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The Role of Oxysterol Binding Proteins in Macrophages

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ACADEMIC DISSERTATION

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List of original publications

This thesis is based on the following publications, which are referred to in text by their roman numerals. In addition, some unpublished data are presented.

I **Vihervaara T**, Uronen R, Wohlfahrt G, Björkhem I, Ikonen E, Olkkonen VM: Sterol binding by OSBP-related protein 1L regulates late endosome motility and function.
Cell Mol Life Sci. 2011 Feb;68(3):537-51

II Béaslas O*, **Vihervaara T***, Li S, Laurila PP, Yan D, Olkkonen VM: OSBP-related Protein 8 (ORP8) is associated with nuclear and centrosome/microtubule regulation in the macrophage.
Exp Cell Res, 2012 Sep 10;318(15):1933-45

III **Vihervaara T**, Käkelä R, Liebisch G, Tarasov K, Schmitz G, Olkkonen VM: Modification of the lipidome in Raw264.7 macrophage subjected to stable silencing of oxysterol-binding proteins.
Biochimie 2012 [Epub ahead of print] *DOI: 10.1016/j.biochi.2012.05.004.*

* These authors contributed equally

Publication I appeared previously in the doctoral thesis "Sterol-binding proteins in late endosomes: Regulation of endosome motility and lipid metabolism" by PhD Riikka-Liisa Uronen (2011).

Abbreviations

AA	arachidonic acid
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
acLDL	acetylated LDL
apo-AI	apolipoprotein AI
BiFC	bimolecular fluorescence complementation
CoA	coenzyme A
DAG	diacylglycerol
DHA	docosahexaenoic acid
EE	early endosome
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
EXO70	exocyst complex 70
FFAT	two phenylalanines in an acidic tract
GFP	green fluorescent protein
GST	glutathione-S-transferase
HDL	high-density lipoprotein
HMG	3-hydroxy-3-methyl-glutaryl
IL	interleukin
iNOS	inducible nitric oxide synthase
IP_3	inositol trisphosphate
KC	ketocholesterol
LD	lipid droplet
LE	late endosome
LPC	lysophosphatidylcholine
LPS	lipopolysaccharide
LTP	lipid transfer protein
LXR	liver X receptor
LY	lysosome
MCP-1	monocyte chemoattractant protein 1
MCS	membrane contact site
MVB	multivesicular body
NPC	nuclear pore complex
NPC1	Niemann-Pick C1
NUP62	nucleoporin p62
NVJ	nucleus-vacuole junction
OHC	hydroxycholesterol
ORD	oxysterol-related domain
ORP	oxysterol binding protein related protein
OSBP	oxysterol binding protein
oxLDL	oxidized LDL

PC	phosphatidylcholine
PE	phosphatidylethanolamine
PE-pl	PE plasmalogen
PG	phosphatidylglycerol
PHD	pleckstrin-homology domain
PI	phosphatidylinositol
PIP	phosphatidylinositol phosphate
PL	phospholipid
PLC	phospholipase C
PLTP	phospholipid transfer protein
PM	plasma membrane
PMA	phorbol myristate acetate
PS	phosphatidylserine
RILP	Rab7 interacting lysosomal protein
ROS	reactive oxygen species
shNT	Non-Targeting shRNA
shRNA	short-hairpin RNA
SM	sphingomyelin
SR	scavenger receptor
SREBP	sterol regulatory element binding protein
TAG	triacylglycerol
TLR	Toll-like receptor
VAP	vesicle-associated membrane protein-associated protein
qPCR	quantitative polymerase chain reaction

Abstract

All biological membranes consist of several types of lipids that have important structural and functional roles. Moreover, these lipids can act as signaling molecules or affect the function of cellular proteins. Regulation of lipid homeostasis is critical for the maintenance of cellular physiology, and disturbances in lipid metabolism aspects are key components behind many metabolic diseases, including atherosclerosis.

Macrophage cells play central roles in the development of atherosclerosis. The excessive uptake of modified lipoprotein particles in the vessel subendothelial space results in a proinflammatory response and the formation of macrophage foam cells. Oxysterols, oxidized cholesterol derivatives that primarily are taken up along with the modified lipoproteins, act at the crossroads of lipid metabolism and inflammation, and are thus important signaling lipids involved in atherogenesis.

In this thesis the functions of three members of the Oxysterol Binding Protein (ORP) Family have been studied. The ORP1L, ORP3, and ORP8 proteins are abundantly expressed in the macrophage cell type, and their endogenous role in macrophages was studied by knocking down the proteins individually using short hairpin RNA encoding lentiviruses. Moreover, the general function of these proteins was studied using other cell models.

In the first part, the targeting of late endosomal protein ORP1L was investigated in HeLa cells. We show that ORP1L affects the motility and function of late endosomes in a sterol dependent manner. ORP1L is reported to mediate the interaction of late endosomes and endoplasmic reticulum, which restricts the motility of the endosomes. Furthermore, we show that cholesterol removal from macrophage foam cells is inhibited in the absence of ORP1L.

In the second part the endogenous role of ORP8 in the macrophage was investigated by transcriptome, biochemical and functional assays. We show that ORP8 knockdown results in activation of several nuclear pathways as well as microtubule and centrosome organization pathways. We further provide evidence that ORP8 affects cellular migration dependent, in part, on its interaction with a nuclear pore complex component, Nucleoporin p62. Moreover, we demonstrate the impact of ORP8 knockdown on microtubule cytoskeleton, which is essential for many processes during migration.

In the third part, the lipidome of macrophage cells subjected to ORP silencing is studied in four conditions relevant for atherogenesis: under basal conditions, or treated with oxidized or enzymatically modified lipoprotein preparations that are known to result in different lipid storage phenotypes, or stimulated with

lipopolysaccharide, which triggers inflammatory responses. The lipidome analysis revealed complex and profound changes in the cellular lipidome in the absence of ORPs, with implications for the inflammatory response.

In addition, some unpublished data are presented regarding the subcellular targeting of ORP3 and ORP8 proteins.

This work has provided information on the cellular functions of macrophage-enriched ORP members, and revealed novel aspects in the function of the protein family in the cellular physiology. These data suggest that the modulation of ORP levels and/or function represents an attractive target for prevention or treatment of atherosclerosis.

Review of the literature

2.1 Cellular lipid homeostasis

2.1.1. Introduction to lipids

Lipids are a heterogeneous group of biomolecules with a hydrophobic moiety. Majority of cellular lipids are present in biological membranes or in special lipid storage organelles. The main membrane lipid classes are glycerophospholipids, sterols and sphingolipids. These have important structural roles, but they also participate in many cellular events, as will be discussed in later sections.

Glycerophospholipids (from now on referred to as phospholipids, PL) are the major structural lipids of eukaryotic membranes. The diacylglycerol (DAG) backbone of phospholipids carries two fatty acyl moieties at sn-1 and sn-2 positions (Figure 1). A phosphate group is bridging the DAG backbone to choline, ethanolamine, serine, inositol, or glycerol headgroups, thus forming phosphatidycholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), or phosphatidylglycerol (PG) lipids, respectively (Figure 1). The fatty acyl moieties can vary greatly in the length (number of carbon atoms) and degree of unsaturation (number of double bonds in the carbon chain). There are thousands of possible combinations of head groups and fatty acyl moieties (Vance, 2008).

The most abundant sterol in mammalian cells is cholesterol. Cholesterol molecule is a rigid and planar 4-ring structure with iso-octyl chain attached to carbon-17 (Figure 1). It is an essential building block of cellular membranes, especially plasma membrane (PM) (Lange, 1991), affecting the membrane fluidity, impermeability and viscosity. In addition, cholesterol acts as precursor for several essential molecules, including steroid hormones, vitamin D, bile acids and oxysterols (chapter 2.1.5.3). The cellular amount of free cholesterol is tightly regulated, and excess cholesterol is stored in an esterified form in the cytoplasmic lipid droplets (LD).

All sphingolipids, including sphingomyelins (SM), ceramides and glycosphingolipids, contain a sphingoid base, most often sphinganine. Sphingolipids are involved in maintaining the cellular structure, cell recognition, adhesion and signaling (Merrill et al., 2007). The most abundant sphingolipid class is SM (Figure 1) that serves both as precursors and end products in ceramide metabolism. Glycosphingolipids arise from addition of carbohydrate structures to the backbone, giving rise to a large amount of distinct sphingolipid molecules. The glycosylation is involved in cell interactions with the extracellular environment (Merrill et al., 2007).

Imbalances in lipid metabolism are a key component of several pathological states, including metabolic syndrome, obesity, cardiovascular diseases and type 2 diabetes (Wymann and Schneiter, 2008). Moreover, upregulation of lipid metabolism is one of the early hallmarks of cancer (Kuhajda et al., 1994; Swinnen et al., 2000). Altogether, these diseases contribute to ever growing health problems in the western world, but also to an increasing extent in the developing countries.

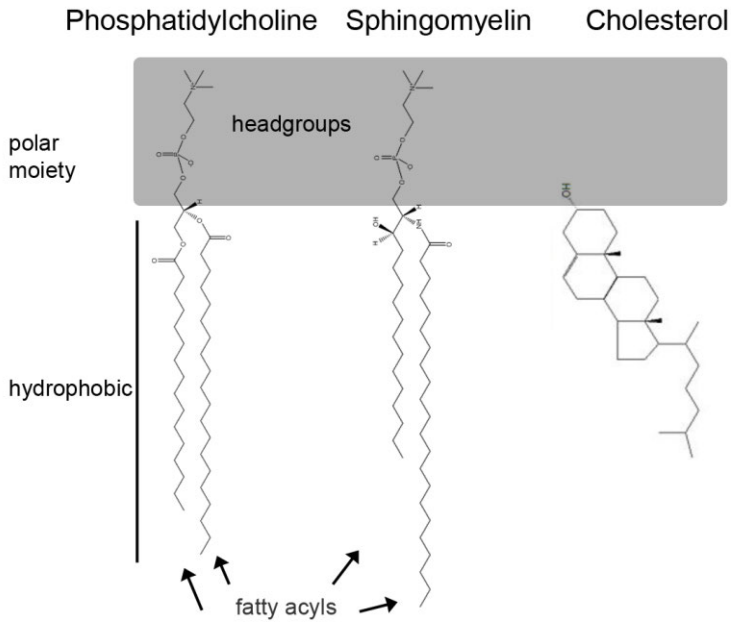

Figure 1. The structures of main membrane lipid classes: glycerophospholipids (PC here as an example), sphingomyelin, and cholesterol. The hydrophilic molecule moieties are highlighted in grey, and the hydrophobic moieties indicated on the left.

2.1.2 Biological membranes

Biological membranes form continuous barriers that surround the cell, nucleus and other cellular organelles. Membranes consist of a phospholipid bilayer, where other lipids and membrane proteins are embedded (Figure 2). In water, phospholipids spontaneously form bilayers or liposomes with the hydrophobic tails facing towards the bilayer or the liposome lumen, and the hydrophilic moieties facing out.

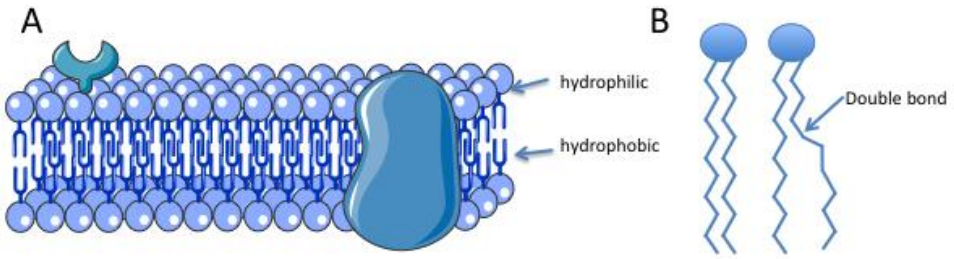

Figure 2. A schematic presentation of A) the lipid bilayer with proteins embedded, and B) the influence of a double bond within the acyl chain on the PL structure. Some figure components were provided by Servier Medical Art (www.servier.com).

Biological membranes are highly dynamic structures. Many physiological processes, including cell growth, cell motility, endocytosis, exocytosis, and phagocytosis require active reshaping of membranes. Moreover, the lipid environment regulates the function of several membrane proteins, including membrane transporters, ion channels, and proteins involved in signal transduction. The regulated import/export of aqueous solutes and larger molecules, unable to passively across the membrane, is an important aspect of cellular homeostasis.

The lipid composition defines dynamic properties of the membrane. The fluidity, resistance and curvature are determined by the lipid constitutions within the membrane: the relative amounts and leaflet asymmetry of lipid classes, their fatty acyl content, and lateral organization. The relative amounts of different lipids vary greatly between organelles and leaflets, and each organelle has a unique lipid composition (Figure 3), necessary for its function. It is, however, poorly understood how the compositions are achieved and maintained.

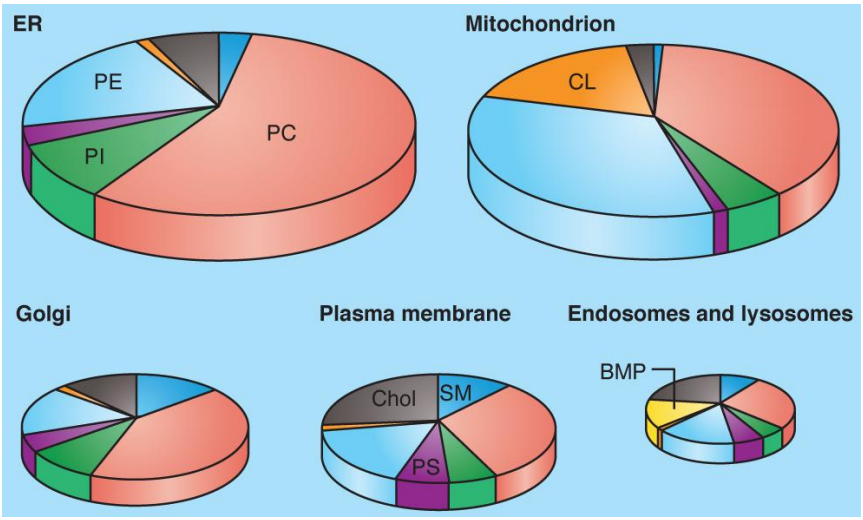

Figure 3. Organelle distribution of different phospholipids, cholesterol, and sphingomyelin, diameters reflect contribution to total cellular lipid. Abbreviations: BMP, bis(monoacylglycero)phosphate; CL, cardiolipin; ER, endoplasmic reticulum; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG,

phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin. Adapted with permission from (van Meer and de Kroon, 2011**).**

The lateral lipid organization is a constantly ongoing process, as the lipids move laterally relatively fast. Mainly hydrophobic forces guide it, and lipids tend to adopt an organization according to the principles of minimal energy (Somerharju et al., 2009). Lipids are not uniformly distributed within the membrane, but form distinct domains that differ in their biophysical properties. The amount of cholesterol is critical for membrane fluidity, as cholesterol rigidifies all membranes by allowing tighter packing of surrounding PLs (Greenwood et al., 2006; Mouritsen and Zuckermann, 2004; Smaby et al., 1994), thus forming more ordered domains in the membrane. The size and shape of phospholipid molecules, defined by the headgroup and fatty acyls, regulate how tightly these lipids can be packed. Long and saturated acyl chains allow for tighter packing, whereas the fluidity increases with the degree of unsaturation of the acyls due to the kinks that double bonds introduce to the molecule (Figure 2).

Cholesterol has a tendency to associate with SM, and membranes enriched in these lipids (such as PM) can form specialized microdomains called lipid rafts. Rafts form platforms for membrane proteins, important in membrane transport and signaling (Hancock, 2006; Lingwood and Simons, 2010).

2.1.3 Cellular lipid trafficking

Due to their hydrophobic nature, lipids cannot move freely between cellular compartments. To maintain correct lipid compositions of membranes, tightly controlled mechanisms for lipid transport have developed. In general, lipid transport can be mediated by vesicular and non-vesicular mechanisms.

2.1.3.1 Non-vesicular trafficking

It has been estimated that majority of cholesterol transport occurs by non-vesicular routes (Ikonen, 2008). Non-vesicular cholesterol transport is mediated by different families of lipid transfer proteins (LTPs), soluble proteins with lipid binding cleft or tunnel that can shuttle the lipid between membranes (D'Angelo et al., 2008). Recently, involvement of membrane contact sites (MCS) in the non-vesicular lipid transfer has been suggested. These are zones where the membranes of two organelles are in a very close proximity (within 10-30 nm), allowing for more efficient lipid transport between the compartments (Levine and Loewen, 2006; Toulmay and Prinz, 2011). At these sites, lipid transfer can occur by action of LTPs or by integral membrane proteins. MCSs are described in further detail in section 2.3.6.

2.1.3.2 Vesicular trafficking

Vesicular trafficking is involved in transport of proteins and lipids between cellular compartments. The transport is controlled at multiple levels to ensure correct localization of biomolecules, and proper membrane composition and function. Vesicular transport assures proper lipid and protein compositions of organelles, essential for their function. Moreover, accurate targeting of transport material is necessary to prevent possibly harmful mixing of organelle constituents. Secreted and membrane proteins and majority of lipids are synthesized in the endoplasmic reticulum (ER), and further post-translational modification of proteins takes place in the Golgi apparatus. From their site of synthesis, molecules are transported to final destinations by vesicular transport (Bonifacino and Glick, 2004, Mellman and Warren, 2000). Vesicular transport is also involved in the trafficking and handling of internalized (endocytosed or phagocytosed) materials. Moreover, cellular migration uses vesicular transport machinery to provide membrane material to dynamically reshape the cell's leading edge (Kriebel et al., 2008).

Vesicular trafficking begins with coat assembly and sorting of the cargo material. Formed vesicles then bud off from the donor compartment, followed by the vesicle transport along cellular cytoskeleton tracks (microtubules) with the help of motor proteins, myosins, kinesins and dyneins (Lippincott-Schwartz, 1998). Vesicle tethering and fusion with the recipient membrane is mediated by SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment) proteins (Bonifacino and Glick, 2004). Finally, the membrane-associated and/or luminal cargo is released to the target organelle (Olkkonen and Ikonen, 2000). Rab GTPases regulate multiple steps of vesicular transport, including tethering and docking, fusion, and motor protein recruitment (Pfeffer, 2001).

2.1.3.3 Endocytosis and phagocytosis

Endocytic machinery is responsible for the "inward" vesicle trafficking, following internalization of soluble materials, lipids, macromolecules and other particles (Huotari and Helenius, 2011). After cargo binding and/or selection, primary endocytic vesicles first deliver their cargo to early endosomes (EE) where molecules are primarily sorted. Proteins to be reutilized (receptors and SNARE proteins, for example) are recycled back to the PM via recycling endosomes or transported to trans-Golgi network (Bonifacino and Rojas, 2006).

Endosomes containing molecules targeted for degradation mature (or are delivered) to late endosomes (LE), multivesicular bodies (MVB), and lysosomes (LY). As the endosomes mature and move towards the cell center, their luminal pH

gradually decreases, internal membranes appear, and the membrane composition changes. While a high content of phosphatidylinositol 3-phosphate (PI3P) and Rab5 are markers for EEs, Rab7 and PI(3,5)P_2 in turn are enriched in LE membranes (Vicinanza et al., 2008). The increased LBPA (lysobisphosphatidic acid) content and decreased pH of LE has been suggested to contribute to the appearance of internal vesicles and membranes of LE (Matsuo et al., 2004). Finally, degradation of endocytosed material occurs at the low pH of lysosomes.

Phagocytosis is an endocytic process for internalization of larger particles, including pathogens, apoptotic cells or cell debris, and it is an important defense mechanism performed by specialized cell types, including monocytes, macrophages, and neutrophils (Conner and Schmid, 2003). Receptor binding at the cell surface triggers a signaling cascade that initiates actin-driven plasma membrane extension formation, followed by engulfment of the particle and formation of a phagosome. The phagosome fuses with endosomes and lysosomes in the cytosolic compartment, followed by enzymatic digestion of the engulfed material (Stuart and Ezekowitz, 2005). As phagocytosing cells are able to engulf relatively large particles compared to their cell size, it is evident that dynamic reshaping of the membrane is needed in the process. Although it is not clear where the extra material originates, ER has been suggested to provide extra membrane (Touret et al., 2005). Similar to endosomal maturation, the membrane lipid composition changes during phagosome maturation (Steinberg and Grinstein, 2008).

2.1.4 Cellular lipid storage

An excess amount of free cholesterol is toxic to cells. Cellular cholesterol levels are dependent on several processes, including the uptake of cholesterol from extracellular compartments, cholesterol biosynthesis, and efflux. These processes are tightly regulated by several feedback mechanisms.

Cells are able to synthesize cholesterol from the ubiquitous precursor, acetyl-coenzyme A (acetyl-CoA) by a series of 30 enzymatic reactions. The cholesterol biosynthetic pathway, described already in 1964 (Bloch, 1965), begins with the synthesis of 3-hydroxy-3-methylglutaryl (HMG-CoA), followed by its reduction by HMG-CoA reductase. This rate-limiting, early step in cholesterol biosynthesis is the target of statins, drugs commonly used in lowering blood cholesterol levels to prevent/treat cardiovascular diseases.

Another way to acquire cholesterol by cells is from extracellular sources, mainly in the form of low-density lipoprotein (LDL). LDL uptake is initiated by binding to LDL receptors at the cell surface, followed by its internalization in clathrin coated pits and formation of vesicles that are transported to endosomes and lysosomes

(Brown and Goldstein, 1986). The amount of LDL uptake depends on the abundance of cellular receptors, which is regulated by a feedback loop by sterol regulatory element binding protein (SREBP) transcription factors. The activity of the pathway is regulated by changes in cellular sterol levels. LDL receptor and other SREBP targets are upregulated when cellular cholesterol levels are low, allowing the cell to take up more cholesterol (Brown and Goldstein, 1997), which is another central aspect of statin function. Other important regulators of cellular cholesterol levels are liver X receptor (LXR) transcription factors. LXRs bind oxysterols and upregulate genes involved in reverse cholesterol transport, a process in which cholesterol is effluxed/taken up from peripheral cells and transported to the liver for excretion (Tontonoz and Mangelsdorf, 2003).

Macrophages are also able to take up LDL particles containing oxidized or enzymatically modified lipids by scavenger receptors. Excessive uptake together with inefficient clearance of modified LDL particles results in accumulation of lipids in macrophages and the formation of foam cells. The significance of this process is further described in section 2.2.2.

The cholesteryl esters (CEs), derived from the internalized LDL, are first hydrolyzed in the lysosomal compartments by lysosomal acid lipase. The free cholesterol then exits lysosomes in a process mediated by Niemann-Pick C proteins NPC1 and NPC2, and is transported to PM and/or ER. The oxysterol binding protein (OSBP) related protein 5 (ORP5) has been implicated in the transport of cholesterol from lysosomes to the ER (Du et al., 2011). In the ER, cholesterol is re-esterified by acyl-coenzyme A:cholesterol acyltransferase (ACAT) enzymes (Chang et al., 1995). The re-esterified cholesterol is then stored in lipid droplets (LD).

Lipid droplets are dynamic lipid and fatty acid storage organelles with a poorly understood function. They consist of a hydrophobic core of neutral lipids, including CE and triacylglycerols (TAG), surrounded by a monolayer of phospholipids. Several LD-associated proteins have been described. These include structural proteins (such as perilipins), proteins involved in lipid homeostasis (for example adipose triglyceride lipase ATGL), and proteins involved in membrane trafficking (including Rab proteins) (Guo et al., 2009). Interestingly, ORP2 has been shown to associate with LD in a sterol-dependent manner, and to regulate TAG and CE metabolism (Hynynen et al., 2009).

Upon demand, cholesterol and fatty acids can be mobilized from LD. Excessive lipid storage in LD plays a central role in the pathogenesis of several metabolic diseases, including obesity, diabetes and atherosclerosis. Reverse cholesterol transport is the only way for non-hepatic cells to get rid of cholesterol. This reverse cholesterol transport pathway is further discussed in section 2.2.1

2.1.5 Lipid signaling

It is becoming evident that, in addition to being cellular building blocks and energy reservoirs, lipids are also heavily involved in maintaining cellular homeostasis. Certain lipids can mediate or act as precursors for biological signaling. Due to the fact that lipids reside in membranes, lipid messengers are not stored but rather produced on demand at the site of intended action. Here, I will discuss the role of some lipids with signaling functions, which are in the scope of the research conducted in this thesis.

2.1.5.1 Phosphatidylinositol phosphates as lipid mediators

Phosphatidylinositol phosphates (PIPs) play central roles in signal transduction, membrane trafficking, cell migration and polarization, actin cytoskeleton remodeling, and sphingolipid metabolism (Saarikangas et al., 2010; Vicinanza et al., 2008). They are constantly phosphorylated and dephosphorylated by specific kinases and phosphatases resulting in seven distinct types of PIPs. These are enriched in specific organelle membranes (Table 1) and act as organizers controlling the recruitment/activation of target proteins (Vicinanza et al., 2008). Moreover, they can act as precursors for second messengers.

Table 1. Enrichment of differenct PIP species at cellular organelles of vesicular trafficking (Vicinanza et al., 2008)**. Abbreviations: LE, late endosome; MVB, multivesicular body; PIP, phosphatidyl inositol phosphate; PM, plasma membrane; TGN, trans-Golgi network.**

PIP species	***Organelle***
PI(3)P	Early endosomes, MVB internal vesicles, phagosomes
PI(4)P	Golgi complex (TGN)
PI(5)P	Cytoplasmic membranes
$PI(3,4)P_2$	PM
$PI(3,5)P_2$	LE (outer membrane)
$PI(4,5)P_2$	PM, clathrin coated pits, Golgi complex,
$PI(3,4,5)P_3$	PM (generated in response to extracellular stimuli)

In response to ligand (e.g. hormones or growth factors) binding, G protein-coupled receptors activate cellular phospholipase C (PLC) that cleave PIP_2 into inositoltrisphosphate (IP_3) and DAG, both of which act as second messengers (Smrcka et al., 2012). IP_3 binds to IP_3 receptors in the ER and triggers the release of Ca^{2+} to the cytoplasm. DAG, in turn, binds and activates protein kinase C (PKC) to control variety of cellular processes (Figure 4).

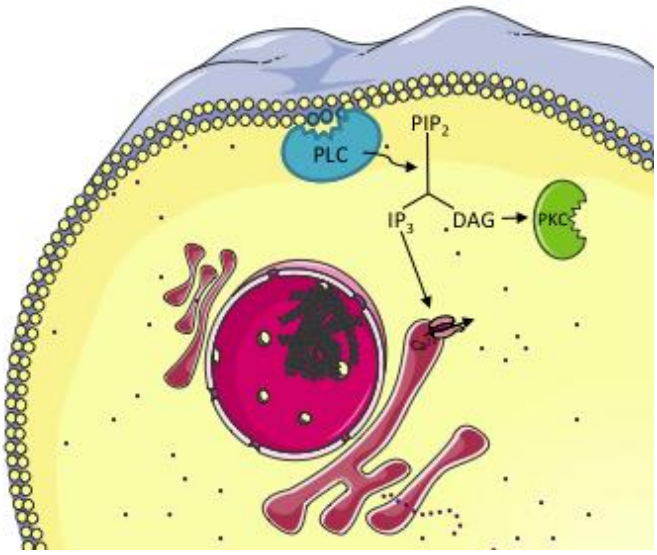

Figure 4. The cleavage of PIP_2 at the plasma membrane by PLC yields second messengers: IP_3 triggers release of Ca^{2+} from the ER, DAG in turn activates PKCs. Some figure components were provided by Servier Medical Art (www.servier.com).

2.1.5.2 Inflammatory lipid mediators

Eicosanoids are potent signaling molecules involved in the inflammatory response (Buczynski et al., 2007; Ma et al., 2012; Wymann and Schneiter, 2008). Polyunsaturated fatty acids, primarily arachidonic acid (AA; 20:4, n-6), eicosapentaenoic acid (EPA, 20:5 n-3), and docosahexaneoic acid (DHA, 22:6 n-3) are the precursors used in the synthesis of eicosanoids. Eicosanoid precursors are released from phospholipids or DAGs by PLA_2 (phospholipase A2) to produce leukotrienes, prostaglandins, thromboxanes, prostacyclins (collectively eicosanoids), and resolvins (non-classical eicosanoids) by the action of lipoxygenases (LOX), cycloxygenases (COX), and cytochrome P450 family members (Lawrence et al., 2002).

In response to cytokine stimulation, mechanical trauma, or Toll-like receptor (TLR) activation and subsequent rise in cellular Ca^{2+} (Buczynski et al., 2007), eicosanoid production and secretion is stimulated (Astudillo et al., 2012). Eicosanoids derived from n-6 fatty acids (e.g. AA) are proinflammatory by nature, whereas the eicosanoids produced from n-3 precursors (DHA, EPA) are counteracting (resolving) these effects (Lawrence et al., 2002; Lee, 2012). Proinflammatory actions of eicosanoids include fever generation, pain responses, leukocyte adhesion and activation, and chemotaxis (Astudillo et al., 2012). Resolvins, on the other hand, function to dampen these inflammatory responses, and by inhibiting leukocyte recruitment and cytokine release (Lee, 2012).

It has been shown that increased eicosanoid levels are associated with pathological disorders including metabolic syndrome and atherosclerosis (Horrillo et al., 2010; Sanchez et al., 2010). Additionally some sphingolipids, including ceramides and sphingosines, as well as lysophospholipids (lyso-PL) are generally regarded as proinflammatory. For example, higher levels of ceramides (Adams et al., 2004) and lyso-PL (Pietiläinen et al., 2007) have been associated with inflammatory conditions such as obesity. Ceramides and sphingosines have pro-apoptotic and anti proliferative effects (Taha et al., 2006). Lyso-PC (LPC) are activators of G-protein coupled receptors to regulate migration and calcium homeostasis (Meyer zu Heringdorf and Jakobs, 2007).

2.1.5.3 Oxysterols

Oxysterols are oxygenated derivatives of cholesterol that rise from both enzymatic and non-enzymatic reactions. The common oxidative modifications include hydroxyl, keto, epoxy, and carboxyl moieties that render the resulting molecule less hydrophobic than cholesterol and thus more easily transportable (Björkhem and Diczfalusy, 2002; Javitt, 2008; Schroepfer, 2000). In biological membranes, oxysterols may impact on membrane lipid packing and lipid draft assembly. Moreover, oxysterols have been reported to impair activity of membrane ion channels (Duran et al., 2010).

Increased oxysterol concentrations have cytotoxic properties (Lordan et al., 2007). However, in physiological concentrations these compounds are potent regulators of cellular cholesterol homeostasis (Gill et al., 2008), and are implicated at the crossroads of lipid metabolism and inflammation in macrophages (Shibata and Glass, 2009, 2010).

In normal conditions, oxysterol concentrations are minute compared to cholesterol, but are elevated in certain pathological situations, such as atherosclerosis, diabetes and obesity (Alkazemi et al., 2008; Murakami et al., 2001, Vaya et al., 2001). Several factors contribute to the cellular oxysterol content, including cholesterol biosynthesis and uptake of oxidized lipoproteins. Oxysterols arise from enzymatic reactions by mitochondrial or ER cholesterol hydroxylases (Russell, 2000). One of the abundant oxysterols, 25-hydroxycholesterol (25-OHC), is secreted by macrophages in response to TLR4 activation (Bauman et al., 2009). Moreover, cholesterol can be autooxidized, for example by action of reactive oxygen species (ROS) or lipid peroxides (Brown and Jessup, 1999). The most important oxysterols arising from cholesterol autoxidation are the 7β-hydroxycholesterol and 7-ketocholesterol (7-KC) (Lordan et al., 2007).

Oxysterol signals are mediated by several cellular effectors with variable downstream outcomes. Nuclear LXR transcription factors, activated by oxysterols

(Janowski et al., 1996), upregulate genes involved in the cholesterol efflux, transport, and excretion (Tontonoz and Mangelsdorf, 2003). Moreover, LXRs negatively regulate macrophage inflammatory gene responses by inhibiting expression of nuclear factor kappa B (NF-κB) activated genes, including interleukin-1β (IL-1β), incucible nitric oxide synthase (iNOS), and monocyte chemoattractant protein-1 (MCP-1) (Joseph et al., 2003). Oxysterols regulate yet another transcriptional machinery involved in lipid homeostasis, namely SREBPs. The insulin induced gene (Insig) proteins were found to bind 25-OHC to regulate SREBP processing, thereby blocking SREBP transport and inhibiting cholesterol biosynthesis (Radhakrishnan et al., 2007; Sun et al., 2007b). Other proteins with reported oxysterol affinity include the late endosomal/lysosomal cholesterol transfer protein NPC1 (Infante et al., 2008), estrogen receptors (Umetani et al., 2007), a putative cholesterol transfer protein StarD5 (Soccio et al., 2005), The retinoic acid receptor-related orphan receptors (RORα/γ) (Wang et al., 2010), Epstein-Barr virus-induced gene 2 EBI2 (Hannedouche et al., 2011; Liu et al., 2011), and oxysterol binding protein family (ORPs), which are discussed in detail in section 2.3.

2.2 Macrophages

Macrophages are phagocytic cells that function both in the innate and adaptive immunity. They derive from blood monocytes, and circulate in the blood until recruited to sites of inflammation, followed by differentiation into macrophages or dendritic cells according to the environmental signals (Gordon and Taylor, 2005). Macrophages contribute to the early host defense mechanism by phagocytosing harmful or foreign material, followed by antigen presentation at their cell surface that can be recognized by other inflammatory cells. Moreover, activated macrophages recruit other cells of the inflammatory system by release of inflammatory mediators.

Macrophages are abundantly present at sites of inflammation, in the liver (so called Kupffer cells), in the white adipose tissue, the intestine, the lung, and the spleen. They are also present already from the early stages of atherosclerotic lesion development, having central role in the lesion development.

Macrophages are able to internalize massive amounts of lipids. They have an important role in clearing accumulated or damaged lipids from tissues, including the subendothelial space of blood vessels (see section 2.2.2). Macrophages are active in reverse cholesterol transport, a process where accumulated lipid from peripheral tissues is delivered to high-density lipoprotein (HDL) particles, to be excreted from the body by the liver (Rader et al., 2008).

The initial step in the reverse cholesterol transport is the mobilization of CEs from LDs, either by neutral hydrolases or autophagosomal degradation of lysosomal compartments (Ouimet and Marcel, 2012). Cholesterol is then transported to extracellular cholesterol acceptors by the action of ATP-binding cassette (ABC) transporters, to lipid-poor apolipoprotein-AI (apo-AI) particles by ATP-binding cassette transporter A1 (ABCA1) or to spherical HDL_2 particles by ATP-binding cassette transporter G1 (ABCG1) (Rader et al., 2008). The resulting HDL particles circulate to the liver, and the cholesterol they contain is excreted as cholesterol or as converted to bile acids.

Macrophages are characterized by active and fast changes in their morphology. The activation of macrophages changes the round morphology of monocytes into amoeboid phenotype (Porcheray et al., 2005), a process that requires active reshaping of cellular cytoskeleton and lipid membranes. Moreover, cell migration, phagocytosis, and infiltration through endothelium are accompanied by dynamic changes in membrane organization and cell shape.

2.2.1 Inflammatory actions of macrophages

Macrophages express several pattern recognition receptors in their cell surface, including TLR and scavenger receptors (SR) (Binder et al., 2002). The ligand binding can result in macrophage activation or internalization of the ligand. SRs, including CD36, SR-B1 and SR-A, recognize modified LDL, including oxidized LDL (oxLDL), and are involved in the internalization of these particles (Sun et al., 2007a).

There are at least ten TLRs in mammalian organisms, with diverse specificity for endogenous and exogenous ligands (Medzhitov, 2001). For example, TLR2 is activated by components of Gram-positive bacteria (Yang et al., 1998), whereas Gram-negative components are recognized by TLR4 (Chow et al., 1999). Moreover, TLR activation can be triggered by free fatty acids (Shi et al., 2006) and necrotic cells (Li et al., 2001). Proinflammatory TLR activation at the cell surface regulates transcription of cytokines, chemokines, iNOS and ROS via activation of NF-κB or interferon regulatory factor 3 (IRF3) pathways (Castrillo et al., 2003; Li et al., 2001; Thoma-Uszynski et al., 2001).

Depending on the activating signals, monocyte-macrophages are polarized into M1 or M2 type of macrophages with different functional properties. Classically activated M1 macrophages are characterized by increased microbicidal capacity, together with increased secretion of proinflammatory cytokines, which further increases the local immune response (Gordon and Taylor, 2005, Gordon, 2007). Classical activation is triggered by pro-inflammatory cytokines, such as interferon-gamma (IFNγ) or tumor necrosis factor-alpha (TNFα), or by recognition of foreign

material, such as bacterial lipopolysaccharide (LPS). On the other hand, anti-inflammatory cytokines, including transforming growth factor beta (TGFβ), IL4, IL10 and IL13, may trigger alternative macrophage activation. These M2-polarized macrophages are involved in the resolution of inflammation and tissue repair (Gordon and Taylor, 2005). Interestingly, saturated fatty acids are reported to promote M1 response (Holland et al., 2011; Nguyen et al., 2007; Shi et al., 2006), while unsaturated fatty acids promote M2 macrophage response (Serhan, 2009).

Another key inflammatory function of macrophages is phagocytosis and killing of pathogens (see section 2.1.3.3). Phagocytic receptors, including TLR and scavenger receptors, recognize foreign particles, which leads to their engulfment and subsequent lysosomal degradation of the particles (Stuart and Ezekowitz, 2005).

2.2.2 Role of macrophages in the development of atherosclerosis

As discussed in the preceding sections, macrophages function at the crossroads of lipid metabolism and inflammation. In several lipid metabolism disorders, including metabolic syndrome, obesity and atherosclerosis, the presence of chronic inflammation is recognized as a key component (Libby, 2002; Ma et al., 2012).

Macrophages play key roles in the initiation and progression of atherosclerotic lesions; they are present already in fatty streaks, the early lesion manifestation. Inflammation (MCP-1) and cholesterol (oxLDL) accumulation into vessel walls promote recruitment and infiltration of macrophages in the subendothelial space. Native and modified (oxidized) LDL particles are taken up by macrophage SRs in excessive amounts, and stored in LDs as CE. As a response, LXR mediated activation of cholesterol efflux attempts to compensate for the lipid overload. Additionally, macrophages recruit more inflammatory cells to the site by secreting inflammatory mediators (Joseph et al., 2003). The overload of the macrophage cholesterol removal capacity results in transformation of macrophages to foam cells, where the cell is characteristically full of LDs. As a result of excessive lipid uptake, foam cells may undergo apoptosis, resulting in a necrotic core and more advanced lesions (Glass and Witztum, 2001).

Interestingly, some reports suggest that macrophages are also able to migrate out of the lesion, which results in lesion regression (Daoud et al., 1981; Llodra et al., 2004). Binding of oxLDL to CD36 scavenger receptors was recently suggested to mediate this emigration event (Park et al., 2009). In that study, CD36 was shown to modulate cellular cytoskeletal dynamics upon oxLDL binding, thereby negatively regulating emigration. OxLDL binding activated a signaling cascade involving activation of Src kinases and FAK, promoting cell spreading and macrophage entrapment. Moreover, LXR mediated upregulation of the chemokine receptor CCR7

was shown to increase emigration of macrophages from atherosclerotic plaques (Feig et al., 2010).

Oxysterols, important regulators of macrophage homeostasis, may have key roles in the lesion development. The uptake of modified lipoproteins by macrophages is a major cellular source of oxysterols, and their significant enrichment has been shown to exist in atherosclerotic plaques (Garcia-Cruset et al., 2001). Moreover, macrophage activation by TLR signaling has been shown to increase 25-OHC production (Bauman et al., 2009). Oxysterols are potent regulators of lipid homeostasis and inflammatory processes (above and chapter 2.1.5.3). Oxysterol effects are partly mediated by the cytosolic oxysterol binding protein family, the topic of this thesis. The protein family is described in detail in the following sections.

2.3 Oxysterol binding proteins

The oxysterol binding protein (OSBP) family is conserved in the eukaryotic kingdom. In addition to mammalian organisms, homologues of OSBP-related proteins (ORP) have been identified in yeast, insects, plants, and worms (Alphey et al., 1998; Avrova et al., 2004; Beh and Rine, 2004; Kobuna et al., 2010; Skirpan et al., 2006; Sugawara et al., 2001), practically in all organisms for which sequence information is available. The studies on yeast ORP homologues (called Osh1-7) have played a pioneering role in understanding the ORP structure and function (Beh and Rine, 2004; Raychaudhuri and Prinz, 2010).

In mammals, including humans, 12 *OSBPL* genes encode for ORP proteins (Figure 5). The number of distinct ORP proteins is, however, much higher than this. Short ("S") and long ("L") variants of some gene products (ORP1S and ORP1L, for example) arise from different promoters (Jaworski et al., 2001; Wang et al., 2002), which differ in expression pattern, subcellular localization and function (Johansson et al., 2003). Moreover, at least eight splice variants of ORP3 have been identified (Collier et al., 2003), highlighting the enormous variability within the protein family.

In general, ORPs differ from one another in their expression pattern, subcellular localization and substrate specificity. Each tissue and cell type expresses a characteristic pattern of ORPs at different quantities (Lehto et al., 2001). The magnitude of ORP homologues in different organisms together with their ubiquitous expression suggests a fundamental role of these proteins in the cellular physiology.

2.3.1 ORP structure

Several characteristic domains are present in a majority of ORPs (Figure 5). A common feature present in all members is an OSBP related domain (ORD), which contains an OSBP fingerprint motif EQVSHHPP. The ORD structure has been determined in complex with several sterols (Im et al., 2005). The structure consists of anti-parallel β-sheets that fold into a barrel-like structure with a hydrophobic core capable of accommodating one lipid molecule at a time, accompanied by a flexible lid which protects the bound lipid molecule from the aqueous environment (Figure 6).

The long ORP variants contain an N-terminal extension with multiple functional domains, including pleckstrin homology domain (PHD), ankyrin repeats ANK, and two phenylalanines in an acidic tract (FFAT) motif (Figure 5). The PHDs bind to PIPs on cellular membranes, and often mediate the subcellular targeting of the proteins (Levine and Munro, 1998; Ngo and Ridgway, 2009). The FFAT motif is a short sequence that binds to integral ER vesicle associated membrane protein-associated proteins (VAP). Interestingly, this motif is also present in other proteins involved in lipid metabolism, including lipid and PI transfer proteins (Loewen et al., 2003), most likely due to the central role of ER in lipid homeostasis. Some ORPs lacking the FFAT motif contain a C-terminal ER-anchoring transmembrane segment (Du et al., 2011; Yan et al., 2008). The ankyrin repeats, typically mediating protein-protein interactions (Li et al., 2006), have been shown to interact with active form of Rab7 in the case of ORP1L (Johansson et al., 2005), or to target the protein to nucleus-vacuole junction (NVJ) in the case of Osh1p (Kvam and Goldfarb, 2004). The significance of multiple domains involved in protein targeting present in ORPs is further discussed in section 2.3.6.

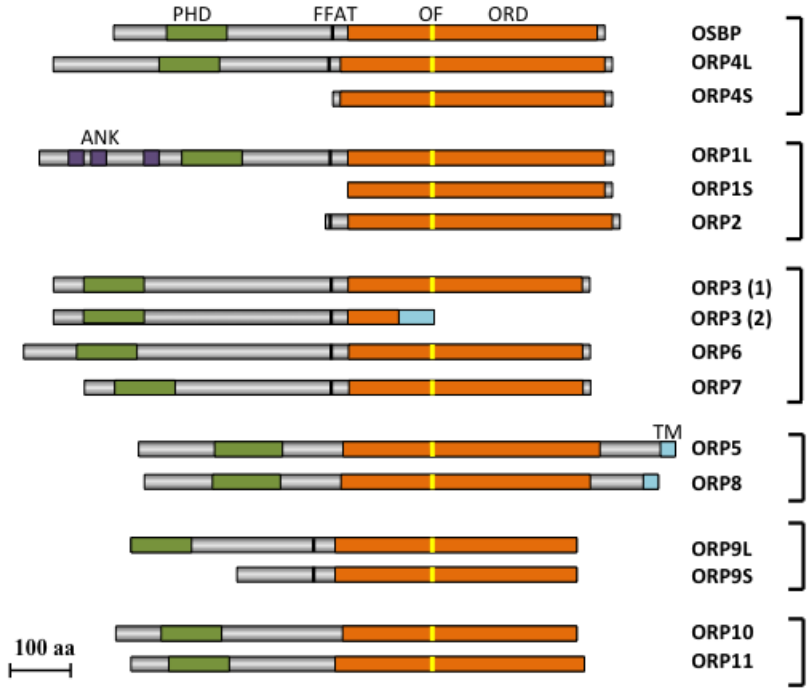

Figure 5. The mammalian ORP protein family with the major structural elements identified. Based on their amino acid secuences, the proteins are divided into 6 subfamilies, indicated on the right. The domains and motifs are color-coded: PHD in green; FFAT motif in black; OF (oxysterol binding protein "fingerprint" sequence) in yellow; ORD in orange; ankyrin repeats (ANK) in purple; trans-membrane segment (TM) in turquoise. See text for more detailed description of the motifs.

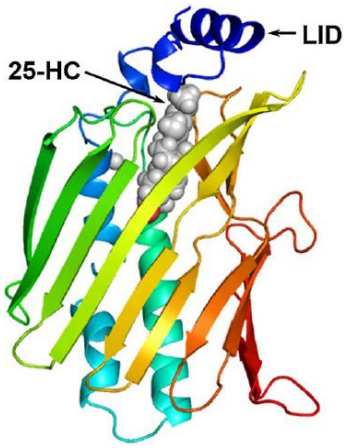

Figure 6. ORP1L ORD as a representative example of the sterol binding fold. The structure is modeled in complex with 25-OHC using Osh4p as template (Im et al., 2005)**. Oxysterol molecule (in grey) is bound in the barrel-like pocket formed by beta strands, and the alpha-helical lid of the pocket is shown in dark blue. The color spectrum of the protein indicates the amino acid positioning; ranging from blue (N-terminal) to red (C-terminal).**

2.3.2 ORP ligands

Osh4p was demonstrated by X-ray crystallographic analysis to bind cholesterol, ergosterol (main sterol in yeast), and several oxysterols (Im et al., 2005). Apart from the 3β-hydroxyl group (facing towards the pocket bottom of the binding pocket), no interaction with the sterol hydroxyl groups and the protein were found. The pocket is thus able to accommodate a variety of sterols, showing a relatively low degree of ligand specificity.

Several mammalian ORPs have been shown to bind oxysterols and cholesterol *in vitro*. Live cell photo-cross-linking with [^{3}H]photo-25-OHC and [^{3}H]photo-cholesterol suggested that all ORPs are able to bind sterols in vitro (Suchanek et al., 2007). This finding has been confirmed for several ORPs (OSBP, ORP2, ORP4, ORP8, ORP9, ORP10) (Hynynen et al., 2009; Ngo and Ridgway, 2009; Suchanek et al., 2007, Wang et al., 2002; Wang et al., 2008; Yan et al., 2007a, 2008; E.Nissilä and V.Olkkonen, unpublished) in sterol binding assays employing purified proteins. These studies have revealed differences in the binding affinities for oxysterols between ORPs. Moreover, a higher affinity for oxysterols than cholesterol was demonstrated for OSBP and ORP4 (Wang et al., 2008; Wyles et al., 2007). Cholesterol binding has been verified for all ORPs tested so far, whereas no oxysterol binding has been observed for certain ORPs (Ngo and Ridgway, 2009; E. Nissilä and V. Olkkonen, unpublished). The ORD is also able to accommodate other ligands besides sterols. A recent study (de Saint-Jean et al., 2011) showed that, PI4P could substitute the sterol bound in the Osh4p pocket. In addition, sterol-resembling small molecular inhibitors of ORPs (ORPhilins, (Burgett et al., 2011)) were shown to compete for 25-OHC binding, suggesting their capacity to bind to the ligand binding pocket.

All ORPs are likely capable of binding several types of ligands. Depending on the ligand bound, the functional consequences could be different. For example, while binding of cholesterol to OSBP induces assembly of a protein phosphatase complex active on the extracellular signal regulated kinases (ERK), the complex is disassembled when 25-OHC binds to OSBP (Wang et al., 2005; Wang et al., 2008). Moreover, the localization of ORP2 in lipid droplets is inhibited by its oxysterol ligand, 22(R)-OHC (Hynynen et al., 2009). These findings suggest that cholesterol is a physiological ligand for all ORPs, and the more abundant availability of cholesterol could compensate for its weaker affinity for ORPs.

2.3.3 The role of ORPs as modulators of lipid homeostasis

Beh et al. showed in 2001 that disruption of all 7 yeast *OSH* genes was lethal, suggesting a common essential function for these proteins (Beh et al., 2001). They

later showed that disruption of *OSH* function altered intracellular sterol-lipid distribution, caused vacuolar fragmentation and accumulation of lipid droplets in the cytoplasm (Beh and Rine, 2004). These studies suggested that *OSH* genes affected intracellular sterol distribution, linking their depletion to multiple cell functions.

In addition, several impacts in lipid homeostasis by mammalian ORPs (OSBP, ORP1L, ORP2, ORP5, ORP8, and ORP10) have been demonstrated. Upon 25-OHC stimulation, OSBP recruits ceramide transfer protein (CERT) to Golgi and thereby controls the transfer of ceramides from ER to Golgi for SM synthesis (Banerji et al., 2010, Perry and Ridgway, 2006), a process regulated by protein kinase D mediated phosphorylation of OSBP (Nhek et al., 2010). Moreover, OSBP has been shown to modify ABCA1 stability (Bowden and Ridgway, 2008), and OSBP overexpression was shown to enhance lipogenesis and VLDL secretion in mice (Yan et al., 2007b).

ORP1L overexpression in macrophages reduced cholesterol efflux to HDL and increased the size of atherosclerotic lesions in a mouse model (Yan et al., 2007a). Silencing of ORP2, which endogenously localizes to LD in an oxysterol-dependent manner, stabilizes cellular triglycerides (Hynynen et al., 2009). Cholesterol accumulation in late endosomal compartments appears in cells silenced for ORP5 (Du et al., 2011). Furthermore, ORP8 overexpression in the mouse liver reduces cholesterol, triglyceride, and phospholipid levels in plasma and the liver tissue by downregulating the activity of SREBP (Zhou et al., 2011).

These examples suggest pleiotropic effects for ORPs in the control of lipid homeostasis. The mechanisms, however, are poorly understood for other members than OSBP.

2.3.4 Are ORPs lipid carriers or sensors?

The structure of the ligand binding pocket, very likely present on all ORPs, is characteristic for all lipid transfer proteins. The structure allows the accommodation of one lipid molecule at a time in a pocket closed by a lid, and transport of this hydrophobic molecule through aqueous environment (D'Angelo et al., 2008). Such a lipid carrier function has been suggested as the common property of ORPs. Indeed, several Osh and ORP proteins have been shown to transfer lipids *in vitro* (Du et al., 2011; Ngo and Ridgway, 2009; Raychaudhuri et al., 2006).

The initial evidence for ORP sterol binding was a study demonstrating the capacity of Osh4p to facilitate the nonvesicular transfer of cholesterol and ergosterol from PM to ER (Raychaudhuri et al., 2006). The investigators also observed a drastic reduction in PM to ER sterol transfer in cells deficient of all Osh proteins. Moreover, involvement of ORP proteins in transfer of newly synthesized cholesterol from ER to PM (Sullivan et al., 2006) has been suggested. These studies, however, provide no

solid evidence for a sterol carrier function. A recent report (Georgiev et al., 2011) suggests that the ORP effect in sterol transfer is indirect, and possibly results from altered membrane sterol organization which, in turn, is mediated by ORPs. Interestingly, Osh4p was recently shown to exchange bound sterol (dehydroergosterol) for PI4P and transport the two lipids between membranes along opposite routes (de Saint-Jean et al., 2011).

Jansen et al. (Jansen et al., 2010) were the first to show an effect for mammalian ORPs in the intracellular cholesterol transfer. Using an assay to monitor the transport of Bodipy-labeled cholesterol from PM to LD, they showed that out of all human ORPs, overexpression of ORP1S or ORP2 stimulated cholesterol transfer. For ORP2 functional sterol binding was shown to be necessary for the observed effect. The study supports the role of ORPs in sterol transfer but does not provide conclusive evidence for a sterol carrier function, similar to Osh proteins. Other mechanisms might be their involvement in MCS (see section 2.3.6), or modulation of membrane lipid composition to enhance sterol transport by ORPs, as earlier suggested (Georgiev et al., 2011; Sullivan et al., 2006).

On the other hand, instead of lipid transfer, ORP sterol binding could execute regulatory functions. Several observations support the role of ORPs as sterol sensors. First, changes in subcellular localization in response to ligand binding have been demonstrated for OSBP (Ridgway et al., 1992) and ORP2 (Hynynen et al., 2009). Second, their mode of action is dependent on the ligand bound, at least in the case of OSBP. While cholesterol-bound OSBP regulated scaffolding of a protein phosphatase complex regulating ERK signaling, upon 25-OHC binding the complex was dissociated, accompanied with ERK activation (Wang et al., 2005). 25-OHC binding by OSBP also mediates activation of cellular sphingomyelin synthesis by recruitment of CERT to Golgi (Perry and Ridgway, 2006). Moreover, manipulation of cellular sterols changed the positioning of LE, an effect regulated by ORP1L on the surface of LE (Rocha et al., 2009). It is likely that similar sterol sensor functions will be revealed for other ORP family members.

2.3.5 The impacts of ORPs on cell signaling

In addition to regulation of ERK signaling (discussed in the previous section), a second signaling function has been described for OSBP. Profilin-1, a protein regulating cytoskeletal architecture is upregulated upon stimulation with 7-KC. The aortic endothelium of diabetic humans expresses increased levels of profilin-1, which contributes to endothelial dysfunction. Furthermore, elevated profiling-1 levels were detected in atherosclerotic plaques compared to adjacent tissue (Romeo et al., 2004). OSBP was shown to mediate the 7-KC induced upregulation of profilin-1 in a process where OSBP is phosphorylated by Janus tyrosine kinase-2 (JAK-2), followed by activation of Signal Transducer and Activator of Transcription-3 (STAT3) (Romeo

and Kazlauskas, 2008). Analogous lipid-specific cell signaling functions probably exist for other ORP family members as well.

PIPs are key components in the communication of cellular compartments and play central roles in a multitude of cellular processes (see section 2.1.5.1). Capability to interact with PIPs, via PHD, ORD, or both, seems to be a common property of the ORPs. Different PIP species are enriched in specific subcellular locations, acting as landmarks and functional regulators for ORPs and other cytosolic proteins that are recruited to membranes (Downes et al., 2005), (Figure 3). Of note, Osh-mediated *in vitro* sterol transfer is dependent on the PIP composition of the lipid vesicles used (Schulz et al., 2009).

On the other hand, ORPs can also modify the cellular levels PIPs. In addition to the suggested function in sterol/PI4P exchange between membranes (de Saint-Jean et al., 2011), Osh4p has been shown to affect the levels and availability of PI4P in the Golgi by inhibiting phosphatidylinositol 4-kinase Pik1p (Fairn et al., 2007). Osh3p in turn was shown to reduce the PM PI4P content, by facilitating the formation of MCS and activation of the ER PIP phosphatase Sac1 (Stefan et al., 2011).

These examples imply that, by modulating the PIP metabolism, the ORPs could impact a variety of signaling processes. Furthermore, lipid rafts (section 2.1.2) have an established role in signal transduction processes (Lingwood and Simons, 2010). Cholesterol is an essential constituent of the lipid rafts, and the altered distribution or levels of cholesterol modify raft formation. Reports showing altered sterol distribution (Beh and Rine, 2004) or intracellular sterol transport (Hynynen et al., 2005; Jansen et al., 2010) by ORP manipulation point out the possibility that ORPs could modify signaling by modification of lipid drafts. Interestingly, ORPs have been suggested to affect the ability of membranes to sequester sterols (Georgiev et al., 2011; Sullivan et al., 2006).

2.3.6 ORP proteins as modulators of membrane contact sites

A key feature in the structure of many ORPs is the presence of several domains/motifs to regulate protein localization and protein-protein/protein-lipid interactions (section 2.3 and Figure 4). Furthermore, most ORPs contain either a FFAT motif to mediate interaction with ER VAP proteins (Loewen et al., 2003), or an ER-anchor (transmembrane) domain (Du et al., 2011; Yan et al., 2008), suggesting that interaction with the ER is a unifying feature of the ORPs and important for their function. Altered protein localization and/or function has been described for ORPs with the FFAT motif inactivated (Hynynen et al., 2009; Loewen et al., 2003), demonstrating the functional importance of the domain.

These features enable ORPs to bind two membranes simultaneously, which has prompted a speculation whether ORPs could be enriched at junctions where ER membranes are at close apposition of other cellular membranes, at so-called MCSs (Levine and Loewen, 2006). At these junctions, signals and small molecules, including lipids and calcium, are transferred between compartments (Toulmay and Prinz, 2011). Due to shorter distance between the organelles (10-30 nm), lipid transfer could be faster and more efficient at MCS. Interestingly, 4 out of 7 Osh proteins were shown to be enriched at sites where two organelles are closely apposed (Schulz et al., 2009). Osh1p is localized to NVJ, but is not necessary for the NVJ formation (Kvam and Goldfarb, 2004). Moreover, Osh3p activates Sac1 at ER-PM junctions to control PM PI4P levels (Stefan et al., 2011).

Several studies support also the MCS localization and function of mammalian ORPs. OSBP localizes at ER-Golgi junctions to control the levels of PI4P, DAG, and SM at the Golgi membranes, essential for Golgi structure and function (Peretti et al., 2008). ORP1L was described to control the motor recruitment at the ER-LE interphase in a cholesterol-dependent manner (Rocha et al., 2009). Knockdown of ORP5, an ER-anchored protein, resulted in cholesterol accumulation in LE/LYs (Du et al., 2011). Moreover, ORP5 was shown to co-immunoprecipitate with NPC1, a protein involved in cholesterol removal from the lysosomes. Accordingly, ORP5 was proposed to act at transient contact sites, where cholesterol is transported from lysosomes to the ER.

These findings highlight the potential role of ORPs at MCSs. It is however unclear, whether the proteins mediate the MCS formation, function as transporters, or execute regulatory functions at these sites.

2.3.7 ORP members enriched in macrophages

Although ubiquitously expressed, ORP isoforms show a high degree of tissue and cell type specificity in their expression patterns (Lehto et al., 2001). In macrophages, in culture, in human coronary lesions, or both, three ORPs (ORP1L, ORP3, and ORP8) are expressed at high levels. The *OSBPL1* gene encodes for two proteins, ORP1L and ORP1S, of which ORP1L is highly expressed in macrophages, brain, and lung (Johansson et al., 2003). The expression of ORP1L during monocyte to macrophage differentiation is heavily (100-160-fold) upregulated, suggesting a macrophage-specific function (Johansson et al., 2003).

ORP3 shows highest expression in the kidney, lymph nodes and thymus (Lehto et al., 2004), and high ORP3 levels in blood leukocytes, including macrophages, T cells, and B cells, have been observed, together with a moderate upregulation upon macrophage differentiation (Johansson et al., 2003). Moreover, ORP3 is highly expressed in certain forms of cancer.

ORP8 is most abundantly expressed in the liver, spleen, kidney, adipose tissue, and macrophages (Yan et al., 2008). Moreover, ORP8 mRNA levels in macrophages from advanced atherosclerotic lesions were approximately 3-fold higher as compared to a healthy arterial wall.

2.3.7.1 ORP1L

ORP1L localizes to the surface of LEs. Overexpression of ORP1L results in altered MVB/LE morphology: LEs were enlarged, abnormally full of internal membranes, and tended to cluster in the perinuclear area (Johansson et al., 2003). The ORP1L LE localization is determined by three N-terminal ankyrin repeats, but a protein containing both the ankyrin repeats and the PHD displays an enhanced LE localization and clustering phenotype is enhanced. In LE, ORP1L is part of a tripartite complex together with Rab7 and Rab7-interacting lysosomal protein (RILP) that acts to recruit a dynein/dynactin motor protein assembly to the surface of LE, followed by the transport of LE along cellular microtubule tracks in the minus-end direction, towards the cell center (Johansson et al., 2005, 2007). The minus-end directed transport of LE results from a cascade, a part of which is an interaction of dynein and betaIII spectrin, apparently facilitated by ORP1L (Johansson et al., 2007).

To characterize cellular ORP1L ligands photo-crosslinking and *in vitro* pull-down assays employing purified protein have been used. In a liposome pull-down assay, the PHD binds several PIPs with a low affinity and specificity (Johansson et al., 2005). Photo-crosslinking experiments suggested the ORP1L ORD is capable of binding both cholesterol and 25-OHC (Suchanek et al., 2007). Affinities for 22(R)-OHC and 25-OHC have also been confirmed with in vitro pull-down assays (Yan et al., 2007a).

Interestingly, macrophages overexpressing human ORP1L displayed reduced ABCG1 mediated cholesterol efflux to HDL_2 acceptors, and caused an increased susceptibility to atherosclerotic lesion development after bone marrow transplantation into LDL receptor knockout ($LDLr^{-/-}$) mice (Yan et al., 2007a)., an established mouse model for atherosclerosis Furthermore, IL-1β and phospholipid transfer protein (PLTP) mRNA levels, and PLTP activity were increased. The authors provided two possible explanations for the increased atherosclerosis susceptibility. First, altered expression of LXR target genes could result from increased ORP1L oxysterol binding. In addition to lipid metabolism related genes, LXRs reciprocally regulate inflammatory gene responses (Joseph et al., 2003; Shibata and Glass, 2009; Zelcer and Tontonoz, 2006). The observed decrease in ABCG1 mRNA and subsequent inhibition of cholesterol efflux, together with the increased pro-inflammatory IL-1β expression, are in line with altered LXR target expression, as suggested. On the other hand, the inhibition of cholesterol efflux could result from a disturbance of endocytic cholesterol transport caused by ORP1L.

2.3.7.2 ORP3

Cellular localization of ORP3 results from a complex interplay between the PHD, ORD and FFAT domain. Our group has shown earlier that the main targeting determinants in the ORP3 structure are the FFAT motif, responsible for ER targeting, and the PHD, that targets the protein to PM (Lehto et al., 2005). In addition to the FFAT motif, a second ER targeting segment (encoded by exons 10 and 11), was also characterized. The role of ORD was suggested to be negative regulation of PM targeting. Moreover, ORP3 localization responded to the cellular lipid status: after 3 days lipid starvation ORP3 localization to ER structures close to the PM was enhanced.

Using purified protein to pull-down PIP containing liposomes, the ORP3 PHD was shown to bind PI(3,4,5)P_3 and PI(3,4)P_2 with highest affinity, and towards other PIP_2 species with moderate affinity (Lehto et al., 2005). For ORP3 ORD, for which binding of cholesterol and 25-OHC have been suggested by photo-crosslinking experiments (Suchanek et al., 2007), no other ligands have been identified. Moreover, 25-OHC treatment failed to induce ORP3 translocation, while a clear shift to the PM vicinity was observed for ORP3:OSBP chimeric protein, where ORP3 ORD was replaced with that of OSBP, capable of responding to stimulus by this oxysterol (Lehto et al., 2005).

Consistent with the role of some ORPs in cell signaling, ORP3 was originally identified as a candidate interaction partner for the small GTPase R-Ras (Goldfinger et al., 2007). Moreover, the role of ORP3 in R-Ras regulated events (Kinbara et al., 2003), including cell adhesion and spreading, organization of the actin cytoskeleton, β1-integrin activity and macrophage phagocytic function was demonstrated (Lehto et al., 2008). In HEK293 cells silencing of ORP3 resulted in impaired cell adhesion, enhanced spreading, and increased β1 integrin activity, while overexpression increased formation of cellular protrusions, at where ORP3 colocalized with R-Ras at the ends. Interestingly, R-Ras has also been implicated to promote neurite outgrowth (Oinuma et al., 2007). ORP3 was further shown to be phosphorylated upon loss of cell adhesion and by stimulation of PKC activators phorbol-12-myristate-13-acetate (PMA) and all-trans retinoic acid (ATRA). Interestingly, induction of phosphorylation enhanced the protrusion formation, suggesting ORP3 localizing at the protrusion tips is the phosphorylated form of ORP3. Conclusions on the effect of ORP3 phosphorylation to R-Ras interaction could not be drawn based on this study.

2.3.7.3 ORP8

A transmembrane span at the ORP8 C-terminus anchors the protein to the ER and nuclear membrane (Yan et al., 2008). Similar to ORP1 and ORP3, ORP8 ORD was by photo-crosslinking suggested to bind both cholesterol and 25-OHC (Suchanek et al., 2007). *In vitro* experiments confirmed cholesterol binding and a weak affinity towards 25-OHC (Yan et al., 2008; Zhou et al., 2011). Other lipid ligands have not been confirmed and the PIP binding capacity/specificity of ORP8 PHD is unknown.

Recently, ORP8 was shown to interact with Nucleoporin p62 (NUP62) at the nuclear membrane (Zhou et al., 2011). NUP62 is part of the nuclear pore complex, which dynamically regulates nucleo-cytoplasmic transport (Tran and Wente, 2006). Adenoviral overexpression of ORP8 in mouse liver markedly decreased cholesterol, TAG and PL in plasma and liver tissue (Zhou et al., 2011). A reduction in nuclear SREBPs and their target gene expression was concomitantly observed. Moreover, the effect of ORP8 on nuclear SREBPs and their target genes was inhibited by NUP62 knockdown, suggesting that ORP8 may together with NUP62 control the nuclear transport of SREBPs.

Earlier studies have shown that ORP8 silencing in the human THP-1 macrophages enhances the expression of ABCA1 and subsequent cholesterol efflux to apo-AI, whereas no effect in ABCG1 expression or cholesterol efflux to HDL_2 was observed (Yan et al., 2008). These effects were suggested to be mediated, in part, by LXR together with E-box regulatory elements present in ABCA1 promoter.

Aims

The aim of this thesis project was to study the role of three oxysterol binding protein (OSBP) related proteins (ORP1L, ORP3, and ORP8) in macrophages and to further characterize these proteins.

The specific aims were the following:

1. To find novel ligands and interaction partners for the ORPs
2. To study the subcellular targeting of these proteins.
3. To clarify the cellular function of these proteins.
4. To study the impacts of ORP silencing on macrophage functions and their lipidome.

Materials and methods

Listed below are described the methods used in the studies that were personally performed by me. The Roman numeral refers to the original publication.

Cell culture and transfection (I, II, III)

Human embryonic kidney HEK293, Huh7 hepatoma cells, and HeLa cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. For mouse macrophage RAW264.7 cell line culture medium contained additionally 10 mM Hepes. For some experiments, RAW264.7 cells were maintained in macrophage serum-free medium (M-SFM, Gibco) supplemented with 10 ng/ml macrophage colony-stimulating factor (M-CSF, Invitrogen).

Transfection of cells with cDNA was performed using Lipofectamine2000 (Invitrogen), FugeneHD (Roche) reagents, or Amaxa nucleofection system (Lonza) according to manufacturer's instructions. HiPerFect transfection reagent (Qiagen) was used to transfect short interfering RNAs (siRNA).

Lentiviral transduction and generation of stably silenced cell pools (I, II, III)

Lentiviruses encoding for short-hairpin RNA (shRNA) against mouse ORP1L, ORP3, and ORP8 were from Sigma MISSION® TRC-Mm 1.0 (Mouse) shRNA library. Stably silenced cell pools were generated by transducing lentiviruses into RAW264.7 cells with multiplicity of infection (MOI) 4 in the presence of 8 µg/ml hexadimethrine bromide. Selection of transduced cells was carried out in the presence of 4 µg/ml puromycin. Out of five shRNA constructs for each gene, two with the best silencing efficiency compared to Non-Targeting shRNA (shNT) control were used in further experiments. The silencing efficiency, determined by quantitative real-time polymerase chain reaction (qPCR), was monitored up to 20 passages.

Purification of recombinant proteins and lipid binding assays (I)

Glutathione-S-transferase (GST) proteins were produced in *E. coli* BL21(DE3) and purified using Glutathione-Sepharose 4B (GE Healthcare) using standard methods.

In vitro oxysterol binding assays were performed as described in (Hynynen et al., 2009). Briefly, 40 nM [^{3}H]-labeled oxysterol were incubated overnight with 500 nM of purified ORP1L-GST or GST protein in the presence or absence of 1-50 fold molar

excess of competing oxysterol. The unbound sterol was then removed with charcoal-dextran, and the amount of protein-bound [^{3}H]-labeled oxysterol was analyzed by liquid scintillation counting.

In vitro cholesterol binding assays were performed by preparing large unilamellar vesicles containing 0.5 mM PC with 1 mol% [^{3}H]cholesterol as in (Ngo and Ridgway, 2009). The prepared vesicles were incubated with GST-ORP1S or GST (1:1 mol protein/mol sterol) for 30 min at 25°C. Liposomes were then ultracentrifuged at 100,000 g for 25 min at 4°C and the radioactivity of the supernatant was measured by liquid scintillation counting.

Coimmunoprecipitation (I)

Co-IP experiments were performed as described in (Johansson and Olkkonen, 2005). HeLa cells transfected with Rab7-Xpress and ORP1L fused to green fluorescent protein GFP) constructs were scraped into 500 µl of lysis buffer (10 mM Hepes pH 7.6, 150 mM NaCl, 0.5 mM $MgCl_2$, 10% glycerol, 0.5% Triton X-100, and protease inhibitor cocktail), kept on ice for 15 min, centrifuged for 15 min at 16,000 g at 4°C. The supernatants were precleared with Protein G-Sepharose 4 fast flow (Amersham) slurry for 30 min, and the supernatants were subsequently incubated with Xpress or control antibodies overnight at 4°C. Protein G-Sepharose 4 fast flow was then added to the mixture for 4 hours, followed by assaying of the bound immunocomplexes by Western blotting using ORP1L or Xpress antibodies.

Labeling of cells with fluorescent probes (I)

To analyze endocytic functionality, HeLa cells were incubated with rhodamine-labeled epidermal growth factor (EGF, Molecular Probes) at 200 ng/ml for 1 h, followed by washes and chase up to 6 hours before fixation. Prior to labeling the cells were incubated for 1 hour in DMEM supplemented with 2 mg/ml bovine serum albumin and 10 mM Hepes. RAW264.7 macrophages were labeled with DiI-acLDL in serum-free medium for 1 hour at 50 µg/ml concentration. Fixed cells were then processed for immunofluorescence microscopy for further analysis.

Fluorescence microscopy and image analysis (I, II)

Cells grown on coverslips were fixed with 4% formaldehyde/PBS for 30 min, followed by 5 min permeabilization with 0.05% Triton X-100/PBS. Nonspecific binding was blocked with 10% FBS/PBS for 30 min, followed by antibody incubations in 5% FBS/PBS (at 37°C for 30 min). Antibodies were visualized with Alexa Fluor secondary antibody conjugates (Invitrogen). Cells were mounted in

Mowiol (Calbiochem) containing 50 mg/ml 1,4-diazocyclo-[2,2,2]octane (Sigma-Aldrich). Coverslips were viewed with Leica SP2 confocal microscope, equipped with 63x/NA 1.40 HCX PL APO CS (oil) Leica objective lens, or Zeiss AxioObserver Z1 microscope, equipped with AxioCam HRm Rev.3 FireWire camera, and Plan-Apochromat 63x/1.40 Oil M27 (oil) or EC Plan-Neofluar 40x/0.75 WD=0.71 M27 objective. The acquisition softwares used were Leica LCS and AxioVision Rel. 4.8. Adobe Photoshop and Image J softwares were used for image processing.

Labeling of cells with radioactive cholesterol and lipid analysis (I, III)

RAW264.7 macrophages were loaded with 25 µg/ml [^{3}H]-cholesterol oleate containing acLDL in the presence of 5% lipoprotein-deficient serum (isolated from FBS by buoyant density centrifugation) for 48 hours.

To analyze cellular cholesterol efflux, medium was replaced after loading. The amount of radioactivity effluxed to the medium containing the cholesterol acceptors, apo-AI (10 µg/ml) or HDL_2 (25 µg/ml), was determined with liquid scintillation counter.

To analyze cellular lipids, cells were washed extensively with PBS before scraping into 0.2% SDS, and extracted using Bligh and Dyer method (Bligh and Dyer, 1959), followed by thin-layer chromatography on silica-gel plates by using petroleum ether/diethyl ether/acetic acid (60:40:1) as the solvent, as described in (Perttilä et al., 2009), or mass spectrometry.

Quantitative real-time PCR (I, II, III)

Total cellular RNA isolation was performed with RNeasy Mini kit (Qiagen). RNA (1 µg) was reverse-transcribed using random hexamer primers and Superscript III (Invitrogen).

For qPCR each RNA sample was analyzed with SYBR Green kit (Applied Biosystems or Finnzymes) and 7000 Sequence Detection System in triplicates for the genes of interest together with housekeeping markers (ribosomal protein 36B4 and Porphobilinogen Deaminase PBGD) for normalization. Relative mRNA quantitations were determined by setting the threshold in the linear range of fluorescence to get the threshold cycle (Ct) for each well, and calculated by the comparative Ct method.

Migration assays (II)

To induce migration, 1 nM N-formylmethionyl-leucyl-phenylalanine (fMLP, 1 nM concentration) or sphingosine-1-phosphate (S1P, 1 µM) were used.

TranswellTM chambers (Corning) with 6.5 mm-diameter and 8 µm pore size were used for migration experiments. The cells in complete medium were added in the upper well and allowed to migrate to the lower compartment for 6 hours. Non-migrant cells were washed, and cells at the bottom of the membrane were fixed, and stained with 0.1% Crystal Violet in 20% (v/v) methanol. After washing and drying of the membranes, the migrated cells were quantitated by dissolving the dye in 10% acetic acid, followed by a measurement of the dye intensity at 560 nm. Migration capacity was assessed as the ratio compared to total amount of cells seeded.

For wound healing experiments, cells were grown on 6-well plates into confluency. The monolayers were then wounded with a pipette tip, followed by washing of detached cells with PBS. The wound closure was monitored up to 0-96 hours with a bright-field EVOS xl microscope (AMG, Bothell, WA).

Cellular migration capacity was additionally monitored by live-cell imaging. Cells were grown in glass-bottom dishes at low confluency, and imaged every 20 seconds by differential interphase contrast microscopy for a total of 30 minutes.

Subcellular fractionation (II)

To separate cellular nuclear and cytosolic fractions, cells were lysed in buffer containing 10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% NP40, pH 7.9, and protease and phosphatase inhibitor cocktails (Roche). The fractions were separated by 10 min centrifugation at 3000 rpm at 4°C, where nuclear fraction was pelleted. Nuclear fraction was further homogenized in a buffer containing 5 mM Hepes, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v), pH 7.9, after adding 4.6 M NaCl. The fractions were analyzed by Western Blotting.

SDS-PAGE and Western Blotting

Cells were harvested in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 10% glycerol, 0.5% NP-40) with protease inhibitor cocktail (Roche). Protein contents were determined by the detergent compatible (DC) protein assay (BioRad). Aliquots of 20 µg of the lysates were electrophoresed on Laemmli gels, followed by transfer to nitrocellulose membranes (Hybond-C Extra). Membranes were blocked for unspecific binding, followed by antibody incubations, all carried out in 5% (w/v) fat-free milk in TBS containing 0.01% Tween-20. The bound antibodies were visualized with HRP-conjugated goat IgG antibodies, and the ECL (Thermo Scientific, Rockford, IL).

Principal component analysis (III)

To analyze the cellular lipidome data obtained by mass spectrometry, principal component analysis (Sirius program package) was used in the analysis of the cellular lipidome data. Prior to the analysis, the molar percentages of lipid species were transformed logarithmically (arc sin) to prevent the abundant components from dominating in the analysis. The samples were plotted into biplots describing largest and second largest variance in the original multidimensional data. The statistical significance of the observations was assessed with soft independent modeling of class analogy (SIMCA, available in the Sirius software package).

Other methods (I, II, III)

All numerical data are presented as average ± stdev or s.e.m. for at least 3 experiments. Depending on the experimental setup, the statistical significance of the data was assessed with Student's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$) or with one-way ANOVA ($p<0.05$), followed by Bonferroni's test for differences between means.

Results and discussion

5.1 Subcellular targeting of ORPs -Lipid and protein interactions

The hallmark feature of all ORPs is the presence of ORD (see section 2.3.1), present in all ORPs. Another noteworthy feature is the presence of several additional motifs, such as ankyrin repeats and PH domains. To understand the protein function, it is important to identify the lipid and protein interactions of the ORP proteins and to clarify the functional consequences of these interactions. In this work, several novel interactions with lipid ligands were identified and the roles of both known and novel interactions were further characterized.

5.1.1 Novel ORP1L interactions

5.1.1.1 ORP1L binds 24(S)-OHC, 7-KC and cholesterol in vitro (I)

In the first part of this work, a competition assay was employed, where ORP1L binding to its established oxysterol ligand, 25-OHC (Yan et al., 2007a), was competed with other oxysterols. This assay identified two novel ORP1L oxysterol ligands, namely 24(S)-OHC, and 7-ketocholesterol (I, Figure 1A). With the experimental setup used, no differences in the binding affinities between oxysterols could be determined. The two newly identified oxysterol ligands are likely to have physiological importance. 7-ketocholesterol is an abundant oxysterol species in atherosclerotic lesions and macrophage foam cells (Garcia-Cruset et al., 2001; Maor et al., 2000), and the binding can thus be important in the context of atherogenesis. 24(S)-OHC (also known as cerebrosterol) is the most abundant oxysterol in the CNS, and it is thought to be important for the cholesterol homeostasis of the brain (for review, see (Björkhem, 2007). This and the fact that ORP1L is expressed at high levels in the central nervous system (Johansson et al., 2003; Laitinen et al., 1999) suggest that ORP1L may play an important role in the cholesterol balance of the brain.

In addition to oxysterols, cholesterol binding by ORP1L was demonstrated using an assay where the ability of purified ORP1 protein to extract cholesterol from large unilamellar vesicles, was determined. In this assay, ORP1 was able to extract cholesterol in a concentration-dependent manner (I, Figure 1B), similar to ORP2, included in the assay as a positive control (Hynynen et al., 2009). However, the affinities could not be compared between oxysterol and cholesterol binding due to the different experimental protocols used in these assays. Cholesterol is likely a physiologically important ORP1L ligand, as biological membranes are far more

enriched in cholesterol than oxysterols (Brown and Jessup, 1999). It is therefore possible that oxysterols are able to replace bound cholesterol from the sterol-binding pocket, serving as a trigger for protein activation or translocation, as described for OSBP, where binding of 25-OHC triggers disassembly of a protein phosphatase complex, induced by binding of cholesterol by OSBP (Wang et al., 2005, 2008).

Whether there is a functional distinction between oxysterol and cholesterol binding of ORP1L, and whether it is a common feature of the ORPs, remain to be investigated in the future studies. Photo-crosslinking experiments have suggested that several ORPs (OSBP, ORP1, ORP2, ORP3, ORP5, ORP6, ORP7, ORP8, ORP10, ORP11) are capable of binding both 25-OHC and cholesterol (Suchanek et al., 2007).

5.1.1.2 Recruitment of kinesin-2 and interaction of VAP by ORP1L (I)

ORP1L has an established role as an effector protein of Rab7, and is involved in the minus-end directed transport of LE (Johansson et al., 2005, 2007). The minus-end directed transport of LE results from a cascade that involves recruitment of dynein-dynactin motor complex to the surface of LE. In addition to its recruitment, dynein needs to interact with betaIII spectrin to translocate LE, a process dependent on ORP1L (Johansson et al., 2007). In (I), we show that in addition to the recruitment of dynein-dynactin complex to LE surface, ORP1L overexpression results in a drastic recruitment of a plus-end directed motor protein, kinesin-2, to the LE surface (I, Figure 3B). The recruitment of both plus- and minus-end motors to LEs was observed to comparable extent regardless of the endosome positioning. This result suggests that the regulation of LE motility is a complex process and that simple motor protein recruitment does not suffice for altering LE motility/distribution. The putative interaction of ORP1L with kinesin-2 was not examined in this study. Moreover, the inactivation of ORP1L FFAT motif and the resulting altered endosome localization and motility (I, Figure 4) suggests a functional interaction between ORP1L and VAP proteins.

The role of ORP1L and its interactions in the LE motility will be further discussed in section 5.3.1.

5.1.2 ORP3

5.1.2.1 Kinases responsible for ORP3 phosphorylation

ORP3 migrates in SDS PAGE as a double band, where the bands represent forms of the protein phosphorylated to various degrees (Lehto et al., 2008). The

phosphorylation is induced when cells upon trypsinisation and replating at a low density, when cells lose their contacts with adjacent cells and the environment.

To identify the kinases catalyzing ORP3 phosphorylation, we performed a screen where human kinases predicted to have target sites in ORP3 sequence, were overexpressed in HEK293 cells, and the degree of the ORP3 phosphorylation was then monitored by Western blotting. Out of the 67 constructs tested, two kinases were able to induce a band shift in ORP3 (Figure 7), namely cyclic AMP dependent kinase PKA (catalytic subunit) and calcium/calmodulin dependent kinase CAMKII (several isoforms). These kinases are activated upon elevated cAMP or calcium levels, respectively. In contrast to previous studies where PMA, a PKC agonist, was able to induce ORP3 band shift, overexpression of PKC α, β, γ, δ, ε, ζ, ι, or ν subunits did not hyper-phosphorylate ORP3 (data not shown). It is, however possible that the subunits do not phosphorylate their targets as efficiently as single subunits.

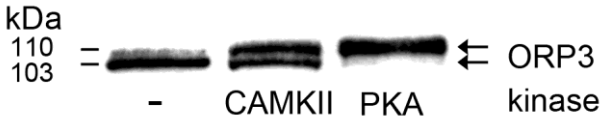

Figure 7. The ORP3 band shift induced by kinase overexpression.

5.2 Subcellular targeting of macrophage ORPs

5.2.1 ORP1L LE localization does not involve ORD or FFAT domains (I)

In the first part of this thesis, we generated several mutant forms of ORP1L to characterize the functionality of its ORD and FFAT motif. To elucidate the role of ORD, we deleted a four amino acid region (amino acids ELSK, 560-563). The leucine residue in this region is completely homologous in the protein family (data not shown), and the deletion of this area has been shown to result in deficient sterol binding for OSBP, ORP4, and ORP9 (Ngo and Ridgway, 2009; Perry and Ridgway, 2006; Wyles et al., 2007). By homology modeling, we further showed that this leucine putatively interacts directly with the side chain of the bound sterol (I, Figure 1C). Moreover, deficient oxysterol binding of the ORP1L Δ560-563 mutant was demonstrated (I, Figure 1D-F). We were not able to assess, whether the mutant is able to bind cholesterol due to an anomalous behavior of the protein in the cholesterol-binding assay, most likely due to conformational changes putatively resulting in increased exposure of hydrophobic residues. Thus, it is possible that the mutant is not defective in cholesterol binding. Based on these binding assays, we concluded that the mutant served its purpose as a tool to study the function of ORP1L ORD.

Using fluorescence microscopy and double staining for endogenous markers (EEA-1 for EE, LAMP-1 for LE), we further showed that the ORP1L Δ560-563 localized to LE membranes (I, Figure 2B), similar to the WT protein (I, Figure 2A). Additionally, ORP1L mFFAT (inactivated FFAT motif) and the double mutant ORP1L Δ560-563 mFFAT localized to LE membranes as well (I, Figure 4E,F). These studies are in line with previous results of Johansson et al. (Johansson et al., 2003), who demonstrated that ORP1L is targeted to LE membranes via N-terminal ankyrin repeats and PHD.

5.2.2 ORP3 and VAP-A interact at the vicinity of plasma membrane

To investigate the interaction of ORP3 and VAP-A, a bimolecular fluorescence complementation (BiFC) technique (Kerppola, 2006) was employed. Huh7 cells were transfected with N- and C-terminal fragments of the fluorescent protein Venus fused with ORP3 and VAP-A, respectively. The BiFC fluorescence arising upon interaction of the ORP3 and VAPA moieties in the cells was imaged. The two proteins were shown to interact at reticular ER locations. Interestingly, BiFC signals could be observed at patches associated with the plasma membrane (Figure 8). Since VAP-A is an integral ER protein, these patches could represent MCSs between PM and ER. Moreover, previous experiments where pull-down assays were employed to investigate the interaction of VAP-A and ORP3, suggested that the hyperphosphorylated ORP3 specifically interacts with VAP-A (Lehto et al., 2005). It will be interesting to study whether the ORP3 phosphorylation controls its association with VAP-A, or whether it controls MCS formation.

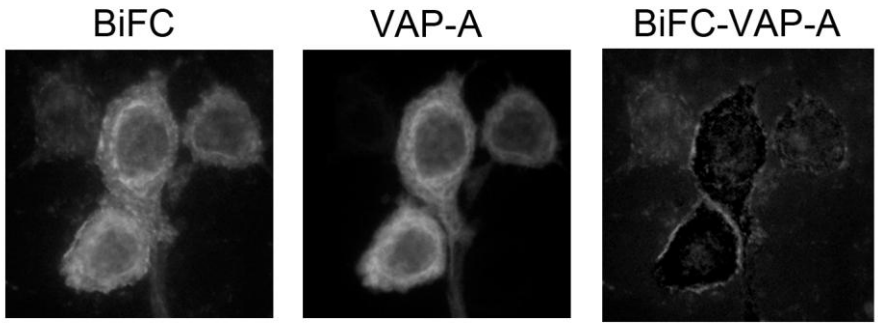

Figure 8. Bimolecular fluorescence complementation technique demonstrates interaction of ORP3 with VAP-A at the reticular ER and at patches at the PM (Left Figure). VAP-A (Middle Figure) was used as a cotransfection marker. The ORP3-VAP-A interaction at the PM is visualized in the right Figure where reticular ER signal (VAP-A) is subtracted from the BiFC signals (BiFC-VAPA). Figures by courtesy of Marion Weber-Boyvat (Minerva Institute).

5.2.3 ORP8

To investigate the role of ORP8 ligand binding, a four amino acid region (Δ377-380, DLSK) encompassing the conserved leucine residue was deleted. Deletion of this region has previously shown to result in deficient sterol binding in other ORP members (Ngo and Ridgway, 2009; Perry and Ridgway, 2006; Wyles et al., 2007) and ORP1L (section 5.2.1). To investigate the subcellular localization of the ORP8 Δ377-380 mutant, HeLa cells overexpressing this mutant were analyzed by fluorescence microscopy. Interestingly, the ORP8 Δ377-380 protein was partially targeted to the PM, where patchy localization was evident (Figure 9). ORP8 has a transmembrane segment, shown to anchor ORP8 to the ER (Yan et al., 2008). Thus, the PM patches could represent PM-ER MCSs. To test this, ORP8 Δ377-380 was cotransfected with VAP-A (ER marker) and SNAP25 (PM marker) constructs. ORP8 Δ377-380 colocalized with both of these markers at the PM (Figure 9A, B), suggesting that they are, in fact, MCSs. To investigate whether the WT protein also targets PM, ORP8 WT was cotransfected with SNAP25. Although majority of ORP8 appeared to localize at the reticular ER, as reported earlier, some localization was observed at the PM vicinity (Figure 9C, arrows). To further confirm PM localization of ORP8 Δ377-380, we used immunoelectron microscopy methods that provides resolution superior to light microscopy. HeLa cells transfected with ORP8 Δ377-380 GFP were processed for electron microscopy, and stained for GFP. Even though the signal is weak, the gold particles specifically localize at the vicinity of PM (Figure 9D).

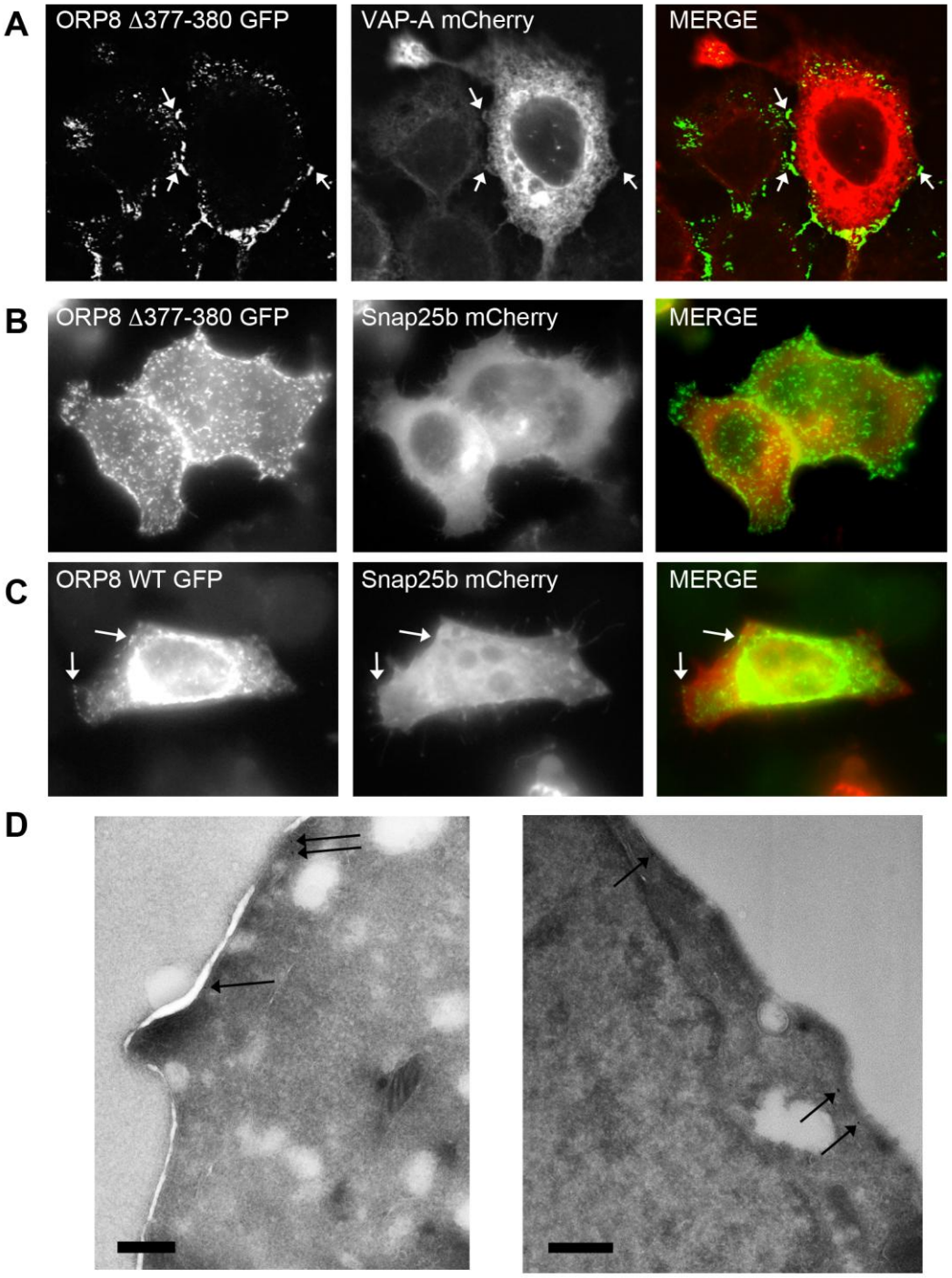

Figure 9. A) Confocal microscopy images of HeLa cells transfected with ORP8 Δ377-380 GFP and VAP-A mCherry showing colocalization at the cell edges. B) Maximal projections of cells overexpressing ORP8 Δ377-380 GFP and Snap25b mCherry demonstrating the plasma membrane localization of ORP8 Δ377-380 GFP, and C) ORP8 WT. D) Immunoelectron microscopy of thin frozen sections of HeLa cells transfected with ORP8 Δ377-380 GFP, followed by detection with anti-GFP antibody and protein A-10 nm gold particles, confirm the plasma membrane proximity of ORP8 Δ377-380 GFP; Bars, 400 nm. EM figures by courtesy of Nils Bäck, University of Helsinki.

5.3 Function of ORPs and their impacts on cell physiology

5.3.1 ORP1L regulates late endosome localization, motility, and function (I)

In the first study of the thesis we characterized the ORD and the FFAT mutant of ORP1L. We demonstrated that even though these mutants localized to LE, the LE distribution was dramatically altered. Whereas overexpression of the WT protein induced perinuclear LE clustering, the ORP1L Δ560-563 decorated LEs were scattered within the cell (I, Figure 2B). A similar LE scattering phenotype was demonstrated by cellular cholesterol depletion (Rocha et al., 2009). We further demonstrated that there was no defect in the interaction between ORP1L Δ560-563 and Rab7 (I, Figure 2D), and showed that the altered localization was not due to an inability to recruit motor complexes to LE surface that was, in fact, enhanced (I, Figure 3). Based on these two studies, ORP1L was suggested to act as a sterol sensor that regulates LE positioning. Furthermore, inactivation of the FFAT motif of ORP1L Δ560-563 mutant resulted in LE clustering, suggesting that this motif is bridging the LEs to ER via VAP interaction in the scattered phenotype.

Importantly, using live cell imaging, we showed that the overexpression of ORP1L or any of its mutant forms resulted in reduced motility of late endosomal compartments compared to mock transfection (I, Figure 4A, red bars). Interestingly, ORP1L mFFAT decorated LE were significantly less immotile than the other ORP1L forms (I, Figure 4A, second green bar from the left), suggesting that the ER interaction restricts motility of these compartments. Silencing of ORP1L, on the other hand, had an opposite effect on LE motility: significantly increased motility of the LE compartments compared to control cells was shown (I, Figure 5B). Moreover, live cell imaging of the LE compartments in ORP1L silenced cells revealed that the LEs spent more time in the cell periphery, suggesting that the missing LE-ER interaction acts to reduce LE motility and to enhance LE centralization.

To study whether the ORP1L overexpression or reduced motility affected the cargo handling of the LE compartments, fluorescently labeled EGF (rhodamine-EGF) was endocytosed, and the degradation was monitored by quantitating the amount of fluorescent signal. The degradative process was inhibited significantly by ORP1L overexpression, and the effect was even more pronounced for ORP1L Δ560-563 mutant (I, Figure 6A). Due to the fact that the rhodamine-EGF accumulated vesicles were not positive for overexpressed ORP1L (I, Figure 6D,E), and the fact that silencing of ORP1L did not affect the EGF degradation (I, Figure 6B), we speculate that the effect of ORP1L on endosomal degradation is an indirect one.

5.3.2 The role of ORP1L in macrophage lipid transport and metabolism (I, III)

To investigate the ORP1L mediated LE dynamics in a physiologically more relevant context, we used RAW264.7 macrophage cells silenced for ORP1L, where foam cell formation was induced by fluorescent acLDL (DiI-acLDL) internalization. Similarly to the previous results, live-cell imaging of the macrophages revealed increased motility of the lipid carrying compartments when ORP1L was silenced (I, Figure 7A). Moreover, a significant inhibition of cholesterol efflux to apo-AI acceptors was observed following loading of the cells with radioactively labeled acLDL (I, Figure 7B). This suggests that in these cells ABCA1 mediated cholesterol efflux is specifically affected, since ABCA1 preferably removes cholesterol from endolysosomal compartments (Azuma et al., 2009; Chen et al., 2001). Consistently, we observed a significant increase in both FC and CE levels in the lipidome analyses of the ORP1L silenced macrophages (III, Figure 2A) at basal situation, suggesting that the putative disturbance of vesicular lipid transport results in the accumulation of cellular cholesterol.

In basal conditions a significant increase in cellular PI levels (III, Figure 4) and an altered PC composition (III, Figure 5), were observed. Cells silenced for ORP1L contained less short (30-32 carbon) and saturated PC species, and more 36-carbon PC species than the control cells. After LPS stimulation decreased PE-plasmalogen levels (PE-pl) were observed in ORP1L silenced cells (III, Figure 7). Plasmalogens are specific phospholipids that contain a vinyl ether moiety at the sn-1 position that is vulnerable to damage or oxidation. Plasmalogens are abundantly present in the PM and in lipid rafts (Pike et al., 2002), and they are suggested to act as membrane antioxidants preventing cholesterol oxidation in the PL bilayers (Maeba and Ueta, 2003), although the evidence is controversial. Interestingly, oxidated plasmalogens are often detected in atherosclerotic lesions (Ford, 2010; Thukkani et al., 2003).

These results indicate that ORP1L is an important player of macrophage lipid metabolism.

5.3.3 Effects of ORP3 silencing on the macrophage lipidome (III)

The specific effects of ORP3 silencing on the macrophage lipidome were fewer than what was observed for ORP1L or ORP8 silenced cells. A significant reduction of PI levels was observed both at basal and LPS stimulated conditions (III, Figure 4, 7), suggesting the role of ORP3 in the PI metabolism. Moreover, decreased levels of PE-pl and glucosyl ceramides were observed (III, Figure 3C, 4, 7).

5.3.4 ORP8 specific effects on the macrophage lipidome (III)

Similar to ORP1L, increased free and esterified cholesterol levels were observed in cells silenced for ORP8 (III, Figure 2A), consistent with the reported capacity of ORP8 to modify cholesterol levels in hepatic cells (Zhou et al., 2011). Moreover, decreased levels of PE-pl and glucosyl ceramides were observed (III, Figure 3C, 4), similar to ORP3. Significantly increased PE levels and decreased PI and PE-pl levels were observed after LPS stimulation (III, Figure 7). A moderate, non-significant increase in total LPC (III, Figure 7) together with significant increases in certain LPC species (III, Figure 8A) observed in ORP8 silenced cells after LPS treatment, could associate with macrophage inflammatory functions. These lipids act as signaling compounds in response to cell stimulation that activate G-protein coupled receptors, and regulate calcium homeostasis and migration (Meyer zu Heringdorf and Jakobs, 2007).

5.3.5 Silencing of ORP8 associates with altered nuclear functions and microtubule organization (II)

The role of ORP8 in the modulation of lipid metabolism of macrophages and hepatic cells was earlier demonstrated (Yan et al., 2008; Zhou et al., 2011). In the second part of this thesis, we wanted to further investigate the function of ORP8 in macrophages; we performed a microarray transcriptome analysis of RAW264.7 macrophage cells silenced for ORP8. Unexpectedly, a pathway analysis of the microarray results did not reveal significant changes in lipid metabolism pathways, but most prominently activated pathways were nuclear pathways (DNA metabolism and chromosome structure) together with centrosome and microtubule organization (II, Table 1). Most downregulated pathways involved lysosome function, growth factor activity and defense response. These changes in gene expression putatively indicate affected cell cycle control and proliferation. However, flow cytometric analysis did not reveal any significant changes in cell cycle progression between the control and the ORP8 silenced cells (II, Supplementary Figure 1A). Furthermore, the MTT assay did not reveal any differences in the proliferation capacity (II, Supplementary Figure 1A).

5.3.6 Silencing of ORP8 enhances macrophage migration (II)

The recently identified interaction between ORP8 and nucleoporin NUP62 (Zhou et al., 2011) led us to investigate the localization of NUP62 in ORP8 silenced cells by immunofluorescence staining of NUP62. In the control cells, NUP62 localizes evenly in the nuclear membranes. In cells silenced for ORP8, NUP62 displayed an abnormal

distribution with extensive intranuclear localization (II, Figure 4A), and could be seen in cytoplasmic vesicles and cell edges. Moreover, NUP62 was demonstrated to be upregulated in ORP8 silenced cells, and to display altered distribution between the nucleus and cytosol: a significantly increased proportion of NUP62 was found in the cytosolic cell fraction (II, Figure 4B,C).

NUP62 has been shown to enhance cell migration in a process where NUP62 is recruited by the exocyst component EXO70 to the leading edge of migrating cells (Hubert et al., 2009). This and the increased amount of cytosolic NUP62 in ORP8 silenced cells led us to investigate their capacity to migrate. Increased migration capacity was demonstrated by three distinct methods: through a porous filter, by a standard wound healing, and by live-cell imaging (II, Figure 5A, C, Supplementary videos 1-2). Furthermore, a functional connection between NUP62 and the increased migration phenotype in these cells was demonstrated by siRNA mediated NUP62 knockdown, which returned the migration capacity to the level of control cell migration (II, Figure 5A).

In the wound healing experiments and live-cell imaging, we observed more active formation of cell extensions, and more prominent lamellipodial formation. Lamellipodial formation requires reorganization of cytoskeletal elements, including actin and microtubules (Mogilner and Keren, 2009; Waterman-Storer et al., 1999). Indeed, microtubule organization was among the altered pathways in the transcriptome analysis. The staining of the microtubule cytoskeleton revealed longer microtubules extending to the cell periphery in cells silenced for ORP8 (II, Figure 5D).

Based on these results we hypothesized that, if NUP62 is recruited by EXO70 in migrating cells, increased interaction between NUP62 and EXO70 must exist in cells silenced for ORP8. Immunofluorescence analysis revealed frequent colocalization of NUP62 and EXO70 at the lamellipodial edges in ORP8 silenced cells (II, Figure 6A). Based on the fact that both EXO70 and ORP8 have been reported to bind the NUP62 coiled-coil domain (Hubert et al., 2009; Zhou et al., 2011), we speculated that these two proteins could bind to NUP62 in a competitive manner, which we confirmed using an in vitro competition assay (II, Figure 6B).

5.3.4 Common ORP impacts on the macrophage lipidome (III)

In the third part of the study, distinct and complex effects on the cellular lipids were demonstrated for cells silenced for individual ORPs (sections 5.3.2-5.3.4). In addition, some lipidome features common for all ORP silenced cells were observed.

All three studied ORPs affected cellular levels of total PIs at least in some conditions. At basal conditions, increased PI levels were observed in ORP1L silenced

cells, whereas a decrease in PI after LPS stimulus was observed when ORP3 or ORP8 was silenced. PIs are present in the cytosolic side of cellular membranes and the phosphorylated forms (PIPs) are important for signaling (section 2.1.5.1). Moreover, majority of long ORP variants (ORP1L, ORP3, and ORP8 included) are capable of interacting with PIPs, and ORPs have been suggested to modify cellular PIP metabolism (see section 2.3.5, third paragraph).

Similarly, cellular levels of total PE-pl were decreased by ORP silencing. At basal conditions, PE-pl levels were decreased in ORP3 and ORP8 silenced cells, and after LPS stimulus decreased levels were observed when ORP1L or ORP8 was silenced.

Interestingly, after LPS treatment all cell pools silenced for ORPs were enriched in 16-carbon sphingolipid species at the expense of the 24-carbon ones (III, Figure 6E). Moreover, an increase in ceramide levels was observed in ORP1L silenced cells (III, Figure 6C). One of the major cellular responses of LPS-mediated TLR4 activation is a rise in ceramide levels (Dennis et al., 2010). This suggests that functional links between sphingolipid metabolism and the studied ORPs may exist. Cer synthases, responsible for producing a spectrum of different Cer species to be incorporated to various SM and glucosyl ceramides, show substrate chain length specificity (Merrill et al., 2007). Moreover, distribution of GPI-anchored proteins in rafts is affected by the SM chain length (Garner et al., 2007). The altered sphingolipid profiles indicate delicate alterations in the sphingolipid biosynthesis. Together with changes in the cellular FC and PE-pl contents, the properties of cholesterol/sphingolipid domains could be altered, and putatively result in modification of raft-dependent signaling. This is consistent with the hypotheses suggesting that the ORPs have the capacity to regulate lipid lateral organization (Georgiev et al., 2011; Sullivan et al., 2006).

Another common trend was observed in the profiles of AA containing phospholipids (III, Figure 8A) that act as precursors for eicosanoids (section 2.1.5.4). Compared to the control cells, ORP silenced cells were enriched in AA containing PE and PC species, and displayed reduced amounts of AA containing PI species, while no difference in the total AA levels was observed. Due to the potent biological activity of eicosanoids, the AA levels, as well as their distribution among PL classes, are tightly controlled. AA is most readily released from PI stores (Astudillo et al., 2011). The decreased amount of PI-derived AA in cells silenced for ORPs may associate with dampened eicosanoid signaling.

In addition to the altered AA distribution, significantly increased total levels of DHA containing phospholipids, precursors for resolvins (section 2.1.5.4), were observed in all the ORP silenced cells (III, Figure 8D). These results imply a capacity of the studied proteins to modify inflammatory responses, possibly by dampening eicosanoid production and enhancing resolvin production.

Conclusions

In this study, macrophage-enriched ORP family members were investigated. Novel sterol ligands and protein interaction partners were identified, and the cellular targeting was investigated. Based on the data obtained, it can be concluded that these proteins perform distinct functions and act, for the most part, at distinct cellular compartments compared to one another. However, apparently all of the ORPs studied localize transiently at MCSs, and putatively mediate the formation of these sites. Moreover, their roles in the macrophage functions and lipid metabolism were demonstrated.

The presence of several targeting domains or motifs is a key feature of long ORP variants (Figure 5), including the ones studied in this thesis. Moreover, their protein structure contains elements that target the proteins to ER membranes, either via interaction of ER VAP proteins, or via a transmembrane segment. In this study, localization at MCS was demonstrated for ORP1L (LE-ER), ORP3 (PM-ER), and ORP8 (PM-ER). The signals mediating MCS formation, as well as the downstream effects differ between the proteins (Figure 10).

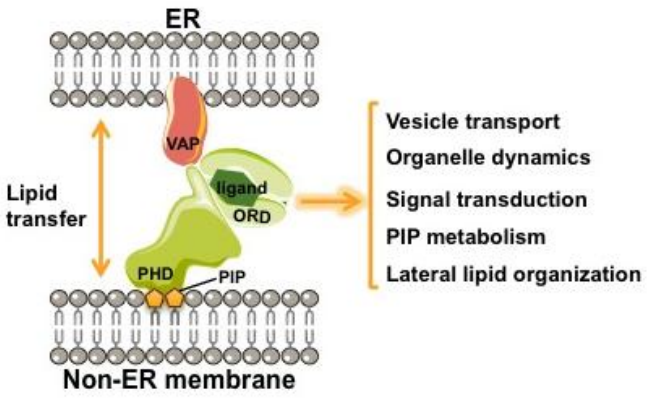

Figure 10. A scheme showing ORP protein at MCS together with putative functions. Some figure components were provided by Servier Medical Art (www.servier.com).

ORP1L, which localizes at LE membranes, was demonstrated to mediate LE-ER interactions in a sterol-dependent manner (I), which was shown to be involved in the regulation of LE motility. However, it is likely that the control of LE motility is not the only function of these MCSs. For example, the maturation of LEs to multivesicular bodies (MVB) requires inward curvature of the vesicle membrane, a process putatively mediated by lipids. A recent study showed that knockdown of ORP1L in HeLa cells led to an impaired multivesicular body formation, which were dramatically increased in size and had significantly less intraluminal vesicles (Kobuna et al., 2010). The same phenotype was observed in *C. elegans* with depletion of ORPs or cholesterol, suggesting the involvement of ORP1L in mediating effects of cholesterol (or other lipids) in MVB formation (Kobuna et al., 2010). Thus, at ER-LE

contact sites, newly synthesized cholesterol could be transported from the ER to LEs, which could facilitate MVB formation.

Targeting of ORP3 to PM or ER (Lehto et al., 2005) and its role as a regulator of cell adhesion (Lehto et al., 2008) have been reported. Here, ORP3 is shown to interact with VAP-A at patchy PM structures. Similarly ORP8, an integral ER-protein, is reported to localize at PM patches, which appears to be regulated by its ligand-binding status. The functions of ORP3 and ORP8 at the ER-PM MCSs remain topics of future research. However, the patchy localization at the PM suggests a specific lipid lateral organization at these sites, and could represent lipid rafts.

At least two events occurring at ER-PM MCSs have been described. Store-operated calcium entry, activated upon depletion of ER calcium stores, requires a complex formation between ER-residing STIM (stromal interaction molecule) proteins and a PM-protein Orai1, to allow entry of calcium from extracellular sources (Shen et al., 2011). Moreover, the PM lipid composition determines targeting of STIM1: it preferentially enriches in lipid rafts (Pani and Singh, 2009). Interestingly, elevated calcium levels have been shown to associate with impaired cell viability induced by oxysterols (Kolsch et al., 1999). Thus, the possible regulation of cellular calcium levels by the ORPs remains an intriguing topic of future investigations.

A tyrosine phosphatase PTP1B is suggested to act at ER-PM MCSs. This ER-residing enzyme has been shown to bind several molecules at the PM, including PKC, tyrosine kinase Src, and insulin receptor (Anderie et al., 2007). ORP8 was previously shown to be required for insulin induced AKT activation and glucose homeostasis (Jordan et al., 2011); raising a possibility that ORP8 could function at the same complex with PTP1B.

In previous reports and in this thesis the roles of ORPs the regulation of essential macrophage processes, including lipid metabolism, cholesterol trafficking, inflammatory response, phagocytosis, and migration, have been demonstrated. Macrophages play key roles in all stages of atherosclerotic lesion development, as discussed in section 2.2.2. Accordingly, the modulation of ORP levels and/or function represents an attractive target for prevention or treatment of atherosclerosis.

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