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REVIEW

Sterol-binding proteins and endosomal cholesterol transport

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Abstract **Endosomal compartments sort and deliver exogenous lipoprotein-derived cholesterol to the endoplasmic reticulum for regulating cellular cholesterol homeostasis. A large number of studies have focused on the removal of endosomal cholesterol, since its accumulation leads to devastating human diseases. Recent studies suggest that cytoplasmic sterol-binding proteins may be involved in endosomal cholesterol transport. In particular, endosome/lysosome-localized or -associated cholesterol-binding proteins may serve as key mediators of cholesterol removal in a non-vesicular manner. Further characterization of these cholesterol-binding proteins will shed light on the molecular mechanisms that regulate endosomal cholesterol sorting.**

Keywords NPC1, NPC2, OSBP/ORP, StAR protein, endosomal cholesterol transport

Introduction

Cholesterol has long been recognized as a fascinating and highly decorated small molecule, representing an indispensable membrane sterol for eukaryotic life. Not only does cholesterol provide membranes with rigidity and fluidity, and help to generate selectively permeable barriers among cellular compartments, but it also participates in the assemblies of lipid rafts (specialized membrane microdomains enriched for particular lipids and proteins), highlighting its importance in membrane trafficking and signaling (Lingwood and Simons, 2010). To ensure proper functioning of membranes, mammalian cells have developed elegant mechanisms to transport cholesterol for maintaining dynamic cellular cholesterol homeostasis. For example, cholesterol synthesis, uptake, esterification and efflux are tightly controlled and carefully regulated at levels from gene transcription to protein post-translational modification (reviewed in Chang et al., 2006; Goldstein et al., 2006; Brown and Goldstein, 2009; Mesmin and Maxfield, 2009; Maxfield and van Meer, 2010).

A challenging subject in cell biology is to understand how cholesterol is transported between different biological membranes. Due to the insolubility of cholesterol in water, cholesterol movement has to be assisted by complex mechanisms. These include vesicular and non-vesicular transport pathways, which have been proposed to ensure heterogeneous distribution of cholesterol among membranes (Soccio and Breslow, 2004; Ikonen, 2008; Mesmin and Maxfield, 2009). Emerging evidence suggests that lipid-binding protein-mediated non-vesicular transport plays important roles in intracellular cholesterol trafficking (Lev, 2010). This brief review will highlight recent advances in understanding some well-known sterol-binding proteins, including but not limited to the Niemann Pick C protein (NPC), START (steroidogenic acute regulatory protein [StAR]-related lipid transfer) domain containing proteins and oxysterol-binding protein (OSBP)-related protein (ORP) families. We discuss how these proteins are involved in endosomal cholesterol transport, a major pathway governing cellular cholesterol homeostasis.

Endosomal cholesterol transport

In addition to *de novo* synthesis from acetic acid in the endoplasmic reticulum (ER), animal cells also acquire cholesterol from exogenous lipoprotein particles. Low-density lipoprotein (LDL), the major source of cholesterol, binds to LDL-receptors on the cell surface, entering cells through receptor-mediated endocytosis (Brown and

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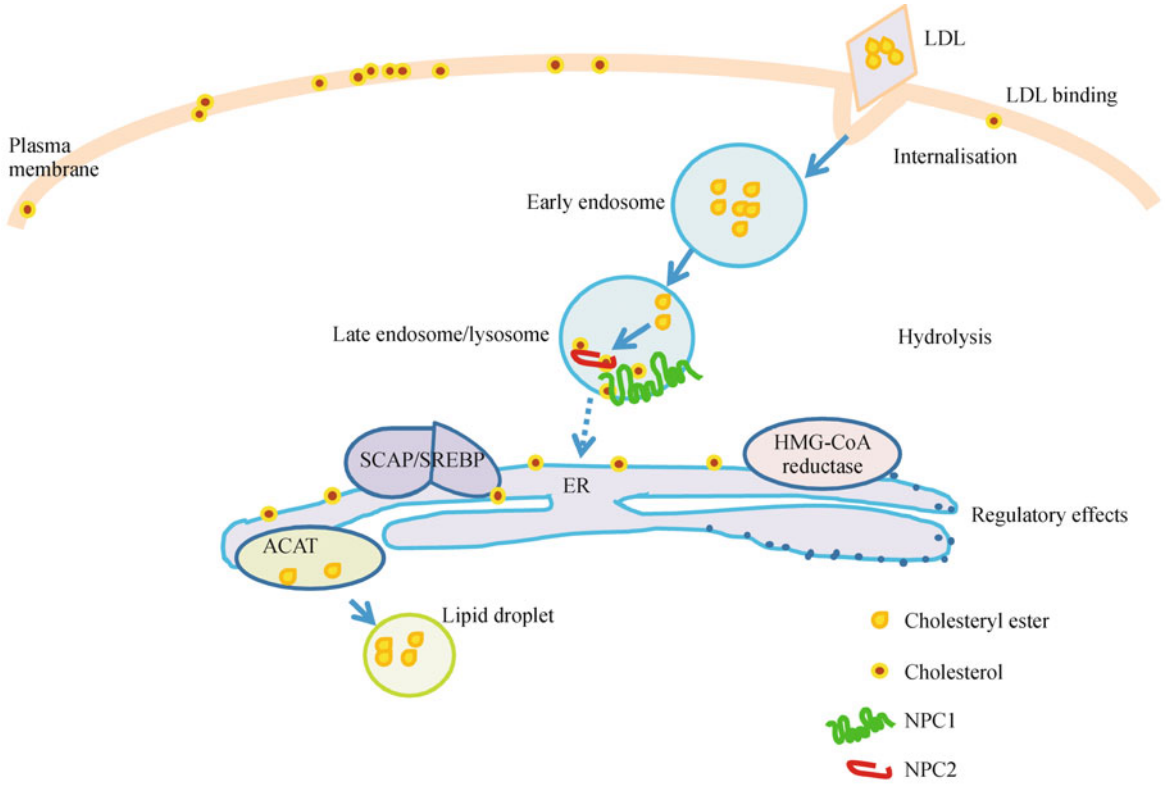

Figure 1 Endosomal transport of low-density lipoprotein (LDL)-derived cholesterol to the endoplasmic reticulum (ER). LDL particles bound to LDL-receptors are internalized and delivered to early and late endosome/lysosome. Cholesteryl ester carried by LDL is hydrolyzed by acid lipase in the late endosome/lysosome. The released free cholesterol is transported to the ER through a process mediated by NPC1, NPC2 and other unknown proteins. In the ER, LDL-derived cholesterol exert its regulatory effects by: 1) inhibiting SCAP/SREBP pathway thereby decreasing cholesterol uptake from LDL and synthesis by HMG-CoA reductase; 2) activating cholesterol esterification by ACAT thereby converting cholesterol back to cholesteryl ester stored in cytosolic lipid droplet. ACAT, acyl-coenzyme A: cholesterol acyltransferase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; NPC, Niemann-Pick type C; SREBP, sterol-regulatory element binding protein; SCAP, SREBP cleavage activating protein.

Goldstein, 1986) (Fig. 1). 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 and the bound LDL-receptor is recycled back to the cell surface. Free cholesterol liberated by hydrolysis egresses from LE/LY and is delivered to the ER, plasma membrane and other cellular compartments for regulatory, functional and structural roles (Chang et al., 2006).

Currently, how endosomal compartments receive cholesterol via the LDL pathway is well understood. However, a clear mechanism underlying the removal of endosomal cholesterol remains somewhat elusive. Important clues come from the two NPC proteins (NPC1, NPC2) and other members of cholesterol-binding proteins, such as the StAR proteins and OSBP/ORP families, which have been implicated in endosomal cholesterol trafficking (Holtta-Vuori and Ikonen, 2006). Recent studies of these sterol-binding proteins have shed light on the endosomal itinerary of LDL-derived cholesterol.

Niemann-pick protein C1 (NPC1) and NPC2

NPC1 and NPC2 are two distinct proteins that are believed to govern the exit of cholesterol from LE/LY. Mutations in either NPC1 or NPC2 lead to an accumulation of unesterified cholesterol in LE/LY (Carstea et al., 1997; Naureckiene et al., 2000). 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 Niemann-Pick type C (NPC) disease. This neurodegenerative disorder is an autosomal recessive, lysosomal storage disease. Approximately 95% of NPC cases are caused by mutations in the NPC1 gene (Carstea et al., 1997) and the rest are related to mutations in NPC2 (Naureckiene et al., 2000). There is currently no cure for NPC disease. However, some recent studies, such as those employing cyclodexstrin administrations in NPC cell lines or NPC mice (Liu et al., 2009; Rosenbaum et al., 2010), have prompted the hopes of finding a more effective treatment for patients.

NPC1

The gene encoding NPC1 with disease-causing mutations was first identified in NPC patients in 1997 by positional cloning methods (Carstea et al., 1997). NPC1 is a 1278-amino acid membrane protein that localizes to LE/LY. Topology 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 (Davies and Ioannou, 2000). 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 (SCAP), 3-hydroxymethylglutaryl-CoA reductase, NPC1-like protein 1 (NPC1L1), as well as the Hedgehog signaling protein, Patched (Davies and Ioannou, 2000). A photoaffinity labeling study reported that a functional SSD within NPC1 is required for direct binding between NPC1 and photoactivated cholesterol (Ohgami et al., 2004). Fluorescent protein-tagged NPC1 was specifically labeled with [^{3}H]7,7-azocholestanol (AC), a photolabile cholesterol analog. The labeling was significantly diminished when loss-of-function mutations were presented within the NPC1 SSD (Ohgami et al., 2004). This report provides the first evidence of NPC1 binding to a cholesterol analog and strongly suggests that NPC1 is a cholesterol-binding protein.

NPC2

The second NPC gene (*NPC2*) was identified in 2000 from a lysosomal proteomic study (Naureckiene et al., 2000). 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 (Okamura et al., 1999). Subcellular fractionation shows that the soluble NPC2 is mainly localized to late endosomal/lysosome (Naureckiene et al., 2000). The lysosomal targeting of NPC2 seems to be strictly dependent on mannose 6-phosphate receptors (MPRs) (Willenborg et al., 2005). When the two receptors (MPR46 and MPR300) are absent, NPC2 fails to reach LE/LY and the majority of the protein is secreted into the culture medium, leading to a massive accumulation of unesterified cholesterol in LE/LY, a phenotype similar to that of the NPC patient fibroblasts (Willenborg et al., 2005). NPC2 has recently been shown to interact with reticulon 4B (Nogo-B) receptor (NGBR), which predominantly localizes to the ER and stabilizes nascent NPC2 (Harrison et al., 2009). Depletion of NGBR decreases NPC2 levels and increases intracellular cholesterol accumulation, mimicking the deficiency effects of an NPC2 mutation (Harrison et al., 2009).

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 (Sleat et al., 2004). The Brown and Goldstein group recently purified NPC1 from rabbit liver membranes (Infante et al., 2008a) and localized the sterol-binding site in NPC1 to the N-terminal domain (NTD), which comprises 240 amino acids, essentially constituting the first large luminal loop (Infante et al., 2008b). NPC1(NTD) contains a deep pocket that surrounds cholesterol with the 3β-hydroxyl group and the tetracyclic ring buried inside, leaving the isooctyl side chain of cholesterol partially exposed (Kwon et al., 2009). The crystallographic structure of NPC2 demonstrates that cholesterol binds in a deep hydrophobic pocket surrounded by the two beta-sheets of NPC2 (Xu et al., 2007). Interestingly, NPC2 binds the isooctyl side chain of cholesterol, leaving the 3β-hydroxyl exposed, in an orientation opposite to NPC1(NTD) (Kwon et al., 2009). Since cholesterol could be transferred between purified NPC1 (NTD) and NPC2 in a bidirectional fashion (Infante et al., 2008c), a working model of lysosomal cholesterol transport was proposed: NPC2 accepts LDL-derived cholesterol in the lysosomal lumen and transports it to membrane-bound NPC1 for export (Infante et al., 2008c; Kwon et al., 2009). This "hand-off" model stands for 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 (Wang et al., 2010). However, the stable physical interaction between NPC2 and NPC1(NTD) has yet 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 (Wang et al., 2010). It should also be noted that, although the current hand-off 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 (Subramanian and Balch, 2008). Further evidence is needed to delineate a *bona fide* cholesterol transfer mechanism by NPC1 and NPC2.

Oxyterol-binding protein (OSBP)-related proteins (ORPs)

OSBP/ORP and cholesterol transport

Putative sterol-carrier proteins may exist for export of LDL-derived cholesterol from LE/LY membrane regardless of the action orders of NPC1 and NPC2 (Subramanian and Balch, 2008; Kwon et al., 2009). 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 (Yan and Olkkonen, 2008; Yang, 2006; Ngo et al., 2010). This family of proteins is conserved evolutionarily and includes 7 members in the budding yeast *Saccharomyces cerevisiae* and 12 in humans (Beh et al., 2001; Lehto et al., 2001). OSBP, the founding member of the family, was first identified as a high-affinity cytosol binding protein specific for oxysterols, such as 25-hydroxycholesterol (Taylor et al., 1984). 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 (Im et al., 2005; Wang et al., 2005b; Suchanek et al., 2007). The ORD in certain ORPs may also simultaneously bind two different organelle membranes, allowing a rapid but regulated sterol transfer at membrane contact sites (Schulz et al., 2009). Moreover, the N terminus of these proteins often possesses a pleckstrin homology (PH) domain and an FFAT motif (diphenylalanine in an acidic tract) for membrane targeting (Wyles et al., 2002; Loewen et al., 2003). The functions of ORD and membrane targeting domain/motifs presented in OSBP/ORPs support possible mechanisms of cholesterol transport: (1) OSBP/ORPs shuttle cholesterol between donor and acceptor membranes and are completely disengaged from membranes after binding and releasing cholesterol; or (2) OSBP/ORPs are simultaneously engaged with both donor and acceptor membranes at contact sites (Ngo et al., 2010).

ORP5 and endosomal cholesterol trafficking

Our laboratory has recently identified ORP5 as a novel protein involved in endosomal cholesterol trafficking (Du et al., 2011). Human ORP5 is a ubiquitous protein containing 879 amino acids. ORP5 belongs to subfamily IV of the OSBP/ORP family along with another member, ORP8 (Yan et al., 2008; Yan and Olkkonen, 2008) (Fig. 2). Both ORP5 and ORP8 localize to the ER via a C-terminal transmembrane domain, a unique feature of this subfamily (Yan et al., 2008; Du et al., 2011). The ORD of ORP5 is capable of binding cholesterol and 25-hydroxycholesterol both *in vivo* and *in vitro* (Suchanek et al., 2007 and our unpublished observations). The role of ORP5 in endosomal cholesterol trafficking is suggested by knockdown studies performed in HeLa cells. ORP5 knockdown led to an accumulation of LDL-derived cholesterol in LE/LY, a phenotype similar to that of NPC1-deficient cells (Du et al., 2011). ORP5 depletion also significantly impaired LDL-derived cholesterol esterification, which is catalyzed by acyl-CoA:cholesterol acyltransferase (an ER resident protein) (Du et al., 2011). These data provide strong evidence that ORP5 is involved in LDL-derived cholesterol transport from LE/LY 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 down-stream of NPC1 to facilitate cholesterol egress from LE/LY membranes. Cholesterol accumulates on the limiting membranes of LE/LY upon ORP5 knockdown, which differs from NPC1 knockdown cells, where cholesterol accumulates in the LE/LY lumen (Du et al., 2011). 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 (Du et al., 2011). These observations support the hypothesis that a functional NPC1 assists LDL-derived cholesterol 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 mannose-6-receptors 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 (Du et al., 2011). We envision that 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.

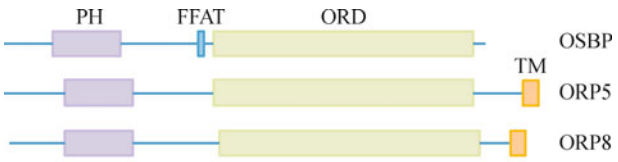

Figure 2 Domain structures of human OSBP, ORP5 and ORP8. PH, pleckstrin homology; FFAT, diphenylalanine in an acidic tract; ORD, OSBP-related domain; TM, transmembrane domain.

ORP5 functions at the membrane contact sites?

ORP5 specifically binds to phosphatidylinositol phosphate enriched in late endosome via its N-terminal PH domain, suggesting that it can interact with endosomal membranes (our unpublished observations). Indeed, when C-terminal transmembrane domain of ORP5 is removed, the truncated protein partially localizes to late endosomes and is enriched in late endosomal cellular fractions (Du et al., 2011 and our unpublished observations). These data lead us to propose a hypothetical model to speculate on how cholesterol can be delivered to the ER from endosomal compartments by ORP5 (Fig. 3). In response to an increased cholesterol level on the limiting membrane, LE/LY may form transient junctions/contact sites with the ER that allow efficient removal of

endosomal cholesterol by ORP5 or other ORPs. As such, 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) VPS4/SKD1 may join to break down such a complex for recycling 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 (Wang et al., 2005a). Moreover, human VPS4 has been shown to regulate endosomal cholesterol trafficking (Bishop and Woodman, 2000) and has been found to interact with NPC1 (Ohsaki et al., 2006). It is now necessary to elucidate the true nature of the interactions between these proteins and to identify other unknown proteins involved in endosomal cholesterol transport to the ER.

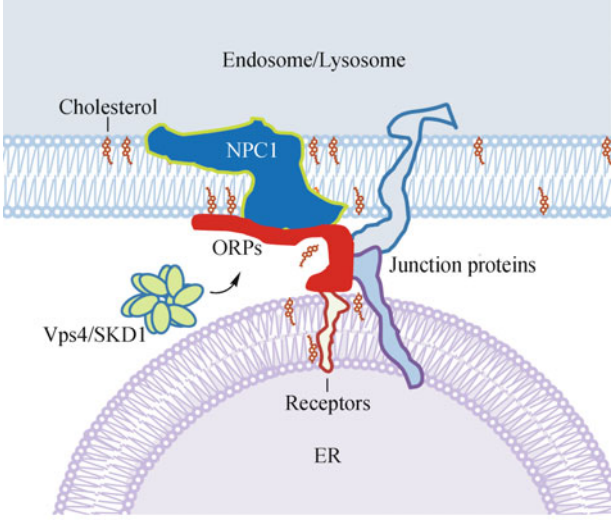

Figure 3 A hypothetical model for cholesterol transport at an endosome-ER junction. ORP5 or other ORPs effieicntly move cholesterol from endosome/lysosome to the ER by forming a complex with membrane lipids, NPC1, putative receptors and junction proteins. Upon completion of cholesterol transfer, the AAA ATPase VPS4/SKD1 disrupts the complex and recycles the ORPs.

Steroidogenic acute regulatory (StAR) proteins

In steroidogenic cells, endosomal cholesterol is also transported to the mitochondrial inner membrane through pathways mediated by StAR proteins (Lavigne et al., 2010). Each of the StAR proteins contains a ~210-amino acid StAR-related lipid transfer (START) domain, which potentially serves as a cholesterol-binding site. The human genome encodes 15 START domain proteins (StARD1-StARD15), five of which have been shown to bind specifically to cholesterol (Soccio and Breslow, 2003; Lavigne et al., 2010). Recently, StARD3 or MLN64 (endosomal metastatic lymph node protein 64) has been documented to mediate endosomal cholesterol egress to the mitochondria independent of the presence of functional NPC1 (Charman et al., 2010). MLN64 depletion by siRNA impaired LDL-derived cholesterol transport to the mitochondrial inner membranes in both wild-type and NPC1 deficient cells (Charman et al., 2010). MLN64 knockdown also alleviated the increased mitochondrial cholesterol contents due to the loss of NPC1, suggesting that MLN64 is involved in mitochondrial cholesterol accumulation found in NPC disease (Charman et al., 2010). Therefore, MLN64 may be pathophysiologically important for understanding NPC disease in terms of mitochondria dysfunction caused by cholesterol accumulation (Rigotti et al., 2010). MLN64 localizes to late endosome via its N-terminal transmembrane domain and binds cholesterol through its START domain (Alpy et al., 2001). It is therefore not surprising that this protein plays an important role in mediating intracellular cholesterol transport en route from LE/LY to the mitochondria. Further studies will be needed to address how MLN64 functions in human disease settings in relation to abnormal mitochondrial cholesterol transport (Rigotti et al., 2010).

Conclusions

During the past few years, significant progresses have been made to understand endosomal cholesterol trafficking. A notable example is the elucidation of the structures and cholesterol-binding and -transfer properties of NPC1 and NPC2, two master regulators controlling the exit of lipoprotein-derived cholesterol from endosomal compartments. However, substantial studies are still needed to further define this pathway. For instance, although NPC1 and NPC2 are known to work in concert to transfer cholesterol to LE/LY membranes, the nature of the interaction between the two proteins has yet to be determined. Moreover, the exact mechanisms underlying cholesterol egress from LE/LY membrane are currently missing. Although the OSBP/ORP and StAR protein families have been shown to participate in this process, putative additional components await to be identified. One would expect that the identification of novel proteins involved in endosomal cholesterol transport will not only shed light on the understanding of this challenging subject in cell biology, but also provide important clues to the treatment of lysosomal storage diseases, such as NPC.

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