Cellular Cholesterol Accumulation and Egress from Endosomal Compartments

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Academic Dissertation

To be publicly discussed with the permission of the Medical Faculty of the University of Helsinki, at the Women’s clinic, Helsinki University Central Hospital, Haartmaninkatu 2, big lecture hall, on April 24th 2009, at 12 o’clock noon.

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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.


ABBREVIATIONS

aa  amino acid
ABCA1  ATP-binding cassette transporter A1
ACAT  acyl-coenzyme A: cholesterol acyltransferase
acLDL  acetylated LDL
apoA-I  apolipoprotein A-I
BODIPY  boron-dipyrromethene
ER  endoplasmic reticulum
GDI  guanosine nucleotide dissociation inhibitor
GSL  glycosphingolipid
HDL  high density lipoprotein
HMGR  3-hydroxy-3-methylglutaryl-CoA reductase
Insig  Insulin-induced gene
LacCer  lactosylceramide
Lamp  lysosome-associated membrane protein
LBPA/BMP  lysobisphosphatidic acid/bismonoacylglycerophosphate
LDL  low density lipoprotein
LDLr  low density lipoprotein receptor
LPDS  lipoprotein-deficient serum
LAL  lysosomal acid lipase
LXR  liver X receptor
MDCK  Madin-Darby canine kidney
mmLDL  minimally modified LDL
MPR  mannose-6-phosphate receptor
M6P  mannose-6-phosphate
mRNA  messenger RNA
NPC  Niemann-Pick type C
NPC1  Niemann-Pick C 1 protein
NPC2  Niemann-Pick C 2 protein
NPC1L1  Niemann-Pick C 1 –like 1 protein
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>oxLDL</td>
<td>oxidized LDL</td>
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<tr>
<td>PM</td>
<td>plasma membrane</td>
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<tr>
<td>RCT</td>
<td>reverse cholesterol transport</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>SCAP</td>
<td>SREBP cleavage activating protein</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>SR-A1</td>
<td>scavenger receptor A1</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>TGN</td>
<td>trans-Golgi network</td>
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<tr>
<td>Tfr</td>
<td>transferrin receptor</td>
</tr>
<tr>
<td>7DHCR</td>
<td>7-dehydrocholesterol reductase</td>
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<td>WB</td>
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INTRODUCTION

Intracellular cholesterol homeostasis is a tightly regulated process aimed at balancing the flux of cholesterol entering and exiting the cell. Defects in cellular cholesterol metabolism have been linked to a variety of diseases ranging from atherosclerosis to Alzheimer’s disease. The pathogenesis of these diseases are, however, quite complex, and it has been challenging to pinpoint the exact cellular processes that lead to disease progression, although the relationship between circulating cholesterol levels and atherosclerosis has been well documented. In general, LDL particles in the circulation are clinically regarded as “bad cholesterol” since these particles get entrapped in the vascular wall, leading to atherosclerosis. Circulating HDL particles are conversely regarded as “good cholesterol” because of their ability to transport cholesterol from peripheral tissues to the liver for secretion as bile salts.

During the progression of atherosclerosis, LDL cholesterol accumulates in the wall of arteries, especially in places with high shear stress. Once inside the vessel intima, the LDL particles undergo a series of chemical modifications either by enzymatic processes or through oxidation. The modified LDL particles are then engulfed by macrophages, resulting in macrophage foam cells. If the macrophage foam cells are not able to efflux the cholesterol back into the bloodstream, the excessive cholesterol ultimately leads to cell death, and the deposition of cellular debris within the atherosclerotic lesion. The cells ability to secrete cholesterol is mainly dependent on the ABCA1 transporter which transfers cholesterol to extracellular apoA-I particles, yielding nascent HDL particles. The nascent HDL particles are then further enriched with cholesterol by the ABCG1 transporter. The process of atherosclerotic plaque development is therefore to a large extent a cellular one, in which the capacity of the macrophages in handling the excessive cholesterol load determines the progression of lesion development. Hence it is of great importance to understand how cholesterol is transported within the cell. To achieve this we have utilized the autosomal recessive cholesterol transport disease Niemann-Pick type C (NPC), as a cellular model and tool to study the processes governing intracellular sterol transport.

In the first part of the study we characterized the mutations in an NPC1 disease patient and studied the consequences of the individual mutations on the function of the NPC1 protein. A
major finding was that mutations in the NPC1 protein cause an upregulation of NPC2 protein gene transcription, lending further support to the theory that these two proteins might function in concert to facilitate cholesterol egress from the endosomal system.

In the second part of the study we used NPC1 disease cells as a cellular model for cholesterol accumulation, in order to identify individual Rab GTPases involved in cholesterol efflux from the endosomal system. Using a combination of fluorescent microscopy and biochemical techniques we identified Rab8 as a key protein in facilitating the transport of endocytosed LDL cholesterol to extracellular acceptors. Knock-down of the Rab8 protein with RNAi resulted in intracellular cholesterol accumulation, while overexpression of Rab8 alleviated the cholesterol storage phenotype seen in NPC1 disease cells.

In the third part of the study we studied the role of Rab8 in macrophage foam cell formation and cholesterol processing. We found that Rab8 is expressed in macrophage foam cells of atherosclerotic lesions. Overexpression of Rab8 in primary human macrophages induced ABCA1-mediated cholesterol efflux to apoA-I. Rab8 facilitated the efflux of cholesterol from macrophage foam cells by regulating ABCA1 protein levels at the cell surface, as well as facilitating cholesterol traffic to ABCA1 substrate pools.
REVIEW OF THE LITERATURE

1. PATHOPHYSIOLOGY OF ATHEROSCLEROSIS

1.1 Atherogenic lipoproteins and macrophage foam cell formation

Although several proatherogenic factors have been identified, the level of circulating LDL particles stands out as the most important determinant of lesion development. Atherosclerotic plaques start out as fatty streaks in the intima of middle and large diameter arteries already at early adulthood. The process of plaque formation is complex but lesions usually form at places with high shear stress. LDL is deposited in the intimal matrix of the artery where it is modified to yield so called minimally modified LDL (mmLDL) and oxidized LDL (oxLDL) (Schwenke and Carew, 1989; Williams and Tabas, 1998). MmLDL can still be recognized by LDL receptors while oxLDL is no longer recognized (Navab et al., 1996). Instead, oxLDL is taken up by macrophages via different receptors, the most important of which are CD36 and scavenger receptor A1 (SR-A1). The lesional macrophages are derived from infiltrating monocytes that are attracted to the site by various chemotactic factors, which can be secreted by other cells in the lesion, or can be components of oxLDL, e.g. lyso-phosphatidylcholine (lyso-PtdCho) (Hansson and Libby, 2006; Quinn et al., 1987).

Blood-borne monocytes infiltrate the arterial intima and differentiate into macrophages, which take up modified LDL particles. This is initially thought to serve as an atheroprotective function. The internalized cholesterol is stored as cholesterol esters in cytoplasmic lipid droplets. Macrophages are, however, not capable of downregulating the scavenger receptors on the plasma membrane, which leads to continued scavenging of lipoprotein particles and expanding intracellular cholesterol stores, giving a foamy appearance of the cytoplasm. The foam cells are not able to efflux cholesterol at the same rate as uptake, leading to cell death, and the deposition of cellular debris in the arterial intima (Figure 1).
Figure 1 Macrophage foam cells in atherosclerotic lesion development. Circulating LDL particles accumulate in the intima of arteries, and undergo chemical modification, resulting in oxidized LDL (oxLDL). Blood-borne monocytes infiltrate the area and differentiate into macrophages that scavenge the oxLDL particles. Macrophage foam cells undergo apoptosis which results in a necrotic core, leading to further lesion development. Modified from (Glass and Witztum, 2001).

1.2 Macrophage reverse cholesterol transport

Reverse cholesterol transport (RCT) is the process of net cholesterol flux from peripheral tissues to the liver (Cuchel and Rader, 2006). In terms of atherosclerosis, the process of macrophage RCT warrants the most attention, although conceivably RCT might also take place from other cells in the atherosclerotic lesion. In terms of macrophage RCT, the combined role of the ATP-binding cassette transporters ABCA1 and ABCG1 seem to be the most important. ABCA1 was found to be mutated in Tangier disease (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999) Although SR-B1 has also been implicated in cholesterol efflux in vitro it is not involved in macrophage RCT in the mouse (Wang et al., 2007).
1.2.1 ABCA1 mediated lipid efflux

The primary function of ABCA1 is thought to be the lipidation of apolipoprotein A-I (apoA-I) at the plasma membrane (Denis et al., 2008), although intracellular compartments may also be involved (Cavelier et al., 2006; Hassan et al., 2008; Neufeld et al., 2004). However, the contribution of intracellular apoA-I to total HDL production has been estimated to be only ~1.4% of total HDL production (Denis et al., 2008). The precise mechanism of apoA-I lipidation is not known. It has been shown in numerous studies that apoA-I interacts directly with ABCA1 (Chroni et al., 2004; Vedhachalam et al., 2004) and that the binding is essential for ABCA1 mediated lipid transfer to ABCA1 (Fitzgerald et al., 2002). However, there seems to be two separate binding sites for apoA-I on the plasma membrane, a low-capacity binding site, thought to represent direct binding to ABCA1, and a high-capacity binding site to membrane domains generated through ABCA1 action (Hassan et al., 2007; Vedhachalam et al., 2007). The binding of apoA-I to ABCA1 is relatively unspecific and apparently involves amphiphatic alpha-helices in apoA-I (Fitzgerald et al., 2002; Vedhachalam et al., 2007). Accordingly, N- and C-terminal domain mutants of apoA-I bind equally well to ABCA1. In contrast, insertion of apoA-I into the plasma membrane domains requires intact hydrophobic C-terminal domain alpha-helices (Gillotte et al., 1999; Saito et al., 2003; Saito et al., 2004). The lipidation process might involve the transfer of phospholipids to apoA-I, generating nascent HDL particles, a process which is defective in Tangier disease patients (von Eckardstein et al., 1998). ABCA1 might function by flipping of phospholipid species from the inner to the outer leaflet (Alder-Baerens et al., 2005), resulting in membrane bending and increased curvature, facilitating the binding and subsequent lipidation of lipid poor apoA-I at the plasma membrane (Vedhachalam et al., 2007).

Binding of apoA-I to ABCA1 might initiate signaling responses via the Janus kinase 2, stabilizing the protein on the cell surface (Tang et al., 2006; Tang et al., 2004). ABCA1 and CDC42 are in close proximity of each other at the plasma membrane and Golgi compartment, and these proteins co-immunoprecipitate (Tsukamoto et al., 2001). It was subsequently shown that binding of apoA-I to ABCA1 also initiates signaling via CDC42 and phosphorylation of PAK-1 and p54^JNK, leading to actin polymerization (Nofer et al., 2006). There is evidence that apoA-I interacts directly with ABCA1 since these proteins cross-link, indicating that they are in close proximity (<7 Å) of each other (Chroni et al., 2004; Denis et al., 2004; Fitzgerald et al., 2002).
2004; Wang et al., 2000). Additionally, binding of apoA-I enhances the interaction between ABCA1 and CDC42 (Nofer et al., 2006)
2. INTRACELLULAR CHOLESTEROL TRAFFICKING AND HOMEOSTASIS

2.1 Uptake and processing of LDL cholesterol in the endosomal system

Regulation of cellular cholesterol homeostasis is mainly achieved through balancing the amount of cholesterol uptake, synthesis and efflux. Circulating LDL particles containing cholesterol bind to the LDL receptor at the cell surface. The LDL receptor complex is then internalized through clathrin-coated pits. Once endocytosed, the LDL particles dissociate from the LDL receptor which then recycles back to the cell surface (Matter et al., 1993). Cholesteryl esters in the LDL particle are then hydrolyzed by lysosomal acid lipase (LAL) to yield free (unesterified) cholesterol. Most of the cholesterol in the endocytic compartments is present in multivesicular endosomes (multivesicular bodies, MVB) and recycling endosomes, ~60% and ~20% respectively (Möbius et al., 2003). The internal membranes of MVBs contain the bulk of cholesterol and are enriched in the endosome specific phospholipid LBPA/BMP (lysobisphosphatidic acid / bismonoacyl-glycerophosphate) (Kobayashi et al., 1999). The free cholesterol exits the endosomal compartments in a process that is dependent on the NPC1 and NPC2 proteins. After exiting the endosomes, cholesterol is mainly distributed to the plasma membrane and Golgi apparatus by yet unidentified pathways, before finally reaching the ER. The ER is usually relatively cholesterol poor and cholesterol entering the ER is rapidly converted to cholesteryl esters by acyl-coenzyme A: cholesterol acyltransferase (ACAT), for storage in lipid droplets (Figure 2).
Figure 2. Cholesterol uptake and compartmentalization. LDL particles bind to the LDL-receptor at the plasma membrane, and are endocytosed through clathrin-coated pits. The LDL particle then dissociates from the LDLr which is recycled back to the plasma membrane. The cholesterol esters derived from the LDL particle is hydrolyzed in the endosomes to yield free cholesterol. The free cholesterol is transported out of the endosomal compartments to the plasma membrane and TGN before reaching the ER (dotted arrows). In the ER, the cholesterol is re-esterified by ACAT, and stored in lipid droplets. (LDLr, LDL receptor; EE, early endosome; RE, recycling endosome; MVB, multi vesicular body; LE, late endosome; LY, lysosome; ER, endoplasmic reticulum; LD, lipid droplet; TGN trans-Golgi network; N, nucleus)

2.2 Regulation of cholesterol biosynthesis in the ER

The ER is the main orchestrator of cellular cholesterol distribution and amount. However, the ER is a relatively cholesterol poor organelle with a cholesterol content of only 1% of total cellular cholesterol (Lange, 1991). The two main proteins mediating cholesterol regulation in the ER are
HMG-CoA reductase (HMGR) and the Sterol response element binding protein (SREBP) Cleavage Activating Protein (SCAP), which forms a complex with SREBP in the ER (Goldstein et al., 2006). HMGR is the rate-limiting protein in de novo cholesterol biosynthesis, while SCAP regulates SREBP processing. Both proteins are regulated by sterol binding to the sterol-sensing domain (SSD) and the ER resident Insig proteins (Insig1 and Insig2). Rising cholesterol levels in the ER induces regulatory mechanisms at both transcriptional and posttranscriptional levels. Cholesterol binds directly to the SSD of SCAP, causing a conformational change in the SSD. This causes SCAP to bind to Insig proteins, leading to retention of the SCAP/SREBP complex in the ER, thus shutting down SREBP dependent gene transcription (Yang et al., 2002). HMGR is also regulated by sterols, but the outcome of Insig binding is entirely different. Binding of HMGR to Insigs leads to ubiquitinylation and subsequent degradation of HMGR in the proteasome, thus reducing de-novo cholesterol synthesis (Espenshade and Hughes, 2007; Sever et al., 2003).

2.3 Transcriptional regulation of cholesterol levels

There are two main transcriptional regulatory systems in the cell which work antagonistically to balance the intake, de novo synthesis and efflux of cholesterol, namely the sterol regulatory element proteins (SREBPs) and liver X receptors (LXRs). As described above, the SREBP system is regulated by ER sterol levels through the SCAP protein. The role of SCAP is to regulate the trafficking of SREBP to the Golgi. Upon cholesterol depletion in the ER, SREBP traffics to the Golgi where it is activated through a proteolytic process by the site1 (S1P) and site2 (S2P) proteases. The active transcription factor travels to the nucleus where it activates the transcription of gene products which increase intracellular cholesterol levels, e.g. HMGR and the LDL receptor (Goldstein et al., 2006). Conversely, activation of LXRs is mediated by oxysterols leading to the transcription of gene products that facilitate cholesterol efflux from the cell, e.g. the ABCA1 transporter (Tontonoz and Mangelsdorf, 2003).
3. NIEMANN-PICK TYPE C DISEASE

3.1 Disease pathophysiology

Niemann-Pick type C disease is a severe lysosomal cholesterol-sphingolipid storage disorder. The progress of the disease is varying with onset of symptoms ranging from early infancy to adulthood. Typically, the disease presents with neurological regression and peripheral manifestations due to massive cholesterol accumulation in the liver and spleen. Clinically the patients present with hepatosplenomegaly, ataxia, dystonia, seizures, supranuclear gaze palsy, and progressive dementia. Early-onset NPC disease is usually more severe and involves peripheral tissues with hepatosplenomegaly often observed. In the brain, there is degeneration of the brain stem and Purkinje neurons of the cerebellum (Walkley and Suzuki, 2004). Why only parts of the brain and specific subsets of neurons are affected is not understood, but the process is likely to involve both apoptosis and autophagy (Ko et al., 2005; Pacheco et al., 2007). A number of biochemical defects have been described, but the traditional hallmark of the disease is cholesterol accumulation in lysosomes combined with decreased esterification of cholesterol in the endoplasmic reticulum (ER) (Blanchette-Mackie et al., 1988). NPC has therefore historically been regarded as a cholesterol transport disorder. This is in contrast to Niemann-Pick types A and B, which are caused by acid sphingomyelinase deficiency. Interestingly, there is concomitant cholesterol accumulation in Niemann-Pick type A and B cells, and the depletion of cholesterol from these cells restores trafficking of fluorescently tagged lactosyl ceramide (BODIPY-LacCer) (Puri et al., 1999). These results emphasize the interdependence of cholesterol and sphingolipids in lysosomal storage disorders. Accordingly, in NPC disease there is a concomitant sphingolipid accumulation and decreased sphingomyelinase activity (Liscum and Klansek, 1998; Lloyd-Evans et al., 2008). The pathophysiology of NPC disease is however quite complex and it is has been challenging to pinpoint the precise pathological mechanism. Nevertheless it is likely that the disease phenotype is the combined result of aberrant cholesterol and sphingolipid metabolism.
One of the main cellular perturbations is a defect in the regulatory responses to cholesterol loading via the LDL pathway. Normally, cholesterol containing LDL particles taken up via the LDL-receptor, where after the cholesterol exits the endosomal system and is transported to the plasma membrane and to the ER, possibly via the trans-Golgi network (TGN). Superfluous cholesterol is then re-esterified by the ER resident protein acyl-CoA acyltransferase (ACAT) for storage in lipid droplets (Chang et al., 2001). In NPC disease cholesterol is trapped in aberrant endosomes and fails to reach the ER (Blanchette-Mackie et al., 1988; Pentchev et al., 1985) (Figure 3). This results in a relative cholesterol deficit in the ER, which is the primary sterol-sensing organelle in the cell. As a result, there is a misregulation of the two major cholesterol homeostasis regulatory machineries i.e. the SREBP and LXR pathways. Thus, de novo cholesterol synthesis is not down-regulated, but rather increased (Liscum and Faust, 1987). Also the cholesterol efflux pathways are not up-regulated appropriately, and the LDL-receptor fails to down-regulate. The failure of down-regulation of the SREBP pathway can easily be attributed to the relative lack of cholesterol in the ER. Concomitantly, there is defective generation of oxysterols, the key activators of the LXR pathway (Frolov et al., 2003).

**Figure 3.** Primary human fibroblasts from control and NPC1 patients. Cells are stained with filipin which visualizes free cholesterol (blue). NPC1 cells accumulate large amounts of cholesterol perinucleally.
3.2 The NPC1 and NPC2 proteins

Niemann-Pick disease type C is comprised of two separate genetic complementation groups with mutations in either of the *NPC1* or *NPC2* genes. Roughly 95% of disease cases are caused by mutations in the *NPC1* gene whilst the remaining cases are accounted for by mutations in the *NPC2* gene (Carstea et al., 1997; Naureckiene et al., 2000). The precise function of the two NPC proteins has not been established. However, lack of either protein leads to a nearly identical cellular phenotype, indicating that these proteins must perform similar functions in sterol egress from the endosomal system. Also, the NPC2/NPC1 double knockout mouse model displays an identical phenotype to that of NPC2 and NPC1 knockout models alone (Sleat et al., 2004). NPC2 is a cholesterol binding protein present in lysosomes. NPC1 has been suggested to function as a transmembrane fatty acid transporter (Davies et al., 2000a), and more recently, as a sphingosine transporter (Lloyd-Evans et al., 2008). The conclusive evidence for a specific transport function for either of the proteins is however still lacking.

3.2.1 Structural features and sterol binding of NPC1

The gene mutated in the major complementation group was identified in 1997 through a positional cloning strategy. The gene product, named NPC1, was found to be a large, 1278 amino acid polytopic membrane protein with 13 transmembrane spans (Carstea et al., 1997) (Figure 4). A number of interesting domains have been identified in the protein, including a putative sterol-sensing domain (SSD) comprising of transmembrane loops three through seven (Davies and Ioannou, 2000). This domain was originally identified in the SCAP (sterol regulatory element binding protein (SREBP) cleavage activating protein) and hydroxymethylglutaryl (HMG)-CoA reductase proteins (HMGR) (Osborne and Rosenfeld, 1998). Other proteins containing a SSD include the Hedgehog morphogen receptor Patched and 7-Dehydrocholesterol reductase (7DHCR) (Kuwabara and Labouesse, 2002), as well as Niemann-Pick type C 1 Like 1 (NPC1L1) (Davies et al., 2000b). The function of the SSD has most extensively been studied in SCAP and HMGR. The SSD is required for cholesterol dependent retention of SCAP in the ER, as well as lanosterol-induced ubiquitinylatation and degradation of HMGR (Nohturff et al., 1999; Song et al., 2005). The evidence regarding a specific sterol-sensing function of the SSD in the NPC1 protein
is still inconclusive. NPC1 is capable of binding to a photoactivatable cholesterol analog, azocholestanol, while mutations in the SSD (P691S, Y634C) abolish this binding (Ohgami et al., 2004). In vitro $[^3H]$-cholesterol binding to the purified SSD of SCAP has also been established, but this was not inhibited by the corresponding Y298C mutation in SCAP (Radhakrishnan et al., 2004). However this mutation in SCAP has previously been shown to inhibit the binding of SCAP to Insig proteins involved in 25-hydroxycholesterol sensing in the ER (Radhakrishnan et al., 2007; Yang et al., 2002). Also, mutations in NPC1, corresponding to gain of function mutations in SCAP, resulted in increased cholesterol trafficking in the cell (Millard et al., 2005). However, in a later study the sterol binding capacity of the NPC1 protein was limited to the N-terminal domain (“NPC domain”) of purified NPC1 (Infante et al., 2008a). Mutations in the SSD are none the less clinically significant, and mutations in the SSD abolish the cholesterol transport function of NPC1 (Watari et al., 1999b). A critical role for the N-terminal domain in NPC1 function has also previously been shown, as well as for the C-terminal LLNF lysosome-targeting motif (Watari et al., 1999b). Deletion of the C-terminal dileucine motif resulted in the retention of the NPC1 protein in the ER. Site directed mutagenesis of cysteins (C63S, C74S/C75S and C97S) resulted in non-functional NPC1 protein that localized to the surface of lysosomes (Watari et al., 1999a).

Figure 4. Schematic representation of the NPC1 protein. The SSD is shown in red. Adapted from (Davies and Ioannou, 2000).
3.2.2 Subcellular localization and trafficking of NPC1

The NPC1 and NPC2 proteins are thought to reside mainly in late endocytic compartments. The proteins show little co-localization, indicating that they are present in different subsets of endosomes. NPC1 colocalizes with the late endosomal/lysosomal marker Lamp2, but not with endocytosed LDL derived cholesterol or the mannose-6- phosphate receptor (MPR) (Neufeld et al., 1999). NPC1 has also been shown to colocalize with LBPA, Rab7, and Rab9 (Higgins et al., 1999; Puri et al., 1999; Zhang et al., 2001b). Drugs that induce lysosomal cholesterol accumulation e.g. progesterone and U18666A redistributes NPC1 to cholesterol laden lysosomes (Neufeld et al., 1999). In normal cells NPC1 is thought to only transiently localize to cholesterol enriched organelles (Higgins et al., 1999; Kobayashi et al., 1999; Neufeld et al., 1999; Patel et al., 1999; Watari et al., 1999a; Zhang et al., 2001b). Also the GM2 ganglioside localizes exclusively with NPC1, but not with endocytosed cholesterol (Osborne and Rosenfeld, 1998).

Live cell microscopy using GFP-tagged NPC1 protein has revealed that NPC1 containing endosomes are highly mobile. NPC1 function seems to be dually required for both lipid sorting and tubular late endocytic trafficking (Ko et al., 2001). Also the motility of NPC1 containing endosomes is modulated by the lipid composition of the membranes, and is inhibited by cholesterol accumulation (Lebrand et al., 2002; Zhang et al., 2001a). NPC1-GFP containing organelles undergo tubulation and fission with both anterograde and retrograde migrations along microtubules. This movement also requires an intact SSD (Zhang et al., 2001a). Newly synthesized NPC1-GFP was also seen by live cell microscopy to be transported from the ER via the Golgi to late endosomes (Zhang et al., 2001a). NPC1 is ubiquitinylated upon cholesterol depletion which might influence the trafficking of NPC1 within different subsets of endosomes (Ohsaki et al., 2006).

3.2.3 Structural features and lipid binding of NPC2

The NPC2 gene product is a 151 amino-acid soluble lysosomal protein containing a 19 amino acid signal peptide which is cleaved off to generate the active 132 amino-acid protein.
Intracellular localization studies have shown that the protein resides both in Lamp1 positive and Lamp1 negative endosomes, as well as the Golgi apparatus. The NPC2 protein is glycosylated and contains a mannose-6-phosphate (M6P) post-translational modification conferring lysosomal targeting through binding to the mannose-6-phosphate receptor (MPR). The NPC2 protein was originally characterized as a cholesterol binding protein found in epididymal fluid named HE1 (human epididymis protein 1) (Okamura et al., 1999). As a result of a screening for M6P containing proteins HE1 was found to localize to lysosomes. This allowed the identification of HE1 as the second protein in NPC disease, and the HE1 protein was renamed NPC2 (Naureckiene et al., 2000).

Structural analysis of the bovine NPC2 protein and site-directed mutagenesis strategies have shown the mechanism of cholesterol binding and identified functional domains in the protein. The bovine orthologue of NPC2, EPV20 had previously been purified from bovine milk (Larsen et al., 1997). The structure of the bovine homolog bNPC2 was elucidated by x-ray crystallography, revealing an Ig-like β-sandwich fold consisting of seven β-strands arranged in two β-sheets (Friedland et al., 2003). Between the β-sheets, three small cavities are formed with a total volume of 158 Å³. The volume of a cholesterol molecule is 741 Å³, indicating that the β-sandwich needs to expand in order to accommodate the cholesterol molecule. The structure of NPC2 bound to cholesterol-3-O-sulfate also supports this model (Xu et al., 2007). X-ray crystallography of NPC2 bound to cholesterol sulfate indicates that the sterol is inserted into the cholesterol binding pocket with the iso-octyl side chain inside the pocket, and the sulfate group at position 3 of the A ring protruding from the entrance (Xu et al., 2007). Such an orientation would explain why NPC2 has low affinity for oxysterols (Infante et al., 2008a). A mutational screen based on evolutionally conserved residues highlights the importance of the cholesterol-binding cavity (Ko et al., 2003) (Figure 5).
Figure 5. Structure of the NPC2 sterol binding pocket. (A) Stereo view of the proposed sterol binding cavity. Sidechains of residues lining the cavity are shown in stick representation. Sidechains required for cholesterol binding (Ko et al., 2003) are shown in red. (B) Space filling model of cholesterol (green) docked in the proposed sterol-binding site. Reprinted from (Friedland et al., 2003) with the permission of the publisher. Copyright (2003) National Academy of Sciences, U.S.A.

The current evidence strongly favors a direct cholesterol shuttling model for NPC2 function. Several studies have now established that NPC2 binds cholesterol (Friedland et al., 2003; Infante et al., 2008a; Ko et al., 2003; Liou et al., 2006). NPC2 is able to extract and insert cholesterol from membranes in vitro (Cheruku et al., 2006) as well as shuttle cholesterol between liposomal vesicles (Babalola et al., 2007). NPC2 can also transfer cholesterol between the N-terminal domain of NPC1 and liposomes in vitro (Infante et al., 2008b). NPC2 is also involved in Thymal T cell selection by loading the MHC class I-like protein CD1d with the selecting glycosphingolipid isoglobotrihexosylceramide (iGb3) in NKT cells (Schrantz et al., 2007). Whereas the in vitro loading of iGb3 to CD1d required dimerization of NPC2, the transfer was not affected by cholesterol binding.
3.2.4 Subcellular localization and trafficking of NPC2

The NPC2 protein resides mainly in the lumen of late endosomes/lysosomes. The intracellular trafficking of NPC2 is governed by the mannose 6-phosphate receptor (MPR) owing to the posttranslational addition of a mannose 6-phosphate moiety to the protein (Naureckiene et al., 2000). The mannose 6-phosphate moiety is recognized by the MPR, which enables trafficking between the Golgi apparatus and lysosomes as well as uptake from the PM. Depletion of the two MPRs, MPR300 (cation-independent) and MPR46 (cation-dependent), in fibroblasts increases the secretion of NPC2 from cells (Willenborg et al., 2005). NPC2 is abundantly present in epididymal fluid and milk (Kirchhoff et al., 1996; Larsen et al., 1997), and can be secreted from cells in the cholesterol bound form (Ko et al., 2003; Okamura et al., 1999). NPC2 is also secreted from cultured glia cells, but does not associate with the major secretory sterol containing lipoprotein particles. Also, overexpression of NPC2 in glia cells does not enhance sterol secretion (Mutka et al., 2004). Secreted NPC2 can be re-endocytosed and is biologically active. Incubation of NPC2 cells with medium preconditioned by incubation with WT cells complements the cholesterol storage phenotype, but the complementation is inhibited by mannose-6-phosphate, indicating that endocytosis by MPR is required (Naureckiene et al., 2000). However overexpression of NPC2 mutants that are not properly secreted still complement the individual transfected cells, indicating that secretion is not an absolute necessity (Ko et al., 2003).

NPC2 is glycosylated, appearing as two individual bands of 21 to 25 kDa in WB of human fibroblast samples. After removal of N-linked glycans with PNGaseF (protein:N-glycosidase F) the protein migrates at 18 kDa (Mutka et al., 2004). A mutational screen of the possible asparagine linked (Asn, N) glycosylation sites revealed that only two of the sites, N58 and N135, are utilized. The study also showed that glycosylation of Asn 58 is required for proper lysosomal targeting of NPC2 (Chikh et al., 2004).
4. RAB GTPases IN MEMBRANE AND CHOLESTEROL TRAFFICKING

4.1 Rab GTPase function

Rab proteins are small GTPases belonging to the Ras superfamily of GTPases (Wennerberg et al., 2005). Rab proteins function as molecular switches, regulating events such as vesicle trafficking, motility, docking and fusion (Pfeffer, 2001; Zerial and McBride, 2001). Rab proteins cycle between the cytosol and target membranes in an active GTP-bound state and an inactive GDP-bound state. The switching to the active state is mediated by guanine nucleotide exchange factors (GEFs), which mediate binding of GTP to the Rab protein. Inactivation is mediated by GTPase-activating proteins (GAPs) which catalyze the intrinsic GTPase activity of the Rab protein, resulting in hydrolysis of the bound GTP to GDP (Pfeffer, 2001; Segev, 2001). In the active GTP bound state, Rab proteins insert into the target membrane by virtue of a prenylation motif located in the C-terminal of the protein. Prenylation of Rab proteins is facilitated by Rab escort protein (REP) which presents newly synthesized Rab protein to Rab geranylgeranyl transferase (GGTase) (Andres et al., 1993). In the ‘soluble’ state Rab proteins are bound and sequestered in the cytosol by GDI (guanosine nucleotide dissociation inhibitor) (Figure 6). Human cells express two isoforms, GDIα which is enriched in the brain and the ubiquitously expressed GDIβ (Alory and Balch, 2001). The binding of Rab proteins to GDI is strongly dependent on prenylation as well as the presence of GDP in the nucleotide binding site. Upon GDP- or GTP binding the Rab proteins change conformation in the highly conserved ‘switch region’, which is recognized by GDI. GDI can also extract Rab proteins from membranes (Sasaki et al., 1990; Ullrich et al., 1993).

The targeting of Rab proteins to specific membranes is thought to be mediated by the C-terminal ‘hypervariable domains’ which are the most divergent amino acid stretches between different Rab proteins (Chavrier et al., 1991). The association of the Rab protein to GDI is probably modulated by the length of the hypervariable domain with affinities of 20-500nM Kd being reported (Schalk et al., 1996; Shapiro and Pfeffer, 1995). The deposition of Rab proteins into the target membrane is facilitated by GDI-displacement factors (GDF).
Figure 6. The Rab cycle. Rab GTPases are activated by guanine nucleotide exchange factors (GEFs). The Rab proteins then bind to effector proteins which can for instance be molecular motors. After fulfilling its task, the Rab protein is deactivated by GTPase activating proteins (GAPs), and removed from the membranes by guanine nucleotide dissociation inhibitor (GDI). Another round of action is initiated by GDI displacement factor (GDF) which facilitates the deposition of Rab proteins into the target membrane.

4.2 Rab proteins in intracellular sterol trafficking

Cholesterol efflux from the endosomal compartments seems to be mediated, at least in part, by Rab protein directed membrane trafficking. It was initially shown that inhibition of Rab protein function by microinjection of Rab-GDI inhibits cholesterol removal from endocytic compartments (Höltta-Vuori et al., 2000). So far, the individual Rab proteins that have been implicated in intracellular cholesterol trafficking localize to the endocytic compartments of the cell (Table 1). Choudhury and colleagues showed that overexpression of dominant negative Rab7 or Rab9 inhibited the Golgi targeting of sphingolipids, and that overexpression of wild-type Rab7 or Rab9 reduced the cholesterol accumulation in NPC1 fibroblasts (Choudhury et al., 2002).
Rab9 was subsequently shown to increase cholesterol efflux in two separate studies (Narita et al., 2005) (Walter et al., 2003). In a recent study, the life span of Rab9 / NPC1-/- transgenic mice was also found to be increased by ~ 22 % as compared to NPC1-/- mice (Kaptzan et al., 2009). The dominant negative form of Rab4 also perturbs cholesterol recycling (Choudhury et al., 2004). Overexpression of Rab11 affects endosomal cholesterol trafficking, resulting in the accumulation of free cholesterol in recycling endosomes (Höltta-Vuori et al., 2002).

**Table 1. Localization and function of Rab proteins which have been shown to affect cholesterol transport in the endocytic compartments.**

<table>
<thead>
<tr>
<th>Rab</th>
<th>Localization</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rab4</td>
<td>Early and recycling endosomes</td>
<td>Rapid endosomal recycling</td>
<td>(van der Sluijs et al., 1992)</td>
</tr>
<tr>
<td>Rab7</td>
<td>Late endosomes, lysosomes</td>
<td>Early to late endosomal transport, Lysosome biogenesis</td>
<td>(Press et al., 1998) (Bucci et al., 2000)</td>
</tr>
<tr>
<td>Rab9</td>
<td>Late endosomes, trans Golgi network</td>
<td>Late endosome to Golgi transport</td>
<td>(Lombardi et al., 1993)</td>
</tr>
<tr>
<td>Rab11</td>
<td>Recycling endosomes</td>
<td>Recycling through the endocytic recycling compartment (ERC)</td>
<td>(Ullrich et al., 1996) (Ren et al., 1998)</td>
</tr>
</tbody>
</table>

**4.3 The Rab8 GTPase**

Two isoforms, Rab8A (referred to here as Rab8) and Rab8b have been identified in humans. Rab8 is ubiquitously expressed in tissues while Rab8b is predominantly expressed in the spleen, testis and brain (Armstrong et al., 1996). There is an 83% sequence identity between Rab8A and Rab8b, with the highest degree of divergence within the C-terminal hypervariable domain (Figure 7) (Armstrong et al., 1996). Rab8 differs from other Rab proteins in that it is geranylgeranylated at a single site as opposed to the usual double geranylgeranylation. This is due to the presence of a CaaL motif instead of the usual CC or CXC motifs (Casey and Seabra, 1996; Wilson et al., 1998).
Rab8 affects cell shape, inducing cellular protrusions when overexpressed (Nachury et al., 2007) (Armstrong et al., 1996; Peränen et al., 1996). In two separate studies Rab8 was found to regulate primary ciliation formation (Nachury et al., 2007; Yoshimura et al., 2007). Ciliation formation was also found to be dependent on the Rab8 specific GAP XM_037557 (Yoshimura et al., 2007). Polarized transport by Rab8 is thought to be partly mediated by reorganization of actin and microtubules (Ang et al., 2003; Peränen et al., 1996). Rab8 also regulates the actin based movement of melanosomes to the PM in MDCK cells (Chabrillat et al., 2005), as well as vesicle transport during neuronal outgrowth. Knock-down of Rab8 with siRNA in neurons results in inhibition of anterograde vesicle movement and disrupts neuronal outgrowth (Huber et al., 1995). In addition to Rab8, vesicle formation and transport to membrane protrusions is dependent on the Rab8 specific GDP/GTP exchange factor Rabin8 (Hattula et al., 2002). Rab8 was shown to mediate apical protein transport in intestinal epithelial cells in a Rab8 -/- mouse model. In intestinal epithelial cells Rab8 might control both direct protein transport from the TGN to the apical membrane, or indirectly via the basolateral membrane. In any case, Rab8 deficiency causes the mislocalization of apical proteins to the lysosomes, and formation of intracellular microvillus inclusions, resulting in dysfunction of apical microvilli and malabsorption (Sato et al., 2007). Reduced Rab8 expression was also found in a human microvillus inclusion disease patient with an identical ultrastructural phenotype to the Rab8-/- mouse (Sato et al., 2007). Recently it was also established that mutations in the MYOB5 gene, which encodes the Rab8 interacting myosinVb motor protein (Roland et al., 2007), also causes microvillus inclusion disease in humans (Muller et al., 2008).
Rab8 is involved in both constitutive transport of proteins from the Golgi (Huber et al., 1993) and basolateral vesicle transport in Madin-Darby canine kidney (MDCK) cells (Ang et al., 2003). In MDCK cells, recycling endosomes might serve as intermediates for Golgi to PM transport, and as a common sorting organelle for the endocytic and secretory pathways (Ang et al., 2004). The polarized transport of proteins to the basolateral membrane is dependent on the AP-1B clathrin adaptor complex, which directs clathrin-coated bud formation at the TGN, and is regulated by Rab8 (Ang et al., 2003). The Rho GTPase CDC42 also selectively regulates the AP-1B pathway, and dominant negative CDC42 causes selective mis-sorting of AP-1B dependent cargo, indicating that Rab8 and CDC42 might function in the same pathway (Ang et al., 2003). In addition, cell morphogenesis is also altered by the Rab8 interacting, TNFα-inducable protein FIP-2 (optineurin), linking Rab8 to huntingtin (Hattula and Peränen, 2000). Optineurin also links Rab8 to myosin VI, which acts as a motor for actin dependent transport of vesicles deriving from the TGN (Au et al., 2007; Sahlender et al., 2005). Mutant huntingtin (mhtt), in turn inhibits Rab8 mediated post-Golgi trafficking, by delocalizing the optineurin/Rab8 complex from the Golgi (Del Toro et al., 2009).

**Table 2. Proteins which have been shown to interact directly with Rab8.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabin8</td>
<td>Rab8 specific GEF</td>
<td>(Hattula et al., 2002)</td>
</tr>
<tr>
<td>XM_037557</td>
<td>Rab8 specific GAP</td>
<td>(Yoshimura et al., 2007)</td>
</tr>
<tr>
<td>Myosin Vb</td>
<td>Molecular motor</td>
<td>(Roland et al., 2007)</td>
</tr>
<tr>
<td>Rabaptin5</td>
<td>Regulator of endocytosis</td>
<td>(Omori et al., 2008)</td>
</tr>
<tr>
<td>FIP-2/optineurin</td>
<td>Vesicle trafficking</td>
<td>(Hattula and Peränen, 2000)</td>
</tr>
</tbody>
</table>
AIMS OF THE STUDY

The aim of the first part of the study was to gain insight into the function of the NPC1 and NPC2 proteins. Making use of a severe form of infantile NPC1 disease the goal was to identify individual disease causing mutations in the NPC1 protein and to study the consequences of the individual mutations at the cellular level. The other key goal of the study was to establish the intracellular location of the NPC2 protein in normal and NPC1 fibroblasts in order to gain further insight into the pathophysiology of NPC disease.

The aim of the second part of the study was to use the cholesterol accumulation found in NPC1 disease as a tool to identify proteins involved in the efflux of cholesterol from the endocytic circuits. Proteins, primarily Rab GTPases, which reduce endosomal cholesterol accumulation in NPC1 cells would then be further characterized in their ability to facilitate intracellular cholesterol transport.

The aim of the third part of the study was to characterize the potential function of Rab8 in cellular cholesterol efflux from primary human macrophages. Macrophage foam cells are key elements in the development of atherosclerotic lesions and prime targets for pharmacological intervention. It is therefore crucial to understand the intracellular processes leading to ABCA1-mediated cholesterol efflux. The goal of this part of the study was therefore to investigate the role of Rab8 in the physiological setting of macrophage foam cell formation.
## METHODS

<table>
<thead>
<tr>
<th>Method</th>
<th>Publication</th>
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</tr>
<tr>
<td>SDS-PAGE, Western blot, ECL (enhanced chemiluminescence)</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Northern blot</td>
<td>I</td>
</tr>
<tr>
<td>Quantitative real-time PCR</td>
<td>III</td>
</tr>
<tr>
<td>Surface biotinylation</td>
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</tr>
<tr>
<td>Adenovirus production and infection</td>
<td>III</td>
</tr>
<tr>
<td>Semliki Forest virus production and infection</td>
<td>II</td>
</tr>
<tr>
<td>RNA isolation</td>
<td>I, II</td>
</tr>
<tr>
<td>Biochemical cholesterol measurements</td>
<td>II, III</td>
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<tr>
<td>Biochemical cholesterol ester measurements</td>
<td>II, III</td>
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<tr>
<td>Direct cholesterol efflux measurements</td>
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<td>GDI extraction of Rab proteins <em>in vitro</em></td>
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<tr>
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<td>Polyclonal antibody production</td>
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<tr>
<td>Immunocytochemistry</td>
<td>I, II, III</td>
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<td>Immunohistochemistry</td>
<td>III</td>
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<tr>
<td>RNA interference</td>
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<tr>
<td>Transient transfection</td>
<td>I, II, III</td>
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<tr>
<td>Electroporation</td>
<td>III</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

1. Defective intracellular trafficking of NPC proteins in NPC disease (I)

1.1 Effects of individual mutations on the NPC1 protein

Since the discovery of the two separate complementation groups in NPC it has been speculated that the two NPC proteins act along the same pathway and might even interact. In order to assess this, we sought to determine the subcellular localization of both NPC proteins, and analyze the effects of disease causing mutations in NPC1 on the distribution of the NPC2 protein. We identified three mutations, two of which were causative of NPC disease, C113R and a c-terminal deletion mutant (delC), and a benign polymorphism P237S. Interestingly, the P237S substitution has previously been regarded as a disease causing mutation (Kaminski et al., 2002). The P237S substitution was present in ~5% of the alleles in the Finnish and Swedish population samples studied. If P237S were to be a disease causing mutation one would expect the incidence of NPC1 disease to be much higher in the Finnish and Swedish populations. Moreover, overexpression of the NPC1 protein harboring the P237S substitution fully restored cholesterol trafficking at the cellular level. Although our results rule out P237S as a disease causing mutation it is still possible that the mutation has a more subtle phenotype not leading to NPC1 disease.

In the case of the C-terminal deletion the case is clear-cut, since it leads to an unstable protein which is rapidly degraded. The C113R substitution, however, results in a non-functional protein that is partially mistargeted. We found the C113R protein to localize mainly to the ER, Rab7-negative endosomes and the plasma membrane. Retarded export from the ER was confirmed by metabolic labeling of the newly synthesized protein with $^{35}$S-methionine followed by immunoprecipitation and endoglycosidaseH (endoH) digestion. The C113R protein showed a decrease in the EndoH resistant fraction indicating that a smaller fraction of NPC1-C113R reached the Golgi compared to the WT protein. What might be the mechanism for the decreased export of NPC1-C113R from the ER? The C113R substitution resides in a highly conserved region of the 240 amino acid (aa) luminal N-terminus containing 13 cysteine residues. Amino
acids 55 through 165 encompass the “NPC” domain, a 112 aa stretch with high conservation between NPC1 orthologues (Watari et al., 1999a). There are 8 conserved cysteine residues within this domain, and site directed mutagenesis of four of these cysteines resulted in an inactive protein that was localized to the limiting membrane of cholesterol-laden lysosomes in NPC cells (Watari et al., 1999a). Presumably, the cysteine at aa position 113 participates in disulfide bridge formation contributing to the tertiary structure of the N-terminal domain. This region was recently shown to mediate oligomerisation of the NPC1 protein as well as to bind to cholesterol and 25-hydroxycholesterol (Infante et al., 2008a). Interestingly the stochiometry of sterol binding indicates that one cholesterol or hydroxycholesterol molecule binds to one dimer of the NPC1 protein. Whether or not dimerization or oligomerization of the NPC1 protein is a requirement for sterol binding has not been assessed. However, it does not seem that sterols are required for the dimerization of the protein since an N-terminal mutant defective in sterol binding (NPC1-Q79A) showed the same pattern of oligomerisation as the WT protein (Infante et al., 2008a) (Linder and Ikonen, unpublished observations). Also the NPC1-C113R protein forms oligomers indicating the presumable changes in the n-terminal conformation are not sufficient to inhibit oligomerisation of the protein (Linder and Ikonen, unpublished observations).

Although the C113R mutation clearly causes a defect in maturation of the protein it is curious that the protein is also mistargeted after leaving the Golgi. The putative dileucine endosomal targeting motif resides in the C-terminal of the protein, and ablation of this motif causes the retention and degradation of the protein in the ER (Watari et al., 1999a). One explanation could be that the C113R substitution interferes with binding to accessory proteins required for the proper targeting of the protein. Alternatively, sterol binding at the N-terminal is required for the proper localization and function of the protein. As stated above, the Q79A mutation causes a defect in sterol binding, whereby 25-HC binding is abolished but residual cholesterol binding capacity is retained. As for the C113R substitution, the cholesterol binding capacity has not been assessed.
1.2 Intracellular localization and regulation of the NPC2 protein in NPC1 cells

Characterization of the NPC2 protein has been hampered by the lack of high quality commercial antibodies against NPC2. We generated polyclonal antibodies using recombinantly expressed NPC2 fused to GST (amino acids 20-151, full length NPC2 w/o signal sequence) as an epitope. The specificity of the antibody was confirmed by preincubation of the antibody with purified recombinant protein prior to IF staining. Also the specificity has been confirmed by Western blot using mouse NPC2 -/- samples (Mutka et al., 2004). In normal human fibroblasts we found the endogenous NPC2 to have a punctate staining pattern. Double immunofluorescence staining indicated that the majority of NPC2 was present in Lamp1-positive lysosomes. On the other hand, in some cells, NPC2 was predominantly colocalized with gamma-adaptin, a marker for the trans-Golgi network (TGN). Taken together, these results reconcile well with MPR regulated shuttling of the NPC2 protein between the Golgi and endo/lysosomes. In contrast, NPC1 cells showed an exclusively lysosomal localization of the NPC2 protein, indicating that there is a sequestration of the NPC2 protein in lysosomes. Consistent with this we saw a roughly 1.5-fold increase in NPC2 protein by Western blotting. This might be a result of decreased degradation of NPC2 or a compensatory upregulation of NPC2 gene transcription. Northern blot analysis showed that the NPC2 transcript was significantly upregulated indicating that the increase in NPC2 protein levels is due to a regulatory increase in NPC2 synthesis.

In the NPC1 fibroblasts the NPC2 protein was sequestered in the cholesterol-laden lysosomes and very little Golgi like staining could be seen. In accordance with this there was also an apparent redistribution of MPR to Lamp-1 positive organelles from the normally predominant TGN localization. Transport of MPRs from late endosomes to the trans-Golgi is mediated by Rab9 (Lombardi et al., 1993; Riederer et al., 1994) and the Rab9 effector TIP47 (Carroll et al., 2001; Diaz and Pfeffer, 1998). Interestingly Rab9 function is perturbed in NPC1 fibroblasts resulting in the accumulation of inactive Rab9 in late endosomes and lysosomal mis-sorting and degradation of MPR (Ganley and Pfeffer, 2006). It is therefore possible that NPC2 accumulates as a result of defective Rab9 mediated transport steps. Whether or not this would have relevance for the disease phenotype is unclear, since the putative site of NPC2 function is in the
endo/lysosomal system, where NPC2 was shown to accumulate. In light of the increased NPC2 mRNA levels in NPC1 fibroblasts it would seem more likely that NPC2 is upregulated by a compensatory mechanism as a result of the endosomal cholesterol accumulation. Alternatively, transcription of NPC2 might fail to be downregulated due to the relative sterol deficiency in the ER. It is therefore possible that the NPC proteins are transcriptionally regulated in concert to maintain cholesterol efflux from the endo/lysosomal compartments, but an increase in one is not sufficient to compensate for the loss of the other.

2. Complementation of Niemann-Pick type C disease by Rab overexpression (II)

Due to the diverse cellular pathology and the abundance of metabolites that accumulate in NPC disease, it seems safe to argue that NPC is to a large extent a disorder of perturbed intracellular membrane trafficking. Whatever the functions of the NPC proteins are and the nature of the primary substrates, there is a gross stagnation of endosomal motility in NPC disease (Ko et al., 2001; Zhang et al., 2001a). As of yet it has been difficult to pinpoint the exact nature of the cellular lesion. It seems likely that the NPC2 protein is a bona fide cholesterol transporter, arguing that in NPC2 disease cholesterol accumulation causes a malfunction in the endosomal compartments. The case for NPC1 is less clear-cut. NPC1 has been proposed to function as a transmembrane pump or transporter of yet unidentified substrates (Davies et al., 2000a), or as a cholesterol transporter in tandem with NPC2 (Infante et al., 2008b).

Inactivation of the NPC1 proteins leads to the stagnation of late endosomal tubulovesicular trafficking (Ko et al., 2001; Zhang et al., 2001a). This phenotype might be a result of impaired function in select Rab GTPases, possibly through altered biophysical properties of the membranes. Global inactivation of Rab GTPases with Rab-GDI causes retarded endosomal cholesterol clearance in cultured human skin fibroblasts loaded with LDL (Höltta-Vuori et al., 2000). Rab inhibition also disrupts complementation of NPC1 fibroblasts with overexpressed NPC1 protein, indicating that this process is dependent on Rab proteins (Höltta-Vuori et al., 2000). It was subsequently shown that overexpression of the late endosomal Rabs Rab7 and Rab9 was also able to complement the NPC1 phenotype. Of the recycling Rabs, only Rab4 has previously been shown to complement the NPC1 phenotype whereas Rab11 does not. We
therefore reasoned that overexpression of Rab proteins in NPC fibroblasts would be suitable for identifying novel pathways of cholesterol transport from the endosomal system.

2.1 Use of filipin staining as a measure of cholesterol efflux from endosomal compartments

The fluorescent antibiotic filipin has been used extensively to visualize free cholesterol in fixed cells. Previous complementation studies on NPC cells have used filipin staining as a measure of cholesterol clearance from cells. In the study we used two NPC1 cell lines and one control cell line. The GM3123 (I1061T/P237S) cell line from ATCC has been used extensively by other laboratories for complementation and was therefore chosen. The F92-116 NPC1 and F92-99 control cell line have been previously characterized in (I). It was immediately apparent by filipin staining that the cholesterol accumulation phenotype varies heavily between the GM3123 and F92-116 cell lines, with much more prominent perinuclear accumulation in the F92-116 line. The heterogeneity of filipin staining in the GM3123 line was also striking. This is noteworthy since previous complementation studies using single cell analysis have utilized this cell line.

We initially determined if filipin could be used as a quantitative measure of cholesterol clearance from endosomes in NPC cells. Since the stochiometry of filipin binding to cholesterol has not been determined we measured the correlation between filipin intensity and cellular free cholesterol amounts. We found that there is good correlation between cellular free cholesterol amounts and filipin intensity, establishing that a decrease in filipin intensity should be a good measure of complementation. Indeed overexpression of Rab4-GFP and Rab7-GFP, which have been previously shown to complement the NPC phenotype, as well as Rab8-GFP decreased whole cell filipin staining. Whole cell filipin intensity can only be used as a measure of loss of free cholesterol i.e. through efflux or esterification, not for redistribution of free cholesterol within the cell. Also, a decrease in whole cell filipin staining might also reflect cholesterol efflux from other compartments than endosomes, e.g. the plasma membrane. Whole cell filipin intensity is therefore not an optimal measure of mobilization of free cholesterol from the endosomal system. To more precisely measure mobilization of free cholesterol from the storage endo/lysosomes we stained the cells with anti-Lamp1 antibodies and used this as a marker for the storage organelles. After image acquisition, the area under the Lamp1 positive organelles was
determined by image analysis and scored for filipin intensity, after subtraction of the PM filipin fluorescence. By specifically scoring the endosomal cholesterol content the difference between control F92-99 and NPC GM3123 fibroblasts becomes more apparent. Using this setup we showed that overexpression of GFP-tagged Rab4, Rab7, and Rab8 specifically reduced the cholesterol content of endosomes in NPC cells.

2.2 Rab8 restores sphingolipid trafficking in NPC fibroblasts

Previous studies have established the restoration of glycosphingolipid transport in parallel with filipin clearance during Rab overexpression (Choudhury et al., 2002; Choudhury et al., 2004). To test whether Rab8 also restored glycosphingolipid trafficking in NPC cells we studied the distribution of the cholera toxin subunit B (CTxB). CTxB binds to GM1 ganglioside and is normally transported to the Golgi upon internalization but is retained in the endocytic compartment in NPC cells (Choudhury et al., 2002; Sugimoto et al., 2001). Fluorescently labeled CTxB localized to punctuate cytosolic structures in GM3123 fibroblasts but in Rab8-GFP overexpressing cells CTxB colocalized with the Golgi marker GM130, indicating that Rab8 restored proper glycosphingolipid trafficking in NPC1 cells.

2.3 Rab8 is resistant to membrane retention during cholesterol loading

Membrane cholesterol loading, as seen in NPC disease, disrupts Rab function by interfering with Rab membrane extraction by Rab-GDI (Choudhury et al., 2004; Ganley and Pfeffer, 2006; Lebrand et al., 2002). In NPC cells, and upon acLDL loading of mouse peritoneal macrophages, we observed an upregulation of the Rab8 protein. To investigate whether this increase in Rab8 was caused by sequestration of Rab8 in cholesterol enriched membranes, we incubated cellular membranes with Rab-GDI. Incubation with 2µM purified GDI solubilized roughly 70% of membrane bound Rab8 independent of cholesterol load. In contrast, Rab7 solubility was reduced in NPC cells in accordance with previous reports (Lebrand et al., 2002). Apparently the membranes that Rab8 resides in do not accumulate cholesterol in NPC cells, and therefore the membrane extractability remains unaltered. Interestingly, the overall membrane extractability of
Rab8 seemed to be greater for Rab8 compared to Rab7. Rab8 is only geranylgeranylated at a single site as opposed to the double prenylation of most other Rab proteins e.g. Rab7, which might confer resistance to membrane sequestration during cholesterol loading. Also, the affinity for Rab-GDI depends on the length of the hypervariable domain of the Rab protein. The high membrane extractability of Rab8 might therefore help Rab8 to stay functional during cholesterol enrichment of membranes.

2.4 Rab8 siRNA induces a cholesterol accumulation phenotype

So far only global inactivation of Rab proteins with Rab-GDI has been shown to induce a cholesterol storage phenotype, whereas RNAi of specific Rabs has been ineffective. Ganley and coworkers showed that specific knock-down of Rab9 did not induce endosomal cholesterol accumulation (Ganley and Pfeffer, 2006). It is therefore possible that overexpression of certain Rab proteins stimulates cholesterol transport in redundant or marginal pathways. To evaluate if Rab8 function is necessary for cholesterol trafficking in normal cells we knocked down the Rab8 protein using RNA interference. Transfection of primary human fibroblasts with Rab8 shRNA resulted in an increase in intracellular cholesterol, as assessed by filipin staining, that partly localized to Lamp1 positive organelles. The cholesterol accumulation was also accompanied by a cholesterol efflux defect to apoA-I. Also, Rab8 RNAi resulted in decreased cholesterol esterification, indicating that the mobilized cholesterol was not targeted to the ER.
3. Rab8 regulates cholesterol processing in primary human macrophages (III)

3.1 Rab8 is expressed in macrophages present in atherosclerotic lesions in vivo

To assess if Rab8 might be beneficial in foam cell reduction we opted to work on primary human macrophages. Initially, we conducted immunohistochemical staining of human coronary artery sections of atherosclerotic lesions. Using a polyclonal antibody against Rab8 we found Rab8 to be expressed in cells surrounding the necrotic core of the lesion. To confirm that these cells were macrophages we double stained the sections with anti-Rab8 and anti-CD68 antibodies. The Rab8 expressing cells showed clear co-localization with CD68 indicating that the cells were indeed macrophages. The results indicate that Rab8 might have a physiological role in the setting of macrophage foam cell formation, but whether this is directly related to cholesterol metabolism can not be determined from these experiments.

3.2 Adenoviral overexpression of Rab8 in vitro increases ABCA1 dependent cholesterol efflux to apoA-I

We used an adenoviral expression system to overexpress Rab8 in primary human macrophages. Infection of macrophages with Rab8 induced marked morphological changes, as has previously been shown in other cell types. The macrophages displayed multiple cell protrusions positive for Rab8. To assess if these cells also showed an increased capacity to withstand foam cell formation we incubated control (GFP) or Rab8-myc infected macrophages in the presence of acLDL and apoA-I. Cells infected with Rab8 accumulated less total cholesterol as compared to the control cells. Interestingly this decrease in total cholesterol could be accounted for by the total decrease in cholesteryl esters indicating that the cholesteryl ester pool is mainly affected. This could be due rapid recycling of cholesterol from the endosomes to the cell surface, thereby decreasing the cholesterol pool that is available for re-esterification in the ER. Alternatively, Rab8 might facilitate the mobilization of cholesterol from lipid droplets. Since the amount of free cholesterol remained constant during Rab8 overexpression it would seem that the mobilized cholesterol is readily effluxed from the cells, suggesting involvement of the ABCA1 transporter. In accordance with this we saw a striking, 2.5-fold increase in the amount of ABCA protein in the Rab8
overexpressing cells. This increase of ABCA1 was unlikely to be caused by an increase in ABCA1 transcription through e.g. LXR activation since we saw no significant upregulation of the ABCA1 mRNA in quantitative real-time PCR. It is therefore logical to assume that Rab8 induces the stabilization of ABCA1 on the cell surface, and thereby retards the degradation of the protein. Stabilization might occur through increased exposition of ABCA1 to extracellular apoA-I through enhanced trafficking of ABCA1 to the PM. It has previously been shown that degradation of ABCA1 is regulated by internalization of the protein, and that apoA-I decreases internalization by stabilizing ABCA1 at the cell surface (Wang et al., 2003). Internalization of ABCA1 is dependent on the C-terminal PEST sequence (Chen et al., 2005). Given the established effects of Rab8 on actin remodelling it is also feasible that ABCA1 stabilization is an actin-mediated effect. ABCA1 has been shown to interact with members of the syntrophin family of proteins (α1, β1 and β2) (Buechler et al., 2002; Munehira et al., 2004; Okuhira et al., 2005). Overexpression of α1- syntrophin increases the half-life of ABCA1 and facilitates cholesterol efflux (Munehira et al., 2004). Co-expression of β1-syntrophin and ABCA1 induces the formation of ABCA1 clusters at the PM, stabilizing the ABCA1 protein and increasing efflux capacity (Okuhira et al., 2005). The syntrophins link ABCA1 to the actin cytoskeleton by forming a complex with the scaffolding protein utrophin. Rab8 might therefore facilitate cholesterol efflux by providing the actin scaffold for stabilization of the ABCA1/syntrophin/utrophin complex.

3.3 Rab8 RNAi inhibits cholesterol efflux and regulates ABCA1 trafficking in primary human macrophages

To establish if Rab8 is essential for cholesterol transport in primary human macrophages we used RNA silencing to knock down the Rab8 protein. We previously showed that Rab8 siRNA in primary human macrophages causes retention of cholesterol within the cells as assessed by filipin staining (II). In the primary human macrophages we opted to biochemically assess the cholesterol amounts in Rab8 knock-down and control cells. Two days after electroporation of the siRNA the macrophages were loaded with 50 μg/ml acLDL. This resulted in a marked, 3-fold increase in total cellular cholesterol and an increase of the cholesteryl ester fraction from ~3.5% to ~45%.
After acLDL loading the cells were incubated with 10 µg/ml apoA-I for 18h. In control cells, the total cellular cholesterol levels decreased by 40% during the incubation period, whereas the Rab8 depleted cells failed to efflux significant amounts of cholesterol. To confirm that the substrate pool for efflux was derived from the endocytosed lipoprotein particles we labeled acLDL with [³H]cholesteryl oleate. Scintillation counting of the cells after loading confirmed equal uptake of the label. However after incubation with apoA-I, the Rab8 depleted cells failed to efflux the [³H]cholesterol, in accordance with the previous results.

After endocytosis the cholesteryl esters of the LDL particle are hydrolysed by lysosomal acid lipase (LAL), and it is conceivable that Rab8 siRNA interferes with this step and therefore inhibits cholesterol mobilization from the cells. This is, however, an unlikely scenario since Rab8 shRNA causes cholesterol accumulation in primary human fibroblasts as assessed by filipin staining, and filipin only stains free cholesterol. There are a number of possibilities how Rab8 siRNA might interfere with cholesterol efflux. Firstly, Rab8 depletion might inhibit the trafficking of recycling endosomes to the cell surface. In accordance, filipin staining of primary human macrophages showed cholesterol accumulation in both Lamp1-positive and Lamp1-negative organelles. This accumulation is separate from that seen in NPC1 disease, which is primarily lysosomal in nature, indicating that Rab8 regulates a down-stream event in cholesterol trafficking. As discussed above, one possibility is that Rab8 controls the stabilization of ABCA1 on the PM. To assess the effect of Rab8 on the plasma membrane localization of ABCA1 we used RNA silencing to knock down Rab8, followed by surface biotinylation of ABCA1. In Rab8 siRNA treated cells there was a significant, 30% decrease in the fraction of ABCA1 on the plasma membrane, indicating that Rab8 regulates the transport of ABCA1 to the PM or stabilization of the protein on the PM.

It is possible that Rab8 controls the trafficking or recycling of ABCA1 to the cell surface. Due to a lack of good antibodies the precise cellular localization of ABCA1 has been hard to pinpoint, although it is clear through surface biotinylation experiments that a large portion of the protein resides at the plasma membrane. By overexpressing GFP-tagged ABCA1 it has also been suggested that ABCA1 resides in endosomal compartments. It seems that a part of this pool of ABCA1 is destined for lysosomal degradation, whereas a part is recycled back to the PM.
RESULTS AND DISCUSSION

(Neufeld et al., 2001). Yet, the specific recycling route has not been identified. Since Rab8 affects the cell surface distribution of ABCA1 we studied whether Rab8 siRNA would also have an effect on the intracellular distribution of ABCA1-GFP. Previous studies on ABCA1-GFP have mostly been carried out in HeLa cells. In a study by Neufeld et al, ABCA-GFP was localized to the plasma membrane and Lamp2-positive vesicles, but not to TfR-positive vesicles (Neufeld et al., 2001). We also found ABCA1-GFP to localize to the PM in HeLa cells, mainly to cellular lamellipodia like protrusions and to intracellular tubules, both colocalizing with β1 integrin. Rab8 siRNA caused a decrease in cellular protrusions in the ABCA1 expressing cells and retention of ABCA1 and β1 integrin in intracellular tubular compartments, indicating that the trafficking of β1 integrin and ABCA1-GFP are interconnected. In accordance with previous results (Neufeld et al., 2001) we did not find ABCA1 to colocalize with TfR. However, in Rab8 siRNA treated cells ABCA1 accumulated perinuclearly, in a partially TfR positive structure. Taken together, the results indicate that Rab8 also regulates the intracellular trafficking of ABCA1.
CONCLUSIONS AND FUTURE PROSPECTS

In this study (I) we have identified a mutation in the NPC1 protein that disrupts the intracellular trafficking of the protein, leading to a severe infantile form of NPC disease. The study demonstrates the importance of the correct localization of NPC1 within the cell. Also, the regulation of NPC2 gene transcription seems to be dependent on proper NPC1 function indicating that these proteins might be functionally linked. Of the various domains in the NPC1 protein, the SSD is of outstanding interest. There is high sequence homology within this domain between SCAP, HMGR, NPC1L1 and NPC1 (Kuwabara and Labouesse, 2002). Therefore, a crucial challenge will be to establish whether the SSD performs a similar sterol-sensing function in NPC1 as has been described for SCAP and HMGR, and if so, to establish the sterols that regulate NPC1 function.

The lessons learned from NPC1 might also have impact on our understanding of the regulation of other SSD containing proteins, e.g. NPC1L1. Conversely, mutational and functional analysis of the SSD within NPC1L1 might shed light on the function of this domain within NPC1. It has already been established that sterols regulate the intracellular trafficking of NPC1L1. Upon sterol depletion NPC1L1 translocates from the endocytic recycling compartment to the plasma membrane where it facilitates cholesterol uptake. It would be interesting to study whether the trafficking of NPC1L1 is dependent on an intact SSD. A recent study also showed that cholesterol induces the internalization of NPC1L1 in intestinal cells, and that the cholesterol lowering drug ezetimibe blocks this internalization (Ge et al., 2008). It would therefore be of interest to determine if ezetimibe added to cells in vitro could block NPC1 mediated tubulovesicular trafficking. If so, this could suggest that the function of NPC1 could be the generation of vesicle budding sites as proposed by Yiannis Ioannou (Ioannou, 2005). In this model NPC1 would function by flipping membrane lipids such as fatty acids or sphingosine, thereby facilitating an outward bending membrane curvature. However, any theory on the function of NPC1 should also take in to account the cholesterol binding properties of the protein. In analogy to NPC1L1 internalization, cholesterol might also serve as an activating trigger for NPC1 function. A regulatory role for cholesterol binding could explain why an NPC1 N-terminal mutant with lack of 25-hydroxycholesterol binding and severely attenuated cholesterol
binding had no apparent cellular phenotype (Infante et al., 2008a). It is feasible that the residual cholesterol binding capacity is sufficient to serve a regulatory function. The bulk cholesterol transport would then be mediated by NPC2 to specific membrane domains created by NPC1. NPC2 is capable of transferring cholesterol from the N-terminal domain of NPC1 to acceptor membranes (Infante et al., 2008b). It is also possible that NPC2 could work in the opposite way, transferring cholesterol from inner membranes to NPC1 at the limiting membrane. In this model cholesterol transfer from inner membranes could be facilitated by LBPA/BMP, which has been shown to increase the sterol transfer capacity of NPC2 when present in the donor membrane (Cheruku et al., 2006).

To date, most studies aimed at elucidating the function of NPC1 have focused on transient transfection to revert the accumulation phenotype. An alternative approach could be the acute knock-down of NPC1 or NPC2, to assess which metabolite(s) accumulate first. This might help to resolve the long standing hen-and-egg issue that has puzzled NPC researchers, i.e. which comes first, cholesterol or sphingolipid accumulation. However, such a study should also take into account the sterol status of the cell, as well as the inherent difference in the sensitivities between assays for cholesterol and sphingolipid accumulation.

Our studies (II, III) are the first to identify a specific Rab-GTPase localized outside the endosomal system that affects the recycling of cholesterol from the endosomes to the PM. We have also shown that Rab8 is involved in the cellular trafficking of ABCA1. It is clear that the processes governing ABCA1-mediated cholesterol efflux to apoA-I are highly complex, involving intracellular signaling cascades and cytoskeleton reorganization. Given the role of Rab8 in inducing cell polarity and actin as well as microtubule reorganization, a likely scenario is that Rab8 promotes cholesterol efflux by creating platforms for cholesterol efflux at the leading edge of the cell. Although the contribution of endocytosed ABCA1/apoA-1 to cellular cholesterol efflux remains to be resolved, it is quite clear that ABCA1 is regulated post-transcriptionally through endocytosis and recycling of the protein. According to our results Rab8 has a role in this process, but the precise mechanism of Rab8 function remains to be established. We have shown that knock-down of Rab8 causes the retention of ABCA1 within the cell in β1 integrin and TfR receptor positive structures. It remains to be established whether ABCA1 containing vesicles
utilize Rab8 directly, e.g. for recruitment of myosin motors, or if the effect is secondary e.g. through altered actin or microtubule organization.

Our results also suggest that Rab8 recycles cholesterol from the endosomal system back to the plasma membrane. The next challenge will therefore be to identify the precise recycling pathway(s) regulated by Rab8 other Rab proteins involved in this process. The strategy adopted in this study i.e. transfection of select Rab proteins and single cell assessment of filipin intensity is quite labor intensive. In order to identify the Rab proteins involved in intracellular cholesterol trafficking there is therefore a need for high throughput approaches, for instance, utilization of siRNA libraries with automated analysis. Alternatively, a Rab-GAP library such as the one used to identify Rab-GTPases involved in primary cilia formation could be used (Yoshimura et al., 2007). The challenge of these approaches is, however, to find a clear read-out for cholesterol trafficking suitable for automated analysis, preferably in living cells. The filipin staining process is not suitable for high throughput applications. To this end one might utilize fluorescent cholesterol analogs to measure changes in uptake, intracellular distribution and efflux. One candidate probe would be BODIPY-cholesterol which has been shown to behave similarly to cholesterol in normal and storage-disease cells (Höltta-Vuori et al., 2008).

Finally, it would be interesting to carry out in vivo studies in mice. The Rab8-/- mice described by Sato and colleagues could be used for such studies, for instance to assess the serum lipoprotein particle composition during low and high cholesterol diet (Sato et al., 2007). Alternatively, generation of transgenic mice between Rab8-/- and LDLr-/- (or apoE-/-) mice might be used to assess the effect of Rab8 deficiency on atherosclerotic plaque development. Also, bone marrow transplants from Rab8 KO mice to LDLr -/- mice might be used to directly assess the contribution of Rab8 in foam cell development.

Macrophage RCT measurements in vivo could also be carried out by adenoviral infection of macrophages with Rab8, and metabolic labeling with [3H]-cholesterol in vitro, followed by intraperitoneal injection of infected macrophages. RCT could then be measured by the appearance of the [3H]-tracer in the plasma, liver and feces (Tanigawa et al., 2007). Together
these approaches should determine the overall contribution of Rab8 in macrophage RCT and atherosclerotic lesion development.
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