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Cholesterol in the endocytic pathway

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ACADEMIC DISSERTATION

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To see a world in a grain of sand

(William Blake, Auguries of Innocence)

Contents

ORIGINAL PUBLICATIONS

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

I Hölttä-Vuori M., Määttä J., Ullrich O., Kaismaneen E. and Ikonen, E. (2000). Mobilization of late-endosomal cholesterol is inhibited by Rab guanine nucleotide dissociation inhibitor. Current Biology 10(2), 95-98.

II Hölttä-Vuori M., Panhaanpää K., Möbius W., Somerharju P. and Ikonen, E. (2002). Modulation of cellular cholesterol transport and homeostasis by Rab11. Molecular Biology of the Cell 13(9), 3107-3122.

III Hölttä-Vuori M., Alpy F., Panhaanpää K., Jokitalo E., Mlatka A-L. and Ikonen, E. (2005). MLN64 is involved in actin-mediated dynamics of late endocytic organelles. Molecular Biology of the Cell, published online June 1, 2005, 10.1091/mbc.E04-12-1105.

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ABBREVIATIONS

ACA	aspartylglucoaminidase
ABCA1	adenosine triphosphate-binding cassette transporter A1
ACAT	acyl-coenzyme A: cholesterol acyltransferase
CTxB	cholera toxin subunit B
DHEC	dehydroergosterol
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine-perchlorate
EEA1	early endosomal autoantigen 1
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERC	endocytic recycling compartment
GDP	guanosine diphosphate
GDI	guanine-nucleotide dissociation inhibitor
GFP	green fluorescent protein
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HDL	high density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
LAMP	lysosome-associated membrane protein
LBPA	lysobisphosphatidic acid
LDL	low density lipoprotein
MLN64	metastatic lymph node 64
NPC	Niemann-Pick type C
PCTP	phosphatidylcholine transfer protein
PEG	polyethylene glycol
PI(3)P	phosphatidylinositol-3-phosphate
$Pyr_{10}SM$	pyrenyldecanoylsphingomyelin
RNAi	RNA interference
SCAP	sterol regulatory element binding protein cleavage activating protein
SR-BI	scavenger receptor B-I
SREBP	sterol regulatory element binding protein
StAR	steroidogenic acute regulatory protein

StARTD domain	steroidogenic acute regulatory protein related lipid transfer domain
TfnR	transferrin receptor
TGN	trans-Golgi network

ABSTRACT

The endocytic pathway delivers extracellular and plasma membrane constituents into the cell. The endocytic pathway consists of different endosomal compartments with distinct compositions and functions. The participation of this route in the regulation of cellular cholesterol homeostasis has been first described in the studies on the cellular delivery and hydrolysis of low density lipoproteins (LDLs) (Brown and Goldstein, 1986). The transport mechanisms used by cholesterol henceforth, as well as the participation of other endocytic processes in cholesterol transport still remain largely unknown. The aim of the present study was to characterize the role of the endocytic pathway in the regulation of intracellular cholesterol transport and homeostasis. Defects in cholesterol metabolism are associated with a variety of human diseases (Vance, 2002a). Elucidating the cellular mechanisms by which cholesterol is distributed should help to understand how these diseases develop and, in the long run, provide new strategies for their treatment.

In the first study we examined the role of vesicular trafficking machinery in the export of late endosomal cholesterol. We found that the microinjection of Rab-guanine-nucleotide dissociation inhibitor (GDI) effectively inhibited cholesterol mobilization indicating that Rab proteins are involved in the process. We also noted that upon overexpression of Niemann Pick C (NPC) 1 protein in NPC1 defective fibroblasts, select lipid and protein constituents were redistributed from late endosomes upon unloading of cholesterol deposits. This observation further links the cholesterol trafficking to the transport of other late endosomal cargo.

To analyze the contribution of specific Rab-regulated trafficking pathways in cellular cholesterol transport we examined the effects of selected endosomal Rab proteins on cholesterol distribution and homeostasis. We found that the overexpression of recycling endosomal Rab11 resulted in prominent accumulation of free cholesterol in Rab11 positive organelles. Rab11 overexpression did not perturb the transport of fluorescently labelled LDL to late endosomes or the NPC1-induced late endosomal cholesterol clearance in NPC patient cells. However, Rab11 overexpression inhibited cellular cholesterol esterification in an LDL-independent manner. This effect could be

overcome by introducing cholesterol to the plasma membrane from a cyclodextrin carrier. These results suggest that in Rab11 overexpressing cells, deposition of cholesterol in recycling endosomes results in its impaired esterification, presumably due to the defective recycling of cholesterol to the plasma membrane. The findings point to the importance of the recycling endosomes in regulating cholesterol and sphingolipid trafficking and cellular cholesterol homeostasis.

Thirdly, we analyzed the role of a late endosomal cholesterol binding protein MLN64 in late endocytic cholesterol transport. We observed that the depletion of MLN64 did not impair cholesterol homeostasis as assessed by cholesterol efflux or esterification, or lead to late endosomal cholesterol accumulation. In contrast, MLN64 overexpression increased sterol deposition in the organelles harbouring the protein. The most obvious phenotype observed upon MLN64 depletion was the dispersal of late endocytic organelles to the cell periphery, indicating that MLN64 participates in late endosome dynamics. We found that upon MLN64 depletion, the association of late endocytic organelles with actin was reduced. In contrast, overexpression of MLN64 increased the association of late endosomes with actin as well as p34-Arc, a component of actin nucleation machinery. We propose that MLN64-mediated local sterol enrichment of late endosomal membranes facilitates the association of late endosomes with the actin cytoskeleton, thereby linking the sterol organization and actin-mediated dynamics of late endocytic organelles in mammalian cells.

REVIEW OF THE LITERATURE

1. Cholesterol in cells

Cholesterol, despite its dubious reputation is an essential component of mammalian cells. In addition to being a membrane building block, cholesterol serves as a precursor for steroid hormones, bile acids and oxysterols. Moreover, cholesterol has a signalling function either by direct modification of signalling molecules as in the case of Hedgehog, a morphogen that covalently binds cholesterol (Incardona and Eaton, 2000); or by participation in the formation of membrane signalling platforms termed rafts (Simons and Ikonen, 1997). What makes cholesterol so special derives from its unusual physical properties. As a molecule it is small and, due to the tetracyclic fused ring skeleton, very rigid. In model membranes sterols have been shown to cause the lipid acyl chains to come closely packed, thus forming a s.c. "liquid-ordered" state of membrane bilayer (Ohvo-Rekila *et al.*, 2002). In comparison to liquid-disordered phase obtained by mere phospholipid bilayers, this would increase the mechanical strength and decrease the permeability of the membrane and yet allow relatively high rates of lateral and rotational diffusion. Those eukaryotes that do not depend on cholesterol utilize other sterols for similar functions: plants synthesize a variety of phytosterols (Schaller, 2004) and yeasts and fungi have ergosterol (Volkman, 2003). Prokaryotes are thought to be mainly devoid of sterols but data exist suggesting that for example, certain methanotrophic bacteria synthesize sterols (Volkman, 2003). However, when present in excess, cholesterol contributes to development of a variety of cellular and whole-body pathologies (Tabas, 2002a). This diversity has earned a reputation for cholesterol as a "Janus-faced molecule".

1.1 Cholesterol in cellular membranes

The amount of cholesterol varies between different organelles in mammalian cells. The current consensus is that the majority of membrane cholesterol resides at the plasma membrane, but the estimates of the precise amount vary from 40% to 90%, depending on the method and cell type used (Liscum and Munn, 1999). In the Golgi apparatus, the amount of cholesterol has been reported to increase in *cis-trans* direction (Coxey *et al.*, 1993). The endocytic

pathway harbours cholesterol in variable amounts: ample cholesterol has been found in early and recycling endosomes and in the internal membranes of multivesicular bodies (Cagesca *et al.*, 2000; Hao *et al.*, 2002; Möbius *et al.*, 2003). Lysosomes and late endosomes do not normally store cholesterol (Möbius *et al.*, 2003). Low concentration of cholesterol is found in the endoplasmic reticulum (ER) with a given estimate of less than 1% of total cellular cholesterol in resting human fibroblasts (Lange *et al.*, 1999). Inner mitochondrial membranes do not incorporate cholesterol except in steroidogenic cells in which cholesterol is imported to mitochondria for steroid production (Jefcoate, 2002).

In addition to the differences in cholesterol distribution between individual organelles, cholesterol is thought to be unevenly distributed within membranes. Lipids have different affinities towards each other; cholesterol has been shown to have high affinity in particular towards sphingomyelin (Ohvo-Rekila *et al.*, 2002). In membranes, sphingolipids associate with each other, presumably e.g. through weak interactions between the sugar moieties of glycosphingolipids, and cholesterol molecules fill the voids between lipid hydrocarbon chains. These lateral assemblies are termed rafts (Simons and Ikonen, 1997). Rafts are thought to provide a platform for various cellular functions such as cell adhesion, signalling, endocytosis and sorting. While the exact properties of rafts still remain to be elucidated, the present idea is that they are actually very small and dynamic, clustering and dissociating upon different cues (Schuck and Simons, 2004).

However, determining the precise distribution of cholesterol among cellular membranes has not been trivial. First, the physiological status of cholesterol metabolism in cells is variable. The amount of cholesterol in a given organelle may vary several folds depending on the metabolic state such as availability of lipoproteins. Second, genuine differences between different cell types exist. As an example the apical membrane of polarized cells differs from the composition of the plasma membrane of non-polarized cells (Schuck and Simons, 2004). Finally, in addition to varying metabolic aspects, part of the controversy arises from technical difficulties. Membranes from given cellular compartments are difficult to purify, and estimates of the amount of cholesterol are easily biased upon contamination (Liscum and Munn, 1999). In past years, new probes have been developed for morphological detection of cholesterol (Ikonen and Hölttä-Vuori, 2004). With these probes new information concerning intracellular cholesterol distribution can be obtained.

Still, it must be noted that studies utilizing different probes have yielded different results. Probably the most reliable information can be obtained by combining several methods, both morphological and biochemical, when possible.

1.2 Cellular cholesterol homeostasis

The cellular cholesterol balance is very tightly regulated. Cells can obtain cholesterol either by *de novo* synthesis or from exogenous sources, usually in the form of plasma-derived lipoproteins. Which one is preferred, depends on the cell type and the availability of exogenous cholesterol. Most cell types are able to synthesize cholesterol themselves. At the whole body level, for instance central nervous system relies practically solely on *de novo* synthesis for cholesterol supply (Dietschy and Turley, 2001). On the other hand, some cell types have specialized in the uptake of lipoproteins, for example hepatic cells and macrophages (Simons and Ikonen, 2000). Too much free cholesterol is toxic to the cells (Tabas, 2002b). When cholesterol is present in excess, it is esterified and stored in cytoplasmic lipid droplets or effluxed out of the cell to extracellular acceptors such as lipoproteins. Cholesterol cannot be broken down in the cells and only the liver is able to remove cholesterol from the metabolic circuit, by converting it to bile acids. Thus, continuous overloading of cells with cholesterol may result in exceeding their capacity to handle the flow. One very common and very dangerous example of such a failure is atherosclerosis (Tabas, 2002b).

1.2.1 Cholesterol biosynthesis and its regulation

Cholesterol is synthesized from acetate in a complex cascade of enzymatic steps. The site of cholesterol biosynthesis is ER from where the newly synthesized cholesterol is relatively quickly (half-time less than 30 min) transported to the plasma membrane (Heino *et al.*, 2000). Many of the biosynthetic enzymes also localize in the ER, albeit localization to other cellular compartments such as lipid droplets has been reported (Ohashi *et al.*, 2003). Cholesterol biosynthesis is vital during development, as mutations in enzymes regulating cholesterol biosynthesis lead to serious malformations (Waterham, 2002). While biosynthetic precursors apparently cannot fully compensate for the lack of cholesterol in mammalian cells, they may have a

specific function of their own. For instance desmosterol has been found in abundance in mouse embryonic astrocytes, pointing to the possibility that there might be differences in sterol requirement in different cell types (Mietka *et al.*, 2004).

ER is also the site of regulation of cholesterol biosynthesis. Cholesterol regulates its own synthesis through sterol regulatory element-binding proteins (SREBPs). SREBPs are transcription factors that regulate the transcription of several cholesterol biosynthetic enzymes (Brown and Goldstein, 1999). When sterol levels in the ER are low, SREBPs are cleaved by SREBP cleavage activating protein (SCAP) in the Golgi and functional transcription factor is released in the nucleus. SREBPs bind to sterol regulatory element in the target gene promoter. Sterol regulatory element has been found for instance in the promoters of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and HMG-CoA reductase that participate in cholesterol biosynthesis. In addition, SREBPs enhance the transcription of the LDL receptor (Brown and Goldstein, 1999), leading to a coordinated increase in cellular cholesterol levels. Conversely, when ER sterol levels are high the complex is retained in the ER and no cleavage occurs. SCAP harbours a conserved sterol-sensing domain and is the target of sterol regulation (Nohturfft *et al.*, 1999).

1.2.2 LDL-internalization pathway

Many cell types acquire cholesterol by uptake of lipoproteins. The pathway utilized by LDL has been well characterized. LDL particles are delivered to the cells by receptor-mediated endocytosis (Brown and Goldstein, 1986). First, LDL binds to the LDL-receptor, is internalized and delivered to the endosomal system. In early endosomes the particle dissociates from the receptor which can be recycled back to cell surface or alternatively, degraded to prevent further influx. The apoprotein and – according to the traditional view - also lipoprotein-derived cholesteryl esters are hydrolyzed in the acidic lysosomes. Cholesteryl ester hydrolysis to free cholesterol and fatty acid is accomplished by acid lipase, a deficiency of which results in the accumulation of cholesteryl esters in late endocytic organelles (Anderson *et al.*, 1994). This enzyme has its activity optimum in an acidic pH (Wakano *et al.*, 1974). However, hydrolysis of lipoprotein-derived cholesteryl esters probably begins before lysosomes as 1) localization of acid lipase has been shown not to be restricted to lysosomes (Sagi *et al.*, 2003) and 2) despite of the fact that

enzyme activity requires acidic pH, de-acidification of lysosomes has been shown not to impair cholesteryl ester hydrolysis (Liscum, 1990).

LDL-receptor mediated pathway is not the only means of importing lipoproteins in the cells. Uptake of LDL can occur for instance via scavenger receptors that are ample in macrophages (Brigatti *et al.*, 2000). Other endocytic processes (pinocytosis, phagocytosis) can contribute to the uptake as well, especially in the case of modified forms of LDL, i.e. aggregated or acetylated (Kruth, 2002). At least in macrophages, macropinocytosis has been shown to operate in the uptake of native LDL too, in the process leading to foam cell formation (Kruth *et al.*, 2005).

1.2.3 Cholesterol esterification

Cholesterol esterification functions in storing cholesterol in cytosolic lipid droplets, supplying lipoproteins with cholesteryl esters, macrophage foam cell formation and absorption of dietary cholesterol (Chang *et al.*, 2001). By large, cholesterol esterification is thought to provide means to detoxify excess free cholesterol that is harmful to cells (Tabas, 2002b). Excess cholesterol is esterified in the ER by the enzyme acyl-coenzyme A: cholesterol acyltransferase (ACAT). Most of the cholesterol delivered for esterification is thought to traverse via the plasma membrane (Lange *et al.*, 1993; Neufeld *et al.*, 1996) but evidence for plasma membrane independent route(s) for delivery of LDL-derived cholesterol to the ER exists (Neufeld *et al.*, 1996; Underwood *et al.*, 1998) (Figure 1). Of cholesterol derived from late endosomes or lysosomes, up to 30% has been reported to reach the ER via plasma membrane-independent pathway (Neufeld *et al.*, 1996). In mammals, two ACAT genes have been cloned (ACAT1 and ACAT2) (Chang *et al.*, 1993; Cases *et al.*, 1998). In humans, ACAT1 has been found in most cell types and tissues studied (Chang *et al.*, 1993). In contrast, ACAT2 expression is mainly observed in the liver and intestine and it is thought to be the major cholesterol esterifying enzyme in the liver (Cases *et al.*, 1998; Parini *et al.*, 2004).

Substrates for ACAT enzymes are sterols and fatty acyl coenzyme A. ACAT enzymes are able to esterify other (hydroxyl)sterols in addition to cholesterol but cholesterol is a superior allosteric activator, at least for ACAT1 (Chang *et al.*, 2003). In atherosclerotic lesions the ability of ACAT to detoxify free cholesterol is saturated. Defective delivery of cholesterol to the ER has

been shown to confer resistance to apoptosis in atherosclerotic lesion macrophages (Feng *et al.*, 2003).

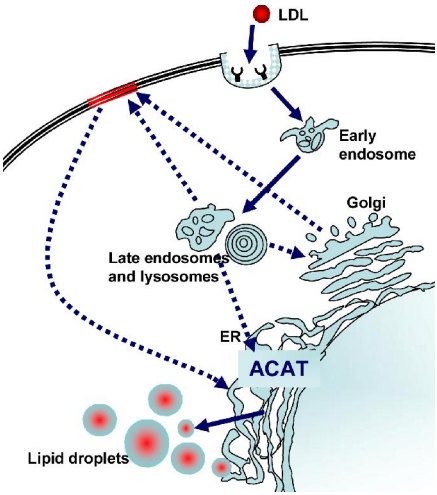

Figure 1. Schematic illustration of pathways for cholesterol esterification. Red colour represents cholesterol; the dashed arrows describe pathways that may not be direct or are not yet conclusively characterized.

1.2.4. Other cholesterol modifications

Cholesterol backbone can serve as a precursor for various biologically active molecules. Examples of such are bile acids, oxysterols and steroid hormones. Bile acids, synthesized by hepatic cells, are detergent-like molecules that facilitate both the secretion of cholesterol out of the body as well as absorption of dietary cholesterol in the intestine (Redinger, 2003; Russell, 2003). The conversion of hydrophobic cholesterol to water-soluble bile acids involves multiple enzymatic steps and can proceed via two alternative pathways. The "classical" route begins with the conversion of cholesterol to 7-hydroxycholesterol by the ER-localized enzyme cholesterol 7-hydroxylase, which is only expressed in the liver (Russell, 2003). The "alternative route" begins with the conversion of cholesterol to 27-hydroxycholesterol by the mitochondrial enzyme sterol 27-hydroxylase. This enzyme is not restricted to hepatic tissues; in macrophages, it is thought to participate in the oxidative elimination of cholesterol and, consequently, have an atheroprotective function (Bjorkhem *et al.*, 1994; Bjorkhem, 2002).

Oxysterols are oxygenated derivatives of cholesterol with a short half-life. In mammals, they are present in very low amounts as compared to cholesterol, but they are thought to be important mediators of cholesterol homeostasis (Bjorkhem, 2002; Olkkonen and Lehto, 2004). Oxysterol production has been suggested to provide a means for the elimination of excess cholesterol, for instance from macrophages and brain (Bjorkhem *et al.*, 1994; Bjorkhem *et al.*, 1997). In addition, oxysterols have been identified as regulators of several genes participating in cholesterol homeostasis, through binding to nuclear liver X receptor (Millatt *et al.*, 2003).

In steroidogenic tissues, cholesterol serves as a precursor for steroid hormone production. The rate-limiting step for steroidogenesis is the delivery of cholesterol to the P450 side-chain cleavage enzyme that converts cholesterol to pregnenolone (Jefcoate, 2002). This enzyme is localized in the mitochondrial inner membrane which is normally cholesterol poor. The key protein facilitating cholesterol transport to mitochondria is steroidogenic acute regulatory protein (StAR) (Soccio and Breslow, 2003; Strauss *et al.*, 2003). This family of lipid-binding proteins is discussed in more detail in Chapter 3.1.

1.2.5 Cholesterol efflux

Cells can lose cholesterol to extracellular acceptors such as lipoproteins. Some tissues, like liver and intestine, synthesize and secrete lipoproteins themselves and incorporate cholesterol in them in an esterified form (Simons and Ikonen, 2000). Cholesterol can access the extracellular acceptors through passive aqueous diffusion from the membrane (Wall *et al.*, 2002). In addition, cholesterol efflux has been shown to be facilitated by specific proteins, such as scavenger receptor B-I (SR-BI) or adenosine triphosphate -binding cassette transporter A1 (ABCA1). SR-BI is a receptor for high density lipoperoteins (HDL) and bidirectional in its function: it participates in both the influx of HDL-derived cholesterol to the cells as well as efflux of cellular cholesterol to lipoprotein acceptors (Connelly and Williams, 2004). ABCA1 was identified as a gene mutated in Tangier's disease. In Tangier's disease, HDL particles do not get properly lipidated and are catabolized as a consequence (Orso, 2002). Both SR-BI and ABCA1 have been proposed to have atheroprotective function in humans (Connelly and Williams, 2004).

2. Endocytosis

The endocytic pathway is a route for delivering of extracellular or plasma membrane constituents into a cell and distributing them to different destinations. Morphologically the endocytic organelles exist in the forms of membrane vacuoles, cisternae, tubules and multilamellar or multivesicular bodies (Miaczynska and Zerial, 2002) (see Figure 2). Cargo in the endocytic pathway can be sorted in different stations for either recycling or degradation. The recycling and degradative pathways are separated both spatially and functionally (Gruenberg, 2001). Endocytic trafficking that was originally thought to be a relatively simple "eating-and-drinking pathway" in the cells has emerged as a complex organization participating in various events of cellular life, such as signal transduction and maintenance of cellular homeostasis.

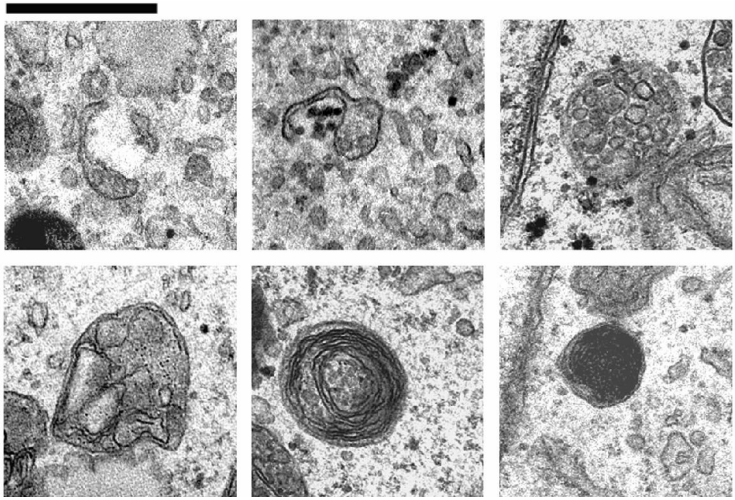

Figure 2. Different types of endosomes in HeLa cells. From top left: recycling and early endosomes, multivesicular body. From down left: multivesicular late endosome, multilamellar lysosome, dense core lysosome. Scale bar 500 nm. The photo is courtesy of Dr. Eija Jokitalo, Institute of Biotechnology, Helsinki.

2.1 Endocytic pathways

2.1.1 Entry to the cells

Endocytosis begins with the internalization of molecules from the cell surface. For this process several mechanisms exist (Conner and Schmid, 2003; Nichols, 2003; Maxfield and McGraw, 2004). Broadly, endocytosis can be divided into two categories: phagocytosis ("cell eating") and pinocytosis ("cell drinking"). Phagocytosis is mainly conducted by cells specialized in clearing large pathogens or debris, such as macrophages, monocytes or neutrophils (Conner and Schmid, 2003). Pinocytosis occurs by at least four different mechanisms. Macropinocytosis involves membrane ruffles that "collapse" onto the plasma membrane and fuse with it to create large endocytic vesicles named macropinosomes (Conner and Schmid, 2003). This mechanism is non-selective and allows a cell to sample large volumes of extracellular material. Other pinocytotic mechanisms include clathrin-mediated endocytosis, caveolar endocytosis and clathrin- and caveolae-independent endocytosis (Conner and Schmid, 2003). The best described of these is the internalization of receptors and their ligands via clathrin-coated pits. In clathrin-mediated endocytosis, receptors and their ligands concentrate into "coated pits" that cover approximately 0.5-2% of the cell surface (Mousavi *et al.*, 2004). The main coat component is clathrin, which under non-physiological conditions is capable of self-assembly into polygonal cages (Conner and Schmid, 2003). In physiological conditions, the cage assembly requires the aid of the adaptor proteins. Clathrin-mediated endocytosis occurs constitutively in mammalian cells and it functions in the delivery of essential nutrients into the cell, intracellular communication and maintenance of cell and serum homeostasis (Conner and Schmid, 2003).

Caveolar uptake is an example of clathrin-independent internalization pathway. Caveolae are flask-shaped invaginations of the plasma membrane that are highly enriched in raft lipids such as cholesterol and sphingolipids. The major structural protein of caveolae is caveolin that binds cholesterol (Ikonen *et al.*, 2004). Caveolae are rather immobile on the cell surface, which has led to the conclusion that caveolae cannot be involved in constitutive endocytosis like clathrin-coated pits (Thomsen *et al.*, 2002). In contrast, caveolar endocytosis is thought to be an inducible process triggered by complex signalling (Pelkmans and Helenius, 2002). Internalization of several

molecules has been reported to occur via caveolae, such as cholera toxin, serum albumin and simian virus 40. Some cell types are devoid of caveolin and in them the internalization of these molecules occurs via non-caveolar mechanisms. Moreover, caveolin-1-knockout mice were viable, suggesting possible redundancy of caveolar endocytosis with other internalization pathways (Razani *et al.*, 2001).

In addition, recent data suggest that internalization mechanisms independent of both clathrin and caveolin exist, but less is known of them. Overexpression of dominant negative mutant of dynamin III guanosine triphosphatase (GTPase) inhibits both clathrin-mediated and caveolar endocytosis, but not the delivery to the endosomes containing the small GTPase Arf6 (Naslavsky *et al.*, 2003). This pathway has been shown to deliver cargo to early endosomes (Nichols, 2003). In cells devoid of caveolae, the internalization of simian virus 40 takes place via a clathrin, dynamin III and Arf6 –independent but cholesterol-dependent mechanism (Damm *et al.*, 2005). Thus, it is likely that clathrin- and caveolin-independent endocytosis is achieved via more than one mechanism.

2.1.2 Endocytic compartments

After internalization, the endocytosed cargo is delivered to further destinations. Different internalization mechanisms can lead to different end stations, but the pathways can also intersect later in the cell. Endosomes can be classified for instance based on their functional properties, such as the kinetics with which the compartments are loaded with endocytosed material (Kleijmeer *et al.*, 1997). In a given pathway, distinct compartments may be relatively easy to distinguish but their precise boundaries may be hard to draw at the molecular level. Components that typically mark a certain organelle are often dynamic and move between many organelles (Maxfield and McCraw, 2004). In addition, organelles themselves may change over time, or maturate, to give rise to another type of organelle. Taken together this means that distinct endosomes are usually defined by relative enrichment of proteins or lipids, even though these components can be found in other compartments too (Maxfield and McCraw, 2004). The endocytic trafficking pathways are summarized in Figure 3.

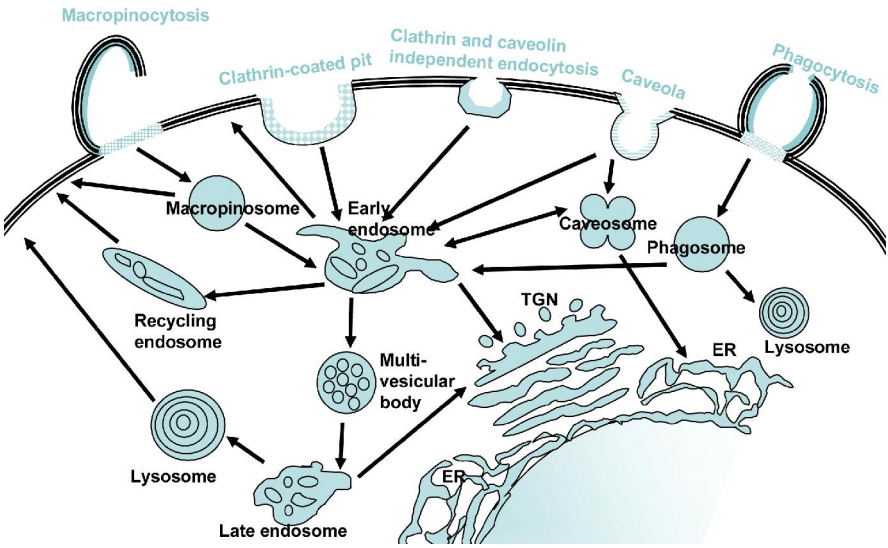

Figure 3. Various endocytic tracks in mammalian cells.

In many cases, internalized cargo is first delivered to early endosomes, also named sorting endosomes. Early endosomes are mildly acidic, tubulating and contain internal membranous structures (Gruenberg, 2001). In early endosomes, cargo entering via receptor-mediated endocytosis is often dissociated from its receptor and sorted either to the plasma membrane, endocytic recycling compartment or late endosomes (Maxfield and McGraw, 2004). In polarized cells, the early sorting endosomes have additional specialized functions. Epithelial cells, for example, have two distinct early endosomal populations (basolateral and apical) that communicate with corresponding plasma membrane domains (Miaczynska and Zerial, 2002).

From early endosomes, two transport routes deliver molecules back to the cell surface. The shorter pathway returns the constituents directly, whereas the longer pathway involves a passage through the endocytic recycling compartment (ERC) (Maxfield and McGraw, 2004). The half-time of the shorter recycling process has shown to be two minutes or less, whereas the slower pathway has a half-time of ~12 minutes (Hao and Maxfield, 2000). The recycling endosomes are less acidic than early endosomes and enriched

with raft lipids cholesterol and sphingomyelin (Cagesca *et al.*, 2000). In addition to endocytosis, recycling endosomes may participate in exocytic transport on the biosynthetic pathway, at least in polarized cells (Ang *et al.*, 2004).

Caveolar endocytosis can lead to delivery to early endosomes or, alternatively, intracellular organelles termed caveosomes. Caveosomes contain caveolin but lack markers for other endosomes, lysosomes, ER or Golgi (Pelkmans *et al.*, 2001). Caveosomes have neutral pH and they are enriched in cholesterol. They were first characterized as intermediates in the trafficking of simian virus 40 from the cell surface to the ER (Pelkmans *et al.*, 2001). The physiological function of caveosomes is at present unknown.

If endocytosed cargo is destined towards degradation, it is sorted from early endosomes to large transport intermediates called multivesicular bodies. In mammalian cells, multivesicular bodies are distinct from both early and late endosomes (Gruenberg, 2001). A protein complex named ESCRT is required both for the formation of internal vesicles of the multivesicular body as well as sorting of ubiquitylated endosomal cargo proteins for the degradative pathway to lysosomes (Katzmann *et al.*, 2002). Proteins that are destined for degradation accumulate within the internal membranes of multivesicular bodies. However, not all the proteins found in the internal structures are degraded. Examples of such proteins are mannose-6-phosphate receptors that recycle to the trans-Golgi network (TGN) from late endosomes (Griffiths *et al.*, 1990) or Major Histocompatibility Complex class II molecules that participate in antigen presentation in antigen-presenting cells (Kleijmeer *et al.*, 1996). Delivery for degradation may also proceed via alternative pathways: for example, caveolar endocytosis has been reported to convey materials for degradation, bypassing early endosomes (di Guglielmo *et al.*, 2003). Whether this pathway involves sorting through multivesicular bodies is so far unclear.

Late endosomes are emerging as sorting organelles much similarly to early endosomes. They are dynamic in nature and have been shown to exhibit tubulation towards the plasma membrane and ER (Ko *et al.*, 2001; Zhang *et al.*, 2001a). These tubules have been postulated to transport lipid and protein cargo. Distinct transport pathways that recycle cargo from late endosomes are not completely elucidated and neither is their regulation. The pathway recycling mannose-6-phosphate receptors from late endosomes to the TGN has been well characterized (Ghosh *et al.*, 2003) but the regulation of trafficking to other intracellular destinations still remains largely unknown.

Cargo not resorted from the degradative pathway is transferred to the lysosomes, the terminal of the endocytic pathway, for degradation. For lysosomes, it has been postulated that they repeatedly fuse with late endosomes to form hybrid organelles from which lysosomes are again reformed (Mazio *et al.*, 2000). In some cell types, for instance hemopoietic cells, the lysosomes can fuse with plasma membrane to release their contents to the extracellular space (Stinchcombe and Griffiths, 1999).

2.2 Endocytic Rab proteins

Rab proteins are small GTPases that act as molecular switches in coordinating vesiculo-tubular transport within a cell (Zerial and McBride, 2001). They localize in distinct intracellular compartments and participate in consecutive stages of transport, such as vesicle formation, vesicle and organelle motility, and tethering to target compartments. The activity of Rab proteins is regulated via their GTP-GDP cycle. The GTP (guanosine triphosphate) -bound form is usually described as the active form of the protein whereas GDP (guanosine diphosphate) -bound would represent the inactive form. The activity, however, is maintained through the ability of Rab proteins to oscillate between GDP-and GTP bound forms (Zerial and McBride, 2001). In the GTP-bound form, Rab proteins recruit a multitude of effector proteins to the cytoplasmic leaflet of the membrane. The effector proteins appear to be very heterogeneous: for example, some are involved in membrane tethering and fusion, some are enzymes and some participate in the regulation of Rab activity (Zerial and McBride, 2001). The combinatorial interactions of Rab proteins and their effectors with the membrane and cargo contribute to the specificity of distinct vesicular transport steps (Pfeffer, 2001).

Rab proteins are prenylated in their carboxyl terminal, which allows them to associate with membranes (Pfeffer and Aivazian, 2004). However, the membrane association is also modulated by the status of bound nucleotides. After the GTP hydrolysis the GDP-bound Rabs are removed from membranes by a protein called Rab GDP dissociation inhibitor (GDI), which also presents "used" Rab proteins back to their compartment of origin for reuse (Dirac-Svejstrup *et al.*, 1994; Pfeffer, 2001). As GDI binds to Rab proteins with high affinity, the dissociation of the Rab-GDI complex requires action of a GDI displacement factor (Dirac-Svejstrup *et al.*, 1997). Such a factor, named Yip3, was recently identified for endosomal Rabs (Sivars *et al.*,

2003). Interestingly, the ability of CDI to extract certain Rab proteins from membranes appears to be sensitive to the cholesterol content of the membrane (Lebrand *et al.*, 2002; Choudhury *et al.*, 2004).

Several Rab proteins have been identified in the endocytic transport route of mammalian cells. Some of those are expressed in a cell or tissue specific manner, whereas others ubiquitously. In polarized and non-polarized cells, the endocytic pathways share many common features and ubiquitous endocytic Rab proteins are thought to regulate analogous pathways in both cell types (Somsel Rodman and Wandinger-Ness, 2000). In addition, a set of distinct Rab proteins facilitate endocytic and transcytotic transport to apical and basolateral plasma membranes in epithelial cells. A list of endosomal Rab proteins is shown in Table 1. The list is not comprehensive; it must be noted that while many Rab proteins have been implicated in endocytosis, only small subset has been extensively characterized.

Perhaps the best characterized endosomal Rab protein up to date is Rab5 that regulates membrane traffic into and between early endosomes. Rab5 participates for instance in homotypic fusion of early endosomes (Stenmark *et al.*, 1994), regulation of motility of early endosomes on microtubules (Nielsen *et al.*, 1999) and signal transduction (Miaczynska *et al.*, 2004). The downstream machinery from Rab5 is extremely complex: there may be over 20 effectors or binding partners for Rab5 (Zerial and McBride, 2001). Diverse functions of Rab5 are achieved by differential recruitment of these effectors, which provides an additional layer of compartment-specific regulation in Rab5 mediated processes (Miaczynska and Zerial, 2002).

From early endosomes, the recycling route proceeds via Rab4 and Rab11 regulated pathways. Rab4 localizes to early endosomes and recycling vesicles and is thought to govern a direct recycling pathway from early endosomes to the cell surface (Daro *et al.*, 1996). Rab11 regulates the slower recycling route via the endocytic recycling compartment (Ullrich *et al.*, 1996). While designated to different intracellular compartments, Rab proteins have partially overlapping distributions along the endocytic pathway. The trafficking of transferrin to the recycling route has been shown to proceed via formation of three distinct Rab domains: one domain containing Rab5 only, second containing Rab5 and Rab4, and third Rab4 and Rab11 (Sonnichsen *et al.*, 2000). Eventually also Rab4 and Rab11 are segregated into separate carriers, and only Rab11 has been found to associate with the exocytic

transport step from the ERC to the plasma membrane (Ren *et al.*, 1998; Ward *et al.*, 2005).

The late endocytic pathway is characterized by two Rab proteins, Rab7 and Rab9. Rab7 has been implicated in the transport from early to late endosomes (Feng *et al.*, 1995; Press *et al.*, 1998), late endosome to lysosome fusion (Bucci *et al.*, 2000) and phagolysosome maturation (Via *et al.*, 1997). Overexpression of constitutively GDP-bound Rab7 has been shown to lead to impaired late endosomal degradation (Vitelli *et al.*, 1997) and dispersion of late endocytic organelles from a perinuclear cluster to the cytoplasm (Bucci *et al.*, 2000). Indeed, analogously to Rab5 in early endosomes, Rab7 has been reported to regulate the transport of late endosomes along the microtubules (Cantalupo *et al.*, 2001; Jordens *et al.*, 2001; Lebrand *et al.*, 2002). Rab9 functions in the recycling pathway from late endosomes to the TGN and it regulates the trafficking of mannose-6-phosphate receptor and its cargo (Lombardi *et al.*, 1993; Riederer *et al.*, 1994). In a recent study, depletion of Rab9 was shown to decrease late endosome size and lead to a reduction in the number of multilamellar and dense tubular late endosomes/lysosomes (Carsley *et al.*, 2004). Like the Rab proteins in the early and recycling pathway, Rab7 and Rab9 occupy distinct domains on the late endosome membranes (Barbero *et al.*, 2002). These observations add support to the idea that Rab proteins and their effectors may act as organizers of distinct membrane domains in endosomes (Zerial and McBride, 2001).

Table 1: Endosomal Rab proteins

Rab	Localization	Function	Cell/tissue specificity	References
Rab4	EE, RE	Direct recycling from EE to PM, sorting at EE to recycling or degradative pathway		(Daro *et al.*, 1996; Sonnichsen *et al.*, 2000; McCaffrey *et al.*, 2001)
Rab5	EE, PM, CCV	Ligand sequestration at plasma membrane, CCV-EE and EE-EE fusion, EE motility, signal transduction		(Stenmark *et al.*, 1994; Nielsen *et al.*, 1999; Miaczynska *et al.*, 2004)
Rab7	LE, LY	EE-LE transport and/or LE-LY fusion, LE motility		(Feng *et al.*, 1995; Press *et al.*, 1998; Bucci *et al.*, 2000; Jordens *et al.*, 2001; Mebrand *et al.*, 2002)
Rab9	LE, TGN	LE to TGN transport, regulation of LE size		(Lombardi *et al.*, 1993; Riederer *et al.*, 1994; Carroll *et al.*, 2004)
Rab11	RE, RE, TGN	Recycling through ERC, exocytic transport from TGN to PM		(Ullrich *et al.*, 1996; Ren *et al.*, 1998; Wilcke *et al.*, 2000; Ward *et al.*, 2005)
Rab15	EE, RE	Inhibition of homotypic EE fusion	Enriched in the neuronal tissue	(Elferink *et al.*, 1992; Zuk and Elferink, 1999, 2000)
Rab17	Apical RE	Regulation of membrane trafficking through apical RE	Epithelial cells	(Zacchi *et al.*, 1998)
Rab18	EE, CCV	Uncharacterized		(Lutcke *et al.*, 1994)
Rab20	EE	Uncharacterized		(Lutcke *et al.*, 1994)
Rab21	EE	Implicated in endocytosis		(Simpson *et al.*, 2004)
Rab22	EE, Golgi	EE dynamics, endocytosis, EE-Golgi communication		(Mesa *et al.*, 2001; Kauppi *et al.*, 2002)
Rab24	ER, LE, auto-phagosomes	Uncharacterized		(Olkkonen *et al.*, 1993; Munafo and Colombo, 2002)
Rab25	Apical RE	Implicated in regulation of transport through the apical RE	Epithelial cells	(Casanova *et al.*, 1999)
Rab27	Melanosomes, secretory granules	Melanosome transport, exocytosis	Melanocytes, endocrine cells, hemopoietic cells	(Bahadoran *et al.*, 2001; Izumi *et al.*, 2003)
Rab34	Golgi, membrane ruffles, macropino-somes	Implicated in LY positioning and macropinosome formation		(Wang and Hong, 2002; Sun *et al.*, 2003)
Rab39	Golgi-associated vesicles	Implicated in endocytosis		(Chen *et al.*, 2003)

Abbreviations used in the table: EE = early endosome, RE = recycling endosome, PM = plasma membrane, CCV = clathrin-coated vesicle, LE = late endosome, LY = lysosome, TGN = trans-Golgi network

2.3 Endosomes – mosaic of domains

It is becoming increasingly evident that proteins and lipids that build up an endocytic organelle are not evenly distributed. Instead, endosomes are thought to consist of a mosaic of structural and functional domains (Gruenberg, 2001). Differences in composition exist both between limiting and internal membranes of endosomes as well as in the plane of a membrane bilayer. Such compartmentalization allows several distinct processes to take place simultaneously and effectively and still maintain the organelle integrity (Miaczynska and Zerial, 2002). How these domains are organized is still a matter of investigation. Various mechanisms are likely to contribute to the selective accumulation of proteins and lipids in a particular membrane region and in the end, the whole cascade may be quite complex.

As described above, Rab proteins have been shown to form separate domains in the organelles in which they reside (Sonnichsen *et al.*, 2000; Barbero *et al.*, 2002). In their GTP-bound state, Rab proteins are able to interact with a variety of effectors that can, in turn, modify the local membrane environment. An example of such a mechanism is the interaction of Rab5 with hVPS34/p150, the type III phosphatidylinositol-3-kinase that generates phosphatidylinositol-3-phosphate (PI(3)P) (Christoforidis *et al.*, 1999). Localized production of PI(3)P leads to the recruitment of proteins that bind to it via specific lipid-binding domains, such as FYVE zinc-finger (Gaullier *et al.*, 1998). These proteins, in turn, participate in the regulation of endosomal dynamics such as membrane fusion (Simonsen *et al.*, 2001). The communication between adjacent Rab domains has been proposed to be regulated by divalent Rab effectors that are able to interact with distinct Rab proteins. Such effectors have been reported for at least Rab4 and Rab5 (Vitale *et al.*, 1998; de Renzis *et al.*, 2002).

Localized lipid production in a certain membrane area provides one way to create lipid asymmetry in the endocytic membranes. In addition, lipids themselves have a tendency to segregate and form distinct domains (see chapter 1.1). The specific lipid enrichment in rafts in turn favours partitioning of some classes of proteins, while others are excluded (Simons and Ikonen, 1997). The existence of rafts in recycling endosomes has been established in several studies (Mayor *et al.*, 1998; Cagesca *et al.*, 2000; Hao *et al.*, 2002). Some studies indicate that normally the degradative pathway is kept largely devoid of rafts (Puri *et al.*, 1999; Lusa *et al.*, 2001). In fact, the accumulation of

raft lipids in late endocytic organelles has been proposed to contribute to the clinical features associated with lysosomal storage diseases (Simons and Gruenberg, 2000). On the other hand, the existence of rafts in late endosomes under physiological conditions has been reported at least in some cell types (Pivaz *et al.*, 2002). As the authors suggest, it is possible that there is a threshold level of raft accumulation that the late endocytic organelles can tolerate. This level would presumably be tightly regulated and exceeding it would lead to pathological development.

2.4 Endosomes and cytoskeleton

Coordinated trafficking of endosomes is made possible by the aid of cytoskeletal networks that participate in the movement of vesicular carriers as well as the maintenance of spatial distribution and morphology of the organelles in a cell. The organelle transport along the microtubule and actin tracks is powered by molecular motor proteins from the dynein, kinesin and myosin families (Kamal and Goldstein, 2000; Vale, 2003).

The kinesin superfamily and cytoplasmic dynein facilitate long-range movement of vesicles along microtubules (Hirokawa, 1998). In non-polarized cells, microtubules radiate from the cell centre to the cell periphery. The peripheral end is termed "plus end" and it represents the site of microtubule growth and shrinkage (Schroer, 2001). The "minus end" is associated with the centrosome in the perinuclear area, although non-centrosomal microtubules do exist and are present in substantial amounts especially in polarized cells (Keating and Borisy, 1999). Conventional kinesin, the founding member of kinesin superfamily, was the first cytoplasmic motor protein identified (Brady, 1985). Conventional kinesin transports vesicles to the plus end of microtubules (Vale *et al.*, 1985). In addition, some kinesins are capable of transporting endosomes towards the minus end (Nielsen *et al.*, 1999). Cytoplasmic dynein drives minus-end motility of endosomes along the microtubules: in cells lacking functional dynein, lysosomes and endosomes that normally reside in a perinuclear cluster were found scattered throughout the cytoplasm (Harada *et al.*, 1998). *In vitro*, dynein has been shown to be required for early to late endosome transport (Aniento *et al.*, 1993). In addition, dynein has been reported to participate in the maturation of phagosomes (Harrison *et al.*, 2003). So far dynein motors have not been shown to participate in the plus-end directed trafficking of vesicles.

In addition to microtubules, actin cytoskeleton participates in vesicle trafficking along the endocytic pathway as well as internalization of cargo (Apodaca, 2001). In yeast, actin cytoskeleton is involved in the early steps of endocytosis (Engqvist-Goldstein and Drubin, 2003; Kaksonen *et al.*, 2003). In mammalian cells, actin dynamics play an essential role in phagocytosis and macropinocytosis and have been implicated at least in caveolar endocytosis (Pelkmans *et al.*, 2002; Engqvist-Goldstein and Drubin, 2003). Actin-based movement of vesicles can occur via actin polymerization propelling organelles within the cytoplasm (Taunton *et al.*, 2000) or by the association of vesicles to actin tracks via myosin motors (Seabra and Coudrier, 2004). In the degradative pathway, the actin network has been implicated in the correct positioning of degradative organelles, maintaining their bidirectional movement, and fusion between late endosomes and lysosomes (van Deurs *et al.*, 1995; Durrbach *et al.*, 1996; Cordonnier *et al.*, 2001; Kjeken *et al.*, 2004). In addition, positioning of recycling endosomes and transport to them has been shown to require an intact actin cytoskeleton (Raposo *et al.*, 1999; Lapierre *et al.*, 2001; Bartz *et al.*, 2003).

In order to achieve coordinated transport of endosomes to various directions the activity of motors must be regulated. The mechanisms of this regulation are at present not completely understood. Recent studies on Rab proteins have led to the suggestion that they might serve as "receptors" for motor proteins (Hammer and Wu, 2002; Seabra and Coudrier, 2004). This idea has been reinforced by several reports linking Rab proteins or their effectors to recruitment of different motors: Rab5 to kinesins via PI(3)P domain (Nielsen *et al.*, 1999; Roepfner *et al.*, 2005), Rab7 to dynein via RILP (Jordens *et al.*, 2001), Rab4 to dynein (Bielli *et al.*, 2001) and Rab11 to myosin Vb via Rab11 Family Interacting Protein 2 (Hales *et al.*, 2002), for example. The idea of utilizing Rab proteins for this function seems reasonable: the large number of Rabs and even larger number of their effectors could probably provide enough diversity to maintain specificity of motor recruitment in different compartments (Hammer and Wu, 2002).

2.5 Lipid sorting in the endocytic pathway

In order to maintain distinct lipid compositions of different endosomes there has to be a mechanism or mechanisms that keep the lipid pools segregated. The different internalization mechanisms provide one way of separation:

lipids from the cell surface are internalized via different endocytic mechanisms, which may lead to delivery to distinct intracellular pools (Marks and Pagano, 2002) (see also chapter 2.1.1). However, this alone is not sufficient as the pathways do intersect later in the cells. There is increasing evidence that different lipids, like proteins, are differentially sorted along the endocytic pathway (Mukherjee and Maxfield, 2000). For proteins, the sorting is achieved through recognition of peptide tags that direct components to correct localizations. For lipids, no such targeting motifs have been identified. It is possible that sorting partially relies on the tendency of some lipids to associate with each other. This characteristic has been highlighted for instance in several lysosomal storage diseases, where accumulation of sphingolipid to lysosomes leads to co-accretion of cholesterol and vice versa (Puri *et al.*, 1999). In addition, sorting has been shown to be influenced by the structural properties of the lipids. Lipids with differences only in the length of their alkyl chains have been reported to be differentially sorted to recycling and late endosomes (Mukherjee *et al.*, 1999), presumably due to their differential association with ordered versus more fluid membrane domains. In addition, lipid shape may be a contributing factor: for instance, the unique late endosomal cone-shaped lipid lysobisphosphatidic acid (LBPA) has been shown to induce the formation of multivesicular inner membrane structures characteristic to late endosomes and multivesicular bodies (Matsuo *et al.*, 2004).

3. Possible mechanisms of cholesterol transfer within the cell

Dissecting the pathways of cholesterol in the cell is complex as there are many aspects to consider. Intracellular cholesterol pools are connected, so trafficking between different compartments may be either direct or indirect (Soccio and Breslow, 2004). In addition, different mechanisms may act together or sequentially, which further adds to the complexity. The possibilities to transport the highly hydrophobic cholesterol within the aqueous cell interior include direct membrane contacts, vesicular transport and cholesterol binding proteins. Evidence for all these mechanisms exist and it is likely that a given means does not exclude the utilization of others.

3.1 Non-vesicular transport

One possibility to move cholesterol from one membrane compartment to another is a direct diffusion between membranes. The studies on this mechanism, however, have mainly been conducted in model membranes. In biophysical studies, cholesterol is able to flip-flop or diffuse very rapidly (nanoseconds) across membranes but its desorption from membrane surface is slow (hours) (Hamilton, 2003). In cells, close apposition of the plasma membrane and the ER could utilize direct cholesterol transfer mechanism(s) (Maxfield and Wustner, 2002; Prinz, 2002). On the other hand, for lysosomal membranes the rate of spontaneous diffusion of cholesterol is extremely slow and thus this mechanism is not considered to be significant in the egress of lysosomal cholesterol (Schoer *et al.*, 2000). In addition, nonvesicular cholesterol transport can be mediated by carrier proteins which transport cholesterol through the aqueous cytosol or pass it from one membrane to another. Examples of such proteins are steroidogenic acute regulatory protein (StAR) and metatstatic lymph node (MLN) 64 that belong to the StAR-related lipid transfer (START) -domain family of lipid-binding proteins.

3.1.1 Overview of START domain proteins

START domain proteins harbour a unique lipid-binding domain. Their function has been implicated in intracellular lipid transport and metabolism as well as signalling events (Soccio and Breslow, 2003). Human and mouse genomes have 15 genes encoding START domain proteins but, for instance, yeasts are devoid of them. Different START proteins have different lipid specificities, but the specific ligands for most of them are yet to be found. Two of the family members, StAR and MLN64, bind cholesterol (Tsujishita and Hurley, 2000; Petrescu *et al.*, 2001). X-ray crystal structures of START domain have been resolved for MLN64, (Tsujishita and Hurley, 2000) phosphatidylcholine transfer protein (PCTP) (Roderick *et al.*, 2002), and StarD4 (Romanowski *et al.*, 2002). They are structurally similar, forming an internal hydrophobic cavity capable of accommodating the ligand. In each structure, lipid entry or egress is likely to require some conformational change in the protein, perhaps to partially unfolded state as has been suggested for MLN64 and StAR (Bose *et al.*, 2000).

For most members of the START protein family, neither the ligand nor the function has been clarified. StarD4 and StarD5 have been shown to promote cholesterol transfer indicating that they are also sterol binding proteins (Soccio *et al.*, 2005). StarD4 expression is repressed in high cholesterol, so it has been speculated to perhaps participate in the transport of cholesterol biosynthetic precursors (Soccio *et al.*, 2002). StarD5 is upregulated in ER stress and it has been proposed to have a protective role in ER-stressed cells (Soccio *et al.*, 2005). As for other START domains, PCTP binds to phosphatidylcholine with a high specificity (Soccio and Breslow, 2003). CERT, a spliced isoform of Goodpasture antigen binding protein, was recently shown to mediate nonvesicular transport of ceramide to the Golgi (Hanada *et al.*, 2003). In several members of this family, other functional domains in addition to the lipid-binding START domain have been described. RhoGAPs are regulators of RhoGTPase-mediated signalling in the organization of actin cytoskeleton and three of them have START domains (Soccio and Breslow, 2003). Acyl-CoA thioesterase subfamily members have type III acyl-CoA thioesterase domain in addition to the START domain. In plants, several START genes are fused to homeodomains suggesting that they may have functions in the regulation of plant development (Schrick *et al.*, 2004).

3.1.2 StAR

StAR mediates transport of cholesterol to the inner mitochondrial membranes in steroidogenic cells (Soccio and Breslow, 2003; Strauss *et al.*, 2003). StAR-mediated delivery of cholesterol to the P450 side-chain cleavage enzyme that converts cholesterol to pregnenolone is the rate-limiting step in steroidogenesis. Mutations in StAR result in congenital lipoid adrenal hyperplasia, a potentially lethal disease characterized by an almost complete lack of steroid production in newborns (Lin *et al.*, 1995).

The precise mechanism by which StAR facilitates cholesterol transport to the inner mitochondrial membranes is not known. The N-terminal signal sequence directs StAR into mitochondria, but a mutated StAR lacking the targeting sequence has been shown to be as effective in increasing steroidogenesis as the wild-type protein (Arakane *et al.*, 1996). Thus, the site of StAR action is likely the outer mitochondrial membrane (Bose *et al.*, 2002). In fact, the mitochondrial import has been suggested to provide a means to

inactivate the protein, as StAR undergoes proteolytic degradation in mitochondria (Granot *et al.*, 2003).

3.1.3 MLN64

MLN64 was originally cloned as a gene amplified in certain breast cancers (Moog-Lutz *et al.*, 1997). MLN64 is a membrane-bound protein consisting of a START domain 37% identical to that of StAR. In addition, MLN64 harbours an N-terminal domain consisting of four transmembrane helices that anchor it to the membrane. MLN64 has been reported to localize mainly to the limiting membrane of late endosomes with both N-terminus and START-domain projecting towards the cytosol (Alpy *et al.*, 2001) (Figure 4).

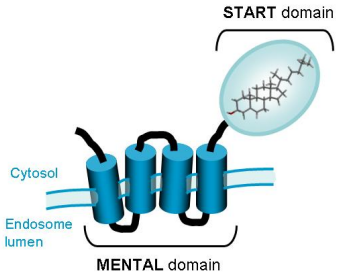

Figure 4. Schematic illustration of the MLN64 protein

Like StAR, MLN64 START domain binds cholesterol (Tsujishita and Hurley, 2000) and is able to stimulate steroidogenesis in cells when co-expressed with the P450 side-chain cleavage enzyme (Watari *et al.*, 1997). In addition, MLN64 has been reported to participate in the unloading of LDL-derived cholesterol from late endosomes to steroidogenic mitochondria (Zhang *et al.*, 2002). These studies have led to the idea that MLN64 may be responsible for shuttling cholesterol to mitochodria for steroidogenesis in placenta where StAR is missing but MLN64 is present in ample amounts (Watari *et al.*, 1997; Strauss *et al.*, 2003). In *Drosophila*, MLN64 homologue START1 has been implicated in the production of the insect hormone ecdysteroid (Roth *et al.*, 2004). However, the expression of MLN64 is not limited to steroidogenic tissues (Watari *et al.*, 1997). In addition, a recent study described a mouse model harbouring a mutated MLN64 incapable of cholesterol binding through the START domain (Kishida *et al.*, 2004). The

mouse was viable and fertile with no discernable sterol phenotype, suggesting that MLN64 may not have an indispensable role in steroidogenesis or maintaining cholesterol homeostasis.

In addition to the START domain, MLN64 harbours the N-terminal transmembrane helices that appear not only to facilitate membrane association but to form an individual functional domain. This MENTAL domain has been shown to be able to bind cholesterol independently of the START domain (Alpy *et al.*, 2005). The physiological significance of this interaction is at present unknown. If the START domain is mutated or deleted, at least the ability of MLN64 to stimulate steroidogenesis is lost (Watari *et al.*, 1997; Zhang *et al.*, 2002). MENTHIO, a protein homologous to MLN64 N-terminus but lacking a START-domain has recently been described (Alpy *et al.*, 2002). MENTHIO localizes in late endosomes like MLN64, and its overexpression has been shown to lead in a pronounced enlargement of these organelles. This observation has led the authors to suggest that MENTHIO may have a function in late endosomal dynamics (Alpy *et al.*, 2002). The same effect has been reported with the overexpression of MLN64 lacking START domain (Zhang *et al.*, 2002). Interestingly, MENTHIO has been shown to interact with MLN64 (Alpy *et al.*, 2005).

3.2 Vesicular trafficking

One clear example of the involvement of vesicular transport in intracellular cholesterol trafficking is the delivery of LDL particles to the cells via endocytosis (Brown and Goldstein, 1986). Cholesterol is present in the intracellular vesicles, so it would seem likely that vesicular transport pathways participate in other cholesterol trafficking itineraries as well. For example, the endocytic recycling compartment has been identified as a major sterol storing organelle within a cell (Hao *et al.*, 2002).

Vesicular trafficking typically requires energy, cytoskeletal elements and is sensitive to temperature blocks. Several studies have shown that upon the utilization of vesicular transport inhibitors, some aspects of intracellular cholesterol trafficking are inhibited whereas others are not (Liscum and Munn, 1999; Soccio and Breslow, 2004). For instance, trafficking of biosynthetic cholesterol to the cell surface has been shown to be only partially sensitive to microtubule depolymerization and low temperature (Heino *et al.*, 2000), while plasma membrane to ER transport step has been reported to

require an intact intermediate filament network (Sarria *et al.*, 1992). In addition, cholesterol delivery from the plasma membrane to the ER for esterification has been suggested to involve atypical vesicular transport carriers that are energy-independent but inhibitable by other vesicular transport inhibitors (Skiba *et al.*, 1996). Vesicular trafficking has been implicated in the cholesterol relocation from late endocytic organelles, as it was blocked at 21°C (Neufeld *et al.*, 1999). Furthermore, a connection between vesicular trafficking and late endosomal cholesterol balance has been implicated in the Niemann Pick type C (NPC) disease, a disorder affecting both cholesterol metabolism and the function of late endocytic organelles.

4. Niemann-Pick type C disease

NPC disease is an autosomally, recessively inherited lysosomal lipidosis characterized by late endosomal/lysosomal accumulation of cholesterol and sphingolipids, leading to a fatal neurological disease (Patterson *et al.*, 2001). As such it resembles other sphingolipid storage diseases which are usually caused by enzyme deficiencies that result in impaired breakdown and subsequent accumulation of lipids in lysosomes (Marks and Pagano, 2002). In NPC disease, however, the primary problem is thought not to arise from compromised lipid catabolism but defective transport through the endosomal system (Neufeld *et al.*, 1999; Ko *et al.*, 2001; Patterson *et al.*, 2001).

The majority of NPC patients (95%) have defects in the NPC1 gene that encodes a polytopic membrane protein with 13 predicted transmembrane spans (Carstea *et al.*, 1997; Davies and Ioannou, 2000). NPC1 protein has mainly been localized to late endosomes, especially in cells exposed to cholesterol load (Higgins *et al.*, 1999; Zhang *et al.*, 2001b). NPC1 has a sterol-sensing domain similar to HMG-CoA reductase, SCAP and the morphogen receptor Patched (Carstea *et al.*, 1997). The smaller portion of NPC patients harbour mutations in NPC2/HE1 gene that encodes a small protein first identified as a major secretory glycoprotein in human epididymis (Kirchhoff *et al.*, 1996). NPC2/HE1 localizes to lysosomes and binds cholesterol with a high affinity (Ko *et al.*, 2003). The precise function for neither of these two proteins has been elucidated so far. NPC1 has been suggested to act as a mediator of vesicular trafficking (Ko *et al.*, 2001) or a transmembrane molecular pump for fatty acids (Davies *et al.*, 2000). It is likely that NPC1 and NPC2 act in sequence in the same pathway, as the phenotype of the double mutant lacking

the function of both proteins does not differ from the phenotype of individual mutations (Sleat *et al.*, 2004).

At present, the studies on NPC disease remain somewhat controversial concerning the primary pathogenesis at the cellular level. Traditional interpretation is that NPC is a disorder affecting first and foremost intracellular cholesterol metabolism. The classical cellular manifestations of NPC disease are the endo/lysosomal cholesterol accumulation and the inability of cells to activate the homeostatic responses upon the influx of LDL (Pentchev *et al.*, 1985; Liscum and Faust, 1987; Blanchette-Mackie *et al.*, 1988) (Figure 5). This view has been challenged by the idea that the cholesterol accumulation is in fact a secondary phenomenon resulting from sphingolipid retention (Cervas *et al.*, 2001; Coudre-Lewis *et al.*, 2003). In yeast, the mutation in the sterol sensing domain of NPC1 orthologue Ncr1 has been shown to affect the recycling of complex sphingolipids but not sterol balance (Malathi *et al.*, 2004). Curiously, Ncr1 null mutant had no discernable phenotype at all. On the other hand, both NPC1 and NPC2 have been shown to bind cholesterol, lending support to the idea that they are modulators of sterol metabolism (Xu *et al.*, 2003; Ohgami *et al.*, 2004).

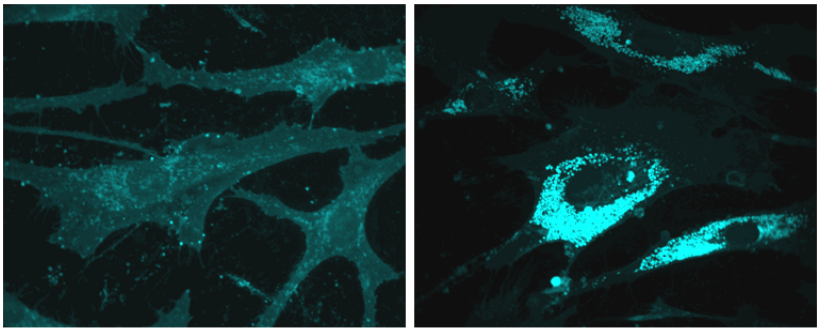

Figure 5. Filipin staining of normal skin fibroblasts (left) and NPC1-deficient fibroblasts (right). Filipin is fluorescent antibiotic that stains cellular free cholesterol (Bornig and Geyer, 1974) and highlights the massive cholesterol storage in NPC1 deficient cells.

The NPC defect has been shown to lead to various deficiencies in the endocytic transport. The clearance of sucrose from NPC1 deficient cells was shown to be retarded, indicating that the trafficking defect is not restricted to sterols (Neufeld *et al.*, 1999). The motility of cholesterol-laden late endocytic organelles has been shown to be reduced, either directly due to the NPC1

defect (Ko *et al.*, 2001) or secondarily due to cholesterol accumulation (Lebrand *et al.*, 2002). Impaired sorting of mannose-6-phosphate receptor from late endosomes has been reported (Kobayashi *et al.*, 1999). In addition to late endocytic compartments, early endosomes have been shown to be affected by NPC1 mutation as demonstrated by the accumulation of CM1 ganglioside and cholesterol in early endosomes (Sugimoto *et al.*, 2001; Choudhury *et al.*, 2004).

AIMS OF THE STUDY

The endocytic pathway receives cholesterol in the process of membrane turnover and recycling as well as the influx of lipoproteins. The cellular cholesterol balance is under a tight regulation, and the amount of cholesterol is uneven between different intracellular compartments. However, little is known of the precise trafficking itineraries that distribute the cholesterol from the endosomal system to other cellular destinations, and the contribution of individual endosomal pathways to cellular cholesterol homeostasis. The aim of this study was to clarify distinct aspects of the role of the endocytic pathway in the regulation of intracellular cholesterol transport and homeostasis. The specific aims of individual projects are listed below.

In the first study the aim was to find out if the Rab-protein mediated vesicular transport machinery participates in the egress of cholesterol from late endosomes/lysosomes. The function of Rab proteins can be inhibited by Rab-GDI. We asked if cholesterol exit from late endocytic organelles could be inhibited by excess Rab-GDI in cells harbouring endo/lysosomal cholesterol accumulation. In addition, we wanted to study if the relocation of late endosomal cholesterol affects other late endosomal constituents.

Rab-GDI is a general inhibitor of Rab-mediated vesicular trafficking. The aim of the next study was to analyze the effects of the overexpression of selected endocytic Rab proteins on cholesterol transport and homeostasis.

The aim of the third study was to characterize the function of the late endosomal cholesterol-binding protein MLN64. By knock-down and overexpression of MLN64 we aimed at pinpointing a process in which its cholesterol transfer activity participates.

METHODS

The methods used in the original articles included in this thesis are summarized in the table below. The roman numerals indicate the number of the original publication in which the method was employed.

Table 2.

Method	Publications
Cell culture and transient transfection	I, III, IV
Immunocytochemistry and microscopy	I, III, IV
Testing the recombinant Rab-CDI	I
Loading of cells with progesterone and LDL	I
Microinjection	I
SDS-page and Western blot analysis	I, III, IV
Cholesterol esterification assays	III, IV
Labelling with fluorescent transferrin	III
Labelling with biotin-2x FVVE	III
Labelling with fluorescent cholera toxin subunit B	III
Labelling with pyrenyldecanoylsphingomyelin	III
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RESULTS

1. Removal of late endocytic cholesterol is inhibited by Rab-GDI

1.1 Microinjection of Rab-GDI inhibits the egress of late endosomal cholesterol

Rab-GDI is a specific inhibitor of Rab-regulated vesicular transport processes, as it is capable of removing Rab proteins from membranes and retaining them inactive in cytosolic complexes (Pfeffer, 2001). We wanted to study if microinjection of recombinant Rab-GDI might have an effect on the removal of late endosomal/lysosomal cholesterol storage. To test this, recombinant Rab-GDI was prepared and introduced into cells by microinjection. We observed that excess Rab-GDI was able to remove Rab7 from the endosomal membranes, as observed by the change of the punctate endosomal staining pattern to a diffuse cytosolic distribution (II, Figure 1). In addition, recombinant Rab-GDI was able to efficiently remove Rab5 from endosomal membranes *in vitro* (II, Figure 1).

Progesterone has been shown to induce lysosomal cholesterol sequestration and inhibit cholesterol esterification. This phenotype is reversible and can be monitored cytochemically by a reduction in filipin staining of lysosomes (Butler *et al.*, 1992). We loaded fibroblasts with LDL in the presence of progesterone for 24 h to induce lysosomal cholesterol accumulation. When cells were fixed after loading and stained with filipin, an NPC-like late endosomal/lysosomal cholesterol sequestration was observed in ~80% of the cells (II, Figure 2). Upon progesterone washout for 4 h, the percentage of cells with endosomal cholesterol accumulation was reduced to ~50%. However, in cells microinjected with recombinant Rab-GDI at the onset of the washout, the cholesterol accumulation was observed in ~70% of the cells, indicating that Rab-GDI inhibited the clearance of late endosomal cholesterol deposits. This effect was not due to the microinjection as such, as injection of buffer alone had no effect on the clearance of sterol accumulation (II, Figure 2).

The same experiment was conducted in NPC1 deficient fibroblasts overexpressing NPC1 and soluble green fluorescent protein (GFP) as a marker. NPC1 overexpression corrects the cholesterol deposition in NPC1-deficient fibroblasts within 2-3 days of expression. After 24 h of transfection, progesterone was added in order to prevent the asynchronous unloading of

cholesterol. After three days of transfection, the cells were microinjected with Rab-CDII and the percentage of cells with cholesterol accumulation was assessed as above. Similarly, the egress of accumulated cholesterol was inhibited by the Rab-CDII but not with the control microinjection (data not shown).

1.2 Complementation of NPC phenotype by NPC1 overexpression results in redistribution of late endosomal constituents

In late endocytic organelles, some constituents are enriched in the outer membrane of the endosomes or lysosomes, whereas others are localized inside the organelle. For instance, lysosome-associated membrane protein 1 (Lamp1) is found predominantly on the outer membrane of late endosomes and lysosomes, whereas mannose-6-phosphate receptors are enriched in the organelle core (Griffiths *et al.*, 1988). In addition, internal membranes of late endosomes are enriched with the unconventional phospholipid LBPA as well as cholesterol (Kobayashi *et al.*, 1999; Möbius *et al.*, 2003). In NPC1 deficient cells, upon transient transfection of the NPC1 the endosomal cholesterol deposits are efficiently cleared. We therefore studied if the unloading of cholesterol would be accompanied by the redistribution of other endosomal constituents.

The results showed that the exit of cholesterol was accompanied by the redistribution of LBPA from the typical punctate to a more diffuse staining pattern, as assessed by the antibody detection (II, Figure 3). In addition, the distribution of lysosomal hydrolase aspartylglucosaminidase (ACA) was altered, leading to a more scattered labelling that partially colocalized with the early endosomal autoantigen 1 (EEA1) in NPC1 overexpressing cells (II, Figure 4). In contrast, the distribution of Lamp1 was not altered upon cholesterol removal (II, Figure 3). Thus, upon the unloading of massive cholesterol deposits, protein and lipid constituents of the internal membranes were relocated along with cholesterol.

2. Rab11 modulates cellular cholesterol homeostasis

2.1 Overexpression of GFP-fused Rab5 and Rab11 alter cholesterol distribution and homeostasis

To address which specific Rab proteins may participate in the transport of endocytosed cholesterol, we applied a strategy of overexpressing GFP-fused forms of several endosomal Rab proteins comprising early (Rab5), recycling (Rab11) and late endosomes (Rab7). In addition, Rab6 that mediates Golgi to ER transport (White *et al.*, 1999) was employed as a non-endosomal control. The effect of Rab overexpression on intracellular cholesterol distribution and homeostasis was assessed morphologically by filipin staining and biochemically by cholesterol esterification.

In untransfected COS-1 cells, filipin stained the plasma membrane and various intracellular compartments. Intracellularly, the perinuclear area corresponding to the Golgi apparatus and pericentriolar recycling compartment was highlighted. In addition, a fraction of early and late endocytic organelles was resolved with filipin staining, as assessed by colocalization of filipin fluorescence with PI(3)P-binding peptide 2xFYVE (Gillooly *et al.*, 2000) and Lamp1, respectively (III, Figure 1).

In transfected cells, Rab6 localized in the Golgi apparatus and Rab7 in punctate endosomal structures, both overlapping the filipin fluorescence in the perinuclear area (III, Figure 2). However, no redistribution of filipin staining was observed upon either Rab6 or Rab7 overexpression in comparison to cells expressing soluble GFP alone. In cells overexpressing Rab5, numerous filipin-positive dots were observed that co-localized with Rab5-GFP indicating that these structures were early endosomes (III, Figure 2). When the GTPase deficient mutant of Rab5 (Rab5Q67L) was used, the early endosomes were massively enlarged and the structures were clearly filipin positive. The most striking effect on filipin staining was, however, observed upon Rab11 overexpression. Rab11 localized in enlarged tubulating structures that were intensely filipin positive (III, Figure 2). The redistribution of filipin staining to Rab11-positive organelles was apparent already at 8 h posttransfection (III, Figure 4). At this time point, Rab11 decorated small punctae in the cytoplasm. With prolonged expression, the organelles became larger and were increasingly filipin positive.

To assess the effect of selected Rab proteins on cholesterol esterification, the overexpressing cells were labelled with [^{14}C]oleic acid in the presence of serum lipoproteins for 4 h. During this time, a fraction of radiolabelled fatty acid was incorporated into cholesteryl esters. This method is not selective to the source from which cholesterol is transported for esterification and can thus be utilized as a measure of the overall rate of cholesterol esterification in cells. With this assay, all of the endocytic Rab proteins were found to inhibit cholesterol esterification to some extent, but Rab11 and Rab5Q79A mutant had the most profound inhibitory effect (III, Figure 3).

In addition to cholesterol, the enlarged tubulo-vesicular structures in Rab11 overexpressing accumulated transferrin and transferrin receptor (TfnR) cells (III, Figure 4). These data indicate that in COS cells, Rab11 regulates the dynamics of recycling compartment. With GTPase deficient Rab11 (Rab11Q70L), the enlarged tubulo-vesicular structures accumulating transferrin were formed very much like with wild-type overexpression, with concomitant redistribution of cholesterol and inhibition of cholesterol esterification (III, Figure 5, and data not shown). With constitutively GDP-bound form of Rab11 (Rab11S25N), the morphology of the recycling compartment was altered (III, Figure 5). In these cells, transferrin was localized in thin tubules radiating from the perinuclear area of the cell. The network was also visualized by filipin staining (III, Figure 5). This phenotype was accompanied by defective cholesterol esterification similarly to overexpression of the wild-type or GTPase deficient Rab11 (data not shown).

2.2 Distribution of select endosomal and plasma membrane lipids in Rab11 overexpressing cells

In addition to cholesterol, we wanted to study if Rab11 overexpression affected the subcellular localization of other lipids. When cells were labelled with the peptide 2xFYVE that binds PI(3)P (Gaullier *et al.*, 1998; Gillooly *et al.*, 2000), the labelling was observed in small vesicular structures characteristic to early endosomes both in control and Rab11 expressing cells (III, Figures 1 and 6). No colocalization with Rab11 was observed. Also the distribution of late endosomal LBPA remained unchanged in Rab11 overexpressing cells, as assessed by antibody staining (III, Figure 6). Because cholesterol is associated with sphingolipids, the distribution of select

glycolipids and sphingomyelin was assessed in Rab11 expressing cells. With antibodies against globotriaosyl ceramide a prominent plasma membrane staining was observed, and this distribution was not altered upon Rab11 overexpression (III, Figure 6). In addition, distribution of GM2 ganglioside that localizes in late endosomes (Zhang *et al.*, 2001b) was not altered in Rab11 overexpressing cells (III, Figure 6).

However, some lipid constituents were affected by Rab11 overexpression. Antibodies against sulfatide gave a heterogeneous distribution in control cells, visualizing plasma membrane and perinuclear organelles that partially overlapped with transferrin (III, Figure 7). In a portion of Rab11 expressing cells, the sulfatide staining was redistributed to Rab11-positive tubular organelles (III, Figure 7). The redistribution of sulfatide was not as marked as that of cholesterol that consistently colocalized with Rab11. To visualize ganglioside GM1, the cells were labelled with Alexa 568-conjugated cholera toxin subunit B (CTxB) on ice, followed by a 2 h internalization at 37°C. In control cells, the toxin localized to the perinuclear area corresponding to the Golgi complex as observed previously (Lencer *et al.*, 1999) (III, Figure 7). However, in Rab11 expressing cells, CTxB was largely redistributed to Rab11-positive organelles. To assess the distribution of sphingomyelin, cells were labelled with fluorescent pyreneyldecanoylsphingomyelin ($Pyr_{10}SM$). In control cells, $Pyr_{10}SM$ was visualized on the plasma membrane and in the perinuclear area, presumably representing the Golgi and late endocytic organelles (III, Figure 7). A similar distribution has previously been reported for BODIPY-sphingomyelin (Puri *et al.*, 2001). In Rab11 expressing cells, a portion of $Pyr_{10}SM$ was found to be distributed to tubular Rab11 containing organelles (III, Figure 7).

2.3 Transport of LDL and LDL receptor is not compromised in Rab11 overexpressing cells

It was possible that the Rab11-induced inhibition of cholesterol esterification was due to Rab11 interfering with LDL-cholesterol internalization. To test this, we added DiI-labelled LDL to living cells for 15 min and either fixed them, or further chased for 2 h prior to fixation. The cells were then stained with antibodies against Lamp1 in order to assess the delivery of DiI-LDL to late endocytic organelles. In control cells, DiI-LDL was present in small vesicular structures not colocalizing with Lamp1 after 15 min, but good colocalization of these two markers was observed after 2 h chase (III, Figure 8).

This pattern was not changed in Rab11 overexpressing cells and no colocalization with Rab11 and DiI-LDL was observed at either time point. These data indicate that the trafficking of DiI-LDL was not compromised upon Rab11 overexpression. In contrast, in Rab5Q67L expressing cells, the DiI-LDL was not able to exit from the enlarged early endosomes but continued to localize in them even after 2 h of chase (III, Figure 8).

In addition, we studied the distribution of the LDL receptor in Rab11 expressing cells. In cells coexpressing soluble CFP and the LDL-receptor, anti-LDL antibodies decorated plasma membrane and small intracellular punctate structures (III, Figure 8). In Rab11 expressing cells, the intracellular staining partially colocalized with Rab11. However, the overall distribution of the LDL receptor was not impaired by Rab11 overexpression; in particular, the prominent plasma membrane staining was still present (III, Figure 8). Together, these results suggest that the cholesterol phenotype observed in Rab11 expressing cells was not due to the impaired trafficking of LDL-receptor or its cargo.

2.4 Rab11 and the NPC phenotype

To further explore the possibility of a link between late endocytic and Rab11-mediated cholesterol trafficking itineraries, we studied the Rab11 phenotype in relation to the NPC phenoptype. As NPC1 and NPC2 proteins are implicated in the trafficking of endocytosed cholesterol, we studied if their localization was altered in Rab11 overexpressing cells. However, overexpression of Rab11 had no effect on the distribution of these two proteins as assessed by antibody staining (III, Figure 9). In contrast, upon overexpression of Rab5Q67L the proteins were partially distributed to the enlargened early endosomes. These data suggest that the trafficking itineraries of NPC1 and NPC2 are perhaps connected to early but not recycling endosomal events.

To ascertain that the Rab11-induced deposition of cholesterol into recycling endosomes was not a phenomenon limited to COS cells, the Rab11 overexpression phenotype was studied in primary fibroblasts overexpressing TfnR as a marker for recycling endosomes. In cells expressing Rab11 and TfnR, cholesterol was deposited in the enlargened Rab11 organelles analogously to what was observed in COS cells (III, Figure 10). In cells expressing soluble CFP and TfnR, such accumulation was not observed. Interestingly, in NPC1 deficient fibroblasts harbouring lysosomal cholesterol

load, Rab11 was able to induce an additional recycling endosomal cholesterol accretion (III, Figure 10). These deposits were more peripherally localized and less intensively stained with filipin than the lysosomal deposits. The same result was observed in COS cells incubated with U18666A, a drug that induces lysosomal cholesterol accumulation (data not shown).

To investigate if Rab11 overexpression would have an effect on the clearance of lysosomal cholesterol deposits, we co-expressed Rab11 and NPC1 in NPC1 -deficient fibroblasts. We found that after three days of transfection, Rab11 and NPC1 were present in distinct compartments and the lysosomal accumulation was cleared in co-expressing cells similarly to cells expressing NPC1 alone (III, Figure 10). Instead, the weaker peripheral filipin staining in Rab11 positive organelles was still present. Thus, Rab11 overexpression had no effect on either the build-up or clearance of late endosomal/lysosomal cholesterol deposits. Moreover, the late endosomal cholesterol transport block did not prevent the formation of Rab11-positive enlargened recycling organelles containing cholesterol.

2.5 Rab11-mediated changes in cholesterol homeostasis are not LDL-dependent

The data described above suggested that Rab11-mediated effects on cholesterol transport might be independent of LDL–cholesterol. We therefore repeated the cholesterol esterification experiment with cells grown in the absence of lipoproteins. The results demonstrated that, albeit the basal rate of esterification in control cells was low with this treatment, Rab11 overexpression was able to significantly suppress it (III, Figure 11). Moreover, the deposition of cholesterol into Rab11 organelles in these cells was evident as assessed by filipin staining. Interestingly, Rab6 overexpression induced a slight increase in the rate of cholesterol esterification in cells grown in the absence of lipoproteins as compared to cells expressing soluble CFP alone (III, Figure 11).

Although seemingly independent of LDL cholesterol, the Rab11 overexpression phenotype appeared to share similar characteristics to the NPC phenotype, namely endosomal cholesterol accumulation and inhibited cholesterol esterification. In NPC1 deficient cells, however, the defective cholesterol homeostatic responses include not only a decreased rate of cholesterol esterification but also an elevated rate of cholesterol biosynthesis (Liscum and Faust, 1987). To analyze if this was the case also in Rab11

expressing cells, we labelled CFP and Rab11-CFP transfected cells with [^{3}H]acetate and analyzed the amount of [^{3}H]cholesterol formed. In contrast to NPC, we found that the reduction in the rate of esterification was not accompanied by an elevated rate of cholesterol biosynthesis in Rab11 expressing cells (III, page 3119).

The majority of cholesterol transported to esterification has been suggested to traffic via the plasma membrane (Neufeld *et al.*, 1996). Since the recycling compartment participates in the delivery of cargo back to the cell surface, we reasoned that the defective esterification upon Rab11 overexpression might be due to impaired delivery of cholesterol to the plasma membrane. If this was the case, one should be able to overcome the Rab11 block by adding more cholesterol to the plasma membrane. To achieve this, the cells were incubated with cholesterol complexed with methyl-β-cyclodextrin that loads cells with cholesterol (Leppimaki *et al.*, 2000) and induces a rapid compensatory increase in the rate of cholesterol esterification, as assessed by the incorporation of [^{3}H]oleic acid into cholesteryl esters (III, Figure 11). This increase was not suppressed by Rab11 overexpression, indicating that supplying the plasma membrane with cholesterol restores the rate of esterification in Rab11 overexpressing cells.

3. Cholesterol binding protein MLN64 participates in actin-mediated endosome dynamics

MLN64 is a cholesterol-binding protein located on the limiting membrane of late endosomes (Alpy *et al.*, 2001). As such, it was hypothesized to participate in the removal of late endosomal cholesterol, possibly in concert with NPC1 and NPC2 (Strauss *et al.*, 2002). On the other hand, mice harbouring a mutation in the sterol-binding domain of MLN64 were shown to be fertile and to lack obvious shortcomings in the whole body cholesterol metabolism (Kishida *et al.*, 2004). We wanted to study the possible contribution of MLN64 to late endosomal cholesterol dynamics by analyzing the effects of i) overexpression of MLN64 and its cholesterol-binding deficient mutants and ii) depletion of MLN64 by RNAi.

3.1. Overexpression of MLN64 results in enlargened late endocytic organelles harbouring cholesterol and LBPA

When overexpressed in COS cells, MLN64 resulted in the enlargement of late endocytic organelles (not, Figure 1). The endosome enlargement was even more pronounced in cells overexpressing mutant MLN64 devoid of cholesterol-binding START domain (ΔSTART). The latter phenotype has been described before and was shown to be accompanied by the accumulation of cholesterol in the enlargened organelles (Zhang *et al.*, 2002). We observed that both in wild-type and ΔSTART MLN64 expressing cells, the enlargened endosomes were often brightly filipin positive (not, Figure 1). In addition, the late endosomal lipid LBPA was frequently observed to be enriched in the enlargened organelles as assessed by antibody staining. The amount of accumulated material was somewhat variable from endosome to endosome and, typically, organelles appeared more compact upon wild-type MLN64 overexpression, as compared to massive endosomes induced upon overexpression of ΔSTART (not, Figure 1).

A protein homologous to the transmembrane sequences of MLN64, named MENTHO, has recently been described (Alpy *et al.*, 2002). The overexpression of MENTHO was shown to result in an endosome enlargement phenotype similar to ΔSTART. Thus, it seems likely that the MLN64 transmembrane region has functional significance independent of the cholesterol-binding START domain. Therefore we created a mutant construct encoding the full length MLN64 but defective in cholesterol binding by START domain. The lipid-binding specificity of START-domain proteins is likely to derive from the interaction of lipid with specific residues lining the lipid-binding cavity. Indeed, a structural divergence was observed when the sequence contributing to the lipid-binding tunnel of MLN64 was compared with the corresponding region of PCTP. Two uncharged amino acid residues conserved in MLN64 and StAR, namely Met 307 and Asn 311, are substituted by charged residues in PCTP. When these two amino acids were changed into Arg 307 and Asp 311 respectively, the cholesterol-binding capacity of MLN64 START domain was significantly diminished (not, Figure 1). These mutations were then introduced into an MLN64 expression vector to create a mutant protein compromised in cholesterol binding.

Filipin staining reveals the steady-state distribution of free cholesterol in fixed cells. To study the effect of wild-type and mutant MLN64

overexpression on sterol distribution in living cells, we utilized the yeast-derived cholesterol analogue DHE. When untransfected COS cells were labelled briefly with DHE and imaged at different time points, DHE was first observed mainly on the plasma membrane (III, Figure 2). Upon prolonged incubation, DHE gained access to intracellular organelles encompassing the perinuclear recycling endosomes. This labelling pattern was similar to what has been previously observed in CHO cells (Mukherjee *et al.*, 1998) and it persisted at least up to 24 h (III, Figure 2). In cells overexpressing CFP-fused MLN64, a fraction of the DHE label started to colocalize with CFP already at early time points (1-15 min). The same phenomenon was observed with the overexpression of both cholesterol-binding deficient mutants of MLN64 (ΔSTARD and M307R, N311D), indicating that the sequestration of sterols might partially be due to the engulfment of sterol-containing membranes upon endosome enlargement (III, Figure 2). However, upon longer chase times (5 or 24 h) the portion of DHE in relation to CFP in endosomes was significantly greater in wild-type MLN64 expressing endosomes versus the endosomes expressing sterol-binding deficient mutants of MLN64. These data indicate that in addition to ΔSTARD, wild-type MLN64 overexpression leads to the enrichment of late endosomal membranes with sterol.

To study the effect of MLN64 in cells harbouring late endosomal cholesterol accumulation we overexpressed both wild-type and ΔSTARD MLN64 in NPC1 deficient fibroblasts. While the overexpression of the wild-type protein was unable to clear the cholesterol accumulation from late endosomes, it often appeared to create big, cholesterol-filled structures harbouring the protein (III, Figure 2). Curiously, ΔSTARD which led to a very pronounced endosome enlargement in COS cells did not have a similar effect on NPC1 deficient cells.

3.2. Depletion of MLN64 does not impair cholesterol homeostasis but results in scattering of late endocytic organelles and inhibition of late endosomal degradation

With overexpression studies alone it is hard to assess the true physiological function of MLN64. With high level of overexpression, the protein may get detached from its normal regulatory circuits. In HeLa cells it was possible to utilize both over- and underexpression strategies to assess the role of MLN64 in endosome dynamics. Similarly to COS cells, in HeLa cells MLN64 localized to late endosomes as assessed by good colocalization with Lamp1

(Fig. 3). However, overexpression of MLN64 or ΔSTART in HeLa cells did not result in a prominent increase in endosome size as it did in COS cells. The reason for this difference is not known but could reflect a more modest level of protein production or intrinsic differences in cell types.

RNAi is a useful method to reduce protein levels in cell culture (Elbashir *et al.*, 2001). By RNAi, we were able to bring down the level of MLN64 protein by 80-90% in HeLa cells (Fig. 3). We observed that the knockdown of MLN64 did not inhibit cholesterol esterification either in the presence or absence of lipoproteins as assessed by the incorporation of radiolabelled oleic acid to cholesteryl esters. Cholesterol efflux to methyl-β-cyclodextrins was even moderately enhanced in cells depleted of MLN64. Moreover, filipin staining of MLN64 knockdown cells was unchanged compared to control cells (Fig. 3). Together, these data indicate that MLN64 depletion did not result in gross alterations in cholesterol homeostasis or cause NPC-like cholesterol accumulation phenotype in late endocytic compartments.

In contrast, MLN64 depletion had a substantial effect on the positioning of a population of late endosomes harbouring endocytosed cargo. When control cells were labelled with DiI-LDL and further incubated for 1.5-2 h, the label was found in a perinuclear cluster of late endocytic organelles in ~85% of cells (Fig. 3). In MLN64 depleted cells, however, the cargo bearing organelles were scattered throughout the cytoplasm and the perinuclear clustering was observed only in ~30% of cells. Both receptor-mediated as well as fluid-phase cargo were similarly affected, as assessed by the distribution of LDL and EGF as well as dextran (Figs. 3,4 and data not shown). The dispersed organelles were devoid of EEA1 and mostly Lamp1-positive, indicating that the phenotype did not reflect impaired cargo sorting from early endosomes (Fig. 4). However, we observed that the degradation of EGF was significantly reduced in MLN64 knockdown cells, suggesting that the dispersed endosomes had reduced hydrolytic capacity (Fig. 4).

3.3 The organelle dispersion in MLN64 depleted cells is not due to impaired Rab7-RILP-dynein machinery

The dispersal of late endocytic organelles and the inhibition of late endocytic degradation have been reported in cells overexpressing dominant negative

mutant of Rab7 (Bucci *et al.*, 2000). Rab7 and its effector RILP regulate the association of the microtubule-binding motor protein dynein to late endocytic organelles (Jordens *et al.*, 2001). Interestingly, the late endosomal cholesterol content has been shown to affect this machinery (Lebrand *et al.*, 2002). Thus, we speculated that cholesterol-binding MLN64 might be involved in this pathway. However, depletion of MLN64 had no effect on the membrane association of Rab7 or dynein as compared to control (not, Figure 5). Upon overexpression of RILP, enlargened perinuclear organelles were frequently observed both in MLN64 depleted and control cells. In contrast, in the majority of MLN64 depleted cells the cargo bearing organelles remained scattered in the cell periphery in spite of RILP overexpression (not, Figure 5). Together, these results suggest that the mechanism behind organelle dispersion in MLN64-depleted cells is distinct from the Rab7-dynein machinery.

3.4 The dispersed organelles are motile and move along microtubules

Next, we studied the motility of dispersed endosomes in MLN64 depleted cells. To achieve this, MLN64 depleted and control HeLa cells were fed DiI-labelled LDL and the probe was allowed to reach late endocytic compartments. The trafficking of labelled endosomes was then monitored with live cell microscopy. Late endosomes are normally motile and move along microtubules in a bidirectional manner, travelling from the cell centre to periphery and back (Ko *et al.*, 2001; Zhang *et al.*, 2001a; Lebrand *et al.*, 2002). When time-lapse images of organelles exhibiting this type of movement are superimposed, they produce a "stellate" pattern of trajectories within a cell. This pattern of endosome movement was observed in the majority of control cells (not, Figure 6). In MLN64 depleted cells, the dispersed organelles were motile too but the pattern of movement was altered. In those cells, DiI-LDL labelled endosomes moved along the cell periphery producing trajectories that outlined the cell (not, Figure 6). The endosome movement could be inhibited with the microtubule depolymerising agent nocodazole in both control and MLN64 depleted cells. This implies that despite altered movement in MLN64 depleted cells, the endosomes were still moving along the microtubules.

In addition to microtubules, the actin cytoskeleton plays a role in the dynamics of late endocytic organelles. Interestingly, the disruption of actin filaments has been reported to result in lost bidirectionality in the movement

of late endocytic organelles as well as their scattering to the cell periphery (Durrbach *et al.*, 1996; Cordonnier *et al.*, 2001). Indeed, upon the treatment of control cells with the actin disrupting drug cytochalasin D, the pattern of endosome movement started to resemble that observed in MLN64 depleted cells (III, Figure 6). This observation raised the question if the modulation of MLN64 affects the association of late endosomes with the actin cytoskeleton.

3.5 MLN64 affects actin-dependent fusion of late endocytic organelles

Actin cytoskeleton has been implicated in the fusion of late endocytic organelles, namely late endosomes with lysosomes and late endosomes with phagosomes or each other (van Deurs *et al.*, 1995; Kjeken *et al.*, 2004). In yeast, homotypic vacuole fusion has been shown to require membrane-bound actin (Eitzen *et al.*, 2002). To study if MLN64 affected the fusion of late endocytic organelles, we monitored the ability of late endosomes/lysosomes to mix in living HeLa cells. This was achieved by labelling the late endosomes/lysosomes with fluorescent dextrans: fluorescein-conjugated for one cell population and rhodamine-conjugated for the other. Cells were then cocultured and fused with polyethylene glycol (PEG). 1 h after PEG treatment, cells were fixed and the degree of fusion of late endocytic organelles was assessed as the degree of red/green signal overlap from confocal micrographs. In control cells, significant colocalization of red and green signals was observed at this time point (III, Figure 7). If the cells were treated with cytochalasin D after PEG treatment, the overlap was considerably reduced. This suggests that disrupting the actin cytoskeleton indeed impairs the ability of late endocytic organelles to fuse *in vivo*. Similarly, MLN64 depletion resulted in a significant reduction in signal overlap (III, Figure 7). In contrast, when cells overexpressing CFP-tagged wild-type MLN64 were fused with cells labelled with red dextran, the red/green overlap was markedly enhanced. This increase in fusion was actin dependent as it was abolished when the cells were treated with cytochalasin D (III, Figure 7). In contrast, ΔSTART overexpression had no significant effect. Together, these results suggest that a) MLN64 depletion impairs fusion of late endocytic organelles similarly to the disruption of actin cytoskeleton and b) MLN64 overexpression enhances fusion of late endocytic organelles in an actin-dependent fashion.

3.6 MLN64 modulates the association of actin and p34-Arc with late endocytic organelles

To further assess the possible link between actin and MLN64, we studied the morphology of the actin cytoskeleton in cells overexpressing or depleted of MLN64. The overall organization of actin was not grossly altered either upon MLN64 depletion or overexpression, as assessed by labelling of filamentous actin with fluorescent phalloidin. However, the association of actin with late endocytic organelles was altered upon modulation of MLN64 protein levels. In control HeLa cells, approximately 20% of late endosomes/lysosomes labelled with fluorescent dextran were decorated with actin patches or extensions (III, Figure 8). This result is in agreement with a recent *in vitro* -study demonstrating that a subset of late endosomes is able to nucleate actin (Kjeken *et al.*, 2004). In MLN64 depleted cells, however, the dextran labelled endosomes were often situated between actin cables but endosome-associated actin patches were only seldom observed (III, Figure 8). In contrast, wild-type MLN64 overexpressing endosomes showed increased actin labelling as compared to control, while ΔSTART overexpression had no significant effect. Thus, we envisioned that MLN64 might be involved in the process leading to actin nucleation in the late endosomal membranes. To further assess this possibility, we analyzed the endosome association of Arp2/3 subunit p34-Arc in MLN64 depleted or overexpressing cells. Arp2/3 complex functions in the regulation of actin polymerization and has been shown to localize to the sites of dynamic actin (Machesky and Gould, 1999). In control cells, p34-Arc antibodies visualized lamellipodia as well as cytoplasmic punctate structures (III, Figure 9). This overall organization was not affected by the modulation of MLN64 protein levels. Similarly to phalloidin labelling, a portion of late endocytic organelles visualized with Lamp1-antibody was decorated with p34-Arc punctae (III, Figure 9). MLN64 depletion resulted in a modest but significant decrease of p34-Arc labelling in late endocytic organelles, whereas MLN64 overexpression increased it. Together, these results indicate that altering MLN64 protein levels modulates the association of actin and p34-Arc with late endocytic organelles.

To analyze the association of late endocytic organelles with the actin cytoskeleton *in vitro*, we fractionated the cells and studied the amount of Lamp2-positive membranes co-sedimenting with actin. To favour the sedimentation of membranes in association with the actin cytoskeleton, the samples were treated with microtubule depolymerising agent nocodazole prior

to pelleting. After a brief centrifugation, a minor fraction of actin and Lamp2 was recovered in the pellet both in control and MLN64 depleted samples (III, Figure 10). While additional treatment with cytochalasin D did not abolish the sedimentation of actin or Lamp2 (data not shown), the fraction of Lamp2 pelleted relative to actin was clearly reduced by this treatment (III, Figure 10). Similarly to actin disruption, the amount of Lamp2 sedimented was decreased upon MLN64 depletion as compared to control. These results suggest that MLN64 depletion reduced the association of Lamp2-positive organelles with the actin cytoskeleton.

DISCUSSION

1. Rab proteins in regulation of late endosomal cholesterol trafficking

The process of cholesterol efflux from the late endosomes and/or lysosomes is still poorly characterized. The majority of LDL-derived cholesterol is eventually found on the plasma membrane (Cruz *et al.*, 2000; Lusa *et al.*, 2001). Elevated cholesterol esterification upon LDL influx suggests that ER cholesterol is increased, too (Brown and Goldstein, 1986). However, little is known of the precise trafficking itineraries that cholesterol follows *en route* to these end destinations. Vesicular trafficking has been one suggested mechanism: tubulating membranous carriers have been visualized to emerge from late endocytic organelles, extending all the way to the cell surface and the ER (Ko *et al.*, 2001). These carriers could deliver cholesterol with them. Our observation that late endosomal cholesterol egress could be inhibited with excess Rab-GDI argues that Rab-mediated vesicular transport processes operate in the removal of late endosomal cholesterol. In addition, upon the clearance of the late endosomal cholesterol accumulation by NPC1 protein in NPC1 deficient cells, we observed a redistribution of other late endosomal/lysosomal lipid and protein constituents (LBPA and ACA, respectively). This would further link the trafficking of cholesterol to the trafficking of other lipid and protein cargo from late endocytic organelles.

This phenotype was not observed in control cells overexpressing NPC1. This suggests that it was the unloading of massive cholesterol accumulation that, perhaps transiently, altered the balance between forward transport and retrieval of cargo in the late endosomes. In contrast, the localization of Lamp1 residing on the outer membrane of the endosomes was not affected by cholesterol unloading. It is possible that this variation just reflects differences in sorting mechanisms: Lamp1 may simply be retrieved more efficiently than LBPA. On the other hand, both LBPA and cholesterol are enriched in the internal membranes of late endocytic organelles (Kobayashi *et al.*, 1999; Möbius *et al.*, 2003). It is possible that upon the cholesterol release, intraendosomal components are differentially affected in comparison to the residents of the limiting membrane.

Rab7 and Rab9 are located in late endosomes and are thus possible candidates for participating in the egress of cholesterol from these organelles. Indeed, in a recent study it was reported that the late endosomal cholesterol

accumulation in NPC1 deficient cells could be overcome by overexpressing either of these two Rab proteins (Choudhury *et al.*, 2002). Later, in another study it was stated that NPC1 deficient cells could be complemented with Rab9 but not with Rab7 (Walter *et al.*, 2003). At the moment this disagreement has not been settled, but differences in the cell and expression systems used have been pointed as one possible reason (Walter *et al.*, 2003). Presumably the overexpression of Rab9 could enhance the late endosome to TGN transport. Thus, it seems feasible that enhancing this transport step would allow cholesterol to recycle more efficiently from late endosomes via the TGN. With Rab7 the interpretation seems a bit more complicated: Rab7 has been suggested to deliver cargo either from early to late endosomes or, alternatively, regulate lysosome biogenesis from late endosomes (Press *et al.*, 1998; Bucci *et al.*, 2000). So far, Rab7 has not been associated with any pathway leading out from the late endosomal compartments. Furthermore, overexpression of Rab7 has been shown to immobilize late endosomes much like what is observed in NPC1 deficient cells (Lebrand *et al.*, 2002). In our studies with COS cells, overexpression of Rab7 resulted in the inhibition of cholesterol esterification when cells were supplied with LDL. These results would seemingly argue against Rab7 participating in the clearance of late endosomal cholesterol. However, comparing the effects of Rab overexpression in normal cells and cells exhibiting late endosomal transport problems (like in NPC disease) may not be feasible. In the latter case, augmenting any membrane movement in the jammed late endosomes might provide enough flexibility for other mechanisms to stand in (Pagano *et al.*, 2000).

Overexpression of endocytic Rab proteins has been suggested as a possible treatment for NPC disease (Choudhury *et al.*, 2002). For the future it would be interesting to see whether there is a mechanistic connection between the Rab-regulated vesicular transport machinery and NPC proteins. At this moment, no such molecular interaction has been established. In addition, it must be noted that the clearance of late endosomal cholesterol deposits, as assessed by filipin staining in NPC patient cells, does not necessarily mean that the molecular defect in NPC has been corrected. While the exact function of the NPC proteins remains unknown, further characterization of Rab overexpression in NPC cells is required to assess the suitability of Rab proteins as a potential cure for NPC patients.

2 Recycling endosomes and cholesterol homeostasis

Recycling endosomes have been shown to be enriched in cholesterol and other raft lipids (Cagesca *et al.*, 2000; Hao *et al.*, 2002). In our study, overexpression of Rab11 in COS cells resulted in a massive enlargement of tubular organelles containing recycling cargo (transferrin and transferrin receptor) as well as substantial amounts of cholesterol, as shown by the intense filipin staining of Rab11 organelles. Concomitantly, a marked decrease in the rate of cholesterol esterification was observed in Rab11 overexpressing cells. These results suggest that the Rab11-regulated recycling pathway participates in the regulation of cellular cholesterol homeostasis. This pathway did not seem to interfere with the trafficking of LDL-derived cholesterol, as judged by several criteria. First, in Rab11 expressing cells, the trafficking of DiI-labelled LDL particles to late endocytic organelles was not compromised. In contrast, in cells expressing the GTPase deficient mutant of Rab5, DiI-LDL was confined to enlargened early endosomes and was not transported normally to late endocytic organelles. Thus, in the case of Rab5, it is possible that the inhibition in the esterification was partially due to the impaired trafficking of LDL cholesterol. Second, in cells depleted of lipoproteins, the rate of esterification was low but Rab11 was still able to further suppress it. The enlargened tubular endosomes collected cholesterol even in the absence of LDL. Finally, the characterization of Rab11 overexpression in NPC1 deficient fibroblasts revealed that 1) filipin-positive Rab11 organelles were formed in spite of the late endosomal cholesterol jam and 2) Rab11 overexpression did not impair the egress of late endosomal cholesterol upon NPC1 overexpression. Together, these results suggest that Rab11-regulated pathway for cholesterol endocytosis is distinct from the LDL-cholesterol pathway. It seems more likely, that the recycling compartment handles the bulk of cholesterol flow from the plasma membrane.

Rab11 is a regulator of recycling membrane trafficking to the cell surface (Ullrich *et al.*, 1996; Ren *et al.*, 1998). We therefore hypothesized that the impaired esterification upon Rab11 overexpression could be primarily due to defective recycling of cholesterol to the plasma membrane. This hypothesis was supported by an earlier study showing that a mutant form of the Rme-1 protein that retards the recycling of transferrin, also slowed the delivery of cholesterol analogue DHE to the cell surface (Hao *et al.*, 2002). Upon overexpression of Rab11 the recycling endosomes were dramatically

expanded in COS cells. As the membranes of recycling endosomes are thought to have high affinity for cholesterol (Hao *et al.*, 2002), they might be able to accommodate accumulating cargo. In such a scenario, even a modest stagnation in recycling could eventually lead to substantial retention of cholesterol within the recycling endosomes. By filipin staining, the plasma membrane of Rab11 expressing cells did not appear grossly depleted of cholesterol. However, already small changes in the plasma membrane cholesterol content have been shown to lead to large responses in the ER (Lange *et al.*, 2004). If Rab11 overexpression in fact retarded cholesterol delivery to the cell surface, one would assume that by plasma membrane cholesterol loading, the Rab11-induced esterification block could be bypassed (Figure 6). Indeed, in cells supplemented with cholesterol-methyl-β-cyclodextrin complex, a rapid and substantial increase in the rate of cholesterol esterification was observed. This increase was not inhibited by Rab11.

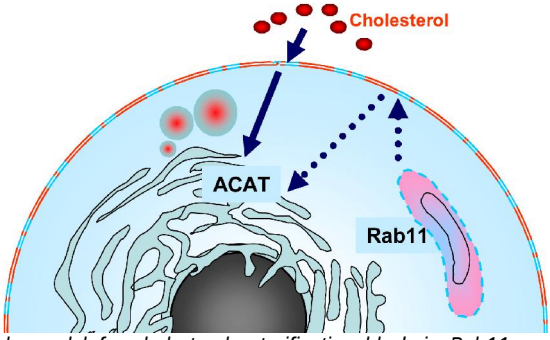

Figure 6. Possible model for cholesterol esterification block in Rab11 overexpressing cells. Rab11 overexpression leads to enlargement of recycling endosomes and stagnation of cholesterol flow to the plasma membrane, which in turn suppresses cholesterol esterification. If cholesterol (indicated in red) is added to the plasma membrane, the block is bypassed.

It is conceivable that several cellular pathways are able to feed ACAT with cholesterol. A pathway transporting cholesterol from late endosomes to the ER bypassing the plasma membrane has been described (Neufeld *et al.*, 1996). From the plasma membrane, cholesterol can be delivered to the ER through a vesiculation process that appears to be energy-independent (Skiba *et al.*, 1996). It is possible that cholesterol from cyclodextrin gets esterified via this pathway as it was not inhibited by Rab11 overexpression. Moreover, in lipoprotein-deprived conditions, overexpression of Rab6 resulted in a small

increase in the rate of cholesterol esterification. In Golgi-to-ER retrograde transport, at least two independent pathways have been implicated: one dependent on the cytosolic coat protein COP-I, the other COP-I independent but specifically regulated by Rab6 (Girod *et al.*, 1999). Cholesterol has been shown to be segregated away from COP-I coated vesicles (Bragger *et al.*, 2000). It is tempting to speculate that Rab6-vesicles would then serve as preferential membrane carriers for cholesterol from the Golgi back to the ER. Increased delivery of cholesterol to the ER by Rab6 overexpression would then lead to increased esterification.

The idea of the differential sorting of lipids along the endocytic pathway is well appreciated, although the mechanisms by which it is achieved have not been conclusively defined. Recycling organelles have been shown to be enriched with other raft lipids in addition to cholesterol, such as sphingomyelin (Cagesca *et al.*, 2000) and GM1 ganglioside, as assessed by the distribution of cholera toxin subunit B that binds to it (Nichols *et al.*, 2001). In agreement, we found that a portion of pyrene-labelled sphingomyelin was enriched in the enlargened Rab11 organelles, as well as the bulk of internalized cholera toxin. In addition, the glycolipid sulfatide was redistributed to Rab11 organelles in a fraction of overexpressing cells. In contrast, Rab11 overexpression did not affect the distribution of globotriaocyl ceramide on the plasma membrane, early endosomal PI(3)P or the late endosomal/lysosomal lipids LBPA and GM2 ganglioside. Thus, the Rab11-induced lipid accumulation in recycling endosomes did not result in a general missorting of lipid constituents along the endocytic pathway. A recent study reported that in NPC1 deficient cells, in addition to late endosomes also early endosomes harbour cholesterol in excess (Choudhury *et al.*, 2004). It was shown that in those cells, Rab4-mediated direct recycling from early endosomes to the cell surface was inhibited. This defect was proposed to be due to impaired extraction of Rab4 by GDI: the *in vitro* extractability of Rab4 was perturbed when cholesterol was present in excess in the membrane. In the same conditions, GDI-mediated extraction of Rab5 and Rab11 was not inhibited (Choudhury *et al.*, 2004). In an earlier study, Rab7 extraction to GDI was shown to be suppressed in excess cholesterol (Lebrand *et al.*, 2002). These results seem interesting as Rab proteins are emerging as organizers of membrane domains within organelles (Zerial and McBride, 2001). If some Rab proteins are more "tolerant" to high cholesterol than others in terms of

their activity cycle, it could have significance in directing the cholesterol flow to certain pathways at the expense of others.

In summary, our data reveal a significant role for recycling endosomes in regulating cellular cholesterol balance. The cholesterol pool flowing through the Rab11-mediated pathway does not seem to derive from LDL, at least directly. Instead, our results point to the importance of continuous membrane cycling through the ERC in the maintenance of cellular cholesterol homeostasis. The cholesterol transport defect observed in Rab11 overexpressing cells shares some similarities with the NPC defect, namely accumulation of cholesterol to the endocytic compartments and impaired cholesterol esterification. This further strengthens the idea that endocytic trafficking and cellular cholesterol balance are interconnected.

3. MLN64 in late endosomal dynamics

When MLN64 was first identified as a resident of late endosomal membranes it was an immediate candidate for a protein unloading the late endosomal cholesterol to other cellular acceptors (Alpy *et al.*, 2001; Strauss *et al.*, 2002). However, when a mouse harbouring a mutant MLN64 incapable of cholesterol binding was studied, no appreciable alterations in cholesterol homeostasis were observed. In addition, the mice were fertile suggesting that MLN64 has no indispensable role in steroid production as does StAR (Kishida *et al.*, 2004). In accordance, we observed no impeding effects on cellular cholesterol homeostasis when cells were depleted of MLN64 by RNAi. Importantly, no NPC-like accumulation of free cholesterol in late endocytic organelles was observed, suggesting that MLN64 is not at least solely responsible for clearing endocytosed cholesterol from late endocytic organelles.

It has previously been reported that upon overexpression of ΔSTARD MLN64, i.e. a construct lacking cholesterol-binding STARD domain, leads to the accumulation of cholesterol in late endocytic organelles in COS cells (Zhang *et al.*, 2002). This led the authors to suggest that ΔSTARD MLN64 would have a dominant negative effect on late endosomal sterol transport. We observed that in COS cells also the wild-type protein was able to induce sterol deposition in the enlargened endosomes harbouring the protein. Moreover, when cells were fed the fluorescent sterol DHE, the amount of sterol deposited in wild-type MLN64 overexpressing endosomes was even greater as

compared to the cholesterol-binding deficient mutants of MLN64. As this effect was observed only with prolonged incubation, it cannot be concluded that we were observing a direct sterol transport by MLN64. It is possible that the sterol deposition could at least partially be explained by membrane engulfment as the organelles enlarged. Nonetheless, the wild-type protein did facilitate the sterol deposition more efficiently than the ΔSTARD or M307R, N311D mutants. Interestingly, in NPC1 deficient cells harbouring late endosomal cholesterol accumulation, wild-type MLN64 seemed to induce endosome enlargement more efficiently than ΔSTARD. These results speak against the idea that the function of MLN64 was mainly to clear the endosomes out of cholesterol. It has been shown that upon overexpression, MLN64 was able to promote cholesterol transport to steroidogenic mitochondria (Zhang *et al.*, 2002). Our results suggest that additionally, MLN64 may promote the transfer of cholesterol within or between endosomal membranes.

Depletion of MLN64 protein in HeLa cells resulted in a striking redistribution of late endocytic cargo bearing organelles to the cell periphery with a concomitant decrease in the late endosomal/lysosomal degradation. The dispersal could be overcome by reintroducing the wild-type protein but not with the sterol-binding deficient mutants. This result pointed to the possibility that MLN64-mediated sterol transfer might participate in the regulation of membrane trafficking. Late endosomes are thought to consist of a "mosaic of domains" with differential protein and lipid compositions (Gruenberg, 2001). The late endosomal Rab proteins Rab7 and Rab9 have been shown to localize in distinct domains on late endosomal membranes (Barbero *et al.*, 2002). Interestingly, cholesterol loading of late endosomes has been shown to modulate their association to microtubules through the Rab7-RILP-dynein machinery, thus affecting endosome positioning (Lebrand *et al.*, 2002). However, we were not able to demonstrate any impairment in the membrane recruitment of Rab7 or dynein in MLN64 depleted cells. Moreover, the endosome dispersion could not be rescued upon RILP overexpression, suggesting that the molecular mechanism underlying organelle dispersion in MLN64-depleted cells is distinct from the Rab7-RILP-dynein machinery.

In addition to microtubules, the actin cytoskeleton participates in the regulation of late endosomal dynamics. Both in yeast and mammalian cells, actin has been shown to participate in the fusion of late endocytic organelles

(van Deurs *et al.*, 1995; Kitzen *et al.*, 2002; Kjeken *et al.*, 2004). Disruption of filamentous actin has been shown to result in altered motility and redistribution of late endocytic organelles to the cell periphery as well as inhibition of late endosomal degradation (van Deurs *et al.*, 1995; Durrbach *et al.*, 1996; Cordonnier *et al.*, 2001). The MLN64 depletion phenotype mimicked actin disruption in regard to late endosomal dynamics in all these respects. In addition, overexpression of MLN64 resulted in enhanced endosome fusion in an actin-dependent manner. Furthermore, we observed that MLN64 depletion decreased the late endosome association of both actin and p34-Arc, a component of Arp2/3 complex that participates in actin nucleation (Machesky and Gould, 1999). In contrast, upon overexpression of MLN64, both actin and Arp2/3 labelling in MLN64 expressing endosomes was increased.

Together, these data point to the idea that MLN64 participates in the regulation of actin association with late endosomal membranes. In mammalian cells, components of the machineries linking late endosomes to the actin cytoskeleton are largely unknown. One myosin (myosin II alpha) has been shown to localize in late endocytic organelles (Raposo *et al.*, 1999). On the other hand, in yeast cells, it has recently been shown that the sterol enrichment of vacuolar membranes promotes actin remodelling and enhances vacuolar fusion (Wedlich *et al.*, 2004). The connection between actin cytoskeleton and membrane cholesterol content has been implicated in several studies (Manes and Martinez-A, 2004). It seems feasible that in mammalian cells, MLN64 modulates locally the cholesterol content of the late endosomal membrane, thereby affecting the recruitment of the actin nucleation machinery and actin itself on late endosomes (Figure 7). This mechanism could involve yet additional components such as phosphoinositides that are known regulators of actin dynamics and sensitive to the cholesterol content in the membrane (Kwik *et al.*, 2003).

In the future, identification of the interaction partners for MLN64 will hopefully help to elucidate the exact molecular sequence of events on the late endosomal membrane in relation to actin dynamics. In addition, further studies are needed to address the question of endosomal domains and the potential role of MLN64 in organizing them. Remodelling of the local cholesterol content might also have actin-independent effects of late endosomal dynamics: for instance, cholesterol has been shown to participate in organizing clusters of SNARE proteins required for vesicle fusion (Lang *et*

al., 2001). Could there in fact be a connection between MLN64 and Rab proteins? While answers to these questions are not yet available, based on the studies described here it can be concluded that cholesterol balance and the endocytic pathway both affect and are affected by one another in a profound way.

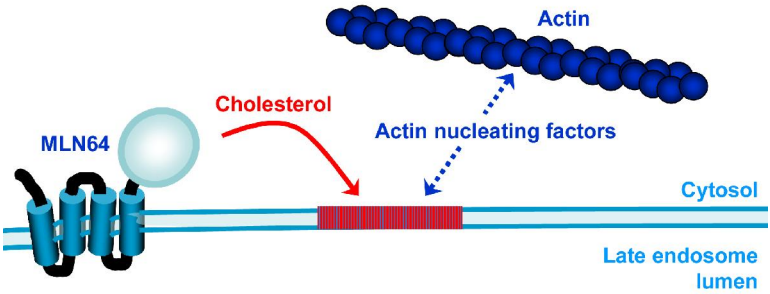

Figure 7. Suggested model for MLN64 action on the late endosome membrane. MLN64 modulates locally the membrane cholesterol content, creating a platform for actin recruitment. It is possible that this association involves additional lipid or protein components.

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