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# OSBP-related protein 2 (ORP2): Unraveling its functions in cellular lipid/carbohydrate metabolism, signaling and F-actin regulation

**Short title:** Unraveling the functions of ORP2

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**Highlights**

- ORP2 is a mammalian oxysterol-binding protein homologue with affinity for cholesterol, oxysterols and phosphoinositides
- ORP2 is suggested to mediate cholesterol transport to the plasma membrane in exchange for $PI4,5P_2$
- ORP2 knock-out in hepatoma cells resulted in defective triglyceride synthesis and storage
- ORP2 knock-out impaired cellular glucose uptake and metabolism, co-indicent with defective Akt signaling
- ORP2 manipulations impact on F-actin, cell adhesion, migration and proliferation

## Abstract

Oxysterol-binding protein (OSBP)-related proteins (ORPs) constitute a family of intracellular lipid-binding/transport proteins (LTPs) in eukaryotes. They typically have a modular structure comprising a lipid-binding domain and membrane targeting determinants, being thus suited for function at membrane contact sites. Among the mammalian ORPs, ORP2/OSBPL2 is the only member that only exists as a 'short' variant lacking a membrane-targeting pleckstrin homology domain.

ORP2 is expressed ubiquitously and has been assigned a multitude of functions. Its OSBP-related domain binds cholesterol, oxysterols, and phosphoinositides, and its overexpression enhances cellular cholesterol efflux. Consistently, the latest observations suggest a function of

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ORP2 in cholesterol transport to the plasma membrane (PM) in exchange for phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$), with significant impacts on the concentrations of PM cholesterol and $PI4,5P_2$. On the other hand, ORP2 localizes at the surface of cytoplasmic lipid droplets (LDs) and at endoplasmic-reticulum-LD contact sites, and its depletion modifies cellular triglyceride (TG) metabolism. Study in an adrenocortical cell line further suggested a function of ORP2 in the synthesis of steroid hormones. Our recent knock-out of ORP2 in human hepatoma cells revealed its function in hepatocellular PI3K/Akt signaling, glucose and triglyceride metabolism, as well as in actin cytoskeletal regulation, cell adhesion, migration and proliferation. ORP2 was shown to interact physically with F-actin regulators such as DIAPH1, ARHGAP12, SEPT9 and MLC12, as well as with IQGAP1 and the Cdc37-Hsp90 chaperone complex controlling the activity of Akt. Interestingly, mutations in *OSBPL2* encoding ORP2 are associated with autosomal dominant non-syndromic hearing loss, and the protein was found to localize in cochlear hair cell stereocilia.

The functions assigned to ORP2 suggest that this protein, in concert with other LTPs, controls the subcellular distribution of cholesterol in various cell types and steroid hormone synthesis in adrenocortical cells. However, it also impacts cellular TG and carbohydrate metabolism and F-actin-dependent functions, revealing a bewildering spectrum of activities.

**Abbreviations used:** BiFC, bimolecular fluorescence complementation; CE, cholesterol ester; ER, endoplasmic reticulum; FC, free cholesterol; FFAT, two phenylalanines in an acidic tract; LD, lipid droplet; LE, late endosome; LTP, lipid-binding/transfer protein; OHC, hydroxycholesterol; KC, ketocholesterol; OSBP, oxysterol-binding protein; ORD, OSBP-related domain; PH, pleckstrin homology; PIP, phosphoinositide; PI4P, phosphatidylinositol-4-phosphate; $PI4,5P_2$, phosphatidylinositol-4,5-bisphosphate; $PI3,4,5P_3$, phosphatidylinositol-3,4,5-trisphosphate; PM, plasma membrane; SPR, surface plasmon resonance; TG, triglyceride

**Key words:** Akt, actin cytoskeleton, energy metabolism, cholesterol transport, OSBPL2, triglyceride

## 1. Introduction

ORP2 was initially discovered in 1999 in a screen for oxysterol-binding protein (OSBP) related expressed sequence tags [1]. The full-length human cDNA was reported in 2001 [2-4] and the

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mouse orthologue in 2002 [5]. The ORP2 mRNA was observed ubiquitously in human [1] and mouse [5] tissues. ORP2 belongs, based on its amino acid sequence and gene structure, to ORP subfamily II, together with the closely related ORP1 [3]. ORPs share a characteristic β-barrel-resembling ligand-binding domain designated OSBP-related domain (ORD) in their carboxy-terminal region [3, 6-8]. While all other mammalian *OSBPL*/Osbpl genes encode ORP proteins of the 'long' subtype, carrying an amino-terminal extension that contains a pleckstrin homology (PH) domain, ORP2 only exists as a 'short' subtype protein lacking a PH domain (Fig. 1; [2, 3, 5]). Another membrane targeting determinant present in most ORPs is called two phenylalanines in an acidic tract (FFAT) which mediates binding of the protein to the integral type 2 membrane proteins of the endoplasmic reticulum (ER), VAMP-associated proteins A and B (VAPA,VAPB; [9]). For two ORPs (ORP5 and ORP8) this functional motif is replaced by an ER-targeting trans-membrane segment [10, 11].

The presence of the ER-targeting motifs and PH domains or a trans-membrane segment confers ORPs the capacity to associate with two distinct membrane organelles at membrane contact sites (MCSs), and the current paradigm postulates that ORPs execute lipid transport and signaling functions at such intimate, direct organelle contacts [12-14]. The lipid transport function of ORPs involves a 'countercurrent' mechanism, in which a substrate lipid (thus far evidence exists for cholesterol or phosphatidylserine) is transferred in exchange for phosphatidylinositol-4-phosphate (PI4P; [15-20]) or another polyphosphoinositide such as $PI4,5P_2$ [21]. A crucial part of the transport cycle is the hydrolysis of the countertransported PI4P by the ER phosphoinositide phosphohydrolase Sac1 [22]. ORP2 does carry close to its amino-terminus a FFAT motif and thus binds to VAP proteins at the ER [23]. However, it lacks the PH domain and associated membrane targeting function. In this focused review we summarize the current knowledge on the function of ORP2.

## 2. Tissue expression pattern and subcellular localization of ORP2

The early reports on the identification of ORP/*OSBPL* sequences demonstrated that the ORP2 mRNA is detectable in all human and mouse tissues [1-3, 5, 24]. In western analysis of mouse tissues also the protein was observed in all tissues, with the strongest signal detected in the brain [24]. Two protein variants, with the apparent molecular masses of 51 and 56 kDa in SDS-

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PAGE were observed, with tissue-specific differences in their abundance. The identity of these protein variants has not been determined.

Cell fractionation by Laitinen et al. [24] suggested that a major portion of the endogenous ORP2 in Chinese hamster ovary (CHO) cells is cytosolic; however, part of the protein was found in the membrane fraction. Early overexpression studies in CHO cells indicated localization of ORP2 at the Golgi complex, consistent with a Golgi vesicle transport defect observed in CHO cells overexpressing this protein [4, 24]. However, later work revealed prominent localization of ORP2 on the surface of cytoplasmic lipid droplets (LDs) in A4312, HeLa, HepG2 and COS-7 cells, with no detectable Golgi targeting [25]. Of interest, incubation of cells with the high affinity ligand of ORP2, 22(R)-hydroxycholesterol (OHC), resulted in a different, diffuse and cell cortex/plasma membrane (PM) localization of the protein [25]. A site-specific mutant deficient in oxysterol binding, ORP2 I249W [26], showed enhanced association with LDs and could not be detached therefrom with 22(R)OHC, suggesting that oxysterol binding controls the conformation of ORP2 and this, in turn, plays a crucial role in its targeting to LDs [25].

Several years later, Weber-Boyvat et al. [23] investigated in HuH7 hepatoma cells the subcellular localization of complexes of ORP2 with its ER anchor, VAPA, by using the bimolecular fluorescence complementation (BiFC) technique. They detected ORP2-VAPA complexes at ER-LD contacts. Moreover, siRNA-mediated knock-down of ORP2 and VAPs enhanced the turn-over of cellular triglycerides. These observations suggested that a major function of ORP2 could involve the ER-LD interfaces and the TG metabolic processes occurring therein. However, the study of Kentala et al. [27] revealed that the loss of ORP2 did not reduce ER-LD contacts, but under basal culture conditions rather increased them. However, this work indicated that the dynamic expansion of the ER-LD MCSs upon fatty acid loading may be disturbed in the ORP2 KO cells. Similar to ORP2 expressed alone, ORP2-VAPA BiFC complexes were upon 22(R)OHC treatment redistributed from LD-associated ER structures to elongated membraneous-appearing elements and occasionally to the cell cortex/PM [28]. Cell cortex localization of ORP2 was also observed in human umbilical vein endothelial cells (HUVECs) that seldom have large LDs. In these cells, the protein was frequently found co-localizing with the actin regulator cortactin in lamellipodia [27].

If cells have large LDs or are subjected to fatty acid loading to increase LD size, ORP2 associates prominently with LDs (Fig. 2A). However, many cultured cell lines display under standard growth conditions only very small or undetectable LDs. Under these conditions the

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cytosolic and other non-LD aspects of ORP2 localization, such as the cell cortex/PM and lamellipodia (Fig. 2B), predominate. The recent study of Koponen et al. [29] revealed a previously undiscovered localization of the protein on late endosomes (LE) labeled with anti-Lamp1 or internalized fluorescent dextran (Fig. 2C).

To summarize, exogenously expressed ORP2 targets LDs or ER-LD contacts, but in the absence of large LDs, it remains largely cytosolic or and/or targets the cell cortex/PM and LEs. When co-expressed with VAPA, ORP2 is seen attached to the ER and prominently at ER-LD contacts. A major problem is that, with the antibodies we have generated or those available commercially it has been difficult to reliably detect the endogenous cellular ORP2. Some efforts have, however, been made. Our study [25] suggested that some endogenous ORP2 may be detectable on the LDs of fatty acid loaded A431 cells. Thoenes et al. [30] reported detection of endogenous ORP2 in the stereocilia of mouse cochlear inner and outer hair cells. Moreover, Escajadillo et al. [31] reported detection of endogenous ORP2 within the nucleus of adrenocortical H295R cells, consistent with their data suggesting its association with the transcription factor liver X receptor (LXR). However, new antibodies of better quality and independent confirmations in different cell types, validated by ORP2 knock-down or knock-out, are required before firm statements can be made on the subcellular localization of endogenous ORP2.

## 3. Ligands of ORP2

A number of reports have addressed the lipid ligands of ORP2 by employing charcoal-dextran, pull-down, lipid-protein overlay assays, extraction of lipids from vesicles, or surface plasmon resonance (SPR) (Table 1). Initially, the group of N. Ridgway investigated the lipidous ligands of ORP2 by using a lipid-protein overlay assay. The results suggested binding of ORP2 to the acidic lipids phosphatidic acid (PA), phosphatidylinositol-3-phosphate (PI3P) and cardiolipin [4]. These results need to be interpreted with caution, since lipids spotted on nitrocellulose surface are presented to proteins in a rather non-physiologic manner. Our group next documented, by using a charcoal-dextran assay, binding of the oxysterol 25OHC to ORP2 produced in *E.coli* as a GST fusion, at a 3.9 x $10^{-6}$ M $K_d$ [26]. In a follow-up study [25] we measured affinities of ORP2 for 22(R)OHC (1.4 x $10^{-8}$ M) and 7-ketocholesterol (7KC; 1.6 x $10^{-7}$ M) and presented evidence that the protein also binds cholesterol, based on a pull-down assay in which ORP2 extracted cholesterol from methyl-β-

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cyclodextrin. Our latest work confirmed the cholesterol binding, by employing a pull-down assay in which radiolabeled cholesterol was solubilized with the zwitterionic detergent CHAPS. However, the binding did not reach saturation, so we were unable to determine a $K_d$ for this interaction [29].

In addition to a PIP-binding PH domain, several ORPs have the capacity to accommodate and extract PI4P and other PIPs by their ORD domain [15-17, 21]. In 2005 we showed that ORP2 is capable of pulling down phosphatidylcholine (PC) vesicles with 2 mol% (unlabeled) multiply phosphorylated PIPs, the pull-down being most efficient with $PI3,4,5P_3$ [32]. This suggested an interaction of ORP2 with the head groups of long-chain PIPs *in vitro* on vesicle surfaces, since extraction of a PIP by the ORD would not result in pull-down of the vesicles labeled with [$^{14}$C]PC. In the latest study we assessed the binding of soluble short-chain (di-C8) PIPs, PI4P, $PI4,5P_2$, and $PI3,4,5P_3$ to Strep-tagged ORP2 immobilized on SPR chips. The signal obtained in this assay may represent binding of the PIPs within the ORD pocket, but also interaction of the PIPs with the surface of ORP2 could contribute to the signal. ORP2 bound $PI4,5P_2$ at 52 $\mu$M, $PI3,4,5P_3$ at 76 $\mu$M, and PI4P at a 305 $\mu$M $K_d$ [29]. However, the data need to be considered with some caution, since the folding status of ORP2 fusion proteins produced in *E. coli* has not been assessed in detail. Importantly, Wang et al. [33] recently solved the high-resolution structure of the ORP2 ORD with bound $PI4,5P_2$ and reported that the protein binds this PIP as a tetramer. They also demonstrated binding of the other six PIPs by employing an *in vitro* assay in which the ORD extracted the PIPs from vesicles (Table 1). The above reports suggest that the ORP2 ORD may show higher affinity for multiply phosphorylated PIPs than for PI4P. Published data suggests that $PI4,5P_2$ and possibly $PI3,4,5P_3$ may be the most important PIP ligands of ORP2 at the PM [29, 32, 33], and we hypothesize that it could bind $PI3,5P_2$ on LE/lysosomes, since ORP2 binds this late endocytic pathway membrane component quite efficiently on vesicle surfaces *in vitro* [32].

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**Table 1.** Lipidous ligands reported to bind to ORP2

| **Ligand** | **Assay method** | **$K_d$** | **Reference** |
|---|---|---|---|
| Phosphatidic acid | Lipid-protein overlay | ND* | [4] |
| Cardiolipin | Lipid-protein overlay | ND | [4] |
| PI3P[#] | Lipid-protein overlay<br>Extraction from vesicles | ND<br>ND | [4]<br>[33] |
| PI4P | SPR[§]<br>Extraction from vesicles | $305 \times 10^{-6}$ M<br>ND | [29]<br>[33] |
| PI5P | Extraction from vesicles | ND | [33] |
| $PI3,4P_2$ | Vesicle pull-down<br>Extraction from vesicles | ND<br>ND | [32]<br>[33] |
| $PI3,5P_2$ | Vesicle pull-down<br>Extraction from vesicles | ND<br>ND | [32]<br>[33] |
| $PI4,5P_2$ | Vesicle pull-down<br>SPR<br>X-ray crystallogr.<br>Extraction from vesicles | ND<br>$52 \times 10^{-6}$ M<br>ND<br>ND | [32]<br>[29]<br>[33]<br>[33] |
| $PI3,4,5P_3$ | Vesicle pull-down<br>SPR<br>Extraction from vesicles | ND<br>$76 \times 10^{-6}$ M<br>ND | [32]<br>[29]<br>[33] |
| 22(R)OHC[€] | Charcoal-dextran | $1.4 \times 10^{-8}$ M | [25] |
| 7KC[&] | Charcoal-dextran | $1.6 \times 10^{-7}$ M | [25] |
| 25OHC | Charcoal-dextran<br>Photo-cross-linking | $3.9 \times 10^{-6}$ M<br>ND | [26]<br>[26] |
| Cholesterol | Pull-down | ND | [25, 29] |
| Dehydroergosterol | Extraction from vesicles | ND | [33] |

*not determined; [#]phosphatidylinositol-3-phosphate; [§]surface plasmon resonance; [€]hydroxycholesterol; [&]ketocholesterol

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## 4. Roles of ORP2 in cellular sterol and steroid hormone metabolism

Overexpression of ORP2 in CHO or HeLa cells was initially found to enhance the efflux of cellular radioisotope-labeled cholesterol to all acceptors, including human serum, apoA-I, and small unilamellar phosphatidylcholine vesicles [24, 32], as well as the appearance of metabolically labeled newly synthesized cholesterol at the cell surface as detected by employing methyl-β-cyclodextrin [32](Fig. 3). These observations suggested that abundant ORP2 in the cytosol of the stably transfected cells facilitated the intracellular transport of cholesterol, resulting in its rapid appearance at the cell surface measured by employing the efflux process or by a wash of cell surfaces with methyl-β-cyclodextrin. The new hallmark study by the group of H. Yang [33] demonstrated the capacity of ORP2 to transport cholesterol to the PM in exchange for $PI4,5P_2$: Its knock-out in HEK293 cells reduced the cholesterol and increased the $PI4,5P_2$ content of the PM, as well as conferred the cells elevated resistance to the cholesterol-binding drug amphotericin B. Further evidence for a direct sterol/$PI4,5P_2$ exchange or 'countercurrent' transfer activity of ORP2 was produced by *in vitro* vesicle-to-vesicle lipid transfer assays. Jansen et al. [34] studied the transport of cholesterol from the PM to lipid droplets in HeLa cells. In an overexpression screen they identified ORP2 and another related 'short' ORP, ORP1S, as gene products whose overexpression speeded up the movement of PM cholesterol to LDs. In more detailed analyses the effect was localized to the initial PM to ER transport step (Fig. 3). To determine whether also depletion of these ORPs would impact the cholesterol transport process, siRNA-mediated knock-down was carried out. Here, the double knock-down of ORP2 and ORP1S resulted in a modest but significant reduction of cholesterol transfer to the ER and LDs, supporting the involvement of the endogenous cellular proteins in PM to ER cholesterol trafficking. Consistent with the observations of Wang et al. [33], Koponen et al. [29] observed in a recent study carried out with HeLa cells that knock-down of ORP2 or overexpression of a mutant defective in PIP binding resulted in the accumulation of cholesterol probes (a derivative of *Clostridium perfringens* toxin, D4H, or filipin) in late endosomes, while overexpression of wild-type ORP2 shifted the D4H probe to the PM. These observations support the idea that ORP2 plays an important role in intracellular cholesterol transport to the PM – from endosomes and possibly other subcellular compartments (Fig. 3). This would be consistent with the enhancement of cholesterol efflux upon ORP2 overexpression [24, 32], but somewhat at odds with the data of Jansen et al. [34]. However, we find it possible that ORP2 may be involved in several routes of cholesterol transport.

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Kentala et al. [27, 35] generated HuH7 hepatoma cells entirely devoid of ORP2 by using the CRISPR-Cas9 technology. Analysis of the lipidome of these cells revealed no change in free cholesterol (FC), cholesterol ester (CE) or oxysterol (25OHC, 27OHC, 7αOHC, 7βOHC, 7KC) content [27]. Even challenge of the cells by sterol depletion by employing lipoprotein-deficient serum plus statin treatment, or by LDL loading, did not change the situation, suggesting that a chronic lack of ORP2 did not distort cholesterol homeostatic regulation in the hepatoma cells. However, next generation RNA sequencing of the cells revealed significant alterations in the mRNA expression of 11 key cholesterol homeostatic genes, including *HMGCR* and *SOAT1*, indicating that the depletion of ORP2 had disturbed the sterol homeostasis, but the effects had been compensated for by adaptation via altered gene expression [27].

The results of Escajadillo et al. [31] in the adrenocortical H295R cell line subjected to stable shRNA-mediated knock-down of ORP2 revealed 2.9-fold and 1.9-fold increases of cellular FC and 25OHC, respectively, as well as significant reductions of 22OHC and 7KC. These changes coincided with significant reductions in multiple steroid metabolites, including progesterone, 11-deoxycortisol and cortisol, but increased concentrations of androgens and estrogens. Moreover, knock-down of ORP2 suppressed the expression of several proteins required for cortisol biosynthesis and of the steroidogenic transcription factor SF1. The authors presented evidence that ORP2 may interact with the nuclear receptor liver X receptor (LXR), and both proteins bind to the promoter of *CYP11B1* in response to cAMP signaling. *CYP11B1* encodes steroid 11β-hydroxylase, a key enzyme converting 11-deoxycortisol to cortisol in the adrenal cortex. However, also a function of ORP2 in the transport of cholesterol, a substrate for the synthesis of steroid hormones, could obviously affect steroidogenesis. The observations are consistent with an earlier finding by the same group identifying ORP2 as an interaction partner of the formin family protein diaphanous homologue 1 (DIAPH1), a Rho GTPase effector which regulates cortisol production by controlling the rate of mitochondrial movement [36]. Of note, DIAPH1 regulates cytoskeletal dynamics by interacting with actin, microtubules and other cytoskeletal-associated regulatory proteins ([37]; see section 7). The above observations suggest that ORP2 executes in steroidogenic cells specific functions in the synthesis of steroid hormones, transcriptional regulation apparently playing an important role therein (Fig. 3).

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## 5. Impacts of ORP2 manipulations on triglyceride metabolism

Discovery of ORP2's affinity for LD surfaces [25] and a reduction of TGs observed in CHO cells stably overexpressing ORP2 [38] suggested that the protein may execute a function in cellular neutral lipid metabolism. We therefore analyzed the impacts of ORP2 knock-down in A431 cells on LD morphology and biochemically on the turn-over of cellular TGs. Upon ORP2 knock-down, the consumption of LDs in response to lipid starvation was slowed down in morphological analyses; Consistently, biochemical analyses revealed that the break-down of radioisotope-labeled TGs was slowed down in the knock-down cells [25]. These observations suggested that ORP2, presumably at the LD surface, may facilitate the consumption of stored TGs upon fatty acid depletion. Weber-Boyvat et al. [23] discovered in HuH7 hepatoma cells that knock-down of ORP2 together with its ER anchors VAPA and -B, speeded up the consumption of radioisotope-labeled TGs upon lipid depletion. Knock-down of ORP2 alone had in this case no significant effect on the TGs. The results appeared contradictory to those of Hynynen et al. [25], perhaps due to a different cell model employed, but nevertheless supported a functional role of ORP2 as a regulator of TG metabolism.

The complete knock-out of ORP2 in the HuH7 hepatoma cell model [27] revealed as the only significant lipidome change, reduced TG storage, and consistently, reduced synthesis of TGs in pulse labeling experiments. RNA sequencing showed a reduced expression of the lipogenic transcription factor SREBP-1 and a large number of its established target genes such as *FASN* and *SCD*, suggesting that the loss of ORP2 may result in transcriptional down-regulation of the hepatocellular lipogenesis. A possible underlying mechanism was proposed: We found defective activity of the Ser/Thr kinase Akt, a key regulator of cell metabolism and viability, in the ORP2 knock-out cells. Since Akt has been documented to control the activity of SREBP-1 [39-41], defective Akt signaling could provide a plausible explanation for the reduction of SREBP-1 dependent transcription and lipogenesis (Fig. 4). To summarize, a number of evidences based on acute knock-down or complete knock-out of ORP2 in different cell types support a functional role of the protein as a regulator of cellular TG metabolism, in addition to a function in cholesterol transport.

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## 6. Function of ORP2 in the carbohydrate metabolism of hepatoma cells

Having consistently observed impacts of ORP2 knock-down/knock-out on TG metabolism, we wanted to investigate whether these impacts actually extend wider in the cellular energy metabolism, including that of carbohydrates. Analyses of the ORP2 knock-out HuH7 hepatoma cells revealed significant defects in glucose uptake, glycogen synthesis and glycolysis [27](Fig. 4). Since glucose metabolism is robustly regulated by insulin via the PI3K/Akt signaling pathway, we assessed the activating Ser473 phosphorylation of Akt and the Ser9 phosphorylation of Akt's target glycogen synthase kinase 3β (GSK-3β), a central regulator of glycogen synthesis [42]. We observed a substantial reduction of both phosphorylations, suggesting that the loss of ORP2 indeed results in defective Akt signaling (Fig. 4). The mechanisms through which ORP2 affects the activity of Akt require further study. However, we managed to find some clues of this: (i) ORP2 was found to physically interact with IQGAP1 [35], a protein acting as a scaffold for phosphoinositide kinases [43] in the cascade that eventually produces $PI3,4,5P_3$, a key messenger in the PI3K/Akt signaling pathway and (ii) ORP2 was found to physically interact with Cdc37 and Hsp90 [27], which form a chaperone complex required for Akt activity [44]. The above results suggested that ORP2 not only regulates the cellular TG metabolism, but has more global impacts on energy metabolism. This function could be in part mediated by ORP2's involvement in inter-organelle lipid transport, potentially resulting in altered lipid domain organization with secondary effects on signaling. However, we also identified protein-protein interactions that most likely are of importance. Of note, defective Akt signaling upon ORP2 depletion was not only observed in the cancerous hepatoma cell line, but also in primary human umbilical vein endothelial cells [27]. It will be extremely interesting to study insulin signaling, lipid and carbohydrate metabolism *in vivo* in Osbpl2 knock-out mice.

## 7. ORP2 in cell F-actin regulation, adhesion, migration and proliferation

The RNA sequencing of ORP2 knock-out hepatoma cells by Kentala et al. [35] revealed significant changes in the expression of genes in the functional categories 'Cellular movement', 'Cell–cell signalling and interaction', 'Cellular development', 'Cellular function and maintenance', 'Cellular growth and proliferation', 'Cell morphology', and 'Cell Death and Survival'. A number of pathways in these categories involved the actin cytoskeleton, cell migration, adhesion or proliferation.

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Moreover, analysis of the ORP2 protein interactome in HuH7 cells uncovered >100 putative new partners, pathway analysis of which revealed 'RhoA signalling' as the most significant ORP2-associated pathway. Interactions of ORP2 with the actin regulatory components SEPT9, MLC12, ARHGAP12 and IQGAP1 were validated by independent assays. Consistently, loss of ORP2 resulted in abnormal F-actin morphology characterized by impaired capacity to form lamellipodia, a migration defect and reduced rates of adhesion to substratum and proliferation. The results point at a novel function of ORP2 as a regulator of the actin cytoskeleton, with impacts on hepatocellular migration, adhesion, and proliferation. However, one has to bear in mind that the above data is based on work with cancerous hepatic cells. It is therefore important to carry out similar analyses in untransformed primary hepatocytes in the future.

## 8. Mutations in *OSBPL2* are associated with hereditary non-syndromic hearing loss

Two studies reported in 2015 that human mutations in *OSBPL2* associate with autosomal dominant non-syndromic hearing loss (ADNSHL)[30, 45]. The syndrome is genetically heterogeneous: 65 loci and more than 30 causative genes have been designated. Thoenes at el. [30] carried out genome-wide linkage analysis and whole exome sequencing, identifying a co-segregating heterozygous frameshift mutation in *OSBPL2*, which truncates the encoded protein. Immunohistochemistry in mouse cochlea by using a commercial ORP2 antibody suggested the presence of ORP2 protein in the inner and outer hair cell stereocilia, structures which strongly depend on F-actin [46]. Similar observations were reported in a large Chinese family by Xing et al. [45], who also found a frameshift mutation in *OSBPL2* co-segregating with the ADNSHL phenotype. Moreover, they found another missense mutation (c.583C>A transversion; p.Leu195Met) in the gene in a sporadic case of the disease. Although the causality of these mutations is not 100% certain, they make *OSBPL2* an obvious candidate gene for the disease, especially since also mutations in *DIAPH1*, an interaction partner of ORP2 [36], are reported to cause hearing loss [47]. We find it likely that the association of *OSBPL2* mutations with hereditary hearing loss reflects a function of the protein in actin cytoskeletal regulation (see section 7). Whether the functional cross-talk of ORP2 with the actin cytoskeleton involves sterol binding or transport by the protein is largely unclear; However, the capacity of ORP2 re-introduced into knock-out HuH7 cells to alter cell surface and F-actin morphology and to replenish migration in a wound healing assay

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depended on the ability of ORP2 to bind phosphoinositides [35], suggesting that the F-actin associated function of ORP2 involves the binding of PIPs by the protein.

## 9. Conclusions and future perspectives

Recent studies employing knock-down or knock-out of ORP2 in different cell types have suggested a bewildering multitude of functions for this lipid-binding protein: Cholesterol and PIP transport, steroid hormone synthesis, TG metabolism, F-actin regulation, cell adhesion, migration and proliferation. Although ORP2 lacks a membrane-targeting PH domain, it can associate with membranes via its FFAT motif and patches of charged amino acid residues [33]. The current data suggests that it is capable of mediating inter-organelle lipid transport by a 'countercurrent' mechanism: the transport of cholesterol to the PM in exchange for $PI4,5P_2$ [33]. In this respect it thus resembles the 'short' yeast ORPs Osh4p, Osh6 and Osh7p [19, 20]. In addition to lipid transport and metabolism, ORP2 also executes functions in cell signaling. This could obviously be secondary to changes in membrane lipid compositions or lateral organization, but our recent findings suggest that protein-protein interactions may play crucial roles here. The role of ORP2 in PI3K/Akt signaling may involve interactions with IQGAP1, Cdc37 and Hsp90, while its functions in F-actin regulation could involve the observed interactions with DIAPH1, SEPT9, ARHGAP12 and MLC12. Moreover, the capacity of ORP2 to bind and transport PIPs is apparently important for its functions in F-actin regulation [35].

To gain a thorough understanding of the physiologic function of ORP2, progress on three major goals is required: (i) The role of lipid binding by ORP2 in terms of the functions of this protein in signaling and F-actin regulation needs to be elucidated. (ii) ORP2/Osbpl2 knock-out mice need to be carefully characterized in terms of their cholesterol/lipid and carbohydrate metabolism, insulin signaling, hearing and other F-actin-dependent physiologic processes. (iii) Since ORP2 is expressed ubiquitously in many cell types, its activities need to be investigated in the functional contexts of distinct cell types both in culture and *in vivo* by employing tissue/cell type-specific knock-out animal models. Additionally, the localization of ORP2 at the ER, LDs and late endocytic compartments raises questions of its possible functions in ER stress responses and lipophagy. Elucidating in detail the functions of ORP2 and other OSBP-related gene products will most certainly open new avenues in the research of organelle lipid homeostasis and their

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coordination with signaling events that control functions such as cell adhesion, motility and proliferation.

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**Disclosure**

The authors have no conflicts of interest to disclose concerning this work.

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**Figure captions**

**Fig. 1.** Alignment of ORP2 protein with the closely related members in subfamily II, ORP1L and ORP1S. ANK, ankyrin repeats; PH, pleckstrin homology domain; FFAT, two phenylalanines in an acidic tract; ORD, OSBP-related (ligand-binding) domain; OF, OSBP fingerprint sequence (EQVSHHPP), a conserved sequence motif with a crucial role in phosphoinositide binding. LE, late endosome; VAP, VAMP-associated protein; ER, endoplasmic reticulum; PIP, phosphoinositide

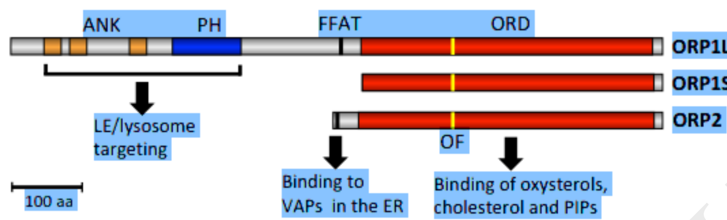




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**Fig. 2.** Fluorescence microscopy images depicting the various subcellular localizations of ORP2. A. Biomolecular fluorescence complementation (BiFC) analysis of ORP2-VAPA complexes in human umbilical vein endothelial cells (HUVECs) transfected for 24 h by electroporation (Nucleofector™, Lonza Bioscience). Note the reticular ER pattern and intense staining at ER domains encircling lipid droplets (LDs) stained with BODIPY-$C_{12}$ (Molecular Probes/Thermo Scientific). B. Localization of ORP2-GFP at the cell cortex, where it co-localizes with the lamellipodial marker cortactin (stained with monoclonal antibody 4F11; Upstate Biotechnology). C. Localization of ORP2-GFP in late endosomes/lysosomes identified by using internalized Alexa Fluor™647-dextran (Molecular Probes/Thermo Scientific).

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Fig. 2

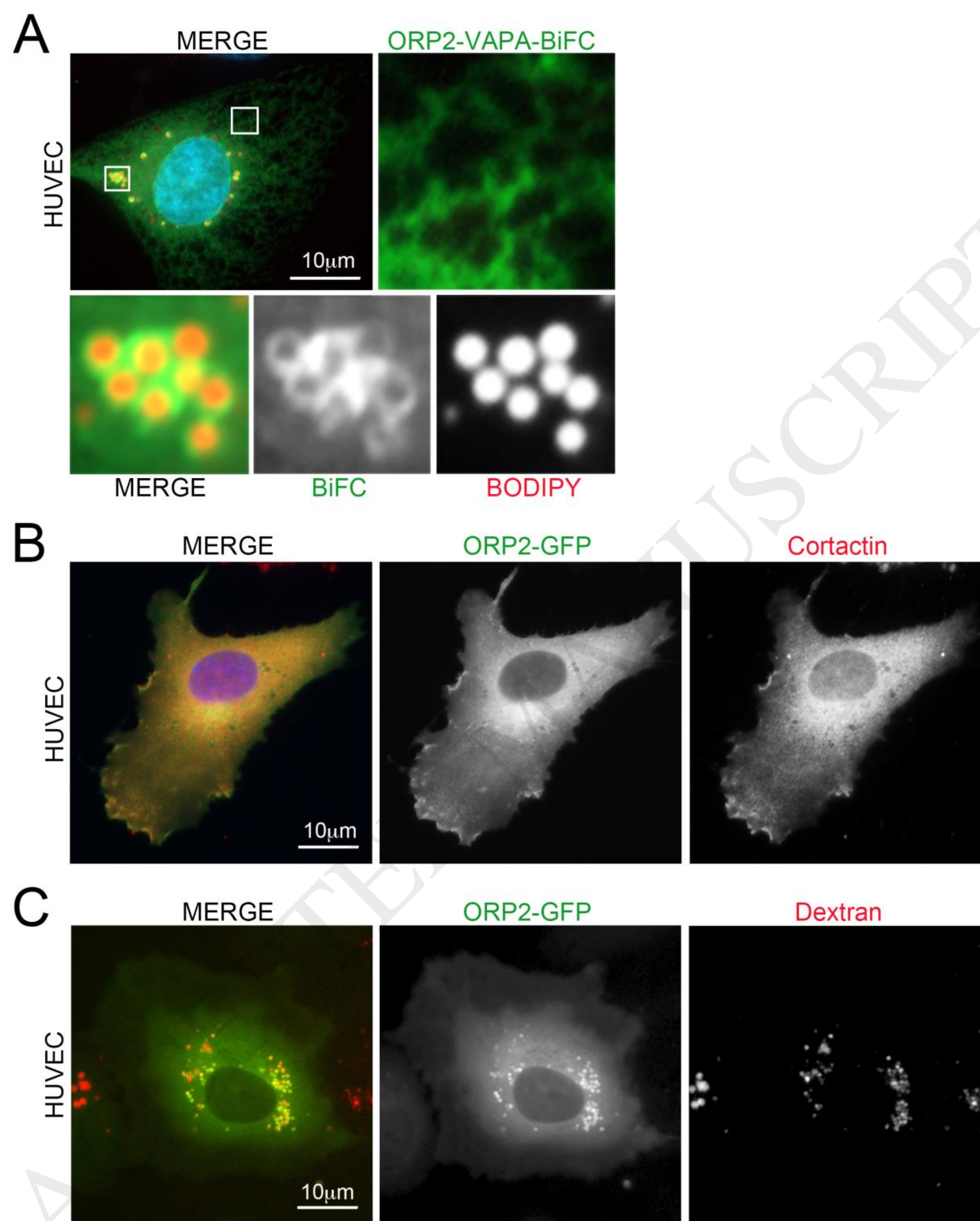

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**Fig. 3.** A schematic image depicting the reported functions of ORP2 in cellular cholesterol and steroid metabolism. The references from which each piece of information originates are indicated in brackets. Published data suggest roles of ORP2 in cholesterol transport from late endosomes (LE)/lysosomes and possibly other compartments to the plasma membrane [29, 33], in transcriptional regulation of sterol metabolism and steroidogenesis [31], in cholesterol efflux from cells [24,32], and cholesterol transport from the plasma membrane to the ER and lipid droplets (LD)[34]. ORP2 is detached from LDs by the high-affinity ligand 22(R)-hydroxycholesterol [22(R)OHC][25]. LXR, liver X receptor; SF1, steroidogenic factor 1; ER, endoplasmic reticulum; CE, cholesterol ester; CYP11B1, cytochrome P450 family 11 subfamily B member 1; ABCA1, ATP-binding cassette transporter A1; HDL, high-density lipoprotein.

Fig. 3

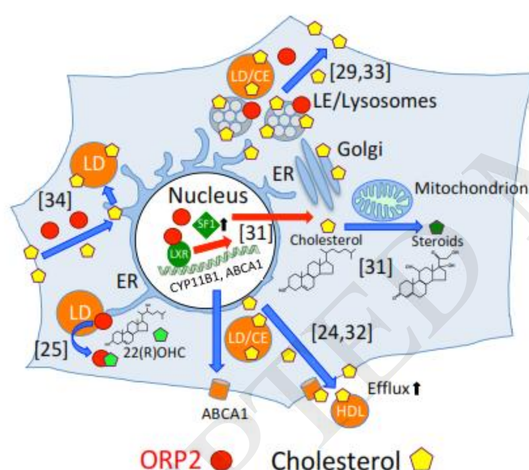

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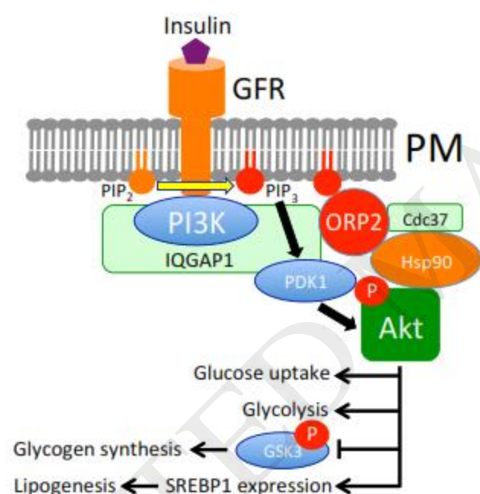
**Fig. 4.** A schematic model on the function of ORP2 in PI3K/Akt signaling and cellular energy metabolism. The Akt signaling pathway is stimulated by growth factors (such as insulin), which bind to plasma membrane (PM) growth factor receptors (GFR) resulting in the activation of phosphoinositide-3-kinase (PI3K). PI3K phosphorylates $PIP_2$ to $PIP_3$, which triggers the recruitment of phosphoinositide-dependent kinase 1 (PDK1) and Akt to the PM. ORP2 interacts with IQGAP1 which scaffolds the phosphoinositide kinases at the PM. Akt interacts at the PM with its effector complex Hsp90-Cdc37-ORP2 and is phosphorylated by PDK1. Active Akt regulates a number of downstream target molecules (including glycogen synthase kinase 3, GSK3), resulting in increased uptake of glucose, glycogen synthesis, glycolysis, and lipogenesis via sterol regulatory element binding protein-1 (SREBP-1).

Fig. 4.

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