₁ **Lysosomal membrane glycoproteins bind cholesterol and**

₂ **contribute to lysosomal cholesterol export**

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₁₀ **Abstract**: LAMP1 and LAMP2 proteins are highly abundant, ubiquitous, mammalian proteins

₁₁ that line the lysosome limiting membrane, and protect it from lysosomal hydrolase action.

₁₂ LAMP2 deficiency causes Danon's disease, an X-linked hypertrophic cardiomyopathy. LAMP2

₁₃ is needed for chaperone-mediated autophagy, and its expression improves tissue function in

₁₄ models of aging. We show here that LAMP1 and LAMP2 bind cholesterol in a manner that

₁₅ buries the cholesterol 3β-hydroxyl group; they also bind tightly to NPC1 and NPC2 proteins that

₁₆ export cholesterol from lysosomes. Quantitation of cellular LAMP2 and NPC1 protein levels

₁₇ suggest that LAMP proteins represent a significant cholesterol binding site at the lysosome

₁₈ limiting membrane, and may signal cholesterol availability. Functional rescue experiments show

₁₉ that the ability of LAMP2 to facilitate cholesterol export from lysosomes relies on its ability to

₂₀ bind cholesterol directly.

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Introduction

Eukaryotic lysosomes are acidic, membrane-bound organelles that contain proteases, lipases and nucleases and degrade cellular components to regenerate catabolic precursors for cellular use (1-3). Lysosomes are crucial for the degradation of substrates from the cytoplasm, as well as membrane bound compartments derived from the secretory, endocytic, autophagic and phagocytic pathways. The limiting membrane of lysosomes is lined with so-called lysosomal membrane glycoproteins (LAMPs) that are comprised of a short cytoplasmic domain, a single transmembrane span, and a highly, N- and O-glycosylated lumenal domain (4-6). Because of their abundance and glycan content, LAMPs have been proposed to serve as a protective barrier to block hydrolase access to the limiting phospholipid bilayer. LAMP1 and LAMP2 are 37% identical and may overlap in function, but knockout of LAMP1 in mouse has a much milder phenotype than depletion of LAMP2 (7): LAMP2-deficient mice have very short lifespans, and show massive accumulation of autophagic structures in most tissues. Indeed, LAMPs are required for fusion of lysosomes with phagosomes (8) and LAMP2 has also been proposed to serve as a receptor for chaperone-mediated autophagy (9-11).

Previous work has implicated LAMP2 in cholesterol export from lysosomes, as LAMP-deficient cells show cholesterol accumulation that can be rescued by LAMP2 expression (12,13). Proteome-wide analysis of cholesterol binding proteins included LAMP1 and LAMP2 among a long list of candidate proteins (14). Despite these hints, the precise function of LAMP proteins has remained unclear, and they are often presumed to be structural components. We show here that LAMP proteins bind cholesterol directly and this capacity contributes to their role in cholesterol export from lysosomes.

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Results and Discussion

We sought to verify direct cholesterol binding to LAMP proteins using LAMP protein lumenal domains, engineered to be secreted from cells by simple deletion of their transmembrane and short cytoplasmic domains (Fig. 1-figure supplement 1; Fig. 1A). Soluble, purified, LAMP1 and LAMP2 proteins appeared to bind ^{3}H-cholesterol saturably, at a stoichiometry comparable to equimolar amounts of purified, NPC1 N-terminal domain (NTD) that contains a single cholesterol binding site (15,16; Figs. 1B, D, E). (Note that this does not provide information about relative binding affinities.) Binding was not especially sensitive to pH (Fig. 1H) and was complete after ~2 hours at 4°C (Fig. 1I).

Cholesterol is poorly soluble, thus binding reactions were carried out in the presence of sub-critical micelle concentration amounts of Nonidet P40 detergent (0.004%) to help solubilize the cholesterol, as worked out by Infante et al. in their studies of cholesterol binding to the N-terminal domain of NPC1 protein (15). Under these conditions, most of the cholesterol remains in a mixed micelle of cholesterol and detergent and is still poorly soluble. Thus, the apparent affinity for cholesterol is likely to be tighter than the curves indicate, as the amounts added do not reflect the concentration of free cholesterol that is actually available for binding.

Preliminary experiments showed that ^{3}H-cholesterol binding was competed by unlabeled cholesterol, 24-hydroxycholesterol, but not cholesterol sulfate (Fig. 1G). This suggested that binding occurs via the 3β-hydroxyl moiety of the cholesterol molecule, similar to the orientation with which the NPC1 N-terminal domain binds cholesterol (16). Consistent with this, LAMP2 also bound [3]H-25-hydroxycholesterol with similar apparent affinity as cholesterol; binding was

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competed by excess cold 25-hydroxycholesterol (Fig. 1C), as would be expected for a specific interaction. Only low levels of background binding were detected using GFP-binding protein or TIP47 as controls (Fig. 1 D,E). More detailed analysis confirmed that 25-hydroxycholesterol (Fig. 2B) and 7-ketocholesterol (Fig. 2D), but not cholesterol sulfate (Fig. 2A), compete with ^{3}H-cholesterol for binding to LAMP2 protein.

Epicholesterol is a cholesterol epimer that differs only in the chirality of carbon 3 such that the hydroxyl group is in the alpha rather than beta conformation. Importantly, epicholesterol failed to compete for cholesterol binding to LAMP2 protein under conditions where cholesterol competed for binding (Fig. 2C). It was not possible to add higher concentrations of sterol competitors due to solubility issues, but significant inhibition was observed. Together, these data strongly support the conclusion that cholesterol binds LAMP2 via its 3β-hydroxyl moiety.

A slight stimulation of binding was seen in reactions containing low levels of competitor cholesterol sulfate or 25-hydroxycholesterol (Fig. 2A,B); this is presumably due to the higher solubility of these sterols, which will help solubilize ^{3}H-cholesterol present in the reaction's mixed micelles, and presumably make it more available for LAMP2 binding (see also ref. 15). Despite its somewhat higher solubility, 25-hydroxycholesterol did not appear to bind LAMP2 much more tightly than cholesterol, at least as inferred from its ability to compete with cholesterol for binding (Figure 2B) or to bind directly (Fig. 1C).

The LAMP protein family includes LAMP1, LAMP2, DC-LAMP, BAD-LAMP and Macrosialin (4). Each of these proteins contains a related "LAMP" domain; LAMP1 and LAMP2 proteins

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each contain two (Fig. 1-figure supplement 1). Soluble versions of the individual, membrane

distal ("domain 1") and membrane proximal ("domain 2") LAMP domains of LAMP2 (Fig. 1A)

bound cholesterol with different capacity: the N-terminal, domain 1 bound more cholesterol than

its membrane proximal, domain 2 counterpart under these conditions (Fig. 1F). It is possible that

both are capable of binding cholesterol within the full length molecule, as total domain 1 binding

was less than that seen with the full length, secreted LAMP2 construct (Fig. 1F).

To verify that LAMP2 binds cholesterol in cells, soluble LAMP2 protein was expressed and

purified from the secretions of HEK293F cells grown in protein-free, FreeStyle 293 Expression

Medium that does not contain cholesterol. Under these conditions, any LAMP2-bound sterol

must come from intracellular sources. We subjected freshly purified LAMP2 protein to

chloroform:methanol extraction and analyzed the extract by thin layer chromatography. As

shown in Fig. 3C, the LAMP2 extract contained a molecular species that co-chromatographed

with cholesterol but not 24-hydroxy-, 25-hydroxy-, or 26-hydroxycholesterol, lanosterol or 7-

beta-hydroxycholesterol. Mass spectrometry of the eluted material (Fig. 3B) confirmed a profile

identical with purified cholesterol standard (Fig. 3C). These experiments show that LAMP2

purified from cell secretions carries primarily, bound cholesterol.

NPC1 and NPC2 proteins mediate cholesterol export from lysosomes (16,17). NPC1 has 13

transmembrane domains, and three large, lumenal domains that are important for its function.

As mentioned earlier, the NPC1 N-terminal domain binds cholesterol directly (16). Because of a

possible connection between LAMP protein cholesterol binding and NPC-mediated cholesterol

export, we checked for an interaction between these proteins. Membrane anchored, endogenous

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LAMP2 co-immuno-precipitated with full length NPC1-GFP but not with the control protein, GFP (Fig. 4A) or the lysosomal membrane protein, MCOLN1 (Fig. 4D), upon expression in HEK293T cells (Fig. 4A). Interestingly, co-immunoprecipitation decreased in cells treated for 24 hours with cyclodextrin to remove cholesterol from lysosomes (18,19) and the plasma membrane (Fig. 4B, C). These conditions (~ 0.1% cyclodextrin) have been shown to be non-toxic (cf. 20) and did not alter cell growth rate or viability in our hands.

Purified, soluble LAMP2 protein also bound very tightly (and directly) to the N-terminal domain of NPC1 protein (Fig. 5A, red) in the presence (or absence, not shown) of cholesterol (K_D=6nM), as monitored by microscale thermophoresis using AF647 dye-conjugated NPC1 protein— binding significantly altered the fluorescence of NPC1 protein (Fig. 5C). No interaction was observed for the control glycoprotein, RNase B (Fig. 5E). The smaller, NPC2 protein (Fig. 5B) also bound to LAMP2 (K_D=122nM), but ~20 fold less tightly that NPC1 N-terminal domain (Fig. 5D); binding was monitored in the presence of cholesterol sulfate which will occupy the binding site of NPC2 but not LAMP2 (Figs. 1 and 2). No binding was seen for NPC2 to the control RNase B protein (Fig. 5F). Thus, LAMP2 binds directly to both NPC1 N-terminal domain and NPC2 proteins. For NPC1, the enhanced binding seen in cells in the presence of cholesterol does not appear to reflect occupancy of NPC1's N-terminal domain, as this variable did not influence LAMP2 binding in solution. However, it is important to note that NPC1 also likely binds cholesterol within its membrane spanning region which may also influence its overall conformation (21,22).

LAMP proteins are highly abundant components of the lysosome membrane and may serve as a reservoir for cholesterol extracted from intra-lysosomal membranes by NPC2, prior to cholesterol export from lysosomes by NPC1. [The term, reservoir, is meant to imply a holding station for cholesterol molecules that have been solubilized from the internal lipid contents of lysosomes by NPC2, and held closer to the limiting membrane, prior to NPC1-mediated export.]

Are LAMP proteins abundant enough to represent a cholesterol reservoir? We used purified LAMP2 and NPC1 proteins as standards to determine their precise abundance in HeLa and HEK293 cell lysates (Fig. 5-Fig. supplement 1). Using the polypeptide molecular weights and cellular protein determinations, we estimate that HeLa cells contain 6.8×10^6 LAMP2 molecules per cell and 3.7×10^5 NPC1 molecules per cell, or 18 fold more LAMP2 than NPC1; HEK293T cells contain 2×10^6 LAMP2 molecules per cell and 5.9×10^5 NPC1 molecules (3.6X fold more LAMP2).

Baby hamster kidney cells have been estimated to contain an absolute volume of $\sim 37\mu m^3$ lysosomes and prelysosomes per cell (3.7×10^{-14} l) and a lysosome membrane area of $370\mu m^2$ (23). Assuming similar values for HeLa cells, this would represent a LAMP2 membrane density of 18,378 or 5676 molecules per μm^2 in HeLa or 293T cell lysosomes, respectively, consistent with previous reports (6). For comparison, tightly packed viral spike glycoproteins occur at a density of 22,000 molecules per μm^2 (24). We assume that LAMP1 will be of similar high density, together with LAMP2, practically lining the interior of the lysosome limiting membrane. In terms of concentrations, 37 femtoliters of lysosome volume would contain 0.3mM LAMP2-associated cholesterol binding sites in HeLa cell lysosomes (assuming one mole cholesterol

213   bound per more LAMP2). It does not seem unreasonable to consider this as a significant

214   reservoir of cholesterol molecules that may be poised for transfer to NPC1 protein prior to

215   export.

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217   **Residues needed for cholesterol binding are needed for LAMP protein function**

218   The structure of an individual LAMP domain from DC-LAMP protein is comprised of a novel,

219   beta-prism fold that appears to contain a hydrophobic pocket (4); we used this structure to model

220   the structure of LAMP2 domain 1 (Fig. 6A). Site directed mutagenesis of hydrophobic residues

221   predicted to line the walls of this cavity yielded purified LAMP2 proteins with impaired

222   cholesterol binding activity. Thus, a soluble, LAMP2 domain 1-I^{111}A/V^{114}A construct yielded a

223   secreted protein (Fig. 6B inset, right lane) that bound significantly less cholesterol than its wild

224   type counterpart (Fig. 6B inset, left lane and panel B). Because these proteins were obtained

225   from cell secretions, they are likely to be properly folded, as they escaped the endoplasmic

226   reticulum's quality control machinery. These experiments show that residues facing the

227   predicted, prism fold pocket are important for cholesterol binding and likely contribute to the

228   cholesterol binding site.

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230   Finally, to verify the importance of cholesterol binding to LAMP2 protein as part of its

231   physiological role, we tested the ability of wild type and mutant LAMP2 constructs to rescue the

232   cholesterol accumulation seen in lysosomes from mouse embryonic fibroblasts missing LAMP1

233   and LAMP2 proteins (12,13). The ability of lysosomes to export cholesterol can be monitored

234   by feeding cells cholesterol in the form of LDL, and using conversion of ^{14}C-oleic acid to

235   cholesteryl oleate that takes place after endocytosed cholesterol is transported to the endoplasmic

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237 reticulum (25). Previous work showed that LAMP1/2 knockout MEF cells were impaired in

238 cholesterol export using this assay (13).

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240 We used lentivirus transduction to test the ability of full length LAMP2, a membrane anchored

241 LAMP2 domain 1 (LAMP2-GFP Δ194-368; Fig. 1-figure supplement 1), or a membrane

242 anchored LAMP2 domain 1-I^{111}A/V^{114}A to rescue the ability of LAMP1/LAMP2 knockout MEF

243 cells to export LDL-derived cholesterol from lysosomes. For these experiments, we used LAMP

244 constructs containing a single LAMP domain, as full length LAMP2 constructs with mutations in

245 both LAMP domains failed to fold properly or be transported efficiently to lysosomes.

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247 It was important to first verify the precise amounts of each construct in lysosomes, to evaluate

248 any functional rescue findings. Flow cytometry analysis showed that the rescue constructs were

249 expressed at comparable levels in each stably expressing cell population (Fig. 6C). Light

250 microscopy confirmed that the constructs were capable of proper lysosome localization, as

251 determined by their colocalization with endogenous LAMP1 protein (Fig. 6E) in HeLa cells.

252 (Similar staining was observed in LAMP knockout MEF cells that lack LAMP protein markers).

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254 To fully confirm the folding of these artificial constructs, we analyzed their glycosylation status

255 and stability after addition of cycloheximide to inhibit new protein synthesis (Fig. 6—Figure

256 supplement 1). Full length GFP-LAMP2 protein migrated at ~140kD and its abundance was not

257 altered after 4 hours cycloheximide treatment, consistent with its long half life in cultured cells

258 (panel B). Similarly, the GFP-domain 1 construct was stable under these conditions and

259 migrated at ~90kD (panels A,B). In contrast, the I^{111}A/V^{114}A mutant domain I protein displayed

261   two distinct bands; the upper band was stable, while the lower band likely corresponded to an ER

262   form that was largely degraded after 4 hours in cycloheximide (panels A,B). From this we

263   conclude that cells expressing membrane anchored LAMP2 domain 1 I^{111}A/V^{114}A are less

264   efficient at folding the protein but some folded protein makes it to lysosomes, where it is stable.

265   This difference was accounted for in subsequent functional rescue experiments (Fig. 6D).

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267   Figure 6D shows that as expected, full length, wild type LAMP2 rescued cholesterol export in

268   LAMP1/2-deficient MEF cells; membrane anchored LAMP2 domain 1 showed a level of rescue

269   consistent with its lower capacity for cholesterol binding (cf. Fig. 1F). Importantly, membrane

270   anchored, LAMP2 domain 1 I^{111}A/V^{114}A failed to rescue cholesterol export from lysosomes

271   (Fig. 6D), consistent with its inability to bind cholesterol; shown are the data corrected for the

272   amount of mature proteins present in lysosomes in these cells. LAMP2 constructs mutated in

273   both cholesterol-binding sites could not be tested, as they were only poorly delivered to

274   lysosomes.

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276   These experiments demonstrate a direct role for LAMP2 in cholesterol export from lysosomes,

277   and confirm that LAMP2's ability to bind cholesterol correlates with its ability to support

278   cholesterol export from LAMP-deficient MEF cells. In addition, LAMP proteins bind tightly to

279   NPC proteins in vitro and in cells, and appear to facilitate cholesterol export from lysosomes.

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281   LAMP proteins are the most highly abundant membrane glycoproteins of the lysosome, and their

282   lumenally oriented cholesterol binding sites represent a significant binding site for this important

283   sterol. We measured ~7 X 10^6 molecules per HeLa cell, representing 0.3mM binding sites in

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lysosomes. A recent cellular mass spectrometry analysis (26) estimated LAMP proteins to be

present at 260,000 copies and NPC1 at 29,193 copies per HeLa cell. While the relative

abundance of these proteins matches the values we report here, their total level was 25 fold lower

in that study. It is possible that these transmembrane glycoproteins were under-represented in

due to their unusual protease resistance as proteins of the lysosome membrane, differences in cell

confluency and/or differences in HeLa cell lines employed.

Lysosomes have recently been shown to sense and signal amino acid availability to influence

lysosome biogenesis in relation to cellular need (27), and LAMP oligomerization has been

reported to correlate with chaperone mediated autophagy (11). Cholesterol levels may influence

LAMP protein conformation or interaction with other partners to signal the availability of

endocytosed cholesterol to influence autophagy and cellular metabolism. The ten fold higher

abundance of LAMP proteins compared with NPC1 protein in HeLa cells suggests that LAMP

proteins may do more than just facilitate NPC1 function in cholesterol export. Future

experiments will be needed to fully understand the roles played by these highly abundant

lysosomal membrane glycoproteins.

We have shown that LAMP2 binds tightly to the N-terminal domain of NPC1 and also binds

cholesterol with the same orientation as that domain. LAMP2 also aids in cholesterol export

from lysosomes. How might LAMP2's cholesterol binding site contribute to cholesterol export?

Current models suggest that the soluble NPC2 protein binds cholesterol from the internal

membranes of lysosomes and delivers it to NPC1 at the limiting membrane of this compartment

(16). One possibility is that NPC2 can deliver cholesterol to both NPC1 and to LAMP2, which

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314 is more abundant. This would help drive the cholesterol export process by moving cholesterol

315 from the accumulated, lumenal lipid stores to the lysosome's limiting membrane. Because

316 LAMP2 and NPC1 N-terminal domains bind cholesterol in the same orientation, it makes sense

317 that NPC2 (which binds in opposite orientation, ref. 28) could transfer the cholesterol between

318 these two proteins. The recent crystal structure of NPC2 bound to the middle, lumenal domain

319 of NPC1 (29) supports a direct handoff between NPC1 and NPC2 (16). In future work, it will be

320 important to elucidate precisely how LAMP2 interacts with both NPC2 and NPC1 to facilitate

321 cholesterol export from lysosomes and how cholesterol binding contributes to LAMP2's other

322 cellular roles.

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Figure 1. Cholesterol binding to LAMP proteins. A. Coomassie-stained SDS-PAGE of purified, secreted human LAMP1, LAMP2 or LAMP domains from LAMP2. B,C. ^{3}H-cholesterol or [3]H-25 hydroxycholesterol binding to soluble LAMP2 (full length protein). Also shown in C is binding in the presence of 50μM cold hydroxycholesterol. D,E, ^{3}H-cholesterol binding to indicated, soluble proteins compared with the soluble NPC1 N-terminal domain. F, Cholesterol binding to LAMP2 domains 1 and 2 compared with full length, soluble LAMP2 protein. P values were determined relative to the full length soluble LAMP2 protein. G, Sterol competition (30μM) for ^{3}H-cholesterol binding to soluble LAMP2. P values were determined

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441   relative to no cold addition. H, I. pH dependence and kinetics of ^{3}H-cholesterol binding to

442   LAMP2. In B, C, E, H and I, a representative experiment is shown; C and E show the average of

443   duplicates. In C, the background counts in reactions containing the control protein, GFP-binding

444   protein, were subtracted; in D and E, the control was TIP47 protein. D, F, and G show the

445   combined results of two experiments in duplicate. Numbers at right (A) indicate mass in kD for

446   this and all subsequent figures. Reactions contained D, E, G, H, I, 500nM total cholesterol; F,

447   5μM total cholesterol; B and C were carried out using increasing concentrations of the indicated

448   sterol.

449

450   **Figure 1-figure supplement 1.** Diagram of constructs used.

451

452   **Figure 2.** Cholesterol binding to LAMP2 is competed by 25-hydroxycholesterol (B), cholesterol

453   (C), 7-ketocholesterol (D) but not cholesterol sulfate (A) or epicholesterol (C). The structures

454   above indicate the regions of the sterol that differ from cholesterol. In C, the background

455   obtained in reactions containing GFP was subtracted. All panels used 50nM ^{3}H-cholesterol and

456   the indicated amounts of competitors.

457

458   **Figure 3.** Mass spectrometry identification of small molecules released from LAMP2 after

459   chloroform:methanol (2:1) extraction. A, masses from cholesterol standard; B, masses of

460   LAMP2-bound material; C, Copper sulfate/phosphoric acid detection of indicated markers after

461   thin layer chromatography compared with material eluted from soluble LAMP2 (50μg). Shown

462   are the results of a representative experiment carried out twice.

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Figure 4. LAMP2 interacts with NPC1 and NPC2 proteins. A, Anti-GFP immunoprecipitation from HEK293T cells grown in FBS containing medium expressing GFP or mouse NPC1-GFP. The blot was developed with ECL. Upper panel, anti-GFP immunoblot (100% elution); lower panel, anti-LAMP2 immunoblot to detect endogenous, full length protein (100% elution). B, C, co-immunoprecipitation of LAMP2 and NPC1-GFP in cells grown in FBS, LPDS (5%) or LPDS + cyclodextrin (1mM) for 24 hours. Left panels, 1% inputs; right panels, 50% elutions. Error bars represent SEM for two combined experiments carried out in duplicate; P value is from comparison with LPDS by two-tailed Student's t-test. B shows duplicate reactions to document reproducibility. D, Anti-GFP immunoprecipitation of HEK293T cells grown in FBS expressing GFP-MCOLN1 or mouse NPC1-GFP. GFP-MCOLN1 occurs as a ~90kD form, a proteolytically processed form, and as higher oligomers (30). Left panels, inputs (2%); right panels, total elution carried out in duplicate. GFP proteins are green; LAMP2 is presented in red. Shown is a representative experiment carried out twice in duplicate.

Figure 5. LAMP2 binds NPC1 and NPC2 proteins. A, B, Structures of NPC1 (31; pdb 3jD8) and NPC2 (28; pdb 2hka) proteins; C,E, Microscale thermophoresis (E) or fluorescence (C) obtained with mixtures of soluble, AF647 labeled-NPC1 N-terminal domain or AF647-RNAase B with increasing concentrations of soluble LAMP2 in 1μM cholesterol. D,F, Microscale thermophoresis of AF647 labeled-NPC2 with increasing concentrations of soluble LAMP2 or RNAase B in 1μM cholesterol sulfate. For C-F, a representative experiment carried out at least twice is shown.

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504   **Figure 5 -- figure supplement 1.** Quantitation of NPC1 (A) and LAMP2 (B) proteins in HeLa

505   and HEK293T cells. The values at the top of each gel represent the amount of purified protein or

506   total cell extract analyzed, determined by BCA protein assay. The immunoblot was developed

507   using rabbit anti-NPC1 or mouse anti-LAMP2 antibodies followed by detection with

508   IRDye800CW goat anti-mouse or donkey anti-rabbit antibodies. Shown is an example of an

509   experiment carried out in duplicate; subconfluent cultures were analyzed. Purified NPC1 is

510   slightly smaller than that in the extract because it was deglycosylated (see Methods).

511

512   **Figure 6.** A, predicted structure model of LAMP2 domain 1; residues I111 and V114 are

513   highlighted in red. B, Relative ^{3}H-cholesterol binding to soluble LAMP 2 domain 1 or LAMP2

514   domain 1-I^{111}A/V^{114}A. Shown is combined data from 5 independent experiments carried out in

515   duplicate in the presence of 50nM ^{3}H-cholesterol. Inset, SDS-PAGE analysis of wild type (left)

516   and domain 1-I^{111}A/V^{114}A (right) proteins analyzed. P value was determined by two-tailed

517   Student's t-test. C, flow cytometry analysis of mean fluorescence of GFP rescue constructs in

518   lentivirus-tranduced cells (>20,000 cells analyzed). D , Cholesteryl oleate synthesis in MEF

519   cells lacking LAMP1 and LAMP2 after rescue with either full length, membrane anchored

520   LAMP2, membrane anchored LAMP2 domain 1, or membrane anchored LAMP2 domain 1-

521   I^{111}A/V^{114}A. C-terminally GFP-tagged, rescue proteins were stably expressed using lentivirus

522   transduction; shown is the combined result of 2 independent experiments, normalized for the

523   amount of mature protein in each sample (Fig. 6-Supp. Fig. 2) relative to the amount of rescue

524   seen with full length LAMP2 protein. P-values are in relation to full length for domain 1, or to

525   domain 1 for the mutant protein, and were determined by one way ANOVA. E, confocal light

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528   microscopic analysis of GFP rescue construct localization (green) and endogenous LAMP1

529   protein (red) in transiently transfected HeLa cells; bars represent 20µm.

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531   **Figure 6 -- figure supplement 1.** Immunoblot analysis of LAMP2 constructs from lentivirus

532   transduced LAMP1/LAMP2-knock out MEF cells. A, total cell extract from cells expressing

533   membrane anchored, GFP-LAMP2 domain 1 or GFP-LAMP2 domain 1-I^{111}A/V^{114}A; B,

534   migration of the constructs indicated, before or after 4 hours cycloheximide treatment (50

535   µg/ml). Panel A was developed as in Fig. 4B; panel B was developed as in Fig. 4A.

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539   **Materials and Methods**

540   Cholesterol, epicholesterol and sodium cholesteryl sulfate were from Sigma (St. Louis, MO); 24-

541   hydroxycholesterol (24-HC) was a gift from Rajat Rohatgi (Stanford University, Stanford, CA);

542   25-hydroxycholesterol and 7-ketocholesterol were from Steraloids (Newport, RI) or Avanti Polar

543   Lipids (Alabaster, AL); [1,2-^{3}H]cholesterol (50 Ci/mmol) and 25-[26,27-^{3}H] hydroxycholesterol

544   were from American Radiolabeled Chemicals (St. Louis, MO). Ni-NTA agarose was from

545   Qiagen (Valencia, CA); freestyle 293™ expression medium and Dulbecco's modified Eagle's

546   medium (DMEM) was from Life Technologies (Carlsbad, CA); lipoprotein deficient serum was

547   from KALEN Biomedical (Montgomery Village, Maryland). Pierce™ Protein Concentrators

548   PES were from Thermo Fisher Scientific (Grand Island, NY); PD-10 desalting columns and Q-

549   Sepharose were from GE Healthcare Life Sciences (Pittsburgh, PA); pFastBac NPC1-N-terminal

550   domain plasmid, LAMP1-mGFP and MCOLN1-pEGFP C3 were from Addgene (Cambridge,

551   MA); pGEM-LAMP2 was from Sino Biological Inc; mouse anti-human LAMP1 and LAMP2

552   antibody culture supernatants were from Developmental Studies Hybridoma Bank (University of

553   Iowa, Iowa city, IA). Chicken anti-GFP antibody was from Aves Labs (Tigard, Oregon); IRDye

800CW donkey anti-chicken and IRDye 680RD donkey anti-mouse antibodies were from LI-COR, Inc. (Lincoln, NE); anti-chicken-HRP conjugate was from Promega (Sunnyvale, CA); goat anti-mouse-HRP conjugate was from BioRad (Hercules, CA); ECL Western Blotting Substrate was from Thermo Scientific (Rockford, IL).

Buffers Buffer A: 50mM ammonium acetate, pH4.5, 150mM NaCl, 0.004% NP-40; buffer B: 50mM MES, pH5.5, 150mM NaCl, 0.004% NP40; buffer C: 50mM MES, pH6.5, 150mM NaCl, 0.004% NP-40; buffer D: 50mM HEPES, pH7.5, 150mM NaCl, 0.004% NP-40; buffer E: 25mM Tris, pH7.4, 150mM NaCl; RIPA buffer: 50mM Tris, pH7.4, 150mM NaCl, 1% NP-40, 0.2% deoxycholic acid, 0.1% SDS.

Plasmids cDNAs encoding full length, soluble human LAMP1(1-382), human LAMP2 (1-375) and domain 1 of human LAMP2 (1-231) were PCR amplified from LAMP1-mGFP and pGEM-LAMP2 respectively. The PCR products were inserted into pEGFP-N3 vector. The constructs were assembled to have an unstructured GSTGSTGSTGA linker at the C terminus, followed by a His_{10} tag and a FLAG tag. For LAMP2, another His_{10} tag was added downstream of the FLAG tag for improved purification. LAMP2 domain 2 was prepared by deleting residues 39-219 from the full length, soluble domain construct. FUGENE6 was used for transient transfection of HeLa cells. Membrane anchored rescue constructs were stably expressed in LAMP1/2 deficient MEF cells by lentivirus transduction and were comprised of full length LAMP2 bearing a C-terminal GFP (LAMP2-GFP), or LAMP2-GFP Δ194-368 (encoding membrane anchored domain 1) or the latter construct carrying point mutations.

Cell culture All cells were cultured at 37°C and under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 7.5% fetal bovine serum, 100U/ml penicillin and 100 μg/ml streptomycin, unless indicated. HEK293F suspension cells were cultured at 37°C under 5% CO2 in Freestyle 293™ medium. Mouse embryonic fibroblasts from LAMP1/LAMP2 double knockout mice (11,12) were the generous gift of Dr. Paul Saftig (Christian-Albrechts-Universität Kiel, Germany). In some experiments, cells were cultured in lipoprotein deficient serum (5%).

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Protein purification pFastBac NPC1-N-terminal domain plasmid was used to make virus for infection of Sf9 insect cells. Seventy-two hours after infection, Sf9 cultures were spun down and ammonium sulfate added to achieve 60% saturation. The resulting precipitate was re-suspended buffer E and incubated with Ni-NTA resin overnight at 4°C. After washing with buffer E with 25mM imidazole, the protein was eluted with buffer E plus 250mM imidazole, and further purified using Q-Sepharose.

HEK293F cells were transfected using 293fection according to the manufacturer. After 72h, supernatants were collected after spinning 3000 rpm for 5min. To purify proteins for [^{3}H] cholesterol binding, supernatants were subjected to 90% ammonium sulfate precipitation. After spinning at 13000 rpm for 30min, pellets were re-suspended in buffer E plus 25mM imidazole and incubated with Ni-NTA resin overnight at 4°C, followed by washing with the same buffer. Bound proteins were eluted with buffer E plus 250mM imidazole. Proteins were concentrated and buffer exchanged into buffer C with Pierce^{TM} Protein Concentrators PES (10kD cut-off). Proteins were either used immediately or stored at -80°C after snap freezing in liquid nitrogen. For cholesterol extraction and thin layer chromatography, supernatants were adjusted to pH 7.4 and incubated with Ni-NTA resin overnight at 4°C; after washing with buffer E plus 25mM imidazole, proteins were eluted with buffer E plus 250mM imidazole. Proteins were desalted into PBS using a PD-10 column.

^{3}H-cholesterol binding Each reaction was carried out in a final volume of 80–100 μl of buffer A, B, C or D containing 0.1-1μg purified His-tagged protein and 10–400 nM **^{3}H**-cholesterol diluted with 0.1-50μM cholesterol. For competition assays, reactions were in 80μl buffer C (50mM MES, 150mM NaCl, 0.004% NP-40, pH6.5) with 0.1μg full length soluble LAMP2 protein and 50nM ^{3}H-cholesterol, competition was started by adding vehicle (ethanol) or different concentrations of competitors as indicated. After incubation overnight at 4°C, the mixture was loaded onto a column packed with 30μl Ni-NTA agarose beads. After incubation for 10min, each column was washed with 5 ml of buffer C plus 10mM imidazole. The protein-bound **^{3}H**-cholesterol was eluted with 250mM imidazole-containing buffer C and quantified by scintillation counting. For competition experiments, assays were carried out in the presence of 30μM unlabeled sterol.

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Mass Spectrometry Samples were analyzed by LC/MS on an Agilent 1260 HPLC and Bruker microTOF-Q II mass spectrometer. Full scan mass and product ion spectra were acquired in positive ion mode, using a Phenomenex Kinetex C18 2.6u 2.1x100mm column, and an initial condition of 30%, 0.1% formic acid in water/70% methanol.

Thin Layer Chromatography Full length soluble LAMP2, and domains 1 and 2 of LAMP2 were purified as described above. Extraction was performed by adding 3 sample volumes of chloroform/methanol (2:1, v/v) to the samples. After repeating once more, extracts were pooled and dried under nitrogen. The extracts were re-dissolved in 50-100μl chloroform/methanol (2:1, v/v). Samples were spotted onto a Silica gel plate. The plate was developed with isopropanol until the front reached 1cm above the loading position; after drying under airflow, the plate was further developed using 2% methanol in chloroform until the front reached the top of the plate. The plate was sprayed with 10% CuSO$_4$ in 4% or 8% phosphoric acid and heated at 180°C to visualize the samples.

Co-immunoprecipitation HEK293T cells expressing pEGFP-N1, pEGFP-N1-mNPC1 or pEGFP-C3-MCOLN1 were harvested 24–48 h post-transfection and lysed in lysis buffer (50 mM MES, pH 5.5, 150 mM NaCl and 0.1% digitonin) supplemented with protease inhibitors. After 30min on ice, lysates were spun at 15,000 g for 15 min, and protein concentrations of the supernatants were measured. Equal amounts of extract protein were incubated with GFP-binding protein–conjugated agarose for 2 h at 4°C. Immobilized proteins were washed 4 times with 1ml lysis buffer, eluted with 2× SDS loading buffer, and subjected to BioRad Mini-PROTEIN TGX 4-20% gradient gels. After transfer to nitrocellulose membrane and antibody incubation, blots were detected with ECL western blotting detection substrate or visualized using LI-COR Odyssey Imaging System.

Microscale Thermophoresis (MST)
MST experiments were performed on a Monolith NT.115Pico instrument (Nanotemper Technologies). Briefly, His$_6$-NPC1 N-terminal domain, RNase B or NPC2 were labeled using the RED-NHS (Amine Reactive) Protein Labeling Kit (Nanotemper Technologies). A constant

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concentration of 6nM labeled protein was mixed with binding partners with a final buffer condition of 50 mM MES, pH 5.5, containing 150 mM NaCl, 0.004% NP-40. Premium coated capillaries contained 16 sequential, 2 fold serial dilutions. Analysis was at 40% laser power for 30 sec, followed by 5 sec cooling. Data were normalized to fraction of bound (0=unbound, 1=bound). The dissociation constant K_D was obtained by plotting the normalized fluorescence F_{norm} against the logarithm of the different concentrations of the dilution series according to the law of mass action.

Quantitation of intracellular LAMP2 and NPC1 protein

HeLa and HEK293T cells were grown to sub-confluence in DMEM supplemented with 7.5% FBS. One 10cm dish of cells was washed 3 times with cold PBS, then lysed with 500μl RIPA buffer with protease inhibitor cocktail (Sigma). After 30min on ice, the lysate was centrifuged at 13000 rpm for 15min at 4°C. The resulting supernatant was transferred to a new tube and protein was measured by BCA assay. Lysates were resolved by SDS-PAGE, using different amounts of purified human LAMP2 or NPC1 protein as standards. After transfer to nitrocellulose, the blot was probed with anti-human LAMP2 or NPC1 antibody followed by IRDye 800CW labeled anti mouse (for LAMP2) or rabbit (for NPC1) secondary antibody, and visualized using a LI-COR Odyssey Imaging System and analyzed using ImageJ software. Calculations were based on molecular weights of 45874 for LAMP2 and 142167 for NPC1 polypeptide chains, and neglected glycan contribution, which is not measured in the protein assay employed. Purified, full length NPC1 protein was the gift of Dr. Xiaochun Li (Rockefeller University) and was N-glycanase treated.

Other Methods Confocal immunofluorescence microscopy was carried out as described (32). Cells grown on coverslips were fixed with 3.7% (vol/vol) paraformaldehyde for 15 min at room temperature. LAMP1 staining was performed with sequential incubation of mouse anti-LAMP1 culture supernate and Alexa Fluor 594 goat anti-mouse antibody (1:1,000, Invitrogen), each for 1 h at room temperature. Coverslips were mounted using Mowiol and imaged using a Leica SP2 confocal microscope and Leica software with a 60×1.4 N.A. Plan Apochromat oil immersion lens and a charge-coupled device camera (CoolSNAP HQ, Photometrics). Flow cytometry was

carried out on a FACScan Analyzer on gently trypsinized cells fixed as described above (32). Structures were presented in drawings created using Chimera software (33).

Cholesterol Ester Formation LAMP1/LAMP2-deficient MEF cells were cultured in DMEM medium with 5% (vol/vol) LPDS for two days and assayed (25, 32) with minor modification. After 48 h, 100 µg/mL LDL, 50 µM lovastatin, and 50 µM sodium mevalonate were added for 5h. Cells were pulse labeled for 4 h with 0.1 mM sodium [1-^{14}C]oleate (American Radiolabeled Chemicals)–albumin complex. Cells were washed two times with 2 mL 50 mM Tris, 150 mM NaCl, 2 mg/mL BSA, pH 7.4, followed by 2 mL 50 mM Tris, 150 mM NaCl, pH 7.4. Cells were extracted and rinsed with hexane-isopropanol (3:2), pooled, and evaporated. After resuspending each sample in 60 µl hexane, 4 µL of lipid standard containing 8 µg/mL triolein, 8 µg/mL oleic acid, and 8 µg/mL cholesteryl oleate was added. Samples were spotted onto a silica gel 60 plastic backed, thin layer chromatogram and developed in hexane. Cholesteryl oleate was identified with iodine vapor, scraped from chromatograms, and radioactivity determined by scintillation counting in 10 mL Biosafe II.

Statistical Analysis-- Minimum sample sizes were determined assuming 5% standard error and > 95% confidence level. P values were determined using Graphpad Prism software and are indicated in all figures according to convention: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. Error bars represent standard error of the mean.

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