Endoplasmic Reticulum - Plasma Membrane cross-talk mediated by the extended synaptotagmins

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Keyword

E-Syts, SMP domain, Ca^{2+}, Non-vesicular lipid transport, Lipid transfer proteins

Abstract

The endoplasmic reticulum (ER) possesses multiplicity of functions including protein synthesis, membrane lipid biogenesis and Ca^{2+} storage and has broad localization throughout the cell. While the ER and most other membranous organelles and membranes are highly interconnected via vesicular traffic that relies on membrane budding and fusion reactions, the ER forms direct contacts with virtually all other membranous organelles, including the plasma membrane (PM) without membrane fusion. Growing evidence suggests that these contacts play major roles in cell physiology, including the regulation of Ca^{2+} homeostasis and signaling and control of cellular lipid homeostasis. Extended-synaptotagmins (E-Syts) are evolutionarily conserved family of ER-anchored proteins that tether the ER to the PM in PM $PI(4,5)P_2$-dependent and cytosolic Ca^{2+}-regulated manner. In addition, E-Syts possess a cytosolically exposed lipid-harboring module that confers the ability to transfer/exchange glycerolipids between the ER and the PM at E-Syts-mediated ER-PM contacts. In this chapter, the functions of ER-PM contacts and their role in non-vesicular lipid transport with special emphasis on the crosstalk between the two bilayers mediated by E-Syts will be discussed.

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1. Introduction

The ER forms a complex network of tubules and sheets that extends throughout the interior of cells. Although ER membranes are functionally connected to all membranes of secretory and endocytic pathways via vesicular transport that relies on membrane budding and fusion reactions, they only fuse with each other and with vesicles involved in retrograde transport from the Golgi complex. However, the close appositions between the ER and the membranes of all other membranous organelles, including the PM, play major roles in cellular physiology without membrane fusion. These sites are involved in numerous functions, including the regulation of Ca^{2+} homeostasis, control of ER-localized enzymes that act *in trans* on the apposed membrane, organelle dynamics and exchange of lipids between bilayers (Holthuis and Levine 2005; Elbaz and Schuldiner 2011; Friedman and Voeltz 2011; Toulmay and Prinz 2011; Stefan et al. 2011; Mesmin et al. 2013; Helle et al. 2013; Prinz 2014; Gallo et al. 2016; Phillips and Voeltz 2016).

ER-PM contacts are universal structures that are present in all eukaryotic cells. In cells of higher eukaryotes, ER-PM contact sites were first described by electron microscopy in the 1950's in muscle (Porter and Palade 1957). They were subsequently also observed in neurons (Rosenbluth 1962; Metuzals et al. 1997; Spacek and Harris 1997). Since then, it has become clear that they represent a general feature of all eukaryotic cells, from unicellular organisms to specialized mammalian cells (Friedman and Voeltz 2011; Phillips and Voeltz 2016; Perez-Sancho et al. 2016).

Although ER-PM contacts had been observed for decades, the molecular mechanisms that govern the formation of these contacts were largely elusive. The distance between the ER and the PM at contact sites is estimated to be 10-30 nm based on electron micrographs, suggesting the presence of molecular tethers that define the distance and functional property of these contacts (Orci et al. 2009; West et al. 2011; Fernandez-Busnadiego et al. 2015; Perni et al. 2015; Varnai et al. 2007).

Identification of molecules that are responsible for ER-PM tethering has contributed significantly to our understanding of the function of ER-PM contacts in cellular physiology. The properties of these molecules are directly linked to numerous functions of ER-PM contacts including the control of Ca^{2+} dynamics, signaling and lipid regulation. For example, physical coupling of ER localized STIM1 and PM-localized Orai Ca^{2+} channel is responsible for Store-Operated Ca^{2+} Entry (SOCE) in all cell types (Liou et al. 2005; Zhang et al. 2005; Feske et al. 2006; Lewis 2007; Orci et al. 2009). A number of ER-localized enzymes including protein and lipid phosphatases have been reported to regulate the function of PM substrates *in trans* (Braithwaite et al. 2006; Stuible and Tremblay 2010; Stefan et al. 2011; Dickson et al. 2016).

Moreover, growing evidence suggests the critical roles of lipid transfer proteins in the regulation of cellular lipid homeostasis without inducing membrane fusion via their property to transfer lipids from one compartment to another at membrane contact sites (Prinz 2014; Holthuis and Menon 2014; Gallo et al. 2016). Some of these lipid transfer proteins localize and function at ER-PM contacts and also act as tethering molecules of these two membranes. The analyses of these tethers have revealed fundamental roles

of the membrane contact sites in lipid regulation. One of these tethers is the E-Syts (tricalbins in yeast). In this chapter, the crosstalk between the ER and the PM mediated by E-Syts and its implication in lipid regulation will be discussed.

2. ER-PM crosstalk mediated by E-Syts

2.1. Identification of E-Syts/Tricalbins as ER-PM tether

While the STIM1-Orai coupling enhances ER-PM contacts, ER-PM contacts exist even before recruitment of STIM1 to the sites. Furthermore, the molecules responsible for this coupling are not conserved in yeast, while ER-PM contacts are evolutionarily conserved in all eukaryotic cells. In yeast, the bulk of the ER (greater than 50%) is cortically localized (West et al. 2011). Further supporting the role of ER-PM contacts in lipid regulation, lipid synthesizing enzymes and lipid transport machinery are enriched at these contacts (Pichler et al. 2001; Tavassoli et al. 2013; Maeda et al. 2013; Chung et al. 2015; Moser von Filseck et al. 2015; Chang and Liou 2015; Kim et al. 2015; Gatta et al. 2015). Thus, proteins that mediate cortical ER formation in yeast might regulate fundamental properties of ER-PM contacts, including lipid regulation. Given the importance of lipid homeostasis in all eukaryotic cells, the mechanisms that regulate ER-PM tethering are likely to be highly conserved.

In 2012, the Prinz and Emr labs reported that three yeast ER proteins, the tricalbins (Tcb1p, Tcb2p, and Tcb3p), are selectively enriched in cortical ER and involved in ER-PM tethering (Toulmay and Prinz 2012; Manford et al. 2012). Mammalian homologs of the tricalbins are E-Syts; both tricalbins and E-Syts share similar domain organization with an N-terminal hydrophobic region, followed by a cytosolic Synaptotagmin-like-Mitochondrial-lipid binding Protein (SMP) domain and several C2 domains.

As their name indicates, E-Syts/tricalbins also share a similar domain organization with classical synaptotagmins (Min et al. 2007). However, E-Syts contain cytosolic SMP domain, which is absent in synaptotagmins, and possess strikingly distinct molecular functions. While synaptogamins are anchored to secretory or synaptic vesicles as Ca^{2+} sensors for SNAP (Soluble NSF Attachment Protein) REceptor (SNARE)-mediated membrane fusion (Chapman 2008; Sudhof 2002, 2012), E-Syts are anchored to ER membranes and involved in ER-PM tethering and in harboring and transferring lipids via their SMP domain (see below). In the following sections, the role of E-Syts in ER-PM tethering as well as in control of PM lipid homeostasis will be discussed in details.

2.2. E-Syts mediate $PI(4,5)P_2$-dependent and Ca^{2+}-regulated ER-PM contacts

There are three E-Syts (E-Syt1, E-Syt2, and E-Syt3) in mammalian cells, which form homo- and heterodimers (Giordano et al. 2013; Saheki et al. 2016). They all localize at the ER through their N-terminal hydrophobic hairpin

sequence that anchors them to ER membrane. The N-terminal hydrophobic region is followed by a SMP domain and multiple C2 domains [i.e. five in the case of E-Syt1 (C2A, C2B, C2C, C2D, C2E) and three in the case of E-Syt2 and E-Syt3 (C2A, C2B, C2C) instead of the two canonical C2 domains of synaptotagmins] (Figure 1a). E-Syts tether the ER to the PM via their C2 domain-dependent interactions with the PM, that critically depends on the presence of PI(4,5)P_2 in the PM (Giordano et al. 2013).

Overexpression of E-Syt2 and E-Syt3 induces massive formation of ER-PM contacts through their C2C domain-dependent interaction with PM PI(4,5)P_2. E-Syt1, on the other hand, recruits the ER to the PM in the presence of high levels of cytosolic Ca^{2+} by Ca^{2+}-sensing property of its C2C domain (Giordano et al. 2013; Fernandez-Busnadiego et al. 2015; Idevall-Hagren et al. 2015). As they form heteromeric complexes, the E-Syts confer cytosolic Ca^{2+} regulation to PI(4,5)P_2-regulated ER-PM contact formation. Supporting the role of E-Syt heteromers in Ca^{2+}-dependent ER-PM contact formation, the depletion of E-Syts leads to the loss of Ca^{2+} sensitivity in ER-PM tethering, which can be rescued by expression of E-Syt1 (Giordano et al. 2013; Saheki et al. 2016).

Measurement of Ca^{2+} concentration required for E-Syt1-recruitment to the PM in semi-intact cells revealed the EC50 of such recruitment in low micromolar Ca^{2+} range, similar to synaptotagmins. Accordingly, E-Syt1 accumulation at ER-PM contact sites occurred only upon experimental manipulations known to achieve these levels of Ca^{2+} via its influx from outside of the cells, such as membrane depolarization in excitable cells and SOCE (Idevall-Hagren et al. 2015). However, E-Syts-dependent contacts are not required for SOCE as reducing the levels of their expression did not affect thin cortical ER formation upon ER Ca^{2+} depletion that is thought to be responsible for SOCE (Giordano et al. 2013). Thus, ER-PM contacts mediated by the E-Syts are functionally distinct from those mediated by STIM1 and Orai (Giordano et al. 2013; Quintana et al. 2015; Jing et al. 2015).

2.3. E-Syts possesses the SMP domain, a lipid-harboring module that belongs to TULIP superfamily

The presence of an SMP domain, a member of a family of lipid binding modules that is often present in proteins localized at membrane contact sites (Kornmann et al. 2009; Kopec et al. 2010; Toulmay and Prinz 2012; Kopec et al. 2011; Alva and Lupas 2016; Reinisch and De Camilli 2016), suggested that E-Syts may transfer lipids between membranes of the ER and the PM. Structural study of the SMP domain of E-Syt2 revealed that the SMP domain has a β-barrel structure and dimerizes to form a ~9 nm long cylinder traversed by a channel lined entirely with hydrophobic residues (Schauder et al. 2014) (Figure 1b). A mass spectrometry analysis of the lipids harbored by the SMP domain revealed the presence of a wide variety of glycerolipids without a particular specificity against their headgroups (Schauder et al. 2014). This is in good agreement with the solved structure of the SMP domain in which acyl chains are inserted to its deep hydrophobic groove while the headgroups are exposed to the outside the groove (Schauder et al. 2014). The similar lipid-harboring property was also reported for the SMP domains of the ERMES

[endoplasmic reticulum (ER)-mitochondria encounter structure] complex, which localizes at ER-mitochondria contact sites (AhYoung et al. 2015).

The structure of the SMP domain of E-Syts confirmed its homology to the *tu*bular *lip*id-binding (TULIP) superfamily (Kopec et al. 2010, 2011; Schauder et al. 2014; Reinisch and De Camilli 2016). Other TULIP domain-containing proteins are also involved in harboring, and at least in some cases, transporting lipids via their TULIP domains. Although many of these proteins act in extracellular environment rather than intracellular environment (like proteins with the SMP domain), the similar structure and the shared lipid-harboring property of the SMP domain and other TULIP domains indicates their common functions in lipid harboring, sensing and transport (Alva and Lupas 2016).

For example, cholesterol ester transfer protein (CETP) is a well-characterized serum protein that transports lipids between high density and low density lipoproteins. Several different lipids, including cholesterol esters, can be harbored by its TULIP domain (Qiu et al. 2007). Other serum proteins, lipopolysaccharide-binding protein (LBP) and bacterial/permeability-increasing protein (BPI), play an important role in the innate immune response against invading Gram-negative bacteria by binding to lipopolysaccharides/endotoxins present in the membrane of the bacteria (Beamer et al. 1997; Weiss 2003). A BPI-family protein NRF-5 has been reported to extract phosphatidylserine (PS) from the surface of apoptotic cells and transport it to engulfing cells during their clearance in *C. elegans* (Zhang et al. 2012b). Interestingly, NRF-5 is also involved in the process of regenerative axonal fusion during injury, which requires PS recognition (Neumann et al. 2015).

These studies suggested that the SMP domain might be involved in direct lipid transport and supported the role of membrane contact sites, which are populated by SMP domain-containing proteins, in lipid exchange reactions without membrane fusion.

2.4. E-Syts transfer glycerolipids *in vitro* via the SMP domain

Given the presence of the SMP domain that can harbor glycerolipids promiscuously, E-Syts were thought to transport multiple lipids between membranes. *In vitro* analysis of lipid transport ability of E-Syt1, the Ca^{2+}-sensing E-Syt, using artificial membranes in cell-free system, revealed that E-Syt1 could indeed transport glycerolipids between membranes regardless of their head groups (Saheki et al. 2016; Yu et al. 2016); E-Syt1 even transports diacylglycerol (DAG), which is most hydrophobic due to the lack of phosphorylated head group (Saheki et al. 2016). In contrast to the ability of SNAREs to induce membrane fusion of two opposing membranes, E-Syts mediate lipid transport between tethered membranes without membrane fusion. The lipid transport ability of E-Syt1 was completely abolished when its lipid-harboring SMP domain was deleted and significantly impaired when the acyl-chain insertion sites of the SMP domain were mutated (i.e. lipids can no longer be accommodated in the lipid-insertion defective mutant protein), while these mutations did not inhibit tethering of the membranes (Saheki et al. 2016; Yu et al. 2016). Synaptotagmin1 (Syt1), on the other hand, only tethers

membranes without lipid transport. However, the SMP domain of E-Syt1 can confer lipid transport ability to Syt1 when it was artificially conjugated to the cytosolic region of Syt1 (Yu et al. 2016). Therefore, the SMP domain is necessary and sufficient for the lipid transport ability of E-Syt1.

E-Syt1-dependent lipid transport is significantly enhanced in the presence of Ca^{2+} in the micromolar range, and this effect requires $PI(4,5)P_2$ in one of the two opposing membranes (Saheki et al. 2016), consistent with the Ca^{2+}-dependency of E-Syt1 recruitment to the PM in semi-intact cells (Idevall-Hagren et al. 2015). To further support the role of Ca^{2+} in this process, E-Syt1 carrying mutations in the Ca^{2+}-binding pockets of C2C domain, the domain responsible for Ca^{2+}-dependent interaction with the PM, show much reduced lipid transfer activity (Yu et al. 2016). Given the $PI(4,5)P_2$-dependent and Ca^{2+}-regulated ER-PM tethering mediated by E-Syts in cells, the major action of the Ca^{2+} is likely to enhance tethering of the opposing bilayers rather than acting directly on the SMP dimer as the SMP domain does not possess Ca^{2+}-binding sites (nor predicted changes in conformation via Ca^{2+}-binding). However, it is also possible that Ca^{2+}-dependent binding to lipid bilayers of its C2A domain (Giordano et al. 2013; Chang et al. 2013), which also contains a Ca^{2+}-binding site, may assist lipid transfer by a direct and local effect on the bilayer. As a Ca^{2+}-sensitive C2A domain is present in E-Syt2 and E-Syt3 (Xu et al. 2014), even these two proteins, whose ER-PM tethering function is not regulated by cytosolic Ca^{2+}, may have a Ca^{2+}-dependent role in lipid transfer.

Collectively, these studies show that the lipid-harboring SMP domain indeed confers E-Syt1, and possibly all the E-Syts, the ability to transport lipids (Saheki et al. 2016; Yu et al. 2016).

2.5. Two models of SMP domain-dependent lipid transfer: shuttle model and tunnel model

How the E-Syt SMP dimer associates with membranes to extract and deliver lipids remains elusive. In one model, termed as “tunnel model”, the SMP domains would act as bridges between the ER and the PM. Lipids could then get transferred along the hydrophobic channel as if they “snorkel” through aqueous cytosol with their hydrophobic fatty acids moieties protected in the channel while their hydrophilic lipid head groups protrude into the cytosol (Reinisch and De Camilli 2016). The similar model was proposed for lipid transfer between lipoproteins by CETP (Qiu et al. 2007; Zhang et al. 2012a). In the tunnel model, the SMP dimer needs to directly contact with the two membranes at its ends, and lipids enter and exit there. For this model to work, the distance between the ER and the PM at ER-PM contacts has to be as close as ~9 nm. However, the electron micrographs of E-Syt-induced contacts do not support such short distance (Fernandez-Busnadiego et al. 2015). Therefore, the second model, termed as “shuttle” model, is more plausible. In this model, SMP dimer is tethered between the ER and the PM by its franking PM interacting modules (C2 domains) and ER membrane anchor, shuttling freely to transfer harbored-lipids between the two bilayers. This is also consistent with the modes of lipid transfer mediated by other lipid transfer proteins (Reinisch and De Camilli 2016; Chiapparino et al. 2016). The

elucidation of the precise mechanisms of lipid extraction and delivery by the SMP domain requires further investigation.

2.6. Control of PM lipid homeostasis by E-Syts

E-Syt1-dependent lipid transport is bidirectional and driven by the concentration gradient of the lipid (Saheki et al. 2016). Microsomal vesicles, representing crude ER fraction, isolated from preadipocytes were able to acquire lipids from artificial membranes; this activity relies on the expression of E-Syt1 (Yu et al. 2016). As E-Syts-dependent lipid transfer between artificial membranes is facilitated by an increase in Ca^{2+}, E-Syts may participate in lipid transport/exchange between the membranes of the ER and the PM particularly when the levels of cytosolic Ca^{2+} increase in cells. In fact, cells lacking E-Syts do not exhibit abnormalities in the major glycerolipids at a resting condition (Saheki et al. 2016), arguing against their primary roles in the steady-state regulation of PM lipid compositions.

Because E-Syts can transfer lipids down their concentration gradient, they may counteract changes in membrane lipid compositions that occur during the increase in cytosolic Ca^{2+} and facilitate the transport of membrane lipids from one membrane to another in order to reset their concentration to the normal levels. For example, the formation of E-Syt-dependent ER-PM tethers in response to stimuli that elevate Ca^{2+} may help reverse accumulation of DAG in the PM by transferring it to the ER for metabolic recycling. Supporting such function, cells lacking E-Syts exhibit enhanced and sustained accumulation of DAG in the PM following $PI(4,5)P_2$ hydrolysis by phospholipase C (PLC) activation (Saheki et al. 2016). This effect can be rescued by expression of E-Syt1, but not by mutant E-Syt1 lacking the SMP domain, supporting the role of the SMP domain in lipid transfer in cells (Saheki et al. 2016).

ER-PM contacts are populated by various ER-associated lipid transfer proteins (Maeda et al. 2013; Chung et al. 2015; Moser von Filseck et al. 2015; Mesmin et al. 2013; Chang et al. 2013; Chang and Liou 2015; Kim et al. 2015; Gatta et al. 2015). Therefore, the function of E-Syts may be coupled with other lipid transfer proteins such as Nir2, which interacts with ubiquitous ER protein VAP (Lev et al. 2008; Murphy and Levine 2016) and interacts with PM lipids such as DAG and PA (Chang and Liou 2015; Kim et al. 2015). In fact, a strong functional coupling between E-Syts and Nir2 was observed at ER-PM contacts (Saheki et al. 2016). In the absence of E-Syts, more Nir2 is recruited to the PM upon PLC activation during an increase in cytosolic Ca^{2+}. Nir2 transfers PA, a phosphorylated product of DAG, to the ER during PLC-dependent $PI(4,5)P_2$ hydrolysis for the recycling of PA for PI resynthesis in the ER (Cockcroft et al. 2016; Kim et al. 2015; Yadav et al. 2015). Therefore, one of the function of E-Syts may be to control the recycling pathway of $PI(4,5)P_2$ metabolites for lipid resynthesis in the ER (Figure 1c).

2.7. Elusive functions of E-Syts

Other functions of E-Syts, including the regulation of signaling, have been also proposed (Herdman and Moss 2016). In plant, all proteins previously

considered synaptotagmin homologues contain SMP domains (Craxton 2001, 2007; Levy et al. 2015). Interestingly, one of these proteins in *Arapidopsis thaliana* is required for plant freezing tolerance as well as for resistance to mechanical stresses (Schapire et al. 2008; Yamazaki et al. 2008; Perez-Sancho et al. 2015). These studies indicate the additional role of this family of proteins in the regulation of lipid integrity in the PM. This is also consistent with the reported function of yeast tricalbins in PM integrity (Aguilar et al. 2007; Omnus et al. 2016).

Mutant mice lacking all the three E-Syts are viable and fertile, and they do not show major abnormality in development (Sclip et al. 2016; Tremblay and Moss 2016), and similar findings are also reported in yeast (Manford et al. 2012). Therefore, these proteins are not essential for life. Given the lack of major phenotypes in E-Syts triple knock-out mice, the function of E-Syts must be at least partially overlapping and redundant with the function of other proteins such as Nir2/3.

Strong evolutionarily conservation of E-Syts from yeast to human indicates important function of these proteins in eukaryotes. In mammals, E-Syts are heterogeneously expressed in different tissues with highest expression in lung, testis and the immune system (Tremblay and Moss 2016). Therefore, functional studies of E-Syts in these tissues may help reveal the still elusive function of these proteins. Elucidating the physiological roles of E-Syts as well as other SMP domain-containing proteins localized at membrane contact sites will be important to gain further insights into how non-vesicular lipid transport contributes to lipid compartmentalization in cells.

3. Future prospective: neuronal function of non-vesicular lipid transport at ER-PM contacts

Brain activity and neuronal survival rely on PM lipids that play a major role in neurotransmission (Saheki and De Camilli 2012). Thus, abnormal lipid homeostasis is linked to numerous neurological disorders (Blackstone et al. 2011; Lev et al. 2008; Di Paolo and Kim 2011).

Neuronal cells are specialized cells with extensions of neuronal processes for efficient neurotransmission; synaptic membranes are highly dynamic and can be separated from the cell body by a significant distance. At distant nerve terminals, vesicular transport is not sufficiently rapid to replenish the loss of PM lipids. Thus, non-vesicular lipid transport is likely to have significant roles in maintenance of the neuronal PM. In fact, neuronal processes, including axons and dendrites, are highly decorated with a continuous network of the ER (Spacek and Harris 1997; Fernandez-Busnadiego et al. 2015) (Figure 2a,b). Significantly, mutations in ER morphogenetic proteins as well as regulators of cellular lipid homeostasis have been identified in neurodegenerative disorders, including hereditary spastic paraplegia (HSP) and amyotrophic lateral sclerosis (ALS) (Blackstone et al. 2011; Lev et al. 2008; Dong et al. 2016). In both HSP and ALS, motor neurons with long axons are selectively lost during disease progression. The communication of neuronal PM, including synaptic membranes, with the ER occurs directly via ER-PM contacts. Therefore, they may play an essential role in the maintenance of PM lipids via regulation of non-vesicular lipid

transport, whose loss might contribute to the reduced viability of neurons with particularly long axons.

Importantly, genetic studies suggest a common genetic basis between these rare disorders and more common neurodegenerative conditions such as Alzheimer's disease and Parkinson's disease (Novarino et al. 2014; Singleton and Hardy 2016; Small and Petsko 2015). Therefore, elucidating the mechanisms of non-vesicular lipid transport and cellular lipid homeostasis will not only advance our knowledge in fundamental cell biology but also our understanding of neurodegeneration in general.

Figure legends

Figure 1. E-Syts mediate ER-PM tethering and regulate lipid transfer.
(**a**) E-Syts, anchored to ER-membrane, form homo- and heterodimer and control ER-PM tethering via their C2 domain-dependent interaction with the PM that is additionally regulated by cytosolic Ca^{2+}. The SMP dimer likely mediates lipid transfer by shuttling the two membranes. (**b**) The crystal structure of the SMP domain. The SMP domain forms a dimer; hydrophobic residues line the channel. Lipid fatty acids are shown in red. Adapted from Schauder et al. (Nature 2014) (**c**) Schematics of the possible role of E-Syts in the control of PM DAG dynamics during PLC activation. E-Syts may cooperate with Nir2 in this process. IP3, inositol 1,4,5-trisphosphate. DAG, diacylglycerol. PA, phosphatidic acid. PLC, phospholipase C.

Figure 2. Maintenance and regulation of the neuronal plasma membrane.
(**a**) The ER extends throughout neuronal processes and forms physical contacts with the PM. These ER-PM contacts are implicated in lipid transfer, signaling, Ca^{2+} homeostasis and synaptic plasticity. Red arrows indicate classical vesicular transport via the Golgi apparatus, which is absent in neuronal processes. (**b**) An electron micrograph showing ER-PM contacts at a dendritic spine. Adapted from Synapse Web, Kristen M. Harris, PI, http://synapseweb.clm.utexas.edu/

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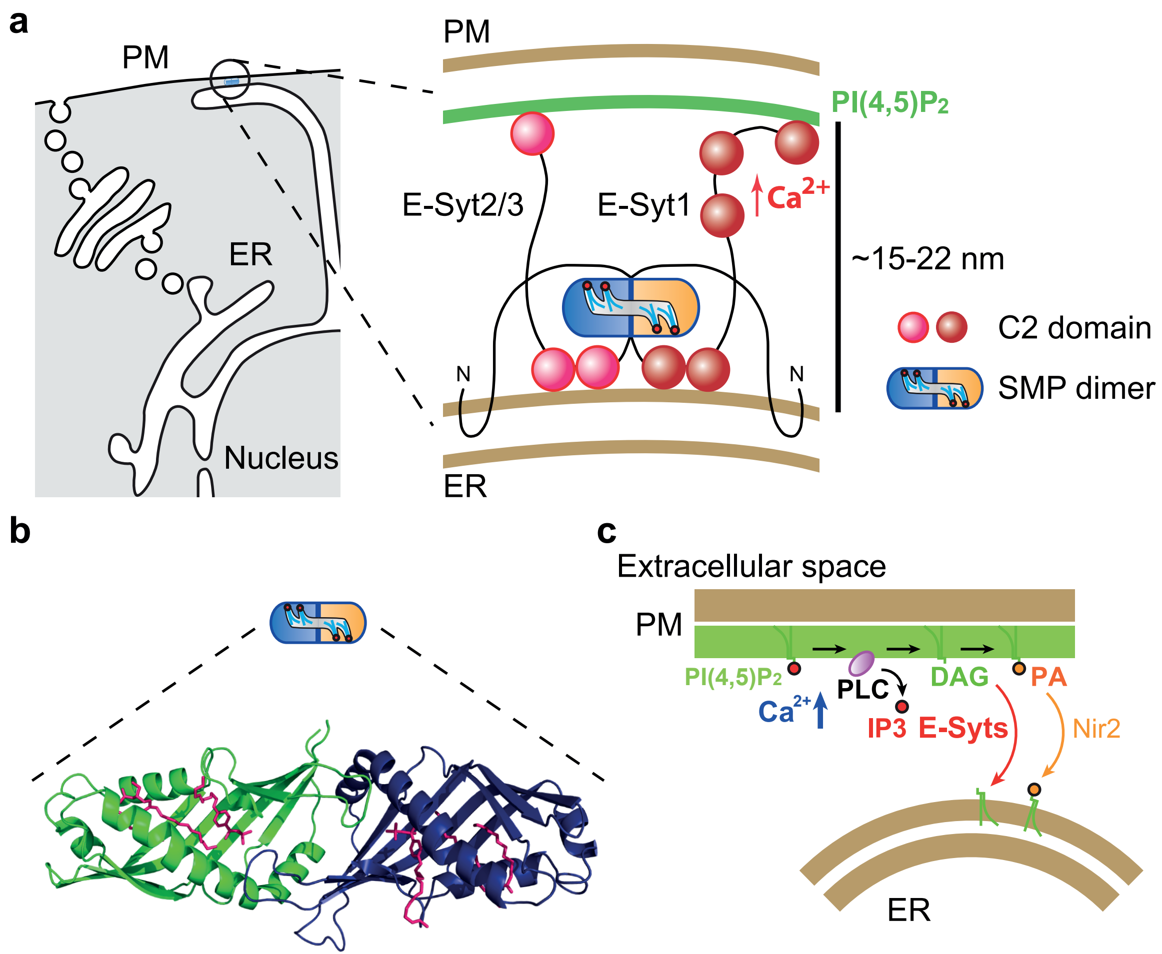

Figure 1, Saheki

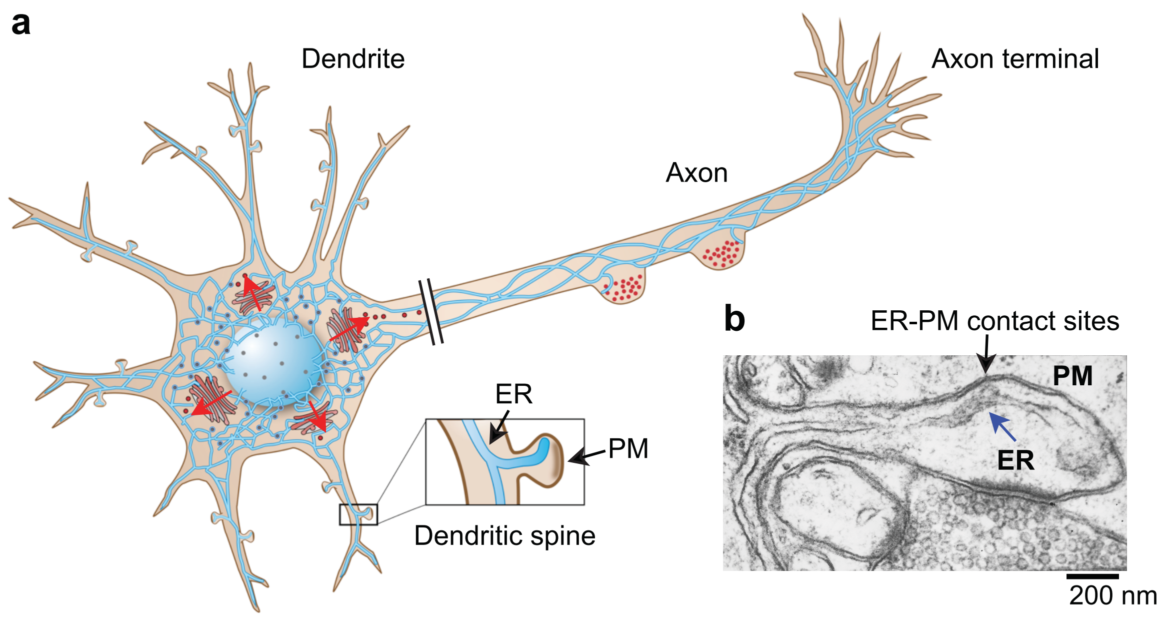

Figure 2, Saheki