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Specificity of lipid transfer proteins: An *in vitro* story

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ABSTRACT

Lipids, which are highly diverse, are finely distributed between organelle membranes and the plasma membrane (PM) of eukaryotic cells. As a result, each compartment has its own lipid composition and molecular identity, which is essential for the functional fate of many proteins. This distribution of lipids depends on two main processes: lipid synthesis, which takes place in different subcellular regions, and the transfer of these lipids between and across membranes. This review will discuss the proteins that carry lipids throughout the cytosol, called LTPs (Lipid Transfer Proteins). More than the modes of action or biological roles of these proteins, we will focus on the *in vitro* strategies employed during the last 60 years to address a critical question: What are the lipid ligands of these LTPs? We will describe the extent to which these strategies, combined with structural data and investigations in cells, have made it possible to discover proteins, namely ORPs, Sec14, PITPs, STARDs, Ups/PRELI, START-like, SMP-domain containing proteins, and bridge-like LTPs, which compose some of the main eukaryotic LTP families, and their lipid ligands. We will see how these approaches have played a central role in cell biology, showing that LTPs can connect distant metabolic branches, modulate the composition of cell membranes, and even create new subcellular compartments.

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1. Introduction

The architecture of a eukaryotic cell is primarily defined by the plasma membrane (PM), which serves as an efficient barrier to

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extracellular medium and membranes that delimit organelles. These membranes are the result of the intricate assembly of lipids, along with proteins. Lipids are extremely diverse, with thousands of subspecies identified to date, categorized into three major classes: glycerophospholipids (GPLs), sphingolipids, and sterols. What must be appreciated is the precision and specificity with which all these lipids are distributed in the cell. This precise distribution ensures that the plasma and organelle membranes have a specific thickness and fluidity, a given surface charge density, and host specific lipids with signaling roles [1]. This gives each organelle membrane a precise molecular identity critical for many cellular processes, including vesicular trafficking, signal transduction, ion exchange, cellular movement, and respiratory function.

At any time, mechanisms are in action to create and maintain this distribution of lipids in the face of constant events that randomize such distribution (e.g., membrane remodeling and exchanges through vesicular trafficking and lipid consumption in signaling pathways). Metabolic pathways ensure lipid synthesis, interconversion, and degradation, while other processes move lipids between and across cell membranes. Whereas metabolic processes are well-defined today, comparatively little is known about how lipids are transported inside the cell.

The distribution of many lipids or lipid precursors among subcellular membranes is quite distinct from that of the synthetic machinery. Notably, many lipids are synthesized in the ER and found concentrated elsewhere in the cell [1]. Therefore, they must move across the cytosol to reach peripheral compartments such as the Golgi apparatus, the PM, or the mitochondria. That is why, early on, it has been suspected that transport routes may be necessary for distributing lipids in the cells [2]. Given the elusive nature of intracellular lipid flow, *in vitro* approaches have been critical in the last 60 years to understand the molecular basis of lipid transport in controlled systems [3]. Today, it is well documented that specialized lipid traffic processes occur independently from membrane-delimited transport vesicles that convey lipids in bulk and cargo proteins between the ER, Golgi, PM, and the endo/lysosomal compartment [4]. This non-vesicular transfer of lipids inside eukaryotic cells is guaranteed by specialized proteins that fall into two main classes: “box-like” and “bridge-like” lipid transfer proteins.

The first category encompasses proteins that comprise a globular domain (210–450 amino acids) with a lipid-binding pocket whose access is usually controlled by a molecular lid [3,5]. These LTPs belong to different families (ORP, Sec14, PITP, START, etc.) [6] and show great structural diversity. Still, they are thought to function similarly by cycling between membranes to transport lipids. During a cycle, an LTP molecule docks to a first membrane and extracts a lipid ligand. The lipid, shielded from the aqueous medium in the LTP's pocket, is delivered into a second membrane. Then, the LTP returns to the first membrane to pick up another lipid and starts a new cycle. Many examples suggest that most LTPs carry out these activities at specific cellular nexuses, which are membrane contact sites (MCSs). These correspond to zones of proximity (10–30 nm) between the ER and the PM or other organelles (e.g., Golgi apparatus, mitochondrion) [3,7–9]. LTPs working in these cellular “short-cuts” are quite sophisticated as they comprise accessory domains to tether organelle membranes, in addition to a lipid transfer module [3,7–9].

The second main category of LTPs is composed of giant proteins (between 1500 and 5000 amino acids). They have been described recently as forming long rod-shaped structures made up of a repetition of a structural pattern named RBG (Repeated β -Groove) and traversed by a long hydrophobic conduit. They also contain additional domains to interact with proteins and membranes. These proteins are all localized at the level of MCSs and are widely

suspected to allow for massive transport of lipids between membranes [10].

As with many proteins, a key step towards defining the function of LTPs is to identify their ligand(s). Here, we review the *in vitro* strategies employed over the last 60 years to identify LTPs, their lipid ligands, and their mode of action. We describe the pros and cons of these strategies and their possible pitfalls. We also illustrate how these approaches have been combined with structural analyses and cellular observations to define how the selectivity of LTPs imparts specialized cellular functions to these proteins.

2. Oxysterol-binding protein-related proteins (ORPs): more than sterol transfer

Oxysterols constitute a family of oxygenated derivatives of cholesterol, which are known to play different cellular functions and, notably, to control intracellular cholesterol levels *via* both transcriptional and post-transcriptional mechanisms [11]. Back in the '70s, very little was known about their modes of action. This led to the identification, purification, and cloning of a cytosolic protein called OSBP (Fig. 1), which could bind 25-hydroxycholesterol (25-HC) with high affinity but not cholesterol [12–15]. In 1992, Ridgway and co-workers identified the C-terminal half of OSBP as the oxysterol-binding domain [16] and observed this protein at the Golgi surface. Later, OSBP was found to recognize PI(4)P and the small GTPase Arf1, two Golgi markers, *via* an N-terminal Pleckstrin-Homology (PH) domain [17–19] and to bind to the ER-resident VAP protein *via* a FFAT motif [20,21]. Eleven proteins in humans, called ORPs (OSBP-related domain-containing proteins), and seven proteins in *S. cerevisiae*, called Osh (Oxysterol-binding protein homologs), were identified to have a lipid-binding module similar to that of OSBP by sequence homology search [22,23]. Most contain extra domains and motifs to target the ER and other organelles, as found with OSBP.

For different reasons, it was progressively considered that OSBP did not play a role in sterol metabolism but in its intracellular distribution. In eukaryotic cells, sterols are scarce in the ER membrane but account for up to 40% of lipids in the PM [24]. Because the final steps of sterol synthesis occur at the ER, it was postulated that sterol fluxes, using mostly non-vesicular routes, could be responsible for building this asymmetric sterol distribution [25]. This prompted the search for proteins specialized in intermembrane sterol transfer and, logically, led to focus on a sterol-binding protein like OSBP. Moreover, the dual ability of OSBP to bind the ER and *trans*-Golgi membrane suggested that it may populate ER-Golgi contact sites, increasingly considered, like other contact sites, to be a hot spot for lipid transfer [26]. Other candidates were Osh proteins, which were found to be collectively important for ergosterol metabolism and distribution in yeast [23,27]. This assumption was supported by the work of Hurley's group, which, in 2005, revealed the structure of Osh4, loaded with ergosterol (the principal sterol in yeast, Figs. 1 and 2A). This protein, which only consists of an ORD, features a β -barrel with an internal pocket closed by a short molecular lid [28]. The sterol contacts the pocket wall and the inner side of the lid, stabilizing the closed conformation of Osh4, and its 3-hydroxyl group contacts the polar residues at the bottom of the pocket. Importantly, binding tests using solubilized radiolabeled compounds showed that Osh4 had almost the same affinities for cholesterol and 25-HC (Table 1) [28]. Because oxysterols are rare in eukaryotic cells, this designated cholesterol/ergosterol as the physiological ligands of ORPs/Osh proteins. Therefore, whether Osh4 and OSBP could transfer sterol was promptly examined. This was done first by measuring the transfer of radiolabeled [3 H] or [14 C] cholesterol from donor to acceptor liposomes (Table 2). One of these liposome populations contained

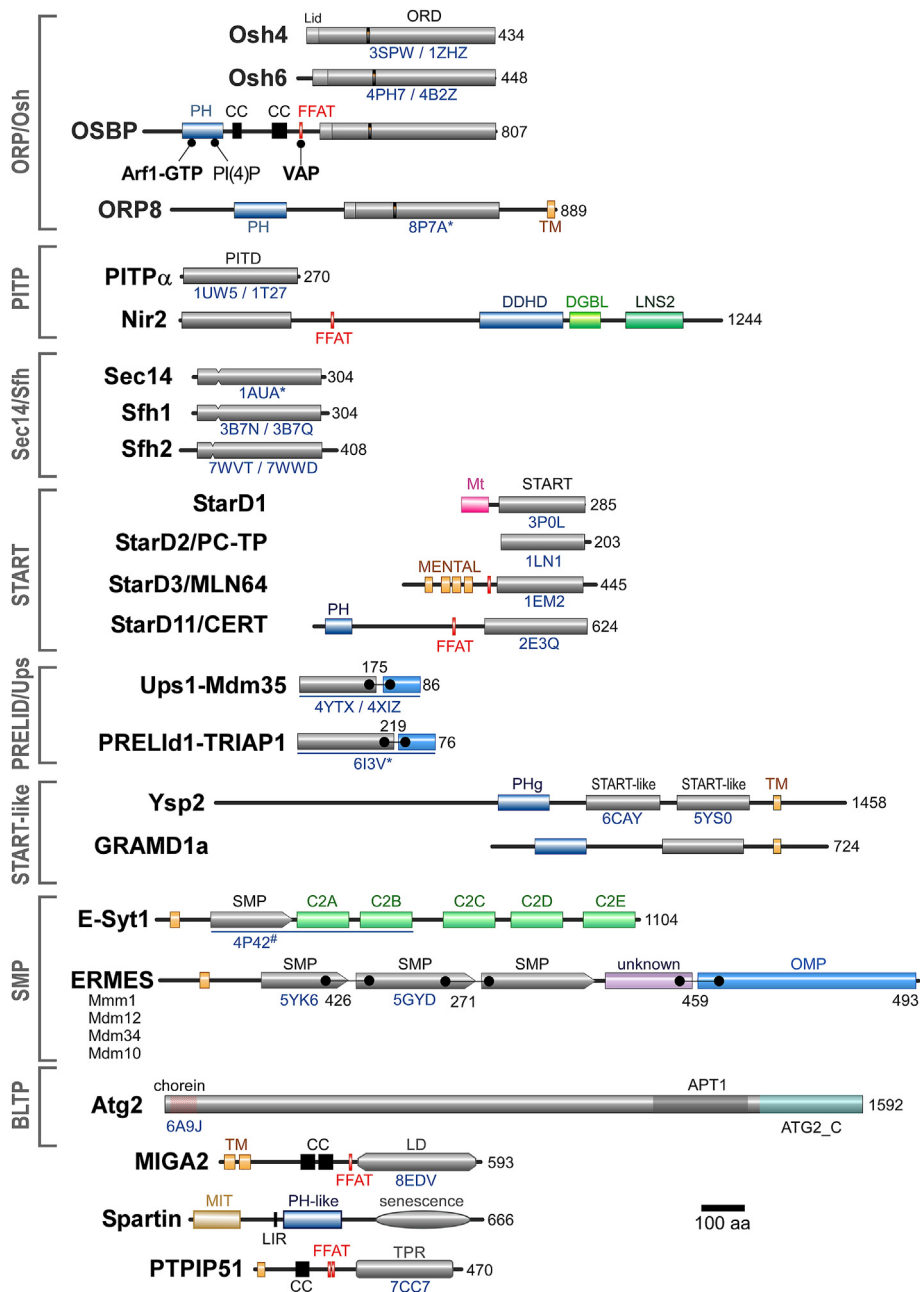


Fig. 1. Domain organization of main eukaryotic LTPs. The sequence length (in amino acids) of each LTP or LTP subunit, is indicated in black on the right or below right of each of them. A black line indicates some protein-protein and lipid-protein interaction. The 4-character alphanumeric PDB identifier (PDB ID) of specific lipid-binding transfer modules or a certain protein region is indicated in dark blue. An asterisk indicates that the structure corresponds to an apo form. #The PDB ID given for E-Syt1 corresponds to the structure of the SMP domain of E-Syt1 in tandem with C2A and C2B domains. CC: coiled-coil, DGBL: DAG-binding-like domain, FFAT: two phenylalanines in an acidic tract, LNS2: Lipin/Nde1/Smp2, MIT: microtubule-interacting and trafficking, Mt: mitochondrial import sequence, OMP: outer membrane protein, ORD: OSBP-related domain, PH: pleckstrin homology, PHg: PH-GRAM, PITD: phosphatidylinositol-transfer domain, SMP: synaptotagmin-like mitochondrial-lipid-binding protein, START: StAR-related lipid-transfer, TM: transmembrane, TPR: tetratricopeptide repeat.

lactosylceramide that could be aggregated using lectin and separated from the other one by centrifugation. Alternatively, one population was loaded with sucrose ("heavy" liposome) and separated from the second one ("light" liposome) by centrifugation. Liposomes were also doped with a specific non-transferable fluorescent lipid to evaluate how well they were recovered and separated from each other. A second type of assay was used, based on dehydroergosterol (DHE), a naturally fluorescent sterol that only differs from ergosterol by the presence of an additional double bond that forms a conjugated triene system. Donor liposomes

containing DHE and a second lipid, dansylphosphatidylethanolamine (DNS-PE), were mixed with acceptor liposomes containing neither of these two lipids. DNS-PE emits fluorescence upon DHE excitation due to energy transfer (FRET) between these lipids, which are, on average, close to each other in the liposome membrane; DNS-PE fluorescence decreases upon DHE transfer to acceptor liposomes (Table 2, DHE can also be incorporated in donor liposomes, and DNS-PE in acceptor liposomes; in this case, DHE transfer results in an increase in FRET). Interestingly, lipid transfer was measured without liposome

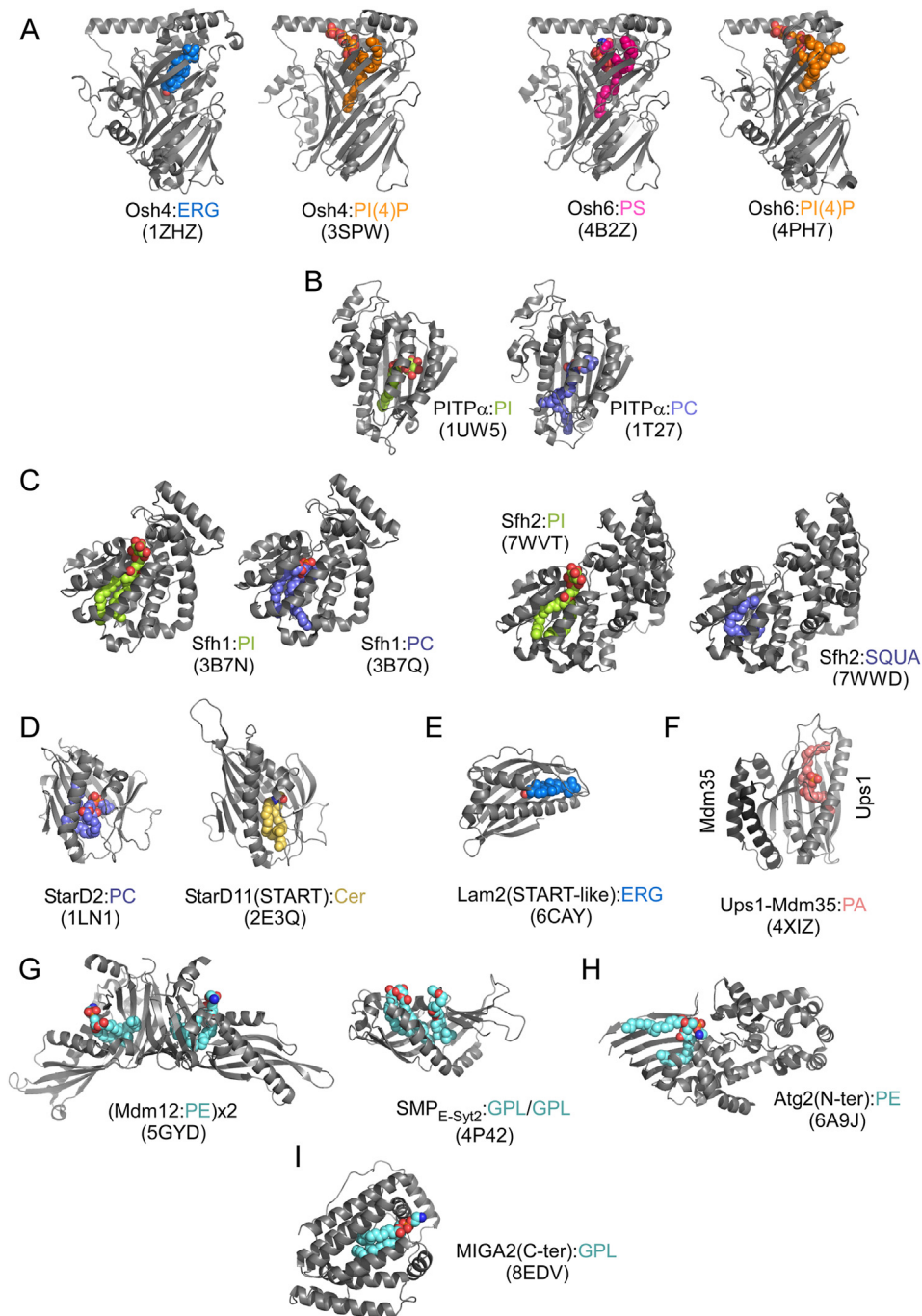


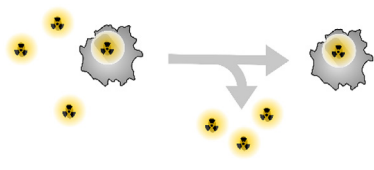
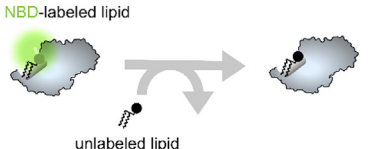

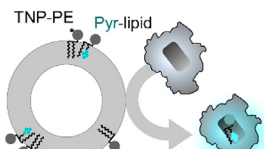
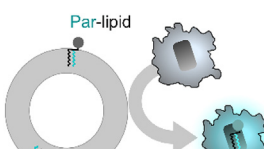
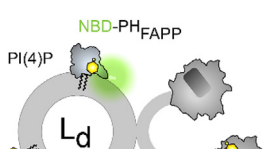
Fig. 2. Structure of protein-lipid or domain-lipid complexes representative of various LTP families. (A) ORP/Osh family: structure of *Saccharomyces cerevisiae* Osh4 and Osh6 proteins, which only consist of an ORD, in 1:1 complex with ergosterol and (16:0/18:1)-PS, respectively, or (18:0/20:4)-PI(4)P. The pocket is closed by an N-terminal lid. (B) PITP family: structure of human PITP α bound to (18:0/20:4)-PI and rat PITP α bound to (18:1/18:1)-PC. (C) Sec14 family: structure of *S.cerevisiae* Sfh1 and Sfh2 proteins bound to (18:0/18:1)-PI or their secondary ligand, either PC or squalene. (D) START family: structure of human PC-TP/StarD2 bound to a (18:2/18:2)-PC molecule and one structure of the START domain of human CERT/StarD11 bound to 18:0 ceramide. (E) Structure of the first START-like domain of the yeast protein Lam2 bound to one ergosterol molecule. (F) *S.cerevisiae* Ups1-Mdm35 heterodimer bound to one molecule of (16:0/16:0)-PA. (G) SMP-domain containing LTPs. ERMES subunit Mdm12 has been crystallized as a dimer, with each monomer bound to a PE molecule. E-Syt2 (human) also has an SMP domain (161–342) into which two GPL molecules are hosted. For clarity, the C2 domains have been removed from the structure. The head group of GPLs is not resolved in the structure. (H) Structure of the 21–240 region of *Schizosaccharomyces pombe* Atg2 bound to one molecule of PE. (I) Structure of the 220–496 region of *Caenorhabditis elegans* MIGA2 in 1:1 complex with PE. Protein's structures are represented as a ribbon mode with the lipid ligand in sphere mode (with carbon atoms in different colors according to the ligand, oxygen atoms in red, nitrogen atoms in blue, and phosphorus atoms in orange). The PDB ID corresponding to each structure is indicated in brackets.

separation, offering excellent time resolution. Osh4 and OSBP were found to transfer sterol using such assays [29,30], suggesting that all ORPs were sterol transporters. However, Osh4 moved sterol slowly [29], and other Osh, except Osh2 and Osh5, had no transfer

activity [31]. Furthermore, it was unclear whether these proteins could substantially transfer sterols into yeast cells, particularly at the ER/PM interface [31,32].

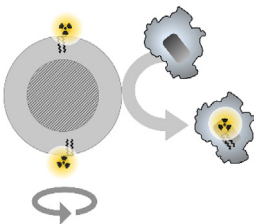
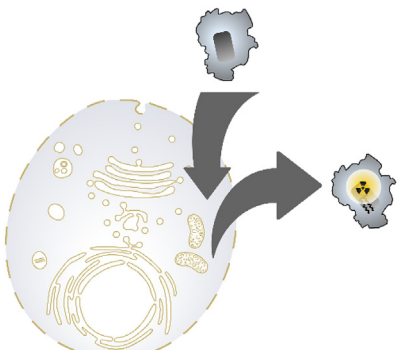
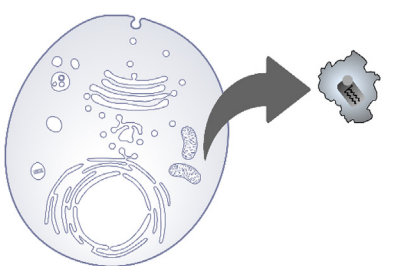

Puzzlingly, Osh4 was reported to decrease the availability of a

Table 1
In vitro approaches to characterize the ligand(s) of LTPs.

Experimental setup	Measurement method	LTPs	Ligand(s)	Reference
Solubilized ligand 	Separation by HiTrap Q resin + scintillation counting	Osh4	[³ H]CHOL	[28]
	Separation by dextran-charcoal method + Scintillation counting	OSBP, ORP9	[³ H]CHOL, [³ H]25-HC	[30, 253]
	Separation by affinity chromatography + Scintillation counting	StarD3 (START), StarD1	[¹⁴ C]CHOL	[141]
	ESR	PC-TP/StarD2 StarD10	5:0 (4 Doxyl)-PC, not 5:0 (4 Doxyl)-PE 5:0 (4 Doxyl)-PC and 5:0 (4 Doxyl)-PE	[155] [155]
	CD-TSA	StarD5, StarD6	Bile acids (StarD5) Pregnenolone, Testosterone (StarD6)	[145-147]
Displacement assays with NBD-labeled lipid 	Fluorescence	Sec14	NBD (12)-PC	[109]
	Non-denaturing gel	ERMES (Mdm12)	Preloaded with NBD-PE (displaced by PC, PG, PE, not by PA, PS, CL)	[189, 190]
	NBD fluorescence	ERMES (Mmm1)	Preloaded with NBD-PE, displaced by PG, PS, PS, PC, weakly by PE, not by CHOL, ERG, CER	[191]
		Mmm1/Mdm12	Preloaded with NBD-PE, displaced by PA, PG, PS, PC, weakly by PE	[191]
		E-Syt2 (SMP)	Preloaded with NBD-PE (displaced by PC, not by SM, CER, CHO)	[197]
		MIGA2	Preloaded with NBD-PC, displaced by PA, PS, PC, PE, not by CHOL	[225]
		MIGA2	Preloaded with NBD-PC, displaced by PC, PA, TAG, FA	[226]
		VPS13	NBD-PS = NBD-PA > NBD-CER > NBD-PE, not NBD-CHOL	[217]
Intrinsic LTP fluorescence → Fluorescent lipid ligand 	Trp/DHE pair (Trp signal)	Osh4, Osh3 (ORD), ORP3(ORD), Lam4S2	Osh4 binds DHE Neither Osh3 nor ORP3 binds DHE	[36, 49, 113, 254]
	Trp/NBD (12)-PS pair (Trp signal)	Osh6	DHE NBD (12)-PS vs various PS and PI(4)P subspecies	[164] [60]
Pyrene-GPL/TNP-PE (FRET) and other FRET-based approaches 	Pyrene fluorescence	Sec14	Pyr (10)-PI > Pyr (10)-PC	[102, 110]
		PITPα	Pyr (8)-PI = Pyr (10)-PI = PI > Pyr (10)-PC = Pyr (8)-PC = PC	[70]
		PITPα	Pyr (10)-PC vs various PC and ParPC	[71]
		PITPα	Pyr (10)-PI > Pyr (10)-PC > Pyr (10)-PA	[92]
		RdgBβ	Pyr (10)-PA > Pyr (10)-PI > Pyr (10)-PC	[92]
		PC-TP/StarD2	Pyr (8)-PC vs various PC and ParPC subspecies	[71]
Parinaroyl-GPL 	NBD-labeled lipids/Rhod-PE DHE (or CTL)/DNS-PE (FRET)	StarD10 OSBP, ORP9	NBD (12)-PC and NBD (12)-PE DHE and CTL	[155] [247]
	Parinaroyl fluorescence	PITPα	parPI > parPC	[68, 69]
Extraction assays with NBD-labeled lipid sensors 	Parinaroyl fluorescence			
	NBD fluorescence	Osh4, Osh6, Osh7	PI(4)P	[50, 53, 255]
	Osh6, Osh7 ORP8 (ORD)	PS PI(4)P, PI(4,5)P ₂		[50, 255] [53]

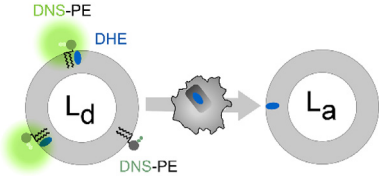
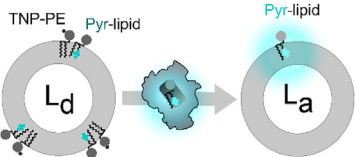
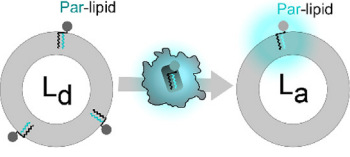
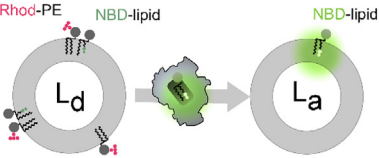
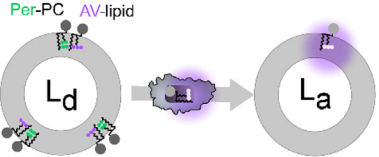
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Table 1 (continued)

Experimental setup	Measurement method	LTPs	Ligand(s)	Reference	
	Separation by centrifugation + scintillation counting	OSBP, ORP9L	[³ H]CHOL	[30]	
	Aggregation by lectin, centrifugation, scintillation counting	ORP9	[³² P]PI(4)P	[247]	
	Separation by centrifugation	Osh4	[¹⁴ C]CHOL	[29, 31]	
	Separation by centrifugation + scintillation counting	Osh4	[³² P]PI(4)P and [³ H]CHO, not [³ H]PI(4,5)P ₂ and [³ H]PI	[36]	
	Separation by centrifugation + HPTLC	Osh6	PS, PG, not PE, PI, CL, ERG	[47]	
	Separation by centrifugation + TSA	Osh6	PS from total yeast extract	[60]	
	Separation by filtration + TLC	StarD7	PC and PE from total lipid extract (HEPA-1 cell)	[154]	
	Separation by centrifugation	CERT/StarD11	[¹⁴ C]CER, [¹⁴ C]SM, [¹⁴ C]sphingosine, [¹⁴ C]PC, [¹⁴ C]CHOL	[161]	
	Separation by centrifugation + TLC	CERT	[¹⁴ C]CER (from ER lipid extract)	[161]	
	Separation by centrifugation + SEC	Ups1	NBD (12)-PA > NBD (12)-PS	[181]	
	Separation by affinity chromatography + HPTLC	Mdm12, Mmm1	PC, PI (from yeast extract)	[189]	
	Separation by centrifugation + Fluorescence	Mmm1	NBD-PE (displaced by PC, PS, PE, PA)	[192]	
	TLC	Lam452, Sfh3	CHOL (from [¹⁴ C] lipids in HL60)	[164]	
	TLC + phosphorimaging	RdgBβ, Dm-RdgBβ(PITD), Nir2 (PITD), PITPα	PC, PI, PA (from [¹⁴ C] lipids in HL60)	[92]	
	Direct infusion ESI MS/MS	RdgBβ	PA and PI from HL60 (only PI for PITPα)	[92]	
	TLC + phosphorimaging	PITPα	PI, CHOL (Sfh3)	[127]	
	TLC + phosphorimaging	Sfh3, Sec14, PITPα	PI, PC (Sec14, PITPα) (from [¹⁴ C] lipids in HL60)	[127]	
	TLC + phosphorimaging	Sfh3, Sfh4	ERG, LAN (from [¹⁴ C] lipids in HL60)	[128]	
		Lipid analysis by LC/MS	TMEM24 (SMP) expressed in Expi293 cells	PC = PI > PE > PS	[204]
		Non denaturing MS	E-Syt2 (SMP) expressed in Expi293 cells	PC = PE > PI > PS	[197]
Lipid analysis by LC/MS		ORP8 (ORD)	PS, PI(4)P	[55]	
Denaturing/Non denaturing MS		ORP10(ORD)	PI(4)P	[56]	
Lipid analysis by LC/MS		Osh4, Osh6	PS (Osh6, Osh7)	[47]	
Lipid analysis by TLC and LC/MS/MS		Sec14, Sfh1-5	ERG (Osh4)	[47]	
HPTLC		ERMES (Mdm12) expressed in yeast	PI (Sec14, Sfh1, Sfh2, Sfh4, Sfh5)	[189]	
ESI-MS		ATG2, VPS13 expressed in Expi293 cells	PC (60%), PI (23%), PE (10.5%), and a small amount of PS (3%)	[217, 218]	
Lipid analysis by LC/MS		ATG2, VPS13 expressed in Expi293 cells	PC ≫ PE > PI > PS (VPS13)	[217, 218]	
Lipid analysis by LC/MS		ATG2, VPS13 expressed in Expi293 cells	PC ≫ PE > PI > PS (ATG2A)	[217, 218]	
	Lipid analysis by LC/LC/MS	Spartin expressed in Expi293 cells	TAG > DAG > sterol ester > GPLs (PC > PE > PS > PI) > SM	[228]	
	Lipid analysis by LC/LC/MS	MIGA2 expressed in Expi293 cells	FA = GPL (PC > PE > PI > PS), TAG	[226]	
	Lipid analysis by LC/MS/MS	PDZD8 expressed in COS7 cells	Lyso-PC, PC, PS, PA, CER, SM	[207]	

CD: circular dichroism, CER: ceramide, CHOL: cholesterol, CL: cardiolipin, CTL: cholestatrienol, DAG: diacylglycerol, DHE: dehydroergosterol, DNS: dansyl, ERG: ergosterol, ESI-MS: electrospray ionisation mass spectrometry, ESR: electron spin resonance, FA: fatty acid, FCCS: fluorescence cross-correlation spectroscopy, FRET: fluorescence resonance energy transfer, GPLs: glycerophospholipids, HPLC: high-performance liquid chromatography, HPTLC: high-performance thin layer chromatography, L_d: acceptor liposome, L_d: donor liposome, LAN: lanosterol, Mic: microsome, Mito: mitochondrion, MS: mass spectrometry, NBD: nitrobenzoxadiazole, PA: phosphatidic acid, Par: parinaroyl, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PI: phosphatidylinositol, PI(4)P: phosphatidylinositol 4-phosphate, PI(4,5)P₂: phosphatidylinositol 4,5-bisphosphate, PIP: phosphoinositide, PS: phosphatidylserine, Pyr: pyrene, Rhod: rhodamine, SEC: size exclusion chromatography, SM: sphingomyelin, SQUA: squalene, TAG: triacylglycerol, TLC: thin layer chromatography, TNP: N-trinitrophenyl, TRP: tryptophan, TSA: thermal shift assay.

Table 2
In vitro approaches for measuring the activity of intracellular LTPs.

Experimental setup	Measurement method	LTP	Transfer activity	Reference
Naturally fluorescent sterol/Fluorescent PE (FRET)	DHE/DNS-PE pair DNS fluorescence	Osh4	DHE ^a	[29, 31, 36, 113, 122, 167]
		OSBP StarD3 (FFAT + START domain) StarD4	DHE ^{a,#} DHE ^{a,#}	[43, 44] [143]
		Ysp2, Lam4 (START-like domain) GRAMD1a-c (START-like domain) PDZD8 (SMP)	DHE ^{a,#} DHE ^{a,#}	[144, 168, 219] [167-169]
	DHE/TNP-PE pair CTL/TNP-PE pair Pyrene fluorescence	Sec14 Sfh1-5 PITP α Sec14 Sfh3 StarD10 PC-TP/StarD2	No DHE transfer ^a Only Sfh3 transfers DHE and CTL Pyr (8)-PI, Pyr (8)-PC, Pyr (10)-PI, Pyr (10)-PC ^a Sec14 transfers Pyr (10)-PC and Pyr (10)-PI, Sfh3 transfers Pyr (10)-PI Pyr (10)-PC, only StarD10 transports Pyr (10)-PE	[206] [122] [70] [102, 122] [155]
Pyrene-GPL/TNP-PE (FRET)				
				
Parinaroyl -GPL	Parinaroyl dequenching	PITP α	parPC, parPI, not parPE, parPG	[68, 69]
				
NBD lipid/Rhod-PE (FRET)	NBD fluorescence	StarD7	NBD (12)-PC, not NBD (12)-PE, NBD (12)-PS and NBD (12)-SM	[154]
		Ups1, PRELID1 Ups2, PRELI3a MIGA2	NBD (12)-PA > NBD (12)-PS NBD (12)-PS = NBD (12)-PA NBD (12)-PS, no transfer of NBD (12)-PA, NBD (12)-PC, NBD-PE, NBD (12)-CER, NBD-CHOL	[181, 185] [183] [225]
		Osh6 MIGA2	NBD (12)-PS, no transfer of NBD (12)-PA, NBD (12)-PC, NBD-PE, NBD (12)-CER, NBD-CHOL NBD (12)-PS, NBD (12)-PA NBD (12)-PE, NBD (12)-PC ^c	[225] [226]
		Spartin	NBD (6)-PC > NBD (6)-PE, no transfer of NBD (6)-PS	[228]
		PTPIP51 ERMES (Mmm1-Mdm12) E-Syt1	NBD (12)-PA NBD-PS > NBD-PE NBD-PE ^c	[231] [192] [199]
		PDZD8 (SMP) PDZD8 (SMP) ATG2, VPS13	NBD labeled PA, not CER and PE NBD labeled PC, Rhod-PE, NBD _{acyl} -CER ^c NBD-PE, NBD-PS ^c	[206] [207] [212, 216-218, 222]
Anthrylvinyl labeled lipid/Perylenoyl labeled lipid (FRET)	AV fluorescence	GLTP CPTP FAPP2	Glycolipid CER-1-phosphate, not CER, GPLs, SM, Galactoceramide Glycolipid	[236] [235] [237]
				
NBD-labeled lipid sensor/Fluorescent-PE (FRET)	NBD/Rhod-PE pair (NBD fluorescence)	Osh4 Osh6 ORP8 (ORD) OSBP ORP10 (ORD) GRAMD1a-c & Ysp2 (START-like domain)	PI(4)P ^a , no PS transfer PS ^a , PI(4)P ^a , PI(4,5)P ₂ ^{a,b} PS ^a , PI(4)P ^a , PI(4,5)P ₂ ^{a,b} PI(4)P ^a PS ^a , PI(4)P Not PS ^a , PI(4)P ^a , PI(4,5)P ₂ ^a except GRAMD1b ^b	[39, 50, 113] [50, 53, 60] [53, 56, 60] [43, 44] [56] [167]

(continued on next page)

Table 2 (continued)

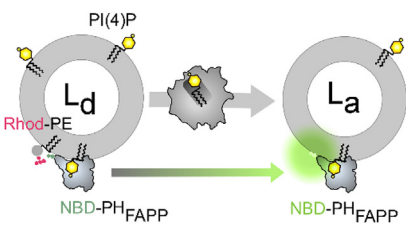
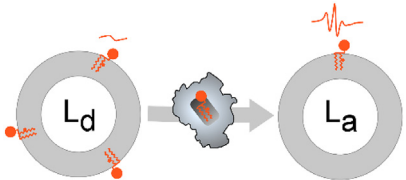
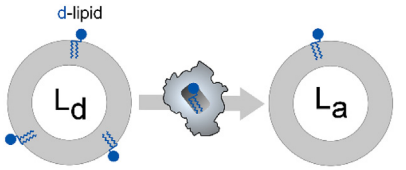
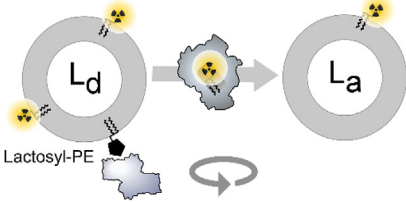
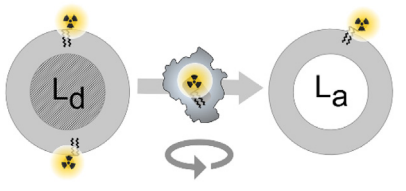
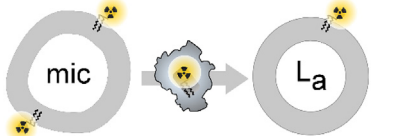
Experimental setup	Measurement method	LTP	Transfer activity	Reference
	CFP/ATTO488-PE pair CFP/DID FCCS	PDZD8 Osh6 Osh6	PI(4,5)P ₂ ^a , various PIPs ^a , PS ^a PS PI(4)P	[206] [61] [61]
Spin labeled-GPL	ESR	PC-TP/StarD2	16:0 (12 Doxyl)-PC, not 16:0 (12 Doxyl)-PE	[149]
	SANS	Sec14	d31-PC ^a	[110]
	Scintillation counting	Osh4 OSBP, ORP9L PITP α CERT/StarD11 Ups1	[¹⁴ C]CHOL [³ H]CHOL [³ H]PI, [³ H]PC [¹⁴ C]CER [¹⁴ C]PA, not [¹⁴ C]PC (from L _d liposome with complex lipid composition)	[29] [30] [66] [161] [179]
	Scintillation counting	Osh1-3 (ORD), Osh4-7 Osh6 ORP8 (ORD) GRAMD1a-c & Ysp2 (START-like domain) Ups1 Ups2, PRELID3b/SLMO2	[¹⁴ C]CHOL only with Osh2 (ORD), Osh4 and Osh5 PS, PG, not PE and ERG PS, PI(4)P [³ H]CHOL, not [¹⁴ C]PE PA PS, PA ^b	[31] [47] [55] [167] [179] [182]
	HPLC Scintillation counting MS Scintillation counting HPLC Scintillation counting NBD fluorescence	E-Syt1 TMEM24 TMEM24 MIGA2	[³ H]DAG transfer ^c PI but no PS transfer ^c [³ H]PI but not [¹⁴ C]-PE transfer ^c NBD (12)-PS > NBD (12)-PE > NBD (12)-PA > NBD (12)-PC > NBD (12)-PS ^c	[199] [204] [204] [226]
	Scintillation counting	PITP α RdgB β , Nir2 (PITD) Sec14, Sfh1-5	[³ H]PI, [¹⁴ C]PC, [³ H]PC [³ H]PI, not [³ H]PC [³ H]PI (only Sec14, Sfh2-4) [¹⁴ C]PC (only Sec14)	[65, 92] [92] [104, 106, 114, 137]

Table 2 (continued)

Experimental setup	Measurement method	LTP	Transfer activity	Reference
Liposome → Mitochondrion	TLC Scintillation counting	Sec14 Sec14, Sfh1-5 Sfh3, Sfh4 StarD1	[³ H]PC, [³ H]PI (from labeled total yeast extract) Only Sfh2 transfers [³ H]SQUA [³ H]ERG, [¹⁴ C]ERG, [¹⁴ C]LAN [¹⁴ C]CHOL	[101] [122] [122, 128] [138]
Micosome → Mitochondrion	TLC, scintillation counting	Supernatant	[³² P]PC > [³² P]PE	[148]
Permeabilized cells → Liposome	Centrifugation + scintillation counting	PITPα, Dm-RdgBα (PITD), RdgBβ	[³ H]PI and [³ H]PC (PITPα), [³ H]PI (Dm-RdgBα)	[92]

CER: ceramide, CHOL: cholesterol, CTL: cholestatrienol, DAG: diacylglycerol, DHE: dehydroergosterol, DNS: dansyl, ERG: ergosterol, ESR: electron spin resonance, FA: fatty acid, FCCS: fluorescence cross-correlation spectroscopy, FRET: fluorescence resonance energy transfer, GPLs: glycerophospholipids, HPLC: high-performance liquid chromatography, L_d: acceptor liposome, L_d: donor liposome, LAN: lanosterol, Mic: microsome, Mito: mitochondrion, MS: mass spectrometry, NBD: nitrobenzoxadiazole, PA: phosphatidic acid, Par: parinaroyl, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PI: phosphatidylinositol, PI(4)P: phosphatidylinositol 4-phosphate, PI(4,5)P₂: phosphatidylinositol 4,5-bisphosphate, PIP: phosphoinositide, PS: phosphatidylserine, Pyr: pyrene, Rhod: rhodamine, SANS: small angle neutron scattering, SM: sphingomyelin, SQUA: squalene, TAG: triacylglycerol, TLC: thin layer chromatography, TNP: N-trinitrophenyl.

^a Available transfer rates.

^b Possible fortuitous lipid ligand.

^c Test of the impact of membrane tethering on lipid transfer rates.

lipid called phosphatidylinositol 4-phosphate (PI(4)P) at the Golgi [33] and to downregulate exocytosis [33,34], a process dependent on PI(4)P. Functional interplays were also unveiled between other Osh proteins and PI(4)P metabolism [35]. When we started working on Osh4, we spent weeks measuring only slow DHE transfers between membranes; changing liposome fluidity or surface charge density offered only marginal gains. One day, by mixing Osh4 with DHE-doped liposomes and PI(4)P-doped liposomes, we measured a high-speed DHE transfer. Next, we found that PI(4)P competes with sterol to occupy Osh4's binding pocket, via an assay in which energy transfer was measured between tryptophan residues around or lying in the binding pocket and DHE (Table 1). We also established the ability of Osh4 to extract PI(4)P by incubating the protein with sucrose-loaded liposomes doped with [³²P]PI(4)P and measuring the associated radioactivity in the supernatant once the liposomes were sedimented by centrifugation (Table 1). These data suggested that Osh4 could capture PI(4)P and sterol in a mutually exclusive manner [36]. Structural analyses clarified these results, revealing that the sterol-binding pocket can host PI(4)P acyl chains, whereas basic residues in an adjacent pocket beneath the lid

coordinate the PI(4)P headgroup in a stereoselective manner (Fig. 2A). Finally, we measured that Osh4 exchanged sterol for PI(4)P between two distinct membranes, with an assay in which "light" DHE-containing liposomes and "heavy" [³²P]PI(4)P-containing liposomes were incubated with Osh4 and then separated by flotation to analyze their respective sterol and PI(4)P content [36].

PI(4)P, like other PIPs, is present in some organelle membranes but absent from others due to the specific localization of enzymes responsible for their synthesis and degradation [37,38]. PI(4)P is produced mostly in the *trans*-Golgi and plasma membranes by PI 4-kinases via phosphatidylinositol (PI) phosphorylation and hydrolyzed by Sac1 into PI in the ER. Considering this, the links between Osh4 and PI(4)P metabolism at the Golgi, and our *in vitro* data, we postulated that Osh4 could transfer neosynthesized sterol from the ER to the Golgi and exchange it for PI(4)P and transfer PI(4)P in the reverse direction. Additionally, we surmised that this sterol/PI(4)P exchange process, coupled with the metabolic maintenance of a PI(4)P concentration gradient, could contribute to sterol build-up in the Golgi membrane.

To explore this scenario in detail, we devised an approach to

measure in real-time the transport of PI(4)P between liposomes to complement DHE transfer assays. We re-engineered the PH domain of the FAPP protein (PH_{FAPP}), which recognizes the PI(4)P head-group specifically, to label it with an NBD fluorophore (7-nitro-2-(1,3-benzoxadiazol-4-yl)). This sensor was mixed with ER-mimetic liposomes containing sterol and Golgi-mimetic liposomes containing PI(4)P and rhodamine-PE (Rhod-PE); its fluorescence was quenched due to energy transfer to Rhod-PE and only recovered if PI(4)P was transferred to ER-mimetic membranes. We found that Osh4 transferred DHE and PI(4)P along opposite routes at full speed between two liposome populations, each initially containing one of the ligands. Moreover, Osh4 could generate a sterol gradient between these membranes by dissipating a pre-existing PI(4)P gradient [39]. Today, it is still unclear how Osh4 translates its capacity to exchange sterol for PI(4)P into a cellular function. Osh4 may deliver sterol to the *trans*-Golgi to support the biogenesis of secretory vesicles [40] or, by extracting PI(4)P, guarantee the docking of these vesicles to the PM [41,42]. However, studies on OSBP and Osh2 have established that some ORPs/Osh proteins use PI(4)P to transfer sterol in the cells *via* the exchange process identified with Osh4 [43–46]. Notably, OSBP transfers significant cholesterol from the ER to the *trans*-Golgi at contact sites between these compartments in exchange for a large part of the Golgi PI(4)P pool [43,44].

In 2013, another breakthrough was achieved by Gavin's team, which found that Osh6 and Osh7 are specialized phosphatidylserine (PS) transporters (Fig. 1) [47]. PS is an anionic lipid that is highly abundant in the PM, where it plays critical roles in signaling [48]. To make this discovery, they used an approach integrating high-performance thin layer chromatography (HPTLC) and mass spectrometry (MS) to identify the lipid(s) captured by tagged LTPs expressed in yeast and purified by affinity and size exclusion chromatography. PS, not ergosterol, was found to be the endogenous ligand extracted by Osh6 and Osh7. *In vitro* assays coupled with HPTLC analyses showed that Osh6 could extract and transfer PS specifically from "heavy" sucrose-loaded liposomes. Solving the structure of the Osh6:PS 1:1 complex revealed how changes in the binding pocket and the lid of an ORD imparts selectivity to PS (Fig. 2A). Finally, Osh6 and Osh7 were found to transfer newly synthesized PS from the ER to the PM in an assay based on a genetically encoded fluorescent PS sensor (C2 domain of lactadherin). For the first time, these data unveiled a mechanism that could explain how PS, originating from the ER, accumulates in the PM [47].

Structural analyses of Osh4 and Osh3 showed that PI(4)P is recognized by residues highly conserved in ORPs/Osh proteins [36,49]. We thus examined whether a link existed between PI(4)P and the Osh6 and Osh7 transport functions. We showed that these proteins extract PI(4)P from liposomes, and we solved the structure of Osh6 bound to PI(4)P (Fig. 2A), suggesting that they may use a PI(4)P gradient to move PS directionally from the ER to the PM. With a novel FRET-based assay using a fluorescent PS sensor (also based on the C2 domain of lactadherin), we observed that Osh6 exchanges natural PS for PI(4)P between membranes. Finally, we established that Osh6 harnesses a PI(4)P gradient at the ER/PM interface to transfer PS from the ER to the PM. De Camilli, Nakatsu, and co-workers identified ORP5 and ORP8, the closest homologs of Osh6/7 in mammals, as PS/PI(4)P exchangers [50]. These are anchored to the ER [51,52] and associate with the PM *via* a PI(4)P/PI(4,5)P₂-specific PH domain to populate ER-PM contact sites [53–55]. To identify the ligands of these proteins, a tagged version of the ORD of ORP8 (ORD_{ORP8}) was expressed in Expi293 cells and purified (Table 1). Next, the molecular weight of the native and denatured form of this construct was compared by mass spectrometry, revealing the existence of two forms of ORD, loaded

either with endogenous PS or PI(4)P species. Lipid transfer assays using "heavy" and "light" liposomes combined with HPLC suggested ORP8 as a PS/PI(4)P exchanger. Finally, ORP5 and ORP8 were found to supply the PM with PS in a PI(4)P-dependent manner [55]. Using some of the approaches mentioned below, ORP10 was later found to be a PS/PI(4)P exchanger working at the ER/endosome interface [56]. These data confirmed sequence analyses suggesting that ORPs, whose lipid-transfer modules contain a conserved LPTFILE motif, specifically bind and transfer PS [47]. More generally, these data established that PI(4)P is a ligand that drives the ability of ORP to ensure not only sterol but also PS fluxes in eukaryotic cells.

It is now well appreciated that ORP5 and ORP8-mediated PS/PI(4)P exchange is at the core of a sophisticated rheostat mechanism that constantly adjusts the levels of PS, PI(4)P in the PM but also of PI(4,5)P₂, which derives from PI(4)P. However, it was reported that ORP5 and ORP8 could directly exchange PS for PI(4,5)P₂ to regulate PM composition [53]. From a structural standpoint, this was a surprise, as previous assays and structural analyses suggested that an ORD could not host PI(4,5)P₂. Indeed, the phosphate group at position 5 on the inositol ring of PI(4,5)P₂ was predicted to make a steric clash with the pocket's wall [36,57]. Nevertheless, the structures of the ORD of ORP1 and ORP2 were obtained in a 1:1 complex with PI(4,5)P₂, showing that the inositol ring of PI(4,5)P₂ adopts an orientation that differs by 180° from that of PI(4)P to avoid this steric clash [58,59]. The lid is partially open and promotes protein oligomerization. In the ORD_{ORP2}:PI(4,5)P₂ complex structure, one of the PI(4,5)P₂ acyl chains is squeezed toward the lid or stretched out along a surface hydrophobic groove. Thus, these structures contrasted with those of ORD in which the PI(4)P, sterol, or PS molecule is deeply buried in the pocket, closed by the lid [28,47,50].

A key argument to suggest that ORP5 and ORP8 use PI(4,5)P₂ for lipid exchange came from *in vitro* measurements showing a faster transfer of PI(4,5)P₂ along its concentration gradient comparatively to PI(4)P by ORD_{ORP8} [53]. We confirmed these data. However, using donor liposomes containing the two phosphoinositides and a specific PI(4,5)P₂ sensor, NBD-PHδ1, we found that the ORD_{ORP8} was unable to transfer PI(4,5)P₂ in the presence of PI(4)P [60]. Moreover, PS was exchanged for PI(4)P even if PI(4,5)P₂ was present. We eventually showed that, comparatively to PI(4)P, the ORD_{ORP8} has a low affinity for PI(4,5)P₂ because this lipid is not secured in the protein's pocket. It may seem counterintuitive that a low-affinity ligand is transported more rapidly *in vitro* than a high-affinity ligand. However, in assays where lipid transfer is measured along a concentration gradient, this can easily be explained by the fact that it is rapidly released into acceptor membranes (high dissociation constant k_{off}) and/or slowly re-extracted once released (low association constant k_{on}) comparatively to a high-affinity ligand. However, if these two ligands are present, the high-affinity ligand is more likely to be captured and transferred by an LTP than the low-affinity ligand, whose transfer is, as a result, inhibited. Given that PI(4)P and PI(4,5)P₂ co-exist in the PM, we concluded that PI(4)P was used by ORP5 or ORP8 in the cell. A study by Humpolickova's group, in which PI(4,5)P₂ transport was measured between liposomes using a fluorescent PI(4,5)P₂ sensor derived from the C2 domain of granuphilin and fluorescence cross-correlation spectroscopy, reached the same conclusion [61]. Finally, the capacity of ORP5 and ORP8 to export PI(4,5)P₂ from the PM has not been confirmed by subsequent cellular studies [54,62]. Therefore, there is a pitfall when analyzing *in vitro* data using membranes of low compositional complexity: measurement of a fast transfer rate for a given lipid and an LTP does not necessarily mean that this lipid is the actual ligand of the LTP.

We also measured the capacity of Osh6 and ORD_{ORP8} to transfer

more than fifteen PS and PI(4)P subspecies whose acyl chains differed in length and saturation degree. Their activities were found to be highly dependent on the nature of the PS and PI(4)P subspecies—notably, big differences were seen between PS with none or only one unsaturation. We established interesting correlations between the activity of these LTPs and their affinity for lipid ligands, which led us to propose new rules for LTPs. First, confirming our results with PI(4,5)P₂, we showed that low-affinity ligands can be transferred more rapidly than high-affinity ligands. Second, we concluded that a high-affinity ligand could be transported more rapidly if exchanged for a second high-affinity ligand. We assume that in the complex context of the cell, LTP can selectively bind a high-affinity ligand but must exchange it with a second high-affinity ligand to work at full speed. Thus, lipid exchange is possibly the best mechanism to ensure fast, accurate, and directional transfer of lipids between organelles.

Our data also indicated that ORPs/Osh proteins optimally function as exchangers with ligands for which they have a comparable affinity, as suggested by previous data on Osh4 [36]. For Osh6 and ORP8, the best exchange rates were measured *in vitro* with unsaturated PS and PI(4)P species, identical or similar to those mostly found inside cells. Like us, other groups have found that brain PI(4)P, a polyunsaturated species (18:0/20:4), completely blocks the exchange activity of Osh6 [53,60,61]. This observation has suggested that PI(4)P inhibits rather than activates Osh6-mediated PS transfer, which led to an alternate model for the coupling between PI(4)P metabolism and PS/PI(4)P exchange [61]. However, this PI(4)P form does not exist in yeast, which challenges this conclusion. Contrastingly, any PI(4)P subspecies, including brain PI(4)P, can be used by Osh4 to perform robust sterol/PI(4)P exchange *in vitro* [36,39], possibly because its binding pocket slightly differs from that of Osh6. Thus, the choice of lipid subspecies and a little bit of chance can be decisive when drawing conclusions on LTPs from *in vitro* assays.

3. PITPs: From the early years to real-time analysis at contact sites

In mammals, five proteins contain a PI transfer domain (PITD) and are referred to as PITPs (Fig. 1, [63]). They have been grouped into two classes based on phylogenetic analyses. The first comprises PITP α and β that only consist of a PITD and share a capacity to transfer PI and phosphatidylcholine (PC) between membranes. The first hint that these proteins exist came from seminal studies in the early '70s reporting PC and PI movements between rat liver microsomes containing radiolabeled lipids and mitochondria or liposomes [64]. Subsequently, a bovine brain protein (known today as PITP α), able to transfer [³H]PI from microsomes to liposomes, was purified under two forms (the PC and PI-bound forms of the protein) [65]. A few years later, Kasper and Helmkamp proved that PITP α is a PC/PI exchanger by measuring PI and PC transfer rates in both directions between two populations of chemically defined liposomes; a separation strategy based on lactosylceramide and lectin was used [66]. The ability of PITP α to bind and transfer PC and PI between membranes was further analyzed using fluorescent analogs of these lipids carrying a parinaroyl chain (i.e., a natural conjugated polyene acyl chain [67]) at the sn-2 position. When this analog was concentrated in donor liposomes, its fluorescence was quenched. Its association with PITP and transfer to acceptor liposome resulted in dequenching. These approaches confirmed the ability of PITP to host PI and PC in a mutually exclusive manner, as well as its ligand selectivity, and its greater affinity for PI than PC (Tables 1 and 2) [68,69]. Other fluorescent PC and PI analogs, whose acyl chain at the sn-2 position (8:0 or 10:0) was labeled with pyrene, were combined with TNP-PE in donor liposomes to develop

FRET-based lipid binding and transport assays (Tables 1 and 2) [70]. Remarkably, competition assays using these analogs showed in the early 90s that the affinity of PITP α for natural PCs is strongly influenced by the length and unsaturation degree of these lipids [71]. In 1989, the gene of rat PITP α was cloned [72], and a few years later, the gene of a second rat PITP (PITP β), sharing 77 % identity with PITP α [73]. Later, the PITP α and PITP β structures were solved empty or loaded with PC or PI (Fig. 2B) [74–77]. The PITD has a fold related to the START domain (see Section 5) consisting of an α/β grip fold made of a central eight-stranded β -sheet and several long helices with an internal lipid-binding cavity. A mobile α helix (G-helix) and 11 C-terminal amino acid residues function as a gate to close the cavity. The head group of PI is coordinated *via* many hydrogen bonds by residues highly conserved in PITDs [74–77]. In contrast, the structural determinants of PC recognition remain unknown. The strong preference of PITPs for PI over PC could be explained by these differences [76].

Interestingly, PITPs were first characterized by *in vitro* approaches, and their *in vivo* functions were only later investigated. Although similar, PITP α and β have distinct expression patterns, subcellular localization, and functions. In developing and adult rats, PITP α is highly enriched in the brain and found in axons with a cytosolic location [78]. In contrast, PITP β is enriched in the liver and seems localized in the cytosol and at the ER and Golgi surface [78–80]. Many recapitulation assays using cytosol-depleted cells showed in the 90s that PITP α could assist the signaling capacity of the cell by enhancing PI(4,5)P₂ or PI(3,4,5)P₃ synthesis [81,82] but also the formation of secretory vesicles from the *trans*-Golgi network [83] or the fusion of secretory granules with the PM [84]. PITP α was found to play a critical role in the development of the nervous system and its function [78,85–87]. PITP β also endorses a critical physiological role as its absence is embryonic lethal in mice [88]. In cells, PITP β might circulate rapidly at the ER/Golgi interface, possibly to execute PC/PI exchange between these compartments [80]. In line with this idea, PITP β was reported to regulate the Golgi PI(4)P level and thereby control retrograde Golgi-to-ER vesicular trafficking at the *cis*-Golgi [89] but also the genesis and trafficking of secretion vesicles at the *trans*-Golgi, critical for the development of the embryonic mammalian neocortex [86]. However, challenging this view, it has recently been reported that the acute pharmacological ablation of PITP activity hardly affects Golgi PI(4)P levels [90]. It is speculated that PITPs supply the PM with PI to assist PI(4)P synthesis and ORP-mediated PS/PI(4)P exchange.

The class II of the human PITP family comprises RdgB β (a.k.a. PITPNC1), Nir2 (PITPNM1), and Nir3 (PITPNM2); as exemplified with Nir2 [91]), their genes were cloned after those of class I PITP and their ligand specificity was fully characterized in 2012. Cockcroft and co-workers found that RdgB β , which consists of a PITD followed by a short extension, transported radiolabeled PI but not PC between microsomes and liposomes, contrary to PITP α [92]. Next, RdgB β was mixed with permeabilized radiolabeled human HL-60 cells, and its lipid content was analyzed by TLC and MS (Table 1). The cellular lipids loaded by the protein were PI and phosphatidic acid (PA). The protein's capacity to bind PA was further assessed using assays with pyrene-labeled GPLs. Comparatively to RdgB β , the PITD of Nir2, expressed in cells, was not prominently copurified with PA. Nevertheless, it showed no PC transport capabilities *in vitro*. These results suggested that Class II PITPs are PI/PA exchangers. Due to the absence of PITD structure bound to PA, why these proteins are selective for this lipid is still unclear.

Nir2 and Nir3 are more sophisticated than RdgB β . In addition to binding PI and PC, they can associate with the ER by interacting with VAPs [93] and with the PM by recognizing diacylglycerol (DAG) and PA *via* a C-terminal LNS2 domain flanked by a DGBL

(DAG-binding-like) motif [94,95]. Thanks to this multidomain organization, Nir2 and Nir3 play a central role in the so-called PI cycle, conceptualized by R. Michell in 1975 [96]. This corresponds to a process by which PI(4,5)P₂, after being hydrolyzed in signaling pathways to generate second messengers (DAG and IP₃) in stimulated cells, is rapidly re-synthesized [94,95,97]. In *Drosophila*, loss-of-function mutants of *Dm-RdgB α* , the Nir2 homolog, were associated with an impaired conversion of light signal into electrical response and a degeneration of photoreceptor cells (hence the name RdgB for retinal degeneration type B). Because photon absorption by the GPCR rhodopsin is transformed into an electrical activity by the PLC β -mediated PI(4,5)P₂-dependent pathway, *Dm-RdgB* and Nir2 were thought to be involved in the maintenance of cellular signaling competencies. Several teams found that in stimulated cells, Nir2 bound to ER-resident VAPs, reached ER-PM contact sites following the conversion of the pool of DAG, arising from PI(4,5)P₂ breakdown, into PA [94,95,97,98]. It was later reported using genetically encoded fluorescent sensors that Nir2 and Nir3 transfer PA from the PM to the ER, where this lipid is converted into PI. Presumably, these proteins also move PI to the PM, where this lipid can be phosphorylated into PI(4,5)P₂. To summarize, Nir2 and Nir3 likely ensure, by PA/PI exchange, a functional connection between two spatially distant branches of the metabolic pathway responsible for PI(4,5)P₂ re-synthesis. *Dm-RdgB α* was known to be localized in the sub microvillar cisternae (SMC), a sub-compartment of the ER adjacent to the microvillar PM of photoreceptor cells [99], *i.e.*, in sites equivalent to ER-PM contact sites in human cells. Recent studies have established that *Dm-RdgB α* sustains the PLC-dependent signal transduction of light *via* its PA and PI transfer capacity [100]. This story shows how the identification of the second ligand of class II PITD was decisive to address a long-standing question about how PI and PA are transferred at the ER-PM interface during the PI-cycle.

4. High diversification of Sec14-like proteins

Sec14 is a yeast protein of ~35 kDa and an archetypical member of the CRAL/TRIO superfamily. In the early 80s, a soluble protein was found to selectively transfer PI and PC from liposomes made of total yeast lipids (labeled by incorporation of [³H] oleic acid or [2-³H]glycerol *in vivo*) to isolated mitochondria [101]. Next, this protein was reported to transport PI more efficiently than PC between liposomes in FRET assays based on pyrene-labeled GPLs [102]. In the late 80s, this protein was identified as Sec14 [103–105] and was found in parallel to play a crucial role in the export of secretory proteins from the Golgi complex and to be essential for yeast viability [104,105]. This unveiled unanticipated links between lipid transfer and vesicular trafficking processes. Next, *via* thorough genetic and functional studies, Sec14 was found in yeast to be critical for maintaining a lipid composition in the Golgi membrane permissive for secretory vesicle biogenesis [33,106]. Most notably, it was concluded that Sec14 aids Pik1, a PI 4-kinase, to produce PI(4)P at the Golgi, thereby promoting vesicle budding. How Sec14 selectively binds PC and PI was explained by structural analyses of this protein and its closest homolog, Sfh1 (Sec fourteen homolog), which binds the same lipids [106,107]. Both comprise a small N-terminal lobe and a larger C-terminal lobe with a longitudinal hydrophobic cavity. PC and PI occupy the same volume in this cavity in an extended conformation, but distinct residues coordinate their headgroups (Fig. 2C). PI is more stabilized than PC, which explains why Sec14 has much more affinity for PI than for PC [108,109].

It is well-accepted that Sec14 must recognize PC and PI to fulfill its biological function [106], but it is unclear what this function is. One scenario is that Sec14 exchanges PI for PC at the ER/Golgi interface. It could transfer PI from the ER, where this lipid is made,

to the Golgi apparatus to supply Pik1 with its substrate. In return, Sec14 could transfer PC to the ER to alleviate the toxic effect of this lipid on post-Golgi trafficking. Contrary to PTP α , no attempts have been made using radiolabeled ligands to demonstrate that Sec14 performs PC/PI exchanges and not simply homotypic PC/PC or PI/PI exchanges between membranes. Some proof has been obtained in fluorescence-based assays showing that Sec14 preferentially releases NBD(12)-PC (a PC analog with a short acyl chain (C12:0) at the sn-2 position bearing an NBD fluorophore) in membranes enriched with PI [109]. Only recently, a method based on small-angle neutron scattering (SANS) using deuterated PC has provided evidence that Sec14 can exchange natural PC and PI molecules between membranes (Table 2) [110]. Nevertheless, an alternative scenario posits that Sec14 exchanges PC and PI locally at the Golgi surface to constantly bring new PI molecules to the catalytic site of Pik1 and allow PI phosphorylation [106]. Arguments in favor of this model have been widely discussed [111,112]. To better understand how Sec14 can support PI(4)P synthesis, we recently reconstituted an artificial ER/Golgi interface with PI-rich liposomes (ER-mimetic) and liposomes coated with a PI 4-kinase (Golgi-mimetic). Using our PI(4)P sensor, we measured that Sec14 supports the production of PI(4)P by transferring PI from the ER to Golgi-mimetic membranes [113]. In contrast, Sec14 cannot activate PI 4-kinase directly associated with PI-rich membranes. These data strengthen the idea that Sec14 transfers PI at the ER/Golgi interface.

As mentioned, Sfh1 highly resembles Sec14 and binds PC and PI. However, it behaves like a stalled LTP [106,114], and its overexpression does not rescue growth defects of Sec14-deficient yeast [106,109,114]. Interestingly, Sfh1 can be converted into a potent LTP by subtle amino-acid substitutions that change the speed at which this protein captures and releases its ligands [115]. The function of Sfh1, found in the nucleus but also at the endosomes, the Golgi complex, and vacuoles, remains unclear [116,117]. Moreover, Sfh1 seems to have a loose selectivity for lipids as it can be crystallized with PE [106] and bind PS and PE in yeast, in addition to PC and PI [117]. The overexpression of Sfh1 promotes the decarboxylation of PS into PE by the PS decarboxylase 2 (Psd2) enzyme, which is localized in the endosomes, suggesting that Sfh1 may supply PS to this compartment. However, no proof has been found that Sfh transports natural PS [117].

There are four other Sfh proteins in yeast that, comparatively to Sfh1, are more divergent from Sec14. They all transfer PI to varying degrees but not PC between membranes [114]. Despite their low sequence identity with Sec14, two of these can rescue growth and secretory defects of Sec14-deficient yeast when overexpressed [114]. Sfh2 is cytosolic, but it also localizes to endosomes, lipid droplets (LDs), and the ER [116,118]. Sfh2 is a homolog of Sec14L2, a mammalian member of the Sec14 superfamily, which was first identified in 1957 as a supernatant protein factor (SPF) promoting the epoxidation of squalene, a linear precursor of sterol, in microsomes [119,120]. Purified SPF was found to transfer [³H]squalene from microsomes harboring no enzymatic activity to microsomes with a squalene epoxidase activity, thereby promoting 2,3-oxidosqualene synthesis [121]. Its ability to aid cholesterol biosynthesis *via* a squalene transfer activity was confirmed *in vivo* [120]. Sfh2 transfers PI and [³H]squalene between membranes, as shown by measuring the mobilization of the radiolabeled form of these lipids from liposomes to mitochondria [122]. However, whether it can exchange these lipids efficiently is unknown. Sfh2 structures, co-crystallized with PI or squalene, have been solved [123]. The head group of PI is engaged in interactions with conserved residues, as identified with Sfh1. Furthermore, Sfh2 has a unique toroid-shaped hydrophobic cavity, as in Sec14L2 [124] that hosts either the PI acyl chains or a squalene molecule (Fig. 2C). Sfh2 may have a role in fatty acid (FA) synthesis in hypoxia and in the regulation of

oxidative stress [125]. However, its exact cellular role and why it needs to bind PI and squalene remain unclear.

Sfh3, a.k.a. PDR16 (Pleiotropic Drug Resistance 16), was first described as protecting yeast against azole treatment, which blocks ergosterol synthesis [126]. Then, Sfh3 was found to bind cholesterol but also an intermediate of the sterol biosynthesis pathway, lanosterol, in addition to PI, using a permeabilized mammalian cell-based lipid-binding assay (Table 1) [127,128]. Its capacity to transfer sterol has been verified using assays based on radiolabeled cholesterol, ergosterol, and lanosterol, as well as DHE and cholestatrienol (CTL, a fluorescent mimetic of cholesterol) [122,128]. Sfh3 has been co-crystallized with PI [129] but not sterol. It is interesting to see how the shape and dimension of the hydrophobic cavity of Sfh3, adapted for the ergosterol-containing rigid hydrocarbon rings, differ from that of Sfh2, which recognizes a linear precursor of this lipid [123]. Some insights into how Sfh3 differentially recognizes PI and sterol have been obtained by testing mutants in transport assays [122]. However, whether Sfh3 can exchange these two lipid types efficiently is unclear. In yeast, Sfh3 is associated with a subpopulation of LDs, which are energy-storage organelles full of triacylglycerol (TAG) and steryl-ester, covered by a lipid monolayer, originating from the ER [130]. Sfh3 may play a key role at LD-vacuole contact sites in growing yeast cells [131] and at the interface between LDs and the prospore membrane in sporulating yeast by regulating the mobilization of ergosterol-ester stored in LDs. Despite thorough *in vitro* investigations revealing Sfh3's features, the cellular role of this LTP remains elusive, and its links with sterol and PI metabolism even more. The reason why Sfh3 changes the yeast response to azole treatment remains enigmatic as well.

Sfh4 is the closest homolog of Sfh3 and recognizes identical ligands [128] but has a different cellular localization, as it is cytosolic and close to the PM [116]. Moreover, Sfh4 is an essential part of a complex that comprises the PI 4-kinase Stt4 and Psd2 and ensures PS-to-PE conversion at the Golgi/endosomal level [132]. Evidence indicates that Sfh4 does not bind PS [133] but interacts physically with Psd2 [134]. Puzzlingly, the PI-binding capacity of Sfh4 is not required for its cellular function, but it is unknown whether its capacity to bind sterol is necessary to its function [134,135].

Sfh5 is cytosolic and was proposed to deliver PI to the PM, resulting in PI(4,5)P₂ synthesis by kinases (Stt4 and Mss4) and PI(4,5)P₂-dependent regulation of actin organization *via* the small G protein Cdc42, during secretion [136]. However, recent mass spectrometry, spectroscopy, and structural analyses have indicated that Sfh5 represents a new class of heme-binding protein with no PI transfer activity [137].

All these *in vitro* data have made it possible to consolidate structural and bioinformatics analyses, showing that the Sec14 and Sfh proteins have a common ability to bind PI while recognizing extremely dissimilar secondary ligands. Yet it remains unclear how Sec14 and Sfh proteins translate this dual lipid binding ability into a cellular function. Notably, a fundamental question is whether some Sfh proteins can exchange their ligands between membranes, as observed for Sec14, ORPs, and PITPs.

5. START domain-containing proteins: association with the three main lipid classes

Mitochondria are double membrane-bound organelles involved in various cellular processes, including energy production and amino acid and lipid metabolism. In all steroidogenic tissues, mitochondria contain the cholesterol side-chain cleavage enzyme (P450_{sc}), which converts cholesterol to pregnenolone, a reaction that constitutes the initial step in steroidogenesis. This process is triggered by tropic hormones within minutes during acute phases and in case of immediate need (e.g., biosynthesis of glucocorticoids

to combat stressful situations). The entry of sterol inside the mitochondria, which is the limiting step of this process, depends on a protein with an N-terminal mitochondrial-signaling sequence called the steroidogenic acute regulatory (StAR) protein (Fig. 1). Its C-terminal region was identified as the sterol transfer module in assays with donor liposomes doped with radiolabeled cholesterol and mitochondria (Table 2) [138]. Another protein, MLN64 (Metastatic Lymph Node 64)/StarD3, overexpressed in HER2-positive cancer, was found to be similar in sequence with this region of StAR and to aid steroidogenesis in cultured cells [139]. Further sequence analyses identified many other proteins with a StAR-related lipid-transfer domain (START, ~210 amino acids) [140]. Solving the crystal structure of the START domain of MLN64 showed it has an α/β helix/grip fold with a hydrophobic cavity [141]. Its ability to directly bind to cholesterol with a 1:1 stoichiometry was established by measuring the radioactivity associated with a tagged version of this domain, mixed with [¹⁴C]cholesterol, immobilized on resin, washed to remove unbound cholesterol, and eluted (Table 1) [141]. Phylogenetic, biochemistry, and structural analyses identified 15 mammalian START-containing proteins classified into six subfamilies, each with a given ligand repertoire, molecular organization, and function.

StarD1 and MLN64/StarD3 constitute the group of membrane-targeted sterol carriers, as StAR is mitochondrial and StarD3 is anchored to the surface of late endosomes *via* an N-terminal transmembrane (TM) segment. StarD3 can also associate with VAPs to form ER-endosome contacts [142,143]. We showed that the cytosolic part of StarD3 could transfer DHE at a high rate between membranes when connecting two of them [143]. Overexpressed in cells, StarD3 massively flows sterol from the ER to the endosomal membranes at the expense of the PM, as visualized by microscopy with fluorescent sterol probes [143]. The reason why StarD3 supports the proliferation of HER2-positive cells remains to be established. The structure of StarD1 and StarD3 bound to sterol has not yet been determined. Conserved residues between the two proteins lining the hydrophobic cavity are thought to interact with this ligand.

A second subfamily includes StarD4, StarD5, and StarD6, which consist almost entirely of a START domain and were initially assumed to be specific LTPs for sterol. StarD4 is still considered to fulfill key roles in cholesterol homeostasis. It swiftly transfers DHE between liposomes [144] and in cells, facilitates cholesterol transport to the ER, supporting cholesteryl ester accumulation in LDs in an acyl-CoA:cholesterol acyl-transferase (ACAT)-dependent manner, and to the endocytic recycling compartment. In contrast, the nature of StarD5 and StarD6's ligands has been largely questioned. First assays showed that StarD5 binds cholesterol [145], but Letourneau et al. found that instead, it binds bile acids, which derive from cholesterol [145]. To obtain this result, they conducted circular dichroism-based thermal shift assays, an approach used to determine the affinity of a protein for a ligand by measuring how much the protein's structure, subjected to increasing temperatures, is stabilized by this ligand. Furthermore, heteronuclear NMR spectroscopy identified residues inside the protein's cavity that recognize bile acids [145]. StarD5 is highly expressed in cell types targeted by these molecules (*i.e.*, liver macrophages and Kupffer cells), suggesting it might be involved in cellular responses triggered by those compounds. Similar approaches revealed a higher affinity of StarD6 for pregnenolone and testosterone than for cholesterol [146,147]. StarD6 may be implicated in cell development and maturation [147].

A third subfamily (StarD2, StarD7, StarD10, and StarD11) comprises LTPs that bind GPLs and/or sphingolipid species but not sterol. StarD2 is the first LTP whose activity was reported in mammals. In 1968, Wirtz and Zilversmit detected radiolabeled PC

and PE exchanges between purified mitochondria and microsomes mixed with rat liver cytosol [148]. This led to the identification of a soluble protein called PC-TP. Pioneer approaches in the late '70s based on electron spin resonance (ESR) and PC with a spin-labeled acyl chain were used to measure the activity of PC-TP between liposomes without a separation step [149]. Later, sequence and structural analyses identified PC-TP as a STAR domain-containing protein (Figs. 1 and 2D) [150]. A triad of aromatic residues at the bottom of the binding pocket provide selectivity for PC via specific interactions with the quaternary amine of this lipid. StarD2 is known today to regulate some aspects of lipid metabolism, notably in the liver. By interacting with the acyl-CoA thioesterase THEM2, it modulates the entry and β -oxidation of FA in mitochondria and, at the transcriptional level, by interacting with PPAR δ in a PC-dependent manner [151,152]. MS analyses of StarD2 incubated with microsomes have shown its preferences for PC with sn-1 palmitoyl (16:0) and sn-2 unsaturated acyl chains [153], in agreement with competition assays using natural and pyrene-labeled PCs [71]. This suggests that StarD2, by discriminating PC within lipid bilayers, functions as a sensor, linking metabolic regulation to membrane composition, rather than as an LTP [151].

StarD7 and StarD10 also recognize PC, and StarD10 additionally binds PE [154,155]. These lipids were found by TLC and MS to be selectively captured by the recombinant forms of these proteins mixed with liposomes composed of radiolabeled cellular lipid extract. Next, StarD10 was shown by ESR and fluorescence to bind PC and PE analogs with one acyl chain labeled with a doxyl moiety, NBD or pyrene [155]. The capacity of StarD7 to selectively transfer PC was established using a FRET-based assay with phospholipids with one acyl chain (C12:0) bearing an NBD fluorophore (Table 2) [154]. Donor liposomes were doped with NBD-labeled lipid and rhodamine-labeled PE (Rhod-PE) and mixed with acceptor liposomes: the emission signal of the NBD-labeled lipid, initially quenched due to its proximity with Rhod-PE was recovered if transported to acceptor liposomes, allowing the measurement of transfer kinetics. Recent works have suggested that StarD7 contains a mitochondrial-targeting signal followed by a TM segment and is processed to be partly released to the cytosol and partly in the inner mitochondrial space (IMS) [156]. Likely, StarD7 transports PC from the ER to the outer mitochondrial membrane (OMM) and then to the inner mitochondrial membrane (IMM) [154,157]. The absence of StarD7 causes defects in the respiratory activity and internal architecture of mitochondria [157] as these organelles lack the enzymes necessary for PC synthesis. StarD7 seems essential to maintain a high PC level and the right balance in the mitochondrial membrane between this lipid and cone-shaped, non-bilayer lipids, such as CL and PE [158]. StarD10 supports the genesis of insulin secretory granules in pancreatic β cells, possibly by transporting phosphoinositides [159]. StarD10 has a wider cavity than StarD2, suggesting it may host such lipids, but this has not been verified [159].

A well-known LTP is StarD11, a.k.a. CERT, whose activity was first identified by investigating why certain cell lines were deficient in SM. This lipid is synthesized in the Golgi, but its precursor, ceramide, is produced in the ER. The absence of CERT was found to impair ceramide delivery to the Golgi and its conversion into SM [160]. *In vitro* assays showed that CERT could trap ceramide from purified ER membranes, in which a radiolabeled form of this lipid was endogenously produced or from liposomes doped with this lipid (Table 1). In contrast, CERT could not extract sphingosine, SM, PC, or cholesterol, which revealed its specificity for ceramide. In these assays, membranes were incubated with recombinant CERT and then separated from it by centrifugation. The amount of ligand bound to the protein, recovered in the supernatant, was determined by scintillation counting or TLC. Next, CERT was found to

transfer radiolabeled ceramide between liposomes [161]. Finally, ceramide conversion into SM was detected upon adding CERT to semi-intact cells. The structure of the START domain of CERT bound to ceramide was solved (Fig. 2D), revealing that the amide and hydroxyl groups of this lipid are stabilized via hydrogen bonds by residues at the bottom of the binding pocket [162]. It is now well understood that CERT joins ER-Golgi contacts by interacting with Golgi PI(4)P via an N-terminal PH domain and with ER-resident VAPs via a FFAT motif. Under the control of OSBP, CERT transfers ceramide to the *trans*-Golgi, synchronizing the production of SM with the delivery of cholesterol in this compartment [112].

Recently, click-chemistry-based approaches have provided new insights into the ligand specificity of STARs [163]. Purified START domains of CERT, StarD2, StarD7, and StarD10 were incubated with liposomes doped with the bifunctional analog of either DAG, PE, PC, ceramide, or SM, whose one acyl chain contained a photo-activatable diazirine and clickable alkyne group. UV irradiation of the diazirine group generated a reactive lipid intermediate that formed a covalent bond with proteins in its vicinity. The alkyne group in the lipid was then labeled by click chemistry with a fluorescent dye, allowing the visualization of protein-lipid complexes by SDS-PAGE. This strategy confirmed data on the ligand selectivity of the tested StarD protein and helped identify ceramide as a new ligand of StarD7. Site-directed mutagenesis and photo-affinity labeling experiments showed that StarD7 has a common binding site for PC and ceramide. StarD7 has no ceramide transfer activity, but its PC transfer capacity can be fine-tuned by ceramides [163]. It was presumed that an increase in the concentration of ceramides at ER-mitochondria contact sites blocks StarD7-mediated mitochondrial PC import, accounting for the detrimental impact of ceramide when it reaches a high level in the cell.

6. LAM/GRAMD1a-c (ASTER): new players in intracellular sterol transfer

The strategy used in 2015 to uncover the existence of the GramD1 (ASTER)/Lam family was very different from that employed earlier to discover other LTP families. Here, it all started when two groups, including Levine's, identified by pure bioinformatics three proteins in humans (GramD1a-c) and six in budding yeast (Ysp1, Ysp2/Lam2, Lam4 and their respective paralogue Sip3, Lam5, and Lam6), distantly related to proteins containing a START domain (Fig. 1) [164,165]. These proteins were predicted to include one or two START-like domains (also termed VASt (VAD1 Analog of StAR-related lipid transfer)) flanked by an N-terminal PH-like domain called GRAM and a C-terminal TM segment. Ysp1 and Sip3 were also predicted to contain PH and BAR (Bin/amphiphysin/Rvs) domains.

To find the lipid ligand(s) of these putative LTPs, the recombinant form of the second START-like domain of Lam4 was purified and incubated with human permeabilized cells, in which all lipids were radiolabeled with [14 C]-acetate. Then, the construct was re-purified and found by TLC to contain cholesterol only, suggesting its ability to capture this lipid selectively among many others [164]. This was supported by a DHE/tryptophane FRET assay previously used to analyze how ORP/Osh proteins capture sterol [28,36]. A distinct study showed that Lam6 (a.k.a. Ltc1), devoid of TM, selectively transferred radiolabeled sterol between "heavy" and "light" liposomes [166]. Teams led by Im, Reinisch, and Menon confirmed the ability of these yeast proteins to transport sterol via DHE transfer assays and solved the structure of the START-like domains of Ysp2 and Lam4 bound to sterol (Fig. 2E) [167–169].

A remarkable facet of this story is that it was possible to rapidly show that these proteins act as sterol transfer proteins in yeast. Because these proteins all contain a TM segment, they were

predicted to be anchored to compartments and possibly confined to contact sites. This was confirmed by microscopy experiments showing the localization of Ysp1, Sip3, Ysp2, and Lam4 at ER-PM contact sites and the presence of Lam5 and Lam6 at the nuclear vacuolar junction (NVJ) and at ER-mitochondria contact sites [164,166,170]. Remarkably, yeast cells with no expression of Ysp1, Ysp2, or Sip3 were sensitive to amphotericin B. Because this antifungal compound forms pores in membranes when associating with ergosterol, this suggested that these cells abnormally accumulated sterol in their plasma membrane. Next, rescue experiments revealed a correlation between the ability of these proteins to lower sterol levels in the PM and to occupy ER-PM contact sites and bind sterol *via* their StART-like domains. As a confirmation, the genetic ablation of Ysp1, Ysp2, and Sip3 was found to slow down PM-to-ER sterol flow [164]. In parallel, Lam6 was found to promote the formation of sterol-enriched membrane domains in the vacuolar membrane in the case of nutritional stress [166,170].

Much data has also been obtained on the human counterpart of LAM, GRAMD1a-c, also referred to as Aster (Greek word for “star”). These proteins populate ER-PM contact sites once they have detected *via* their GRAM domain a surge of accessible cholesterol in the PM coinciding with the high level of PS in this membrane [171–173]. This expansion arises from sterol uptake by endocytosis of low-density or high-density lipoproteins [171,173–175]. Next, GRAMD1/Asters convey cholesterol to the ER, and this cholesterol is subsequently esterified and stored in LDs [174,175]. Noticeably, this sterol movement also notifies a central regulatory system present at the ER, the SREPB2 pathway, about the overload of PM with sterol, preventing a poisonous intracellular accumulation of this lipid. This leads to the transcriptional repression of genes coding for sterol synthesis and uptake [171,172,174,175]. Defects in GRAMD-dependent sterol transfer can result in a lack of steroid hormone synthesis in model animals [174] and may be linked to intellectual disability in humans [172].

To conclude, the discovery of the Lam/GRAMD1 family shows how *in silico* investigations, coupled with stringent *in vitro* analyses, have provided decisive results regarding the selectivity of these new LTPs, leading to the identification of important physiology-relevant regulatory mechanisms.

7. Ups/PRELI family: specific PA and PS transfer across the mitochondrial intermembrane space

Cardiolipin (CL) and PE are enriched in the mitochondria membrane and are critical for the proper architecture and function of this organelle, notably due to their conical shape [176,177]. CL derives from the combination of two PA molecules, whereas PE is obtained *via* PS decarboxylation. PA and PS originate from the ER, and data suggest they are conveyed to the OMM by LTPs at ER-mitochondria contact sites (for instance, in yeast by the ER-Mitochondria Encounter Structure (ERMES), see the next section [178]). Once these lipids have been delivered in the OMM, they must cross the IMS to reach the IMM CL and PE synthesis machinery. Over the last decade, the Ups1/PRELI-like proteins and their mitochondrial chaperones (TRIAP1/Mdm35) have been identified as evolutionarily conserved devices that convey these lipids across the IMS (Fig. 1).

Ups1–Mdm35 is a heterodimer that shuttles PA from the OMM to the IMM in yeast. The first clue that led to this discovery came from observations that in cells devoid of Ups1, the level of CL decreased in the mitochondria to the benefit of PA [179]. The ability of Ups1–Mdm35 to selectively transfer PA was then established by stringent *in vitro* lipid transfer assays [179]. Purified Ups1–Mdm35 was mixed with “heavy” donor liposomes composed of all the major GPL species, mimicking the lipid composition of the OMM,

and “light” acceptor liposomes composed of PC, PE PI, and CL, mimicking the IMM. Next, the lipid content of these liposomes, once separated from each other, was quantified by MS, showing the build-up of PA in the acceptor liposomes. Transfer assays based on radiolabeled [¹⁴C]-PA confirmed the role of Ups1 in capturing PA [179]. Crystal structures of the Ups1–Mdm35 bound to one PA molecule [180,181] were obtained with purified protein mixed with liposomes or micelles doped with PA (Fig. 2F). Ups1 was found to have a remote structural similarity with the START domain and PITD despite no significant sequence homology. Remarkably, bacterial PA was detected by MS inside the Ups1–Mdm35 complex, directly purified from *E. coli*, validating all the other approaches [181].

Ups2–Mdm35, a second heterodimer, transfers PS from the OMM to the IMM to feed PS decarboxylase 1 (Psd1), supporting PE production [182,183]. Metabolic pulse-chase unraveled its capacity to connect two spatially distant enzymatic reactions (PS and PE synthesis) *via* its lipid transfer capacity in assays like those employed for Ups1–Mdm35. Furthermore, Ups2 was found to be able to selectively export PS from complex membranes, even doped with PA [182]. However, Ups2 was also observed to substantially transport PA in assays using PS-free membranes (this illustrates once again the risk of using low-complexity membranes to determine LTP selectivity) [183]. It was also established that, in humans, a PRELID1–TRIAP1 homolog transfers PA to the CL synthetic pathway, using strategies close to those used to characterize Ups proteins [184]. PRELID3b–TRIAP1 heterodimer was found to be the human equivalent of Ups2–Mdm35 [182]. There is no structural data on how Ups2 and PRELID3b bind PS; nevertheless, structure-function analyses have provided ideas on how PS may be recognized.

Once the Ups/PRELI ligands were known, real-time assays based on NBD-labeled PS or PA species were devised to characterize these LTPs further. In these assays, donor liposomes doped with NBD(12)-PS or NBD(12)-PA and Rhod-PE were mixed with acceptor liposomes containing natural PS or PA. Ups1–Mdm35 and PRELID1–TRIAP1 were found to shuttle NBD(12)-PA more rapidly than NBD(12)-PS, in line with kinetic data collected with unmodified lipids [181,185]. What was likely measured is the intermembrane exchange between the NBD-labeled ligand and its natural form: the latter, in excess, set the speed for the process, which provided reliable results. This strategy based on NBD-labeled ligand was then helpful to test Ups/PRELI mutants and thereby define the role of conserved residues and structural elements (Ω -loop) in PA and PS recognition but also to investigate whether these LTPs can be regulated by mitochondrial CL levels [182,185].

8. SMP-domain containing proteins: a new group of LTPs with low selectivity for GPLs

As mentioned, PS must be transferred from the ER to the mitochondria to be decarboxylated into PE. This lipid, once produced, must travel in the reverse direction to be methylated into PC in the ER. In 2009, Kornmann et al. identified a four-subunit complex called ERMES that connects the ER and mitochondria inside yeast [178]. This complex comprises the OMM protein Mdm10, the ER-resident Mmm1, Mdm34 and Mdm12. The loss of one subunit was enough to cause a deceleration of PS to PC conversion in yeast, suggesting ERMES may transport lipids in addition to bridging membranes. However, it remained unclear whether this was true [186]. The solution to this question came from bioinformatics analyses predicting the presence of an SMP (synaptotagmin-like, mitochondrial-lipid binding protein) domain in Mmm1, Mdm12, and Mdm34. SMP domains were identified as part of the TULIP domain superfamily [187], known to comprise proteins with a

central, tubular cavity to host lipids or other hydrophobic ligands (like the extracellular Cholesteryl Ester Transfer Protein [188]). *In vitro* approaches were decisive to show that ERMES subunits bind GPLs *via* their SMP domain and that ERMES conveys lipids between membranes. In 2015, Egea and co-workers purified Mdm12 expressed in *E. coli*, extracted the lipids bound to the protein, and determined their nature and abundance using TLC and MS [189]. These lipids were primarily PE and PG (phosphatidylglycerol), in a similar proportion to that found in bacterial membranes where they are prominent. Interestingly, when Mdm12 was purified from yeast, it mainly contained PC, PI, PE, and a small amount of PS. PC is absent in *E. coli*, explaining why it was not detected before. PC was enriched in the Mdm12 sample compared with its abundance in yeast membrane, suggesting it may be the *bona fide* ligand of that protein.

The SMP domain of Mdm12 has a conical shape and is traversed by a groove lined with hydrophobic residues, open toward the solvent, and occupied by one GPL (Fig. 2G) [190]. The lipid is inserted in the cavity *via* its acyl chains, whereas its head group protrudes above the groove and is solvated, making almost no contact with the protein. Identification of this structure explained why Mdm12 could bind various GPLs with low selectivity. Lipid displacement assays were employed to analyze this further. Mdm12 was mixed with NBD-PE, a PE species whose polar head is labeled with NBD, and then mixed or not with natural lipids. The capacity of these lipids to compete with NBD-PE loaded in Mdm12 was analyzed by native PAGE and detection of NBD fluorescence. Mdm12 was shown to bind GPLs with different polar heads, so using NBD-PE was not problematic. This analysis showed that Mdm12 preferentially recognizes PC, PE, PG, and, to a lesser extent, PI, but neither PA, PS, nor CL [190].

Another ERMES subunit, Mmm1, was also extensively studied. Structural analyses revealed that its SMP domain can host GPLs in a way similar to that identified for Mdm12 but possibly *via* more specific interactions [191]. Mmm1 was thus presumed to act as a filter for screening GPLs among the pool of cellular lipids. Displacement assays indicated that it markedly differs from Mdm12 in specificity. Indeed, it can preferentially recognize PA and PG, slightly less PC and PS, and not PE [191]. Thus, it was found that Mmm1 and Mdm12 can accommodate some of the lipids known to transit between the ER and the mitochondria, namely PA, PC, PS, and PE.

Nevertheless, whether the ERMES complex was directly involved in lipid transport at the ER/mitochondria interface was still unclear. To solve this question, Endo and co-workers used FRET-based assays with NBD-labeled lipids to show that the assembly of Mmm1 and Mdm12 is critical for efficient lipid transport [192]. Next, they employed a complex reconstitution system using mitochondria and ER membranes isolated from yeast expressing normal or mutated versions of Mmm1 or Mdm12: after adding [¹⁴C]-serine, they measured how the full ERMES complex supported PS synthesis and conversion into PC. This process was impaired if Mmm1 and Mdm12 carried mutations found to stall their transfer activity *in vitro*. This correlation established ERMES as a lipid trading complex at ER-mitochondria contact sites [192]. It is still unclear whether ERMES solely participates in PS-to-PC conversion by trafficking PS and PE, as Mmm1 and Mdm12 have no apparent selectivity (and even affinity) for these ligands. Also, the lipid-binding properties of Mdm34 have not been defined. A new strategy called METALIC (Mass tagging-Enabled TrAcking of Lipids in Cells) has recently been used to track PE and PC transfer between these organelles based on the enzymatic addition of mass tags to GPLs at the ER and mitochondria. ERMES has been identified as critical for these processes, with, interestingly, some aptitude to select lipids according to their unsaturation degree [193].

Therefore, *in vitro* and *in vivo* investigations suggest that ERMES is a general, non-selective transporter for glycerolipids, except CL. Presumably, lipid fluxes between the ER and mitochondria are primarily dictated by the activity of lipid synthesis and modifying enzymes present in the two organelles.

It is still unknown how the ERMES complex between membranes transfers lipids. First, structural analyses showed that purified Mmm1 and Mdm12 could form an elongated complex with two Mmm1 copies at the center and one Mdm12 copy at each end [189,191]. It was suggested that these lipids could slide along the hydrophobic groove, traversing the Mmm1-Mdm12 complex to flow between membranes. However, as Mmm1 and Mdm12 could also form homodimers separately, and Mdm12 could bridge Mmm1 with Mdm34, other mechanistic models were envisioned [189,191]. In view of the recent data obtained on the *in situ* native architecture of ERMES at ER-mitochondria contact sites, it appears today that it was a near-impossible task to understand how ERMES transfers lipids in reconstitution assays with liposomes and an incomplete set of ERMES subunits. Indeed, by combining quantitative live imaging, cryo-correlative microscopy, sub-tomogram averaging, and molecular modeling, Kukulski and co-workers established that Mmm1, Mdm12, and Mdm34 form a trimer in which the subunits are oriented in a zig-zag arrangement. Mmm1 is anchored in the ER and associated with Mdm12, which in turn binds to Mdm34, anchored to the OMM by Mdm10. The cavities of the three SMP-domain-containing proteins form a hydrophobic conduit between the ER and mitochondrial membranes, through which lipids can continuously flow. These data rule out previous models, notably those assuming that the SMP domains of ERMES, somehow connected to membranes, move between membranes to shuttle lipids. The zig-zag arrangement of ERMES and its apparent flexibility raises questions about how lipids slide through the complex [194]. Using functional approaches to measure the lipid transfer activity of mutated versions of ERMES at ER-mitochondrion contact sites coupled with *in situ* structural analyses could provide critical insight into how this complex shuttles lipids.

In 2012–2013, Emr and De Camilli's groups described the contribution of three mammalian proteins called extended-synaptotagmin (E-Syt1/2/3) (tricalbin in yeast) to the formation of ER-PM contacts [195,196]. These proteins comprise an N-terminal hydrophobic hairpin segment anchored to the ER membrane, followed by a linker, an SMP domain, and several C2 domains with a regulatory function or the capacity to dock to the PM by recognizing PI(4,5)P₂ [195]. Reinisch's team, by solving the structure of a fragment of E-Syt2 comprising an SMP and two C2 domains, revealed that the SMP domain forms a dimer, which allows E-Syt2 to homo or heterodimerize Fig. 2G. A hydrophobic seam running along the dimer can host the hydrophobic moieties of four GPL molecules (the electron density of two GPLs *per* SMP domain was observed). A tagged version of the E-Syt2 fragment, expressed in Expi293 cells, was purified, and quantitative lipidomic analysis revealed that it contained mainly PC but also PE, PI, and PS in a proportion similar to that found in the cell. These results, remarkably similar to those obtained with ERMES subunits, showed that E-Syt2 has no apparent GPL specificity [197]. Consequently, this made it possible to study the lipid transfer activity of E-Syts *in vitro* using GPLs with an NBD-labeled head group. The simplicity of FRET measurement allowed for the design of complex experiment settings that aimed to uncover some of the mechanistic aspects of E-Syts. Such assays served to uncover how Ca²⁺ regulates the capacity of E-Syt1 to form ER-PM contacts and transfer lipids, to define the ideal intermembrane distance at which transfer occurs, and to provide insights into how lipids are conveyed by the SMP dimer [198–201]. E-Syts, anchored to the ER and the PM *via* their N-terminal end and C2 domain, respectively, likely function according to two regimes: if

the gap between the ER and PM is exceptionally narrow, the tips of the SMP dimer contact the two membranes, allowing lipids to slide from one to another; if the distance is larger, the SMP dimer moves between membranes to shuttle lipids.

Interestingly, the ligand of E-Syts was not identified by *in vitro* approaches but by in-cell investigations. The absence of E-Syts was found to delay the clearance of DAG from the PM following the breakdown of PI(4,5)P₂ in stimulated cells [199]. The ability of E-Syts to transfer DAG was next established using “light” liposomes functionalized with the cytosolic portion of E-Syt1 and connected with “heavy” liposomes doped with [³H]DAG and PI(4,5)P₂ in the presence of Ca²⁺. DAG transfer was measured by counting the radioactivity associated with each liposome population once disconnected by enzymatic cleavage and separated by centrifugation [199]. Since then, couplings between E-Syt-mediated DAG transfer and signaling pathways have been confirmed in other cellular systems [202,203].

Approaches similar to those used for ERMES and E-Syt were employed to show the activity of other SMP-containing LTPs. TMEM24, which functions at ER-PM contact sites in insulin secretory cells, was copurified with different GPLs and yet, it showed some preference for PI. This led to the finding that TMEM24 transfers PI to the PM to replenish PI(4,5)P₂ levels once insulin has been released by exocytosis [204]. Another SMP-containing protein, PDZD8, was first described as promoting the formation of ER-mitochondria contacts and regulating mitochondrial Ca²⁺ import [205]. It was next found at ER-endosome contact sites where it binds various GPLs, ceramide, and SM but not sterol [206,207]. Despite the use of many *in vitro* transport assays using DHE, NBD-labeled lipids, and lipid sensors, no clear consensus was reached on the specificity of PDZD8. It seems that it can transport PA, PS, and phosphoinositides, including PI(4,5)P₂, PE, and possibly ceramide [206,207]. However, data obtained in early *C. elegans* embryos suggested that PDZD8 transfers PI(4,5)P₂ from the endosomes to the ER and, with the help of PI(4,5)P₂ phosphatases, maintains a low level of this lipid in the endosomal compartment [206]. In yeast, the SMP-domain-containing protein Nvj2 has been reported to form ER-Golgi contacts upon ER stress to transfer ceramide from the ER to the Golgi for conversion into sphingolipids. Thus, the cell is prevented from accumulating ceramide and being intoxicated [208]. *In vitro* assays showed that a recombinant form of Nvj2 added to yeast lysate promotes sphingolipid synthesis; however, there is no direct proof that it binds nor transfers ceramide. Tex2, the Nvj2 homolog in metazoans, was proposed to function like PDZD8 to regulate endosomal PI(4,5)P₂ level in *C. elegans* [206]. In humans, Tex2 was found to regulate the lipid composition of late endosomes/lysosomes via distinct modalities [209]. Its SMP domain was found to trap endogenous PC, sphingosine, and ceramide but not PIPs [209]. Overall, the lipid selectivity of Nvj2/Tex2 remains quite mysterious.

9. Bridge-like LTPs: a new mode of lipid transfer between membranes

In the last decade, five families of new LTPs, unified under the name of bridge-like LTPs (BLTPs), have been discovered in eukaryotes: VPS13, ATG2, BLTP1, BLTP2, and BLTP3 ([210,211]). Cryo-electron microscopy combined with structural prediction algorithms has shown that the core of these giant proteins (2000–5000 aa) corresponds to a slightly twisted rod-like structure resulting from the assembly of Repeating β -Groove domains (RBG, 150 aa). Each RBG domain contains a hydrophobic cavity which together form a long hydrophobic groove running along the entire rod, ideally suited for the passage of lipids [212,213]. The chorein and APT1 regions, encompassing RBG domains at the N-terminal and C-

terminal ends of the rod, respectively, are evolutionarily conserved. The length of the rod, which depends on the number of repeated RBG domains (from 6 to 17), is predicted to vary between 12 and 33 nm [10]. Bridge-like LTPs can thus span the distance between organelles at contact sites, as verified *in situ* with human VPS13 [214]. Thanks to motifs and domains appended to the central rod-like structure, they can engage with contact sites by associating with lipids and protein partners. Extensive works on the VPS13 and ATG2 family have suggested that bridge-like LTPs flow lipids in bulk between connected organelles but are also crucial for the *de novo* formation of cell compartments. In particular, ATG2 is critical during autophagy, a process by which a portion of cytoplasm can be sequestered and conveyed to the lysosomes for degradation. By providing lipids, ATG2 guarantees the expansion of an isolation membrane adjacent to the ER to form the autophagosome (see recent review [215]).

The discovery of VPS13 and ATG2 as a family of LTPs came partly from approaches similar to those used for characterizing SMP-containing proteins (identification of lipids bound to protein purified from cells, native gel assays to measure how NBD-labeled lipid species are trapped). N-terminal fragments of ATG2 and VPS13 encompassing the chorein region were copurified almost exclusively with GPLs, which were bound according to their cellular abundance, and no other lipid classes [216–218]. Remarkably, the full-length human ATG2A (~1940 aa) was loaded with ~20 GPLs at once (and a 1400 aa-fragment of fungus VPS13 was bound with ~10 GPLs) [217,218], pointing to a role of these proteins in bulk lipid transfer. The crystal structure of the chorein region of yeast Atg2 bound to one PE molecule and of VPS13 revealed a lipid binding mode akin to that reported for SMP domains (Fig. 2H) [216,217]. The cryo-EM structure of the N-terminal region of Vps13 revealed that a large section of its hydrophobic tunnel is too narrow in diameter to let more than one lipid at a time, suggesting that lipids can only move directionally. Much evidence on ATG2 suggests that this movement occurs from the N- to the C-terminal tip of the rod [215]. All these findings have led to the idea that BTLPs ensure massive and vectorial transfer of GPLs in eukaryotic cells.

This assumption was indirectly validated by experiments showing that mutations disrupting the continuity of the hydrophobic groove of VPS13 block its capacity to provide lipids for the expansion of prospore membranes in sporulating yeast [213]. FRET-based assays using NBD-labeled lipids were also used to show that the chorein region or longer N-terminal fragment of ATG2 and VPS13, artificially confined between liposomes, can extract and deliver lipids [216–218]. More realistic assays with full-length yeast or human ATG2 were conducted in conditions where the C-terminal region of ATG2 was bound to PI(3)P-rich acceptor liposomes, mimicking the phagophore membrane via its interaction with PROPPIN proteins (yeast ATG18 or human WIPs) [212,216]. In these conditions, which favor the ability of ATG2 to connect membranes, lipid transfer was faster. However, the few attempts made to calculate lipid transfer rates from fluorescence traces have provided a maximal value of ~0.017 s⁻¹ per copy of BLTP (i.e., ATG2) [212], lower than those measured with ORPs (0.4–0.5 s⁻¹) [39,43,60] or StarD4 (0.1–0.2 s⁻¹) [144,219]. This contradicts the expectation that BLTPs function as “firehoses” for lipids, working much faster than classical box-like LTPs. However, several points must be considered. First, BLTPs can recognize GPLs with low selectivity; therefore, in FRET-based assays, unlabeled GPLs of the donor liposomes are likely transported along with NBD-labeled ligands, leading to an underestimation of the transfer rate. In the reference [212], NBD-PE represents only 2 % of GPLs in donor liposomes, meaning that ATG2’s actual lipid transfer rate can be, in theory, much higher, equal up to 50 × 0.017 = 0.85 s⁻¹. Second, in this experimental setup, lipids can potentially move both ways in ATG2’s tunnel.

Given donor and acceptor liposome concentration, NBD-PE might represent only 1 % of transferable lipid, meaning that the lipid transfer rate is potentially the double, close to 2 s^{-1} . Finally, the estimation of the transfer rates is possibly distorted as Rhod-PE, which must remain in the donor liposomes to provide an interpretable fluorescence signal, is probably transported by ATG2 to acceptor liposomes. Using non-transferable fluorescent membrane-spanning lipids could partially help overcome this particular issue [220]. However, even after refining some numbers, we cannot conclude today from *in vitro* assays that a BLTP such as ATG2 transfers lipids much faster than a box-like LTP does. Presumably, the measurement of very high-speed vectorial lipid transfer by BLTPs will require much more sophisticated reconstitution systems, whose design has just started to be imagined [221]. Notably, a strong assumption is that BTLs interact and function with scramblases, transmembrane proteins that can swiftly equilibrate lipids between membrane leaflets to ensure fast lipid transfer between membranes and *de novo* formation of subcellular compartments. First attempts have been made to examine this partnership *in vitro* (e.g., ATG2 with ATG9) [222].

10. Newcomers with elusive lipid specificity at the mitochondrial surface

Mitoguardins 1 and 2 (MIGA) are proteins that maintain the capacity of mitochondria to fuse with each other [223]. Besides this, in white adipocytes, MIGA2 functionally links the mitochondrial and ER branches of a metabolic pathway that produces FAs from carbohydrates and incorporates them in TAGs (*de novo* lipogenesis); as a result, MIGA2 promotes LDs biogenesis in which TAGs are ultimately stored [224]. MIGA2 is anchored in the OMM *via* two N-terminal TM segments and associates with the ER by interacting *via* a FFAT motif with VAPs (Fig. 1). It also contains a C-terminal domain to interact with LDs (LD targeting domain), which has recently been found to be an all-helical structure with an L-shaped hydrophobic cavity [225,226]. A GPL, likely a bacterial PE or PC, co-purified with the protein, is found in this cavity (Fig. 2I). The absence of electrostatic or hydrogen-bonding interactions between the GPL head group and the protein suggests a non-specific binding mode mediated by hydrophobic interaction. Moreover, MS analyses have revealed that MIGA2 isolated from cells is loaded with free FAs and GPLs, mostly PC and PE, and, to a lesser extent, TAG. Lipid competition and transfer assays using NBD-labeled lipids have confirmed that MIGA2 recognizes these lipids and can transport diverse GPLs [226]. However, using similar assays, MIGA2 has also been reported to be a specific PS transfer protein [225]. Such discrepancies likely come from differences in experimental design (in one case, the LD domain is artificially attached to liposomes). Introducing mutations in MIGA2 that change its capacity to transfer lipids *in vitro* affects mitochondria morphology and LD biogenesis and growth. MIGA2 presumably acts as a non-specific transporter of FA and GPLs at three-way contact sites between the LDs, mitochondria, and the ER. It may supply mitochondria with PA to maintain their fusogenic properties or PS to promote PE synthesis. It may also facilitate PC/PE and FA transport between LDs and mitochondria and FA transport between mitochondria and the ER to aid TAG synthesis. Therefore, a future step is to precisely determine the physiological ligands of MIGA2 to better define its function.

Another newly discovered LTP is Spartin, which is localized on LDs and involved in their autophagy (mitophagy), a process allowing the mobilization of TAGs. Defects in this protein are associated with the Troyer syndrome, a form of complex hereditary spastic paraplegia. Spartin is recruited *via* its LC3A/C-interacting region (LIR) motif by the core autophagy machinery and can bind to LDs thanks to its C-terminal senescence domain [227]. An MS

analysis has recently revealed that Spartin, purified from cells, is loaded with lipids usually enriched in LDs (DAG, TAG, sterol esters, PC, PE) [226]. *In vitro* investigations have shown that Spartin transfers NBD-PC better than NBD-PE and NBD-PS between liposomes *via* its senescence domain [228]. Remarkably, a mutation that disables the transfer capacity of Spartin profoundly impacts LD turnover; it has thus been suggested that Spartin may promote the breakdown of LDs during lipophagy by transferring GPLs from or into LD's monolayer. It is unclear whether the senescence domain transfers TAGs, with which it is predominantly copurified from cells. It is also unknown how lipids are hosted in this domain, of which neither a structure nor a reliable structural model have been established [228].

A third, potentially new LTP anchored at the mitochondria surface is PTPIP51 (Protein Tyrosine Phosphatase Interacting Protein-51), which contributes to tethering ER and mitochondria by binding to VAPs. It facilitates IP₃ receptor-mediated delivery of Ca²⁺ from ER stores to mitochondria and its metabolism [229,230]. Recently, it has been reported that the tetratricopeptide repeat (TPR) domain at the C-terminal half of PTPIP51 is a PA transfer module [231]. From a biochemistry perspective, this is not well ascertained. First, although a ligand is associated with a solvent-exposed cavity of the TPR domain, whether this is a genuine lipid is unknown. Second, there is no assessment *via* an unbiased approach that PTPIP51 preferentially binds PA among other lipids. Third, the use of non-normalized transfer assays based on fluorescently labeled PA, high LTP: ligand molar ratios, and/or conducted over periods of hours does not certify that PTPIP51 is a PA transporter. Nevertheless, distinct investigations in cells support the notion that PTPIP51 transfers PA at the ER-mitochondrion interface to promote CL synthesis [231,232].

11. Conclusion - *In vitro* veritas?

Over the last 60 years, *in vitro* approaches have been vital to identifying proteins that transfer lipids in eukaryotic cells with diverse degrees of selectivity. Due to space limitations, we could not present the full story of many of them, and we apologize for this. It is worth mentioning the essential investigations by Brown and co-workers on the functional features of glycolipid transfer protein (GLTP) in the '90s and, more recently, their contribution to identifying close homologs of this LTP, notably ceramide-1-phosphate transfer protein (CPTP) and FAPP2 (see review [233]). This work was done *via* structural analyses [234,235] combined with lipid transfer assays based on FRET between lipid ligands labeled with anthrylvinyl and perylenoyl-labeled lipids [235–237]). Mention should also be made of the non-specific lipid transfer Sc2p on which important studies have been carried out (see review [238]). Likewise, we would like to report the recent discovery of a yeast ceramide transporter, Svf1, which plays a role analogous to CERT's [239]. Interestingly, it seems that a standardized way to identify LTPs is emerging today. It consists in identifying the lipid(s) bound to a putative LTP expressed in cells, assessing whether the protein transfers fluorescently labeled lipids between liposomes, and analyzing how the inactivation of its transfer capacity affects a cellular function. However, more detailed investigations are needed to understand the function of several newly discovered LTPs, as their lipid specificity is not fully delineated.

In vitro veritas? Undoubtedly, the answer is partially yes. *In vitro* approaches have greatly aided in defining whether potential LTPs can transport lipids and in determining their lipid selectivity and mode of functioning. But of course, by design, they are not suitable for discovering the intracellular lipid transfer routes these proteins mediate or how they are regulated inside the cells. Therefore, solid

insights into the biological functions of LTPs have only been obtained by cross-validating *in vitro* data with in-cell investigations. As highlighted by Levine and collaborators, straight conclusions can be obtained for LTPs that functionally link spatially distant steps of lipid metabolism pathways by moving lipids (e.g., CERT, Ups/PRELI) [3]. It is more difficult to be conclusive when the role of LTPs is to regulate the composition and bulk properties of membranes, like their surface charge density or fluidity, as this implies challenging investigations using microscopy and *ad hoc* fluorescent sensors to monitor lipid movement and accumulation. Considerable efforts are continuously being made to accurately measure the transfer activity and lipid selectivity of LTPs directly in cells. How can this be achieved? Ideally, by applying the basic principles of *in vitro* transfer assays. The first condition is to have an identifiable pool of the lipid species whose transfer is to be monitored in an organelle membrane, defined as a donor membrane. The second is to have a quantitative measure of the passage of this lipid pool to an acceptor membrane. The third is to determine the origin of this lipid flux by modifying the expression or functionality of candidate LTPs. The fourth condition is to determine when the measurement of the lipid flow starts.

In our opinion, pulse-chase experiments based on traceable lipid precursors and their modification by spatially localized enzymes coupled to genetic or pharmacological manipulations of LTPs remain among the best ways to measure intracellular lipid transfer [161,179,192]. In particular, as mentioned below, using assays based on radiolabeled precursors has been particularly fruitful in capturing the kinetics of the lipid transfer and/or exchange activity of many LTPs at contact sites. However, these assays are based on identified endogenous metabolic pathways and can only be used to examine specific lipid fluxes. The METALIC approach based on endogenous and targetable lipid-modifying enzymes and deuterated precursors constitutes a promising step towards developing protocols to track the transfer of any GPL and possibly sphingolipids at different intracellular interfaces. Importantly, it offers a high-resolution view of the transferred species, notably their acyl chain profile [193,240]. Chemically or light-based dimerization strategies to trigger the translocation and activity of LTPs at contact sites coupled with genetically encoded fluorescent lipid sensors are also powerful strategies to identify intracellular lipid transfer routes [50,54,56,94,204]. It is worth mentioning the assay used to measure Osh-mediated PS transfer, which can be viewed as “hybrid” as it combines the pulse-chase principle with *in situ* and real-time lipid detection and might inspire the design of other protocols. In this hybrid experiment, the restoration at a given time of a PS pool at the ER through the acylation of exogenously added lyso-PS and the use of a fluorescent PS sensor made it possible to measure the kinetics of PS transfer mediated by Osh6 and Osh7 at the ER/PM interface [47,50].

Yet, despite their robustness, it is not yet sure that these approaches in cells might allow LTP chemistry to be dissected as precisely as in *in vitro* assays. For instance, these approaches rarely offer an absolute quantification of the lipid flux and LTP responsible for this flux and/or lack temporal resolution and thus cannot provide lipid transfer rates. It is also unclear whether they can explain how membrane features impact LTP activity. However, the most problematic issue is that the analysis of intracellular lipid fluxes is always associated with uncertainties due to side processes, such as the constant remodeling of lipids and redundant or compensatory lipid transfer pathways. Thus, *in vitro* approaches remain invaluable in offering solid hallmarks on LTP's biochemistry and in helping us navigate the complexity of intracellular lipid transfer.

What information could we obtain in the future from *in vitro* assays? What technical improvements could be made? First, we lack numbers for LTP's activity rates *in vitro*, as data are not

systematically normalized in terms of transferred lipids *per* protein and time unit. It is critical to establish more precisely the speed at which box-like and bridge-like LTPs function to better define their respective role in cells. Besides this, we still do not know whether several LTPs belonging to “old” families (e.g., Nir2/Nir3, Sfh proteins), which bind two lipid ligands, act as lipid exchangers, *i.e.*, transfer these ligands efficiently between membranes, as shown for Osh proteins. For instance, can we prove *in vitro* that Nir2/Nir3 act as PA/PI exchangers as proposed in current models of the PI cycle [112]? So far, these potential exchangers have been tested for their capacity to transfer each lipid ligand separately and often in assays with a low temporal resolution and/or with membranes that were not chemically defined (e.g., microsomes or mitochondria). It is thus necessary to further determine *in vitro* how these LTPs function to shed light on their cellular roles. We have also noted intriguing mismatches between *in vitro* data, structural analyses, and cellular observations related to SMP-domain-containing LTPs, initially suspected to be poorly selective, considering their structural features. For instance, it is surprising to see that *in vitro* ERMES subunits weakly bind to PE and PS compared to PA and PC [190,191] and that E-Syts and TMEM24 are selective for DAG and PI in cells [199,204]. The features of cellular membranes depend on the nature of the polar head and the acyl chains of lipids. Many efforts have been made to understand how enzymes use and produce lipids with specific lengths and unsaturation degrees [241]. In contrast, only a few studies [162,242–244], including ours, have addressed how LTPs transfer lipids as a function of their acyl chain; thus, efforts have to be made to define to what extent LTPs can fine-tune the acyl chain composition of cell membranes. Finally, we could still make surprising discoveries relative to the selectivity of well-known transporters, as exemplified with StarD7 [163] and recently with NPC1. Using click-chemistry, Höglinger and co-workers revealed that this vital transporter responsible for the egress of free sterol from lysosomes may also transport sphingosine out of this compartment [245]. It has also been recently reported that several human fatty-acid binding proteins act as sterol transport proteins [246]. We believe it necessary to emphasize once again that the identification of new LTPs and their ligand must be based on *in vitro* assays using membranes of high compositional complexity. Indeed, the risk is too great of considering a fortuitous ligand as the authentic ligand of an LTP, when using assays with membranes that are too minimalist in composition.

Certainly, from a technical standpoint, spectroscopy approaches are the most desirable for accurately measuring lipid flow in various and sophisticated experimental setups, notably to investigate the interplay between the membrane tethering and lipid transfer capacities of LTPs. Lipids bearing an extra fluorescent moiety have been extensively used to obtain a spectroscopic signal. In the case of SMP-domain-containing LTPs, bridge-like LTPs, or MIGA2, with a low selectivity for GPLs, lipids with a labeled head group have been used with reasonably good results. In parallel, lipids with labeled acyl-chains have been widely used for LTPs such as ORPs, Sec14/Sfh, PITP, and other START-like proteins with a high selectivity for GPLs. Some of these have been reported to similarly bind and transfer natural ligands and analogs bearing different types of labels (*i.e.*, NBD, pyrene, or doxyl) [69,71,102,109]. DHE, CTL, and parGPLs are a particular class of fluorescent lipids, as they have no extra moiety and strongly resemble natural lipids. However, doubts have been raised about the capacity of these molecules to be reliable mimetics of sterol and GPLs [71,247]. For instance, parPC is less well recognized than regular PC by PITP α and StarD2, suggesting that a small network of double bonds in one acyl chain can make a difference [71]. Using competition assays, Ridway's team observed that OSBP has much less affinity for DHE and CTL than cholesterol [247]. We and others have also found that small changes

in the length and unsaturation degree of GPLs can drastically change how they are bound and transported by LTPs [60,71]. Thus, we must consider that using fluorescent lipids, even chemically close to their natural counterparts, will never be ideal and can provide outcomes that intimately depend on the nature of the LTP. It is also worth noting that adding a fluorescent dye can strongly disturb the intrinsic behavior of a lipid. Note the case of 22-NBD-cholesterol, which has a spontaneous transfer rate much higher than that of cholesterol and is thus not suitable for transfer assays [248]. In the future, as exemplified by a study on Sec14 [110], the SANS approach based on deuterated lipids may be the method of choice to measure LTP activity in real-time.

Another way to track the movement of natural lipids between membranes is the utilization of fluorescent lipid sensors. A few sensors have been developed to measure the transfer of PS, PI(4)P, and PI(4,5)P₂ between liposomes [39,60]; others, derived from known lipid-binding domains (C1, Spo20, D4H [249]) to follow the movement of lipids like DAG, PA, and sterol, respectively have yet to be developed or used *in vitro*. However, these sensors have limitations. Notably, caution must be exercised when varying liposome compositions in the assays, as the binding of these probes to their ligand can be significantly impacted by various membrane features (negative charge, lipid packing); each time a new lipid composition is tested, control experiments must be conducted to correctly interpret and convert the fluorescence signal in terms of transferred lipid. These sensors cannot easily be used to analyze the activity of LTPs in artificial contact sites. Indeed, in these heterogeneous systems where liposomes connected by LTPs tend to aggregate massively over time, it is difficult to predict how these sensors can detect lipid movement. Sensors that track the movement of lipids like PC and PI are still lacking, but efforts have been made to fill this gap. Recently, it has been demonstrated that PI sensors for in-cell investigations can successfully be derived from an inactivated PI phospholipase [250].

Beyond the question of selectivity, there are other future challenges. In addition to better tracking of lipid movements between membranes to obtain kinetic parameters, it would be useful to analyze LTPs in different and/or more sophisticated reconstitution systems. For instance, it is essential to develop assays to measure lipid transfer between artificial LDs and bilayers and to analyze the functions of various LTPs [228]. Another key issue is to understand how most LTPs interact with lipids and protein partners to connect membranes and transfer lipids at contact sites. For instance, one of the biggest challenges would be to reconstitute the partnership of bridge-like LTPs, difficult to purify, with their various partners such as scramblases [210] and to determine whether parameters like osmotic membrane tension can drive a fast and unidirectional transfer of lipids by these proteins, as proposed recently [221].

Many LTPs function together in the same contact sites, coupled to metabolic pathways (e.g., OSBP and CERT, Nir2, and E-Syt1 [112]). An important challenge is to deeply analyze *in vitro* the links between lipid synthesis and different transfer pathways to understand how lipid metabolism relates to lipid fluxes within the cell. Recently, using functionalized liposomes and fluorescence tools, we managed to reconstitute the functional interplay between Osh4, Sec14, Sac1, and PI 4-kinase using a liposome-based system, showing that PI transfer by Sec14 can assist Osh4-mediated sterol/PI(4)P exchange by promoting PI(4)P synthesis [113]. To produce the most reliable kinetic data, the design of these reconstitution systems must take advantage of the most recent lipidomic (e.g., Ref. [251]) and microscopy data [252], which provide an unprecedented view of the lipid composition and shape of the organelle membranes (i.e., curvature) to which LTPs are bound.

The activity of LTPs is still investigated *in vitro* in shaking/

stirring conditions. Most often, the concentrations of protein and lipids in these assays are similar to those found in cells. However, the distance between membranes constantly fluctuates, and the volume explored by LTPs is incredibly vast compared to cell dimensions. A daunting task is to invent much more realistic setups in which the volume explored by an LTP and the distance between membranes are fixed and similar to those measured in authentic cells, possibly using microfluidic approaches.

Competing interests

The authors declare that they have no competing interests.

CRediT authorship contribution statement

Amazigh Hamai: Writing – review & editing, Writing – original draft, Conceptualization. **Guillaume Drin:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

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