Oxysterol binding protein homologues: Novel functions in intracellular transport And adipogenesis

YOU ZHOU

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine, University of Helsinki for public examination in Lecture Hall 2, Biomedicum Helsinki on January 25th, 2013, at 12 noon.

Helsinki 2013
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ISBN 978-952-10-8594-9 (paperback)
ISBN 978-952-10-8595-6 (pdf)
Cover designer: You Zhou
Helsinki University Print (Unigrafia)

HELSINKI 2013
To Eunjee and Joel
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ABSTRACT

Lipids, as the major components of cell membranes, are multi-functional molecules that also critically contribute to signal transduction, transcriptional regulation and membrane trafficking. The voice from single lipid molecule mixes together to create a “balancing symphony or chorus” called lipid homeostasis. Disturbances in the “molecular song” by lipids trigger profound physiological responses, which are reflected as metabolic disorders, such as obesity, metabolic syndrome, and atherosclerosis. Hence, it is important to understand the semiotics of this molecular language.

Lipid homeostasis is context-dependent and regulated coordinately by a variety of lipids per se and proteins. Oxysterol binding protein (OSBP) and its homologues (ORPs) are implicated to regulate lipid homeostasis, sterol transfer and cell signaling. In this thesis, two ORPs, ORP11 and ORP7 have been extensively studied. ORP11 is abundant in human ovary, testis, kidney, liver, stomach, brain and adipose tissue and resides at the Golgi-Late endosome interface. ORP11 forms a dimer with its close homologue, ORP9, the interaction occurring in the region of aa154-292 in ORP11 and 98-372 in ORP9, which maintains the subcellular distribution of ORP11.

ORP7 interacts with GATE-16 and might be involved in autophagosome biogenesis. Excess ORP7 induces recruitment of GATE-16 from Golgi to autophagosomes. ORP7 thereby regulates the proteosome-dependent degradation of Golgi v-SNARE protein, GS28, another binding partner of GATE-16. 25-hydroxycholesterol, a ligand of ORP7, modifies GS28 protein stability, an effect which is influenced by ORP7.

Being the largest endocrine organ in human body, adipose tissue is the primary place to store fat. However, there is no study to compare expression patterns of ORPs in white adipose depots and adipose cells. In this thesis, we showed that human subcutaneous and visceral adipose depots as well as Simpson–Golabi–Behmel syndrome (SGBS) adipocytes share similar ORP expression patterns, indicating that the mRNA signals of ORPs in adipose tissues predominantly originate from adipocytes. During adipogenesis, ORP2, ORP3, ORP4, ORP7 and ORP8 mRNA levels were downregulated, whereas ORP11 was upregulated. Silencing of ORP11
resulted in a decreased expression of adiponectin and aP2, while overexpression of ORP8 down-regulated the aP2 mRNA. Interestingly, silencing of ORP11 and overexpression of ORP8 significantly impaired triglyceride storage in the adipocytes.

Taken together, the work in this thesis identifies protein binding partners of ORPs and indicates their potential roles in protein distribution and stability, intracellular trafficking, sterol sensing, and the adipocyte phenotype.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter A1</td>
</tr>
<tr>
<td>ACAT</td>
<td>acyl-CoA:cholesterol acyltransferase</td>
</tr>
<tr>
<td>ANK</td>
<td>ankyrin repeat</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>apolipoprotein A-I</td>
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<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
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<tr>
<td>Atg8</td>
<td>autophagy-related protein 8</td>
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<tr>
<td>BiFC</td>
<td>bimolecular fluorescence complementation</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BSEP</td>
<td>bile salt export pump</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>CERT</td>
<td>ceramide transfer protein</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<td>COP</td>
<td>coatomer protein</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<td>DHE</td>
<td>dehydroergosterol</td>
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<td>EPOX</td>
<td>epoxycholesterol</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-activated kinase</td>
</tr>
<tr>
<td>FAPP2</td>
<td>four-phosphate-adaptor protein 2</td>
</tr>
<tr>
<td>FCH</td>
<td>familiar combined hyperlipidemia</td>
</tr>
<tr>
<td>FFAT</td>
<td>two phenylalanines in an acidic tract</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glycolipid transfer protein</td>
</tr>
<tr>
<td>GATE-16</td>
<td>Golgi-associated ATPase enhancer of 16 kDa</td>
</tr>
<tr>
<td>GLTP</td>
<td>glycolipid transfer protein</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>HMGCoAR</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A reductase</td>
</tr>
<tr>
<td>Insig</td>
<td>Insulin-induced gene</td>
</tr>
<tr>
<td>KC</td>
<td>ketocholesterol</td>
</tr>
<tr>
<td>LC3</td>
<td>microtubule-associated protein 1 light chain 3</td>
</tr>
<tr>
<td>LD</td>
<td>lipid droplet</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>LE</td>
<td>late endosome</td>
</tr>
<tr>
<td>LTP</td>
<td>lipid transfer protein</td>
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<tr>
<td>LXR</td>
<td>liver X receptor</td>
</tr>
<tr>
<td>MCS</td>
<td>membrane contact site</td>
</tr>
<tr>
<td>NPC</td>
<td>Niemann-Pick type C</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide sensitive factor</td>
</tr>
<tr>
<td>NVJ</td>
<td>nucleus-vacuole junction</td>
</tr>
<tr>
<td>OHC</td>
<td>hydroxycholesterol</td>
</tr>
<tr>
<td>OSBP</td>
<td>oxysterol binding protein</td>
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<tr>
<td>ORP</td>
<td>oxysterol binding protein related protein</td>
</tr>
<tr>
<td>ORD</td>
<td>OSBP-related (ligand binding) domain</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PDK-2</td>
<td>phosphoinositide-dependent kinase-2</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>phosphatidylglycerol</td>
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<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PI4P</td>
<td>phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PIP</td>
<td>phosphoinositide</td>
</tr>
<tr>
<td>PITP</td>
<td>PI transfer protein</td>
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<tr>
<td>PL</td>
<td>phospholipid</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>RILP</td>
<td>Rab-interacting lysosomal protein</td>
</tr>
<tr>
<td>ROR</td>
<td>receptor-related orphan receptor</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage activating protein</td>
</tr>
<tr>
<td>SCP</td>
<td>sterol carrier protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDHA</td>
<td>succinate dehydrogenase subunit alpha</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SGBS</td>
<td>Simpson–Golabi–Behmel syndrome</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>SM</td>
<td>sphingomyelin</td>
</tr>
<tr>
<td>SNAP</td>
<td>soluble NSF attachment protein</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble NSF attachment protein receptors</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol regulatory element binding protein</td>
</tr>
<tr>
<td>STAR</td>
<td>steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>START</td>
<td>STAR related lipid transfer protein</td>
</tr>
<tr>
<td>T2D</td>
<td>type 2 diabetes</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>t-SNARE</td>
<td>target SNARE</td>
</tr>
<tr>
<td>VAP</td>
<td>vesicle associated membrane-associated protein</td>
</tr>
<tr>
<td>VLDL</td>
<td>very-low-density lipoprotein</td>
</tr>
<tr>
<td>vesicle SNARE</td>
<td>v-SNARE</td>
</tr>
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</table>
1 INTRODUCTION

The incidence and prevalence of metabolic disorders have increased drastically during the recent decades. Owing to their characteristics such as chronicity and the long-term interventions required, metabolic disorders constitute a serious health threat and pose a heavy economic burden on societies. These disorders such as obesity, diabetes, atherosclerosis, high blood pressure and cardiovascular disease are tightly connected with the metabolism of lipids, molecules playing essential roles in the control of energy homeostasis, organ physiology and cellular metabolism. Disturbances of lipid homeostasis can provoke a variety of cellular responses, and further trigger life-threatening metabolic diseases. It is hence important to understand lipid signaling, transport and related cellular processes.

Oxysterols are 27-carbon oxygenated derivatives of cholesterol, which are present at low concentrations in healthy mammalian tissues. However, they are enriched in atherosclerotic lesions, macrophage foam cells, gall stones, cataracts and specific low-density lipoprotein (LDL) subfractions. Oxysterols play important roles in signaling and development through regulation of Hedgehog signaling pathway and estrogen receptor function. Furthermore, oxysterols mediate lipid metabolism by controlling a number of transcription factors. In addition, oxysterols associate with a variety of complex metabolic diseases as well as the monogenic Niemann-Pick type C disease (NPC).

Oxysterol binding protein (OSBP) was originally identified because of its ability to bind oxysterols. OSBP and its homologues constitute a large family of lipid transfer proteins. They are conserved throughout the eukaryotic kingdom and play diverse functions in a wide variety of cellular processes including lipid metabolism, cell signaling, vesicular and non-vesicular trafficking. In humans, 12 ORP genes encode more than 16 different protein products through differential pre-mRNA splicing and use of alternate promoters. The core lipid binding OSBP-related domain (ORD) at the C-terminus containing the conserved motif EQVSHHPP is the feature common for all ORPs. Many ORPs are dual membrane-targeting proteins: This means that they carry a two phenylalanines in an acidic tract (FFAT) motif which anchors the proteins to
the ER membrane; a pleckstrin homology (PH) domain at the N-terminus of many ORPs binds phosphoinositides, thus also contributing to the subcellular localization of the proteins. This specific characteristic suggests that ORPs have profound roles at membrane contact sites, at which ER is closely apposed with other organelles. ORPs regulate cellular lipid homeostasis and signaling and their roles in human disease are emerging. It is thus extremely interesting and of importance to investigate the function of ORP in cellular metabolism and in pathways disturbed in human disease.
2 REVIEW OF THE LITERATURE

2.1 Lipids

More than 1,000 chemically distinct lipid species are organized into the separate membrane-bound compartments or organelles of eukaryotic cells (Sleigh. 1987). The primary cellular role of lipids is to form the bilayer barrier of cells and organelles and further to contribute to the intrinsic properties of membranes, such as thickness, permeability, asymmetry and curvature. Thus, different biological membranes vary in lipid composition. For example, plasma membrane (PM) is enriched in sterols and sphingolipids and exhibits a transverse lipid asymmetry, whereas the endoplasmic reticulum (ER), which contains a low level of both of the above lipids, displays a symmetrical transbilayer lipid distribution (Pomorski, et al. 2001).

Lipids in mammalian cell membranes are divided into three major groups: glycerolipids, sphingolipids and sterols (Sprong, et al. 2001). Glycerophospholipids, derived from glycerol-3-phosphate with two fatty acid chains, are the major building blocks of membranes (Figure 1). Another important group of glycerolipids are the mono-, di- and triacylglycerols. Glycerophospholipids and sphingomyelin (SM), collectively designated phospholipids (PLs) such as phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylycerol (PG), phosphatidylinositol (PI), and cardiolipin are defined based on their head groups. PLs are distributed asymmetrically in the PM. For example, PC and SM are mainly found in the outer leaflet while PE and PS are generally concentrated in the inner leaflet (van Meer, et al. 2008). In contrast, ER membrane has a symmetric lipid arrangement with enriched unsaturated glycerophospholipids which maintain the flexible property of membrane and facilitate the transportation of synthesized proteins.

Sphingolipids (Figure 1) based on a C18 sphingoid base are enriched in the PM, Golgi and endosomal membranes. Different classes of sphingolipids are defined according to the structure of their head groups. The head groups phosphocholine and phosphoethanolamine form SM and ethanolaminephosphorylceramide, respectively. Alternatively, glucose or galactose in this position produces different forms of glycosphingolipids (Figure 1) which are abundant in the outer leaflet of the PM and
luminal leaflet of intracellular vesicles. Glycosphingolipids are capable of mediating cell-cell interactions and signal transduction (Schnaar. 2004, Lahiri & Futerman. 2007).

Figure 1.
The three main classes of mammalian membrane lipids. C stands for a variety of polar head group according to species. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol (Reproduced with permission, van Meer & de Kroon. 2011). Please see text for details.

Sterols are present as different forms with comparable functions in most eukaryotic cells. Cholesterol is found in mammals, sitosterol in plants and ergosterol in fungi. Based on a planar four-ring structure (Figure 1), these sterols have similar physical properties and intracellular distribution (Bagnat, et al. 2000, Bhat & Panstruga. 2005, Prinz. 2007). Cholesterol shows a gradient distribution across the secretory system, with the lowest concentrations in the ER and the highest in the PM. Cholesterol is necessary for the viability of mammalian cells and maintenance of the permeability barrier function of the PM (Prinz. 2007).
2.2 Intracellular lipid transport

Because the concentrations of lipids differ largely in various subcellular organelles, intracellular lipid trafficking has been extensively investigated. However, how the lipids are transported to their target destinations remains not fully understood. Increasing lines of evidence suggest that lipids are delivered to their destinations through both vesicular and non-vesicular pathways (Funato & Riezman. 2001, Lev. 2010, Lev. 2012).

2.2.1 Vesicular transport

As the primary constituent of vesicles, large amounts of lipids are transported by vesicles between the various subcellular compartments. Transport of sphingolipids and glycerolipids is to a large extent based on vesicular transport. To maintain the high concentration of cholesterol in the PM, cholesterol is incorporated into vesicles destined to the PM. However, a major part of cholesterol is known to bypass the vesicle transport through the Golgi apparatus (Heino, et al. 2000). Sphingomyelin and sterols are partially segregated from Coatomer protein (COP)I-coated vesicles. This segregation process is important for the intra-Golgi transport and retrograde transport from Golgi to ER (Prinz. 2007, Holthuis & Levine. 2005). In addition, the affinity to the microdomains and the extent of membrane curvature are suggested to drive the sorting of lipids (Schmitz & Grandl. 2009a). The key regulators involved in vesicular trafficking are Rab GTPases that provide compartmental specificity for membrane trafficking (Fukuda. 2008), while vesicle fusion is mediated by soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) (Pfeffer. 1996).

2.2.2 Non-vesicular transport

Although vast majority of lipid trafficking occurs between organelles participating in vesicle transport, substantial lipid exchange has been detected between compartments which are not directly connected by the vesicular transport machinery. Such are the ER and the PM, the ER and the trans-Golgi, the ER and endosomes, the ER and lipid
droplets, as well as the ER and mitochondria (Hanada, et al. 2003, Raychaudhuri, et al. 2006). Lipid transport has also been seen under conditions in which vesicular transport was abrogated by either ATP depletion, temperature reduction or treatment with specific pharmacological inhibitors (such as brefeldin A and colchicine) (Lev. 2010, Kaplan & Simoni. 1985, Vance, et al. 1991). These findings suggest that non-vesicular transport plays an important role in intracellular lipid trafficking (e.g. Ceramide transfer protein (CERT), Funato & Riezman. 2001).

Three mechanisms, monomeric lipid exchange, lateral diffusion and transbilayer flip-flop are involved in non-vesicular lipid transport (Lev. 2010). Monomeric lipid exchange is thought to represent a major route for the transport of lipids between membranes, and is promoted by close apposition of the two membranes. Lipid molecules are moved across an aqueous phase from the outer leaflet of the donor membrane to the outer leaflet of the acceptor membrane without the requirement for metabolic energy. This process could be either spontaneous or assisted by lipid transfer proteins (LTPs). Lateral diffusion mediates lipid transport in the lateral plane of the bilayer, which majorly occurs within membranes. Transbilayer flip-flop occurs spontaneously or is mediated by proteins such as flippases and translocases. The flip-flop of lipids between the inner and outer leaflets of the membrane bilayer would further ensure monomeric lipid exchange.

Intracellular movement of lipids is greatly facilitated by the membrane contact sites (MCSs) via a non-vesicular transport mechanism (Lev. 2010). MCSs are small cytosolic gaps (10-30 nm) between the membranes of the ER and most other organelles including the PM, Golgi apparatus, lipid droplets, late endosomes, lysosomes and mitochondria. These dynamic structures have been identified in all eukaryotes and are typically enriched in proteins involved in lipid synthesis and transport or channels responsible for ion transport (Gillon, et al. 2012). MCSs are formed by the tethering of adjacent membranes via protein-protein or protein-lipid interactions. Such tethers regulate lipid composition of the tethered membranes and affect calcium homeostasis (Levine & Loewen. 2006).

In principle, non-vesicular lipid transport could occur spontaneously by lipid desorption. However, the rate of this process is slow and not sufficient to support
substantial transport of most lipids. Wirtz and Zilversmit (1968) et al. initially discovered that lipid transfer proteins (LTPs) are able to accelerate exchange of different lipid species between membranes \textit{in vitro}. Since then more and more lipid transfer proteins have been characterized according to their lipid-transfer domain (D’Angelo, et al. 2008). In addition, LTPs have been found throughout the eukaryotic kingdom and have been subdivided into different families on the basis of their sequence and structural similarity, such as SEC14, PI transfer protein (PITP), steroidogenic acute regulatory protein (STAR)-related lipid transfer (START) proteins, glycolipid-transfer protein (GLTP), sterol carrier protein 2/non-specific lipid transfer protein (SCP2/NSLTP), oxysterol-binding protein (OSBP) and its homologues (D’Angelo, et al. 2008). Commonly, they have a lipid-binding/transfer domain and most of them have additional domains with different functions, responsible for e.g. membrane targeting.

LTPs are present in two distinct conformations: a “closed” or a transport-competent confirmation in which a hinged lid domain covers the lipid accommodating tunnel, and an “open” confirmation (Prinz. 2007). The interaction between an LTP and a membrane is thought to stimulate the transformation between the two conformations. The stability of the closed lid is secured by hydrophobic interactions. The tunnel opening is induced by polar interactions between the LTP and membrane phospholipids. LTPs interact with a donor membrane, open the lipid-binding tunnel, absorb a lipid, dissociate from the donor membrane, and are found in the close conformation in the aqueous phase. At the destination on an acceptor membrane, the lipid molecule is desorbed or exchanged with another lipid while the tunnel is opening. The whole sequential process facilitates inter-organelle lipid transport and maintains the overall lipid homeostasis. In addition, some LTPs could bind both acceptor and donor membranes simultaneously (Prinz. 2007). Moreover, it is of interest to note that the membrane binding motifs of LTPs are thought to affect the transport of specific lipids through binding to other lipids/proteins on the membranes.

LTPs show different binding affinities to the headgroup and backbone of lipid substrates (Kumagai, et al. 2005). Based on their lipid-binding specificity and transfer capability, LTPs can be categorized in three major classes: phospholipid-, sterol- and sphingolipid-transfer proteins. Phospholipid-transfer proteins in mammals
represent three major classes: PC-transfer protein (PCTP), PI-transfer protein (PITP) and nonspecific LTP (NSLTP), which is, however, also capable of sterol transfer and has thus also been called sterol carrier protein 2 (SCP2) (Prinz. 2007). OSBP and OSBP-related proteins (ORP), certain members of START family (e.g., STARD3/MLN64), Niemann-Pick type C (NPC) 2 are considered to be sterol-transfer proteins. Ceramide transfer protein (CERT) and four-phosphate-adaptor protein 2 (FAPP2), which transfer ceramide and glucosylceramide respectively, and glycolipid transfer protein (GLTP) are examples of sphingolipid-transfer proteins (Yamaji, et al. 2008, Malinina, et al. 2004).

A number of LTPs have been detected at MCSs with diverse functions. For example, oxyterol binding protein related protein ORP1L was reported to induce and maintain the formation of ER-late endosome MCSs under low cholesterol conditions (Rocha, et al. 2009, Vihervaara, et al. 2011b). CERT, which shuttles ceramide between the ER and the trans-Golgi, could specifically target the ER-Golgi MCSs for transportation. It has two targeting domains – that is a pleckstrin homology (PH) domain which binds phosphatidylinositol 4-phosphate (PI4P) and a FFAT motif that interacts with the ER resident protein of vesicle associated membrane-associated proteins (VAPs) family (Hanada, et al. 2003).

### 2.3 Lipid signaling

Lipids have been recognized as signaling molecules and their levels contribute to crucial functional consequences. A major discovery in the 1980s was that diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (Ins(1,4,5)P$_3$), the products of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P$_2$ or PtdIns(4,5)P$_2$), were identified as second messengers to provoke the activation of protein kinase C (PKC) and calcium release into the cytosol (Nishizuka. 1992). Over the past decades, the eicosanoids produced from arachidonic acid have also been identified as the key signaling lipids that have the capacity to control inflammatory responses (Serhan & Savill. 2005). Specific eicosanoids apparently bind to peroxisome proliferator-activated receptors (PPARs), which further modulate lipid homeostasis (Chawla, et al. 2001). Further studies have reported that phosphatidylinositol-3,4,5-trisphosphate, a minor product
of inositol phospholipid metabolism, serves as a signaling lipid that mediates cell growth, proliferation and motility (Engelman, et al. 2006).

Sphingolipids are an important group of bioactive lipids. Of these, sphingosine is the first one to be identified. The phosphorylation of sphingosine forms sphingosine-1-phosphate that exerts important functions in cell survival, growth, migration, and inflammation (Hannun & Obeid. 2008). Ceramide plays crucial roles in cell differentiation, senescence and apoptosis. It can be generated rapidly from sphingomyelin in response to the activation of sphingomyelinase. Under different cellular stress conditions, such as DNA damage or lysosome disruption, ceramide concentrations increase. Moreover, ceramide has the capacity to regulate insulin signaling in regulating several crucial intermediates in the insulin signaling pathway (Summers. 2006).

Disturbances of the lipid signaling pathways exert pleiotropic effects on inflammatory processes and contribute to a variety of diseases such as cancer, atherosclerosis, hypertension, type 2 diabetes and cardiovascular diseases. In addition, increased lipid signaling is suggested to play a role in the development of tumors from benign to malignant stage (Wymann & Schneiter. 2008).

2.4 Oxysterols

Oxysterols are 27-carbon oxygenated products of cholesterol that arise through enzymatic or non-enzymatic oxidation processes, or are absorbed from the diet (Vaya, et al. 2011). Several major oxysterols are produced as intermediates in the biosynthesis of bile acids or steroid hormones. The common modifications of cholesterol occurring in oxysterols are hydroxyl, keto, hydroperoxy, epoxy and carboxyl moieties. The most abundant oxysterols in human serum are 24(S)-, 27-, 7α-, and 4β-hydroxycholesterol (OHC) (Figure 2), which are generated in reactions catalyzed by mitochondrial or ER cholesterol hydroxylases belonging to the cytochrome P450 family (Olkkonen. 2009). 24(S)-OHC is exclusively present in neurons of the central nervous system (CNS). The synthesis of 24(S)-OHC catalyzed by the cholesterol hydroxylase CYP46A1 maintains the CNS sterol homeostasis.
27-OHC, which is generated in the liver, catalyzed by CYP27A1, serves as an intermediate of the alternative, so called acidic bile acid synthetic pathway (Bjorkhem & Eggertsen. 2001). CYP27A1, is however also expressed by other, non-hepatic cells (Babiker, et al. 1997). Similarly, 7α-OHC is an intermediate in the neutral pathway of the bile acid synthesis. Thus, 7α-OHC is primarily produced in the liver catalyzed by CYP7A1. 4β-OHC is converted from cholesterol by the drug metabolizing enzyme CYP3A4 which is induced by certain anti-epileptic pharmaceuticals (Bjorkhem & Eggertsen. 2001).

Oxysterols can also be synthesized through non-enzymatic, free radical or lipid peroxide processes, which are often designated cholesterol autoxidation. For example, 1% of the sterols in the western diet has been estimated to be non-enzymatically oxidized (van de Bovenkamp, et al. 1988). The autoxidation commonly occurs at the 7-position of the cholesterol B-ring, which yields 7-ketocholesterol (7-KC), 7α-OHC or 7β-OHC (Figure 2).
2.4.1 Oxysterols in Signaling and Development

2.4.1.1 Oxysterols regulate Hedgehog signaling pathway

The Hedgehog (Hh) signaling pathway plays a fundamental role in embryonic development, physiological processes and stem-cell renewal. Cororan and Scott first reported that cholesterol or certain oxysterols are critically required for Sonic Hh pathway signal transduction (Corcoran & Scott. 2006). Dwyer et al. demonstrated that 20(S)- and 22(S)-OHC induce the osteoinductive effects through the activation of Hh signaling in pluripotent mesenchymal cells (Dwyer, et al. 2007). Furthermore, Parhami's group found that 22R-OHC, 20(S)-OHC and its analogues exert effects of inhibiting adipocyte differentiation through the Hh signaling pathway (Kha, et al. 2004, Kim, et al. 2007, Johnson, et al. 2011). Very recently, Nachtergaele et al. presented the evidence that the enantiomer and an epimer of 20(S)-OHC regulate Hh
signaling pathway through activation of a specific target protein Smoothened (Nachtergaele, et al. 2012). These lines of evidence established a novel role of oxysterols as developmental regulators.

2.4.1.2 27-OHC regulates estrogen receptor function

Two different forms of the estrogen receptor, ER-α and ER-β, are members of nuclear hormone superfamily that mediate a number of physiological processes. One important role of estrogen receptors is the regulation of cardiovascular system where estrogens exert protective effects on the blood-vessel wall (Mendelsohn & Karas. 1999). Umetani et al. reported that 27-OHC directly antagonizes the transcriptional and non-transcriptional functions of the estrogen receptors, resulting in a loss of the cardioprotective function of estrogen (Umetani, et al. 2007). Furthermore, they demonstrated that 27-OHC acts as an endogenous selective estrogen receptor modulator based on its cell-type specific proestrogenic action.

2.4.2 Oxysterols mediate regulation of lipid metabolism

2.4.2.1 Oxysterols act as the ligands of Liver X receptor

There are two isoforms of nuclear receptors called liver X receptors (LXRs) in mammals, LXRα and LXRβ. LXRα is abundant in liver, intestine, spleen, kidney, macrophages and adipose tissue, while LXRβ is ubiquitously expressed at a low level (Beltowski. 2008). LXRs form heterodimers with retinoid X receptor (RXR), a common partner for several nuclear receptors such as peroxisome proliferator activated receptors (PPARs) and vitamin D receptor. The formed complex can be activated through liganding of either partner to regulate gene expression in response to the recruitment of coactivators or corepressors. The activation further regulates the intestinal cholesterol absorption, reverse cholesterol transport, bile acid synthesis, cholesterol synthesis, hepatic lipogenesis, high density lipoprotein (HDL) formation and inflammatory conditions (Beltowski. 2008). In addition, several studies have reported that LXR agonists reduce the size of atherosclerotic lesions in mouse models (Joseph, et al. 2002, Terasaka, et al. 2003). This effective protection is valid not only
during the development of lesions but also in the established atherosclerosis (Levin, et al. 2005). Increasing lines of evidence clearly show that LXR agonists are thus potential drugs for atherosclerosis.

Janowski et al. first demonstrated that oxysterols are physiological activating ligands for LXR (Janowski, et al. 1996). Since then, the interest towards oxysterols has greatly intensified. It has been reported that 24(S)-OHC, 22(R)-OHC, 20(S)-OHC, 27-OHC and 24(S),25-epoxycholesterol (EPOX), are the common endogenous LXR agonists (Janowski, et al. 1996, Lehmann, et al. 1997, Fu, et al. 2001). Triple-knockout mice lacking CYP46A1, CYP27A1 and cholesterol 25-hydroxylase responsible for the biosynthesis of three oxysterols, 24S-OHC, 25-OHC, and 27-OHC, showed impaired responses of LXR target genes to dietary cholesterol, confirming the view that endogenous oxysterols have an essential role in LXR activation (Chen, et al. 2007). However, it should be noted that not all oxysterols are LXR ligands. Most non-enzymatically produced oxysterols such as 7α-OHC and 7β-OHC are not ligands for the LXR (Janowski, et al. 1999).

### 2.4.2.2 Oxysterols regulate sterol regulatory element binding protein pathway

Sterol regulatory element binding proteins (SREBPs), designated as SREBP-1a, SREBP-1c, and SREBP-2, are transcriptional factors and control fatty acid and cholesterol synthesis. SREBPs are synthesized in the ER as membrane-bound precursors and form complexes with another membrane protein, SREBP cleavage activating protein (SCAP). SCAP is considered to be a cholesterol-sensor protein which controls the intracellular localization and proteolytic maturation of SREBPs. In the conditions of low sterol levels, SREBP-SCAP are delivered to the Golgi apparatus by COPII vesicles, where SREBP are proteolytically cleaved to release a soluble N-terminal domain that is translocated to the nucleus and binds to specific sterol regulatory element DNA sequences TCACNCCAC in the promoter regions of target genes. This binding triggers the expression of encoded proteins such as LDL-receptor and enzymes involved in sterol synthesis. When sterol levels are sufficient in the cells, the sterols induce SCAP to bind to ER anchor proteins called Insig (Insulin-induced gene), and COPII can no longer bind to SCAP. Hence, SREBP are inhibited from
moving to Golgi and are retained in the ER. Although cholesterol and oxysterols both stimulate the interaction between SCAP and Insig proteins, they operate in two different, converging mechanisms. Cholesterol binds to SCAP whereas 25-OHC directly binds to the Insig proteins, eliciting their conformational change, which increases the binding affinity of SCAP-Insig complexes and prevents SREBP exit from the ER (Adams, et al. 2004). Oxysterols associated with Insig also induce the degradation and ubiquitination of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR), a key rate limiting enzyme of the sterol biosynthetic pathway, in a sterol-dependent manner. 24(S)-25 EPOX, a side product of cholesterol biosynthesis (Figure 2), works as a potent warning system to acutely regulate cholesterol synthesis by suppressing SREBP2 processing and HMGCoAR activity (Janowski, et al. 2001, Wong, et al. 2007).

2.4.2.3 Oxysterols regulate other transcriptional factors

As discussed in chapter 2.4.2.1, it is well established that oxysterols act as agonists for LXRs. Moreover, oxysterols have been reported as the ligands for certain other transcriptional factors. Several specific oxysterols could increase steroidogenic factor-1 (SF-1)-dependent steroidogenic acute regulatory protein (StAR) promoter activity, as well as promoters of P450 enzymes in the specific cell types (Reyland, et al. 2000). StAR enhances the delivery of cholesterol to the mitochondrial membrane and regulates the synthesis of steroid hormones in steroidogenic tissues.

It is widely accepted that bile salt export pump (BSEP) is responsible for the bile acid secretion. 22(R)-OHC has been reported to markedly induce BSEP expression through binding to the nuclear receptor farnesoid X receptor (FXR) instead of LXR (Deng, et al. 2006).

The retinoid acid receptor-related orphan receptors (RORs), including the α, β, γ isoforms, are important nuclear receptors: RORα regulates cerebellum development, bone formation and immune response while RORγ plays an essential role in the development of lymphoid tissues and T cells. Both of these receptors are able to operate as regulators of circadian rhythms (Kang, et al. 2007). Wang et al. found that
the transcriptional activity of RORα and RORγ could be suppressed by direct binding of the antagonists 7α-OHC or 24(S)-OHC, respectively (Wang, et al. 2010b, Wang, et al. 2010a). Moreover, they found that 24(R)-OHC and 24(S),25-EPOX both regulated RORγ activity selectively. These findings suggest that RORα and RORγ may serve as sensors of oxysterols and have overlapping ligand preference and functional cooperation with LXR (Wang, et al. 2010a).

2.4.3 Oxysterols induce apoptosis

Apoptosis is a genetically controlled process of programmed cell death characterized by distinct morphological characteristics and energy-dependent mechanisms. Normally, apoptosis occurs during embryonic development and aging. It is well accepted as a homeostatic mechanism to maintain cell populations in tissues and a defense mechanism in immune responses (Elmore. 2007). The apoptosis process includes the activation of a family of cysteine proteases called “caspases” and a complex of events related to the cell death in response to a wide variety of stimuli, irradiation, hormones and oxysterols (Elmore. 2007).

Oxysterols modify the biophysical and biochemical properties of membranes and have the capacity to induce cell death through two major apoptotic pathways, the mitochondrial (intrinsic) pathway and the receptor-dependent (extrinsic) pathway. Notably, the apoptotic effects and employed pathways of apoptosis depend on the particular oxysterol and the cell type. Hence, it is most likely that there is no universal mechanism of apoptosis induced by oxysterols (Lukyanenko & Lukyanenko. 2009, Olkkonen, et al. 2012a). Because of the cytotoxic and pro-apoptotic functions, oxysterols are suggested to be involved in the progress of diseases such as atherosclerosis and biliary tract diseases (Shibata & Glass. 2010, Wang & Afdhal. 2001).

2.4.4 Oxysterols and metabolic diseases

A number of studies suggest that the plasma concentrations of oxysterols could be used as indicators of oxidative stress in vivo. Oxysterols have been detected to be
remarkably accumulated in LDL fractions of coronary artery disease (CAD) patients compared to the controls (Olkkonen. 2009). Moreover, it was found that plasma 7-KC and 7β-OHC were significantly higher in patients with stable CAD compared to controls with normal coronary arteries (Rimner, et al. 2005). It was reported that the levels of 7-KC and 7β-OHC were remarkably elevated in the plasma of familiar combined hyperlipidemia (FCH) patients compared to sex- and age-matched healthy controls (Arca, et al. 2007). 7-KC concentration in plasma was found to be positively associated with risk of type 2 diabetes (T2D) mellitus, especially in the group with multiple coronary risk factors (Endo, et al. 2008). The plasma concentration of 7β-hydroxycholesterol was reported higher in the patients with T2D than those without T2D, or with impaired glucose tolerance (Ferderbar, et al. 2007). 27-OHC is regarded as the most abundant oxysterol in atherosclerotic lesions, the concentration of which is associated with the severity of atherosclerosis (Garcia-Cruset, et al. 2001).

Several oxysterols have been proposed as disease markers. However, reliable quantification of oxysterol levels in clinical samples depends on sample freshness, storage method, assay technique and sample cholesterol levels (Lukyanenko & Lukyanenko. 2009). Notably, it is not reliable to quantify the levels of non-enzymatically formed oxysterols in clinical samples because they easily arise artefactually (Gill, et al. 2008). For example, concerning the measurement of 7-KC in the cerebrospinal fluid from patients, the results from two groups varied by more than three orders of magnitude (Diestel, et al. 2003, Leoni, et al. 2005). Hence, it is important to carefully optimize the methodology employed to quantify the levels of oxysterols in clinical samples.

### 2.4.5 Oxysterols in Niemann-Pick type C disease

Niemann-Pick type C (NPC) disease is a rare autosomal recessive neurodegeneration disorder characterized by accumulation of cholesterol and other lipids in late endosomal/lysosomal compartments (Vazquez, et al. 2012). Approximately 95% of NPC cases are caused by mutations in the NPC1 gene while the remaining 5% are caused by NPC2 gene. The NPC1 gene encodes a membrane protein that localizes in the late endosomal compartment. The NPC1 protein has 13 trans-membrane domains.
that have homology to HMGCoAR and SCAP. The NPC2 gene encodes a soluble lysosomal protein that binds cholesterol. In the late endosomal/lysosomal compartments, NPC2 delivers LDL-derived cholesterol to the N-terminal domain of NPC1, which facilitates the insertion of cholesterol to the outer lysosome membrane (Kwon, et al. 2009). Subsequently, NPC1 mediates the exit of cholesterol from lysosomes, a process in which the OSBP homologue ORP5 is suggested to play an important role (Du, et al. 2011).

The markedly reduced ability to esterify LDL-derived cholesterol is a defining characteristic of NPC disease. Remarkably, the production of oxysterols is impaired in the NPC disease cells, which might be accounted by the disturbances of cholesterol homeostasis (Frolov, et al. 2003). In addition, Xie et al. (2003) observed decreased production of 24(S)-OHC in the NPC1 knockout mice. Furthermore, Infante and colleagues found that 24(S)-OHC, 25-OHC and 27-OHC bind to the N-terminal luminal loop of NPC1, suggesting a direct functional cross-talk between oxysterols and NPC1, which may represent a regulatory ligand interaction (Infante, et al. 2008).

Moreover, oxysterols have been suggested to play roles in the pathology of other degenerative disorders such as Alzheimer’s disease and age-related macular degeneration (Jeitner, et al. 2011, Javitt & Javitt. 2009).
2.5 Cytoplasmic oxysterol binding proteins

A number of LTPs are suggested to transfer sterols between cellular compartments through non-vesicular pathways. As a large family of LTPs, the cytoplasmic oxysterol binding protein, OSBP, and its homologues are conserved in the eukaryotic kingdom from yeast to humans (Olkkonen & Levine. 2004, Olkkonen. 2009). In mammals, 12 ORP genes encode a large number of proteins designated as OSBP-related (ORP) or OSBP-like (OSBPL) proteins. The ORPs have been implicated in many cellular processes including lipid metabolism, cell signaling, vesicular trafficking and non-vesicular sterol transfer. Most ORPs genes are expressed ubiquitously. However, the distinct tissues and cell types display marked quantitative differences in the expression pattern of ORP variants (Johansson, et al. 2003, Lehto, et al. 2004).

2.5.1 Identification of OSBP and ORPs

Four decades ago, scientists found that oxysterols were much more potent than cholesterol itself in suppressing the activity of HMGCoAR, thus thought to act as feedback signals for the inhibition of cholesterol synthesis (Brown & Goldstein. 1974, Kandutsch & Chen. 1974, Kandutsch, et al. 1978). These findings prompted a search for oxysterol responsive proteins that led to the identification of OSBP by Taylor and co-workers (Taylor, et al. 1984, Taylor & Kandutsch. 1985). Subsequently, OSBP protein was purified and cDNAs were cloned from rabbit and human (Dawson, et al. 1989a, Dawson, et al. 1989b). OSBP was identified as a cytosolic protein which binds to oxysterols with high affinity (Levanon, et al. 1990). Treatment of cells with 25-OHC resulted in the translocation of OSBP from a cytosolic or vesicular compartment to Golgi membranes rather than the nucleus, indicating that OSBP did not regulate transcription in a direct manner (Ridgway, et al. 1992).

The cloning of OSBP subsequently led to the identification of its homologues from a spectrum of eukaryotic organisms. To date, ORPs have been most studied in mammals and the yeast Saccharomyces cerevisiae. In addition, OSBP and ORPs have been investigated in Drosophila melanogaster (Alphey, et al. 1998), Caenorhabditis elegans (Sugawara, et al. 2001, Kobuna, et al. 2010), Dictyostelium discoideum
The existence of ORPs throughout the eukaryotic kingdom reveals a fundamental and important role of the ORPs family in eukaryotic evolution.

2.5.2 Structure of ORPs

In humans, 12 ORP genes encode more than 16 different protein products through the use of different promoters and via differential pre-mRNA splicing. The proteins can be divided into 6 subfamilies according to the sequence homology (Figure 3). The mouse genome contains 12 ORP genes with high homology to human ORPs. The yeast *S. cerevisiae* genome encodes 7 OSBP homologues called Osh1p-7p, the pioneering studies of which have provided the evidence to deeply understand the ORP structure and functions (Im, et al. 2005, Georgiev, et al. 2011, de Saint-Jean, et al. 2011). The short yeast ORP, Osh4p, carrying 435 amino acids, is the only ORP the structure of which has been solved at atomic resolution (Im, et al. 2005). Structure of Osh4p was determined in complex with cholesterol, several oxysterols and ergosterol, the main sterol in yeast. All these virtually identical structures revealed that the protein has an almost complete β-barrel formed by 19 anti-parallel β sheets, which operates as a lipid binding pocket or tunnel to accommodate sterols. Moreover, the structure contains a flexible lid domain that shields the bound sterol from the solvent. The interaction between sterol side chains and the hydrophobic inner surface of the lid stabilizes the closed confirmation. Notably, the ligand interactions are substantially flexible because of the mediating effects by water molecules within the pocket, explaining the ability of the protein to bind a wide range of sterols. Sterols could enter and exit the binding pocket when the lid is open, which may be triggered by the interaction with membranes. Hence, the conformational change in the protein is stimulated upon binding and release of sterols. In addition, structures of the OSBP-related ligand binding domains (ORDs) of mammalian ORPs have been modeled on the basis of the Osh4p structure (Vihervaara, et al. 2011b, Suchanek, et al. 2007). This structure suggests that ORPs might act as sterol transporters or mediators of sterol signals.
The common feature for all ORPs is the core lipid binding ORD domain at the C-terminus, which contains the conserved OSBP signature motif EQVSHHPP. A majority of ORPs in mammals, and some in yeast, carry long N-terminal extensions containing a pleckstrin homology (PH) domain. The PH domains of ORPs have been found to bind phosphoinositides (PIPs) (Johansson, et al. 2005, Lemmon. 2007, Niisila, et al. 2012), which control the subcellular localization of proteins. The proteins containing only the ORP domain are called “short ORPs”, while those carrying a PH domain are designated “long ORPs”. Moreover, many ORPs also carry a two phenylalanines in an acidic tract (FFAT) motif in the region between the PH domain and ORD, which commonly exists in the proteins involved in lipid metabolism and is found to bind by ER-resident proteins called VAPs. Hence, some ORPs, with both PH and FFAT domains, act as dual membrane-targeting proteins. However, not all ORPs have these domains: ORP1S and ORP4S lack both, ORP2 and ORP9S have FFAT motifs but no PH domain, whereas ORP10 and ORP11 lack FFAT motifs but have PH domains. Moreover, some ORPs have ankyrin repeats (ANK) in their N-terminal regions, which are thought to mediate protein-protein interactions (Figure 3).
Figure 3.

A schematic presentation of mammalian ORP protein structures. The roman numerals on the right side indicate six subfamilies I-VI of ORP proteins. The color codes are: green, ORP domain; OSBP-related (ligand binding) domain; yellow, OSBP-fingerprint (OF) motif; red, pleckstrin homology (PH) domain; purple, ankyrin repeats; black, FFAT motif, two phenylalanines in an acidic tract targeting VAP of the ER; turquoise, transmembrane domain (TM).

2.5.3 Cellular sterol ligands of ORPs

OSBP was originally identified because of its ability to bind oxysterols (Dawson, et al. 1989b). Subsequently, OSBP was found to bind cholesterol as well (Wang, et al. 2005). The modeling of mammalian ORP structures using Osh4p suggests that they all have the ability to bind sterols. Using photo-cross-linkable sterol derivatives in live cells, Suchanek and coworkers found that most of the mammalian ORPs may bind
sterols (Suchanek, et al. 2007). The results have been confirmed in *in vitro* experiments by using sterol binding assays for ORP1 (Yan, et al. 2007a), ORP2 (Hynynen, et al. 2009), ORP4 (Moreira, et al. 2001, Wang, et al. 2002), ORP8 (Yan, et al. 2008), ORP9 (Ngo & Ridgway. 2009) and ORP10 (Nissilä, et al. 2012). However, the binding affinity to individual sterols varies among the ORPs. OSBP and ORP4 have been found to show higher affinity to oxysterols than cholesterol (Wang, et al. 2008, Wyles, et al. 2007). Importantly, all ORPs appear to be able to bind cholesterol. For instance, ORP9 and its close relative ORP10 were recently found to bind cholesterol but not oxysterols (Nissilä, et al. 2012, Ngo & Ridgway. 2009).

Fragments of OSBP have been found to retain ability to bind sterol, indicating that the intact ORD of OSBP may not in all cases be required for sterol binding (Wang, et al. 2008). Indeed, the authors suggest that the lid domain of OSBP is not only the region which controls the entrance and exit of sterols but also acts as the primary sterol-binding site. The sterol might first anchor to the lid domain and subsequently be moved into the binding pocket. Moreover, the authors found that a glycine/alanine rich region near the N-terminus of OSBP, together with PH domain, appears to regulate cholesterol binding (Wang, et al. 2008).

### 2.5.4 Subcellular distribution of ORPs

The subcellular localization is important to identify the cellular functions of ORPs. Binding of specific PIPs by PH domains of ORPs, in some cases, contributes to the localization of the proteins in the cells (Levine & Munro. 2002). For instance, the localization of OSBP in the trans-Golgi network (TGN) is regulated by the interaction between its PH domain and PI4P. Addition of 25-OHC to the cells induces the translocation of OSBP from cytosol to TGN possibly through a ligand-activated conformational change that exposes the PH domain (Ridgway, et al. 1992). The TGN localization of OSBP also depends on ADP-ribosylation factor (ARF) 1, a small GTPase (Levine & Munro. 2002). Moreover, targeting of yeast Osh1p to Golgi occurs in a similar manner (Roy & Levine. 2004). Very recently, Ridgway and coworkers found that phosphorylation on two serine-rich motifs specifically controls the localization of OSBP in the ER via interaction with VAP (Goto, et al. 2012). The PH
domains of certain other “long” ORPs such as ORP1L (Johansson, et al. 2003, Johansson, et al. 2005), ORP3, ORP6, ORP7 (Lehto, et al. 2004) and ORP9 (Ngo & Ridgway. 2009, Wyles & Ridgway. 2004) have prominent influences on their localizations.

Most mammalian ORPs, OSBP, ORP1, 2, 3, 4, 6, 7, and 9 contain a FFAT motif that binds VAPs, ER-resident integral membrane proteins. In yeast, Osh1p, Osh2p and Osh3p, contain a FFAT-motif. They bind suppressor of choline sensitivity 2 (Scs2p), a primary VAP homolog in the yeast which is regulated by the phospholipid composition in the ER (Loewen & Levine. 2005). ORP5 and ORP8, lacking a FFAT motif, anchor to the ER by a C-terminal transmembrane segment (Ngo, et al. 2010).

Mammalian ORP1L and S. cerevisiae Osh1p and Osh2p, have motifs with ankyrin repeats (ANK) in their N-terminal regions, which are involved in protein-protein interactions (Li, et al. 2006). The ANK region of ORP1L binds to the GTP-bound active form of Rab7, a late endosomal (LE) small GTPase. The interaction contributes to the localization of ORP1L to a MCS between the ER and LE. Under low cholesterol conditions, a conformational change of ORP1L allows its ANK region and FFAT motif to bind Rab7 and VAP protein, respectively, at the same time, which induces the formation of contact sites between the ER and the LE (Rocha, et al. 2009). In analogy with ORP1L, OSBP and ORP9 may localize to the MCSs between the ER and the Golgi (Peretti, et al. 2008, Ngo & Ridgway. 2009, Raychaudhuri & Prinz. 2010).

The multiple targeting domains found in most ORPs facilitate them to shuttle between two different compartments or to be enriched in MCS regions. In addition, binding of the FFAT motif and the PH domain to ER and non-ER membrane compartments simultaneously might facilitate the formation of close contact sites between the ER and a second organelle (Olkkonen & Levine. 2004, Raychaudhuri & Prinz. 2010). Increasing lines of evidence suggest that ORPs might operate as LTPs at the MCSs, to regulate lipid transport or directly move lipids between the apposed membranes of the two organelles (Du, et al. 2011, Ngo & Ridgway. 2009).
2.5.5 ORPs act as sterol transporters

The yeast ORP homologue Osh4p is able to transfer sterols from liposomes to acceptor membranes. Moreover, Raychaudhuri et al. (2006) reported that depletion of all seven Osh proteins decreased transfer of cholesterol or ergosterol from the PM to the ER by 80%. Subsequently, Georgiev et al. (2011) used the fluorescent sterol dehydroergosterol (DHE) to monitor the lipid traffic from the PM to the lipid droplets. They found that cells deficient of all 7 Osh proteins displayed only a 50% reduction of DHE transfer rate and a minor effect on ergosterol movement from the ER to the PM. The authors thus concluded that the small effect in transport rate reflects the dispensable role of Osh proteins for intracellular sterol transport, bringing up the possibility that Osh proteins might rather regulate undefined downstream events. Interestingly, de Saint-Jean et al. (2011) recently demonstrated that Osh4p efficiently exchanges DHE for phosphatidylinositol-4-phosphate (PI4P) in vitro and is thus able to transport these two lipids between membranes along opposite routes. The authors proposed a model: on one hand, Osh4p transfer sterols from ER to the trans-Golgi; on the other hand, it transports PI4P in the backward direction. The sterol/PI4P exchange activity of Osh4p promotes sterol enrichment of membranes of the late secretory pathway and regulates distribution of PI4P. In addition, most of the residues responsible for PI4P recognition are conserved in Osh/ORP proteins, indicating that ORPs potentially act as sterol/phosphoinositol phosphate exchangers (de Saint-Jean, et al. 2011).

Several groups have reported that ORPs, OSBP, ORP1S, ORP2, ORP9L, and the ORP5 ORD are able to transfer cholesterol between membranes in vitro (Du, et al. 2011, Ngo & Ridgway. 2009, Jansen, et al. 2011). However, only limited evidence shows that ORPs play roles as cholesterol transporters in live cells. Du et al. (2011) found that knocking down ORP5, an ER membrane protein, resulted in cholesterol accumulation in late endosomes and lysosomes. The authors afterward proposed a model that ORP5, in collaboration with NPC1, might regulate cholesterol transport from the endosomes/lysosomes to the ER. Jansen et al. recently reported that overexpression of two short ORPs, ORP1S and ORP2, significantly enhanced cholesterol transport from the PM to the ER. Silencing of ORP1S and ORP2 simultaneously attenuated cholesterol transport from the PM to the ER and lipid
droplets (LDs), suggesting a function of these proteins in intracellular cholesterol trafficking (Jansen, et al. 2011).

Despite the fact that purified ORPs can act as sterol transporters in vitro, scientists have not reach a consensus on the putative role of ORPs as sterol transporters in vivo. Schulz and colleagues found that all 7 Osh proteins could contact two distinct membranes simultaneously, an activity which might efficiently regulate sterol extraction and delivery between the donor and acceptor membranes (Schulz, et al. 2009). Through interaction with two membranes simultaneously, ORPs, hence, have the potential to transport sterols at MCSs via flipping the sterols across the aqueous phase. However, Geogiev et al. recently provided data suggesting that ORPs impact sterol fluxes through altering the lateral organization of membrane lipids (Georgiev, et al. 2011). Concerning the discrepant evidence, one cannot exclude the possibility that ORPs could have an indirect, regulatory function in sterol trafficking.

2.5.6 ORPs regulate cellular lipid homeostasis

OSBP overexpression was reported to stimulate cholesterol biosynthesis and reduce cholesterol esterification (Lagace, et al. 1997). Cellular sterol status affects the localization of OSBP at Golgi, suggesting that OSBP is a Golgi sterol content sensor. In addition, OSBP is involved in activation of ceramide transport from ER to trans-Golgi by CERT and SM synthesis (Perry & Ridgway. 2006, Banerji, et al. 2010). OSBP phosphorylation by protein kinase D inhibits its Golgi localization in response to 25-hydroxycholesterol stimulation or cholesterol depletion, impairs transport of ceramide from the ER to the Golgi and induces Golgi fragmentation (Nhek, et al. 2010). Independently of this function, Bowden and Ridgway reported that OSBP regulates cholesterol efflux and the stability of ATP-binding cassette transporter A1 (ABCA1) (Bowden & Ridgway. 2008). Furthermore, our group showed that adenoviral overexpression of OSBP in mouse liver enhances plasma lipogenesis and very-low-density lipoprotein (VLDL) secretion, the underlying mechanism apparently being impact on the transcription factor SREBP-1c (Yan, et al. 2007b).
In addition to OSBP, increasing lines of evidence suggest that also other ORPs are associated with lipid homeostasis. The localization of ORP2 on the surface of cytoplasmic LDs depends on oxysterol binding (Hynynen, et al. 2009). Silencing of ORP2 affects TG hydrolysis and cholesterol esterification, while overexpression of ORP2 stimulates cellular cholesterol efflux (Hynynen, et al. 2005). Furthermore, our group found that macrophage ORP1L overexpression in LDL receptor deficient mice reduced cholesterol efflux to HDL and increased the size of atherosclerotic lesions (Yan, et al. 2007a). Silencing of ORP1L in macrophage foam cells inhibits cholesterol efflux to apolipoprotein A-I (apoA-I) (Vihervaara, et al. 2011b). Moreover, ORP8 in human macrophages was reported to modulate ABCA1 expression and cholesterol efflux to apoA-I (Yan, et al. 2008). Zhou et al. recently reported that ORP8 overexpression in mouse liver reduced levels of cholesterol, phospholipids and TG in plasma and liver tissues. They found that ORP8 could moderately mediate the expression of SREBP-1 and SREBP-2 (Zhou, et al. 2011). Interestingly, our group recently demonstrated that silencing of 3 ORPs individually has distinct effects on the lipidome of mouse macrophage cells (Vihervaara, et al. 2012). These findings indicate that ORPs are involved in the control of cellular sterol and lipid homeostasis. However, the underlying mechanisms remain poorly understood. It is likely that altered sterol status or distribution upon ORP manipulation diversely affect a wide range of events involved in lipid metabolism such as the endogenous oxysterol syntheses, the lipid composition of subcellular membranes and the abundance of MCSs.

### 2.5.7 The role of ORPs at MCSs (membrane contact sites)

The ORP proteins comprise several motifs that control subcellular localization and regulate their interactions with proteins or lipids (Figure 3). Most ORPs have an ER-anchored fragment, either a FFAT motif which associates with ER membrane proteins, VAPs or an ER-targeting membrane segment (Du, et al. 2011, Yan, et al. 2008, Olkkonen, et al. 2012b). ER membrane, hence, is likely to be one target for the ORPs, while PH domain in the N-terminal, in several cases, acts as a determinant for targeting ORP proteins to a second membrane enriched in PIPs. This implies that ORPs might, via membrane interactions, localize at MCSs between ER and the
apposed organelle (Figure 4). Schulz et al. reported that 4 Osh proteins, Osh2p, Osh3p, Osh6p, and Osh7p, are enriched in ER-PM junctions (Schulz, et al. 2009). Stefan and co-workers showed that the yeast ORP Osh3p acts at ER/PM contact sites as a sensor of PM PI4P and an activator of the ER Sac1 phosphatase (Stefan, et al. 2011). Moreover, Osh1p was found to localize at the nucleus-vacuole junction (NVJ), where the nucleus and the vacuole are closely apposed. NVJ is a structure unique for yeast cells, the formation of which, however, does not depend on Osh1p (Kvam & Goldfarb. 2004).

In mammalian cells, several lines of evidence suggest that some ORPs such as OSBP (Peretti, et al. 2008), ORP1L (Rocha, et al. 2009, Vihervaara, et al. 2011b) and ORP5 (Du, et al. 2011) locate at MCSs. Three LTPs, OSBP, CERT and Nir2 at the ER–Golgi membrane contact sites, coordinately regulate lipid composition of the Golgi (Peretti, et al. 2008). Rocha and colleagues reported that ORP1L acts as a cholesterol sensor that regulates the formation of ER-LE contact sites (Rocha, et al. 2009). Under high cholesterol conditions, the Rab7 effector Rab7-interacting lysosomal protein (RILP), in complex with ORP1L and Rab7, interacts with the subunit of dynein-dynactin motor, p150(Glued). Under low cholesterol conditions, ORP1L undergoes a conformational change to bind the ER membrane protein VAP, which induces the formation of a junction between the ER and the LE, and removes p150(Glued) and associated motors. ORP5 has a C-terminal trans-membrane segment which anchors it in the ER. Moreover, ORP5 associates with the LE through an interaction with the late endosomal cholesterol egress mediator NPC1 protein. Du et al. proposed a model that ORP5 and NPC1 might coordinately induce the formation of LE-ER membrane contact sites which facilitate the transport of endosomal cholesterol by ORP5 from LE to the ER (Du, et al. 2011). These pieces of evidence suggest that certain ORP family members are involved in MCS formation, or operate as regulatory factors or lipid transporters at such sites.

2.5.8 ORPs modulate signaling processes

OSBP acts as a cholesterol-sensing protein that regulates the activity of the extracellular signal-activated kinases (ERK) signaling pathway (Wang, et al. 2005).
Furthermore, OSBP modifies JAK2/STAT3 signaling in response to 7-ketocholesterol, which in turn controls the expression of the proatherogenic protein profilin-1 (Romeo & Kazlauskas. 2008). Interestingly, increasing lines of evidence indicate that also other members of the ORP family may exert similar scaffolding functions in cell signaling pathways. Lessmann and coworkers first reported that ORP9 plays a role as a novel modifier of the phosphatidylinositol 3-kinase (PI3K) pathway in different cell types. According to them, ORP9 acts as a substrate of phosphoinositide-dependent kinase-2 (PDK-2) to negatively regulate Akt phosphorylation (Lessmann, et al. 2007). Furthermore, Goldfinger et al. identified ORP3 and ORP7 as R-Ras-interacting proteins by immunoprecipitation and mass spectrometry (Goldfinger, et al. 2007). R-Ras is a small GTPase of the Ras family that regulates cell adhesion, spreading and migration. Interestingly, our group found that ORP3 exerts similar cellular effects as R-Ras by regulating cell adhesion, spreading and integrin activity (Lehto, et al. 2008). In addition, recent findings on yeast Osh proteins suggest that ORPs are potentially involved in diverse signaling processes via modulation of PIP metabolism (de Saint-Jean, et al. 2011).

Cholesterol, a major constituent of cell membrane, exerts essential functions in a wide variety of signal transduction processes (Lingwood and Simons, 2010). Overexpression or silencing of ORPs affects cholesterol distribution or intracellular transportation (Hynynen, et al. 2009, Jansen, et al. 2011). Disturbance of ORP functions, hence, is likely to impinge on signal transduction via modification of lipid microdomain-associated processes. However, further investigations are required to elucidate this issue in detail.

2.5.9 The roles of ORPs in human diseases

The functions of ORPs proposed above raise the question of their putative roles in disease. In fact, polymorphisms of several ORPs have already been reported to be associated with metabolic disorders. Our group found that intronic variants of ORP10 associate with high plasma TG levels in Finnish dyslipidemic patients, the underlying mechanism possibly involving a functional role of ORP10 in hepatocyte apolipoprotein B-100 (apoB-100) secretion (Nissilä, et al. 2012, Perttilä, et al. 2009).
Subsequently, ORP10 variants were reported to associate with elevated plasma levels of LDL-cholesterol (Koriyama, et al. 2010a) and peripheral artery disease (Koriyama, et al. 2010b) in the Japanese population. These findings support an idea that ORP10 may play an important role in the development of dyslipidemias and atherosclerosis.

The closest relative of ORP10, ORP11, is analogously associated with risk factors of metabolic diseases. Bouchard et al. reported that ORP11 was up-regulated in the visceral adipose tissue of obese Canadian men with metabolic syndrome as compared to obese subjects without the syndrome (Bouchard, et al. 2009). The authors further showed that ORP11 variants in obese patients associated with a number of cardiovascular risk factors such as LDL-cholesterol levels, diastolic blood pressure, hyperglycemia/diabetes as well as metabolic syndrome *per se*. Subsequently, the follow-up study by the same group showed that ORP11 displayed different expression levels in subcutaneous adipose tissue between the high and low responders to caloric restriction (Bouchard, et al. 2010). The findings raise the possibility that ORP11 is likely to be involved in the events occurring during adipocyte differentiation which are connected with cardiovascular risk factors. Furthermore, there is another ORP member, ORP7, the polymorphisms of which were reported to be associated with serum total and LDL-cholesterol by genome-wide association analysis (Teslovich, et al. 2010). However, there is no study assessing the functional role of ORPs in adipocytes (Figure 4).

Alterations in the expression level of certain ORPs have been detected in cancerous tumors or cells. For instance, OPR3 mRNA was found to be up-regulated in B-cell-associate malignancies (Sander, et al. 2005, Chng, et al. 2006). Increased expression of ORP4 mRNA was detected in circulating tumor cells in blood samples, indicating that it might be a putative marker for tumor dissemination (Fournier, et al. 1999). Koga et al. reported the association of ORP5 expression level with invasion and poor prognosis of human pancreatic cancer (Koga, et al. 2008). These pieces of evidence provide a notion that abnormal ORP expression levels may be functionally associated with uncontrolled cell proliferation or tumor cell invasion.
Figure 4.
A schematic presentation summarizing the biological functions of ORPs. The localization of ORPs at a membrane contact site is depicted. The role of ORPs in adipose tissue is an unknown territory. ER, endoplasmic reticulum; VAP, VAMP-associated protein anchoring the ORP at the ER; ORD, OSBP-related (ligand binding) domain; PHD, pleckstrin homology domain; PIP, phosphatidylinositol phosphate.

2.6 GATE-16, GS28 and intracellular trafficking

Several cellular proteins such as GATE-16 and GS28 are involved in the intracellular trafficking. The Golgi SNARE complex 1 (GS28), which is also designated GOS28 or GOSR1, is a 28-kDa Golgi vesicle SNARE (v-SNARE) protein. GS28 is involved in intra-Golgi and ER–Golgi vesicular transport (Subramaniam, et al. 1996, Nagahama, et al. 1996). GS28 is able to bind its cognate target SNARE (t-SNARE) syntaxin-5 at the cis-Golgi and forms a SNARE complex for docking between donor and acceptor membranes.

GATE-16 is a Golgi-associated ATPase enhancer that belongs to Autophagy-related 8 (Atg8) protein family. Mammalian Atg8 proteins comprise three subfamilies (Table 1), microtubule-associated protein 1 light chain 3 (LC3), γ-aminobutyric acid receptor-associated protein (GABARAP) and GATE-16 (also called GABARAPL2) (Shpilka, et al. 2011). GATE-16 is primarily expressed in the brain, similar to
GABARAP-L1, which is mainly expressed in the central nervous system. As an essential factor for intra-Golgi protein transport, GATE-16 modulates late stages of intra-Golgi protein transport by coupling N-ethylmaleimide sensitive factor (NSF) activity and SNARE activation. Subsequently, soluble NSF attachment proteins (SNAPs) bound to NSF, which facilitates the release of GATE-16 from the complex and stimulates interaction of GATE-16 with GS28 in an ATP-dependent manner. The specific interaction in turn precludes GS28 from binding its t-SNARE partner and protects it from proteolysis (Sagiv, et al. 2000, Muller, et al. 2002). Therefore, GATE-16 is likely to work as a v-SNARE protector and controls the appropriate assembly of SNARE complexes.

Table 1. Human Autophagy-related 8 (Atg8) protein family

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<tr>
<td>GATE-16</td>
<td>GATE-16/GABARAPL2/GEF2</td>
<td>16q22.1</td>
</tr>
<tr>
<td>GABARAP</td>
<td>GABARAPL1/GEC1</td>
<td>12p13.31</td>
</tr>
<tr>
<td></td>
<td>GABARAP</td>
<td>17p13.1</td>
</tr>
<tr>
<td>LC3</td>
<td>MAP1LC3A</td>
<td>20q11.22</td>
</tr>
<tr>
<td></td>
<td>MAP1LC3B</td>
<td>16q24.2</td>
</tr>
<tr>
<td></td>
<td>MAP1LC3B2</td>
<td>12q24.22</td>
</tr>
<tr>
<td></td>
<td>MAP1LC3C</td>
<td>1q43</td>
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2.7 Adipocytes and the Simpson-Golabi-Behmel syndrome (SGBS) model

The adipose tissue is the largest endocrine organ in human body, which comprises 20% of total body weight in lean adults and almost 50% or more in severely obese individuals. The adipose tissue is constituted of multiple depots of white adipose tissue and small depots of brown adipose tissue. White adipose tissue, a key endocrine organ which contributes to energy storage and hormone production, is distributed in
the two major divisions throughout the body: subcutaneous adipose tissue and visceral adipose tissue (also called abdominal or central adipose tissue). These two major adipose tissues display distinct metabolic effects. Epidemiologists have demonstrated that visceral adipose tissue exerts detrimental effects based on a positive association between the tissue and metabolic disorders such as insulin resistance, T2D mellitus, dyslipidemia, hypertension, atherosclerosis, and metabolic syndrome and further, overall mortality (Wang, et al. 2005, Zhang, et al. 2008, Tran & Kahn. 2010). By contrast, subcutaneous adipose tissue appears to be associated with insulin sensitivity and a lower risk of T2D development, suggesting its protective role via improving metabolic function (Misra, et al. 1997). Brown adipose tissue, which is innervated by the sympathetic nervous system, is primarily responsible for energy expenditure and heat production (Kozak & Young. 2012). As a highly vascularized tissue, brown adipose tissue is characterized by multilocular lipid droplets and abundant expression of uncoupling protein-1 (UCP1) in the mitochondria (Gilsanz, et al. 2012).

A marked cellular heterogeneity is commonly found in adipose tissue: preadipocytes, adipocytes, fibroblasts, macrophages, endothelial cells and multipotent stem cells are the components of adipose tissue. Adipocytes, also called fat cells, are the major cells in adipose tissue. In the medical field, increase in adipocyte number (hyperplasia) and in adipocyte volume (hypertrophy) are two major types of processes to characterize obesity. Hypertrophy is commonly detected in all overweight and obese individuals, while hyperplasia is associated with severe obesity. At the cellular level, adipocytes act as essential regulators of energy homeostasis. They secrete adipocyte-specific or enriched protein factors that regulate a wide variety of processes such as energy balance, metabolic events, immune function and angiogenesis. For instance, leptin is a highly conserved hormone which is primarily expressed in adipose tissue and tightly correlated with body mass index (BMI). Leptin acts as a regulator of food intake, body weight, energy expenditure and normal immune function (Rajala & Scherer. 2003). Adiponectin, the most abundant hormone secreted from adipose tissue, is negatively associated with adiposity, insulin resistance, T2D and metabolic syndrome (Rabe, et al. 2008).

Adipocyte differentiation (adipogenesis) consists of two phases, determination and termination. These processes involve complex gene-expression events which are
regulated by a cascade of transcription factors such as peroxisome proliferator-activated receptor-γ (PPARγ). PPARγ, a member of the nuclear-receptor superfamily, is a master regulator of adipocyte differentiation in vivo and in vitro. Apart from its necessary role in adipogenesis, PPARγ is critical for maintenance of the differentiated state of mature adipocytes (Rosen & MacDougald. 2006). Two kinds of cell lines, preadipocyte and multipotent stem cell lines, are currently used to investigate the molecular mechanisms involved in adipocyte differentiation. 3T3-L1 and 3T3-F442A, mouse preadipocyte cell lines, are popular models to study the profile of adipocytes. However, using rodent cells as models might produce different results compared with ones derived from human source in respect to gene expression and regulation in adipocytes. In 2001, Wabitsch’s group established the Simpson-Golabi-Behmel syndrome (SGBS) pre-adipocyte model which could be effectively used to investigate human adipocyte biology. SGBS cells are originated from subcutaneous adipose tissue of an infant with Simson-Golabi-Behmel syndrome, an overgrowth syndrome with macrostomia (large mouth), macroglossia (large tongue), hepatosplenomegaly (enlarged liver and spleen), and renal and skeletal abnormalities (Wabitsch, et al. 2001, Fischer-Posovszky, et al. 2008). In the presence of PPARγ agonists in culture medium without serum and albumin, the cells display efficient differentiation and retain this capacity for up to 50 generations. The SGBS model, hence, has been widely used to profile adipokine secretion, to identify drug effects on adipogenesis, and to understand the mechanisms of human adipogenesis (Rosenow, et al. 2012, Fischer-Posovszky, et al. 2012).
3 AIMS OF THE STUDY

I. To map the mRNA and protein expression patterns of OSBP/ORPs in subcutaneous and visceral white fat of human subjects

II. To determine the functional effects of ORP genetic manipulation on the adipocyte differentiation

III. To characterize the expression pattern, subcellular localization and function of ORP11

IV. To identify protein binding partners of ORP11 and ORP7

V. To investigate the cellular role of ORP7
4 Materials and methods

4.1 List of Published methods

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*: methods used by collaborators

4.2 Brief descriptions of the methods employed

Details regarding the materials and methods are presented in the original articles (I-III).
Western blot analysis (I, II, III)

The cultured cells or tissues were homogenized in 250 mM Tris-HCl, pH 6.8, 8% SDS and protease inhibitor cocktail. The crude extracts were cleared by centrifugation and the protein concentration was subsequently measured. The proteins were electrophoresed on Laemmli gels and electrotransferred onto Hybond-C Extra nitrocellulose membranes. The bound primary antibodies were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and the enhanced chemiluminescence system. ImageJ (http://rsbweb.nih.gov/ij/) was used to quantify the production levels after normalization of the data according to the β-actin signal.

Co-immunoprecipitation (I, II)

After transfection overnight, cells were washed twice with ice-cold PBS and dissolved in the lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.5 mM MgCl₂, 10% glycerol, 0.5% Triton X-100, pH 8.0 and protease inhibitor cocktail). After centrifugation, the supernatant was preabsorbed with protein G-Sepharose 4 Fast Flow. The recovered supernatant was incubated with antibodies overnight and protein G-Sepharose was added. Beads were washed three times with the lysis buffer and boiled for western blot analysis.

Immunohistochemistry (II)

Immunohistochemical stainings were performed using affinity-purified rabbit antibodies. The primary antibody was detected using the avidin-biotin complex system. Brown 3,3’-diamino-benzidine was used as chromogen. After preincubation with the immunizing polypeptide or with a non-related control polypeptide at 4 °C overnight, the primary antibodies were used to simultaneously stain adjacent sections in order to verify the specificity of the immunostaining signals.
**Fluorescence microscopy (I, II)**

For fixation, cells were fixed with 4% paraformaldehyde and then permeabilised with 0.1% Triton X-100 in PBS for 20 min. The cells were then incubated with primary antibodies. After washing with PBS, the specimens were incubated with Alexa Fluor secondary antibody conjugates. After washing with PBS, the specimens were mounted on glass slides for the analysis by a laser scanning confocal microscope system.

**Electron microscopy (I)**

The cells after different treatments were fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide. Subsequently, the cell specimens were dehydrated and embedded in the epoxy resin (Epon). Sections were post-stained with uranyl acetate and lead citrate and analyzed with an electron microscope.

Bimolecular fluorescence complementation (BiFC) assay (II)

Human BIFC constructs were generated to obtain fusions of the target genes with the N- and C-terminal fragments of the fluorescent protein Venus. Plasmids were transfected into the cells which were analyzed by a fluorescence microscope.

**Yeast two-hybrid analysis (I and II)**

Full-length human ORP11 and ORP7 cDNAs in the bait vector pGBK7T7 were used to screen a human normalized cDNA library (Clontech). Two-hybrid library screening was conducted by using the yeast mating method. Briefly, the concentrated bait was mixed with human cDNA library in yeast strains and incubated overnight until diploid yeast clones formed. SD/-Trp-Leu-His-Ade (SD/4-) selective plates and β-galactosidase (x-gal) filter assays were used to select positive colonies.
Quantitative RT-PCR (qPCR) analyses (II, III)

Total RNAs from tissues or cells were reverse transcribed by using the VILO kit or random hexanucleotide primers in the presence of RNase inhibitor (Takara Bio, Shiga, Japan). The full-length ORP cDNA inserts were excised from constructs and used as calibrators to estimate the corresponding mRNA copy numbers in tissues and cells as previously described (Johansson, et al. 2003). Succinate dehydrogenase subunit alpha (SDHA), β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as housekeeping genes for the PCR runs. After measurement of threshold cycle (C_T) of each well, relative mRNA levels of target genes were quantified by using the ΔΔC_T method.

Human tissue specimens (I, III)

For immunohistochemistry, the specimens were obtained from medico-legal autopsies. Human epicardial fat was obtained from a transplant recipient at Helsinki University Central Hospital upon cardiac transplantation. Human subcutaneous and visceral adipose tissue specimens were obtained from morbidly obese female patients upon bariatric surgery at Peijas Hospital, Finland. The average BMI of the subjects (n=4) was 46.9 kg/m^2 (range 43.7 to 50.8 kg/m^2) and waist to hip ratio 0.91 (range 0.88-0.96).

Human SGBS preadipocyte/adipocyte culture (III)

Human SGBS preadipocytes were cultured as described by Wabitsch et al. (2001) and Fischer-Posovszky et al. (2008). To induce differentiation, the cells at 80%-90% confluence were incubated with serum-free DMEM/F12 medium supplemented with 0.01 mg/ml human apo-transferrin (Sigma-Aldrich), 20 nM insulin, 100 nM cortisol (Sigma-Aldrich), 0.2 nM triiodothyronine (Sigma-Aldrich), 25 nM dexamethasone (Sigma-Aldrich), 250 µM 1-methyl-3-isobutyl-xanthine (IBMX, Sigma-Aldrich) and 2 µM rosiglitazone (Sigma-Aldrich) for 4 days. Subsequently, the cells were changed
to serum-free DMEM/F12 medium containing 20 nM insulin, 100 nM cortisol, and 0.2 nM triiodothyronine and incubated for additional 18 days. In the adipocytes, multiple lipid droplets were visible (Figure 5).

![Image](image-url)

**Figure 5.**

**Mature SGBS adipocytes after adipogenic differentiation.** Oil Red O staining of SGBS adipocytes differentiated for 22 days. The cells are filled with high amounts of visible lipid droplets. A: scale bar = 400 μm. B: scale bar= 200 μm.

*Lentiviral/adenoviral transduction (III)*

SGBS cells were transduced with the shRNA lentiviruses for 72 hrs and stable cell pools were selected by puromycin at 0.5 μg/ml. SGBS adipocytes under differentiation on day 10 were transiently transduced by adenovirus at a multiplicity of 50 PFU/cell for 72 hrs. The silencing or overexpression effects were verified by qPCR and Western blotting.

*Oil Red O staining of neutral lipids (III)*

Cells were fixed with 10% formalin and subsequently washed with water. Then 60% isopropanol was added on the cells for 5 min. 1.8 mg/ml Oil Red O in 60%
isopropanol was used to stain the cellular neutral lipids. Haematoxylin was used to
stain the nuclei of the cells.

Assay for cellular triglyceride content (III)

The cells were lysed and the supernatant was harvested after centrifugation. A diluted
series of glycerol (2300, 1150, 575, 287.5, 143.75 and 71.88 µmol/L) was used as the
reference. Each sample was mixed with GPO-PAP Triglyceride kit (Cobas,
Roche/Hitachi) reagent on a 96-well plate for 20 min. The quantification of TG was
carried out by colorimetric assay at 510 nm. The triglyceride levels were normalized
according to total cell protein concentrations.

Bioinformatics analysis of OSBP/ORP mRNA expression (III)

ORP mRNA expression was analysed based on the microarray dataset of Human
U133A/GNF1H Gene Atlas at the BioGPS web site (http://biogps.org/downloads/).
Information of the probe sets in different tissues was extracted separately according to
the individual ORP. In the case that the signal was throughout the tissue selection not
more than 3 folds from the median value, the probe set were estimated as “non-
functional” and eliminated. The functionality of probe sets was further verified
according to the results on ORP mRNA or protein expression from previously
published reports. R program (http://www.r-project.org/) was used for the data
analysis and generation of a heat map.

Statistical analysis

The data are presented as mean ± standard deviation (SD) in publication II and mean
± standard error of mean (SEM) in Publication III. Student’s T-test was applied for
comparisons between groups of data points. All statistical tests were two-sided and p
values <0.05 were considered significant.
5 RESULTS AND DISCUSSION

5.1 Characterization of ORP11

5.1.1 Tissue and cell type specific expression patterns of ORP11

ORP11 is a previously unexplored ORP family member. Using a 66-amino acid region at the N-terminal of the protein, we generated an affinity-purified ORP11 rabbit antiserum for the characterization of ORP11. The Western blotting analysis of the hepatoma cell line Huh7 and the embryonic kidney cell line HEK293 showed a major protein band with the apparent molecular mass of 85 kDa. This is consistent with the ORP11 mass predicted from the cDNA sequence, 83.6 kDa (I, Figure 2A).

On a commercial human multiple tissue blot filter, we detected ORP11 at highest levels in ovary, testis, brain, kidney, liver and stomach, in contrast to its low levels found in the small intestine, skeletal muscle, and spleen, and none in heart or lung (I, Figure 1B). Brain is the most cholesterol-enriched organ in human. The oxysterol 24S-OHC is important for the elimination of the excess cholesterol in the brain (Dietschy & Turley. 2004). Gonads are involved in extensive cholesterol uptake, intracellular transport, and conversion to steroid hormones. Moreover, the central roles of liver, kidney and adipose tissue in lipid homeostasis have been well accepted. The abundant expression of ORP11 in these tissues would thus be consistent with a role in active lipid metabolism or lipid sensing.

In addition, we observed abundant expression of ORP11 in a human pericardial adipose tissue specimen by Western blotting (I, Figure 1B). Intriguingly, our bioinformatics analysis showed that ORP11 is expressed in the adipocytes at markedly high levels as compared to almost all the other tissues, based on analysis of the human U1333A/GNF1H Gene Atlas microarray dataset (III, Figure S1). ORP11 was found abundantly expressed in visceral adipose tissue of obese Canadian men with metabolic syndrome (Bouchard, et al. 2009). Therefore, we found it of interest to investigate the functional role of ORP11 in adipose tissue.
The immunohistochemical staining of tissue sections from human autopsy material displayed a strong ORP11 immunoreactivity in the epithelial cell layers of tubules in kidney medulla (I, Figure 2A), of testicular seminiferous tubules (Figure 2B), of caecum (I, Figure 2C), of skin epidermis and sebaceous gland (I, Figure 2D). The abundant expression of the protein in epithelial cells indicates that ORP11 may play pivotal roles in specific polarized cell types with the responsibility for directed transport of cellular components. Skin epidermis and sebaceous gland epithelium are important sites for lipid synthesis, storage or secretion, in which ORP11 might regulate a wide variety of functions. Moreover, we observed strong immunostaining in stroma of the ovary (I, Figure 2E), in liver hepatocytes (I, Figure 2F) as well as in para-aortic lymph notes (Figure 6A) in contrast to the weak staining in sections of skeletal muscle (Figure 6B) and lung (Figure 6C). Interestingly, the strongest staining of ORP11 in liver hepatocytes localized to regions with lipid droplets reflecting hepatic steatosis. Hence, excess neutral lipid accumulation might up-regulate or stabilize ORP11. These findings suggest ORP11 may regulate cellular lipid metabolism and are thus consistent with the previous findings that ORP11 gene variants are associated with plasma LDL-cholesterol levels and several other cardiovascular risk factors (Bouchard, et al. 2009).

Figure 6. Immunohistochemical analysis of ORP11 protein in human tissues showing strong staining in para-aortic lymph notes (A) and weak staining in skeletal muscle (B) and lung (C). Bar = 100 µm
5.1.2 Subcellular localization of ORP11

In contrast to most ORP family members, ORP11 lacks a FFAT motif which potentially targets the ER. Immunofluorescence microscopy with the affinity-purified ORP11 antiserum revealed that ORP11 localizes on Golgi membranes, specifically to the medial-trans parts of the organelle (I, Figure 3). Moreover, we found that ORP11 showed significant co-localization with Rab6, Rab7 and Rab9 proteins, and occasional co-localization with lysosome/late endosome markers, suggesting that ORP11 localizes in the LE-TGN region (I, Figure 4). Rab7 and Rab9 are involved in the communication between the LE and the Golgi complex. As a regulator of membrane traffic, Rab9 binds to its effector TIP47, which enhances the recycling of cation-independent mannose-6-phosphate receptor from the LE to the TGN (Hutagalung & Novick. 2011). Rab7 plays a central role in trafficking from LE to lysosome but also in LE-TGN recycling (Seaman, et al. 2009). ORPs are implicated to be involved in non-vesicle communication rather than vesicle transport, which might affect the lipid composition of subcellular organelle membranes (Rocha, et al. 2009, Olkkonen & Levine. 2004, Perry & Ridgway. 2006, Peretti, et al. 2008). Our localization data suggested that ORP11 might play a role in non-vesicular communication between the LE and the medial-trans-Golgi. Even though the physiologic function of such communication has not been established, intimate contacts between LE and trans-Golgi have been described in electron microscopic studies at the so-called GERL (Golgi-ER-late endosome/lysosome) structures (Novikoff, et al. 1971, Oliver, et al. 1980) in which lipid transport might occur (Levine & Loewen. 2006).

Overexpression of EGFP-ORP11 resulted in an even cytosolic-appearing pattern with a weak Golgi staining aspect. The cytosolic pattern suggests that ORP11 anchors at the membrane at saturable sites. Unlike for the endogenous protein, we observed significant colocalization between overexpressed ORP11 and all Golgi markers: the cis-Golgi marker GM130 (I, Figure 5A-C), TGN marker TGN46 (I, Figure 5D-F) and the medial-trans-Golgi marker GT-CHERRY (Figure 7A-C). The overexpressed ORP11 also overlapped with GFP-Rab9 and GFP-Rab7, similarly to the endogenous ORP11 (I, Figure 5J-O). The N-terminal fragment ORP11 (aa 1-292) displayed a prominent LE localization and the C-terminal fragment ORP11 (aa 273-747) appeared
totally cytosolic, indicating that the C-terminal ORD domain negatively regulates the LE targeting of ORP11. However, the PH domain failed to target the LE. This raises the question how ORP11 targets the LE, which remains a subject of future studies.

Figure 7. Confocal microscopy images of HEK293 cells transfected with EGFP-ORP11 and GT-CHERRY showing colocaliation at the medial-trans-Golgi. Bar = 10 µm

5.2 Cellular roles of ORP11

5.2.1 Overexpression of ORP11 induces the formation of lamellar lipid bodies

We frequently observed brightly stained vacuolar-like structures of ectopically expressed ORP11, which colocalized significantly with GFP-Rab7 (I, Figure S1 G-I) and GFP-Rab9 (I, Figure S1 J-L). Furthermore, electron microscopy showed structures containing multilamellar membrane accumulation. The multilamellar lipid bodies were found to be associated with vacuolar structures and the Golgi complex (I, Figure S1). This indicates a disturbance of lipid trafficking on the endosome-Golgi axis. Similar multilamellar membranes are formed in the endo-lysosomal system where excess lipids accumulate because of disturbances in lipid transport. For example, several group observed multilamellar membranes arises upon incubation with oxidized LDL or induced by inheritable lysosomal storage disorders (Anderson & Borlak. 2006, Schmitz & Grandl. 2009b). Moreover, this finding is in good agreement with the localization data of ORP11 in the LE-Golgi region.
The localization of OSBP and ORP9 to the TGN is mediated by their PH domains, which bind to PI(4)P. An in vitro binding assay showed that a GST fusion protein of the ORP11 the PH domain (aa 60-157) specifically bound to PtdIns(3)P, -4P, -5P, -(3,4)P$_2$, (4,5)P$_2$, and phosphatidic acid (I, Figure 7A). A fluorescent fusion carrying two ORP11 PH domains in tandem appeared largely cytosolic and intra-nuclear and occasionally displayed weak staining in the Golgi (I, Figure 7B-D). Hence, the interaction between the PH domain and PI(4)P might partially regulate ORP11 targeting to the Golgi, but cannot be entirely responsible for this localization.

5.2.2 ORP11 interacts with ORP9

To identify the determinants that might impact the cellular localization of ORP11, we carried out a yeast two-hybrid analysis using full-length ORP11 as a bait to screen a library consisting of human cDNAs from a wide spectrum of tissues. ORP9 was identified as one candidate to interact with ORP11. Immunoprecipitation using lysate of HEK293 cells after cotransfection with Xpress-ORP11 and Flag-tagged ORP9 further confirmed the interaction between the two proteins (I, Figure 8A).

To determine the specific interacting region, we made serial deletion constructs of ORP9 in the prey plasmid pGADT7 and of ORP11 in the bait vector pGBK7T7 (I, Fig 9A, C). The yeast two-hybrid assay showed that aa 154-747 of ORP11 dimerized with ORP9 within aa 98-372, possibly 106-195 (I, Figure 9B, D). The cutpoint at aa 367 aligned exactly with the cutpoint 372 in ORP9. However, two-hybrid tests failed to detect interaction between the N-terminal ORP11(1-367) and ORP9. Interestingly however, the N-terminal fragment ORP11(1-292) could be co-immunoprecipitated with ORP9 (I, Figure 9E), suggesting that the result in the two-hybrid assay with ORP11(1-367) was a false negative. Taken together, ORP11 aa 154-29 is most likely to be the dimerization region.

ORP9 has a FFAT motif and a PH domain which determine the targeting of the protein to ER and Golgi membranes (Ngo & Ridgway. 2009, Wyles & Ridgway. 2004). Overexpression of ORP9 recruited the largely cytosolic EGFP-ORP11 to the ER and Golgi membranes (I, Figure 8B-D). Almost 100% overlap between ORP9 and
ORP11 demonstrated that the recruitment of ORP11 by ORP9 is highly efficient, indicating that the presence of ORP9 protein in trans-Golgi may contribute to the ORP11 Golgi association. ORP11 has no FFAT motif that would specifically interact with ER membrane protein VAP-A. Our results confirmed that ORP11 has no association with ER membranes via VAP-A, and suggest that ORP9 recruits ORP11 directly to specific membranes (I, Figure 8E-G).

**5.2.3 ORP11 Golgi association is facilitated by ORP9**

The expression of ORP9 protein was reduced by 85-90%, while ORP11 was reduced by 75-80% in HEK293 cells after siRNA transfections (I, Figure 10A). After significant reduction of ORP9, we failed to observe the typical distribution of ORP11 in the Golgi. Instead, we detected ORP11 distributed in dispersed, vesicular structures (I, Figure 10D and E) different from the control (I, Figure 10C), which might be caused by a shift towards an endosomal localization. However, remarkable reduction of ORP11 had no effect on the distribution of ORP9 (I, Figure 10F and G), which remained similar to the control (Figure 10B). Furthermore, Western blotting analysis showed that silencing of ORP9 or ORP11 had no impact on the levels of the other protein in the cells (I, Figure 10A).

Ridgway et al. observed Golgi fragmentation after knockdown of ORP9L expression (Ngo & Ridgway, 2009). In our hands ORP9 deficiency had only a minor effect on TGN integrity as judged from co-staining for TGN46 (Figure 8A-C), and absolutely no effect on cis-Golgi morphology, as judged from GM130 staining (data not shown). At the level of ORP11 silencing reached we observed no disturbance of Golgi complex integrity by reduction of the cellular ORP11 protein (Figure 8D-F).

Overexpressed ORP9 was able to recruit ORP11 to ER and Golgi membranes through dimerization between the two proteins, suggesting that the presence of ORP9 in trans-Golgi might regulate ORP11 targeting. Silencing of ORP9 resulted in a dispersed, vesicular distribution of ORP11, supporting the important impact of ORP9 on ORP11 Golgi association. Hence, Golgi localization of ORP11 appears to be determined by complex membrane targeting through dimerization with ORP9 and interaction of the
PH domain with acidic phospholipids. Interestingly, the localizations of certain other ORPs are reported to be determined in a similar manner, by complex membrane targeting. For instance, interactions with PI(4)P and with the small GTPase ARF drive the localization of OSBP to TGN membranes (Levine et al., 1998; Levine et al., 2002). The ankyrin repeat region of ORP11 interacts with Rab7 and the PH domain binds to phosphoinositides, which contribute to the localization of ORP1L to the LE (Johansson, et al. 2003, Johansson, et al. 2005).

5.3 ORPs in human adipose depots and cultured adipocytes

OSBP acts as a sterol sensor that integrates the cellular sterol status with sphingomyelin metabolism by regulating ceramide transport from the endoplasmic

5.3.1 OSBP/ORP copy numbers in human adipose depots and SGBS adipocytes.

To date, no group has compared the expression of OSBP/ORP in human visceral and subcutaneous adipose tissues, or assessed the functional role of ORPs in adipocytes. We measured copy numbers of ORP mRNAs in human subcutaneous and visceral adipose tissue specimens from 4 obese female patients, as well as in Simpson-Golabi-Behmel syndrome (SGBS) cells. We found that OSBP/ORP displayed almost identical expression patterns in the subcutaneous (s.c.) and visceral fat depots (III, Figure 1A, B), and a highly similar pattern in the SGBS adipocytes (III, Figure 1C). This suggests that the ORP mRNA signals in the human adipose tissue specimens mainly derive from adipocytes, and are not strongly affected by mRNAs of ORPs from other cell types included in the adipose tissues such as fibroblasts, macrophages, and endothelial cells (Gimeno & Klaman. 2005). In fact, the expression profiles of different ORPs were markedly uneven in the adipose tissues, with up to 100-fold differences between the distinct ORP mRNAs detected.

Among the ORPs the mRNAs of which were abundant in the adipose depots (III, Figure 1A, B), OSBP, ORP2, ORP8, and ORP9 were detected at relatively equal levels in the two depots by Western blotting of total protein specimens (III, Figure 2). Although ORP11 mRNA was present at relatively low levels in the adipose depots, we were able to detect the protein by Western blotting indicating that our antiserum was highly sensitive.
Bioinformatics analysis of human U133A/GNF1H Gene Atlas dataset showed that OSBP, ORP4 and ORP9 mRNAs were expressed at relatively even levels across different tissues and cell types (III, Figure S1). ORP8 was found abundantly expressed in adipocytes, CD14(+) monocytes (III, Figure S1) and in tissue macrophage (result by Yan et al., 2008), suggesting that a proportion of the ORP8 mRNA signals in the adipose depots of patients might come from monocyte-macrophages within the inflamed adipose tissues.

Interestingly, even though ORP11 has a relative low mRNA copy number in adipose depots, its mRNA expression in adipocytes was markedly higher as compared to most of the other tissues/cell types, suggesting that it might have important specific functions in the adipocytes. Importantly, ORP11 mRNA was found to be elevated in the visceral adipose tissue of obese patients with metabolic syndrome compared to the obese individuals without the syndrome, bringing up the possibility that ORP11 might adversely affect adipose tissue metabolism or signaling (Bouchard, et al. 2009).

5.3.2 OSBP/ORP expression during SGBS adipocyte differentiation

After 22 days of differentiation, we observed that 80% of SGBS cells were filled with lipid droplets (III, Figure 3A, B). In good accordance with previous reports (Wabitsch et al., 2001; Fischer-Posovszky et al., 2008), extensive up-regulation of adiponectin, aP2, leptin and PPARγ at the mRNA level was detected (III, Figure 3C), consistent with the view that a large proportion of the cells were differentiated to adipocytes.

As compared to preadipocytes, five ORPs, ORP2 (-54%), ORP3 (-86%), ORP4 (-74%), ORP7 (-56%), and ORP8 (-67%) were significantly down-regulated at the mRNA level in adipocytes. In contrast, only one mRNA, ORP11, was upregulated 3.5-fold upon SGBS cell adipocytic differentiation (III, Figure 4A). Of these six ORPs, ORP3, ORP8 and ORP11 were selected as the candidates for the further analysis due to the availability of excellent molecular and immunological tools in the lab. Furthermore, we observed by Western blotting a 2-fold increase of ORP11 protein, a 55% reduction of ORP8, and a 60% reduction of ORP3 upon SGBS adipocytic
differentiation (III, Figure 4B, C), confirming the changes in ORP3, ORP8 and ORP11 expression at the protein level (Figure 4B).

Time course data showed that the down-regulation of ORP3 and ORP8 occurred between days 0 and 4 without further progress during the late stages. This might be associated with the cessation of cell proliferation due to the adipogenesis. However, the ORP11 mRNA was elevated at the late time points between days 16 and 22. It is thus likely that ORP11 exerts a role during late stages of adipogenesis, or might be essential for maintenance of the mature adipocyte phenotype.

5.3.3 Effect of ORP silencing/overexpression on adipocyte differentiation

ORP11 was overexpressed in the visceral adipose tissue of obese Canadian men with metabolic syndrome (Bouchard, et al. 2009). We found during this study ORP11 localizes at the Golgi-late endosome interface in HEK293 cells, and demonstrated its dimerization with ORP9 (I). However, the precise function of ORP11 remains obscure.

Transduction of SGBS preadipocytes with a lentivirus expressing ORP11 shRNA resulted in 60-70% reduction of the ORP11 protein as compared to the controls (III, Figure 6A). Interestingly, a significant reduction of adiponectin and aP2 mRNAs was detected in the ORP11 knock-down cells as compared to the controls (III, Figure 6B). Adiponectin is an adipocyte-specific protein, which in humans is encoded by the ADIPOQ gene (Scherer, et al. 1995). As an abundant circulating adipocytokine, adiponectin has anti-inflammatory properties negatively associated with cardiovascular disease, T2D, and obesity. Because of its anti-inflammatory and anti-atherogenic effects, adiponectin is commonly considered an important potential target for pharmacologic treatment of T2D and metabolic syndrome (Lago, et al. 2007). aP2, also called FABP4, is a 15-kD cytosolic fatty acid binding protein which is primarily expressed in adipocytes and macrophages (Coe, et al. 1999). The protein has important regulatory functions in glucose and lipid homeostasis. Lipolysis efficiency was reported to be markedly reduced in the adipocytes derived from aP2-deficient mice (Coe, et al. 1999). Increasing lines of evidence suggest that aP2 is closely
associated with insulin resistance, metabolic syndrome, type 2 diabetes, and atherosclerosis (Terra, et al. 2011). In addition, serum aP2 level is also associated with the development of metabolic syndrome, T2D and atherogenic dyslipidemia (Xu, et al. 2007, Tso, et al. 2007, Cabre, et al. 2012). Apart from the impacts on adiponectin and aP2 expression, silencing of ORP11 markedly reduced the storage of triglycerides in the SGBS adipocytes, as compared to the controls transduced with a non-targeting shRNA lentivirus (III, Fig 6C). In addition, a HuH7 hepatoma cell line with stably silenced ORP11 was generated by lentiviral transduction. ORP11 was stably silenced at least until passage 10 (Figure 9). This stable hepatoma cell model could be used to investigate potential role of ORP11 in hepatocytes.

![Western Blotting Analysis](image)

**Figure 9. The Western blotting analysis of Huh7 cells demonstrates that ORP11 was stably silenced until passage 10.** HuH7 cells were transduced with an ORP11 shRNA (Sh-ORP11) or non-targeting (Sh-NT) shRNA lentivirus. P3, P6, P8, P10: passage 3, passage 6, passage 8 and passage 10.

According to the results of the differentiation time course experiments (III, Figure 5), endogeneous ORP3 and ORP 8 mRNAs were firmly down-regulated and cytoplasmic LDs were rapidly generated on day 10 (III, Figure 5). Therefore, we started to overexpress ORP3 or ORP8 on this time point of SGSB adipogenesis. After 3 days' transduction, ORP8 protein was markedly overexpressed (III, Figure 6A), resulted in a significant reduction of ap2 mRNA levels, and impaired the storage of cellular triglycerides (III, Figure 6B). ORP8 has been reported to regulate lipid metabolism
and nuclear functions (Beaslas, et al. 2012). As a target of microRNA (miR)-143, ORP8 exerts an important function to mediate insulin signaling in mouse adipocytes (Jordan, et al. 2011). Therefore, ORP8 overexpression might impair the insulin stimulation of the adipogenic process. Further studies on the connection between ORP8 and insulin signaling pathways in adipocytes are therefore warranted.

In contrast, ORP3 overexpression affected significantly neither mRNA levels of adipogenesis markers mRNA levels nor cellular triglyceride storage (III, Figure 6B), suggesting a non-essential role of ORP3 upon the adipogenesis. As a phosphoprotein, ORP3 regulates cell adhesion (Lehto, et al. 2008). ORP3 protein was reported to be up-regulated in adipocytes differentiated from human mesenchymal stem cells, which is inconsistent with our results (Lee, et al. 2006). The use of different cell models in the two studies is mostly likely to result in the inconsistency. We found that ORP3 was abundantly expressed in the adipose depots and adipocytes, which is consistent with the results in (Lee, et al. 2006). We therefore find it likely that ORP3 could be up-regulated at an earlier phase of mesenchymal stem differentiation into the adipocytic lineage, and not at the preadipocyte-adipocyte conversion.

5.4 ORP7 and GATE-16

5.4.1 ORP7 interacts with GATE-16

ORP7 was found predominantly expressed in gastrointestinal tract including stomach, duodenum, jejunum and ascending colon as well as in fetal lung. In human intestinal samples, ORP7 is exclusively expressed in the epithelial cells indicating that it might be involved in the lipid absorption occurring at the intestinal epithelium (Lehto, et al. 2004). It was previously reported that ORP7 protein is distributed between the cytosol and ER membranes (Lehto, et al. 2004).

Using full-length ORP7 as bait and a normalized universal human cDNA library as prey, we carried out a yeast two-hybrid screen. We identified two positive clones encoding GATE-16, which is a Golgi-associated ATPase enhancer (Sagiv, et al. 2000). The results from yeast two-hybrid analysis provided a clue that ORP7 interacts
with GATE-16. BiFC assay further confirmed the interaction in live cells, which is visualized in the figure (II, Figure 1C, D).

To identify specific regions in GATE-16 and ORP7 responsible for the interaction, we made serial deletion constructs of GATE-16 in the prey vector pGADT7 (II, Figure 3A) and of ORP7 in the bait plasmid pGBK7 (II, Figure 3C). In combination with empty prey vector, neither full length nor deletion constructs of ORP7 bait could grow on SD/4- agar plates or activate the β-gal reporter gene. As prey constructs, GATE-16 (aa 30-117) but not GATE-16 (1-45) were able to grow on SD/4- agar plates and to activate the transcription of β-gal, suggesting that amino acids 30-117 of GATE-16 interact with ORP7 (II, Figure 3B). As the bait, ORP7 (aa 1-142) and ORP7 (aa 1-472) constructs grew on SD/4- agar and activated transcription of the β-gal reporter gene. In contrast, amino acids 470-842 and 142-828 in the C-terminal region containing the ORD of ORP7 failed to activate the reporter (II, Figure 3D). This finding implicates that amino acids 1-142 of ORP7 are sufficient to mediate its interaction with GATE-16. The binding region of ORP7 contains its PH domain which might play a role as a determinant for membrane targeting via interaction with phospholipids and proteins such as GATE-16 (Vihervaara, et al. 2011a).

5.4.2 Subcellular localization of ORP7, GATE-16 and GS28

As a member of the ubiquitin-fold (UF) protein family, GATE-16 is located to the Golgi complex and interacts with GS28 (Muller, et al. 2002). In 293A cells, overexpressed GFP-GATE-16 displayed a pattern consisting of largely cytosolic distribution with some Golgi staining (II, Figure 2A-C). Overexpressed ORP7 displayed some ER-like staining but was prominently distributed as large, vacuolar-like elements co-localizing with the autophagosome marker RFP-LC3 (II, Figure 2D-F). However, overexpression of RFP-LC3 alone resulted in a pattern consisting of largely cytosolic staining and small punctate structures, smaller than the ones in the presence of the excess ORP7 (II, Figure 2G-I). The findings implicated that overexpressed ORP7 induced formation of vacuolar-like structures representing autophagosomes.
As described in 5.4.1, ORP7 interacts with GATE-16. We further investigated the impact of the interaction on their localization. The brightly stained vacuolar-like structures represented ORP7 co-localizing strongly with GFP-GATE-16 (II, Figure 2J-L). In contrast, the endogeneous GS28 did not overlap with vacuolar-like elements containing overexpressed ORP7 (II, Figure 2M-O). This suggests that ORP7 is able to recruit GATE-16 but not GS28 to the large autophagosomal structures.

**5.4.3 ORP7 regulates GS28 protein level through transcriptional regulation**

ORP7 overexpression reduced the GS28 protein level by 25% in 293A cells (II, Figure 4C, E). Moreover, the ORP7 protein was reduced by approximately 90% after transfection of the most efficient ORP7-specific shRNA (sh3638) (II, Figure 4A), which resulted in a 40% upregulation of cellular GS28 protein (II, Figure 4B, D). Therefore, not only the excess ORP7 upon overexpression but also the endogenous ORP7 modulates the stability of GS28 protein. However, overexpression of ORP7 failed to reduce the mRNA levels of GS28 as compared to the control (II, Figure 5F), suggesting that the regulation operates through a post-transcriptional mechanism.

**5.4.4 ORP7 affects GS28 degradation on proteasomes via GATE-16**

As a component of the intra-Golgi transport machinery, GATE-16 binds to GS28, a Golgi v-SNARE in an ATP-dependent but not ATP hydrolysis dependent manner, protecting GS28 from proteolysis and maintaining GS28 in a competent state for promoting Golgi vesicle transport (Sagiv, et al. 2000, Muller, et al. 2002). We found that silencing of GATE-16 resulted in a significant reduction of GS28 by 85% (II, Figure 5A, C, E).

To investigate whether GATE-16 regulates the effect of ORP7 on GS28 protein levels, we generated a truncated construct of ORP7 (aa 1-142) lacking the region binding to GATE-16. Interestingly, overexpression of the truncated construct had no effect on GS28 protein levels, differing from the impact of full-length ORP7 protein (II, Figure 5B, D). The finding suggests that GATE-16 is involved in the functional crosstalk between ORP7 and GS28.
Based on the previous results, we envisioned that ORP7 and GS28 might act as competitive partners for GATE-16. Their competitive interactions with GATE-16 might attenuate the protection of GATE-16 on GS28 and in turn result in the proteolysis of GS28. To investigate whether the reduction of GS28 follows the mechanism of proteasome-dependent degradation or lysosomal degradation, we treated cells with the proteasome inhibitor MG132 or the lysosomal inhibitor chloroquine, respectively. MG132 effectively blocks proteolytic activity of the proteasome complex (Myung, et al. 2001), while chloroquine accumulates in lysosomes and impairs lysosomal protein degradation via inhibition of endo-lysosomal acidification (Gonzalez-Noriega, et al. 1980). Overexpression of ORP7 resulted in a significant reduction of GS28 protein levels, consistent with the result in 5.4.3. MG132 treatment for 16 h significantly inhibited the degradation of GS28 (III, Figure 5G). In contrast, in the cells treated with chloroquine, GS28 was degraded as efficiently as in the controls. The results suggest that ORP7 affects GS28 protein levels via a mechanism of proteasomal and not lysosomal degradation.

5.4.5 ORP7 potentiates 25-OH-induced degradation of GS28

After treatment of cells with serial dilutions of 25-OHC for 48 h, we observed a inverse association between GS28 protein levels and 25-OHC concentrations, as compared to the control (II, Figure 6A, C). Increasing 25-OHC concentrations induced reduction of GS28 protein, an effect that was further strengthened upon overexpression of ORP7 (II, Figure 6B, C). In contrast, silencing of ORP7 abolished the impact of 25-OHC on GS28 protein levels (II, Figure 6D-F). Thus ORP7 overexpressing and silencing strengthen or abolish the effect of 25-OHC, demonstrating that ORP7 acts a potential mediator of the oxysterol effect of GS28 stability.

To investigate whether GS28 degradation upon 25-OHC treatment is due to proteasomal or lysosomal degradation, we treated the cells with MG132 or chloroquine similarly to the previous experiment. GS28 protein degradation was eliminated after treatment with MG132 but not chloroquine, indicating that 25-OHC induces GS28 degradation which is dependent on the proteasome but not lysosome hydrolytic activity.
When 25-OHC or cholesterol binds to the ORD domain of OSBP, the protein undergoes a conformational change to facilitate its cellular function (Wang, et al. 2005, Wang, et al. 2008). ORP7 has been reported to have the capacity to bind to 25-OHC (Suchanek, et al. 2007). Interestingly, 25-OHC was reported to diminish the GS28 protein but not the lysosome-associated protein LAMP1 in melanocytes through an unknown mechanism (Hall, et al. 2004). We postulated, therefore, that 25-OHC might regulate the function of ORP7 in a similar manner to OSBP. ORP7 could undergo a conformational change upon binding of 25-OHC in the ORD region. The conformational change may enhance the affinity of ORP7 binding to GATE-16, which competitively abolishes the protection of GS28 afforded by GATE-16 and results in the proteolysis of the t-SNARE. Therefore, the crosstalk between ORP7 and its oxysterol ligand can be envisioned to maintain the stability of GS28.
6 CONCLUSIONS AND PERSPECTIVES

Metabolic disorders such as obesity, metabolic syndrome, T2D, dyslipidemia, hypertension and atherosclerosis seriously erode human life quality. They result in an extremely heavy burden on global socioeconomics because their characteristics - high prevalence and incidence, chronicity, and long-term health care implications. Obesity is an essential independent risk factor of cardiovascular disease, T2D and certain other chronic disorders. It is characterized by status, distribution and amount of adipose tissue which is the largest endocrine organ in the human body. The function of this endocrine organ is primarily regulated by a variety of events occurring in the adipocytes. According to the previous evidences, OSBP/ORP proteins sense the intracellular concentrations of different sterols and relay information to cellular machineries that contribute to cellular lipid homeostasis, intracellular vesicle transport, and cell signaling. Disturbances of these functions result in the interruption of cellular events and are most likely to play critical roles in metabolic disorders.

Data presented in this thesis showed that OSBP/ORPs have similar mRNA expression patterns in the human s.c. and visceral adipose depots, as well as in human SGBS adipocytes. During adipocytes differentiation, ORP2, ORP3, ORP4, ORP7 and ORP8 mRNA levels are upregulated while ORP11 is downregulated. Importantly, this work is the first one to reveal the function role of ORP proteins during adipocytes differentiation: ORP8 affects aP2 expression and ORP11 modulates aP2 and adiponectin mRNA levels, and both of them play roles as regulators of adipocyte TG storage. Moreover, disturbance of the functions of ORP proteins might have the potential to influence expression of some other key genes and additional cellular events during adipogenesis. Further study is hence warranted.

According to our data, OPR11, among all the ORP family members, is the only one induced during adipogenesis. The present work reports the tissue and cell type-specific expression patterns of ORP11. The protein localizes in the Golgi-LE interface and dimerizes with its close relative ORP9. The dimerization regulates the subcellular targeting of ORP11. Therefore, we envision that the ORP9-ORP11 dimer might act as a sterol sensor or transporter to regulate lipid homeostasis and further influence
metabolic disorders. It will be interesting to investigate whether the dimerization of ORP11 with ORP9 plays a role in adipocyte differentiation.

Common ORP7 genetic variants significantly associate with plasma LDL and total cholesterol in large population samples, indicating that also this protein might be functionally correlated with metabolic disorders (Teslovich, et al. 2010). Here, we first report that GATE-16 as a binding partner of ORP7. The work in this thesis reveals that ORP7 modulates the stability of GS28 via binding to GATE-16. Moreover, ORP7 modulates the effect of its ligand 25-OHC on proteasome-dependent degradation of GS28, which may result in modulation of Golgi transport functions.

As a conclusion, the lines of evidence from previous reports and this thesis suggest that ORPs regulate lipid metabolism, intracellular membrane trafficking and adipocyte differentiation via dimerization with their homologues, interactions with specific membrane proteins and lipid components, and via regulatory impacts on proteins characteristic of adipogenesis. Further exploration of the detailed mechanisms of ORP action and their roles in molecular pathways disturbed in human disease are warranted in future studies.
7 ACKNOWLEDGEMENTS

“It is good to have an end to journey toward; but it is the journey that matters, in the end.”

- Ernest Hemingway

The completion of this PhD thesis represents the landmark of a long journey’s end. However, I believe the challenging journey is best measured in friends rather than miles. It was only through the help and support from the following people that I could walk along this arduous but fulfilling road and reach the end.

I would like to express my heartfelt gratitude to my supervisor, Adjunct Professor Vesa Olkkonen for his thoughtful guidance, warm encouragement and constant support. What I learned from him is not only scientific knowledge but also organizing skills, sanguine attitude, great patience and scientific motivation. I deeply thank him for encouraging me to take a variety of lectures and seminars and guild me to organize multiple tasks effectively: You set up as one of the best role models in my life.

I wish to express my deep gratitude to Adjunct Professor Anna-Liisa Levonen for being as my opponent and Prof. Elina Ikonen as my Kustos. I gratefully acknowledge Prof. Pekka Lappalainen and Adjunct Professor Pirkko Pussinen for reviewing the thesis and for constructive discussions and valuable suggestions in such a tight schedule. I warmly thank my thesis committee members, Adjunct Professor Matti Jauhiainen and Dr. Saara Laitinen for your great contribution on following up the progress of my work.

Without excellent technical support from Seikku Puomilahti, Liisa Arala, Eeva Jääskeläinen, Pirjo Ranta, Sari Nuutinen and Jari Metso, close collaboration with Prof. Daguang Yan, Prof. Hannele Yki-Järvinen, Prof. Christian Ehnholm, Prof. Martin Wabitsch, Marius Robciuc, Shiqian Li, Mikko Mäyränpää, Nils Bäck, Wenbin Zhong, and effective secretarial assistances from Carita and Cia, no piece
of work in this thesis could be completed. I owe a great deal of appreciation for all of you.

Words are short to express my deep sense of gratitude towards my present and former colleagues, Marion, Markku, Olivier, Terhi, Julia, Eija, Riikka, Raghu, Henriikka, Hanna, Raisa and all the members in Minerva. I am grateful for Jarkko and Pirkka-Pekka for creating funny topics. I would like to express my sincere thanks to my present and former “office mates”, Jenny, Noora, Alise, Sami, Jani and Timofey for all the pleasing time.

This work was carried out in the National Institute for Health and Welfare (2007-2009) and Minerva Foundation Institute for Medical Research (2010-2012). I would like to thank following organisations for their generous financial support of the work: the Sigrid Jusélius Foundation, the Finnish Foundation for Cardiovascular Research, the Magnus Ehrnrooth Foundation, the Liv och Hälsa Foundation, the Novo Nordisk Foundation, the Academy of Finland, the EU FP7 (LipidomicNet), the Paulo Foundation, the Orion-Farmos Research Foundation, the University of Helsinki Medicine Fund and Jubilee Fund, the Finnish Atherosclerosis Society and the University of Helsinki Chancellor travel grant.

I am cordially thankful to Prof. Deyin Guo of Wuhan University, for his warm advice and support during my master study from 2005-2007. Dr. Chunmei Li is deeply thanked for her kind help in Finland. My thanks go to all my Chinese and Finnish friends, in particular to Lu Cheng & Danmei Huang, Erkka & Anna-Maria Tuomela.

I would like to extend huge, warm thanks to all the Finnish people – this friendly and respectful nation plays one of the most influential part in my life. Our baby, hence, has a Finnish name, Joel.

The road of the PhD journey would not be contemplated without thoughtful love and encouragement from my parents, Jian-ping Zhou and Li Wang as well as my parents-in-law, Wan Goo Cho and Kyung Hee Cho. To all of you, a thousand thanks!
At last but not least, to Eunjee for sharing all my passions and dreams. Words are not enough to express how lucky, grateful and overwhelmed I am for being your soul mate. I dedicate this thesis to you and our little Joel with all my love.

In Helsinki, January 2013

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