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REVIEW ARTICLE

Illuminating the membrane contact sites between the endoplasmic reticulum and the *trans*-Golgi network

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Membrane contact sites (MCSs) between different organelles have been identified and extensively studied over the last decade. Several classes of MCSs have now well-established roles, although the contacts between the endoplasmic reticulum (ER) and the *trans*-side of the Golgi network (TGN) have long remained elusive. Until recently, the study of ER–TGN contact sites has represented a major challenge in the field, as a result of the lack of suitable visualization and isolation techniques. Only in the last 5 years has the combination of advanced technologies and innovative approaches permitted the identification of new molecular players and the functions of ER–TGN MCSs that couple lipid metabolism and anterograde transport. Although much has yet to be discovered, it is now established that ER–TGN MCSs control phosphatidyl-4-phosphate homeostasis by coupling the *cis* and the *trans* activity of the ER-resident 4-phosphatase Sac1. In this review, we focus on recent advances on the composition and function of ER–TGN MCSs.

Keywords: endoplasmic reticulum; lipid exchange; membrane contact sites; membrane trafficking; PI4P homeostasis; *trans*-Golgi network

Membrane contact sites: old discovery for a young cell biology field

Membrane contact sites (MCSs) are areas of juxtaposition between multiple membrane systems (two organelles or an organelle and lipid droplets) that, although in contact, conserve their identity. They are defined as homotypic if occurring between identical organelles or heterotypic when they occur between different organelles [1]. This close apposition between membranes unequivocally implicates a contact site if the distance between the organelles spans 10–30 nm, allowing membrane tethering in the absence of fusion [1,2]. Despite their very early discovery during the 1960s ([3–6], they remained almost ignored for decades. Over the last 10 years, MCSs have become a hot topic in cell biology and many different classes were first identified and then characterized in terms of localization, function and composition [1,2].

The main features of membrane contact sites

All MCSs between the endoplasmic reticulum (ER) and other organelles, including the *trans*-Golgi network (TGN), mitochondria and the plasma membrane (PM), share two main features: (i) the presence of tethers, meaning proteins that can simultaneously contact the two organelles and/or are necessary for contact site establishment

Abbreviation

ApoB100, apolipoprotein B100; CERT, ceramide transfer protein; DAG, diacylglycerol; ER, endoplasmic reticulum; LTP, lipid transfer protein; MCS, membrane contact site; OSBP1, oxysterol-binding protein 1; PI, phosphatidylinositol; PI4P, phosphatidyl-4-phosphate ; PKD, protein kinase D; PM, plasma membrane; PS, phosphatidylserine; SM, sphingomyelin; SMS, sphingomyelin synthase; TGN, *trans*-side of the Golgi network.

and maintenance [7] and (ii) the function of lipid exchange between the two apposed membranes [8].

Protein tethers

Among the most studied and characterized tethering complexes are those involving the ER proteins VAPA and VAPB and their yeast homologues Scs2/22. These have been found to be involved in the formation of MCSs between the ER and almost every other organelle as a result of their ability to function as the ER determinant for a variety of proteins working at the ER–organelle interface [9]. The VAP orthologues Scs2/22 play an important role in ER–PM contact site formation in yeast because their depletion results in a reduction of ~50% in contact site number [10].

Unlike its yeast counterpart, mammalian VAP does not appear to be strictly required for ER–PM contact maintenance, probably because many described ER–PM tethers are ER transmembrane proteins and hence do not require VAP for ER targeting [11–13]. At the ER–mitochondria interface, VAPs represents the ER-anchor for the tether VPS13A [14] and, by binding the mitochondrial protein PTPIP51, they also directly regulate ER–mitochondria associations that are reduced by 30% under VAP depletion [15]. Multiple roles for VAP have been described also at the level of ER–endosome contact sites, where VAPs represent the ER determinant for the localization of a variety of ER–endosome tethers, such as protrudin [16], ORP1L [17], STARD3 [18] and VPS13C [14]. However, although, for example, STARD3-induced contacts are VAP-dependent [18], no direct studies have yet reported a role for VAPs in basal ER–endosome contact site establishment.

At the level of ER–TGN contact sites, VAPs represent the ER-anchor for proteins required for ER–TGN contact site formation and maintenance, not only for phosphatidylserine (PS) transfer protein ORP10 and the tethering factors oxysterol-binding protein 1 (OSBP1) and ORP9 [19], but also for all the lipid transfer proteins (LTPs) that take advantage of ER–TGN contact sites to mediate non-vesicular trafficking of lipids between the two membranes, such as ceramide transfer protein (CERT), Nir2 and OSPB1 [9]. As a consequence, the role of VAPs at ER–TGN contact sites is crucial and cells lacking VAPs show a strong impairment in ER–TGN contact site establishment ([19]).

Lipid exchange

Another common feature shared by ER–TGN and other ER–organelle contact sites is that they are ‘used’ for lipid exchange between the two organelles by LTPs.

Phosphatidylinositol (PI) has been shown to be transported by non-vesicular trafficking at the ER–PM interface by the LTPs Nir2 and TMEM24 to replenish the PI(4,5)P2 PM pool following receptor and glucose stimulation, respectively [13,20]. In addition, Nir2 transports PI from the ER to the Golgi, where PI is converted into phosphatidyl-4-phosphate (PI4P) and diacylglycerol (DAG) [21]. Sterol and PS transport also occur at contact sites and can be coupled with the counter-exchange of PI4P. ORP family members ORP5/ORP8 and their yeast counterparts Osh6/7 mediate the transfer of PS from the ER to the PM and of PI4P in the opposite direction at the ER–PM interface [11,22]. Something similar occurs at ER–TGN contact sites where OSBP1 and its yeast orthologue Kes1/Osh4 mediate the counter-exchange of PI4P with cholesterol and ergosterol, respectively [23,24]. Members of the Lam/Ltc protein family in yeast, which localize at ER–PM contact sites, have been shown to mediate PM to ER sterol transport [25], even though this has not yet been coupled with PI4P homeostasis. At ER–endosome contact sites, the lipid transfer protein STARD3 mediates cholesterol delivery to endosomes [18], whereas OSBP1 controls the homeostasis of a local pool of PI4P, presumably by a mechanism analogous to the one observed at ER–TGN contact sites [26].

Among the many different types of contact sites, little is known about MCSs between the ER and the *trans*-Golgi network (TGN). In this review, we discuss the limitations that impeded the study of ER–TGN contact sites, as well as the tools that have been developed recently to study them. Moreover, we focus on recently identified features, components and functions of ER–TGN contact sites, as well as highlight open questions in the field.

ER–TGN contact sites from yeast to mammals

The association between the ER and the Golgi in different organisms is marked by some peculiarities, mainly as a result of the characteristic architecture and organization of the Golgi complex, whereas the organization of the ER in tubules and cisternae is conserved from yeast to higher animals and plants [27]. The budding yeast *Saccharomyces cerevisiae* and *Pichia pastoris* differ in the organization of Golgi cisternae that are isolated and scattered throughout the cytoplasm in *S. cerevisiae* but organized in ordered and polarized stacks in *P. pastoris* [28,29]. A scattered Golgi distribution is observed in plants [30], whereas, in *Drosophila*, the Golgi cisternae are assembled in

coupled and polarized stacks that, in contrast to those in mammals, are not interconnected but diffuse in the cytoplasm [31]. In yeast, *Drosophila* and *Caenorhabditis elegans*, the Golgi elements are strictly associated with the ER via their *cis* face forming specialized domains known as tER–Golgi units, the basic secretory units [28,29,32,33]. Some recent findings have demonstrated how yeast ER–Golgi contact sites dynamically reorganize upon perturbations as a result of the ER-resident protein Nvj2p, which relocalizes to ER–Golgi contacts upon ER stress, acting as a tether to stabilize them [34]. In plants, the use of laser trapping technology allowed the study of the Golgi units and revealed that Golgi stacks are motile and physically associated with ER tubules [35,36].

By contrast to other eukaryotic cells, mammalian cells have a complex membrane system between the *cis*-Golgi and the ER, identified in the late 1980s and called ERGIC (ER–Golgi intermediate compartment) [37]. In mammals, the Golgi complex comes into close apposition with the ER through its *trans* face and MCSs between these two organelles have been recently defined as ER–TGN contact sites [19]. Mammalian ER–TGN contact sites have been documented from the ultrastructural point of view from the 1960s onward as a result of advances in microscopy techniques that have allowed the precise description of the morphology of the TGN and ER areas involved in the contacts [3,38,39]. Indeed, the ER side apposed to the TGN cisternae was described as devoid of ribosomes, while the opposite side was defined as decorated by ribosomes [39].

Limitations of the early studies on ER–TGN contact sites

Until recent years, visualizing and studying ER–TGN contact sites has been very challenging because they are located in a very crowded perinuclear area, where it is often difficult to distinguish individual subcellular components. For this reason, electron microscopy (with its specialization 3D tomography) has been until recently the only way to explore ER–TGN MCSs [39]. This technique allows a very detailed visualization of ER tubules, of the *cis*, *medial* and *trans* Golgi cisternae and, obviously, of the sites of apposition and tethering between ER and TGN, although it cannot provide an overview of the contact sites in the whole cell. On the other hand, confocal microscopy, which would ideally provide a nice overview of MCSs in the cell, is suitable for studying ER–TGN contact sites because its resolution (200 nm) far exceeds ER–TGN contact site width (10–30 nm). Furthermore, both microscopy methods often require fixation steps that may alter the contacts or introduce artifacts in their visualization.

Thus, considering the transient and highly dynamic nature of ER–TGN contact sites and their ability to change upon perturbations, the best method for identifying and characterizing these dynamic structures should provide a resolution sufficient to appreciate in detail the area involved in the contacts combined with non-invasive procedures for sample preparation. Ideally, an imaging method able to capture the contacts in live cells would be appropriate and definitely helpful. Indeed, in recent years, much effort has been made by researchers in the field to conceive and develop a suitable tool to unambiguously identify ER–TGN contact sites and follow their dynamics.

New methods and tools for studying ER–TGN contact sites

A step forward in the field of ER–TGN contact site visualization was made by Mesmin *et al*. [23] who used a fragment of the OSBP1 encompassing the PH domain and the FFAT domain (PH-FFAT) that is able to bridge ER and TGN membranes, as demonstrated by the relocalization of an ER marker, VAPA, in the Golgi area and the presence of large patches of ER–TGN apposition visualized by electron microscopy. Although this was instrumental in identifying one of the functions of ER–TGN contact sites, namely the counter-exchange of PI4P for cholesterol (see below), this tool artificially induces stable ER–TGN contact sites and is not suitable for screening possible physiological components that establish and maintain the contacts. There was a major breakthrough in ER–Golgi contact site biology very recently as a result of work conducted by Venditti *et al*.[19,40], which combined the classical electron microscopy approach with innovative fluorescence-based techniques, thus providing a powerful tool for ER–TGN contact site visualization and study. On the one side, single-stack resolution was achieved using an electron microscopy approach that provides a comprehensive characterization of ER–Golgi contact sites in their native state, showing that only a fraction of Golgi stacks is engaged in contacts with the ER (~55%), with an average of 24% of the TGN surface being involved and a contact width ranging from 5 to 20 nm. This characterization was complemented by a 3D view of ER–TGN contact sites obtained by focused ion beam–scanning electron microscopy, which allows 3D imaging at electron microscopy resolution power and enables the detection of a surface area of up to 0.2 μm^2 for each organelle at the ER–TGN interface [19]. On the other side, a

new fluorescence resonant energy transfer–fluorescence lifetime imaging microscopy technique was developed to screen multiple conditions. The fluorescence lifetime imaging microscopy measured changes in the lifetime (ns) of a donor fluorophore (GFP) fused to a TGN marker in the presence of an acceptor molecule (mCherry) fused to an ER marker. When two membranes are sufficiently close (within 10 nm), an energy transfer occurs, and the lifetime of the donor fluorophore becomes significantly shorter. This powerful tool was used to screen for putative candidates and led to the identification of new proteins required for contact site formation [19]. Finally, the ER (mCherry-Cb5) and TGN (GFP-TGN46) reporters were further engineered with the addition of the FKBP and FRB domains, respectively, allowing the visualization of ER–TGN contact sites by regular confocal microscopy. Rapamycin induces the dimerization of FKBP and FRB and it was found that even very low concentrations and a short time (200 nM for 2 min) induce the redistribution of the ER marker to the Golgi area that can be easily visualized by fluorescence [19,40]. It thus represents a method for capturing the presence/absence of pre-existing ER–TGN contact sites because only regions of close proximity allow the heterodimerization of the two reporters. Further supporting these conclusions, VAP depleted cells failed to show any ER–TGN contact site formation upon this short rapamycin treatment. This innovative tool will allow fast, high content screening to identify ER–TGN contact site determinants and regulators, without affecting or stabilizing ER–TGN contact sites over time, in contrast to the constitutive expression of a tethering factor (e.g. overnight expression of the PH-FFAT domain of OSBP) that stably connects the membranes of the two organelles.

New molecular players at ER–TGN contact sites: tethering and regulatory proteins

To better understand how the membranes of these two distinct organelles can communicate, proteins involved in maintenance and function of ER–TGN contact sites need to be identified. Regarding all of the other MCSs, tethering components and proteins that act specifically at the ER–Golgi interface have been discovered recently. However, if it is true that the list of identified proteins involved in other contact sites is continuously growing, this is definitively not the case for contact sites occurring between the ER and the TGN. Only proteins that affect the formation and number of contacts can be defined as tethering molecules.

A few proteins have been described as acting as molecular tethers at ER–TGN contact sites: the ER-resident VAP proteins (both VAPA and VAPB) and OSBP1 and ORP9 LTPs [19]. VAP (VAMP-associated proteins, as well as their yeast homologs Scs2 and Scs22) proteins represent a hallmark of other ER-mediated contact sites in cells [9]. They are small ER-resident C-tailed anchored proteins (VAPA is a 33 kDa protein, whereas VAPB is a 27.2 kDa protein), possessing a MSD domain at the cytosolic N terminus. Proteins are recruited and concentrated to ER–organelle contacts through an interaction between the MSP domain of the VAPs and the FFAT motif (phenylalanine in an acidic tract motif) of the proteins [9]. VAP-KD or VAP-KO cells exhibit an almost complete loss of ER–TGN contact sites, with a concomitant large increase in PI4P at the TGN and in other cellular compartments (e.g. endosomes), as expected considering the importance of VAP proteins in regulating several MCS functions [10,15,26,40].

In recent decades, proteins belonging to the LTPs class, such as CERT, OSBP1, some ORP proteins, FAPP2 and Nir2, have been often classified as ER–TGN contact site markers because they can localize at the interface between the two organelles (Fig. 1) [8,9,41]. This, however, can be ascribed to the intrinsic nature of these proteins that possess a dual-targeting motif, a FFAT domain that binds the ER-resident proteins VAPs and a PH domain binding PI4P in the TGN [41], although it does not rely on any systematic study of ER–TGN composition and function.

Coordination of lipid transfer activity: the case of CERT

An important function of ER–TGN contact sites is the regulation of lipid homeostasis. This action is tightly regulated by the VAP proteins, which ensure the correct localization of proteins at the ER–TGN interface, and by the LTPs that possess both lipid binding domains (e.g. the PH domain), providing the proper molecular targeting to the TGN by recognition of PI4P, and lipid transfer domains responsible for the extraction and physical transfer of lipids [8,41]. The first mechanism of lipid transfer at the ER–TGN contact sites was described early in the 2000s, as a result of the identification of CERT [42]. CERT was identified by Hanada *et al.* [42] using a functional rescue cloning strategy: one variant of CHO with defective sphingomyelin (SM) synthesis was found to also show defective ER–Golgi ceramide transport. Screening of cDNAs that were able to revert this phenotype led to the discovery of CERT, which catalyzes the

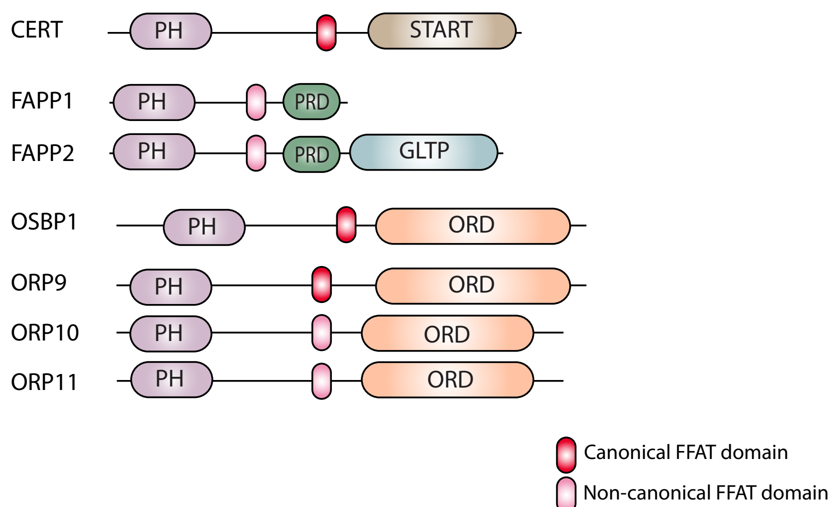

Fig. 1. LTPs acting at the ER–TGN contact sites. Representation of LTPs acting at ER–TGN contact sites. LTPs possess a Golgi targeting motif represented by the PH domain binding PI4P at the TGN and an ER targeting motif represented by the two phenylalanine in an acidic tract (FFAT) domain that mediates the interaction with the ER-localized VAP proteins. The FFAT domain can be either canonical (CERT, OSBP1 and ORP9) or non-canonical (FAPP1, FAPP2, ORP10 and ORP11) [9]. A peculiarity of LTPs is the presence of a lipid binding and transfer domain at the C terminus. The START and the GLTP domains of CERT and FAPP2 bind ceramide and glucosylceramide, respectively [42,96]. The ORD domain of the OSBP-related proteins is specific for different lipids: cholesterol for OSBP1 and ORP9 and PS for ORP10 and ORP11 [64].

non-vesicular transport of ceramide from the ER to the TGN [42].

To exert this function, CERT has dual-targeting motifs, a (canonical) FFAT domain that binds both VAPA and VAPB and a PH domain that allows TGN recognition via PI4P [42,43]. At its C terminus, CERT contains a START domain that appears to be quite flexible: it binds several ceramide species and, in the resolved crystal structure, it was found to bind C16-ceramide, a long-chain ceramide species (Fig. 2A) [44].

Intriguingly, CERT transfers ceramide at ER–TGN contact sites without any other lipid counter-exchange (see below), at least *in vivo*. *In vitro*, CERT has been shown to recognize DAG when loaded with ceramide, supporting the fascinating hypothesis of a counter-transport of DAG from the TGN to the ER, a process that might operate to control SM synthase (SMS) function in the Golgi because DAG inhibits SMS synthase activity *in vitro* [42,45]. Further and more extensive studies will be needed to clarify whether this mechanism occurs in cells.

Another peculiar feature of the CERT protein resides in its high degree of phosphorylation, highlighting how cells control the activity of this protein. CERT is inhibited by phosphorylation of its serine-rich region (located next to its PH domain) by protein kinase D (PKD) [46]. The phosphorylation prevents CERT binding to PI4P, switching off its transfer capacity [46]. This phosphorylation is reversible as a result of the action of protein phosphatase 2C epsilon, which occurs at ER–TGN contact sites in a VAP-directed manner [47]. Moreover, the interaction between VAP and CERT has been found to be positively regulated by an additional phosphorylation at S315 of CERT, which occurs in conditions of reduced SM production [48]. This mechanism highlights the importance of keeping CERT fully active at the ER–TGN contact sites to sustain SM production. An excellent and exhaustive review by Kumagai and Hanada [49] in this issue of *FEBS* describes all of the features of the CERT protein in detail.

Regulation of PI4P homeostasis

PI4P-cholesterol counter-exchange at the ER–TGN interface

PI4P levels at the TGN are crucial for many cellular functions (e.g. intracellular trafficking), although they are also pivotal in directing the localization of LTPs that function at ER–TGN contact sites [50]. Besides these important functions, recent work has demonstrated that PI4P is more than a localization signaling molecule, although it behaves as ‘fuel’ for counter-exchange of lipids at the MCS interface. One well-characterized example is represented by the OSBP1 protein [23].

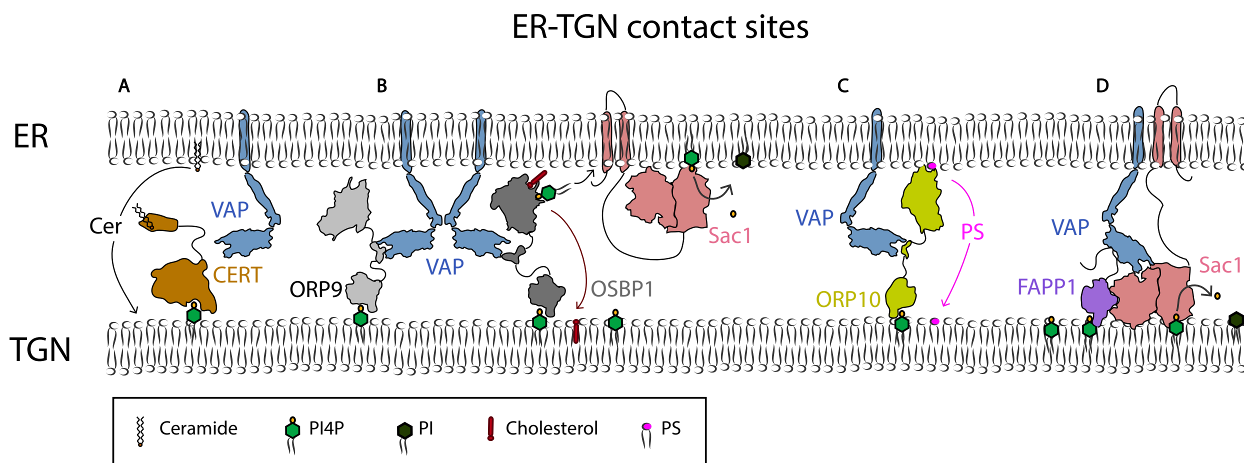

Fig. 2. Molecular machinery acting at ER–TGN MCSs. (A) CERT acts at the ER–TGN interface driving non-vesicular trafficking of ceramide from the ER to the TGN [51]. (B) OSBP1 and ORP9 act as redundant tethering factors at ER–TGN contact sites [19]. Additionally, OSBP1 operates a counter-transport of cholesterol from ER to the TGN with PI4P in the opposite direction to drive *in cis* dephosphorylation of the latter by its phosphatase Sac1 [23]. (C) ORP10 mediates ER to TGN transfer of PS, which is necessary for ER–TGN contact site formation [19]. (D) At the level of ER–TGN contact sites, the PI4P sensor FAPP1 binds and orients the ER-localized Sac1 towards its substrate in the TGN, thus promoting *in trans* PI4P dephosphorylation [40].

First identified as an oxysterol sensor, OSBP1 is a lipid transfer protein exerting its function at the ER–TGN contact sites [23]. OSBP1 exhibits a clear cytosolic, Golgi and vesicular localization, and can quickly re-localize to the Golgi upon oxysterol addition [23,51]. OSBP1 encodes an ORD domain at its C terminus, responsible for sterol transfer, and a PH domain and a FFAT domain at its N-terminal part [52–54]. A chimera encoding the OSBP1 PH-FFAT sequence has been shown to act as a tethering factor at the ER–TGN contact sites [23] and it can be used to visualize those contacts. OSBP1 exchanges PI4P from the TGN to the ER for sustaining sterol transfer from the ER to the TGN (Fig. 2B) [23,55]. The ORD domain is very well conserved among all the other members of the OSBP-related proteins, the ORP proteins [64]. Some ORPs (ORP9 and ORP10) were found at the ER–TGN contact sites [19,56–58]. Combined silencing of OSBP1 and ORP9 led to a complete disruption of ER–TGN contact sites, showing that they act as redundant molecular tethers for this class of contacts (Fig. 2B) [19].

Similar to CERT, OSBP1 is also subjected to phosphorylation by PKD but, different from CERT, the effect of this modification on OSBP1 activity and/or localization is not well defined [59]. In recent work, it was proposed that OSBP1 phosphorylation by PKD may sustain the transfer of PI4P to the ER to lower its level upon ceramide load with a consequent stimulation of SM synthesis [60]. More extensive studies will be mandatory to better characterize the full mechanisms of regulation of OSBP1 modification at ER–TGN contact sites and to formally demonstrate that the lipid transfer activity occurs *in vivo*. A recent review by Antonny *et al.* [61] provides an extensive description of all the features of the OSBP1 protein.

PI4P–phosphatidylserine counter-exchange at the ER–TGN interface

Recently, another non-vesicular lipid transport activity has been proposed for the ER–TGN contact sites: the transfer of PS from the ER to the TGN. PS is synthesized into the ER but is enriched in the PM, endosomes and TGN [62,63]. Curiously, PS shows an asymmetrical distribution, being restricted to the cytosolic leaflet of the membranes. The question concerning the enrichment of PS at the TGN was partially addressed with the observation that altering ORP10 levels results in an alteration of the concentration of PS in this compartment [19]. ORP10 was first described as a microtubule-associated protein that localized also to the Golgi complex [19,58]. Similar to its close relative family members, ORP10 possesses a very weak FFAT domain [9] that promotes binding to the VAP proteins and an ORD domain that is very similar to the ORD domain of ORP5 and ORP8, proteins responsible for the counter-exchange of PS from the ER to the PM [11]. ORP10 was shown to transfer PS *in vitro*, similarly to ORP5 and ORP8 [64].

Knocking down ORP10 not only alters the PS content at the TGN, but also results in a severe loss in the number of ER–TGN contact sites, as measured both by electron microscopy and using a fluorescence system of reporters [19]. Moreover, it has been shown that ORP10 is not an ER–TGN tethering molecule because the re-expression of mutants in the ORD domain in ORP10-KD cells was not able to restore ER–TGN contact site number [19], highlighting the importance of PS content in TGN membranes. The addition of exogenous PS to ORP10-KD cells partially restores ER–TGN contact site formation, supporting the idea that this lipid is crucial for ER–TGN contact site stability [19]. Further studies will be aimed at formally demonstrating that ORP10 physically transfers PS *in vivo*, as well as addressing the question of the role of PS in maintaining ER–TGN contacts. One possibility might be related to TGN membrane composition *per se*, whereas a different hypothesis might be related to the fact that PS at the TGN is recognized by tethering proteins required for ER–TGN contact site formation (Fig. 2C).

Cis versus *trans* activity of Sac1 at ER–TGN contact sites

All studies performed on ER–TGN contact sites so far have led to the common conclusion that these structures are needed to regulate PI4P homeostasis at the TGN [23,40], coherent with the evidence that PI4P and the 4-phosphatase Sac1 reside on different membranes (TGN and ER, respectively). However, the mechanism of Sac1-mediated PI4P regulation in this context is not unequivocal and at least two modes of action can be described for Sac1 at the level of ER–TGN contact sites: the *cis* and the *trans* mode.

In the *cis* mode, Sac1 and its substrate PI4P meet on the same membrane as a result of the action of specialized LTPs that bind, extract and deliver PI4P into the ER where it is dephosphorylated by Sac1 [11,23,26]. This mechanism has been described to occur at the level of different ER–organelle contact sites, such as ER–PM or ER–endosome contacts, where Sac1 regulates local pools of PI4P [11,26]. In many cases, PI4P delivery to the ER occurs by counter-exchange of PI4P with another lipid, such as PS, exchanged for PI4P at the level of ER–PM contacts [11] or cholesterol, counter-transported with PI4P from the ER to the TGN [23].

The presence of an unstructured linker of ~70 residues located between the catalytic motif and the transmembrane domain in the crystal structure of Sac1 predicts that it could act *in trans* to dephosphorylate its substrate on a different, juxtaposing membrane [65]. Although a subsequent study demonstrated that a significant portion of this region is required for PI4P recognition and for catalytical activity, thus making the remaining available free portion shorter [66], this flexible linker would still be able to span the distance between the narrowest contact sites, allowing Sac1 to dephosphorylate its substrate *in trans* on another juxtaposed membrane. However, for a long time, the only evidence for Sac1 *in trans* activity was one work in yeast by Stefan *et al*. [10] in which the OSBP1 Osh3 was reported to stimulate Sac1 activity in the ER to dephosphorylate its substrate *in trans* on the PM. The first evidence of a putative *trans* activity of Sac1 in mammalian cells came from a study performed in neurons in which Dickson *et al*.[67] demonstrated that, under Ca^{2+} stimulation, the ER–PM contact site component E-Syt2 (extended-synaptotagmin 2) physically recruits Sac1 at PI(4,5)P2/E-Syt-mediated ER–PM contact sites where it dephosphorylates PI4P on the PM. However, although the data suggest that Sac1 in the ER could dephosphorylate PI4P *in trans* on the PM in this system, it is also possible that PI4P could be delivered to the ER to allow Sac1-mediated dephosphorylation *in cis*. Subsequent work by Zewe *et al*. [68] failed to observe an enrichment of Sac1 at the level of ER–PM MCSs both under steady-state conditions or after stimulation, suggesting that this could be a mechanism specific for neurons, which could respond differently to Ca^{2+} signaling. Accordingly, using a chimeric construct to mimic the *cis* and *trans* configuration of Sac1 at ER–PM contact sites, it was concluded that Sac1 *in trans* activity is much lower than the *cis* one at the ER–PM interface and can be boosted only in the presence of a linker between the TM and the catalytic domain [68].

The formal proof that Sac1 can also act *in trans* in mammalian cells was described only very recently thanks to the work of Venditti *et al*. [40] who showed that the PI4P-binding protein FAPP1, for which the function had remained elusive for many years, simultaneously binds VAP and Sac1 and stimulates the *in trans* phosphatase activity of the latter towards its substrate at the TGN (Fig. 2D). It was proposed that the two modes of action of Sac1, *cis* and *trans*, coexist at the level of ER–TGN MCSs. Given the structural constraints of Sac1 and the low affinity of FAPP1 for PI4P [69], FAPP1-mediated stimulation of Sac1 *in trans* can only occur at the level of tighter contact sites and only at sites with high levels of PI4P, whereas OSBP1-mediated dephosphorylation of PI4P *in cis* can occur at the level of contact sites with a greater

distance between the ER and the TGN because OSBP1 can shuttle between the two membranes [40].

Interplay between ER–TGN contact sites, the PI4P pool and Golgi exit

The evidence that ER–TGN MCSs are needed to control PI4P homeostasis cleared the way for the identification of putative physiological roles of these structures. PI4P is able to coordinate a large variety of functions at the Golgi complex via binding and activation of different class of effectors [50]. One of the best characterized roles of the Golgi pool of PI4P is the regulation of anterograde membrane trafficking of cargo from the Golgi to PM, as highlighted by the work of Szentpetery *et al.* [70] in which the selective, acute depletion of the Golgi PI4P pool by recruiting Sac1 at the TGN with a rapamycin-inducible system virtually abolishes the trafficking of cargoes to the PM. This depends on PI4P-mediated Golgi recruitment of a number of effectors involved in different steps of post-Golgi carrier formation. This is the case of FAPP2, required for post-Golgi trafficking to the PM [71,72] as a result of the ability of its PH domain to induce membrane tubulation and/or the establishment of specific microdomains at the TGN [73] and GOLPH3, which simultaneously binds PI4P at the TGN and the actin cytoskeleton via the unconventional MYO18A, thus creating a mechanical force that induces post-Golgi carrier detachment [74]. On the other hand, cellular secretion is stimulated by conditions that increase PI4P levels in the Golgi, such as PI4KIIIIβ overexpression [75] and Sac1 down-regulation [76]. Another role for PI4KIIIβ in cellular secretion is indicated by its interaction with the 14-3-3γ proteins, which are responsible for the stabilization of the kinase, and with the fission-controlling protein BARS [77]. In this model, a PI4KIIIβ-14-3-3-BARS complex forms selectively at the TGN under a trafficking wave, and the disruption of this complex results in elongated tubular carriers that do not undergo fission [77]. Much evidence suggests that PI4P at the Golgi not only regulates general secretion, but, depending on upstream regulators and downstream effectors, also modulates selective Golgi export of specific cargoes. This has been shown for the PI4P effector Arfaptin-1, which was demonstrated to be required for the trafficking of chromatogranin A but not for general secretion [78]. Similarly, overexpression of Arfaptin-1 reduces glucose-stimulated insulin secretion without affecting constitutive secretion [79]. Arfaptin-2, in complex with ARL1, ARF1 and PKD2, selectively regulates the secretion of MMP2 and MMP7 [80], thus confirming a role for PI4P in Golgi export of selected cargos. A recent study by Judith *et al.* [81] defined a role for Arfaptin-2 in the redistribution of the autophagy protein ATG9 from the TGN, where it is localized in control fed conditions, to the ATG9-positive compartments formed under starvation, a step required for autophagy initiation, thus expanding the role of PI4P and its effectors in the Golgi export of cargoes not destined for secretion. Because PI4P is regulated at the level of MCSs, one logical consequence of contact site modulation would be an alteration in cargo trafficking and secretion. This has been shown to occur at the level of ER–endosome contact sites that regulate an endosomal pool of PI4P required for endosome-to-TGN retrograde trafficking of cargoes [26]. The role of ER–TGN contact sites in cargo secretion remained elusive for much longer as a result of the lack of tools to destabilize them. Wakana *et al.* [82] demonstrated that PI4P at the Golgi is required for post-Golgi trafficking of a particular class of carriers named CARTS (carriers of the TGN to cell surface) that exclude VSV-G and require a dedicated machinery, including PKD2 and kinesin-5, for their biogenesis and transport. It was shown that PI4P depletion via overexpression of Sac1 reduces CARTS biogenesis and secretion, and this process was placed at the level of ER–TGN contact sites because contact site stabilization by overexpressing a PH-FFAT mutant of OSBP1 also impairs CARTS secretion [82]. Because a similar reduction in CARTS secretion is observed under depletion of VAPs or the LTPs CERT and OSBP1, it was proposed that ER to TGN nonvesicular trafficking of ceramide and cholesterol operated by CERT and OSPB1 at the level of contact sites is needed to create the lipid microenvironment for correct CARTS biogenesis [82]. In recent studies, Venditti *et al.* [19,40] have demonstrated that the depletion of both ER–TGN contact sites determinants, such as ORP10, and regulators, such as the sensor FAPP1 (see above), results in an uncontrolled increase in PI4P at the Golgi. Such work took advantage of these findings to expand the previous evidence that ORP10 depletion in hepatocytes increases the secretion of the apolipoprotein B100 (ApoB100), an essential component of very low-density lipoproteins [58]. Using a combination of pulse-chase, ELISA and temperature block synchronization assays to specifically analyze the Golgi exit, it was shown that FAPP1, similar to ORP10, acts as a negative regulator of the Golgi export of ApoB100, and this depends on the regulation of PI4P levels at ER–TGN contact sites [40]. FAPP1 is thus proposed to act as a PI4P sensor that is able to regulate Golgi export and secretion of selected cargos

by finely controlling PI4P levels at ER–TGN contact sites via stimulation of Sac1 *in trans* activity. Further studies will be needed to understand the mechanism of selective ApoB100 trafficking regulation by FAPP1 and PI4P, determine whether other cargoes require this system to be secreted, and identify the eventual PI4P effectors involved. Considering that very low-density lipoprotein trafficking through the ER and the Golgi has been shown to occur via incorporation into dedicated carriers [83] and that ORP10 mutations are associated with dyslipidemia [84,85], a deeper understanding of these processes would be of crucial importance both from a mechanistic and an applied perspective.

Comparing ER–TGN contact sites with other ER–organelle contact sites: lessons to be learnt

ER–TGN contact sites appear to diverge from the other contacts that involve the ER. A distinctive feature of some contact sites resides in their ability to be dynamically regulated under calcium stimulation. This occurs at the ER–PM interface where extended-synaptotagmin-like proteins (E-syts) have been shown to form contacts in response to increased levels of cytosolic Ca^{2+} [12], as well as the ER–PM tether TMEM24, which is recruited to transfer PI at the ER–PM interface in response to Ca^{2+} stimulation [13]. Interestingly, from the opposite perspective, Ca^{2+} homeostasis can be controlled at the level of ER–organelle MCSs. This was one of the first characterized functions described for ER–PM contacts where, upon depletion of ER calcium stores, the PM calcium channel Orai1 is bound and activated by the ER-localized protein STIM1, so that the extracellular calcium reservoirs can be used to replenish the ER stores [86]. Something analogous occurs in skeletal muscle cells where ER–PM contact sites control calcium flux to drive muscle contraction as a result of the binding of the ryanodine receptor on ER membranes with Cav1.1, a subunit of a voltage-dependent calcium channel, on the PM [87]. Calcium signaling is one of the best characterized functions of ER–mitochondria contact sites where a conduit is generated by the inositol 1,4,5-triphosphate receptor channel in the ER and the voltage-dependent anion-selective channel in the outer mitochondrial membrane to transport high concentration of Ca^{2+} from the ER to the mitochondria [88]. The dynamin-like protein mitofusin 2 is one of the proposed tethers required to bind the two organelles, thus allowing Ca^{2+} transport at ER–mitochondria contact sites in response to stimuli that generate inositol-1,4,5-trisphosphate [89]. Recently, PDZD8, a mammalian orthologue of the yeast tether Mmp1, was shown to be required for ER–mitochondria contact formation and to regulate Ca^{2+} homeostasis in neurons where PDZD8 mediates the mitochondrial uptake of Ca^{2+} released by the ER in response to synaptic stimulation [90].

Currently, there is no clear association between ER–TGN contact sites and the regulation of Ca^{2+} signaling. This may partly be a result of the temporal gap with respect to characterizing the contacts between the ER and the TGN relative to the ER with other organelles: tools required for ER–TGN contact site visualization and investigations have been developed only recently [19,40]. However, the existence of Ca^{2+} pumps mediating Ca^{2+} influx into the Golgi lumen is well established [91] and, over the last decade, interesting evidence has been obtained suggesting that modulating Ca^{2+} levels in the TGN has an impact on TGN sorting and the consequent secretion of specific cargos [92,93]. In the light of the recent evidence of ER–TGN function in post-Golgi trafficking [40], a possible role of ER–TGN contact sites in the regulation of calcium homeostasis at the TGN and/or a modulation of contacts in response to calcium levels will certainly be worthy of investigation.

Interorganellar contact sites have been shown to be important for organelle fission and movement. This is the case for mitochondria, where contact sites with the ER define the sites where the fission machinery is recruited for mitochondrial division to take place [93], as well as for ER–endosome contact sites that have been shown to have a role in defining the timing and sites of endosomal fission, a process required for the segregation and trafficking of cargos [94,95]. Finally, ER–endosome contact sites have been shown to be important for late endosome movement to the cell periphery. This process can be mediated by the interaction between VAPA and the ER protein protrudin, which recruits Rab7 and kinesin-1 to promote the anterograde trafficking of LEs [16], or can occur in response to cholesterol levels that modulate the interaction between VAPA and the cholesterol sensor ORP1L, thus resulting in the induction of ER–endosome contact sites, dissociation of retrograde motors and subsequent anterograde movement of LEs to the cell periphery [17]. These latter functions appear to be very specific for contacts involving ER and organelles that rely on fission/fusion and movement along the cytoskeleton to exert their function and, thus, at the moment, a similar role at the ER–TGN contact site level has not been described.

Conclusions and perspectives

Recent decades have seen an ever-growing interest in membrane contact site biology, thus revolutionizing the collective perception of inter-organelle communication. Contact sites between the ER and the TGN have been visualized very early on via electron microscopy, although they remained uncharacterized for a long time in terms of composition and function because of important technical limitations for their visualization by regular fluorescence microscopy. The last 5 years have marked a breakthrough in ER–TGN contact site biology as a result of the introduction of tools to artificially stabilize them, and, very recently, the development of cutting-edge reporter-based strategies to visualize ER–TGN contact sites by fluorescence microscopy. This technological advance allowed the identification of structural determinants of ER–TGN contact sites, such as the VAP proteins and the redundant tethering factors OSBP1/ORP9, as well as the lipid transfer protein ORP10 that maintains ER–TGN contact sites by acting on the lipid environment as a result of its ability to transfer PS and, finally, functional proteins that, although not structurally required to establish the contacts, work at the level of ER–TGN contact sites to ensure their correct functioning, as is the case of the sensor FAPP1. New light has been shed on ER–TGN contact site functions and it is now clear that these structures are needed to control the homeostasis of PI4P at the TGN through at least two mechanisms that coexist at ER–TGN contact sites: (i) the OSPBP1-mediated counter-exchange of cholesterol for PI4P, leading to the transfer of the latter to the ER to induce its dephosphorylation *in cis* by the phosphatase Sac1 and (ii) PI4P consumption orchestrated by the PI4P sensor FAPP1 that binds Sac1 in the ER at the level of ER–TGN contact sites and orients it to dephosphorylate its substrate *in trans* at the TGN. Intriguingly, the regulation of PI4P homeostasis at ER–TGN contact sites is instrumental for ensuring the correct Golgi export and secretion of selected cargos, such as the lipoprotein ApoB100, for which secretion is markedly increased under ER–TGN contact site deregulation and subsequent PI4P accumulation at the TGN. These novel findings open interesting scenarios for consideration. One issue that needs to be addressed concerns the impact of PI4P regulation at ER–TGN contact sites on cell physiology: what are the consequences of increased ApoB100 secretion in a living organism, are there cargoes other than ApoB100 that rely on PI4P for Golgi export, and which other cellular functions, apart from anterograde trafficking respond to PI4P levels, are key point to assess in the near future. On the other hand, nothing is currently known about ER–TGN contact site modulation. Studies in our group suggest that they can be regulated in response to phosphorylation/dephosphorylation (R. Venditti, unpublished observations) and future studies are mandatory to understand which signaling pathways, intracellular messengers and/or physiological context may play a role in the regulation of ER–TGN contact site structure and function.

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