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ER-mitochondrial contact-ology: structure and signaling functions

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Summary

Interorganellar contacts are increasingly recognized as central to the control of cellular behavior. These contacts, which typically involve a small fraction of the endomembrane surface, are local communication hubs that resemble synapses. We propose the term ‘contactology’ to denote the analysis of interorganellar contacts. Endoplasmic reticulum (ER) contacts with mitochondria were recognized decades ago; major roles in ion and lipid transfer, signaling and membrane dynamics have been established, while others continue to emerge. The functional diversity of ER-mitochondrial contacts is mirrored in their structural heterogeneity, with sub-specialization likely supported by multiple, different linker-forming protein structures. The nanoscale size of the contacts has made studying their structure, function and dynamics difficult. This review focuses on the structure of the ER-mitochondrial contacts, methods for studying them, and the contacts’ roles in Ca^{2+} and reactive oxygen species signaling.

Keywords

MAM; linkers; sarcoplasmic reticulum; Ca^{2+}; IP3 receptor; Ryanodine receptor

Introduction

Several disciplines have provided clues to the existence of interorganellar contacts, including biochemistry, which showed the co-purification of membranes of different organelles, biophysics and cell biology that provided visualization of close associations and interorganellar linkers, and cell physiology that described functions requiring local interactions of adjacent organelles. These clues came together to reveal a diversity of nanometer scale structures with distinctive functions but also indicate features shared among contacts. Building on these observations and employing specialized methods that are powerful in the study of the various contacts, a new field of study is emerging that we refer to as contactology.

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Here we focus on the contacts of the ER and sarcoplasmic reticulum (SR) with mitochondria, which were among the first to be noticed and which have generated progressively growing interest in the past several decades. Fundamental functions of ER-mitochondrial contacts (henceforth, ER-mito contacts) have been established in biosynthetic processes, cell signaling, cells' execution, and mitochondrial dynamics. We summarize here, the methods that are available to study the nanometer scale ER-mito contacts, their structure and the role of these contacts in Ca^{2+} and ROS signaling. While we try to comprehensively describe these matters in Metazoa, in terms of other aspects of the ER-mito contacts, including their role in lipid transfer, membrane dynamics, cell survival and disease we refer readers to recent reviews [1–6].

Methods for detecting ER-mito contacts and identifying relevant proteins

Many approaches to elucidate the structure of ER-mito contacts can be found in the literature. For direct visualization, there is no substitute for electron microscopy (EM), because its resolution matches the size of the nm scale contacts. As far back as 1958, EM was used to visualize co-sedimentation of ER and mitochondria in cell homogenates [7]. For the most complete and detailed view of ER-mito contacts, three-dimensional (3-D) reconstructions may be generated by electron tomography [8–12]. Several groups have produced whole-cell scale, 3-D images of ER-mito contacts using serial, tilt-angle tomography in yeast cells [11], focused ion beam scanning EM in neurons at 4 nm resolution [13] and, recently, soft x-ray tomography with 50 nm resolution in lymphoblastoid cells [14]. The acquisition and reconstruction processes for these 3-D approaches remain quite laborious and therefore not yet widely applicable as an approach generating sufficient data for statistical comparisons of organelle geometry.

Using transmission EM several groups have measured parameters of ER-mito contacts to generate statistical comparisons, though the specific measurements have varied, making cross-study comparisons difficult. The fundamental parameters are the gap width between OMM and ER membrane and the length of the interface. Some have counted the number or frequency of ER-mito contacts [15, 16], usually normalized to the number of mitochondria, while others have measured the lengths of cross-sections normalized to the mitochondrial perimeters [17–20]. In either case, a decision must be made as to a threshold distance that constitutes an interaction, typically between 20 and 100 nm. In order to determine the presence of particular proteins at the ER-mito contacts, immuno-gold labeling in EM has also been used [21–23]. A major limitation of the above approaches is that they cannot be used in living samples.

Many investigators have used fluorescence microscopy to assess ER-mito contacts in live and fixed samples. Assays based on colocalization of ER and mitochondrial markers in confocal micrographs [10, 15] and with structured illumination microscopy (SIM) [24, 25] have been used, though it is doubtful whether these approaches provide sufficient resolution to detect the physiologically relevant interfaces, which are believed to have gaps of less than 100 nm. Particularly the axial (z) resolution limit of confocal microscopy is at least 700 nm, which SIM and other new techniques such as Zeiss' Airyscan can improve to ~300–400 nm [26]. Thus, a decrease in colocalization might be used as a marker for less contacts but

'colocalized' objects, as visualized by these techniques, may in fact be separated by hundreds of nanometers, leading to much false-positive detection.

Techniques of optical microscopy with higher resolution are also now available. ER-mito contacts at 100 nm resolution in all dimensions have been visualized by combining total internal reflectance (TIRF) with SIM [27]. Super-resolution imaging by stimulated emission depletion (STED) with <50 nm radial resolution has been demonstrated [28], and technology now exists to obtain similar resolution in the axial dimension [29]. Stochastic optical reconstruction microscopy (STORM) with a theoretical resolution of ~20 nm has also been used to visualize ER-mito contacts [30]. The combination of super-resolution techniques and confocal microscopy was employed to describe the clustering of vMIA, the viral mitochondria-localized inhibitor of apoptosis, at ER-mito contacts [31], but none of these approaches has yet been applied to quantify the overall extent or geometry of the interfaces.

There are also several approaches that detect points of contact without trying to describe their architecture. First is the system of rapamycin-inducible linkers, tagged with a pair of fluorophores capable of generating Förster resonance energy transfer (FRET) [17]. One half of the linker is targeted to the outer mitochondrial membrane (OMM) and is tagged with, e.g., cyan fluorescent protein (CFP), while the other half is targeted to the ER surface and tagged with yellow fluorescent protein (YFP). A short treatment with rapamycin or its analogues causes linkage of the two halves where they are in sufficiently close proximity. The linkage can be visualized as an increase in FRET signal. Similarly, in fixed cells, the proximity ligation assay, which relies on amplification of DNA oligonucleotides in places where two target proteins are in sufficient proximity, may be used [32, 33].

Finally, there are approaches to quantify the extent of ER-mito contacts without direct visualization. One simple method is immunochemical detection of the amount of ER present in a crude mitochondria preparation from fractionated cells, either by western blot or imaging [9]. A splitluciferase assay has also been used [34, 35]. Functional assays have been used as indirect measures of contacts. The earliest reported function of mitochondrial associated membrane (MAM) was in the trafficking and synthesis of phospholipids that depend on both mitochondria- and ER-resident enzymes, as detected by radio-labeled lipids [36]. Measurements of the local transfer of Ca^{2+} from ER to mitochondria have also been employed. These can be done by single-cell imaging using mitochondria-targeted fluorescent or luminescent Ca^{2+} sensors [37, 38] or in permeabilized cell suspensions where sources of Ca^{2+} capture other than mitochondria are eliminated [38]. A difficulty with each of these techniques is controlling for the ER Ca^{2+} content and the kinetics of its release, as well as the mitochondrial membrane potential and Ca^{2+} uptake machinery.

To identify novel proteins resident at ER-mito contacts or relevant to their formation and function, several approaches have been employed. In yeast, the ER-mitochondrial encounter structure (ERMES) was discovered in a screen for mutants that could be complemented by expression of a synthetic linker [39], while a role for the ER membrane protein complex (EMC) was uncovered by a screen based on the role of ER-mito contacts in lipid transfer [40]. Yeast two-hybrid screening of suspected linker components was used to find both the VDAC1-GRP75-IP3 receptor (IP3R) [41] and VAPB-PTPIP51 [32] interactions. Several

studies on mass spectrometry (MS) evaluation of ER-mito contacts have been published based on MAM fractions purified by gradient centrifugation from mouse brain and liver [42] and in virus-infected cells [43, 44]. Recently, two groups employed proximity biotinylation to identify proteins present at ER-mito contacts by MS. In one case, the ER (microsomal) fraction was purified after biotinylation from cells expressing engineered ascorbate peroxidase (APEX) localized in the OMM [35]. In the other, APEX2 labeling was used to generate separate, putative proteomes of the ER and OMM surfaces, the intersection of which were considered ER-mito contact-localized [45]. Discrepancies between these two studies show that caution is warranted in interpreting them. Only two proteins were found in common (TMX1 and CNX) and, while one study is limited to the ER fraction, the other seems biased toward OMM proteins with 48 of 69 found in the MitoCarta2 mitochondrial proteome [46]. Indeed, the ER component of the novel complex described in that paper, RRBP1, was not identified by their screening approach [45].

Structural organization of ER-mito contacts

Local signaling and molecular traffic between two dynamic organelle networks is most effective when supported by physical coupling mechanisms. Physical links between ER and mitochondria were first proposed over 40 years ago, based on transmission EM of liver mitochondria [47, 48]. In 2006, an electron tomography study revealed variously shaped and sized ‘tethers’ connecting the mitochondrial surface (OMM) with subdomains of smooth and rough ER (sER and rER) [9]. Here, we refer to connecting proteins as linkers, to indicate their flexibility and probable mutability. The necessity of protein linkage for communication at ER-mito contacts has been demonstrated by disruption of Ca^{2+} transfer by limited proteolytic treatment of permeabilized cells [9] and by rescuing genetically disrupted communications by synthetic linkers [39, 49].

Linkage mechanics

Morphologically, ER-mito contacts can be characterized by their lateral extent and gap distance, both of which are largely determined by the local selection of membrane proteins. Regarding gap distance, membrane proteins can limit the maximum distance (flexible linkers) or minimum distance (spacers, represented by bulky, rigid structures) or both (linker-spacers, e.g. rigid or composite linkers). Fig1 shows a 3D reconstruction of the interface between a rER sheet and OMM (from [9]). Some of the ER-OMM linkers incorporate ribosomes while others attach to ribosome-free membrane. Given their large size, ribosomes act as spacers, limiting the minimum distance between rER and OMM to ~20 nm. The OMM-sER gap distance on the other hand can be <10 nm [9]. However, the massive cytoplasmic domains of the ER/SR Ca^{2+} release channels IP3Rs, Ryanodine receptors (RyRs), which bulge out from the sER membrane surface by ~10–12 nm [50–52], also limit the minimum gap distance. The confinement of Ca^{2+} channels to wider contacts explains why Ca^{2+} sensors targeted to tight ER-mito contacts via engineered short (~5 nm) linkers detected smaller local [Ca^{2+}] rise upon IP3R activation than those targeted via long (~15 nm) linkers [17].

The schematics in Fig 1 illustrate how physical properties of the linkers could limit the ER-mito contacts. In this simplified model, linkers can be flexible, thus limiting how far apart, but not how close, the connected membranes can go (Fig1E i) or how rigid they may become, thus fixing the distance between the interconnected membrane points (Fig1E ii). Fully rigid linkers are likely rare and most linkers incorporate both flexible and rigid (spacer) components at varying proportions. Recently, FATE1, an OMM/MAM protein resident in the testis and up-regulated in certain cancers, has been proposed to function as a fully or mostly rigid spacer-linker that interferes with ER-mito contact formation, local Ca^{2+} communication and related apoptotic signaling [53]. Besides the linkers' intrinsic properties, their membrane attachment is also likely to limit ER-mito contacts (Fig1E iii). Thus, removal or addition of one particular linker species may have varying impact on the measures of ER-mito contacts, depending on the variety and distribution of co-existing linkers (Fig1E). This exposes an inherent difficulty in the research efforts to reveal the molecular identity of native, bona fide ER/SR-OMM linkers. Most of these efforts utilized genetic ablation or overexpression of candidate protein(s) followed by microscopic evaluation of the changes in the ER-mito contacts. However, interpretation of these approaches is error-prone. For example, removal of a long flexible linker from an ER-mito contact, which also hosts shorter linkers, may not affect the metrics of the interface (Fig.1Ei, 'Subtraction' lower). On the other hand, removing a long rigid linker (spacer-linker) may give way for shorter linkers to tighten the gap (Fig.1E ii, 'Subtraction' lower). Overexpression of a linker may or may not expand the area of the ER-mito contacts depending whether or not the linker's membrane anchorage is limited to a particular membrane microdomain (e.g. lipid rafts) (Fig1E i-ii vs. iii, 'Addition'). Thus, the 3D (or 4D, spatio-temporal) extent of ER-mito contacts is likely an integrated function of an array of different linker (flexible or rigid or both) and non-linker spacer protein elements as well as their lateral mobility limitations in their host membrane.

Synthetic linkers

Our group constructed artificial monomeric linkers by extending a fluorescent protein with OMM and ER/SR membrane anchor domains [9]. When overexpressed, these linkers effectively expanded the area of contact and tightened the ER-mito contacts [9]. These linkers also complemented a lethal phenotype in yeast lacking different components of an endogenous linker complex (ERMES) [39]. Engineered bipartite linkers have also been developed using rapamycin-inducible heterodimerizing tags FKBP12 and FRB, each targeted to either the OMM or the ER/SR membrane and also tagged with a fluorescent protein suitable to monitor the dimerization (e.g. via FRET) or the local [Ca^{2+}], ROS, pH etc.[17, 54]. These inducible linkers first, accumulate at and selectively label the preexisting contacts (in 3–10min) and subsequently, start to expand the contact area to enwrap mitochondria by ER [17].

Linker forming proteins

In this review, we focus on proteins as linkers and regulators of linkage, though phospholipid bilayer 'stalks' have also been proposed as ER-mitochondrial connectors and means of inter-organellar lipid exchange (reviewed in [55]). An inter-membrane linker can be a single protein with two membrane-interacting domains, like some plasma membrane (PM)-docking

proteins (e.g. junctophilins, STIM1, extended synaptotagmins). Currently, ATAD3 is the only native, single-protein linker candidate for ER-mito contacts [56]. Alternatively, a linker can be an oligomeric complex, many of which have been reported at ER-mito contacts. The number of proteins put forward as contributors to membrane docking at ER-mito contacts has been continuously growing (Table 1). The majority of these proteins have been described as **linker** components (see column entitled “ER-mito contact role”). Many of these proteins tighten the ER-mito contacts, while others force a relatively wide gap between the ER and OMM (IP3R, RyR, FATE1/EMD). FATE1/EMD was also shown to inhibit contact functions that require tight associations (**linker/spacer**). Another group of these proteins is referred to as **promoter** or **disruptor**, which increase or decrease ER-mito contacts acting either locally at the organellar interface or at a distance (e.g. Fus1 acts via GSK3β that phosphorylates the PTPIP51/VAPB linker). Lastly, Table 1 describes a group of **resident** proteins, which are localized at contacts and affect some contact functions without known impact on the contact structure (e.g. DGAT2 that facilitates lipid transfer). Table 1 also lists the relevant interactions and functions, organellar localization, presence of the mitochondrial proteins in Mitocarta2 (MC2), the number of transmembrane domains (TM), relevant interactions and functions for each.

In the engagement of bi/multipartite linkers, promiscuity and/or competition may occur. PTPIP51 (OMM) and VAPB (ER), when overexpressed in a motor-neuron-like neuroblastoma cell line, strongly expanded the ER-mito contacts [19]. The oxysterol binding protein ORP5, another tail-anchored PTPIP51 binding partner in the ER has been suggested as a ‘conditional’ ER-mito contact linker that would associate with the VAPB-PTPIP51 complex and mediate ER-OMM lipid transfer. However, ORP5 also connects ER to PM via its pleckstrin homology domains to mediate PI4P/PS counter-transport [57]. Co-overexpression of ORP5 with PTPIP51 expanded the ER-mito contacts to similar extent as VAPB with PTPIP51 [23]. However, up or down-regulation of ORP5 alone did not alter the extent of ER-mito contacts, but did affect ER-PM contacts, suggesting that the native VAPB-PTPIP51 interaction is dominant over ORP5-PTPIP51 at the ER-mito contacts. To further complicate matters, PTPIP51 is named for its interaction with protein tyrosine phosphatase 1B (PTP1B), a tail-anchored protein present in both ER and mitochondria [58, 59], though this interaction has not yet been investigated as an ER-mitochondrial linker, per se. Thus, with multi-partite linkers there may be situations when independent components in one membrane have the same linkage partner in the interfacing membrane, and in such situation one component might mask the other’s role as linker in genetic up/down-regulation assays.

Competition may occur at either side of the membrane interface between different multi-partite linker partners. VAPB seemed to out-compete ORP5 as an ER linkage partner of PTPIP51 and so VAPB besides being a linker component can also be considered as a suppressor of the ORP5-PTPIP51 linker assembly. A bipartite ER-mito contact linker, mitofusin 2 (MFN2) plays a central role in OMM fusion, including the inter-mitochondrial linkage step (via *trans* homo-dimerization or hetero-dimerization with MFN1) [60, 61], but also localizes to the ER and thus can link the ER with the OMM [10]. However, lately it has been under intense debate whether MFN2 promotes or hinders ER-mito contact formation (pro: [10, 62–64], contra: [15, 18, 65, 66]) owing to seemingly conflicting outcomes of over-

expression and silencing experiments. The anti-ER-mito contact role of MFN2 has been attributed to lateral interference with other bi/multipartite linker components in the same membrane by OMM- and/or ER-localized MFN2. This interference might be suspended by sequestration of 'free' MFN2 mediated by ER-localized presenilin 2 (PS2) [65], but opposite interplay between these proteins (PS2 interfering with MFN2 as a linker) has also been proposed [67]. MFN2's function in membrane shaping and fusion is a confounding factor in understanding its role in ER-mito contacts, as discussed in 2.4. Future works will need to clarify the 'hierarchy' amongst different ER-OMM linkers in the establishment of various ER-mito contacts. The currently available information on the contribution of mitochondrial, ER and cytosolic proteins to ER-mitochondrial linker complexes is illustrated by the Venn diagram in Fig 2.

Lipid membranes and lipid biosynthesis

Organelle interfaces are formed by bringing two fluid and dynamic lipid bilayers into close proximity. Also, several lipid metabolic pathways involve lipid transport from the ER membrane to the OMM and IMM and back, including the synthesis of phosphatidylethanolamine (PE) from phosphatidylserine (PS) and steroid production (reviewed in [68]). In yeast, the ERMES complex is believed to act as a lipid transferase by lipid binding to the SMP domains of Mmm1, Mdm12 and Mdm34 [69]. Many SMP-domain containing proteins are found in the mammalian genome [70]— e.g. the extended synaptotagmins that have been shown to link ER to the PM [71]—but only very recently was one, PDZD8, identified at ER-mito contacts [72] and its ability to transport lipids has not been shown. The synthesis of PE in the mitochondria was exploited in a yeast genetic screen to uncover a role for the EMC in lipid transfer [40]. In mammals, SLC25A46 was recently described as an OMM protein that interacts with EMC components and with architectural components of the IMM (e.g. the mitochondrial contact site and cristae organizing system, MICOS), and functions in inter-organelle phospholipid transfer ([73] and Fig. 3). Similarly, ATAD3A—an IMM AAA+ ATPase, whose C-terminal, ATP binding domain resides in the matrix—seems to coordinate the interactions among the IMM, OMM and ER membrane [74] and has been shown to affect cholesterol delivery to the mitochondria [75]. The MAM fraction is enriched in cholesterol and ceramides [67, 76] dependent on the presence of caveolin 1, at least in hepatocytes [77]. These domains have features reminiscent of lipid rafts—relatively less fluid membrane microdomains [78]. Indeed multiple MAM proteins have been reported to locate to 'internal lipid rafts' (detergent resistant membranes) including TMX1 [21], Sigma1 receptor [79], presenilin 2 [67] and the cytomegalovirus anti-apoptotic protein vMIA [80].

Organelle shaping and dynamics

ATAD3A and SLC25A46, besides their roles in lipid transport are among the many ER-mito contact factors that influence the morphology of the organelles. To date, little is known about the reciprocal relationships between ER-mito contact formation, lipid homeostasis and organelle morphology. ATAD3A regulates mitochondrial morphology via interaction of its N-terminal domain with the OMM [74]. SLC25A46 is reported to affect the morphology of both the ER and mitochondria via interactions with cristae organizing structures of the IMM and the OMM fusion effectors, MFN1/2 [73, 81]. MFN2 has also been found to be involved

in the removal of defective/stressed mitochondria by mitophagy [82], a process intimately connected with morphology regulation [83]. Additionally, ER-resident MFN2 regulates the ER network morphology [10]. Genetic removal of MFN2, thus, may affect ER-mito contacts based on its linker function or by changes in ER and mitochondrial shape or impairments in mitophagy. The initiation of mitochondrial fission occurs at sites where ER tubules constrict the mitochondria [12]. Actin appears to be a key mediator in these processes wherein ER-bound INF2 and OMM-bound Spire1c cooperate to polymerize actin to promote constriction [84, 85]. Dynamic cycling of actin onto the OMM in concert with morphological changes has been observed, but the structural implications of this with regard to ER-mito contacts are unknown [86, 87]. Among ER-shaping proteins implicated in ER-mito contact formation, in addition to MFN2, are REEP1 [88], which has been proposed to form ER-mitochondrial linkers via *trans* homodimerization [34], and RTN1A, which was identified as an ER-mito contact promoter in a proteomic screen [35].

Besides shape and proximity, organelle motility may also be a factor in ER-mito docking; moving the partner organelles close to each other and stopping them in the vicinity of each other should favor docking. Both ER and mitochondria utilize microtubules (MTs) as major transport tracks in mammalian cells and have been shown to preferentially localize along MTs with acetylated tubulin, which may serve to establish the proximity necessary to form ER-mito contacts [89]. ER stress was demonstrated to drive an MT-dependent relocation of ER and mitochondria to the perinuclear area, concomitant with an increase in ER-mito contacts [90]. Furthermore, $[Ca^{2+}]_c$ increases have been shown to slow/stop mitochondrial motility via Ca^{2+}-sensitive uncoupling of the connection with the MTs, in which the small GTPase MIRO1 plays central role [91–94]. This Ca^{2+}-sensitive stoppage of mitochondrial movement might in turn promote the engagement of bipartite linkers between ER Ca^{2+} release nanodomains and mitochondria. Colocalization of MIRO1 with ER-mito contacts has been reported in mammalian cells, and the yeast homologue of MIRO1, Gem1 has been shown to be a regulatory component of the ERMES complex, supporting a role for MIRO/Gem1 in the ER-mito contact formation [95].

Contacts with the ribosome-bound ER

Factors such as cell type, growth/cell cycle stage and differentiation status are also known to affect ER-mito contacts. In some cases, this might be mediated by differences in the ER type. Mitotic cells and steroid hormone producing endocrine cells have mostly reticulated ER tubules while cells with intense protein synthesis like exocrine pancreatic cells are loaded with rER [96]. While sER tubules form the ER-mito contacts where local phospholipid exchange takes place and those at the mitochondrial fission sites [97], the roles and dynamics of rER-mito contacts is less defined to date. Some sER-mito contact linkers are too short to operate at rER-mito contacts as they would not reach across the gap space occupied by the ribosomes; however, patches of sER-mito contact occur commonly in the plane of rER sheets ([98] and our unpublished observations). A bipartite OMM-rER linker complex formed by synaptojanin-2 binding protein (SYNJ2BP/OMP25) and the ribosome binding protein RRBP1, linked via respective PDZ/PDZ-binding domains, has been recently proposed [45], though its function remains unknown. It was also recently proposed that ubiquitination by the autocrine motility factor receptor AMFR/GP78 and ER-associated

degradation of MFN2 would promote rER-mito contacts but not sER-mito contacts [66]. This would put MFN2 into the “anti-linker” role and it is tempting to speculate that it might interfere with the SYNJ2BP-RRBP1 linker complex.

The mammalian target of rapamycin complex mTORC2, a master-regulator of growth and nutrient signaling, has been shown to bind ribosomes, likely via one of its constituents, Rictor [99], and also to functionally depend on ribosome binding [100]. mTORC2 has also been proposed to promote MAM formation and the assembly of the IP3R/GRP75/VDAC1 linker complex via direct interactions, and it was suggested that this involved the active mTORC2, thus also depended on ribosome binding [22]. However, the latter suggestion was based on the observation that in crude mitochondrial fraction of cell homogenate removal of ribosomes also caused loss of mTORC2. Considering size constraints, it does not seem likely that the IP3R/GRP75/VDAC1 linker would form right where a ribosome (big spacer) is also present. Thus, it is yet to be determined if mTORC2 contributes to a rER-mitochondrial linker or rather mTORC2 activated in a ribosome-dependent manner could shuttle between adjacent rER and sER domains. Calnexin (CNX), an ER membrane protein that locates to MAM in an S-palmitoylation-dependent manner [21], is also a key component of the ribosome-translocon complex, again S-palmitoylation-dependently [101]. This might reflect a role for CNX in rER-mito contacts (or rough-ER-containing MAM); however, S-palmitoylation is a more generic molecular tool that serve to increase (for CNX) protein life time and stabilize membrane location. The palmitoyl transferase (ZDHHC6) that palmitoylates CNX has been shown to widely distribute over the ER [102]. Hence, it is yet to be clarified if CNX in the rER locates to or promotes rER-mito contacts.

Signaling at ER-mito contacts: Ca^{2+} and ROS

Functional roles of ER-mito contacts were first uncovered in phospholipid biosynthesis and transport (reviewed in [103, 104]). Subsequently, ER-mito contacts were implicated in cell signaling (Ca^{2+}, ROS, and phosphoregulation) [105, 106], organelle shaping (mitochondrial fission and autophagy) [3] and inheritance [107, 108] as well as innate antiviral immunity [44, 109] (Fig 3). We have touched on the function of ER-mito contact proteins in organelle dynamics and lipid biosynthesis in the context of structural organization (2.3–5.) and here, we focus on recent progress in Ca^{2+} and ROS signaling at ER-mito contacts.

Ca^{2+} signaling

The ER/SR serves as the major intracellular Ca^{2+} store that, upon exposure of the cells to various stimuli, releases Ca^{2+} via the IP3Rs or RyRs to create a cytoplasmic [Ca^{2+}] signal and, in turn, to control almost every aspect of cell function. Local ER-mitochondrial Ca^{2+} transfer was demonstrated in the ‘90s [37, 38, 110] but how this communication is affected by ER-mito contact dynamics is only beginning to be unraveled. Mitochondria do not, as a rule, store Ca^{2+} under physiological conditions but have a tremendous driving force for electrogenic cation entry (~ −180 mV IMM potential) and contain several physiologically and also pathophysiologically relevant proteins that can be controlled by an increase in matrix [Ca^{2+}]. The Ca^{2+}-gated Ca^{2+} uniporter (mtCU) of the IMM is maintained closed at resting cytoplasmic [Ca^{2+}]. To relay rapid cytoplasmic [Ca^{2+}] signals to the matrix,

activation of the mtCU in most cell types requires [Ca^{2+}] above the levels achieved by the cytoplasmic peaks. This can be attained at ER/SR-mito contacts, where IP3R/RyR-derived high [Ca^{2+}] nanodomains can locally expose the mtCU (reviewed in [111]).

The extensive ER/SR membrane network and the mitochondrial membranes, particularly the greatly folded IMM, represent large surfaces which are close to each other only in small areas. For effective local communication, the ER/SR Ca^{2+} release channels, the mtCU and the porins (VDACs) that provide Ca^{2+} permeation through the OMM all need to be at <100 nm proximity [112] and so these components have to be concentrated at the ER/SR-mito contacts. Recruitment of proteins to ER-mito contacts can happen via linking to the opposing membrane or via stabilization in linked membrane subdomains. The IP3R has been described to link with the OMM via VDAC1 and the chaperone GRP75 [41, 113] as well as with PM lipid rafts via ankyrin B and PM Ca^{2+} channels via homer [114]. Whether the IP3R/GRP75/VDAC1 complex operates as a stable inter-organelle linker or just as a channel alignment tool is yet to be clarified. The latter hypothesis seems to be supported by recent data showing a dependence of IP3R–VDAC1 interactions on the PTPIP51-VAPB linkage [49]. Besides the Grp75/VDAC1 complex, IRBIT (AHCYL1), an IP3Rs binding protein released with IP3 has also been shown to promote ER-to-mitochondrial Ca^{2+} signal propagation and ER-mito contact formation dependent on its phosphorylation [115]. Very recently, FUNDC1, a small integral OMM protein earlier known as a MAM-associated mitophagy receptor (reviewed in [116]) has been put forward as a binding partner with IP3R (IP3R2), MAM promoter and IP3R–to-mitochondria Ca^{2+} signaling promoter in cardiac muscle [117]. However, the significance of this potential interaction in local Ca^{2+} communication will need further elucidation since FUNDC1 deletion seemed to be associated with diminished levels of IP3R2 and the MAM regulator PACS2, and caused diminished IP3R-mediated cytosolic [Ca^{2+}] signals [117]. Of the three IP3R isoforms, IP3R3 has been put forward most often as ER-mito contact-associated [113, 118, 119], either via the linkage to VDAC or via retention by Sigma1-receptor-mediated local protection from proteasomal degradation [118].

RyRs of the striated muscle are clustered to specialized SR sub-regions (terminal cisternae) that frequently form SR-mito contacts, to which MFN2 [62, 63] and the PTPIP51/VAPB [120] linkers have been reported to contribute. However, RyRs face the PM (T-tubule), where their activators (L-type Ca^{2+} channels) locate. Since SR terminal cisternae are ‘inflated’ in the skeletal muscle, RyR1 are >100 nm from the OMM [121]. In cardiac muscle, the cisternae are more ‘flattened’ thus the cardiac RyR2 is close enough (<50 nm nearest OMM distance [122, 123]) to create a high [Ca^{2+}] nanodomain in the SRMCS .

On the mitochondrial side, VDAC1 is the most abundant protein of the OMM; yet its availability may limit local Ca^{2+} transfer from IP3R as overexpression studies [124] and disruption of the IP3R/GRP75/VDAC complex demonstrated. It has been suggested that RyR2-derived Ca^{2+} signals use mainly VDAC2 to cross the OMM in the heart [125–127] and a direct RyR2-VDAC2 interaction has been proposed [127] that is unlikely because RyR2s in the SR terminal cisternae face away from the OMM. As such, this mechanism may be more relevant in atrial cells, in which RyR2 frequently locate to the so-called corbular SR that do not interface the PM [128]. mtCU density is variable amongst mitochondria of

different tissues, and is particularly low in murine heart [129] despite the relatively large organelle size and IMM surface. This setting likely requires strategic recruitment of mtCU to the SR-mito contacts for effective local Ca^{2+} transfer from the dyadic RyR2 [130].

Local ER/SR-mito Ca^{2+} transfer also depends on ER/SR Ca^{2+} loading mediated by SERCA pumps. Local SERCA activity at ER-mito contacts may control the background [Ca^{2+}] and filter out slow Ca^{2+} release signals [131]. Notably, there are multiple MAM-bound mechanisms that regulate SERCA activity. The transmembrane chaperone calnexin (CNX) has been proposed to inhibit SERCA activity in a phosphorylation-dependent manner, and IP3R-mediated Ca2+ release activity to lead to dephosphorylation of CNX, promoting Ca^{2+} re-accumulation [132], although later CNX was also suggested to be a positive SERCA2b regulator [133]. In conjunction with CNX, thioredoxin-related TMX1 has also been proposed to interact with and inhibit SERCA2b activity [20]. TMX1 also seemed to promote ER-mito contact formation, thus inhibiting SERCA2b might be a compensatory measure against mitochondrial Ca^{2+} overload as discussed in [134]. Very recently another ER membrane and MAM resident redox regulator, glutathione peroxidase 8 (GPX8) has also been shown decrease SERCA2b activity [135]. Notably, deletion of MFN2, an ER-mitochondrial linker was also associated with increased ER Ca^{2+} loading [10]; yet it is for future studies to establish if this is a direct regulatory effect on SERCA or on other factors of ER Ca^{2+} storage.

ROS signaling

Sources and targets of ROS, which include several interconvertible molecules with often different effects, are abundant both in the ER and mitochondria [105]. Furthermore, spatially confined effects of ROS have been reported on both SR and mitochondrial targets [136, 137]. However, it has been difficult to test whether ER-mito contacts display localized ROS signaling. Using synthetic linkers, we enriched a genetically encoded H_2O_2 sensor at the ER-mito contacts to show high H_2O_2 nanodomains that are evoked by Ca^{2+} signal delivery to the mitochondrial matrix [54]. ROS originating from the intermembrane space seems to be delivered through the cristae openings to the area of the ER-mito contacts. A functionally relevant target of the H_2O_2 nanodomain is the IP3R that utilizes ROS to sustain cytoplasmic Ca^{2+} oscillations [54]. Potential sources of ER-mito contact ROS also include ROS producers of the OMM, like MAO-B and ERO1alpha, a key controller of oxidative folding in the ER lumen, which seems to be enriched at ER-mito contacts [138, 139]. However, specific anchoring mechanisms to target ROS generating enzymes to the area of ER-mito contacts remain to be identified. Both local Ca^{2+} and ROS signals at ER-mito contacts may be self-reinforcing by slowing down mitochondrial motility and preserving contact sites [140–142].

ROS nanodomains are competent to affect selectively the ER-mito contact localized subsets of ER and mitochondrial proteins. Local ROS signaling at ER-mito contacts could also be established without ROS nanodomains by concentrating ROS targets at the contact areas. Indeed, many of the proteins that show some localization to ER-mito contacts have redox sensitive thiols. For example the localization of ERO1 alpha to the MAM [139], the channel activity of every mammalian IP3R and RyR isoform [143, 144], and the fusion activity of

MFNs [145] are all controlled by ROS. Studies of cysteine-less mutants of putative targets and identification of oxidized thiols by proteomics upon ER-mito contact-localized ROS exposure will help to identify the ER-mito contact proteins which have physiologically relevant local control by ROS.

Concluding Remarks

In recent years, concentration of research efforts on interorganellar contact sites employing powerful new technologies has resulted in some mechanistic insights to the structure and function of ER-mito contacts. At the same time, the current limitations of contactology have also been better defined (see ‘Outstanding Questions’). Answering these questions will help to establish that many aspects of cell function, including intracellular transport, signaling and dynamics are largely mediated or organized by structurally and functionally diverse interorganellar contacts. This information will facilitate the sorting of many pathologies that currently, are loosely linked to ER-mitochondrial contacts.

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Highlights

- ER-mitochondrial (ER-mito) contacts permit efficient local interorganellar communication without altering the whole cell environment.
- The relevance of ER-mito contacts has been broadly recognized in both physiology and pathophysiology.
- New approaches have recently emerged for the visualization, morphometry as well as for identification of the supporting proteins of the ER-mito contacts.
- Measurements of Ca2+ and reactive oxygen species dynamics at the ER-mito contacts indicates fundamental similarities between interorganellar solute signaling and synaptic transmission.
- The diversity of the reported ER-mitochondrial contact structures, compositions and functions indicate specialization of the contacts at the level of individual mitochondria, cells and tissues.

Outstanding Questions

- Can sub-classes of ER-mito contacts be defined according to their functions, molecular constituents and/or physical characteristics?
- How can methods to quantify the geometry and extent of ER-mito contacts be further improved and standardized? How can advances in high-resolution 3D microscopy be utilized and how can throughput be increased?
- What is the life-cycle of ER-mito contacts? How are they formed, reshaped and removed?
- What linkers and ER-mito contact regulatory proteins remain to be discovered? How can candidate proteins be validated with high confidence given the complexity and variety of participants?
- How does ER-mito contact formation influence lipid homeostasis and vice versa? What are the roles of particular lipid species in contact formation?
- What are the mechanisms of Ca^{2+} and ROS signal integration at ER-mito contacts?
- What is the role of rER-mito contacts in the pathogenesis of diseases, including many neurodegenerative conditions associated with derangements of the ER-mito contacts?

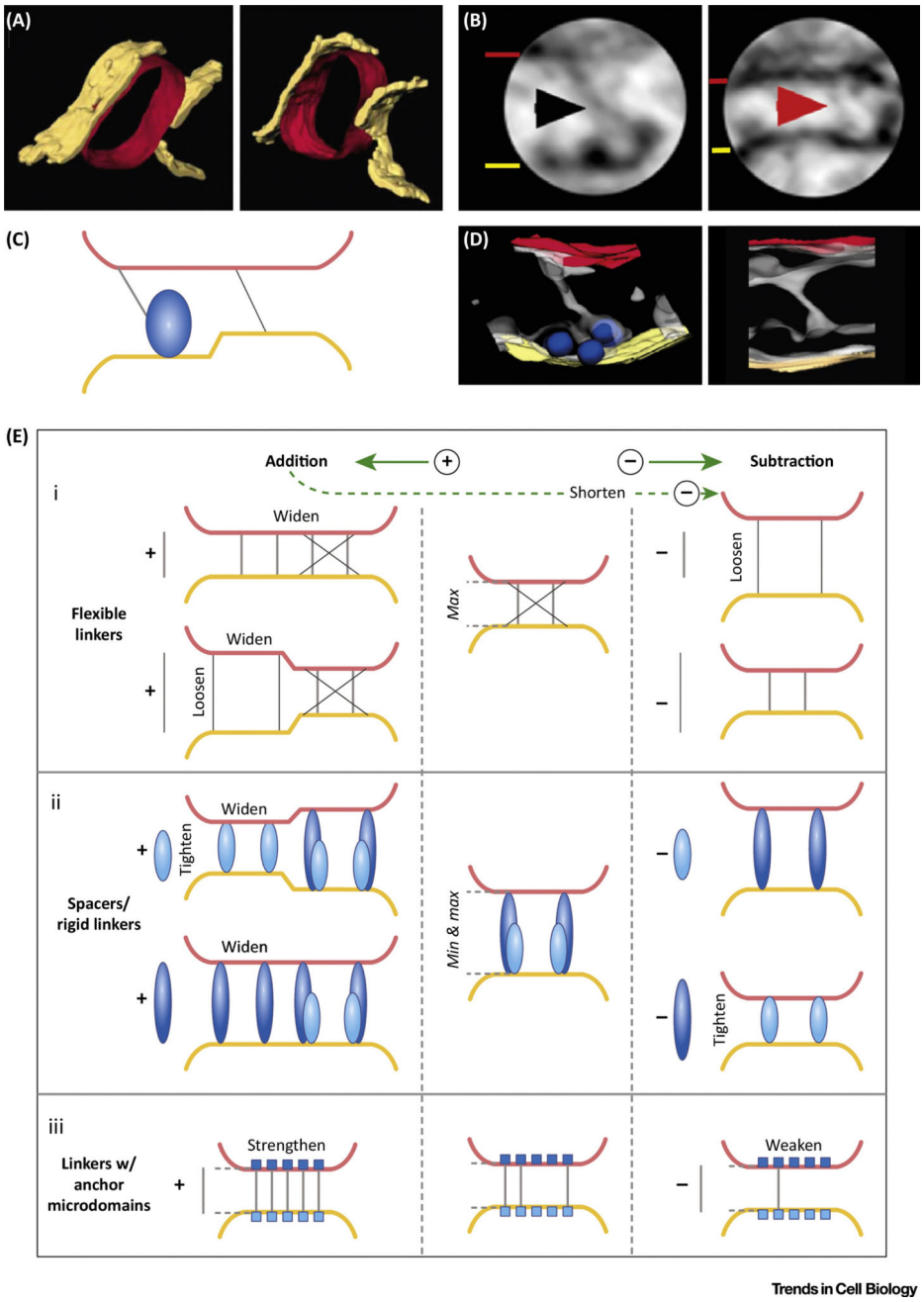

Figure 1. Shaping of the ER-mitochondrial contacts by linkers

A. Pseudo-colored electron-tomographic reconstructions of a 180 nm thick slice of a mitochondrion's outer membrane (OMM, red) that forms contact with two ER sheets (yellow), from two different view angles. **B**. Zoomed-in image segments from the tomography slices used for A, showing electron-dense tethering structures (arrow heads) between the OMM and ER membranes (respectively indicated with the red and yellow lines). **C**. Tomographic reconstruction of the areas in B showing isodensity surfaces corresponding to 'tethers' (gray), OMM (red), ER (yellow) and ribosomes (blue ellipsoids). A–C are excerpts (from [9]) using specimen from DT40 chicken B cells. **D**. Simplified

diagram for the ER-mito contact segments shown in C, with respective numbering. E. Diagrams illustrating how ER-mito contact dynamics is regulated by the membrane connecting elements. See text for details.

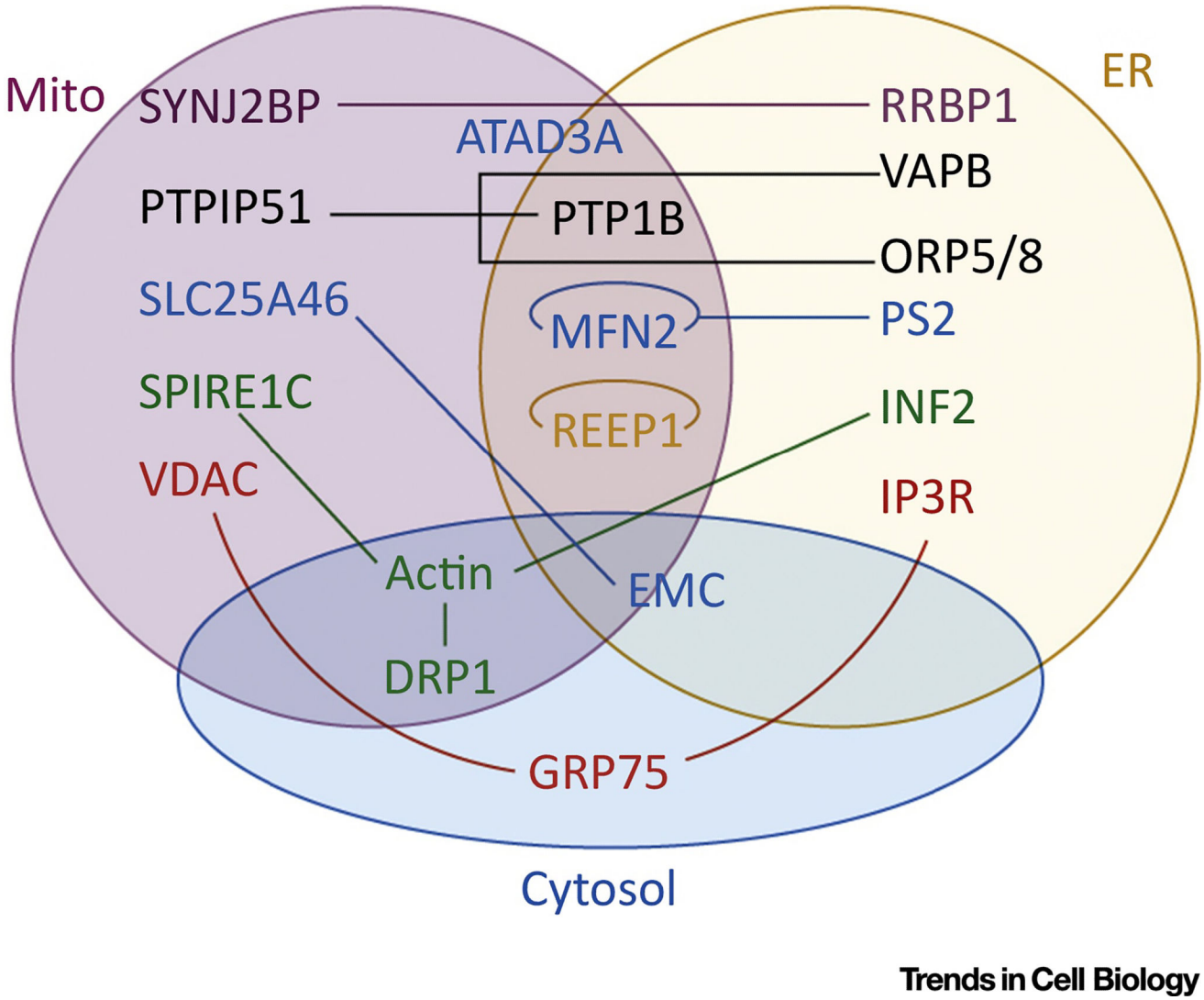

Figure 2. Diversity of distribution and interactions of ER-mito contact linker proteins

Venn diagram showing the subcellular distributions of a variety of ER-mito contact linker protein complexes between mitochondria, ER and cytosol; lines indicate protein-protein interactions. (The multimeric EMC is shown as a single entity for simplicity.)

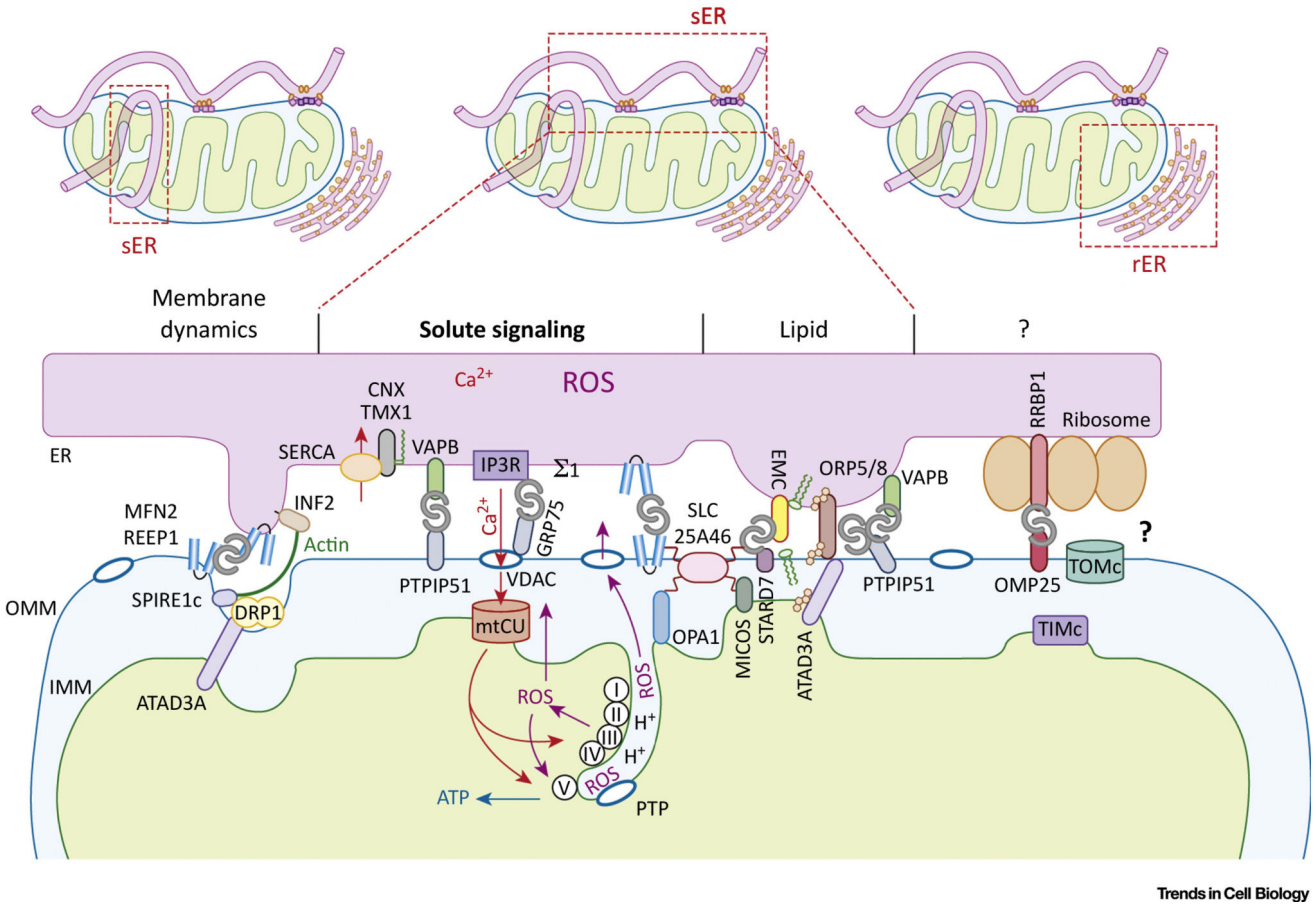

Figure 3. Functions of the ER-mito contacts: membrane dynamics, signaling, lipid transfer

Top, schematized mitochondrion with a fission groove engaged to various contacts with sER tubules and a rER sheet. **Below**, on a schematized mitochondrial and interfacing ER segment, the molecular contributors of the main known functional interactions at ER-mito contacts are summarized. For bi/multipartite linkers the coupling domains are shown as blue clasps. Local Ca^{2+} fluxes are indicated with transparent red arrows while main mitochondria-derived ROS ($\cdot O_2^-$, H_2O_2) fluxes with transparent purple arrows. Abbreviations not yet introduced: PTP, permeability transition pore; Σ1, sigma-1 receptor; Roman I-V, respective respiratory chain complexes; TIMc/TOMc, protein translocase complexes of the IMM/OMM mediating mitochondrial protein import.

Table 1

Membrane proteins reported to directly mediate and/or regulate ER-mito contacts/MAM structure and/or function.

Protein	Location UniProt, Biotinylomes[], Other[]	MC2	TM	ER-mito Contact Role	Relevant Interactions	Relevant Functions	ER-mito Contact Ref.
AMFR	ER, ER[45]	n/a	7	**Promoter**	MFN2 ubiquitination/degradation	E3 ubiquitin ligase, ERAD	[66, 146, 147]
ATAD3A	IMM, ERxOMM[45]	+	1? 2?	**Linker** (IMM-OMM-ER)?	Interacts with DRP1	Sterol transport	[56, 74, 75]
B(C)AP31	ER, ER_{mt}[35], ER[45]	n/a	3	**Linker**	Interacts with FIS1; recruits procaspase 8	Apoptosis	[148]
CAV1	PM, Golgi, ER/MAM [77]	n/a	1 M	**Promoter**		Cholesterol binding and transfer	[77]
DGAT2	ER, ER&OMM [149]	n/a	2	**Resident**		Lipid transfer; LD generation	[149]
DISC1 DISC1Boymaw	Cy, OMM	−	0	**Disruptor** (mutant DISC1-Boymaw)	Interacts w/MIRO, TRAK	Axonal mitochondrion transport	[25]
DRP1	Cy,OMM,prx	+	0	**Promoter** if SUMOylated by MAPL [150]	Interacts with SPIRElc, INF2, ATAD3A	Mitochondrial fission and more	[151]
EMC1	ER	n/a	1 M		EMC1-10: EMC	PL transfer	[152] [40]
EMC2	Mt, ER, Nc, Cy, ER[45]	+	0	**Linker?**	EMC2: Interacts with TOM5, EMC		
EMC3/4	ER	n/a	2		EMC3/4: EMC	ER protein folding Me^{2+} ion transport protein folding, autophagosome	
EMC5	ER, Golgi, Endosome	n/a	2		EMC5: EMC		
EMC6	ER, Autophagosome	n/a	2		EMC6: EMC		
EMC7/10	ER	n/a	1 M		EMC7/10: EMC		
EMC8/9	Cy	n/a	0		EMC8/9: EMC		
EMD/Emerin	Nc, ER[45]	n/a	1C	**Spacer-Linker**	Anchors FATE1	Actin polymerization	[53]
FATE1	OMM/MAM	−	1C	**Spacer-Linker**	Binds EMD	Anti-apoptotic	[53]
FIS1	OMM, Prxsm, OMM[45]	+	1C	**Linker**	Binds BAP31	Apoptosis	[148]

Protein	Location UniProt, Biotinylomes[], Other[]	MC2	TM	ER-mito Contact Role	Relevant Interactions	Relevant Functions	ER-mito Contact Ref.
FUNDC1	OMM	+	3	**Linker?**	Binds IP3R(2);	Mitophagy receptor [116]	[117]
FUS	Nc, ER[45]	n/a	0	**Disruptor**	GSK3 activation	DNA, RNA binding	[24]
GPX8	ER[45], MAM	n/a	1 M	**Resident**		Inhibits SERCA2b, decreases Ca^{2+}ER	[135]
GRP75	Mt, nucleolus	+	0	**Linker?**	Complex w/ IP3R and VDAC1	Chaperone	[41]
GSK3β	PM, Nc, Cy	n/a	0	**Disruptor**	↓.PTPIP51-VAPB interaction	Kinase	[18][21]
INF2(-CAAX)	ER [82]	n/a	0	**Linker**	Binds actin & SPIRE1c	Mito fission grove formation	[84, 85]
IP3R1	ER, ER[45]	n/a	6	**1: Linker-Spacer**	w/VDAC&GRP75	1-3: ER Ca^{2+} release;	See text
IP3R2	ER, ER_{mt}[35], ER[45]	n/a	6	**2: Linker-Spacer**	w/FUNDC1 (in heart)		
IP3R3	ER, ER_{mt}[35], ER[45]	n/a	6	**3: Linker-Spacer**	w/VDAC&GRP75	3: Σ1R stabilizes it in MAM	
IRBIT/AHCYL1	ER	n/a	0	**Promoter**	IP3R binding	Promotes ERMCS and Ca^{2+} transfer	[115]
MAVS	OMM, Prxsm, ERxOMM[45]	+	1 C	**Resident**	Interacts w cytoplasmic antiviral response proteins	Innate antiviral immune response	[44, 109]
MARCH5/ MITOL	ER, OMM, ERxOMM[45]	+	4	**Promoter?**	MFN2 recruitment to MAM and ubiquitination in OMM	E3 Ub ligase	[153]
MFN2	OMM, ERxOMM[45]	+	2	**Linker**	Binds MFN2/1; might subdue other linkers	Multi-functional	See text
MIRO1	OMM, ERxOMM[45, 95]	+	1 C	**Promoter?**		Ca^{2+}-effector in stopping mito motility	[91–94]
MOGAT2	ER	n/a	3	**Resident**	Interacts w/ DGAT2	Lipid transfer	[154]
mTORC2:				**Promoter**	Interacts with IP3R/GRP75/VDAC1		[22]
mTOR	Many organelles	−	0			Core of mTORC	
mLST8	Cy	n/a	0			PK activator	
Protor/PRR5	mTORC2, Mt	−	0				

Protein	Location UniProt, Biotinylomes[], Other[]	MC2	TM	ER-mito Contact Role	Relevant Interactions	Relevant Functions	ER-mito Contact Ref.
Rictor	mTORC2, Cy	n/a	0		Ubiquitin ligase binding	Enzyme activator	
SIN1	PM, Nc, Ves	n/a	0		Binds kinases, ribosome (GO) Binds PA, PIP2/3, kinases, Ras		
ORP5 ORP8	ER ER_{mt}[35]	n/a n/a	1 C	**Linker**	Binds PTPIP51	Sterol transport	[23]
PACS2	ER, Mt, Cy[45]	−	0	**Promoter**	Stabilizes BAP31	ER shaping?	[155]
PDZD8	ER	n/a	1	**Linker**			[72]
PS2	ER, Golgi, ER[45]	n/a	8.5	**Promoter OR Disruptor**	Binds MFN2	Supports non-MFN2 linkers? [65] MAM limiter? [67]	
PTP1B	ER, ER[45]	n/a	1 C	**Linker?**	Interacts w/ PTPIP51 (hence its name)		[58, 59]
PTPIP51	OMM, ERxOMM[45]	+	1 N	**Linker**	Binds VAPB, ORP5	Lipid/sterol transfer	[23, 32]
RAB32	OMM, ER[45], MAM	+	0	**Resident**	DRP1 recruitment	Acts as an AKAP	[156]
REEP1	ER, OMM, ER[45]	−	2	**Linker**	*Trans*-homodimer (like MFN2)	Membrane bending	[34]
RRBP1	rER, ER[45]	n/a	1 N	**Linker**	Binds SYNJ2BP (via PDZ-binding domain)		[45]
SIGMA1R	ER, Nc, LD, ER[45]	n/a	1 N	**Promoter**	Protects IP3R3 from ERAD	Chaperon	[118]
SLC25A46	OMM, ERxOMM[45]	+	6	**Linker?**	Binds MFN2, OPA1, MICOS, EMC1,2	Phospholipid exchange	[73]
SPIRE1C	OMM[85]	−	2?	**Linker**	Binds actin-INF2	Mito fission grove	[85]
STARD7	OMM, OMM[45]	+	0	**Resident**		PC transfer, redox homeostasis	[157]
SYNJ2BP	OMM, ERxOMM[45]	+	1 C	**Linker**	Binds RRBP1 via PDZ domain		[45]
TDP-43	Nc	n/a	0	**Disruptor**	GSK3 β activation	DNA, RNA binding	[19]

Protein	Location UniProt, Biotinylomes[], Other[]	MC2	TM	ER-mito Contact Role	Relevant Interactions	Relevant Functions	ER-mito Contact Ref.
TMX1	ER, ER_{mt}[35], ERxOMM[45]	−	1 M	**Resident** (palmitoylated)	Binds and inhibits SERCA, binds calnexin	Disulfide isomerase	[20, 21]
Trichoplein	Multi; MAM/OMM[158]	+	0	**Disruptor**	Binds and interferes with MFN2		[158]
VAPB	ER, ER_{mt}[35], ER[45]	n/a	1 C	**Linker**	Binds PTPIP51	Vesicle fusion	[19, 32]
VDAC1	OMM, ERxOMM[45]	+	NA	**Linker?**	may form complex with IP3R and GRP75	1-3 OMM porins	[41, 113]
VDAC2	OMM	+					
VDAC3	OMM	+					

Proteins are listed alphabetically by their UniProt entry names. Cellular locations are listed per UniProt, the two recent APEX(2)-based proximity biotinylomes [35, 45], or in certain cases other specified publications. For mitochondria-located proteins, it is indicated whether or not listed in MitoCarta 2 (MC2) [46]. The number of predicted or confirmed transmembrane helices (TM), based on UniProt, is also listed; for single TMs the position relative to the termini is indicated as N, C for N/C-terminal (0-10aa away from N/C-terminus), and M (Mid, >10aa away from both termini). "?" indicates the lack of structural evidence. In the Function/Interactions/Special features column the structural contribution to ER-mito contact dynamics is bolded. Cy, cytoplasmic; ERAD, ER-associated (protein) degradation; ERmt, mitochondria-associated ER (from [35]); ERxOMM, cross-section of ER membrane and OMM biotinylome from [45]; LD, lipid droplet; Me^{2+}, bivalent metal ions; Mt, mitochondrial (submitochondrial not specified); Nc, nuclear; Prxsm, peroxisome.