Building ER-PM Contacts: Keeping Calm and Ready on Alarm

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Short title: ER-PM Contacts: Principals of Membrane Organization

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Abstract

It is well over half a century since contacts between organelles such as the endoplasmic reticulum (ER), mitochondria, and the plasma membrane (PM) were first observed in electron microscopy studies. Still, these early images of seemingly rare organelle interactions continue to capture the attention and curiosity of cell biologists even today. From seminal studies first proposing roles for organelle cross talk in excitable cells, the field has now expanded to cover nearly all aspects of eukaryotic cell biology, from calcium and membrane lipid transport to vesicular trafficking, cell signaling, metabolism, and homeostasis. This review highlights recent discoveries pointing to vital roles for ER-PM contacts in membrane lipid dynamics and organization.

Introduction

Regions of the peripheral endoplasmic reticulum (ER) closely apposed to the plasma membrane (PM) were revealed in elegant detail by electron microscopy (EM) studies on muscle cells [1], where they play important roles in calcium (Ca^{2+})-mediated excitation-contraction events [2]. A few years later, Jack Rosenbluth reported "subsurface cisterns" in neurons [3], which are now recognized as ER-PM contacts and have been described in greater morphological detail with recent advances in cryo-EM tomography [4,5]. At the time of their discovery the role of "subsurface cisterns" were obscure, but because these structures were prominent in neurons and muscle cells Rosenbluth surmised that they might be integral to metabolic 'peculiarities', including ion transport and rapid membrane lipid dynamics, occurring in excitable cells. This prediction has turned out to be the case, and ER-PM contacts are now widely accepted as important sites for non-vesicular Ca^{2+} and membrane lipid transport. Moreover, it is now evident that ER-PM contacts are ubiquitous structures that function not only in excitable cells but also numerous cell types across single- and multi-cellular eukaryotic organisms.

Conserved roles for ER-PM contacts in membrane lipid dynamics and homeostasis have been elucidated by the identification of proteins that form and function at these important cellular structures. In particular, the identification of lipid transfer proteins that function at these sites has greatly aided our understanding of how ER-PM contacts control membrane lipid composition and organization. Within the past few

years, we have learned a great deal more about the selective transport of lipids between the ER and PM. Cells have a vast toolkit of lipid transfer proteins on hand for the discrete movement of lipids, for anterograde transport to the PM versus retrograde transport to the ER, and for the establishment of lipid concentration gradients versus lipid equilibration. Importantly, we are now learning more about the regulatory mechanisms that control the directional movements of lipids at ER-PM contacts as needed. As such, a key concept is emerging in the field. Namely, distinct ER-PM contacts with unique features and functions are built on demand, in response to intrinsic and extrinsic physiological cues, to balance membrane lipid homeostasis and dynamics as needed.

Keeping Calm: ER-PM Contacts in Membrane Organization and Homeostasis

Membrane integrity ensures cellular integrity. As such, membrane status is continuously assessed and maintained through careful control of membrane protein and lipid composition. Lipid molecules have distinct physical properties that determine their organization in a cellular membrane bilayer. Accordingly, the PM has a unique membrane lipid composition that determines its mechano-physical properties, organization, and identity from other membrane compartments in the cell [6]. For example, the PM is enriched in sterol, sphingolipids, and certain phospholipids as compared to other cellular membranes (Figure 1) [7,8]. In particular, high levels of the phospholipid phosphatidylserine maintain an overall negative charge at the cytosolic face of the PM, a key feature of the PM [9]. The PM displays

unique features at the lipid species level as well, represented in distinctive acyl chain properties. First, the PM is enriched in long acyl chain sphingo- and phospholipid species in order to suit the length of PM transmembrane domain proteins [7,8,10]. Moreover, the PM is enriched in lipids with saturated acyl chains thought to preferentially associate in liquid-ordered domains stabilized by sterol lipids [7,8]. In contrast, the ER membrane is less ordered, more fluid, thinner, and less charged than the PM. The chemical and physical properties of the ER membrane are defined by low sterol, sphingolipid, and phosphatidylserine content and, in converse, higher levels of neutral aminophospholipids with shorter, unsaturated acyl chain species (Figure 1).

The conserved PM and ER lipid identity codes are created by the selective delivery of lipids between the ER and PM by specific lipid transfer proteins and possibly by vesicular trafficking (Figure 1), as spontaneous lipid transfer between membranes is energetically unfavorable and potentially non-specific [11]. In particular, members of a conserved family of lipid transfer proteins, the oxysterol-binding protein related proteins (ORP), are thought to be crucial for the establishment and maintenance of PM lipid identity (Figure 2). ORP family members transfer newly synthesized cholesterol and phosphatidylserine from the ER in exchange for phosphatidylinositol 4-phosphate (PI4P) at the PM [12-15]. In this manner, cells utilize PI4P metabolism for directional transport of lipids from the ER to the PM (*i.e.* transport from the ER to the PM against a concentration gradient). While exchange of one lipid molecule for

another lipid molecule may not result in net lipid gain, this mechanism effectively results in the enrichment of sterol and phosphatidylserine at the PM.

ER-PM contacts may then have essential roles in PM organization and homeostasis (keeping calm). In support of this notion, loss of proteins previously shown to form and function at ER-PM contacts in yeast, namely Scs2/22 (VAP orthologs), the Tcb1/2/3 tricalbin proteins (Extended synaptotagmin orthologs, E-Syt proteins), and Ist2 (a TMEM16 family member), results in loss of PM integrity upon membrane stress conditions [16]. Upon physiological membrane stress conditions, phosphoinositide kinase signaling at the PM triggers TORC2-induced sphingolipid synthesis in the ER necessary to maintain cellular integrity. Defects in ER-PM contact formation results in mis-regulation of phosphoinositide lipid metabolism, Ca^{2+} signaling, sphingolipid synthesis, and ultimately loss of cellular integrity [16]. Another cellular stress response pathway in yeast, the Rim101-ESCRT coupled membrane sensing and sculpting pathway, is activated upon disruptions in ER-PM contacts further suggesting important roles for these structures in the control of membrane organization [17].

Membrane homeostasis requires both the selective delivery and removal of lipids to adjust membrane lipid composition as needed. In particular, sterol lipids are key determinants of membrane bilayer packing order (*i.e.* liquid ordered and disordered domains), fluidity, and integrity [6]. As such, their levels and distribution within a

membrane bilayer must be tightly regulated. Conserved ER-localized StART-like (StAR-related lipid-transfer) domain proteins, named Lam/Ltc/Ysp proteins in yeast and collectively referred to as the StARkin domain family, are suggested to direct retrograde transfer of sterol lipids from the PM to the ER [18-22]. This activity may oppose ORP-mediated sterol transport to the PM. In line with this, ORP and StARkin transport activities occur at spatially distinct ER-PM contacts (Figure 2) [19]. This implies that specific sterol transfer systems are built and coordinated as needed and that the balance of sterol delivery and removal ensures PM homeostasis. Consistent with this, loss of StARkin proteins at ER-PM contacts in yeast results in TORC2-mediated stress responses potentially due to perturbations in PM sterol organization [23].

Specialized ER-PM contacts are also formed in response to changes in ER status. A well-studied example is store-operated calcium entry (SOCE) whereby the ER-localized STIM proteins bind and activate the PM-localized Orai1 channel to maintain ER Ca^{2+} homeostasis [2]. The unfolded protein response (UPR) sensor PERK is recently implicated in this process [24]. Upon depletion of ER Ca^{2+} stores and ER stress, PERK signaling drives actin network reorganization to assist STIM protein ER-PM targeting and SOCE. As PERK is also activated by ER membrane perturbations, particularly the accumulation of ‘PM-like’ saturated acyl chain lipid species [25], this stress response pathway may modulate ER and PM lipid homeostasis as well. While this remains speculative, PERK facilitates targeting of the lipid transfer protein E-Syt1 to ER-PM contacts [24] (see below for further discussion of the E-Syt proteins).

Taken together, recent studies establish that ER-PM contacts are built as needed to control PM lipid composition, ensuring membrane homeostasis and integrity.

Ready on Alarm: Inducible ER-PM Contacts in Regulated Exocytosis and Endocytosis

In addition to membrane organization, ER-PM contacts support dynamic vesicular trafficking events taking place at the PM [2]. Phosphoinositide lipid and Ca^{2+} dynamics regulated at ER-PM contacts are vital for modulatory neurotransmitter release, pulsatile insulin secretion from pancreatic β cells, and regulated exocytosis in immune cells. It is generally understood that receptor-mediated phospholipase C (PLC) activation results in phosphatidylinositol 4,5 bis-phosphate hydrolysis, $PI(4,5)P_2$, generating second messenger molecules including diacylglycerol and Ca^{2+} signals that trigger exocytic events (Figure 3) [26]. However, we are learning more about how $PI(4,5)P_2$ hydrolysis, even upon slight physiological reductions in cellular pools [27], sets forth a chain of lipid transfer and metabolic reactions needed to sustain $PI(4,5)P_2$ pools for multiple rounds of exocytosis in response to extracellular stimuli [28,29]. These events are mediated by inducible, regulated lipid transfer protein activities that are activated in response (ready on alarm) to the same physiological stimuli that trigger exocytosis (*e.g.* elevated glucose in the case of β cells, and antigen stimulation in mast cells).

A striking and yet underappreciated observation is that levels of PI4P, a precursor to $PI(4,5)P_2$, rise simultaneously with $PI(4,5)P_2$ hydrolysis at the PM (Figure 3) [30,31]. This implies that robust systems for $PI(4,5)P_2$ re-synthesis are engaged upon PLC activation. Rapid recycling of diacylglycerol, a direct product of PLC-mediated $PI(4,5)P_2$ hydrolysis, facilitates efficient $PI(4,5)P_2$ re-synthesis in a process known as the phosphoinositide cycle [26]. Because diacylglycerol is generated from $PI(4,5)P_2$ at the PM and phosphatidylinositol, the precursor to PI4P and $PI(4,5)P_2$ is synthesized by the CDP-diacylglycerol pathway in the ER, lipid transfer protein activities at ER-PM contacts are instrumental during the phosphoinositide cycle. Recent studies on the Nir2 phosphatidylinositol transfer protein and its ortholog RdgB in *Drosophila* have provided mechanistic insight into this process [32-36]. Diacylglycerol produced from $PI(4,5)P_2$ hydrolysis is first converted to phosphatidic acid by diacylglycerol kinase at the PM. Nir2 then translocates to the PM by binding phosphatidic acid. At ER-PM contacts, Nir2 transfers phosphatidic acid to the ER for phosphatidylinositol synthesis (via the CDP-diacylglycerol pathway) and in turn transports phosphatidylinositol back to the PM for PI4P and $PI(4,5)P_2$ synthesis. Interestingly, targeting of Nir2 to ER-PM contacts is further specified by binding the ER-localized VAP proteins that also recruit certain ORP isoforms [35]. Thus in response to stimuli that trigger PLC-regulated exocytosis, ER-PM contacts may switch from sites of PI4P exchange (*e.g.* PI4P and sterol, Figure 2) to sites that promote PI4P and $PI(4,5)P_2$ synthesis (Figure 3).

Additional lipid transfer proteins function at ER-PM contacts in response to PLC-generated signals. The extended synaptotagmin (E-Syt) proteins are Ca^{2+}-activated ER-PM tether proteins that transfer PLC-generated diacylglycerol from the PM to the ER [37], potentially working in concert with the Nir2 protein during the phosphoinositide cycle [32]. E-Syts utilize their SMP domain (synaptotagmin-like mitochondrial lipid-binding protein domain) to transfer lipids [37-39], but while this activity is apparently selective for diacylglycerol *in vivo* [37], the E-Syts are not selective *in vitro*. Both biochemical and structural studies demonstrate that SMP domains bind various glycerolipids and phospholipids and transfer them down a concentration gradient (from high to low) [37-39]. This implies that E-Syt activity must be tightly regulated and only allowed to proceed in response to specific stimuli. Otherwise E-Syt proteins may non-selectively transfer phospholipids enriched at the PM, including phosphatidylserine, back to the ER. This would potentially undue PI4P- and ORP-mediated phosphatidylserine transfer to the PM, a critical determinant for PM identity and function. Consistent with this, cells lacking E-Syt proteins do not display changes in major glycerolipid species in the absence of stimuli that trigger PLC signaling [37].

Accordingly, both *in vitro* and *in vivo* studies have uncovered important E-Syt regulatory mechanisms. The crystal structure of the SMP domain from E-Syt2 revealed a β-barrel structure similar to other tubular lipid-binding (TULIP) domain proteins [38]. Interestingly, the SMP domain dimerizes forming a cylindrical channel lined with hydrophobic residues that can accommodate glycerophospholipids.

However, the SMP dimer spans approximately 9 nm in length, suggesting that E-Syt lipid transfer activity may only occur at sites where the ER and PM are closely tethered and may not take place at ORP-mediated ER-PM contacts typically 25-30 nm apart (Figure 2). Indeed, SMP domain-mediated lipid transfer *in vitro* requires tight membrane tethering by Ca^{2+}-bound C2 domains in the E-Syt proteins [37,39]. Notably, E-Syt1 undergoes autoinhibition and Ca^{2+} binding relieves autoinhibitory interactions between the C2A and SMP domains as well as between C2C and C2E that prevent binding to $PI(4,5)P_2$ in the PM [40]. In support of the *in vitro* evidence, *in vivo* studies show that Ca^{2+} influx by the SOCE pathway and voltage gated Ca^{2+} channels triggers E-Syt1 recruitment to ER-PM contacts [41]. Moreover, Ca^{2+} influx shortens the distance between the ER and PM at E-Syt1-dependent contact sites, as revealed by cryo-EM tomography [4]. Thus, E-Syt proteins specifically respond to external stimuli that trigger PLC signaling, Ca^{2+} influx, and regulated exocytosis. Importantly, this shared Ca^{2+} regulatory mechanism indicates vital cross talk between ER-associated PM zones and ER-free vesicular trafficking zones (Figure 3).

While ER-PM contacts are crucial sites for SOCE and $PI(4,5)P_2$ re-synthesis following PLC activation, another recent study has suggested that ER-PM contacts are important sites for PI4P homeostasis by E-Syt2 and the PI4P phosphatase Sac1 [42]. PI4P directly activates SOCE via the Orai1 channel [43] and is central to $PI(4,5)P_2$ synthesis; as such, PI4P levels must be tightly regulated. In response to intense PLC activity and $PI(4,5)P_2$ hydrolysis, E-Syt2-dependent ER-PM contacts are depleted facilitating PI4P-mediated SOCE and $PI(4,5)P_2$ re-synthesis [42]. Following

SOCE and $PI(4,5)P_2$ re-synthesis, E-Syt2 ER-PM contacts are reformed allowing Sac1-mediated PI4P regulation preventing excessive Ca^{2+} and $PI(4,5)P_2$ overshoot [42]. Altogether, recent findings have highlighted several key roles in the homeostatic control of $PI(4,5)P_2$ and Ca^{2+} signaling. Disruptions in ER-PM cross talk result in impaired $PI(4,5)P_2$ synthesis [32,33,35,36,44] and mis-regulation of PI4P-dependent Ca^{2+} signaling [16,42,44,45] (Figure 3).

Considering the vital roles the E-Syt proteins serve in $PI(4,5)P_2$ and Ca^{2+} regulation, one might expect they are essential for cellular function and viability. Surprisingly, two recent studies independently report that mice lacking E-Syt proteins do not display obvious defects [46,47]. This may suggest that the E-Syt proteins have highly specialized roles. Another plausible explanation is additional lipid transfer proteins compensate for loss of E-Syt protein function. Interestingly, the SMP domain protein TMEM24 controls Ca^{2+} and $PI(4,5)P_2$ signaling necessary for regulated insulin secretion in pancreatic β cells [48]. The TMEM24 SMP domain preferentially binds and transfers phosphatidylinositol from the ER to the PM. Upon loss of TMEM24 in β cells, $PI(4,5)P_2$ synthesis is impaired decreasing the generation of Ca^{2+} signals that trigger insulin release. It remains unknown whether TMEM24 controls phosphoinositide and Ca^{2+} signaling in other cell types. Moreover, additional members of the SMP/TULIP superfamily exist and their potential roles at ER-PM contacts remain to be explored [49,50].

Previous studies implicate Ca^{2+}, $PI(4,5)P_2$, and actin oscillations during regulated exocytosis [51,52]. Given the important roles the Nir2, E-Syt, and TMEM24 proteins serve in $PI(4,5)P_2$ and Ca^{2+} regulation, ER-PM contacts may be generally involved in vesicular trafficking events at the PM. In support of this, the majority of exocytic and clathrin-mediated endocytic events in HEK293 cells appear to occur in close proximity to ER-PM contacts [53]. Another recent study suggests that ER-PM contacts promote actin polymerization during late stages of clathrin-mediated endocytic internalization in yeast [54]. Intriguingly, ER-PM contacts are also implicated in clathrin-independent epidermal growth factor receptor internalization [55], likely through modulation of phosphoinositide and Ca^{2+} signaling. Thus while ER-PM contacts and ER-free PM zones may appear to be spatially distinct and have different physiological roles (non-vesicular transport versus vesicle trafficking), it is becoming increasingly clear that these PM domains are functionally interconnected (Figure 3).

Summary

ER-PM contacts are important sites for membrane lipid homeostasis and organization. Because PM composition is continuously adjusted according to physiological cues, distinct ER-PM contacts are assembled and disassembled as needed. This is accomplished by the specific recruitment and regulation of lipid transfer proteins that function at these sites. Consequently, differential activities at ER-PM contacts can effectively influence sterol and phospholipid distribution, phosphoinositide metabolism, and vesicle trafficking events. We must continue to

examine the interplay between lipid transfer proteins and how they may act in concert for directional net movements of lipids between the ER and PM. As such, future studies on ER-PM contacts will have tremendous impact on our understanding of cellular architecture and physiology.

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Competing interests

The author declares no conflicts of interest.

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Figures and Figure Legends

Stefan, Figure 1

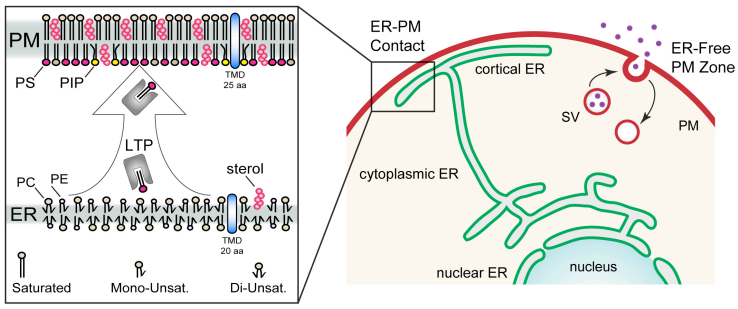

Figure 1. The plasma membrane is organized into ER-associated domains, termed ER-PM contacts, and ER-free domains. At ER-PM contacts, close apposition of the cortical ER to the PM (spanning 10 to 30 nm apart) facilitates non-vesicular lipid exchange by specific lipid transfer proteins. The selective delivery of newly synthesized lipids from the ER to the PM by lipid transfer proteins establishes the unique lipid composition of the PM including high sterol lipid content and phosphatidylserine species with long saturated acyl chains. This endows the PM with its unique chemical and physical properties including high negative charge, high membrane lipid packing order, and a bilayer thickness to accommodate PM proteins with long transmembrane domains. In contrast, the ER has a very different membrane lipid identity with low sterol levels and phospholipids with short unsaturated acyl chains resulting in decreased membrane lipid packing order and bilayer thickness. Vesicular trafficking events occur in ER-free PM domains. For this reason, ER-PM contacts and ER-free PM zones are often considered to be spatially and functionally distinct (*i.e.* sites for non-vesicular transport versus vesicular trafficking, respectively). However, it is becoming more and more clear that membrane lipid dynamics taking place at ER-PM contacts generate lipid and calcium signals necessary to sustain vesicle trafficking in ER-free zones. Mono-Unsat., mono-unsaturated lipid species; Di-Unsat., di-unsaturated lipid species; LTP, lipid transfer protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP, phosphatidylinositol phosphate; PS, phosphatidylserine; SV, secretory vesicle; TMD, transmembrane domain.

Stefan, Figure 2

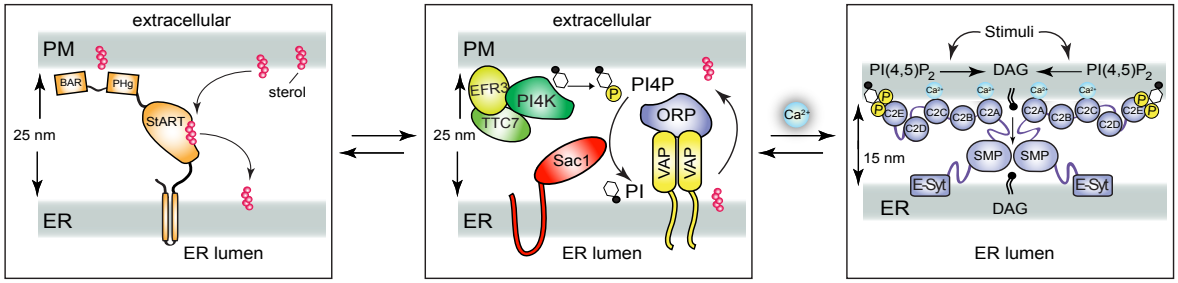

Figure 2. ER-PM contacts are assembled as needed by the specific recruitment of distinct lipid transfer proteins. **(Center)** The ORP (oxysterol-binding related proteins) lipid transfer proteins control plasma membrane lipid composition, organization, and identity. Certain ORP family members (including the yeast Osh2 protein) bind the ER-localized VAP proteins to form ER-PM contacts spanning approximately 25 to 30 nm in distance. ORP isoforms bind newly synthesized sterol lipids (*e.g.* ergosterol or cholesterol) at the ER and deliver the bound sterol lipid to the PM. The ORP lipid transfer proteins then bind PI4P generated by a phosphatidylinositol 4-kinase (PI4K) complex at the PM to prevent removal of sterol molecules enriched at the PM. The bound PI4P is hydrolyzed by the ER-localized Sac1 phosphatase for continued rounds of sterol delivery to the PM. In addition to sterols, the PM is highly enriched in negatively charged phosphatidylserine. ORP isoforms (the Osh6 and Osh7 proteins in yeast and the ER-localized ORP5 and ORP8 human orthologs) function at ER-PM contacts as PS/PI4P exchange proteins to deliver newly synthesized PS from the ER to the PM (not shown; see main text for additional details). **(Left)** Conserved StARkin protein family members function as sterol transfer proteins at ER-PM contacts. PM organization and homeostasis not only requires delivery of sterol lipids to the PM but also the selective removal of sterol molecules from the PM. In yeast, StARkin family members bind sterol species (*e.g.* dihydroergosterol) using their StART-like (StAR-related lipid-transfer) domains for retrograde transport of sterol from the PM to the ER. The StARkin family member Ysp1 is represented. Importantly, ER-PM contacts formed by the StARkin proteins are spatially distinct from ER-PM contacts where ORP family members function. In this manner, the StARkin and ORP lipid transfer protein activities may precisely balance sterol lipid levels, species composition, and distribution in the PM as needed. **(Right)** The extended synaptotagmins (E-Syts) function as inducible lipid transfer proteins at ER-PM contacts. E-Syt proteins use their SMP (synaptotagmin-like and mitochondrial lipid-binding protein) domains to dimerize and transfer glycerolipids and glycerophospholipids down a concentration gradient (from high to low concentrations). Consequently, E-Syt proteins could potentially undue the phosphatidylserine gradient set up by ORP isoforms at ER-PM contacts. Accordingly, E-Syts proteins require close membrane tethering to function as lipid transfer proteins and may not be active at the 25 to 30 nm contacts formed by ORP-VAP assemblies (center panel). Moreover, E-Syts are tightly regulated and specifically activated upon receptor signaling coupled to phospholipase C activity and calcium (Ca^{2+}) influx. Ca^{2+} binding to the C2A and C2C domains as well as $PI(4,5)P_2$ binding to the C2E domain of the E-Syt1 protein results in the formation of specialized close ER-PM contacts for the retrograde recycling of phospholipase C-generated diacylglycerol (DAG) from the PM to the ER. In this manner, the E-Syts may specifically participate in lipid and Ca^{2+} signaling events at the PM.

Stefan, Figure 3

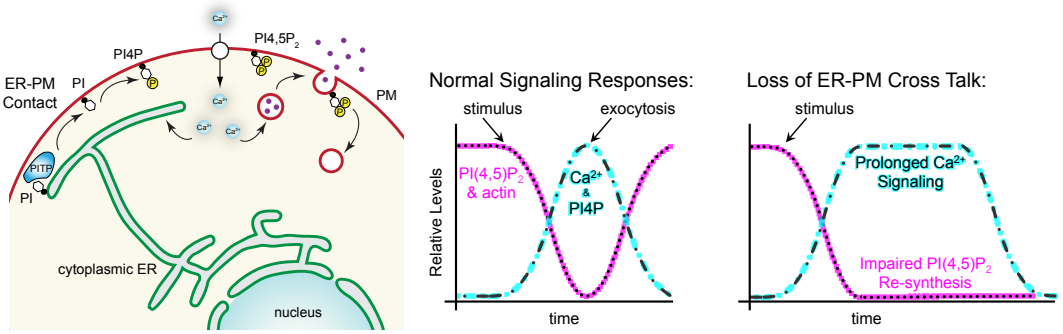

Figure 3. ER-PM contacts modulate phosphoinositide lipid and Ca^{2+} oscillations during regulated exocytosis. **(Left)** Ca^{2+} influx by the store-operated Ca^{2+}-entry (SOCE) pathway and voltage-gated Ca^{2+} channels triggers the release of secretory cargo and the formation of specialized ER-PM contacts. This coordinated regulatory mechanism controls phosphoinositide lipid and Ca^{2+} dynamics necessary to sustain exocytic events at the PM. **(Center)** Stimuli that activate phospholipase C and $PI(4,5)P_2$ hydrolysis subsequently result in Ca^{2+} influx (by SOCE) and increase PI4P levels necessary for $PI(4,5)P_2$ re-synthesis. The Nir2 and Ca^{2+}-regulated TMEM24 phosphatidylinositol transfer proteins (PITP, left panel) facilitate PI4P synthesis at the PM following phospholipase C activation. In turn, PI4P activates the SOCE pathway and serves as a substrate for $PI(4,5)P_2$ re-synthesis. Importantly, these dynamic fluctuations in phosphoinositide lipid and Ca^{2+} signals, even while seemingly slight (less than two-fold changes), modulate the timing and extent of exocytic events at the PM. **(Right)** Transient disruptions in the homeostatic regulation of phosphoinositide and Ca^{2+} signaling at ER-PM contacts results in prolonged PI4P-regulated Ca^{2+} influx and impaired $PI(4,5)P_2$ re-synthesis. Long-term interruptions in ER-PM cross talk eventually lead to significant depletion of $PI(4,5)P_2$ pools and failure to generate cytosolic Ca^{2+} signals, a system-wide collapse of the PI kinase-Ca^{2+} signaling paradigm. Consequently, ER-PM contacts impact vital physiological processes including neurotransmission, insulin secretion, and immune cell signaling.