STEROL-BINDING PROTEINS IN LATE ENDOSONES: REGULATION OF ENDOSONE MOTILITY AND LIPID METABOLISM

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Sterol-Binding Proteins in Late Endosomes: Regulation of Endosome Motility and Lipid Metabolism

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Original Publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.


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<th>Abbreviation</th>
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<tr>
<td>ABCA1</td>
<td>ATP Binding Cassette Transporter A1</td>
</tr>
<tr>
<td>ACAT1</td>
<td>Acyl-coenzyme A Cholesterol Acyltransferase 1</td>
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<tr>
<td>acLDL</td>
<td>acetylated LDL</td>
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<tr>
<td>ApoAI</td>
<td>Apolipoprotein A1</td>
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<tr>
<td>BODIPY</td>
<td>Boron-Dipyrromethene</td>
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<td>CHO</td>
<td>Chinese Hamster Ovary</td>
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<tr>
<td>DHE</td>
<td>Dehydroergosterol</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>EEA1</td>
<td>Early Endosomal Antigen 1</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<td>HDL</td>
<td>High Density Lipoprotein</td>
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<tr>
<td>HPTLC</td>
<td>High Performance Thin Layer Chromatography</td>
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<tr>
<td>LAMP1</td>
<td>Lysosome Associated Membrane Protein 1</td>
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<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
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<tr>
<td>LDLR</td>
<td>Low Density Lipoprotein Receptor</td>
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<tr>
<td>LPDS</td>
<td>Lipoprotein Deficient Serum</td>
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<tr>
<td>LUV</td>
<td>Large Unilamellar Vesicles</td>
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<tr>
<td>LXR</td>
<td>Liver X Receptor</td>
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<tr>
<td>NPC</td>
<td>Niemann-Pick disease type C</td>
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<tr>
<td>OHC</td>
<td>Hydroxycholesterol</td>
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<td>OSBP</td>
<td>Oxysterol Binding Protein</td>
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<td>ORP</td>
<td>OSBP Related Protein</td>
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<tr>
<td>ORP1L</td>
<td>OSBP Related Protein 1 Long isoform</td>
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<tr>
<td>SCAP</td>
<td>SREBP Cleavage Activating Protein</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<tr>
<td>SREBP</td>
<td>Sterol Regulatory Element Binding Protein</td>
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<tr>
<td>SSD</td>
<td>Sterol Sensing Domain</td>
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<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>ULSAM</td>
<td>Uppsala Longitudinal Study of Adult Men</td>
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<tr>
<td>VAP</td>
<td>Vesicle Associated Protein</td>
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<tr>
<td>VLDL</td>
<td>Very Low Density Lipoprotein</td>
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<td>WT</td>
<td>Wild Type</td>
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Abstract

Despite its bad reputation in the mass media, cholesterol is an indispensable constituent of cellular membranes and vertebrate life. It is, however, also potentially lethal as it may accumulate in the arterial intima causing atherosclerosis or elsewhere in the body due to inherited conditions. Studying cholesterol in cells, and research on how the cell biology of cholesterol affects on system level is essential for a better understanding of the disease states associated with cholesterol and for the development of new therapies for these conditions. On its way to the cell, exogenous cholesterol traverses through endosomes, transport vesicles involved in internalizing material to cells, and needs to be transported out of this compartment. This endosomal pool of cholesterol is important for understanding both the common disorders of metabolism and the more rare hereditary disorders of cholesterol metabolism.

The study of cholesterol in cells has been hampered by the lack of bright fluorescent sterol analogs that would resemble cholesterol enough to be used in cellular studies. In the first study of my thesis, we present a new sterol analog, Boron-Dipyrromethene (BODIPY)-cholesterol for visualizing sterols in living cells and organism. This fluorescent cholesterol derivative is shown to behave similarly to cholesterol both by atomic scale computer simulations and biochemical experiments. We characterize its localization inside different types of living cells and show that it can be used to study sterol trafficking in living organisms.

Two sterol binding proteins associated with the endosomal membrane; the Niemann-Pick type C disease protein 1 (NPC1) and the Oxysterol Binding Protein Related Protein 1 long isoform (ORP1L) are the subjects of the rest of this study. Sensing cholesterol on endosomes, transporting lipids away from this compartment and the effects these lipids play on cellular metabolism are considered.

In the second study we characterize how the NPC1 protein affects lipid metabolism. We show that this cholesterol binding protein affects synthesis of triglycerides and that genetic polymorphisms or a genetic defect in the NPC1 gene affect triglyceride on the whole body level. These effects take place via regulation of carbon fluxes to different lipid classes in cells.

In the third part we characterize the effects of another endosomal sterol binding protein, ORP1L on the function and motility of endosomes. Specifically we elucidate how a mutation in the ability of ORP1L to bind sterols affects its behavior in cells, and how a change in ORP1L levels in cells affects the localization, degradative capacity and motility of endo-
somes. In addition we show that manipulation of ORP1L affect cholesterol balance also in macrophages, a cell type important for the development of atherosclerosis.
Review of the Literature

1. Cholesterol in Cells

The most important role of cholesterol is as a structural building block and a functional unit of cellular membranes. In addition, cholesterol is needed as a precursor for the synthesis of steroid hormones, bile acids and vitamin D. Cholesterol is a very rigid, planar molecule (Figure 1.) and therefore brings rigidity, impermeability, viscosity and stiffness to the membrane. These characteristics are especially important for the outer membrane of cells, the plasma membrane, which functions as a barrier between the cell and its surroundings. The plasma membrane also contains more cholesterol than other cellular membranes (Lange, 1991).

Figure 1. Structure of cholesterol

Cellular cholesterol levels are determined by cholesterol intake (from lipoprotein particles), cholesterol synthesis and cholesterol efflux. A lot of cholesterol is absorbed from the diet, but all nucleated cells are also capable of synthesizing cholesterol for their own needs. The central nervous system, however, does not utilize dietary cholesterol as lipoproteins do not cross the blood brain barrier. The cells of the central nervous system are thought to synthesize all the cholesterol the brain needs (Dietschy, 2009). Cholesterol cannot be broken down by our cells, and must therefore be effluxed out of cells and excreted out of the body when not needed.
1.1. Cholesterol in Cellular Membranes

The basic structure of biological membranes consists of a phospholipid bilayer. When the membrane contains large amounts of cholesterol and sphingolipids, as in the case of the plasma membrane, the so called lipid rafts are formed. Rafts are nano scale clusters of these rigid lipids that form functional domains on the membrane (Simons and Ikonen, 1997; Simons and Gerl, 2010). Rafts are believed to swim around the more fluid “sea” of the phospholipid membrane. Rafts are important at least in membrane trafficking (Romer et al., 2010) and signaling (Suzuki et al., 2007). For a broader survey of lipid raft functions in cells, see a recent review (Simons and Gerl, 2010).

In addition to the plasma membrane, cholesterol can be found in other cellular compartments in varying degrees (reviewed in (Ikonen, 2008)). The endoplasmic reticulum (ER) only contains ~1% of total cellular cholesterol (Lange, 1991), but some endosomal compartments (Möbius et al., 2003) and the trans-side of the Golgi complex (Coxey et al., 1993) contain significant amounts of cholesterol, the specific amounts depending on the metabolic state of the cells (Coxey et al., 1993). The ER, normally poor in cholesterol, contains the homeostatic machinery for maintaining intracellular cholesterol levels (see section 1.2. “Metabolic Homeostasis of Cholesterol”). Due to its low cholesterol content, even small changes in cholesterol levels can be sensed, enabling the cell to respond to small changes in sterol levels (Radhakrishnan et al., 2008). Mitochondria are the site for steroid hormone synthesis and some cholesterol oxidation reactions, so cholesterol is actively transported also to mitochondria, especially in steroidogenic cells (Kallen et al., 1998).

Lysosomes in a steady state are cholesterol-poor (Möbius et al., 2003). Some compartments, like multivesicular endosomes and the recycling compartment do contain larger amounts of cholesterol (Hao et al., 2002; Möbius et al., 2003). How much cholesterol resides in a given compartment also depends greatly on the cell type and its homeostatic state.

1.2. Metabolic Homeostasis of Cholesterol

At high concentrations, cholesterol forms cholesterol crystals, which are toxic to cells. High concentrations of cholesterol can also harm cells e.g. by causing ER stress (Feng et al., 2003). Too little cholesterol is, however, also detrimental to cells. This is demonstrated in inherited defects of cholesterol synthesis (reviewed in (Porter, 2002)) where function of
the plasma membrane, functions of many sterol associated proteins, synthesis of sterol derivatives and signaling pathways are perturbed. This has led to the evolution of tightly regulated cholesterol balancing systems, best characterized in fibroblasts and hepatocytes (Brown and Goldstein, 2009).

When cellular cholesterol levels decrease, cells respond by increasing cholesterol synthesis and by taking up more cholesterol as lipoprotein particles. All cells can synthesize cholesterol from acetate, but many peripheral cells also take up Low Density Lipoproteins (LDL) by receptor mediated endocytosis, and use cholesterol contained in these particles. When cholesterol concentration in cells decrease, a protein complex in the ER senses this change and activates transcription of genes involved in acquiring more cholesterol, i.e. genes of cholesterol synthesis and uptake (Figure 2). The core part of this machinery and the actual transcription factor is called the Sterol Regulatory Element Binding Protein 2 or SREBP2. When sterol levels are low, SREBP2 is transported to the Golgi where it is cleaved to yield an active transcription factor that enters the nucleus and activate genes that have the Sterol Regulatory Element in their promoter (Wang et al., 1994). SREBP2 itself is not, however, sensitive to the surrounding sterol levels. Instead, the scaffolding protein of SREBP2, the SREBP Cleavage Activating Protein (SCAP) has a Sterol Sensing Domain (SSD) that is believed to convey the sterol sensitivity of the system (Nohturfft et al., 2000). When sterol levels in the ER are sufficient, SCAP binds to yet another protein, Insig, which blocks the exit of the SREBP-SCAP complex from the ER and hence inhibits transcription of genes needed for increasing cholesterol levels (Yang et al., 2002). When sterol levels decrease, the conformation of SCAP changes and it no longer binds Insig. This frees the SREBP-SCAP complex to be transported to the Golgi, where SREBP2 is cleaved to an active transcription factor. Another form of SREBP, SREBP1C is involved in the regulation of neutral lipid synthesis, but has less effect on cholesterol balance (Liang et al., 2002) (Figure 2).

If, on the other hand, cholesterol levels in the cell are too high, another transcription factor system is activated, leading to elimination of cholesterol from cells (Venkateswaran et al., 2000). The main components of this system are the Liver X Receptors (LXRs). These are activated by several different oxysterols (Janowski et al., 1999), i.e. oxidized derivatives of cholesterol that form when cholesterol levels increase. LXRs form heterodimers with a Retinoid X Receptor (RXR) to activate transcription (Willy et al., 1995). LXR target genes include several ATP Binding Cassette (ABC) transporters such as ABC-A1 and ABC-G1 transporting cholesterol out of cells, but also many genes necessary for eliminating cholesterol from the body, such as genes involved in bile acid synthesis and secretion (Figure 2). LXR activation thus leads to elimination of cholesterol at both cellular and whole body level. In addition, LXRs also activate fatty acid and triglyceride synthesis, but the signific-
ance of this somewhat paradoxical phenomenon has remained unclear. A more complete list of LXR targets and mechanism of function is presented in (Millatt et al., 2003).

**Figure 2.** Transcriptional regulation of lipids

In addition to activating the LXR pathway, cells are also able to neutralize cholesterol by esterifying it with a fatty acid. These cholesteryl esters are generated by the ER resident protein Acyl-coenzyme A Cholesterol Acyltransferase 1 (ACAT1) (Stokke and Norum, 1970) and the esters are mainly stored in cytoplasmic lipid droplets. This enzyme is not regulated by the transcriptional systems described above, but allosterically by cholesterol so that when abundant in the ER, cholesterol becomes esterified (Chang et al., 1998).
2. Cholesterol Trafficking in Cells

Due to its high hydrophobicity, cholesterol does not dissolve in water, and therefore cannot travel freely through the cytosol by aqueous diffusion. Cholesterol transport in cells may occur through several different mechanisms. It can travel along with cellular membrane trafficking, it can be transported by cytosolic carrier proteins or it can move from membrane to membrane via membrane contact sites (Ikonen, 2008). There is evidence to support the existence of all three routes, and it is probable that all of these routes contribute to the distribution of cholesterol.

Intracellular membrane trafficking is a major means to transfer cargo in cells. In this process vesicles bud off from one membrane compartment (e.g. ER), travel to the target membrane (e.g. the Golgi complex) and fuse with it. Cargo can travel along inside the vesicle or on the vesicle membrane. Many membrane trafficking routes have been characterized in detail. In general, membrane trafficking needs machineries for vesicle budding from the donor membrane (e.g. coat proteins such as clathrin) (Kirchhausen, 2000), transport machineries (e.g. molecular motors) to travel from the original membrane to the target (Ross et al., 2008) and fusion machineries (e.g. the Soluble NSF Attachment Protein Receptor (SNARE) proteins) to fuse with the target membrane (Jahn and Scheller, 2006). Long range movement of membranes takes place via microtubules. Molecular motors exhibit the ability to walk along the microtubules, but are restricted in their directionality. One family of proteins called dyneins is responsible for transport towards the microtubule minus ends (towards the centrosome). Another family of proteins, kinesins, are responsible for transport towards the microtubule plus ends (towards the cell periphery) as in the case for endosomes (Loubery et al., 2008). Both proteins also need several accessory proteins, such as dynactin, to function in cells. Plus and minus end directed movement of organelles is known to be coregulated and different motors are known to exist simultaneously on the same organelles (Loubery et al., 2008; Ross et al., 2008). Cholesterol does travel passively along with intracellular membrane trafficking. The transport of cholesterol does, however, seem to also depend on membrane trafficking, at least when exiting from endosomes (Hölttä-Vuori et al., 2000).

Another possible way for cholesterol to move inside cells is via cytosolic carrier proteins. Several families of cytosolic sterol binding proteins could potentially bind cholesterol on one membrane and hand over the cholesterol molecule to the target membrane. These families include the Oxysterol Binding Protein (OSBP) Related Proteins (ORP), the Star-related lipid transfer domain containing proteins (STARTs) and the Sterol Carrier Proteins (SCPs). Of these, the ORPs are described in more detail under “ORP Family of Proteins” and START and SCP family of proteins are more extensively covered in a recent review (Lev, 2010).
The third mode to transfer cholesterol from membrane to membrane is via membrane contact sites. These are locations in cells where membranes of two organelles come close together, but do not fuse or necessarily touch physically (Levine and Loewen, 2006). The physical closeness of the membranes increases the probability for lipids to transfer from one membrane to another, and this process may be aided by proteins localized to the contact sites. This cholesterol transport route, however, has the least experimental evidence to support it (Lev, 2010).

2.2. Endosomal Cholesterol Trafficking

One of the trafficking routes of cholesterol is the endocytic transport of LDL particles to lysosomes. Endosomes constitute a membrane transport pathway starting at the plasma membrane and ending in lysosomes, where cargo is degraded (Saftig and Klumperman, 2009). Endocytic cargo is first transported to early/sorting endosomes, then further into late endosomes and finally to lysosomes. In this endosomal progression the pH of the organelles gradually decreases and the outer endosomal membrane buds inwards forming internal membranes (Saftig and Klumperman, 2009). On light microscopy level it is difficult to separate late endosomes from lysosomes as they contain a lot of the same markers. These markers include Lysosome Associated Membrane Proteins (Lamps) and many endocytosed fluorescent markers (such as small molecular weight dextran used in this thesis) which reside in both compartments. The term endolysosome is used in this thesis to refer to late endosomes and lysosomes together. LDL particle bound to the LDL receptor at the plasma membrane is transported along this pathway to endolysosomes where the LDL-derived cholesteryl esters are hydrolyzed, followed by efflux of cholesterol to other cellular membranes (Ikonen, 2008).

The transport of cholesterol out of endolysosomes appears to be slow (Schoer et al., 2000) and to some extent depend on membrane trafficking (Hölttä-Vuori et al., 2000). In contrast, the transport of cholesterol from the ER to the plasma membrane (Urbani and Simon, 1990; Heino et al., 2000) and from the plasma membrane to ER and lipid droplets (Raychaudhuri et al., 2006; Jansen et al., 2010) has been shown to be mostly independent of vesicular trafficking. Many disorders of endosomal function such as Niemann-Pick diseases (described below), Gaucher disease (Vaccaro et al., 2010) and Mucolipidosis type IV (Soyombo et al., 2006) lead to accumulation of free cholesterol in endosomes. Reasons to why sterol transport in/from endolysosomes is so different from other cellular membranes may include the highly glycosylated limiting membrane of lysosomes. This rigid membrane functions to protect the lysosomal outer membrane from degradation, isolat-
ing the outer membrane from the lysosomal lumen and possibly affecting cholesterol mobility.

### 2.2.1. NPC Family of Proteins

Niemann-Pick type C (NPC) disease (Online Mendelian Inheritance in Man (OMIM) IDs #257220 and #607625) is an autosomal recessive disorder characterized by accumulation of cholesterol and other membrane constituents in all tissues (Figure 3, reviewed in (Vanier, 2010)). The disease manifests itself most commonly as a neurodegenerative disease leading to death often in late childhood. Mutations in either of the two causative genes NPC1 (Carstea et al., 1997) or NPC2 (Naureckiene et al., 2000) lead to indistinguishable clinical and biochemical diseases. The major early finding of the disease was that NPC patient fibroblasts failed to respond to increased levels of LDL by increasing cholesterol esterification (Pentchev et al., 1985). Control cells started to increasingly esterify cholesterol in order to limit the amount of cellular free cholesterol, in response to an increase in cholesterol levels, but NPC cells were unable to accomplish this. Later it was shown that NPC cells are unable to suppress the biosynthesis of cholesterol (Liscum and Faust, 1987) and to generate oxysterols (Frolov et al., 2003) in response to LDL and are not able to efflux cholesterol out of the cells (Choi et al., 2003). It is therefore thought that NPC is primarily a cholesterol retention disease and that NPC1 and NPC2 function in the mobilization of LDL-derived cholesterol (Wang et al., 2010). NPC1 is a member of the resistance-nodulation-cell division (RND) permease family of proteins known to transport small hydrophobic molecules in bacteria, thereby causing multidrug resistance (Blair and Pidcock, 2009). Whether NPC1 could also function in pumping cholesterol or other small hydrophobic molecules, such as fatty acid (Davies et al., 2000) or sphingosine (Lloyd-Evans et al., 2008) has long been discussed, without convincing results (Lloyd-Evans and Platt, 2010).

NPC1 is a late endosomal protein containing 1278 amino acids and 13 putative transmembrane helices (Davies and Ioannou, 2000), a SSD (Carstea et al., 1997) and an N-terminal cholesterol binding domain (Kwon et al., 2009). The SSD is homologous to the SSD of SCAP and of the rate limiting enzyme of cholesterol synthesis, 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase. Both of these proteins are regulated through the sterol binding by SSD. This domain is also shared by the morphogen receptor Patched that is essential for animal development. The precise function of SSD in NPC1 and Patched is unknown, but mutations in the SSD render both proteins non-functional (Watari et al., 1999; Martin et al., 2001).
NPC2 is a small soluble lysosomal protein containing 132 amino acids that bears no homology to NPC1. It is targeted to lysosomes via the mannose-6-phosphate and its receptor (Naureckiene et al., 2000) and is also secreted to many physiological fluids such as plasma and bile (Klein et al., 2006). The protein consists mostly of a cholesterol binding cavity and is able to transfer cholesterol between membranes in vitro. The transfer is highest at low pH and in the presence of lipids typical for the lysosomal inner membranes (Cheruku et al., 2006), rendering the protein most active in late endosomes and lysosomes. NPC2 is the causative gene in only ~5% of all NPC cases and the rest are believed to be caused by NPC1.

Once NPC2 has bound cholesterol, it can transfer it to the NPC1 N-terminal domain in vitro. A model of “hydrophobic handoff” was proposed based on these data. In this model NPC2 binds cholesterol immediately after it has been released by Acid Lipase, and transfers it to NPC1, that is then able to place the cholesterol molecule on the lysosomal outer membrane (Wang et al., 2010). The N-terminal domain used in these studies, however, is only 17% of the total protein, so there is probably more to NPC1 function than known to date.

**Figure 3.** Filipin staining of an epidermal carcinoma cells (A431) treated with control small interfering RNA (siRNA) or siRNA against NPC1.

![control](image1.png) ![NPC1](image2.png)

### 2.2.2. ORP Family of Proteins

OSBP related proteins (ORPs) are a family of 12 ORP proteins, all bearing a common oxysterol binding domain (ORD; OSBP-related ligand-binding domain), enabling ORPs to bind oxysterols. The crystals structure of the yeast OSH4 together with its sterol ligand
has been resolved (Im et al., 2005). In addition to the ORD domain, most ORPs contain a pleckstrin homology domain that binds phosphatidylinositol lipids allowing ORPs to associate with membranes. Some ORPs also contain a FFAT (two phenylalanines in an acidic tract) domain involved in binding the ER resident Vesicle Associated Protein (VAP) (reviewed in (Yan and Olkkonen, 2008)). Distinct functions for several ORP proteins have been described; OSBP functions in the integration of cholesterol balance and sphingomyelin synthesis (Perry and Ridgway, 2006), ORP1L has a role in motor recruitment to endosomes (Johansson et al., 2007) and ORP2 regulates neutral lipid homeostasis (Hynynen et al., 2009) to mention but a few. The ORP family is conserved throughout the eukaryotic lineage. The yeast Saccharomyces cerevisiae has seven Oxysterol Binding Protein Homologs (OSH1-OSH7), indicating a fundamental role for these proteins in eukaryotic cell biology (Yan and Olkkonen, 2008).

Whether ORPs transfer sterols from one membrane to another or function as sterol sensors regulating other cellular processes has remained a matter of debate. In yeast it was shown that knocking out all of the seven yeast ORP homologues, impairs the transport of cholesterol (Raychaudhuri et al., 2006). No effect was, however, achieved by knocking down single ORP-homologues (Raychaudhuri et al., 2006). Some ORPs have also been shown to transfer cholesterol between liposomes in vitro (Ngo and Ridgway, 2009; Jansen et al., 2010) suggesting that at least some ORPs may transfer cholesterol between membranes also in vivo. Whether cholesterol transport is the physiological role of mammalian ORPs, remains to be elucidated.

ORP1L (ORP1 Long isoform), the ORP family member studied in this thesis is a late endosomal protein needed for normal perinuclear localization of late endosomes (Johansson et al 2007). It is the longer product of the ORP1 gene, which also gives rise to ORP1S (ORP1 Short isoform). ORP1L and ORP1S have different intracellular localizations and different tissue distributions, indicating diverging functions (Johansson et al., 2003). ORP1L forms a complex with Rab7, its effector RILP and dynein, driving the minus-end directed movement of late endosomes (Johansson et al 2007). ORP1L has also been shown be involved in the development of atherosclerotic lesions in vivo (Yan et al 2007).

2.3. Uptake of Cholesterol into Cells

When cells need cholesterol, they can acquire more by de novo synthesis, or by taking up cholesterol from various lipoprotein particles that serve as cholesterol donors to cells. Most peripheral cells take up cholesterol by receptor mediated endocytosis of LDL-particles. The internalization of LDL starts when the major protein of LDL-particles, apo-
lipoprotein B (ApoB) binds to cell surface LDL Receptor (LDLR) which triggers internalization of this complex (Ho et al., 1976). The complex is endocytosed in clathrin coated pits and vesicles, and transported to early endosomes. In early endosomes the LDLR is released and is ready to recycle back to the plasma membrane via endocytic recycling pathways. The LDL particle is transported along the endocytic pathway to late endosomes and lysosomes, where the enzyme acid lipase hydrolyses the cholesteryl ester molecules to yield free cholesterol and free fatty acids (Ikonen, 2008). How exactly cholesterol then leaves the endosomal system has remained unclear, except that NPC1 and NPC2 are probably involved in the placing of cholesterol to the lysosomal outer membrane.

Special cell types: hepatocytes, macrophages and steroidogenic cells can take up cholesteryl esters from High Density Lipoprotein (HDL) particles via scavenger receptor B1 through selective lipid uptake where the cholesteryl ester molecules enters the cell, but the protein does not (Acton et al., 1996). Scavenger Receptor B1 is also used by macrophages in the uptake of LDL particles modified in the arterial intima. These modified particles are no longer recognized by LDLR (this process is described in more detail under “Role of macrophages in atherosclerosis”). A special case is also presented by enterocytes that are able to absorb diet-derived cholesterol from bile salt micelles in the gut lumen.

2.4. Secretion of Cholesterol from Cells

Just as cholesterol cannot freely move through the cytosol, it also cannot freely move in the circulation. Lipoproteins have evolved to transport cholesterol and other lipids from one tissue to another; to transport diet and liver-derived lipids to peripheral cells and cholesterol from peripheral cells to the liver. Lipoproteins are droplets of neutral lipids (ranging from 5-500nm) surrounded by a monolayer of phospholipids, free cholesterol, and an apolipoprotein (Figure 4).

The cycle of cholesterol in lipoproteins starts in enterocytes that pack diet-derived lipids into chylomicron particles and secrete the particles into the lymph. Chylomicrons consist mostly of triglycerides. Most of this triglyceride is hydrolyzed by endothelial lipase and stored in the adipose tissue and the chylomicron remnants are taken up by the liver. The liver can use the cholesterol derived from chylomicron remnants for its own needs, store it, or pack it further in Very Low Density Lipoprotein (VLDL) particles. These particles are again secreted into the circulation. The triglyceride in VLDL particles is hydrolyzed to yield free fatty acids by members of the lipase-family proteins in the capillary endothelium (Hasham and Pillarisetti, 2006). What remains after this process is the LDL particle. Peripheral cells can take up these particles and use the cholesterol in them as their cholesterol
source, but the majority of LDL is taken up to the liver (Spady et al., 1983). The so called reverse cholesterol transport pathway starts when the peripheral cells have more cholesterol than they need. This cholesterol is secreted into circulating HDL particles that are taken up by the liver which is able to secrete cholesterol to the bile and out of the system. For long it was believed that the liver is the only organ capable of secreting cholesterol out of the body, but it has been recently shown that the gut also contributes to fecal cholesterol excretion (van der Velde et al., 2007).

**Figure 4.** Common lipoproteins.

The assembly of the chylomicron particles in enterocytes and VLDL particles in hepatocytes is largely similar. The process begins when ApoB mRNA is translated on the ER membrane. In enterocytes ApoB mRNA is modified to yield a message 48% of the full length, so that chylomicrons produce this shorter version of the protein, called ApoB48. In the human liver, this modification does not take place, and the full length ApoB, ApoB100 associates with the VLDL particle. ApoB48 lacks the LDLR binding site, and chylomicrons are thus not able to enter cells via the LDLR pathway. The lipidation of ApoB starts already during translation as the newly formed polypeptide is inserted into the ER (Figure 5). This lipidation is mediated by the Microsomal Triglyceride transfer Protein (MTP) (Ingram and Shelness, 1997). The particles are then transported via the
secretory pathway to the Golgi and secreted to the lymph (chylomicrons) or into the blood stream (VLDL). How and where the final lipidation of the particle happens has remained elusive, but cytosolic lipid droplets probably provide the bulk triglyceride (Oh-saki et al., 2008; Ye et al., 2009).

**Figure 5.** Cholesterol secretion from cells. Left: VLDL secretion starts when the ApoB mRNA is transcribed on the ER membrane. The particle is gradually lipidated and secreted out of the cell (Arrows). Right: ApoAI binds to cell surface ABCA1 that transports membrane cholesterol to ApoAI. Dashed arrows indicate possible routes of cholesterol to the lipoproteins.

The route of HDL also starts from the liver or enterocytes that secrete Apolipoprotein AI (ApoAI) proteins. ApoAI is secreted in a lipid-poor form that is able to collect cholesterol from peripheral cells in a process called reverse cholesterol transport. Cholesterol is secreted to ApoAI as free cholesterol and subsequently esterified by Lecithin-cholesterol
acyltransferase (LCAT), a protein associated with HDL. As cholesterol accumulates on the ApoAI it becomes a spherical, mature HDL particle (Zannis et al., 2006). The secretion of cholesterol to lipid-poor ApoAI and mature HDL happens via several plasma membrane cholesterol transporters of the ATP Binding Cassette (ABC) family (Cavelier et al., 2006). The most studied one of these is ABCA1 (ATP Binding Cassette transporter A1). The absence of functional ABCA1 causes Tangier disease that is characterized by a lack of HDL particles (Brooks-Wilson et al., 1999). ABCA1 mediated cholesterol efflux takes place on the plasma membrane and possibly in endosomes after endocytosis of ApoAI (Chen et al., 2005b), but the exact localization of cholesterol transfer to ApoAI remains debated (Kang et al., 2010) (Figure 5). ABCA1 prefers to efflux cholesterol to lipid-poor ApoAI and ABCG1 transfers cholesterol to the more mature, spherical HDL (Wang et al., 2004). The precise molecular mechanism of lipid transfer by these proteins remains to be elucidated.

3. Studying Cholesterol Trafficking in Cells

A major obstacle in studying intracellular cholesterol transport has been the lack of good methods. The interest in lipid cell biology has been rather low until the 21st century, and (cell biological) lipid methodology has therefore developed slowly. Lipids were long considered to be merely a source of energy and an inactive layer on the cell surface. With the emergence of the raft theory (Simons and Ikonen, 1997), increasing knowledge on atherosclerosis, and new connections between lipids and other diseases, such as Alzheimer’s disease (Corder et al., 1993), the field of lipid research is gaining increasing interest. This is, to the benefit of a lipid scientist, also leading to new advances in technology in the field.

Several challenges are ahead when one takes up the analysis of lipids; they cannot be chemically fixed, they easily oxidize during sample preparation if not handled correctly and their analytical methods are painstaking and involve many hazardous organic solvents. These difficulties have led to conflicting results and many unanswered questions. To overcome these handicaps, several different methodologies need to be used in parallel, to produce reliable results.

3.1. Biochemical Methods to Study Cholesterol

Cholesterol and other lipids can be biochemically separated from other organic matter by using chemical extraction by organic solvents and from each other by using chromato-
graphic separation. For cholesterol this procedure is relatively easy as it can be separated from other cellular lipids by Thin Layer Chromatography (TLC). This method, however, does not separate cholesterol from its closest precursor sterols. However, in most cases cholesterol constitutes the absolute majority of sterols, and the precursors may often be ignored. For precise analysis of many other lipids, more sophisticated analysis such as mass spectrometry needs to be used.

To study specific pools of cholesterol (or other lipids) one can use radioactively labeled lipids or lipid precursors and feed them to cells. The kinetics of metabolism and to some extent also transport can then be calculated from the results. While precise information about metabolism can be achieved using these methods, it should be carefully considered how the radioactive tracer is fed to the cells - the only physiological way for exogenous cholesterol to enter most cells is via lipoprotein particles.

While radioactive cholesterol can help elucidate metabolism of cholesterol very accurately, less information can be drawn about its transport. To analyze where radioactive cholesterol was transported to during the experiment, one must separate cellular compartments biochemically. This is most often done with density gradient ultracentrifugation where different compartments separate according to their density e.g. on a sucrose matrix. Cholesterol can, however, move between compartments during sample preparation and the presence of cholesterol in a certain compartment in a biochemical experiment is not a guarantee that it has been transported there physiologically. Alternatively, the localization of radioactive cholesterol can be deduced from enzymatic processes. If, for example, radioactive cholesterol is esterified, it must have reached the ER where the esterification takes place. Esterification cannot, however, be used as an absolute measure of transport, as not all cholesterol transported to the ER is esterified (Jansen et al., 2010). Plasma membrane localized cholesterol can be determined by extracting cholesterol by methyl-\(\beta\)-cyclodextrin (Pörn and Slotte, 1995; Heino et al., 2000) or by oxidizing cell surface cholesterol after fixation of cells (Lange, 1991). Methyl-\(\beta\)-cyclodextrin was, however, recently shown to be internalized to cells by endocytosis, and may also extract cholesterol therein (Rosenbaum et al., 2010).

Sterol binding and transfer by sterol binding proteins can be studied in vitro. A sterol binding protein can extract cholesterol from unilamellar vesicles (Ngo and Ridgway, 2009). Furthermore, the efficacy of a potent sterol transporter can be studied in a similar system where one pool of vesicles contains radioactive lipid and the other does not (Jansen et al., 2010). These methods, however, apply preferentially to soluble proteins as membrane proteins cannot easily be inserted into liposomes. Protein binding to cholesterol in vivo can be studied using photocholesterol (7,7-azocholestanol) that is first incorporated into living
cells. This method makes it possible to study sterol binding of membrane protein such as NPC1 (Ohgami et al., 2004) and MLN64 (Hölttä-Vuori et al., 2005).

3.2. Imaging Methods to Study Cholesterol

While biochemical methods cannot provide high resolution data about cholesterol localization in cells, methods using microscopy do this much more precisely. Cholesterol itself cannot, however, be imaged with traditional microscopy. One has to rely on either molecules that bind cholesterol or on using fluorescent cholesterol analogs that behave similarly to the natural lipid (Table 1).

Table 1. Fluorescent compounds to study cholesterol localization in cells. (modified from (Ikonen and Hölttä-Vuori, 2004)). EM= Electron microscopy.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Characteristics</th>
<th>Living Cells?</th>
<th>EM?</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filipin</td>
<td>Fluorescent antibiotic</td>
<td>No</td>
<td>Yes</td>
<td>(Linder et al., 2007)</td>
</tr>
<tr>
<td>θ-toxin</td>
<td>Derived from Clostridium perfringens toxin, perfringolysin-O</td>
<td>No</td>
<td>Yes</td>
<td>(Möbius et al., 2003; Sugii et al., 2003)</td>
</tr>
<tr>
<td>Dehydroergosterol (DHE)</td>
<td>A yeast natural sterol</td>
<td>Yes</td>
<td>No</td>
<td>(Mukherjee et al., 1998; Hao et al., 2002)</td>
</tr>
<tr>
<td>Dansyl cholesterol</td>
<td>A cholesterol with a fluorescent dansyl group on position 6</td>
<td>Yes</td>
<td>Possible but no reported so far</td>
<td>(Wiegand et al., 2003)</td>
</tr>
<tr>
<td>NBD-cholesterols</td>
<td>Several conjugates of NBD and cholesterol</td>
<td>Yes</td>
<td>Possible but no reported so far</td>
<td>(Alecio et al., 1982)</td>
</tr>
</tbody>
</table>

The most widely used cholesterol staining agent is an antibiotic named filipin (Drabikowski et al., 1973) that binds covalently to cholesterol 3'-OH group and is intrinsically fluorescent. Because of its antibiotic properties, it cannot, however, be used in living cells as while binding to cholesterol it perforates membranes. Filipin is considered to be a ra-
ther faithful indicator of cholesterol distribution in cells, albeit it recognizes other 3'-hydroxylated sterols in addition to cholesterol. As cholesterol comprises the majority of sterols in cells, the lack of specificity may often be ignored. Potential problems in using filipin include fixation of cells using paraformaldehyde which does not fix cholesterol. One cannot therefore be sure that cholesterol did not move from its original position during sample preparation. The binding of filipin to cholesterol may also be dependent on the membrane environment of cholesterol (Blau and Bittman, 1977). \(\Theta\)-toxin is another molecule that binds to cholesterol. It can be fluorescently labeled and can therefore be used as a marker for cholesterol (Möbius et al., 2003). It does not, however, penetrate the cells so it also cannot be used for living cells (Table 1).

The other alternative is to use cholesterol-mimicking fluorescent tracers. The advantage in using fluorescent sterols is that they do not require fixation of cells and enable the follow up of sterol trafficking in living cells. The use of fluorescent sterols is discussed in more detail in the Results part of this book.

### 4. Metabolic Diseases Affected By Endosomal Cholesterol

Consuming excess amounts of lipid and cholesterol has led to the emergence of metabolic diseases in the western world. The most commonly known of these is atherosclerosis, caused by the retention of modified lipoproteins in the arterial intima, but atherosclerosis is often a part of a more complex disorder; the metabolic syndrome. Metabolic syndrome is characterized by central obesity, dyslipidemia and insulin resistance and creates a risk for developing diabetes and cardiovascular disease.

#### 4.1. Role of Hepatocytes in Metabolic Disease

The liver is the major logistic station in cholesterol homeostasis at the whole body level. Modern western diet contains approximately 400mg cholesterol per day, and our bodies produce an additional 10mg/kg/day (Dietschy et al., 1993). Diet-derived cholesterol is transported to the liver in chylomicrons, and the liver is also responsible for most of cholesterol synthesizes (outside the central nervous system). The liver also converts cholesterol to bile acids and secretes bile acids and cholesterol into the gall bladder from where cholesterol eventually ends up in the small intestine and is excreted out of the body. Some of the cholesterol is reabsorbed back to the enterocytes and cycle back to the liver, contributing to the enterohepatic cycle.
The liver is severely affected by excess consumption of lipid due to its central role in lipid metabolism. As natural selection has led to a system optimizing usage of energy and cholesterol, the liver is not equipped to function with this kind of excess. Three things therefore happen; 1) cholesterol is secreted into bile in excess leading to cholesterol crystallization in the gall bladder creating gall stones, 2) increasing storage of cholesterol and other lipid in the liver causing nonalcoholic fatty liver disease and rendering the liver insulin resistant (Seppälä-Lindroos et al., 2002) and 3) increased secretion of cholesterol and other lipids in VLDL particles (Adiels et al., 2006), leading to increased circulating LDL cholesterol.

Increased cholesterol leads to increased LXR activation causing increased hepatic de novo lipogenesis. Hepatocytes receive lipid from the diet, but also from the adipose tissue and from the so called “spill over pathway” where peripheral tissues are not able to receive all of the lipoprotein-derived fatty acids and this lipid ends up in the liver (Donnelly et al., 2005). This lipid is stored in hepatic lipid droplets.

4.2. Role of Macrophages in Atherosclerosis

Atherosclerosis is a widely studied disease where excessive consumption of cholesterol is considered to be the major contributor. Excess dietary cholesterol, together with de novo synthesized cholesterol lead to surplus LDL in the circulation. Circulating LDL passes through the endothelium to reach target tissues that use LDL. In the arterial intima, however, the internal elastic lamina prevents LDL particles from reaching the underlying tissue (Smith, 1990). LDL therefore becomes trapped in the intima. These LDL particles get oxidized and further modified by resident enzymes and aggregate. Accumulating cholesterol in the intima attracts macrophages that try to clear out this cholesterol (Tabas et al., 2007). While failing at this task the macrophages internalize toxic amounts of cholesterol, store this cholesterol in their lipid droplets and become so called “foam cells”. These foam cells attract more immune cells and eventually die by apoptosis or necrosis, contributing to the accumulation of debris in the intima. As this process proceeds over decades the intima thickens, develops a necrotic core consisting of cholesterol and dead cells. These atherosclerotic plaques may eventually rupture causing a heart attack or stroke.

As is evident from the above description, macrophages play a key role in the development of atherosclerosis. While lowering circulating LDL is the main clinical target in atherosclerosis management, pharmacological modifications of macrophage function are attracting increasing interest (Tabas et al., 2007). Macrophages are able to internalize modified lipoproteins found in atherosclerotic lesions very efficiently and very efficiently metabolize
this cholesterol and store it in lipid droplets. Higher levels of HDL are associated with smaller risk of atherosclerosis (Gordon et al., 1977), presumably because of the ability of HDL to enhance reverse cholesterol transport, i.e. cholesterol transport from macrophages to the liver.

Uptake of cholesterol is somewhat different in macrophages compared to other peripheral cells, as they are able to endocytose not only native LDL, but also oxidatively modified and aggregated LDL. The Scavenger Receptor B1 is mostly responsible for this uptake (Vainio and Ikonen, 2003). The LDLR is downregulated when cellular cholesterol levels increase (through a decrease in SREBP signaling), which prevents excessive accumulation of cholesterol in most cells. The SR-B1, however, is not downregulated in a similar manner, but the macrophages internalize toxic amounts of cholesterol eventually leading to apoptosis (Vainio and Ikonen, 2003). Macrophage cholesterol efflux is therefore a highly studied subject as enhancement of this efflux could decrease the advancement of atherosclerosis.
Aims of the Study

This study aimed to shed light on how endosomal cholesterol and endosomal cholesterol binding proteins NPC1 and ORP1L affect biological processes on the cellular and whole body level. The individual aims of the three studies making up this thesis are listed below:

I) We aimed to analyze whether a new fluorescent sterol analog, BODIPY-cholesterol would be suitable for visualizing cholesterol in living cells and organisms and to bring a new tool to the cholesterol field. The chemical synthesis and physical behavior of BODIPY-cholesterol has been reported (Li et al., 2006) making it an interesting candidate to be used for visualizing cholesterol.

II) The aim of the second study was to characterize the interplay between Niemann-Pick type C disease and triglyceride homeostasis. This is a so far unexplored area, but receives increasing interest as our knowledge of the integration of metabolic pathways increases. In this study we analyzed the effects of common polymorphisms in the NPC1 gene on serum lipids at the population level and lipid metabolism in the liver and hepatocytes of Npc1+/+ and Npc1-/- mice.

III) The aim of the third study was to characterize how ORP1L and its sterol binding status affect endosomal processing and motility. It has been previously shown that this protein is involved in endosomal motor recruitment and localization and that it is involved in the development of atherosclerotic lesions. This study aimed at integrating previous cell biological findings with further characterization of the cell biology and effects of ORP1L in macrophages.
5. Materials and Methods

5.1. Animal Work

The Npc1-/- mouse has a spontaneous mutation in its Npc1 gene, and the mouse model was characterized already in 1980s (Miyawaki et al., 1982). The animals were maintained and used for experiments according to the guidelines of the institutional animal care and use committee. They were maintained on a chow diet (Altromin 1320) in standard conditions. Roughly equal amounts of female and male mice were used as the results were similar in both genotypes, except in work II Figure 5 where only male mice were used.

Primary mouse hepatocytes were isolated using the collagenase perfusion system (Smets et al., 2002). The mice were anesthetized using Mebunat 60mg/kg. The inferior vena cava was cannulated, the portal vein of the liver was sectioned and Earles balanced Salt solution (EBSS) with 0.5mM EGTA and 10mM Hepes, excluding Ca\(^{2+}\) and Mg\(^{2+}\) was flowed through the liver at 5ml/minute. After 6 minutes, the solution was changed into EBSS including Ca\(^{2+}\) and Mg\(^{2+}\) and this was flowed through the liver for 3 minutes. Finally, EBSS including collagenase was allowed to flow through the liver for approximately 6 minutes to collapse the liver macrostructure. The collapsed liver was then filtered through a 100µm nylon mesh to separate cells. The hepatocytes were washed 3-5 times with DMEM containing 10% Fetal Bovine Serum (FBS), 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin and 10mM Hepes and plated on cell culture dishes coated with gelatin. One million cells were plated on a Ø 6 cm dishes and 500 000 cells on Ø 3.5cm dishes. Hepatocytes were maintained in culture for no more than 24 hours.

5.2. Cell Culture and Transfection

Wild Type (WT) Chinese Hamster Ovary (CHO), M12 (Millard et al., 2000), and M19 (Hasan et al., 1994) cells were cultured in DMEM supplemented with 5% FBS, 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin. HeLa cells were cultured similarly, except with 10% FBS. Raw264.7 cells were cultured as HeLa cells but including 10mM Hepes. Cell culture plastics were from Gibco or Sarstedt. Lipoprotein deficient serum (LPDS) was prepared by sequential KBr gradient ultracentrifugation of FBS (Krieger, 1986).

cDNA constructs were transfected to cells using LipoFectamine2000. For 0.5ml of cells, 3µl LipoFectamin2000 (Invitrogen) was diluted in 100µl and 1µg DNA was diluted in 100µl OPTIMEM and these complexes were incubated for 5 minutes. The LipoFecta-
mine2000 solution was then added to the DNA solution and this was incubated further for 15 minutes. The transfection complex was then added to freshly divided cells in antibiotic free medium. The complex was removed after 4 hours and the overexpressing cells were analyzed the following day. For transfections with small interfering RNAs (siRNA) on Ø 3.5cm dishes in 1ml of culture medium, 20pmol of the siRNA duplex and 6µl of the HiPerFect reagent (Qiagen) were diluted in 100µl OPTIMEM and the complex was incubated 5-10 minutes. The complex was then added to cells cultured in complete medium. The cells were analyzed after 3 days of siRNA transfection.

5.3. Labeling of Cells with Fluorescent Probes

BODIPY-cholesterol was fed to cells dissolved in DMSO (dimethyl sulphoxide) 0.5µM in LPDS overnight to two days. Alternatively, BODIPY-cholesterol was complexed with methyl-β-cyclodextrin with a final calculated concentration of 25µg/ml BODIPY-cholesterol (with molar ratio of sterol/ cyclodextrin 1/10 as in (Leppimäki et al., 2000)). Rhodamine-dextran was fed to cells overnight at 1mg/ ml and Alexa 568-dextran overnight at 50µg/ ml concentration, both were from Molecular Probes. Alexa 568-transferrin (Molecular probes) was fed to cells at 50µg/ ml for 30 minutes. Prior to transferrin labeling the cells were starved in serum free medium for 1 hour. Macrophages were labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine-perchlorate labeled acetylated LDL (DiI-acLDL) in serum free medium at 50µg/ ml for 1 hour.

5.4. Labeling of Cells with Radioactive Probes

Cells were labeled with [³H]cholesterol 1µCi/ ml overnight (Raw264.7 macrophages) or two days (CHO cells). [³H]oleate was fed to cells in a complex with BSA. A serum free medium containing 2% BSA and 1µCi/ ml [³H]oleate was incubated for 30 minutes at +37°C prior to feeding to cells. This complex was then incubated with hepatocytes for 5 hours. [³H]acetate(10µCi/ ml) was fed to cells for 16 hours in serum free medium.

To achieve LDL labeling with radioactive cholesteryl esters, 1mg of LDL (isolated from healthy human donors as previously described in (Havel et al., 1955)) was incubated with 40µCi of [³H]cholesteryl oleate or cholesteryl [³H]oleate dissolved in DMSO for 2 hours at 40°C, followed by extensive dialysis against PBS. Cells were then incubated with these particles for varying times in the presence of the ACAT1 inhibitor Sandoz 58-035 (2µg/ ml) to prevent re-esterification to cholesteryl esters.
5.5. Lipid Extraction and Analysis

Lipids were extracted from cell and tissue samples, adjusted for protein concentration, using organic solvents by the method by (Bligh and Dyer, 1959). The material to be extracted (cell or tissue lysates) was adjusted to 1600µl of 2% NaCl. 4ml of methanol and 2ml chloroform was added, the sample was vortexed and centrifuged to pellet precipitated protein material. 2ml chloroform and 2ml water was then added, and the sample was again vortexed and centrifuged. After this procedure the sample was in two phases, the lower of which was the organic phase containing the lipids.

For analyzing distribution of radioactivity in different lipid classes, the lipids were resolved on a regular TLC with appropriate standards, the silica scraped and radioactivity counted in a liquid scintillation counter. For analyzing lipid masses, a High Performance Thin Layer Chromatography (HPTLC) Camag system was used. Samples were extracted as above, run on a HPTLC along with appropriate standards. The plate was then stained with 3% copper sulfate 8% phosphorous acid solution and charred at 180°C for 5 minutes to visualize the lipids. For analysis of BODIPY-cholesterol, the plates were imaged under a UV-table prior to staining and burning. The amount of lipid in each lane was then assessed by densitometric analysis using ImageJ software. Similar mobile phases were used on both TLC systems: Hexane: diethyl ether: acetic acid 80:20:1 was used to separate, cholesteryl esters and triglycerides from each other, petroleum ether: diethyl ether: acetic acid 40:60:1 was used to separate free cholesterol from other lipids in work II.

Cholesterol efflux was analyzed by first labeling cells with [³H]cholesterol (1µCi/ ml) overnight to two days. The label was then removed, and the medium replaced with fresh medium including or excluding cholesterol acceptors (0,2% BSA, ApoAI 10µg/ ml or HDL₂ 25µg/ ml). After 16-24h the radioactivity in cells and culture media was determined by liquid scintillation counting.

To analyze detergent insolubility of sterols, cells were labeled with BODIPY-cholesterol, Compound 1, [³H]cholesterol or [³H]desmosterol overnight. Cells were lysed in cold 1% Triton-X-100, 150mM NaCl, 10mM Tris pH8.0, 1mM ethylenediaminetetraacetic acid and centrifuged at 16 000 x g for 30 minutes at +4° to pellet the detergent insoluble material. The detergent soluble material was removed and the pellet was solubilized into the same buffer as above. The radioactivity in insoluble and soluble samples was determined by liquid scintillation counting and the fluorescence with a fluorometer (Varian Cary Eclipse).
5.6. Immunohistochemistry, Microscopy and Image Analysis

To fix cultured cells and tissues, they were incubated in 4% paraformaldehyde dissolved in 250mM Hepes including 100µM MgCl₂ and 100µM CaCl₂ for 30 minutes (cell culture samples) or overnight (tissue samples) followed by quenching of the formaldehyde autofluorescence by 50mM NH₄Cl. Filipin staining was performed with 0,05% filipin (Sigma) in 10% FBS 30 minutes at +37°C. When necessary, filipin staining was followed by incubations with primary antibodies in 5% FBS 1h in +37°C and secondary antibodies in 5% FBS 30 minutes in +37°C. For Oil Red O (Sigma-Aldrich) staining tissue sections were incubated with 60% isopropanol including 0.6% Oil Red O. All samples were mounted using Mowiol and 1,4 diazobicyclo-(2.2.2) octane (DABCO, an antifading reagent).

Imaging was performed using one of the following systems: an inverted epifluorescent microscope IX70 (Olympus) equipped with a Polychrome IV monochromator (TILL Photonics), an Olympus AX70 Provis epifluorescent system, a Leica TCS SP2 AOBS confocal microscope or a Zeiss LSM 5 Live confocal equipped with a line scanner for fast imaging. Both confocal systems and the IX70 epifluorescent system were equipped with a heating unit for live cell imaging. When imaged live, cells were incubated in CO₂ independent medium (Gibco). For imaging with the inverted microscopes (Olympus IX70 and Zeiss LSM5 Live) the cells were plated and imaged on glass bottom Ø 3.5cm dishes (MatTek). Time lapse sequences were acquired with the Zeiss LSM5 Live system at 1frame/s. Image analysis was performed with Image Pro Plus software (Media Cybernetics).

5.7. Other Methods

Proteins were determined from all samples using a DC Protein Assay from Bio Rad. Western Blotting was performed harvesting samples in 1% Nonidet-P40 supplemented with a protease inhibitor cocktail (25 µg/ml of chymostatin, leupeptin, antipain, and pepstatin). 20µg of protein was then loaded to an SDS-PAGE gel of appropriate density and transferred to Hybond-C Extra membrane (Amersham Biosciences). The membrane was then blocked with 5% milk in 0,1% Tween in Tris-buffered saline for 1h at +37°C, followed by primary antibody in the same solution overnight at +4°C and secondary antibody in 0,1% Tween in Tris-buffered saline 2 hours at room temperature. The antibodies were then visualized using enhanced chemiluminescence detection (ECL).
Results and Discussion

6. BODIPY-Cholesterol as a Tool to Visualize Cholesterol in Cells and Tissues

6.1. Atomic Scale Computer Simulations of BODIPY-Cholesterol in Membranes

As cholesterol resides within the bilayer in biological membranes, it is important to establish if a fluorescent sterol behaves similarly in a membrane compared to cholesterol. It is not, however, possible with current techniques to experimentally directly address interactions and orientation of cholesterol or its analogues in vivo in cellular membranes. We therefore utilized atomic scale computer simulations to study how BODIPY-cholesterol (Figure 6.) behaves in a phospholipid membrane together with sphingomyelin and cholesterol. Although computer simulations do not provide real information about the behavior of BODIPY-cholesterol in the membrane, they do provide a useful proxy for what happens on the atomic scale. In the simulations, BODIPY-cholesterol occupies a similar area on the membrane compared to cholesterol, does not affect membrane thickness and only minimally perturbs neighboring lipids. Importantly, the simulations also show that the sterol backbone of BODIPY-cholesterol is positioned similarly to that of cholesterol so that the BODIPY-group most of the time points toward the inside of the membrane, the sterol backbone residing upright and the OH-group on the same level as it is for cholesterol (I, Figure 1 and Supporting Information).

**Figure 6.** Structure of BODIPY-cholesterol
6.2. Biochemical Characteristics of BODIPY-Cholesterol

Next, we analyzed the biochemical behavior of BODIPY-cholesterol in living cells to address if the behavior of BODIPY-cholesterol in cells is similar to that of cholesterol. We utilized either wild type CHO cells or CHO cells lacking a functional NPC1 protein (M12-cells). The cells were incubated with 0.5µM BODIPY-cholesterol overnight. Use of this protocol resulted in bright labeling of cells, with only ~1% addition of sterol to the total cellular sterol pool (I, Figure 2A). This is important, as we did not want to load cells with sterol and because high levels of the unnatural sterol might interfere with cellular functions. Additionally, no metabolism of the probe could be detected during this time, as assessed by TLC (not shown), implicating that what was seen by microscopy was intact BODIPY-cholesterol. To test if BODIPY-cholesterol could substitute for cholesterol in living cells with no other source of sterol, we utilized a CHO cell line called M19. These cells do not synthesize any sterols and are unable to survive without exogenously added cholesterol (Hasan et al., 1994). We cultured these cells in 5% LPDS and 35µM oleate and supplemented the medium with 1µM sterol: cholesterol, BODIPY-cholesterol or DHE or only DMSO and assessed their growth by determining the amount of protein in each sample. The control cells with no supplementation slowed down their growth after a day in culture, as their cholesterol stores were depleted, whereas all supplemented cells kept growing with no significant differences between sterols supplemented (I Figure 2B). This experiment suggests that BODIPY-cholesterol is able to support cell growth.

Next, to address if BODIPY-cholesterol behaves biochemically similarly to cholesterol, we labeled cells with both BODIPY-cholesterol and [3H]cholesterol overnight and compared their behavior. When the postnuclear supernatant was subjected to sucrose density gradient ultracentrifugation as in (Hao et al., 2002), BODIPY-cholesterol and [3H]cholesterol separated very similarly with no significant differences in their appearance on different sucrose densities, implicating that the subcellular distribution of BODIPY-cholesterol is similar to that of [3H]cholesterol (I Figure 2C).

Resistance of biological membranes and their constituents to the detergent extraction at +4° is one of the fundamental characteristics of cholesterol in biological membranes and is often used as a proxy for rafts. We next tested if BODIPY-cholesterol is found in detergent resistant membranes. When compared to [3H]cholesterol, BODIPY-cholesterol was resistant to detergent extraction similarly to cholesterol (I Figure 2D). We also tested two other sterols for their detergent resistance in this assay. [3H]desmosterol, previously shown to be slightly less detergent resistant (Vainio et al., 2006) was also slightly less detergent resistant in this experiment. Another BODIPY-derivative of cholesterol where the BODIPY is joined to the sterol nucleus with an ester linkage (Compound 1) was almost
completely detergent soluble in line with results from an earlier study (Li et al., 2006). In addition, when M12 cells were used, the detergent insolubility of both [3H]cholesterol and BODIPY-cholesterol was increased, consistent with earlier results showing more detergent resistant cholesterol in NPC1 deficient cells (Lusa et al., 2001).

Effluxing and esterifying cholesterol are the two principal ways for cells to lower their sterol levels. We therefore tested if these reactions would occur also for BODIPY-cholesterol. WT CHO and M12 cells labeled two days with both BODIPY- and [3H]cholesterol were incubated in serum free medium in the presence or absence of physiological cholesterol acceptors (0.2% BSA or 10µg/ml ApoAI) for an additional 24 hours and the fluorescence and radioactivity in cells and culture medium were determined to calculate the fraction effluxed. In these experiments, cells effluxed BODIPY-cholesterol much more avidly than they do [3H]cholesterol (Figure 2E). However, the highest efflux rates were to the specific acceptor ApoAI. In addition the defect in cholesterol efflux seen in NPC1 deficient cells (Chen et al., 2001) can also be seen with BODIPY-cholesterol indicating that although higher than for natural cholesterol, efflux of BODIPY-cholesterol still follows physiological regulation.

To test if cells are able to esterify BODIPY-cholesterol we used a mouse macrophage cell line RAW264.7 as these cells esterify large amounts of cholesterol, especially when challenged by LDL loading. The cells were labeled with BODIPY- or [3H]cholesterol overnight in the presence or absence of 10µg/ml acetylated LDL, the lipids were extracted and separated by TLC. However, only a small amount of BODIPY-cholesterol was found in esterified form (Figure 2F). It is possible that the BODIPY-group inhibits the reaction catalyzed by ACAT1 or that the cells prefer to efflux BODIPY-cholesterol or store it unesterified in lipid droplets (Jansen et al., 2010).

Together, these results show that some of the biochemistry of BODIPY-cholesterol follows that of [3H]cholesterol, while in some aspects BODIPY-cholesterol behaves differently. This biochemical characterization is important as fluorescent probes always differ from their natural counterparts. It is therefore essential to know which aspects are comparable and in which aspects one needs to be cautious when making conclusions.

6.3. BODIPY-Cholesterol Imaging in Living Cells and Organisms

To study the localization and behavior of BODIPY-cholesterol in living cells and organisms, we labeled cells with the probe and studied them under the microscope. First we
labeled WT CHO cells with BODIPY-cholesterol from a methyl-β-cyclodextrin complex which donates the sterol to the plasma membrane. After only 2 minutes of labeling, we could already see a bright labeling of cells (I Figure 3A). This labeling was initially restricted to the plasma membrane, but as the cells were chased, they internalized the probe and intracellular vesicles and tubules became visualized. To identify these structures we localized them with cellular markers for organelles known to contain cholesterol. Small molecular weight dextran is internalized to cells via fluid phase endocytosis. We fed fluorescent (rhodamine labeled) dextran to cells overnight to label late endosomes and lysosomes. Alexa 568 transferrin that fluorescently labels the endocytic recycling compartment was fed to CHO cells overexpressing transferrin receptor at 50µg/ml for 30 minutes at +37°C. Similarly to filipin, steady-state BODIPY-cholesterol labels many endosomal structures (I Figure 3B). When colocalizations of BODIPY-cholesterol with these organelar markers were quantified in control and NPC1 deficient cells, we could see more BODIPY-cholesterol in dextran-positive organelles in the NPC1 deficient cells. This indicates that BODIPY-cholesterol can trace the endosomal cholesterol accumulation seen in NPC disease. The accumulation of BODIPY-cholesterol in M12 cells was not, however, as dramatic as it is when visualized with filipin (I Supplementary Figure 8). This possibly reflects the fact that the majority of cholesterol accumulating in NPC cells is derived from LDL and as our probe does not specifically trace this pathway, it is less readily visualized. In addition to endocytic structures we could also localize BODIPY-cholesterol to lipid droplets and the Golgi complex and to a lesser extent also to ER and even to mitochondria (Figure 7 and R-L.U. unpublished results). Different compartments are differentially visualized depending on the cell type and its metabolic status (Figure 7).

**Figure 7.** BODIPY-cholesterol in (A) a primary human fibroblast, arrow pointing to the Golgi complex (epifluorescent image) and (B) in HeLa cells, arrows pointing to lipid droplets (confocal scan).
Another feature of NPC cells is that motility of endosomes is hampered (Ko et al., 2001). The precise reason for this phenomenon is not known, but it is considered to be a secondary defect of endosomal cholesterol accumulation as Rab proteins (Lebrand et al., 2002) and SNAREs (Fraldi et al., 2010) become trapped in high cholesterol membranes. When the motility of BODIPY-cholesterol containing organelles was quantified in WT CHO and M12 cells we observed a defect in NPC1 deficient cells also with BODIPY-cholesterol (I Figure 3C). This result for the first time demonstrates the motility of sterol containing organelles and confirms impaired motility of cholesterol containing organelles in NPC1 deficient cells. To study sterol exchange between cells we cocultured rhodamine-dextran labeled WT CHO cells with non-rhodamine-dextran labeled M12 cells and labeled the coculture with BODIPY-cholesterol from DMSO overnight. Initially BODIPY-cholesterol intensity in both cell types was similar, but when chased, M12 cells started to accumulate more BODIPY-cholesterol where as BODIPY-cholesterol intensity in WT CHO cells decreased (I Figure 4A). To more precisely quantify how this shuffling between cells happened, we cocultured BODIPY-cholesterol and rhodamine-dextran labeled WT CHO or M12 cells with unlabeled CHO or M12 cells. During the first hours of coculture BODIPY-cholesterol started to appear in the non-labeled cell population and after 24 of coculture, BODIPY-cholesterol was spread between the two cell populations (I Figure 4B). However, M12 cells acquired more of the sterol independent of if they were donors or acceptors. These results demonstrate that cells in culture can and do exchange sterol between them and balance out sterol levels. It also demonstrates that NPC1 deficient cells collect more cholesterol, and when cocultured with WT cells always end up with more sterol, presumably due to their lower ability to efflux cholesterol to the culture medium (I Figure 2E).

Next we labeled mouse embryonic brain cell culture with BODIPY-cholesterol, again from DMSO overnight. These cultures were obtained from hippocampi of E17½ mouse embryos. In such cultures, most of the BODIPY-cholesterol partitioned into neurons (I Figure 4C). To verify that this result was not due to the use of BODIPY-cholesterol we repeated the experiment and stained the cells with filipin. Indeed, also filipin stains neurons more brightly than astrocytes (I Figure 4D). Finally, to demonstrate the partitioning of BODIPY-cholesterol in a living organism, we injected fertilized zebra fish embryos at one- to two-cell stage with 20-40 nmol BODIPY-cholesterol or FITC-BSA (0.8-1.5pmol per embryo) and followed the development of the embryo until 7 days post fertilization. Importantly, the fish developed normally in the presence of BODIPY-cholesterol and no toxic effects were seen. BODIPY-cholesterol was gradually mobilized from the yolk sack and incorporated into organs known to contain large amounts of cholesterol such as the gut and the central nervous system, whereas the FITC-BSA disappeared within 7 days (I...
Figure 5). These results demonstrate that BODIPY-cholesterol can be used to visualize sterol compartmentalization and trafficking also in living organisms.

This study provides a new tool for visualizing sterol trafficking in living systems. While previous fluorescent sterol probes either have weak fluorescent properties or behave very differently from natural cholesterol, BODIPY-cholesterol is both bright and mimics cholesterol in many aspects. As with any fluorescent probe, one needs to keep in mind that a probe never behaves exactly like its natural model. With this in mind, BODIPY-cholesterol can be used to study the cell biology and physiology of cholesterol in living cells and organisms.

7. Triglycerides in Niemann-Pick Type C Disease

7.1. NPC1 Single Nucleotide Polymorphisms (SNPs) Associate with Altered Triglyceride Levels in Man

The initial hypothesis of this study was that variations in the NPC1 gene would be associated with altered cholesterol levels in serum at the population level. Since NPC1 binds cholesterol and its deficiency causes a cholesterol storage disease, we thought it probable that its genetic variations could affect circulating lipids. This hypothesis was tested first with 19 NPC1 SNPs in a population cohort of Swedish men involved in the Uppsala Longitudinal Study of Adult Men (ULSAM) (Hedstrand, 1975; Zethelius et al., 2005) consisting of 1053 individuals. To our surprise, there were only weak associations between NPC1 genotype and plasma cholesterol levels. Instead, there were several SNPs strongly associated with altered triglyceride levels (II Table). To further validate this unexpected result, we tested the SNP that gave the most significant p-value in the ULSAM cohort (rs1429934) in two additional cohorts; 2882 individuals from The Framingham and Fast Revascularization During Instability in Coronary Artery Disease II (FRISCII) (Investigators, 1999) and the Sweden Women and Men and Ischemic Heart Disease (SWISCH) (Johnston et al., 2004) studies, and 5159 men participating in the Malmö Diet and Cancer Study (MDC) Cardiovascular Cohort (Berglund et al., 1993). The result was confirmed as the SNP was associated with increased triglyceride levels also in these additional population cohorts (II Supplementary Table III). This suggests that genetic variations in NPC1 are indeed one of the determinants for circulating triglyceride levels on the population level.
7.2. Lipid Balance in \textit{Npc1-/-} Mouse Serum, Liver and Hepatocytes

After the initial results from the genetic studies, we turned our attention to the NPC mouse model to establish whether there is a triglyceride imbalance also in the NPC mouse. We used 7 week old mice throughout this study, as at this age the accumulation of cholesterol is prominent, but no effects on food intake (Xie et al., 1999), intestinal fatty acid absorption (Dixit et al., 2007) and body weight (Voikar et al., 2002) are yet seen. The major finding in NPC liver is a massive accumulation of free cholesterol. Liver sections from \textit{Npc1+/-} and \textit{Npc1-/-} were stained with filipin, a fluorescent antibiotic that binds cholesterol or Oil-Red-O, a neutral lipid stain visualizing lipid droplets (II Figure 1A). Filipin staining revealed a massive increase in free cholesterol in \textit{Npc1-/-} versus \textit{Npc1+/-} livers. This result was further confirmed by determining sterol masses from the same livers by extracting the liver lipids and analyzing them by HPTLC and indeed there is a great increase in free cholesterol levels in the \textit{Npc1-/-} liver (II Figure 1B). In addition to the change in free cholesterol, however, we also detected a substantial decrease in the levels of triglycerides in the \textit{Npc1-/-} liver (II Figure 1B). Furthermore, Oil-Red-O staining of liver sections showed practically no lipid droplets in the \textit{Npc1-/-} liver (II Figure 1A). Prompted by this finding, we analyzed the levels of Perilipin 2, a protein known to associate with lipid droplets in the liver. This was done by analyzing liver lysates on Western Blot with an anti-Perilipin 2 antibody. Perilipin 2 was indeed almost undetectable in the \textit{Npc1-/-} liver (II Figure 1C) in line with low neutral lipid storage.

\textbf{Figure 8.} Filipin staining of \textit{Npc1+/-} and \textit{Npc1-/-} hepatocytes demonstrating massive cholesterol storage in \textit{Npc1-/-} cells.

To study if these changes in intact liver tissue have an effect on lipid secretion from hepatocytes, we isolated primary hepatocytes from \textit{Npc1+/-} and \textit{Npc1-/-} mice. These cells
exhibited a massive increase in free cholesterol levels, but also a significant decrease in triglyceride levels, as analyzed by HPTLC (II Figure 2A) and seen by filipin staining (Figure 8). In addition, when the culture media of these cells was analyzed, we noted an increase in free cholesterol secretion, but a decrease in the secretion of triglycerides. As previously published (Kulinski and Vance, 2007), these cells secreted more ApoB, as assessed by western blotting (II Figure 2A). To test if the lipid and ApoB found in the culture media were associated with secreted VLDL particles, the medium was subjected to a sucrose density gradient fractionation to separate VLDL particles (Asp et al., 2000). In these gradients we found 98% of lipid and 60% of apoB floating on top of the gradient, indicating that the majority of lipid and apoB was associated with VLDL particles. To study if these changes in hepatic lipid balance are also reflected in circulating lipid levels we analyzed lipids in the serum of \( \text{Npc1}^{+/-} \) and \( \text{Npc1}^{-/-} \) mice as previously described (Jaari et al., 2001). Indeed, we also found increased total cholesterol, but decreased triglyceride levels in the serum of the \( \text{Npc1}^{-/-} \) mice (II Figure 2B). Together, these results demonstrate a severe dysregulation of neutral lipids in the \( \text{Npc1}^{-/-} \) mouse.

7.3. Fatty Acid Metabolism in \( \text{Npc1}^{-/-} \) Hepatocytes

NPC1 has been suggested to function as an endosomal fatty acid transporter due to its homology with bacterial RND permeases (Davies et al., 2000). This theory, if correct, could help us explain the decreased neutral lipid storage in the \( \text{Npc1}^{-/-} \) hepatocytes as the fatty acid not able to leave endosomes would not be able to reach the ER to be esterified to form triglycerides. We therefore labeled LDL particles with cholesteryl \(^{3} \text{H}\)oleate, i.e. a cholesterol ester molecule with the radioactive atoms in the fatty acid part of the molecule. These particles were fed to \( \text{Npc1}^{+/-} \) and \( \text{Npc1}^{-/-} \) hepatocytes in serum free medium for 3 hours, and chased for an additional 3 hours, during which time the cells start to hydrolyze the cholesteryl \(^{3} \text{H}\)oleate to yield free cholesterol and \(^{3} \text{H}\)oleate and metabolize them further. The distribution of the radioactivity in different lipid classes was then analyzed by extracting lipids, separating them on TLC and counting radioactivity in each lipid class. The \( \text{Npc1}^{-/-} \) cells were slightly slower in hydrolyzing the esters with 68%±1.8% hydrolyzed after the three hour chase in \( \text{Npc1}^{-/-} \) cells and 82%±3.5% hydrolyzed in \( \text{Npc1}^{+/-} \) cells. However, in both genotypes, the vast majority of the released fatty acid became incorporated in triglyceride (II Figure 3A). In order to be incorporated into triglyceride, the fatty acid must leave the endosome and traffic to the ER as this is where the triglyceride synthesizing enzymes acyl-CoA:monoacylglycerol acyltransferase (MGAT) and acyl-CoA:diacylglycerol acyltransferase (DGAT) reside (Yen et al., 2002; Kuerschner et al., 2008). Our data therefore indicate that the cholesteryl ester-derived fatty acid is not trapped in endosomes in \( \text{Npc1}^{-/-} \) hepatocytes. This is in accordance with previous results from NPC1 deficient fibroblasts (Passeggio and Liscum, 2005). However, there was a
small but significant decrease in the incorporation of the LDL-derived fatty acid into triglyceride, possibly contributing to the decrease in triglyceride levels. In addition, there was an increase in the amount of radioactivity discovered in free fatty acid in Npc1-/- hepatocytes, consistent with the increased free fatty acid levels reported in NPC liver endosomes (Chen et al., 2005a).

As we did not detect a defect in how Npc1-/- hepatocytes handle endosome-derived fatty acids, we next tested if the metabolism of free [3H]oleate complexed to albumin was different between the genotypes. This is the major pathway of lipid entry to the liver during fasting. The hepatocytes were incubated in serum free medium supplemented with [3H]oleate-BSA for 5 hours, and the distribution of radioactivity between lipid classes was analyzed. Most radioactivity was found in triglyceride in both cell types (II Figure 3B). However, a significant decrease was again seen in triglyceride synthesis in Npc1-/- cells.

These experiments suggested that there was no severe defect in how Npc1-/- cells handled the incoming fatty acid, but rather that the decrease in triglyceride was due to a more subtle regulation of triglyceride synthesis. We therefore studied triglyceride synthesis from its core building block acetate. To this end, Npc1+/+ and Npc1-/- hepatocytes were cultured in the presence of [3H]acetate for 16 hours in serum free medium and the incorporation of radioactivity to different lipid classes was analyzed as described above. Indeed, there was a large decrease in the incorporation of [3H]acetate into triglyceride in Npc1-/- cells (II Figure 3C). In parallel there was an increase in the incorporation of [3H]acetate into cholesterol and to choline phospholipids. This increase in carbon unit flux to cholesterol synthesis was evident also from [3H]oleate (II Figure 3B) and cholesteryl [3H]oleate (II Figure 3A). For fatty acid-derived carbon to end up in free cholesterol, the fatty acid has to be degraded in beta oxidation to yield Acetyl Coenzyme A which can then serve as a building block for cholesterol biosynthesis.

Finally, to test if Npc1-/- cells have the capacity to synthesize triglyceride in the presence of ample exogenous substrate, we incubated Npc1+/+ and Npc1-/- hepatocytes with 500µM oleate-BSA complex. After 16 hour incubation with exogenous fatty acids, the triglyceride levels were determined by HPTLC. We found similar triglyceride levels in both genotypes, indicating no fundamental defect in the triglyceride or lipid droplet machinery in Npc1-/- cells (II Figure 4). However, when the exogenous fatty acid was removed, Npc1-/- cells immediately ceased synthesizing more triglyceride, unlike cells from their Npc1+/+ littermates. Together, these experiments indicate that Npc1-/- cells have a decreased triglyceride synthesis that is not due to a defect in fatty acid transport nor to the triglyceride synthesis machinery. Rather, as the decreased carbon flux to triglyceride is paralleled with an increased carbon flux to cholesterol synthesis, these data suggest that
the metabolic flux of carbon units is guided towards cholesterol at the expense of triglyceride.

7.3. Inhibition of Cholesterol Synthesis Corrects the Triglyceride Reduction in \textit{Npc1-/-} Hepatocytes

If indeed the increased cholesterol synthesis was the explanation for low triglyceride levels in the \textit{Npc1-/-} liver, then blocking cholesterol synthesis should normalize triglyceride levels in \textit{Npc1-/-} cells. \textit{Npc1+/+} and \textit{Npc1-/-} hepatocytes were therefore incubated with 20\textmu M lovastatin and 50\textmu M mevalonate (to support cell viability) for 16 hours, and lipid masses were determined by HPTLC. This treatment reduced cholesterol amounts in both genotypes, but increased triglyceride levels in \textit{Npc1-/-} hepatocytes (II Figure 5A). Incubation with statin increased the secretion of triglyceride from \textit{Npc1-/-} hepatocytes, whereas the effect in \textit{Npc1+/+} hepatocytes was the opposite (II Figure 5B). In addition, when \textit{Npc1-/-} cells incubated with exogenous free fatty acid were treated with statin, they kept synthesizing more triglyceride after the exogenous fatty acid was removed, similarly as the \textit{Npc1+/+} cells (II Figure 4). These results further support the idea of the highly increased cholesterol synthesis in \textit{Npc1-/-} cells being responsible for the decreased triglyceride levels. When the synthesis of cholesterol is blocked with statin, the carbon that would have been used to produce more and more cholesterol can now be used for triglyceride synthesis.

Interestingly, while this work was underway and after its publication, additional reports have linked NPC1 function to neutral lipid balance. Triglycerides were found to be increased in a subset of NPC patients (Garver et al., 2010), \textit{Npc1} haploinsufficiency (\textit{=heterozygocity}) was found to promote weight gain (Jelinek et al., 2010) and a \textit{NPC1} SNP was found to be associated with early onset and morbid obesity in humans (Meyre et al., 2009). The SNP found associated with morbid obesity was also tested in our initial screen with 19 \textit{NPC1} SNPs (rs1805081 II Table). We found no associations for circulating lipid levels nor to obesity (data not shown) with this SNP. This is not surprising, as the amount of the morbidly obese in this data set was very small. These studies together with our work open new possibilities for NPC1 functions in cells. Does NPC1 have a direct regulation of neutral lipid balance, and is the NPC disease affected by this phenomenon, will be the subject of further research.
8. ORP1L Function in Endosomal Trafficking and Function

8.1. Sterol Binding by ORP1L

Previously, ORP1L has been shown to bind 22(R) - and 25-hydroxycholesterol (OHC) (Suchanek et al., 2007; Yan et al., 2007). We first wanted to characterize the sterol binding specificity of ORP1L more comprehensively. For this, ORP1L-GST was produced in an E. Coli expression system, and the protein was purified. The ability of the purified protein to bind oxysterols was tested on an in vitro charcoal-dextran based assay. ORP1L was first bound to \[ ^{3}H \]25-OHC, a known ORP1L ligand, and this binding was competed with different oxysterols. 7-ketocholesterol, 22(R)OHC, 24(S)OHC and 25OHC were all able to compete out the \[ ^{3}H \]25-OHC, indicating that ORP1L is able to bind all of these oxysterols with roughly equal efficiencies (III Figure 1A). Many ORPs are also able to bind cholesterol, and ORP1L has also been suggested to do so (Suchanek et al., 2007). We therefore tested if we could demonstrate this binding in vitro. As cholesterol is more poorly soluble in water than its oxidized forms, this experiment was done on large unilamellar vesicles (LUV). In such experiments \[^{3}H\]cholesterol is placed into the vesicle, from which a cholesterol binding protein should be able to extract it. However, GST-ORP1L cannot be produced without the use of detergent, the presence of which would collapse the LUVs. Therefore we used the shorter splice variant of ORP1, ORP1S to perform these experiments, as ORP1S can be produced without detergent and the sterol binding pocket in the two variants is identical. Indeed, we were able to show binding of cholesterol to ORP1 as efficiently as published earlier for ORP2 (Hynynen et al., 2009) (III Figure 1B).

To further study the sterol binding of ORP1L, and how its sterol binding state affects cells, we generated a mutant of ORP1L possibly unable to bind oxysterols. By homology modeling of the ORP1L sterol binding pocket we deduced that amino acids 560-563 (ELSK) would localize to the lid of the sterol binding pocket and that deletion of these amino acids would render the protein unable to bind sterols (III Figure 1C). We found that the \[ \Delta \]560-563 binds significantly lower to 22(R), 24(S) and 25-hydroxycholesterols indicating that this mutant is indeed defective in sterol binding.

8.2. Endosomal Functions by ORP1L

To study how the defect in ORP1L sterol binding affects its behavior in cells, we overexpressed a Green Fluorescent Protein (GFP) fusion construct of this mutant in HeLa cells
and compared its localization to WT ORP1L-GFP. WT ORP1L overexpression creates large perinuclear clusters of late endosomes where it is able to recruit its binding partner Rab7 (Johansson et al., 2005). In contrast, the Δ560-563 ORP1L was found in smaller endosomes dispersed throughout the cell (III Figure 2A, d and g). A similar result was recently achieved by expressing an ORP1L mutant with the whole ORD deleted (Rocha et al., 2009). The localization of WT ORP1L containing organelles has been linked to increased recruitment of dynein-dynactin motor complex driving the motility of endosomes toward the center of the cell (Johansson et al., 2007). To investigate if also the mutant ORP1L localized to late endosomes, the cells were costained for the Early Endosomal Antigen-1 (EEA1) or Lysosome Associated Membrane Protein 1 (LAMP1) to visualize early endosomes and late endosomes and lysosomes, respectively. As seen in the case of WT ORP1L, the Δ560-563 mutant showed no colocalizations with EEA1 (III Figure 2B d-f), but partial colocalization with LAMP1 (III Figure 2B g-i). In addition, both constructs colocalized extensively with Rab7 (III Figure 2A and B a-c), indicating that despite the different morphology, the ORP1L containing organelles were still late endosomes. Similar results with perinuclear WT ORP1L and scattered Δ560-563 ORP1L that partially colocalized with LAMP1 were observed when the constructs were expressed in a mouse macrophage cell line RAW264.7 (III Figure S1).

To test if the sterol binding affects the functions of ORP1L, we tested if Δ560-563 ORP1L is also capable of binding Rab7. Both WT ORP1L and Δ560-563 mutants coimmunoprecipitate equally well with Rab7, indicating that the ability of ORP1L to interact with Rab7 is not dependent on its sterol binding capacity (III Figure 2D). ORP1L also binds to the ER resident VAPs. This binding is mediated through the FFAT motif and can be abolished by mutating this domain. When the Δ560-563 ORP1L was mutated also in its FFAT motif, the scattering phenotype disappeared and instead ORP1L was found again on perinuclear clusters, indicating that ER association via binding to VAP is the force holding the organelles in their peripheral location (III Figure 2C).

As the peripheral localization of the WT ORP1L organelles is considered to be due to increased recruitment of dynein (Johansson et al., 2007), we tested if the more peripheral localization of Δ560-563 ORP1L containing organelles was caused by a defect in recruiting dynein, despite the ability of the mutant to bind Rab7. This was tested by staining cells with antibodies against the dynein subunit P150glued. This staining is mostly diffuse cytosolic in control cells but in both WT and Δ560-563 ORP1L the staining was clearly punctuate, colocalizing with ORP1L (III Figure 3A). This indicates that the sterol binding ability of ORP1L does not affect its association with dynein. As Δ560-563 ORP1L had clearly more peripheral (and thus not dynein-driven) localization, we tested if also other motor types would be recruited with ORP1L overexpression to explain the altered localization. Indeed, also a late endosomal microtubule plus end directed motor Kinesin-2 was similar.
ly recruited to ORP1L containing organelles of both WT and Δ560-563 ORP1L as judged by staining the cells with antibodies against the Kinesin-2 subunit KIF3A (III Figure 3b). These data suggest that ORP1L and its sterol binding status could be one of factors regulating the directionality of endosomal movement.

To more precisely assess how ORP1L affects motility of late endosomes, we overexpressed both WT ORP1L-GFP and Δ560-563 ORP1L-GFP in HeLa cells. The cells were labeled with Alexa 568 dextran overnight in order to visualize the movement of late endosomes and lysosomes. The cells were then imaged live to determine the motility of these organelles. Organelles containing ORP1L-GFP exerted very little motility. This was particularly clear for the large clusters of endosomes seen in WT ORP1L-GFP overexpressing cells, but also for the Δ560-563 ORP1L-GFP (III Figure 4A, green bars). The colocalization between ORP1L-GFP and dextran was poor, indicating that the endocytosed dextran did not reach the WT ORP1L or Δ560-563 ORP1L containing organelles efficiently. There were, however, several instances where a dextran-positive organelle could be seen on the surface of an ORP1L containing organelle (III Figure 4C) and moving along with it, without fusing with it. When the Δ560-563 mFFAT ORP1L double mutant was similarly expressed, all of the dextran became associated with the ORP1L-GFP containing clusters, with no dextran-positive organelles remaining free of ORP1L. How this “attachment” of endolysosomes to ORP1L containing organelles takes place, is so far unknown.

The motility of dextran positive organelles was also decreased in all of the ORP1L overexpressing cells compared to cells expressing GFP only (III Figure 4A red bars). This is partly because of their attachment to the ORP1L containing organelles, but also because the motility of the “free” dextran organelles was also decreased. This might be because there is still ORP1L-GFP on these organelles, although below detection limit, or because a large amount of the vesicle movement machineries (e.g. motor proteins) are being recruited to the ORP1L containing endosomes thus reducing their availability to other organelles. When the double mutant Δ560-563 mFFAT ORP1L was expressed in a similar setup, we again saw clusters of endosomes that exerted very little motility. However, when the mFFAT ORP1L was expressed alone, the motility of ORP1L containing organelles was significantly higher than with WT or the other mutants. Their motility also resembled that of regular late endosomes with typical back-and forth movements and longer vectorial movements. These organelles also colocalized with the endocytosed dextran, possibly due to their increased motility and increased communication with other endosomes.

As the overexpression of ORP1L or its mutant forms decreased the motility of endosomes, we next tested if its depletion by siRNA would have an effect on endosomal motility. We transfected HeLa cells with RNA sequences against ORP1 or with a non-
specific RNA sequence (control). This method resulted in a 75-80% decrease in the levels of ORP1L (III Figure 5A). The cells were again labeled with Alexa 568 dextran overnight to label late endosomes and lysosomes and imaged live. The motility of dextran containing organelles was indeed increased when ORP1L was removed from the cells (III Figure 5B). This is an opposite result from the overexpression studies indicating that ORP1L has a true effect on endosome motility, inhibiting the motility of organelles it is associated with. In addition to the extent of motility, also the quality was different, as more peripheral organelles were seen in the ORP1 knock down condition (III Figure 5C), consistent with the more distributed location reported earlier (Johansson et al., 2007).

To test if the altered localization and motility also affects the degradative function of endosomes, we asked whether the ORP1L overexpressing or silenced cells were still capable of degrading epidermal growth factor (EGF). EGF is normally degraded in lysosomes and fluorescently labeled EGF provides a simple means to study the integrity of the endocytic system. This was assessed by feeding cells with rhodamine-labeled EGF and chasing them. Control cells degrade EGF in hours, which can be seen as disappearance of fluorescence from cells. WT ORP1L and Δ560-563 ORP1L overexpressing cells had a severe defect in EGF degradation, probably due to the decreased motility and communication of endosomes (III Figure 6A). EGF was able to reach LAMP1 positive organelles also when ORP1L was expressed (III Figure S3), but EGF, like dextran did not reach the ORP1L containing endosomes (III Figure 6C-E). ORP1 silencing, however, had no effect on EGF degradation (III Figure 6B) suggesting that the highly moving endosomes in ORP1 depleted cells are functional.

Together, these results indicate that ORP1L functions in the regulation of motility and localization of endolysosomes. ORP1L stalls the motility of these organelles probably by binding to VAP, and this leads to functional defects in endo-lysosomes when ORP1L is overexpressed.

8.3. ORP1L in Macrophages

To analyze the consequences of ORP1L silencing in a more physiological setup, we used RAW264.7 macrophages as ORP1L is highly induced when monocytes differentiate into macrophages (Johansson et al., 2003). Earlier it was found that overexpression of ORP1L in bone marrow-derived macrophages enhances atherosclerotic lesion formation apparently by reducing cholesterol efflux to HDL (Yan et al., 2007). We therefore generated two cell lines stably expressing shRNA constructs containing a non-targeting or ORP1 targeting sequences. This method yielded a ~75% decrease in both ORP1L mRNA (not
shown) and protein (III Figure 7A) levels. We first determined if this manipulation affects endosome motility. We used acLDL fluorescently labeled with a DiI probe to track the movement of endosomes and, furthermore, to track the organelles specifically involved in cholesterol handling. We assessed the motility of the organelles by a method where sequential frames are subtracted from one another leaving only organelles that moved during the video. We found that the DiI containing organelles moved faster in the cells where ORP1 was depleted (III Figure 7A).

To test whether depletion of ORP1L and the increased motility of cholesterol containing organelles affects cholesterol in these cells, we labeled the cells with acLDL containing $[^3]$Hcholesteryl oleate. Efflux of $[^3]$Hcholesterol was then followed to two different physiologic acceptors: lipid-poor ApoAI and spherical HDL. We found that efflux to ApoAI was severely impaired in cells depleted of ORP1L, but efflux to HDL was not (III Figure 7B). As different cellular proteins are responsible for efflux to these acceptors, the results suggest that the one responsible for efflux to ApoAI, i.e. ABCA1 protein, is not functioning properly in cells lacking ORP1L. No changes were observed in the uptake of the probe, or in the hydrolysis of the cholesteryl ester molecule (data not shown). The difference was also only seen when cholesterol was internalized in acLDL, and not when cells were labeled with free $[^3]$Hcholesterol. This result indicates that the efflux problem is specific to endosomal, lipoprotein-derived cholesterol, which makes sense since ORP1L is an endosomal protein.

The results of this work implicate that ORP1L regulates the motility and localization of endolysosomes depending on its sterol binding status. A recent work using ORP1L lacking the entire ORD domain found that sterols regulate endosomal localization such, that endosomes loaded with cholesterol are found close to the nucleus (Rocha et al., 2009). This pattern in localization was reversed when truncated ORP1L mutant was expressed, indicating that ORP1L sterol binding is needed for this phenomenon. Rocha et al suggested a model where sterol free ORP1L is bound to VAP on the ER and sterol binding to ORP1L replaces VAP with the dynein motor complex and endosomes are transported to the perinuclear area (Rocha et al., 2009). The present work, together with previous studies (Johansson et al., 2007; Rocha et al., 2009), suggests that endolysosomal localization and motility is regulated by ORP1L and its sterol binding status. When ORP1L is sterol free, it binds to VAP proteins on the ER and inhibits the motility of these organelles. When cholesterol levels in cells increase, ORP1L binds cholesterol (or one of the oxysterols), detaches from VAP and the motility of these organelles is released. Both processes are needed for proper handling of cholesterol in endosomes since both ORP1L overexpression (Yan et al., 2007) and silencing (this study) perturbs cholesterol efflux.
Conclusions and Future Prospects

This work describes the effects of two endosomal sterol binding proteins, ORP1L and NPC1 on endosomal transport and lipid metabolism. These studies suggest that cholesterol and endosomal cholesterol binding proteins have a crucial role in regulating endosomal movement, but also more generally in lipid metabolism. These effects are mediated at least by NPC1 and ORP1L, but possibly also by other endosomal sterol binding proteins, such as MLN64 (Hölttä-Vuori et al., 2005) or NPC2. In addition, this study provides a new tool, BODIPY-cholesterol, for studying cholesterol in living cells and tissues.

I) BODIPY-cholesterol mimics cholesterol on cellular membranes and can be used to study in cholesterol trafficking. BODIPY-cholesterol is an important new tool for lipid cell biology and will help elucidate new aspects of sterol trafficking (as already observed in (Jansen et al., 2010)). BODIPY-cholesterol may greatly help scientists to unravel new aspects of cholesterol in cells, but the imaging of cholesterol and other lipids is continually improving. First, the only physiological way for cholesterol to enter cells is from lipoprotein particles. However, BODIPY-cholesterol can be esterified to a fatty acid creating BODIPY-cholesteryl esters. These molecules can then be incorporated into LDL particles and fed to cells in a physiological manner. Importantly, this allows one to specifically follow the trafficking of LDL-derived cholesterol (Uronen et al manuscript in preparation). Also other ways to develop the methodology of BODIPY-cholesterol can be thought of, such as oxidized forms of BODIPY-cholesterol to study oxysterol trafficking or using BODIPY-cholesterol to visualize sterol trafficking in live mammals. In the long haul however, it would be attractive to develop techniques, that would enable one to visualize cholesterol itself, without using fluorescent analogs or sterol binding compounds (Lim et al., 2010).

II) NPC1 genetic status was found to be associated with changes in triglyceride balance in both mice and men. Several NPC1 SNPs were associated with increased triglyceride levels in man, and the NPC1 deficient mouse had decreased triglyceride levels as a result of decreased triglyceride synthesis. This work, together with other recent findings in the field (Meyre et al., 2009; Garver et al., 2010; Jelinek et al., 2010) provide a new participant to triglyceride metabolism and obesity. How does this endosomal cholesterol binding protein affect how the body handles and stores triglycerides? The data presented in this thesis supports a view where NPC1 regulates changes in cholesterol metabolism, which then lead to changes in triglyceride metabolism. The integration of cholesterol and triglyceride metabolism is not fully understood, but does involve e.g. the LXR-SREBP1c pathway.
III) ORP1L functions as sterol dependent regulator of endosome motility and localization. In this study, we showed that ORP1L has a negative effect on endosome motility dependent on its ability to bind VAP, and that the localization of endosomes is dependent on the ability of ORP1L to bind sterols. We also provide evidence that ORP1L changes the functional status of endo-lysosomes. An interesting aspect of ORP1L, regarding future studies, is that in addition to differentiated macrophages it is also highly expressed in the central nervous system (Johansson et al., 2003). The present study brings more insight into this since the brain-specific sterol 24(S)OHC (Lutjohann et al., 1996) seems to be the best of the tested ligands for ORP1L. It is tempting to speculate therefore, that ORP1L might have a role in sterol regulated transport of endosomes in neurons.

Integrating knowledge from cell biology and lipid metabolism is of outstanding importance when we try to understand the mechanisms of common metabolic diseases. The studies presented in this work provide two models of endosomal sterol binding proteins affecting phenomena important in such states. The NPC1 protein has a surprising role in triglyceride metabolism and obesity, and the ORP1L protein may regulate cholesterol efflux from foam cells via endo-lysosomal dynamics. To gain further insight into the mechanisms of metabolic diseases, one must understand the mechanisms governing the cell biological processes behind them.
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