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Cholesterol transport between cellular membranes:

a balancing act between interconnected lipid fluxes

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Abstract

Cholesterol represents the most abundant single lipid in mammalian cells. How its asymmetric distribution between subcellular membranes is achieved and maintained, attracts considerable interest. One of the challenges is that cholesterol is rarely transported alone, but rather coupled with heterotypic transport and metabolism of other lipids, in particular phosphoinositides, phosphatidylserine and sphingolipids. This perspective summarizes the major exo-and endocytic cholesterol transport routes and how lipid transfer proteins at membrane contacts and membrane transport intersect along these routes. It discusses the co-transport of cholesterol with other lipids in mammalian cells and reviews emerging evidence related to the physiological relevance of this process.

Introduction

Cholesterol is an essential component of mammalian cell membranes. All nucleated cells are capable synthesizing it *de novo* from the central 2-carbon metabolic precursor acetate that is processed to the unique 27-carbon 4-ring structure via a complex series of enzyme reactions. In addition, animal-based food products provide a source of exogenous cholesterol, which after entero-hepatic processing reaches peripheral cells via circulating low-density lipoprotein (LDL) particles. In the receiving cells, receptor-mediated endocytosis of LDL and hydrolysis in acidic compartments transiently enriches late endosomes and lysosomes (LE/Ly) with exogenous cholesterol to be redistributed to other subcellular membranes.

Within cells, the distribution of cholesterol is markedly uneven, being highly enriched in the plasma membrane (PM) where it constitutes roughly 40 mol% of all lipids, but low in the endoplasmic reticulum (ER), where it only represents less than 5 mol% of lipids (Ikonen, 2008; Mesmin and Maxfield, 2009) (**Figure 1**). This is critical for the ER cholesterol sensing SREBP/SCAP (sterol regulatory element binding protein/SREBP cleavage activating protein) machinery. Membrane trafficking routes communicating with the PM, such as *trans*-Golgi network (TGN) and recycling endosomal compartments, are cholesterol enriched and there is a decreasing cholesterol content towards the *cis*-Golgi. As most of the cholesterol synthesizing enzymes localize to the ER, efficient export mechanisms for ER cholesterol and retention mechanisms for PM cholesterol must be in place.

Cholesterol harbors a small polar head group (a single hydroxyl moiety at carbon-3) and is therefore intercalated in the phospholipid bilayer and moves readily between leaflets with an estimated sub-second time scale (Steck and Lange, 2018). Cholesterol can be lifted from the bilayer to the aqueous, cytosolic environment with the help of lipid transfer proteins (LTPs) whose hydrophobic cavity shields the lipid from water and can catalyze lipid transfer between

organelles. Such transfer processes are often concentrated at membrane contact sites (MCSs) **(Figure 2)**, i.e. regions where membranes are tethered at a close (~10 nm or more) distance (for a recent review, see (Prinz et al., 2020). The dynamic nature of such contacts is emerging as an important principle for metabolic rewiring (Bohnert, 2020).

It is also becoming increasingly evident that inter-organelle cholesterol transport can no longer be considered in isolation: cholesterol transfer is often coupled to and geared by the transport and metabolism of other lipids, in particular phosphoinositides (PIPs), phosphatidylserine (PS) and sphingolipids. Keeping these inter-dependencies in mind, the present review aims to provide a brief account of the major exo-and endocytic cholesterol transport routes and how LTPs at membrane contacts and membrane trafficking intersect along these routes.

How to keep the ER cholesterol low?

OSBP-dependent cholesterol export

Early studies indicated that efficient export of cholesterol from the ER mostly relies on non-vesicular trafficking (Heino et al., 2000; Urbani and Simoni, 1990) and identified a protein with an oxysterol-binding domain, OSBP, bridging between the Golgi and ER membranes (Ridgway et al., 1992; Wyles et al., 2002). Further work revealed that the yeast OSBP homolog Osh4p can exchange sterols for phosphatidylinositol 4-phosphate (PI4P) between membranes (de Saint-Jean et al., 2011) and is in fact 10 times more efficient as a lipid exchanger than as a plain transporter (Von Filseck et al., 2015). However, some of the Osh4p biology cannot easily reconciled with this counter-current model of lipid transport (Georgiev et al., 2011; Quon et al., 2018).

Importantly, PI(4)P is enriched in the Golgi and PM (Di Paolo and De Camilli, 2006) but absent in the ER due to the activity of the ER-localized phosphatase Sac1 that dephosphorylates PI(4)P

to PI (Foti et al., 2001). Thus, Osh4p can generate a sterol gradient between ER and Golgi membranes by exporting sterol from the ER to the Golgi and transferring PI(4)P to the opposite direction in repeated exchange cycles. Despite being a one-by-one transfer, this process could be efficient enough to account for up to 60% of the sterol delivery that yeast needs, in order to double its PM area during asymmetric cell division (Sullivan et al., 2006).

Remarkably, a similar sterol/PI(4) counter-exchange was found to be carried out by OSBP for cholesterol delivery at ER-trans-Golgi contact sites in mammalian cells (Mesmin et al., 2013) (**Figure 2B**, panel 1). The domain architecture of OSBP supports a mechanism by which the protein connects to the ER through its FFAT motif binding to the ER VAP-A protein, and to the TGN via its PH domain that binds to the TGN via dual interaction with PI(4)P and the small GTPase Arf1, transferring cholesterol between these juxtaposed membranes via the OSBP-related domain (ORD) (Levine, 2004). Upon acute chemical inhibition of OSBP, sterol accumulates in the ER and lipid droplets (LDs) at the expense of the TGN (Mesmin et al., 2017). Considering the low levels of PI(4)P (roughly 1% of total phospholipids) compared to sterol, an efficient phosphorylation-dephosphorylation cycle of PI(4)P is needed to energize cholesterol delivery against its concentration gradient. Curiously, loss of Sac1 has a minimal impact on monophosphorylated PIPs (Charman et al., 2017), but this may be due to compensatory effects.

These findings prompt the next question, i.e. how cholesterol gets onwards from the TGN to reach the PM. Interestingly, lipid transfer at ER-Golgi membrane contacts is proposed to promote the biogenesis of TGN derived carrier vesicles called CARTS (carriers of the trans-Golgi network to the cell surface) that ferry selective cargoes to the PM, through organization of cholesterol and sphingomyelin (SM) enriched nanodomains at the TGN (Wakana et al., 2015). Recent results showed that in cholesterol-rich conditions, the ER cholesterol sensor

SCAP interacts with Sac1 and promotes the formation of TGN-PM carriers depending on ER cholesterol (Wakana et al., 2021). Whether CARTS represent major carriers for cholesterol delivery from the TGN to the PM remains to be addressed.

ORP5/ORP8-dependent export of PS

PI(4)P exchange between membranes and Sac1 dependent PI(4)P dephosphorylation are not only involved in ER cholesterol export but also drive other lipid transport steps, such as the transfer of PS from the ER, its site of synthesis, to the PM where it is enriched in the inner leaflet (**Figure 2B**, panel 2). This transfer is mediated by ORP5 and ORP8 mediated counter-transport of PS and PI(4)P (Chung et al., 2015). The dynamic recruitment of ORP5/8 to the PM depends on PI(4)P and PI(4,5)P_2 (Ghai et al., 2017), with ORP8 being recruited when PI(4,5)P_2 is increased (Sohn et al., 2018). This provides a mechanism to exquisitely regulate PM PI(4)P, PI(4,5)P_2 and PS levels. With high PM PI(4,5)P2, ORP8 and ORP5 are recruited to supply PS and remove PI(4)P, limiting PI(4,5)P_2 synthesis from PI(4)P. At low PI(4,5)P_2 levels, only ORP5 docks and transfers PS to the PM, until PM PI(4)P is exhausted. Why the PM delivery of PS is relevant for cholesterol transport is discussed in later sections of this perspective.

Cholesterol delivery from the ER towards lysosomes

Increasing evidence indicates that LTPs also deliver cholesterol from the ER toward endo-lysosomal compartments via MCSs. This may help e.g. to support the formation of intraluminal vesicles in late endosomes under low cholesterol levels, as shown for ORP1L (Eden et al., 2016), to modulate the sterol content of late endosomal internal membranes, as proposed for StARD3 (Wilhelm et al., 2017), or to orchestrate nutrient signaling by delivering cholesterol to the limiting membrane to activate mTORC1 kinase, as in the case for OSBP (Lim et al., 2019).

How does cholesterol partition among plasma membrane lipids?

The distribution and behavior of cholesterol in membranes depends heavily on other lipids, both on their compositions and in their dynamic arrangements between membrane leaflets and in the lateral plane of the membrane. The majority of studies on membrane lipid compositions have focused on the PM, dictated in part by convenience, i.e. its accessibility and relatively planar structure. Importantly, it is also the membrane where cholesterol not only has the highest concentration but also important functional roles.

In the PM, cholesterol partitions with sphingolipids and other saturated lipids into transient, ordered nanodomains termed rafts that affect the sorting and interactions of membrane proteins (Goñi, 2019; Kusumi et al., 2020; Simons and Ikonen, 1997). Sphingolipids, with SM as the dominant species, constitute 10-15% of PM lipids (**Figure 1**), are mostly saturated thanks to the ceramide backbone and are enriched in the exoplasmic leaflet (**Figure 3**). The cytoplasmic PM leaflet is approximately twofold more unsaturated than the exoplasmic one, with PS representing a dominant, highly unsaturated phospholipid class (Lorent et al., 2020) (**Figure 3**). Phosphatidylethanolamine (PE) is also almost exclusively confined to the inner leaflet and typically polyunsaturated (**Figure 3**). Overall, because of these marked asymmetries, the outer PM leaflet is likely to be more packed and less diffusive than the inner leaflet (Lorent et al., 2020).

Despite the seminal role of cholesterol, its transbilayer distribution is not firmly established, and considering the facile flip-flop, might not be fixed (Steck and Lange, 2018). Nevertheless, significant progress in understanding cholesterol PM partitioning has been made by using protein domains from cholesterol-binding bacterial and fungal proteins, as tools. This has enabled the classification of PM cholesterol into three operational pools (**Figure 4**). A cholesterol pool recognized by the D4 domain of perfringolysin O (PFO) and anthrolysin O

(ALOD4) represents roughly 10 mol% of PM lipids (this pool becomes inaccessible when PM cholesterol falls below 30 mol%) and is highly mobile, moving rapidly to the ER to signal cholesterol surplus to the sterol-regulatory element binding protein 2 (SREBP2) machinery (Das et al., 2014; Infante and Radhakrishnan, 2017). Ostreolysin A (OlyA) (Endapally et al., 2019) and Nakanori (Makino et al., 2017) in turn recognize SM/cholesterol complexes. This pool represents about 15 mol% of PM lipids and forms the basis of the SM-sequestered pool of cholesterol (ordered nanodomain or raft cholesterol, if you will). The remainder of PM cholesterol (about 15 mol% of PM lipids) is sequestered by other membrane factors, critical for cell viability and currently lacks probes.

It is important to note that the cholesterol-binding protein domains are just not passive reporters of cholesterol. Rather, their binding to live cells actively configures PM cholesterol distribution: for instance, binding of OlyA to the PM outer leaflet depletes the mobile, ALOD4 accessible pool, possibly by stabilizing SM/cholesterol complexes, increasing their equilibrium distribution and thereby depleting the uncomplexed cholesterol (Johnson et al., 2019).

How is LDL-cholesterol redistributed in cells?

LDL-cholesterol enters cells by receptor mediated endocytosis of LDL and free cholesterol released from the particles upon acid hydrolysis along the endocytic pathway accumulates in late endosomal compartments (**Figure 5**). The NPC1 protein in the limiting membrane of these organelles is a key gatekeeper for the release of cholesterol from lysosomes (Ikonen, 2018). Rab GTPases and PIPs represent key players in determining endosome identities and coordinating endo-lysosomal functions (Jean and Kiger, 2012). It is therefore not surprising that they also affect cholesterol delivery. Early work showed that lysosomal cholesterol export depends on the Rab GDP/GTP cycle (Hölttä-Vuori et al., 2000) and overexpression of Rab

proteins alleviated cholesterol accumulation in NPC1-deficient lysosomes (Choudhury et al., 2002; Linder et al., 2007). Recently, an activator (guanidine exchange factor complex) of Rab7 was shown to control NPC1-dependent lysosomal cholesterol export (van den Boomen et al., 2020).

The trafficking of NPC1 organelles and at least part of LDL-cholesterol recycling toward the PM is controlled by a Rab8a-myosin Vb-actin dependent membrane trafficking route (Kanerva et al., 2013). This route bears intriguing similarities to the transport of a related cholesterol transporter NPC1L1 that is critical for cholesterol absorption in enterocytes: The recycling of NPC1L1 also requires actin and myosin Vb via the actin-binding protein LIMA (Chu et al., 2009; Zhang et al., 2018).

Post-lysosomal destination of LDL-cholesterol

The pathways that transport cholesterol downstream of NPC1 are not fully characterized. For instance, which cholesterol transporters contribute to post-NPC1 cholesterol delivery to the PM, has remained open. ORP2 was shown to exchange cholesterol for PI(4,5)P_2 and regulate PM cholesterol levels (Wang et al., 2019). Our recent data provide evidence that ORP2-dependent cholesterol/PI(4,5)P_2 exchange takes place between late and recycling endosomes, spreading LDL-derived cholesterol to recycling circuits on its way to the PM (Takahashi et al.). Indeed, recycling endosomes are sterol enriched (Hao et al., 2002) and have been implicated in cholesterol delivery. Rab11 controls cholesterol recycling (Hölttä-Vuori et al., 2002) and the Rab11 and OSBP-binding protein RELCH can tether RE and TGN to each other and facilitate OSBP-dependent cholesterol transfer to TGN (Sobajima et al., 2018).

Upon reaching the PM, LDL-derived cholesterol is likely rapidly incorporated into cholesterol/SM complexes (Johnson et al., 2019) and replenishes the accessible pool of cholesterol that has become depleted in lipoprotein-deficient conditions (Takahashi et al.).

Importantly, if PM PS levels are low, LDL-cholesterol accumulates in the PM and fails to reach the ER, arguing that cholesterol delivery from the PM to the ER requires PS (Trinh et al., 2020). This most likely reflects the requirement of Aster/GramD proteins for PS (see below).

How is plasma membrane cholesterol delivered to the ER?

The Aster/GramD proteins (encoded by GramD1a, b and c genes) facilitate PM-to-ER sterol trafficking (Sandhu et al., 2018). These proteins are anchored to the ER by a transmembrane domain, contain a central cholesterol binding StART-like/Aster domain and an N-terminal GRAM domain that binds PS and mediates Aster recruitment to PM-ER contact sites upon PM cholesterol accumulation (**Figure 2B**, panel 3). Indeed, Aster proteins were shown to regulate the movement of the PM accessible cholesterol pool to the ER (Ferrari et al., 2020; Naito et al., 2019). Aster-dependent PM-to-ER cholesterol transfer is relevant both for the transport of HDL-derived cholesterol that is captured by SR-B1 receptors at the PM (Ikonen and Kanerva, 2019; Sandhu et al., 2018) and for the transport of LDL-derived cholesterol downstream of NPC1 after it has reached the PM (Xiao et al., 2021).

Recent results suggest that the GRAM domain is a co-incidence detector for cholesterol and anionic lipids, with distinct binding sites for both (Ercan et al., 2021) (**Figure 2B**, panel 3). A fraction of cholesterol is closely associated with PS (and possibly other anionic lipids, such as PIPs) without being sequestered by them and SM can lower this codistribution due to its cholesterol sequestering potential. Vice versa, SM hydrolysis liberates cholesterol for detection by the GRAM domain (Naito et al., 2019). Together, these findings strengthen the idea that unsequestered cholesterol can rapidly exchange between the outer and inner PM leaflet.

Increasing structural information on cholesterol binding pockets of proteins will enable comparisons of cholesterol binding and release mechanisms between LTPs, such as ORD

domains of ORP family, START domains of StAR family and StART-like/Starkin/Aster of Aster/GramD proteins. Of note, for the newcomer, StARkin domain an unexpected water-controlled mechanism for sterol acquisition/discharge was recently proposed (Khelashvili et al., 2019). Despite structural similarities between ligand binding domains, some inhibitors show selectivity among Aster/GramD proteins (Laraia et al., 2019). In this context, it is also worthwhile noting that the compound U18666A that inhibits NPC1 (as well as select sterol biosynthesis enzymes) also inhibits Asters (Xiao et al., 2021).

The accessible pool of cholesterol is chemically more active than the sphingolipid sequestered pool, and is thus more avidly available for cytoplasmic acceptors, such as LTPs. It may also be more accessible for release out of cells, perhaps to dispose surplus cholesterol under lipid-laden conditions (He et al., 2018). Interestingly, oxysterols are potent modulators of cholesterol accessibility. As little as 1% of 25-hydroxycholesterol (25-HC) can produce a detectable increase in cholesterol accessibility, probably in part by the membrane disordering effects of 25-HC (Bielska et al., 2014). Recent observations highlight how pathogens, both bacteria and viruses, including coronaviruses, rely on accessible cholesterol for cell entry (Abrams et al., 2020; Wang et al., 2020). In defense, the immune system props up the enzyme cholesterol 25-hydroxylase as one of the interferon-stimulated genes, the produced 25-HC allosterically activates the cholesterol esterifying enzyme ACAT/SOAT (Cheng et al., 1995) and triggers rapid internalization of PM accessible cholesterol, thus conferring resistance to pathogen entry. The ER membrane resident SOAT1/ACAT1 enzyme catalyzes the esterification of cholesterol in the ER membrane and provides an important safety valve to prevent ER cholesterol levels from rising. The generated cholesteryl esters are diffusible in the ER membrane but become packaged into lipid droplets deriving from the ER, by so far incompletely understood mechanisms (Thiam and Ikonen, 2021) (**Figure 2B**, panel 4).

Conclusions and future prospects

This perspective provides a brief overview of major cellular cholesterol trafficking routes, highlighting the role of the OSBP in ER cholesterol export toward the PM and Asters/GramDs in PM cholesterol import to the ER and storage of surplus cholesterol in lipid droplets. LDL-cholesterol routing intersects with the latter circuit, by the bulk of LDL-cholesterol initially directed to the PM. This safeguards PM cholesterol as a barrier and organizer of ordered domains, while the remaining, chemically active cholesterol is readily mobilized to the ER.

This simplified view does not do justice to the full repertoire of players involved. Indeed, the richness of proteins belonging to several evolutionarily conserved families and capable of mediating cholesterol delivery at contact sites between heterologous membranes has steadily increased. Even if most of the relevant LTPs might now be identified, their mutual co-operation, interplay between membrane trafficking routes and physiologically relevant context(s) will continue to entice researchers. How the dynamic regulation of lipid transfer at such contacts is achieved, is only beginning to be unraveled, as exemplified by the emerging roles of phosphorylation (Di Mattia et al., 2020) and cytoplasmic calcium (Malek et al., 2020). The human disease relevance of the LTPs involved is also expected to increase once we understand more of their tissue and cell-type specific functions. Finally, the field needs to spur further development of techniques both for acute manipulation of lipid transfer proteins and related enzymes (e.g. light or drug inducible protein relocalization or degradation) and for sensitive tracking of lipids by imaging and mass spectrometry.

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Declaration of Interests

The authors declare no competing interests.

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Figure legends

Figure 1. Comparison of the lipid composition between endoplasmic reticulum and plasma membrane. Pie charts show the fractions of the lipids indicated in the PM and ER membrane, according to (Vance, 2015). PC (phosphatidylcholine), PE (phosphatidylethanolamine), PIs (phosphoinositides), PS (phosphatidylserine), SM (sphingomyelin), Chol (cholesterol), O (other lipids).

Figure 2. Lipid transfer at membrane contact sites related to cholesterol trafficking. (A) ER connects to the nuclear envelope and continuously contacts with other cellular membrane compartments, such as the Golgi complex, lipid droplets (LDs) and plasma membrane (PM). (B) At ER-TGN contacts (panel 1), OSBP bridges the membranes by interacting with ER membrane protein VAP-A and TGN-enriched PI(4)P, respectively, to exchange cholesterol and PI(4)P. At ER-PM contacts (Panel 2), the PS and PI(4)P exchange is executed by ORP5/8 that harbor a single-pass ER transmembrane domain and are recruited by PI(4)P (or ORP8 by PI(4,5)P_2) to PM contacts. Both PI(4)P-coupled cholesterol and PS transfers are regulated by the hydrolysis of PI(4)P by phosphatase Sac1 in the ER. At ER-PM contacts (Panel 3), the ER-resident lipid transfer protein Aster-B/GramD1B recognizes PI(4)P and PS simultaneously in the PM and transports accessible cholesterol to the ER. Excessive cholesterol in the ER is esterified by SOAT1/ACAT1 and stored in the lipid droplets as cholesteryl esters (Panel 4).

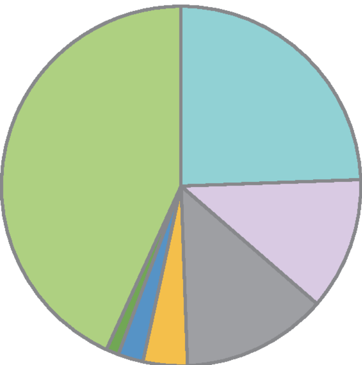
Figure 3. Plasma membrane phospholipid composition and saturation. Relative abundancy of phospholipids in the exoplasmic (red) and cytoplasmic (blue) PM of erythrocytes (adapted from (Lorent et al., 2020)). Number of double bonds in the acyl chains are shown by a color gradient. PC (phosphatidylcholine), PCp (phosphatidylcholine plasmalogen), PE

(phosphatidylethanolamine), PEp (phosphatidylethanolamine plasmalogen), PI (phosphatidylinositol), PS (phosphatidylserine), PA (phosphatidic acid), Cer (ceramide), SM (sphingomyelin).

Figure 4. Three cholesterol pools in the plasma membrane. (A) Relative abundancy of the three cholesterol pools (modified from (Das et al., 2014)), with the accessible cholesterol pool recognized by a series of bacterial cholesterol binding toxin-derived probes indicated, the SM-sequestered cholesterol pool recognized by OlyA (ostreolysin A) and Nakanori, and the essential cholesterol pool. (B) Structures and corresponding RCSB accession codes of PFO (perfingolysin O, 1M3I), ALO (anthrolysin O, 3CQF), OlyA (6MYI), and Nakanori (5H0Q). D4, cholesterol binding domain of PFO. D4H, QYDA and YDA are derived from D4 with higher sensitivity to the accessible cholesterol. PFO*, non-lytic PFO mutant. ALOD4, cholesterol binding domain of ALO.

Figure 5. Routing of LDL-derived cholesterol and effects in the ER. (a) Low-density lipoprotein (LDL) enters the cell via receptor -mediated endocytosis and releases free cholesterol in the acidic lumen of late endosome/lysosome (a). (b) NPC2 and NPC1 cooperate to facilitate cholesterol transit until the limiting membrane of lysosome. Subsequently, most LDL-derived cholesterol is transferred to the plasma membrane, where cholesterol in the exoplasmic PM leaflet can be recognized by cholesterol binding probes. (c) Accessible cholesterol in the PM continuously flows back to the ER at membrane contact sites and inactivates SREBP. (d) Excessive cholesterol in the ER is converted by SOAT1/ACAT1 to cholesteryl ester and stored in lipid droplets. (a') In a cholesterol limited environment, SREBP and SCAP are dissociated from Insig, (b') sorted into the COPII vesicles, and transported to the Golgi. (c') SREBP is cleaved by the proteases S1P and S2P to release its bHLH domain,

(d') which transcriptionally activates the expression of genes involved in lipid uptake and synthesis.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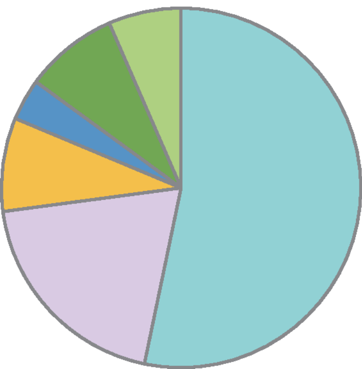
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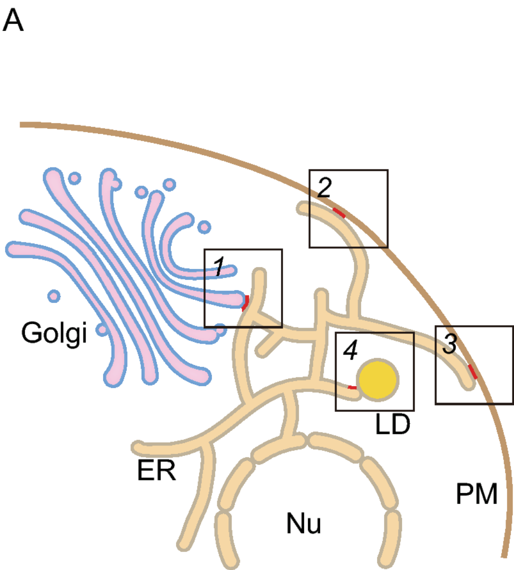
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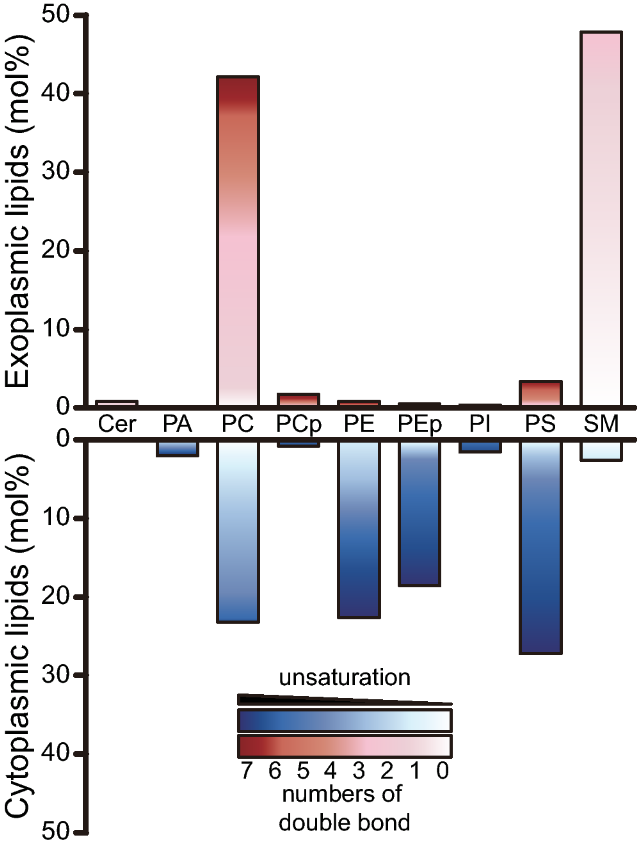

Exoplasmic lipids (mol%)
Cytoplasmic lipids (mol%)
50
40
30
20
10
0
Cer
PA
PC
PCp
PE
PEp
PI
PS
SM
0
10
20
30
40
50
unsaturation
7 6 5 4 3 2 1 0
numbers of
double bond

A

Plasma membrane cholesterol (mol% of total PM lipids)

50
40
30
20
10
0

Accessible

PFO, D4, D4H, QYDA, YDA
PFO* (non-lytic PFO mutant),
ALOD4

SM-sequestered

OlyA
Nakanori

Essential

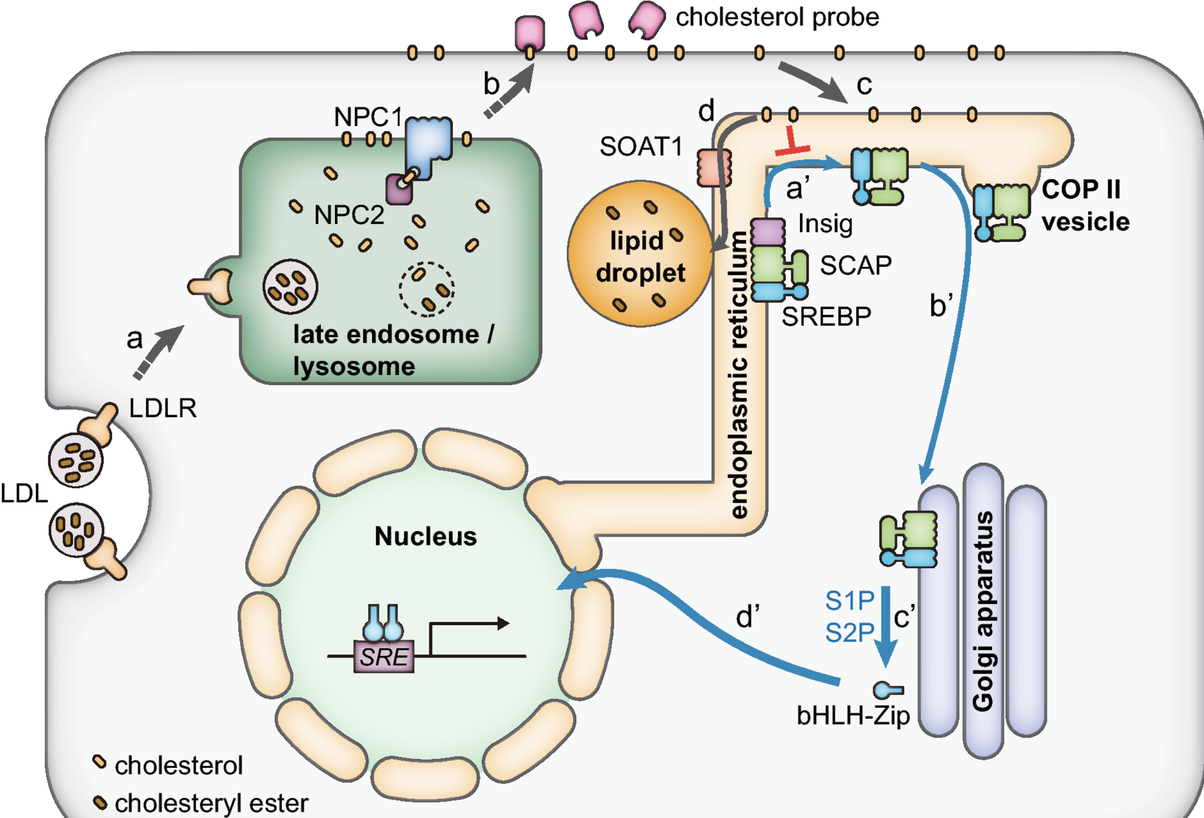
B

PFO (1M3I)

ALO (3CQF)

OlyA (6MYI)

Nakanori (5H0Q)


cholesterol probe
b
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SOAT1
NPC2
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COP II
vesicle
lipid
droplet
SCAP
SREBP
endoplasmic reticulum
b'
late endosome /
lysosome
a
LDLR
LDL
Nucleus
Golgi apparatus
S1P
S2P
c'
d'
SRE
bHLH-Zip
cholesterol
cholesteryl ester