

Review

Endosomal cholesterol trafficking: protein factors at a glance

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The delivery of low-density lipoprotein-derived cholesterol (LDL-C) from endosomal compartments to the plasma membrane and the endoplasmic reticulum (ER) is an important yet poorly understood cellular process. Niemann-Pick C1 (NPC1), a multi-pass integral membrane protein on the limiting membranes of late endosomes (LE)/lysosomes (Ly), is known to insert luminal LDL-C to the limiting membrane of LE/Ly. Recent progress has identified novel cytoplasmic proteins that regulate the exit of LDL-C from LE/Ly, such as ORP5, a member of the oxysterol-binding protein-related protein (ORPs) family, and Hrs/VPS27, a well-established regulator of the endosomal sorting complex required for transport pathway. Whereas ORP5/ORPs may serve as cytosolic cholesterol carriers and deliver cholesterol in a non-vesicular manner, how Hrs/VPS27 regulate endosomal cholesterol sorting remains enigmatic. We discuss the functional relationship between NPC1, Hrs, and ORP5, and formulate possible schemes on how LDL-C may be moved from endosomal compartments to other cellular organelles.

Keywords NPC1; NPC2; OSBP/ORP; Hrs; endosomal cholesterol transport

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Introduction

Cholesterol is an essential membrane lipid for animal cells that require cholesterol together with other phospholipids for membrane synthesis and expansion. Importantly, cholesterol participates in the assembly of lipid rafts (specialized membrane microdomains enriched for particular lipids and proteins), which provides platforms necessary for membrane trafficking and signaling [1]. In some specialized tissues and cells, cholesterol also serves as a precursor for the biosynthesis of steroid hormones, bile acids, and vitamin D. In addition, cholesterol can be oxidized to generate oxysterols, which are important in many physiological processes [2].

Cholesterol is synthesized in the endoplasmic reticulum (ER) from a simple two-carbon substrate acetic acid through a complex pathway involving the action of at least 30 enzymes. Mammalian cells also acquire exogenous cholesterol mainly from circulating low-density lipoprotein (LDL) through receptor-mediated endocytosis [3]. This well-known endocytic pathway sorts and delivers LDL from early endosome to late endosome/lysosome (LE/Ly), where cholesteryl esters carried by LDL are hydrolyzed by acid lipase. Freed cholesterol egresses from LE/Ly and is delivered to the ER, plasma membrane, and other cellular compartments for regulatory, functional, and structural roles [4]. In most cases, cellular cholesterol homeostasis is controlled by feedback regulation of cholesterol biosynthesis and uptake from LDL. This is crucial since, despite its vital role in cell survival, too much cholesterol is toxic to cells; and at the human body level, imbalanced cholesterol metabolism/homeostasis has been linked to many diseases, such as cardiovascular diseases, the leading causes of death worldwide.

Upon arrival at the ER from LE/Ly, LDL-derived cholesterol (LDL-C) inhibits the sterol regulatory element-binding protein (SREBP) pathway, subsequently decreasing the transcription of the genes responsible for cholesterol synthesis and uptake [5]. The accumulation of LDL-C in the ER also blunts cholesterol synthesis by accelerating the proteasomal degradation of key enzymes including 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase [6] and squalene monooxygenase [7]. Furthermore, LDL-C induces the activity of ACAT1, an ER-resident enzyme that catalyzes the conversion of cholesterol into cholesteryl esters stored in cytosolic lipid droplets [8], directly lowering the ER free cholesterol levels. Therefore, the endosomal transport of LDL-C to the ER is of critical importance to the regulation of cellular cholesterol homeostasis.

Currently, how endosomal compartments receive cholesterol via the LDL pathway is well understood. However, a clear mechanism underlying the removal of endosomal cholesterol remains elusive. Recent progress in the field has identified novel proteins that may directly or indirectly regulate the transport of LDL-C to the ER. This review

examines these protein factors and discuss how they are involved in endosomal cholesterol trafficking.

Niemann-Pick type C Proteins

Niemann-Pick C1 and Niemann-Pick C2

Niemann-Pick C1 (NPC1) and NPC2 are two distinct proteins that are known to govern endosomal cholesterol transport. Mutations in either NPC1 or NPC2 lead to an accumulation of unesterified cholesterol in LE/Ly [9,10]. The sequestration of free cholesterol and other lipids in LE/Ly due to NPC1 or NPC2 mutations is a typical cellular phenotype of a rare, fatal medical condition termed NPC disease. This neurodegenerative disorder is an autosomal-recessive, lysosomal storage disease that mainly afflicts teenagers and is devastating since there is currently no cure. Approximately 95% of NPC cases are caused by mutations in the NPC1 gene [9] and the rest are related to mutations in NPC2 [10]. Some recent studies, such as those employing the administrations of cyclodextrin or histone deacetylase inhibitors in NPC cell lines or NPC mice [11,12], have raised the hopes of finding an effective treatment for NPC patients. It is also hoped that more effective treatment will stem from the understanding of molecular functions of NPC1 and NPC2.

The gene-encoding NPC1 with disease-causing mutations was first identified in 1997 [9]. Since then, the structure of NPC1 protein has been well characterized. NPC1 is a 1278-amino acid membrane protein that mainly localizes to limiting membrane of LE/Ly. Topological analysis of the protein predicts 13 transmembrane domains (TMDs), three large luminal loops including an N-terminal domain

(NTD), six smaller cytosolic loops, and a C-terminal cytoplasmic tail (**Fig. 1**) [13]. The TMDs 3–7 of NPC1 share strong sequence homology to the sterol-sensing domains (SSD) of other cholesterol-responsive proteins, such as SREBP cleavage-activating protein; HMG-CoA reductase, NPC1-like protein 1 (NPC1L1), as well as the Hedgehog signaling protein, Patched [13,14]. A photoaffinity labeling study reported that a functional SSD within NPC1 is required for direct binding between NPC1 and photoactivated cholesterol [15]. This was the first evidence that NPC1 may be able to directly bind cholesterol. However, the exact role of the SSD in NPC1 function is still uncertain. To date, there is little evidence for the similarity of the SSD among above-mentioned proteins. It is noteworthy that, in response to sterols, both the SSDs of HMG-CoA reductase and SCAP binds to Insig, an ER membrane protein that plays a crucial role in feedback regulation of cholesterol synthesis [16,17]. Whether this domain in NPC1 is also responsible for protein–protein interaction remains to be elucidated.

The second NPC gene (*NPC2*) was identified in 2000 from a lysosomal proteomic study [10]. NPC2 is a 151-amino acid glycoprotein containing a 19-amino acid signal peptide. The protein has previously been known as HE1, a major secretory protein of the human epididymis that binds cholesterol with high affinity [18]. Subcellular fractionation shows that the soluble NPC2 is mainly localized to late endosomal/lysosomal lumen [10]. The lysosomal targeting of NPC2 seems to be strictly dependent on mannose 6-phosphate receptors (MPRs) [19]. When the two receptors (MPR46 and MPR300) are absent, NPC2 fails to reach LE/Ly and the majority of the protein is secreted into

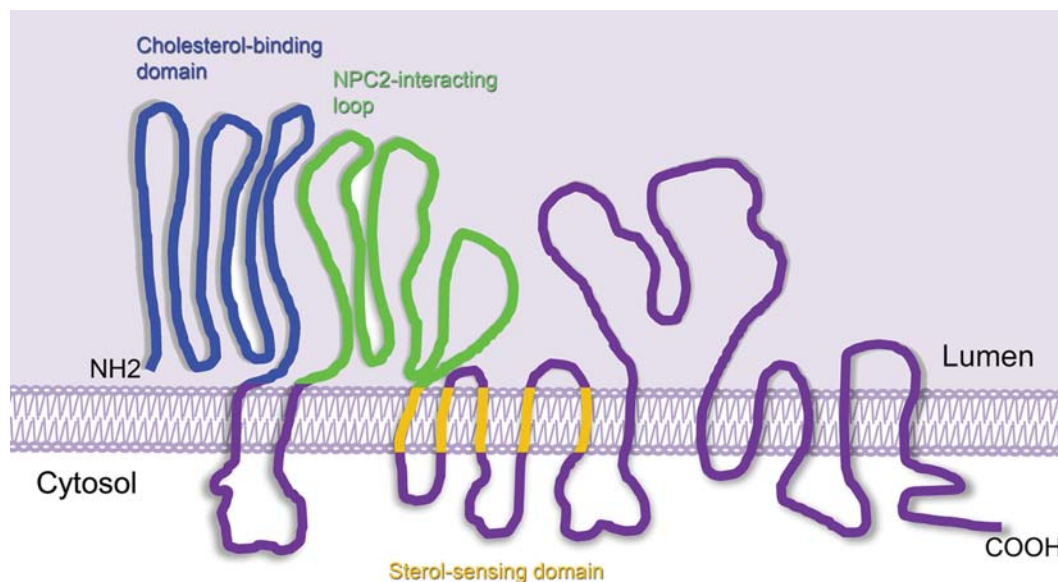


Figure 1 Membrane topology of NPC1 The amino-terminal cholesterol-binding luminal domain is shown in blue. The NPC2-interacting domain (the middle large luminal loop) is shown in green. The sterol-sensing domain consists of transmembrane domains 3–7 and is shown in yellow.

the culture medium, leading to a massive accumulation of unesterified cholesterol in LE/Ly, a phenotype similar to that of the NPC patient fibroblasts [19]. NPC2 has been shown to interact with reticulon 4B (Nogo-B) receptor (NGBR), which predominantly localizes to the ER and stabilizes nascent NPC2 [20]. Depletion of NGBR decreases NPC2 levels and increases intracellular cholesterol accumulation, mimicking the deficiency effects of an NPC2 mutation [20].

Cholesterol 'hand-off' between NPC1 and NPC2

Homozygous mutations in either NPC1 or NPC2 gene produce an undistinguishable clinical phenotype and the same pattern of cholesterol accumulation in LE/Ly, indicating that both proteins function in the same pathway to facilitate cholesterol egress [21]. NPC1 has been purified from rabbit liver membranes [22] and the sterol-binding site in NPC1 has been localized to the NTD, which comprises 240 amino acids, essentially constituting the first large luminal loop [23] (Fig. 1). NPC1(NTD) contains a deep pocket that surrounds cholesterol with the 3 β -hydroxyl group and the tetracyclic ring buried inside, leaving the isooctyl side chain of cholesterol partially exposed [24]. The crystallographic structure of NPC2 demonstrates that cholesterol binds in a deep hydrophobic pocket surrounded by the two beta-sheets of NPC2 [25]. Interestingly, NPC2 binds the isooctyl side chain of cholesterol, leaving the 3 β -hydroxyl exposed, in an orientation opposite to NPC1(NTD) [24]. Since cholesterol could be transferred between purified NPC1(NTD) and NPC2 in a bidirectional manner [26], a working model of lysosomal cholesterol transport was proposed: NPC2 accepts LDL-C in the lysosomal lumen and transports it to membrane-bound NPC1 for export [24,26].

The 'hand-off' model predicts a direct transfer and/or interaction between the two proteins, thus avoiding the necessity for insoluble cholesterol to transit the water phase. By generating mutant forms of NPC2 and NPC1(NTD) that are capable of binding cholesterol but not engaging in transfer from one protein to the other, a recent study pinpointed discrete regions on the surface of the two proteins as the possible interacting sites [27]. However, the stable physical interaction between NPC2 and NPC1(NTD) has been difficult to be demonstrated. It is likely that the interactions between the two proteins are transient and may only be supported by functional and kinetic evidence [27]. Interestingly, by using surface plasmon resonance and affinity chromatography, a recent study showed that NPC2 directly binds to the second luminal domain of NPC1 [28] (Fig. 1). Importantly, this interaction only occurs at acidic pH, a condition that mimics lysosomal lumen environment and is optimal for cholesterol binding to NPC2 and transfer to NPC1 [28]. It is possible that the second luminal domain of NPC1 holds NPC2 in position to facilitate directional

cholesterol transfer from NPC2 onto NPC1, in a way supporting the hand-off model. However, it should be noted that although the current model favors the NPC2 to NPC1 direction of cholesterol transport, the reverse direction could also be true: cholesterol in LE/Ly is bound by NPC1(NTD) first and then transferred to NPC2 for delivery to a cholesterol efflux transporter [29]. Further evidence is needed to delineate a *bona fide* cholesterol transfer mechanism by NPC1 and NPC2.

NPC1 and LDL-C transport to the ER

Although the NPC1/NPC2 hand-off model well depicts cholesterol transport inside lysosome lumen, the big question still remains: how LDL-C is exported from LE/Ly membranes and transported to the ER? Because NPC1 localizes to the limiting membrane of LE/Ly, it is conceivable that NPC1 may sense LDL-C on LE/Ly membrane and directly mediates LDL-C transport to the ER. The transport of LDL-C by NPC1 may mainly rely on vesicular mechanism, a pathway involving vesicles that bud off from LE/Ly and fuse with the ER. This hypothesis is supported by an earlier study, which reported that NPC1-containing organelles undergo dynamic movement toward the ER [30], although whether such movements can directly transfer cholesterol remains questionable. Another indication of vesicular transport of LDL-C to the ER comes from overexpression studies of Rab proteins which are key mediators of vesicle trafficking. In NPC1 deficient cells, overexpression of Rab7 or Rab9 could significantly reduce cholesterol accumulation and restore LDL-C egress from LE/Ly [31,32]. Further indication may come from NPC1L1, a close homolog of NPC1 that mediates dietary cholesterol absorption through vesicular endocytosis from the plasma membrane to the endocytic recycling compartment (reviewed in ref. [33]). Given the structural similarities between the two proteins, it is plausible that NPC1 may employ an NPC1L1-like mechanism to facilitate LDL-C transport to the ER.

NPC1 may also function at LE/Ly and ER membrane contact sites to elicit non-vesicular cholesterol transport. Such membrane contact sites have been demonstrated in other cellular events. For instance, epidermal growth factor receptor (EGFR) signaling is mediated by the ER-localized PTP1B (protein tyrosine phosphatase 1B), which dephosphorylates EGFR to down-regulate this signaling pathway [34]. PTP1B on the cytoplasmic face of the ER interacts with endocytosed, multiple vesicular body/LE-localized EGFR via direct membrane contacts sites between the ER and endosomes [34]. This inter-organellar junction may provide potential sites of protein-protein interaction, as well as cholesterol and other lipid exchange between organelles. On a more related note, cholesterol status in LE prompts the cholesterol sensor oxysterol-binding protein-

related protein 1 long form to adopt a conformational change, which induces the formation of ER–LE membrane contact sites [35]. The existence of ER–LE membrane contact sites potentially provides platforms for NPC1 and other proteins, which may act together to transport cholesterol directly to the ER. Indeed, it has been suggested that putative cytosolic proteins may exist for export of LDL-derived cholesterol from LE/Ly membrane [24,29].

ORP5

In search of candidates responsible for protein-mediated endosomal cholesterol transport, increasing attention has been focused on the OSBP (oxysterol-binding protein)/ORP (OSBP-related protein) family [36–38]. This family of proteins is conserved evolutionarily and includes seven members in the budding yeast *Saccharomyces cerevisiae* and 12 in humans [39,40]. OSBP, the founding member of the family, was first identified as a high-affinity cytosolic-binding protein specific for oxysterols, such as 25-hydroxycholesterol [41]. Other members have subsequently been isolated in most eukaryotes. All OSBP/ORPs share a conserved ~400-amino acid OSBP-related domain (ORD) found at the C-terminus of OSBP, which has been shown to bind cholesterol and oxysterols [42–44] (Fig. 2). The ORD in certain ORPs may also simultaneously bind two different organelle membranes, allowing a rapid but regulated sterol transfer at membrane contact sites [45]. Moreover, the N-terminus of these proteins often possesses the pleckstrin homology (PH) domain and an FFAT motif (diphenylalanine in an acidic tract) for membrane targeting [46,47] (Fig. 2). The functions of ORD and membrane targeting domain/motifs presented in OSBP/ORPs support possible mechanisms of cholesterol transport: (i) OSBP/ORPs shuttle cholesterol between donor and acceptor membranes and are completely disengaged from membranes after binding and releasing cholesterol or (ii) OSBP/ORPs are simultaneously engaged with both donor and acceptor membranes at contact sites [37]. An important recent study has demonstrated that Osh4p (a yeast ORP) can also bind PI4P, and couple cholesterol and PI4P transfer between

membranes with high efficiency *in vitro* [48]. This lends further support to an *in vivo* role of ORPs as sterol carriers.

ORP5 has been identified as a novel protein involved in endosomal cholesterol trafficking [49]. Human ORP5 is a ubiquitous protein containing 879 amino acids. The ORD of ORP5 is capable of binding cholesterol and 25-hydroxycholesterol both *in vivo* and *in vitro* [42]. The role of ORP5 in endosomal cholesterol trafficking is suggested by knockdown studies performed in HeLa cells [49]. ORP5 knockdown leads to an accumulation of LDL-C in LE/Ly and significantly impaired LDL-C transport to the ER. The potential role of ORP5 in endosomal cholesterol trafficking may reflect a functional interaction between ORP5 and NPC1. Indeed, ORP5 appears to function downstream of NPC1 to facilitate cholesterol egress from LE/Ly membranes. Cholesterol appears to accumulate on the limiting membranes of LE/Ly upon ORP5 knockdown, which differs from NPC1 knockdown cells, where cholesterol accumulates in the LE/Ly lumen. Interestingly, ORP5 and NPC1 double-knockdown cells exhibit a phenotype that is similar to NPC1 knockdown alone, i.e. cholesterol accumulation predominantly in the LE/Ly lumen. These observations support the hypothesis that a functional NPC1 assists LDL-C in reaching the limiting membrane of LE/Ly, where ORP5 may be required to form a transport machinery to remove cholesterol. Further evidence supporting the NPC1/ORP5 functional link comes from examining the localization of DsRed-Golgi and other retrograde transport cargos, e.g. the cation-independent MRPs and TGN46. These markers localize to the Golgi at steady state in normal cells, but are mis-localized to LE/Ly upon ORP5 depletion. Remarkably, the mis-localization of DsRed-Golgi is corrected in ORP5/ NPC1 double knockdown cells [49]. Cholesterol accumulating on the limiting membrane upon ORP5 depletion may severely disturb the physical properties of the limiting membrane. Therefore, ORP5 depletion is more disruptive to the retrograde transport pathway, which involves endosomal limiting membranes. On the other hand, when NPC1 is compromised, cholesterol may not reach the limiting membrane but accumulate in the lumen of LE/Ly, leaving membrane trafficking largely unaffected.



Figure 2 Domain structures of human OSBP, ORP5, and ORP8 PH, pleckstrin homology; FFAT, diphenylalanine in an acidic tract; ORD, OSBP-related domain; TM, transmembrane domain.

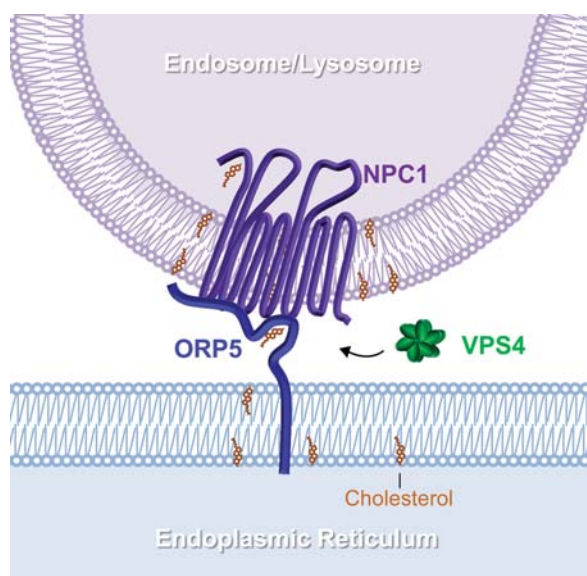


Figure 3 A hypothetical model for the transport of LDL-C at an endosome-ER junction by a multiple protein complex In response to increasing cholesterol content on the limiting membranes, ORP5 efficiently move cholesterol from endosome/lysosome to the ER by forming a complex with membrane lipids, NPC1, and putative receptors/junction proteins (not depicted). Upon completion of cholesterol transfer, the AAA ATPase VPS4 disrupts the complex and recycles the ORPs.

ORP5 specifically binds to phosphatidylinositol phosphate enriched in LE via its N-terminal PH domain, suggesting that it can interact with endosomal membranes (our unpublished observations). ORP5 may form a transient protein complex with NPC1 at the membrane contact sites connecting LE/Ly and the ER. Other yet-to-be-identified proteins are required to form such a junction in order to help ORP5/NPC1 complex carry out non-vesicular sterol transport. Upon completion of sterol transfer, AAA ATPases (ATPase associated with a variety of cellular activities) like VPS4/SKD1 may join to break down such a complex for recycling the ORP5 and other components (Fig. 3). In support of this model, our laboratory has previously found for the first time a link between yeast ORPs (Osh6/7p) and the Vps4p [50]. Importantly, we recently demonstrated that VPS4 is indeed required for proper transport of LDL-C from LE/Ly to the ER [51]. Since VPS4 is also functionally associated with NPC1 [52], it has become necessary to elucidate the true nature of the interactions between these proteins and to identify other unknown proteins involved in LDL-C transport to the ER.

Hrs

In a proteomic analysis, out of 48 000 independent human brain cDNA clones, ORP5 was identified as one of 48 distinct proteins that specifically bound to the ubiquitin-interacting motif of hepatocyte growth factor-regulated tyrosine kinase

substrate (Hrs, called VPS27 in yeast for vacuolar protein sorting 27) [53]. Hrs interacts with signal transducing adaptor molecule to make up endosomal sorting complex required for transport (ESCRT)-0, which plays a crucial role in initiating the ESCRT pathway [54,55]. ESCRT-0 recruits ESCRT-I possibly through the direct interaction between Hrs and Tsg101 (an ESCRT-I component that also binds ubiquitinated proteins), and hands the ubiquitinated cargo to ESCRT-I [56–59]. The possible link between ORP5 and Hrs [53] raised an interesting and important question: do Hrs and other ESCRT components regulate intracellular cholesterol transport and homeostasis?

We recently reported that Hrs indeed plays an essential role in the transport of LDL-C to the ER [60]. In HeLa cells, when Hrs is depleted by RNA interference, LDL-C is trapped in LE/Ly and its delivery to the ER is almost completely blocked, an effect essentially reminiscent of that found in NPC1 deficient cells. Interestingly, NPC1 and NPC2 expressions and localizations are not affected upon Hrs depletion, suggesting that Hrs has an indirect effect on LDL-C sorting. This effect seems to be distinct in that knocking down of other ESCRT subunits has no effect on LDL-C transport to the ER. How Hrs regulates endosomal cholesterol trafficking is intriguing. In one hypothetical scenario, Hrs may selectively facilitate the removal of cholesterol by cytoplasmic carriers such as ORP5 from specific regions of the limiting membrane, thereby generating a cholesterol-rich membrane microdomain [60]. Further studies are needed to investigate the existence of the possible cholesterol-rich microdomain mediated by Hrs, and whether NPC1 employs such a microdomain to facilitate LDL-C transport to other organelles, including the ER [33]. Alternatively, Hrs may be required to generate a specific set of intraluminal vesicles that are required for the storage and trafficking of cholesterol within endosomal lumen [61].

Conclusion

A challenging subject in cell biology is to understand how cholesterol is transported between different biological membranes. This is mainly due to the water-insoluble nature of cholesterol and the constraints of proper methodological approaches. Despite years of intensive studies, a unifying mechanism for LDL-C exit from LE/Ly is still not available. Nevertheless, tremendous progresses in recent years have been made to understand this pathway. The most notable examples are the elucidation of the structures and cholesterol-binding/transfer properties of NPC1 and NPC2, and the identification of some novel regulators that potentially work together with NPC1 to deliver LDL-C to the ER. Since non-vesicular and vesicular mechanisms may co-operate in endosomal sterol trafficking [62], one would expect that rather complicated mechanisms for this process

may exist, and that substantial studies are still required to delineate the network of protein factors governing endosomal cholesterol trafficking.

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