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# Nonvesicular sterol transport: two protein families and a sterol sensor?

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**Sterols, essential components of eukaryotic membranes, are actively transported between cellular membranes. Although it is known that both vesicular and non-vesicular means are used to move sterols, the molecules and molecular mechanisms involved have yet to be identified. Recent studies point to a key role for oxysterol binding protein (OSBP) and its related proteins (ORPs) in nonvesicular sterol transport. Here, evidence that OSBP and ORPs are *bona fide* sterol carriers is discussed. In addition, I hypothesize that ATPases associated with various cellular activities regulate the recycling of soluble lipid carriers and that the Niemann Pick C1 protein facilitates the delivery of sterols from endosomal membranes to ORPs and/or the ensuing membrane dissociation of ORPs.**

## Introduction

Sterols (a subgroup of steroids with a hydroxyl group in the 3 position of a hydrocarbon ring) are indispensable components of eukaryotic membranes and serve to modulate membrane rigidity, fluidity and permeability. Membrane sterols have also been shown to have key roles in several important cellular processes, such as signal transduction and vesicular trafficking [1,2]. Eukaryotic cells have developed elegant mechanisms to maintain a constant level of free sterol. In mammalian cells, cholesterol is obtained either through *de novo* synthesis in the endoplasmic reticulum (ER) or through the uptake of plasma lipoproteins. Excess cholesterol is delivered to acyl CoA cholesterol acyl transferase (ACAT) for esterification and subsequent storage in lipid particles [3]. The ER is a key site where the sterol level is monitored and feedback regulatory cascades are initiated to control cholesterol biosynthesis and uptake so that cellular sterol homeostasis is maintained [4].

Although sterols are essential components of eukaryotic membranes, they are not uniformly distributed throughout the cell, and the amount of free membrane sterol in each subcellular organelle is unique [5]. For instance, the sterol level is low at its site of synthesis in the ER, but it is concentrated in the plasma membrane (PM). Mechanisms must therefore exist to regulate the transport and distribution of cellular sterol to maintain a distinct sterol level in each membrane. The importance of such transport and sorting mechanisms is highlighted in several human diseases. For instance, accumulation of cholesterol in endosomal compartments is associated with Niemann Pick Disease type C and possibly Alzheimer's disease [6,7].

The molecular mechanisms underlying sterol biosynthesis, esterification and cellular uptake are relatively well established. Two recent reviews have discussed the intracellular distribution of cholesterol and cholesterol-rich membrane domains, general pathways of intracellular sterol movement and methods for studying sterol transport and distribution [8,9]. However, how the non-homogenous distribution of sterols within the cell is maintained and how sterols move between cellular membranes is not fully understood in molecular terms. Here, I focus on recent insights into the molecular mechanisms of nonvesicular sterol transport. I propose a transport scheme involving the participation of oxysterol binding protein (OSBP) and its related proteins (ORPs), ATPases associated with various cellular activities (AAA ATPases) and Niemann Pick C1 protein (NPC1) in ATP-dependent, nonvesicular sterol transport. In particular, I discuss the possibility that ORPs are *bona fide* sterol transfer proteins and that an *N*-ethylmaleimide sensitive factor (NSF)-like mechanism is used to recycle lipid carriers, such as OSBP and the ORPs.

## Intracellular transport of sterols and lipids

Several themes have emerged in the past few decades for intracellular sterol transport. Sterols can be moved by membrane transport vesicles, by diffusible carrier proteins or by putative multi-protein scaffolding complexes between donor and acceptor membranes [8,10]. With the exception of mitochondria, most organelles in eukaryotic cells are connected by small, membrane-enclosed vesicles. For cargo delivery, vesicles bud from the donor membranes assisted by coat proteins, move along microtubules or actin filaments and fuse to target membrane through the action of soluble NSF attachment protein receptors (SNAREs) [11,12]. Vesicular transport requires metabolic energy and an intact cytoskeleton. Given the dynamic nature of vesicular transport, it is conceivable that a significant amount of lipids and/or sterols could be moved between organelle membranes in transport vesicles (Box 1).

Nonvesicular transport mechanisms for sterol transport have also been proposed. Such mechanisms are also employed for the transport of other lipids, such as ceramide and phosphatidylserine (PS) [13,14]. Diffusible carrier proteins are known to facilitate the nonvesicular transport of lipids [8]. The best characterized carrier protein is the ceramide transport protein CERT, which catalyzes the ATP-dependent, nonvesicular transport of ceramide from

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**Box 1. Membrane vesicles and sterol transport**

Several studies have implicated the involvement of both endocytic and secretory pathways in sterol transport. The delivery of newly synthesized cholesterol to the PM was partially inhibited when Golgi function was disrupted by brefeldin A [43]. The delivery of lipoprotein-derived sterol from late endosomes or lysosomes to the ER was sensitive to ATP depletion and to agents known to disrupt vesicular transport in macrophages [44]. Overexpression of the small GTPases Rab5 or Rab11 altered the distribution of cholesterol among cellular membranes [45]. Interestingly, elevated levels of Rab7 or Rab9 induced the egress of cholesterol from NPC1 endosomes [46,47].

It is clear that sterols can be moved in transport vesicles; however, it remains to be determined whether the main function of the generation of any of such vesicles is to deliver sterols and/or other lipids. Nor is it clear how sterols are actively sorted during budding and fusion events [11,42]. The existence of various membrane domains can cause lateral segregation of lipids, and these segregated lipid domains can be selectively included or excluded from transport vesicles [48]. Complex sphingolipids, which localize predominantly to the luminal leaflet of cell membranes and therefore have no access to carrier-mediated nonvesicular transport, are sorted by selective incorporation into anterograde vesicles and exclusion from retrograde vesicles. Consistent with this, there was much less sphingomyelin in COPI-coated vesicles than in their donor Golgi membranes [49]. The transport of yeast sphingolipids from Golgi to the PM also depends on an intact secretory pathway [50]. In contrast to sphingolipids, a significant amount of cholesterol can be found in the cytoplasmic leaflets, and sterols are delivered from the ER to the PM predominantly in a Golgi-independent, nonvesicular manner [35,41,42]. Therefore, the significance of phase separation and vesicle transport in the sorting and transport of sterols requires further investigation.

ER to the Golgi [13]. To fulfil its ceramide carrier function, CERT has a pleckstrin homology (PH) domain for Golgi targeting, a 'two phenylalanines in an acidic tract' (FFAT) motif for ER targeting and a 'steroidogenic acute regulatory protein (StAR)-related lipid transfer' (START) domain for ceramide binding and extraction [9,13,15]. Alternatively, nonvesicular transport could also take place directly between closely opposed donor and acceptor membranes using multiprotein complexes [14].

One of the better characterized sterol binding proteins in higher eukaryotes is StAR (also called StarD1), which is required for the delivery of cholesterol to the inner mitochondrial membrane for the biosynthesis of steroid hormones. Although it is clear that StAR has an essential role in delivery of cholesterol to mitochondria, it remains to be determined whether StAR and other members of the START domain-containing proteins are sterol or lipid carrier proteins or alternatively, are primarily regulatory proteins [9]. Caveolins can bind cholesterol directly and have been implicated in the delivery of cholesterol from the ER to the plasma membrane [16]. However, it seems that caveolins could have an indirect role in sterol transport, given that caveolin-deficient cells do not show any defects in cellular sterol transport [17].

## OSBP and ORPs: potential sterol transporters?

Recently, OSBP and ORPs have been shown to mediate nonvesicular sterol trafficking [18,19]. OSBP was first identified as a high affinity cytosolic receptor for oxysterols, such as 25-hydroxycholesterol. Proteins homologous to OSBP have subsequently been identified in most eukaryotes including 12 members in humans and seven in the budding yeast *Saccharomyces cerevisiae* [19,20]. These proteins all share a conserved ~400 amino acid OSBP-related domain (ORD) found at the C-terminus of OSBP, which binds oxysterols. Members of the ORP family vary in length; the short ORPs comprise primarily the ORD whereas the long ones often possess additional functional domains, including a PH domain and ankyrin repeats. Both the long and short ORPs appear to associate with membranes, possibly through binding to phosphoinositides [21–23].

## OSBP and ORPs: roles in cellular sterol transport

Many lines of evidence suggest that OSBP and ORPs have a direct role in cellular sterol transport.

### *ORPs in yeast*

Aspects of sterol homeostasis in *S. cerevisiae* share striking similarities with those in animal cells [24]. The predominant sterol in yeast is ergosterol, whose structure is only slightly different from cholesterol: it has additional double bonds at C7 and C22 and an extra methyl group at C28. Like cholesterol in mammalian cells, ergosterol is synthesized in the ER and concentrates at the yeast plasma membrane (PM) [25]. The movement of sterols between the ER and PM in yeast uses nonvesicular mechanisms [26,27]. The yeast genome does not encode homologues to most putative sterol carriers, including START domain-containing proteins, sterol carrier protein 2 (SCP2) or caveolins. Interestingly, however, there are seven ORPs, Osh1p–Osh7p (Osh stands for OSBP homologue) encoded in a small genome of ~6000 genes. None of the individual *OSH* genes is essential, but deletion of all seven genes is lethal and is accompanied by a 3.5-fold increase in the cellular ergosterol level [20]. More recent work showed that intracellular sterol distribution was also altered upon elimination of all *OSH* function [28]. Therefore, the Osh protein family could be responsible for the transport and proper distribution of ergosterol.

### *Membrane targeting*

The localization of OSBP and some ORPs with membranes is controlled by sterol homeostasis. For example, soluble OSBP associates with the Golgi in response to oxysterol loading [29]. Oxysterol loading probably affects OSBP targeting indirectly by altering cholesterol content [30]. Membrane targeting is determined by specific motifs present in OSBP and some ORPs. Osh1p associates with Golgi through its PH domain and with the nuclear–vacuole junction through its ankyrin repeats [31]. Osh1p contains a FFAT motif that interacts with Scs2p, a resident ER protein and a homologue of mammalian VAMP-associated protein-A (VAP-A) [15,32]. This feature is conserved, as CERT also contains a FFAT domain that mediated its interaction with VAP-A in the ER [33]. The existence of these targeting motifs indicates that non-vesicular sterol trafficking can be regulated to take place between specific donor and acceptor membranes.

### *Structural evidence*

Crystallographic analysis of the yeast ORP Osh4p revealed that the sterol is bound within a hydrophobic cavity and is

protected from the aqueous environment by a flexible lid [34]. It was proposed that sterol and membrane binding stimulates reciprocal conformation changes of Osh4p that promote a sterol transfer cycle.

### *Functional evidence*

Two recent studies in yeast have confirmed the essential role of Osh proteins in nonvesicular sterol transport. Prinz and colleagues showed that the nonvesicular transport of ergosterol from the PM to the ER requires the Osh proteins [18]. Importantly, Osh4p can specifically facilitate the *in vitro* nonvesicular transport of cholesterol and ergosterol between model membranes. Interestingly, evidence from experiments *in vivo* and *in vitro* points to an important role of phosphoinositides in normal PM-to-ER sterol transport. Short ORPs without a PH domain, such as Osh4p and

**Box 2. An NSF-like mechanism for nonvesicular lipid transport?**

For ORPs and CERT to fulfil their role as lipid carriers, they must associate with and dissociate from donor and acceptor membranes in an efficient and regulated manner. It is conceivable that desorption or extraction of lipids mediated by ORPs or CERT at the donor membrane and unloading at the acceptor membrane is assisted by other receptors or anchors and adaptor proteins (which are yet to be identified) and is ATP-dependent. We have recently established a link between two ORPs (Osh6p and Osh7p) and an AAA ATPase, Vps4p, in yeast [51]. A different ORP (Osh1p) also co-purified with another AAA ATPase (Afg2p), further highlighting the intimate relationship between AAA ATPases and ORPs [52,53].

The key mode of action of AAA ATPase family members is the energy-dependent unfolding of proteins and disassembly of protein complexes [54,55]. One of the most well-known AAA ATPases is NSF (Sec18p in yeast), which disrupts SNARE complexes after transport vesicles fuse with the target membrane, thereby allowing the recycling of the SNAREs for future rounds of membrane fusion. Vps4p functions in a similar way: it disrupts the endosomal sorting complex required for transport (ESCRT) complexes after each round of membrane invagination and formation of lumenal vesicles in the multivesicular body sorting pathway [56]. Similar to SNAREs, Osh7p forms a high molecular weight protein complex that is resistant to detergent solubilization in the absence of Vps4p, suggesting that Vps4p is required to disrupt a membrane-associated protein complex containing Osh7p. In addition, Osh6p and Osh7p interact with Vps4p only after the lipid cargo is unloaded [51]. Furthermore, deletion of *VPS4* in yeast reduces sterol esterification, and overexpression of a mutant human VPS4 causes cholesterol accumulation in late endosomes [57]. The effect of Vps4p on sterol metabolism appears to be independent of its role in MVB sorting since deletion of other ESCRT components has no effect on sterol homeostasis ([51] and H.Y., unpublished). Conversely, Osh proteins do not seem to serve as sterol sensors to modulate the activity of AAA ATPases and vesicular transport, as deletion of all *OSH* genes had no impact on protein secretion or vacuolar protein sorting [28]. Taken together, it is logical to hypothesize that AAA ATPases function to directly regulate the recycling of soluble lipid carriers, such as ORPs, in a manner similar to how NSF recycles SNAREs. Our working model is summarized in Figure I.

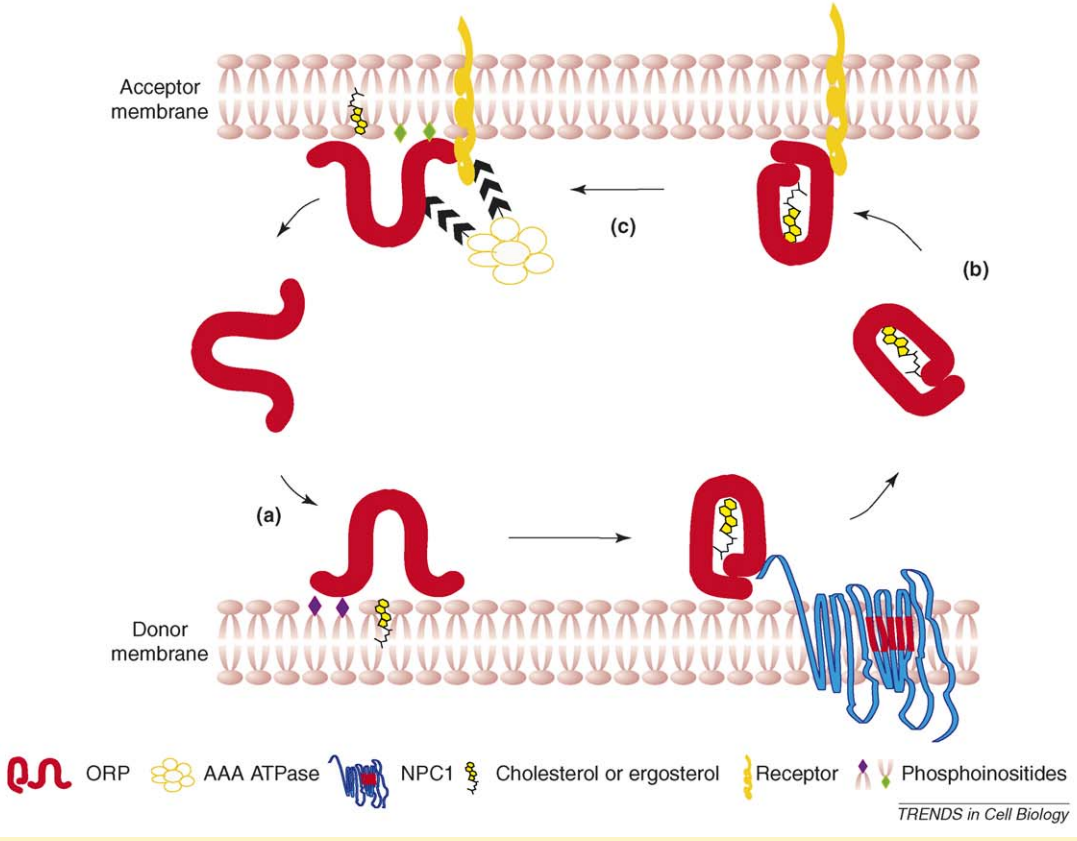


**Figure I**. AAA ATPases regulate ORP-mediated sterol transport. ORPs shuttle sterols between membranes and AAA ATPases and possibly NPC1 might regulate this process. **(a)** A ligand-free ORP might bind to phosphoinositides at the donor membrane. **(b)** After lipid extraction and 'lid' closure, the ligand-bound ORP heads for the acceptor membrane and is tethered by a membrane receptor or anchor. Such a receptor or anchor does seem to exist, for instance, OSBP in mammals and Osh1p in yeast interact with the ER-resident protein VAP-A and Scs2p, respectively [14,32]. **(c)** The receptor and ORP work together to unload the lipid cargo, possibly with the assistance of other yet-to-be identified proteins. Upon unloading, an AAA ATPase is recruited to disrupt the protein complex at the acceptor membrane to allow the recycling of ORPs. [51–53,57]. NPC1 probably facilitates the extraction of lipids by ORPs and/or the membrane dissociation of ORPs if the donor membrane is from lysosomes or vacuole (Figure I, Box 3).

Osh6p, are known to bind phosphoinositides [22,23]. Given that each cellular organelle has a unique profile of membrane phosphoinositides, the phosphoinositide-binding property of ORPs might guide the targeted delivery of sterols. Interestingly, Menon and colleagues showed that Osh proteins are also important for the transport of ergosterol in the opposite direction, from the ER to the PM [35]. Taken together, these functional results are consistent with a role for Osh proteins as sterol carriers. In summary, although OSBP/ORPs have acquired additional functions, such as modulating extracellular regulated kinase (ERK) signaling or inhibiting vesicle budding from the Golgi [36,37], the basic and primordial duty of this family of proteins could be serving as cytoplasmic sterol transporters.

## Energy dependence of nonvesicular sterol transport

Nonvesicular sterol transport can occur by both ATP-dependent and ATP-independent mechanisms. Many lines of evidence indicate that the default mechanism for intracellular sterol transport could be a passive, ATP-independent process. By following the trafficking in live cells of a fluorescent sterol, dihydroergosterol (DHE), Maxfield and colleagues have elegantly demonstrated that DHE was efficiently transferred from the plasma membrane to lipid particles and endosomal recycling compartment (ERC) after energy depletion [38,39]. Moreover, the influx of plasma membrane sterol was not dependent on ongoing ATP synthesis [40]. ATP-independent sterol transport could be facilitated by soluble sterol carriers or, alternatively, through proteinaceous membrane contact sites. Intriguingly, accumulation of DHE in the ERC was observed in permeabilized cells by using an artificial carrier (methyl-β-cyclodextrin), indicating that specific targeting by a soluble carrier was unnecessary [8,38]. These studies suggest that lipid particles and ERC membranes could simply be thermodynamic sinks for sterols. In this case, the sterol carriers need not themselves be specifically targeted or regulated.

The existence of ATP-dependent, nonvesicular sterol transport has also been proposed. ER-to-PM sterol transport in both yeast and mammalian cells requires ATP but not transport vesicles, and has a half-time of more

### Box 3. NPC1, a sterol sensor for nonvesicular sterol transport?

NPC1 is one of the best-known regulators of intracellular sterol transport and is essential for the egress of cholesterol from endosomes and lysosomes [6,58]. NPC1 has a sterol sensing domain that is conserved in several proteins implicated in sterol metabolism and signalling. Despite intense research efforts, the exact molecular mechanisms underlying NPC1 function and cholesterol exit from endosomes and lysosomes remain elusive. NPC1 binds sterol through its sterol sensing domain and, like sterol regulatory element-binding protein cleavage activating protein (SCAP), might adopt different conformations in response to changes in membrane sterol levels [4,59]. SCAP interacts with Insulin-induced (Insig) proteins when the ER sterol levels are high but with COPII proteins upon sterol depletion [4]. The sterol-triggered conformational change in SCAP represents a key feature of how cells monitor sterol concentrations and adjust sterol biosynthesis and uptake. Likewise, NPC1 might assume a conformation upon sterol loading that enables it to interact with cytoplasmic lipid carriers such as the ORPs. It is also interesting to note that, as yeast contains NPC1 and ORP orthologues but none of the other proteins implicated in mammalian sterol transport, NPC1 and ORPs are likely to have a fundamental and conserved role in sterol transport.

I propose that an NPC1–ORP interaction could activate ORPs and facilitate extraction of lipids and/or the ensuing dissociation of ORPs from endosomal membranes (Figure I). An important future direction would be to investigate the functional and physical interactions between NPC1 and ORPs. It should be pointed out that the hydrophobic cavity of Osh4p is rather large and that parts of the cholesterol binding site are not well conserved in all ORPs [34]. Therefore, the ORP proteins might bind different lipid species. For instance, ORPs could extract membrane sterols with the help of NPC1, but it is also possible that they extract and transport sphingolipids directly but that sterols are released from endosomal membranes as by-products [27,60].

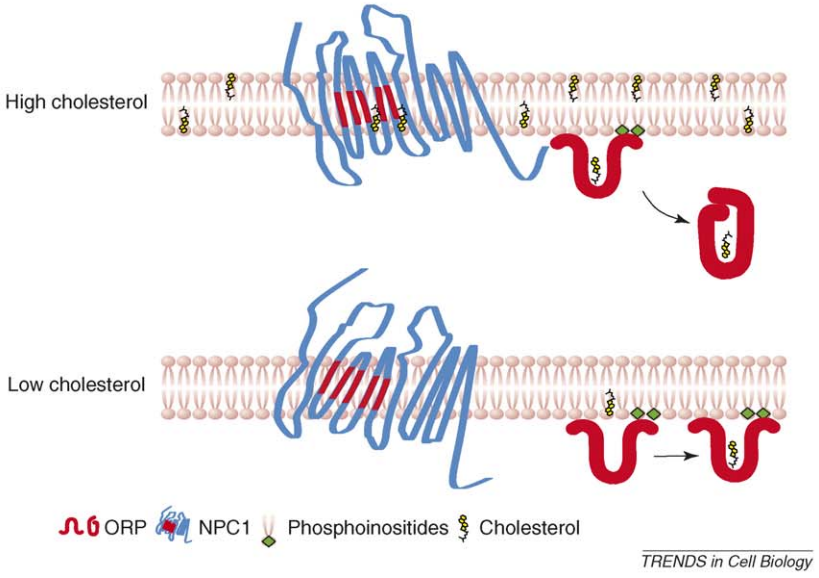


**Figure I**. NPC1 might be a membrane sterol sensor that regulates ORPs-mediated, nonvesicular sterol transport. NPC1 senses membrane sterol levels through its sterol sensing domain and changes its conformation. At high sterol level, NPC1 adopts a conformation that enables it to interact with cytosolic lipid carriers, such as ORPs, thereby facilitating sterol extraction by ORPs and/or the subsequent membrane dissociation of the ORPs. The putative sterol sensing domain of NPC1 is in red.

than 10 minutes [27,41]. The maintenance of a high concentration of PM sterols is believed to result from the affinity of sterols for an equally high amount of PM sphingolipids, and metabolic energy is not required for maintaining a concentration gradient of sterol between the ER and the PM [27,41,42]. Therefore, the ATP requirement for this otherwise spontaneous reaction might be for regulatory or membrane-targeting purposes. The best characterized nonvesicular lipid transport process, CERT-mediated ceramide transport (as described above), requires ATP [13]. Given the structural and functional similarities between CERT and ORPs, an ATP-dependent nonvesicular mechanism might also apply to ORPs. ATP could be required to synthesize phosphoinositides, as CERT and most ORPs tested to date can bind to phosphoinositides. Furthermore, additional levels of control could be exerted over the sterol transport process through phosphorylation of ORPs and CERT, a process that also consumes ATP ([30] and M. Nishijima and H. Yang, unpublished). Finally, the association of an AAA ATPase with yeast ORPs suggests a general ATP-dependent recycling mechanism for lipid carriers during ceramide and sterol transport (Box 2).

## Concluding remarks and future directions

The sorting and transport of intracellular sterols has been a recalcitrant problem. In the past few years, exciting molecules and mechanisms have been discovered in the broad area of intracellular lipid transport. The identification of CERT and ORP proteins as potential lipid transport carriers represents a major breakthrough that will open new research opportunities to identify other transport components and to understand the targeting and regulation of these transporters. The interaction between AAA ATPases and ORPs suggests a novel mode of regulation for this important family of intracellular trafficking pathways. However, many questions remain to be answered. Both CERT and some ORPs are phosphorylated, but the physiological significance of the phosphorylation remains to be elucidated. Likewise, little is known about the functional implications of the interaction between members of the AAA ATPases and Osh proteins. In addition, NPC1 is a key regulator of intracellular sterol transport but its precise role in vesicular or nonvesicular sterol transport remains to be determined. In light of recent progress made on OSBP and ORPs, the relationship between NPC1 and ORPs deserves closer study (Box 3).

Recently, yeast genetics has contributed to the identification of candidate sterol transporters [18,35]. It is therefore time to develop reliable *in vitro* assays with isolated cell membranes as donors and acceptors to measure nonvesicular sterol transport. If successful, such transport assays would allow us to further examine the role of ORPs, AAA ATPases and NPC1 in sterol transport, to isolate new components and to dissect the functional relationship between various transport partners. Ultimately, it is hoped that intermembrane sterol transport can be reconstituted *in vitro* with defined components.

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