

# Membrane Contact Sites: Complex Zones for Membrane Association and Lipid Exchange

## Supplementary Issue: Cellular Anatomy of Lipid Traffic

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**ABSTRACT:** Lipid transport between membranes within cells involves vesicle and protein carriers, but as agents of nonvesicular lipid transfer, the role of membrane contact sites has received increasing attention. As zones for lipid metabolism and exchange, various membrane contact sites mediate direct associations between different organelles. In particular, membrane contact sites linking the plasma membrane (PM) and the endoplasmic reticulum (ER) represent important regulators of lipid and ion transfer. In yeast, cortical ER is stapled to the PM through membrane-tethering proteins, which establish a direct connection between the membranes. In this review, we consider passive and facilitated models for lipid transfer at PM–ER contact sites. Besides the tethering proteins, we examine the roles of an additional repertoire of lipid and protein regulators that prime and propagate PM–ER membrane association. We conclude that instead of being simple mediators of membrane association, regulatory components of membrane contact sites have complex and multilayered functions.

**KEYWORDS:** membrane contact sites, membrane lipids, nonvesicular transport, membrane-tethering proteins, lipid transfer proteins, plasma membrane, endoplasmic reticulum

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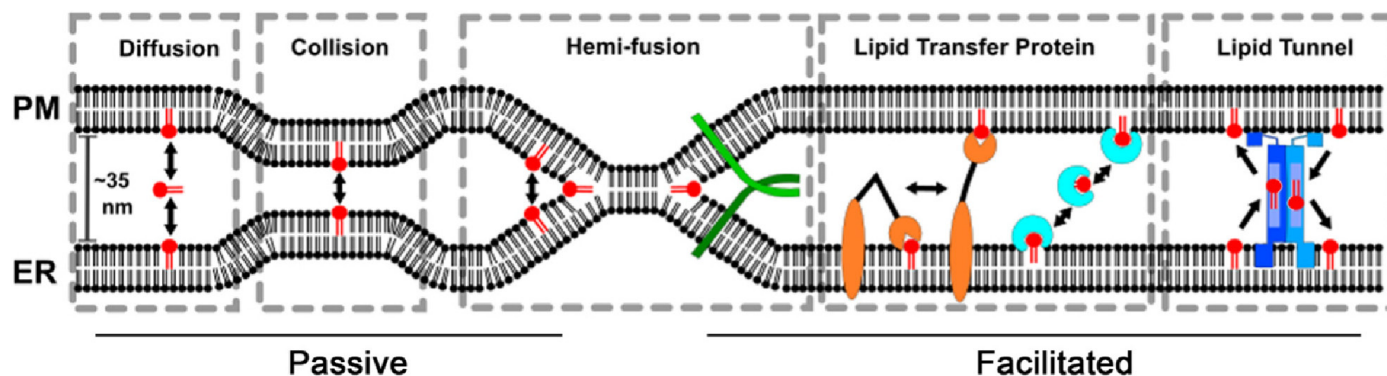
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## Membrane Contact Sites, Stapled Together by Tethering Proteins, Serve as Zones for Nonvesicular Transfer

Two general modes of transfer dictate lipid exchange between membranes within the cell: vesicular and nonvesicular lipid transport.<sup>1–3</sup> Given that secretory vesicles are inherently composed of lipids, it is not surprising that vesicles mediate the bulk of lipid transport. In contrast, lipid transfer can also occur in the absence of vesicles through mechanisms that are less well defined. In addition to bona fide soluble lipid transfer proteins (LTPs) that shuttle lipids between membranes, lipid exchange also involves specific sites where intracellular membranes are closely apposed (Fig. 1). In fact, LTPs and membrane contact sites appear to be interrelated mechanisms that together mediate nonvesicular transport.

Membrane contact sites are distinct regions between heterotypic organelle membranes that align within close proximity of one another, ~10–50 nm apart.<sup>4</sup> This review focuses on the mechanisms promoting membrane contact between the plasma membrane (PM) and cortical endoplasmic reticulum (ER) in yeast. However, contact sites are observed in all eukaryotes between the mitochondria and ER, between

the Golgi and ER, or between the nucleus and vacuole.<sup>5–10</sup> A diverse set of membrane-tethering proteins participate in the direct attachment of the PM and ER membranes, as discussed later. Of those identified to date, several tethering proteins are necessary for PM–ER contact, and their deletion, either individually or in combination, reduces the number of discrete membrane contact sites. While these primary tethering proteins establish initial membrane contact, still other proteins might maintain membrane association at contact sites. For example, secondary tethering proteins or ancillary regulators might fortify or expand the association of membrane around the established contact sites. Such secondary tethering proteins and regulators would be predicted to be sufficient for promoting membrane contact, but not necessary. In other words, they would be dispensable for establishing contact but might enhance PM–ER membrane association if overexpressed. Membrane association conferred by these secondary tethering proteins and regulators would also be predicted to be dependent on the primary tethering proteins. In this context, we examine both PM–ER membrane-tethering proteins and potential ancillary factors, including LTPs, that affect nonvesicular lipid transport. We also note that tethering



**Figure 1.** Mechanistic models for nonvesicular lipid transport between the ER and PM at membrane contact sites. Hypothetically, lipids' exchange (red) between membranes involves passive or facilitated mechanisms. Overcoming a large energy barrier due to lipid exposure to the aqueous cytoplasm, passive lipid exchange models (left) involve spontaneous lipid transfer in the absence of a protein intermediate. *Diffusion* occurs when a lipid is ejected into the aqueous phase, followed by its diffusion and reinsertion to an acceptor membrane. *Collision* of membranes might permit a direct exchange of lipids when bilayers transiently collide, thereby minimizing exposure to the aqueous cytoplasm. *Hemifusion* might involve a more persistent collision whereby direct lipid exchange occurs by *cis*-leaflet fusion between the PM and ER membranes. The hemifusion intermediate might also be facilitated by proteins (green), leading to incomplete SNARE-mediated fusion (eg, between the ER-resident R-SNARE Sec22p and a PM-localized Q-SNARE). Facilitated mechanisms might also involve the active exchange of lipids between membranes via membrane-bound or soluble *lipid transfer proteins* (LTPs) or *lipid tunnels* (blue) that contain putative hydrophobic channels mediating a rapid lipid flux between membranes.

proteins are not just membrane staples that join membranes but also have distinct specific functions, such as in nonvesicular transport.

### Soluble LTPs Mediate One Aspect of Nonvesicular Transport at Membrane Contact Sites

Soluble LTPs, such as the ceramide transfer protein (CERT) and the sterol carrier (STARD4), represent paradigms for nonvesicular lipid transport. As reviewed by others,<sup>11,12</sup> their mechanisms of lipid capture, lipid shielding from the aqueous cytoplasm, and trafficking to target membranes are well described. CERT and other soluble LTPs are also enriched at membrane contact sites, suggesting an additional complexity in the mechanism of nonvesicular lipid transport.<sup>7,13–17</sup> The oxysterol-binding protein-related proteins (ORPs) represent yet another potential class of LTPs, and a number of yeast and mammalian ORPs are recruited to the ER and PM–ER contacts,<sup>14,15,17–21</sup> At these sites, recent studies showed that the mammalian ORP5 and ORP8 and the yeast ORP Osh6p act as phosphatidylserine (PtdSer)/phosphatidylinositol 4-phosphate (PtdIns4P) transfer proteins.<sup>18,19</sup> It is proposed that these ORPs move PtdSer against a concentration gradient from the ER to the PM by coupling its transfer to the energetically favorable transport of PtdIns4P in the reverse direction. The concept of this *powered exchange mechanism* is based on the *in vitro* liposome experiments involving the reciprocal exchange of sterols and PtdIns4P by another yeast ORP, Osh4p.<sup>20,22</sup> However, unlike the ORPs that exchange PtdSer for PtdIns4P, the role of Osh4p in sterol/PtdIns4P exchange is unclear. Deletion of *OSH4* or, for that matter, elimination of all yeast ORPs has no impact on nonvesicular sterol transport from the ER, where sterols are synthesized, to the PM where sterols are concentrated.<sup>23</sup> In hypoxic cells forced to take

up exogenous sterols, the redistribution of sterols from the PM to internal lipid droplets (involving several intermediary steps, including ER sterol esterification) slows by ~50% when ORPs are eliminated.<sup>23</sup> This small effect was suggested to be a downstream consequence of eliminating Osh protein function.<sup>23</sup> In contrast, the elimination of all yeast ORPs greatly increases PtdIns4P levels, which led to the proposal that the primary and collective function of yeast ORPs actually involves phosphoinositide regulation.<sup>17,24,25</sup> Phosphoinositides play an important role in regulating membrane contact by tethering proteins, as discussed later. Regardless of how sterol distribution is affected, LTPs, like the ORPs, seem to regulate lipid transfer and phosphoinositide metabolism, which affect the bilayer properties of the PM and ER membranes.

### Contact Sites Between Organelles Allow Lipid and Second Messenger Exchange and Membrane Modification *in Trans*

Classically, membrane contact sites were defined by direct observation or through biochemical fractionation. In muscle cells, Porter and Palade<sup>5</sup> reported early descriptions of ER (sarcoplasmic reticulum) membrane associations with the PM. Later, the biochemical purification of *Fraction X* identified a distinct microsomal *membrane preparation* that had phospholipid enzymatic activities corresponding to contact sites at mitochondria-associated ER membranes (MAMs).<sup>26</sup> MAMs are presumed to provide the conduit through which phospholipid precursors (ie, PtdSer) are transferred from the ER to the mitochondria, enzymatically converted to the next pathway intermediate (ie, phosphatidylethanolamine [PtdEtn]), and then returned back to the ER to complete the phosphatidylcholine (PtdCho) synthesis.<sup>10,27–30</sup> A similar PM-associated ER membranes' (PAMs) subfraction is also enriched for



phospholipid and sterol synthetic machinery.<sup>31</sup> Although the nonvesicular transfer of sterols is postulated to occur at these PAM/PM–ER contact sites,<sup>1,32</sup> direct experimental tests of this proposal are still needed. At contact sites, lipid substrates can be exchanged between membranes, or alternatively, lipid-modifying enzymes resident on one membrane can reach across to act on another. As an example, the ER-associated PtdIns4P phosphatase, Sac1p, is proposed to act (in conjunction with yeast ORPs) in *trans* to dephosphorylate PtdIns4P present within the PM.<sup>17</sup> These examples underscore the importance of contact sites as membrane zones for integrating different aspects of lipid metabolic pathways.

Apart from lipids, membrane contact sites also provide restricted zones for specific and efficient transfer of second messengers in particular calcium. Contact sites between the ER and mitochondria or between the ER/sarcoplasmic reticulum and PM create a signaling microdomain to localize the diffusible signaling potential of calcium ions.<sup>9</sup> These localized calcium signals drive downstream responses, including the regulated release of vesicles, the stimulation of mitochondrial metabolic processes, and the release of intracellular (ER or sarcoplasmic reticulum) calcium stores.<sup>33,34</sup> In some cases, calcium regulation and lipid organization are integrated to maintain membrane tethering at contact sites. For instance, in mammalian cells, the ER transmembrane Ca<sup>2+</sup> sensor, STIM1, clusters in response to low calcium levels within the ER lumen.<sup>35</sup> This clustering extends STIM1 toward the PM where it interacts with the Ca<sup>2+</sup> release-activated Ca<sup>2+</sup> channel subunit, Orai1, to form the PM–ER contact sites.<sup>35</sup> At these sites, Orai1 mediates calcium influx through the PM and across the small gap between the PM and ER membranes, which enables calcium uptake into the ER to replenish its stores.<sup>35–37</sup> The activity of the Orai1–STIM1 complex at these membrane contact sites is regulated by PtdIns(4,5)P<sub>2</sub>,<sup>38</sup> which is also a lipid regulator of other membrane-tethering proteins (see section “Membrane Domains and Bilayer Asymmetry as Determinants of PM Contact with Cortical ER”).

### Passive Lipid Exchange Between Membranes at Contact Sites: How Close is Close Enough?

The physical properties and morphological architecture of membranes at contact sites appear to be conserved from yeast to humans.<sup>39</sup> In yeast, PM–ER contact is extensive where ~45% of the inside surface of the cortex is covered with ER tubules, and PM and ER membranes are separated on average by 33 nm.<sup>4</sup> Given that calcium can readily traverse this negligible distance at membrane contact sites in muscle cells, it is tempting to speculate that lipids might as well. Without the assistance of an associated protein transporter, spontaneous lipid exchange between two membranes could theoretically occur through several mechanisms: (i) aqueous diffusion; (ii) membrane collision; and (iii) transient bilayer hemifusion (Fig. 1).

As one of the least hydrophobic lipids, unesterified cholesterol is arguably a prime candidate for a lipid that can be

transferred by aqueous diffusion between membranes at contact sites. Aqueous diffusion involves the first-order kinetics of lipid desorption from a donor membrane into the aqueous cellular milieu, followed by diffusion and reinsertion of the lipid into an acceptor membrane (Fig. 1).<sup>2</sup> However, because the step of lipid desorption from the donor membrane is rate limiting, this type of spontaneous lipid transfer is not contingent on membrane proximity to the acceptor. As determined *in vitro*, the half-time of spontaneous cholesterol transfer between donor (20 mol% cholesterol) and acceptor vesicles is 2.3 hours,<sup>40</sup> but *in vivo*, the approximate half-time of cholesterol exchange between the ER and PM is 4 minutes.<sup>23</sup> Thus, the amount of cholesterol that is spontaneously ejected into the aqueous phase can only account for 3% of the observed amount transferred *in vivo*. For more hydrophobic lipids, half-times for transfer are much longer,<sup>41</sup> precluding aqueous diffusion as a general mode of lipid transfer. For example, the half-times for spontaneous transfer between membranes for phospholipids, such as 1-palmitoyl-2-oleoyl PtdCho and dipalmitoyl PtdCho, are 48 hours and 83 hours, respectively.<sup>40,42</sup> It is true that for measurements involving biological membranes containing embedded proteins, the physicochemical nature of the bilayer might have an unpredicted effect on lipid ejection. However, the data suggest that even for less hydrophobic lipids, aqueous diffusion across the aqueous gap at contact sites is insufficient to meet the cellular requirement for lipid exchange.

In contrast to aqueous diffusion, a direct exchange of lipids might result from stochastic collisions of two membranes, without exposing hydrophobic lipid side chains to the cytoplasm (Fig. 1). Contact sites could promote direct membrane interactions as required by both lipid collision and facilitated collision models.<sup>43</sup> In these models, lipid exchange proceeds either through superficial surface interactions or through partial fusion between donor and acceptor membranes.<sup>2</sup> Facilitated collision also involves stressing the packing order of lipids to cause their protrusion from the bilayer, thereby decreasing the energy required for transfer. Through facilitated or passive mechanisms, donor and acceptor membranes might completely or partially (hemifusion) fuse, allowing lateral diffusion of lipids between bilayers (Fig. 1).<sup>43</sup> For instance, it has been shown that the ER-resident R-SNARE Sec22p interacts with the PM-localized Q-SNARE to facilitate an interaction between PM and ER membranes, which, based on the known SNARE interactions, could be postulated to promote partial membrane fusion.<sup>44</sup> However, Sec22p was experimentally shown not to mediate the partial fusion because membranes at contact sites where it is present do not get closer than ~15 nm apart, and lipid mixing does not occur.<sup>44</sup> Although these facts alone do not preclude the existence of short-lived membrane fusion bridges, the inability to detect them suggests that they cannot be the major conduits for lipid exchange. More likely are models in which lipids are actively transported by protein shuttles, such as LTPs, or possibly through proteinaceous tunnels that span the gap between membranes at contact sites (Fig. 1). In agreement





with these models, isolated PM–ER membrane contact sites from *unroofed cells* (where the cell membrane is sheared away, dispersing the cytoplasm and leaving the inner face of the cell surface) exhibited no detectable passive sterol or phospholipid transfer.<sup>45</sup> However, these results would also seem to preclude the possibility of any lipid-transferring tunnels or membrane-bound LTPs, which ought to have been present and active in these isolated contact sites (assuming that sterol transfer occurs at these contact sites in the first place). Unfortunately, soluble ancillary regulators required by membrane-bound transfer proteins would also be lost using this experimental approach. These results notwithstanding, several membrane-associated LTPs are found at membrane contact sites.

### Lipid-Transferring Tunnel Proteins can Theoretically Bridge the PM and ER Membranes for a Direct Channeling of Lipids

In contrast to LTPs, the *Tunnel model* postulates that protein bridges span the distance between the PM and ER and facilitate direct lipid transfer through a hydrophobic channel (Fig. 1).<sup>46</sup> Examples of potential tunnel proteins include the E-Syts, a family of *extended synaptotagmin-like proteins* anchored to the ER membrane.<sup>47–49</sup> E-Syts are integral membrane proteins that contain multiple Ca<sup>2+</sup>-binding C2 domains involved in phospholipid binding<sup>48</sup> and a synaptotagmin-like mitochondrial lipid-binding protein (SMP) domain that forms a hydrophobic groove when dimerized.<sup>46–51</sup> Similar SMP domains are also found in the Mmm1p/Mdm12p/Mdm34p core components of ER–mitochondria encounter structure (ERMES) complex,<sup>50</sup> which tethers the ER and outer mitochondrial membranes together and is implicated in direct phospholipid exchange between these membranes.<sup>52</sup> It was initially proposed that the ERMES complex forms a lipid-binding tunnel for shuttling lipids between the ER and mitochondria,<sup>10,46</sup> but an in-depth structural analysis of the core ERMES complex components sheds doubt on whether any contiguous tunnel or channel can form through these proteins.<sup>46,53</sup> In so far as the E-Syts are concerned, it was argued that the tunnel structural conformation might be too short to span the observed gap between the PM and ER, but in an alternative *shuttle conformation*, the E-Syts might reach across.<sup>46</sup> These structural models suggest that the ERMES complex and the E-Syts might act more like transmembrane domain-tethered shuttles/LTPs than tunnels (Fig. 1).

### Membrane-Bound LTPs as Shuttles for Direct Transfer of Lipids Between the PM and ER Membranes

An example of such a membrane-bound LTP is the mammalian integral membrane protein, steroidogenic acute regulatory (StAR)-related lipid transfer (StART) domain protein 3 (STARD3).<sup>12</sup> STARD3 is a representative of a large protein family in which the prototypical member, StAR, transfers cholesterol from the outer- to the inner-mitochondrial

membrane.<sup>12,54–56</sup> STARD3 is tethered to the late endosomal membrane through its MLN64 N-terminal (MENTAL) domain, and it uses its C-terminal StART domain to capture cholesterol from the late endosomal membrane and then to transfer it to the ER.<sup>12,57–60</sup> For STARD3, the StART domain has been proposed to swing between these membranes to mediate sterol transfer,<sup>12</sup> though direct experimental evidence is needed.<sup>61</sup> Even though STARD3 transfers cholesterol in vitro,<sup>60,62</sup> disruption of its StART domain in vivo has modest physiological consequences.<sup>61</sup> In this regard, STARD3 might be functionally redundant for cholesterol transport in vivo, or STARD3 might have another important function independent of its StART domain. STARD3 has a close homolog called *STARD3 N-terminal like* (STARD3NL), which completely lacks a StART domain.<sup>58</sup> Through their FFAT (two phenylalanines in an acidic track) domains, both STARD3 and STARD3NL bind “VAMP/synaptobrevin-associated protein.” VAP-A and -B, two mammalian “*VAMP/synaptobrevin-associated protein*” homologs.<sup>59</sup> The FFAT (two phenylalanines in an acidic tract) motif links STARD3 and STARD3NL to the ER via VAP, and the MENTAL domain anchors STARD3 and STARD3NL to late endosomes. Recently, several yeast StART protein homologs, Ysp1p, Ysp2p, Sip3p, and Lam4p–Lam6p, have been identified.<sup>63,64</sup> All members contain one or more ER transmembrane domains, and several have PH domains permitting *trans* association with other membranes. These properties suggest a mechanism of action similar to STARD3, where LTP function is coupled with the capability as *secondary* tethering proteins to bring acceptor membranes closer for direct lipid transfer. In fact, it has been shown that Lam6p regulates the size and expansion of membrane contact sites for multiple interorganelle associations.<sup>65</sup> As far as some StART domain proteins are concerned, it is clear that they have a complex range of functions.

### PM–ER Membrane Contact Requires not Only Tethering Proteins but Also Ancillary Lipid and Protein Regulators

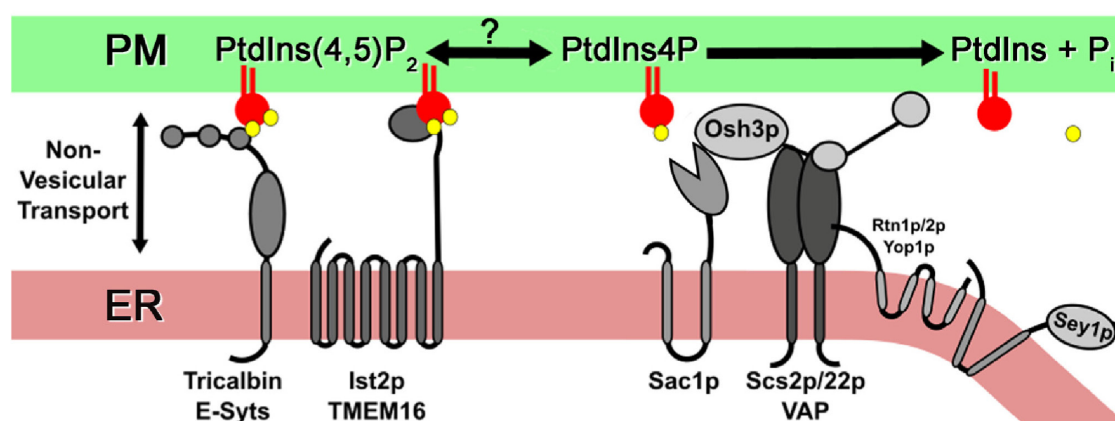
Whether or not they form contiguous tunnels between membranes, it is perhaps not surprising that E-Syt orthologs in yeast were identified as PM–ER membrane-tethering proteins.<sup>66</sup> The yeast tricalbins Tcb1p–Tcb3p are members of the E-Syt/SMP family proteins,<sup>67</sup> and together with Ist2p and the VAP orthologs Scs2p/Scs22p, they mediate contact between the PM and cortical ER.<sup>66</sup> Based on proteomic interactions, one can weave an *interactome* between these tethering proteins that might represent a single complex, but it seems more likely that there are several tethering complexes perhaps with subunit exchange between them.<sup>66,68–70</sup> Although yeast cells lacking all these *primary* tethering proteins have a significant reduction in PM–ER association, some PM–ER contact still remains. It is, therefore, likely that additional tethering proteins are yet to be found that will also help staple the PM and ER membranes together. As reviewed

elsewhere, all the identified tethering proteins are conserved in mammalian cells and relatively well understood.<sup>39</sup> Perhaps less understood, however, are the contributing mechanisms that facilitate the priming and propagation of PM–ER membrane association by tethering proteins. In terms of regulatory proteins and membrane structure and composition, what is required to nucleate tether assembly at presumptive membrane contact sites? Do tethering proteins require modulations in organelle morphology and bilayer curvature to bring membranes close enough for initiating membrane capture? Following the establishment of membrane contact, what lipids and proteins affect the spread of membrane apposition around contact sites, and what governs contact stability? If these contributing mechanisms are significant, we predict that yeast cells lacking known tethering proteins might be hypersensitive to additional mutational defects in such ancillary lipid and protein regulators.

In addition to promoting lipid extraction from the bilayer by LTPs, membrane curvature and bilayer distortion can affect the plasticity of interacting membranes to bend them for closer apposition.<sup>71</sup> Unlike the ER, the PM is inherently inflexible due its relative enrichment in PtdCho and PtdSer, which preferentially form flat bilayers, and cholesterol, which fortifies lipid packing.<sup>72–74</sup> In contrast, ER membrane structure is more malleable and ductile reflecting a lipid composition and organization that is more pliant for shaping.<sup>29,75,76</sup> These divergent properties of the ER and PM suggest that different mechanisms operate to deform and bring these membranes in juxtaposition for tethering proteins to establish contact.<sup>71</sup> In addition to the physical effects of lipid composition and bilayer asymmetry on membrane architecture, a greater impact on membrane architecture seems to be conferred by protein regulators recruited to those membranes.

In yeast, the induction of membrane curvature in the ER is largely due to the action of the reticulons Rtn1p and Rtn2p and the reticulon-like protein Yop1p.<sup>77,78</sup> These proteins insert wedge-like amphipathic helices into the cytosolic face of the ER membrane and thereby generate positive curvature.<sup>79</sup> The yeast atlastin homolog Sey1p is a dynamin-like GTPase that also contributes to ER reticulation and membrane remodeling by facilitating ER–ER homotypic membrane fusion.<sup>80,81</sup> Rtn1p/2p, Yop1p, and Sey1p are all cortically localized, and their combined disruption causes increased cortical ER and the creation of a sheet of ER over the internal face of the PM, instead of the normal tubular lattice.<sup>4,77,78,80,81</sup> The presence of Sey1p is also required for nuclear ER association during yeast mating because its ER remodeling activity is required to allow nuclear membranes to come close enough for tethering.<sup>82</sup> The reticulon genes *YOP1* and *SEY1* also genetically interact with genes encoding ERMES complex components, which reflect a role for reticulons in lipid exchange between the ER and mitochondria.<sup>83</sup> Based on these findings, a reasonable prediction is that the reticulons together with Yop1p and Sey1p help shape the cortical ER along the PM so that tethering proteins can then staple the membranes together (Fig. 2). This model also predicts that ER shaping by these proteins might play a role in the frequency and stability of PM–ER membrane contact. Consistent with this notion, Rtn1p/2p and Sey1p physically interact with Scs2p, a key tethering protein.<sup>66</sup> This interaction might represent a mechanism by which Scs2p recruits ER-remodeling regulators to expand or stabilize the zone of cortical ER association with the PM beyond the point of contact.

Given the comparative rigidity of the PM, gross changes in membrane architecture are less likely to play a significant role in its association with other organelles. Instead, PM interactions



**Figure 2.** Tethering proteins, membrane, and lipid regulators of PM–ER membrane contact sites. Regulation of PtdIns(4,5)P<sub>2</sub> in the PM (green) is required to recruit tricalbins/E-Syts and Ist2p/TMEM16 tethering proteins, which are anchored in the ER membrane (coral colored) and make contact with the PM. The PtdIns(4,5)P<sub>2</sub> precursor PtdIns4P is dephosphorylated in the PM *in trans* by the ER-resident PtdIns4P phosphatase, Sac1p. In yeast, Sac1p activity is regulated by its interactions with ORP homologs, such as Osh3p, and the VAP homologs, such as Scs2p/Scs22p. Scs2p is itself a tethering protein that interacts with other tethering proteins, several ORPs, lipid regulators, and ER membrane-remodeling proteins. The reticulons Rtn1p and Rtn2p, the reticulon-like protein Yop1p, and the dynamin-like GTPase Sey1p are membrane-remodeling proteins that induce ER membrane plasticity and potentially affect the juxtappositioning of the ER near the PM.



with other membranes appear to be governed by lipid domains within the lateral section of the bilayer, and the active control of bilayer asymmetry. Unlike in the ER membrane that contains small amounts of PtdSer found mainly within the luminal bilayer leaflet,<sup>84,85</sup> in the PM, PtdSer is highly enriched and exclusively resides in the cytoplasmic face of its bilayer<sup>72,76</sup> and promotes the recruitment of proteins through both specific (eg, with discoidin-type C2 domains) and low-affinity interactions (eg, lipidated polybasic proteins).<sup>86</sup> Likewise, another charged phospholipid, PtdIns(4,5)P<sub>2</sub>, is also enriched in the PM exclusively in the cytoplasmic bilayer leaflet.<sup>87,88</sup> This anionic charge density makes the PM a unique target for protein regulators, not least of which are tethering proteins themselves.

### Membrane Domains and Bilayer Asymmetry as Determinants of PM Contact with Cortical ER

The tethering proteins Tcb1p, Tcb2p, Tcb3p, and Ist2p bind to PtdIns(4,5)P<sub>2</sub> in the PM to form a protein bridge between the PM and ER to establish membrane association (Fig. 2).<sup>89</sup> The affinity for PtdIns(4,5)P<sub>2</sub> is shared by mammalian E-Syt tethering proteins,<sup>49</sup> including the ER-localized STIM1 and STIM2 tethering proteins that regulate store-operated Ca<sup>2+</sup> channels in the PM.<sup>90</sup> Thus, the localization and activity of phosphoinositide kinases and phosphatases at PM-ER contacts are likely to be ubiquitous regulators of tethering protein recruitment.

Of all the defined primary tethering proteins, the elimination of the VAP ortholog Scs2p causes the greatest reduction in PM-ER membrane contact sites.<sup>66,91</sup> Scs2p may be of particular importance because of its direct interactions and recruitment of other tethering proteins, interactions with a number of lipid regulatory proteins, and physical interactions with several phosphoinositide species.<sup>14,17,66,91-94</sup> Scs2p binds the ER-localized PtdIns4P phosphatase, Sac1p, which in turn interacts with and is activated by specific yeast ORPs.<sup>17</sup> Some of these ORPs also interact with Scs2p and bind PtdIns(4,5)P<sub>2</sub> and/or PtdIns4P (Fig. 2).<sup>24,25,95,96</sup> In addition, Tcb1p-Tcb3p and Ist2p tethering to PtdIns(4,5)P<sub>2</sub> in the PM might be controlled by phosphoinositide regulation, similar to the E-Syts.<sup>97</sup> In this regard, there is conflicting evidence that reductions in PtdIns4P by Sac1p affect the PtdIns(4,5)P<sub>2</sub> levels in such a way as to affect LTP and tethering protein binding to the PM.<sup>98,99</sup> However, if PtdIns(4,5)P<sub>2</sub> levels decrease at contact sites because of increased PtdIns4P turnover by Sac1p, then this might represent a mechanism for Sac1p to limit the extent of PM-tethering protein interactions. An experimental prediction of this *feedback model* would be that PM-ER membrane association would increase in cells lacking Sac1p (and decrease upon Sac1p overexpression). Besides regulating Sac1p activity, Scs2p directly binds and modulates the activity of Opi1p, a transcription factor that represses Ino1p expression and phospholipid biosynthesis.<sup>14,100</sup> In the absence of inositol, Scs2p sequesters Opi1p on the ER membrane, and target genes, such as *INO1*, are derepressed. The multilayered functions of Scs2p

suggest that one of its primary roles is to integrate the many processes affecting the PM-ER membrane contact sites.

The yeast ORPs, Osh2p and Osh3p, bind to Scs2p and are recruited to the ER and PM-ER contacts,<sup>14,15,17</sup> whereas other ORPs (Osh4p, Osh6p, and Osh7p) also appear to be recruited to the ER but through an Scs2p-independent mechanism.<sup>15,20,21</sup> At these sites, the role of these proteins appears to involve lipid transfer, but another function may be to regulate the PM lipid composition and organization. For example, in living cells, yeast ORPs were largely dispensable for PM/ER intermembrane sterol exchange,<sup>23</sup> but in cells lacking ORPs, the organization of sterols within the PM is grossly altered.<sup>23,24,101</sup> Based on the cyclodextrin extractability, two interchangeable pools of ergosterol (the major yeast sterol) are detectable in yeast within the PM.<sup>102</sup> In the absence of yeast ORPs, the cyclodextrin-extractable pool increases by 25-fold.<sup>23</sup> This shift reflects a substantive change either in lateral domain organization in the PM or in bilayer asymmetry causing an increase in sterols in the extracellular leaflet. While transbilayer movement of sterols in synthetic membranes and red blood cells is rapid,<sup>103</sup> in yeast, the general diffusion of membrane components is slow, and sterol movement between leaflets might require facilitation.<sup>104</sup>

Another finding, suggesting a role for yeast ORPs in regulating transbilayer asymmetry, involves the antagonistic interaction between Osh4p and the P4-ATPase lipid flippase Drs2p.<sup>105</sup> Drs2p flipping in the Golgi creates a PtdSer and PtdEtn bilayer asymmetry that is preserved in post-Golgi vesicles destined to fuse with the PM.<sup>106-109</sup> Drs2p in the Golgi thereby contributes to lipid asymmetry in the PM. Apart from Osh4p, it is unclear if other yeast ORPs affect transbilayer asymmetry or if this function impacts membrane contact sites. However, it is noteworthy that Drs2p, despite its localization to the *trans*-Golgi, physically interacts with both Sac1p and the PM-ER tethering protein Tcb3p.<sup>110</sup> These findings hint at the possibility that PM-ER contact sites bring together LTPs, phosphoinositide regulators, and possibly transbilayer asymmetry regulators.

Scramblases also translocate phospholipids between bilayer leaflets, but, unlike P4-ATPase flippases, the transfer is bidirectional and generally dissipates bilayer asymmetry.<sup>106-109,111</sup> The PM-ER tethering protein, Ist2, is a homolog of the *Aspergillus fumigatus* TMEM16 membrane scramblase.<sup>39</sup> However, when directly tested, Ist2p did not exhibit TMEM16 phospholipid scramblase activity.<sup>112</sup> In budding yeast at least, Ist2p so far appears only to be a membrane-tethering protein,<sup>66</sup> but in other eukaryotes, Ist2p/TMEM16 homologs might have additional functions that coordinate tethering, intermembrane, and transbilayer exchange.

### Conclusion and Perspectives

Membrane contact sites provide nucleating loci for the integrated regulation of several different lipid pathways. Lipid metabolic pathways, where the biosynthetic machinery is



anchored on different organelles, necessitate lipid precursor exchange between different membranes at contact sites. Therefore, membrane contact sites are centers not only for lipid biosynthesis but also for lipid regulation of protein interactions. In the PM–ER membrane contact sites, phosphoinositides appear to be the key regulators because of the dependence of LTPs and tethering proteins on PtdIns4P and PtdIns(4,5)P<sub>2</sub>. As a result, we predict that phosphoinositide regulators, such as Sac1p, might have a major impact on the establishment and propagation of PM–ER membrane association. The importance of phosphoinositide levels at membrane contact sites is also evident in the specific context of PtdIns(4,5)P<sub>2</sub> depletion in response to phospholipase C.<sup>113,114</sup> To restore PtdIns(4,5)P<sub>2</sub> to the PM, the mammalian protein Nir2 and its *Drosophila* homolog RDGB $\alpha$  appear to act as bidirectional phosphoinositide/phosphatidic acid transporters at PM–ER membrane contact sites.<sup>51,113–115</sup> All told, the regulatory mechanisms of both phosphoinositide metabolism and PM–ER membrane contact sites appear to be intertwined.

In general, the evidence we have presented here challenges a simplified mechanistic view of the roles played by LTPs and tethering proteins. As opposed to being simple carriers involved in intermembrane lipid transport, LTPs such as the yeast ORPs clearly affect the bilayer organization of their target membranes. These changes might facilitate lipid presentation at the membrane surface for capture and transfer by other LTPs, either soluble or membrane bound. Other potential LTPs, such as Lam6p, have additional regulatory functions in controlling the extent of membrane association as secondary PM–ER membrane-tethering proteins. Even primary tethering proteins are not just simple staples for adhering different membranes together. Tethering proteins, such as Scs2p, can serve as scaffolds for regulatory proteins, including membrane-anchored transcription factors and proteins that induce organelle shaping. Moving forward then, to understand the complex roles performed by membrane contact sites, the diverse functions of each constituent protein and lipid must be embraced.

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## Author Contributions

Contributed to the writing of the manuscript: EQ and CTB. Made critical revisions and approved final version: EQ and CTB. Both authors reviewed and approved of the final manuscript.

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