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Endolysosomal cholesterol export: More than just NPC1

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Abstract

NPC1 plays a central role in cholesterol egress from endolysosomes, a critical step for maintaining intracellular cholesterol homeostasis. Despite recent advances in the field, the full repertoire of molecules and pathways involved in this process remains unknown. Emerging evidence suggests the existence of NPC1-independent, alternative routes. These may involve vesicular and non-vesicular mechanisms, as well as release of extracellular vesicles. Understanding the underlying molecular mechanisms that bypass NPC1 function could have important implications for the development of therapies for lysosomal storage disorders. Here we discuss how cholesterol may be exported from lysosomes in which NPC1 function is impaired.

KEYWORDS
cholesterol homeostasis, cholesterol transport, endolysosome, NPC1, NPC1 bypass

INTRODUCTION

Cholesterol plays a vital role as a structural constituent of cellular membranes and as a building block for important biomolecules such as steroid hormones or bile acids. However, sustained cholesterol excess leads to deleterious consequences at both cellular and organismal levels, thus cholesterol levels are subject to tight regulation by multiple mechanisms. Except for the central nervous system that relies on de novo cholesterol biosynthesis, most cells in our bodies rely on circulating low density lipoprotein (LDL) as their primary source of cholesterol. After receptor-mediated endocytosis (Figure 1A; step 1), LDL is delivered to endolysosomes (ELs) where LDL-derived cholesteryl esters are hydrolyzed by lysosomal acid lipase to yield free cholesterol and fatty acids (Figure 1A; step 2). It is at this point that the intricate journey of intracellular cholesterol transport begins. Two highly conserved gene products: Niemann-Pick C2 (NPC2) and Niemann-Pick C1 (NPC1)[1] mediate the next step of exporting cholesterol from the lysosome (Figure 1A). The importance of the NPC1/2 transport system is underscored by loss-of-function mutations of these genes causing Niemann-Pick type C1 disease (NPC), a fatal lysosome storage disorder characterized, at the cellular level, by abnormal cholesterol accumulation in ELs.

Like most lysosomal proteins, upon translation NPC1 and NPC2 enter the classical secretory pathway where they become glycosylated at multiple residues.[2] In addition to this post-translational modification, NPC1 can also be ubiquitylated, a modification important for its folding quality control[3] and turnover.[4] Once properly folded and glycosylated, NPC1/2 proteins are targeted to ELs by distinct mechanisms. While NPC2 uses the cation-dependent mannose-6-phophate receptor,[5] NPC1 harbors a dileucine repeat motif within its c-terminus conserved in other lysosomal proteins.[6] This motif together with additional signals in the sterol-sensing domain are thought to regulate the transport of NPC1 from the trans-Golgi network (TGN) to ELs.[7] Apart from ELs, NPC1 has been reported to localize to the endoplasmic reticulum (ER),[8] the TGN,[9] and caveolin-1-positive multivesicular endosomes.[10]

Recent biochemical and structural studies have provided novel mechanistic insight into the NPC1/2-mediated cholesterol

Abbreviations: ELs, endolysosomes; ER, endoplasmic reticulum; GAP, GTPase activating protein; HDL, high density lipoprotein; ILV, intralumenal vesicle; LD, lipid droplet; LDL, lipid transfer protein; NPC, low density lipoprotein; LTP, Niemann-Pick type C1 disease; PG, phosphatidylglycerol; PM, plasma membrane; TGN, trans-Golgi network.

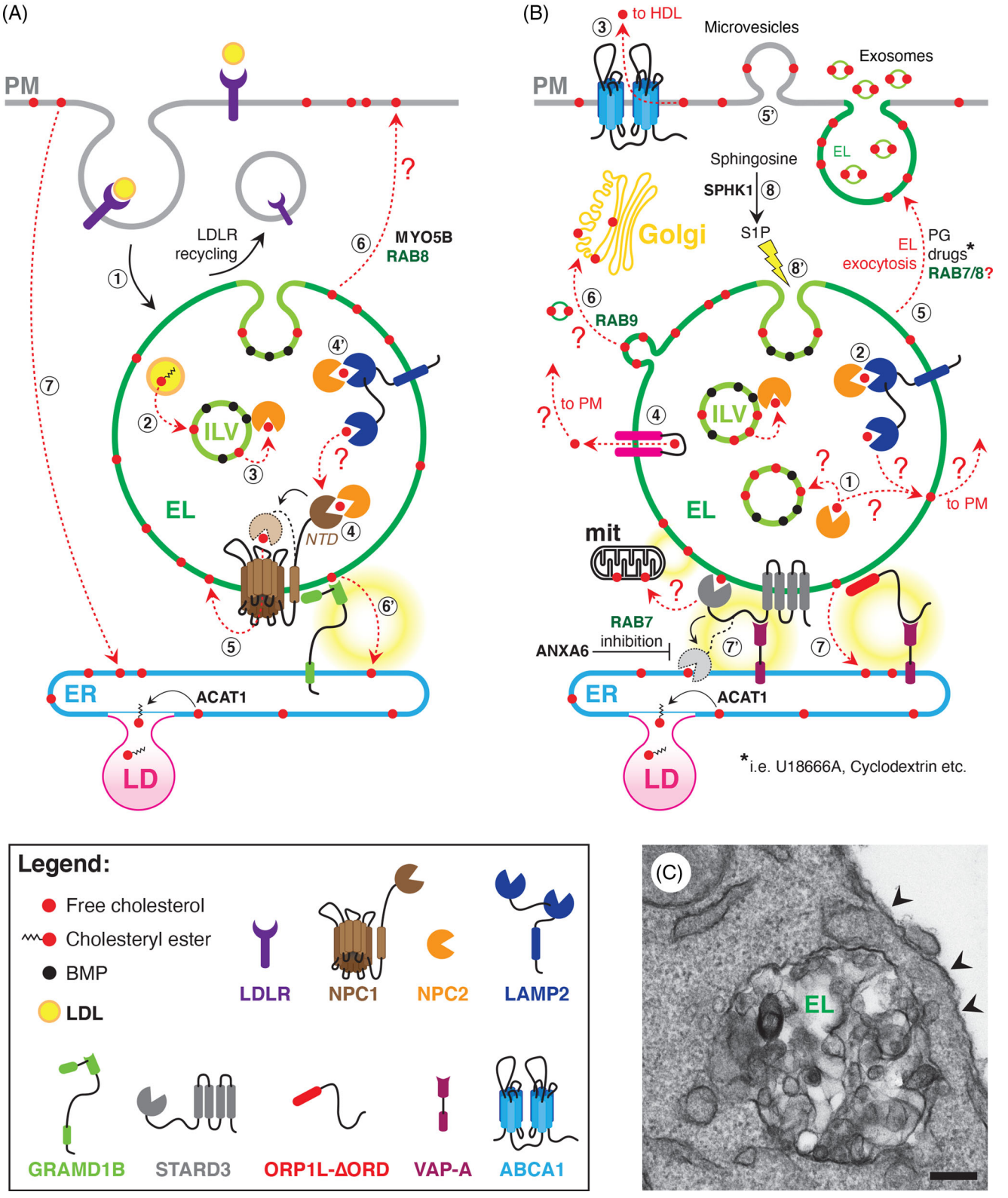

FIGURE 1 **Endolysosomal cholesterol egress pathways.** (**A**) NPC1/2-mediated cholesterol export from endolysosomes. (**B**) NPC1-independent routes. (**C**) Transmission electron micrograph of an NPC1$^{-/-}$ HeLa cell showing an endolysosomal structure (EL) in close proximity to the plasma membrane (PM; arrowheads). Note the highly abundant and heterogeneous ILVs (image credit: Dr Carlos Enrich, University of Barcelona). Regions of membrane contact sites are represented in yellow background. Scale bar, 200 nm. EL, endolysosome; ER, endoplasmic reticulum; PM, plasma membrane; LD, lipid droplet; mit, mitochondria; BMP, bis(monoacylglycero)phosphate; LDL, low density lipoprotein; LDLR, LDL receptor; HDL, high density lipoprotein; VAP-A, vesicle-associated membrane protein-associated protein A; MYO5B, myosin-5B.

export pathway.[11–14] NPC2 extracts free cholesterol from intralumenal membranes of ELs (Figure 1A; step 3), a process facilitated by NPC2 binding to the endolysosomal phospholipid bis(monoacylglycero)phosphate[15] (BMP; also known as LBPA). Next, NPC2 transfers cholesterol directly to the lumenal, N-terminal domain of NPC1 (Figure 1A; step 4), which undergoes a pH-dependent conformational change that aligns bound cholesterol towards an internal structural tunnel through which it is then channeled to the endolysosomal outer leaflet[12,16] (Figure 1A; step 5).

The concerted activities of NPC1 and NPC2 proteins may be facilitated by the highly abundant endolysosomal protein LAMP2, which interacts with NPC1 and may serve as a reservoir of cholesterol molecules solubilized by NPC2[17] (Figure 1A; step 4′). Previous studies showed abnormal accumulation of unesterified cholesterol and preserved hydrolytic activities in ELs from fibroblasts derived from LAMP1/2 double-knockout mice. Notably, overexpression of LAMP2, but not LAMP1, rescued this phenotype and retarded endolysosomal cholesterol build-up upon pharmacological inhibition of NPC1.[18,19] Nevertheless, the exact function of LAMP2 in cholesterol export from ELs still needs to be determined. Finally, once transferred to the endolysosomal limiting membrane, cholesterol is distributed to different membrane compartments by mechanisms that remain largely uncharacterized and may involve both formation of interorganelle membrane contact sites (Figure 1A; step 6′) and vesicular transport (Figure 1A; step 6; for excellent reviews see [20,21]).

Despite the central role that the NPC1 plays in cholesterol transport from ELs, NPC fibroblasts are still able to mobilize their entire cytoplasmic cholesterol pool at a rate 4 to 5 times slower than wild type cells,[22] suggesting that alternative egress pathways exist. Indeed, a number of NPC1-independent routes have been recently described. Known molecules and mechanisms independent of NPC1 function are discussed below.

NPC2, BMP, AND COLLABORATORS

BMP is a unique acidic phospholipid exclusively present in endolysosomal intralumenal vesicles (ILVs) where it enhances the activity of lipid hydrolases and together with NPC2, solubilizes LDL-derived cholesterol.[15,23] Indeed, blocking BMP function with endocytosed monoclonal antibodies results in cholesterol accumulation and impaired EL function.[24] While it is known that BMP de novo biosynthesis can be stimulated by the BMP precursor, phosphatidylglycerol (PG), BMP's biosynthetic and degradation pathways remain elusive.[23,25] As previously reported by McCauliff et al. supplementation of NPC1-deficient cells with PG increased BMP levels and concomitantly reversed endolysosomal cholesterol accumulation in an NPC2-dependent manner. A hydrophobic knob on the NPC2 surface mediates binding to BMP, enhancing cholesterol solubilization[15]; mutations in the NPC2 knob prevented this interaction and reversal of cholesterol accumulation. Perhaps upregulation of BMP levels also triggers back-fusion of cholesterol-rich ILVs,[23,26] directly delivering it on the surface of ELs for subsequent transport to other compartments.

More recently, Ilnytska et al. showed induction of exosome release in NPC1 knockout fibroblasts treated with PG[27] (Figure 1B; steps 1 and 5). Consistent with this, microscopy analysis of these cells revealed increased number of endolysosomes in close proximity to the cell periphery. Remarkably, exosomes released by PG-treated cells harbored elevated levels of free cholesterol compared to untreated cells. Thus, PG supplementation upregulates BMP biosynthesis and exocytosis of cholesterol-enriched exosomes in NPC1-deficient cells (Figure 1B; step 5), ameliorating cholesterol accumulation in ELs. In line with these findings, others have shown that supplementation of NPC2-deficient cells with recombinant NPC2 also increases extracellular vesicle release.[28]

As discussed earlier, one would expect that NPC2, together with BMP, should solubilize free cholesterol from ILVs[15,29] (exosomes when released), rather than promoting its enrichment in these structures. On the other hand, since NPC2 interacts with LAMP2,[17] upregulation of BMP levels should promote cholesterol hand-off between NPC2 and LAMP2 (Figure 1B; step 2), increasing the pool of free cholesterol bound to the endolysosomal limiting membrane. Fusion of this compartment with the PM would then place cholesterol on the cell surface from which it could be released in shedding microvesicles, as previously proposed[28] (Figure 1B; step 5′). Alternatively, PM-localized ABCA1 could also mediate efflux of the incoming endolysosomal cholesterol pool[30] (Figure 1B; step 3). Indeed, overexpression of ABCA1 reduced endolysosomal cholesterol accumulation in NPC1- but not in NPC2-deficient fibroblasts,[31] suggesting that ABCA1 and NPC2 may operate in the same pathway. Finally, since LAMP proteins are also enriched in exosomes,[32,33] NPC2 may also transfer cholesterol to these internal membranes before being released[34] (Figure 1B; step 1). In conclusion, how NPC2, BMP and associated proteins can mediate NPC1-independent cholesterol export is still unclear.

LIMP2/SCARB2: MORE THAN JUST A GBA1 TRANSPORTER

The CD36 scavenger receptor protein superfamily is comprised of three members: CD36, a fatty acid transporter also involved in phagocytosis[35]; scavenger receptor B1 (SR-B1; also known as SCARB1), that binds to and exchanges cholesterol from high density lipoprotein (HDL) for transport into the liver and steroidogenic organs[36]; and LIMP-2/SCARB2, which serves as a phospholipid receptor and also mediates endolysosome delivery of β-glucocerebrosidase,[37] an essential lysosomal lipid hydrolase that maintains glycosphingolipid substrate homeostasis. These membrane-anchored proteins have two transmembrane domains, a large ectodomain and short cytoplasmic tails. Previous structural studies showed that LIMP-2 and related family members SR-B1 and CD36 contain an intramolecular tunnel that traverses the entire length of the molecule. In the case of SR-B1, this large cavity funnels selective uptake of cholesterol from HDL to the PM.[38]

A recent study described a role for LIMP-2 in endolysosomal cholesterol egress independent of NPC1 function.[39] Schwann cells

of peripheral nerves from LIMP-2$^{-/-}$ mice were found to exhibit membrane-enclosed cholesterol-like crystals within the cytoplasm, a phenotype similar to that observed in macrophages surrounding atherosclerotic lesions. Moreover, embryonic fibroblasts from LIMP-2 knockout mice displayed endolysosomal cholesterol accumulation, as seen in NPC cells. It is worth noting that these defects could also be secondarily triggered by a lack of β-glucocerebrosidase in ELs, which leads to deleterious accumulation of GBA1 substrates and lysosomal dysfunction.[37] Nevertheless, overexpression of wild-type LIMP-2, but not a mutant form harboring mutations in the intramolecular tunnel, decreased the endolysosomal cholesterol accumulation of NPC1-deleted cells. Together, these results suggest that LIMP-2 may contribute to cholesterol export from endolysosomes in an NPC1-independent manner (Figure 1B; step 4). In subsequent experiments, a cell surface-localized LIMP-2 construct was shown to bind lipoproteins from which a fluorescent cholesterol analog was transferred to the plasma membrane. Lipoprotein binding was efficiently achieved at acidic pH (as in ELs), and mutations in the LIMP-2 tunnel prevented cholesterol translocation to the PM. Finally, LIMP-2 depleted cells treated with LDL exhibited significantly slowed EL-to-ER trafficking and cholesterol esterification prior incorporation to LDs. Accordingly, sterol regulatory element binding protein 2 (SREBP2) was also dysregulated, a phenotype that was further accentuated upon loss of NPC1. Taken together, these data indicate that in addition to NPC1, LIMP2 is also needed for proper control of cholesterol transport regulation.

Despite this unexpected role for LIMP-2, it is likely that this alternative pathway operates at a much lower rate than that regulated by NPC1.[39] Paradoxically, a HeLa cell contains 262,901 copies of LIMP-2, compared to 29,193 copies of NPC1.[40] However, the fact that LIMP-2 expression is not sufficient to restore cholesterol traffic defects in NPC patients supports the notion that the NPC1/2 transport system represents the primary cholesterol export pathway from ELs (Figure 1A).

EXPORTING CHOLESTEROL ON VESICLES: RABs CAN ALSO DO THE JOB

RAB GTPases are master regulators of intracellular membrane trafficking including transport throughout the endocytic pathway.[41] Normal functioning and homeostasis of ELs relies mainly on RAB7 and RAB9.[42–44] We and others recently reported a regulatory role for RAB7 in endolysosomal cholesterol export from ELs.[45,46] Promoting RAB7 inactivation by expression of its cognate GTPase activating protein (GAP), TBC1D15, induced a phenotype reminiscent of NPC1 mutant cells.[45] A similar phenotype was caused after deleting any of the subunits of the RAB7 guanine nucleotide-exchange factor (GEF) MON/CCZ1/C18orf8 complex.[46] In contrast, overexpression of RAB7 reduced endolysosomal cholesterol levels in NPC patient fibroblasts,[46,47] indicating that RAB7 may regulate endolysosomal cholesterol egress in an NPC1-independent manner. While RAB7-GTP appears to interact with NPC1, this interaction was independent of NPC1 function or cholesterol availability and the precise residues mediating this interaction have yet to be defined.[46] Nevertheless, it is likely that RAB7 drives the recruitment of membrane contact site proteins such as PDZD8 and protrudin.[48,49] Protrudin, in turn, interacts with kinesin-1 and couples movement of endolysosomes towards the cell periphery where cholesterol may be transferred to the PM.[50]

Recently, we conducted genome-wide CRISPR/Cas9 genetic screens to characterize globally, homeostatic regulators of cholesterol and BMP.[25] Screens were performed under both control and NPC1-inhibition conditions to identify genes that, when deleted, could rescue the NPC1-deficiency phenotype. One of these genes was RAB7, whose deletion increased both cholesterol and BMP under control conditions but decreased both lipids in cells treated with the specific NPC1 inhibitor U18666A.[25] This suggests that perhaps RAB7 plays a dual role in cholesterol egress from ELs depending on NPC1 functional status. Nevertheless, contrary to overexpression studies,[46,47] it is unclear why in our screens RAB7 deletion reversed U18666A-induced cholesterol accumulation. We cannot discard the possibility that this apparent discrepancy may reflect cell type-specific differences. Intriguingly, knocking out different subunits of the RAB7 effector, homotypic fusion and vacuole protein sorting (HOPS) complex, consistently behaved similarly to RAB7 under both screen conditions.[25] The HOPS complex, is involved in tethering and fusion between ELs, and also heterotypic fusion between ELs and autophagosomes.[51,52] In this scenario, under normal conditions, loss of either RAB7 or HOPS complex subunits may cause cholesterol accumulation in small vesicles unable to undergo fusion. On the other hand, cholesterol accumulation in NPC cells inhibits RAB7 GTP hydrolysis, increasing its membrane association on ELs. This defect restrains EL transport towards the cell periphery.[53] Moreover, since sensing endolysosomal cholesterol levels by ORPL1 is coupled to transport and tethering of ELs via coordinated functions of RAB7 and HOPS complex,[54,55] loss of these proteins in NPC$^{-/-}$ cells may trigger EL mobility towards and fusion with the PM (Figure 1B; step 5), ultimately releasing cholesterol-rich exosomes. Supporting this idea, a subpopulation of multivesicular endosomes enriched in cholesterol-laden ILVs appeared to be more susceptible to undergoing fusion with the cell surface of B cells.[56] In these cells, cholesterol also appeared to be highly enriched in recycling endosomes rather than in LAMP-positive endolysosomes enriched in BMP. It is possible that aside from its role in lipid catabolism, the presence of BMP favors rapid cholesterol egress from this compartment via NPC1/2.[15,26] Once placed on the endolysosomal outer leaflet oxysterol-binding protein-related protein 2 (ORP2/OSBPL2) may transfer cholesterol to recycling endosomes from which it may be ultimately delivered to the PM.[57]

In addition to RAB7, overexpressing RAB4, RAB8, or RAB9 also reduced endolysosomal cholesterol in NPC1-deficient cells[47,58,59] (Figure 1B; steps 5 and 6). Ikonen and colleagues reported a RAB8-dependent vesicular pathway transporting cholesterol from ELs to the PM from which ABCA1 promoted its efflux.[58,60] As observed for RAB7, overexpression of RAB8 decreased endolysosomal cholesterol levels in NPC1-deficient cells. Conversely, depletion of RAB8 in NPC1-expressing cells significantly delayed LDL-derived cholesterol clearance from ELs, as similarly reported upon RAB9 knockdown.[60]

Silencing RAB8 and RAB9 simultaneously compromised even further this process, suggesting that these two RABs control different cholesterol efflux routes from ELs. Even though RAB8 overexpression was shown to bypass NPC1 function,[58] recruitment of RAB8 to cholesterol-laden CD63-positive ELs required presence of NPC1. Microtubule-mediated movement of these cholesterol carriers towards the cell periphery was shown to rely on myosin-5B (Figure 1A; step 6). Finally, kiss-and-run fusion of these vesicles with the PM was proposed to deposit cholesterol.[60]

RAB9-mediated cholesterol transport was proposed to reflect vesicle transport from ELs to the TGN[61] (Figure 1B; step 6). The role of RAB9 in retrograde transport from ELs has been extensively studied.[62] Of note, this route is largely affected in the absence of NPC1.[43] As mentioned earlier, RAB9 overexpression efficiently rescued the endolysosomal cholesterol accumulation in NPC1 but not NPC2 mutant cells, suggesting the existence of a RAB9-mediated NPC1-alternative pathway that requires NPC2 function. Previous work proposed a cholesterol trafficking pathway from ELs to the TGN controlled by soluble NSF attachment protein receptors (SNAREs) syntaxin 6, and syntaxin 16 and VAMP4.[61] However, a requirement for RAB9 in this pathway was not tested. Moreover, this set of SNARES has been implicated in RAB6-, but not RAB9- mediated, retrograde transport from early endosomes to the TGN.[62]

Altogether, while RAB-mediated transport may regulate cholesterol egress at a low rate from ELs, it is not sufficient to rescue loss of NPC1 function at steady state. Overexpression of RABs may simply upregulate vesicular transport and thus provide cholesterol an alternative route out of this compartment. In our screens, we showed that under NPC1-inhibition conditions, deletion of different RAB genes including RAB7 and RAB9 (but not RAB8), decreased free cholesterol levels in myeloid K562 cells.[25] It is worth noting that cholesterol transport may be controlled by different subsets of RABs depending on the cell type under study. Future research will be needed to clarify the role of RABs and vesicle-mediated cholesterol transport in the presence and absence of NPC1 function.

BYPASSING NPC1 FUNCTION THROUGH MEMBRANE CONTACT SITES

Once transferred to the endolysosomal outer leaflet, cholesterol can transit to other membrane compartments via non-vesicular mechanisms involving membrane contact sites. We and others recently described membrane contact site-mediated EL-to-ER cholesterol transport routes in NPC1-deficient cells. Höglinger et al. showed that NPC1 may also function as an organelle tether by interacting with ER-localized lipid transfer protein (LTP) GRAMD1B although the precise binding interface is yet to be identified.[63] This interaction results in the formation of membrane contact sites through which GRAMD1B extracts cholesterol from ELs and transfers it to the ER (Figure 1A). Loss of either NPC1 or GRAMD1B decreased EL-ER membrane contact sites while promoting the formation of EL-mitochondria membrane contact sites via STARD3 (Figure 1B; step 7′), an EL-localized lipid transfer protein. Such an increase in membrane contact sites was hypothesized to account for abnormally increased cholesterol levels observed in the mitochondria of NPC1-deficient cells.[64] Interestingly, overexpression of ORP1L, another EL-localized lipid transfer protein that contributes to cholesterol egress,[65] not only expanded EL-ER membrane contact sites but also restored endolysosomal cholesterol export in NPC1$^{-/-}$ cells. Moreover, this effect was replicated by an ORP1L mutant that lacks cholesterol transporting activity but preserves tethering function via ER-localized VAP-A (Figure 1B; step 7), indicating that artificial membrane contact sites expansion overcomes loss of NPC1 function.[63]

Complementing these findings, we described an NPC1 bypass pathway mediated by membrane contact sites in cells lacking both NPC1 and the Ca^{2+}-dependent membrane-binding protein, Annexin-A6 (ANXA6).[45] Previous studies showed that ANXA6 is recruited to EL membranes of cells treated with LDL or with the NPC1 inhibitor U18666A,[66,67] and that its overexpression induces an NPC-like phenotype.[68] This provides evidence for a role of ANXA6 in cholesterol transport regulation.[69] Deletion of ANXA6 reversed endosomal cholesterol accumulation and redistributed ELs towards the cell periphery of NPC1 mutant cells.[45] Moreover, these cells exhibited elevated levels of GTP-loaded RAB7. A yeast two-hybrid screen identified the RAB7 GAP TBC1D15 as an ANXA6-interacting protein, providing a functional link to our previous observations. Accordingly, in ANXA6-overexpressing cells, depletion of TBC1D15 restored cholesterol transport from ELs to the ER, further suggesting that ANXA6-mediated recruitment of TBC1D15 onto ELs inactivates RAB7, resulting in defective cholesterol egress. Finally, we showed that in the absence of ANXA6, NPC cells display decreased extension of EL-ER membrane contact sites mediated by STARD3, which binds VAP-A on the ER (Figure 1B; step 7′). These results suggest that ANXA6 may regulate the formation of STARD3-mediated, EL-ER membrane contact sites by modulating RAB7 activation, dysregulated in NPC cells[53] (Figure 1B; step 7′). Analogous to this model, FIS1-dependent mitochondrial recruitment of TBC1D15 was previously shown to be required for RAB7 inactivation, which induced disassembly of EL-mitochondria membrane contact sites.[70] Altogether, these studies provide evidence for membrane contact site-mediated NPC bypass pathways, which deserve further attention in future research.

SORTING CHOLESTEROL FROM ENDOLYSOSOMES WITH SNX13

Our recently developed CRISPR screens revealed a number of genes that when deleted, restored endolysosomal cholesterol levels in cells with inhibited NPC1 function.[25] Such genes could be involved in NPC1 bypass pathways and could represent potential NPC therapeutic targets. Among all the identified hits that behaved in this manner (including RAB7), we identified SNX13 and SNX14, two members of the sorting nexin (SNX)-RGS family of SNX proteins. SNX14 mutations cause autosomal recessive spinocerebellar ataxia-20 (SCAR20), a recently described neurodegenerative disorder characterized by disrupted endolysosomal cholesterol export.[71]

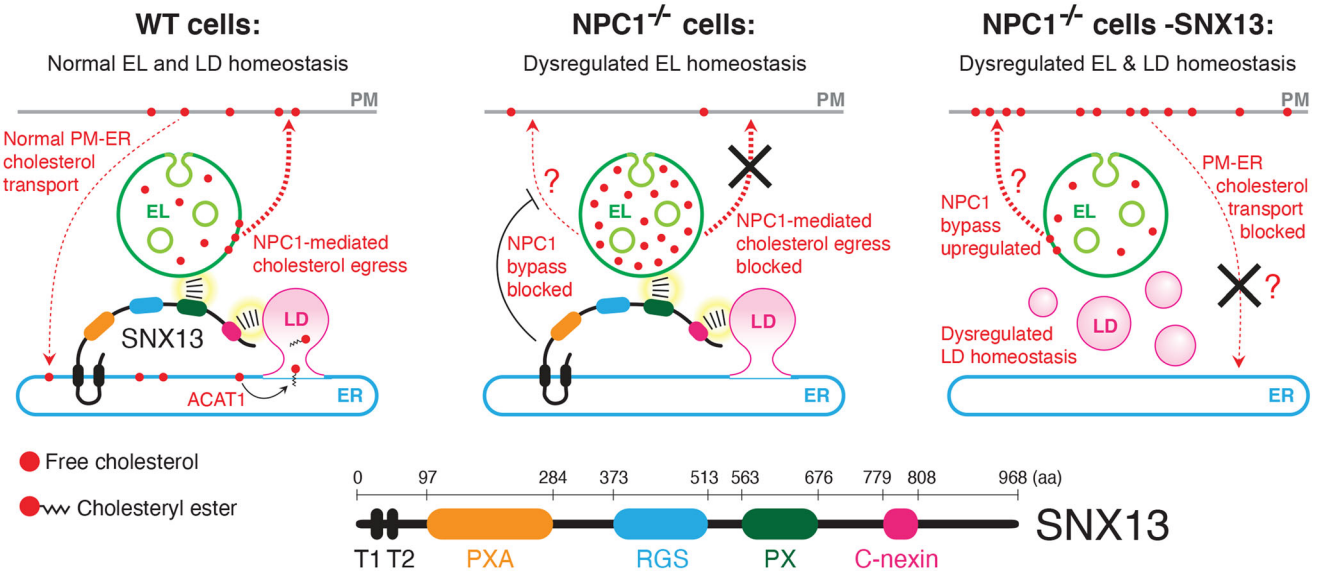

FIGURE 2 **Model depicting proposed SNX13-mediated cholesterol transport regulation**. (**A**) Under normal conditions, endolysosomal cholesterol export is mainly regulated by NPC1. SNX13 is an endoplasmic reticulum (ER)-localized transmembrane protein that mediates ER-endolysosome (EL) and ER-lipid droplet (LD) membrane contacts. (**B**) In NPC1$^{-/-}$ cells, cholesterol accumulates in ELs and SNX13 represses NPC1 bypass pathways. (**C**) In the absence of both NPC1 and SNX13, upregulation of an unknown NPC1-independent pathway allows cholesterol egress from ELs towards the PM. In addition, mobilization of PM-accessible cholesterol to the ER is blocked and LD homeostasis is dysregulated. SNX13 multidomain structure (bottom) consists of two putative transmembrane domains (T1, T2), a poorly described PX-associated (PXA), followed by a regulator of G-protein signaling domain (RGS) domain.[57] The C-terminal region contains a PX homology domain that binds different lipid species including endosomal phosphoinosites and phosphatidylserine[72]. Finally, an uncharacterized C-Nexin domain is placed at the end of the sequence.

Validation experiments confirmed that cholesterol accumulation induced by NPC1 inhibition or deletion was rescued in the absence of SNX13 and, to a lesser extent, SNX14. Moreover, loss of SNX13 resulted in rescuing normal cholesterol redistribution to the PM, accompanied by an increase in lipid droplet (LD) biogenesis independent of ACAT1-mediated cholesterol esterification. Cellular lipid content analysis of SNX13-depleted cells revealed significant alterations of triacylglycerols, free fatty acids, and BMP, while total cholesterol content remain unchanged. These data suggested that SNX13 functions as a negative regulator of an NPC1 bypass pathway towards the PM (Figure 2). In addition to these findings, our lipidomics analysis also revealed the surprising result that total BMP content does not change much upon NPC1 inhibition, contrary to what our microscopy data indicated. These results indicate that, as proposed for free cholesterol, BMP exists in at least 2 pools: an "antibody-accessible" pool likely to represent active BMP[24]; and an antibody non-accessible pool that may be unable to interact with NPC2.

RGS-SNX genes encode for a family of ER-anchored multidomain-containing proteins predicted to function as LTPs.[73,74] Previous work in yeast showed that the SNX13 ortholog, MDM1 mediates ER-vacuole membrane contact sites important for fatty acid homeostasis regulation.[75,76] In mammals, the paralogs SNX14 and SNX19 mediate ER-LD and ER-early endosome membrane contact sites, respectively.[77,78] Analogously, we found that overexpression of SNX13 increased the number and duration of EL-ER membrane contact sites. Moreover, in cells treated with oleic acid, SNX13 was dramatically redistributed to LD-associated ER domains that were very often in close apposition with BMP-positive ELs. Altogether, our investigations suggest that SNX13 may work as tri-organelle tether important for maintaining lipid trafficking between ELs, ER and LDs (Figure 2). Given these connections, how might loss of SNX13 allows cholesterol transport to the PM in NPC1-deficient cells? One explanation is that analogous to SNX19, SNX13-mediated membrane contact sites could restrict EL motility; loss of SNX13 may enable ELs to move towards the cell surface where cholesterol could be exchanged via membrane contact sites or upon EL fusion with the PM.

The striking redistribution of cholesterol to the PM and increased LD biogenesis independent of cholesterol esterification observed in SNX13-depleted cells suggest that SNX13 may promote PM-to-ER transport. Trinh et al. recently uncovered a role for phosphatidylserine synthase 1 (PTDSS1) in cholesterol trafficking from the PM to the ER.[79] Note that PTDSS1 appeared as hit in our screens, and SNX13 was also identified in the Trinh et al. study. PTDSS1-mediated phosphatidylserine (PS) synthesis in the ER was found to be required for PM-to-ER transport of free cholesterol. Trinh et al. showed that ER-anchored GRAMD1 protein that translocates PM-cholesterol in a PS-dependent manner to the ER via membrane contact sites.[80] Binding of PS to human SNX13 and its Drosophila ortholog, Snazarus, has been also reported. While SNX13 does not contain a sterol-sensing domain like GRAMD1s, its paralog SNX14 binds cholesterol. Since SNX13 heterodimerizes with SNX14 ([81]; Henne personal communication), perhaps a SNX13-SNX14 functional interplay regulates

exchange of PS for cholesterol at ER-PM membrane contact sites. Further research is needed to determine whether PS binding to SNX13 is part of this regulatory circuit.

EXOSOME RELEASE: DUMPING EXCESS CHOLESTEROL OUTSIDE OF THE CELL

The endolysosomal compartment is characterized by the presence of ILVs which are targets for degradation by lipid hydrolases. When ELs undergo fusion with the PM, ILVs become extracellular vesicles (EVs) known as exosomes[82] (Figure 1B). Initially thought to represent a mechanism for cellular waste disposal or plasma membrane repair, it is now widely accepted that this and other types of EVs mediate intercellular communication by transferring biologically active molecules harbored in their lumen and associated membranes.[82] The lipid bilayer of exosomes is characterized by a unique lipid composition enriched in cholesterol, sphingolipids and glycerophospholipids such as phosphatidylserine or BMP.[83–85] Some of these lipids (including cholesterol and BMP) play important roles in ILV/exosome biogenesis, which is mediated by ESCRT-dependent and -independent mechanisms.[82] Remarkably, our genetic screening approach revealed that deletion of several endosomal sorting complex required for transport (ESCRT) subunits consistently aggravated endolysosomal cholesterol accumulation caused by NPC1 inhibition.[25] This observation suggests that ILV/exosome formation is important for maintaining cholesterol levels in ELs. Other work showed that siRNA depletion of the ESCRT-0 subunit HRS, but not other ESCRT subunits, induces an NPC-like phenotype.[86] Perhaps the absence of ESCRT function up-regulates an alternative pathway that generates ILVs highly enriched in cholesterol. Indeed, cells lacking different key subunits of all four ESCRTs still generated ILVs that were bigger in size and perhaps different in lipid composition.[86,87] Further work is needed to unravel the exact role of ESCRTs in endolysosomal cholesterol transport.

A common feature of most lysosomal storage diseases (including NPC) is the presence of accumulated cytotoxic material in the lumen of ELs that is also accompanied by highly abundant and heterogeneous ILVs (Figure 1C). Previous work characterized a transcriptionally-regulated exocytic pathway whereby ELs undergo fusion with the PM to release pathologic endolysosomal content in lysosomal storage disorder cells, a process coined as "cellular clearance". Subsequent studies showed that both NPC fibroblasts or wild type cells treated with the NPC1 inhibitor U18666A, exhibit increased exosome release[88–90] (Figure 1B; step 5), ameliorating abnormal cholesterol storage. In one study, U18666A-induced EL exocytosis was proposed to depend on flotillins,[90] previously proposed to participate in intestinal cholesterol uptake by NPC1L1, a homolog of NPC1.[91,92] Nevertheless, other studies reported opposite effects under U18666A conditions but using different cell types,[93,94] suggesting that not all cells respond similarly to endolysosomal cholesterol accumulation.

Interestingly, a number of drugs including cyclodextrin,[95] δ-Tocopherol[96] or BK channel agonists[97] enhance EL exocytosis from NPC cells (Figure 1B; step 5), decreasing intracellular cholesterol overload. Spiegel and colleagues recently reported that pharmacological activation of sphingosine kinase 1 (SPHK1) was sufficient to bypass NPC1 function.[98] An isoform-specific agonist of SPHK1 rescued cholesterol accumulation in NPC1 mutant fibroblasts. Sphingolipid catabolism in ELs yield sphingosine which is translocated into the cytoplasm where it is converted into sphingosine-1 phosphate (S1P) by SPHK1 and SPHK2 (Figure 1B; step 8). Previous studies showed that S1P-mediated activation of EL-localized S1P receptors promotes exosome biogenesis and release[99] (Figure 1B; steps 8′ and 5). S1P supply on EL membranes relied on the presence of SPHK2 isoform but not SPHK1. Moreover, while SPHK2 localized to ELs, SPHK1 did not. However, others have shown that SPHK1 also localizes to EL structures[100] and that inhibiting its activity impairs EL biogenesis.[101] On the other hand, expression of SPHK2 prevents cholesterol accumulation in macrophages of atherosclerosis mouse models.[100] All of this evidence supports the idea that SPHK-mediated S1P production may up-regulate an exosome release pathway that enables cholesterol export independent of NPC1. Finally, as mentioned earlier, augmenting BMP levels in NPC cells treated with PG induces secretion of cholesterol-laden exososomes[27] (Figure 1B; step 5). Altogether, induction of exosome release may represent a potential therapeutic strategy for NPC and other lysosomal storage disorders.

RESCUING NPC1-DEFFICIENCY WITH A VIRAL PROTEIN

In recent years it has become increasingly appreciated that many viruses harness cholesterol metabolism and transport during host cell infection.[102] A remarkable example is an NPC1-independent cholesterol transport pathway induced by the adenovirus protein Ridα.[103] Cells infected with Ridα-deficient adenoviruses exhibited aberrant LC3-positive autolysosome compartments that contain accumulated free cholesterol. Expression of Ridα in cells induced formation of autophagy-like vesicles that, in addition to Ridα, were also positive for several EL markers including RAB7 or ORP1L (Figure 3). In addition, stable expression of Ridα in cells treated with U18666A ameliorated endolysosomal cholesterol accumulation. Importantly, cholesterol reduction was similarly observed in Ridα-expressing NPC fibroblasts, which also showed increased formation of autophagy-like BMP-positive compartments decorated with Ridα, RAB7, ORP1L. Finally, inhibition of class III phosphoinositide 3-kinase activity, essential for autophagy, prevented Ridα-mediated NPC1 bypass in these cells. In earlier work, Carlin and colleagues showed that Ridα controls EL maturation by hijacking RAB7 effectors.[104] An interaction between the LTP protein ORP1L and Ridα was reported.[104] Subsequent studies suggested that Ridα may stabilize ORP1L-mediated ER-EL membrane contact sites through which cholesterol is mobilized in a RAB7- and NPC1-independent manner.[55,105] Overall, these studies suggest that Ridα up-regulates an NPC1 bypass pathway in which autophagy may be involved. But how can this benefit adenovirus infection?

Cholesterol is used by many viruses for fueling replication and other activities inherent to their life cycles.[102] Since NPC1-mediated

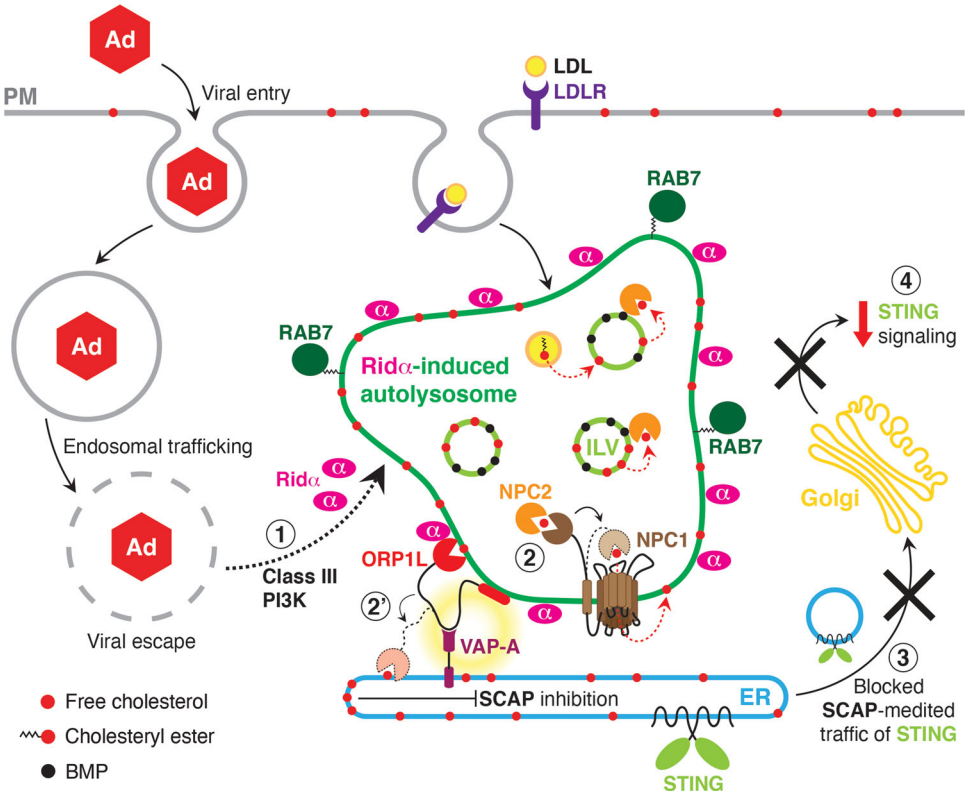

FIGURE 3 **A cholesterol egress pathway mediated by adenoviral Ridα protein.** Adenoviruses (Ad) enter cells via the endocytic pathway. Once endocytosed, adenoviruses disrupts the endosomal membrane to escape into the cytoplasm, where virions are uncoated and their respective genomes delivered into the host cell nucleus to initiate viral gene expression. Upon translation, Ridα initiates a process that involves hijacking of host cell factors, including RAB7 effectors (ORP1L), and class III phosphoinositide 3-kinase (PI3K) activity (**step 1**). As a result, autolysosomes enriched in cholesterol and positive for Ridα, ORP1L, BMP, and RAB7 are formed. From this compartment, cholesterol may be exported by NPC1-dependent (**step 2**) and -independent mechanisms; the latter involving EL-ER membrane contacts sites (represented in yellow background) established by ORP1L and ER-localized VAP-A (**step 2′**). Once transported to the ER, cholesterol inactivates SCAP which in turn blocks SCAP-mediated ER-to-Golgi transport of STING (**step 3**). Decreased STING transport diminishes Golgi-localized STING signaling (**step 4**) needed for triggering antiviral innate immune responses. ER, endoplasmic reticulum; PM, plasma membrane; BMP, bis(monoacylglycero)phosphate; VAP-A, vesicle-associated membrane protein-associated protein A; SCAP, sterol regulatory element-binding proteins cleavage activating protein; STING, stimulator of interferon genes.

cholesterol export is rate-limiting, Ridα may induce the formation an autophagy-like compartment to more efficiently mobilize cholesterol. Such an enlarged compartment may concentrate cholesterol and an elevated number of lipid transfer protein molecules (such as ORP1L) that increase ER-EL membrane contact sites through which cholesterol is transported as discussed above[63] (Figure 3). While Ridα is dispensable for adenovirus replication,[106] its deficiency increases antiviral immune responses.

A link between NPC1 function and inflammation triggered via stimulator of interferon genes (STING) was recently reported.[107] Mechanistically, NPC1 may recruit STING to the lysosome for degradation. In the absence of NPC1, STING is no longer targeted to lysosomal down-regulation, which consequently increases inflammation in NPC mouse models.[107] Earlier work uncovered an interaction between STING and Sterol regulatory element-binding proteins (SREBP) cleavage activating protein (SCAP) that was required for STING-mediated signaling needed for innate immune responses against viruses including adenoviruses.[108,109] Under normal conditions, NPC1 allows endolysosomal cholesterol to move from ELs to the ER where it engages with SCAP, preventing ER-to-Golgi transport of SREBPs. Indeed, inhibiting SCAP-mediated trafficking of SREBPs blocked priming of STING signaling,[107] suggesting that SCAP also chaperones STING transport from the ER to the Golgi, where STING signaling is triggered. Taken together, these data suggest that adenoviruses may have evolved a Ridα-mediated NPC1 bypass that ensures efficient cholesterol transport to the ER, ultimately inhibiting STING-mediated antiviral immune responses (Figure 3). Deciphering the molecular mechanisms that regulate NPC1-independent cholesterol export pathways may also contribute to the design of antiviral therapies.

CONCLUDING REMARKS

I have summarized here, a number of molecules and pathways that contribute to NPC1-independent cholesterol egress from endolysosomes.

Some of these routes work in parallel to the classical NPC1-mediated route, while others may work independently and only under specific conditions, such as viral infection. Despite the existence of these alternative pathways, it is important to remember that NPC patients fatally succumb to the disease due to the lack of NPC1 function. Nevertheless, milder or chronic cases of the disease may represent up-regulation of NPC1 bypass mechanisms. Unravelling the molecular mechanisms that regulate cholesterol export in the absence of NPC1 function may certainly offer avenues for the design of novel therapeutic interventions for not only lysosomal storage disorders, but also for viral infections including COVID-19.[110] Overall, cholesterol trafficking from ELs is a more complex process than previously anticipated, and unexpected discoveries surely await.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors do not have a conflict of interest.

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