Role for LAMP-2 in endosomal cholesterol transport

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Abstract

The mechanisms of endosomal and lysosomal cholesterol traffic are still poorly understood. We showed previously that unesterified cholesterol accumulates in the late endosomes and lysosomes of fibroblasts deficient in both lysosome associated membrane protein-2 (LAMP-2) and LAMP-1, two abundant membrane proteins of late endosomes and lysosomes. In this study we show that in cells deficient in both LAMP-1 and LAMP-2 (LAMP-/-/LAMP-/-), low-density lipoprotein (LDL) receptor levels and LDL uptake are increased as compared to wild-type cells. However, there is a defect in esterification of both endogenous and LDL cholesterol. These results suggest that LAMP-/-/LAMP-/- cells have a defect in cholesterol transport to the site of esterification in the endoplasmic reticulum, likely due to defective export of cholesterol out of late endosomes or lysosomes. We also show that cholesterol accumulates in LAMP-2 deficient liver and that overexpression of LAMP-2 retards the lysosomal cholesterol accumulation induced by U18666A. These results point to a critical role for LAMP-2 in endosomal/lysosomal cholesterol export. Moreover, the late endosomal/lysosomal cholesterol accumulation in LAMP-/-/LAMP-/- cells was diminished by overexpression of any of the three isoforms of LAMP-2, but not by LAMP-1. The LAMP-2 luminal domain, the membrane-proximal half in particular, was necessary and sufficient for the rescue effect. Taken together, our results suggest that LAMP-2, its luminal domain in particular, plays a critical role in endosomal cholesterol transport and that this is distinct from the chaperone-mediated autophagy function of LAMP-2.

Keywords: LAMP-2 • LAMP-1 • cholesterol • LDL • lysosome • late endosome • NPC2

Introduction

Lysosomes are acidic, membrane-bound organelles rich in hydrolytic enzymes, responsible for the degradation of macromolecules derived from the extracellular space through endocytosis or phagocytosis, and from the cytoplasm through autophagy. The membrane limiting late endosomes and lysosomes separates the potent activities of lysosomal acid hydrolases from other cellular constituents [1]. Several highly glycosylated proteins of the lysosomal membrane have been identified, but the specific functions of these proteins are largely unknown [2].

Lysosome associated membrane protein-1 (LAMP-1) and LAMP-2 are major protein components of the lysosomal membrane. They are type I transmembrane proteins with a large heavily

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glycosylated luminal domain, one transmembrane domain and a C-terminal cytoplasmic tail. The conserved cytosolic tails of LAMP-1 and LAMP-2 are 11 residues long and contain necessary information for their intracellular targeting after biosynthesis [3]. Despite their 37% amino acid sequence homology, LAMP-1 and LAMP-2 are distinct proteins [4]. The molecular mass of the polypeptide backbone of human LAMP-1 and LAMP-2 is 40 to 45 kD; however after glycosylation the mass of the glycoproteins is approximately 120 kD [5, 6]. N-glycosylation seems to be important for the stability of the proteins [7, 8]. Both LAMPS also have O-linked carbohydrates in the ‘hinge’ region of the luminal domain [9]. LAMP-2 undergoes alternative splicing that produces three isoforms called LAMP-2A, LAMP-2B and LAMP-2C [10].

Mutations in LAMP-2 gene cause Danon disease, a fatal cardiomyopathy and myopathy associated with mental retardation. The disease is characterized by the accumulation of late autophagic vacuoles in the heart and skeletal muscle [11, 12]. Most of the detected mutations lead to complete or nearly complete absence of LAMP-2 protein in muscle tissue.

To address the specific functions of LAMP-1 and LAMP-2, we generated mice deficient in each of these proteins. LAMP-1 deficient mice showed a very mild phenotype, and lysosomal properties were similar to controls in LAMP-1 deficient cells [13]. However, deficiency of LAMP-2 caused a severe phenotype. Fifty percent of the mice died at the age of 20 to 40 days [14]. Electron microscopy revealed a massive accumulation of autophagic vacuoles in several tissues including liver, pancreas, kidney, skeletal muscle, heart and neutrophilic leucocytes. We also generated mouse embryonic fibroblast (MEF) lines double deficient in both LAMP-1 and LAMP-2. The double deficient MEFs showed increased accumulation of late autophagic vacuoles and a prominent accumulation of unesterified cholesterol in late endosomes/lysosomes [15]. MEFs deficient in LAMP-1 showed no cholesterol accumulation, while MEFs deficient in LAMP-2 showed an accumulation that was intermediate to the LAMP double deficient (LAMP\(^{-/-}\)) and wild-type MEFs.

Niemann-Pick C (NPC) disease is a neurodegenerative disorder characterized by a late endosomal/lysosomal accumulation of unesterified cholesterol and other lipids [16]. At cellular level the phenotype thus resembles the LAMP\(^{-/-}\) MEFs. NPC disease is caused by mutations in either gene encoding NPC1 or NPC2. NPC1 is a large multispans membrane protein localized in late endosomes, while NPC2 is a small soluble protein localized in lysosomes and late endosomes. NPC1 and NPC2 proteins are thought to function in cholesterol transport out of late endosomes/lysosomes [17–19]. Although the phenotypes of NPC patient fibroblasts and LAMP-2 deficient fibroblasts resemble each other, the symptoms of NPC disease and Danon disease are different. NPC patients have hepatosplenomegaly and a neurologic disorder consisting of cerebellar ataxia, dysarthria, dysphagia and progressive dementia [16]. The symptoms of Danon disease, caused by mutations in \(\text{lamp}-2\), include hypertrophic cardiomyopathy, myopathy and a variable extent of mental retardation [20]. So far, there are no reports on cholesterol accumulation in Danon patients.

The aim of the present study was to further elucidate the role of LAMP-1 and LAMP-2 in intracellular cholesterol traffic. Our data support the conclusion that LAMP proteins, LAMP-2 in particular, are involved in the export of cholesterol out of late endosomes/lysosomes.

**Materials and methods**

**Cell lines**

MEF lines were generated as described [15]. Cells were grown in Dulbecco’s MEM containing 10% foetal calf serum and antibiotics. At least two independent cell lines for each genotype were used in most experiments.

**Biochemical cholesterol assays**

For analysis of liver and serum cholesterol levels, LAMP-2 deficient and wild-type mice (4–5 months old) were starved for 4 hrs. Lipids were extracted from liver [21] and total cholesterol was measured using an enzymatic cholesterol assay [22] (Roche Diagnostics, Mannheim, Germany). To analyse serum lipoprotein distribution, plasma samples from four mice per group were pooled and separated by fast performance liquid chromatography using a Superox 6 10/30 column (GE Healthcare, Hamburg, Germany). The fractions were analysed for cholesterol concentration as above.

**DNA constructs and transfections**

LAMP-1 and LAMP-2A cDNAs were subcloned into pCneo (Promega, Mannheim, Germany). Mouse LAMP-2A cDNA was inserted using NotI restriction site. Together with the LAMP-2A cDNA, restriction sites for SacII and EcoRI were inserted upstream, and for EcoRI and SpeI downstream, of LAMP-2A. To construct mouse LAMP-2B and LAMP-2C in pCneo, the part common for all three LAMP-2 cDNA sequences was amplified by PCR as EcoRI-XbaI fragment and inserted in front of fragments containing the unique sequences for LAMP-2B and LAMP-2C in pBlueScriptSK. LAMP-2 XbaI-Apal fragment was excised from pAMC63 containing mouse LAMP-2B (from Ana Maria Cuervo, Albert Einstein College of Medicine, NY, USA) and LAMP-2C XbaI-Apal fragment was assembled from synthetic oligonucleotides by overlap extension PCR. Both genes were then recloned into pCneo as EcoRI-Apal fragments and inserted in front of fragments containing the unique sequences for LAMP-2B and LAMP-2C in pBlueScriptSK. LAMP-2 XbaI-Apal fragment was excised from pAMC63 containing mouse LAMP-2B (from Ana Maria Cuervo, Albert Einstein College of Medicine, NY, USA) and LAMP-2C XbaI-Apal fragment was assembled from synthetic oligonucleotides by overlap extension PCR. Both genes were then recloned into pCneo as EcoRI-Apal fragments. The unified nomenclature recommended by Eskelinen et al. [10] is used for the mouse LAMP-2 isoforms.

The chimeric constructs between LAMP-1 and LAMP-2A were generated by fusion PCR, using full length rat LAMP-1 (from Stefan Höning, University of Cologne, Germany), mouse LAMP-1 and mouse LAMP-2A cDNAs as templates. The chimeric constructs are schematically presented in Fig. 5F, and the boundaries between LAMP-1 and LAMP-2 are presented in Table 1.

Green fluorescent protein (GFP) tagged Rab7 and RILP constructs in pEGFP-C (Invitrogen, Paisley, UK) were from Cecilia Bucci (University of Lecce, Italy), GFP tagged Rab8 and Rab9 constructs in pEGFP-C plasmid were from Johan Peränen (University of Helsinki, Finland) and Suzanne
Table 1 Construction of the LAMP-1/LAMP-2 chimeras

| Chimera | Luminal domain | Transmembrane domain* | Cytoplasmic domain* | Vector | Inserted with |
|---------|----------------|------------------------|---------------------|--------|---------------|
| #1      | M1-N379 mLAMP-2A | M372-I395 rLAMP-1      | G396-I407 rLAMP-1   | pCIneo | NotI          |
| #2      | M1-M372 rLAMP-1  | F380-G404 mLAMP-2A     | L405-F415 mLAMP-2A  | pCIneo | XhoI          |
| #3      | M1-N379 mLAMP-2A | F380-G404 mLAMP-2A     | G396-I407 rLAMP-1   | pCIneo | NotI          |
| #4      | M1-N371 rLAMP-1  | M372-I395 rLAMP-1      | L405-F415 mLAMP-2A  | pCIneo | XhoI          |
| #5      | M1-W94 mLAMP-2A K96-N371 rLAMP-1 | M372-I395 rLAMP-1 | G396-I407 rLAMP-1 | pCIneo | NotI          |
| #6      | M1-T190 mLAMP-2A S192-N371 rLAMP-1 | M372-I395 rLAMP-1 | G396-I407 rLAMP-1 | pCIneo | NotI          |
| #7      | M1-T298 mLAMP-1  | Y307-N373 mLAMP-2A     | G396-I407 rLAMP-1   | pcDNA3.1/Zeo(-) | EcoRI 5’ BamHI 3’ |
| #8      | M1-P190 mLAMP-1  | P191-N379 mLAMP-2A     | G396-I407 rLAMP-1   | pcDNA3.1/Zeo(-) | EcoRI 5’ BamHI 3’ |

* The amino acid sequences of rat (r) and mouse (m) LAMP-1 are identical in the transmembrane and cytoplasmic domains.

Pfeffer (Stanford University, CA, USA), respectively. Transfections were performed with Fugene 6, Fugene HD (Roche), Lipofectamine 2000, or Lipofectamine LTX (Invitrogen) according to manufacturer’s instructions.

Antibodies

The following primary antibodies were used: rat antismouse LAMP-1 and rat antismouse LAMP-2 (1D4B and Ab1B9, respectively, Developmental Studies Hybridoma Bank, IA, USA); rabbit anti-rat LAMP-1 and rabbit anti-rat lysosomal integral membrane protein (LIMP)-2/LGP-85 (Yoshitaka Tanaka, University of Kyushu, Japan); mouse anti-BMP (also called anti-LBPA, Jean Gruenberg, University of Geneva, Switzerland), rabbit anti-human NPC2 (Shutish Patel, Newington, USA), and rabbit antismouse NPC2 generated against a mixture of peptides CKDKVYSYLNKLPVKC and KLVVEWLEDQDKNLFC. Anti-LDLR (low-density lipoprotein [LDL] receptor) was from R&D Systems (Wiesbaden, Germany), and anti-β-actin was from Sigma (Munich, Germany). Anti-LRP1 (LDL receptor related protein 1) has been described [23].

Immunofluorescence

Cells were grown on cover slips and fixed in cold methanol or 4% paraformaldehyde in phosphate buffered saline (PBS). For NPC2 staining, the cells were fixed in Bouin’s fixative (picric acid:formalin:acetic acid 15:5:1). After aldehyde fixation, cells were permeabilized in 0.1% Triton X-100 or 0.2% saponin in PBS. Primary and secondary antibodies were diluted in 3% bovine serum albumin (BSA) in PBS without or with saponin. Goat anti-rabbit, rat, or mouse conjugated to Alexa Fluor 488 or 594 (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. Filipin or Nile Red staining were done as described [15]. The samples were viewed with one of the following fluorescence microscopes: ZEISS Axiovert 200M (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with or without an Apotome device for optical sectioning, Olympus AX79 fluorescence microscope (Olympus Corporation, Tokyo, Japan), or Leica TCS SP 5 confocal microscope (Leica Microsystems CMS GmbH, Mannheim, Germany).

For filipin staining of liver sections, mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The liver was dissected and post-fixed in the same fixative overnight at 4°C. Vibratome sections (35 μm thick) were cut, permeabilized in 0.2% saponin/PBS, and incubated in filipin (0.05 mg/ml) for 20 min. Finally the sections were washed in 0.02% saponin/PBS and in PBS, and mounted in Prolong antifade mounting solution (Invitrogen, Karlsruhe, Germany).

Quantification of cells with late endosomal/lysosomal cholesterol accumulation or lipid droplets

Cells were stained with filipin and anti-LAMP-2 or anti-LAMP-1. Cells expressing LAMPs at levels similar to endogenous proteins were screened using the immunofluorescence staining. Photographs were taken with 60× objective and filter sets detecting the immunofluorescence and filipin stainings, and used for the quantification. The intensity and localization of filipin staining was scored for the presence or absence of late endosomal/lysosomal cholesterol accumulation as demonstrated in Fig. 5A–D. A minimum of 50 cells for each sample were scored in each experiment. The percentage of cells with lipid droplets was estimated using a similar procedure. Cells with one or more brightly stained droplets were scored positive.

Lipid analysis by thin layer chromatography

The lipids were extracted in chloroform:methanol:water (2:5:1, v/v/v) at 37°C for 24 hrs. Same amount of cell protein was applied to each lane on the thin layer chromatography (TLC) plates. The chromatograms were developed in chloroform/methanol/glacial acetic acid (190:9:1). To visualize
the lipids, the TLC plates were sprayed with an aqueous solution containing 8% H2PO4 and 10% CuSO4, charred for 10 min. at 140°C or at 180°C for sterols or the other lipids, respectively, and quantified by scanning densitometry at 595 nm (Shimadzu CS 9301 PC, Kyoto, Japan). For metabolic labelling, fibroblasts were fed with [1-14C] acetate (cholesterol) or [14C] serine (sphingolipids) (61 Ci/mol; 1 μCi/ml, Amershams, Braunschweig, Germany) in a medium containing 0.3% foetal calf serum for 48 or 24 hrs, respectively. Same amounts of radioactivity or cell protein were applied to each lane on the TLC plates. The chromatograms were developed in chloroform/methanol/glacial acetic acid (190:9:1) for cholesterol and chloroform/methanol/0.2% CaCl2 in water (60:35:8) for sphingolipids. Radioactive bands were visualized with a Bio Imaging Analyser 1000 (Fuji, Japan), and quantification was performed with Tina software (Raytest, Staufenhardt, Germany). Lipids were identified and quantified using commercially available standards.

**LDL uptake and degradation**

LDL was prepared from plasma of normolipidemic donors by two sequential density-gradient ultracentrifugations in KBr gradients [24]. LDL was radiolabelled with 125I by the iodine monochloride method [25]. Reproducibly 95% of the radiolabelled LDL was trichloroacetic acid-pre- cipitable. The protein content of 125I-LDL in two different labelled preparations was 1.1 ± 0.1 mg/ml. To determine 125I-LDL internalization, MEFS were washed with PBS and incubated with 125I-LDL (5-50 μg/ml; in triplicates) in Dulbecco’s minimal essential medium (DMEM), 1% BSA. Cells were then incubated for 4 hrs at 37°C. After incubation, the medium was harvested and the protein-associated radioactivity was removed by trichloroacetic acid precipitation. To determine the radioactivity associated with 125I-tyrosine, free iodine was separated by chloroform extraction [24]. Cells were washed with ice-cold PBS and subsequently with PBS/Heparin (100 units/ml) to remove surface-bound 125I-LDL, and the cell monolayers were solubilized in 0.1 N NaOH. The radioactivity of the medium and cell extract, and protein concentration of the lysate, were determined in order to calculate specific uptake and degradation of LDL.

**Esterification of LDL cholesterol**

Cells were seeded on 3.5 cm dishes and incubated in DMEM supplemented with 5% lipoprotein-deficient serum (LPDS) for 3 days. The cells were washed twice with PBS and incubated in DMEM supplemented with 2% defatted BSA and 5 μg/ml [3H] oleic acid (Perkin Elmer, Waltham, MA, USA), with or without 50 μg/ml LDL prepared as described [26], for 6 hrs. The cells were harvested, and lipids were extracted and cholesterol esterification measured as described previously [27].

**Western blotting**

To analyse lipoprotein receptor expression, cells were lysed in lysis buffer (2 mM CaCl2, 80 mM NaCl, 1% TritonX-100, 50 mM Tris/HCl, pH 8.0). Proteins were separated by SDS-PAGE in 4–12% gradient gels (Invitrogen), transferred to nitrocellulose and incubated with specific antibodies against LDLR, β-actin and LRP1. The secondary horse radish peroxidase-labelled antibodies were from Jackson ImmunoResearch (Dianoova, Hamburg, Germany). Signals were detected with enhanced chemiluminescence. Western blotting of NPC2 was performed as described [15, 28].

**Results**

**LAMP double deficient MEFS have a defect in cholesterol esterification**

We reported earlier that LAMP−/− MEFS show increased total cholesterol levels, accumulate free cholesterol in late endosomes/lysosomes, and contain reduced amounts of lipid droplets [15] (Fig. 1A). We confirmed the latter finding by quantitative analysis which showed that lipid droplets were present approximately 3.5 times more often in wild-type MEFS than in LAMP−/− MEFS (Fig. 1B). Similar results were obtained using Sudan III and Bodipy 493/503 staining (not shown). We also used TLC to analyse the proportions of free and esterified cholesterol. As expected, we observed that in LAMP−/− MEFS, the percentage of free cholesterol was increased to 67.3% when compared to the 46.1% in wild-type MEFS (Fig. 1C). These findings suggest that cholesterol esterification is defective in LAMP−/− MEFS. In order to test this, we labelled the cells metabolically with [14C] acetate to follow the esterification of newly-made cholesterol. The results showed a substantial defect in the esterification of cholesterol in LAMP−/− MEFS (Fig. 1D). The metabolic labelling also suggested that LAMP deficient MEFS synthesized less triacylglycerol than wild-type MEFS (Fig. 1D), which is consistent with the decreased amount of lipid droplets.

**Uptake and esterification of LDL cholesterol in LAMP−/− MEFS**

A defect in cholesterol esterification could be due to a defect in transport of newly-made and/or endocytosed cholesterol from late endosomes/lysosomes to the endoplasmic reticulum, the main site of cholesterol ester formation. Notably, NPC mutant cells also show a defect in cholesterol esterification [29, 30]. Therefore, we decided to analyse the uptake, degradation and esterification of exogenous LDL cholesterol. Cells maintain sterol homeostasis by feedback mechanisms that involve the membrane-bound transcription factor, sterol regulatory element binding proteins (SREBP), as the principal regulator of LDL receptor (LDLR) levels [31]. Low cholesterol level in the endoplasmic reticulum triggers the synthesis of new LDL receptors in order to stimulate the uptake of LDL cholesterol. We compared LDLR expression as well as LDLR mediated uptake and degradation of radiolabelled LDL in wild-type and LAMP−/− MEFS. We observed approximately twofold expression levels of LDLR in LAMP−/− MEFS (Fig. 2A), suggesting that these cells have a defect in transport of cholesterol to the endoplasmic reticulum. In agreement with previous reports on elevated LDLR levels in LDL receptor related protein 1 (LRP1) deficient hepatocytes [32], we observed a concomitant reduction of LRP1 expression in LAMP−/− MEFS (Fig. 2A). As expected, the uptake and degradation of radiolabelled LDL were also increased twofold, in a dose-dependent manner, in the LAMP−/− MEFS (Fig. 2B, C).
We then analysed cholesterol esterification as a response to LDL loading, using an assay that is used in the diagnostics of NPC disease. Cholesterol esterification was measured by incorporation of \(^{3}H\) oleate into cholesteryl esters. MEFs were grown in LPDS for 3 days, and then incubated with \(^{3}H\) oleate in the presence or absence of LDL. As expected, we found that the rate of cholesterol esterification was markedly reduced in LAMP
\(^{-/-}\)/H11002/MEFs, both under lipoprotein deprivation and upon LDL loading (Fig. 2D).

Taken together, our results indicate that the deficiency of LAMP proteins has functional consequences for the regulation of cellular cholesterol homeostasis, which lead to elevated LDLR expression levels, increased LDL uptake and degradation, and decreased cholesterol esterification. The data support the conclusion that this phenotype is due to a defect in cholesterol transport to the endoplasmic reticulum. Since we also observe a prominent accumulation of free cholesterol in late endosomes/lysosomes, we propose that LAMP proteins function in the export of cholesterol out of these organelles.

**Lipid storage in LAMP\(^{-/-}\) MEFS differs from the storage in NPC cells**

NPC patient cells store other lipids, particularly sphingolipids, in addition to cholesterol [33]. A recent study suggested that NPC is a primary sphingosine storage disease that causes secondary storage of sphingolipids and cholesterol [34]. We used TLC to analyse the levels of sphingolipids in wild-type and LAMP\(^{-/-}\) MEFS. The cells were metabolically labelled with \(^{14}C\) serine, and lipids were extracted and analysed using TLC. We detected no significant differences in the levels of sphingomyelin, ceramide and gangliosides between wild-type and LAMP\(^{-/-}\) MEFS (not shown). This suggests that sphingolipids do not accumulate in LAMP\(^{-/-}\) MEFS.

Oxysterols play an important role in cholesterol homeostasis by suppressing cholesterol synthesis and activating the liver X receptors [35]. Certain oxysterols, such as 25-hydroxycholesterol (25-HC), signal cholesterol excess and down-regulate its accumulation through several homeostatic mechanisms including stimulation of cholesterol esterification [36–38]. Production of LDL-derived oxysterols is defective in NPC1 and NPC2 mutant cells, and exogenous oxysterols such as 25-HC reduce or abolish late endosomal/lysosomal cholesterol accumulation and increase cholesterol esterification in NPC mutant cells [29, 39]. We tested the effect of 25-HC on the cholesterol accumulation in LAMP\(^{-/-}\) MEFS. The cells were cultured in the presence of 5 \(\mu\)M or 10 \(\mu\)M 25-HC for 2 days, and stained with filipin to detect free cholesterol. Both 5 \(\mu\)M and 10 \(\mu\)M 25-HC reduced filipin staining on the plasma membrane and in the perinuclear region, but filipin staining of late endosomes/lysosomes was not significantly reduced.
The percentage of cells containing late endosomal/lysosomal cholesterol accumulation decreased only moderately, from approximately 80% in non-treated cells to 69% in cells treated with 10 μM 25-HC (not shown). We also monitored the effect of 25-HC on the level of total cholesterol using the Amplex Red assay [15], and observed that the level of total cholesterol did not change during 25-HC treatment (not shown). The results indicate that, unlike in NPC patient cells, the late endosomal/lysosomal cholesterol accumulation of LAMP−/− MEFs cannot be alleviated by oxysterol treatment.

NPC2 expression and localization in LAMP−/− MEFs

NPC1 and NPC2 proteins are thought to function in cholesterol export from late endosomes/lysosomes. We showed earlier that overexpression of neither NPC1 nor NPC2 was able to decrease the late endosomal/lysosomal cholesterol accumulation in LAMP−/− MEFs [15]. In LAMP−/− MEFs NPC1 was located in the large cholesterol-containing late endosomes/lysosomes, but the expression levels of NPC1 as detected by Western blotting were not different from wild-type MEFs. Therefore, we studied the expression of NPC2 in LAMP−/− MEFs. Western blotting showed substantial variation in NPC2 expression levels between individual cell lines. Quantification of signals from several wild-type and LAMP−/− MEF lines showed that the LAMP−/− MEFs possessed less NPC2 than wild-type MEFs (71% of the wild-type levels), but the difference was not statistically significant (Fig. 3A). Notably, all of the LAMP−/− cell lines, including lines #5 and #79 that showed the highest level of NPC2, showed late endosomal/lysosomal cholesterol accumulation as detected by filipin staining. We also studied the subcellular localization of NPC2 in wild-type and LAMP−/− MEFs. Immunofluorescence staining in wild-type MEFs showed colocalization of endogenous NPC2 with LAMP-2 (Fig. S1). Reexpression of LAMP-2A in the LAMP−/− MEFs did not change the immunofluorescence pattern of endogenous NPC2 (Fig. 3B, left panel), and the re-expressed LAMP-2A colocalized with NPC2.

Fig. 2 Uptake, degradation and esterification of LDL-cholesterol in wild-type and LAMP−/− MEFs. (A) The expression levels of LDLR and LRP1 were determined by Western blotting in non-transformed MEFs and in MEFs transformed using the Simian virus large T antigen (SV). β-actin is shown as a loading control. (B) To determine LDL uptake, MEFs were incubated with 5–50 μg/ml [125I]-LDL for 4 hrs at 37°C. Internalized radioactivity was determined. (C) To estimate the degradation of internalized LDL, the content of [125I]-tyrosine in the supernatant was determined after 4 hrs incubation. In (B) and (C), the values of specific uptake and degradation represent the mean and S.E.M. of three independent experiments. (D) Cholesterol esterification as a response to LDL loading. Cells grown in lipoprotein deprived medium were pulsed for 6 hrs with [3H] oleic acid in the absence (LPDS) or presence (LDL) of 50 μg/mL LDL. The radioactivity incorporated into CE, per microgram of cell protein, was analysed. The bars represent mean and S.E.M. of two to three individual samples.
We also studied the colocalization of NPC2 and BMP (also called LBPA) in wild-type and LAMP−/− MEFs. These antigens showed considerable colocalization that was similar in both genotypes (not shown). These results indicate that the absence of LAMP proteins does not significantly affect the expression level and localization of NPC2.

Elevated cholesterol levels in LAMP-2 deficient liver and circulating LDL

We showed previously that, in LAMP−/− MEFs, the filipin staining (detecting free cholesterol) was increased and the level of total cholesterol was approximately twofold higher than in wild-type MEFs [15]. In LAMP-2 single deficient MEFs, the filipin staining intensity and cholesterol level were intermediate to wild-type and LAMP−/− MEFs. In order to test whether this phenotype would be observed in LAMP-2 deficient tissue, we investigated cholesterol levels in the liver of mice deficient in LAMP-1 or LAMP-2. Quantitative cholesterol assay showed that the level of total cholesterol was approximately twofold in the LAMP-2 deficient liver compared to the wild-type liver, whereas the level of LAMP-1 deficient liver was not different from the wild-type (Fig. 4A). Filipin staining of liver sections showed that LAMP-2 deficient liver accumulated unesterified cholesterol (Fig. 4A). The results indicate that, similar to LAMP-2 single deficient and LAMP double deficient MEFs, LAMP-2 deficiency leads to an accumulation of free cholesterol and increase in total cholesterol also in liver tissue. This finding suggests that LAMP-2 is more critical than LAMP-1 for the liver cholesterol homeostasis.

In order to test whether LAMP-2 deficiency influences the plasma lipoprotein profile in vivo, gel filtration analysis was performed for plasma samples of LAMP-2 deficient mice and LAMP-2 heterozygous littermate controls. Compared to the controls, both female and male LAMP-2 deficient mice showed an approximately 50% increase in cholesterol levels in the plasma LDL fraction (Fig. 4B). Altogether these findings suggest that LAMP-2 plays an important role in cholesterol homeostasis at the organ level, and through plasma LDL cholesterol levels also at the organismal level.

Overexpression of LAMP-2 retards U18666A-induced cholesterol accumulation

U18666A is a steroid and belongs to class 2 amphiphiles. Treatment of cultured cells with this drug causes a phenotype that...
Fig. 4 Expression of LAMP-2 influences cholesterol levels. (A) LAMP-2 deficient liver accumulates cholesterol. Left panel: Total cholesterol was estimated from five wild-type, three LAMP-1−/−, and three LAMP-2−/− livers. The error bars represent standard deviation. The difference between the control and LAMP-2−/− is statistically significant ($P < 0.001$). Middle and right panels: Filipin staining of liver sections from six-month-old wild-type and LAMP-2−/− mice. (B) Gel filtration analysis of plasma lipoprotein profiles of LAMP-2−/− mice and heterozygote littermate controls (please note that lamp-2 gene is located in the Y chromosome). Total cholesterol levels in the fractions are shown. Plasma samples from four mice were pooled for each group. Note that both female and male LAMP-2−/− mice show elevated LDL cholesterol levels. (C, D) Overexpression of LAMP-2A retards the U18666A induced cholesterol accumulation. (C) HeLa cells were transfected with mouse LAMP-2A and grown for 29 hrs. The cells were then treated with increasing concentrations of U18666A for 15 hrs, and processed for filipin staining and immunolabelling with antimouse LAMP-2. Asterisks indicate the LAMP-2A overexpressing cells in the left panels. (D) The percentage of cells with late endosomal/lysosomal cholesterol accumulation was estimated. None, HeLa cells with no overexpression; mLAMP-2A, HeLa cells overexpressing mouse LAMP-2A. The results are the mean and standard deviation from one experiment with two parallel samples. The experiment was repeated with similar results.
Luminal domains of LAMP-2A and LAMP-1 (Fig. 5F, chimeras #1 and #2), and tested their effects on cholesterol accumulation. The correct late endosomal/lysosomal localization of the expressed chimeras was checked by double staining with anti-LIMP-2 or anti-BMP, depending on the eligibility of the antibodies for double labelling. We observed that the chimera containing the luminal domain of LAMP-2 (#1) was able to abolish late endosomal/lysosomal cholesterol accumulation, while the chimera containing LAMP-1 luminal domain (#2) showed no rescue effect (Fig. 5C–E). We also tested chimeras where the cytoplasmic tails of LAMP-2A and LAMP-1 were switched (chimeras #3 and #4). Again, the chimera containing the LAMP-2 luminal domain abolished cholesterol accumulation, while the chimera containing LAMP-1 luminal domain showed no effect (not shown). These results indicate that the luminal domain of LAMP-2, when correctly targeted to late endosomes/lysosomes, is necessary and sufficient to abolish the cholesterol accumulation in LAMP−/− MEFs.

**Re-expression of LAMP-2A, but not LAMP-1, is able to abolish the late endosomal/lysosomal cholesterol accumulation in LAMP−/− MEFs**

We showed earlier that LAMP-2 single deficient MEFs showed a moderate cholesterol accumulation in late endosomes/lysosomes, while LAMP-1 single deficient MEFs were not different from wild-type cells [15]. We wanted to test whether we can rescue the phenotype of LAMP−/− MEFs by re-expression of the missing LAMP proteins. Transient overexpression of LAMP-2A abolished the late endosomal/lysosomal cholesterol accumulation in LAMP−/− MEFs, while rat or mouse LAMP-1 showed no effect (Fig. 5A, B, E, and not shown). Double immunofluorescence labelling was used to check that the expressed proteins were correctly localized in late endosomes/lysosomes. Endogenous LAMP-2 showed nearly complete overlap with endogenous LIMP-2 (Fig. S2A). Our primary antibodies did not allow double labelling of rat LAMP-1 and LIMP-2. However, endogenous LAMP-1 showed a high degree of colocalization with the late endosomal/lysosomal lipid BPI (Fig. S2B). Transiently expressed mouse LAMP-2 and rat LAMP-1 in LAMP−/− MEFs showed similar overlap with endogenous LIMP-2 and BMP, respectively (Fig. 5A, B), indicating that the expressed proteins were correctly located in late endosomes and lysosomes.

**Luminal domain of LAMP-2 is necessary and sufficient for the rescue effect**

Our results showed that overexpression of LAMP-2 abolished cholesterol accumulation in LAMP−/− MEFs, while expression of rat or mouse LAMP-1 was without effect. This allowed us the use of LAMP-2/LAMP-1 chimeric proteins to test which region of the LAMP-2 molecule is critical for the rescue. We first switched the luminal domains of LAMP-2A and LAMP-1 (Fig. 5F, chimeras #1 and #2), and tested their effects on cholesterol accumulation. The correct late endosomal/lysosomal localization of the expressed chimeras was checked by double staining with anti-LIMP-2 or anti-BMP, depending on the eligibility of the antibodies for double labelling. We observed that the chimera containing the luminal domain of LAMP-2 (#1) was able to abolish late endosomal/lysosomal cholesterol accumulation, while the chimera containing LAMP-1 luminal domain (#2) showed no rescue effect (Fig. 5C–E). We also tested chimeras where the cytoplasmic tails of LAMP-2A and LAMP-1 were switched (chimeras #3 and #4). Again, the chimera containing the LAMP-2 luminal domain abolished cholesterol accumulation, while the chimera containing LAMP-1 luminal domain showed no effect (not shown). These results indicate that the luminal domain of LAMP-2, when correctly targeted to late endosomes/lysosomes, is necessary and sufficient to abolish the cholesterol accumulation in LAMP−/− MEFs.

**LAMP-2A and LAMP-2B are equally effective in abolishing the late endosomal/lysosomal cholesterol accumulation**

All three LAMP-2 isoforms have the same luminal domain, while the transmembrane and cytosolic domains differ (Fig. 6A) [10]. We tested the effect of overexpression of each isoform on the cholesterol accumulation in LAMP−/− MEFs. The rescue was equally effective with LAMP-2A and LAMP-2B, while LAMP-2C was less effective (Fig. 6B, C). This may be related to the subcellular localization of LAMP-2C. LAMP-2B showed a prominent colocalization with endogenous LIMP-2, similar to LAMP-2A (Figs 5A and 6B). However, LAMP-2C showed a less complete colocalization with endogenous LIMP-2, and it was also detected on the plasma membrane (Fig. 6B). This may be due to decreased internalization from the plasma membrane, or alternatively to increased recycling from endosomes to the plasma membrane. To conclude, the result shows that all isoforms of LAMP-2 are able to diminish the late endosomal/lysosomal cholesterol accumulation in LAMP−/− MEFs, supporting the role of the LAMP-2 luminal domain in the rescue.

**Membrane proximal half of LAMP-2 luminal domain is critical for the rescue effect**

In order to clarify which region of the LAMP-2 luminal domain is most critical for the rescue effect, we generated chimeras using LAMP-1 as basis, and exchanged varying portion of the luminal domain with the corresponding region of LAMP-2 (see Figs 5F, 7A). Chimeras containing part of the N-terminal half of the luminal domain from LAMP-2 showed no rescue effect on the late endosomal/lysosomal cholesterol accumulation (Fig. 7A, chimeras #5 and #6). Also the chimera containing the fourth disulphide bond region from LAMP-2 showed no rescue effect (Fig. 7A, chimera #7). Only the chimera containing the hinge region and the mem-
brane-proximal half of the luminal domain from LAMP-2 showed a rescue effect (Fig. 7A, chimera #8, amino acids P191 – N379 from LAMP-2). Double immunofluorescence staining with BMP or LIMP-2 showed that all these chimeras were correctly localized in late endosomes and lysosomes (Fig. 7B).

We also constructed chimeras in which the following region of the luminal domain in LAMP-1 was replaced by LAMP-2: amino acids P191-V306; amino acids A95 – N379; and amino acids A95-V306. These chimeras were, however, not targeted to lysosomes.

Fig. 5 Effect of LAMP-2A and LAMP-1 re-expression on late endosomal/lysosomal cholesterol accumulation in LAMP double deficient MEFs. (A–D) LAMP−/− MEFs were transfected with mouse LAMP-2A (A), rat LAMP-1 (B), chimera #1 (C) or chimera #2 (D), as indicated. One day after transfection, the cells were fixed and stained with filipin to detect cholesterol, and with antimouse LAMP-2 or anti-rat LAMP-1 to detect cells that express the transfected protein. Filipin staining (left panels) and LAMP staining (middle panels) are shown for the same fields. Asterisks indicate the expressing cells in the filipin panels. Immunofluorescence staining was used to study the lysosomal localization of the expressed proteins (right panels in A–D). The samples were double stained for LAMP-2 or LAMP-1 (green) and LIMP-2 or BMP (red). (E) The percentage of wild-type and LAMP−/− MEFs containing late endosomal/lysosomal cholesterol accumulation was estimated. The LAMP−/− cells were analysed either without re-expression (None), or when re-expressing LAMP-2A (LA-2), LAMP-1 (LA-1) or one of the LAMP-1/LAMP-2 chimeras #1 or #2. Cells expressing the LAMP protein or chimera were selected for the analysis using LAMP immunofluorescence staining. The results are the mean and S.E.M. from two or three independent experiments. (F) Schematic presentation of the LAMP-1/LAMP-2A chimeras used in this work. Black and green represent LAMP-1 and LAMP-2A, respectively. The red bar represents the lysosomal membrane, with lysosomal lumen above the membrane. The N terminus of LAMP-2 is in the lysosome lumen. The exact location of the boundaries between LAMP-1 and LAMP-2 are presented in Table 1.
and/or they were unstable in lysosomes, since we observed low expression and localization only in the endoplasmic reticulum and/or Golgi apparatus. The same was observed for C185S and C237S mutants were one of the cysteines needed for the disulfide bonds was mutated to serine. Therefore, these chimeras were not used for rescue experiments.

**Overexpression of Rab proteins or RILP does not abolish the cholesterol accumulation**

Overexpression of Rab7 diminishes the cholesterol accumulation in NPC1 mutant cells [41, 42]. We reported earlier that overexpression of wild-type Rab7 had no effect on the late endosomal/lysosomal cholesterol accumulation in LAMP−/− MEFs [15]. We also tested the overexpression of constitutively active Rab7 (Q67L), and dominant negative Rab7 (T22N). Quantitative analysis of late endosomal/lysosomal cholesterol accumulation showed no difference between LAMP−/− MEFs with no overexpression and those overexpressing GFP only, GFP-Rab7, GFP-Rab7Q67L, or GFP-Rab7T22N (not shown).

The cholesterol-loaded late endosomes/lysosomes in LAMP−/− MEFs localize to the cell periphery [15]. We tested whether normalization of the late endosome/lysosome localization by overexpression of the Rab7 effector RILP would have an effect on the cholesterol accumulation. RILP overexpression caused concentration of filipin-positive vesicles to the perinuclear region both in wild-type and LAMP−/− MEFs (Fig. S3A, B). In wild-type MEFs the RILP and cholesterol-positive vesicles accumulated very close to the nucleus, in line with earlier reports on RILP overexpression [43]. In LAMP−/− MEFs the number of RILP and cholesterol-positive vesicles was much higher. These vesicles were concentrated around the nucleus, but less tightly than the vesicles in wild-type cells. However, instead of a rescue effect, overexpression...
of RILP increased the percentage of cells with late endosomal/lysosomal cholesterol accumulation both in wild-type and LAMP\(^{-/-}\) MEFs (Fig. S3E).

Since overexpression of wild-type RILP seemed to increase the late endosomal/lysosomal cholesterol accumulation, we also tested the effect of the dominant negative RILP construct, RILP-C33 [43]. This form lacks the N-terminal part of RILP and binds active, prenylated Rab7 but does not recruit the dynein microtubule motor. In wild-type MEFs, RILP-C33 was localized in vesicles scattered around the cytoplasm, as expected (Fig. S3C). In LAMP\(^{-/-}\) MEFs, however, RILP-C33 was predominantly diffuse, and it tended to induce large vacuoles in the cytoplasm (Fig. S3D). Overexpression of RILP-C33 also increased the percentage of cells with late endosomal/lysosomal cholesterol accumulation both in wild-type and LAMP\(^{-/-}\) MEFs (Fig. S3E). Taken together, the results show that the late endosomal/lysosomal cholesterol accumulation in LAMP\(^{-/-}\) MEFs cannot be diminished by activation or inhibition of Rab7 or RILP function. Notably, we have earlier reported that neither Rab7 nor RILP overexpression rescued the maturation of plasma-membrane derived phagosomes in LAMP\(^{-/-}\) cells [44, 45].

Rab9 functions in transport from late endosomes to the Golgi apparatus [46], while Rab8 functions in endocytic recycling and exocytosis [47–49]. Overexpression of Rab9 or Rab8 diminishes the lysosomal cholesterol accumulation in NPC1 mutant cells [41, 42]. We analysed their effects on cholesterol accumulation in LAMP\(^{-/-}\) MEFs. LAMP\(^{-/-}\) MEFs were transfected with GFP-Rab9, or GFP-tagged wild-type or constitutively active mutants of Rab8A and Rab8B (Q67L mutants), and stained with filipin 2 days later. Rab9 localized to the cholesterol-loaded late endosomes/lysosomes, but quantitative analysis showed that Rab9 overexpression did not rescue the late endosomal/lysosomal cholesterol accumulation (not shown). Similarly, overexpression of wild-type or constitutively active Rab8 isoforms did not change the percentage of cells with late endosomal/lysosomal cholesterol accumulation (not shown).

**Discussion**

Our results showed that both LAMP-2 single deficient tissue and MEFs, and LAMP double deficient MEFs accumulated free cholesterol. Filipin staining of MEFs indicated that free cholesterol accumulated in late endosomes/lysosomes. We also observed an increase in the expression levels of LDL receptors and a twofold increase in LDL uptake in LAMP\(^{-/-}\) MEFs. Taken together, our data suggest that LAMP\(^{-/-}\) MEFs have a defect in transport of cholesterol to the endoplasmic reticulum. This is in agreement with the finding that LAMP\(^{-/-}\) MEFs have a defect in the esterification of both endogenous and LDL cholesterol, and contain significantly less lipid droplets than control MEFs. Our data support the conclusion that LAMP\(^{-/-}\) cells accumulate free cholesterol in late endosomes/lysosomes because they have a defect in export of cholesterol from these organelles. Of note, we were able to abolish the endosomal/lysosomal cholesterol accumulation and elevated levels of total cholesterol in LAMP\(^{-/-}\) cells by simultaneous inhibition of cholesterol synthesis and cultivation in LDL-deficient medium, but not by the latter treatment alone [15, 45]. This
suggests that both endogenous cholesterol synthesis and uptake of LDL cholesterol contribute to the endosomal/lysosomal cholesterol accumulation. Both of these processes are stimulated by low cholesterol levels in the endoplasmic reticulum.

Unlike NPC patient cells, LAMP\(^{-/-}\) MEFs did not show a prominent accumulation of sphingolipids. We considered that this could be due to a milder cholesterol accumulation in LAMP\(^{-/-}\) MEFs. However, the level of total cholesterol in LAMP\(^{-/-}\) MEFs was slightly higher than in those NPC1 patient cells we tested (our unpublished results). Thus, the lack of sphingolipid accumulation is not likely due to a more moderate cholesterol accumulation, but rather speaks for a specific role for LAMPS in cholesterol trafficking.

Fibroblasts deficient in LAMP-2 showed a phenotype that is between wild-type cells and LAMP double deficient fibroblasts, while LAMP-1 deficient cells were similar to wild-type fibroblasts [15]. Further, LAMP-2 deficient liver showed a prominent accumulation of cholesterol and increased filipin staining, while LAMP-1 deficient liver was not different from the wild-type. These results suggest that LAMP-2 is more critical for the cholesterol transport, while LAMP-1 deficiency has a tendency to enhance the phenotype of LAMP-2 deficiency in fibroblasts. Indeed, the accumulation of free cholesterol in late endosomes/lysosomes of LAMP\(^{-/-}\) MEFs was readily abolished by re-expression of LAMP-2, while re-expression of LAMP-1 had no effect. U18666A causes accumulation of unesterified cholesterol in lysosomes and blocks LDL-stimulated cholesterol esterification [40]. An identical phenotype is observed in fibroblasts of NPC1 and NPC2 patients [29, 39]. U18666A is thought to act on the NPC1 protein itself or somewhere along the cholesterol efflux pathway involving NPC1 [39]. Remarkably, overexpression of LAMP-2 was able to retard the late endosomal/lysosomal cholesterol accumulation caused by U18666A. This supports the conclusion that LAMP-2 plays a role in intracellular cholesterol traffic, likely in the efflux of cholesterol from late endosomes and lysosomes.

The luminal domain of LAMP-2, when targeted to late endosomes/lysosomes by the transmembrane and cytosolic domains of LAMP-1, was critical for the cholesterol transport. Thus the region of LAMP-2 needed for the cholesterol transport is common to all three isoforms LAMP-2A, B and C. In agreement with this result, all three isoforms A, B and C were able to diminish the late endosomal/lysosomal cholesterol accumulation, although LAMP-2C was less effective than LAMP-2A and LAMP-2B. Together these results indicate that chaperone-mediated autophagy, for which the LAMP-2A isoforms are critical [50], is not likely to be involved in the cholesterol transport function of LAMP-2.

We observed increased plasma membrane localization of LAMP-2C isoform when compared to the isoforms LAMP-2A and 2B. This is in agreement with previous results obtained using the expression of avian LAMP-1/LAMP-2 chimeric molecules in mouse L cells [51]. The chimera containing the cytoplasmic tail of LAMP-2C (formerly called LAMP-2b, see [10]), showed the highest plasma membrane localization and lowest internalization rate compared with chimeras containing the cytoplasmic tails of the other two LAMP-2 isoforms. LAMP-2 isoforms show differences in their tissue specificity. LAMP-2A is predominant in the liver, while LAMP-2B predominates in muscle and heart [52, 53]. The LAMP-2C (formerly called m-LAMP-2b) transcript seems to have a restricted expression compared to the other two isoforms [53].

Our results showed that the membrane-proximal half of the luminal domain in LAMP-2, amino acids p\(^{191} – N\(^{279}\), was critical for intracellular cholesterol traffic. This region contains the hinge domain with potential O-glycosylation sites, two of the four potential disulfide bonds (C\(^{237} – C^{270}\) and C\(^{326} – C^{373}\)) and nine potential N-glycosylation sites. The membrane-proximal half of LAMP-2 luminal domain shows more conservation than the N-terminal half with the other members of the family including LAMP-1, LAMP-3 and CD68. However, BLAST search did not reveal homology to any known cholesterol-binding proteins. The LAMP-2 luminal domain, including the membrane-proximal half, is heavily glycosylated. According to the Swiss Prot database, mouse LAMP-2 luminal domain has 16 potential N-glycosylation sites, and experimental evidence suggests that most of these sites are used for glycosylation. The molecular weight of LAMP-2 reduces approximately 50%, from 100 to 50 kD, when carbohydrates are removed. This suggests that the luminal domain, including the membrane-proximal half, is tightly covered by carbohydrates. Therefore, it seems unlikely that the LAMP-2 luminal domain could bind cholesterol directly but the glycocalyx might be important indirectly, e.g. in the localization of cholesterol-binding proteins.

We suggest that LAMP-2 luminal domain possibly affects the stability, localization or function of other factor(s) that are needed for cholesterol traffic. The soluble NPC2 is one candidate for a factor that could be influenced by LAMP-2 [54]. NPC2 has been proposed to act in cholesterol efflux by binding cholesterol in the lysosomal lumen and delivering it to other proteins, such as the membrane-bound NPC1, for transport through the lysosomal membrane [55]. Recent studies showed that NPC2 facilitated the delivery and removal of cholesterol from NPC1, and transport of cholesterol from and between membranes [56–58]. NPC2 showed colocalization with LAMP-2 (Figs 3B and S1) [59]. However, the expression level and localization pattern of NPC2 were not significantly altered in LAMP\(^{-/-}\) MEFs, rather suggesting that LAMP-2 is not critical for the stability or localization of NPC2. Further, our co-immunoprecipitation experiments failed to show interaction between NPC2 and LAMP-2 (unpublished results). Notably, in the LAMP-deficient MEFs both endogenous (unpublished) and GFP-tagged NPC1 [15] were localized to the limiting membranes of the cholesterol-loaded late endosomes/lysosomes, indicating that NPC1 was recruited to these vesicles but was obviously unable to export the extra cholesterol out of them. Re-expression of LAMP-2A rescued the NPC1 localization back to small endosomal cholesterol-negative vesicles, which is normally observed in wild-type cells [15]. Thus, it appears that LAMP-2 is more critical for the correct localization of NPC1 than NPC2.

Cholesterol accumulation in NPC patient cells or NPC mutant cells can be diminished by overexpressing several Rab proteins including Rab7, Rab8 and Rab9 [41, 42]. In addition, cholesterol mobilization from late endosomes/lysosomes is inhibited by Rab guanine nucleotide dissociation inhibitor that removes Rab proteins from membranes [60]. These results indicate that the
Rab-related membrane trafficking machinery is involved in late endosomal/lysosomal cholesterol efflux. However, none of the proteins Rab7, Rab8, or Rab9 was able to diminish the late endosomal/lysosomal cholesterol accumulation in LAMP−/− MEFs. These results suggest that LAMP-2 is necessary for a cholesterol efflux step or pathway that cannot be overcome by Rab7, Rab8 or Rab9 overexpression. In addition, while overexpression of the Rab7 effector RILP was able to correct the aberrant peripheral localization of cholesterol-laden organelles from the cell periphery to the cell centre, it did not abolish the cholesterol accumulation, suggesting that the altered subcellular localization of the vesicles is not the crucial defect in LAMP−/− cells.

In addition to the endosomal/lysosomal cholesterol accumulation, LAMP double deficient MEFs also showed a prominent defect in the maturation of autophagosomes and phagosomes to degradative autolysosomes and phagolysosomes [15, 44, 45, 61]. However, unlike in the case of cholesterol accumulation, the maturation defect was not observed in MEFs single deficient in LAMP-1 or LAMP-2. Further, re-expression of either LAMP-1 or LAMP-2 was able to at least partially rescue the defect. We suggest that in the autophagosome and phagosome maturation the cytoplasmic tail of LAMP-2 (or LAMP-1) is more likely to be critical. Notably, our data showed that correcting the endosomal/lysosomal cholesterol accumulation in LAMP−/− MEFs by inhibiting cholesterol biosynthesis and cultivation in lipoprotein-depleted medium, did not rescue the defect in phagosome maturation [44, 45]. This indicates that cholesterol accumulation is not the cause of the phagosome maturation defect, and that these two phenotypes are likely separate from each other.

To conclude, our data are in favour of the idea that LAMP-2 has a role in intracellular cholesterol traffic, most likely at the level of efflux from late endosomes/lysosomes. Further studies are needed to clarify the exact function of LAMP proteins in cholesterol export.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 NPC2 and LAMP-2 show colocalization in wild-type MEFs. The cells were fixed and double labelled with anti-NPC2 (green) and anti-LAMP-2 (red). Yellow colour in the lower panel indicates colocalization.

Fig. S2 In wild-type MEFs, LAMP-2 colocalizes with LIMP2, and LAMP-1 colocalizes with the lipid BMP. The cells were fixed and double labelled with (A) anti-LAMP-2 (green) and anti-LIMP2 (red), or (B) anti-LAMP-1 (green) and anti-BMP (red).

Fig. S3 Overexpression of RILP or RILP-C33 did not abolish the cholesterol accumulation in LAMP deficient MEFs. Wild-type and LAMP double deficient MEFs were transfected with GFP-tagged RILP (wild-type RILP), or the truncated form RILP-C33. Two days after transfection the cells were fixed and stained with filipin. Asterisks indicate the RILP expressing cells in the left panels. Note that overexpression of wild-type RILP caused translocation of the filipin-positive vesicles towards the nucleus (A, B), while RILP-C33 did not show this effect (C, D). (E) Quantification of cells with endothelial cholesterol accumulation after transfection with wild-type RILP or the truncated RILP-C33. ‘None’ indicates cells with no overexpression.

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