

Regulation of Plasma Membrane Sterol Homeostasis by Nonvesicular Lipid Transport

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Abstract

Sterol contributes to the structural integrity of cellular membranes and plays an important role in the regulation of cell signaling in eukaryotes. It is either produced in the endoplasmic reticulum or taken up from the extracellular environment. In most eukaryotic cells, however, the majority of sterol is enriched in the plasma membrane. Thus, the transport of sterol between the plasma membrane and other organelles, including the endoplasmic reticulum, is crucial for maintaining sterol homeostasis. While vesicular transport that relies on membrane budding and fusion reactions plays an important role in bulk sterol transport, this mode of transport is slow and non-selective. Growing evidence suggests a critical role of nonvesicular transport mediated by evolutionarily conserved families of lipid transfer proteins in more rapid and selective delivery of sterol. Some lipid transfer proteins act primarily at the sites of contacts formed between the endoplasmic reticulum and other organelles or the plasma membrane without membrane fusion. In this review, we describe the similarities and differences of sterol biosynthesis and uptake in mammals and yeast and discuss the role of their lipid transfer proteins in maintaining plasma membrane sterol homeostasis.

Keywords

cholesterol, endoplasmic reticulum, plasma membrane, nonvesicular, lipid transfer protein, endosome

Introduction

Sterol is an essential component of the eukaryotic membranes, playing important roles in maintaining membrane organization and modulating signal transduction. The main sterols found in eukaryotes are represented by three predominant forms: ergosterol in fungi, phytosterols in plants, and cholesterol in animals (Moreau et al., 2002; van Meer et al., 2008). Sterol synthesized in the endoplasmic reticulum (ER) is known to equilibrate between the ER and the plasma membrane (PM) with a half-time ($t_{1/2}$) of approximately 10–20 min in both yeast (Baumann et al., 2005; Georgiev et al., 2011) and mammalian cells (DeGrella & Simoni, 1982; Kaplan & Simoni, 1985), although how the synthesis of sterol in the ER is coupled with its transport to the PM is not well understood. Importantly, the rate of sterol transport between the ER and the PM is not significantly altered in yeast mutants defective in vesicular transport (*sec* mutants) (Baumann et al., 2005; Schnabl et al., 2005) or in mammalian cells that are treated with brefeldin A (a fungal toxin that disassembles Golgi complex and inhibits vesicular transport between the ER and the PM [Fujiwara et al., 1988]) (Heino et al., 2000; Urbani & Simoni, 1990). Thus, nonvesicular transport plays an important role in maintaining sterol transport in both yeast and mammalian cells (Liscum & Munn, 1999; Maxfield & Menon, 2006). Nonvesicular

lipid transport is greatly accelerated by lipid transfer proteins (LTPs), which possess hydrophobic cavities that can capture and transport specific lipid species. LTPs allow the hydrophobic membrane lipids to be selectively and efficiently transported between different membrane compartments across the hydrophilic environment of cytosol (Holthuis & Menon, 2014; Reinisch & Prinz, 2021; Wong et al., 2019). LTP-mediated nonvesicular lipid transport is often facilitated at membrane contact sites, where the ER and various other organelles as well as the PM are in close appositions of 10–30 nm apart without membrane fusion (Figure 1) (Prinz et al., 2020; Saheki & De Camilli, 2017; Wu et al., 2018), although LTP-mediated nonvesicular lipid transport can

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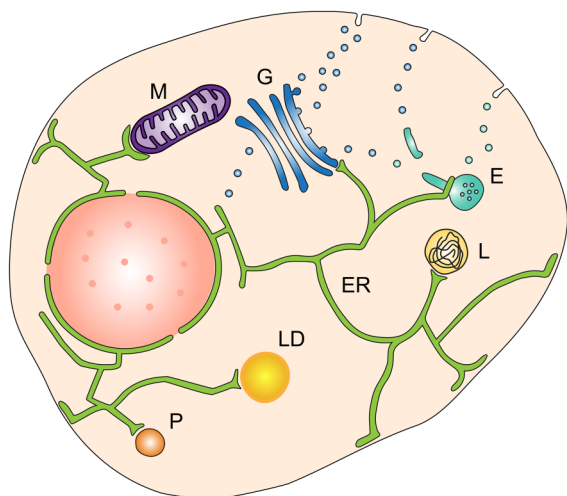


Figure 1. Organelles contact with each other without membrane fusion. Schematic drawing depicting organelle contacts formed between the ER and other membranous organelles and the plasma membrane. Note. E = endosome; ER = endoplasmic reticulum; G = Golgi complex; L = lysosome; LD = lipid droplet; M = mitochondria; P = peroxisome.

also occur at the wider gaps between organelles and membranes that are not in close contact.

In this review, we briefly summarize the mechanisms that regulate the biosynthesis and uptake of sterol in mammals and yeast and discuss how nonvesicular sterol transport mediated by subsets of evolutionarily conserved LTPs contributes to PM sterol homeostasis.

Sterol Biosynthesis and Uptake in Mammals and Yeast

Cholesterol, the major sterol in mammals, accounts for approximately 20% of the total cellular lipids (van Meer et al., 2008; Vance, 2015). It is either synthesized *de novo* in the ER or taken up from external sources. Extracellular cholesterol uptake is primarily mediated through receptor-mediated endocytosis of low-density lipoprotein (LDL), which is filled with cholesterol esters (Brown & Goldstein, 1986; Goldstein & Brown, 2009, 2015). LDL binds to the LDL receptor on a cell surface and eventually reaches lysosomes, where the cholesterol esters are released from LDL and hydrolyzed to cholesterol. Cholesterol is then released from lysosomes and delivered to other organelles, including the trans-Golgi network (TGN), recycling endosomes, peroxisomes, and the ER, either directly or indirectly (Goldstein et al., 1982; Goldstein & Brown, 2009; Luo et al., 2019). Cells also receive cholesterol from high-density lipoprotein (HDL) via a distinct pathway known as reverse cholesterol flux (Acton et al., 1996; Phillips, 2014). Regardless of the source, up to 90% of total cellular cholesterol is enriched in the PM, where it contributes to

approximately 40% of the total lipids in this bilayer (de Duve, 1971; Ikonen, 2008; Lange et al., 1989; Maxfield & Mondal, 2006; Ray et al., 1969; van Meer et al., 2008). However, the regulatory network that controls both cholesterol synthesis and uptake resides in the ER (Brown & Goldstein, 1997; Brown et al., 2018; Goldstein & Brown, 1990) (Figure 2A). Master transcription factors of such regulatory network are sterol regulatory element-binding proteins (SREBPs), which consist of an N-terminal transcription factor domain, two transmembrane segments, and a C-terminal regulatory domain (Brown & Goldstein, 1997; Hua et al., 1993). The mammalian genome encodes three SREBP isoforms, designated SREBP-1a, SREBP-1c, and SREBP-2, among which cholesterol homeostasis is primarily controlled by the SREBP-2 isoform (Horton et al., 2002). The SREBP-2 normally localizes to the ER by associating with a cholesterol-sensing protein, SREBP-cleavage activating protein (SCAP), and insulin-induced gene (INSIG) complexes (Brown et al., 2018) (Figure 2A). When the levels of cholesterol in the ER fall below a critical threshold level (~5% of the total lipid in the ER), the SREBP-2-SCAP complex is released from INSIG, transported to the Golgi apparatus, and cleaved by proteases (S1P and S2P) in a sequential two-step reaction (Brown & Goldstein, 1999; Sakai et al., 1996). The resulting active fragment from these cleavage reactions then enters the nucleus and binds to the sterol regulatory element (SRE) sequence in the promoter region of its target genes and activates transcription of various cholesterol synthesizing enzymes and LDL receptors (Luo et al., 2020) (Figure 2A). Upon elevation of cholesterol in the ER, SREBP-2-SCAP complex is trapped in the ER by INSIG, leading to suppression of SREBP-2 cleavage, thereby shutting down cholesterol biosynthesis and uptake (Radhakrishnan et al., 2008; Steck & Lange, 2010). Therefore, the ER needs to closely monitor the levels of PM cholesterol in order to maintain cellular cholesterol homeostasis. This is mediated partly by the controlled transport of a biochemically distinct fraction of cholesterol, known as “accessible cholesterol” (also known as chemically active cholesterol), from the PM to the ER. Most cholesterol in the PM forms complexes with sphingomyelin and other phospholipids, being “inaccessible” (also known as chemically inactive) for transport at steady state (Chakrabarti et al., 2017; Das et al., 2014; Gay et al., 2015; Lange et al., 2013, 2004; McConnell & Radhakrishnan, 2003; Ohvo-Rekila et al., 2002; Radhakrishnan et al., 2000; Sokolov & Radhakrishnan, 2010). However, a fraction of PM cholesterol (approximately 16% of the total lipids in the PM) remains accessible for transport (Das et al., 2014). When the levels of PM cholesterol rise beyond a certain threshold, a newly expanded pool of the “accessible” cholesterol is rapidly transported to other organelles, including the ER, and increase the levels of cholesterol in the ER to shut down cholesterol biosynthesis and uptake by suppressing the cleavage of SREBP-2, thereby avoiding cholesterol

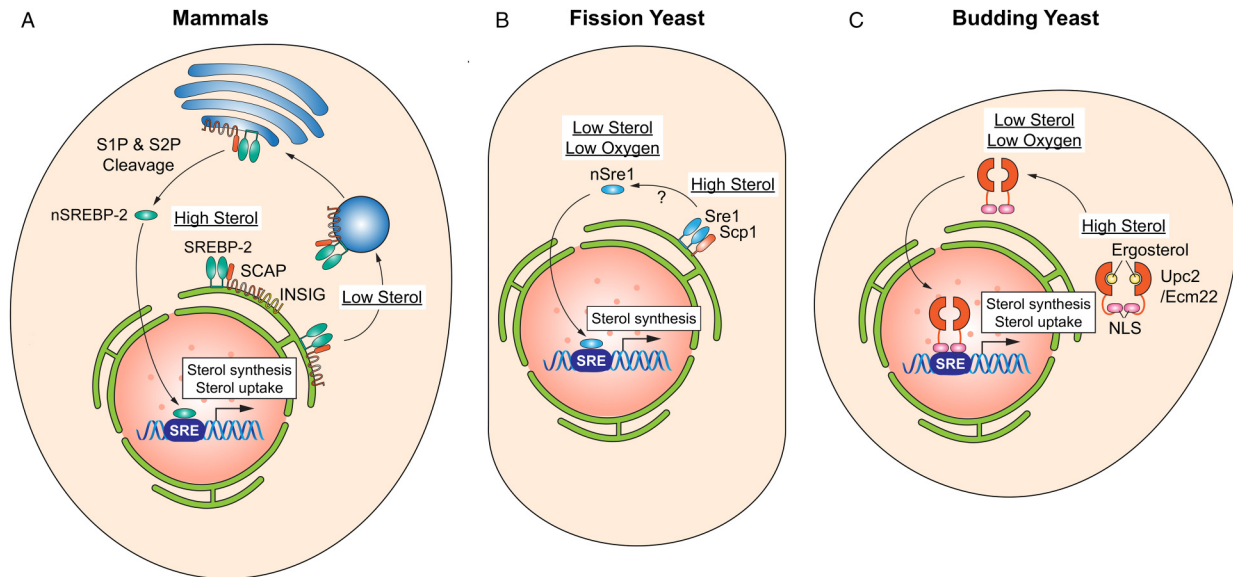


Figure 2. Schematic drawing illustrating the regulation of sterol biosynthesis and uptake in mammals and yeast. (A) In mammals, the SREBP-2-SCAP complex is retained in the ER via its interaction with INSIG at steady state. When the levels of cholesterol fall below a certain threshold, SREBP-2-SCAP complex translocates to the Golgi via COPII, and SREBP-2 undergoes sequential cleavage by proteases (S1P and S2P), releasing nSREBP-2. nSREBP-2 then translocates into the nucleus and binds SRE to upregulate expression of various genes involved in cholesterol biosynthesis and uptake, including LDL receptor. (B) In fission yeast, Sre1-Scp1 complex mainly localizes to the ER at steady state. When the levels of cholesterol or oxygen fall below a certain threshold, Sre1 is cleaved, and nSre1 translocates into the nucleus to upregulate expression of various genes involved in ergosterol biosynthesis. The mechanisms that regulate Sre1 cleavage remain elusive. Fission yeast does not take up sterol from extracellular environment. (C) In aerobic growth conditions, budding yeast does not take up sterol (a condition known as “aerobic sterol exclusion”), and ergosterol-bound Upc2/Ecm22 remains in the cytosol. When the levels of cellular sterol become low under anaerobic conditions, ergosterol is released from Upc2/Ecm22, leading to its conformational change. Upc2/Ecm22 then translocates into the nucleus to induce expression of genes involved in ergosterol biosynthesis and uptake, including ABC transporters (e.g., Aus1 and Pdr11). Note. ABC = ATP-binding cassette; ER = endoplasmic reticulum; LDL = low-density lipoprotein; SCAP = SREBP-cleavage activating protein; SRE = sterol regulatory element; SREBP = sterol regulatory element-binding protein.

overaccumulation and maintaining PM cholesterol levels (Das et al., 2014; Infante & Radhakrishnan, 2017; Lange & Steck, 1997; Lange et al., 2014; Scheek et al., 1997; Slotte & Bierman, 1988).

In yeast, ergosterol is the major sterol. Similar to mammals, up to 70% of the total cellular ergosterol resides in the PM, where it contributes to approximately 40% of total PM lipids (Solanko et al., 2018; van Meer et al., 2008; Zinser et al., 1993). De novo biosynthesis of ergosterol occurs in the ER and requires a large amount of oxygen (12 oxygen molecules per ergosterol in yeast) (Espenshade & Hughes, 2007). However, the regulation of ergosterol synthesis and uptake differs depending on the species of yeast. Fission yeast possesses homologs of SREBPs and SCAP, called Sre1 and Scp1, respectively (Figure 2B). Similar to SREBP-2, Sre1 localizes to the ER and acts as a transcription factor upon its cleavage in low sterol conditions, although the precise mechanisms of how Sre1 is cleaved remain a mystery (Hughes et al., 2005). Once cleaved, the fragment of Sre1 enters the nucleus, binds to SREs, and upregulates the expression of sterol synthesizing genes (Hughes et al., 2005) (Figure 2B). Cleavage of Sre1 is also induced in hypoxic conditions, which upregulates the expression of

oxygen-dependent enzymes in the ergosterol biosynthesis pathway, such as Erg3 and Erg25 (Bien & Espenshade, 2010; Todd et al., 2006). Unlike mammalian cells that take up exogenous cholesterol, fission yeast does not import exogenous sterol. Accordingly, fission yeast lacking both Sre1 and Scp1 cannot grow in an anaerobic condition due to its inability to synthesize ergosterol in low oxygen conditions (Hughes et al., 2005).

In aerobic or normal growth conditions, sterol in budding yeast is supplied through ergosterol biosynthesis as it does not take up exogenous sterol (a phenomenon known as “aerobic sterol exclusion”) (Lorenz & Parks, 1991; Trocha & Sprinson, 1976). Unlike fission yeast, however, budding yeast do take up exogenous sterol in hypoxic/anaerobic conditions; sterol uptake is indeed essential for the viability of budding yeast during anaerobic growth when sterol biosynthesis is compromised due to limited availability of oxygen (Trocha & Sprinson, 1976). Budding yeast lacks -homologs of SREBPs but possesses a distinct sterol regulatory mechanism to regulate ergosterol synthesis and uptake, namely, Upc2/Ecm22 paralog that belongs to a fungal specific family of zinc finger transcription factors (Lewis et al., 1988; Maguire et al., 2014; Shianna et al., 2001; Vik &

Rine, 2001) (Figure 2C). Deletion of both Upc2 and Ecm22 leads to lethality, suggesting an essential role of these proteins in sterol regulation in budding yeast (Shianna et al., 2001). Upc2 possesses a hydrophobic pocket in the C-terminal domain, which binds to sterol and regulates shuttling of Upc2 between cytosol and nucleus (Yang et al., 2015). When sterol is abundant in cells, sterol-bound Upc2 remains mostly in the cytosol (Yang et al., 2015). Under anaerobiosis or low sterol conditions, the sterol is released from Upc2, and free Upc2 enters the nucleus to induce expression of enzymes for ergosterol biosynthesis (Vik & Rine, 2001) as well as ATP-binding cassette (ABC) transporters, Aus1/Pdr11 paralog, which mediate ergosterol uptake in anaerobic conditions (Kohut et al., 2011; Wilcox et al., 2002) (Figure 2C). Once taken up by Aus1 and Pdr11, the sterol is then transported from the PM to the ER via nonvesicular transport (Li & Prinz, 2004). Details of cholesterol and ergosterol biosynthesis pathways have been extensively reviewed in other outstanding articles (Espenshade & Hughes, 2007; Goldstein & Brown, 1990; Jorda & Puig, 2020; Liu et al., 2019).

Recent studies have identified several families of evolutionarily conserved LTPs that play significant roles in nonvesicular sterol transport. Despite some differences in the regulatory mechanisms for sterol biosynthesis and uptake in yeast and mammals, some of the LTPs have been shown to have conserved functions in maintaining the levels of PM sterol.

Oxysterol-binding Homology/Oxysterol-binding Protein-Related Protein Family Proteins

The Osh/ORPs (Oxysterol-binding Homology [Osh] in yeast and Oxysterol-binding Protein [OSBP] and OSBP-Related Protein [ORP] in mammals) are evolutionarily conserved LTPs that are present in all eukaryotes. These proteins have been shown to bind and transport various lipid species, including sterol, anionic phospholipids, and phosphoinositides (PIPs) (Delfosse et al., 2020; Mesmin et al., 2013a; Raychaudhuri & Prinz, 2010). There are seven members of Osh proteins in yeast and 12 ORP genes in humans that generate up to 16 different ORP variants (Beh et al., 2001; Jaworski et al., 2001; Lehto et al., 2001). The Osh proteins include four subfamilies (I to IV) (Beh et al., 2001) while ORPs include six subfamilies (I to VI) depending on their sequence similarities and ligand specificity (Lehto et al., 2001). The key signature of all Osh/ORPs is the presence of a conserved OSBP-related domain (ORD), consisting of a β -barrel core and an N-terminal lid, which extracts and transports lipids, including sterol and phospholipids (Delfosse et al., 2020; Im et al., 2005). Except Osh4, Osh5, Osh6, Osh7, and some spliced ORP isoforms (ORP1S and ORP4S), all other Osh/ORPs possess additional

domains and/or sequence elements at their N-terminus. They include a pleckstrin homology (PH) domain that recognizes and binds to PIPs in membranes (Balla, 2005; Lemmon, 2008) and a two phenylalanine-in-an-acidic tract (FFAT) motif that interacts with ER-localized vesicle-associated membrane protein-associated protein (VAP) (or Scs2 and Scs22 in the case of yeast) (Loewen et al., 2003). These elements often allow Osh/ORPs to be anchored to the ER (via VAP/Scs2) and be targeted to membrane contact sites with other organelles that are enriched in PIPs, including the Golgi, endosomes, and the PM (Delfosse et al., 2020).

Budding yeast mutants lacking all the Osh proteins are not viable, but expressing any one of the seven Osh proteins in these mutants restores viability (Beh et al., 2001). Furthermore, acute depletion of Osh proteins leads to growth arrest and massive accumulation of sterol in cells (Beh & Rine, 2004), suggesting that Osh proteins have overlapping functions to maintain cell viability, one of which is likely to maintain cellular sterol distribution. Osh4 and other Osh proteins, including Osh3 and Osh5, were initially reported to mediate sterol transport from the PM to the ER (Raychaudhuri et al., 2006). Some of the Osh proteins, including Osh1, Osh2, Osh4, and Osh5, transport sterol between artificial membranes *in vitro*, making Osh proteins major candidates for LTPs that may mediate nonvesicular sterol transport between the PM and the ER (Manik et al., 2017; Moser von Filseck et al., 2015; Raychaudhuri et al., 2006; Schulz et al., 2009; Tian et al., 2018). However, another study showed that Osh proteins do not play a major role in nonvesicular PM to ER sterol transport but rather they regulate the organization of sterols in the PM (Georgiev et al., 2011). In fact, growing evidence suggests that many Osh/ORPs bind PI4P (and possibly other PIPs) as their shared ligand (Delfosse et al., 2020). Thus, Osh/ORPs may contribute to the cellular distribution of sterol primarily through their properties to regulate the levels of PI4P and other phospholipids in various membrane compartments.

Some Osh/ORPs exchange PI4P with sterol to mediate the directional transport of sterol between two different membrane compartments. Osh4, also known as Kes1, is one of the best characterized Osh with such properties, consisting of the ORD with amphipathic α -helix termed ArfGAP1 lipid packing sensor motif at its N-terminus, which may promote its interaction with highly curved membranes and/or lipid packing defects (Drin et al., 2007). Osh4 localizes to the Golgi (Fairn et al., 2007; Li et al., 2002) and plays a role in regulating the levels of PI4P in this organelle (Fairn et al., 2007) (Figure 3A). Osh4 binds sterol and PI4P in a mutually exclusive manner, and Osh4 counter transports sterol in exchange for PI4P between artificial membranes *in vitro* (de Saint-Jean et al., 2011; Moser von Filseck et al., 2015). Ectopic overexpression of Osh4 in HeLa cells leads to an increase in sterol and decrease in PI4P in the Golgi, supporting the role of Osh4 in nonvesicular sterol transport from the ER to the Golgi in exchange for PI4P (Moser von Filseck

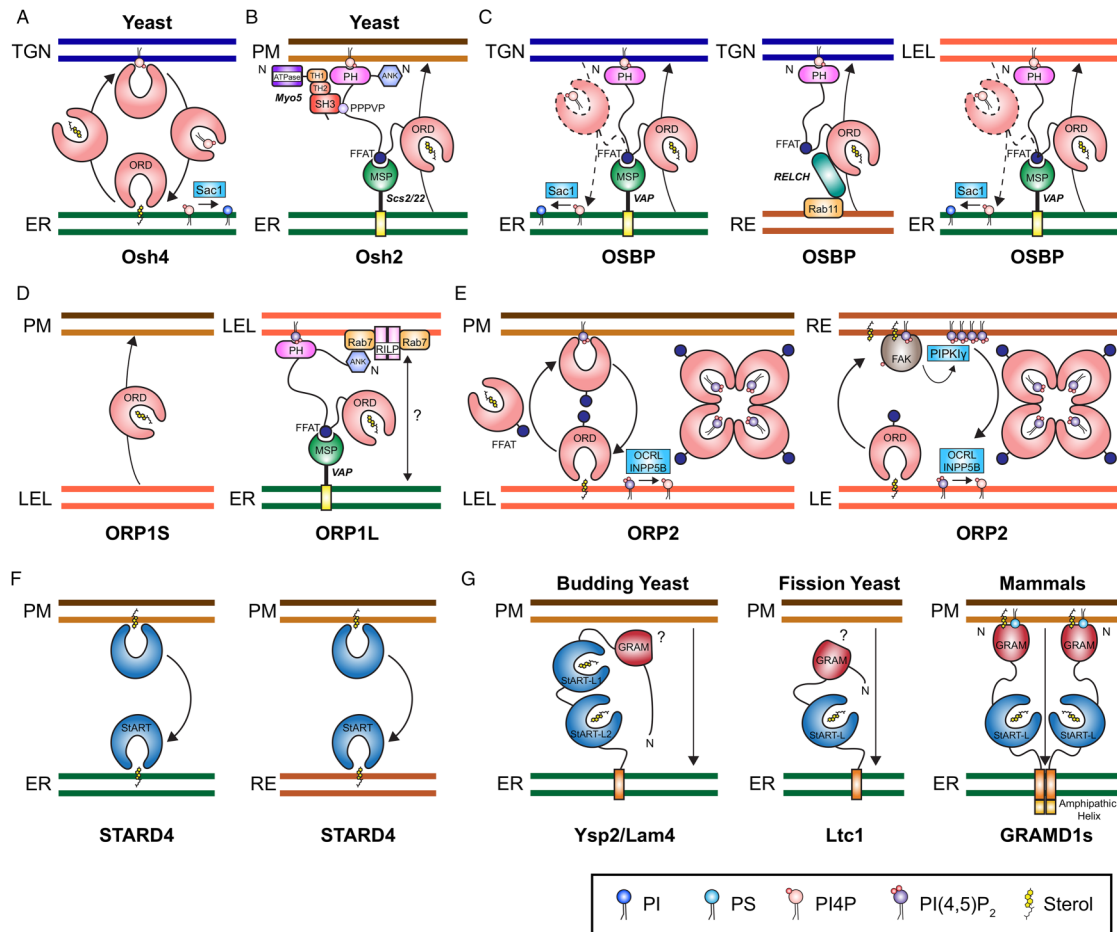


Figure 3. Schematic drawings of lipid transfer proteins that contribute to plasma membrane sterol homeostasis via nonvesicular lipid transport. (A) Osh4 transports ergosterol from the ER to the TGN in exchange for PI4P. PI4P that is transported to the ER is then dephosphorylated to PI by ER-anchored Sac1 phosphatase. (B) Osh2 bridges the PM and the ER by interacting with both Myo5 and Scs2/22 (homologs of VAP in yeast). Osh2 localizes to the ER via interaction of its FFAT motif to the MSP domain of Scs2/22 and interacts with PM PI4P via its PH domain. Osh2 interacts with the SH3 domain of Myo5 through its polyproline PPPVP motif. The ORD domain of Osh2 extracts and transports sterol from the ER to the PM to trigger localized actin polymerization. (C) Left: OSBP localizes to the ER via interaction of its FFAT motif to the MSP domain of VAP and interacts with PM PI4P via its PH domain to transport cholesterol to the TGN in exchange for PI4P. Middle: At the RE-TGN contacts, the ORD domain of OSBP interacts with RELCH-Rab11 complex on the RE to facilitate cholesterol transport to the TGN. Right: At ER-LEL contacts, OSBP transports cholesterol from the ER to the LEL in exchange for PI4P. PI4P that is transported to the ER is dephosphorylated to PI by ER-anchored Sac1 phosphatase (left and right). (D) Left: ORP1S transports cholesterol from the LEL to the PM. Right: ORP1L localizes to the LEL through its interaction with Rab7 and PIPs via its Ankyrin repeat and the PH domain, respectively. The FFAT motif of ORP1L interacts with the MSP domain of ER-localized VAP, allowing ORP1L to tether the LEL to the ER. Whether ORP1L mediates cholesterol transport at LEL-ER contacts remains elusive. (E) Left: ORP2 transports cholesterol from the LEL to the PM in exchange for PI(4,5)P₂. ORP2 forms a tetramer to efficiently transport PI(4,5)P₂ to the LEL. Right: ORP2 transports LDL-derived cholesterol from the LE to FAK/integrin-containing RE. Phosphorylated FAK increases endosomal PIPK1 γ activity to increase PI(4,5)P₂ levels, which are transported back to the LE by ORP2. PI(4,5)P₂ that is transported to the LEL or LE by ORP2 is hydrolyzed to PI4P by phosphoinositide 5-phosphatases (e.g., OCLR and INPP5B). (F) STARD4 transports cholesterol from the PM to the ER (left) or to the RE (right) via its StART domain. (G) Left: In budding yeast, ER-anchored Ysp2 and its paralog, Lam4, facilitate sterol transport from the PM to the ER via its StART-L domain. The presence of polybasic residues after the second StART-like domain may facilitate targeting of Ysp2/Lam4 to the PM, where anionic lipids are enriched. Middle: Fission yeast Ltc1 localizes to ER-PM contacts and facilitates sterol transport from the PM to the ER upon acute inhibition of the Arp2/3 complex. Right: In humans, GRAMD1s form homomeric or heteromeric complexes via interaction of their transmembrane domains and amphipathic helices. GRAMD1s sense a transient expansion of the accessible pool of PM cholesterol by the GRAM domain and facilitate accessible cholesterol transport from the PM to the ER by the StART-like domain at ER-PM contacts. The GRAMD1 GRAM domain acts as a coincidence detector for both accessible cholesterol and anionic lipids, including PS, within the PM. The role of yeast GRAM domain in membrane recognition remains elusive. *Note.* ER = endoplasmic reticulum; FAK = focal adhesion kinase; FFAT = two phenylalanine-in-an-acidic tract; LE = late endosome/lysosome; MSP = major sperm protein; ORD = OSBP-related domain; OSBP = oxysterol-binding protein; PI = phosphatidylinositol; PI4P = phosphatidylinositol 4-phosphate; PI(4,5)P₂ = phosphatidylinositol 4,5-bisphosphate; PIPK1 γ = phosphatidylinositol phosphate kinase type I γ ; PM = plasma membrane; PS = phosphatidylserine; StART-L = StART-like; RE = recycling endosome; TGN = trans-Golgi network; VAP = vesicle-associated membrane protein-associated protein.

et al., 2015). Given the abundance of PI4P in the PM, it was proposed that Osh4 may mediate nonvesicular sterol transport from the ER to the PM for its exchange for the PI4P at the PM; however, direct evidence supporting this function is missing. Interestingly, the deletion of Osh4 makes yeast resistant to nystatin, a polyene antibiotic whose toxicity is proportional to the amount of ergosterol in the PM (Beh et al., 2001). Further, yeast Δ -s-tether mutants that lack majority of ER-PM contacts are synthetic lethal with deletion of Osh4, suggesting the possible redundancy of the functions of Osh4 and ER-PM contacts (Quon et al., 2018). It is also plausible that Osh4 may maintain the levels of PM ergosterol indirectly through its role in regulating the pool of sterol and PI4P in the Golgi.

Recent studies showed that Osh2 and Osh3 bridge the PM and the ER by linking the endocytic myosin-I/Myo5 to Scs2/Scs22 (VAP homologs in yeast) and facilitate actin polymerization at endocytic sites (Encinar Del Dedo et al., 2017). Osh2 contains the Ankyrin repeats, the PH domain, and FFAT motif in addition to the C-terminal ORD and interacts with the SH3 domain of Myo5 through its polyproline motif (PPPVP) (Encinar Del Dedo et al., 2017) (Figure 3B). Osh2 interacts with the PM via its PH domain, which binds PIPs, including PI4P (Balla & Varnai, 2009; Roy & Levine, 2004), and associates with ER-localized Scs2/Scs22 via its FFAT motif (Encinar Del Dedo et al., 2017) (Figure 3B). Osh2 extracts sterol from the unique subdomains of the ER, which are populated by the enzymes for ergosterol synthesis, and promotes transfer of sterol to the PM to trigger localized actin polymerization (Encinar Del Dedo et al., 2021). Thus, Osh2 plays an important role in mediating nonvesicular sterol transport from the ER to the PM at ER-endocytic contacts for normal endocytosis.

In mammals, OSBP, ORP1, ORP2, ORP4, and ORP9 have been reported to harbor and transport cholesterol (Antonny et al., 2018; Aw et al., 2020; Delfosse et al., 2020). Among these proteins, OSBP is functionally most similar to Osh4, acting as a major PI4P/cholesterol exchanger in mammalian cells. OSBP contains additional motifs in its N-terminus (PH domain and FFAT motif) and localizes to membrane contact sites formed between the ER and other organelles, including the TGN, endosomes, and lysosomes (Dong et al., 2016; Lim et al., 2019; Mesmin et al., 2013b). OSBP interacts with the ER by binding to ER-anchored VAPs via its FFAT motif and tethers the ER to these other organelles by the interaction of its PH domain with PI4P (and Arf1) (Figure 3C). At ER-TGN contacts, OSBP transports cholesterol from the ER to TGN against its concentration gradient in exchange for PI4P that is generated by specific TGN-enriched PI4 kinases. PI4P that is transported from the TGN to the ER is then hydrolyzed by ER-anchored Sac1 phosphatase before OSBP binds and transports another cholesterol (Mesmin et al., 2013b, 2017) (Figure 3C). Acute inhibition of OSBP in HeLa cells by a

chemical inhibitor, OSW-1, leads to reduction of cholesterol in the TGN and accumulation of cholesteryl ester in lipid droplets, consistent with overaccumulation of cholesterol in the ER (Mesmin et al., 2017). Reducing the expression of OSBP as well as depletion of VAPs (VAPA and VAPB) leads to massive accumulation of PI4P in endosomes, supporting an important role of OSBP in maintaining endosomal PI4P levels (Dong et al., 2016). In addition, TGN-localized OSBP was shown to interact with RELCH, a Rab11 effector adaptor protein that links OSBP to recycling endosomes. The TGN-recycling endosome tethering mediated by the OSBP-RELCH-Rab11 complex is implicated in OSBP-dependent cholesterol transport from recycling endosomes to TGN (Figure 3C). This complex is also required for the efficient transport of cholesterol from lysosomes to recycling endosomes or the TGN. However, how nonvesicular cholesterol transport mediated by the OSBP-RELCH-Rab11 complex at TGN-recycling endosome contacts affects the transport of cholesterol from lysosomes remains elusive (Sobajima et al., 2018). Notably, OSBP also localizes to ER-lysosome contacts and mediates cholesterol transport from the ER to the limiting membrane of the lysosomes to activate the mTORC1 signaling pathway (Lim et al., 2019) (Figure 3C). Depletion of OSBP leads to PI4P accumulation in lysosomes, suggesting its role in PI4P counter transport at these contacts (Lim et al., 2019). Interestingly, acute inhibition of OSBP by OSW-1 reduces the lipid order of the PM compared to untreated control cells, indicating a role of OSBP in the establishment of the gradient of lipid order along the secretory pathway (Mesmin et al., 2017). Thus, OSBP may act together with vesicular transport systems and contribute to PM cholesterol homeostasis by its property to mediate counter transport of PI4P and cholesterol at various membrane contact sites. In addition to OSBP, many other Osh/ORPs harbor PI4P (and possibly other PIPs) as their common ligand (Delfosse et al., 2020). Thus, some of these proteins may regulate sterol transport rather indirectly by transporting PI4P (or other PIPs). In the light of this concept, some of the previous results that indicated the direct role of Osh/ORPs in sterol transport via cell-based or cell-free lipid transport assays may need to be re-interpreted.

Among other ORP family proteins, ORP1 (spliced into ORP1S and ORP1L isoforms) and ORP2 have been implicated in influencing the levels of cholesterol in the PM. Early studies proposed that ORP1S (ORD-only short isoform) and ORP2 mediate nonvesicular sterol transport from the PM to the ER, thereby facilitating sterol loading into lipid droplets (Jansen et al., 2011). However, recent studies support the functions of these proteins in transporting sterol from endosomal compartments to the PM (Wang et al., 2019; Zhao et al., 2020). Individual overexpression of ORP1S or ORP2 in HeLa cells was shown to increase the levels of PM cholesterol (Wang et al., 2019). HeLa cells lacking ORP1 show a reduction in the levels of cholesterol in the PM and accumulation of cholesterol in late

endosomes/lysosomes (LELs), both of which were restored by re-expression of ORP1S. Similarly, depletion of ORP2 in HEK293 cells results in a reduction in the levels of cholesterol in the PM and some increase of cholesterol in LELs, indicating the role of ORP2 in cholesterol transport from LELs to the PM (Wang et al., 2019). Such delivery of cholesterol to the PM by ORP2 is coupled with removal of PM phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) and driven by hydrolysis of PI(4,5)P₂ by PI(4,5)P₂ 5-phosphatases OCRL and INPP5B (Wang et al., 2019) (Figure 3E). Another study showed that ORP2 transports LDL-derived cholesterol from the late endosome to focal adhesion kinase (FAK)/integrin-containing recycling endosomes, facilitating FAK association with the PI(4,5)P₂-containing membrane. This allows FAK activation, which increases the activity of endosomal phosphatidylinositol phosphate kinase type I γ (PIP1 γ) activity to generate more PI(4,5)P₂ on the recycling endosome. The PI(4,5)P₂ are then counter transported by ORP2 back to the late endosome (Figure 3E). This coupling of ORP2 and FAK activity is also implicated in facilitating cholesterol delivery to the PM (Takahashi et al., 2021). Whether ORP2 primarily acts between LELs and the PM (Wang et al., 2019) or between late endosomes and recycling endosomes (Takahashi et al., 2021) requires further investigation. It remains possible that ORP2 may act between several different membrane compartments.

In contrast, ORP1L, which contains the Ankyrin repeats, the PH domain, and FFAT motif in addition to the C-terminal ORD, localizes to both LELs and multivesicular endosomes/bodies (MVBs) through its interaction with Rab7 via Ankyrin repeats and PIPs via the PH domain (Johansson et al., 2003, 2005; Ma et al., 2018; Rocha et al., 2009; Tong et al., 2019) (Figure 3D). Through its FFAT motif-dependent binding to ER-localized VAP, ORP1L also mediates ER-LEL/MVB tethering (van der Kant et al., 2013), and ORP1L has been implicated in promoting bidirectional sterol transport between the ER and LELs/MVBs (Eden et al., 2016; Zhao & Ridgway, 2017) (Figure 3D). However, it is still unclear whether ORP1L primarily acts as a tethering factor (by its ability to sense endosomal cholesterol levels) or directly mediates nonvesicular cholesterol transport as an LTP at these contacts (Aw et al., 2020). Cholesterol transport activity of ORP1 ORD is strongly enhanced by PI(4,5)P₂ and PI(3,4)P₂ on membranes *in vitro*, suggesting both ORP1L and ORP1S may mediate cholesterol transport at PI(4,5)P₂ and PI(3,4)P₂-rich membranes, such as the PM and LELs (Dong et al., 2019).

Steroidogenic Acute Regulatory Protein-related Lipid Transfer Domain Family Proteins

As described in the previous section, some Osh/ORPs participate in nonvesicular sterol transport and influence the levels

of PM sterol either directly or indirectly. However, the majority of Osh/ORPs have not been implicated in direct sterol transport between the PM and ER. Thus, other LTPs are likely involved in more direct sterol transport between these membrane compartments.

The mammalian Steroidogenic Acute Regulatory Protein (StAR)-related Lipid Transfer (START) Domain (STARD) family contains 15 proteins, which all possess a lipid-harboring START domain, an α -helix/ β -grip fold comprising of an antiparallel β -sheet flanked by N- and C-terminal α -helices (Alpy & Tomasetto, 2005; Tsujishita & Hurley, 2000). They are divided into six subfamilies according to their sequence similarity and ligand binding properties of the START domain (Alpy & Tomasetto, 2014; Soccio & Breslow, 2003). Among these proteins, five STARDs (STARD1, STARD3, STARD4, STARD5, and STARD6) have been shown to harbor and transport cholesterol (Alpy & Tomasetto, 2014; Clark, 2012; Rodriguez-Agudo et al., 2019; Stocco, 2001; Voilquin et al., 2019). However, only STARD4 has been implicated in transporting cholesterol between the PM and other organelles, such as the ER and recycling endosome (i.e., endocytic recycling compartment) (Iaea et al., 2017, 2020; Mesmin et al., 2011). Although STARD4 does not contain any organelle-targeting motif, it possesses a surface-exposed basic patch that facilitates interactions with membranes that are enriched with anionic lipids, such as the PM and endosomes (Iaea et al., 2015; Mesmin et al., 2011). This facilitates the insertion of its C-terminal α -helix into the membrane and allows sterol to diffuse into its hydrophobic sterol binding pocket (Iaea et al., 2015). The hydrophobic pocket is closed by the β 5– β 6 loop known as the Ω 1 loop, which acts as a flexible lid for the sterol binding and release (Iaea et al., 2015; Tan et al., 2019).

Overexpression of STARD4 facilitates delivery of sterol to the recycling endosome and enhances Acyl-coenzyme A:cholesterol acyltransferases (ACAT)-mediated cholesterol ester formation in the ER and incorporation of sterol into lipid droplets (Mesmin et al., 2011; Rodriguez-Agudo et al., 2011), supporting the role of STARD4 in transporting cholesterol from the PM to the recycling endosome and to the ER (Figure 3F). In particular, STARD4 mediates a large fraction of sterol transport between the PM and recycling endosomes (estimated to be ~25% of total sterol transport that occurs between these compartments) (Iaea et al., 2017). In addition, STARD4 overexpression traps SCAP in the ER, further supporting the role of STARD4 in increasing the levels of cholesterol in the ER (Mesmin et al., 2011). STARD4 expression is transcriptionally regulated by SREBP-2. Therefore, a negative feedback loop, in which cholesterol transported by STARD4 to the ER contributes to suppression of SREBP-2 cleavage, results in reduced expression of STARD4 (Horton et al., 2003; Iaea et al., 2020; Soccio et al., 2005). Depletion of STARD4 in U2OS cells shows complex phenotypes, one of which is an increase in free cholesterol levels, possibly by modulating the

response of SREBP-2 (Mesmin et al., 2011). These STARD4-depleted cells also show changes in the PM fluidity and accumulation of cholesterol in late endosomes, which is accompanied by altered lipid homeostasis (Iaea et al., 2020). Collectively, these studies support the role of STARD4 in mediating nonvesicular transport of cholesterol between the PM and other cellular compartments, such as the ER and the recycling endosome.

STARD4 is widely expressed with the highest expression in the liver (Riegelhaupt et al., 2010). STARD4 knock-out mice are viable, and they show no major changes in liver cholesterol metabolism or plasma lipids dynamics despite a slight decrease in body size and weight compared to wild-type mice (Riegelhaupt et al., 2010). This suggests that there is functional redundancy among STARDs and possibly overlapping functions with other families of sterol transporters in mammals. Importantly, yeast does not possess STARD homologs. This pointed to a possibility that there might be a more ancient family of lipid transfer proteins that may participate in PM to ER transport of sterol in eukaryotes.

GRAMD1/Lam/Ltc Family Proteins

The search for yeast proteins that contain the StART-like domain identified a new evolutionarily conserved family of LTPs, namely, Lam/Ltc proteins (GRAMD1s/Asters in mammals) (Gatta et al., 2015). In budding yeast, this family consists of three pairs of paralogs (Ysp1/Lam1 and Sip3/Lam3, Ysp2/Lam2/Ltc4 and Lam4/Ltc3, and Lam5/Ltc2 and Lam6/Ltc1) (Gatta et al., 2015; Murley et al., 2015). These proteins all contain an N-terminal Rab-like GTPase activators, and myotubularins (GRAM) domain, followed by one or two StART-like domains and a C-terminal transmembrane region that anchors these proteins to the ER (Gatta et al., 2015; Murley et al., 2015). The GRAM domain is structurally similar to the PH domain, which often binds anionic lipids, including PIPs (Begley et al., 2006, 2003; Lemmon, 2008). The StART-like domain shares overall structural similarity to the StART domain and contains a deep hydrophobic groove for capturing sterol (Horenkamp et al., 2018; Jentsch et al., 2018; Laraia et al., 2019; Sandhu et al., 2018; Tong et al., 2018). Ysp1 and Sip3 also possess a Bin/Amphiphysin/Rvs (BAR) domain and a PH domain, suggesting their possible role in membrane curvature generation/sensing via the BAR domain (Itoh & De Camilli, 2006; Peter et al., 2004). Ysp1, Sip3, Ysp2, and Lam4 localize to ER-PM contact sites (Gatta et al., 2015) (Figure 3G), while Lam5 and Lam6 are targeted to various membrane contacts, such as the nucleus-vacuole junctions (NVJs) (ER-lysosome contact equivalent in animals) and ER-mitochondrial contacts (Elbaz-Alon et al., 2015; Gatta et al., 2015; Murley et al., 2015). The molecular mechanisms that mediate the constitutive targeting of Ysp1, Sip3, Ysp2, and Lam4 to

ER-PM contacts remain elusive, although Ysp2 was shown to require only its transmembrane domain and luminal region to localize to ER-PM contacts (Gatta et al., 2015). The localization of Lam6 to NVJs requires a vacuolar protein, Vac8, but occurs without relying on its GRAM domain (Jeong et al., 2017; Murley et al., 2015; Wang et al., 1998). In contrast, both the GRAM domain and mitochondrial preprotein import receptors Tom70/71 are required for the targeting of Lam6 to ER-mitochondrial contacts (Murley et al., 2015; Schlossmann et al., 1996; Schmidt et al., 2010). In fission yeast, two StART-like domain-containing proteins are expressed, namely, Ltc1 (Lam5 and Lam6 homolog) and Ltc2 (Ysp1 and Sip3 homolog). Fission yeast Ltc1 constitutively localizes to ER-PM contacts (Marek et al., 2020) (Figure 3G). Polybasic residues that are present after the second StART-like domains of budding yeast Ysp2 and Lam4 (Tong et al., 2018) and after the StART-like domain of fission yeast Ltc1 may potentially play a role in constitutive localization of yeast Lam/Ltc proteins to the PM, where anionic lipids are particularly abundant (Doktorova et al., 2020; Ingolfsson et al., 2014; Leventis & Grinstein, 2010). Supporting this notion, the polybasic residues promote the binding of purified budding yeast StART-like domains to artificial membranes containing phosphatidylserine (PS), a major anionic lipid in the PM (Tong et al., 2018). Collectively, the unique cellular localization of Lam/Ltc proteins, which all contain the StART-like domain, suggested a major role of this family of LTPs in non-vesicular sterol transport at membrane contact sites, including ER-PM contacts.

StART-like domains transport sterols between membranes *in vitro* (Gatta et al., 2015; Horenkamp et al., 2018; Jentsch et al., 2018; Laraia et al., 2019; Naito et al., 2019; Sandhu et al., 2018), and the flexibility of the Ω 1 loop located between the β 2 and β 3 strands in yeast (between the β 3 and β 4 strands in human) was shown to be important for sterol binding and release, similar to mammalian STARD proteins (Horenkamp et al., 2018; Naito et al., 2019). Budding yeast lacking either Ysp1, Ysp2, or Sip3 shows slower sterol traffic from the PM to the ER as well as increased sensitivity to an antifungal drug, Amphotericin B (a polyene drug that binds PM sterols and eventually causes cell death [Kinsky, 1970]), indicating chronic accumulation of PM ergosterol in these yeast mutants (Gatta et al., 2015). In fission yeast, acute blockade of the activity of the Arp2/3 complex, a critical mediator of actin assembly, results in Ltc1-dependent transport of sterol from the PM to endosomes (possibly via its property to promote PM to ER sterol transport) (Marek et al., 2020). Fission yeast lacking Ltc1 shows the accumulation of sterol in the PM upon blockade of the Arp2/3 complex and increased sensitivity to Amphotericin B, consistent with the accumulation of sterol in the PM (Marek et al., 2020). Collectively, these studies show that Lam/Ltc proteins transport sterol from the PM to the ER, thereby contributing to PM sterol homeostasis in yeast.

Mammalian homologs of yeast Lam/Ltc proteins are GRAMD1s (GRAMD1a/Aster-A, GRAMD1b/Aster-B, and GRAMD1c/Aster-C), which comprise an N-terminal GRAM domain, followed by a StART-like domain and a C-terminal transmembrane ER anchor (Besprozvannaya et al., 2018; Naito et al., 2019; Naito & Saheki, 2021; Sandhu et al., 2018). GRAMD1s bind one another through their transmembrane domains and luminal amphipathic helices and form homo- and heteromeric complexes (Naito et al., 2019) (Figure 3G). Despite their structural similarity to budding yeast Lam5 and Lam6 and fission yeast Ltc1, they localize throughout the ER at steady state. However, they rapidly move to ER-PM contacts upon elevation of the levels of PM cholesterol either by cholesterol loading or expansion of the accessible pool of PM cholesterol induced by hydrolysis of sphingomyelin (Naito et al., 2019; Sandhu et al., 2018). This is mediated by the GRAM domain of GRAMD1s, which acts as a coincidence detector of both anionic lipids, including PS, and accessible cholesterol in the PM (Ercan et al., 2021; Ferrari et al., 2020; Naito et al., 2019) (Figure 3G). The GRAMD1b GRAM domain (and possibly other GRAM domains of GRAMD1s) possesses distinct sites, which synergistically contribute to sensing accessible cholesterol and anionic lipids (Ercan et al., 2021). This ability of the GRAM domain allows GRAMD1b to bind the cytoplasmic leaflet of the PM, where PS is abundant, only when the pool of accessible PM cholesterol is acutely expanded (such as when additional cholesterol is supplied through LDL or HDL). Interestingly, the amino acid residues that are critical for anionic lipid sensing and sterol sensing properties of the GRAM domain are highly conserved from yeast Lam/Ltc proteins to human GRAMD1s (Ercan et al., 2021). Currently, it is unknown whether GRAM domains of yeast Lam/Ltc proteins are involved in sensing ergosterol (or any anionic lipids) in cellular membranes. The molecular mechanisms of reversible (in the case of mammalian GRAMD1s) versus constitutive (in the case of some yeast Lam/Ltc proteins) recruitment of GRAMD1/Lam/Ltc family proteins to ER-PM contact sites needs further investigation.

Purified near-full length GRAMD1b proteins transport cholesterol between PM-like and ER-like membranes *in vitro*, and such transport activities are strongly influenced by the accessible cholesterol-sensing property of the GRAM domain (Ercan et al., 2021). Thus, the GRAM domain contributes to fine-tuning of StART-like domain-dependent sterol transport of GRAMD1b through facilitation of membrane tethering at ER-PM contacts. The StART-like domain of GRAMD1b also transports PI(4,5)P₂ *in vitro* (Horenkamp et al., 2018), although its physiological implication needs to be elucidated. Experimental manipulations that deplete or inhibit GRAMD1s in various mammalian cell types, including fibroblasts and macrophages, result in inefficient suppression of SREBP-2 cleavage upon cholesterol

loading or expansion of the accessible pool of PM cholesterol (Naito et al., 2019; Sandhu et al., 2018; Xiao et al., 2021). Furthermore, these manipulations also lead to chronic expansion of the accessible pool of PM cholesterol at steady state, consistent with less efficient cholesterol transport from the PM to the ER in the absence of GRAMD1s (Ferrari et al., 2020; Naito et al., 2019). Therefore, GRAMD1s play a major role in nonvesicular cholesterol transport from the PM to the ER at ER-PM contact sites and contribute to cholesterol homeostasis in mammalian cells (Figure 3G).

Although some ORPs, including OSBP, ORP1L, and ORP5, have been implicated in acting at ER-lysosome contacts (Du et al., 2011; Johansson et al., 2003; Lim et al., 2019), the pathways that control the delivery of LDL-derived cholesterol downstream of lysosomes remain elusive. GRAMD1b was reported to interact with Niemann-Pick type C protein 1 (NPC1), a lysosome membrane protein involved in cholesterol egress from lysosomes, and localize to ER-lysosome contacts. Thus, it may transport LDL-derived cholesterol directly from lysosomes to the ER (Hoglinger et al., 2019). However, it has been also suggested that some fractions of LDL-derived cholesterol first reach the PM before being transported to the ER (Infante & Radhakrishnan, 2017). PM to ER transport in this process requires PS (Trinh et al., 2020). GRAMD1s might be involved in delivering LDL-derived cholesterol from the PM to the ER after cholesterol reaches the PM because GRAMD1s detect the presence of both PS and accessible cholesterol in the PM as a coincidence detector. Chemical inhibition of GRAMD1s blocks the suppression of the SREBP-2 cleavage by LDL treatment, implicating the role of GRAMD1-mediated nonvesicular transport in the delivery of LDL-derived cholesterol to the ER (Xiao et al., 2021). Whether GRAMD1s act primarily at ER-PM contacts or ER-lysosome contacts for this process requires further investigation. Further, both depletion of Lam6 in budding yeast and removal of GRAMD1c in mammalian cells alters mammalian target of rapamycin (mTOR) signaling, whose activity is controlled at various membrane compartments, including the PM and lysosomes/vacuoles (Murley et al., 2017; Zhang et al., 2020). Thus, more studies are required to dissect the function of GRAMD1s at different cellular sites.

GRAMD1a is expressed ubiquitously with high levels of expression in the central nervous system (Sandhu et al., 2018; Yue et al., 2014). The highest levels of GRAMD1b expression are detected in steroidogenic tissues, such as testes and adrenal glands, but GRAMD1b is also highly expressed in the brain. On the other hand, GRAMD1c is expressed at much lower levels compared to other GRAMD1s, but is expressed in the testes and liver (Sandhu et al., 2018; Yue et al., 2014). Rodent adrenal glands rely on a scavenger receptor, SR-BI, for HDL cholesterol uptake for steroidogenesis. Depletion of GRAMD1b in mice results in major defects in cholesteryl ester storage in adrenal glands as well as the

reduction in corticosterone levels. Thus, GRAMD1b is critical for mediating PM to ER transport of HDL-derived cholesterol for its esterification and subsequent production of steroid hormones (Sandhu et al., 2018). Human genetic studies identified possible links between GRAMD1b and various neurodevelopmental disorders, including schizophrenia and intellectual disability (Reuter et al., 2017; Santos-Cortez et al., 2018; Schizophrenia Working Group of the Psychiatric Genomics, 2014; Thyme et al., 2019). An intellectual disability-associated missense mutation in GRAMD1b GRAM domain (R189W) disrupts the cholesterol-sensing ability of this domain and impairs GRAMD1b-mediated cholesterol transport (Ercan et al., 2021). Zebrafish knock-out of GRAMD1b shows reduced brain activity (Thyme et al., 2019), suggesting a conserved role of GRAMD1b in the development and function of the brain in animals. Future studies are required to better understand the physiological functions of GRAMD1s in the brain and other organs.

Discussion

Despite some differences in the regulatory mechanisms of sterol biosynthesis and uptake, nonvesicular sterol transport mediated by LTPs plays a major role in maintaining PM sterol homeostasis in both yeast and mammals. Osh/ORPs were initially proposed to participate in sterol transport between the PM and the ER. In yeast, Osh2 transports sterol from the unique subdomains of the ER to the PM by acting at ER-endocytic sites to facilitate actin polymerization. Although some other Osh/ORPs also bind and transport sterol, more recent findings support the role of most of these proteins in nonvesicular lipid transport at other membrane interfaces. Growing evidence supports a major role of GRAMD1/Lam/Ltc family proteins in mediating nonvesicular PM to ER sterol transport to contribute to PM sterol homeostasis. In mammals, STARD4 also participates in nonvesicular sterol transport between the PM and other organelles, including the ER and the recycling endosome.

Several outstanding questions remain in the field. While it is clear that nonvesicular sterol transport plays a major role in the cellular distribution of sterol, vesicular transport also participates in bulk sterol transport from one compartment to another. How these two transport pathways are coupled to maintain the levels of sterol in different cellular compartments, including the PM, remains incompletely understood. Forward sterol traffic assays that have been widely used in yeast and mammalian cells typically rely on metabolic labeling, and hence, they are specifically measuring the transport of newly synthesized sterol. Whether different pools of sterol (e.g., pre-existing vs. newly synthesized) are transported via distinct mechanisms remains to be determined.

GRAMD1/Lam/Ltc family proteins have been implicated in PM to ER sterol transport but not in ER to PM sterol transport. It is unclear whether other LTPs may mediate the

transport of sterol from the ER to the PM. In yeast, Osh2 has been implicated in this process, but it remains elusive if any one of the mammalian ORPs may mediate direct ER to PM cholesterol transport. ORP1L is structurally similar to Osh2, but its role in ER to PM cholesterol transport has not been reported. Furthermore, some LTPs, including STARD4, ORP1S, and ORP2, do not necessarily rely on membrane contact sites for lipid transport. Thus, it is important to understand how much of nonvesicular sterol transport actually occurs at membrane contact sites. In mammalian cells, LTPs have been shown to participate in the cellular distribution of LDL-derived cholesterol downstream of lysosomes, but the precise mechanisms remain largely elusive. A number of new studies have shown that different membrane compositions affect the efficiency of LTP-mediated lipid transport, although how such effects are linked to the activities of various LTPs in cellular contexts needs further investigation. Future studies are needed to better understand the molecular mechanisms of intracellular sterol transport. Visualization of cholesterol transport with a novel cholesterol biosensor in living cells may help tackle some of these important questions.

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