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Review

A Tether Is a Tether Is a Tether: Tethering at Membrane Contact Sites

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Membrane contact sites enable interorganelle communication by positioning organelles in close proximity using molecular "tethers." With a growing understanding of the importance of contact sites, the hunt for new contact sites and their tethers is in full swing. Determining just what is a tether has proven challenging. Here, we aim to delineate guidelines that define the prerequisites for categorizing a protein as a tether. Setting this gold standard now, while groups from different disciplines are beginning to explore membrane contact sites, will enable efficient cooperation in the growing field and help to realize a great collaborative opportunity to boost its development.

Introduction

Organellar membranes are crucial features of eukaryotic cells, but they also impose obstacles for the flow of material and information between cellular regions. A first hint of how communication across membranes might be accomplished came from early electron microscopy studies that noted an unexpected level of spatial organization whereby organelles were found frequently very closely opposed to each other at defined foci (Bernhard and Rouiller, 1956; Copeland and Dalton, 1959; Gray, 1963; Porter and Palade, 1957; Rosenbluth, 1962). Studies on the triadic muscle junction between the endoplasmic reticulum (ER) and plasma membrane (PM) invaginations in skeletal muscle cells revealed that these mysterious interorganellar connections involve specialized spanning proteins bridging the two membranes (Kawamoto et al., 1986). However, electron microscopy images, although essential for the discovery of contact sites, dramatically underestimated the number of contact sites that exist in the cell.

A further example of interoganellar contact was discovered at the beginning of this century, when the yeast vacuolar protein Vac8 was shown to form a complex with the integral nuclear envelope protein Nvj1. The interaction of Nvj1/Vac8 results in physical tethering of the two organelles and formation of structurally confined membrane contact sites (Pan et al., 2000). Such spanning proteins that physically tether two organelles have since been colloquially termed "tethers." Identification of the Nvj1/Vac8 tether pair kick-started a hunt for novel contact sites and their molecular tethers.

It is now becoming clear that many, possibly all, organelles communicate by means of interorganellar tethering. The list of proteins suggested to be involved in mediating contact sites or residing in contacts is continuously expanding (Table 1 for yeast proteins and Table 2 for metazoan proteins). From this growing list, it is becoming apparent that contact site resident proteins may contribute to one of at least three molecular functions. First, some resident proteins act as the molecular tethers that physically bridge the respective membranes and attach them to each other (Csordas et al., 2006; Helle et al., 2013). Furthermore, some proteins have a specific function that is required at the contact sites, such as transfer of small molecules in a non-vesicular manner. To date, such functional proteins that have been well defined are factors mediating ion and lipid transfer (Henne, 2016; Prinz, 2014). Finally, regulatory components have been identified that likely integrate environmental signals and mediate adaptation of contact site size and abundance to cellular needs, or coordinate the functions of different contact site machineries with each other (Elbaz-Alon et al., 2014; Giordano et al., 2013; Henne et al., 2015; Kornmann et al., 2011; Mesmin et al., 2013; Murley et al., 2015; Stroud et al., 2011). Importantly, some contact site proteins may harbor several of these functions in one molecule; for example, tethering and lipid transfer. Due to this, it is becoming increasingly hard to tease apart the roles of the various resident proteins.

While regulatory and functional molecules are not necessarily involved in all types of organellar contact sites, interorganellar tethering is the basic defining feature of a contact site, and tethering components are thus expected to be an essential part of the machinery for each and every contact site. Such tethering without the presence of any other functional module may serve to correctly position organelles in the cell for division or inheritance. Regardless, despite the obvious requirement for tethers at each contact site, and although the knowledge of the molecular tether has huge experimental advantages in controlling the contact site formation, most tethers have not yet been identified. Moreover, it has proven to be a challenging task to experimentally prove the tethering function of a protein. The difficulty seems to arise due to limited availability of suitable techniques and due to redundancy in interorganellar tethering machineries that tends to mask the effects of single tether disruptions. Hence, to date, the assignment of a tether role to a protein is not always sufficiently experimentally supported, leading to confusion in the field.

In this review, we clarify the definition of the widely used, yet ill-defined, term of "tether" in membrane contact sites in the hope that at a time when the field is still developing, the community can reach agreement on what is the gold standard for proving that a tether is a tether is a tether.

Table 1. Contact Site Proteins in *Saccharomyces cerevisiae*

Contact Site	Protein Name	Description	Reference
ER-PM	Scs2	ER receptor for numerous proteins containing a FFAT motif, principal tether in ER-PM contact sites	Murphy and Levine, 2016
	Scs22	Scs2 homolog	
	Ist2	integral ER protein, binds PM via a polybasic region	Manford et al., 2012
	Tcb1/2/3	SMP-domain-containing auxiliary tethers	Toulmay and Prinz, 2011; Manford et al., 2012
	Osh2/3	LTPs, structurally equipped for tethering (FFAT motif, PH domain)	Levine and Munro, 2001
	Lam1/2/3/4	LTPs, structurally equipped for tethering (TMD, GRAM domain)	Gatta et al., 2015
ER-mitochondria	Mmm1, Mdm10/12/34	ER-mitochondria encounter structure (ERMES), Mmm1, Mdm12, and Mdm34 contain SMP domain	Kornmann et al., 2009
	Gem1	Mitochondrial GTPase, ERMES interactor	Kornmann et al., 2011; Stroud et al., 2011
	Emc1/2/3/4/5/6	ER membrane complex (EMC), interacts with Tom5	Lahiri et al., 2014
	Tom5	TOM complex subunit, interacts with EMC	
	Tom70/71	TOM complex subunits, interact with Lam6	Elbaz-Alon et al., 2015; Murley et al., 2015
	Lam5/6	LTPs, structurally equipped for tethering (TMD, GRAM domain); Lam6 is an auxiliary tether and interacts with Tom70/71	Gatta et al., 2015; Elbaz-Alon et al., 2015; Murley et al., 2015
Nucleus-vacuole	Nvj1, Vac8	nucleus-vacuole junction (NVJ) principal tether pair	Pan et al., 2000
	Osh1	LTP, structurally equipped for tethering (FFAT motif, PH domain)	Levine and Munro, 2001
	Tsc13	ER protein required for fatty acid elongation	Kohlwein et al., 2001
	Nvj2	contains SMP domain, structurally equipped for tethering (TMD, PH domain)	Toulmay and Prinz, 2011
	Lam5/6	LTPs, structurally equipped for tethering (TMD, GRAM domain); Lam6 interacts with Vac8	Elbaz-Alon et al., 2015; Gatta et al., 2015; Murley et al., 2015
	Mdm1	auxiliary NVJ tether, contains TMD and PX domain	Henne et al., 2015
	Nvj3	NVJ component, interacts with Mdm1	
	Vps13	vacuolar protein localizing to NVJ and vCLAMP	Lang et al., 2015b
Mitochondria-cortex	Num1	binds PM via PH domain and mitochondria via CC domain, required for mitochondrial retention in mother cell	Klecker et al., 2013; Lackner et al., 2013
	Mdm36	Num1 partner protein	
	Mmr1	required for mitochondrial transmission to bud, may be required for anchoring at the bud cortex	Swayne et al., 2011
	Mfb1	required for retention of a high-functioning mitochondrial population in mother cells	Pernice et al., 2016
Mitochondria-vacuole	Vps39	component of the vacuole and mitochondria patch (vCLAMP) and HOPS subunit	Elbaz-Alon et al., 2014; Honscher et al., 2014
	Ypt7	vacuolar Rab GTPase, vCLAMP component	
	Vps13	vacuolar protein localizing to vCLAMP and NVJ	Lang et al., 2015b
ER-peroxisome	Pex3, Inp1	ER-peroxisome tether proteins with a role in peroxisome inheritance	Munck et al., 2009; Knoblach et al., 2013
Mitochondrial IM-OM	Mic60/27/26/19/12/10	mitochondrial contact site and cristae organizing system (MICOS), an integral protein complex of the inner membrane	Harner et al., 2011; Hoppins et al., 2011; von der Malsburg et al., 2011
	Sam50 TOM Ugo1 Por1	MICOS interaction partners in the outer mitochondrial membrane	

FFAT, phenylalanine in an acidic tract, SMP, synaptotagmin-like mitochondrial lipid-binding protein, LTP, lipid transfer protein, PH, pleckstrin homology, TMD, transmembrane domain, CC, coiled-coil, HOPS, homotypic fusion and protein sorting, IM, mitochondrial inner membrane, OM, mitochondrial outer membrane.

Table 2. Contact Site Proteins in Metazoans

Contact Site	Protein Name	Description	Reference
ER-PM	VAPs	ER receptors for numerous proteins containing FFAT motif	Murphy and Levine, 2016
	STIM1, Orai	dynamic tether: PM Ca^{2+} channel Orai binds to ER integral STIM1 at low luminal Ca^{2+}	Liou et al., 2007
	E-Syt1/2/3	SMP-domain-containing tethers; E-Syt1 is a Ca^{2+}-dependent dynamic tether	Giordano et al., 2013
	Junctophilin1/2/3/4	ER residents, bind PM via MORN domains	Takeshima et al., 2015
	DHPR, RyR	PM and ER Ca^{2+} channels, interact and function in a concerted way	Rebbeck et al., 2011
	ORP5, ORP8	LTPs, dynamic tethers, contain TMD and PH domain	Chung et al., 2015
ER-mitochondria	MFN1/2	mitochondrial fusion GTPase; an ER MFN2 pool mediates tethering by interacting with mitochondrial MFN1/2	de Brito and Scorrano, 2008
	IP3R, VDAC, Grp75	ER Ca^{2+} release channel IP3R and mitochondrial metabolite channel VDAC are connected, Grp75 may be involved	Szabadkai et al., 2006
	Fis1, BAP31	mitochondrial Fis1 and ER BAP31 interact for transmission of apoptotic signals	Iwasawa et al., 2011
	PTPIP51, VAP	PTPIP51 is a mitochondrial LTP structurally equipped for tethering via VAP binding	De Vos et al., 2012
ER-endosome	VAPs	ER receptors for numerous proteins containing FFAT motif	Murphy and Levine, 2016
	StARD3, StARD3NL	integral endosomal proteins, interact with ER protein VAP via FFAT motif	Alpy et al., 2013
	ORP1L, ORP5	LTPs active at the ER-endosome interface	Rocha et al., 2009; Du et al., 2011
	PTP1B, EGFR, Annexin A1	components mediating interplay between ER and multivesicular bodies	Eden et al., 2010; Eden et al., 2016
	Protrudin, Rab7	ER protrudin interacts with Rab7 and phosphatidylinositol-3-phosphate on late endosomes	Raiborg et al., 2015
ER-Golgi	VAPs	ER receptors for numerous proteins containing FFAT motifs	Murphy and Levine, 2016
	OSBP	LTP, dynamic phosphatidylinositol-4-phosphate dependent tether, contains FFAT motif and PH domain	Mesmin et al., 2013
	CERT	LTP, putative dynamic tether, contains FFAT motif and PH domain	Peretti et al., 2008
	FAPP2	LTP, structurally equipped for tethering (FFAT motif, PH domain)	D'Angelo et al., 2007
	Nir2	phosphatidylinositol transfer protein, contains FFAT motif	Peretti et al., 2008
Lysosome-peroxisome	Synaptotagmin-7	mediates lysosome-peroxisome tethering important for cholesterol transfer	Chu et al., 2015
ER-lipid droplet	DGAT2, FATP1	lipid droplet resident DGAT2 and ER resident FATP1 interact and coordinate lipid droplet biogenesis	Xu et al., 2012
Mitochondria-lipid droplet	Perilipin-5	lipid droplet scaffold protein involved in interaction with mitochondria	Wang et al., 2011
Mitochondrial IM-OM	Mic60/27/26/25/19/10, Qil1	mitochondrial contact site and cristae organizing system (MICOS), an integral protein complex of the inner membrane	Zerbes et al., 2012b
	SAMM50, Metaxin1/2	MICOS interaction partners in the outer mitochondrial membrane	

FFAT, phenylalanine in an acidic tract, SMP, synaptotagmin-like mitochondrial lipid-binding protein, MORN, membrane occupation and recognition nexus, LTP, lipid transfer protein, TMD, transmembrane domain, PH, pleckstrin homology, IM, mitochondrial inner membrane, OM, mitochondrial outer membrane.

How to Prove a Tether

Since the membranes of organelles at contact sites must be actively positioned in very close vicinity (usually 10–40 nm distance), while maintaining the structural integrity of each organelle and disenabling fusion, the primary job of a tether is to bridge the respective membranes and to mediate their physical attachment. Thus, the biological function of a tether is inherently connected to its specific positioning in a unique niche within the cell and its presence, or at least enrichment, at contact sites must be demonstrated. Moreover, its ability to somehow influence the extent of proximity or the distance between the two membranes must be shown.

Experimentally exploring the role of proteins that function in highly ordered environments always brings its own challenges, for instance, generation of a simple cell lysate might generally compromise an organellar protein more than a resident of the cytosol as it involves complete destruction of the protein's native environment. Studying membrane contact sites as highly organized focal structures connecting organellar subdomains currently imposes novel technological challenges and again asks for development of new creative experimental strategies. In this section, we discuss the characteristics of molecular tethers that have been identified and summarize the experimental strategies used to analyze these special proteins.

Tethers under the Microscope: Puncta and Patches

Contact sites are generally spatially confined focal structures, and thus, contact site residents tagged with a fluorescent marker often show characteristic focal patterns. In yeast, the ER-mitochondria contact site can be visualized by tagging one of the tethering complexes that have been discovered for this contact, the ER-mitochondria encounter structure (ERMES). Tagging any one of the four ERMES constituents (Mdm12, Mdm10, Mmm1, or Mdm34) results in one to five fluorescent puncta per cell (Kornmann et al., 2009). Tagging the nucleus-vacuole junction (NVJ), tether component Nvj1 uncovers a single, more extended patch where a vacuole contacts the nucleus (Pan et al., 2000). Visualizing the highly abundant contact sites between the cortical ER and the PM demonstrates that they cover nearly half of the PM area (Manford et al., 2012). Thus, each contact site has a unique visual shape under the fluorescence microscope and resident proteins in these contact sites are expected to either uniquely reside in similar visual structures or at least be enriched in them.

Electron microscopy combined with immunogold labeling or related techniques can be used to get an even better sense of the contact site architecture. Demonstrating that a protein is indeed enriched in the sites of close apposition between distinct membranes provides strong support for it to be a contact site resident (Daniele et al., 2014; Elbaz-Alon et al., 2014, 2015; Giordano et al., 2013; Honscher et al., 2014; Kvam and Goldfarb, 2004). These techniques are the most powerful way currently available to prove that a protein is localized within a contact site.

Hence, the first prerequisite of a tether is that it be found at its contact site. However, since some contact site proteins may not function as tethers but have other roles, such as that of a regulator, it is not enough to determine that a protein is a resident of a contact to assign to it the role of a tether. For example, the GTPase Gem1 can be co-isolated with the ERMES machinery and is highly enriched in ER-mitochondria contact sites but is dispensable for the ERMES tethering function (Kornmann et al., 2011; Stroud et al., 2011). Using microscopy alone, it is therefore impossible to distinguish between the different classes of contact site residents.

A Glimpse at Tethers In Situ: Defining the Architecture of Tethers

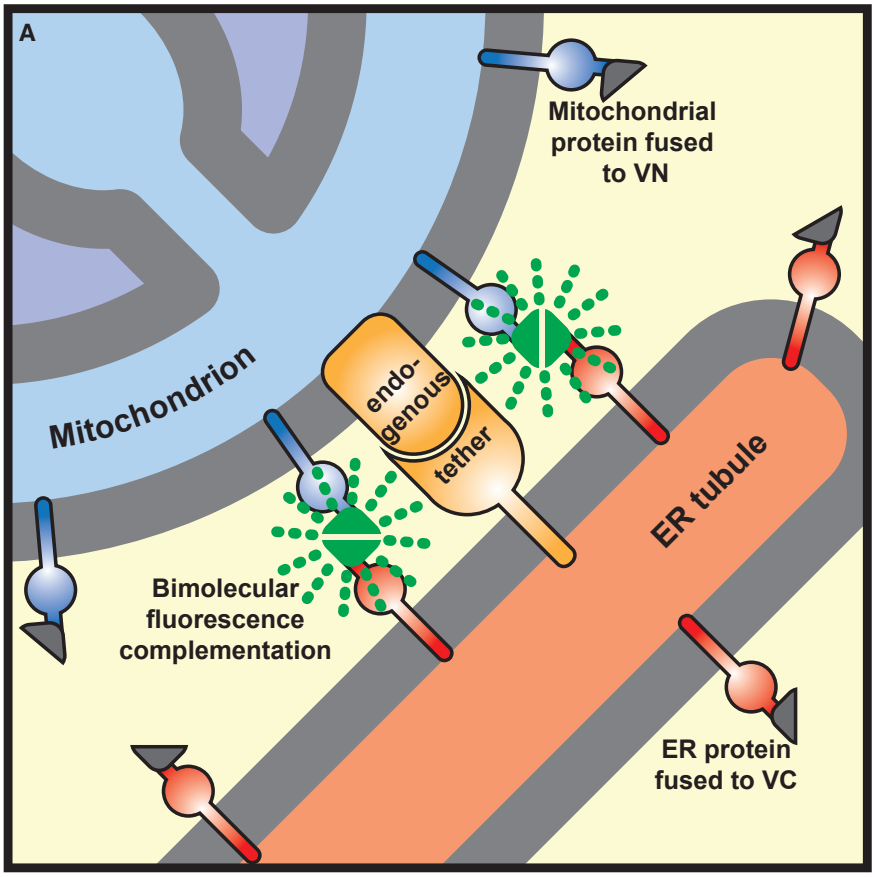
Electron cryo-tomography (cryo-ET) has recently strongly promoted our understanding of contact site morphologies. Most strikingly, visualization of the three-dimensional distribution of ER-mitochondria contact sites unveiled ER tubules that tightly wrap around the whole circumference of mitochondria, leading to the notion that these contact sites play an active role in the process of mitochondrial division (Friedman et al., 2011; Murley et al., 2013). Similarly, cryo-ET was helpful in understanding the role of ER-endosome contact sites in endosome fission (Friedman et al., 2013; Rowland et al., 2014).

However, the real strength of cryo-ET in studying tethering molecules is their potential to directly visualize tether structures bridging two organellar membranes in situ. Structures of unknown molecular identity connecting the mitochondrial outer membrane to ER membranes have been observed by cryo-ET in fractionated rat liver cells (Csordas et al., 2006). In a recent study, the interface between two mitochondria about to fuse was analyzed by cryo-ET (Brandt et al., 2016). This structure is not a regular contact site but an intermediate of a membrane fusion process. Despite this important functional difference, it is topologically identical to a contact site. Indeed, in this case, cryo-ET enabled visualization of numerous densities between the closely aligned mitochondrial outer membranes, which were identified as the outer membrane fusion GTPase, Fzo1, by quantum dot labeling (Brandt et al., 2016). Intriguingly, the distribution of Fzo1 in the contact zone between the two mitochondria had a functional significance: In one unordered state, Fzo1 bridges were randomly distributed in the contact site, and in a second phase, an ordered state appeared, in which the Fzo1 tethers were organized in a ring shape at the outer rim of the membrane interface (Brandt et al., 2016). This opens the question whether and how the constituents of regular contact sites are spatially organized. The recent technological advances in the field of cryo-ET promise great progress in visualization of tethers in situ at high resolution in the near future, which might present many surprising observations and add a distinct spatial organization requirement for a protein to be defined as a tether.

Tether Mutants: Loss of Membrane Proximity… or Not

The molecular function of tethers is to physically hold distinct cellular membranes at very close distance. Theoretically, their depletion should thus result in loss of either the extent of the physical proximity of the respective membranes or alterations to their distance.

With a width of just a few tens of nanometers, contact sites are below the diffraction limit of light microscopy. Therefore, in order to assess changes in contact site size or number, fluorescent labeling of the respective organelle pair is usually not sufficient to reliably detect alterations of contact sites. Instead, visualization of the contact site by markers is required, ideally fluorescently tagged proteins proven to localize exclusively to the contact site of interest. If no such protein is known, synthetic contact site sensors can be used as a powerful tool to visualize contact sites. In the past, complementary FRET (fluorescence resonance energy transfer) pairs fused to proteins residing in the



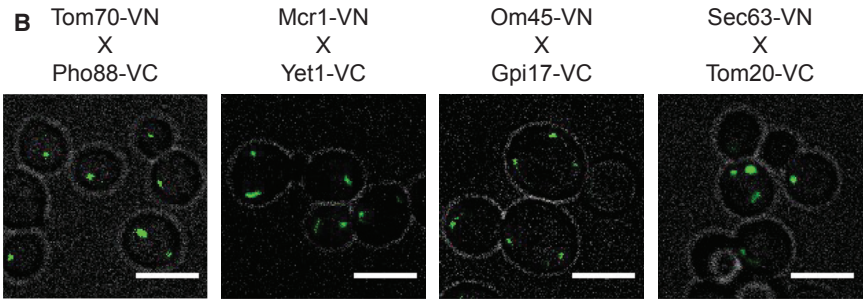


Figure 1. Split Venus Fluorescence Complementation at Contact Sites Depends on Membrane Proximity but Not on Inherent Affinity of Tagged Protein Pairs

(A) VN- and VC-tagged membrane proteins are uniformly distributed all over the organellar surfaces. At contact sites, adjacent to the endogenous tether, organellar membranes are in very close proximity, so that fluorescence complementation between tagged proteins present in this area can occur even for unrelated proteins that are not enriched in contact sites.

(B) Tagging of four unrelated pairs of ER membrane and mitochondrial outer membrane proteins results in labeling of ER-mitochondria contact sites by bimolecular fluorescence complementation. VN, N-terminal half of Venus protein; VC, C-terminal half of Venus protein. Scale bar, 5 μm.

membranes of two organelles were used to detect contact sites (Csordas et al., 2010). Another possibility is the use of bimolecular fluorescence complementation systems, such as "split Venus," to label areas of membrane proximity (Figure 1 and see below). Both endogenous and synthetic contact site markers allow detection of changes in contact site number and also pronounced changes in contact site area but do not allow for quantification of contact extent due to the limited diffraction. Currently, electron microscopy combined with statistical analysis is the method of choice for quantitative comparison of contact site area. Likewise, electron microscopy is required to assess changes in membrane proximity within the contact site (widening/tightening of membrane distance), although FRET contact site sensors offer the possibility to measure changes in membrane distance as well.

In addition to changes in their visual appearance, alterations in contact site architecture are also expected to detectibly affect the contact site's physiological role, be it a structural role, a role in signaling, or a role in non-vesicular material transport. Indeed, this was the case in early experiments on the NVJ whereby deletion of the tether component *VAC8* was shown to lead to both loss of organelle proximity and loss of one physiological function of the contact, which is piecemeal microautophagy of the nucleus (Pan et al., 2000; Roberts et al., 2003).

However, it is now known that loss of the NVJ tether machinery, Nvj1/Vac8, does not result in complete loss of proximity between ER and vacuolar membranes (Henne et al., 2015). Moreover, in almost all other contact sites studied, profound alterations in organellar distribution upon depletion of bona fide tethers have not been demonstrated. The reason for this is simple; due to their essential functions, contact sites often do not rely on a single type of tether but instead on several independent tethering machineries resulting in a functional redundancy that masks the morphological loss-of-function phenotypes upon depletion of a single set of tethers. A prime example for this phenomenon is the interface between the ER and the PM. In wild-type yeast cells, the peripheral or cortical ER is directly adjacent to the PM, and the contact area of these two membranes covers up to half of the PM (Pichler et al., 2001; West et al., 2011). In an attempt to reduce the area of contact between these two membranes, it was shown that six different tethering components (*SCS2*, *SCS22*, *IST2*, *TCB1*, *TCB2*, and *TCB3*) had to be deleted in order for 90% of the cortical ER to collapse away from the PM into the cytoplasm. Moreover, expression of single tether components resulted in persistence of substantial contact areas and correct localization of the respective tether (Manford et al., 2012). Of note, even in the

strain bearing the six deletions, small ER-PM contact sites were reproducibly observed, and in a later study, a family of putative sterol transfer proteins involved in ER-PM contact sites was found to localize to these remaining contact areas and most probably exert a tethering force in these domains (Gatta et al., 2015). This indicates that multiple independent tethering machineries act in parallel at the ER-PM interface and explains why deletion of single tethers does not necessarily result in dramatic phenotypes. Hence, when loss of a contact site protein does not lead to a reduction in apparent organelle proximity, this does not rule it out as a potential tether. Importantly, contact sites with multiple redundant tether machineries have a higher degree of plasticity compared with simple contact sites relying on a single tether. Therefore, long-term genetic ablation of tethers that allow the rest of the contact site machinery to adapt to this perturbation might not be the most promising approach to uncover the individual contribution of a protein to membrane tethering. Instead, analyzing membrane proximity directly after tether inactivation without time for adaptation could potentially yield results resembling the physiological state more closely. One experimental possibility would be to introduce a high specificity protease cleavage site such as the tobacco etch virus (TEV) protease site into the protein of interest, rapidly cleave the potential tether by inducing TEV protease expression, and then follow the immediate effect on membrane proximity before back-up proteins have had a chance to be recruited.

A complementary approach to depleting/inactivating tethers and expecting a reduction in contact area is to overexpress the putative tether and search for expansion of the contact site area. There are indeed numerous examples of contact site proteins that when overexpressed lead to substantial contact site enlargement (Chung et al., 2015; Elbaz-Alon et al., 2015; Giordano et al., 2013; Henne et al., 2015; Honscher et al., 2014; Pan et al., 2000). For example, overexpression of Vps39 dramatically enhances the extent of the VaCuoLe And Mitochondria Patch (vCLAMP), the contact site between mitochondria and the vacuole in yeast (Honscher et al., 2014). However, lack of expansion upon overexpression does not mean that a protein is not a tether as there may be additional limiting factors to contact site expansion (Giordano et al., 2013; Mesmin et al., 2013). Furthermore, showing that a contact site expands upon overexpression of a protein does not automatically make it a tether as expansion of contact sites can also be caused by other means such as overexpressing a positive regulator of the respective contact site.

Some Synthetic Biology: What Artificial Tethers Teach Us about Contact Sites

A conceptually very simple and elegant approach in contact site research is based on the idea that it should be possible to compensate for loss of a tether protein by expressing any synthetic component designed to tether the respective membranes at a precise distance. The first such synthetic protein was designed to bridge the ER and mitochondria and consisted of a monomeric red fluorescent protein moiety and membrane targeting domains destined to the ER and mitochondrial outer membrane at the C and N termini, respectively. Overexpression of this synthetic tether indeed caused hyper-tethering of the ER with mitochondria (Csordas et al., 2006). The second generation of such a tether, called ChiMERA (construct helping in mitochondria-ER association), was utilized in a genetic screen to identify mutants that relied on expression of the synthetic tether for efficient growth, and this strategy resulted in identification of the native tether complex ERMES (Kornmann et al., 2009). Expression of ChiMERA both suppressed the growth phenotype of mutants lacking the ERMES components Mdm12 or Mdm34 and reverted their mitochondrial morphology phenotype (large spherical mitochondria) to a tubular, wild-type-like morphology. However, in contrast to the efficient rescue of *mdm12*Δ and *mdm34*Δ mutants, ChiMERA only partially alleviated the respective phenotypes in strains lacking the other ERMES components, Mdm10 and Mmm1 (Kornmann et al., 2009). Mdm10 is not only an ERMES subunit but is found in a second pool at the Sorting and Assembly Machinery (SAM), where it is involved in mitochondrial protein biogenesis (Meisinger et al., 2004), and loss of this second molecular function might well cause defects that are not expected to be compensated for by ChiMERA. This suggests that Mmm1 also has a, yet undiscovered, function in addition to tethering at this contact site.

More generally, there are two possible reasons for the inability of a synthetic tether to compensate for the loss of a tether protein: first, as described above, if the protein has another cellular function outside of the contact; second, if the tethering protein has an additional functional role in the contact site itself. For example, in addition to acting as a physical tether, ERMES is also believed to be involved in lipid transfer between the ER and mitochondria (AhYoung et al., 2015; Elbaz-Alon et al., 2014; Kopec et al., 2010; Kornmann et al., 2009; Lang et al., 2015a; Reinisch and De Camilli, 2016). It is therefore possible that Mmm1 might have a key role in the lipid transfer function of ERMES that cannot be replaced by an artificial tether. Importantly, many known tether components have domains such as lipid-binding domains (Kopec et al., 2010) that suggest that they additionally fulfill lipid transfer functions.

Another example of the use of synthetic tethers to demonstrate an endogenous tethering role is the case of Num1, a mitochondria-PM tether, whose tether function is strongly supported by the fact that the defects caused by its deletion can be compensated for by expression of an artificial mitochondria-PM tether (Klecker et al., 2013; Lackner et al., 2013), and also by a truncated "minimal version" of Num1 consisting just of the two membrane-binding domains spaced by a GFP moiety (Ping et al., 2016).

Rescue of the loss-of-function phenotypes of a potential tether by a synthetic tether construct is therefore a very strong indication that the protein in question indeed is a tether or part of a tethering machinery; but lack of rescue does not indicate that a protein is not a tether but merely that it may not be only a tether. In these cases, a detailed structure-function analysis of the respective tether candidates is a promising approach.

A Typical Tether: Characteristic Features of Tether Proteins and the Importance of Structure-Function Analyses

Membrane tethering can be accomplished by single proteins or by oligomeric protein complexes. No matter how many components form the functional tether, it has to comprise at least two domains that ensure anchoring to two distinct membranes. These elements can be transmembrane domains (TMDs) that are integrated inside the lipid bilayer or domains that mediate

binding to membrane surfaces, for example, lipidated domains or pleckstrin homology (PH) domains that bind to phosphatidylinositides.

The ERMES is a well-studied example for an oligomeric tether (Lang et al., 2015a). It consists of Mmm1, which is anchored in the ER membrane by an α-helical TMD, two mitochondrial proteins Mdm34 and Mdm10 (an outer membrane β-barrel protein), and a further component Mdm12 that has no membrane integral part but is required for structural integrity of the complex (Lang et al., 2015a). Deletion of any ERMES subunit leads to disintegration of the complex, which can be seen by fluorescence microscopy as loss of the typical ERMES foci and uniform re-distribution of the remaining subunits within the respective membranes they are anchored in (Kornmann et al., 2009). Another example for an oligomeric tether is the Nvj1/Vac8 pair with Vac8 being anchored to the vacuole membrane by a palmitoyl moiety (Wang et al., 1998) and Nvj1 to both the inner and outer nuclear membrane by two TMDs (Millen et al., 2008). Similarly to ERMES, in the absence of Vac8, its nuclear interaction partner Nvj1 loses its focal pattern and is dispersed through the nuclear membrane (Pan et al., 2000).

A large group of contact site proteins are based on protein-protein interactions but are not in dedicated oligomeric complexes. These contact site proteins are targeted to the ER via a phenylalanine in an acidic tract (FFAT) motif. The FFAT motifs bind to VAMP-associated proteins (VAP-A and VAP-B in human; Scs2 and Scs22 in yeast), small ER membrane proteins with a C-terminal TMD and an N-terminal major sperm protein domain exposed to the cytosol, that act as protein receptors (Loewen et al., 2003; Murphy and Levine, 2016).

Single protein tethers are often anchored in one membrane by TMDs and associate with the second membrane through domains that bind membrane surfaces, for example, the Tricalbins (TCBs) in the ER-PM contact site that have ER membrane spanning TMDs and bind the PM via C2 domains (Manford et al., 2012; Toulmay and Prinz, 2011). However, some tethers seem to only bind membrane surfaces. For example, Num1, a tether component that anchors mitochondria to the PM, has recently been suggested to mediate tethering independently of any transmembrane structures via two domains that bind to membrane surfaces: a PH domain that recognizes phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) in the PM and a novel type of membrane-binding domain that binds to the mitochondria-specific cardiolipin (Ping et al., 2016).

For single protein tethers as well as tether components with interaction partners with unknown identity, a structure-function analysis is the method of choice to demonstrate their affinity to two distinct membranes. A straightforward way to show that a protein is bridging the membranes of two organelles is to mutate either putative membrane-binding domain of the potential tether tagged with a fluorescent marker. There are numerous examples of contact site proteins that are seen as foci at organellar interfaces when both membrane-binding domains are intact and diffusely re-distribute within either organellar membrane upon deletion of single membrane-binding domains (Gatta et al., 2015; Henne et al., 2015; Klecker et al., 2013; Lackner et al., 2013). Very detailed structure-function analysis has been performed on the mitochondria-cortex tether Num1 (Klecker et al., 2013; Lackner et al., 2013; Ping et al., 2016). Indeed, while full-length Num1 is found at regions of proximity between mitochondria and the PM, Num1 lacking its N-terminal coiled-coil domain relocalized to the cell cortex, whereas deletion of its PH domain resulted in accumulation of Num1 on mitochondria (Lackner et al., 2013). Interestingly, in both cases, Num1 partially conserved its focal distribution, indicating that in this specific case, it is not only the spatial restriction to focal contact sites that is causing the clustering of the tether protein (Lackner et al., 2013). Moreover, since Num1 is a very large protein (313 kDa), simultaneously expressing two Num1 variants with different fluorescent tags on each terminus enabled direct demonstration that the C terminus is closer to the cell cortex, while the N terminus is oriented toward mitochondria (Ping et al., 2016).

In summary, detailed structure-function analyses can be very powerful in determining if and how proteins mediate membrane tethering.

Proof of Proximity: Useful and Less-Useful Approaches to Study Intermembrane Protein-Protein Interactions

Physical proximity of a membrane protein to a known resident of a different membrane has previously been used to suggest its involvement in a tethering complex. One way by which proximity of proteins can be measured is by bimolecular fluorescence complementation assays such as the popular split Venus system (Kerppola, 2006). The split Venus technique is based on the fact that two fragments of the fluorescent protein Venus corresponding to its N- and C-terminal halves are non-fluorescent but can bind to each other and thus form the functional, fluorescent Venus when they are in close proximity to each other. When proteins reside in the same organelle, such an interaction is taken as strong proof of their physical association. Theoretically, one could thus argue that it should be possible to tag tether candidates of two different membranes with the N- and C-terminal Venus halves and to interpret fluorescence complementation at organellar interfaces as identification of a novel tether pair. However, one must use this methodology with extreme care. In our hands, tagging of virtually any protein pair on membranes that form a contact site results in efficient labeling of the contacts by fluorescence complementation (Figure 1). For example, randomly picking four unrelated membrane proteins on the ER and on mitochondria (chosen only based on the fact that their tagged terminus was predicted to face the cytosol and that the proteins were abundant) and tagging them with split Venus pairs shows that all combinations label the contact between mitochondria and the ER (Figure 1B). We reason that irrespective of which membrane protein is tagged, over time, some N- and C-terminal Venus halves will meet each other at the contact sites next to the native tethers where the two membranes are closest to each other, and consequently, diffusion of the tagged proteins will be restricted to the contact site area by binding of the Venus half to its counterpart on the opposing membrane (Figure 1A). Therefore, split Venus can and should be used as a powerful tool to label any contact sites without prior information on their proteinaceous machinery. However, binding of proteins by the split Venus system at contact sites should not be interpreted to support that the tagged protein is a native contact site resident, let alone that it has a tethering role.

So, how can proximity be followed? The whole toolbox of protein biochemistry techniques is available. One example of a

machinery that has extensively been studied in such a way is the contact site machinery between the inner and outer mitochondrial membranes. The mitochondrial contact site and cristae organizing system (MICOS) is a large protein complex of the inner mitochondrial membrane that physically interacts with a number of outer membrane residents, the Translocase of the Outer Membrane (TOM) complex, the Sorting and Assembly Machinery (SAM) complex, the β-barrel protein porin, and the fusion machinery component Ugo1 (Bohnert et al., 2012; Ding et al., 2015; Harner et al., 2011; Hoppins et al., 2011; Korner et al., 2012; Ott et al., 2012; von der Malsburg et al., 2011; Xie et al., 2007; Zerbes et al., 2012a). The resulting contact sites are peculiar because they are not established between two organelles but between two distinct membrane systems within one organelle. To identify and characterize the proximity of MICOS on the inner membrane to proteins of the outer membrane, a combination of enrichment of the contact site fraction (by affinity purification or by sub-organellar fractionation) and mass spectrometry was a key tool (Alkhaja et al., 2012; Harner et al., 2011; Hoppins et al., 2011; von der Malsburg et al., 2011). Furthermore, for characterization of MICOS, a broad set of tools was employed, and its functional organization and physical proximity to outer membrane components was analyzed in detail by native PAGE, chemical crosslinking, and affinity chromatography (Bohnert et al., 2012, 2015b; Korner et al., 2012; Ott et al., 2012; Zerbes et al., 2012a, 2016). Similar tools can be used to characterize each tethering molecule.

Beyond Proximity: Measuring Membrane Tethering Forces

In order to be categorized as a tether, a protein needs to be part of a structure that connects two separate organelle membranes, i.e., it necessarily needs to physically interact with the two phospholipid bilayers themselves or with proteins bound to them. But is every protein that fulfills this criterion automatically a tether? Proteins residing in distinct organelle membranes might be in close contact to each other for many reasons. For example, components of the TOM complex, the translocase of the outer mitochondrial membrane, which is the entry gate for most mitochondrial proteins, and the TIM23 complex, the main translocase of the inner membrane, can be crosslinked to each other (Shiota et al., 2011; Tamura et al., 2009; Waegemann et al., 2015). The physical interplay between these two protein translocases is modulated by incoming precursor proteins (Shiota et al., 2011; Waegemann et al., 2015) and is believed to allow direct handover of substrate proteins and shielding from the aqueous intermembrane space (Bohnert et al., 2015a). Thus, there are cases where residents of different membranes interact for reasons other than physical tethering. In order to experimentally distinguish between tethers and other intermembrane interactions, another parameter in addition to physical proximity has to be taken into account: the ability of a proteinaceous intermembrane bridge to withstand a force trying to displace the membranes.

For most suggested tethers, this ability has not been tested directly. However, one case in which this has been addressed elegantly is the ER-chloroplast contact site of plants (Andersson et al., 2007). First, it was demonstrated that rupturing protoplasts using a laser scalpel maintains the close physical proximity of the ER with chloroplasts. Then, optical tweezers were used to pull the two organelles away from each other demonstrating that the interorganellar tethers could withstand pulling forces of up to 400 pN (Andersson et al., 2007).

Other examples of force measurements come from the plant Golgi-ER contact site (Sparkes et al., 2009) and peroxisome-chloroplast contact site. The latter was measured by both optical tweezers (Gao et al., 2016) and by femtosecond laser technology, an approach in which small, femtosecond laser-induced shock waves are used to estimate physical association of objects upon physical disturbance by the laser (Oikawa et al., 2015). Addressing the adhesion strength of different contact site complexes will be important for getting a better mechanistic understanding of contact site machineries and for assessing the individual contributions of different tether components to interorganellar communication. For example, it would be expected that a tether that has a TMD would be able to withstand stronger pulling forces than a tether that is only binding to phospholipid head groups via small interaction domains.

Is There a Gold Standard to Naming Something a Tether?

In conclusion, it is no wonder that tethering molecules have been hard to discover and hard to prove. The presence of back-up tethers, functional and structural diversity, and lack of tools all are contributing factors. Hence, proof of a tethering function must be done very carefully so that when something is called a tether, it will have a high probability of indeed being one. Importantly, no single piece of evidence is enough to assign a protein or protein complex as a contact site tether. We suggest that the following minimal requirements must be fulfilled:

(1) Defined location: A tether must reside exclusively in, or be enriched in, the contact site. This can be determined by methods such as fluorescence or electron microscopy.
(2) Structural capacity: A tether/tether complex must mediate binding to the membranes of the opposing organelles forming the contact site. Structure-function analyses are currently the best way to prove this point.
(3) Functional activity: A tether must exert a tethering force. This can either be measured directly in vivo or in reconstituted systems or indirectly by the effect of the tether on the extent of the contact site or the rescue of tether loss by artificial tethers.

Tethering in Membrane Fusion: Different Game, Same Players?

Tethers are not only the key players of organelle contact sites. In fact, tethers also exist in contacts between cells organized in tissues (such as tight junctions, adherence junctions, and desmosomes; Steed et al., 2010) and in structures such as the immunological (Dustin, 2007) and neurological (Dalva et al., 2007) synapse, where they are called adhesion molecules. Moreover, tethering occurs within the cell during each membrane fusion event, be it between a small vesicle and its target membrane or between structures as large as mitochondria, lysosomes, or yeast vacuoles. Each fusion event is preceded by a "docking stage," in which the two phospholipid bilayers are still discontinuous but physically tethered by specialized protein machinery. Morphologically, docked membranes about to fuse fulfill all the characteristics of a canonical contact site; they are positioned at a close distance in the range of a few nanometers

and are physically connected by proteinaceous tethers. However, functionally, these structures are fundamentally different from contact sites, because pre-fusion docking will at some point (although in some cases, such as synaptic vesicle fusion, the docking stage can last for hours and days) lead to coalescence of the docked entities, resulting in one physically continuous, uniform organelle. In contrast, contact sites are structures that offer a very defined limited exchange between opposing membrane-bound entities, and it is these limitations that turn contact sites into highly specialized cellular subdomains that are inherently different from both interaction partners. Despite this key functional difference, the morphological similarities between these two cases make it unsurprising that the molecular tethers underlying the formation of these two types of membrane interfaces show extensive similarities and even overlapping functions. For this reason, it is important to remember the commonalities and similarities as they can teach us a lot about contact sites. For example:

(1) The ER-PM tether family of extended Synaptotagmins (E-Syts) has relatives in the secretory fusion machinery, the Synaptotagmins, which are key players in neurovesicular fusion. Both types of proteins can interact with two membranes via transmembrane segments and C2 domains. Synaptotagmin-1 is located on secretory vesicles and acts as a key Ca^{2+} sensor during Ca^{2+}-dependent vesicle fusion. While this function had previously been related to an interaction with both the soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) machinery and target membrane phospholipids, it was recently found that, at tightly controlled physiological ionic conditions, Synaptotagmin-1 did not interact with SNAREs but just bound efficiently to PM $PI(4,5)P_2$ (Park et al., 2015). Synaptotagmin-1 thus acts as a Ca^{2+}-dependent membrane tether, a mechanism that highly resembles Ca^{2+}-dependent contact site formation by E-Syt1 (Giordano et al., 2013).

(2) An incomplete, apparently non-fusogenic, SNARE complex has recently been proposed to function in ER-PM tethering. This complex consists of Sec22b, a SNARE protein with an established role in the early secretory pathway, and the PM Syntaxin1 but lacks any component of the SNAP23/25/29 family (Petkovic et al., 2014).

(3) Vps39 has originally been described as a component of the homotypic fusion and protein sorting (HOPS) complex, a multisubunit tethering complex that acts as a Rab7 effector in the endolysosomal pathway where it mediates membrane tethering prior to SNARE-dependent fusion (Wickner, 2010). Intriguingly, Vps39 has a second important function as a component of the vCLAMP, the contact site between the yeast vacuole and mitochondria (Elbaz-Alon et al., 2014; Honscher et al., 2014).

(4) Two homologous dynamin-related GTPases, Mitofusin 1 and 2 (MFN1 and 2), are residents of the outer mitochondrial membrane where they mediate a two-step process of docking and subsequent fusion in mammalian mitochondria (Hoppins and Nunnari, 2009). Intriguingly, there is evidence for a second pool of MFN2 on ER membranes, which forms ER-mitochondria contact sites by interacting with mitochondrial MFN1 or MFN2 proteins (de Brito and Scorrano, 2008). Hence, under some conditions, this molecule promotes only the first docking step and in others also the second fusion step.

In summary, there are manifold interrelations between contact site and fusion machineries, and more likely await discovery. These findings and the obvious shared structural and functional requirements on tethering components in either context make it tempting to speculate that contact sites and fusion machineries might also be evolutionarily closely related.

A Tether Is a Tether Is a Tether: Are All Tethers the Same?

While all tethers in membrane contact sites share the ability to bridge two membranes and to exert a tethering force, it is becoming clear that not all tethers are alike. Instead, there is a fascinating variety of distinct tethering structures in the cell that differ in the quality of their tethering activity and can have additional functions and regulatory modules. Different classes of tethers can be defined based on three criteria (Figure 2).

Principal or Auxiliary

There are tethers that are both necessary and sufficient to form and maintain contact sites independently of other components. These proteins may be referred to as "principal tethers" (Figure 2A). Depletion of principal tethers generally leads to loss of their respective contact site (unless a functionally redundant principal tether exists). An example of such a tether is the NVJ tether pair Nvj1/Vac8. On the other hand, many, possibly all, contact sites additionally host proteins with limited tethering activity. These contact site residents are able to bridge two membranes, however, they have a tendency to join pre-existing contact sites established by principal tethers rather than inducing membrane proximity de novo. Their tethering activity becomes apparent when the principal tethers fail to function (or are mutated or lost) or under special experimental conditions. An example of such behavior is Mdm1. Mdm1 localizes to the NVJ, but its deletion does not affect NVJ formation by the principal tethering machinery Nvj1/Vac8. Mdm1 has been shown to have a domain organization that is compatible with a function as an intermembrane tether with its two N-terminal TMDs anchoring it in the ER and the C-terminal phosphoinositide binding PX domain attaching it to the vacuolar membrane (Henne et al., 2015). Indeed, Mdm1 overexpression results in drastic expansion of the contact site with vacuoles encircling the entire nucleus and Nvj1 redistributing over the expanded nuclear-vacuolar contact area, showing that Mdm1 has the potential to modulate the size of its host contact site (Henne et al., 2015). Interestingly, under conditions where the Nvj1/Vac8 interaction is abolished, Mdm1 maintains its focal distribution typical for contact site components, although under these conditions, Mdm1 foci are smaller and localize to areas of contact between vacuoles and the cortical and cytoplasmic ER instead of the nuclear envelope (Henne et al., 2015). Thus, Mdm1 preferentially joins pre-existing contact sites established by the Nvj1/Vac8 principal tethering pair, but its localization to contact sites is not dependent on their presence.

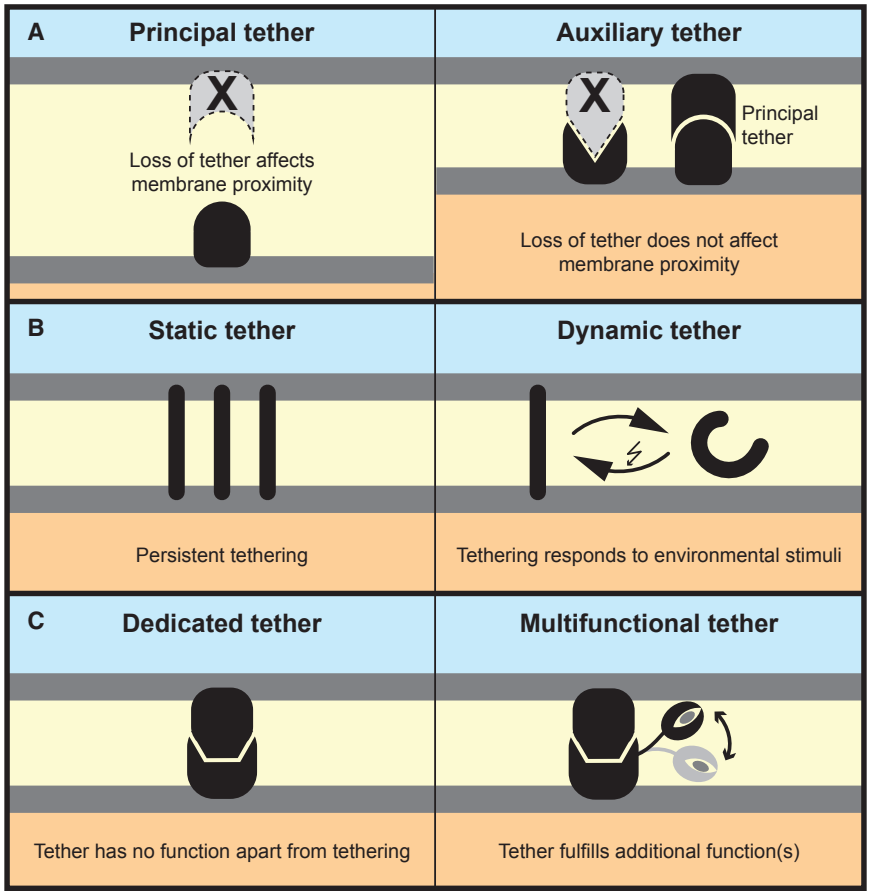


Figure 2. A Tether Is a Tether Is a Tether
Schematic representation of different types of contact site tethers defined by three criteria: (A) principal versus auxiliary; (B) static versus dynamic; (C) dedicated versus multifunctional. X represents loss-of-function mutation. Flash symbolizes an environmental stimulus. Yellow, cytosol; blue and orange, lumens of two distinct organelles.

Another example can be found in the yeast *Tricalbins*. Concomitant deletion of all three TCB genes (*TCB1*, *TCB2*, and *TCB3*) does not significantly affect the extent of ER-PM contact (Manford et al., 2012; Toulmay and Prinz, 2011). Nevertheless, in the absence of the ER-PM tether proteins, Scs2 (likely the principal tether in this contact site), Scs22, or Ist2, loss of TCBs leads to a further reduction of ER-PM contact.

Finally, a further good example is the contact site resident Lam6/Ltc1 that localizes to the ER/mitochondria contact and the NVJ (Elbaz-Alon et al., 2015; Gatta et al., 2015; Murley et al., 2015). *LAM6* deletion does not affect the formation of either of its host contact sites, suggesting that it is not the principal tether (Elbaz-Alon et al., 2015). However, overexpression of Lam6 results in dramatic enlargement of both contact sites that Lam6 associates with (Elbaz-Alon et al., 2015). Indeed, Lam6 appears structurally equipped to mediate membrane tethering (Gatta et al., 2015): it contains two domains at its termini that might mediate membrane binding, one C-terminal transmembrane segment and an N-terminal GRAM domain, a type of PH-related domain that has been shown to bind to membrane surfaces via interaction with phosphoinositides or proteins (Begley et al., 2003; Doerks et al., 2000). Furthermore, Lam6 has been reported to interact with the mitochondrial proteins Tom70 and Tom71 as well as with the vacuolar protein Vac8 (Elbaz-Alon et al., 2015; Murley et al., 2015). Indeed, we have recently found that Lam6-dependent mitochondria-ER hyper-tethering happens as efficiently in an ERMES mutant as in the presence of ERMES tethers (Figure 3). Thus, although Lam6 it is not a principal tether required for contact site formation, it may have an auxiliary tethering ability, that might also be important for regulating contact site size and/or activity.

Interestingly, there are more examples of proteins that are known to join pre-existing contact site, but appear structurally equipped to have tethering activities of their own. These include, for example, Osh1, 2, and 3, Nvj2, and FAPP2. These proteins may thus be termed "auxiliary tethers" (Figure 2A). It is conceivable that the basis of the contact site landscape in the cell might be laid out by principal tethers that are optimized to seed and maintain contact sites, which are then fine-tuned in size, shape, and/or function by additional auxiliary tethers that can modulate contact sites in order to adapt their extent to the respective physiological state of the cell.

Static or Dynamic

Tethers may not always be static but rather be dynamic and regulated. There are many examples of proteins that fulfill all prerequisites for being termed a tether but only function as tethers in response to special environmental stimuli. A well-described example is the tether pair STIM1 (an integral ER protein) and Orai (a PM Ca^{2+} channel), which enables efficient Ca^{2+} transfer from the extracellular space into the ER in response to low luminal Ca^{2+} levels (Liou et al., 2007). Another example is E-Syt1, which is spread over the whole surface area of the ER at resting conditions but specifically recruited to ER-PM interfaces by a Ca^{2+}-dependent membrane-binding C2 domain upon elevation of cytosolic Ca^{2+} levels (Chang et al., 2013; Giordano et al., 2013; Saheki et al., 2016) (Figure 4A). A further example is the tethering activity of mammalian oxysterol binding protein (OSBP), which becomes sequestered at the Golgi-ER interface alongside its ER binding partner, VAP, in response to the presence of oxysterols (Mesmin et al., 2013; Ridgway et al., 1992) (Figure 4B). OSBP binding to the Golgi membrane is mediated by a phosphatidylinositol 4-phosphate (PI4P)-binding PH domain. OSBP transfers PI4P from the PI4P-rich Golgi membranes to the ER and counter-transfers sterols from the ER to the Golgi against a concentration gradient, and the decline in Golgi PI4P levels acts as an intrinsic "OFF" switch

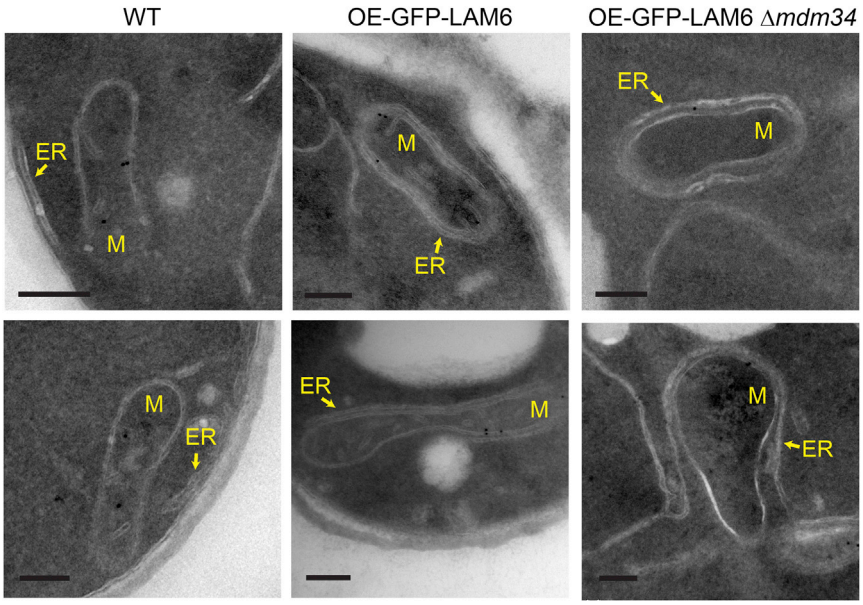


Figure 3. Lam6 Promotes Contact Site Formation Independently of ERMES
Electron microscopy shows that Lam6 overexpression (OE-GFP-LAM6) leads to massive expansion of ER-mitochondria contact sites, resulting in mitochondria completely wrapped in ER sheets, both in a wild-type background and in a strain deleted for *MDM34*, a component of the ER-mitochondria tether ERMES. Lam6 is present within the contact site, as detected by immunogold labeling. WT, wild-type. Scale bar, 200 nm.

for OSBP-dependent lipid transfer by abolishing tethering. Hence, OSBP has a highly regulated tethering activity that depends on its lipid transfer activity (Mesmin et al., 2013). The OSBP-related proteins ORP5 and ORP8 function in a similar way as regulated ER-PM tethers and mediate PI4P/phosphatidylserine (PS) counter-transport at this contact site (Chung et al., 2015). The ceramide transfer protein CERT is a further regulated tether at the interface between the ER and the trans-Golgi. Phosphorylation of a serine residue adjacent to its FFAT motif was found to occur under conditions of pronounced requirement for sphingolipid biosynthesis and enhance the interaction between CERT and VAP (Kumagai et al., 2014). In contrast, phosphorylation of a serine-rich patch close to the PH domain negatively regulates CERT activity (Kumagai et al., 2007). Hence, CERT function appears tightly regulated by differential phosphorylation. Interestingly, numerous further tether factors have been proposed to be subject to phosphorylation, among them Osh2 and Osh3, OSBP, the vCLAMP component Vps39, and the ERMES subunit Mdm34 (Honscher et al., 2014; Nhek et al., 2010; Swaney et al., 2013).

These tethers may be termed "dynamic tethers" as opposed to unregulated "static tethers" (Figure 2B).

Dedicated or Multifunctional

The simplest tether that can be imagined would be a protein that is solely dedicated to holding two membranes together. However, it seems that much of the tethering force in contact sites is exerted by multifunctional tethers that also harbor an additional biological role on top of tethering (Figure 2C).

One clue for the widespread occurrence of multifunctional tethers comes from the presence of lipid-binding domains in numerous tethers (e.g., ERMES, TCBs/E-Syts, ORPs, LAM proteins). Lipid-binding domains generally have a hydrophobic cavity that can accommodate a lipid molecule. Many of the lipid-binding domain-containing proteins have also been suggested to act as lipid transfer proteins (LTPs) as in vitro experiments showed that many proteins with a lipid-binding domain have the ability to transport specific lipids from one liposome to another (Lev, 2010). It is, however, a matter of debate to what extent LTPs are involved in bulk lipid transport in vivo. Alternatively, they have been suggested to function as lipid sensors, as membrane modulators that induce specific local membrane alterations rather than mediating bulk lipid flow, or as scaffolds that present membrane lipids to the cytosol (Lev, 2010). It is thus very well possible that components like the TCBs, LAM proteins, and ORPs exert their functional role in contact sites mainly through their lipid binding instead of their tethering function but that their presence at the contact site in large amounts increases the general avidity between the two membranes (Figure 2C).

In a broader view, it is not surprising that contact sites, as specialized domains where two organelles collaborate, host many membrane proteins that have a functional relationship with residents of the opposing membrane. For example, the voltage-dependent anion channel VDAC1, a key player in metabolite flow across the outer mitochondrial membrane, has been suggested to be physically connected to the Ca^{2+}-release channel inositol triphosphate receptor (IP3R) in the ER membrane through the chaperone glucose-regulated protein 75 (Grp75), a functional connection that might control mitochondrial Ca^{2+} uptake (Szabadkai et al., 2006). Such collaborative exertion of a common function will inherently create physical interactions that will contribute some tethering forces between the membranes. In many cases, the tethering function of a membrane bridging structure might be the (evolutionarily consolidated) by-product of a primary functional interaction between residents of the two organelles. It remains to be determined to what extent function-based interactions influence contact site architecture as opposed to specialized components dedicated specifically to membrane tethering.

In summary, while the textbook view of contact sites was built on the idea of a single, principle, static, dedicated tether, it is conceivable that some contact sites might not harbor any such tether but rather be structurally maintained by a concerted tethering force exerted by the combined avidity of numerous functional interactions.

Conclusions

The discovery of contact site machineries caused a stir because it offered a new perspective on the functioning of a cell. The

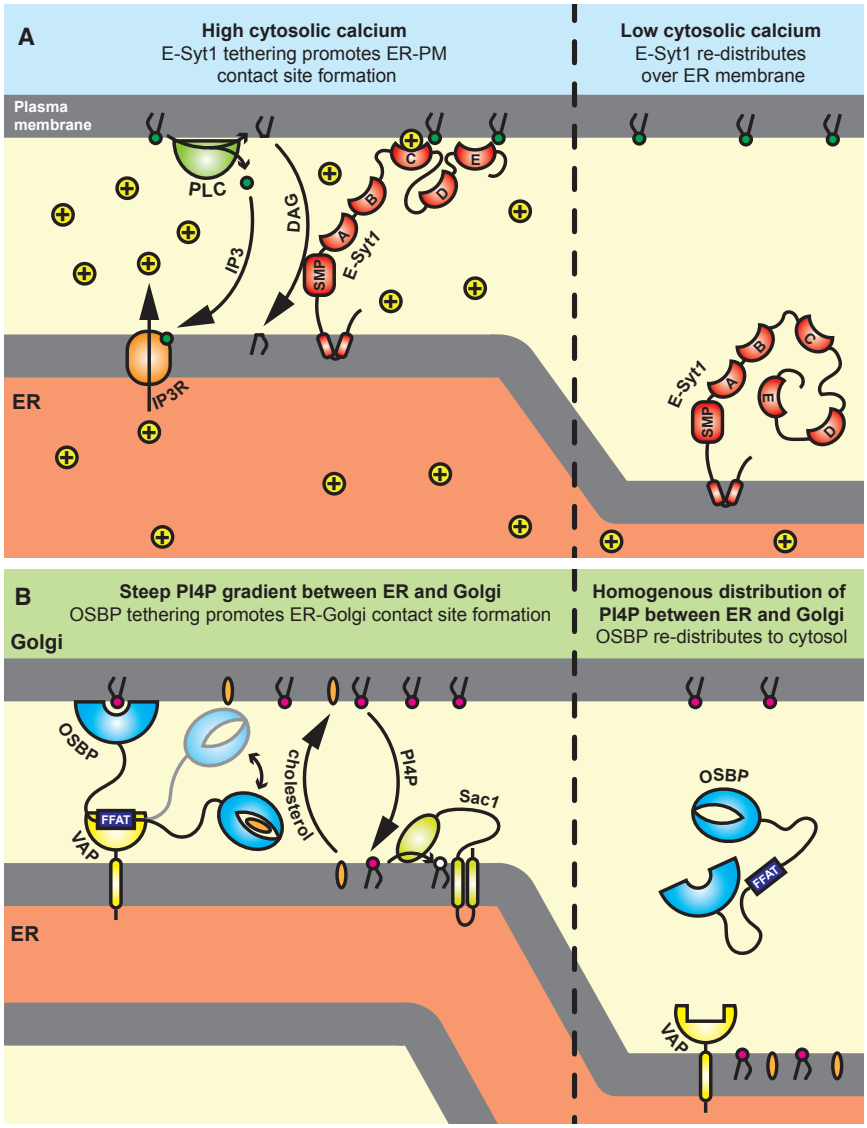


Figure 4. Examples of Dynamic Tethers

(A) The integral ER protein extended Synaptotagmin-1 (E-Syt1) acts as an ER-plasma membrane (PM) tether at high cytosolic calcium (symbolized by +) levels dependent on calcium binding to one of its five C2 domains, the C2C domain (left). At low cytosolic calcium, the tethering function of E-Syt1 is abolished (right). Calcium release from the ER via the inositol triphosphate receptor (IP3R) depends on activity of phospholipase C (PLC), which catalyzes conversion of PM phosphatidylinositol-4,5-bisphosphate (green head group) to inositol triphosphate (IP3) and diacylglycerol (DAG; no head group). The synaptotagmin-like mitochondrial lipid-binding protein (SMP) domain of E-Syt1 is important for removal of DAG from the PM.

(B) Oxysterol binding protein (OSBP) alternates between ER-Golgi tethering (left) and a cytosolic form (right). Its phenylalanine in an acidic tract (FFAT) motif binds to the integral ER protein VAP (VAMP-associated protein). A PH domain mediates binding to the Golgi membrane dependent on the presence of phosphatidylinositol-4-phosphate (PI4P; pink head group). Via its lipid-binding domain, tethered OSBP pumps cholesterol (orange oval) against a concentration gradient into the Golgi membrane, fueled by a counterflow of PI4P. The PI4P gradient is being maintained by the activity of the PI4P phosphatase Sac1, which converts PI4P to phosphatidylinositol (white head group) in the ER.

notion that many, possibly all organelles are physically tethered led to the understanding that the interior of each cell is at any given moment more spatially organized and functionally interconnected than ever anticipated. While many novel contact sites and their components are still awaiting discovery, recent studies are now starting to zoom in on the molecular mechanisms of individual contact sites in order to address their spatial, temporal, and functional organization. We are beginning to understand that each contact site is a functional microdomain based on the concerted activity of distinct types of tether and non-tether components. The recent finding of biologically meaningful positioning of tethers during mitochondrial fusion (Brandt et al., 2016) imply that another aspect of contact sites that awaits further investigation is the spatial organization of contact site residents. At this current stage, it is essential that we clearly define the molecular role of contact site residents, so that we will be able to mechanistically understand how contact sites work and realize their collective functional role. This enterprise requires that we first experimentally pin down each contact site's core, which after all is a tether, is a tether, is a tether.

AUTHOR CONTRIBUTIONS

Conceptualization: M.E.B., N.S., M.S., and M.B.; Methodology: N.S. and M.S.; Investigation: M.E.B. and N.S.; Visualization: M.E.B., N.S., and M.B.; Writing – Original Draft: M.S. and M.B.; Writing – Review and Editing: M.E.B., N.S., M.S., and M.B.; Supervision: M.S. and M.B.; Funding Acquisition: M.S.

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