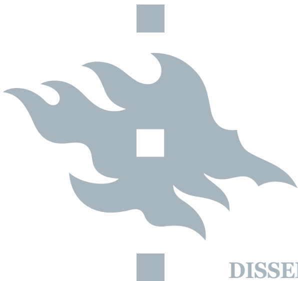

DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

47/2018

HENRIIKKA KENTALA

# ORP2 – A Sterol Sensor Controlling Hepatocellular Bioenergetics and Actin Cytoskeletal Functions

MINERVA FOUNDATION INSTITUTE FOR MEDICAL RESEARCH
DIVISION OF BIOMEDICINE
DEPARTMENT OF ANATOMY
FACULTY OF MEDICINE
DOCTORAL PROGRAMME IN BIOMEDICINE
UNIVERSITY OF HELSINKI

**Henriikka Kentala**

# ORP2 – a sterol sensor controlling hepatocellular bioenergetics and actin cytoskeletal functions

ACADEMIC DISSERTATION

*To be presented with the permission of Faculty o Medicine of the University of Helsinki, for public examination in Seth Wichmann Hall at Women's Hospital, on August 18th 2018, at 12 noon.*

Minerva Foundation Institute for Medical Research

and

Doctoral School in Health Sciences (DSHealth), Doctoral Programme in Biomedicine (DBPM)

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Helsinki 2018

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ISBN 978-951-51-4380-8 (paperback)
ISBN 78-951-51-4381-5 (PDF, http://ethesis.helsinki.fi)

ISSN 2342-3161 (paperback) and ISSN 2342-317X (PDF)

*"If you expect to see the final result of your work,*
*you simply have not asked a big enough question"*
*-I.F. Stone*

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## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals:

**I** **Kentala H**, Pfisterer SG, Olkkonen VM, Weber-Boyvat M. Sterol liganding of OSBP-related proteins (ORPs) regulates the subcellular distribution of ORP-VAPA complexes and their impacts on organelle structure. Steroids 2015; 99:248–258

**II** **Kentala H**, Koponen A, Kivelä AM, Andrews R, Li C, Zhou Y, Olkkonen VM. Analysis of ORP2 knockout hepatocytes uncovers a novel function in actin cytoskeletal regulation. FASEB J 2018; 32(3):1281–1295

**III** **Kentala H**, Koponen A, Vihinen H, Pirhonen J, Liebisch G, Pataj Z, Kivelä AM, Li S, Andrews R, Meriläinen L, Matysik S, Ikonen E, Zhou Y, Jokitalo E, Olkkonen VM. OSBP-realted protein 2 (ORP2): A novel Akt effector that controls cellular energy metabolism. Cel Mol Life Sci 2018; in press

Author's contribution to the publications:

I Contributed to the design of the experiments, performed the experimental work with exclusion of TLC and the TLC quantification, analysed the data and participated in writing the manuscript.

II Contributed to design of the project and the experimental work, performed most of the experiments, analysed most of the data and wrote the manuscript.

III Contributed to design of the project and the experimental work, performed most of the experiments, analysed most of the data and wrote the manuscript.

# ABSTRACT

OSBP-related proteins (ORPs) are lipid binding and transport proteins expressed ubiquitously in eukaryotic cells. ORPs localize at specific organelle interfaces designated membrane contact sites (MCSs). They possess a characteristic ligand-binding domain with specificity for oxysterols, cholesterol and phospholipids. Some ORPs have the capacity to mediate bi-directional transfer and exchange of two different lipids over MCSs. However, a number of findings suggest that a majority of the ORP family members act as lipid sensors with cell signalling properties rather than actual lipid transporters. ORP2 localizes on the surface of cellular lipid droplets. Earlier finding suggests that ORP2 contributes in triglyceride (TAG) and cholesterol homeostasis but the comprehensive knowledge of the cellular function of ORP2 is deficient.

In my thesis I investigated the sterol regulation of the subcellular localization ORP–VAP complexes and the role of ORP2 in cellular physiology by generating a stable ORP2 knock out (KO) cell line in HuH7 hepatocytes. I demonstrated that the subcellular distribution of OSBP–, ORP4L– and ORP2–VAP-A complexes at MCSs are regulated by ORP oxysterol liganding and by the cellular cholesterol status. Moreover, ORP2-KO impaired a diversity of cellular functions. The ORP2-KO affected mRNAs of >2000 genes, including TAG and glucose processing metabolic enzymes, components of several cell signalling processes and actin cytoskeleton regulators.

Analysis of ORP2 interaction partners revealed novel connections of ORP2 with PI3K/Akt and RhoA signalling, important regulators of cell metabolic processes and the actin cytoskeleton. Consistently, the ORP2-KO impaired the hepatocellular energy metabolism by inhibiting the synthesis of TAGs and glycogen, and by reducing the rates of glucose uptake and glycolysis, suggesting an extensive contribution of ORP2 to cellular bioenergetics.

In addition, I present novel clues of ORP2 function beyond the lipid and energy metabolism. In migrating cells, ORP2 was found to localize to lamellipodial cell surface protrusions, and the ORP2-KO, on the other hand, was found to impair the lamellipodia formation. Moreover, ORP2-KO resulted in an inhibition of cell adhesion, migration and proliferation, important cell physiological processes involving function of the actin cytoskeleton.

The results point at a novel function of ORP2 as a lipid-sensing regulator of the actin cytoskeleton, which importantly involves the integration of cellular metabolism with growth, migration and cell signalling.

## ABBREVIATIONS

| | |
|---|---|
| ACC | acetyl-CoA carboxylase |
| ACL | ATP citrate lyase |
| ADP | adenosine diphosphate |
| AMP | adenosine monophosphate |
| Arhgap12 | Rho GTPase activating protein 12 |
| Arp2/3 | actin related protein 2/3 |
| ATP | adenosine triphosphate |
| BiFC | bimolecular fluorescence complementation technique |
| CERT | ceramide transporter |
| ChREBP | carbohydrate response element binding protein |
| CPT | carnitine palmitoyltransferase |
| DAG | diacylglycerol |
| DGAT | diacylglycerol acyltransferase |
| DHPR | dihydropyridine receptor |
| DIAPH1 | diaphanous-1 (gene) |
| DRP1 | dynamin-related protein 1 |
| ER | endoplasmic reticulum |
| ERK | extracellular signal-regulated kinase |
| ERM | ezrin/radixin/moesin family |
| ERMES | ER-mitochondria encounter structure |
| E-Syt | extended synaptotagmin |
| FAS | fatty acid synthase |
| FFAT | two phenylalanines in an acidic tract motif |
| GK | glucokinase |
| GKRP | glucokinase regulatory protein |
| GLUT | glucose transporter |
| GPAT | glycerol-3-phosphate acyltransferase |
| gRNA | guide RNA |
| GSK-3 | glycogen synthase kinase-3 |
| GST | glutathione S-transferase |
| GYS | glycogen synthase |
| G-6-P | glucose 6-phosphate |
| G6Pase | glucose-6-phosphatase |
| HA | hemagglutinin |
| HOPS | homoptypic fusion and vacuole protein sorting |
| HSL | hormone sensitive lipase |
| Hsp90 | heat shock protein 90 |
| HUVEC | human umbilical vein endothelial cells |
| IQGAP1 | Ras GTPase-activating-like protein |
| IPA | Ingenuity pathway analysis software |
| $IP_3R$ | inositol triphosphate receptor |
| -KC | ketocholesterol |
| KO | knock out |
| LD | lipid droplet |
| LDL | low-density lipoprotein |
| LE | late endosome |
| LPA | lysophosphatic acid |

| | |
|---|---|
| LTP | lipid transfer protein |
| LXR | liver X receptor |
| MAPK | mitogen-activated protein kinase |
| MCS | membrane contact site |
| MCU | mitochondrial $Ca^{2+}$ uniporter |
| mDia1 | diaphanous-1 |
| MFN | mitofusin |
| MLC | myosin light chain |
| mTOR | mammalian target of rapamycin |
| MVB | multivesicular body |
| NPC | Niemann-Pick C |
| -OHC | hydroxycholesterol |
| ORD | OSBP-related domain |
| ORP | OSBP-related protein |
| OSBP | oxysterol-binding protein |
| Osh | *S. cerevisiae* OSBP/ORP ortholog |
| PA | phosphatidic acid |
| PBS | phosphate-buffered saline |
| PC | phosphatidycholine |
| PDK | protein kinase D |
| PE | phosphatidylethanolamine |
| PEPCK | phophoenolpuruvate carboxykinase |
| PFK-1 | phosphofructokinase |
| PFKFB | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase |
| PHD | pleckstrin homology domain |
| PI | phosphatidylinositol |
| PIP | phosphatidylinositol phosphate |
| PI3K | phosphoinositide 3-kinase |
| PI4P5K | phosphatidylinositol-4-phosphate 5-kinase |
| PK | pyruvate kinase |
| PS | phosphatidylserine |
| PTPIP51 | protein tyrosine phosphatase-interacting protein 51 |
| PYGL | glycogen phosphorylase |
| ROCK | Rho-associated kinase |
| RyR | ryanodine receptor |
| SCD1 | stearoyl-CoA desaturase-1 |
| SM | sphingomyelin |
| SOCE | store-operated $Ca^{2+}$ entry |
| SREBP | sterol regulatory element binding protein |
| STARD3 | StAR-related lipid transfer domain protein 3 |
| STIM1 | stromal interaction molecule-1 |
| SR | sarcoplasmic reticulum |
| TAG | triacylglycerol |
| TCA | tricarboxylic acid |
| TGN | *trans*-Golgi network |
| VAP | VAMP-associated protein |
| VDAC | voltage-dependent anion channel |
| VLDL | very-low-density lipoprotein |
| WASP | Wiskott-Aldrich syndrome protein |

# 1 INTRODUCTION

Oxysterol-binding protein (OSBP) was initially discovered by A. Kandutsch and co-workers in the turn of 1980s, from a cytosolic protein fraction containing 25-hydroxycholesterol (Kandutsch *et al.*, 1977; Kandutsch and Thompson, 1980). Around the turn of the millennium and also by the efforts of our group, additional 11 mammalian OSBP-related proteins (ORPs) were characterized (Jaworski *et al.*, 2001; Laitinen *et al.*, 1999; Lehto *et al.*, 2001). The OSBP/ORP protein family is found to be present in all studied eukaryotes. In addition to 12 mammalian proteins, *Danio rerio* has 14 OSBP/ORP encoding genes (Zhou *et al.*, 2014), *Drosophila melanogaster* and *Caenorhabditis elegans* four genes (Alphey *et al.*, 1998; Ma *et al.*, 2010; Sugawara *et al.*, 2001), *Arabidopsis thaliana* 12 genes (Skirpan *et al.*, 2006) and *Saccharomyces cerevisiae* has seven ORP orthologs (Beh *et al.*, 2001).

A few decades of dedicated research on OSBP/ORPs have shed light on their ligand binding properties: They are currently known to bind various different types of lipids, not only oxysterols to which their name refers, but also cholesterol and glycerophospholipids including phosphoinositides (Olkkonen, 2013). However, there is no unambiguous perception of their cellular functions, as researches struggle to understand the relation between their function in intracellular lipid transport and in lipid sensing through which they apparently impact a broad spectrum of cell signalling events (Vihervaara *et al.*, 2011a). Nevertheless, their ubiquitous expression profile and their existence in all eukaryotes implicate a fundamental role for OSBP/ORPs in the cell physiology. This is supported by an early finding by Beh *et al.* (2001), describing that simultaneous deletion of all seven yeast proteins is lethal implying an essential role for ORPs in cell viability.

The general interest on OSBP/ORPs among researchers' have increased considerably in the recent years as the understanding of the role of organelle contacts sites in the intracellular communication has begun to emerge. Currently these specialized membrane structures are accepted as important mediators of a number of cellular processes, including the transmission of calcium signals and lipid trafficking (Helle *et al.*, 2013; Prinz, 2014). ORP family of proteins are implicated as prominent effectors in the communication of endoplasmic reticulum (ER) with other membranous organelles, such as Golgi apparatus, mitochondria, plasma membrane, endosomes and lipid droplets, due to their capacity to localize to distinct organelle contacts. In principle, ORP family have a potential capacity to bridge ER with almost all cellular membrane compartments, making them highly intriguing subjects in the research field of inter-organelle communication over membrane contact sites.

In the present series of studies I have mainly focused on ORP2. I have established a complete ORP2 knock out cell line by means of CRISPR-Cas9 mediated genome editing, in order to comprehensively study the effects of ORP2 depletion on cell

physiology. The cellular function of ORP2 was in previous studies linked to the metabolism of neutral lipids (Hynynen *et al.*, 2009; Weber-Boyvat *et al.*, 2015b), but the detailed mechanism of its action remained poorly understood. The present studies elucidate novel connections of ORP2 with the cell metabolic processes, not only in the regulation of cellular lipid storage, but also in cellular glucose homeostasis and in the signalling processes which ultimately control the cellular bioenergetics. Furthermore, these studies provide novel information of the involvement of ORP2 in cytoskeletal functions, including cell migration and proliferation. Such a comprehensive involvement of ORP2 in these cellular metabolic and cytoskeletal functions is not previously reported, for which reason the basic scientific knowledge of these cellular processes is summarized in the literature review. These studies also focused on to elucidating the role of ORP high-affinity sterol liganding on their targeting to membrane contact sites. The study employed the Bimolecular Fluorescence Complementation (BiFC) technique, which enabled me to limit the analysis to ORPs in interaction with their ER anchor protein VAP-A. The present studies imply the importance of ORP2 in the coordination of different cell physiological processes suggesting their intimate regulatory connections.

# 2 REVIEW OF THE LITERATURE

## 2.1 Mammalian oxysterol binding proteins

### *2.1.1 Structural features of ORPs*

Twelve *OSBP* genes encode a conserved family of mammalian proteins termed oxysterol-binding protein-related proteins (ORPs; Figure 1) (Anniss *et al.*, 2002; Jaworski *et al.*, 2001; Lehto *et al.*, 2001; Levanon *et al.*, 1990), named after the discovery of their ability to bind oxysterols as their ligands (Taylor *et al.*, 1984). OSBP/ORPs are, apart from ORP5 and ORP8, cytosolic proteins, but also localize to distinct cellular membranes via lipid or protein interactions enabled by their specific structural features. The unifying feature of all ORPs is a conserved carboxy-terminal OSBP-related domain (ORD) with a signature motif EQVSHHPP, which has been used for identifying the ORP family members (Jaworski *et al.*, 2001; Laitinen *et al.*, 1999). The domain itself includes a hydrophobic ligand-binding cavity and a mobile lid, which encloses the OSBP/ORP lipid ligand within the pocket and shields the hydrophobic lipid from the hydrophilic environment of cytosol by blocking the cavity entrance.

The detailed structures of mammalian ORPs have not been determined, but erudite deductions of ORPs structural characteristics have been made by exploiting structural information from *S. cerevisiae* OSBP/ORP orthologs termed Osh. Crystallographic characterization of Osh4 by Im. *et al.* (2005) led to a fundamental proposition that the ligand binding is important for determining the conformation of ORPs. The study concluded that the ligand binding immobilizes the otherwise instable lid region resulting in a more soluble protein conformation.

With the exception of ORP2, OSBP/ORPs contain an amino-terminal pleckstrin homology (PH) domain, which binds phosphatidylinositol phosphates (PIPs) at the surface of cellular membranes, which have an important role in subcellular targeting of the ORPs (Lagace *et al.*, 1997; Lehto *et al.*, 2005; Levine and Munro, 1998; Levine and Munro, 2002; Musacchio *et al.*, 1993; Ngo and Ridgway, 2009; Ridgway *et al.*, 1992). ORP1, ORP4 and ORP9 are expressed as two variants, long (L) and short (S), encoded by transcripts derived from different promoters, and the short variant similarly to ORP2 lacks this membrane-targeting PH-domain (Jaworski *et al.*, 2001). Moreover, the subcellular targeting of ORP1L is not mediated by a PH-domain but via protein interactions enabled by an additional ankyrin motif (Johansson *et al.*, 2005).

Furthermore, eight out of twelve ORPs contain a two phenylalanines in an acidic tract (FFAT) motif, which interacts with type-2 ER trans-membrane proteins VAMP-associated proteins (VAPs), thus attaching ORPs to a close association with the ER

(Kaiser *et al.*, 2005; Loewen *et al.*, 2003). On the other hand, ORP5 and ORP8 contain an ER trans-membrane domain, which docks these proteins predominantly to the ER (Du *et al.*, 2011; Yan *et al.*, 2008). Hence, ORP10 and ORP11 are the only family members, which are not shown to interact with the ER.

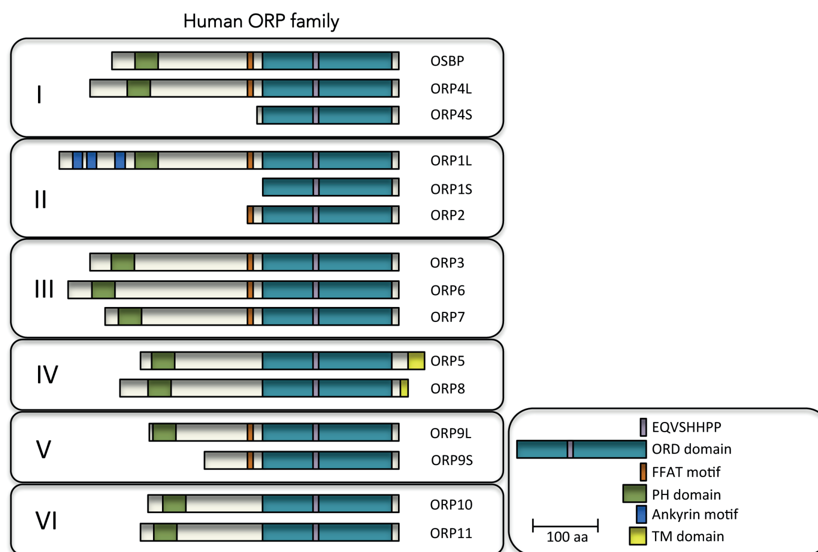


Figure 1. Schematic figure of the structural features of human ORP family. The roman numerals designates to different protein subfamilies defined by their sequence homology. Shown are ORP signature motif EQVSHHPP, OSBP-related domain (ORD), two phenylalanines in an acidic tract motif (FFAT), plecstrin homology domain (PHD), Ankyrin motif and transmembrane domain (TM). Letter 'L' indicates for long protein variant and 'S' for short.

### *2.1.2 Ligands of the ORPs*

Oxysterol-binding proteins were named after their first identified ligands, oxysterols, which are oxygenated derivatives of cholesterol – generated by cholesterol autoxidation or by enzymatic processing – with a number of reported cell signalling and cholesterol homeostatic properties (Olkkonen *et al.*, 2012; Schroepfer, 2000). Later ORPs were found to bind also cholesterol (usually with lower affinity) and glycerophospholipids. The currently characterized lipid ligands of the mammalian ORPs' ORD are summarized in the Table 1. ORPs binds lipids with a 1:1 stoichiometry and most likely the hydrophobic surface of the lid domain accessible in the open conformation helps to extract the lipid from membranes (Wang *et al.*, 2008a). The Osh4 structure revealed that the bound sterol is oriented in the ligand-binding pocket with the 3β-hydroxyl group facing to the bottom and the side chains interact with the lid at its inner hydrophobic surface. Importantly, the structure showed no direct interaction between hydroxyl-groups of the sterol and the protein

suggesting that Osh4 can bind a variety of different sterols with a low degree of ligand specificity. (Im *et al.*, 2005)

The ORPs' PH domain binds PIPs at organelle membranes, but later discoveries indicated that the ORD is capable of binding PIPs as well (de Saint-Jean *et al.*, 2011). In fact, the structure of Osh3 suggested that its ligand binding cavity is too narrow for sterols but binds PI(4)P instead (Tong *et al.*, 2013). In fact, it is suggested that PI(4)P binding is a unifying feature for the ORDs of all Osh/ORPs, recognized by highly conserved amino acid residues, and the sterol binding is an additional feature of only some of the family members (de Saint-Jean *et al.*, 2011; Tong *et al.*, 2013).

| | LIGANDS | REFERENCES |
|---|---|---|
| OSBP | 25OHC ($K_d$ 10 nM), cholesterol ($K_d$ 173 nM), 20OHC, 7KC, PI(4)P | (Dawson *et al.*, 1989; Mesmin *et al.*, 2013; Ridgway *et al.*, 1992; Wang *et al.*, 2005; Wang *et al.*, 2008a) |
| ORP1 | 24(S)OHC, 22(R)OHC, 25OHC ($K_d$ 97 nM), 7KC, cholesterol, PA, PS, PG, cardiolipin, PI(3)P, PI(4)P, PI(5)P, PI(3,4)$P_2$, PI(3,5)$P_2$, PI(4,5)$P_2$, PI(3,4,5)$P_3$ | (Fairn and McMaster, 2005a; Suchanek *et al.*, 2007; Vihervaara *et al.*, 2011b; Xu *et al.*, 2001; Yan *et al.*, 2007) |
| ORP2 | 22(R)OHC ($K_d$ 14 nM), 25OHC ($K_d$ 3.9 μM), 7KC ($K_d$ 140 nM), cholesterol, PA, cardiolipin, PI(3)P, PI(3,4)$P_2$, PIP$(3,5)_2$, PI(3,4,5)$P_3$ | (Hynynen *et al.*, 2005; Hynynen *et al.*, 2009; Suchanek *et al.*, 2007; Xu *et al.*, 2001) |
| ORP3 | ? | |
| ORP4 | 25OHC ($K_d$ 10 nM), 7KC, 20OHC, 22(R)OHC, 22(S)OHC, 7OHC, cholesterol, PI(4)P | (Charman *et al.*, 2014; Wang *et al.*, 2002; Wyles *et al.*, 2007) |
| ORP5 | PS, dehydroerhosterol, PI(3)P ($K_d$ 9 μM), PI(4)P ($K_d$ 6.7 μM), PI(5)P ($K_d$ 26.8 μM), PI(3,4)$P_2$ ($K_d$ 0.8 μM), PI(3,5)$P_2$ ($K_d$ 5.9 μM), PI(4,5)$P_2$ ($K_d$ 6.7 μM), $PIP_3$ ($K_d$ 1.1 μM) | (Du *et al.*, 2011; Ghai *et al.*, 2017; Maeda *et al.*, 2013) |
| ORP6 | ? | |
| ORP7 | ? | |
| ORP8 | PS?,cholesterol, 25OHC, PI(3)P ($K_d$ 1.7 μM), PI(4)P ($K_d$ 2.3 μM), PI(5)P ($K_d$ 5.9 μM), PI(3,4)$P_2$ ($K_d$ 2.5 μM), PI(3,5)$P_2$ ($K_d$ 3.6 μM), PI(4,5)$P_2$ ($K_d$ 5.3 μM), $PIP_3$ ($K_d$ 6 μM) | (Ghai *et al.*, 2017; Yan *et al.*, 2008; Zhou *et al.*, 2011) |
| ORP9 | cholesterol, PI(4)P, PI(5)P, PI(3)P,PI(3,5)$P_2$, PI(4,5)$P_2$ | (Fairn and McMaster, 2005b; Liu and Ridgway, 2014; Ngo and Ridgway, 2009) |
| ORP10 | PS, cholesterol, PI(3)P | (Fairn and McMaster, 2005b; Maeda *et al.*, 2013; Nissila *et al.*, 2012) |
| ORP11 | ? | |

Table 1. Identified mammalian OSBP/ORPs ORD ligands with known dissociation constants ($K_d$)

### *2.1.3 ORPs in intracellular lipid transport*

OSBP/ORPs are suggested to function in inter-organelle lipid transport or in lipid sensing with regulatory functions (D'Angelo *et al.*, 2008). Lipid trafficking is essential for constructing and maintaining the composition of organelle lipid membrane bilayers. The inter-organelle lipid transport occurs by vesicular or by non-vesicular mechanisms, but non-vesicular lipid trafficking is suggested to be more dominant (Lev, 2010). The non-vesicular transport upholds lateral diffusion of lipids and lipid transport by carrier proteins, of which the latter enables lipid movement across greater distances, as the lipid-enclosing proteins shield the hydrophobic lipids from the aqueous cytosol (D'Angelo *et al.*, 2008; Lev, 2010).

The most revealing evidences for OSBP/ORPs lipid transporter functions are derived from *in vitro* studies by using purified recombinant proteins. Actual lipid transfer activity *in vitro* has been shown for OSBP, ORP5, ORP8 and ORP9L (Chung *et al.*, 2015; Du *et al.*, 2011; Mesmin *et al.*, 2013; Ngo and Ridgway, 2009), but manipulation of the cellular levels of ORP1S/ORP2 was shown to affect the transport of exogenously administered cholesterol between plasma membrane (PM) and ER or lipid droplets (LDs) (Jansen *et al.*, 2011). Intriguingly, Mesmin *et al.* (2013) showed by using liposomes mimicking the ER and *trans*-Golgi membranes, that OSBP is capable of transporting cholesterol from ER to Golgi and exchanging it to PI(4)P which is then transported in opposite – from Golgi to ER – direction. After reaching ER, PI(4)P is dephosphorylated to phosphatidylinositol (PI) by the PI-phosphatase Sac1, and the energy consumed is utilised by OSBP for the continuum of cholesterol transport against its chemical gradient. On the other hand, the transport of PI from the ER back to the Golgi is suggested to occur via the PI-transporter Nir2, which is shown to restore Golgi PI(4)P levels and to support OSBP PHD Golgi targeting, presumably via PI phosphorylation by Golgi-localized PI4-kinases (Peretti *et al.*, 2008). The study by Mesmin *et al.* also pinpointed the importance of OSBP's structural features for this transporter function: In order to transport and exchange cholesterol for PI(4)P, OSBP needs both the FFAT motif and PHD to connectedly bind to ER and sense PI(4)P at Golgi membranes (Mesmin *et al.*, 2013). Furthermore, 25OHC binding to OSBP was shown to inhibit this bi-directional lipid transportation (Mesmin *et al.*, 2013).

The OSBP/ORP4L antagonist OSW-1, a drug that binds to the same ligand-binding cavity of OSBP as sterols, was established to inhibit OSBP function and promote its protein degradation (Burgett *et al.*, 2011). In line with reduction of OSBP-mediated cholesterol transport by 25OHC liganding, the same group later showed that inhibition of the endogenous OSBP by OSW-1 impaired cholesterol transport from ER to other membranes, including *trans*-Golgi network (TGN), resulting in cholesterol accumulation in the ER (Mesmin *et al.*, 2017). Moreover, OSW-1 treatment increased the cellular levels of PI(4)P and also its amount in TGN (Mesmin *et al.*, 2017). The studies of OSBP counter-transport function refers that cholesterol

transport against its chemical gradient is enabled by the energy provided by the retrograde transport and subsequent hydrolysis of PI(4)P, which is in line with the observed increment of PI(4)P as a result of OSBP inhibition (Mesmin *et al.*, 2017). This model could also serve as a plausible explanation for an earlier report observing that the knock down of Sac1 causes a disorganization of the Golgi membranes by an unknown mechanism (Liu *et al.*, 2008).

Similarly, the group of De Camilli and co-workers (2015) showed that ORP5 and ORP8 also exploit PI(4)P hydrolysis derived energy by Sac1 to counter-transport PI(4)P and phosphatidylserine (PS) between the ER and the PM, thus sustaining sufficient PM PS levels (Chung *et al.*, 2015). Importantly, a recent study by Ghai *et al.* (2017) demonstrated that the PHDs/ORDs of ORP5 and ORP8 do not exclusively bind PI(4)P but are able to bind all PIPs with a similar affinity, and moreover, the PM–ER PS counter-transport activity was shown to be even higher with PI(4,5)$P_2$ than with PI(4)P. In addition, ORP5/8 double knock down was shown to decrease the PM PS levels and increase the PI(4,5)$P_2$, further supporting a function of ORP5/8 in bi-directional transport of PS and PI(4,5)$P_2$ (Ghai *et al.*, 2017).

It is still unclear whether the lipid cargo transport by PIP counter-exchange is a universal function for ORP family members, but studies with yeast Osh proteins also support the role of a number of Osh's in bi-directional lipid/PIP transport (de Saint-Jean *et al.*, 2011; Maeda *et al.*, 2013; Moser von Filseck *et al.*, 2015). In addition, the above finding that PIPs other than PI(4)P could also serve as counter-transport substrates expands the possibility that such a function could be a general property among ORPs.

### *2.1.4 ORPs in cell signalling*

In addition to the above described findings of ORPs in lipid transport, various studies implicate OSBP/ORPs in cellular processes in which the role of their lipid transport activity is unknown. Hence various studies suggest that ORPs could in addition function as lipid sensors or receptors regulating cell signalling via effector proteins.

#### O S B P

OSBP – the archetype of oxysterol-binding proteins – is suggested to act as a sterol sensor regulating sphingomyelin (SM) synthesis at ER–*trans*-Golgi interfaces via protein kinase signalling. Cellular cholesterol depletion or external 25OHC treatment is shown to promote OSBP *trans*-Golgi localization (Mohammadi *et al.*, 2001; Ridgway *et al.*, 1992; Ridgway *et al.*, 1998; Storey *et al.*, 1998), mediated by the interaction of its PHD with PI(4)P and with ADP-ribosylation factor (Arf1) (Godi *et al.*, 2004; Levine and Munro, 1998; Levine and Munro, 2002; Mesmin *et al.*, 2013).

The 25OHC liganding by OSBP was found to inhibit the cholesterol–PI(4)P counter-transport activity as described above, apparently by occupying the OSBP ORD. In turn, in the presence of 25OHC, OSBP was shown to facilitate association of a ceramide transporter CERT with *trans*-Golgi, required for the transport of ceramides from ER to Golgi apparatus for SM synthesis (Banerji *et al.*, 2010; Hanada *et al.*, 2003; Perry and Ridgway, 2006). This process is additionally regulated phosphorylation of OSBP at Ser240 by protein kinase D (PDK). OSBP phosphorylation inhibits its localization to Golgi in response to 25OHC or cholesterol depletion, which consequently reduces the ceramide transport by impairing the Golgi localization of CERT and promotes Golgi fragmentation (Nhek *et al.*, 2010).

On the other hand, when bound with cholesterol instead of 25OHC, OSBP was found to form a complex with active form of PP2A and HePTP phosphatases (Wang *et al.*, 2005; Wang *et al.*, 2008a), which regulate the activity of extracellular signal-regulated kinases ERK1/2, important factors in the mitogen-activated protein kinase (MAPK) signalling pathway. Increment of 25OHC or cholesterol depletion was found to dissociate OSBP from the complex and disrupt the complex assembly resulting in the phosphorylation of ERK1/2. OSBP is also reported to mediate a 7-ketocholesterol (7KC) induced up-regulation of the actin binding protein Profilin-1 via a JAK-2/STAT3 signalling pathway (Romeo and Kazlauskas, 2008). However, the affinity of OSBP for 7KC is only moderate (Dawson *et al.*, 1989), thus it is possible that other unidentified factors participate in this process.

Regardless, it is evident that OSBP executes its cell signalling functions in a sterol-dependent manner, showing different modes of action in the presence of 25OHC or cholesterol, supporting the idea that OSBP is a multifunctional sterol sensor/transporter affecting various cellular processes including cell signalling and lipid homeostasis.

### ORP3

Unlike OSBP, the ORP3 ORD is unlikely to accommodate sterols but presumably binds PI(4)P (Zhou *et al.*, 2014), and the PHD is shown to interact with $PI(3,4)P_2$ and $PI(3,4,5)P_3$ at the PM (Lehto *et al.*, 2005). ORP3 also interacts with VAPs at the ER, the affinity of which was shown to be regulated by ORP3 phosphorylation status (Weber-Boyvat *et al.*, 2015a). ORP3 interacts with a small GTPase R-Ras, and manipulation of ORP3 controls various R-Ras downstream signalling processes including cell adhesion and migration, organization of the actin cytoskeleton, Akt phosphorylation and β1-integrin activity (Lehto *et al.*, 2008; Weber-Boyvat *et al.*, 2015a). Moreover, R-Ras signalling was suggested to originate at ER–PM membrane contact sites (MCS), as the activation of R-Ras by ORP3 was found to be dependent on ORP3 targeting to both the ER and the PM (Weber-Boyvat *et al.*, 2015a).

### O R P 9

ORP9 is also suggested to regulate Akt activity, thus potentially controlling a variety of functions subjected to regulation by PI3K/Akt signalling. ORP9S/L was identified as a substrate for phosphoinositide-dependent kinase-2 (PDK-2) at Ser287 (Lessmann *et al.*, 2007), which also phosphorylates Akt at Ser473 (Kawakami *et al.*, 2004). Phosphorylation of ORP9 by PDK-2 was found to depend on protein kinase Cβ or mammalian target of rapamycin (mTOR), and negatively regulate Akt phosphorylation at Ser473 (Lessmann *et al.*, 2007).

### O R P 5

A recent study by Du *et al.* (2018) suggested that the cellular function of ORP5 is not limited to PS transport at ER–PM MCSs, but it regulates mTORC1 signalling. The study suggested, that dependent on its lipid-binding pocket, ORP5 interacts with mTORC1. Moreover, ORP5 overexpression was found to induce mTORC1 signalling activity, and to enhance cell migration and proliferation of HeLa cells, also dependent on ORP5 ability for ligand binding. ORP5 silencing, on the other hand, was found to inhibit mTORC1 and disrupt mTOR localization to lysosomes. (Du *et al.*, 2018) This further suggests that the lipid transporting is nonexclusive function of ORPs, but in addition they are lipid sensors contributing to cell signalling processes, which direct connection to the lipid transporting is rather undecipherable.

### O R P 2

Similar to OSBP, sterol binding was also shown to regulate the subcellular targeting of ORP2. ORP2 is a cytosolic protein which also targets the ER via VAP-binding and is also found on the surface of the intracellular LDs, a targeting which is abolished by an external 22(R)OHC treatment (Hynynen *et al.*, 2009). The co-expression of ORP2 and VAP-A was observed to enhance the LD targeting of ORP2, and a sterol-binding deficient mutant of ORP2 also displayed enhanced LD association (Hynynen *et al.*, 2009; Weber-Boyvat *et al.*, 2015b). It is however unclear what physiological function of ORP2 this sterol liganding regulates.

The manipulations of ORP2 cellular levels correlate with the homeostatic regulation of trigacylglycerols (TAGs) (Hynynen *et al.*, 2009; Weber-Boyvat *et al.*, 2015b), which together with cholesterol esters constitute the lipophilic core of LDs. In addition, ORP2 promotes transport of newly synthesized cholesterol to the PM as well as cholesterol efflux (Hynynen *et al.*, 2005; Jansen *et al.*, 2011; Laitinen *et al.*, 2002). Consistently, co-suppression of ORP2 and ORP1S expression was found to inhibit cholesterol transport from the PM to the ER and LD (Jansen *et al.*, 2011). In adrenocortical cells, ORP2 was suggested to be important for LXR nuclear

localization and ORP2 silencing was shown to increase the levels of cellular cholesterol while decreasing 22(R)OHC and 7KC (Escajadillo *et al.*, 2016). Furthermore, adrenocortical cells have an important physiological role in the production of steroid hormones, and ORP2 was shown to be physically involved in a protein complex, which regulates cortisol synthesis (Li *et al.*, 2013). Consistently, silencing of ORP2 in adrenocortical cells was shown to reduce expression of proteins involved in cortisol synthesis as well as the amounts of several steroid metabolites, and increase the concentrations of androgens and estrogens (Escajadillo *et al.*, 2016).

In addition, ORP2 interacts with diaphanous-1 (mDia1) (Li *et al.*, 2013), an important molecular component in RhoA signalling. mDia1/RhoA also regulate the steroid hormone production of adrenocortical cells but additionally have a pivotal role in the actin cytoskeleton and microtubule organization (Geneste *et al.*, 2002; Li *et al.*, 2013; Nakano *et al.*, 1999; Palazzo *et al.*, 2001). Mutations in mDia1 are connected with autosomal dominant hearing loss (Kang *et al.*, 2017; Neuhaus *et al.*, 2017; Stritt *et al.*, 2016), where it is suggested to play a role in actin polymerization in inner ear hair cells (Stritt *et al.*, 2016). Intriguingly, ORP2 was also found to be expressed in inner and outer hair cell stereocilia, and a frameshift mutation in the ORP2 encoding gene was suggested to cause a form of autosomal dominant hearing loss (Thoenes *et al.*, 2015; Xing *et al.*, 2015), consistent with a functional linkage between ORP2 and mDia1.

## 2.2 Membrane contact sites in mammals

### *2.2.1 Biology of membrane contact sites*

Eukaryotic cells are compartmentalized into intracellular organelles specialized in different biological processes encompassing organelle specific proteins and other components necessary for their function. These organelles are usually membrane enclosed regions separated from the cytosol in order to have their own optimal physical and chemical micro-environments. However, for a cell to operate as a functional integrity and sustain life, all cellular compartments need to be connected and co-operated optimally. One of the mechanisms how organelles are able to communicate with each other is over membrane contact sites, which are described as close appositions (10–30 nm) between membranes of two organelles without involving membrane fusion (Helle *et al.*, 2013; Prinz, 2014). The existence of these sites has been known for decades (Copeland and Dalton, 1959; Porter and Palade, 1957; Robertson, 1960), yet the knowledge of the function of these membrane domains is still more or less insufficient. Currently there is no comprehensive information explaining the molecular machineries responsible for MCS biogenesis or the mechanisms of MCS regulation and function. MCSs are, however, found to be

universal architectures present in yeast, mammalian and plant cells, and similar structures are also found to exist in prokaryotes.

Most MCS are sites between ER and a second membranous organelle, such as Golgi apparatus, mitochondria, endosomes or plasma membrane. However, MCS also exist between non-ER organelles, such as lysosomes and peroxisomes, and between LD and mitochondria (Chu *et al.*, 2015; Jagerstrom *et al.*, 2009). The two membranes are kept in close proximity by tethering components allowing steady but temporally regulated connections. Up to this point, there is no indisputable evidence, which defines proteins involved in the MCS formation. The first intrinsic MCS tethers were identified by Kornmann *et al.* (2009) in yeast *S. cerevisiae* ER–mitochondria encounter structure (ERMES), in which a protein complex composed of four subunits [maintenance of mitochondrial morphology protein 1 (Mmm1) and mitochondrial distribution and morphology protein 10/12/34 (Mdm10/Mdm12/Mdm34)] tethering between the ER and mitochondria, was suggested to play a role in phospholipid synthesis and $Ca^{2+}$signalling. An additional groundbreaking achievement was made by the group of S. Emr by managing to abolish MCS between ER and PM in yeast by deleting six individual genes (Manford *et al.*, 2012). This study implicated that the proteins required for the existence of PM–ER contact sites in yeast are cortical ER protein Ist2 (related to mammalian anoctamin/TMEM16), tricalbin 1–3, and ER trans-membrane proteins Scs2/22. Of those Scs2, which is homologous to mammalian VAPs, was suggested to be the most essential (Manford *et al.*, 2012). Importantly, the study emphasised that MCSs are not necessarily formed by a single factor but by multiple determinants.

In mammalian cells the involvement of tricalbin homologues termed extended synaptotagmins (E-Syts) have been shown to play a role in the formation of MCSs between PM and ER (Chang *et al.*, 2013; Giordano *et al.*, 2013). E-Syts, like many other MCS tethering factors, have domains targeting both membranes, and the localization of E-Syts at PM–ER junctions are regulated by calcium and binding to PI(4,5)$P_2$ (Chang *et al.*, 2013; Giordano *et al.*, 2013; Idevall-Hagren *et al.*, 2015; Min *et al.*, 2007). Another family of established mammalian MCS promoters are junctophilins. Also junctophilins have structural features enabling them to bind two membranes simultaneously, and they are found to be responsible for the formation of contact sites between PM and sarcoplasmic reticulum (SR) in muscle cells (Garbino *et al.*, 2009; Takeshima *et al.*, 2000). The deletion of junctophilins does not, however, completely prevent the SR–PM contact site formation, which implies that also in myocytes the MCSs are rather formed by groups of proteins or protein complexes (Hirata *et al.*, 2006; Ito *et al.*, 2001).

To this day, dozens of proteins are found to localize and function at MCSs, and some of them presumably stabilize already formed contacts. However, the role of those tethering factors in the *de novo* MCSs formation is either unclear or for some of them even unlikely. The proteins found to localize at MCS and later discussed in this

chapter are listed in Table 2. Of note, MCSs can also form between compartments in the same organelle or inside organelles, for example between *cis*-, medial-, and *trans*-Golgi cisternae or between the inner and outer mitochondria membrane (Harner *et al.*, 2011; Schorr and van der Laan, 2018; Wollweber *et al.*, 2017; Xiang and Wang, 2010). This chapter, however, focuses on MCSs mediating communication between different organelles.

| PM–ER MCS | *PM* | *ER* | | References |
|---|---|---|---|---|
| | | junctophilins | Section 2.2.1 | (Garbino *et al.*, 2009; Takeshima *et al.*, 2000) |
| | | E- Syts | Section 2.2.1 | (Chang *et al.*, 2013; Giordano *et al.*, 2013) |
| | DHPRs | RyRs | Section 2.2.2 | (Rebbeck *et al.*, 2011) |
| | Orai1 | STIM1 | Section 2.2.2 | (Korzeniowski *et al.*, 2009; Maleth *et al.*, 2014; Park *et al.*, 2009; Wu *et al.*, 2006) |
| | | ORP5, ORP8 | Section 2.2.3 | (Chung *et al.*, 2015; Ghai *et al.*, 2017) |
| | Nir2 | VAPs | Section 2.2.3 | (Chang *et al.*, 2013; Chang and Liou, 2015; Kim *et al.*, 2015) |
| Mitochondria–ER MCS | *Mitochondria* | *ER* | | |
| | MFN2/MFN1 | MFN2 | Section 2.2.4 | (de Brito and Scorrano, 2008) |
| | PTPIP51 | VAP-B | Section 2.2.2 | (De Vos *et al.*, 2012) |
| | PTPIP51 | ORP5/8 | Section 2.2.3 | (Galmes *et al.*, 2016) |
| | VDAC1 | $IP_3R$ | Section 2.2.2 | (Csordas *et al.*, 2010; De Stefani *et al.*, 2016; Szabadkai *et al.*, 2006) |
| | DRP1 | | Section 2.2.4 | (Friedman *et al.*, 2011) |
| Golgi–ER MCS | *Golgi* | *ER* | | |
| | OSBP | VAPs | Section 2.2.3 | (Mesmin *et al.*, 2013; Mesmin et al., 2017) |
| | CERT | VAPs | Section 2.2.3 | (Hanada *et al.*, 2003; Perry and Ridgway, 2006) |
| LE–ER MCS | *Endosome* | *ER* | | |
| | ORP1L | VAPs | Section 2.2.4 | (Johansson *et al.*, 2007; Rocha *et al.*, 2009; van der Kant *et al.*, 2013) |
| | NPC1 | ORP5 | Section 2.2.3 | (Du *et al.*, 2011) |
| | STARD3 | VAPs | Section 2.2.3 | (Alpy *et al.*, 2013; Wilhelm *et al.*, 2017) |
| | Annexin A1 | S100A1 | Section 2.2.4 | (Eden *et al.*, 2016) |
| | Protrudin | VAPs | Section 2.2.4 | (Raiborg *et al.*, 2015) |

Table 2. Summary of the proteins and protein complexes tethering at distinct membrane contact sites discussed in this chapter.

*2.2.2 Cellular calcium homeostasis at organelle contacts*

One of the best known roles for MCSs is in the regulation of cellular calcium homeostasis. $Ca^{2+}$ is an important second messenger that, if uncontrolled, causes cell toxicity, and hence it needs to be conducted impeccably to its action site. MCSs provide solid pipelines for calcium crossover between membranes. One important site for calcium signalling is at the ER–PM MCSs, especially in muscle cells, where $Ca^{2+}$ has a crucial role in triggering muscle contractions. In dormant state the cytoplasmic $Ca^{2+}$ concentration is relatively low, but to activate muscle contraction, the $Ca^{2+}$ quickly flows into the cytosol from the sarcoplasmic reticulum and from the extracellular space through the PM, which allows myosin – an actin motor protein – to manoeuver along actin filaments in the sarcomeres. This process is enabled by PM $Ca^{2+}$ channels, dihydropyridine receptors (DHPRs), and ER $Ca^{2+}$ channels, ryanodine receptors (RyRs), which form a coupled gating at SR–PM contacts and in strict cooperation regulate the cytosolic $Ca^{2+}$ increase (Bannister, 2007; Bannister, 2016; Rebbeck *et al.*, 2011). The modulation of DHPR and RyR1 levels have no effect on the quantity of SR–PM contacts, suggesting only a functional role at these sites (Franzini-Armstrong *et al.*, 1991; Ikemoto *et al.*, 1997; Takekura *et al.*, 1995a; Takekura *et al.*, 1995b).

$Ca^{2+}$ homeostasis is also regulated at PM–ER contacts sites in non-contractile cells. ER is the storage compartment for cellular $Ca^{2+}$ and many cellular calcium-dependent processes takes place at this site. ER has a mechanism to preserve adequate levels of luminal calcium without significantly elevating cytosolic $Ca^{2+}$ via a process called store-operated $Ca^{2+}$ entry (SOCE): When the ER calcium levels reduce, the integral ER calcium sensor stromal interaction molecule-1 (STIM1) oligomerizes and undergoes a conformational change which allows it to bind $PI(4,5)P_2$ at PM and interact and activate the PM calcium channel Orai1, which mediates the calcium transmission into the ER lumen (Korzeniowski *et al.*, 2009; Liou *et al.*, 2005; Liou *et al.*, 2007; Luik *et al.*, 2008; Maleth *et al.*, 2014; Park *et al.*, 2009; Stathopulos *et al.*, 2008; Walsh *et al.*, 2009; Wang *et al.*, 2009; Wu *et al.*, 2006). The direct interaction between STIM1 and Orai1 at membrane contacts allows straight calcium influx into the ER (Jousset *et al.*, 2007).

$Ca^{2+}$ is important also for mitochondrial function. Mitochondria contain enzymes with important functions in cellular respiration which are activated by $Ca^{2+}$ (Denton, 2009). $Ca^{2+}$ is pumped from ER to mitochondria via channels which are interacting at ER–mitochondria MCSs (Rizzuto and Pozzan, 2006). The ER inositol triphosphate receptor ($IP_3R$) and outer mitochondria voltage-dependent anion channel (VDAC1) are found to bridge ER–mitochondria membranes with the cytosolic chaperone glucose-regulated protein 75 (GRP75) and mitochondrial $Ca^{2+}$ uniporter (MCU) complex to induce the $Ca^{2+}$ exchange (Csordas *et al.*, 2010; De Stefani *et al.*, 2016; Szabadkai *et al.*, 2006). In addition, ER resident VAP-B is found to interact with the mitochondrial membrane protein tyrosine phosphatase-interacting protein 51

(PTPIP51) and similarly regulate the $Ca^{2+}$ uptake at ER–mitochondria contacts (De Vos *et al.*, 2012).

### *2.2.3 Role of MCSs in lipid biosynthesis and transport*

Lipids are the major compounds of cellular membranes and different organelles have their own characteristic lipid composition affiliated with their function. The maintenance of the adequate lipid concentration of membranes requires lipid trafficking from their synthesis site, most commonly from the ER, to their destinations. However, due to their hydrophobic nature, maverick diffusion of lipids is limited and therefore lipids are transported intracellularly via non-vesicular or vesicular pathways. The non-vesicular lipid transfer is suggested to cover the major proportion of the cellular lipid transportation (Lev, 2010) in which they are often transported via carrier proteins enclosed with a hydrophobic ligand-binding pocket characteristic of these proteins.

Lipid transfer protein (LTP) is a generic name covering multiple protein families functioning in the intracellular lipid transport: PITP/Nir, START, GLTP, SEC14, SCP2, Ups/PRELI, TULIP, LAM and OSBP/ORP (Alva and Lupas, 2016; Burgardt *et al.*, 2017; D'Angelo *et al.*, 2008; Hsuan and Cockcroft, 2001; Lev, 2010; Wong *et al.*, 2017). Although LTPs carry lipids by moving within the cell between donor and acceptor membranes, there is emerging evidence implicating that a proportion of the lipid transfer occurs at closely apposed membranes that LTPs are bridging (Helle *et al.*, 2013). In fact, various LTPs from different protein families are found to tether at MCSs, in example PITP/Nir, START and OSBP/ORP (Alpy *et al.*, 2013; Chang and Liou, 2015; Chung *et al.*, 2015; Mesmin *et al.*, 2013; Peretti *et al.*, 2008). The studies of OSBP at *trans*-Golgi–ER interfaces and ORP5/ORP8 at ER–PM contacts have provided novel information of the mechanism of lipid transport over MCSs. These proteins are found to bi-directionally transport PI(4)P to ER for its hydrolysis and exploit the from the PI(4)P hydrolysis for the transport of counter lipid against its chemical gradient (Chung *et al.*, 2015; Ghai *et al.*, 2017; Mesmin *et al.*, 2013). The more detailed description of this bi-directional lipid transport as well as the role of OSBP/CERT in the synthesis of SM at *trans*-Golgi–ER contacts are described in chapters 2.1.3 and 2.1.4, respectively. These findings, however, illustrate examples of how MCSs orchestrate the maintenance of lipid compositions of cellular membranes.

Furthermore, ER–PM MCSs are suggested to regulate the $PI(4,5)P_2$ levels of the PM. $PI(4,5)P_2$ is an important signalling molecule that controls various cellular functions. The activation of G-coupled receptors at PM reduces the $PI(4,5)P_2$ which causes a disruption of E-Syt2 mediated ER–PM contacts and also reduces the interaction of phosphoinositide phosphatase Sac1 with the PM. The impaired association of Sac1 with the PM increases the PI(4)P precursor pool resulting in $PI(4,5)P_2$ elevation. The opposite effect is caused by activation of SOCE after ER luminal $Ca^{2+}$ depletion, in

which the Sac1 enhances its interaction with the PM at the E-Syt2 mediated ER–PM contacts, resulting to depletion of the PM PI(4)P. (Dickson *et al.*, 2016) On the other hand, the PM PI(4)P is generated from PI, which itself is produced at ER from phosphatidic acid (PA). For the sequential generation of PI(4,5)$P_2$, PI needs to be transported from the ER to the PM. The reduction of PM PI(4,5)$P_2$ via its hydrolysis to inositol 1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG) is found to induce Nir2 translocation to E-Syt1 mediated ER–PM contacts for the enhancement of PI transport to the PM via exchange for PA (Chang *et al.*, 2013; Chang and Liou, 2015). The localization of Nir2 to the PM is mediated via binding to PA, which is generated from DAG after the hydrolysis of PI(4,5)$P_2$, and at the ER–PM contacts, Nir2 exchanges the ER PI for the PM PA and counter transports PA to the ER (Figure 2) (Chang and Liou, 2015; Kim *et al.*, 2015).

The synthesis of phosphatidylethanolamine (PE) and phosphatidycholine (PC) are also suggested to occur via MCSs (Dimmer and Rapaport, 2017; Flis and Daum, 2013; Osman *et al.*, 2011). PS is the precursor for PE and PC, and is synthetized in the ER from the PA, which is then passed to inner membranes of mitochondria for PS decarboxylase to convert it to PE (Emoto *et al.*, 1999; Osman *et al.*, 2011). In addition, in hepatocytes the synthesis of PC takes place at the ER, hence, the precursor PE needs to be transferred back to the ER from the mitochondria for methylation via *N*-methyltransferase to generate PC (Figure 2). This phospholipid exchange between ER and mitochondrial membranes is suggested to occur through ER–mitochondria contacts (Daum and Vance, 1997; Dennis and Kennedy, 1972; Flis and Daum, 2013; Osman *et al.*, 2011; Rusinol *et al.*, 1994; Vance, 1990; Vance, 2014; Voelker, 1989; Wu and Voelker, 2004). A study by Galmes *et al.* (2016) suggested that in addition to localization of ORP5/8 to ER–PM MCSs, these PS transporting proteins also found at the ER–mitochondria contacts by interaction with the mitochondrial PTPIP51. The study reported that the localization of ORP5/8 to ER–mitochondria MCSs was dependent on the ORD, but direct PS transport of ORP5/8 from ER to mitochondria was not addressed in the study (Galmes *et al.*, 2016). The study, however, did not exclude the possibility that the PS transport from ER to mitochondria could also be mediated by ORP5/8. In fact, the finding that the PS transport over ER–PM MCS was shown to be higher with PI(4,5)$P_2$ counter-transport rather than with PI(4)P is further supporting the possibility (Ghai *et al.*, 2017), since PI(4,5)$P_2$ but not PI(4)P is found from the outer mitochondrial membrane (Rosivatz and Woscholski, 2011).

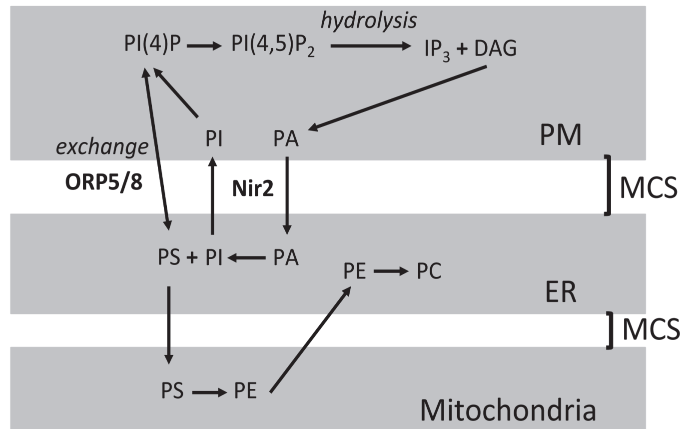


Figure 2. Schematic model of phospholipid transfer over ER–PM and ER–mitochondria MCSs. $PI(4,5)P_2$ is generated at PM by phosphorylation of PI to PI(4)P and then to $PI(4,5)P_2$. The hydrolysis of $PI(4,5)P_2$ generates $IP_3$ and DAG, from which the DAG is converted to PA. The PA at PM is exchanged for PI at ER via Nir2. At the ER, the PA is used for the synthesis of PS and PI. The PS is counter transported to PM via ORP5/ORP8, and the PI(4)P is transported in opposite direction. Alternatively, the PS is transported to mitochondria for the synthesis of PE. The newly formed PE is then transferred to the ER for the synthesis of PC.

Finally, the cholesterol transfer from the late endosome (LE)/lysosome to the ER is also suggested to occur directly through MCSs between ER and LE. Luminal endosomal cholesterol egress factor Niemann-Pick C2 (NPC2) transfers LDL-derived cholesterol to the LE limiting membrane cholesterol transporter NPC1, which subsequently transfers cholesterol to the ER (Infante *et al.*, 2008; Kwon *et al.*, 2009). This transfer is suggested to occur via interaction of NPC1 with ORP5 at ER (Du *et al.*, 2011), and via ORP1L–VAP mediated LE association with ER (Zhao and Ridgway, 2017). This is supported by findings that silencing of NPC1 or ORP5, or ORP1L knock out, results in cholesterol accumulation at LE and inhibition of its esterification, which occurs in the ER (Du *et al.*, 2011; Zhao and Ridgway, 2017). An other LTP found to transport cholesterol between ER–LE contacts is STARD3, which localizes to LEs, and similar to ORP1L, interacts with VAPs at the ER (Alpy *et al.*, 2013; Wilhelm *et al.*, 2017). Wilhelm *et al.* (2017) found that STARD3 transports cholesterol to opposite direction compared to NPCs, from the ER to the LE, and STARD3 overexpression was found to increase cholesterol accumulation in endosomes by routing its transport from the ER to LE and concomitantly decreasing the cholesterol from PM.

*2.2.4 Role of MCSs in organelle dynamics*

Endosomes traffic along microtubules during their maturation, which allows them to distribute endocytic cargo to different cellular compartments. Trafficking of endosomes is also associated with ER contacts, which at least in the case of endocytic transport of cholesterol is associated with endosome motility. Via its ankyrin motif, the cholesterol sensor ORP1L interacts with the LE GTPase Rab7 and Rab-interacting lysosomal protein (RILP). Upon high cholesterol conditions, this LE protein complex recruits dynein-dynactin motor complex and homotypic fusion and vacuole protein sorting (HOPS) on LE, which promotes LE trafficking towards the cell centre where it fuses with lysosome (Johansson *et al.*, 2007; Rocha *et al.*, 2009; van der Kant *et al.*, 2013). Conversely, when endosomal cholesterol is depleted, ORP1L is suggested to undergo enhanced interaction with ER-bound VAP increasing its association with ER–LE contacts. This results in the dissociation of dynein–dynactin motor complexes from LE and hence restricts the dynein-mediated LE mobility (Rocha *et al.*, 2009).

The formation and regulation of ER–LE contacts is suggested to occur via interactions of the endosome-localized Annexin A1 and its ER resident ligand S100A1 (Eden *et al.*, 2016). When the endosomal cholesterol depletes and the LE motility is restricted, Annexin A1 regulates the transfer of ER cholesterol to multivesicular endosome/bodies (MVBs) through the ER–LE contacts, which also requires the ORP1L–VAP interaction (Eden *et al.*, 2016). However, enhancement in ER–LE contacts does not necessarily implicate reduced LE motility. In fact, Friedman *et al.* (2013) showed that the ER–LE contacts are enhanced during LE maturation. Moreover, the above studies puzzlingly suggested roles of ORP1L the transport of cholesterol both from LE to ER and in the opposite direction. Of note, Vihervaara *et al*. (2011) showed that ORP1L knock down inhibited the efflux of endocytosed cholesterol from macrophages, supporting a function of the protein in the egress of cholesterol from LE. Future study is warranted to reach a full understanding of the function of ORP1L in intracellular cholesterol trafficking.

LE trafficking is also linked to ER–LE MCSs upon neurite outgrowth via the ER trans-membrane protein protrudin. Protrudin interacts on LE with Rab7 and PI(3)P, and with VAPs in the ER. In association with LEs, protrudin recruits kinesin-1 which promotes LE trafficking towards cell periphery resulting in endosome fusion with the PM, and overexpression or down-regulation of protrudin are found to result LE accumulation on cell periphery or in cell centre, respectively (Raiborg *et al.*, 2015). Finally, ER–LE contacts are also found as key components in the determination of the time and position of endosome fission (Rowland *et al.*, 2014).

Similarly, also the mitochondrial fission is associated with ER contacts. Mitochondrial fission is dependent on dynamin-related protein 1 (DRP1), which localizes to mitochondria–ER junctions before initiating the mitochondrial division

(Friedman *et al.*, 2011). In yeast the mitochondrial fission is mediated by the ERMES, the counterpart of which has not been found in mammals (Kornmann *et al.*, 2009; Murley *et al.*, 2013). Moreover, dynamin-like GTPase mitofusin 2 (MFN2) has been suggested tether between mitochondria and the ER, and at the ER, it homo- or heterodimerizes with mitochondrial MFN2 or MFN1, respectively (de Brito and Scorrano, 2008). Mitofusins are known to be involved in mitochondrial fusion (Chen *et al.*, 2003), but it is however unclear whether MFN2 at ER–mitochondria contacts promotes this fusion. Finally, ER-mitochondria contacts are also found to play an important role in the formation of autophagosomes (Hamasaki *et al.*, 2013).

## 2.3 Hepatic energy metabolism

### *2.3.1 Glucose uptake into hepatocytes*

Liver is an essential metabolic organ that for provides energetic substrates for other tissues such as skeletal muscle and adipose tissue. Hepatic energy metabolism is tightly controlled by neuronal, hormonal and nutrient signals, and the dysfunction of liver metabolic processes predisposes to various metabolic disorders such as type 2 diabetes and non-alcoholic fatty liver disease (NAFLD). One of the most essential nutrient is glucose since it can serve as a precursor for amino acids and lipids, or for the energy production. Glucose uptake from the bloodstream into the hepatocytes occurs largely via the GLUT2 plasma membrane glucose transporter (Nordlie *et al.*, 1999). After entering the hepatocytes, the glucose is rapidly phosphorylated to glucose 6-phosphate (G-6-P) by a glucokinase (GK) to prevent its exit back into the bloodstream, but in fasted state it can also be dephosphorylated back to glucose. G-6-P is a precursor for pyruvate and glycogen, which are discussed later in this chapter.

In non-hepatic insulin sensitive cells glucose uptake occurs largely via the GLUT4 glucose transporter, the translocation of which from intracellular vesicles to the PM is regulated by insulin (Watson *et al.*, 2004). In hepatocytes the insulin does not regulate the glucose uptake by mobilizing glucose transporters, but it stimulates GK expression and counteracts glucose absorption via an indirect mechanism (Kim *et al.*, 2004): The conversion of glucose to G-6-P reduces the concentration of intracellular glucose which promotes the glucose uptake by generation of a concentration gradient.

### *2.3.2 Glycogenesis and glycogenolysis*

Glucose cannot be stored within hepatocytes in long term, hence, it is processed and stored as the polysaccharide glycogen for its later demand. Glycogen is the main source of liver glucose during short-term fasting. In the synthesis of glycogen, G-6-P is first converted to glucose 1-phosphate and then activated by an addition of uridine

nucleotide to form UDP-glucose. Multiple UDP-glucoses are then chained by glycogenin and fully polymerized by glycogen synthase (GYS) (Adeva-Andany *et al.*, 2016). The reverse catabolic reaction is called glycogenolysis, in which the glycogen chain is degraded back to free glucose subunits by a glycogen phosphorylase (PYGL) (Agius, 2015).

G-6-P acts as an allosteric activator for GYS and allosteric inhibitor for PYGL (Agius, 2008), but the activity of GYS and PYGL are primarily regulated by kinases and phosphatases, as phosphorylation of GYS inhibits its activity and activates PYGL. GYS is a downstream target of phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathway which is regulated by insulin and other growth factors. Active Akt phosphorylates and inactivates the GYS phosphorylating kinase glycogen synthase kinase 3 (GSK-3), thus increasing glycogen synthesis via GYS as a downstream effect of insulin (Cross *et al.*, 1995). In the fasted state the levels of glucose and insulin are depleted, and glucagon increases, resulting in the activation of PYGL to elevate the intracellular glucose concentration (Agius, 2015).

### *2.3.3 Glycolysis*

The hepatic glucose in not only stored as glycogen, but it is also metabolized in a process termed glycolysis, which importantly provides both energy fuel and various building blocks for hepatocytes. In the glycolysis glucose is metabolized through various enzymatic steps finally to pyruvate. The rate limiting steps in this multiphase process are glucose phosphorylation to G-6-P by GK, fructose 6-phosphate phosphorylation to fructose 1,6-bisphosphate by phosphofructokinase (PFK-1), and phosphoenolpyruvate conversion to pyruvate by liver pyruvate kinase (L-PK), processes which are induced in the fed state by glucose and insulin and conversely inhibited during fasting. (Rui, 2014) In addition to glycolysis, the G-6-P can be alternatively catabolized in a parallel process termed pentose phosphate pathway, which generates NADPH required for lipogenesis.

The expression level of GK is markedly elevated after ingestion of carbohydrates. The transcription factor sterol regulatory element binding protein 1c (SREBP-1c) induces the gene expression of GK as a consequence of activated insulin/Akt signalling (Fleischmann and Iynedjian, 2000; Foretz *et al.*, 1999a; Kim *et al.*, 2004; Ribaux and Iynedjian, 2003). The GK is also regulated by glucokinase regulatory protein (GKRP): Under low cellular glucose conditions glycolysis is unwarranted causing GK to interact with GKRP, leading to the nuclear localization of this protein complex and preventing the cytosolic function of GK (Brouwers *et al.*, 2015). When the level of glucose is elevated, GK is enabled to dissociate from GKRP permitting its release from the nucleus to the cytosol.

PFK-1 primarily admits glucose to glycolysis in order to create more ATP, hence PFK-1 is allosterically activated by high concentrations of AMP and conversely inactivated by ATP. The final rate limiting enzyme regulating the levels of glycolysis is L-PK. L-PK converts phosphoenolpyruvate to pyruvate, which can be then passed to mitochondria. The expression of L-PK is also nutritionally regulated – glucose activates the transcription factor Carbohydrate response element binding protein (ChREBP), which binds to the *L-PK* promoter and permits its expression (Dentin *et al.*, 2004; Iizuka *et al.*, 2004; Kawaguchi *et al.*, 2001; Yamashita *et al.*, 2001). In addition, L-PK is subjected to allosteric regulation by fructose-1,6-bisphosphate. Fructose-1,6-bisphosphate binds to L-PK resulting in a conformational change, which activates L-PK (Jurica *et al.*, 1998). L-PK transcription is repressed by glucagon, which acts through its second messenger cyclic AMP (cAMP) (Vaulont *et al.*, 1984). cAMP also inactivates ChREBP by phosphorylation thus preventing its nuclear function (Denechaud *et al.*, 2008; Dentin *et al.*, 2012).

The cytosolic pyruvate formed as an end product of glycolysis enters mitochondria where it is decarboxylated by the key mitochondrial enzyme complex, pyruvate decarboxylase (PDC), to acetyl-CoA. The mitochondrial acetyl-CoA is then either completely oxidized in tricarboxylic acid (TCA) cycle and oxidative phosphorylation to generate high energetic ATP molecules, or alternatively, the acetyl-CoA is removed from the TCA cycle and carried back into the cytosol to be used for fatty acid synthesis. (Rui, 2014) In addition to pyruvate, the glycolysis provides also other important intermediates, which can be used for the synthesis of different metabolic compounds such as glycerol, which is required for fatty acid esterification. Moreover, glycolysis also provides precursors for amino acid and nucleotide synthesis.

### *2.3.4 Gluconeogenesis*

*De novo* glucose synthesis or gluconeogenesis is the main source of glucose after the depletion of stored glycogen during long term fasting or starvation. Liver is able to synthetize glucose from lactate, pyruvate, glycerol and amino acids. Lactate and pyruvate are converted to oxaloacetate in mitochondria, and then to phophoenolpuruvate by cytoplasmic phophoenolpuruvate carboxykinase (PEPCK). Phophoenolpuruvate is further converted to G-6-P and finally to glucose by glucose-6-phosphatase (G6Pase) through enzymatic steps reverse to those of glycolysis. Amino acids, on the other hand, are first converted to α-ketoacids, which can be then converted to gluconeogenic substrates such as pyruvate and oxaloacetate. Glycerol can be used for glucose synthesis by conversion to glycol-3-phosphate by glycerol kinase, which is a gluconeogenic intermediate. (Rui, 2014)

The regulation of gluconeogenesis depends on the metabolic state and the availability of gluconeogenic substrates, affecting the expression of gluconeogenic enzymes. Insulin suppresses gluconeogenesis and glucagon stimulates the expression of the rate

limiting enzymes PEPCK and G6Pase (Hatting *et al.*, 2018; Oh *et al.*, 2013; Sutherland *et al.*, 1996).

### *2.3.5 De novo lipogenesis*

Another important metabolic function of the liver is to convert absorbed carbohydrates into fatty acids. When carbohydrates are abundant, only part of the absorbed carbohydrates are stored as hepatic glycogen and the excess is used for *de novo* lipogenesis. Lipogenesis requires release of the acetyl-CoA from glucose through glycolysis and mitochondrial pyruvate decarboxylation, after which it enters the TCA cycle. Fatty acid synthesis occurs in the cytosol, but mitochondrial acetyl-CoA cannot directly switch over back to the cytosol from the mitochondrial TCA cycle, therefore it is first converted to citrate before transfer into the cytosol, after which it is converted back to acetyl-CoA by ATP citrate lyase (ACL). In the first enzymatic reactions of the lipogenesis, the cytosolic acetyl-CoA is carboxylated to malonyl-CoA via acetyl-CoA carboxylase (ACC), and the malonyl-CoA is then converted to palmitic acid by fatty acid synthase (FAS), which is the precursor molecule for the synthesis of the other phospholipid species and TAG. In the synthesis of TAGs, the other intermediate fatty acids are then produced by palmitic acid elongation and desaturation, which involves stearoyl-CoA desaturase-1 (SCD1). In the final stage, the fatty acids are esterified with glycerol-3-phosphate by glycerol-3-phosphate acyltransferase (GPAT) to lysophosphatic acid (LPA). LPA is used for the synthesis of DAG followed by the synthesis of TAG by diacylglycerol acyltransferase (DGAT). The TAGs produced in lipogenesis are either stored within hepatic LDs or secreted into bloodstream as very-low-density lipoprotein (VLDL) particles for the usage of the extrahepatic tissues. (Rui, 2014)

Dietary carbohydrates promote the lipogenesis since the rate of lipogenesis is highly reliant of the availability of lipogenic precursors (Figure 3). Hence it is not surprising that the *de novo* lipogenesis is principally regulated by the same two transcription factors as the glycolysis – SREBP-1c and ChREBP (Dentin *et al.*, 2004; Dentin *et al.*, 2005; Foretz *et al.*, 1999a). SREBP-1c and ChREBP themselves are subject to regulation by insulin and glucose, respectively, ensuring coordination between glucose and fatty acid metabolism. Together SREBP-1c and ChREBP regulate the expression of all pivotal lipogenic enzymes: ACL, ACC, FAS, SCD1, GPAT and DGAT (Berwick *et al.*, 2002; Dentin *et al.*, 2004; Dentin *et al.*, 2006; Foretz *et al.*, 1999a; Foufelle and Ferre, 2002; Horton, 2002; Iizuka *et al.*, 2004; Ishii *et al.*, 2004; Magana and Osborne, 1996). Conversely, the rate of lipogenesis is inhibited by dietary fats, polyunsaturated fatty acids in particular, which suppress the production of lipogenic enzymes (Blake and Clarke, 1990; Landschulz *et al.*, 1994).

*2.3.6 Lipolysis and fatty acid β-oxidation*

In the fasted state the acquisition of necessary energy from nutrients diminishes, hence the metabolic fuel needs to be released from the intracellular energy reservoirs. Lipolysis is a catabolic process which breaks down store stored TAGs from LDs by hydrolase enzymes termed lipases. Lipolysis occurs primarily in adipocytes, but also in other high-energy demanding cells such as muscle cells and hepatocytes. Adipose triglyceride lipase (ATGL), monoacylglycerol lipase (MGL) and hormone sensitive lipase (HSL) hydrolyse TAGs, DAGs and monoacylglycerols to release non-esterified fatty acids and glycerol (Zechner *et al.*, 2009). Those can then be exploited as precursors for membrane lipid synthesis or to be used for energy production via fatty acid oxidation.

For the mitochondrial fatty acid oxidation, fatty acids are esterified by fatty acyl-CoA synthase to create long-chain acyl-CoAs. Long-chain fatty acyl-CoAs are, however, unable to pass the mitochondrial membrane, thus carnitine palmitoyltransferase-1 (CPT1) converts long-chain acyl-CoA to long-chain acylcarnitine, which can be substituted with intra-mitochondrial carnitine. In mitochondria, long-chain acylcarnitine is transported across the inner mitochondrial membrane via carnitine translocase, after which it is converted back to long-chain acyl-CoA by carnitine palmitoyltransferase-2 (CPT2) for its complete oxidation. Four main enzymatic steps are required for β-oxidation in which long-chain acyl-CoAs are catalysed to acyl- and acetyl-CoAs, which can then be passed on to the TCA cycle and oxidative phosphorylation. (Rui, 2014) The main oxidative enzymes are acyl-CoA dehydrogenase, enoyl-CoA hydratase, hydroxyl acyl-CoA dehydrogenase and ketoacyl-CoA thiolase, of which the activity of the last three can be covered by mitochondrial trifunctional enzyme. Instead of the complete oxidation, the formed acyl- and acetyl-CoA molecules can also be used for the formation of ketone bodies through ketogenesis, which liver releases into the bloodstream for the use of other organs such as brain, heart and skeletal muscle.

Similar to gluconeogenesis, lipolysis and fatty acid β-oxidation are enhanced by glucagon and inhibited by insulin (Longuet *et al.*, 2008; Sidossis and Wolfe, 1996). Fasting also suppresses ChREBP and activates the nuclear receptor Peroxisome proliferator-activated receptor α (PPARα), which induces genes involved in β-oxidation and ketogenesis (Dentin *et al.*, 2006; Kersten *et al.*, 1999; Kersten, 2014). It is vital that the rates of catabolic and anabolic processes are tightly co-regulated by the availability of external nutrients for the liver to ensure constant but efficient supply of energy compounds for other tissues.

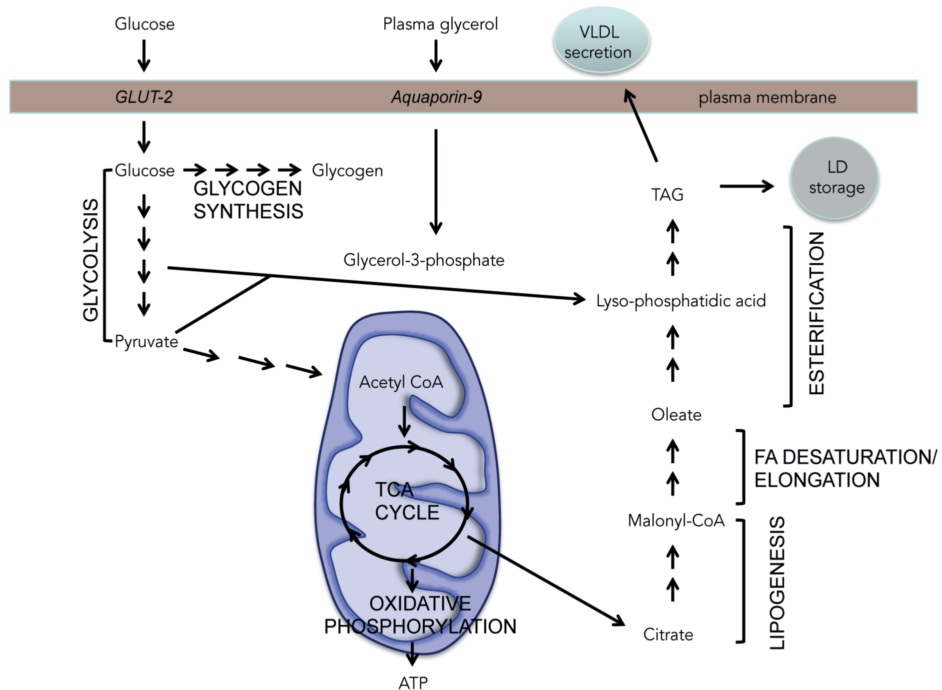


Figure 3. Schematic figure of glucose conversion into fatty acids in hepatocytes. Glucose enters to hepatocytes via plasma membrane GLUT2 transporter, after which glucose is either subjected to glycogens synthesis or glycolysis to generate pyruvate. The pyruvate is channelled into TCA cycle and completely oxidized in oxidative phosphorylation to create ATP, or the pyruvate is converted to citrate for the synthesis of fatty acids (FA). The lipogenesis involves FA desaturation and elongation, and in the final stage in the formation of triglycerides (TAG) FA is esterified with glycerol-3-phosphate, an intermediate of glycolysis or absorbed from plasma via aquaporin-9. TAGs are stored within hepatocytes in lipid droplets (LD) or secreted into circulation in very-low-density lipoprotein VLDL particles.

### 2.3.7 *PI3K/Akt-signalling in metabolic regulation*

As described already in the previous chapters, PI3K/Akt signalling is a signal transduction pathway regulating a variety of cellular functions, including glucose and fatty acid metabolism, in order to maintain cellular growth and survival. The signalling cascade is triggered by an external insulin-like growth factor stimuli, which leads to phosphoinositide 3-kinase (PI3K) activation via cell surface receptors. Activated PI3K phosphorylates plasma membrane $PI(4,5)P_2$ to $PI(3,4,5)P_3$, which leads to PM recruitment of certain PH-domain containing proteins (Lietzke *et al.*, 2000). Particularly, this attracts Akt and 3-phosphoinositide-dependent kinase (PDK1) to re-localize and bind $PI(3,4,5)P_3$ at PM, where PDK1 phosphorylates Akt at residue Thr308 (Alessi *et al.*, 1997; Franke *et al.*, 1995). The full activation of Akt-

kinase requires phosphorylation at second residue (Ser473) by mammalian target of rapamycin (mTOR) (Sarbassov *et al.*, 2005). The activation of Akt consequently results to phosphorylation of dozens of Akt downstream targets leading to activation or inactivation of signalling cascades regulating various distinct functions including protein synthesis, cell cycle, apoptosis and metabolism.

The regulation of cellular glucose and fatty acid metabolism by PI3K/Akt signalling are mainly triggered by the anabolic signals of insulin. By communicating through PI3K/Akt signalling pathway insulin is able to promote the expression of glycolytic and lipogenic genes required for the complete glucose utilization (Berwick *et al.*, 2002; Deprez *et al.*, 1997; Gottlob *et al.*, 2001; Semenza *et al.*, 1996). This is primarily mediated via transcription factor SREBP-1c, which synthesis and nuclear localization Akt induces (Bauer *et al.*, 2005; Berwick *et al.*, 2002; Fleischmann and Iynedjian, 2000; Porstmann *et al.*, 2005; Porstmann *et al.*, 2008; Ribaux and Iynedjian, 2003; Yecies *et al.*, 2011). Conversely, PI3K/Akt inhibits catabolic processes including the production and release of glucose by down-regulating the expression of pivotal enzymes of gluconeogenesis, glycogenolysis and also lipolysis (Barthel *et al.*, 2001; Li *et al.*, 2007; Nakae *et al.*, 2001; Sutherland *et al.*, 1996).

PI3K/Akt also regulates carbohydrate metabolism by non-transcriptional means, as it increases glucose uptake by stimulating the PM translocation of GLUT4 and GLUT1 (Barthel *et al.*, 1999; Kohn *et al.*, 1996; Rathmell *et al.*, 2003), and furthermore, PI3K/Akt signalling also promotes glucose storage by GSK-3 phosphorylation (Cross *et al.*, 1995). The activation of PI3K/Akt signalling as a downstream response of extracellular insulin is essential for cells to be able to adapt their metabolism and growth to correspond to the availability of external energetic substrates. Thus it is not surprising that dysfunctional PI3K/Akt signalling is associated with various different types of human diseases (Follo *et al.*, 2015; Jha *et al.*, 2015; Matsuda *et al.*, 2013; Patel and Mohan, 2005).

## 2.4 The actin cytoskeleton

### *2.4.1 Actin architecture and dynamics*

Actin is one of the most abundant proteins in all cell types having a fundamental role in the organization of the cellular cytoskeleton. Actin networks provide mechanical strength, motility and internal communication routes for the cell. Actin is largely accompanied with actin-binding proteins, which for example, orchestrate actin assembly from actin monomers to polymeric filamentous actin (F-actin). Among the most important actin regulators are Rho GTPases, which in regard, play a pivotal role in the regulation of actin transitions between its mono- and polymeric states as a response to extracellular signals. External plasma membrane receptors transmit

signals to intracellular Rho GTPases, which distribute the messenger information via signalling cascades throughout the cell finally to actin effectors (Hall, 1994; Ridley, 2001).

In the generation of filamentous actin through polymerization of actin monomers, an important actin effector is Arp2/3 complex (Higgs and Pollard, 1999; Machesky and Gould, 1999; Pollard and Borisy, 2003). The actin filament nucleation is an energetically unfavourable process hence restricted from spontaneous occurrence. The Arp2/3 complex acts as a nucleation factor to join free actin monomers to initiate filament growth (Pollard, 2007). The actin monomers are asymmetric and bind to each other in the filament in the same orientation, making the two ends of the filament structurally different. The slower growing terminus of the filament is termed a pointed end and the faster growing side a barbed end. The elongation of actin polymers requires the actin-binding protein profilin, which recruits monomeric actin to bind to the barbed end of the growing filament and inhibits monomer association with the pointed end (Goldschmidt-Clermont *et al.*, 1991). In addition, each actin monomer is an ATPase which hydrolyses ATP. When the concentration of free actin subunits are optimal and the barbed end is in ATP-bound state and the pointed end is in ADP-bound state, the filament adopts a property termed actin filament treadmilling, in which new monomeric actin is added to the barbed end at the same rate as the pointed end looses its subunits, dependent on the energy consumption from the constant hydrolysis of ATP (Wegner, 1976). The shrinkage of the pointed end is often mediated by actin-depolymerizing factor (ADF)/cofilin, which binds the ADP-bound actin at the filament pointed end and causes a depolymerization (Carlier *et al.*, 1997; Carlier *et al.*, 1999; Ichetovkin *et al.*, 2000; Lappalainen *et al.*, 1998). Together with profilin, the detached actin monomers can be recycled back into filaments after being re-energized through exchange of bound ADP to ATP (Pollard *et al.*, 2000).

Via the regulation by actin effectors, the filamentous actin together with actin-binding proteins organizes to different higher-order architectures with different biochemical and mechanical properties. Multiple actin filaments form close parallel or antiparallel alignments termed bundles and more loosely orthogonally oriented branched networks, which define the cell shape by supporting the plasma membrane. Actin also provides contractile force for the cell via cross-linking of actin bundles with myosin II. Myosin binds to ATP-bound actin and uses the energy of ATP hydrolysis to move towards the barbed end of the actin filament, regulated by the myosin light chain phosphorylation. These contractile structures present in most cells are termed stress fibers, which also participates in connecting the cell to the extracellular matrix. Actin also assembles into transient structures such as lamellipodia, filopodia and focal adhesions – important elements in the cell physiology that permit cell contacts, movement, cell to cell signalling and sensing of cell environment. The important property of actin filament is its dynamicity, as it can rapidly undergo disassembly, reassembly or depolymerisation based on the cell's demands to adapt to the fluctuating changes of its external environment. (Lee and Dominguez, 2010)

### *2.4.2 Cell migration*

Cell migration plays an important role during development in embryonic morphogenesis, but cells also migrate in fully developed organisms during the normal life cycle (e.g. the migration of immune cells) and in diseases (e.g. by promoting cancer cell invasion and metastasis). During migration, cells forms a polarized leading edge and membrane protrusions, as well as new cell adhesions, which all involve cytoskeletal reorganization via effector proteins. (Svitkina, 2018)

Cell migration is commonly driven by actin-rich plasma membrane extensions called lamellipodia composed of branched actin networks, which pushes the leading edge by extensive actin polymerization at the front (Abercrombie *et al.*, 1971; Abercrombie *et al.*, 1972; Pollard and Borisy, 2003). The leading edge then forms new adhesions to the substratum, termed focal adhesions, and to move forward, the lamellipodium fills with cytosol and the membrane at the trailing edge de-adheres and retracts towards the cell body, executed by a pulling force of actin stress fibers. (Lauffenburger and Horwitz, 1996; Medalia and Geiger, 2010; Mitchison and Cramer, 1996; Palecek *et al.*, 1998; Ridley and Hall, 1992)

Another important structure mediating cell migration is a fingerlike protrusion of actin bundles termed filopodium. Filopodia are organized at the leading edge lamellipodium, and they project out of the cell body and mediate the sensing of the extracellular matrix (Mattila and Lappalainen, 2008). Compared to lamellipodia, the protrusion forces generated by filopodia are much weaker, but some cells lacking lamellipodia can predominantly migrate via filopodia (Suraneni *et al.*, 2012; Wu *et al.*, 2012).

A less common mechanism of cell migration is via cell surface protrusions termed blebs. Blebs are formed when actin cortex is locally detached from the plasma membrane (Charras *et al.*, 2008). The intracellular hydrostatic pressure forces the non-actin bound parts of the plasma membrane to push out as a hernia (Charras *et al.*, 2005; Charras *et al.*, 2006). After their extension, cell surface blebs are filled with filamentous actin and myosin to form new actin cortex (Charras *et al.*, 2006). Lamellipodia or bleb-based cell motility are not necessary exclusive, but instead can occur concurrently or in certain conditions some cells can switch between these two modes of action (Bergert *et al.*, 2012; Wolf *et al.*, 2003).

### *2.4.3 Actin in cell proliferation*

Cell division requires extensive re-organization of the cytoskeleton. The most notable process is the re-organization of microtubules into mitotic spindle, which orchestrates chromosome segregation into daughter nuclei, but the cell cycle progression also involves contribution of the actin network. During mitosis, cell migration is arrested

and the actin cytoskeleton is reorganized to give the cells the characteristic round shape with reduced cell-matrix adhesions (Cramer and Mitchison, 1997; Dao *et al.*, 2009). Actin also contributes to the cell cycle control and centrosome separation, evident by drugs inhibiting actin polymerization, which result in delayed progression of mitosis or unseparated centrosomes (Lee and Song, 2007; Uzbekov *et al.*, 2002). This is associated with an actin binding protein cortactin, which is found to connect the actin filaments to centrosomes to drive the centrosome separation (Wang *et al.*, 2008b).

The rounding of the cell is achieved by cross-linking actin beneath the plasma membrane to form an actomyosin cortex, which increases cell rigidity (Bray and White, 1988; Dao *et al.*, 2009). The actin cross-linking is mediated by ezrin/radixin/moesin (ERM) family of actin-binding proteins, which have the ability to interact with trans-membrane proteins and PI(4,5)$P_2$ of the PM hence stabilizing the cortical actin network (Fehon *et al.*, 2010; Hao *et al.*, 2009; Niggli and Rossy, 2008; Wang *et al.*, 2008a). In the end of mitosis, actin–myosin bundles play a role in the formation of a contractile ring pivotal for cytokinesis by producing the force to divide the cell into two (Mabuchi, 1986). After the complete cell division, the two daughter cells re-establish their actin networks in order to migrate apart and re-adhere with the substratum.

### *2.4.4 Rho GTPases in actin cytoskeleton regulation*

Actin is involved in the dynamic regulation of various cellular functions, which is enabled by co-operation of a multitude of actin regulators and cell signalling processes. Rho GTPases are designated as the most pivotal factors in actin cytoskeleton regulation, in particular RhoA, Rac1 and Cdc42, which are the most studied members of the Rho GTPase family and highly conserved across eukaryotes (Boureux *et al.*, 2007; Hall, 1994). Here I describe the central properties of RhoA, Rac1 and Cdc42, principally their role in the assembly of actin cytoskeleton during cell locomotion. These proteins are active when bound to GTP promoted by guanine nucleotide exchange factors (GEFs) and are inactivated by the hydrolysis of bound GTP to GDP (Rossman *et al.*, 2005). The key function of the active RhoA, Rac1 and Cdc42 signalling pathways is to distribute external messenger information to downstream effectors, which consequently orchestrate the actin organization into higher-order structures important for cell locomotion: Rac1 is mainly responsible for the formation of lamellipodia at the leading edge and the activation of Cdc42 triggers the formation of filopodia organized by bundled actin (Gupton and Gertler, 2007; McCarty *et al.*, 2005), while RhoA permits the cross-linking of actin bundles with myosin II into stress fibers (Figure 4) (Ridley and Hall, 1992).

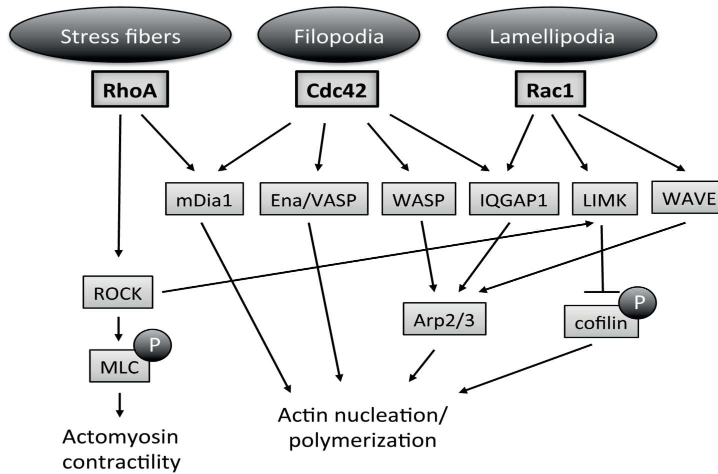


Figure 4. Schematic figure of actin cytoskeleton regulation via RhoA, Cdc42 and Rac1 signalling, which are responsible for stress fiber, filopodia and lamellipodia formation, respectively. RhoA activates Rho-associated kinase (ROCK), which phosphorylates myocin light chain (MLC), thus increasing actomyosin contractility. RhoA polymerisizes actin via mDia1. Cdc42 also increases actin polymerization via mDia1, Ena/VASP, WASP and IQGAP1, which activates Arp2/3 complex. Rac1 involves actin polymerization via IQGAP1 and WAVE. Rac1 and ROCK also activate LIM-kinase, which inactivates cofilin by phosphorylation.

The lamellipodia formation is mainly enabled by actin nucleation and polymerization via the Arp2/3 complex, which is activated by a WAVE complex – downstream of Rac1 (Chen *et al.*, 2017; Chen *et al.*, 2010; Miki *et al.*, 1998). Rac1 also inhibits cofilin-mediated actin depolymerization by stimulating the cofilin phosphorylating LIM-kinase (Nishita *et al.*, 2005; Stanyon and Bernard, 1999). Rac1 also interacts with Ras GTPase-activating-like protein (IQGAP1) scaffold, which stabilizes Rac1 (Briggs and Sacks, 2003). Similarly, the extension of filopodia requires extensive actin nucleation and polymerization. Cdc42 activates the formin family member diaphanous-1 (mDia1) and Wiskott-Aldrich syndrome proteins (WASP, N-WASP), which consequently activates the Arp2/3 complex supported by IQGAP1 (Ismail *et al.*, 2009; Le Clainche *et al.*, 2007; Peng *et al.*, 2003; Peng *et al.*, 2003; Rohatgi *et al.*, 1999; Symons *et al.*, 1996). Cdc42 also mediates actin filament elongation through Ena/VASP family of proteins, which are able to bind to the barbed end of bundled actin filaments and catalyse their growth (Disanza *et al.*, 2013; Sechi and Wehland, 2004).

Similar to Cdc42, also RhoA activates the formin mDia1, which is important for the polymerization of unbranched actin filaments and formation of actin bundles (Narumiya *et al.*, 2009; Pollard, 2007; Watanabe *et al.*, 1999). RhoA also activates the Rho-associated kinase (ROCK), which increases myosin light chain phosphorylation

enabling myosin motor protein binding to actin bundles, hence stimulating the actomyosin contractility (Kimura *et al.*, 1996; Narumiya *et al.*, 2009). Furthermore, ROCK inhibits cofilin via the LIM-kinase to stabilize actin filaments (Maekawa *et al.*, 1999). The induced actin polymerization and actomyosin tension are important for stress fiber contraction that mediates the retraction of the rear end of the cell during the cell migration. Furthermore, ROCK also promotes the ERM family proteins to induce association of the actin cortex with PM via PIP5K (Lacalle *et al.*, 2007; Lokuta *et al.*, 2007; Niggli and Rossy, 2008; Yamazaki *et al.*, 2002). High levels of RhoA/ROCK activity are associated with the inhibition of lamellipodia and observed upon bleb-based cell motility (Aoki *et al.*, 2016; Charras and Paluch, 2008).

# 3 AIMS

1. To determine sterol regulation of ORP–VAP-A complexes at MCSs
2. To shed light to ORP2 intracellular function and role in cell pysiology
3. To determine ORP2 role in the hepatocellular energy metabolism

# 4 MATERIALS AND METHODS

## 4.1 List of used methods

The methods explained here are the ones that I have personally used for my thesis. The complete description of all used methods can be found from the original publications I, II, III.

| **METHOD** | **ORIGINAL PUBLICATION** |
|---|---|
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## 4.2 Description of the methods used

*Antibodies*

The following primary antibodies were used in the experiments:

| Antibody | Source | Type | Manufacturer |
| --- | --- | --- | --- |
| anti-Akt | rabbit | polyclonal | Cell Signaling |
| anti-Akt(Ser473) | rabbit | polyclonal | Cell Signaling |
| anti-β-actin | rabbit | polyclonal | Millipore-Sigma |
| anti-Cdc37 | rabbit | polyclonal | Cell Signaling |
| anti-Cdc37 | mouse | monoclonal | Thermo Scientific |
| anti-cortactin | mouse | monoclonal | Millipore-Sigma |
| anti-GFP | rabbit | polyclonal | BD Bioscience |
| anti-GM130 | mouse | monoclonal | BD Bioscience |
| anti-GSK-3β | rabbit | polyclonal | Cell Signaling |
| anti-GSK-3β(Ser9) | rabbit | polyclonal | Cell Signaling |
| anti-Hsp90 | rabbit | polyclonal | Cell Signaling |
| anti-IQGAP1 | mouse | monoclonal | BD Biosciences |
| anti-ORP2 | rabbit | polyclonal | (Laitinen *et al.*, 2002) |
| anti-PDI | mouse | monoclonal | Enzo Life Sciences |
| anti-vimentin | mouse | monoclonal | Millipore-Sigma |

Cell Signaling (Danvers, MA); Millipore-Sigma (Billerica, MA); Thermo Scientific (Waltham, MA); BD Bioscience (San Jose, CA); Enzo Life Sciences/Stressgen (New York, NY)

*Anti-HA co-immunoprecipitation*

Cells transiently overexpressing HA-ORP2 or ORP2 cultured in 10 cm were lysed in lysis buffer [10 mM HEPES (pH 7.6), 150 mM NaCl, 0.5 mM $MgCl_2$, 10% glycerol, 0.5% Triton X-100, 0.5% Saponin, Protease inhibitor cocktail] and the unbroken cells were removed by centrifugation at 20 000 x g for 10 min. To reduce the unspecific binding to the beads, the obtained lysates were first incubated with 30 µl of Protein G Magnetic Beads (Pierce/Thermo Scientific) for 30 min at 4 °C, before adding the magnetic anti-hemagglutinin (HA) beads (Pierce/Thermo Scientific) and incubated for 1.5 h on rotation at 4 °C. The beads were washed three times with lysis buffer and two times with deionized $H_2O$. Bound proteins were eluted with 100 µl of 0.1 M glycine (pH 2.0) for 30 min at room temperature and the alkaline elution was neutralized with 8 µl of 1 M Tris base (pH 9.0). The eluted proteins were identified by mass spectrometry [Meilahti Clinical Proteomic Core Facility (University of Helsinki, Finland)]. The non-spesific interactions identified in the ORP2 sample lacking a HA epitope tag were subtracted from the proteins identified in the anti-HA-precipitated HA-ORP2 sample.

*Bligh and Dyer lipid extraction*

To extract cellular cholesterol and triglycerides, cells were scraped in 2% NaCl and collected by centrifugation at 700 x g in 4 °C. 2:1 methanol-chloroform was added to the cells, followed by separation of insoluble material by centrifugation. For the extraction of hydrophilic lipids, 1:1 chloroform-$H_2O$ was added, followed by centrifugation and collection of the hydrophobic phase. The solvent was evaporated under gaseous nitrogen at 37 °C, and the lipids were re-dissolved to chloroform for TLC.

*Cell culture and transfection*

Human hepatoma cell line (HuH7) was cultured in Eagle's minimal essential medium with Earle's salts (AQmedia™, Millipore-Sigma) supplemented with 10% heat inactivated fetal bovine serum (Millipore-Sigma), 4 mM L-Glutamine (pH 7.35) (Millipore-Sigma), 100 µg/ml streptomycin and 100 U/ml penicillin (Millipore-Sigma). Cells were transfected with Lipofectamine® 2000 (Invitrogen, Carlsbad, CA) using manufacturer's instruction for 24 h in non-antibiotic containing media.

*Cellular sterol manipulation*

Cellular cholesterol was depleted from HuH7 cells by culturing the cells in AQmedia depleted from fetal bovine serum but containing 5% lipoprotein-deficient serum instead (LPDS, from Matti Jauhiainen, THL, Helsinki) for 96 h. Alternatively 5 µM Mevastatin (Millipore-Sigma) supplement was included at the end for 24 h. To increase the cellular cholesterol, 20–50 µg/ml human low-density lipoprotein [LDL; as total protein, from Matti Jauhiainen, THL, Helsinki) was added at the end for 24 h. For the oxysterol treatment, the cultured cells were loaded with 6 µM 22(R)OHC, 25OHC or 7KC or vehicle for 24 h.

*Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technique*

The endogenous ORP2 was stably knocked out (KO) from HuH7 using RNA-guided CRISPR-Cas9 genome editing with double nicking. The *OSBPL2* targeted guide RNAs (gRNAs) were designed using optimized CRISPR design tool (http://crispr.mit.edu/) with Nickase analysis. The gRNA primers were cloned into pGL3-puro-sgRNA vector (obtained from Dr. Shiqian Li, Department of Anatomy, University of Helsinki, Finland) and the acquired plasmids were verified by sequencing. Cells transfected with gRNA plasmids and Cas9 (pcDNA4-Cas9n-2A-GFP, obtained from Dr. Shiqian Li), or only Cas9 for the generation of control cells, were collected as single cells using fluorescence-activated cell sorting (FACSAriaII,

BD Biosciences, Franklin Lakes, NJ) at the Biomedicum Flow Cytometry Core Facility, Helsinki, Finland. Single cell clones were expanded for 3 weeks in cell culture to generate stable cell lines. The complete deletion of ORP2 from the HuH7 cells was ensured by western blotting with anti-ORP2 antibody and by sequencing of the targeted PCR product.

*Co-immunoprecipitation*

HuH7 cells were transfected with ORP2-V5 and GFP-MLC12 or GFP-MLC12 alone as a negative control. Cells were lysed in lysis buffer [10 mM HEPES pH 7.6, 150 mM NaCl, 0.5 mM $MgCl_2$, 10% glycerol, 0.5% Triton X-100, 0.5% Na-deoxycholate, Protease inhibitor cocktail (Roche Diagnostics)] and the intact cells were removed by centrifugation at 20 000 x g. Lysates were incubated with 15 µl anti-HA Magnetic Beads (Pierce/Thermo Scientific) to reduce the unspecific binding for 30 min at 4 °C. The unbound proteins were incubated with 40 µl anti-V5 Magnetic Beads (MBL, Woburn, MA) overnight at 4 °C. The beads were washed four times with lysis buffer, resuspended in 30 µl Laemmli sample buffer and boiled for 5 min.

*Co-precipitation using recombinant protein*

HuH7 cells were transfected with GFP-SEPT9 or GFP-ARHGAP12 plasmid or alternatively left untrasfected for the pull-down of endogenous proteins, and lysed in lysis buffer [10 mM HEPES pH 7.6, 150 mM NaCl, 0.5 mM $MgCl_2$, 10% glycerol, 0.5% Triton X-100, 0.5% Na-deoxycholate, Protease inhibitor cocktail (Roche Diagnostics)] and the intact cells were removed by centrifugation at 20 000 x g. The obtained lysates were mixed with 300 µmol GST-ORP2 or plain GST (produced in insect cells by the BioMediTech Protein Technologies core facility) and 20 µl Glutathione-Sepharose 4B (GE Healthcare, Buckinghamshire, UK) for 2 h at 4 °C. The beads were the washed thoroughly and resuspended in 30 µl Laemmli sample buffer and boiled for 5 min.

*Fluorescence microscopy*

Fluorescence signals were visualized with a Zeiss Axio Observer Z1 microscope with an ECPInN 40x/0.75 DICII or PInApo x63/1.40 oil DICII objectives and a Colibri laser (Zeiss, Oberkochen, Germany). Images were recorded with Axio Visio Rel. 4.8.1 or Zen 2 v.2.0.0 softwares (Carl Zeiss Imaging Solutions GmbH, Oberkochen, Germany). For image processing Adobe Photoshop 7 (San Jose, CA) and Image J softwares (National Institutes of Health, Bethesda, MD) were used.

*Glucose and fatty acid uptake*

The cells were initially starved for 3 h in glucose and serum free media [Dulbecco's modified Eagle medium (DMEM) Gibco, Grand Island, NY]. To measure the fatty acid uptake, the cells were pulse labeled with a mixture of 20 mM glucose, 5 nM oleic acid-BSA and 1 µCi [$^{3}$H]-oleic acid for 15 min. In turn to measure the glucose uptake, the cells were pulse labelled with 10 µM [$^{3}$H]-2-deoxy-D-glucose (100 mCi/mmol) (PerkinElmer, Waltham, MA) supplemented with 10 µM 2-Deoxy-D-glucose (Millipore-Sigma) for 15 min. To determine the rate of non-specific glucose uptake, the control cells were treated with 40 µM Cytochalasin B (Millipore-Sigma) for 45 min prior the pulse labelling. After the pulse labelling, the cells were washed thoroughly with PBS and lysed in 0.03% SDS. The radioactivity was measured using liquid scintillation counting in Optiphase HiSafe 3 scintillation cocktail (PerkinElmer) and normalized to total cellular protein (BCA assay, Thermo Scientific). To determine the intrinsic glucose uptake, the rate of non-specific glucose uptake was subtracted from the total rate of glucose uptake.

*Glycogen synthesis*

The cells were starved for 2 h in glucose and serum free media (DMEM, Gibco), followed by a pulse labeling with 0.18 µCi/µmol D-[U-$^{14}$C]-glucose (PerkinElmer) for 1 h. After the pulse labelling, the cells were washed thoroughly with PBS, lysed in 0.03% SDS, and the cellular glycogen was precipitated with a 1 mg of carrier glycogen (Millipore-Sigma) by 30 min boiling followed by addition of ethanol. The glycogen precipitate was washed with 70% ethanol, centrifuged at 20,000 x g for 15 min and dried from the excess ethanol. The radioactivity was measured using liquid scintillation counting in Optiphase HiSafe 3 scintillation cocktail (PerkinElmer) and normalized to total cellular protein (BCA assay, Thermo Scientific).

*Glycolytic activity*

The cells were cultured on Seahorse XF96 cell culture microplates in basal growth medium (Agilent Tecnologies, Santa Clara, CA) for 24 h. One hour prior the measurements the medium was changed to Seahorse XF base medium with 2 mM L-glutamine and the cells were incubated at 37 °C without $CO_2$. Extracellular acidification rate (ECAR) was measured in real time 3 x 3 min cycles with Seahorse XFe Analyzer (Agilent Technologies) with addition of following compounds prior single measurement cycle: 10 mM glucose (Millipore-Sigma), 1 µM Oligomycin (Millipore-Sigma) and finally 50 mM 2-Deoxy-D-glucose (Millipore-Sigma). ECAR values were normalised to the corresponding total protein concentrations (BCA assay, Thermo Scientific) and the glycolysis was calculated by subtracting the non-glycolytic ECAR from the maximum ECAR after glucose injection.

*Immunofluorescence staining*

Cells were fixed with 4% paraformaldehyde, additionally combined with 0.02% glutaraldehyde, for 20 min. Aldehyde groups were blocked with 50 mM $NH_4Cl$ for 10 min and the cells were permeabilized with 0.1% Triton X-100 for 5 min. Unspecific binding of antibodies were blocked with 10% fetal bovine serum for 45 min at 37 °C, followed by incubation with the primary antibody in 5% fetal bovine serum for 1 h and the secondary antibodies [fAlexa Fluor-568 goat anti-rabbit (Molecular Probes/Invitrogen, Carlsbad, CA), [fAlexa Fluor-594 goat anti-mouse (Thermo Scientific)], Cy5® goat anti-mouse [Life Technologies, Eugene, OR] or Oregon green 488 phalloidin (Molecular Probes, Eugene, OR) in the same conditions. The cells were mounted in Mowiol (Calbiochem, LA Jolla, CA)/1,4-Diazabicyclo-[2.2.2] octane (50 mg/ml, Millipore-Sigma) supplemented with or without DAPI (5ug/ml, Molecular Probes).

*Lipid droplet staining*

Cellular lipid droplets were labelled with 5 nM Bodipy-$C_{12}$ (Molecular Probes) in serum-free medium at 37 °C for 1 h, followed by 4 h chase in basal growth medium and cell fixation.

*Thin layer chromatography (TLC)*

TAGs and cholesterol esters were analysed by thin layer chromatography (TLC). After the Bligh and Dyer lipid extraction described above, the radioactively labelled lipids were applied to analytical silica gel glass plates (Merck, Kenilworth, NJ) and separated in glass chamber in solvent containing 80% of hexane, 18% of diethylether, 0.01% of acetic acid and 0.003% of $H_2O$. The segregated lipids were visualized with iodine and scraped in Optiphase HiSafe 3 scintillation cocktail (PerkinElmer). The radioactivity was measured using liquid scintillation counting and the count of lipids was normalized to total cellular protein (BCA assay, Thermo Scientific).

*Triglyceride synthesis*

The cells pulse labelled with 3.5 µCi [$^3$H]oleic acid (PerkinElmer) in ethanol for 3h, after which they were washed thoroughly and scraped into 2% NaCl. The TGs were extracted using the Bligh and Dyer method and [$^3$H]TGs separated by TLC (described above).

*Western blotting*

Cells were lysed in lysis buffer [10 mM HEPES pH 7.6, 150 mM NaCl, 0.5 mM $MgCl_2$, 10% glycerol, 0.5% Triton X-100, 0.5% Na-deoxycholate, Protease inhinbitor cocktail (Roche Diagnostics, Mannheim, DE)], mixed thoroughly by vortexing and incubated on ice for 10 min. Intact cells were removed by centrifugation at 20 000 x g for 10 min. Total cellular proteins were measured with BCA assay (Thermo Scientific) and lysates containing 10–20 µg of total cellular proteins were boiled with Laemmli sample buffer for 5 min. After boiling, the lysates were subjected to SDS-PAGE followed by transfer to membranes [Hybond-C Extra nitrocellulose (GE Healthcare) or Trans-Blot Turbo LF PVDF, Bio-Rad (Hercules, CA)]. Unspecific binding was blocked with 5% fat-free milk in TBS containing 0.01% Tween-20 at room temperature for 1 h, followed by primary antibody treatment in the same blocking buffer at 4°C over night. HRP-conjugated secondary antibodies were incubated in the same blocking buffer at room temperature for 1 h. Bound antibodies were detected with enhanced chemiluminescence system ECL (Thermo Scientific).

*Wound healing assay*

HuH7 cells were grown in the basal growth medium and the confluent cell monolayers were wounded with a small pipet tip. The cells were washed extensively with PBS to remove the detached cells, and the wound was marked at the bottom of the dish. The wound closure was monitored for 22 h with by using the bright-field Evos XL Cell Imaging Systerm (AMG, Thermo Scientific).

*xCelligence Real-Time Cell Analysis*

xCELLigence® Real-Time Cell Analysis system with DP instrument (ACEA Bioscience, San Diego, CA) was employed to analyse the cell adhesion to substratum, proliferation and cell migration. For the analysis of adhesion and proliferation, cells were seeded at 8 x $10^3$ cells per well on electronic microtiter plates with noninvasive gold microelectrode biosensors (E-plate® 16, ACEA Bioscience) and electrical impedance was monitored in real-time with RTCA Software 1.2 (ACEA Bioscience) for 85 h. For the analysis of migration cells were seeded at 3 x $10^4$ cells per chamber in upper chambers in medium depleted of fetal bovine serum (CIM-plate® 16, ACEA Bioscience) and the lower electronically integrated Boyden chambers contained basal growt medium. Cell movement from the upper to the lower chamber was monitored in real-time by electrical impedance with the RTCA Software 1.2 for 24 h.

# 5 RESULTS AND DISCUSSION

## 5.1 The localization of ORP–VAP complexes is regulated by cellular sterol content

### *5.1.1 Subcellular targeting of ORP–VAP-A complexes alters upon cellular sterol manipulations (I)*

Several studies indicate that OSBP/ORPs sterol liganding regulates their intracellular localization (Hynynen *et al.*, 2009; Mesmin *et al.*, 2013; Nhek *et al.*, 2010; Ridgway *et al.*, 1992; Storey *et al.*, 1998). However, the impacts of sterol liganding have not been carried out as a systematic screen among the ORP family, and moreover, the sterol effects on the localization of ORPs in a complex with VAPs, integral trans-membrane proteins of the ER, are not comprehensively described. In this study we screened the sterol manipulation effects on the ORP–VAP complexes, limiting the analysis to ORPs which i) are known to bind sterols and ii) contain the VAP-interacting FFAT-motif. The ORPs included in the study were OSBP, ORP1L, ORP2, ORP4L and ORP9L.

To focus the analysis to ORP–VAP interactions, we employed the Bimolecular Fluorescence Complementation technique (BiFC), which enables fluorescent microscopy visualization of protein interactions in live cells. BiFC is based on a formation of a fluorescent complex when two truncated non-fluorescent fluorophore fragments are organized in close association with each other (Kerppola, 2008). To visualize protein–protein interactions in live cells, the interaction candidates are fused with the non-fluorescent fragments and the formed protein interactions permit the association between the two fragments, which then generate a fluorescent complex (Kerppola, 2008). The specificity of the BiFC signal is reasonably good but not absolute, requiring verification of the positive interaction and exclusion of putative false positive signals (Kerppola, 2008). In our study we used ORP8 as a negative control for ORP–VAP interaction, since ORP8 lacks FFAT-motif and is incapable of binding to VAPs. In addition, the abolishment of BiFC signal by using ORP constructs with FFAT-motifs inactivated by mutations is established previously (Weber-Boyvat *et al.*, 2015b).

Moreover, when using the BiFC technique, it is important to take into account that the BiFC complex formation is irreversible *in vitro* (Shyu and Hu, 2008), and this irreversible nature of the BiFC requires careful evaluation because BiFC is able to trap transient and strengthen weak interactions, which sets limitations to the analysis of dynamic interactions (Kerppola, 2008; Kodama and Hu, 2012). Here, however, we did not employ the BiFC to study the ORP–VAP interaction dynamics, and the cellular sterol manipulations were carried out before the generation of the BiFC interactions.

To study the sterol manipulation effects on ORP–VAP BiFC distribution, HuH7 hepatocytes were treated for 24 h with 6 µM oxysterols [25OHC, 22(R)OHC or 7KC], or alternatively, the cellular cholesterol level was manipulated by 96 h lipoprotein starvation, with an additional 5 µM Mevastatin or 50 µg/ml human LDL treatment for 24 h.

The studied BiFC complexes were OSBP–, ORP1L–, ORP2–, ORP4L– and ORP9–VAP-A, from which three were found to be sensitive to the sterol manipulations (OSBP–, ORP2– and ORP4L–VAP-A), while no effects were observed in ORP1L– or ORP9–VAP-A BiFC complexes upon any of the cellular sterol manipulations (I: Fig. 1A & 2A). Moreover, no detectable BiFC signal was observed upon ORP8–VAP-A expression, indicating specificity of the observed ORP–VAP BiFC signals.

The observation that none of the above described sterol manipulations affected the ORP1L– or ORP9L–VAPA-A BiFC distributions is to some extent conflicting, as it has previously suggested that the sterol binding deficient mutants of ORP1L [ORP1L(ΔELSK)] and ORP9L [ORP9L(ΔDLTK)] alter the subcellular targeting of these proteins in complex with VAP-A (Weber-Boyvat *et al.*, 2015b). The wild type ORP1L–VAP-A was found to localize to ring-like structures surrounding the LEs, but overexpression of ORP1L(ΔELSK)–VAP-A was suggested to narrow down the diameters of the LE-surrounding ring-like BiFC-positive structures (Weber-Boyvat *et al.*, 2015b). Furthermore, the wild type ORP9L–VAP-A BiFC expressed as dots, blobs and perinuclear lines, but the ORP9L(ΔDLTK)–VAP-A displayed an altered distribution co-localizing with a Golgi marker, which was not observed with the wild type ORP9L (Weber-Boyvat *et al.*, 2015b). It is, however, possible, that ORP1L and ORP9L are subjective to regulation by other ligands not included in this study (PIPs for example), or alternatively, the altered localization of sterol binding mutants can be a result of structural alterations or improper folding, putatively resulting in unnatural properties of these mutated proteins.

### *5.1.2 Sterol manipulation effects on cholesterol and TAG content (I)*

To analyse the above described sterol manipulation effects on the concentration of cellular lipids, the content of free and esterified cholesterol and TAGs were measured by thin-layer chromatography. The lipoprotein starvation reduced the cellular content of cholesterol esters by 35% and the Mevastatin treatment by 50%, and conversely, the LDL loading increased cholesterol esters by 29% (I, Supplementary Figure S1 A). However, the Mevastatin treatment did not reduce the amount of free cholesterol compared to basal control, and the lipoprotein starvation in fact increased the free cholesterol by 17% (I, Supplementary Figure S1 C). Also the LDL treatment increased the free cholesterol compared to basal control by 35%, but the increment of free cholesterol was not significant as compared to the lipoprotein starvation (I, Supplementary Figure S1 C).

The analysis clearly indicated that the cellular cholesterol depletion with lipoprotein starvation and additional Mevastatin treatment diminished the level of total cellular cholesterol by an efficient reduction of cholesterol esters, but the level of free cholesterol was not reduced and during the lipoprotein starvation the level of free cholesterol was even moderately increased. We have to assume that the lipoprotein starvation resulted in an activation of compensatory mechanisms to elevate the cellular cholesterol levels, thus increasing the levels of free cholesterol. This is supported by the finding that the elevation in the free cholesterol was abolished by the additional Mevastatin treatment, which effectively down-regulates the hepatic *de novo* cholesterol synthesis.

Moreover, all these cholesterol manipulations increased the cellular levels of TAGs (I, Supplementary Figure S1 B), but on the contrary, the oxysterol treatments [22(R)OHC and 25OHC] decreased the TAG content (I, Supplementary Figure S2 B). The oxysterols also reduced the level of free cholesterol by 27–55% (I, Supplementary Figure S2 C), but the levels of cholesterol esters, on the other hand, were increased with 25OHC by 20% (I, Supplementary Figure S2 A). The modulation of cellular cholesterol content by the 25OHC or 22(R)OHC increment is probably due to their cholesterol homeostatic properties. 25OHC is able to reduce cholesterol synthesis, and its formation is implicated as a feed-back control mechanism to inhibit excess cholesterol synthesis, but 25OHC is also found to increase cholesterol esterification, putatively explaining the observed effects of 25OHC treatment on the levels of free and esterified cholesterol (Du *et al.*, 2004; Kandutsch and Chen, 1975; Lange *et al.*, 1999). Moreover, 22(R)OHC is also suggested to reduce the cellular cholesterol content by suppressing cholesterol synthesis via LXR and by enhancing cholesterol efflux via increased expression of ATP-binding cassette transporter A1 (ABCA1) (Janowski *et al.*, 1996; Koldamova *et al.*, 2003; Lehmann *et al.*, 1997; Wang *et al.*, 2008b).

#### *5.1.3 Sterol liganding regulates OSBP–VAP-A Golgi localization (I)*

The effects of the cellular sterol manipulations on the cellular distribution of OSBP–VAP-A BiFC were investigated. In the basal control cells, the OSBP–VAP-A complex displayed a scattered perinuclear localization, which partly co-localized with the *trans*-Golgi/TGN marker GalT1(1-81) and only weakly with *cis*-Golgi visualized by GM130 staining (I, Figure 3A,B). Upon 25OHC loading – which is a high-affinity oxysterol ligand of OSBP – in 80% of the cells the OSBP–VAP-A BiFC was clustered at one pole of the nucleus, significantly distinct compared to the diffuse BiFC signal observed in the control cells (I, Figure 1A). By the mCherry-GalT1(1-81) overexpression or by GM130 staining, the clustered OSBP–VAP-A BiFC was found to co-localize completely with the condensed Golgi apparatus (I, Figure 3A,B), indicating enhanced Golgi localization of OSBP–VAP-A complexes. Importantly, the

25OHC treatment did not only affect the distribution of OSBP–VAP-A but also the organization of the Golgi apparatus, by clustering the *cis-/trans*-Golgi at a juxtanuclear position. On the other hand, the low-affinity oxysterol ligands of OSBP – 22(R)OHC and 7KC – had no significant effects on the OSBP–VAP-A BiFC localization compared to control cells.

The cellular cholesterol depletion resulted in a similar clustering of OSBP–VAP-A BiFC at *cis/trans*-Golgi as that observed with 25OHC (I, Figure 3A,B). To be precise, the lipoprotein starvation induced the clustering in 36% of the cells and Mevastatin in 49% (I, Figure 2A), indicating that cellular cholesterol depletion enhances the OSBP–VAP-A Golgi targeting similar to 25OHC. The LDL loading, however, did not affect the OSBP–VAP-A BiFC localization compared to control cell: In both conditions the OSBP–VAP-A clustering was observed only in ≈10% of the cells (I, Figure 1A, 2A).

To verify that the 25OHC effect on the OSBP–VAP-A BiFC distribution is a result of OSBP ligand binding, effects on a sterol-binding deficient mutant (ΔELSK) of OSBP were investigated upon 25OHC treatment. Interestingly, the 25OHC treatment condensed the Golgi markers close to the nucleus, but OSBP(ΔELSK)–VAP-A BiFC was unaffected by the 25OHC treatment, displaying a scattered perinuclear localization similar to the basal control. As a result, OSBP(ΔELSK)–VAP-A BiFC showed no co-localization with Golgi markers (I, Figure 3A,B). The result indicates that 25OHC regulates the subcellular localization of OSBP–VAP-A by directly binding to OSBP, but overexpression of the OSBP–VAP-A complex is not required for the Golgi clustering. Consistently, the 25OHC treatment induced the GM130 and GalT1(1-81) clustering also in the absence of OSBP–VAP-A BiFC overexpression (I, Supplementary Figure S3).

However, Nhek and co-workers previously indicated that LDL treatment resulted in a scattered distribution of the *trans*-Golgi marker TGN46, and as a result, they implied that the inefficient localization of the OSBP to the Golgi in response to the LDL treatment promoted the Golgi fragmentation (Nhek *et al.*, 2010). The HuH7 cells' Golgi is naturally highly fragmented and thus unaffected by the LDL treatment, but is condensed upon 25OHC treatment or cholesterol depletion. We observed clustering of the *cis*- and *trans*-Golgi upon 25OHC also in the untransfected cells. However, we have to take into account that the cells contained endogenous OSBP and VAP-A, thus the involvement of OSBP in the Golgi clustering cannot be reliably excluded based on our observation.

Also the ligand binding dependency of the OSBP Golgi localization has been previously investigated, and several studies support our findings that the OSBP Golgi targeting is promoted by 25OHC or cholesterol depletion, or by altered plasma membrane cholesterol trafficking, and conversely, cholesterol increment reduces the OSBP Golgi localization (Nhek *et al.*, 2010; Ridgway *et al.*, 1992; Storey *et al.*, 1998). In addition, Storey *et al.* (1998) indicated that the Golgi localization of OSBP,

in response to 25OHC, increased the Golgi SM synthesis, which was conversely inhibited by cholesterol. Mesmin *et al.* (2013) suggested that the 25OHC treatment additionally modifies the localization of VAP-A when co-expressed with OSBP. The study reported that in the basal conditions, VAP-A was evenly distributed along the ER membrane, but upon the 25OHC treatment, it co-localized with OSBP at the perinuclear Golgi structures. Hence, it was suggested that the 25OHC treatment promotes the VAP-A translocation towards the Golgi via the OSBP interaction. The study also showed that mutation of the PHD of OSBP shifted the OSBP localization to the ER, and the FFAT mutation towards the Golgi, implicating an important role for PHD PIP-binding in the OSBP Golgi targeting and VAP-binding in the ER association. (Mesmin *et al.*, 2013) Later it was also shown that OSW-1 treatment, which blocks the OSBP ligand binding, also induced OSBP translocation to the Golgi accompanied with VAP-A (Mesmin *et al.*, 2017).

The observed BiFC signals in our experiments implicate that the interaction of OSBP with VAP-A is not compromised upon any of the cellular sterol manipulations, but the localization of the OSBP–VAP-A interaction is altered depending on the cellular cholesterol or 25OHC levels. One could criticise, that the cellular oxysterol treatments using micromolar concentrations represent an unnaturally high dose compared to the levels of endogenously produced oxysterols, the concentrations of which in the plasma are in nanomolar range. However, our unpublished observations suggest that oxysterols rapidly precipitate in aqueous solutions, i.e. when added to the culture media, thus the actual concentrations obtained by the cells are expected to be significantly lower.

### *5.1.4 Sterol manipulations regulate ORP4L–VAP-A localization to vimentin filaments (I)*

ORP4L is the closest homologue of OSBP and similarly found to bind 25OHC with high-affinity (Wang *et al.*, 2002). In addition, both OSBP and ORP4L contain a dimerization motif enabling the formation of OSBP–ORP4L heterodimers (Wyles *et al.*, 2007). ORP4 was previously shown to localize to vimentin intermediate filaments and to regulate the vimentin filament organization (Wyles *et al.*, 2007). Here in the basal conditions, the ORP4L–VAP-A BiFC complexes showed localization to vimentin but also to a lesser extent at the PM (I, Figure 1A). At the PM, the ORP4L–VAP-A BiFC signals co-localized with the ER–PM MCS marker E-syt2 (I, Figure 4A). The 25OHC treatment, but not 22(R)OHC or 7KC, caused a re-localization of ORP4L–VAP-A to punctate cytosolic-like structures in 62% of the treated cells (I, Figure 1B), which did not co-localize with the vimentin (I, Figure 4B). Also the cholesterol depletion through lipoprotein starvation or Mevastatin resulted in similar punctate elements in 40% and 50% of the cells, respectively (I, Figure 2B). Furthermore, upon the LDL treatment, the ORP4L–VAP-A BiFC was found at the PM and did not display vimentin-associated structures (I, Figure 2B).

The sterol-binding deficient mutant of ORP4L displayed enhanced PM targeting compared to wild-type ORP4L–VAP-A BiFC, as the co-localization of ORP4L(ΔELSR)–VAP-A with the vimentin filaments was abolished (I, Figure 4A,B). The punctate elements induced by 25OHC or sterol depletion, which co-localized with the ER marker protein disulphide isomerase (PDI) (I, Figure 4C), were also observed with ORP4L(ΔELSR)–VAP-A after the 25OHC treatment (I, Figure 4A,B), suggesting that the localization of ORP4L–VAP-A complexes to the condensed ER elements is not a result of direct binding of 25OHC to ORP4L. It is possible that the 25OHC elevation regulates the ORP4L–VAP-A localization via an indirect mechanism, or that the ORP4L(ΔELSR) mutant does not completely prevent the 25OHC binding to ORP4L.

ORP4L was previously shown to localize to vimentin, and a leucine repeat motif of ORP4L was suggested to be important for the native organization of the vimentin filaments, since the overexpression of ORP4S or ORP4L mutant, with a leucine repeat deletion, aggregates the vimentin filaments close to the nucleus (Wang *et al.*, 2002; Wyles *et al.*, 2007). On the other hand, another study implicated that the PHD of ORP4L is important for the impact of ORP4L on vimentin organization, since the overexpression of mutant ORP4L lacking the PHD strongly induced vimentin aggregation (Charman *et al.*, 2014).

In controversy with our observation, the study by Wyles *et al.* (2007) indicated that the 25OHC or cholesterol binding does not affect the ORP4L localization to vimentin. In our observations the 25OHC and the cholesterol depletions accumulated ORP4L–VAP-A BiFC to condensed ER elements, and the LDL treatment induced PM targeting of the ORP4L–VAP-A complexes. However, the conclusion of the study was acquired by using a very different approach to ours: In the earlier study the affinity of recombinant wild type and a sterol binding deficient mutant ORP4L(Δ501-505) proteins for vimentin binding was analysed using a pull-down assay, combined with 25OHC and cholesterol treatments. The pull-downs showed similar affinity of wild type ORP4L and ORP4L(Δ501-505) for the vimentin binding, and furthermore, no difference was observed for the vimentin binding of ORP4L with the sterols compared to the non-sterol containing controls, resulting to a hypothesis that the vimentin targeting of ORP4L is not regulated by sterols (Wyles *et al.*, 2007).

However, consistent with our observations, in live cell imaging the sterol binding deficient mutant (Δ501-505) of ORP4L was not observed to co-localize with vimentin (Wyles *et al.*, 2007), and in our studies the ORP4L(ΔELSR)–VAP-A BiFC signal localized at the PM instead. It is possible that, despite the affinity of sterol binding mutants of ORP4L for vimentin, in live cells other cellular mechanisms are reducing its vimentin targeting. Our observation suggests that the sterol binding domain is important for the ORP4L vimentin targeting, but is not however necessarily due to its sterol binding capacity, as the ORP4L(ΔELSR) was not completely immune to the 25OHC treatment. Possibly the 4-aa deletion mutation caused a conformational

change, which permitted more efficient targeting to the PM. On the other hand, the discrepancy with the earlier study addressing ORP4L vimentin targeting (Wyles *et al.*, 2007) could also be a result of the sterol manipulations affecting specifically the interaction site in the ORP4L–VAP-A complex but not in ORP4L alone.

Here we did not analyse the putative effect of ORP4L–VAP-A BiFC on the vimentin filament organization. Our result, however, provides a novel clue implicating that the ORP4L function is not limited to the interaction with vimentin, but instead ORP4L also plays a role at the ER–PM MCSs. In fact, ORP4 silencing causes a growth arrest and proliferation defect, that were suggested to be independent of the ORP4L vimentin targeting (Charman *et al.*, 2014). In addition, in T-cell acute lymphoblastic leukemia cells ORP4L is shown co-localize and activate phospholipase C β3 (PLCβ3) at the PM by scaffolding PLCβ3 with CD3ε and $G\alpha_{q/11}$ into a signalling complex, rquired for $IP_3$ production and $Ca^{2+}$ release from the ER (Zhong *et al.*, 2016).

*5.1.5 ORP2–VAP-A targeting to LDs is inhibited by 22(R)OHC (I)*

In basal HuH7 cells, the ORP2–VAP-A BiFC displayed a blob-like perinuclear localization, which was found to associate with ER–LD contacts visualized with Bodipy-$C_{12}$ and the ER marker PDI (I, Figure 5A,B). In a previous study ORP2 was suggested to localize on the surface of the LDs (Hynynen *et al.*, 2009), but here the ORP2–VAPA BiFC was often observed in enlarged ER patches surrounded by LDs. This is, however, most likely a result of a BiFC overexpression artefact since ER does not reportedly form large blob-like structures surrounded by LDs.

The treatment with the ORP2 high-affinity ligand, 22(R)OHC, reduced the BiFC complexes' LD targeting in 76% of the cells, and promoted a diffuse ER localization of the BiFC, and also to a lesser extent localization at filament-like structures and the PM (I, Figure 1D). The filmentous ORP2–VAP-A structures most likely represent localization to ER sheets or tubules, as there was no co-localization observed with tubulin, actin, vimentin nor the Golgi, and VAP-A is predominantly attached to the ER. The low-affinity ligand 25OHC had a similar, but much weaker effect on the ORP2–VAP-A distribution as compared to 22(R)OHC.

The 7KC treatment or the cellular cholesterol manipulations did not affect ORP2–VAP-A BiFC localization compared to basal control (I, Figure 1D, 2D). The BiFC was, however, distributed more to ring-like structures surrounding the LDs and not vice versa, suggesting that the sterol manipulations reduce the formation of artefactual blob-like ER structures.

Similar to the OSBP and the ORP4L analysis, the ligand-binding dependency of ORP2–VAP-A localization was studied by using the sterol binding deficient mutant

of ORP2. Importantly, the ORP2(ΔELSK)–VAP-A BiFC was targeted to LDs regardless of the 22(R)OHC or 25OHC treatments, indicating that the subcellular localization of ORP2–VAP-A is determined by the ORP2 oxysterol liganding. This is consistent with the earlier finding by Hynynen *et al.* (2009), which showed that the 22(R)OHC treatment reduces the LD targeting of ORP2 and consistently, the sterol binding deficient mutant of ORP2 (I249W) displayed enhanced LD targeting.

Our results indicate that the ORP2 high-affinity oxysterol ligand binding affects the LD targeting of ORP2 also in complex with VAP-A. ORP2 is intrinsically a cytosolic protein but the dispersed cytosolic-like distribution of the ORP2–VAP-A caused by 22(R)OHC treatment indicated that ORP2 remains also under these conditions associated with the ER via VAP-A. Interestingly, the localization of ORP2–VAP-A BiFC to the PM indicates that ORP2 localizes to ER–PM MCSs as a result of ORP2 ligand binding. The ligand binding most likely results in a conformational change that inhibits the exposure of an ORP2 LD targeting motif, albeit the precise mechanism of ORP2 LD targeting is yet unknown.

## 5.2 ORP2-KO hepatocytes display altered expression of thousands of genes

### *5.2.1 ORP2-KO dysregulates mRNAs involved in actin regulation (II)*

To extensively analyse the ORP2-related cellular functions, we established a stable ORP2 knock out (KO) cell line in HuH7 hepatocytes by employing CRISPR-Cas9 mediated gene editing. Two different KO cells lines, derived from different gRNAs used in the genome manipulation, were used in the studies to minimize the possible off-target effects which may have occurred during the genome manipulation process. The viability of the KO cells importantly indicated that ORP2 is not a prerequisite for viability. The ORP2-KO cells, however, displayed an altered growth pattern upon culture, as the KO cells were forming ‘islet-like’ cell clusters instead of growing as an even monolayer like the maternal HuH7 cells (II, Figure 1C).

To gain clues of the cellular effects of ORP2 depletion, mRNA levels of four parallel cultures of both ORP2-KO cell lines and controls were analysed using RNA sequencing. The RNA sequencing of the ORP2 depleted cells revealed altered expression of >2000 mRNAs, 579 of which had at least 2-fold expression changes. Importantly, the analysis showed that the ORP2 abrogation did not affect the mRNA levels of other ORP family members or VAPs (II, Supplemental Figure S2).

The transcriptome of ORP2-KO cells was analysed using the Ingenuity pathway analysis (IPA) software, which predicted a number cellular functions and cell signalling pathways to be dysregulated in the ORP2-KO cells. These included several

pathways involving the actin cytoskeleton (II, Figure 1A; Table 2). Of note, IPA identified significant dysregulation in 12 mRNAs involved in the RhoA signalling pathway and 16 mRNAs of Rac1 signalling (II, Figure 4A; Supplementary Figure S5). These pathways are key components in the organization of F-actin into stress fibers and lamellipodia, respectively.

The up-regulated RhoA signalling components were Rhotekin 1 and 2, phosphatidylinositol-4-phosphate 5-kinase (PI4P5K), DIAPH1 and actin related protein 2/3 (Arp2/3) complex subunits 1A and 5, while the down-regulated ones were lysophosphatidic acid receptor (LPAR), profilin 1 and 2, Rho-associated protein kinase 2 (ROCK), G-actin 1 and cofilin-2. From those PI4P5K, cofilin-2 and Arp2/3 complex subunits are also subject to regulation by Rac1 signalling. Additional components up-regulated in the Rac1 signalling pathway were Abl interactor 2 (ABI2), mitogen-activated protein kinase kinase kinase 1 (MEKK1), protein kinase C (PKC), NADPH oxidase 1 (Nox1), guanine nucleotide exchange factor (DBS) and cytoplasmic FMR1 interacting protein 1 (CYFIP1), and down-regulated were N-Ras, mitogen-activated protein kinase 8 (JNK1), mitogen-activated protein kinase kinase 1 (MEK), mitogen-activated protein kinase 1 (ERK), nuclear factor κB subunit 1 (NF-κB), par-3 family cell polarity regulator (PAR3) and phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2α (PI3K).

Extensive disturbance in the genetic regulation of RhoA and Rac1 signalling associated genes suggests that ORP2 has a functional role in the actin cytoskeleton regulation. Interestingly, down-stream of RhoA signalling the DIAPH1 mRNA was found to be up-regulated which encodes a protein mDia1. mDia1 is in previous study indicated as an interaction partner of ORP2 (Li *et al.*, 2013). mDia1 plays a role in the actin filament polymerization and provides and additional clue to a putative role of ORP2 in actin cytoskeleton regulation.

### *5.2.2 ORP2-KO dysregulates TAG metabolic genes (III)*

In addition to genes involved in the cytoskeletal regulation, the ORP2 knock out cells displayed significant alterations in the expression of TAG metabolic enzymes. The up-regulated genes in TAG synthesis were GPAM, MBOAT1, AGPAT3 and LPGAT1, while the down-regulated ones were GPAT4 and lipin1–2. In addition, up-regulated enzymes involved in TAG hydrolysis were PNPLA4, CES1 and NDST1, and down-regulated PNPLA3 and DAGLα (III, Figure 5F).

Glycerol-phosphate acyltransferases (GPAM/GPATs) are implicated as rate limiting enzymes in the TAG synthesis through glycerol phosphate pathway, but genetic defects in other TAG biosynthetic enzymes have also been suggested to exert rate-limiting regulation in later stages of the biosynthetic pathway (Lewin *et al.*, 2008;

Wendel *et al.*, 2009). GPAM/GPATs execute the initial step of TAG synthesis by esterifying long-chain fatty acids with glycerol-3-phosphate.

The analysis of ORP2 knock out cells' transcriptome showed 20% up-regulation of the mRNA for mitochondrial GPAM, and oppositely a 10% reduction in the expression of the the mRNA for GPAT4. GPAT4 is localized to ER membrane and was only later identified as an isoform of GPAT proteins (Nagle *et al.*, 2008). The role of GPAT4 in TAG synthesis is not yet comprehensively characterized, but *GPAT4*$^{-/-}$ mice display 49% reduction in the liver GPAT activity and 45% diminished hepatic TAG content accompanied with resistance to diet-induced and genetic obesity (Nagle *et al.*, 2008; Vergnes *et al.*, 2006).

In addition to GPATs, lipins are important in determining the rate of TAG synthesis. In the mRNA profile of ORP2-KO cells the expressions of lipin-1 and lipin-2 were significantly down-regulated, by 27% and 39%, respectively. Studies of lipin-1 deficient mice point to reduced adipose tissue TAG storage (Phan *et al.*, 2004; Reue *et al.*, 2000), and in line, silencing of lipin-1 in hepatobastoma cells reduces cellular TAGs (Ishimoto *et al.*, 2009). The role of lipin-2 is less studied, but the protein is enriched in the liver and silencing of lipin-2 correlates with reduced TAG synthesis (Donkor *et al.*, 2007; Gropler *et al.*, 2009).

The ORP2 knock out cells also displayed small but significant reduction (-13%) in the expression of SREBP-1 (III, Figure 5E). SREBP-1 is expressed as an inactive membrane-bound precursor located in the ER, but during its activation, SREBP-1 undergoes a proteolytic cleavage and re-localization into nucleus where it binds to SRE elements in the promoters of lipogenic genes (Brown and Goldstein, 1997). However, despite its proteolytic processing, the activity of SREBP-1c, which is the major SREBP-1 isoform responsible for the regulation of glucose and fatty acid metabolism, is suggested to be mainly regulated transcriptionally by insulin via the PI3K/Akt pathway (Azzout-Marniche *et al.*, 2000; Fleischmann and Iynedjian, 2000; Foretz *et al.*, 1999b; Kim *et al.*, 1998; Porstmann *et al.*, 2005; Ribaux and Iynedjian, 2003). The ORP2-KO transcriptome also displayed significant alterations in the mRNA levels of 28 established SREBP-1 target genes (III, Figure 5E). Reduced expression of SREBP-1 in the ORP2 knock out cells implies defects in cellular fatty acid and TAG synthesis, as diminished SREBP-1 is associated with reduced expression of a range of lipogenic enzymes, including FAS and SCD1, the levels of which were reduced by 31% and 17%, respectively. In addition, silencing of SREBP-1 in hepatoblastoma cells correlates with reduced Lipin-1 expression (Ishimoto *et al.*, 2009).

The altered expression of TAG biosynthetic enzymes and SREBP-1 in ORP2 knock out cells implies effects on the cellular levels of TAGs, which are described in the section 5.3.3. However, transcriptional data cannot necessarily be used to explain physiological effects, as transcriptional regulation is only one mechanism among

others to regulate enzymatic reactions. In fact, several metabolic enzymes are regulated allosterically or by post-translational modifications, which permits more rapid adaptation to fluctuating availability of substrates (Jensen-Urstad and Semenkovich, 2012; Munday and Hemingway, 1999). In addition, the knowledge of the role of microRNA guided regulation of lipid metabolism is emerging. Several microRNAs are currently identified as important regulators of TAG metabolism by inhibiting the translation of their sequence-specific target genes in metabolic pathways (Horie *et al.*, 2013; Shirasaki *et al.*, 2013; Yang *et al.*, 2015). Moreover, in the ORP2-KO transcriptome all enzymes in TAG synthesis or hydrolysis were not consistently up- or down-regulated, making it difficult to directly predict the consequence on the levels of TAGs. The non-systematic alteration in the mRNA expression levels of the enzymes suggests that some compensatory effects may have arisen to moderate the enzymatic defects at the cellular level.

### *5.2.3 ORP2-KO reduces expression of glycolytic enzymes (III)*

The RNA sequencing of ORP2-KO cells also displayed down-regulation of enzymes involved in glycolysis. In fact, loss of ORP2 resulted in significant reduction of the transcripts of 12 glycolytic enzymes, which ultimately cover the whole glycolytic pathway. The enzymes affected were glucose-6-phosphate isomerase (GPI), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3–4 (PFKFB3–4), fructose-bisphosphate aldolase (ALDOA), triosephosphate isomerase 1 (TPI1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 1 (PGK1), phosphoglycerate mutase 1 (PGAM1), enolase 1–3 (ENO1–3) and muscle pyruvate kinase (PKM) (III, Figure 4E). Most of the mRNAs were reduced only moderately (by 10–30%), but the gene expression of PFKFB4 was reduced by 65%.

PFK-1 and L-PK are the rate limiting enzymes in the glycolytic pathway. PFK-1 is, however, not regulated at the transcriptional level, but by the cellular ATP/AMP ratio. In addition, fructose 2,6-bisphosphate is suggested to act as an allosteric activator of PFK-1 thus stimulating glycolysis (Rider *et al.*, 2004). The formation of fructose 2,6-bisphosphate from fructose-6-phosphate is catalysed by PFKFBs, from which the isoforms 3 and 4 were significantly down-regulated by the loss of ORP2, suggesting a decreased rate of glycolysis. In addition, the isoenzyme of L-PK, PKM, was found to be down-regulated in ORP2-KO cells. In total, the reduced expression of 12 glycolytic enzymes indicates a diminished rate of glycolysis in ORP2-KO cells, which is described in the section 5.3.2.

## 5.3 ORP2 knock out reduces the availability of energetic substrates

### *5.3.1 Membrane lipid and sterol content of ORP2-KO cells (III)*

In previous studies ORP2 was suggested to regulate cholesterol metabolism and silencing of ORP2 was shown to reduce the cellular levels of 22(R)OHC and 7KC, and increase cholesterol (Escajadillo *et al.*, 2016; Hynynen *et al.*, 2005; Laitinen *et al.*, 2002). To comprehensively examine the possible effects of ORP2 depletion on the concentration of membrane lipids and sterols we exploited mass spectrometric lipidome analysis. The cellular lipid levels were measured from cells cultured under basal conditions, but analysis of free and esterified cholesterol levels were additionally performed after modulating the cellular cholesterol metabolism by lipoprotein starvation and by Mevastatin or LDL treatments prior to the measurements. The analysis covered different cellular lipid classes and the molecular species of following lipid classes: PCs, lyso-PCs, PEs, PE-plasmalogens, PIs, PSs and phosphatidylglycerols. In addition, ceramides, sphingomyelins and oxysterols (25OHC, 27OHC, 7αOHC, 7βOHC and 7KC) were analysed. Unexpectedly, the results showed no significant alterations in any of the above lipid classes or oxysterol species in the ORP2-KO cells compared to the controls (III, Supplementary Figure S3). The result indicates that either (i) in hepatocytes the ORP2 function is not directly connected to membrane lipid or sterol homeostasis or (ii) that the HuH7 cells chronically lacking ORP2 have developed compensatory adaptations.

Furthermore, we cannot exclude the possibility that ORP2 executes functions in a cell type specific manner, and in some cell types ORP2 may have a tighter connection to sterol metabolism. This is implicated by the study by Escajadillo *et al.* (2016), which showed that the modulation of ORP2 expression affects the cellular levels of cholesterol and certain oxysterols in adrenocortical cells, which have an important role in steroid hormone biosynthesis. Cholesterol is the precursor for steroid hormone synthesis and also oxysterols are well established intermediates in steroid hormone production (Hanukoglu, 1992; Olkkonen *et al.*, 2012), hence the ORP2 silencing effects on the adrenocortical sterol concentrations could reflect a specific steroidogenic function of ORP2 in certain tissues.

### *5.3.2 ORP2-KO reduces glucose utilization (III)*

Inhibition in the expression of glycolytic enzymes in ORP2-KO cells suggests that ORP2 may have a role in cellular glucose homeostasis. The putative effects of ORP2 depletion in the glucose processing were studied by measuring the rate of glucose uptake, glycolysis and glycogen synthesis in KO cells. Importantly, the study indicated that all of these cellular glucose handling processes were significantly impaired due to loss of ORP2. The uptake of $[^3H]$-2-deoxy-D-glucose in a 15 min

period was reduced by 28–33% (III, Figure 4A), the glycolysis measured by extracellular acidification rate was decreased by 22–37% (III, Figure 4F,G) and the synthesis of [$^{14}$C]glycogen from D-[U-$^{14}$C]-glucose in a 1 h period was reduced by 50–58% (III, Figure 4B). To further confirm the direct involvement of ORP2 in glucose handling, the glycogen synthesis was measured in ORP2-KO cells with ORP2 reintroduced by overexpression. The ORP2 overexpression in the KO cells increased the glycogen synthesis by 37 % (III, Figure 4C).

Moreover, the effects of two previously established ORP2 mutants, deficient in VAP- or PIP-binding, on the putative rescue of glycogen synthesis in the KO cells were analysed (III, Supplementary Figure S1B). Similar to the wild type, the VAP-binding deficient mutant rescued the KO phenotype, suggesting that the interaction with the ER or the VAPs are not a prerequisite for ORP2 to contribute the cellular glucose handling. The effects of the PIP-binding mutant varied between the experiments, thus the role of ORP2's association with PIPs in the context of glucose metabolism could not be reliably determined.

The reduced rate of glycolysis in ORP2-KO cells is consistent with the observed reduction of the mRNAs of glycolytic enzymes. The impaired synthesis of glycogen suggests impaired activity of GK, GYS or GSK-3. The GK provides substrates for glycogen synthesis by phosphorylating glucose to G-6-P, which also promotes glucose uptake via GLUT2 by maintaining a glucose concentration gradient. However, GK is transcriptionally regulated, and the ORP2-KO cells' transcriptome did not indicate inhibition in the GK nor in the GLUT2 expression. GYS is principally regulated via phosphorylation by GSK-3, which is a down-stream target of PI3K/Akt signalling, as described in section 5.4.2. GLUT2 is the major PM glucose transporter of hepatocytes, but also GLUT1 is expressed in the liver (Karim *et al.*, 2012). The expression of GLUT1 is also regulated by the PI3K/Akt signalling (Barthel *et al.*, 1999), and the ORP2-KO cells' transcriptome displayed a 30% reduction in the GLUT1 mRNA, which is in line with the ≈30% defect of glucose uptake by the KO cells.

### *5.3.3 ORP2-KO reduces TAG content and TAG synthesis (III)*

The involvement of ORP2 in TAG synthesis is suggested by earlier studies (Hynynen *et al.*, 2009; Weber-Boyvat *et al.*, 2015b), and the dysregulation of the TAG metabolic genes in the ORP2-KO cells' transcriptome further supported an involvement of ORP2 in TAG metabolism. Thus, the TAG content of ORP2-KO cells was analysed. The analysis showed that, as compared to the normal ORP2 expressing cells, the ORP2-KO reduced the basal cellular TAG content by 21–28% (III, Figure 5A). Moreover, analysis of the KO cells' capacity to synthesize [$^{3}$H]TAGs in a 3 h period from externally administered [$^{3}$H]oleic acid also revealed diminished TAG synthesis by the KO cells (III, Figure 5B). Similar to glycogen synthesis, a direct

connection of ORP2 protein with the TAG synthesis was confirmed by an overexpression rescue study, which displayed a 31% increment in the TAG synthesis of the ORP2 overexpressing KO cells (III, Figure 5D).

Additionally, no significant alteration was observed in the rescue effect between the wild type and VAP- or PIP-binding deficient mutants of ORP2 (III, Supplementary Figure S1C). This indicates that neither is the VAP- nor PIP-binding indispensable for ORP2 mediated enhancement in TAG synthesis. This is to some extent contrary to the previous indication by Weber-Boyvat *et al.* (2015b), who concluded that ORP2 regulates TAG metabolism in complex with VAPs. The conclusion in the earlier study was result from a notion that triple-silencing of ORP2 together with VAP-A/-B had a greater impact on TAG synthesis and degradation than the double-silencing of ORP2 with VAP-A or VAP-B. However, now taking into account our ORP2(mFFAT) rescue experiments in the ORP2-KO cells, it is possible that the knock-down of VAP-A/-B in Weber-Boyvat *et al.* (2015b) affected the TAG synthesis via a different mechanism that is at least partially independent of their interaction with ORP2.

One of the main functions of glycolysis is to provide carbons and energy to be used for fatty acid synthesis. Hence, it not surprising that the reduced glycolytic capacity of ORP2-KO cells also results in a reduced content of TAGs. It is, however, important to take into account that glycolysis also provides precursors for glycerophospholipid and cholesterol synthesis, the concentrations of which were not affected in the KO cells. Moreover, the TAG synthesis of ORP2-KO cells from externally added fatty acids was also significantly reduced, but the uptake of fatty acids to KO cells was shown not be affected (III, Figure 5C). This suggests that ORP2 affects the regulation of TAG synthesis also at a later stage independent of the availability of glycolytic substrates. However, it is possible that the impaired synthesis of TAGs correlates with the reduced release of glycolytic energy utilized for the *de novo* lipogenesis. Moreover, the observed reduction in the levels of basal TAGs and in the TAG synthesis could be a result of down-regulation in TAG synthesizing enzymes GPAT4 and Lipin1–2 in the ORP2-KO cells.

#### *5.3.4 ORP2-KO and ER–lipid droplet contacts (III)*

ORP2 is previously suggested to enhance MCSs between LDs and ER when in complex with VAP-A (Weber-Boyvat *et al.*, 2015b). We analysed the quantity of ER–LD MCSs in ORP2-KO and control cells under basal conditions or after 3 h fatty acid loading by transmission electron microscopy. The analysis showed that the total LD area or the total length of the ER–LD contacts per cell were both increased in the ORP2-KO cells compared to the control cells. (III, Figure 6B,C). Moreover, after fatty acid loading, these parameters were significantly increased in the control cells, but remained unaltered in the KO cells, suggesting that the ER–LD contacts are not directly dependent on the LD area. Importantly, these results indicate that the ORP2–

VAP complexes at ER–LD interfaces are not a requirement for MCS formation, but vice versa, under the basal culture conditions the quantity of the ER–LD contacts was greater in the KO cells than within the controls.

These results do not support the earlier observation, which was also made in the HuH7 cell model (Weber-Boyvat *et al.*, 2015b). It is however important to take into account that these studies were carried out by different methodological approaches. Here we used ORP2-KO cells and in the previous study the ER–LD contacts were measured after ORP2–VAP-A BiFC overexpression. The problem of the BiFC approach in the analysis of MCS is its irreversible nature (Kerppola, 2008; Kodama and Hu, 2012). MCSs are suggested as highly dynamic and temporal connections, but when using the BiFC approach with two interacting proteins which localize to the apposing membranes, the protein interaction formed between the two membranes is stabilized by the BiFC complex, which once formed can no longer dissociate (discussed in the section 5.1.1). Thus the MCSs containing BiFC protein complexes are transformed to stable contacts, and during 24 h overexpression carried out in the earlier study, the number of contacts presumably accumulates over the time. We speculate that the measurement of contacts sites in ORP2-KO cells provides a more accurate perspective to the intrinsic MCSs as compared to the BiFC overexpression. Considering this, the involvement of ORP2 in the ER–LD contact site formation is not sufficiently justified.

## 5.4 Novel ORP2 interaction partners and cell signalling

### *5.4.1 ORP2 interacts with Hsp90 and Cdc37 (III)*

In addition to gaining novel understanding on the intracellular role of ORP2 by analysing the ORP2-KO effects on the global mRNAs, the putative ORP2-related functions were studied by screening of novel ORP2 protein–protein interactions. The identification of ORP2 interaction partners was performed by transient expression of HA-ORP2 construct in HuH7 cells followed by HA co-immunoprecipitation and identification of eluted proteins by mass spectrometry proteomics. The interactome analysis identified 107 putative novel interaction partners for ORP2 (II: Supplemental data).

Among the 107 identified proteins, one of the putative new partners of ORP2 was Cdc37. Cdc37 is a co-chaperone of heat shock protein 90 (Hsp90), which adapts client protein kinases to Hsp90 kinase activation system (Silverstein *et al.*, 1998). To confirm the validity of the interaction we carried out pull-down experiments from HuH7 cell lysates using purified recombinant GST-ORP2 or plain GST as negative control. The experiments indicated a direct interaction between endogenous Cdc37 and GST-ORP2. Interestingly, the pull-downs also showed an interaction of ORP2

with Hsp90 and Akt (III, Figure 2F). Akt is one of the Hsp90–Cdc37 chaperone clients, and disruption of Akt–Hsp90 interaction promotes Akt dephosphorylation and kinase inhibition via protein phosphatase 2A (Sato *et al.*, 2000). Moreover, Akt and its phosphorylating kinase PDK1 are sensitive to Hsp90 inhibitors (Basso *et al.*, 2002).

As Hsp90 and Cdc37 are found within cells as a binary complex and thus it is possible that Hsp90 was recognized in the pull-down sample due to its affinity for Cdc37. However, in the ORP2 pull-down sample compared to the starting lysate the band intensity was stronger for Hsp90, but with Cdc37 the band intensity was rather similar in both samples, suggesting that ORP2 may interact with Hsp90 directly and not via Cdc37. The interaction was further confirmed by treating the HuH7 cells with Withaferin A prior to the pull down – a drug which reportedly disrupts the Cdc37–Hsp90 complex (Yu *et al.*, 2010). The pull-down showed that the interaction of neither Hsp90 nor Cdc37 with ORP2 was abolished or reduced compared to the untreated cell control, indicating that both Cdc37 and Hsp90 interact directly with ORP2 (III, Figure 2G).

No similar drug that would abolish the interaction between Hsp90 and Akt is known, complicating the identification of direct interaction of Akt with individual proteins in the Hsp90–Cdc37–ORP2 ternary complex. However, Akt was only barely visible in the ORP2 pull-down sample, which could indicate that ORP2 does not directly interact with Akt, but through Hsp90. The pull-down experiments also showed that the Withaferin A treatment promoted Akt dephosphorylation, detected initially by a band-shift and confirmed by lambda phosphatase treatment (III, Figure 2G,H). Importantly, in the GST-ORP2 pull-down sample only the hyperphosphorylated form of Akt was detected (III, Figure 2H). This finding strongly suggests that ORP2 does not interact with the inactive form of Akt but is physically involved in a complex in which Akt adopts its phosphorylated form, since dephosphorylated Akt was not detected in GST-ORP2 pull down. Hence it is possible that ORP2 is involved in the Akt activation process.

### *5.4.2 ORP2-KO inhibits PI3K/Akt-signalling (III)*

To further analyse the role of ORP2 in the Akt activation, the Akt-signalling activity was assessed in ORP2 knock out cells with Akt(Ser473) and with its down-stream target GSK-3β(Ser9) antibodies in both basal and fatty acid starved conditions followed by insulin stimulations. Western blot analysis of KO cells showed a significant reduction in the phosphorylation of both Akt and GSK-3β in all conditions (III, Figure 1A,B,C,D), indicating suppression of Akt signalling in ORP2 depleted cells compared to controls. The knock out cells were, however, not completely immune to the insulin stimulus, as insulin was able to increase the protein phosphorylations in the KO cells. Furthermore, the ORP2-KO cells' transcriptome

analysis showed dysregulation of 20 mRNAs of the PI3K/Akt signalling pathway (III, Figure 1A,B), IPA analysis of which indicated PI3K/Akt signalling as a significant ORP2-associated pathway and predicting it to be suppressed due to the loss of ORP2, consistent with the observed reduction in the Akt and GSK-3β phosphorylations.

The analysis was expanded to non-cancerous cells, to avoid effects which could be caused by a point mutation in the p53 tumour suppressor gene of HuH7 cells. ORP2 was knocked down with a lentivirus expressing ORP2-specific shRNA in primary human umbilical vein endothelial cells (HUVECs), followed by epidermal growth factor stimulation and analysis of Akt(Ser473) phosphorylation. Moreover, HUVECs were subjected to transduction using lentiviral vectors expressing wild type or two previously described ORP2 mutants, followed by the Akt(Ser473) quantification. Similar to HuH7 cells, depletion of OPR2 reduced the phosphorylation of Akt (-65%) in HUVECs (III, Figure 2E), and on the contrary, ORP2 overexpression increased the Akt phosphorylation by 18-fold (Supplemental Figure S1A). Importantly, similar to the TAG synthesis rescue in the ORP2-KO cells, both VAP- and PIP-binding deficient mutants were able to increase the Akt(Ser473) phosphorylation in HUVECs. The results imply that the role of ORP2 in the stimulation of Akt phosphorylation is not limited to immortalized cancer cells, and neither is the affinity of ORP2 for PIPs nor VAPs necessary for this function.

The analysis of GSK-3β phosphorylation also provided a plausible explanation of the observed reduction of glycogenesis in the KO cells. Insulin/Akt signalling is designated as the main regulator of glycogenesis via GSK-3 phosphorylation at Ser9, which directly activates GYS. Impaired GSK-3 phosphorylation results in the inactivation of GYS and reduced glycogen synthesis, which was shown in section 5.3.2.

### *5.4.3 ORP2 interacts with components of Rac1 and RhoA signalling pathways (II)*

As described previously, the ORP2-KO cells' transcriptome analysis revealed dysregulation in dozens of components involved in the RhoA and the Rac1 signalling pathways. Consistently, analysis of the ORP2 interactome revealed 107 potential ORP2 protein interactions, of which the IPA analysis suggested 'RhoA signalling' as the most significant putative ORP2-associated pathway. The proteins identified in the ORP2 interactome and involved in RhoA signalling were Septin 9, ezrin, myosin light chain 12 (MLC12), Rho GTPase activating protein 12 (Arhgap12) and PI4Kα (II, Figure 4A). Furthermore, IQGAP1 of the Rac1 signalling was suggested as an interaction partner of ORP2 (II, Supplemental Figure S5). The interaction of ORP2 with Septin 9, MLC12, Arhgap12 and IQGAP1 were validated by independent pull-down or co-immunoprecipitation assays (II, Figure 4B,C; Supplemental Figure S5).

Septins are filament forming GTP-binding proteins, which are indicated as regulators of cytokinesis. Septin 9 localizes to the contractile ring of mitotic cells and disruption of septin 9 function is found to cause an arrest in cytokinesis (Chircop, 2014). Arhgap12 is a member of Rho GTPase activating proteins which negatively regulates the activity of Rho GTPases by promoting the hydrolysis of GTP and hence have an important role in cytoskeletal regulation (Moon and Zheng, 2003). Furthermore, myosins constitute a large family of actin binding proteins. MLC12 is one of regulatory light chains of the non-muscle myosins, the phosphorylation of which controls actin–myosin interaction and provides the contraction force for the cell. MLC phosphorylation is regulated by ROCK downs-stream of RhoA, and in non-muscle cells the acto-myosin bundles produce force for cell division through the contractile ring, and for cell migration in the form of stress fibers. Consistently, MLC12 knock down is shown to disrupt actin stress fiber formation (Park *et al.*, 2011). Moreover, IQGAP1 is a protein scaffold down-stream of Rac1/Cdc42 and suggested as an important factor in the regulation of cadherin-mediated cell adhesions, cell polarization, lamellipodia formation and cell migration (Noritake *et al.*, 2005; Owen *et al.*, 2008). Interestingly, the scaffolding function of IQGAP1, which brings protein kinases in close proximity, was found to be important for regulation of Akt signalling. IQGAP1 was found to scaffold PI3K, PI4KIIIα and PIPKIα for efficient production of PI(3,4,5)$P_3$ and secondly, to scaffold PDK1 and Akt to provide functional proximity for PI3K/Akt signalling activation (Choi *et al.*, 2016).

## 5.5 ORP2 and the actin cytoskeleton

### *5.5.1 ORP2 impacts actin organization (II,III)*

IPA analysis of the >500 gene transcripts with at least 2-fold expression changes in the ORP2-KO cells as compared to controls suggested a close link between the ORP2 and a number of cellular functions involving actin cytoskeleton dynamics. Moreover, RhoA and Rac1 signalling, additionally connected to ORP2 by the identification of novel protein interactions, are important factors in the regulation of actin dynamics. To analyse the putative effects of ORP2 depletion on the actin organization, the KO cells were stimulated with insulin and the F-actin was visualized with fluorescent phalloidin. Interestingly, the fluorescence microscopy analysis showed that the cells lacking ORP2 had a significant defect in the formation of lamellipodia (II, Figure 2C). As HuH7 cells are not described as highly migrating cells, the fluorescence microscopy analysis was expanded to HUVECs. Consistent with the results in the ORP2-KO cells, the overexpression of ORP2 in HUVECs displayed a significant enhancement of lamellipodia formation compared to the controls (II, Supplemental Figure S4). The formation of lamellipodia requires active Rac1 (McCarty *et al.*, 2005), suggesting that ORP2 depletion interferes with Rac1 signalling. Presumably the dysregulated mRNAs of the Rac1 signalling components and/or the impeded

interaction of IQGAP1 with ORP2 results in unbalanced Rac1 signalling consequently disturbing lamellipodia formation in the ORP2 depleted cells.

Moreover, the fluorescence microscopy analysis of F-actin showed a significant induction of cell surface blebs in the ORP2-KO cells, which were only seldom detectable in the control cells (II, Figure 2D). Knock down of ORP2 in the HUVECs resulted in the same phenotypic increment in bleb formation, suggesting that this ORP2 disruption phenotype is not limited to HuH7 cells. The bleb-based cell motility is associated with a high amount of active RhoA (Charras and Paluch, 2008). Some cells are found to switch to bleb-based motility under higher mechanical resistance of the substratum, which requires myosin II mediated actomyosin contractility (Ruprecht *et al.*, 2015; Zatulovskiy *et al.*, 2014), concomitant with increased RhoA activity. The interactome and transcriptome analyses strongly imply involvement of ORP2 in RhoA signalling, but further studies are required to confirm the putative enhancement in the RhoA signalling activity due to loss of ORP2.

Moreover, the effect ORP2 overexpression to the F-actin organization in the KO cells was analysed accompanied with the VAP- or PIP-binding deficient mutants. Intriguingly, the overexpression of wild type ORP2 and the VAP-binding deficient mutant resulted in an enhancement in formation of filopodia-like cell surface protrusions (II, Figure 3A,B,D), suggesting increased actin polymerization. However, the F-actin of the KO cells transfected with the ORP2 mutant incapable of PIP-binding did not differ from the untransfected KO cells (II, Figure 3C). This indicates that the ability of ORP2 to bind PIPs is important for its actin regulatory function.

Further analysis of insulin stimulated HuH7 cells and HUVECs showed that GFP-ORP2 localized in cortactin-positive lamellipodia, accompanied with phosphorylated Akt(Ser473) and Cdc37 (III, Fig 3A,B; Supplementary Figure S2). It is already previously suggested that PI3K and active Akt localize to the lamellipodia of migrating cells, and facilitate cell migration by inducing actin polymerization and actin reorganization into lamellipodia and filopodia (Qian *et al.*, 2004; Ridley *et al.*, 2003; Xue and Hemmings, 2013). Furthermore, PI3K and Akt are found to activate Rac1 (Henderson *et al.*, 2015; Scita *et al.*, 2000). Hence, the suppression of PI3K/Akt signalling mediated by ORP2-KO is fully consistent with the dysregulation of mRNAs involved in Rac1 signalling and with the observed inhibition of lamellipodia formation.

### *5.5.2 ORP2-KO impairs cell adhesion and proliferation (II)*

The RNA sequencing and the IPA analysis uncovered, as a result of ORP2 depletion, dysregulation in 194 mRNAs encoding proteins involved in cell migration, 50 mRNAs involved in cell adhesion and 223 mRNAs involved in cell proliferation processes, suggesting involvement of ORP2 protein in these cell physiological functions.

The ORP2-KO effects on cell adhesion and proliferation were measured using the xCELLigence® Real-Time Cell Analysis system. During the 5 h measurement, cell adhesion to the substratum was decreased in ORP2-KO cells by 44–49% as compared to control (II, Figure 6B). Additionally, the slope of cell proliferation curve during the 45 h measurement was reduced by 38–64% (II, Figure 6D). These results indicate that ORP2 is required for cell adhesion and proliferation, and the defects in these processes due to ORP2 depletion most likely result from disturbed expression of necessary genes and disrupted actin organization. Moreover, Septin9 is suggested to be important for cytokinesis and MLC phosphorylation in the contractile ring producing the force for the cell to divide, hence it is possible that ORP2 also participates in cell proliferation via its novel protein interactions.

### *5.5.3 ORP2-KO mediated inhibition of cell migration (II)*

The migration of ORP2-KO cells was studied using a wound healing assay and xCELLigence® trans-filter migration application. Both analyses showed a significant defect in the migration of ORP2-KO cells as compared to ORP2 expressing control cells. The trans-filter migration analysis showed 65–77% reduction in the slopes of ORP2-KO cells' migration curves (II, Figure 5E), and consistently, ORP2-KO cells' wound closure was inhibited by 30–49% (II, Figure 5B). The impact of ORP2 on cell migration was further studied by overexpression analysis. Wild type ORP2 and mutants deficient in VAP- or PIP-binding were overexpressed in the ORP2-KO cells. Overexpression of the wild type ORP2 and the VAP-binding deficient mutant facilitated the wound closure by 74–130% compared to ORP2-KO cells, further supporting an important role for ORP2 in cell migration (II, Figure 5C). However, the ORP2 PIP-binding deficient mutant was unable to rescue the wound healing defect of the ORP2-KO cells (II, Figure 5C). This indicates an important role for ORP2 PIP liganding in the cell migration process, which is consistent with the observed defect of PIP-binding mutant to induce filopodia formation described above. Of note, it is possible that the phenotypic feature of ORP2-KO cells to grow as 'islet-like' cell clusters (described on page 53) is also a result of impaired migration of the cells, as one could postulate that the atypical clustering of cells upon culture is a consequence from proliferating cells' inability to migrate apart after cell division.

The observed cell migration defect is consistent with the described defect of lamellipodia formation by the ORP2-KO cells, a process that is dependent on active Rac1. Altogether 194 mRNAs involved in cell migration and 16 mRNAs of Rac1 signalling were found to be dysregulated in ORP2-KO cells, which possibly explains the defect in lamellipodia formation resulting in reduced cell locomotion. In addition, the inhibition of lamellipodia formation and cell migration can be a result of supressed PI3K/Akt signalling of the ORP2 KO cells.

A number of studies connect PI3K/Akt signalling to the regulation of actin cytoskeleton organization and to cell migration. Both PI3K and Akt are found to induce actin filament reorganization and increase lamellipodia formation, as well as cell migration. On the contrary, drugs inhibiting PI3K and Akt are found to have the opposite effects. (Kim *et al.*, 2001; Qian *et al.*, 2004; Usatyuk *et al.*, 2014; Zhao *et al.*, 2016) In fibroblasts the Akt mediated enhancement in cell motility is suggested occur via Rac/Cdc42 (Higuchi *et al.*, 2001), and PI3K/Akt are suggested to activate Rac1 (Henderson *et al.*, 2015; Scita *et al.*, 2000). Furthermore, Enomoto *et al.* (2005) showed that Akt phosphorylates an actin binding protein girdin, the phosphorylation of which increases migration of kidney epithelial cells. Furthermore, the study showed that Akt and phosphorylated girdin localizes to the lamellipodia of migrating cells, and in line, silencing of girdin disrupted the lamellipodia formation (Enomoto *et al.*, 2005). Girdin also induces the migration of glioblastoma cells via PI3K/Akt (Gu *et al.*, 2014; Ni *et al.*, 2015). These studies indicate that the suppressed Akt signalling of ORP2-KO cells may be responsible for the inhibition of cell migration, and intriguingly, girdin was one of the putative ORP2 interaction partners identified in the interactome analysis. The interaction between ORP2 and girdin, however, is not yet confirmed.

Hsp90 is also suggested to bind and crosslink actin filaments via the N-WASP and ARP2/3 complexes (Koyasu *et al.*, 1986; Park *et al.*, 2007), and Hsp90 is found to promote filopodia formation via N-WASP (Park *et al.*, 2007). Furthermore, inhibition of Hsp90 is found to decrease actin polymerization and the formation of filopodia and lamellipodia, as well as to inhibit cell motility (Taiyab and Rao, 2011). Hence, it is possible that dysfunctional Rac1 signalling and supressed PI3K/Akt signalling together impair the lamellipodia formation and migration of the ORP2 depleted cells, which, on the other hand, can be a result of impeded ORP2 protein interactions.

# 6 SUMMARY AND CONCLUSIONS

In my thesis, I examined the effects of ORPs' high-affinity oxysterol ligands and modulation of the cellular cholesterol content on the intracellular localization and distribution of ORP–VAP-A BiFC complexes, to understand how the fluctuation in the intracellular sterol concentrations regulates the association of these complexes with distinct MCSs. In our investigations we did not observe alterations in the subcellular distributions of ORP1L–VAP-A or ORP9L–VAP-A upon sterol manipulations, suggesting that these complexes are not under the regulation of the cellular sterol content, or alternatively, these protein complexes are sensitive to regulation by sterol(s) not analysed in this study.

The OSBP–VAP-A BiFC complexes were found to translocate to the *trans*-Golgi upon 25OHC loading or upon cellular cholesterol depletion, and the LDL treatment was found to have the opposite effect, resulting in a diffuse ER localization of OSBP–VAP-A BiFC, consistent with earlier observations on the regulation of OSBP localization via sterols (Mesmin *et al.*, 2013; Mesmin *et al.*, 2017; Nhek *et al.*, 2010; Ridgway *et al.*, 1992; Storey *et al.*, 1998). The effect of 25OHC on OSBP Golgi targeting was found to be dependent on the OSBP ligand-binding domain, suggesting that the ligand binding causes a conformational change in the OSBP that facilitates PI4P-targeting by the PH domain. In summary, our results provide strong evidence that the 25OHC or cholesterol depletion enhances the OSBP–VAP-A tethering at ER–*trans*-Golgi MCSs. Importantly, Mesmin *et al.* (2013) indicated, that in the ER–Golgi interfaces, OSBP exchanges PI4P and cholesterol bi-directionally between these organelles. It is reasonable that upon low cellular cholesterol, enhanced *de novo* cholesterol synthesis at the ER promotes cholesterol transport to the *trans*-Golgi, but what could be the functional rationale of the effect of 25OHC on OSBP Golgi targeting? To continue, Mesmin *et al*. also indicated that the 25OHC binding inhibits the OSBP counter-transport activity, hence it is unlikely that the 25OHC mediated Golgi targeting of OSBP is important for the maintenance of the adequate Golgi cholesterol levels. It is possible, despite their similar impacts on OSBP Golgi targeting, that cholesterol depletion and 25OHC regulate distinct properties of OSBP. To support this, 25OHC was found to increase the association of CERT with *trans*-Golgi via OSBP, required for the transport of ceramides from ER to the Golgi apparatus for SM synthesis (Banerji *et al.*, 2010; Hanada *et al.*, 2003; Perry and Ridgway, 2006).

Similar to OSBP, ORP4L–VAP-A was found to be susceptible to regulation by 25OHC or cellular cholesterol manipulations. In basal conditions the ORP4L–VAP-A complexes were found to localize to vimentin filaments and to the ER–PM MCSs, but the vimentin targeting was reduced upon all of the sterol treatments. The 25OHC treatment or the cellular cholesterol depletion were found to strengthen the ORP4L–VAP-A association with the ER, and in turn, the LDL treatment caused enhanced PM

targeting of ORP4L–VAP-A. ORP4L is suggested to be important for cell proliferation, which is apparently independent of its property to localize to vimentin (Charman *et al.*, 2014). Hence, it is possible that the PM localization of ORP4 is important for its function in the maintenance of cell proliferation, possibly for its involvement in signal transduction.

Furthermore, we observed that the ORP2–VAP-A complexes exclusively localized to the interfaces of LDs and the ER, but the ER–LD targeting was severely compromised by the 22(R)OHC treatment, which caused the dispersal of the interaction along the ER network and also translocated ORP2–VAP-A complexes to ER–PM MCSs. The diverse localizations of ORP2 are consistent with our functional observations, that the cellular role of ORP2 is not limited to the TAG metabolism, but involves a wide spectrum of cell physiological functions the connections of which to LDs are unknown or even unlikely.

The ubiquitous expression pattern of ORPs in eukaryotic tissues and their capacity to target MCSs of various different organelles in complex with VAPs provides an interesting hypothesis that these protein complexes could be important for the formation of new contacts. However, our investigations in the ORP2 knock out hepatocytes do not support a role for ORP2 in the formation of ER–LD MCSs, but it does not exclude that ORP2 could play a role in the inter-organelle communication at the ER–LD interfaces. This is not necessarily the case for all ORPs, since Mesmin *et al.* have indicated that OSBP does not only execute a lipid transfer function at the ER–Golgi interfaces but it is also involved in the formation of new contacts (Mesmin *et al.*, 2013; Mesmin *et al.*, 2017).

In our analysis of the stable ORP2 knock out cells, we revealed new evidences of the metabolic functions of ORP2. We observed that the ORP2 depletion reduced the cellular TAG content and acute TAG synthesis, but the levels of membrane lipids and sterols remained unaffected, implicating a role of ORP2 in the maintenance of cellular energy reservoirs rather than in the homeostasis of membrane lipid compositions. This was further endorsed by the findings that the ORP2 depletion additionally reduced the uptake and the cellular processing of glucose, implicating a functional role of ORP2 in cellular anabolic processes. Importantly, our observations indicated that ORP2 depletion affects the TAG and glucose homeostasis by inhibiting the transcription of TAG and glucose metabolic enzymes as well as the transcription factor SREBP-1, and it also reduces the phosphorylation of GSK-3β – down-stream of PI3K/Akt signalling.

The PI3K/Akt signalling route is an important regulator of cellular glucose and fatty acid metabolism, and intriguingly, we detected a novel connection of ORP2 with the PI3K/Akt signalling. We were able to show that ORP2 physically interacts with the Hsp90–Cdc37 chaperone complex, which promotes Akt phosphorylation and activation of the signalling cascade. Consistently, the ORP2 knock out resulted in

dysregulation of the mRNAs of various PI3K/Akt signalling components and reduced the activating phosphorylation of Akt at Ser473.

Intriguingly, ORP2 was found to co-localize with phosphorylated Akt and Cdc37 at lamellipodia, and consistently, the formation of lamellipodia were severely compromised in the ORP2-KO cells, which also displayed a defect in migration. Furthermore, ORP2-KO was found to interact with components of the RhoA and the Rac1 signalling routes, and various mRNAs of the components of these signalling pathways were dysregulated as a result of ORP2-KO. In fact, the impaired lamellipodia formation and migration suggest compromised Rac1 signalling as a result of ORP2-KO, but most likely these phenotypic effects are also connected with the impaired Akt signalling. Various studies implicate to functional connections of PI3K and Akt with lamellipodia formation, cell migration or Rac1 regulation (Henderson *et al.*, 2015; Kim *et al.*, 2001; Qian *et al.*, 2004; Ridley *et al.*, 2003; Scita *et al.*, 2000; Usatyuk *et al.*, 2014; Xue and Hemmings, 2013; Zhao *et al.*, 2016). On the other hand, ORP2 was also found to associate with the Rac1 effector IQGAP1, with an established role in the cell migration (Noritake *et al.*, 2005), but which is also suggested to scaffold phosphoindositide kinases, PDK1 and Akt into functional proximity, by which it permits PI(3,4,5)$P_3$ formation and Akt activation (Choi *et al.*, 2016). In relation to the previous, ORP2 is indicated to bind PI(3,4,5)$P_3$ (Hynynen *et al.*, 2005), and here we observed that the ORP2 mutant defective in PIP-binding was unable to rescue the ORP2 depletion effects on cell migration. This indicated that the PIP-binding property is important for the actin regulatory function of ORP2. For example, it is possible that due to its PI(3,4,5)$P_3$ binding capacity, ORP2 is involved in the regulation of PI(3,4,5)$P_3$ second messaging and consequently the abolishment of ORP2 results in disrupted down-stream signalling processes observed as actin-based cell regulatory defects described here. The PIP-binding mutant of ORP2 was, despite this, able to increase the phosphorylation of Akt in HUVECs, suggesting that the mutation in the PIP-binding cleft may not prevent interaction of ORP2 with the Hsp90–Cdc37 chaperone complex. This hypothesis, however, requires future investigations.

ORP2 depletion also interfered with the cell adhesion and proliferation, and furthermore, the KO cells displayed increased formation of cell surface blebs, which indicates an impaired association of F-actin with the plasma membrane (Charras *et al.*, 2006). The increased blebbing implicates a high activity of RhoA (Aoki *et al.*, 2016; Charras and Paluch, 2008), and in addition to altered mRNA levels of RhoA signalling components, ORP2 was found to physically interact with several RhoA down-stream targets. Moreover, interaction of ORP2 with mDia1 is previously shown (Li *et al.*, 2013). Together these observations suggests that via various protein interactions, ORP2 plays important roles in several cell signalling processes, and the abrogation of ORP2 results in severe defects in cell physiology, indicating that disturbed function of ORP2 may contribute to pathological conditions. In support of this notion, our bioinformatics analyses revealed a significant up-regulation in ORP2

in malignant chromophore kidney cancer.

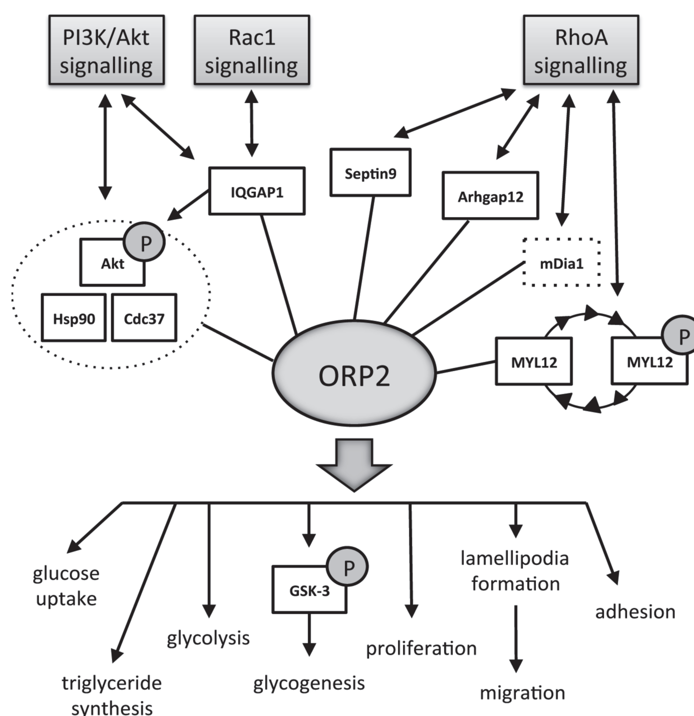


Figure 5. A schematic model of novel ORP2 interaction partners and the putative role of ORP2 interactions in different cellular processes. ORP2 was found to interact with Hsp90–Cdc37 complex, IQGAP1, Septin9, Arhgap12 and MYL12, molecular components of PI3K/Akt, Rac1 and RhoA signalling. ORP2 knock out was found to reduce the synthesis of glycogen and triglycerides, inhibit glucose uptake and glycolysis, and furthermore, impair lamellipodia formation, cell migration, adhesion and proliferation, suggesting a functional role of ORP2 in these cell physiological processes.

Finally, the present observations of the novel connections of ORP2 with the actin cytoskeleton, and the previously reported causation of an autosomal dominant hearing loss as a result of a frameshift mutation in *OSBPL2* encoding ORP2 (Thoenes *et al.*, 2015; Xing *et al.*, 2015), strongly suggests that ORP2 plays an important role in the regulation of actin polymerization in hair cell stereocilia, which could depend on the observed interaction of ORP2 with mDia1 (Li *et al.*, 2013), since mutations in DIAPH1 similarly result in an autosomal dominant non-syndromic hearing loss (Kang *et al.*, 2017; Neuhaus *et al.*, 2017; Stritt *et al.*, 2016).

To conclude, the present series of studies implicates a novel function of ORP2, which via PI3K/Akt signalling, acts as a regulatory link between cellular energy metabolism and cytoskeletal organization, and hence plays important roles in various cell physiological processes. Interestingly, a very recent study by Du *et al.* (2018) suggested that also ORP5 is involved in cell migration and proliferation, raising the possibility that this is a more general property among the OSBP/ORP family.

# 7 ACKNOWLEDGEMENTS

The present series of studies was carried out during the years 2012–2018 at the Minerva Foundation Institute for Medical Research, which is thanked for providing excellent research facilities. The University of Helsinki Doctoral Programme in Biomedicine (DPBM) is thanked for the educational and financial support.

I would warmly like to thank my supervisor Professor Vesa Olkkonen for giving me the opportunity to do my doctoral thesis in his research group and for giving me scientific guidance and support during these years. I appreciate how he always found time to help me through even the smallest problems encountered during the studies, and the freedom he gave me in designing and implementing my projects.

I would like to express my appreciation to Docent Varpu Marjomäki for accepting the role of opponent, and Professor Matti Airaksinen for accepting the role of custos in the thesis dissertation. I would also like to thank my thesis reviewers Professor Neale Ridgway and Professor Pekka Lappalainen for giving me helpful comments on my thesis. The thesis committee members, Professor Elina Ikonen and Docent Honqxia Zhao, are thanked for giving me valuable advice and support during the meetings.

I wish to acknowledge all the co-authors for their contribution and I am also equally grateful to all the people that I have worked with. Special gratitude belongs to Eeva Jääskeläinen, Liisa Arala and Riikka Kosonen for their great technical assistance. Without Eeva I wouldn’t have made it through, I have been very fortunate to be able to share this time with her.

I am greatly indebted to my former colleague Docent You Zhou, whose help in my final two studies has been invaluable. Annika is thanked for her genuine interest towards my research and the great help she gave me which made it possible to finish my projects. For me it has been a great pleasure to work and spend time with her, I consider us to be a perfect team. Selina is thanked for her friendship and company both in and out of the lab. Nidhi is thanked for her support and advice for all these years. I feel very honoured how she has always believed in me and to my ability to conduct science. Raghu is thanked for his company in the meetings and for our countless laughs in the lab. I also want to thank the rest of the “Minerva girls”, Kata, Annukka, Enni, Johanna and Hanna, for our fun and loud nights out together.

My deepest gratitude belongs to my beloved parents who have always supported me in all possible ways, and have made sure that I have the best possible premises to pursue my dreams. I also thank Mummi and Satu, and my siblings Samuel, Julkku and Rasse, and their families Aleksi, Maukka, Otava, Sanna-Maaret and Paavo.

I also want to thank all my friends outside the lab, especially ‘the yam-yam cats’: Marikki, Hanni, Kari, Karo, Anna, Jesu, Emmi, Linda, Oona and Sanna for helping me to forget the work routines, their company gives so much joy, laughter, entertainment and compassion to my life.

I would also like to thank the following organisations for their financial support of the work: the University of Helsinki Doctoral Programme in Biomedicine, the Finnish Concordia Fund, the Ida Montin Foundation sr, the Finnish-Norwegian Medical Foundation, the Aarne Koskelo Foundation, the Orion Research Foundation sr, the Päivikki and Sakari Sohlberg Foundation, the Aarne and Aili Turunen’s Foundation, the Eemil Aaltonen Foundation, The Niilo Helander Foundation, the Pulsus Foundation, the Finnish Atherosclerosis Society and the University of Helsinki Chancellors travel grant.

In Helsinki, June 2018

Henriikka

# 8 REFERENCES

Abercrombie, M., Heaysman, J. E. & Pegrum, S. M. 1972. Locomotion of fibroblasts in culture. V. Surface marking with concanavalin A. *Experimental cell research* **73:** 536-539.

Abercrombie, M., Heaysman, J. E. & Pegrum, S. M. 1971. The locomotion of fibroblasts in culture. IV. Electron microscopy of the leading lamella. *Experimental cell research* **67:** 359-367.

Adeva-Andany, M. M., Gonzalez-Lucan, M., Donapetry-Garcia, C., Fernandez-Fernandez, C. & Ameneiros-Rodriguez, E. 2016. Glycogen metabolism in humans. *BBA clinical* **5:** 85-100.

Agius, L. 2015. Role of glycogen phosphorylase in liver glycogen metabolism. *Molecular aspects of medicine* **46:** 34-45.

Agius, L. 2008. Glucokinase and molecular aspects of liver glycogen metabolism. *The Biochemical journal* **414:** 1-18.

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B. & Cohen, P. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Current biology* **7:** 261-269.

Alphey, L., Jimenez, J. & Glover, D. 1998. A Drosophila homologue of oxysterol binding protein (OSBP)--implications for the role of OSBP. *Biochimica et biophysica acta* **1395:** 159-164.

Alpy, F., Rousseau, A., Schwab, Y., Legueux, F., Stoll, I., Wendling, C., Spiegelhalter, C., Kessler, P., Mathelin, C., Rio, M. C., Levine, T. P. & Tomasetto, C. 2013. STARD3 or STARD3NL and VAP form a novel molecular tether between late endosomes and the ER. *Journal of cell science* **126:** 5500-5512.

Alva, V. & Lupas, A. N. 2016. The TULIP superfamily of eukaryotic lipid-binding proteins as a mediator of lipid sensing and transport. *Biochimica et biophysica acta* **1861:** 913-923.

Anniss, A. M., Apostolopoulos, J., Dworkin, S., Purton, L. E. & Sparrow, R. L. 2002. An oxysterol-binding protein family identified in the mouse. *DNA and cell biology* **21:** 571-580.

Aoki, K., Maeda, F., Nagasako, T., Mochizuki, Y., Uchida, S. & Ikenouchi, J. 2016. A RhoA and Rnd3 cycle regulates actin reassembly during membrane blebbing. *Proceedings of the National Academy of Sciences of the United States of America* **113:** E1863-71.

Azzout-Marniche, D., Becard, D., Guichard, C., Foretz, M., Ferre, P. & Foufelle, F. 2000. Insulin effects on sterol regulatory-element-binding protein-1c (SREBP-1c) transcriptional activity in rat hepatocytes. *The Biochemical journal* **350:** 389-393.

Banerji, S., Ngo, M., Lane, C. F., Robinson, C. A., Minogue, S. & Ridgway, N. D. 2010. Oxysterol binding protein-dependent activation of sphingomyelin synthesis

in the golgi apparatus requires phosphatidylinositol 4-kinase IIalpha. *Molecular biology of the cell* **21:** 4141-4150.

Bannister, R. A. 2016. Bridging the myoplasmic gap II: more recent advances in skeletal muscle excitation-contraction coupling. *The Journal of experimental biology* **219:** 175-182.

Bannister, R. A. 2007. Bridging the myoplasmic gap: recent developments in skeletal muscle excitation-contraction coupling. *Journal of muscle research and cell motility* **28:** 275-283.

Barthel, A., Okino, S. T., Liao, J., Nakatani, K., Li, J., Whitlock, J. P.,Jr & Roth, R. A. 1999. Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. *The Journal of biological chemistry* **274:** 20281-20286.

Barthel, A., Schmoll, D., Kruger, K. D., Bahrenberg, G., Walther, R., Roth, R. A. & Joost, H. G. 2001. Differential regulation of endogenous glucose-6-phosphatase and phosphoenolpyruvate carboxykinase gene expression by the forkhead transcription factor FKHR in H4IIE-hepatoma cells. *Biochemical and biophysical research communications* **285:** 897-902.

Basso, A. D., Solit, D. B., Chiosis, G., Giri, B., Tsichlis, P. & Rosen, N. 2002. Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. *The Journal of biological chemistry* **277:** 39858-39866.

Bauer, D. E., Hatzivassiliou, G., Zhao, F., Andreadis, C. & Thompson, C. B. 2005. ATP citrate lyase is an important component of cell growth and transformation. *Oncogene* **24:** 6314-6322.

Beh, C. T., Cool, L., Phillips, J. & Rine, J. 2001. Overlapping functions of the yeast oxysterol-binding protein homologues. *Genetics* **157:** 1117-1140.

Bergert, M., Chandradoss, S. D., Desai, R. A. & Paluch, E. 2012. Cell mechanics control rapid transitions between blebs and lamellipodia during migration. *Proceedings of the National Academy of Sciences of the United States of America* **109:** 14434-14439.

Berwick, D. C., Hers, I., Heesom, K. J., Moule, S. K. & Tavare, J. M. 2002. The identification of ATP-citrate lyase as a protein kinase B (Akt) substrate in primary adipocytes. *The Journal of biological chemistry* **277:** 33895-33900.

Blake, W. L. & Clarke, S. D. 1990. Suppression of rat hepatic fatty acid synthase and S14 gene transcription by dietary polyunsaturated fat. *The Journal of nutrition* **120:** 1727-1729.

Boureux, A., Vignal, E., Faure, S. & Fort, P. 2007. Evolution of the Rho family of ras-like GTPases in eukaryotes. *Molecular biology and evolution* **24:** 203-216.

Bray, D. & White, J. G. 1988. Cortical flow in animal cells. *Science* **239:** 883-888.

Briggs, M. W. & Sacks, D. B. 2003. IQGAP proteins are integral components of cytoskeletal regulation. *EMBO reports* **4:** 571-574.

Brouwers, M. C., Jacobs, C., Bast, A., Stehouwer, C. D. & Schaper, N. C. 2015. Modulation of Glucokinase Regulatory Protein: A Double-Edged Sword? *Trends in molecular medicine* **21:** 583-594.

Brown, M. S. & Goldstein, J. L. 1997. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **89:** 331-340.

Burgardt, N. I., Gianotti, A. R., Ferreyra, R. G. & Ermacora, M. R. 2017. A structural appraisal of sterol carrier protein 2. *Biochimica et biophysica acta* **1865:** 565-577.

Burgett, A. W., Poulsen, T. B., Wangkanont, K., Anderson, D. R., Kikuchi, C., Shimada, K., Okubo, S., Fortner, K. C., Mimaki, Y., Kuroda, M., Murphy, J. P., Schwalb, D. J., Petrella, E. C., Cornella-Taracido, I., Schirle, M., Tallarico, J. A. & Shair, M. D. 2011. Natural products reveal cancer cell dependence on oxysterol-binding proteins. *Nature chemical biology* **7:** 639-647.

Carlier, M. F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G. X., Hong, Y., Chua, N. H. & Pantaloni, D. 1997. Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. *The Journal of cell biology* **136:** 1307-1322.

Carlier, M. F., Ressad, F. & Pantaloni, D. 1999. Control of actin dynamics in cell motility. Role of ADF/cofilin. *The Journal of biological chemistry* **274:** 33827-33830.

Chang, C. L., Hsieh, T. S., Yang, T. T., Rothberg, K. G., Azizoglu, D. B., Volk, E., Liao, J. C. & Liou, J. 2013. Feedback regulation of receptor-induced Ca2+ signaling mediated by E-Syt1 and Nir2 at endoplasmic reticulum-plasma membrane junctions. *Cell reports* **5:** 813-825.

Chang, C. L. & Liou, J. 2015. Phosphatidylinositol 4,5-Bisphosphate Homeostasis Regulated by Nir2 and Nir3 Proteins at Endoplasmic Reticulum-Plasma Membrane Junctions. *The Journal of biological chemistry* **290:** 14289-14301.

Charman, M., Colbourne, T. R., Pietrangelo, A., Kreplak, L. & Ridgway, N. D. 2014. Oxysterol-binding protein (OSBP)-related protein 4 (ORP4) is essential for cell proliferation and survival. *The Journal of biological chemistry* **289:** 15705-15717.

Charras, G. & Paluch, E. 2008. Blebs lead the way: how to migrate without lamellipodia. *Nature reviews. Molecular cell biology* **9:** 730-736.

Charras, G. T., Coughlin, M., Mitchison, T. J. & Mahadevan, L. 2008. Life and times of a cellular bleb. *Biophysical journal* **94:** 1836-1853.

Charras, G. T., Hu, C. K., Coughlin, M. & Mitchison, T. J. 2006. Reassembly of contractile actin cortex in cell blebs. *The Journal of cell biology* **175:** 477-490.

Charras, G. T., Yarrow, J. C., Horton, M. A., Mahadevan, L. & Mitchison, T. J. 2005. Non-equilibration of hydrostatic pressure in blebbing cells. *Nature* **435:** 365-369.

Chen, B., Chou, H. T., Brautigam, C. A., Xing, W., Yang, S., Henry, L., Doolittle, L. K., Walz, T. & Rosen, M. K. 2017. Rac1 GTPase activates the WAVE regulatory complex through two distinct binding sites. *eLife* **6:** 10.7554/eLife.29795.

Chen, H., Detmer, S. A., Ewald, A. J., Griffin, E. E., Fraser, S. E. & Chan, D. C. 2003. Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *The Journal of cell biology* **160:** 189-200.

Chen, Z., Borek, D., Padrick, S. B., Gomez, T. S., Metlagel, Z., Ismail, A. M., Umetani, J., Billadeau, D. D., Otwinowski, Z. & Rosen, M. K. 2010. Structure and control of the actin regulatory WAVE complex. *Nature* **468:** 533-538.

Chircop, M. 2014. Rho GTPases as regulators of mitosis and cytokinesis in mammalian cells. *Small GTPases* **5:** 10.4161/sgtp.29770.

Choi, S., Hedman, A. C., Sayedyahossein, S., Thapa, N., Sacks, D. B. & Anderson, R. A. 2016. Agonist-stimulated phosphatidylinositol-3,4,5-trisphosphate generation by scaffolded phosphoinositide kinases. *Nature cell biology* **18:** 1324-1335.

Chu, B. B., Liao, Y. C., Qi, W., Xie, C., Du, X., Wang, J., Yang, H., Miao, H. H., Li, B. L. & Song, B. L. 2015. Cholesterol transport through lysosome-peroxisome membrane contacts. *Cell* **161:** 291-306.

Chung, J., Torta, F., Masai, K., Lucast, L., Czapla, H., Tanner, L. B., Narayanaswamy, P., Wenk, M. R., Nakatsu, F. & De Camilli, P. 2015. INTRACELLULAR TRANSPORT. PI4P/phosphatidylserine countertransport at ORP5- and ORP8-mediated ER-plasma membrane contacts. *Science* **349:** 428-432.

Copeland, D. E. & Dalton, A. J. 1959. An association between mitochondria and the endoplasmic reticulum in cells of the pseudobranch gland of a teleost. *The Journal of biophysical and biochemical cytology* **5:** 393-396.

Cramer, L. P. & Mitchison, T. J. 1997. Investigation of the mechanism of retraction of the cell margin and rearward flow of nodules during mitotic cell rounding. *Molecular biology of the cell* **8:** 109-119.

Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. & Hemmings, B. A. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378:** 785-789.

Csordas, G., Varnai, P., Golenar, T., Roy, S., Purkins, G., Schneider, T. G., Balla, T. & Hajnoczky, G. 2010. Imaging interorganelle contacts and local calcium dynamics at the ER-mitochondrial interface. *Molecular cell* **39:** 121-132.

D'Angelo, G., Vicinanza, M. & De Matteis, M. A. 2008. Lipid-transfer proteins in biosynthetic pathways. *Current opinion in cell biology* **20:** 360-370.

Dao, V. T., Dupuy, A. G., Gavet, O., Caron, E. & de Gunzburg, J. 2009. Dynamic changes in Rap1 activity are required for cell retraction and spreading during mitosis. *Journal of cell science* **122:** 2996-3004.

Daum, G. & Vance, J. E. 1997. Import of lipids into mitochondria. *Progress in lipid research* **36:** 103-130.

Dawson, P. A., Ridgway, N. D., Slaughter, C. A., Brown, M. S. & Goldstein, J. L. 1989. cDNA cloning and expression of oxysterol-binding protein, an oligomer with a potential leucine zipper. *The Journal of biological chemistry* **264:** 16798-16803.

de Brito, O. M. & Scorrano, L. 2008. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature* **456:** 605-610.

de Saint-Jean, M., Delfosse, V., Douguet, D., Chicanne, G., Payrastre, B., Bourguet, W., Antonny, B. & Drin, G. 2011. Osh4p exchanges sterols for

phosphatidylinositol 4-phosphate between lipid bilayers. *The Journal of cell biology* **195:** 965-978.

De Stefani, D., Rizzuto, R. & Pozzan, T. 2016. Enjoy the Trip: Calcium in Mitochondria Back and Forth. *Annual Review of Biochemistry* **85:** 161-192.

De Vos, K. J., Morotz, G. M., Stoica, R., Tudor, E. L., Lau, K. F., Ackerley, S., Warley, A., Shaw, C. E. & Miller, C. C. 2012. VAPB interacts with the mitochondrial protein PTPIP51 to regulate calcium homeostasis. *Human molecular genetics* **21:** 1299-1311.

Denechaud, P. D., Bossard, P., Lobaccaro, J. M., Millatt, L., Staels, B., Girard, J. & Postic, C. 2008. ChREBP, but not LXRs, is required for the induction of glucose-regulated genes in mouse liver. *The Journal of clinical investigation* **118:** 956-964.

Dennis, E. A. & Kennedy, E. P. 1972. Intracellular sites of lipid synthesis and the biogenesis of mitochondria. *Journal of lipid research* **13:** 263-267.

Dentin, R., Benhamed, F., Hainault, I., Fauveau, V., Foufelle, F., Dyck, J. R., Girard, J. & Postic, C. 2006. Liver-specific inhibition of ChREBP improves hepatic steatosis and insulin resistance in ob/ob mice. *Diabetes* **55:** 2159-2170.

Dentin, R., Girard, J. & Postic, C. 2005. Carbohydrate responsive element binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c): two key regulators of glucose metabolism and lipid synthesis in liver. *Biochimie* **87:** 81-86.

Dentin, R., Pegorier, J. P., Benhamed, F., Foufelle, F., Ferre, P., Fauveau, V., Magnuson, M. A., Girard, J. & Postic, C. 2004. Hepatic glucokinase is required for the synergistic action of ChREBP and SREBP-1c on glycolytic and lipogenic gene expression. *The Journal of biological chemistry* **279:** 20314-20326.

Dentin, R., Tomas-Cobos, L., Foufelle, F., Leopold, J., Girard, J., Postic, C. & Ferre, P. 2012. Glucose 6-phosphate, rather than xylulose 5-phosphate, is required for the activation of ChREBP in response to glucose in the liver. *Journal of hepatology* **56:** 199-209.

Denton, R. M. 2009. Regulation of mitochondrial dehydrogenases by calcium ions. *Biochimica et biophysica acta* **1787:** 1309-1316.

Deprez, J., Vertommen, D., Alessi, D. R., Hue, L. & Rider, M. H. 1997. Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. *The Journal of biological chemistry* **272:** 17269-17275.

Dickson, E. J., Jensen, J. B., Vivas, O., Kruse, M., Traynor-Kaplan, A. E. & Hille, B. 2016. Dynamic formation of ER-PM junctions presents a lipid phosphatase to regulate phosphoinositides. *The Journal of cell biology* **213:** 33-48.

Dimmer, K. S. & Rapaport, D. 2017. Mitochondrial contact sites as platforms for phospholipid exchange. *Biochimica et biophysica acta* **1862:** 69-80.

Disanza, A., Bisi, S., Winterhoff, M., Milanesi, F., Ushakov, D. S., Kast, D., Marighetti, P., Romet-Lemonne, G., Muller, H. M., Nickel, W., Linkner, J., Waterschoot, D., Ampe, C., Cortellino, S., Palamidessi, A., Dominguez, R., Carlier, M. F., Faix, J. & Scita, G. 2013. CDC42 switches IRSp53 from inhibition

of actin growth to elongation by clustering of VASP. *The EMBO journal* **32:** 2735-2750.

Donkor, J., Sariahmetoglu, M., Dewald, J., Brindley, D. N. & Reue, K. 2007. Three mammalian lipins act as phosphatidate phosphatases with distinct tissue expression patterns. *The Journal of biological chemistry* **282:** 3450-3457.

Du, X., Kumar, J., Ferguson, C., Schulz, T. A., Ong, Y. S., Hong, W., Prinz, W. A., Parton, R. G., Brown, A. J. & Yang, H. 2011. A role for oxysterol-binding protein-related protein 5 in endosomal cholesterol trafficking. *The Journal of cell biology* **192:** 121-135.

Du, X., Pham, Y. H. & Brown, A. J. 2004. Effects of 25-hydroxycholesterol on cholesterol esterification and sterol regulatory element-binding protein processing are dissociable: implications for cholesterol movement to the regulatory pool in the endoplasmic reticulum. *The Journal of biological chemistry* **279:** 47010-47016.

Du, X., Zadoorian, A., Lukmantara, I. E., Qi, Y., Brown, A. J. & Yang, H. 2018. Oxysterol-binding protein-related protein 5 (ORP5) promotes cell proliferation by activation of mTORC1 signaling. *The Journal of biological chemistry* **293:** 3806-3818

Eden, E. R., Sanchez-Heras, E., Tsapara, A., Sobota, A., Levine, T. P. & Futter, C. E. 2016. Annexin A1 Tethers Membrane Contact Sites that Mediate ER to Endosome Cholesterol Transport. *Developmental cell* **37:** 473-483.

Emoto, K., Kuge, O., Nishijima, M. & Umeda, M. 1999. Isolation of a Chinese hamster ovary cell mutant defective in intramitochondrial transport of phosphatidylserine. *Proceedings of the National Academy of Sciences of the United States of America* **96:** 12400-12405.

Enomoto, A., Murakami, H., Asai, N., Morone, N., Watanabe, T., Kawai, K., Murakumo, Y., Usukura, J., Kaibuchi, K. & Takahashi, M. 2005. Akt/PKB regulates actin organization and cell motility via Girdin/APE. *Developmental cell* **9:** 389-402.

Escajadillo, T., Wang, H., Li, L., Li, D. & Sewer, M. B. 2016. Oxysterol-related-binding-protein related Protein-2 (ORP2) regulates cortisol biosynthesis and cholesterol homeostasis. *Molecular and cellular endocrinology* **427:** 73-85.

Fairn, G. D. & McMaster, C. R. 2005a. Identification and assessment of the role of a nominal phospholipid binding region of ORP1S (oxysterol-binding-protein-related protein 1 short) in the regulation of vesicular transport. *The Biochemical journal* **387:** 889-896.

Fairn, G. D. & McMaster, C. R. 2005b. The roles of the human lipid-binding proteins ORP9S and ORP10S in vesicular transport. *Biochemistry and cell biology = Biochimie et biologie cellulaire* **83:** 631-636.

Fehon, R. G., McClatchey, A. I. & Bretscher, A. 2010. Organizing the cell cortex: the role of ERM proteins. *Nature reviews. Molecular cell biology* **11:** 276-287.

Fleischmann, M. & Iynedjian, P. B. 2000. Regulation of sterol regulatory-element binding protein 1 gene expression in liver: role of insulin and protein kinase B/cAkt. *The Biochemical journal* **349:** 13-17.

Flis, V. V. & Daum, G. 2013. Lipid transport between the endoplasmic reticulum and mitochondria. *Cold Spring Harbor perspectives in biology* **5:** 10.1101/cshperspect.a013235.

Follo, M. Y., Manzoli, L., Poli, A., McCubrey, J. A. & Cocco, L. 2015. PLC and PI3K/Akt/mTOR signalling in disease and cancer. *Advances in biological regulation* **57:** 10-16.

Foretz, M., Guichard, C., Ferre, P. & Foufelle, F. 1999a. Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. *Proceedings of the National Academy of Sciences of the United States of America* **96:** 12737-12742.

Foretz, M., Pacot, C., Dugail, I., Lemarchand, P., Guichard, C., Le Liepvre, X., Berthelier-Lubrano, C., Spiegelman, B., Kim, J. B., Ferre, P. & Foufelle, F. 1999b. ADD1/SREBP-1c is required in the activation of hepatic lipogenic gene expression by glucose. *Molecular and cellular biology* **19:** 3760-3768.

Foufelle, F. & Ferre, P. 2002. New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. *The Biochemical journal* **366:** 377-391.

Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R. & Tsichlis, P. N. 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81:** 727-736.

Franzini-Armstrong, C., Pincon-Raymond, M. & Rieger, F. 1991. Muscle fibers from dysgenic mouse in vivo lack a surface component of peripheral couplings. *Developmental biology* **146:** 364-376.

Friedman, J. R., Dibenedetto, J. R., West, M., Rowland, A. A. & Voeltz, G. K. 2013. Endoplasmic reticulum-endosome contact increases as endosomes traffic and mature. *Molecular biology of the cell* **24:** 1030-1040.

Friedman, J. R., Lackner, L. L., West, M., DiBenedetto, J. R., Nunnari, J. & Voeltz, G. K. 2011. ER tubules mark sites of mitochondrial division. *Science* **334:** 358-362.

Galmes, R., Houcine, A., van Vliet, A. R., Agostinis, P., Jackson, C. L. & Giordano, F. 2016. ORP5/ORP8 localize to endoplasmic reticulum-mitochondria contacts and are involved in mitochondrial function. *EMBO reports* **17:** 800-810.

Garbino, A., van Oort, R. J., Dixit, S. S., Landstrom, A. P., Ackerman, M. J. & Wehrens, X. H. 2009. Molecular evolution of the junctophilin gene family. *Physiological genomics* **37:** 175-186.

Geneste, O., Copeland, J. W. & Treisman, R. 2002. LIM kinase and Diaphanous cooperate to regulate serum response factor and actin dynamics. *The Journal of cell biology* **157:** 831-838.

Ghai, R., Du, X., Wang, H., Dong, J., Ferguson, C., Brown, A. J., Parton, R. G., Wu, J. W. & Yang, H. 2017. ORP5 and ORP8 bind phosphatidylinositol-4, 5-biphosphate (PtdIns(4,5)P 2) and regulate its level at the plasma membrane. *Nature communications* **8:** 757-017-00861-5.

Giordano, F., Saheki, Y., Idevall-Hagren, O., Colombo, S. F., Pirruccello, M., Milosevic, I., Gracheva, E. O., Bagriantsev, S. N., Borgese, N. & De Camilli, P. 2013. PI(4,5)P(2)-dependent and Ca(2+)-regulated ER-PM interactions mediated by the extended synaptotagmins. *Cell* **153:** 1494-1509.

Godi, A., Di Campli, A., Konstantakopoulos, A., Di Tullio, G., Alessi, D. R., Kular, G. S., Daniele, T., Marra, P., Lucocq, J. M. & De Matteis, M. A. 2004. FAPPs control Golgi-to-cell-surface membrane traffic by binding to ARF and PtdIns(4)P. *Nature cell biology* **6:** 393-404.

Goldschmidt-Clermont, P. J., Machesky, L. M., Doberstein, S. K. & Pollard, T. D. 1991. Mechanism of the interaction of human platelet profilin with actin. *The Journal of cell biology* **113:** 1081-1089.

Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R. B. & Hay, N. 2001. Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes & development* **15:** 1406-1418.

Gropler, M. C., Harris, T. E., Hall, A. M., Wolins, N. E., Gross, R. W., Han, X., Chen, Z. & Finck, B. N. 2009. Lipin 2 is a liver-enriched phosphatidate phosphohydrolase enzyme that is dynamically regulated by fasting and obesity in mice. *The Journal of biological chemistry* **284:** 6763-6772.

Gu, F., Wang, L., He, J., Liu, X., Zhang, H., Li, W., Fu, L. & Ma, Y. 2014. Girdin, an actin-binding protein, is critical for migration, adhesion, and invasion of human glioblastoma cells. *Journal of neurochemistry* **131:** 457-469.

Gupton, S. L. & Gertler, F. B. 2007. Filopodia: the fingers that do the walking. *Science's STKE : signal transduction knowledge environment* **2007:** re5.

Hall, A. 1994. Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Annual Review of Cell Biology* **10:** 31-54.

Hamasaki, M., Furuta, N., Matsuda, A., Nezu, A., Yamamoto, A., Fujita, N., Oomori, H., Noda, T., Haraguchi, T., Hiraoka, Y., Amano, A. & Yoshimori, T. 2013. Autophagosomes form at ER-mitochondria contact sites. *Nature* **495:** 389-393.

Hanada, K., Kumagai, K., Yasuda, S., Miura, Y., Kawano, M., Fukasawa, M. & Nishijima, M. 2003. Molecular machinery for non-vesicular trafficking of ceramide. *Nature* **426:** 803-809.

Hanukoglu, I. 1992. Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. *The Journal of steroid biochemistry and molecular biology* **43:** 779-804.

Hao, J. J., Liu, Y., Kruhlak, M., Debell, K. E., Rellahan, B. L. & Shaw, S. 2009. Phospholipase C-mediated hydrolysis of PIP2 releases ERM proteins from lymphocyte membrane. *The Journal of cell biology* **184:** 451-462.

Harner, M., Korner, C., Walther, D., Mokranjac, D., Kaesmacher, J., Welsch, U., Griffith, J., Mann, M., Reggiori, F. & Neupert, W. 2011. The mitochondrial contact site complex, a determinant of mitochondrial architecture. *The EMBO journal* **30:** 4356-4370.

Hatting, M., Tavares, C. D. J., Sharabi, K., Rines, A. K. & Puigserver, P. 2018. Insulin regulation of gluconeogenesis. *Annals of the New York Academy of Sciences* **1411:** 21-35

Helle, S. C., Kanfer, G., Kolar, K., Lang, A., Michel, A. H. & Kornmann, B. 2013. Organization and function of membrane contact sites. *Biochimica et biophysica acta* **1833:** 2526-2541.

Henderson, V., Smith, B., Burton, L. J., Randle, D., Morris, M. & Odero-Marah, V. A. 2015. Snail promotes cell migration through PI3K/AKT-dependent Rac1 activation as well as PI3K/AKT-independent pathways during prostate cancer progression. *Cell adhesion & migration* **9:** 255-264.

Higgs, H. N. & Pollard, T. D. 1999. Regulation of actin polymerization by Arp2/3 complex and WASp/Scar proteins. *The Journal of biological chemistry* **274:** 32531-32534.

Higuchi, M., Masuyama, N., Fukui, Y., Suzuki, A. & Gotoh, Y. 2001. Akt mediates Rac/Cdc42-regulated cell motility in growth factor-stimulated cells and in invasive PTEN knockout cells. *Current biology* **11:** 1958-1962.

Hirata, Y., Brotto, M., Weisleder, N., Chu, Y., Lin, P., Zhao, X., Thornton, A., Komazaki, S., Takeshima, H., Ma, J. & Pan, Z. 2006. Uncoupling store-operated Ca2+ entry and altered Ca2+ release from sarcoplasmic reticulum through silencing of junctophilin genes. *Biophysical journal* **90:** 4418-4427.

Horie, T., Nishino, T., Baba, O., Kuwabara, Y., Nakao, T., Nishiga, M., Usami, S., Izuhara, M., Sowa, N., Yahagi, N., Shimano, H., Matsumura, S., Inoue, K., Marusawa, H., Nakamura, T., Hasegawa, K., Kume, N., Yokode, M., Kita, T., Kimura, T. & Ono, K. 2013. MicroRNA-33 regulates sterol regulatory element-binding protein 1 expression in mice. *Nature communications* **4:** 2883.

Horton, J. D. 2002. Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. *Biochemical Society transactions* **30:** 1091-1095.

Hsuan, J. & Cockcroft, S. 2001. The PITP family of phosphatidylinositol transfer proteins. *Genome biology* **2:** REVIEWS3011.

Hynynen, R., Laitinen, S., Kakela, R., Tanhuanpaa, K., Lusa, S., Ehnholm, C., Somerharju, P., Ikonen, E. & Olkkonen, V. M. 2005. Overexpression of OSBP-related protein 2 (ORP2) induces changes in cellular cholesterol metabolism and enhances endocytosis. *The Biochemical journal* **390:** 273-283.

Hynynen, R., Suchanek, M., Spandl, J., Back, N., Thiele, C. & Olkkonen, V. M. 2009. OSBP-related protein 2 is a sterol receptor on lipid droplets that regulates the metabolism of neutral lipids. *Journal of lipid research* **50:** 1305-1315.

Ichetovkin, I., Han, J., Pang, K. M., Knecht, D. A. & Condeelis, J. S. 2000. Actin filaments are severed by both native and recombinant dictyostelium cofilin but to different extents. *Cell motility and the cytoskeleton* **45:** 293-306.

Idevall-Hagren, O., Lu, A., Xie, B. & De Camilli, P. 2015. Triggered Ca2+ influx is required for extended synaptotagmin 1-induced ER-plasma membrane tethering. *The EMBO journal* **34:** 2291-2305.

Iizuka, K., Bruick, R. K., Liang, G., Horton, J. D. & Uyeda, K. 2004. Deficiency of carbohydrate response element-binding protein (ChREBP) reduces lipogenesis as

well as glycolysis. *Proceedings of the National Academy of Sciences of the United States of America* **101:** 7281-7286.

Ikemoto, T., Komazaki, S., Takeshima, H., Nishi, M., Noda, T., Iino, M. & Endo, M. 1997. Functional and morphological features of skeletal muscle from mutant mice lacking both type 1 and type 3 ryanodine receptors. *The Journal of physiology* **501:** 305-312.

Im, Y. J., Raychaudhuri, S., Prinz, W. A. & Hurley, J. H. 2005. Structural mechanism for sterol sensing and transport by OSBP-related proteins. *Nature* **437:** 154-158.

Infante, R. E., Wang, M. L., Radhakrishnan, A., Kwon, H. J., Brown, M. S. & Goldstein, J. L. 2008. NPC2 facilitates bidirectional transfer of cholesterol between NPC1 and lipid bilayers, a step in cholesterol egress from lysosomes. *Proceedings of the National Academy of Sciences of the United States of America* **105:** 15287-15292.

Ishii, S., Iizuka, K., Miller, B. C. & Uyeda, K. 2004. Carbohydrate response element binding protein directly promotes lipogenic enzyme gene transcription. *Proceedings of the National Academy of Sciences of the United States of America* **101:** 15597-15602.

Ishimoto, K., Nakamura, H., Tachibana, K., Yamasaki, D., Ota, A., Hirano, K., Tanaka, T., Hamakubo, T., Sakai, J., Kodama, T. & Doi, T. 2009. Sterol-mediated regulation of human lipin 1 gene expression in hepatoblastoma cells. *The Journal of biological chemistry* **284:** 22195-22205.

Ismail, A. M., Padrick, S. B., Chen, B., Umetani, J. & Rosen, M. K. 2009. The WAVE regulatory complex is inhibited. *Nature structural & molecular biology* **16:** 561-563.

Ito, K., Komazaki, S., Sasamoto, K., Yoshida, M., Nishi, M., Kitamura, K. & Takeshima, H. 2001. Deficiency of triad junction and contraction in mutant skeletal muscle lacking junctophilin type 1. *The Journal of cell biology* **154:** 1059-1067.

Jagerstrom, S., Polesie, S., Wickstrom, Y., Johansson, B. R., Schroder, H. D., Hojlund, K. & Bostrom, P. 2009. Lipid droplets interact with mitochondria using SNAP23. *Cell biology international* **33:** 934-940.

Janowski, B. A., Willy, P. J., Devi, T. R., Falck, J. R. & Mangelsdorf, D. J. 1996. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* **383:** 728-731.

Jansen, M., Ohsaki, Y., Rita Rega, L., Bittman, R., Olkkonen, V. M. & Ikonen, E. 2011. Role of ORPs in sterol transport from plasma membrane to ER and lipid droplets in mammalian cells. *Traffic* **12:** 218-231.

Jaworski, C. J., Moreira, E., Li, A., Lee, R. & Rodriguez, I. R. 2001. A family of 12 human genes containing oxysterol-binding domains. *Genomics* **78:** 185-196.

Jensen-Urstad, A. P. & Semenkovich, C. F. 2012. Fatty acid synthase and liver triglyceride metabolism: housekeeper or messenger? *Biochimica et biophysica acta* **1821:** 747-753.

Jha, S. K., Jha, N. K., Kar, R., Ambasta, R. K. & Kumar, P. 2015. p38 MAPK and PI3K/AKT Signalling Cascades inParkinson's Disease. *International journal of molecular and cellular medicine* **4:** 67-86.

Johansson, M., Lehto, M., Tanhuanpaa, K., Cover, T. L. & Olkkonen, V. M. 2005. The oxysterol-binding protein homologue ORP1L interacts with Rab7 and alters functional properties of late endocytic compartments. *Molecular biology of the cell* **16:** 5480-5492.

Johansson, M., Rocha, N., Zwart, W., Jordens, I., Janssen, L., Kuijl, C., Olkkonen, V. M. & Neefjes, J. 2007. Activation of endosomal dynein motors by stepwise assembly of Rab7-RILP-p150Glued, ORP1L, and the receptor betalll spectrin. *The Journal of cell biology* **176:** 459-471.

Jousset, H., Frieden, M. & Demaurex, N. 2007. STIM1 knockdown reveals that store-operated Ca2+ channels located close to sarco/endoplasmic Ca2+ ATPases (SERCA) pumps silently refill the endoplasmic reticulum. *The Journal of biological chemistry* **282:** 11456-11464.

Jurica, M. S., Mesecar, A., Heath, P. J., Shi, W., Nowak, T. & Stoddard, B. L. 1998. The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate. *Structure* **6:** 195-210.

Kaiser, S. E., Brickner, J. H., Reilein, A. R., Fenn, T. D., Walter, P. & Brunger, A. T. 2005. Structural basis of FFAT motif-mediated ER targeting. *Structure* **13:** 1035-1045.

Kandutsch, A. A. & Chen, H. W. 1975. Regulation of sterol synthesis in cultured cells by oxygenated derivatives of cholesterol. *Journal of cellular physiology* **85:** 415-424.

Kandutsch, A. A., Chen, H. W. & Shown, E. P. 1977. Binding of 25-hydroxycholesterol and cholesterol to different cytoplasmic proteins. *Proceedings of the National Academy of Sciences of the United States of America* **74:** 2500-2503.

Kandutsch, A. A. & Thompson, E. B. 1980. Cytosolic proteins that bind oxygenated sterols. Cellular distribution, specificity, and some properties. *The Journal of biological chemistry* **255:** 10813-10821.

Kang, T. H., Baek, J. I., Sagong, B., Park, H. J., Park, C. I., Lee, K. Y. & Kim, U. K. 2017. A novel missense variant in the DIAPH1 gene in a Korean family with autosomal dominant nonsyndromic hearing loss. *Genes & genetic systems* **91:** 289-292.

Karim, S., Adams, D. H. & Lalor, P. F. 2012. Hepatic expression and cellular distribution of the glucose transporter family. *World journal of gastroenterology* **18:** 6771-6781.

Kawaguchi, T., Takenoshita, M., Kabashima, T. & Uyeda, K. 2001. Glucose and cAMP regulate the L-type pyruvate kinase gene by phosphorylation/dephosphorylation of the carbohydrate response element binding protein. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 13710-13715.

Kawakami, Y., Nishimoto, H., Kitaura, J., Maeda-Yamamoto, M., Kato, R. M., Littman, D. R., Leitges, M., Rawlings, D. J. & Kawakami, T. 2004. Protein kinase C betaII regulates Akt phosphorylation on Ser-473 in a cell type- and stimulus-specific fashion. *The Journal of biological chemistry* **279:** 47720-47725.

Kerppola, T. K. 2008. Bimolecular fluorescence complementation (BiFC) analysis as a probe of protein interactions in living cells. *Annual review of biophysics* **37:** 465-487.

Kersten, S. 2014. Integrated physiology and systems biology of PPARalpha. *Molecular metabolism* **3:** 354-371.

Kersten, S., Seydoux, J., Peters, J. M., Gonzalez, F. J., Desvergne, B. & Wahli, W. 1999. Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *The Journal of clinical investigation* **103:** 1489-1498.

Kim, D., Kim, S., Koh, H., Yoon, S. O., Chung, A. S., Cho, K. S. & Chung, J. 2001. Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **15:** 1953-1962.

Kim, J. B., Sarraf, P., Wright, M., Yao, K. M., Mueller, E., Solanes, G., Lowell, B. B. & Spiegelman, B. M. 1998. Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *The Journal of clinical investigation* **101:** 1-9.

Kim, S. Y., Kim, H. I., Kim, T. H., Im, S. S., Park, S. K., Lee, I. K., Kim, K. S. & Ahn, Y. H. 2004. SREBP-1c mediates the insulin-dependent hepatic glucokinase expression. *The Journal of biological chemistry* **279:** 30823-30829.

Kim, Y. J., Guzman-Hernandez, M. L., Wisniewski, E. & Balla, T. 2015. Phosphatidylinositol-Phosphatidic Acid Exchange by Nir2 at ER-PM Contact Sites Maintains Phosphoinositide Signaling Competence. *Developmental cell* **33:** 549-561.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A. & Kaibuchi, K. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase. *Science* **273:** 245-248.

Kodama, Y. & Hu, C. D. 2012. Bimolecular fluorescence complementation (BiFC): a 5-year update and future perspectives. *BioTechniques* **53:** 285-298.

Kohn, A. D., Summers, S. A., Birnbaum, M. J. & Roth, R. A. 1996. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *The Journal of biological chemistry* **271:** 31372-31378.

Koldamova, R. P., Lefterov, I. M., Ikonomovic, M. D., Skoko, J., Lefterov, P. I., Isanski, B. A., DeKosky, S. T. & Lazo, J. S. 2003. 22R-hydroxycholesterol and 9-cis-retinoic acid induce ATP-binding cassette transporter A1 expression and cholesterol efflux in brain cells and decrease amyloid beta secretion. *The Journal of biological chemistry* **278:** 13244-13256.

Kornmann, B., Currie, E., Collins, S. R., Schuldiner, M., Nunnari, J., Weissman, J. S. & Walter, P. 2009. An ER-mitochondria tethering complex revealed by a synthetic biology screen. *Science* **325:** 477-481.

Korzeniowski, M. K., Popovic, M. A., Szentpetery, Z., Varnai, P., Stojilkovic, S. S. & Balla, T. 2009. Dependence of STIM1/Orai1-mediated calcium entry on plasma membrane phosphoinositides. *The Journal of biological chemistry* **284:** 21027-21035.

Koyasu, S., Nishida, E., Kadowaki, T., Matsuzaki, F., Iida, K., Harada, F., Kasuga, M., Sakai, H. & Yahara, I. 1986. Two mammalian heat shock proteins, HSP90 and HSP100, are actin-binding proteins. *Proceedings of the National Academy of Sciences of the United States of America* **83:** 8054-8058.

Kwon, H. J., Abi-Mosleh, L., Wang, M. L., Deisenhofer, J., Goldstein, J. L., Brown, M. S. & Infante, R. E. 2009. Structure of N-terminal domain of NPC1 reveals distinct subdomains for binding and transfer of cholesterol. *Cell* **137:** 1213-1224.

Lacalle, R. A., Peregil, R. M., Albar, J. P., Merino, E., Martinez-A, C., Merida, I. & Manes, S. 2007. Type I phosphatidylinositol 4-phosphate 5-kinase controls neutrophil polarity and directional movement. *The Journal of cell biology* **179:** 1539-1553.

Lagace, T. A., Byers, D. M., Cook, H. W. & Ridgway, N. D. 1997. Altered regulation of cholesterol and cholesteryl ester synthesis in Chinese-hamster ovary cells overexpressing the oxysterol-binding protein is dependent on the pleckstrin homology domain. *The Biochemical journal* **326:** 205-213.

Laitinen, S., Lehto, M., Lehtonen, S., Hyvarinen, K., Heino, S., Lehtonen, E., Ehnholm, C., Ikonen, E. & Olkkonen, V. M. 2002. ORP2, a homolog of oxysterol binding protein, regulates cellular cholesterol metabolism. *Journal of lipid research* **43:** 245-255.

Laitinen, S., Olkkonen, V. M., Ehnholm, C. & Ikonen, E. 1999. Family of human oxysterol binding protein (OSBP) homologues. A novel member implicated in brain sterol metabolism. *Journal of lipid research* **40:** 2204-2211.

Landschulz, K. T., Jump, D. B., MacDougald, O. A. & Lane, M. D. 1994. Transcriptional control of the stearoyl-CoA desaturase-1 gene by polyunsaturated fatty acids. *Biochemical and biophysical research communications* **200:** 763-768.

Lange, Y., Ye, J., Rigney, M. & Steck, T. L. 1999. Regulation of endoplasmic reticulum cholesterol by plasma membrane cholesterol. *Journal of lipid research* **40:** 2264-2270.

Lappalainen, P., Kessels, M. M., Cope, M. J. & Drubin, D. G. 1998. The ADF homology (ADF-H) domain: a highly exploited actin-binding module. *Molecular biology of the cell* **9:** 1951-1959.

Lauffenburger, D. A. & Horwitz, A. F. 1996. Cell migration: a physically integrated molecular process. *Cell* **84:** 359-369.

Le Clainche, C., Schlaepfer, D., Ferrari, A., Klingauf, M., Grohmanova, K., Veligodskiy, A., Didry, D., Le, D., Egile, C., Carlier, M. F. & Kroschewski, R. 2007. IQGAP1 stimulates actin assembly through the N-WASP-Arp2/3 pathway. *The Journal of biological chemistry* **282:** 426-435.

Lee, K. & Song, K. 2007. Actin dysfunction activates ERK1/2 and delays entry into mitosis in mammalian cells. *Cell cycle* **6:** 1487-1495.

Lee, S. H. & Dominguez, R. 2010. Regulation of actin cytoskeleton dynamics in cells. *Molecules and cells* **29:** 311-325.

Lehmann, J. M., Kliewer, S. A., Moore, L. B., Smith-Oliver, T. A., Oliver, B. B., Su, J. L., Sundseth, S. S., Winegar, D. A., Blanchard, D. E., Spencer, T. A. & Willson, T. M. 1997. Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *The Journal of biological chemistry* **272:** 3137-3140.

Lehto, M., Hynynen, R., Karjalainen, K., Kuismanen, E., Hyvarinen, K. & Olkkonen, V. M. 2005. Targeting of OSBP-related protein 3 (ORP3) to endoplasmic reticulum and plasma membrane is controlled by multiple determinants. *Experimental cell research* **310:** 445-462.

Lehto, M., Laitinen, S., Chinetti, G., Johansson, M., Ehnholm, C., Staels, B., Ikonen, E. & Olkkonen, V. M. 2001. The OSBP-related protein family in humans. *Journal of lipid research* **42:** 1203-1213.

Lehto, M., Mayranpaa, M. I., Pellinen, T., Ihalmo, P., Lehtonen, S., Kovanen, P. T., Groop, P. H., Ivaska, J. & Olkkonen, V. M. 2008. The R-Ras interaction partner ORP3 regulates cell adhesion. *Journal of cell science* **121:** 695-705.

Lessmann, E., Ngo, M., Leitges, M., Minguet, S., Ridgway, N. D. & Huber, M. 2007. Oxysterol-binding protein-related protein (ORP) 9 is a PDK-2 substrate and regulates Akt phosphorylation. *Cellular signalling* **19:** 384-392.

Lev, S. 2010. Non-vesicular lipid transport by lipid-transfer proteins and beyond. *Nature reviews. Molecular cell biology* **11:** 739-750.

Levanon, D., Hsieh, C. L., Francke, U., Dawson, P. A., Ridgway, N. D., Brown, M. S. & Goldstein, J. L. 1990. cDNA cloning of human oxysterol-binding protein and localization of the gene to human chromosome 11 and mouse chromosome 19. *Genomics* **7:** 65-74.

Levine, T. P. & Munro, S. 2002. Targeting of Golgi-specific pleckstrin homology domains involves both PtdIns 4-kinase-dependent and -independent components. *Current biology* **12:** 695-704.

Levine, T. P. & Munro, S. 1998. The pleckstrin homology domain of oxysterol-binding protein recognises a determinant specific to Golgi membranes. *Current biology* **8:** 729-739.

Lewin, T. M., de Jong, H., Schwerbrock, N. J., Hammond, L. E., Watkins, S. M., Combs, T. P. & Coleman, R. A. 2008. Mice deficient in mitochondrial glycerol-3-phosphate acyltransferase-1 have diminished myocardial triacylglycerol accumulation during lipogenic diet and altered phospholipid fatty acid composition. *Biochimica et biophysica acta* **1781:** 352-358.

Li, D., Dammer, E. B., Lucki, N. C. & Sewer, M. B. 2013. cAMP-stimulated phosphorylation of diaphanous 1 regulates protein stability and interaction with binding partners in adrenocortical cells. *Molecular biology of the cell* **24:** 848-857.

Li, X., Monks, B., Ge, Q. & Birnbaum, M. J. 2007. Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1alpha transcription coactivator. *Nature* **447:** 1012-1016.

Lietzke, S. E., Bose, S., Cronin, T., Klarlund, J., Chawla, A., Czech, M. P. & Lambright, D. G. 2000. Structural basis of 3-phosphoinositide recognition by pleckstrin homology domains. *Molecular cell* **6:** 385-394.

Liou, J., Fivaz, M., Inoue, T. & Meyer, T. 2007. Live-cell imaging reveals sequential oligomerization and local plasma membrane targeting of stromal interaction molecule 1 after Ca2+ store depletion. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 9301-9306.

Liou, J., Kim, M. L., Heo, W. D., Jones, J. T., Myers, J. W., Ferrell, J. E.,Jr & Meyer, T. 2005. STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. *Current biology* **15:** 1235-1241.

Liu, X. & Ridgway, N. D. 2014. Characterization of the sterol and phosphatidylinositol 4-phosphate binding properties of Golgi-associated OSBP-related protein 9 (ORP9). *PloS one* **9:** e108368.

Liu, Y., Boukhelifa, M., Tribble, E., Morin-Kensicki, E., Uetrecht, A., Bear, J. E. & Bankaitis, V. A. 2008. The Sac1 phosphoinositide phosphatase regulates Golgi membrane morphology and mitotic spindle organization in mammals. *Molecular biology of the cell* **19:** 3080-3096.

Loewen, C. J., Roy, A. & Levine, T. P. 2003. A conserved ER targeting motif in three families of lipid binding proteins and in Opi1p binds VAP. *The EMBO journal* **22:** 2025-2035.

Lokuta, M. A., Senetar, M. A., Bennin, D. A., Nuzzi, P. A., Chan, K. T., Ott, V. L. & Huttenlocher, A. 2007. Type Igamma PIP kinase is a novel uropod component that regulates rear retraction during neutrophil chemotaxis. *Molecular biology of the cell* **18:** 5069-5080.

Longuet, C., Sinclair, E. M., Maida, A., Baggio, L. L., Maziarz, M., Charron, M. J. & Drucker, D. J. 2008. The glucagon receptor is required for the adaptive metabolic response to fasting. *Cell metabolism* **8:** 359-371.

Luik, R. M., Wang, B., Prakriya, M., Wu, M. M. & Lewis, R. S. 2008. Oligomerization of STIM1 couples ER calcium depletion to CRAC channel activation. *Nature* **454:** 538-542.

Ma, Z., Liu, Z. & Huang, X. 2010. OSBP- and FAN-mediated sterol requirement for spermatogenesis in Drosophila. *Development* **137:** 3775-3784.

Mabuchi, I. 1986. Biochemical aspects of cytokinesis. *International review of cytology* **101:** 175-213.

Machesky, L. M. & Gould, K. L. 1999. The Arp2/3 complex: a multifunctional actin organizer. *Current opinion in cell biology* **11:** 117-121.

Maeda, K., Anand, K., Chiapparino, A., Kumar, A., Poletto, M., Kaksonen, M. & Gavin, A. C. 2013. Interactome map uncovers phosphatidylserine transport by oxysterol-binding proteins. *Nature* **501:** 257-261.

Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K. & Narumiya, S. 1999. Signaling from Rho to the actin

cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* **285:** 895-898.

Magana, M. M. & Osborne, T. F. 1996. Two tandem binding sites for sterol regulatory element binding proteins are required for sterol regulation of fatty-acid synthase promoter. *The Journal of biological chemistry* **271:** 32689-32694.

Maleth, J., Choi, S., Muallem, S. & Ahuja, M. 2014. Translocation between PI(4,5)P2-poor and PI(4,5)P2-rich microdomains during store depletion determines STIM1 conformation and Orai1 gating. *Nature communications* **5:** 5843.

Manford, A. G., Stefan, C. J., Yuan, H. L., Macgurn, J. A. & Emr, S. D. 2012. ER-to-plasma membrane tethering proteins regulate cell signaling and ER morphology. *Developmental cell* **23:** 1129-1140.

Matsuda, S., Kobayashi, M. & Kitagishi, Y. 2013. Roles for PI3K/AKT/PTEN Pathway in Cell Signaling of Nonalcoholic Fatty Liver Disease. *ISRN endocrinology* **2013:** 472432.

Mattila, P. K. & Lappalainen, P. 2008. Filopodia: molecular architecture and cellular functions. *Nature reviews. Molecular cell biology* **9:** 446-454.

McCarty, O. J., Larson, M. K., Auger, J. M., Kalia, N., Atkinson, B. T., Pearce, A. C., Ruf, S., Henderson, R. B., Tybulewicz, V. L., Machesky, L. M. & Watson, S. P. 2005. Rac1 is essential for platelet lamellipodia formation and aggregate stability under flow. *The Journal of biological chemistry* **280:** 39474-39484.

Medalia, O. & Geiger, B. 2010. Frontiers of microscopy-based research into cell-matrix adhesions. *Current opinion in cell biology* **22:** 659-668.

Mesmin, B., Bigay, J., Moser von Filseck, J., Lacas-Gervais, S., Drin, G. & Antonny, B. 2013. A four-step cycle driven by PI(4)P hydrolysis directs sterol/PI(4)P exchange by the ER-Golgi tether OSBP. *Cell* **155:** 830-843.

Mesmin, B., Bigay, J., Polidori, J., Jamecna, D., Lacas-Gervais, S. & Antonny, B. 2017. Sterol transfer, PI4P consumption, and control of membrane lipid order by endogenous OSBP. *The EMBO journal* **36:** 3156-3174

Miki, H., Suetsugu, S. & Takenawa, T. 1998. WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *The EMBO journal* **17:** 6932-6941.

Min, S. W., Chang, W. P. & Sudhof, T. C. 2007. E-Syts, a family of membranous Ca2+-sensor proteins with multiple C2 domains. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 3823-3828.

Mitchison, T. J. & Cramer, L. P. 1996. Actin-based cell motility and cell locomotion. *Cell* **84:** 371-379.

Mohammadi, A., Perry, R. J., Storey, M. K., Cook, H. W., Byers, D. M. & Ridgway, N. D. 2001. Golgi localization and phosphorylation of oxysterol binding protein in Niemann-Pick C and U18666A-treated cells. *Journal of lipid research* **42:** 1062-1071.

Moon, S. Y. & Zheng, Y. 2003. Rho GTPase-activating proteins in cell regulation. *Trends in cell biology* **13:** 13-22.

Moser von Filseck, J., Vanni, S., Mesmin, B., Antonny, B. & Drin, G. 2015. A phosphatidylinositol-4-phosphate powered exchange mechanism to create a lipid gradient between membranes. *Nature communications* **6:** 6671.

Munday, M. R. & Hemingway, C. J. 1999. The regulation of acetyl-CoA carboxylase--a potential target for the action of hypolipidemic agents. *Advances in Enzyme Regulation* **39:** 205-234.

Murley, A., Lackner, L. L., Osman, C., West, M., Voeltz, G. K., Walter, P. & Nunnari, J. 2013. ER-associated mitochondrial division links the distribution of mitochondria and mitochondrial DNA in yeast. *eLife* **2:** e00422.

Musacchio, A., Gibson, T., Rice, P., Thompson, J. & Saraste, M. 1993. The PH domain: a common piece in the structural patchwork of signalling proteins. *Trends in biochemical sciences* **18:** 343-348.

Nagle, C. A., Vergnes, L., Dejong, H., Wang, S., Lewin, T. M., Reue, K. & Coleman, R. A. 2008. Identification of a novel sn-glycerol-3-phosphate acyltransferase isoform, GPAT4, as the enzyme deficient in Agpat6-/- mice. *Journal of lipid research* **49:** 823-831.

Nakae, J., Kitamura, T., Silver, D. L. & Accili, D. 2001. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *The Journal of clinical investigation* **108:** 1359-1367.

Nakano, K., Takaishi, K., Kodama, A., Mammoto, A., Shiozaki, H., Monden, M. & Takai, Y. 1999. Distinct actions and cooperative roles of ROCK and mDia in Rho small G protein-induced reorganization of the actin cytoskeleton in Madin-Darby canine kidney cells. *Molecular biology of the cell* **10:** 2481-2491.

Narumiya, S., Tanji, M. & Ishizaki, T. 2009. Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. *Cancer metastasis reviews* **28:** 65-76.

Neuhaus, C., Lang-Roth, R., Zimmermann, U., Heller, R., Eisenberger, T., Weikert, M., Markus, S., Knipper, M. & Bolz, H. J. 2017. Extension of the clinical and molecular phenotype of DIAPH1-associated autosomal dominant hearing loss (DFNA1). *Clinical genetics* **91:** 892-901.

Ngo, M. & Ridgway, N. D. 2009. Oxysterol binding protein-related Protein 9 (ORP9) is a cholesterol transfer protein that regulates Golgi structure and function. *Molecular biology of the cell* **20:** 1388-1399.

Nhek, S., Ngo, M., Yang, X., Ng, M. M., Field, S. J., Asara, J. M., Ridgway, N. D. & Toker, A. 2010. Regulation of oxysterol-binding protein Golgi localization through protein kinase D-mediated phosphorylation. *Molecular biology of the cell* **21:** 2327-2337.

Ni, W., Fang, Y., Tong, L., Tong, Z., Yi, F., Qiu, J., Wang, R. & Tong, X. 2015. Girdin regulates the migration and invasion of glioma cells via the PI3K-Akt signaling pathway. *Molecular medicine reports* **12:** 5086-5092.

Niggli, V. & Rossy, J. 2008. Ezrin/radixin/moesin: versatile controllers of signaling molecules and of the cortical cytoskeleton. *The international journal of biochemistry & cell biology* **40:** 344-349.

Nishita, M., Tomizawa, C., Yamamoto, M., Horita, Y., Ohashi, K. & Mizuno, K. 2005. Spatial and temporal regulation of cofilin activity by LIM kinase and

Slingshot is critical for directional cell migration. *The Journal of cell biology* **171:** 349-359.

Nissila, E., Ohsaki, Y., Weber-Boyvat, M., Perttila, J., Ikonen, E. & Olkkonen, V. M. 2012. ORP10, a cholesterol binding protein associated with microtubules, regulates apolipoprotein B-100 secretion. *Biochimica et biophysica acta* **1821:** 1472-1484.

Nordlie, R. C., Foster, J. D. & Lange, A. J. 1999. Regulation of glucose production by the liver. *Annual Review of Nutrition* **19:** 379-406.

Noritake, J., Watanabe, T., Sato, K., Wang, S. & Kaibuchi, K. 2005. IQGAP1: a key regulator of adhesion and migration. *Journal of cell science* **118:** 2085-2092.

Oh, K. J., Han, H. S., Kim, M. J. & Koo, S. H. 2013. CREB and FoxO1: two transcription factors for the regulation of hepatic gluconeogenesis. *BMB reports* **46:** 567-574.

Olkkonen, V. M. 2013. OSBP-related proteins: liganding by glycerophospholipids opens new insight into their function. *Molecules* **18:** 13666-13679.

Olkkonen, V. M., Beaslas, O. & Nissila, E. 2012. Oxysterols and their cellular effectors. *Biomolecules* **2:** 76-103.

Osman, C., Voelker, D. R. & Langer, T. 2011. Making heads or tails of phospholipids in mitochondria. *The Journal of cell biology* **192:** 7-16.

Owen, D., Campbell, L. J., Littlefield, K., Evetts, K. A., Li, Z., Sacks, D. B., Lowe, P. N. & Mott, H. R. 2008. The IQGAP1-Rac1 and IQGAP1-Cdc42 interactions: interfaces differ between the complexes. *The Journal of biological chemistry* **283:** 1692-1704.

Palazzo, A. F., Cook, T. A., Alberts, A. S. & Gundersen, G. G. 2001. mDia mediates Rho-regulated formation and orientation of stable microtubules. *Nature cell biology* **3:** 723-729.

Palecek, S. P., Huttenlocher, A., Horwitz, A. F. & Lauffenburger, D. A. 1998. Physical and biochemical regulation of integrin release during rear detachment of migrating cells. *Journal of cell science* **111:** 929-940.

Park, C. Y., Hoover, P. J., Mullins, F. M., Bachhawat, P., Covington, E. D., Raunser, S., Walz, T., Garcia, K. C., Dolmetsch, R. E. & Lewis, R. S. 2009. STIM1 clusters and activates CRAC channels via direct binding of a cytosolic domain to Orai1. *Cell* **136:** 876-890.

Park, I., Han, C., Jin, S., Lee, B., Choi, H., Kwon, J. T., Kim, D., Kim, J., Lifirsu, E., Park, W. J., Park, Z. Y., Kim, D. H. & Cho, C. 2011. Myosin regulatory light chains are required to maintain the stability of myosin II and cellular integrity. *The Biochemical journal* **434:** 171-180.

Park, S. J., Suetsugu, S., Sagara, H. & Takenawa, T. 2007. HSP90 cross-links branched actin filaments induced by N-WASP and the Arp2/3 complex. *Genes to cells : devoted to molecular & cellular mechanisms* **12:** 611-622.

Patel, R. K. & Mohan, C. 2005. PI3K/AKT signaling and systemic autoimmunity. *Immunologic research* **31:** 47-55.

Peng, J., Wallar, B. J., Flanders, A., Swiatek, P. J. & Alberts, A. S. 2003. Disruption of the Diaphanous-related formin Drf1 gene encoding mDia1 reveals a role for Drf3 as an effector for Cdc42. *Current biology* **13:** 534-545.

Peretti, D., Dahan, N., Shimoni, E., Hirschberg, K. & Lev, S. 2008. Coordinated lipid transfer between the endoplasmic reticulum and the Golgi complex requires the VAP proteins and is essential for Golgi-mediated transport. *Molecular biology of the cell* **19:** 3871-3884.

Perry, R. J. & Ridgway, N. D. 2006. Oxysterol-binding protein and vesicle-associated membrane protein-associated protein are required for sterol-dependent activation of the ceramide transport protein. *Molecular biology of the cell* **17:** 2604-2616.

Phan, J., Peterfy, M. & Reue, K. 2004. Lipin expression preceding peroxisome proliferator-activated receptor-gamma is critical for adipogenesis in vivo and in vitro. *The Journal of biological chemistry* **279:** 29558-29564.

Pollard, T. D. 2007. Regulation of actin filament assembly by Arp2/3 complex and formins. *Annual Review of Biophysics and Biomolecular Structure* **36:** 451-477.

Pollard, T. D., Blanchoin, L. & Mullins, R. D. 2000. Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annual Review of Biophysics and Biomolecular Structure* **29:** 545-576.

Pollard, T. D. & Borisy, G. G. 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell* **112:** 453-465.

Porstmann, T., Griffiths, B., Chung, Y. L., Delpuech, O., Griffiths, J. R., Downward, J. & Schulze, A. 2005. PKB/Akt induces transcription of enzymes involved in cholesterol and fatty acid biosynthesis via activation of SREBP. *Oncogene* **24:** 6465-6481.

Porstmann, T., Santos, C. R., Griffiths, B., Cully, M., Wu, M., Leevers, S., Griffiths, J. R., Chung, Y. L. & Schulze, A. 2008. SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. *Cell metabolism* **8:** 224-236.

Porter, K. R. & Palade, G. E. 1957. Studies on the endoplasmic reticulum. III. Its form and distribution in striated muscle cells. *The Journal of biophysical and biochemical cytology* **3:** 269-300.

Prinz, W. A. 2014. Bridging the gap: membrane contact sites in signaling, metabolism, and organelle dynamics. *The Journal of cell biology* **205:** 759-769.

Qian, Y., Corum, L., Meng, Q., Blenis, J., Zheng, J. Z., Shi, X., Flynn, D. C. & Jiang, B. H. 2004. PI3K induced actin filament remodeling through Akt and p70S6K1: implication of essential role in cell migration. *American journal of physiology. Cell physiology* **286:** C153-63.

Raiborg, C., Wenzel, E. M., Pedersen, N. M., Olsvik, H., Schink, K. O., Schultz, S. W., Vietri, M., Nisi, V., Bucci, C., Brech, A., Johansen, T. & Stenmark, H. 2015. Repeated ER-endosome contacts promote endosome translocation and neurite outgrowth. *Nature* **520:** 234-238.

Rathmell, J. C., Fox, C. J., Plas, D. R., Hammerman, P. S., Cinalli, R. M. & Thompson, C. B. 2003. Akt-directed glucose metabolism can prevent Bax

conformation change and promote growth factor-independent survival. *Molecular and cellular biology* **23:** 7315-7328.

Rebbeck, R. T., Karunasekara, Y., Gallant, E. M., Board, P. G., Beard, N. A., Casarotto, M. G. & Dulhunty, A. F. 2011. The beta(1a) subunit of the skeletal DHPR binds to skeletal RyR1 and activates the channel via its 35-residue C-terminal tail. *Biophysical journal* **100:** 922-930.

Reue, K., Xu, P., Wang, X. P. & Slavin, B. G. 2000. Adipose tissue deficiency, glucose intolerance, and increased atherosclerosis result from mutation in the mouse fatty liver dystrophy (fld) gene. *Journal of lipid research* **41:** 1067-1076.

Ribaux, P. G. & Iynedjian, P. B. 2003. Analysis of the role of protein kinase B (cAKT) in insulin-dependent induction of glucokinase and sterol regulatory element-binding protein 1 (SREBP1) mRNAs in hepatocytes. *The Biochemical journal* **376:** 697-705.

Rider, M. H., Bertrand, L., Vertommen, D., Michels, P. A., Rousseau, G. G. & Hue, L. 2004. 6-Phosphofructo-2-Kinase/fructose-2,6-Bisphosphatase: Head-To-Head with a Bifunctional Enzyme that Controls Glycolysis. *The Biochemical journal* **381:** 561-579.

Ridgway, N. D., Dawson, P. A., Ho, Y. K., Brown, M. S. & Goldstein, J. L. 1992. Translocation of oxysterol binding protein to Golgi apparatus triggered by ligand binding. *The Journal of cell biology* **116:** 307-319.

Ridgway, N. D., Lagace, T. A., Cook, H. W. & Byers, D. M. 1998. Differential effects of sphingomyelin hydrolysis and cholesterol transport on oxysterol-binding protein phosphorylation and Golgi localization. *The Journal of biological chemistry* **273:** 31621-31628.

Ridley, A. J. 2001. Rho family proteins: coordinating cell responses. *Trends in cell biology* **11:** 471-477.

Ridley, A. J. & Hall, A. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70:** 389-399.

Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T. & Horwitz, A. R. 2003. Cell migration: integrating signals from front to back. *Science* **302:** 1704-1709.

Rizzuto, R. & Pozzan, T. 2006. Microdomains of intracellular Ca2+: molecular determinants and functional consequences. *Physiological Reviews* **86:** 369-408.

Robertson, J. D. 1960. The molecular structure and contact relationships of cell membranes. *Progress in biophysics and molecular biology* **10:** 343-418.

Rocha, N., Kuijl, C., van der Kant, R., Janssen, L., Houben, D., Janssen, H., Zwart, W. & Neefjes, J. 2009. Cholesterol sensor ORP1L contacts the ER protein VAP to control Rab7-RILP-p150 Glued and late endosome positioning. *The Journal of cell biology* **185:** 1209-1225.

Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T. & Kirschner, M. W. 1999. The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* **97:** 221-231.

Romeo, G. R. & Kazlauskas, A. 2008. Oxysterol and diabetes activate STAT3 and control endothelial expression of profilin-1 via OSBP1. *The Journal of biological chemistry* **283:** 9595-9605.

Rosivatz, E. & Woscholski, R. 2011. Removal or masking of phosphatidylinositol(4,5)bisphosphate from the outer mitochondrial membrane causes mitochondrial fragmentation. *Cellular signalling* **23:** 478-486.

Rossman, K. L., Der, C. J. & Sondek, J. 2005. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nature reviews. Molecular cell biology* **6:** 167-180.

Rowland, A. A., Chitwood, P. J., Phillips, M. J. & Voeltz, G. K. 2014. ER contact sites define the position and timing of endosome fission. *Cell* **159:** 1027-1041.

Rui, L. 2014. Energy metabolism in the liver. *Comprehensive Physiology* **4:** 177-197.

Ruprecht, V., Wieser, S., Callan-Jones, A., Smutny, M., Morita, H., Sako, K., Barone, V., Ritsch-Marte, M., Sixt, M., Voituriez, R. & Heisenberg, C. P. 2015. Cortical contractility triggers a stochastic switch to fast amoeboid cell motility. *Cell* **160:** 673-685.

Rusinol, A. E., Cui, Z., Chen, M. H. & Vance, J. E. 1994. A unique mitochondria-associated membrane fraction from rat liver has a high capacity for lipid synthesis and contains pre-Golgi secretory proteins including nascent lipoproteins. *The Journal of biological chemistry* **269:** 27494-27502.

Sarbassov, D. D., Guertin, D. A., Ali, S. M. & Sabatini, D. M. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307:** 1098-1101.

Sato, S., Fujita, N. & Tsuruo, T. 2000. Modulation of Akt kinase activity by binding to Hsp90. *Proceedings of the National Academy of Sciences of the United States of America* **97:** 10832-10837.

Schorr, S. & van der Laan, M. 2018. Integrative functions of the mitochondrial contact site and cristae organizing system. *Seminars in cell & developmental biology* **76:** 191-200

Schroepfer, G. J.,Jr. 2000. Oxysterols: modulators of cholesterol metabolism and other processes. *Physiological Reviews* **80:** 361-554.

Scita, G., Tenca, P., Frittoli, E., Tocchetti, A., Innocenti, M., Giardina, G. & Di Fiore, P. P. 2000. Signaling from Ras to Rac and beyond: not just a matter of GEFs. *The EMBO journal* **19:** 2393-2398.

Sechi, A. S. & Wehland, J. 2004. ENA/VASP proteins: multifunctional regulators of actin cytoskeleton dynamics. *Frontiers in bioscience : a journal and virtual library* **9:** 1294-1310.

Semenza, G. L., Jiang, B. H., Leung, S. W., Passantino, R., Concordet, J. P., Maire, P. & Giallongo, A. 1996. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *The Journal of biological chemistry* **271:** 32529-32537.

Shirasaki, T., Honda, M., Shimakami, T., Horii, R., Yamashita, T., Sakai, Y., Sakai, A., Okada, H., Watanabe, R., Murakami, S., Yi, M., Lemon, S. M. & Kaneko, S.

2013. MicroRNA-27a regulates lipid metabolism and inhibits hepatitis C virus replication in human hepatoma cells. *Journal of virology* **87:** 5270-5286.

Shyu, Y. J. & Hu, C. D. 2008. Fluorescence complementation: an emerging tool for biological research. *Trends in biotechnology* **26:** 622-630.

Sidossis, L. S. & Wolfe, R. R. 1996. Glucose and insulin-induced inhibition of fatty acid oxidation: the glucose-fatty acid cycle reversed. *The American Journal of Physiology* **270:** E733-8.

Silverstein, A. M., Grammatikakis, N., Cochran, B. H., Chinkers, M. & Pratt, W. B. 1998. p50(cdc37) binds directly to the catalytic domain of Raf as well as to a site on hsp90 that is topologically adjacent to the tetratricopeptide repeat binding site. *The Journal of biological chemistry* **273:** 20090-20095.

Skirpan, A. L., Dowd, P. E., Sijacic, P., Jaworski, C. J., Gilroy, S. & Kao, T. H. 2006. Identification and characterization of PiORP1, a Petunia oxysterol-binding-protein related protein involved in receptor-kinase mediated signaling in pollen, and analysis of the ORP gene family in Arabidopsis. *Plant Molecular Biology* **61:** 553-565.

Stanyon, C. A. & Bernard, O. 1999. LIM-kinase1. *The international journal of biochemistry & cell biology* **31:** 389-394.

Stathopulos, P. B., Zheng, L., Li, G. Y., Plevin, M. J. & Ikura, M. 2008. Structural and mechanistic insights into STIM1-mediated initiation of store-operated calcium entry. *Cell* **135:** 110-122.

Storey, M. K., Byers, D. M., Cook, H. W. & Ridgway, N. D. 1998. Cholesterol regulates oxysterol binding protein (OSBP) phosphorylation and Golgi localization in Chinese hamster ovary cells: correlation with stimulation of sphingomyelin synthesis by 25-hydroxycholesterol. *The Biochemical journal* **336:** 247-256.

Stritt, S., Nurden, P., Turro, E., Greene, D., Jansen, S. B., Westbury, S. K., Petersen, R., Astle, W. J., Marlin, S., Bariana, T. K., Kostadima, M., Lentaigne, C., Maiwald, S., Papadia, S., Kelly, A. M., Stephens, J. C., Penkett, C. J., Ashford, S., Tuna, S., Austin, S., Bakchoul, T., Collins, P., Favier, R., Lambert, M. P., Mathias, M., Millar, C. M., Mapeta, R., Perry, D. J., Schulman, S., Simeoni, I., Thys, C., BRIDGE-BPD Consortium, Gomez, K., Erber, W. N., Stirrups, K., Rendon, A., Bradley, J. R., van Geet, C., Raymond, F. L., Laffan, M. A., Nurden, A. T., Nieswandt, B., Richardson, S., Freson, K., Ouwehand, W. H. & Mumford, A. D. 2016. A gain-of-function variant in DIAPH1 causes dominant macrothrombocytopenia and hearing loss. *Blood* **127:** 2903-2914.

Suchanek, M., Hynynen, R., Wohlfahrt, G., Lehto, M., Johansson, M., Saarinen, H., Radzikowska, A., Thiele, C. & Olkkonen, V. M. 2007. The mammalian oxysterol-binding protein-related proteins (ORPs) bind 25-hydroxycholesterol in an evolutionarily conserved pocket. *The Biochemical journal* **405:** 473-480.

Sugawara, K., Morita, K., Ueno, N. & Shibuya, H. 2001. BIP, a BRAM-interacting protein involved in TGF-beta signalling, regulates body length in Caenorhabditis elegans. *Genes to cells : devoted to molecular & cellular mechanisms* **6:** 599-606.

Suraneni, P., Rubinstein, B., Unruh, J. R., Durnin, M., Hanein, D. & Li, R. 2012. The Arp2/3 complex is required for lamellipodia extension and directional fibroblast cell migration. *The Journal of cell biology* **197:** 239-251.

Sutherland, C., O'Brien, R. M. & Granner, D. K. 1996. New connections in the regulation of PEPCK gene expression by insulin. *Philosophical transactions of the Royal Society of London.Series B, Biological sciences* **351:** 191-199.

Svitkina, T. 2018. The Actin Cytoskeleton and Actin-Based Motility. *Cold Spring Harbor perspectives in biology* **10:** 10.1101/cshperspect.a018267.

Symons, M., Derry, J. M., Karlak, B., Jiang, S., Lemahieu, V., Mccormick, F., Francke, U. & Abo, A. 1996. Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. *Cell* **84:** 723-734.

Szabadkai, G., Bianchi, K., Varnai, P., De Stefani, D., Wieckowski, M. R., Cavagna, D., Nagy, A. I., Balla, T. & Rizzuto, R. 2006. Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca2+ channels. *The Journal of cell biology* **175:** 901-911.

Taiyab, A. & Rao, C. 2011. HSP90 modulates actin dynamics: inhibition of HSP90 leads to decreased cell motility and impairs invasion. *Biochimica et biophysica acta* **1813:** 213-221.

Takekura, H., Nishi, M., Noda, T., Takeshima, H. & Franzini-Armstrong, C. 1995a. Abnormal junctions between surface membrane and sarcoplasmic reticulum in skeletal muscle with a mutation targeted to the ryanodine receptor. *Proceedings of the National Academy of Sciences of the United States of America* **92:** 3381-3385.

Takekura, H., Takeshima, H., Nishimura, S., Takahashi, M., Tanabe, T., Flockerzi, V., Hofmann, F. & Franzini-Armstrong, C. 1995b. Co-expression in CHO cells of two muscle proteins involved in excitation-contraction coupling. *Journal of muscle research and cell motility* **16:** 465-480.

Takeshima, H., Komazaki, S., Nishi, M., Iino, M. & Kangawa, K. 2000. Junctophilins: a novel family of junctional membrane complex proteins. *Molecular cell* **6:** 11-22.

Taylor, F. R., Saucier, S. E., Shown, E. P., Parish, E. J. & Kandutsch, A. A. 1984. Correlation between oxysterol binding to a cytosolic binding protein and potency in the repression of hydroxymethylglutaryl coenzyme A reductase. *The Journal of biological chemistry* **259:** 12382-12387.

Thoenes, M., Zimmermann, U., Ebermann, I., Ptok, M., Lewis, M. A., Thiele, H., Morlot, S., Hess, M. M., Gal, A., Eisenberger, T., Bergmann, C., Nurnberg, G., Nurnberg, P., Steel, K. P., Knipper, M. & Bolz, H. J. 2015. OSBPL2 encodes a protein of inner and outer hair cell stereocilia and is mutated in autosomal dominant hearing loss (DFNA67). *Orphanet journal of rare diseases* **10:** 15-015-0238-5.

Tong, J., Yang, H., Yang, H., Eom, S. H. & Im, Y. J. 2013. Structure of Osh3 reveals a conserved mode of phosphoinositide binding in oxysterol-binding proteins. *Structure* **21:** 1203-1213.

Usatyuk, P. V., Fu, P., Mohan, V., Epshtein, Y., Jacobson, J. R., Gomez-Cambronero, J., Wary, K. K., Bindokas, V., Dudek, S. M., Salgia, R., Garcia, J. G. & Natarajan, V. 2014. Role of c-Met/phosphatidylinositol 3-kinase (PI3k)/Akt signaling in hepatocyte growth factor (HGF)-mediated lamellipodia formation, reactive oxygen species (ROS) generation, and motility of lung endothelial cells. *The Journal of biological chemistry* **289:** 13476-13491.

Uzbekov, R., Kireyev, I. & Prigent, C. 2002. Centrosome separation: respective role of microtubules and actin filaments. *Biology of the cell* **94:** 275-288.

van der Kant, R., Fish, A., Janssen, L., Janssen, H., Krom, S., Ho, N., Brummelkamp, T., Carette, J., Rocha, N. & Neefjes, J. 2013. Late endosomal transport and tethering are coupled processes controlled by RILP and the cholesterol sensor ORP1L. *Journal of cell science* **126:** 3462-3474.

Vance, J. E. 2014. MAM (mitochondria-associated membranes) in mammalian cells: lipids and beyond. *Biochimica et biophysica acta* **1841:** 595-609.

Vance, J. E. 1990. Phospholipid synthesis in a membrane fraction associated with mitochondria. *The Journal of biological chemistry* **265:** 7248-7256.

Vaulont, S., Munnich, A., Marie, J., Reach, G., Pichard, A. L., Simon, M. P., Besmond, C., Barbry, P. & Kahn, A. 1984. Cyclic AMP as a transcriptional inhibitor of upper eukaryotic gene transcription. *Biochemical and biophysical research communications* **125:** 135-141.

Vergnes, L., Beigneux, A. P., Davis, R., Watkins, S. M., Young, S. G. & Reue, K. 2006. Agpat6 deficiency causes subdermal lipodystrophy and resistance to obesity. *Journal of lipid research* **47:** 745-754.

Vihervaara, T., Jansen, M., Uronen, R. L., Ohsaki, Y., Ikonen, E. & Olkkonen, V. M. 2011a. Cytoplasmic oxysterol-binding proteins: sterol sensors or transporters? *Chemistry and physics of lipids* **164:** 443-450.

Vihervaara, T., Uronen, R. L., Wohlfahrt, G., Bjorkhem, I., Ikonen, E. & Olkkonen, V. M. 2011b. Sterol binding by OSBP-related protein 1L regulates late endosome motility and function. *Cellular and molecular life sciences* **68:** 537-551.

Voelker, D. R. 1989. Reconstitution of phosphatidylserine import into rat liver mitochondria. *The Journal of biological chemistry* **264:** 8019-8025.

Walsh, C. M., Chvanov, M., Haynes, L. P., Petersen, O. H., Tepikin, A. V. & Burgoyne, R. D. 2009. Role of phosphoinositides in STIM1 dynamics and store-operated calcium entry. *The Biochemical journal* **425:** 159-168.

Wang, C., JeBailey, L. & Ridgway, N. D. 2002. Oxysterol-binding-protein (OSBP)-related protein 4 binds 25-hydroxycholesterol and interacts with vimentin intermediate filaments. *The Biochemical journal* **361:** 461-472.

Wang, P. Y., Weng, J. & Anderson, R. G. 2005. OSBP is a cholesterol-regulated scaffolding protein in control of ERK 1/2 activation. *Science* **307:** 1472-1476.

Wang, P. Y., Weng, J., Lee, S. & Anderson, R. G. 2008a. The N terminus controls sterol binding while the C terminus regulates the scaffolding function of OSBP. *The Journal of biological chemistry* **283:** 8034-8045.

Wang, W., Chen, L., Ding, Y., Jin, J. & Liao, K. 2008b. Centrosome separation driven by actin-microfilaments during mitosis is mediated by centrosome-

associated tyrosine-phosphorylated cortactin. *Journal of cell science* **121:** 1334-1343.

Wang, Y., Deng, X., Zhou, Y., Hendron, E., Mancarella, S., Ritchie, M. F., Tang, X. D., Baba, Y., Kurosaki, T., Mori, Y., Soboloff, J. & Gill, D. L. 2009. STIM protein coupling in the activation of Orai channels. *Proceedings of the National Academy of Sciences of the United States of America* **106:** 7391-7396.

Wang, Y., Litvinov, R. I., Chen, X., Bach, T. L., Lian, L., Petrich, B. G., Monkley, S. J., Kanaho, Y., Critchley, D. R., Sasaki, T., Birnbaum, M. J., Weisel, J. W., Hartwig, J. & Abrams, C. S. 2008a. Loss of PIP5KIgamma, unlike other PIP5KI isoforms, impairs the integrity of the membrane cytoskeleton in murine megakaryocytes. *The Journal of clinical investigation* **118:** 812-819.

Wang, Y., Rogers, P. M., Su, C., Varga, G., Stayrook, K. R. & Burris, T. P. 2008b. Regulation of cholesterologenesis by the oxysterol receptor, LXRalpha. *The Journal of biological chemistry* **283:** 26332-26339.

Watanabe, N., Kato, T., Fujita, A., Ishizaki, T. & Narumiya, S. 1999. Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. *Nature cell biology* **1:** 136-143.

Watson, R. T., Kanzaki, M. & Pessin, J. E. 2004. Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes. *Endocrine reviews* **25:** 177-204.

Weber-Boyvat, M., Kentala, H., Lilja, J., Vihervaara, T., Hanninen, R., Zhou, Y., Peranen, J., Nyman, T. A., Ivaska, J. & Olkkonen, V. M. 2015a. OSBP-related protein 3 (ORP3) coupling with VAMP-associated protein A regulates R-Ras activity. *Experimental cell research* **331:** 278-291.

Weber-Boyvat, M., Kentala, H., Peranen, J. & Olkkonen, V. M. 2015b. Ligand-dependent localization and function of ORP-VAP complexes at membrane contact sites. *Cellular and molecular life sciences* **72:** 1967-1987.

Wegner, A. 1976. Head to tail polymerization of actin. *Journal of Molecular Biology* **108:** 139-150.

Wendel, A. A., Lewin, T. M. & Coleman, R. A. 2009. Glycerol-3-phosphate acyltransferases: rate limiting enzymes of triacylglycerol biosynthesis. *Biochimica et biophysica acta* **1791:** 501-506.

Wilhelm, L. P., Wendling, C., Vedie, B., Kobayashi, T., Chenard, M. P., Tomasetto, C., Drin, G. & Alpy, F. 2017. STARD3 mediates endoplasmic reticulum-to-endosome cholesterol transport at membrane contact sites. *The EMBO journal* **36:** 1412-1433.

Wolf, K., Mazo, I., Leung, H., Engelke, K., von Andrian, U. H., Deryugina, E. I., Strongin, A. Y., Brocker, E. B. & Friedl, P. 2003. Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *The Journal of cell biology* **160:** 267-277.

Wollweber, F., von der Malsburg, K. & van der Laan, M. 2017. Mitochondrial contact site and cristae organizing system: A central player in membrane shaping and crosstalk. *Biochimica et biophysica acta* **1864:** 1481-1489.

Wong, L. H., Copic, A. & Levine, T. P. 2017. Advances on the Transfer of Lipids by Lipid Transfer Proteins. *Trends in biochemical sciences* **42:** 516-530.

Wu, C., Asokan, S. B., Berginski, M. E., Haynes, E. M., Sharpless, N. E., Griffith, J. D., Gomez, S. M. & Bear, J. E. 2012. Arp2/3 is critical for lamellipodia and response to extracellular matrix cues but is dispensable for chemotaxis. *Cell* **148:** 973-987.

Wu, M. M., Buchanan, J., Luik, R. M. & Lewis, R. S. 2006. Ca2+ store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane. *The Journal of cell biology* **174:** 803-813.

Wu, W. I. & Voelker, D. R. 2004. Reconstitution of phosphatidylserine transport from chemically defined donor membranes to phosphatidylserine decarboxylase 2 implicates specific lipid domains in the process. *The Journal of biological chemistry* **279:** 6635-6642.

Wyles, J. P., Perry, R. J. & Ridgway, N. D. 2007. Characterization of the sterol-binding domain of oxysterol-binding protein (OSBP)-related protein 4 reveals a novel role in vimentin organization. *Experimental cell research* **313:** 1426-1437.

Xiang, Y. & Wang, Y. 2010. GRASP55 and GRASP65 play complementary and essential roles in Golgi cisternal stacking. *The Journal of cell biology* **188:** 237-251.

Xing, G., Yao, J., Wu, B., Liu, T., Wei, Q., Liu, C., Lu, Y., Chen, Z., Zheng, H., Yang, X. & Cao, X. 2015. Identification of OSBPL2 as a novel candidate gene for progressive nonsyndromic hearing loss by whole-exome sequencing. *Genetics in medicine : official journal of the American College of Medical Genetics* **17:** 210-218.

Xu, Y., Liu, Y., Ridgway, N. D. & McMaster, C. R. 2001. Novel members of the human oxysterol-binding protein family bind phospholipids and regulate vesicle transport. *The Journal of biological chemistry* **276:** 18407-18414.

Xue, G. & Hemmings, B. A. 2013. PKB/Akt-dependent regulation of cell motility. *Journal of the National Cancer Institute* **105:** 393-404.

Yamashita, H., Takenoshita, M., Sakurai, M., Bruick, R. K., Henzel, W. J., Shillinglaw, W., Arnot, D. & Uyeda, K. 2001. A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 9116-9121.

Yamazaki, M., Miyazaki, H., Watanabe, H., Sasaki, T., Maehama, T., Frohman, M. A. & Kanaho, Y. 2002. Phosphatidylinositol 4-phosphate 5-kinase is essential for ROCK-mediated neurite remodeling. *The Journal of biological chemistry* **277:** 17226-17230.

Yan, D., Jauhiainen, M., Hildebrand, R. B., Willems van Dijk, K., Van Berkel, T. J., Ehnholm, C., Van Eck, M. & Olkkonen, V. M. 2007. Expression of human OSBP-related protein 1L in macrophages enhances atherosclerotic lesion development in LDL receptor-deficient mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* **27:** 1618-1624.

Yan, D., Mayranpaa, M. I., Wong, J., Perttila, J., Lehto, M., Jauhiainen, M., Kovanen, P. T., Ehnholm, C., Brown, A. J. & Olkkonen, V. M. 2008. OSBP-related protein

8 (ORP8) suppresses ABCA1 expression and cholesterol efflux from macrophages. *The Journal of biological chemistry* **283:** 332-340.

Yang, Z., Cappello, T. & Wang, L. 2015. Emerging role of microRNAs in lipid metabolism. *Acta pharmaceutica Sinica.B* **5:** 145-150.

Yecies, J. L., Zhang, H. H., Menon, S., Liu, S., Yecies, D., Lipovsky, A. I., Gorgun, C., Kwiatkowski, D. J., Hotamisligil, G. S., Lee, C. H. & Manning, B. D. 2011. Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. *Cell metabolism* **14:** 21-32.

Yu, Y., Hamza, A., Zhang, T., Gu, M., Zou, P., Newman, B., Li, Y., Gunatilaka, A. A., Zhan, C. G. & Sun, D. 2010. Withaferin A targets heat shock protein 90 in pancreatic cancer cells. *Biochemical pharmacology* **79:** 542-551.

Zatulovskiy, E., Tyson, R., Bretschneider, T. & Kay, R. R. 2014. Bleb-driven chemotaxis of Dictyostelium cells. *The Journal of cell biology* **204:** 1027-1044.

Zechner, R., Kienesberger, P. C., Haemmerle, G., Zimmermann, R. & Lass, A. 2009. Adipose triglyceride lipase and the lipolytic catabolism of cellular fat stores. *Journal of lipid research* **50:** 3-21.

Zhao, H. F., Wang, J., Jiang, H. R., Chen, Z. P. & To, S. S. 2016. PI3K p110beta isoform synergizes with JNK in the regulation of glioblastoma cell proliferation and migration through Akt and FAK inhibition. *Journal of experimental & clinical cancer research* **35:** 78-016-0356-5.

Zhao, K. & Ridgway, N. D. 2017. Oxysterol-Binding Protein-Related Protein 1L Regulates Cholesterol Egress from the Endo-Lysosomal System. *Cell reports* **19:** 1807-1818.

Zhong, W., Yi, Q., Xu, B., Li, S., Wang, T., Liu, F., Zhu, B., Hoffmann, P. R., Ji, G., Lei, P., Li, G., Li, J., Li, J., Olkkonen, V. M. & Yan, D. 2016. ORP4L is essential for T-cell acute lymphoblastic leukemia cell survival. *Nature communications* **7:** 12702.

Zhou, T., Li, S., Zhong, W., Vihervaara, T., Beaslas, O., Perttila, J., Luo, W., Jiang, Y., Lehto, M., Olkkonen, V. M. & Yan, D. 2011. OSBP-related protein 8 (ORP8) regulates plasma and liver tissue lipid levels and interacts with the nucleoporin Nup62. *PloS one* **6:** e21078.

Zhou, Y., Wohlfahrt, G., Paavola, J. & Olkkonen, V. M. 2014. A vertebrate model for the study of lipid binding/transfer protein function: conservation of OSBP-related proteins between zebrafish and human. *Biochemical and biophysical research communications* **446:** 675-680.