
Over Six Decades of Discovery and Characterization of the Architecture at Mitochondria-Associated Membranes (MAMs)

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Abstract

The discovery of proteins regulating ER-mitochondria tethering including phosphofurin acidic cluster sorting protein 2 (PACS-2) and mitofusin-2 has pushed contact sites between the endoplasmic reticulum (ER) and mitochondria into the spotlight of cell biology. While the field is developing rapidly and controversies have come and gone multiple times during its history, it is sometimes overlooked that significant research has been done decades ago with the original discovery of these structures in the 1950s and the first characterization of their function (and coining of the term mitochondria-associated membrane, MAM) in 1990. Today, an ever-increasing array of proteins localize to the MAM fraction of the endoplasmic reticulum (ER) to regulate the interaction of this organelle with mitochondria. These mitochondria-ER contacts, sometimes referred to as MERCs, regulate a multitude of biological functions, including lipid metabolism, Ca^{2+} signaling, bioenergetics, inflammation, autophagy, mitochondrial structure, and apoptosis.

Keywords

Mitochondria-associated membrane • Lipids • Calcium • Endoplasmic reticulum • Mitochondria

2.1 Introduction

In the early 1980s, most cell biologists dismissed the idea that the endoplasmic reticulum and mitochondria make physical contacts as an

artifact (Katz et al. 1983). There was little indication that these contacts exist outside of obscure model systems such as teleosts or onions (Bracker and Grove 1971; Copeland and Dalton 1959; Morre et al. 1971). This explains why Jean Vance's biochemical description of the mitochondria-associated membrane (MAM, originally called fraction X) from rat liver in 1990 only received relatively modest numbers

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of citations in the beginning (Vance 1990). However, today this publication is seen as the seminal work that described ER-mitochondria membrane contact sites (MCS) for the first time as essential structures needed for the activities of phospholipid biosynthetic enzymes. Vance's work demonstrated that MAMs co-fractionate with mitochondria and that they house enzymes synthesizing phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) (Vance 1991). Others extended these discoveries to demonstrate that PS can translocate between the ER and mitochondria directly (Voelker 1989). Today, the term MAM is often used when referring to ER subdomains, which are biochemically distinct from the remainder of the ER, but which remain attached to mitochondria as mitochondria-ER contacts (MERCs) (Sood et al. 2014).

Curiously, many cell biologists are unaware of much earlier studies of electron microscopy (EM) images from the 1950s that identified ER-mitochondria contacts. It appears safe to say that these contacts had been seen for the first time on electron micrographs of rat liver by Wilhelm Bernhard in 1952 (Bernhard et al. 1952) and 1956 (Bernhard and Rouiller 1956a, b). His studies also led to the insight that ER-mitochondria contacts depend upon feeding and fasting, a remarkable finding that was solidified only during the past 5 years (Theurey and Rieusset 2017). Following these pioneering studies, liver MERCs were also seen by others (Fawcett 1955) and were also discovered in mouse lung cells (Karrer 1956), *Tetrahymena* ciliates (Franke and Kartenbeck 1971), fungi (Bracker and Grove 1971), and some types of plants (Morre et al. 1971). Compared to this ease of detecting ER-mitochondria contacts via EM, biochemical experiments were more difficult to perform and, the co-fractionation of the ER with mitochondria was often interpreted as a contamination problem (Lever and Chappell 1958; Siekevitz 1963). A first step forward was achieved with the insight that the biochemical ER moiety can be separated into two pools, each with distinct levels of association with mitochondria (Shore and Tata 1977). Further experiments showed that the portion of the ER, which remains attached to mitochondria,

is biochemically distinct from the bulk ER (Meier et al. 1981) and is mostly of the smooth subtype (Pickett et al. 1980).

Further research has shown that ER-mitochondria contacts result when the ER approaches mitochondria to a distance of about 20 nm on 20% of the mitochondrial surface (Rizzuto et al. 1998). An important recent insight is that the extent and length of these contacts depends on cellular conditions such as ER stress (Bravo et al. 2011; Csordas et al. 2006). Physical, proteinaceous tethers mediate MAM contacts, since proteinases can detach the ER from mitochondria (Achleitner et al. 1999; Csordas et al. 2006), and artificial tethers can boost ER-mitochondria ion flux (Csordas et al. 2006). The past 5 years have seen the discovery of a novel, unexpected role of proteinaceous components of the MAM in mitochondrial dynamics. From their location at ER-mitochondria contacts, human dynamin-related protein 1 (Drp1) (Friedman et al. 2011) and mitofusins determine mitochondrial fission and fusion (Klecker et al. 2014).

Today, we can look back to well over three decades of research on ER-mitochondria contacts (Fig. 2.1). These have led to the insight that the MAM accommodates the exchange of lipids, ions, and second messengers (Levine and Patel 2016), houses the machinery for mitochondrial fission (Friedman et al. 2011), serves as a point of origin for autophagosome formation (Hamasaki et al. 2013), and makes connections to signaling pathways and the cytoskeleton.

2.2 The Study of Lipid Metabolism Discovered Functional Significance of Mitochondria-ER Contacts (MERCs)

The fundamental requirement for ER-mitochondria interaction in phospholipid metabolism has been discovered around 1970 through the observation that liver-derived mitochondria themselves are unable to synthesize PS, but rather must rely on import of this lipid from the ER (McMurray and Dawson 1969; Sauner and Levy 1971; Wirtz and Zilversmit

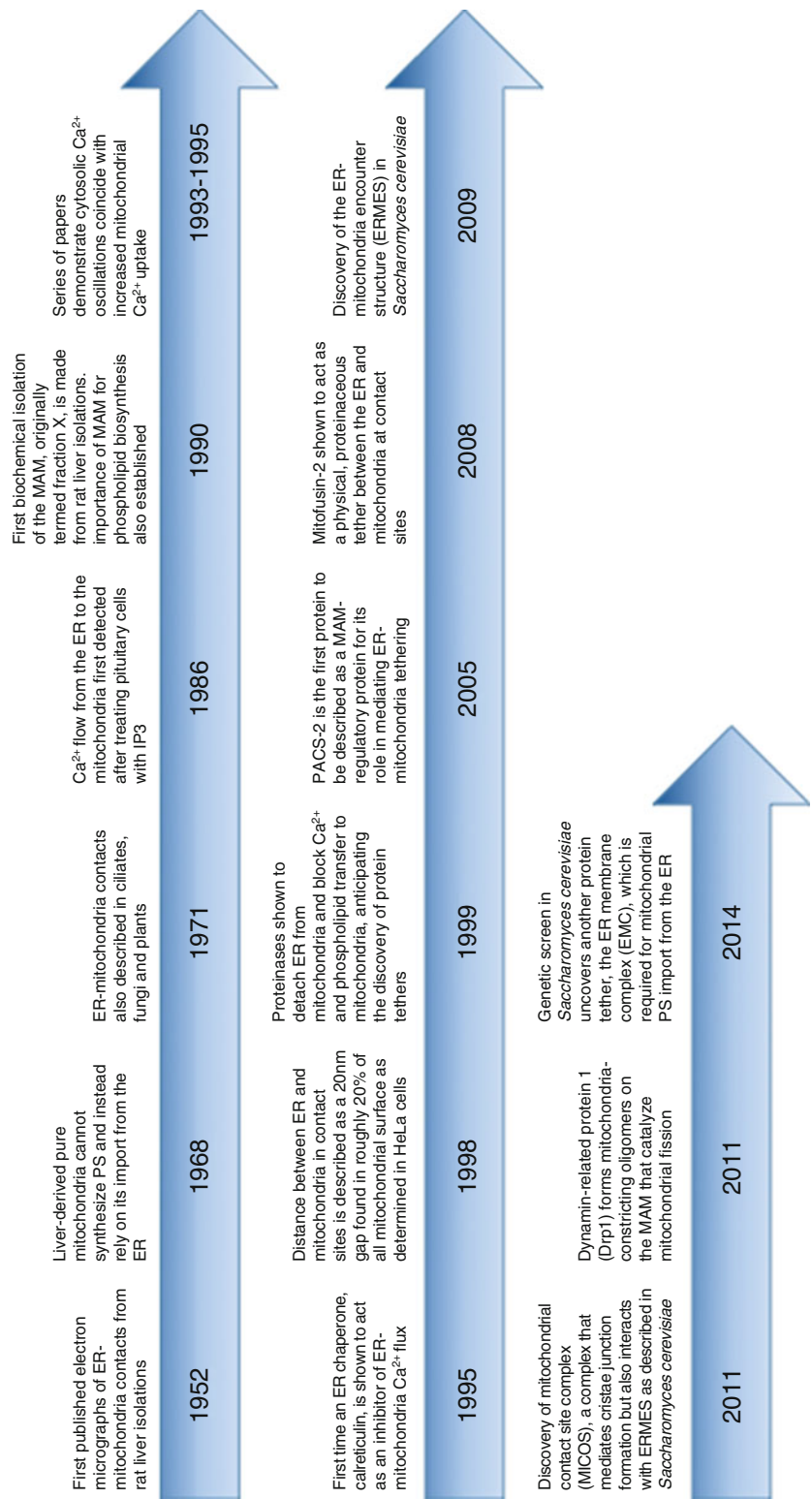


Fig. 2.1 A timeline of the key discoveries in the field of mitochondria-associated membrane (MAM) from 1952 to 2016

1968). While initial hypotheses proposed a cytoplasmic transfer mechanism based on proteinaceous shuttles between the ER and mitochondria (Dennis and Kennedy 1972), it became clear later that PS shuttles directly over to mitochondria, where it is decarboxylated to yield PE (Voelker 1989, 1990). Interestingly, this transfer does not use a vesicular transport step (Shiao and Vance 1995; Vance 1991; Voelker 1989). PS transfer likely occurs on a triple contact site where ER membranes and mitochondrial inner and outer membranes meet and house enzymes that produce PE (Ardail et al. 1991, 1993). While the mechanism for PE transfer to the ER is still unknown, the enzymes catalyzing PE methylation to yield PC localize to the MAM (Vance 1991), as determined via biochemical and EM techniques (Rusinol et al. 1994). Interestingly, MAM sorting signals in the form of cryptic mitochondrial targeting sequences have been detected in some of these enzymes, including acyl-CoA:diacylglycerol acyltransferase 2 (Stone et al. 2009).

An interesting observation is that ER-mitochondria membrane apposition (Flis and Daum 2013) and PS transfer from the ER to mitochondria require ATP in liver cells (Tatsuta et al. 2014). This finding is in contrast to what is known in yeast, where PS and PE shuttle between the two organelles (Gnamusch et al. 1992) without the need for ATP (Achleitner et al. 1999; Simbeni et al. 1993). Ubiquitination of MAM proteins could regulate this lipid shuttling, as shown by the requirement of the yeast SCF ubiquitin ligase Met30p for PS transfer from the MAM to mitochondria (Schumacher et al. 2002) and of the human ubiquitin ligase MITOL for ER-mitochondria apposition (Sugiura et al. 2013).

Lipid transfer between the ER and mitochondria is critical for mitochondria metabolism, albeit for unclear reasons (Vance 2015). For instance, PE deficiency in mammalian mitochondria impairs mitochondrial bioenergetics (Tasseva et al. 2013). Another example is cardiolipin, synthesized from phosphatidic acid (PA) within mitochondria, which is essential for mitochondrial oxidative phosphorylation,

apoptosis, mitochondrial protein import, and mitochondrial membrane dynamics (Claypool and Koehler 2012). Similarly, the intracellular PC transfer protein StarD7, which controls both ER and mitochondria structure and function (Flores-Martin et al. 2016). However, further research is needed to fully explain these tight links between mitochondria metabolism and ER-mitochondria lipid transfer.

2.3 Mitochondria-ER Contacts (MERCs) Accumulate Sterols and May Orchestrate the Trafficking of Sterol Towards Mitochondria

Besides PA production, the ER also houses sterol synthesis (Porter 2015). Although not abundant within mitochondria, the sterols cholesterol and ergosterol are important for mitochondrial structure and function in mammalian cells (Lucken-Ardjomande et al. 2008) and yeast (Altmann and Westermann 2005). Research performed by several labs using human cells has identified the MAM as a cholesterol-rich membrane marked with the sterol-interacting protein caveolin (Bosch et al. 2011a, b; Sano et al. 2009). Moreover, the MAM remarkably exhibits lipid raft-like properties (Area-Gomez et al. 2012; Fujimoto et al. 2012; Hayashi and Fujimoto 2010; Williamson et al. 2011). Importantly, caveolin appears to be central to lipid-related functions of the MAM, since it helps to enrich intracellular lipid and sterol metabolism-related processes to the MAM (Sala-Vila et al. 2016). Consistent with these findings, methyl- β -cyclodextrin, an agent that depletes membrane cholesterol, disrupts the MAM platform (Ciarlo et al. 2010), leading to disrupted mitochondrial bioenergetics (Ziolkowski et al. 2010).

This suggests that sterols might use MAM contacts to enter mitochondria. Consistent with this hypothesis, mitochondria use the steroidogenic acute regulatory (StAR) protein D1 (STARD1), which binds free cholesterol in the cytoplasm and then shuttles it toward the MAM

in a PKA-dependent manner (Miller 2013). Here, sterols bind to the voltage-dependent anion channel (VDAC), a component of mitochondrial Ca^{2+} import and known MAM protein (Szabadkai et al. 2006), which imports them to mitochondria (Bose et al. 2002) and distributes them within the organelle (Campbell and Chan 2007). Another example is yeast Lam6p/Ltc1p, a StAR-domain-containing protein, which partially localizes to ER-mitochondria contacts (Gatta et al. 2015). Lam6p/Ltc1p interacts with the mitochondrial Tom70/71 complex and might tether the two organelles independently from other molecular tethers (Elbaz-Alon et al. 2015; Murley et al. 2015).

Another candidate mechanism for mitochondrial sterol import is based on a family of proteins originally thought to sense cytosolic sterols, the oxysterol-binding protein (OSBP)-related proteins (ORPs) (Dawson et al. 1989; Taylor et al. 1984). Their 16 human and 7 yeast family members (called oxysterol homology or Osh proteins) frequently localize to MCS (Du et al. 2015). In order to achieve sterol translocation, ORPs translocate from the ER and exchange sterols produced at the ER for phosphatidylinositol 4-phosphate at their cognate target membrane (Drin et al. 2016). ORP5 and ORP8 are of particular interest. These two family members partially target to the MAM, but also interact with a suspected MAM tether and mitochondrial membrane protein, PTPIP51. While it is currently unknown whether these two ORPs mediate lipid or cholesterol trafficking to mitochondria, their knockdown alters biochemical activities, as well as membrane dynamics of mitochondria (Galmes et al. 2016). Together, despite a proven role of sterols in ER-mitochondria contact formation, a number of questions remain on their role for the MAM. Further research will have to investigate the relationship between plasma membrane caveolin and MAM caveolin, as well as the role of ORP proteins in E tethering.

2.4 MAM Proteins Mediate Calcium Signaling: Mitochondria-ER Contacts (MERCs) Emerge as an Intracellular Signaling Hotspot

While the functional significance of the MAM is historically best tied to lipid metabolism, this structure has broader significance for intracellular ion flux, since four mitochondrial dehydrogenases require Ca^{2+} ions to allow for respiration and the Krebs cycle (Denton 2009). Another functional connection exists between Ca^{2+} and the permeability transition pore that controls the mitochondrial protein content, as well as the mitochondrial membrane potential (Hurst et al. 2017). Early in mitochondrial research, Ca^{2+} had been identified as a factor that opens the pore (Hunter et al. 1976). In all cases, the ER acts as a point of origin for Ca^{2+} (Bravo-Sagua et al. 2013).

First evidence for this connection was seen with the observation that the ER and mitochondria cooperate to regulate cytosolic Ca^{2+} amounts (Becker et al. 1980). Moreover, an early study suggested in 1981 that the isolation of ER-mitochondria membrane contacts drastically decreases in the presence of the Ca^{2+} chelator EDTA (Meier et al. 1981). A flux of Ca^{2+} ions between the ER and mitochondria was described for the first time in 1986 using a Ca^{2+} -selective mini-electrode in digitonin-permeabilized rat pituitary GH₃ cells as a discrete pool of ER Ca^{2+} that is transferred to mitochondria upon incubation of a mixed organellar preparation with inositol 1,4,5-trisphosphate (IP₃) (Biden et al. 1986). Following these early discoveries, apoptosis research showed that mitochondria receive Ca^{2+} from the ER and that this abnormally high flux of the ion leads to apoptosis (Baffy et al. 1993).

However, the true magnitude of these discoveries and speculations became clear only through further research by the laboratories of Tullio Pozzan and Andrew Thomas. Their studies demonstrated that cytosolic Ca^{2+} oscillations

coincided with mitochondrial Ca^{2+} increases in live human hepatocytes and HeLa cells, using the Rhod-2 dye or mitochondrially targeted aequorin, a Ca^{2+} -activated photoprotein (Hajnóczky et al. 1995; Rizzuto et al. 1993, 1994). Moreover, this Ca^{2+} transfer required the formation of highly concentrated microdomains used by mitochondrial Ca^{2+} uniporters (MCUs) to import Ca^{2+} (Rizzuto et al. 1993). This observation was refined by the Nabi lab, demonstrating that concentrations below 100 nM favor dissociation of contacts, while concentrations above 1 μM favor contact formation (Wang et al. 2000). Using green fluorescent protein (GFP)-tagged fusion proteins targeted to the ER and mitochondria, Rosario Rizzuto then pushed the field further by showing that mitochondria form an interconnected network that shows close apposition to the ER on 5–20% of its surface (Rizzuto et al. 1998). These points of apposition were also proposed as the location of Ca^{2+} microdomains in the same study. Here, mitochondrial ATP fuels ER Ca^{2+} uptake (Landolfi et al. 1998) upon formation of Ca^{2+} microdomains (Hajnóczky et al. 1999). The complex relationship between the two organelles regarding Ca^{2+} leads to the establishment of Ca^{2+} signal transmission between the ER and mitochondria similar to what occurs at a synapse, as formulated by the Hajnóczky lab in 1999 upon their analysis of experiments with fluorescent, mitochondria-targeted probes (Csordas et al. 1999).

ER-mitochondria Ca^{2+} crosstalk boosts mitochondrial bioenergetics (Cardenas et al. 2010), and studies by Murphy and Finkel using MCU knockout (ko) cells show that in vivo, mitochondrial Ca^{2+} import is critical for mitochondria peak performance (Pan et al. 2013). The MCU molecular discovery in 2011 jointly by the Rizzuto and Mootha labs (Baughman et al. 2011; De Stefani et al. 2011) showed that this protein forms a complex with mitochondrial calcium uptake 1 and 2 (MICU1 and MICU2) (Mallilankaraman et al. 2012; Perocchi et al. 2010; Plovanich et al. 2013). These two regulatory proteins likely perform vital functions in the control of ER-mitochondria Ca^{2+} flux as well,

although this has not yet been fully investigated (Antony et al. 2016). Interestingly, *S. cerevisiae* mitochondria have lost the MCU during evolution, suggesting that *S. cerevisiae* has a drastically reduced and simplified Ca^{2+} machinery (Carafoli and Lehninger 1971). Moreover, yeast stores the majority of its Ca^{2+} inside vacuoles, not the ER (Belde et al. 1993). More detailed characterizations need to demonstrate whether this organism is able to faithfully reproduce ER-mitochondria Ca^{2+} flux as observed in metazoan cells (Plattner and Verkhratsky 2016).

On the ER side of the MAM, the investigation of cytosolic Ca^{2+} waves by Lechleiter and Camacho identified additional players. These excitatory events are triggered via the activation of IP_3Rs and depend on the presence of the Ca^{2+} pump sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA). The properties of Ca^{2+} waves suggest they are generated from a cross-reaction with oscillating cytosolic $[\text{Ca}^{2+}]$ and Ca^{2+} -regulated IP_3Rs (Camacho and Lechleiter 1993). Moreover, Ca^{2+} waves are tightly connected to mitochondria metabolism: while mitochondrial Ca^{2+} import and mitochondrial ROS can boost Ca^{2+} waves (Walsh et al. 2009), a block of mitochondrial respiration reduces their amplitude and frequency (Jouaville et al. 1995). The mechanistic basis for this connection has recently been elucidated in a series of elegant experiments by the Hajnóczky lab. ER-mitochondria Ca^{2+} transfer leads to the release of cristae-stored ROS that subsequently diffuse over to the ER and converge on the MAM, where they chemically modify Ca^{2+} -handling proteins (Booth et al. 2016). One group of these are inositol 1,4,5-trisphosphate receptors (IP_3Rs), which localize to special subdomains of the ER and possibly concentrate in portions that have the tendency to appose to mitochondrial membranes (Takei et al. 1994). ROS modifications result in the sensitization of IP_3Rs , thus strengthening cytosolic Ca^{2+} waves (Walsh et al. 2009). Further research needs to address the role of ER-derived ROS in this process (Appenzeller-Herzog et al. 2016). Nevertheless, these results mechanistically tie ER-mitochondria Ca^{2+} flux to ER energy

demand (Bravo et al. 2011). In further research, ER chaperones, including calreticulin (Camacho and Lechleiter 1995), calnexin (Roderick et al. 2000), and ERp57 (Li and Camacho 2004) were identified as inhibitors of cytosolic Ca^{2+} waves and MAM Ca^{2+} crosstalk (Appenzeller-Herzog and Simmen 2016). Consistent with these observations, calnexin reduces the amount of Ca^{2+} that can reach mitochondria, but increases the amount of Ca^{2+} that can be taken up to the ER (Lynes et al. 2013). This function of calnexin depends on its localization to the MAM and is dependent on its phosphorylation and palmitoylation state (Lynes et al. 2012; Myhill et al. 2008). Opposing these inhibitors of ER-mitochondria Ca^{2+} flux, the ER oxidoreductase TMX1/TXNDC1 interacts in a thiol-dependent manner with SERCA2b to reduce ER Ca^{2+} import and thus augment MAM Ca^{2+} flux (Raturi et al. 2016). By doing so, TMX1 concomitantly increases mitochondrial respiration and ATP production, while mitochondria metabolism is blocked in its absence, resulting in a Warburg-style acceleration of cancer cell growth (Raturi et al. 2016).

The functional connection between ER protein folding and ER-mitochondria Ca^{2+} flux is not limited to the regulation of SERCA activity, but also extends to modulatory interactions between ER chaperones and IP_3 Rs. For instance, the ER oxidoreductases ERp44 and Ero1 α inhibit or activate IP_3 Rs, respectively, and, by doing so, ER-mitochondria Ca^{2+} flux (Higo et al. 2005; Li et al. 2009). Again, at least in the case of Ero1 α , this property is tied to its localization to the MAM (Anelli et al. 2011; Gilady et al. 2010).

Proteins of the Bcl2 family mediate further regulation of ER Ca^{2+} release and uptake (Vervliet et al. 2016). For example, Bcl-x_L interacts with IP_3 R1 to activate this channel when [IP_3] is low, but to inactivate it when [IP_3] is high (Yang et al. 2016). The BH3-only protein Bid and pro-apoptotic Bax inhibit this regulatory interaction (White et al. 2005). The regulatory mechanism extends to the mitochondrial membrane, where Bcl-x_L blocks mitochondrial Ca^{2+} import via VDAC1 (Monaco et al. 2015). In contrast, the interaction between Bcl2 and the IP_3 R largely serves to block

pro-apoptotic Ca^{2+} transfer (Chen et al. 2004; Hanson et al. 2008; Monaco et al. 2012; Rong et al. 2009), similar to Bax inhibitor-1 (BI-1) (Sano et al. 2012). A similar inhibitory interaction exists between Bcl2 and the ryanodine receptor (Vervliet et al. 2014).

A prototype regulatory protein of ER-mitochondria Ca^{2+} flux is the MAM-localized sigma-1 receptor (SIGMAR1), a transmembrane protein that can act as a chaperone for IP_3 Rs under conditions of low ER [Ca^{2+}] (Hayashi and Su 2007). Through this activity and its localization to detergent-resistant membranes at the MAM (Hayashi and Fujimoto 2010), a property shared with mitofusin-2 and TMX1 (Lynes et al. 2012), SIGMAR1 controls ER-mitochondria Ca^{2+} flux and mitochondria lipid metabolism (Bernard-Marissal et al. 2015; Marriott et al. 2012).

The sum of these chaperone/oxidoreductase interactions with Ca^{2+} -handling proteins allows ER protein folding to increase ER-mitochondria Ca^{2+} flux under conditions of the unfolded protein response (UPR). This boosts ATP import into the ER, which can then alleviate folding problems or trigger apoptosis, should these problems persist (Bravo et al. 2011). There is therefore a tight link between ER protein folding and redox and the MAM that can telegraph the state of the ER to mitochondria (Simmen et al. 2010).

2.5 Proteins Mediating Formation of the MAM

Proteinases have been shown to untether the ER and mitochondria (Achleitner et al. 1999; Csordas et al. 2006) and thus disrupt Ca^{2+} flux as well as phospholipid import into mitochondria. This insight led to the postulate that proteinaceous tethers rather than interorganellar membrane fusion events mediate ER-mitochondria apposition (de Brito and Scorrano 2008). The past decade has seen the discovery of MAM-forming and MAM-regulating proteins that tether ER and mitochondria membranes and regulate the composition of contact sites (Fig. 2.2).

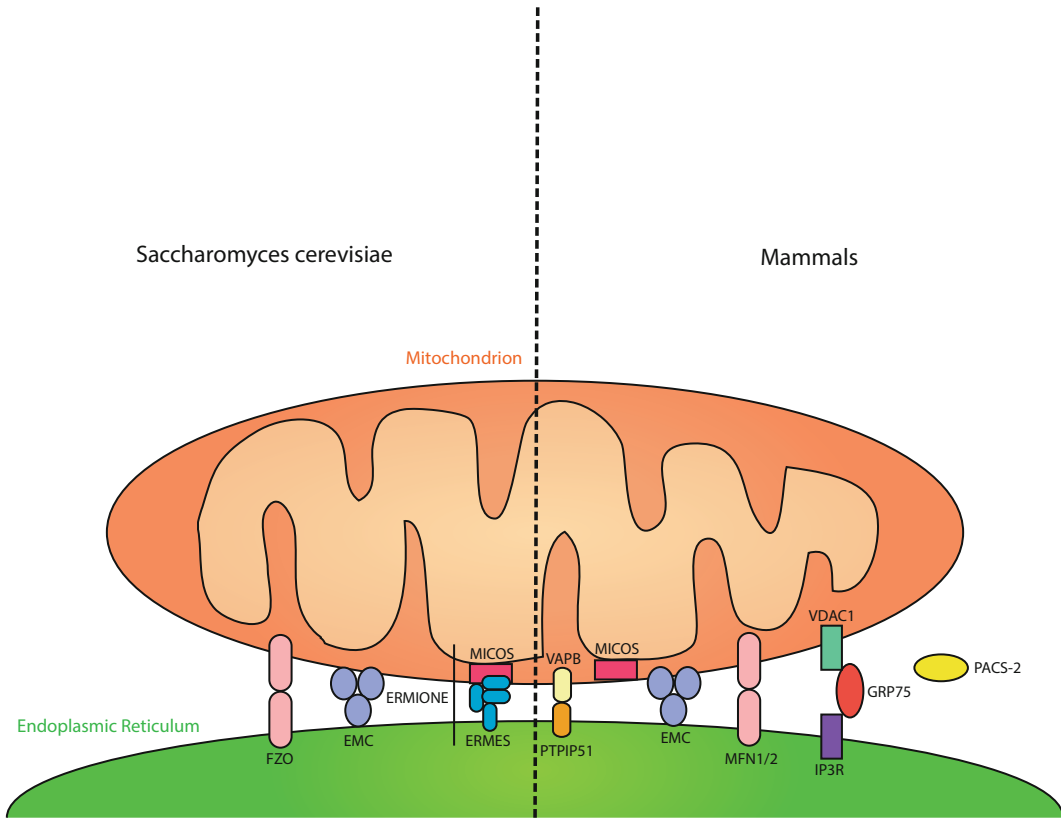


Fig. 2.2 Representation of ER-mitochondria tethers identified in yeast and mammalian models (See text for details)

A genetic screen in *S. cerevisiae* expressing an artificial ER-mitochondria tether led Benoît Kornmann to discover the ER-mitochondria encounter structure (ERMES) (Kornmann et al. 2009). This heterotetrameric protein complex is composed of Mmm1p, an ER transmembrane protein; Mdm12p, a cytosolic protein; as well as Mdm34p and Mdm10p, which target to the mitochondrial outer membrane (Lang et al. 2015). Interestingly, the ERMES subunits Mdm12p, Mdm34p, and Mmm1p all contain synaptotagmin-like lipid-binding protein domains (SMP), known modules that mediate MCS formation (Reinisch and De Camilli 2016). The Ca^{2+} -binding, rho-like GTPase Gem1p regulates the number and activity of ERMES contact points between the ER and mitochondria (Kornmann et al. 2011). Questions arose in the past whether ERMES is dispensable for PS transport from the ER to mitochondria

(Nguyen et al. 2012), but this appears now firmly established (Kojima et al. 2016). Curiously, despite this strong evidence for an essential role of ERMES in yeast, this complex is not conserved in metazoa (Wideman et al. 2013). In yeast cells, the mitochondrial contact site complex (MICOS) genetically interacts with ERMES (Hoppins et al. 2011). MICOS comprises the six proteins Mic60p, Mic10p, Mic12p, Mic19p, Mic26p, and Mic27p (Harner et al. 2011; Hoppins et al. 2011; von der Malsburg et al. 2011) and is critical for the formation of cristae junctions within mitochondria (Ardail et al. 1990; Hackenbrock 1968). Since MICOS is not restricted to yeast (Kozjak-Pavlovic 2017), its role in aligning the MAM with cristae junctions could be conserved.

More recently, Prinz and Loewen identified another potential tether structure in a yeast genetic screen using *CHO2* that negatively

interacts with *PSD1*. This screen identified the ER membrane protein complex (EMC), composed of Emc1p, Emc2p, Emc3p, Emc4p, Emc5p, and Emc6p, all of which are necessary for mitochondrial PS import from the ER (Lahiri et al. 2014). Subsequent work has identified the additional components Emc7, Emc8, Emc9, and Emc10 in human cells, but these proteins are absent in yeast (Wideman 2015). The EMC complex is far better conserved throughout evolution than ERMES. However, it is currently unclear how important its role in tethering is compared to its other roles in ER protein folding, since EMC catalyzes the assembly of proteins that span the membrane multiple times as well (Satoh et al. 2015).

In mammalian models, defects in MAM Ca^{2+} flux or the optical apposition between the ER and mitochondria have been used frequently to identify tethers. One example of a human protein implicated in ER-mitochondria tethering is phosphofurin acidic cluster sorting protein 2 (PACS-2), a cytosolic protein that regulates MAM formation (Simmen et al. 2005). It is unclear whether PACS-2 is a MAM tether per se, but its knockdown or knockout nevertheless uncouples the ER from mitochondria, as seen by light and electron microscopy (Simmen et al. 2005). Consistent with a role in MAM formation that is required for Ca^{2+} moving from the ER towards mitochondria, PACS-2 knockdown blocks Ca^{2+} -mediated apoptosis progression (Myhill et al. 2008). The functions of PACS-2 at the MAM are under the control of its serine 437 residue, where Akt-mediated phosphorylation enables PACS-2 to maintain the MAM (Betz et al. 2013). Interestingly, Akt is downstream of mammalian target of rapamycin complex 2 (mTORC2) that localizes to the MAM itself and activates PACS-2 via Akt (Betz et al. 2013). PACS-2 interacts with calnexin, another known regulator of ER-mitochondria Ca^{2+} flux (Lynes et al. 2013; Myhill et al. 2008), and prevents BAP31 processing mediated by caspase-8 (Simmen et al. 2005). Potentially, this interaction is the true basis of PACS-2-mediated ER-mitochondria tethering, since BAP31 directly interacts with the mitochondrial Drp1

docking protein Fis1 (Iwasawa et al. 2011). This complex becomes associated with procaspase-8 under conditions of cell stress. Under that condition, active caspase-8 triggers the formation of the BAP31 p20 fragment, an activator of mitochondrial fission (Breckenridge et al. 2003). Importantly, PACS-2 knockdown leads to this latter event by itself and thus detaches the ER from mitochondria (Simmen et al. 2005).

A second mechanism that influences ER-mitochondria tethering was discovered by the lab of Luca Scorrano in the form of mitofusin-2 (de Brito and Scorrano 2008). When examining the structures of mitofusin-2 ko cells, light and electron microscopy showed a detachment of the ER and mitochondria in mouse embryonic fibroblasts, neurons (Schneeberger et al. 2013), and cardiomyocytes (Chen et al. 2012). Mitofusin-2 ko cells also show inhibited Ca^{2+} transfer from the ER to mitochondria upon Ca^{2+} release from the ER. Consistent with this observation, ER stress does not result in efficient apoptosis onset in mitofusin-2 ko cells (Munoz et al. 2013). Further demonstrating the role for the formation of ER-mitochondria contacts, mitofusin-2 ko cells show a decrease in mitochondrial respiration capacity (Mourier et al. 2015). A characteristic shared between PACS-2 and mitofusin-2 is that their knockdown results in ER stress (Sebastian et al. 2012; Simmen et al. 2005), but silencing of the kinase PERK can reverse the mitofusin-2 ko phenotype partially (Munoz et al. 2013). Some doubts on the role of mitofusin-2 for the MAM arose with the observation that some ER tubules can be found in closer contact with mitochondria in mitofusin-2 ko cells (Cosson et al. 2012; Filadi et al. 2015). However, recent results suggest that this effect could be due to culture conditions (Naon et al. 2016), consistent with cell stress arising in mitofusin-2 ko cells quicker than in wild-type cells (Bucha et al. 2015).

Additional tethers include a protein complex between IP_3Rs , VDAC, and the outer mitochondrial membrane chaperone Grp75 (Szabadkai et al. 2006) as well as a novel tethering complex formed between the ER vesicle-associated

membrane protein- associated protein B (VAPB) and PTPIP51 (De Vos et al. 2012). TDP-43 can disrupt the VAPB-PTPIP51 tethers and results in disrupted cellular Ca^{2+} homeostasis (Stoica et al. 2014). Fused in sarcoma (FUS) is another inhibitor of these tethers and similarly results in clean detachment of the ER from mitochondria (Stoica et al. 2016), suggesting that the VAPB-PTPIP51 tethers are currently among the best characterized.

2.6 The MAM Regulates Mitochondrial Dynamics

The past 5 years have seen the discovery of novel, unexpected connections between the MAM and mitochondrial dynamics. These are based on both the mitochondrial fission and fusion machinery. Connections with mitochondrial fusion could be based on the equilibrium of mitofusins mediating mitochondrial fusion and mitofusins localizing to the MAM. Here, the human ubiquitin ligase MITOL regulates MAM formation and activates mitofusin-2 by ubiquitinating its mitochondrial moiety. Apparently, this function is restricted to MAM-specific functions of mitofusin-2 and does not apply to the role of mitofusin-2 in mitochondrial fusion (Sugiura et al. 2013). A similar case is the E3 ubiquitin ligase Gp78. This ER protein normally targets mitofusin-2 for degradation, but its function appears to predominantly determine the ratio between rough ER (rER) and smooth ER (sER)-mitochondria contacts (Li et al. 2015). This finding suggests that mitofusin-2 could also serve to keep the rER apart from mitochondria while promoting the formation of contacts between mitochondria and the sER (Wang et al. 2015).

The best-studied connection between the MAM and mitochondrial dynamics involves mitochondrial fission. At mitochondria-ER contacts, human dynamin-related protein 1 (Drp1) forms mitochondria-constricting oligomers that dock to the mitochondrial proteins Fis1, Mff, and MiD49/MiD51 (Friedman et al. 2011), all localized to the MAM prior to

mitochondrial fission (Murley et al. 2013). The actin-remodeling proteins WAVE-1 (Sung et al. 2008) and ER-associated inverted formin 2 (INF2) (Korobova et al. 2013) form a construction site onto which Drp1 can oligomerize (Hatch et al. 2014). A number of mitochondrial protein kinase A (PKA)-anchoring proteins (AKAPs) like the MAM trafficking GTPase Rab32 control this mechanism (Bui et al. 2010), since PKA acts as an on/off switch for Drp1 (Cribbs and Strack 2007). Another candidate is AKAP121, which can target to either ER or mitochondria (Huang et al. 1999), from where it fine-tunes the availability of Drp1 (Kim et al. 2011).

2.7 There Is More to the MAM Than MERCs the Eye

The new millennium has seen a further expansion of the palette of functions of ER-mitochondria contacts with the discoveries that important cellular signaling mechanisms use mitochondria-ER contacts as a home base for their activities. These include the formation of inflammasomes, which form here upon production of ROS within the ER or mitochondria (Zhou et al. 2011); PKA, which operates from this structure upon binding to the small GTPase Rab32 in HeLa cells (Bui et al. 2010); or the mammalian target of rapamycin complex 2 (mTORC2) that can be co-isolated with the MAM, from where it controls Akt phosphorylation of IP₃Rs and hexokinase 2 (Betz et al. 2013).

A connection between the MAM and autophagy has been made in the context of syntaxin-17, a master regulator of autophagosome formation and progression (Itakura et al. 2012). The significance of syntaxin-17 for the MAM is demonstrated by its localization to mitochondrial rafts and its requirement for Drp1 localization and activity (Arasaki et al. 2015). These activities of syntaxin-17 depend on its inhibitory action on the MAM AKAP Rab32 that uses Drp1 as an effector (Ortiz-Sandoval et al. 2014).

Autophagy is a highly conserved process that involves the formation of a double-membrane

vacuole to capture cytosolic contents and deliver them to lysosomes for degradation (Ashrafi and Schwarz 2013; Lamb et al. 2013; Yang and Klionsky 2010). Autophagy is initiated with the formation of an isolation membrane or phagophore, which expands as it engulfs contents and seals to form a double-membrane vacuole known as the autophagosome (Shibutani and Yoshimori 2014). The current paradigm in the field is that the isolation membrane is derived from a core existing membrane and then formed largely de novo with the addition of vesicles and lipids (Klionsky 2007) to yield a platform for autophagosome biogenesis as an Ω -shaped membrane (Axe et al. 2008). Consistent with the importance of the MAM for autophagosome formation, multiple lines of evidence show that mitochondrial and autophagosomal membranes are transiently linked in a mitofusin-2-dependent manner (Hailey et al. 2010). These autophagosomal membranes containing the ganglioside GD3 become enriched with the MAM protein calnexin (Lynes et al. 2012) and then participate in the early autophagic process (Garofalo et al. 2016). Importantly, calnexin serves as a docking site to target the OMM protein FUNDC1 to the MAM, where it interacts with Drp1 (Wu et al. 2016). Although ER autophagic receptors Atg39p, Atg40p (yeast), and FAM134B (human) have been described recently, it is unclear whether they play a role for MAM-initiated autophagy (Khaminets et al. 2015; Mochida et al. 2015). In yeast, ERMES is critical for the progression of mitophagy (Bockler and Westermann 2014), suggesting that proteins mediating or regulating the tethering of the ER to mitochondria could play a similar role.

2.8 Conclusion

As our chapter shows, ER-mitochondria contacts have come a long way from being seen for the first time in the 1950s on EM micrographs by Bernhard and others. Following their first assignment of a biochemical function by Vance in 1990, the field has exploded (Fig. 2.1) and has

served as a paradigm for the ongoing characterization of MCS between a multitude of organelles.

Although not the topic of this review, it comes therefore as no surprise that the MAM plays important roles in many diseases. Again, this was interestingly anticipated by the pioneers of the 1950s, who saw that MAM formation changes during feeding (Bernhard and Rouiller 1956b) and also during the progression of cancer (Howatson and Ham 1955). Today, researchers are unraveling the molecular mechanisms behind these early observations (Giorgi et al. 2015; Raturi et al. 2016; Sood et al. 2014; Theurey et al. 2016), showing that the MAM machinery is intricately linked to diabetes (Arruda et al. 2014; Tubbs et al. 2014) and cancer (Bononi et al. 2013). Emerging areas of research show that the MAM is likely also playing a prominent role in infection (Horner et al. 2011), as well as neurodegeneration (Area-Gomez et al. 2012; Paillusson et al. 2016). We therefore predict that the ongoing identification of MAM tethers, MAM targeting mechanisms, and novel MAM signaling mechanisms will lead to important insight into these diseases.

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