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Piecing together the patchwork of contact sites

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Abstract:	Contact sites are places where two organelles join together to carry out a shared activity requiring non-vesicular communication. A large number of contact sites have been discovered, and almost any two organelles can contact each other. General rules about contacts include constraints on bridging proteins, with only a minority of bridges physically creating contacts by acting as "tethers". The downstream effects of contacts include changing the physical behaviour of organelles, and also forming biochemically heterogeneous sub-domains. However, some functions typically localised to contact sites, such as lipid transfer, have no absolute requirement to be situated there. Therefore, the key aspect of contacts is the directness of communication, which allows metabolic channelling and collective regulation.

Piecing together the patchwork of contact sites

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Abstract

Contact sites are places where two organelles join together to carry out a shared activity requiring non-vesicular communication. A large number of contact sites have been discovered, and almost any two organelles can contact each other. General rules about contacts include constraints on bridging proteins, with only a minority of bridges physically creating contacts by acting as "tethers". The downstream effects of contacts include changing the physical behaviour of organelles, and also forming biochemically heterogeneous sub-domains. However, some functions typically localised to contact sites, such as lipid transfer, have no absolute requirement to be situated there. Therefore, the key aspect of contacts is the directness of communication, which allows metabolic channelling and collective regulation.

Key Words

Non-vesicular traffic; biological transport; intracellular membranes/metabolism; membrane lipids/metabolism; vesicular transport.

Miscellaneous membranes mix at contact sites

Each intracellular **organelle** (see Glossary) carries out a limited set of reactions. When one biochemical pathway is distributed across multiple organelles there must be intracellular communication. While a subset of organelles can communicate by vesicular traffic, all organelles can communicate via non-vesicular traffic. This involves proteins or metabolites dissociating from one organelle, diffusing across intracellular gaps, and binding to receptors on another organelle. Special cases of intracellular communication occur where two distinct (not **homotypic**) organelles form a **contact site**. The contact site field began in 1956 with ultrastructural studies by Bernhard and Rouiller [1]. Afterwards contact sites were largely overlooked for 50 years [2], and are still missing from text books. The last decade has seen much progress, and there are many excellent reviews that describe these developments as a whole [3,4], or at a single contact site [5] or for one family of components [6]. This article has a dual focus: in this section we describe how almost every pair of organelles now appears to form contacts (Figure 1, Key Figure); later we make general inferences about contact site function.

Contacts formed by mitochondria, a second intracellular network

Early on the ER appeared as a common partner to most other organelles, including mitochondria, plasma membrane, endosomes/phagosomes/lysosomes, Golgi apparatus and lipid droplets. This led us to suggest a model that all organelles had constitutive contacts with the ER, which acts as a pan-cellular conduit for small metabolites such as Ca^{2+} and lipids [2]. However, the demonstration that many non-ER organelles directly contact each other has shown that our model was wrong. After the ER, a second intracellular reticulum is formed by mitochondria. Although mitochondria vary in size and inter-connectedness, when viewed as a whole they can form an intracellular network that is almost as extensive as the ER. Like the ER, the mitochondrial network contacts most other organelles. Here we review the evidence for the different contacts formed by mitochondria.

Endosome/lysosome–mitochondrion

contacts between endo-/lysosomes and mitochondria have been seen in several mammalian cell types, where they mediate direct traffic of material. In red blood cell precursors early endosomes contact mitochondria to transfer endocytosed iron for mitochondrial heme synthesis [7]. In hypoxic cancer cells similar contacts allow partial “kiss and run” fusion that transfers endocytic

proteolytic enzymes to mitochondria [8]. Contact sites have also been found in pigmenting cells joining melanosomes, which are lysosome-related organelles, to mitochondria, possibly for production of ATP close to sites of melanization [9].

The best characterised contacts of this type are in budding yeast, where they are called vaCuoLe And Mitochondria Patches (vCLAMPs), vacuoles being the yeast degradative compartment equivalent to lysosomes [10,11]. The first vCLAMP component discovered was Vps39p (also called Vam6p), already known as a component of complexes that **tether** late endosomes to vacuoles. Additionally the cytoplasmic protein Vps13p targets vCLAMPs (and others including endosome-mitochondrial contacts [12]), and it may be recruited by Vps39p [13]. vCLAMPs may provide a route for lipid traffic into mitochondria, however this becomes obvious only when mitochondria cannot acquire lipids directly from the ER. A minority of mitochondrial-ER contacts contain the ER-mitochondrial encounter structure (ERMES) complex, three subunits of which are members of the tubular lipid transfer protein (TULIP) family (Figure 2A) [14-19]. When ERMES is destabilised, mitochondria swell into spheroids >1 μm in diameter to accommodate massively expanded vCLAMPs, and both Vps39p and Vps13p become essential for mitochondrial function. This suggests that when lipids cannot flow directly from ER to mitochondria, they take a circuitous route via vacuoles and vCLAMPs. Even though some TULIPs transfer lipids [18,19], generating proof that ERMES or any lipid transfer proteins transfer lipids across contact sites is not trivial [20].

Peroxisome–mitochondrion

Vesicular traffic between peroxisome-mitochondria [21] is supplemented by contact sites that have been partially characterised in yeast. Mitochondria are affected by the presence of peroxisomes, since the mitochondrial matrix near to the peroxisomal contact site accumulates the pyruvate dehydrogenase enzyme complex [22]. Both this complex and peroxisomes produce acetyl CoA, indicating that there is integrative control of acetyl-CoA production across the contact (Figure 2A). Pex11p is required for their maximal formation, although it is not clear if Pex11p itself is the tether (Table 1) [23].

Chloroplast–mitochondrion

Chloroplasts not only make contacts with ER [24], but also with mitochondria, creating a potential route for lipid traffic. During starvation of plants for phosphate, chloroplast enzymes convert mitochondrial phospholipids to galactolipids. The evidence for lipid traffic across chloroplast–mitochondrial contacts is that they increase 3-fold in size during phosphate starvation [25].

Plasma Membrane–mitochondrion

Mitochondria are found close to the plasma membrane in many cell types. In yeast such attachment is proposed to organize inheritance of mitochondria, and there are two different attaching complexes, one for daughter buds, and another for mother cells, the latter tethered by Num1p (Table 1) [26]. Sub-plasma membrane mitochondria occur in mammalian cells too, where they preferentially take up Ca^{2+} entering the cell nearby. In both yeast and mammalian cells these contacts are typically accompanied by ER that contacts both plasma membrane and mitochondria, making a three-way contact called mitochondria–ER–cortex-anchor (MECA) [26].

One unusually distant "contact" is found in presynaptic termini in neurons, where mitochondria are anchored ~200 nm from the plasma membrane by a filamentous structure of unknown composition, with many synaptic vesicles filling the gap [27]. Does this arrangement meet the definition of contact site? We suggest that the answer is yes. Local communication occurs, although the main relationship may be between mitochondria and the intervening vesicles to optimise ATP supply for neurotransmitter accumulation.

Autophagosome–mitochondrion

Compared to other cellular membranes, the isolation membrane surrounding autophagosomes is enriched in lipid over proteins. The source of the lipid is controversial, but non-vesicular delivery of mitochondrial lipids is one mechanism, though as in MECA the ER may intervene [28,29].

Lipid droplet–mitochondrion

ATP production by beta-oxidation of fatty acids occurs in mitochondria of animal cells, particularly in myocytes and brown adipocytes. The fatty acids are stored in lipid droplets as triacylglycerol, which is converted to fatty acids on the lipid droplet surface. Five related perilipins bind to lipid droplets, inhibiting lipolysis by displacing lipases. Uniquely, perilipin-5 mediates lipid droplet-mitochondrion contacts via a hydrophilic motif that binds to an unknown mitochondrial binding partner [30]. Fatty acids traffic from perilipin-5-positive lipid droplets to nearby mitochondria, but whether fatty acid traffic occurs across contacts as proposed for bilayer lipids is unknown [31].

Mitochondrial inner membrane–mitochondrial outer membrane

It has long been known the two perimeter mitochondrial membranes form contacts without fusing. The molecular basis for this has now begun to be described. The mitochondrial contact site (MICOS) complex embedded in the mitochondrial inner membrane was discovered initially as a

regulator of crista junctions through its homotypic interactions. In addition, MICOS has heterotypic interactions with three outer membrane complexes (Figure 2A) [32-35]. It is not yet clear if MICOS acts primarily as a tether (Table 1). The largest MICOS subunit (Mic60p in yeast, mitofilin in mammals) contributes to cardiolipin transfer possibly by binding the lipid headgroup to reduce bilayer stability (Table 1) [33].

Contact sites locally modulate organelle networks

The mitochondrial network is heterogeneous. One source of this heterogeneity are the different contacts that locally modulate aspects of mitochondrial function, including, metabolic activity [27,31], accumulation of pro-apoptotic signals [8], inheritance [36], and licensing mtDNA replication [37] prior to network fission [4]. Another feature that is coming to the fore is the formation of three-way contacts containing mitochondria, ER plus one other organelle all contributing to one pathway [23,26,28].

Contacts between two multi-copy spheroidal organelles

There are many organelles that exist as multiple copies of isolated spheroidal bodies: either membrane bound vesicles (peroxisomes, late endosomes, lysosomes, also including lipid droplet) or liquid drops (nucleoli, P-bodies, inclusion bodies *etc*.). The distribution and relationships of each of these organelles to each other has until recently appeared quite random. Now different types of organelle have been found to contact one another. Here we review the different contacts formed between lipid droplets, peroxisomes, endo-/lysosomes and others.

Lipid droplet–peroxisome

Lipid droplets not only contact the ER as they form [38], and also mitochondria (above), but also they contact peroxisomes both in many eukaryotic cell types [39]. Yeast growing on lipid as their sole energy source break down fatty acids stored in lipid droplets by beta-oxidation in peroxisomes (not in mitochondria as in metazoa). Here lipid droplet–peroxisome contacts enhance fatty acid traffic and eventually allow peroxisomal beta-oxidative enzymes to access the core of the lipid droplet, indicating a slow fusion process [40].

Lipid droplet-endosome and peroxisome-endosome

In fungal hyphae these contacts mediate long-range co-transport of both lipid droplets and peroxisomes with endosomes. The latter have a microtubule motor, while the other organelles

attach to the endosomes (Figure 2B) [41,42]. Such indirect attachment for cytoskeletal-based transport may act in other elongated cell processes, for example in neurons.

Lipid droplet–inclusion body

Contacts form in yeast between lipid droplets and inclusion bodies, focal cytoplasmic accumulations of aggregated, misfolded proteins that have escaped degradation by proteasomes or autophagy, which might be considered to be organelles or **compartments** even though they are not membrane-bounded. The inclusion body protein Iml2p interacts with lipid droplet proteins, and recruits lipid droplets to inclusion bodies [43]. Intriguingly, normal clearance of inclusion bodies requires not only Iml2p but also lipid droplet proteins that produce or transfer ergosterol, the yeast equivalent of cholesterol. This suggests that a sterol-derived lipid acts as a natural detergent to unfold misfolded proteins.

Lysosome-peroxisome contact sites

LDL cholesterol is released from lysosomes and eventually reaches the plasma membrane or ER [44]. Unexpectedly, lysosome-peroxisome contact sites are involved in this cholesterol traffic [45]. The peroxisome is tethered by the lysosomal transmembrane protein synaptotagmin-7, normally found at synapses but with a subpopulation on lysosomes (Table 1). How peroxisomes mediate sterol traffic is still unknown, and it could be that the peroxisomal contribution is to handle free fatty acid.

Everywhere there is difference

The list of organelles that contact each other is expanding rapidly. The multitude of contacts means that each peroxisome, lipid droplet and endosome (*etc.*) is heterogeneous purely on the basis of its contacts. The causes and effects of this are unknown.

Contact sites as biochemical hubs

B**ridges** at contact sites define and organize biochemically distinct sub-regions in two different ways.

Organization across contacts: metabolic channeling between organelles

Contact sites promote direct communication of material or signals between organelles. The communication can be focused 100% on the target, particularly if the protein that enacts the communication also forms a bridge. For Ca^{2+} traffic in muscle cells, voltage-dependent Ca^{2+} channels on the plasma membrane directly bind to Ca^{2+}-induced Ca^{2+}-release proteins in the sarcoplasmic reticulum, amplifying the initial depolarization signal to activate acto-myosin throughout the cell (Figure 3A). Other bridging proteins implicated in traffic include lipid transfer proteins, which can be recruited from the cytoplasm (Figure 3B), or may have a permanent membrane **anchor** (Figure 3C) [46,47]. Additional pathways localised to contacts include: cyclic AMP signalling [48,49], acetyl-CoA generation [22], lipid synthesis [50], and the acquisition of proteins for movement [51], fission [4], or both [52]. In addition, "transcatalysis" takes place, where enzyme and substrate are separated [53].

An increasingly prominent idea is that bi-specific lipid traffic proteins exchange one lipid down a steep gradient to force another lipid up a less steep gradient (Figure 3C) [6]. The identification of the second lipid may be quite complex. For example, the oxysterol binding protein (OSBP) homologue Osh4p in yeast can counter-transfer not only sterol and phopshoinositide 4-phosphate (PI4P) (Figure 3D), but also phosphatidylserine (PS) and $PI(4,5)P_2$ [54]. Counter-current exchange has been associated with contact sites, but it has no absolute need for them. Even though contact sites create conditions for maximum and regulatable efficiency [55], counter-currents work for lipid transfer proteins that have no strong membrane targeting, such as Osh4p, although these tend to be expressed at >10-fold greater levels than other family members, possibly to compensate for the inefficiency of increased **diffusion** (Figure 3D).

In summary, an absolutely key aspect of contact sites is the creation of a unique space that excludes other organelles. This is equivalent to metabolic channeling between enzymes, but on a larger scale [3,56]. Together, the combination of directness and the short distance between donor and acceptor may create sites where transfer is highly efficient and easily regulated *en bloc*. These advantages may have brought into existence the large number of contacts between so many organelle pairs.

Lateral organisation: subdomains within organelles

Bridging complexes can recruit other proteins to nearby portions of the organelle. Thus, contact sites organise functionally distinct sub-domains even within continuous organellar networks such

as the ER and mitochondria. For example, mitochondrial contacts with ER and peroxisomes colocalize with adaptive specializations in the contacting organelles (Figure 2A) [22,57]. To study such lateral organisation, membranes enriched for contact sites have been purified, in particular mitochondrial associated ER membranes (MAMs). Many proteins localised to MAMs are lipid biosynthetic enzymes [58-61], but ≥30 other proteins are enriched there, including gamma-secretase and TORC2 (reviewed in [5]).

Since contacts recruit lipid modifying enzymes, contact site-associated subdomains may have specific lipid compositions. The best known example is MAMs, which are enriched for sterol in metazoa [62], but not in yeast [63]. The adjacent region of mitochondria contains mtDNA, and is also rich in sterols [64]. MAMs are also enriched both in Acyl-CoA:cholesterol acyltransferase (ACAT), a key regulator of cellular free sterol [44], and in nascent lipoproteins that mediate cholesterol secretion [65]. This shows that cholesterol has a high concentration in and high flux through MAMs. However, claims that sterols drive protein partitioning in MAMs to form “lipid rafts” are unvalidated [66]. As yet sterol based lipid-lipid interactions that partition membrane domains have only been found where there is no supporting actin and low membrane protein concentration [67].

The physicality of direct contact

Among contact site functions, there are multiple aspects that simply describe the physicality of pairs of organelles being joined together. Here we identify some general principles of these physical relationships.

Anchoring

Contact allows organelles to exert force on each, for example so that one can pull another. Organelles known to interact physically and move together include endosomes plus ER [68] and endosomes plus peroxisomes or lipid droplets (Figure 2B) [41,42].

Untangling tubular organelles by marking sites of fission

As organelles that form three-dimensional tubular networks, for example the ER and mitochondria, change shape and move they will cross each other and potentially form tangles. Like DNA, an extended biomolecule with specific untangling enzymes, there is untangling machinery for extended organelles. Sites of mitochondrial fission are found near a sub-set of ER-mitochondrial contacts where ER tubules partly encircle the mitochondrial network. Fission requires sequential recruitment of two constriction machineries, one that reduces the diameter from 300-500 nm to less than 150 nm, and a second that induces severing (reviewed in [4]). Endosomes also form extended tubules, which also undergo fission near ER contact sites that recruit specific fission machinery [4,52]. Contacts involved in organelle fission must have a limited life-time, so that they may turn over faster, and move more rapidly, than other contact sites.

That the ER marks sites of fission on other tubular organelles, but it is not divided itself, can be linked to the evolution of contemporary ER from the ancestral plasma membrane, which would have strongly resisted fission. As envisaged by Baum and Baum, early eukaryotes evolved “inside-out”, having an intermediate stage with ER-like functions residing in deep plasma membrane clefts linked to the nuclear envelope (Figure 2C) [69]. This plasma membrane would not have undergone fission, but would have marked other tubular organelles for fission in primordial cells. This may have evolved into the ER marking other tubular organelles for fission now.

Tethering (and the making of a tether)

Physical contact requires organelles to be tethered, but criteria for defining contact site “tethers” have not yet been decided by cell biologists. “Tethers” in a vesicular pathway are proteins that capture vesicles prior to fusion, often ≥100 nm away from their target, and so these proteins inform our understanding of highly extended contact site proteins (see section on Long Linkers below). We propose that tethers at contacts are those proteins for which the main function is the creation of a significant proportion of the contact structure. Tethering might best be estimated from studying the loss of contact when a protein is missing. The early discovery of Nvj1p in yeast as a protein that is not only required for formation of the nucleus vacuole junction (NVJ) but also contains no other active domains suggested that tethers might be common [2]. However, this is not so: proteins that meet the criteria to be pure tethers are relatively rare (Table 1A). Many contact site components that have been called tethers only have this function partially or to a small degree (Table 1B/C). Instead they contain domains that strongly point to other functions. Clear examples of this can be found among six bridging proteins at ER-plasma membranes

contacts in yeast that have been deleted to reduce cortical ER by 90% [70]. Only the yeast VAP homologs Scs2/22p are clearly responsible for tethering, but there is no obvious effect on the extent of cortical ER with single deletions of the other bridging proteins: tricalbins and Ist2p (Table 1). These proteins contribute significantly to ER-plasma membrane tethering only when VAP/Scs2p is missing, suggesting that they may not be primarily tethers. Our null hypothesis is that their easily identifiable other domains mediating lipid transfer (tricalbins) [18,19], or ion flux (Ist2p) [71] dominate their function. These proteins may adopt a tethering role only when other bridges are deleted. Such redundancy is found at other contacts [72], so the effect of protein loss on contact formation cannot be the sole determinant of whether a protein is a tether. Making things harder still, not all proteins that are required for contact act by tethering. Instead their specific functions may alter organelle properties (*e.g.* lipid composition) to affect contact site formation indirectly. An example is Ice2p in yeast, which affects ER-plasma membrane contact sites [73] and has pleiotropic interactions in the ER [74]. Although Ice2p may be able to bridge from the ER to other organelles, rather than functioning as a tether, Ice2p appears to have a primary function in channelling lipids [75], which is supported by its remote homology to Serinc proteins (TL, unpublished observation) which alter membrane properties widely [76]. Therefore, the term "tether" should be reserved for cases where it clearly warranted, with care to avoid over-simplifications.

Linkers constrain contact site proteins

Many bridging proteins including lipid transfer proteins and enzymes that work *in trans* are embedded on one side of the contact site by transmembrane helices. They share a common form with their active domain separated from the transmembrane helix by a **linker** predicted to be unstructured (Figure 4A). If the active domains functions on the far side of the contact site, the linker must be able to stretch across the gap. How big are the gaps? And are the linker regions adapted for working *in trans*?

To answer the first question, structural studies of contact sites are scarce. ER-PM contacts are among the best visualized by electron microscopy of well preserved cells; their median gap is 29 nm in yeast (range 16-45 nm) [77], and 23-25 nm in mammalian cells (neurons and COS7) [78]. The yeast nucleus vacuole junction is narrower at 18 nm [79]. Some contacts contain electron dense material corresponding to bridging proteins [9,53], and some bridging proteins produce characteristic appearances [78].

Next, looking at linkers of contact site components, we identify two different categories:

Long linkers

Our definition of contact site merely stipulates the presence of molecular bridges, with gaps at most contact sites so far studied in the range 15-30 nm [77-79], although there are gaps that are much wider [27]. To span an inter-organellar gap ≥15 nm, based on the Cα-Cα distance in unstructured polypeptide loops ≤0.38 nm [80], a linker must have at least 40 amino acids. As an example, the phosphatase domain of PTP1B has a predicted unstructured linker of 126 residues. With a maximum reach of 48 nm it clearly can dephosphorylate EGFR, its substrate on endosomes (Figure 4A) [53]. Other contact site components with long unstructured linkers include STIM1 and Ist2p. Both are integral ER proteins with polybasic regions that bind anionic lipids in the plasma membrane [81]. The unstructured linker in STIM1 can stretch up to 80 nm (Figure 4A). This long reach may enhance the ability of STIM1 to attach ER tubules to the plasma membrane when it is activated by emptying ER Ca^{2+} stores [82].

Short linkers

Several contact site components have linkers that appear only just long enough for the protein to reach across the gap. Extended-synaptotagmin-2 (E-syt2) has 44 residues, Ysp2p has 59 residues, with maximum reach 17 and 23 nm respectively (Figure 4B) [16,47]. For Ysp2p, we showed that its linker needed at least 40 residues for activity, which was maximal with ≥70 residues [47]. Ysp2p is one of those ER embedded proteins where the linker contains a polybasic region [83]. At contacts that are narrow enough, these regions may bind anionic lipids of the plasma membrane inner leaflet. This leaves fewer residues to form the unstructured linker: for Ysp2p the linker would be only 41 residues (≤ 16 nm, Figure 4B). One possibility that might still allow Ysp2p to function is if single contact site components are focally enriched to create a local region where the gap matches its linker. This is supported by experiments where over-expression of individual components alters the contact site width: narrowing has been seen with E-Syt1 using an extra C2 domain in the presence of Ca^{2+} [78]; widening has been seen by adding rigid helices to Sec22b in a trans-SNARE complex with syntaxin-1 [84]. The existence of contacts with different gaps is supported by experiments on the long linker of Ist2p, which can extend up to 130 nm (Figure 4A). Shortening from 340 to 58 residues (≤22 nm) redistributes Ist2p from extended ER-plasma membrane contacts to punctate contacts [71], suggesting that contact site components match the length of their linkers to specific zones of contact.

Can linkers be too short to reach across a contact site?

It possible that a linker could be too short for the active domain to act *in trans* across a contact site, especially if that domain does not have a high affinity interaction with the opposing membrane that might trap a transient motion into close proximity. This question is most controversial for the PI4P-phospatase Sac1, which is embedded in the ER and is sometimes modelled as reaching out from there and hydrolyze PI4P in the plasma membrane [85] and NVJ [86]. Our own analysis of this region, based on two crystal structures [87,88] and sequence conservation with other 4-phosphatases, predicts that Sac1 has a linker up to 7 nm long, and so cannot work across ER-plasma membrane contacts or NVJ *in vivo* (Figure 5). Unless Sac1 creates narrower contacts than have yet been reported, this analysis strongly supports models where PI4P traffics to the ER to drive other lipids in the opposite direction (Figure 3C, reviewed in [6]).

Concluding Remarks

In the last decade we have moved from asking which organelles can form contact sites, to looking for explanations for the lack of contact formation by a few unusual organelles, including ER-Golgi intermediate compartment (ERGIC), *cis* and medial Golgi cisternae. An underlying reason for the large variety of contacts may be to create unique spaces for operating and regulating pathways. Having such a wide range of intracellular routes leads to circularity, which may explain how cells survive when one individual route is lost [10,11,13]. This circularity also means that intracellular traffic is not genetically straightforward [89]. In the next decade, after defining contact site components, we can move to describing their regulation.

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Glossary

Anchor: proteins on the outer face of membrane-bound organelles can be integral to the membrane, *i.e.* with one or more transmembrane helix which is an irreversible anchor. The alternative is peripheral attachment by binding a protein or lipid.

Bridge: a protein that crosses from one organelle to another. The simplest bridge has one integral membrane protein that binds a membrane lipid in the other organelle. More complicated bridges could include additional protein(s) and lipid(s).

Compartment: this term is exchangeable with organelle (see below).

Contact site: any place where a physical bridge links two organelles with functional consequences. Strict definitions beyond this have not been formalised, so both contacts that lead to fusion in the exo-/endo-cytic pathways and homotypic contacts might be included in this category by other authors. We have excluded these categories and focussed on heterotypic, non-fusogenic contact sites. We have also included contacts with inclusion bodies, which are not membrane-bound, so we have avoided the term “membrane contact site”. Gaps between organelles at these contact sites are mostly in the range 10-30 nm, but this is not part of a definition. One feature that we suggest should be included in a definition is that the linkage across a contact site creates a biochemically unique zone, typically by excluding other organelles and even excluding large protein complexes such as ribosomes. In addition to such exclusions, contact sites often specifically include components involved in communication of material or signals between the two organelles.

Diffusion: time t for diffusion in 3 dimensions across a distance x from a point source is described by the equation: $t=x^2/6D$. For a small protein in cytoplasm the diffusion constant (D)≈10 μm^2/sec, so diffusion across 1 µm of cytoplasm takes 16 msec (not 160 msec as we recently published in error [20,49]); by comparison diffusion across a contact site (≤30 nm) takes ≤0.02 msec. Such rapidity is important for Ca^{2+} signalling, which is affected by the precise contact site gap [55]**.** However, for lipids the data is not yet available to say how frequently they go through a lipid transfer cycle *in vivo*, or the extent to which this rate is limited by diffusion versus dwell time at donor/acceptor membranes.

Homotypic contacts: contacts formed by two membranes of the same organelle, found for ER, mitochondria, Golgi apparatus, lipid droplets and peroxisomes. These mediate fusion, as well

regulating other aspects of organellar structure and biochemistry, and presumably affect heterogeneity. They have not been considered here.

Linker: the portion of a protein that links its active domain to its membrane anchor. While the linker may fold back on itself, *i.e.* zero extension, its maximal extension is 0.38 nm per residue [80]. This sets the range within which the domain can access binding partners.

Organelle: a region of a cell boundaried in three dimensions within which multiple reactions co-occur in loose but highly predictable ways. This includes nucleoli and other liquid drops that are phase separated but not membrane-bounded. Here we use interchangeably with compartment (above, but see [90]). We also apply the term to inclusion bodies

Tether: not all bridges are tethers. We use the term for bridges that create a significant proportion of the structure of a contact site under physiological conditions. *A priori* we consider that proteins containing a domain that functions at contact sites, *e.g.* lipid transfer domains, are unlikely to be tethers. Instead, we assume they have a primary function linked to that domain until proven otherwise.

Legends

Figure 1, Key Figure. The patchwork of contact sites

Diagram of 19 different contact sites involving the plasma membrane and nine other organelles in yeast or mammalian cells (see key). Contact sites formed are ER (x8 = number of contact sites), mitochondria (x7), lipid droplets (x5), peroxisomes (x5), endosomes (x4), lysosome/vacuoles (x3), plasma membrane (x2), autophagosomes (x2), late Golgi (x1) and inclusion bodies (x1). The presence of contact sites is indicated by ring bindings. More than half of the pairwise combinations formed by these organelles have so far been demonstrated. We have excluded intra-Golgi contacts as being homotypic. Also not shown are chloroplasts, which may form many additional contact sites in plant cells; and contact sites between the ER and both secretory granules and phagosomes, which extend the STIM-Orai1 domain. This diagram resembles budding yeast, where contact sites are best known, although other cell types have provided major insights, and a similar large range of contacts is found in all eukaryotic cell types, even if the specific amounts vary between cells. In yeast the nuclear envelope is a specialised zone of ER that forms unique contacts with the degradative vacuole and lipid droplets.

Figure 2. Illustrative examples of physical contact site functions

(A) Lateral organisation of contact site allows long distance communication from the contact site to adjacent parts of each organelle, here showing two contact sites in yeast. **TOP:** Mitochondrial-ER contact sites enriched with sterol (yellow dots) are spanned by multiple complexes including ERMES and EMC-TOM, and Lam6p-TOM. MICOS makes multiple interactions across the intermembrane space (black arrows). mtDNA (dark red circle) is anchored by an unknown protein (orange) in a sterol-rich domain. There appears to be long-range communication of sterol enrichment (dotted arrow). **BOTTOM:** Pyruvate dehydrogenase complex accumulates in the mitochondrial matrix near to mitochondrial-peroxisomal contact sites, possibly allowing communication of Ac-CoA status between the two organelles (dotted arrow). (B) Endosomes act as carriers for peroxisomes and lipid droplets for movement along microtubules in hyphae of fungal cells of *Ustilago maydis*, with no significant contribution by the ER (see key to Figure 1). (C) Model of how eukaryotic evolution has led to the ER marking fission sites. Ancestral pre-eukaryotic cells are proposed to have had clefts lined by plasma membrane (an outgrowth of the nuclear envelope) and prototypic mitochondria. Plasma membrane would be expected to mark sites of fission of internal tubular organelles, including mitochondria. Functions that include

marking sites of fission were inherited by the ER of the last eukaryotic common ancestor (LECA).

Figure 3. Vertical organisation of contact sites leads to metabolic channelling

Examples of non-vesicular traffic both at contact sites (A-C), and not at contact sites (D). (A) T-tubule DHPR channels (light blue) bind to Ca^{2+}-responsive RyR channels (red) in the sarcoplasmic reticulum, an expansion of the ER (red). Ca^{2+} entry (arrow "1") is sensed rapidly, and amplified by secondary Ca^{2+} release (arrow "2"). This arrangement creates excitation-contraction coupling in skeletal myocytes, where pan-cytoplasmic Ca^{2+} rises within 10-50 milliseconds of depolarization. (B) Lipid transfer proteins such as ceramide transferase (CERT) can bind both sides of a contact site simultaneously, forming bridges. Note that CERT might feasibly also use diacylglycerol as a second (counter-current) ligand. Such lipid transfer proteins bind VAP (red) in the ER (pink) and a lipid (blue) in a membrane of the late secretory pathway (light blue). Both VAP and the linkers for attached lipid transfer domains can span ≥20 nm. (C and D) Counter-current exchange of sterol and PI4P by OSBP related proteins (ORPs): ORP5 at ER-plasma membrane contact sites (C) or Osh4p at post-Golgi secretory vesicles (D). The lipid transfer domain binds one lipid at a time, either sterol, PI4P (blue) or others (not shown). PI4P synthesised in the Golgi is dephosphorylated by Sac1 in the ER (scissors). The PI4P gradient (blue–white arrows) can drive sterol up a gradient (light-dark yellow arrows). Sec14 homologues can exchange PI, PC and in some cases sterol (not shown). At contact sites (A, B and C) components of different organelles directly impinge on each other, and they can be regulated collectively. However, lipid transfer proteins that do not obviously target contact sites, such as yeast Osh4p and Sec14p (D) can carry out similar counter-current transfer. The absence of a contact site prevents *en bloc* regulation of the pathway, and imposes additional diffusion steps that are likely to reduce efficiency. Possibly related to this, both Osh4p and Sec14p are present in much higher copy number (> 10x) than anchored transfer proteins such as Osh1-3p and Lam1-6p.

Figure 4. Linkers across contact sites

(A) contact site components with linkers easily long enough to cross contact sites. The topology of some of these (for example Ist2p and STIM1) is similar with long, mainly unstructured linkers and a terminal polybasic region (PBR, with net charge shown). (B) contact site components with short linkers. All are embedded in the ER, except StARD3 (late endosomes); except VAP and PTP1B, all are lipid transfer proteins: Ysp2p, Lam1p and GramD1b are in the LAM family, ORP8 (or its homologue ORP5) is an OSBP homologue, Mmm1p (in ERMES) and E-Syt2 are TULIPs. Details of the linker regions (right hand side) show polybasic regions as in A, and the maximum distance

spanned by the remaining unstructured linker. Note that although over-expression of E-Syt1 alters the recruitment and function of other contact site proteins, this effect might result from Ca^{2+}-dependent lipid traffic, not from narrowing of the contact site.

Figure 5. Does Sac1 function across contact sites?

(A) Domain map of Sac1 (yeast), with detail of C-terminus. Residues 1-456 produced diffraction data in two crystal structures, with the C-terminus forming a phosphatase domain, and the N-terminus forming a uniquely folded accessory domain. This suggested that the linker is ~70 residues long. However, residues 457-502 are not only highly conserved in all PI4Pases, including those without transmembrane helices, but this region is required for catalysis. Also, these residues are predicted to form a sheet (arrow) and two helices (according to PSI-PRED) following helix-9 in the solved structure. The failure of residues 457-502 to diffract may be explained by them adopting multiple conformations, but forming an unstructured loop appears unlikely. The detail shows that after the catalytic domain ends at residue 502 the remaining C-terminus is predicted as: a linker of 19 residues (503-521, maximum reach 7.2 nm), two transmembrane helices (residues 522-544 and 556-573), and a cytoplasmic extreme C-terminal domain that is typically short (*e.g.* 19 residues in humans) but uniquely extended in yeast (31 extra residues, grey), containing 11 conserved residues (grey lines), and with multiple predicted β-sheets (not shown).

(B) Scale diagram of Sac1 at a ER-plasma membrane contact site. Given the lack of evidence that residues 457-502 can unfold completely, we have assumed that they are positioned close to the catalytic site. Thus, the active site (orange circles) can only reach out slightly over 7 nm. This implies that transcatalysis cannot occur at ER-plasma membrane contact sites (gap ≥16 nm) or NVJ gap (18 nm).

Table 1: Proteins suggested as tethers at contact sites

A. Strong validity as tether

contact site protein	contact site studied (organism)	(i) Effect of loss on contact site (and over-expression‡)	(ii) Interactions at contact site (binding domains). Other domains.	Ref
Junctophilin (JP)-1 to 4	triad & dyad junctions in myocytes (vertebrates)	Loss strongly affects extensive myocyte ER-plasma membrane (PM) contact site. Some overlap (e.g. JP3/JP4). Also in non-excitable cells.	Integral to ER envelope, binds PM lipids esp. PI4,5P_2 (multiple MORN motifs). No other domains.	[91] [92] [93]
Num1p	mito-PM ± ER → MECA (yeast)	Deletion of Num1p regions involved in bridging reduces cortical tethering of mitochondria in yeast mother cells.	Binds: PIP2 on PM (PH domain); Scs2p on ER (possible FFAT); Mdm36p and cardiolipin on mitochondria (N-terminal helical region N.B. not a BAR domain¶*)	[26] [94] [95] *[96]
Nvj1p	NVJ (yeast)	Deletion reduces NVJ almost to nothing.	Integral to nuclear envelope, binds Vac8p on vacuole. No other domains	[97] [98]
Perilipin-5	Lipid-droplet-mitochondria (vertebrates)	Loss of identified mitochondrial interaction domain reduces lipid droplet recruitment.	Peripheral on lipid droplets (amphipathic helices) and binds mitochondria (hydrophilic motif). No other domains.	[30]
PTPIP51 (also called RMD3)	ER-mitochondria (vertebrates)	Loss reduces contacts by 50%.	Integral to outer mitochondrial membrane, binds VAP on ER (FFAT motif). No other domains	[99] [100]
Synapto-tagmin-7	Lysosome-peroxisome (vertebrates)	Over-expression of a dominant negative construct reduces contact site formation in vitro and in vivo.	Integral to lysosome; binds PIP2 on peroxisome (C2 domains). No other domains.	[45]
VAP (Scs2p)	ER + 7 other organelles (widely conserved)	Deletion of Scs2p (yeast) reduces cortical ER by 50%; loss of VAP-B (human) reduces ER-mito contact site by 30%.	Integral to ER + binds ≥100 partners, 50% of which have FFAT motifs (MSP domain). Can extend ≤27 nm (Figure 4B). No other domains.	[99] [100] [101] [102]

B. Equivocal validity as tether

E-Syt1–3	ER-PM (mammals, but see Tcb1–3)	Loss of all three E-Syts in humans reduces contact site by >50%. This differs from observed effect of deleting homologs in yeast.	Integral to ER, bind anionic PM lipids (C2 domains). Contain TULIP lipid transfer domains (Fig. 4B)§.	[103]
EMC (≤9 proteins)	ER-mitochondria (yeast)	Deletions of five components reduces extent of contacts.	Many subunits are integral to ER, several bind TOM. EMC1 has one or two predicted 6-bladed β-propellers related to PQQ quinoproteins (Pfam 13360)¶.	[104]
ERMES (5 proteins)		Single deletions reduce extent of contacts.	Mmm1p and Mdm10p integral to ER and mitochondria respectively; other subunits bridge. Mmm1p, Mdm12p and Mdm34p contain TULIP domains§.	[10] [14]
Ist2p	ER-PM (yeast)	Deletion reduces closely adherent cortical ER (gap ≤30 nm) by 80%, but total cortical ER (gap ≤200 nm) either by 30% or 0% (depending on study). ‡	Integral to ER, binds anionic PM lipids (polybasic region on extended linker, Figure 4A). Channel in TMEM16 family.	[70] [105]
MICOS	Intra-mito (widely conserved)	Deletions do not affect relationship between inner and outer membranes.	Deletions affect crista morphology and mitochondrial function. Largest subunit, Mic60/mitofilin, has a C-terminal domain unrelated to any lipid transfer protein ¶.	[35]
Mitofusin-2	ER-mitochondria (vertebrates)	Unclear; loss reduces or increases close contact (with differential effects on distant contacts) depending on study. Same variation in effect on Ca^{2+} traffic.	Peripheral mitochondrial protein, possibly a small proportion on ER, forms homodimers. Contains dynamin-like GTPase.	[106] [107] [108]
Pex11p	mito-peroxisome (yeast)	Deletion reduces contact by 50%.	Peripheral on peroxisome, partner unknown; comes close to Mdm34p (but not to other ERMES components). No other domains.	[23] [109]
SNX2	ER-endosome (metazoa)	Loss appears to prevent local PI4P traffic to ER, indicating possible loss of contact site, but structural effects not reported.	Binds: PI3P on endosomes (BAR-PX); VAPA/B (FFAT-like motifs x2). No other domains.	[52]
Vps39	mitochondria-vacuole (yeast)	Not known if deletion reduces contacts. ‡	Peripheral vacuolar protein via binding Rab7, mitochondrial partner not known. No other domains.	[10] [11]

C. Weak validity as tether

Lam6p	ER-mito and ER-vacuole (yeast)	Deletion does not reduce either contact. ‡	Integral to ER; binds Tom70/71p and Vac8p (PH domain). Contains StARkin lipid transfer domain.	[20] [110] [111]
Mdm1p	NVJ (yeast)	Deletion has no effect on NVJ. ‡	Integral to ER; binds PI3P (PX domain). 2 other domains of unknown function.	[86]
OSBP and other ORPs	ER-PM (ER-TGN, endosome, NVJ, *etc.*)	Deletion has no effect on contacts (*e.g.* ΔΔΔosh123 in yeast. Partial constructs with mutated or missing OSBP-related lipid transfer domain expand contact sites, especially with co-overexpression of VAP.	Mostly peripheral to ER (FFAT motif, except ORP5/8 integral); PI4P and ARF GTPase on other membranes (PH domain). Contain OSBP-related lipid transfer domains, which transfer PI4P away.	[112]
Tcb1–3p	ER-PM (mammals, but see E-Syt1–3)	No effect of deleting Tcb1–3p unless Scs2p absent. This differs from observed effect of deleting human homologs.	Integral to ER, bind anionic PM lipids (C2 domains). Contain TULIP lipid transfer domains§.	[70]

20 bridging proteins/complexes proposed to have tether properties, categorised by extent to which they meet two overall criteria: (i) their effect on contact structure, especially whether loss of protein diminishes contact; effect of overexpression is given where known‡, but this may simply indicate which proteins are contact site components; (ii) analysis of their domains, especially means of membrane attachment, and lack of a domain that suggests a primary function in traffic or signalling. (A) 7 bridges meet all criteria. Where reductions from deletion are partial (*e.g.* VAP) it is probable that other complexes bridge the same contact sites, possibly taking on the role of tether under experimental conditions. (B) 9 bridges meet some criteria and crucially do not fail through presence of a trafficking/signaling domain. (C) 4 bridges do not meet criteria, though there may be contact site expansion on over-expression. All contain additional relevant domains, typically capable of lipid transfer. Remote homologies for domains of unknown function were predicted using HHpred. They have either been verified§. or are our unpublished observations¶. For the proposed BAR domain at the N-terminus of Num1*, we supplemented HHpred with I-TASSER, which predicted helices, bundled possibly two or three together, but with no sequence or structural homology to BAR domains.

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Figure 1

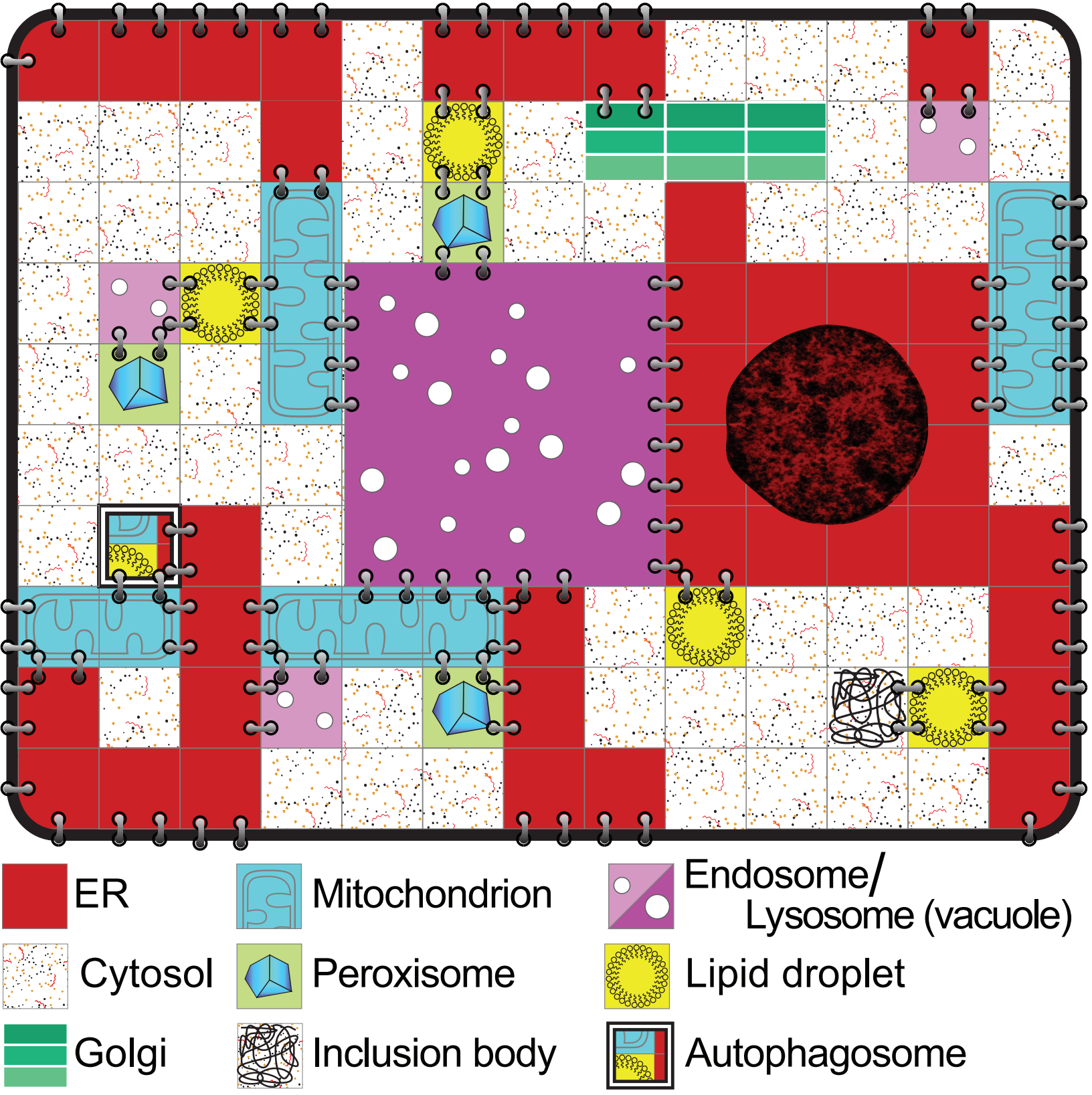

ER
Mitochondrion
Endosome/
Lysosome (vacuole)
Cytosol
Peroxisome
Lipid droplet
Golgi
Inclusion body
Autophagosome

Figure 2

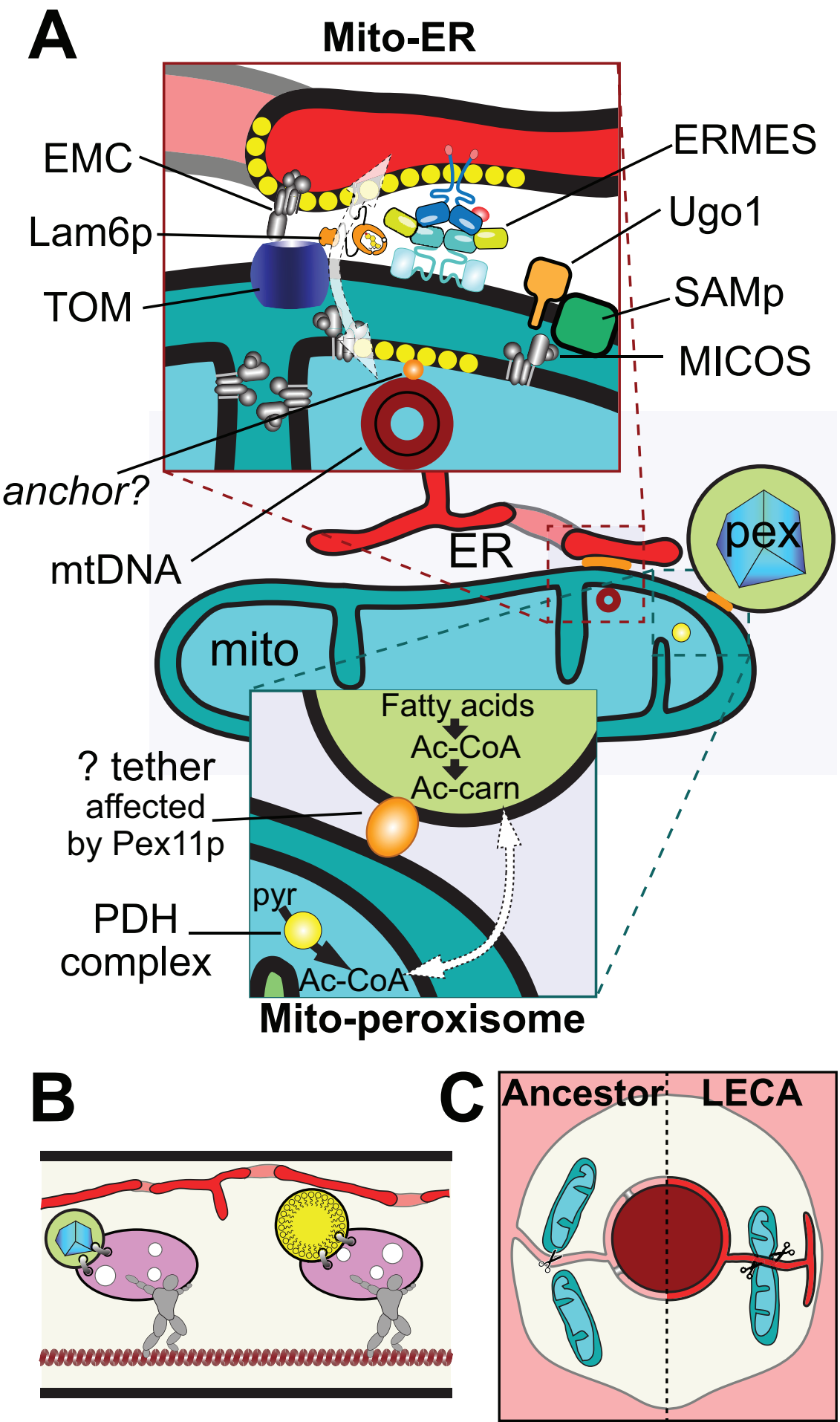

A
Mito-ER
EMC
Lam6p
TOM
ERMES
Ugo1
SAMp
MICOS
anchor?
mtDNA
ER
pex
mito
Fatty acids
Ac-CoA
Ac-carn
? tether
affected
by Pex11p
pyr
PDH
complex
Ac-CoA
Mito-peroxisome
B
C
Ancestor
LECA

Figure 3

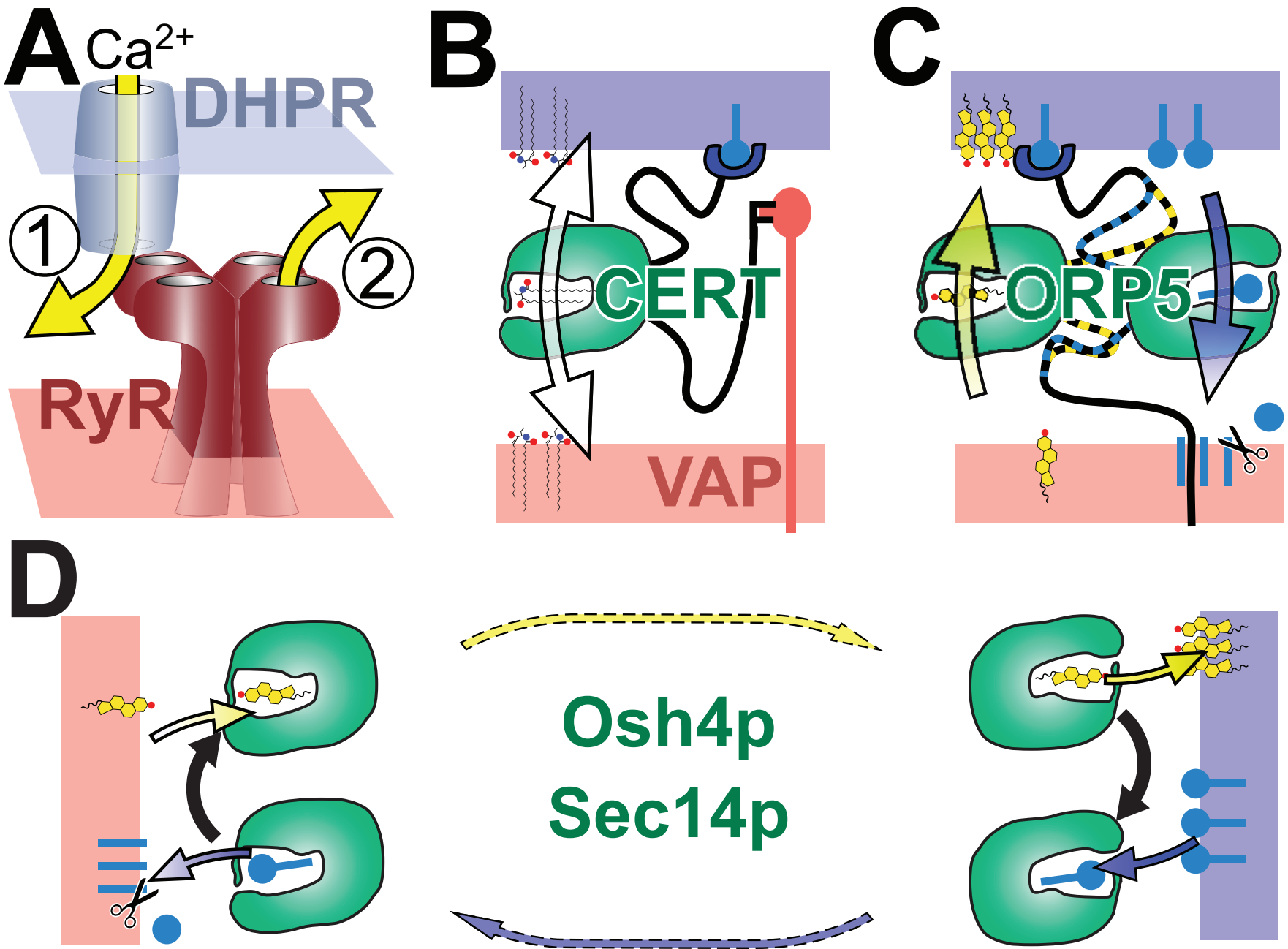


Figure 4

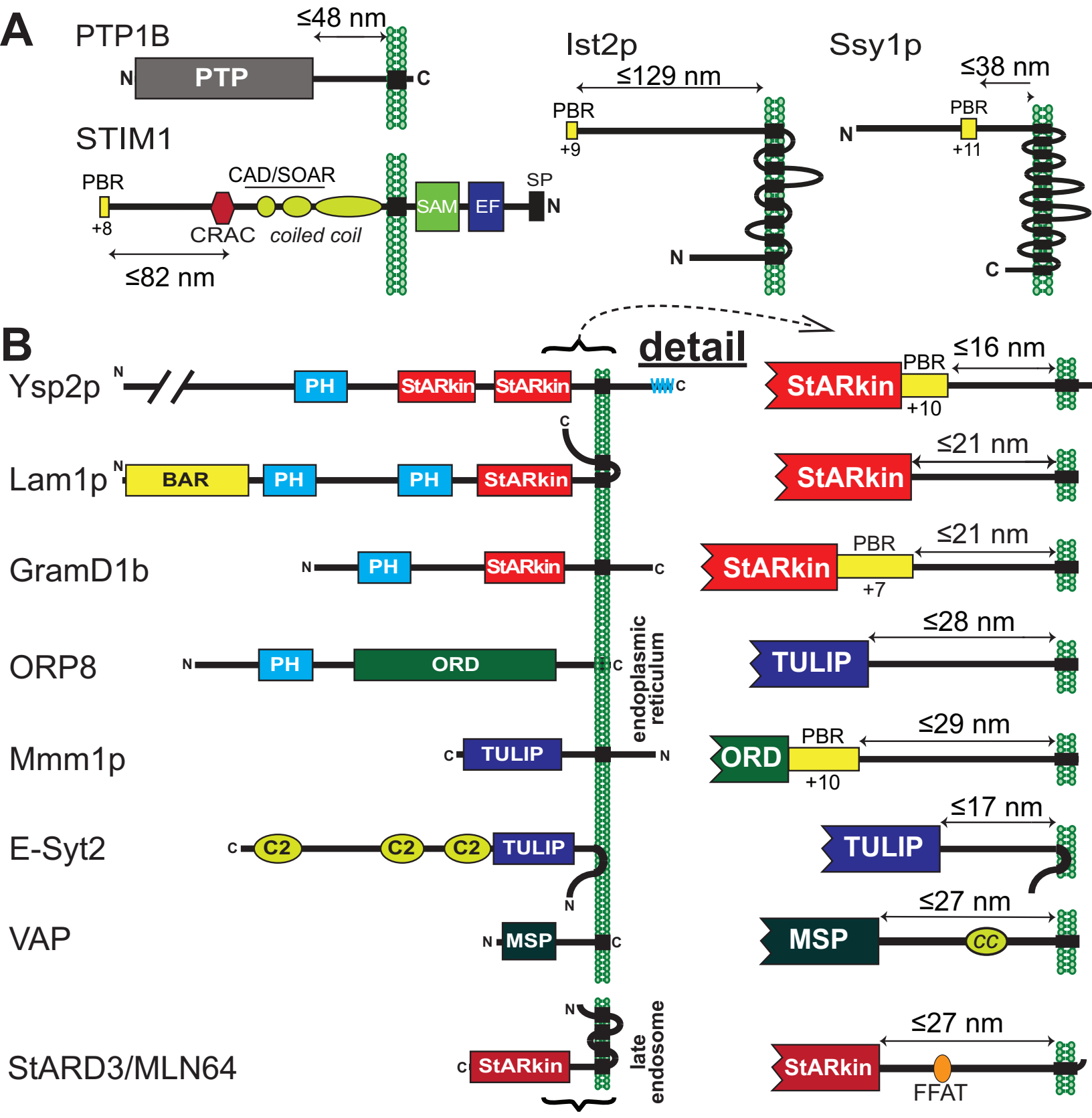
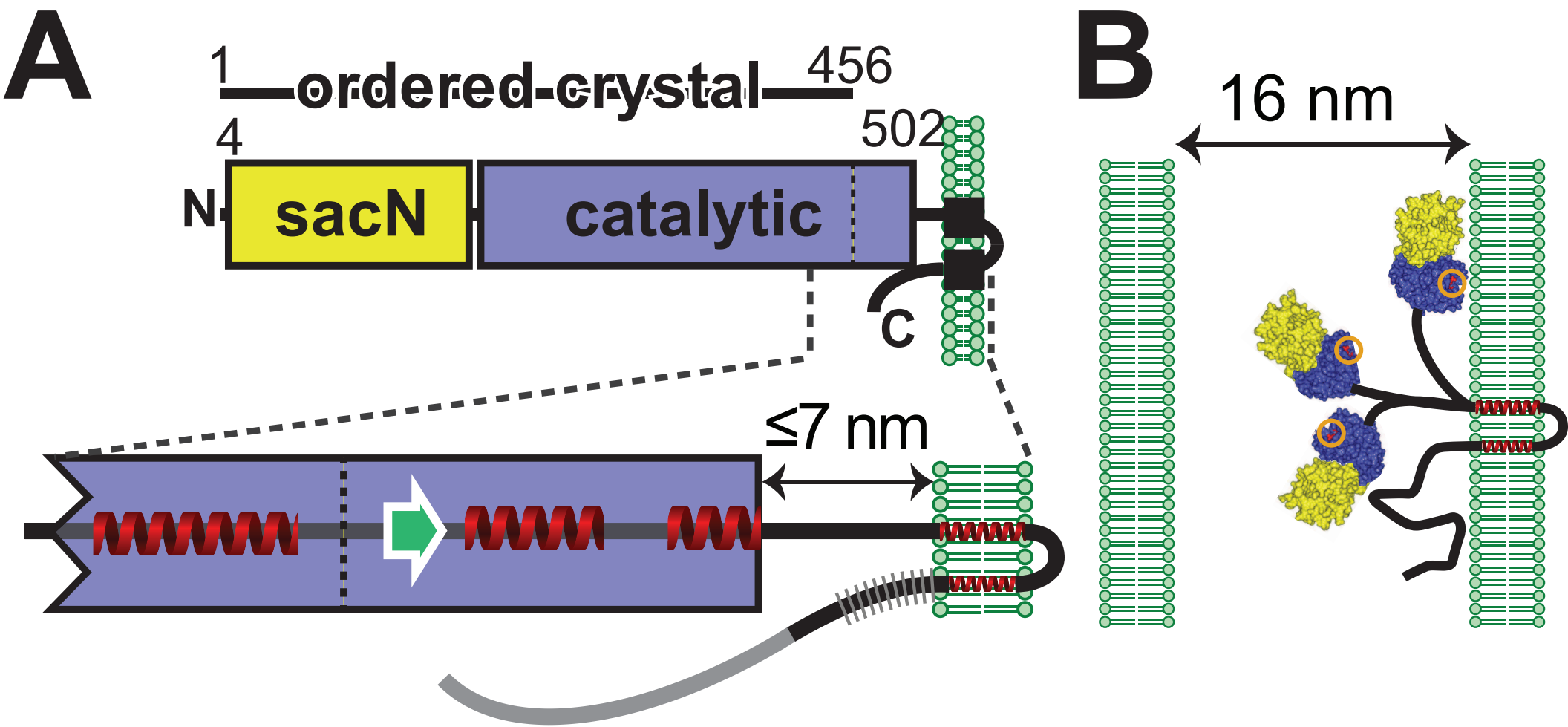

A
PTP1B
≤48 nm
N
PTP
C
STIM1
PBR
+8
CAD/SOAR
CRAC
coiled coil
SAM
EF
SP
N
≤82 nm
Ist2p
≤129 nm
PBR
+9
N
Ssy1p
≤38 nm
PBR
+11
N
C
B
detail
Ysp2p
N
PH
StARkin
StARkin
C
StARkin
PBR
+10
≤16 nm
Lam1p
N
BAR
PH
PH
StARkin
C
StARkin
≤21 nm
GramD1b
N
PH
StARkin
C
StARkin
PBR
+7
≤21 nm
ORP8
N
PH
ORD
C
endoplasmic reticulum
TULIP
≤28 nm
Mmm1p
C
TULIP
N
ORD
PBR
+10
≤29 nm
E-Syt2
C
C2
C2
C2
TULIP
N
TULIP
≤17 nm
VAP
N
MSP
C
MSP
CC
≤27 nm
StARD3/MLN64
N
C
StARkin
late endosome
StARkin
FFAT
≤27 nm

Figure 5



TRENDS BOX

• Many membrane contact sites have been discovered in the past decade, particularly those not involving the ER.

• Diffusion across an MCS occurs in the order of microseconds, and this is an important aspect for the flow of ions such as Ca^{2+}.

• The single most important general function common to all MCSs is metabolic channelling, enabling material and signals to be focussed directly from one organelle (or compartment) to another with nothing else getting in the way.

• MCS components with short linkers may be sorted to, or even create, zones within an MCS that match the distance they can reach out.

OUTSTANDING QUESTIONS BOX

• Which proteins function *in trans* across MCSs? It is particularly important to establish this for Sac1 in relation to OSBP and its homologues which can transfer PI4P to the ER.

• How do linkers work at MCSs? For example, can a protein with a short linker like Sac1 create an MCS small enough to work *in trans*?

• Lipid transfer proteins might be true to their in vitro-derived name, picking up and dropping off the many thousands of lipid molecules each second. Alternately, they may act only as sensors. Can new technologies be developed that show the rate at which lipids are transferred by lipid transfer proteins *in situ*?

• Constitutive MCS that form between larger organelles such as the ER, mitochondria and plasma membrane tend to have multiple bridging complexes. Do MCSs between multi-copy spheroidal organelles (for example peroxisomes, lipid droplets, autophagosomes) have more simple structures (*i.e.* single protein bridges)? This would allow such contacts to respond more flexibly to specific metabolic states, a flexibility that could be determined by studies of the regulation and dynamics of MCSs.

• How do cells handle the heterogeneity within organelles imposed by MCSs? This is particularly obvious for MCSs between multi-copy organelles. Are some organelles biochemically distinct before they make contacts (*i.e.* contact formation is intrinsic)?

• When three (or more) organelles contribute to a single pathway (*e.g.* mitochondria, peroxisomes, lipid droplets and ER in fatty acid metabolism) are there specific mechanisms to bring multiple topologically different MCSs together to create three-way contacts?

Figure360 - mp4 format (from iMovie)

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Figure360 - m4v format (from iMovie)

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