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