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A vertebrate model for the study of lipid binding/transfer protein function: Conservation of OSBP-related proteins between zebrafish and human

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ABSTRACT

Oxysterol-binding protein (OSBP) and OSBP-related (ORP) or OSBP-like (OSBPL) proteins constitute a family of lipid-binding/transfer proteins (LTPs) present in eukaryotes from yeast to man. The mechanisms of ORP function have remained incompletely understood. However, several ORPs are present at membrane contact sites and act as either lipid transporters or sensors that control lipid metabolism, cell signaling, and vesicle transport. Zebrafish, *Danio rerio*, has gained increasing popularity as a model organism in developmental biology, human disease, toxicology, and drug discovery. However, LTPs in the fish are thus far unexplored. In this article we report a series of bioinformatic analyses showing that the *OSBPL* gene family is highly conserved between the fish and human. The *OSBPL* subfamily structure is markedly similar between the two organisms, and all 12 human genes have orthologs, designated *osbpl* and located on 11 chromosomes in *D. rerio*. Interestingly, *osbpl2* and *osbpl3* are present as two closely related homologs (*a* and *b*), due to gene duplication events in the teleost lineage. Moreover, the domain structures of the distinct ORP proteins are almost identical between zebrafish and man, and molecular modeling in the present study suggests that ORD liganding by phosphatidylinositol-4-phosphate (PI4P) is a feature conserved between yeast Osh3p, human ORP3, and zebrafish Osbpl3. The present analysis identifies *D. rerio* as an attractive model to study the functions of ORPs in vertebrate development and metabolism.

1. Introduction

Lipid synthetic processes in eukaryotic cells are compartmentalized and the different organelles have distinct lipid compositions [1]. Moreover, the roles of lipids and products of their metabolism as signaling compounds are increasingly appreciated in cell biology and biomedicine [2,3]. These features of the lipidome necessitate efficient and well controlled inter-organelle lipid fluxes. While lipid trafficking is in part mediated by carriers of the vesicle transport routes [4,5], non-vesicular mechanisms play an increasingly appreciated role in lipid trafficking. The hydrophobicity of membrane lipids makes their diffusion through the cytosol energetically unfavorable. Therefore, non-vesicular lipid transfer is facilitated by close proximity of the two membranes and/or the involvement of LTPs carrying bound lipid across the cytosolic phase [6]. Endoplasmic reticulum (ER) membranes, at which many major lipid biosynthetic processes take place, form zones of contact (10–30 nm distance) with other organelle limiting membranes. Such zones are designated membrane contact sites (MCS), and have established roles in lipid syntheses, non-vesicular lipid transport, Ca^{2+} signaling, and ER function [7–10].

Oxysterol-binding protein homologs, designated OSBP-related (ORP) or OSBP-like (OSBPL) proteins, are LTPs characterized by a carboxy-terminal OSBP-related ligand-binding domain (ORD). Most of them have in their N-terminal part a pleckstrin homology (PH) domain that binds phosphoinositides, and either a short amino acid motif (FFAT, two phenylalanines in an acidic tract) that binds to ER VAMP-associated proteins (VAPs) or a C-terminal trans-membrane segment mediating association of the proteins with ER membranes [11,12]. The protein family is in mammals encoded by 12 genes [13–15], while in yeast *Saccharomyces cerevisiae* there are 7 OSBP homologs (*OSH*) [16]. The ORDs of a number of ORPs accommodate a variety of sterols [17,18]. The hallmark study on yeast Osh4p revealed a β-barrel like fold with a lipid-binding pocket, in which the bound sterol is oriented with its 3β-hydroxyl group facing the bottom of the pocket. The bound sterol stabilizes a closed conformation of a lid which makes contacts with the sterol iso-octyl side chain [17]. Studies in yeast have provided evidence for a function of Osh proteins in sterol transport from the plasma

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membrane (PM) to the ER [19,20]. However, there are contradicting observations that rather suggest function of the Osh proteins as regulators of the lateral sterol organization at the PM [21], secretory vesicle transport [22], or nutrient signaling [22,23]. Although a number of studies on mammalian ORPs have indicated functional roles of ORPs as sterols sensors with signaling functions [18], the latest report suggests that OSBP acts as a lipid transporter that moves cholesterol from the ER to *trans*-Golgi and PI4P in the opposite direction [24].

2. Methods

2.1. Bioinformatic analyses

Sequence alignment, clustering, and dendrogram generation were carried out with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) by using the ligand-binding (ORD) domain sequences of the proteins. Functional domains/motifs of the proteins were predicted at http://smart.embl-heidelberg.de and http://www.ebi.ac.uk/interpro/. The amino acid sequence identity between human and zebrafish ORPs was determined by the BLAST algorithm at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2. Homology modeling of human ORP3 and zebrafish Osbpl3

The structure of yeast Osh3p [25] was used as a template for modeling the C-terminal OSBP-related domain (ORD) of human ORP3. Prime, version 3.3, (Schrödinger, LLC, New York, NY, 2013) was used to align the human ORP3 sequence with Osh3p (PDB code 4inq) obtained from the wwPDB [26]. The final structural model of ORP3 consists of amino acids A508 to W887. Prime 3.3 was used to build surface loops for 1 insertion and 4 deletions in the alignment, and after this, new side chains were placed and their conformations optimized. PI4P was taken from the template (4inq) and its position was adjusted in the final energy minimization of the complete model. The model of zebrafish Osbpl3 built in the same way consists of amino acids F503 to W882.

3. Localization and function of ORPs at membrane contact sites

The yeast ORP Osh1p was found to localize at a MCS, the nucleus-vacuole junction [27,28], and four other our yeast ORPs, Osh2p, Osh3p, Osh6p and Osh7p, were reported to be enriched at the cortical ER or ER-PM contacts [20,29]. Insight into ORP function at a MCS was provided by Stefan et al. [30], who showed evidence that Osh3p acts to recruit the PI4P phosphatase Sac1p anchored at ER membranes, at PM sites where it has access to its substrate, PI4P. Related observations were reported by Tavassoli et al. [31]. These findings suggested Osh3p acts as an organizer of protein complexes at ER-PM MCSs, with potential impacts on PM signal transduction and vesicle transport.

In mammalian cells, evidence for a MCS-associated function has been reported for OSBP and ORP1L. OSBP was recently shown to bridge ER and *trans*-Golgi membranes or artificial membranes by binding to VAPs in the ER and either PI4P or the small GTPase Arf1 in *trans*-Golgi [24]. Substantial evidence was presented for a 4-step functional cycle in which OSBP transfers cholesterol forward from the ER to the Golgi and PI4P in the backward direction (Fig. 1). ORP1L resembles OSBP in that it interacts with ER VAP proteins through its FFAT motif and with a small GTPase at a non-ER membrane location: In this case the GTPase is the late endosomal (LE) Rab7 [32]. ORP1L acts as a sterol-controlled switch that controls LE association with ER membranes and the interactions of the Rab7 effector RILP with dynactin and the homotypic vacuole fusion and protein sorting (HOPS) complex mediating tethering of late endocytic compartments [33,34]. Why the LE-ER contacts and LE positioning/motility need to be regulated according to sterol cues, as well as the possible function of ORP1L as a lipid transporter at these contacts, have remained poorly understood. However, ORP1L binds sterols [35], and we observed in Raw264.7 macrophage subjected to ORP1L knock-down a defect in the efflux of endocytosed cholesterol to extracellular apolipoprotein A-I as well as cellular accumulation of cholesterol esters [35,36], suggesting a function associated with LE sterol transport.

4. Liganding by glycerophospholipids opens new insight into ORP function

De Saint-Jean et al. [37] determined the high-resolution structure of yeast Osh4p with PI4P inserted in the ORD, and demonstrated that a bound sterol is readily exchanged for PI4P. They suggested that Osh4p could transport the two lipids in opposite directions, i.e. sterol from the ER to the *trans*-Golgi/PM and PI4P backwards. The data by Mousley et al. [23], on the other hand, suggested that a sterol-binding deficient mutant *osh4/kes1*^{Y97F} disturbs cell proliferation due to its enhanced PI4P-dependent association with *trans*-Golgi network (TGN) and endosomes. They suggested that binding of sterol by Osh4p has a negative regulatory role, detaching the protein from TGN/endosome membranes [23], rather than a sterol transport function.

Tong et al. [25] recently solved the structure of the Osh3p ORD with bound PI4P, strongly suggesting that the ligand cavity of Osh3p is too narrow to accommodate bulky sterol molecules. Importantly, the authors identified, consistent with the study of De Saint-Jean et al. [37], a cleft at the entrance of the ligand cavity that accommodates the phosphoinositol moiety of PI4P, and

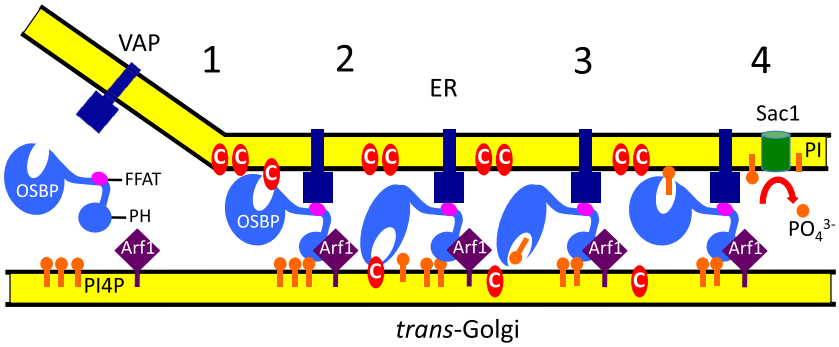

Fig. 1. A model for OSBP function. (1) OSBP mediates the tethering of ER and *trans*-Golgi at a membrane contact site via binding the VAMP-associated proteins (VAP) at the ER, and phosphatidylinositol-4-phosphate (PI4P) and the small GTPase Arf1 in the Golgi. C, cholesterol; PH, pleckstrin homology domain; FFAT, two phenylalanines in an acidic tract. (2) OSBP transfers cholesterol from the ER to Golgi membranes. (3) OSBP transfers PI4P from the Golgi to the ER. (4) The phosphatase Sac1hydrolyzes the PI4P in the ER, thus providing the energy for the transport process (based on [24]).

indicated that amino acid residues lining this cleft are conserved among all ORPs. We have modeled the structures of the closest homologs of yeast Osh3p in human and zebrafish, ORP3 and Osbpl3, respectively, to assess the putative conservation of the ORD glycerophospholipid binding specificity (see 5.3.).

The report by Maeda et al. [38] suggested that yeast Osh6p and Osh7p bind phosphatidylserine (PS), and that disruption of *osh6/osh7* impairs PS transport from the ER to the PM. Together with the study by Mesmin et al. [24], these reports on yeast Osh proteins bring up the idea that ORPs could mediate the non-vesicular inter-organelle transport of either sterols or glycerophospholipids/sphingolipids in exchange for phosphoinositides [24,25,37,38].

5. The ORP/Osbpl family is highly conserved between human and zebrafish

5.1. Zebrafish – a model organism for the study of development, metabolism, and disease

Thus far only two *Osbpl* gene knock-out mouse models have been reported [39,40] and the phenotypic characterization of mammalian knock-out or knock-down models is likely hampered by the presence of several closely related *Osbpl* genes with overlapping functions [13–16]. Therefore, it is desirable to devise for the study of ORP function an alternative vertebrate model that offers an ease of genetic manipulation and is more rapid and less expensive to develop, maintain, and characterize than mammals. The past two decades have seen a dramatic increase in the use of zebrafish, *Danio rerio*, as a model organism in biomedical research. This booming popularity is due to the numerous advantages zebrafish can offer to study developmental biology, human disease, toxicology, and drug discovery. Female zebrafish can lay hundreds of eggs weekly. The externally fertilized embryos develop remarkably rapidly and hatch from their chorions two days post fertilization. Their internal yolk reserves are depleted in approximately a week, after which the fish depend on external food sources. Zebrafish reach adulthood at three months. For research purposes adults aged 6–18 months are typically used. Great numbers of fish that are easily obtained and develop rapidly allow research to progress at a faster pace than with other vertebrate model organisms. Along with the small size of zebrafish, these factors account for the relative affordability of husbandry and maintenance compared to mammals. However, the main strengths of zebrafish lie in their small size, external development, and genetic tractability. The optical transparency of larval zebrafish enables high-resolution *in vivo* imaging. Furthermore, many chemical interventions are possible by simply adding the desired compounds into the bath water. Importantly, the zebrafish genome is sequenced (http://www.sanger.ac.uk/Projects/D_rerio/). Numerous transgenic zebrafish lines, many with fluorescent markers, as well as an increasing amount of knock-out zebrafish lines [41] are available from the Zebrafish International Resource Center (http://zebrafish.org). Recently, techniques for producing targeted gene knock-out have been introduced [42,43]. Additionally, gene knock-down with morpholino oligonucleotides is a well-established method to silence genes of interest for 3–5 days in embryonic fish [44]. These numerous advantages of zebrafish contribute to its increasing attractiveness as a model to study lipid metabolism and lipid-related diseases including atherosclerosis, obesity, diabetes and hepatic steatosis [45,46].

5.2. Conservation of the ORP/Osbpl family between human and zebrafish

The 12 human *OSBPL* genes have 14 orthologs in *D. rerio*, designed *osbpl*, located in 11 different chromosomes (Table 1). Among them, *osbpl2* and *osbpl3* are present as two closely related homologs (*a* and *b*), which apparently reflect gene duplication events in the teleost lineage. Of *osbpl8*, only partial sequence (encoding 561 amino acids) is available in the NCBI database.

Table 1
The zebrafish *osbpl* gene family.

Gene	chr	Refseq	Protein ID*
osbp	1	XM_005160138	B8A443
osbp2	10	XM_001334348	E9QE50
osbpl1a	22	XM_005161723	F1QS47
osbpl2a	23	XM_005162340.1	E7F4S3
osbpl2b	23	NM_199872	Q7ZVL1
osbpl3a	19	XM_005170028	E7F4U6
osbpl3b	16	NM_001256189	F1R2E5
osbpl5	25	XM_005170705	E7FGK0
osbpl6	9	XM_005172507	E7F2M8
osbpl7	12	XM_005156241	Q5XIY7
osbpl8	4	XM_005164481	XP_005164538
osbpl9	8	XM_005166900	A0JMN8
osbpl10	16	XM_005158241	E7FE64
osbpl11	9	XM_683921	F1QCI1

* The protein identifiers are from UniProt except for Osbpl8, which is from NCBI.

The individual Osbpl/ORP proteins in *D. rerio* and *Homo sapiens*, respectively, display a markedly high similarity of amino acid sequence. Clustering analysis using the most conserved part of the protein sequences, the ORD, and the ClustalW algorithm reveals three major phylogenetic branches and remarkable conservation of the Osbpl/ORP phylogenetic tree between the two species (Fig. 2), evidencing for an origin of the various Osbpl/ORP subfamilies early in vertebrate evolution (>400 My ago). The branch containing Osbp/OSBP, Osbp2/ORP4, Osbpl1a/ORP1, and Osbpl2/ORP2 contains the family members demonstrated to display high affinity for sterol ligands [18]. Of note, zebrafish has two *osbpl2* genes located on chromosome 23 (Table 1, Fig. 2), reflecting a gene duplication in the teleost lineage. The next branch represents the clade (Osbpl/ORP5, 8, 9, 10 and 11) suggested by Maeda et al. [38] to bind PS. The 3rd branch consists of Osbpl/ORP3, 6 and 7. Unlike human, zebrafish has here two *osbpl3* genes, of which *osbpl3a* is located on chromosome 19 and *osbpl3b* in chromosome 16 (Table 1) – the b isoform is clearly more closely related with human ORP3 (Fig. 2) and was therefore chosen for further analysis by homology modeling in the present study (see 5.3.). The amino acid sequence identity between human and zebrafish varies from 60% for ORP5/Osbpl5 to 83% for ORP9/Osbpl9.

The organization of functional domains in the Osbpl/ORP proteins is highly conserved between zebrafish and human, the locations of the ligand-binding ORDs, PH domains, the ER-targeting FFAT motifs, and ankyrin repeats in the human and fish orthologs being almost identical (Fig. 3). Like most human ORPs, all Osbpl proteins of zebrafish except for Osbpl2a and 2b have PH domains in their N-terminal region. These two closely related proteins lacking the N-terminal PH-domain region are classified in the 'short' (S) ORP subtype that in humans also includes ORP1S, ORP4S, and ORP9S arising via alternate promoter usage or splice variation (Fig. 3). Whether other short Osbpls besides Osbpl2 exist in zebrafish is as yet unclear. A minor species difference in domain organization is that zebrafish Osbpl1a has 4 predicted ankyrin repeats while the human ortholog ORP1L has only 3; In human ORP1L the ankyrin repeat segment mediates interaction with the small GTPase Rab7 [32]. Similar to human ORP5, the fish Osbpl5 carries a predicted C-terminal trans-membrane segment which in the case of the human ortholog targets the protein to the ER [47]. Whether such segment also exists in zebrafish Osbpl8 remains open, since the available mRNA sequence (RefSeq accession number: XP_005164538) is truncated at the 3′ end.

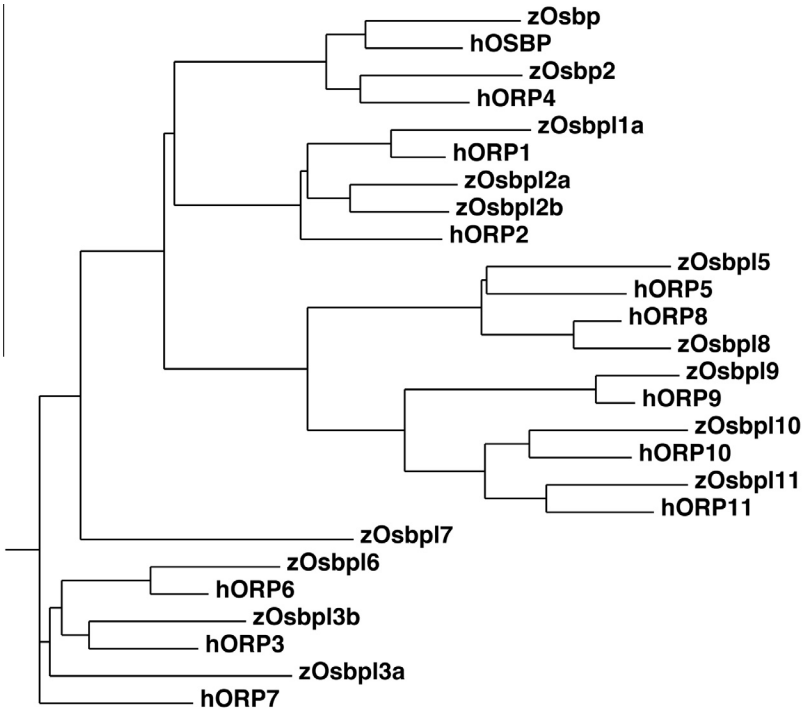

Fig. 2. A dendrogram displaying the relatedness of *Homo sapiens* ORP and *Danio rerio* Osbpl amino acid sequences. The data was generated by using the ligand-binding (ORD) domain sequences of the proteins. Human sequences are indicated with h and zebrafish ones with z.

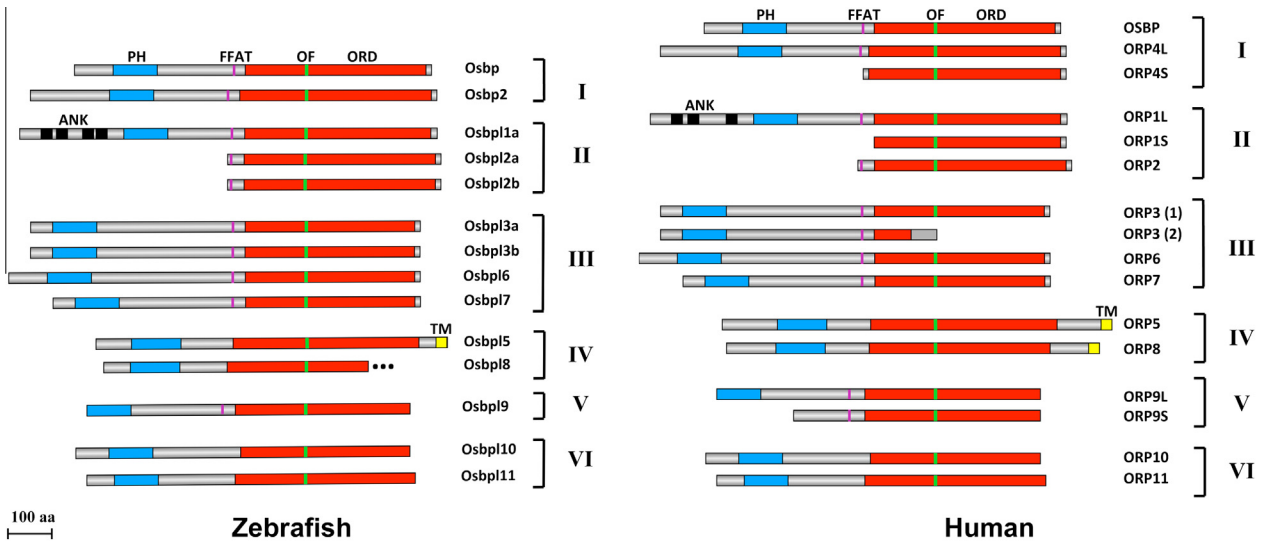

Fig. 3. A schematic diagram of zebrafish and human Osbpl/ORP protein structures. Predicted functional domains/motifs are indicated: PH, pleckstrin homology domain; FFAT, two phenylalanines in an acidic tract; OF, OSBP fingerprint motif; ORD, OSBP-related ligand-binding domain; ANK, ankyrin repeats; TM, trans-membrane segment. The dotted line indicates missing C-terminal sequence of zebrafish Osbpl8. The Osbpl/ORP subfamilies are identified by Roman numerals on the right and the amino acid scale in the bottom left-hand corner.

5.3. Homology modeling supports conserved liganding by PI4P from yeast Osh3p to zebrafish Osbpl3 and human ORP3

Prompted by the report by Tong et al. demonstrating specific liganding of yeast Osh3p by PI4P [25], we undertook homology modeling of the human and zebrafish orthologs of this protein to investigate whether the observed glycerophospholipid specificity might be conserved in evolution. The overall sequence identity between human ORP3 and the structural template, *S. cerevisiae* Osh3p, is 34% with larger differences present in surface loops (Fig. 4A), while the putative lipid-binding pocket exhibits a higher degree of conservation (~70%) than the rest of the protein (Fig. 4B). A difference that could be of functional importance is substitution of yeast Osh3p S657 by K542 in ORP3 close to the inositol-4-phosphate head group of the PI4P ligand (Fig. 4C), which may affect the tolerated phosphate substitution pattern of the inositol. Three of the amino acids in Osh3p, which were speculated by Tong et al. [25] to prevent ergosterol binding, are conserved in human ORP3 and zebrafish Osbpl3 (L523, Y588, R693). Additional differences are found around one of the acyl chains, where especially substitution of Osh3p L673by R553 in Osbpl3, while not sterically blocking the pocket but being more polar, might have an influence on the

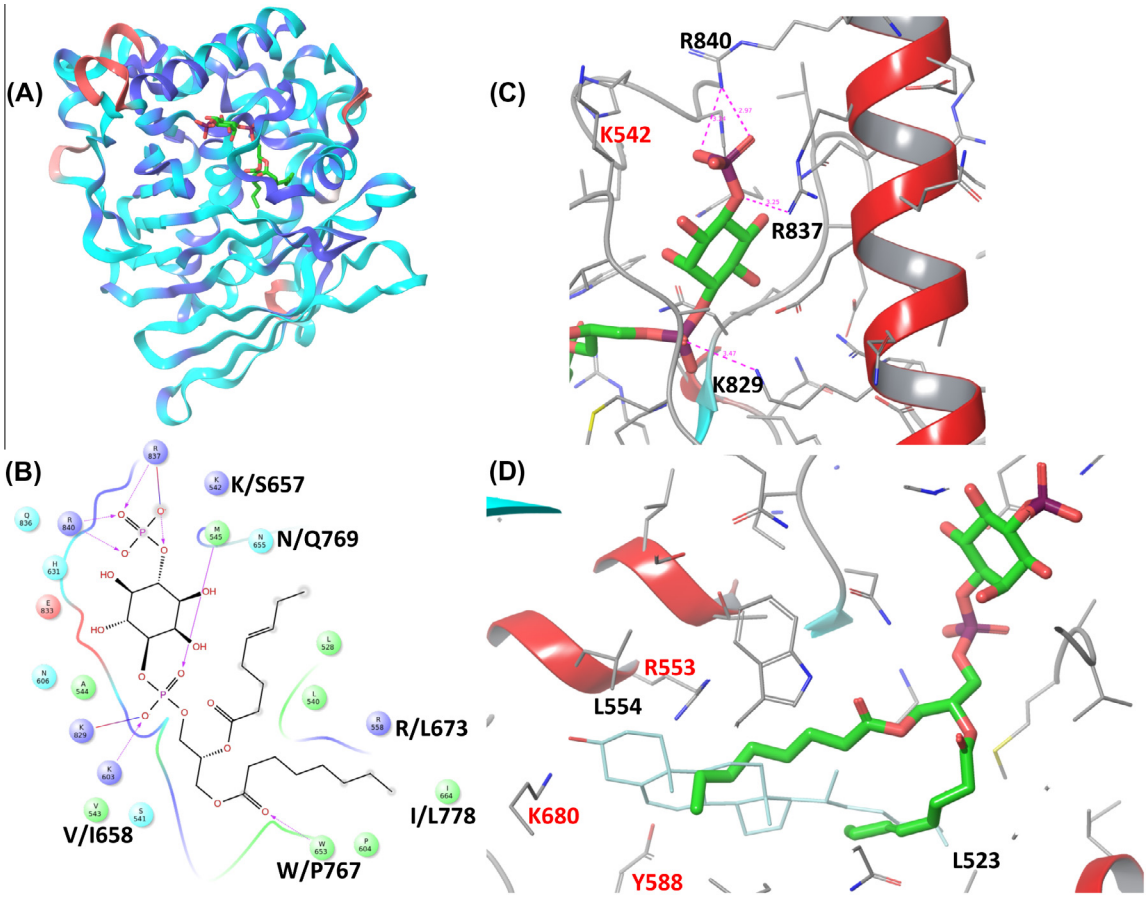

Fig. 4. Homology modeling of human ORP3 and zebrafish Osbpl3 ORD structures. (A) The overall structure of the homology model of human ORP3. Regions conserved with respect to the modeling template Osh3p are shown in dark blue (34%), non-conserved side chains in light blue (62%) and gaps in the sequence alignment (4%), which were modeled as new loops, are indicated in red. The phospholipid ligand PI4P is shown in green. (B) Ligand interaction diagram of human ORP3 with PI4P. Differences compared to Osh3p are indicated in black print, the 1st amino acid residue referring to Osh3p and the 2nd to human sequence. (C) Conserved interactions of human ORP3 with the PI4P head group. (D) Comparison of the zebrafish Osbpl3b ligand pocket with the oxysterol binding mode from Osh4p (thin, turquoise). Residues with short contacts to the modeled oxysterol are labelled and strong overlaps are marked in red.

tolerated lipid profile (Fig. 4D). The lipid pocket size and shape of human ORP3 and zebrafish Osbpl3 are similar, or even smaller as compared to Osh3p (Fig. 4D; [25]). This suggests that, similar to Osh3p, the human and zebrafish orthologs may not be able to accommodate sterol ligands. The sequence identity of the human and fish proteins is 71%, but all potential phospholipid contacting residues are conserved between the two species, suggesting that zebrafish would indeed be a useful model system to study the physiologic function of ORP3/Osbpl3.

6. Concluding remarks

Intracellular LTPs are key players in cellular lipid metabolism, signaling and vesicle transport. Several ORPs are present at membrane contact sites and act there as either lipid transporters or sensors that control the activity of enzymatic effectors or assembly of protein complexes, with impacts on lipid metabolism, cell signaling, and vesicle transport. The zebrafish and human Osbpl/ORP proteins share the same functional domains and are markedly conserved between the two species, consistent with fundamental functions of the distinct ORP subfamilies in vertebrate physiology. Furthermore, our homology modeling suggests that the binding of PI4P within the ORD ligand pocket is an evolutionarily conserved property shared by yeast, zebrafish, and human Osh3/Osbpl3/ORP3 proteins. Despite the marked gene conservation, Osbpl function in zebrafish has remained totally unexplored. As a conclusion, we propose *D. rerio* as an attractive model for the study of ORP function in vertebrate development and lipid metabolism.

Conflict of interest

The authors have no conflicts of interest to disclose.

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