4 Endoplasmic Reticulum-Mitochondria Communication Through Ca^{2+} Signaling: The Importance of Mitochondria-Associated Membranes (MAMs)

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Abstract

The execution of proper Ca^{2+} signaling requires close apposition between the endoplasmic reticulum (ER) and mitochondria. Hence, Ca^{2+} released from the ER is "quasi-synaptically" transferred to mitochondrial matrix, where Ca^{2+} stimulates mitochondrial ATP synthesis by activating the tricarboxylic acid (TCA) cycle. However, when the Ca^{2+} transfer is excessive and sustained, mitochondrial Ca^{2+} overload induces apoptosis by opening the mitochondrial permeability transition pore. A large number of regulatory proteins reside at mitochondria-associated ER membranes (MAMs) to maintain the optimal distance between the organelles and to coordinate the functionality of both ER and mitochondrial Ca^{2+} transporters or channels. In this chapter, we discuss the different pathways involved in the regulation of ER-mitochondria Ca^{2+} flux and describe the activities of the various Ca^{2+} players based on their primary intra-organelle localization.

Keywords

Endoplasmic reticulum (ER) • Mitochondria • Mitochondria associated membranes (MAMs) • Calcium • ROS • ER-mitochondria contact sites • Cell death • Apoptosis • Autophagy

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4.1 Introduction

The endoplasmic reticulum (ER) and the mitochondrion in living cells are two essential organelles with roles that are classically quite distinct. Evidence has been accruing over the years that points to a specific interplay and cooperation between these compartments that is essential for several cellular functions, such as Ca^{2+} signaling, lipid metabolism, autophagy, inflammation, cell survival, and cell death (Decuypere et al. 2011; Lamb et al. 2013; Marchi et al. 2014; Vance 2014; Patergnani et al. 2015). These close appositions between the ER and mitochondria represent a site where microdomains with a high Ca^{2+} concentration ($[Ca^{2+}]$) are generated upon IP_3-mediated Ca^{2+} release (Rizzuto et al. 1998). Ca^{2+} accumulation in the mitochondrial matrix occurs through the mitochondrial calcium uniporter (MCU) and by the membrane potential ($\Delta\Psi_m$) that exists across the inner mitochondrial membrane (IMM) (Marchi and Pinton 2014). The MCU has a low Ca^{2+} affinity (K_d approximately 10–20 μM), and thus, the Ca^{2+} uptake rate, particularly under resting conditions, is extremely slow (Gunter and Gunter 2001). Based on this consideration, it was long assumed that mitochondrial Ca^{2+} uptake was of little importance in cell physiology. Although initial experiments suggested that mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) could increase up to ~10 μM, subsequent research revealed that $[Ca^{2+}]_m$ may in fact transiently reach near mM levels in different systems (Montero et al. 2000). It is the ER-mitochondria connection that allows for fast uptake of a large amount of Ca^{2+} inside the mitochondrial matrix, where the rapid diffusion of ions within it permits the swift tuning of mitochondrial metabolism to address the needs of the cell (Rimessi et al. 2008). ER-mitochondria Ca^{2+} transfer is fundamental to ensure the cellular energy supply by modulating key enzymes involved in mitochondrial ATP production (Bonora et al. 2012). However, excessive transfer of ER Ca^{2+} to mitochondria is a pro-apoptotic signal with important consequences for cell fate (Giorgi et al. 2012). In other words, ER-mitochondria connections are relevant for cell survival, and the maintenance of the proper spacing between the ER and mitochondria appears critical for proper cell functioning (Naon and Scorrano 2014). Tomography analysis has shown that tethers of ~10 nm or ~25 nm adjoin the two organelles, depending on whether smooth ER and/or rough ER are implicated (Csordas et al. 2006). These sites of contact are typically termed mitochondria-associated ER membranes (MAMs) (Giorgi et al. 2015c).

The development and expression of ER-mitochondria artificial linkers revealed that the length of the tethers is strategic. Using rapamycin-inducible fluorescent inter-organelle linkers, Csordas et al. (Csordas et al. 2010) elegantly demonstrated that the spatial relationship between the ER and the outer mitochondrial membrane (OMM) is a critical factor in the efficient transfer of Ca^{2+} and is likely to affect the other functions of the junction in various ways. The distance between the ER and the OMM may vary in different pathophysiological situations. In response to apoptotic agents, the ER-mitochondria gap narrows (Csordas et al. 2006). Also, during the early phases of ER stress, the number of ER-mitochondria contacts increases and their Ca^{2+} transfer is enhanced, helping the cell to overcome this emergency state through the modulation of key mitochondrial metabolic events (Bravo et al. 2011, 2012). These observations reflect the extremely dynamic nature of MAMs, which is now generally assumed to be a strategic intracellular platform that employs Ca^{2+} flux to regulate a wide range of biological processes (Naon and Scorrano 2014). For example, ER-mitochondria connections are essential for modulating mitochondrial fission. In the close appositions between the ER and mitochondria, organelle constriction occurs via a Ca^{2+}-dependent mechanism that involves recruitment of the cytosolic dynamin-related protein 1 (Drp1) and the mitochondria-associated membrane protein syntaxin17 (Friedman et al. 2011; Arasaki et al. 2015). Mitochondrial fission, in turn, is an

essential event that is involved in mitochondrial network shaping, and it is required to generate small organelles to be transported or to facilitate the removal of damaged organelles by a selective form of autophagy, termed mitophagy (Haroon and Vermulst 2016). Bulk autophagy directly depends on ER-mitochondria juxtaposition. Not only does the ER membrane supply material for the formation of autophagosomes (Tooze et al. 2010), but the ER-mitochondria contacts may also be the specific regions where autophagosomes start to form, due to the MAM localization of the pre-autophagosomal ER protein Atg14 (Hamasaki et al. 2013; Lamb et al. 2013).

Therefore, the correct organization, the mutual interactions between the ER and mitochondria, and their Ca^{2+} crosstalk are linked events aimed at coordinating important functions of the two organelles, and these events determine key aspects of cell fate. For these reasons, a huge number of proteins are gathered at the ER-mitochondria interface to regulate MAM dynamics, thereby preserving the intracellular equilibrium. Indeed, alterations in both MAM architecture and composition lead to different pathological conditions, which in many cases are accompanied by a drastic dysregulation of the intracellular Ca^{2+} homeostasis and dynamics (Patergnani et al. 2011). In this chapter, we will discuss the different pathways controlling Ca^{2+} flux from the ER to the mitochondria and their impact on the physiological state of the cell, and the activities of the various Ca^{2+} players will be distinguished based on their primary intra-organelle localization.

4.2 Ca^{2+} Signaling on the ER Side

The ER is the largest store of Ca^{2+} inside the cell. In resting condition, the ER may contain hundreds of μM free Ca^{2+} (in order of magnitude, nearly three- to fourfold higher compared to the cytosol) (Hofer and Schulz 1996; Bonora et al. 2013). Specific Ca^{2+} pumps and channels operate to maintain the correct luminal $[Ca^{2+}]$ by executing the correct balance between ER Ca^{2+} uptake and release (Ashby and Tepikin 2001) (Fig. 4.1).

ER Ca^{2+} uptake is exclusively performed by the sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps, which actively pump Ca^{2+} into the ER in an ATP-dependent manner (Vandecaetsbeek et al. 2011). Three SERCA genes (ATP2A1, ATPA2, and ATP2A3) are present in the human genome. They generate various splice variants that differ in their C-terminal regions, and their expression is dependent on tissue type and development stage (Papp et al. 2012). Generally, SERCA1a and 1b are widely present in adult and neonatal skeletal muscle. The isoform 2a is highly expressed in cardiomyocytes, while SERCA2b is ubiquitously expressed, functioning as the housekeeping isoform. Finally, SERCA3 is the least studied and gives rise to six isoforms. Among the different SERCAs, the 2b isoform displays the highest Ca^{2+} affinity and, thus, is the main isoform involved in Ca^{2+} uptake in the ER in virtually all cells, except skeletal and cardiac muscle. Because SERCAs are the only pumps regulating ER Ca^{2+} entry, it is not surprising that their activity mediates a wide range of cellular functions controlled by proper ER Ca^{2+} homeostasis, including protein folding, lipid and steroid synthesis, and cell death and survival processes like proliferation, apoptosis, growth, and differentiation (Vandecaetsbeek et al. 2011). These functions are regulated by the luminal ER $[Ca^{2+}]$ ($[Ca^{2+}]_{ER}$) and by intracellular Ca^{2+} peaks and oscillations. SERCAs influence the amplitude, the shape, and the frequency of these modulatory events. Their Ca^{2+}-sequestering activity is regulated by several physiological actors, such as i) proteins, ii) posttranslational modifications, and iii) microRNAs (miRNAs) (Harada et al. 2014; Melo et al. 2015). Their activity can also be modulated by natural compounds and pharmacological tools that either inhibit SERCA like thapsigargin, BHQ, and CPAE (Lytton et al. 1991; Vangheluwe et al. 2009) or promote SERCA like CDN1163 (Kang et al. 2016). Important biological modulators of SERCA are phospholamban (PLN), sarcolipin (SLN), calreticulin, calnexin, TMX1, and ORMD1.

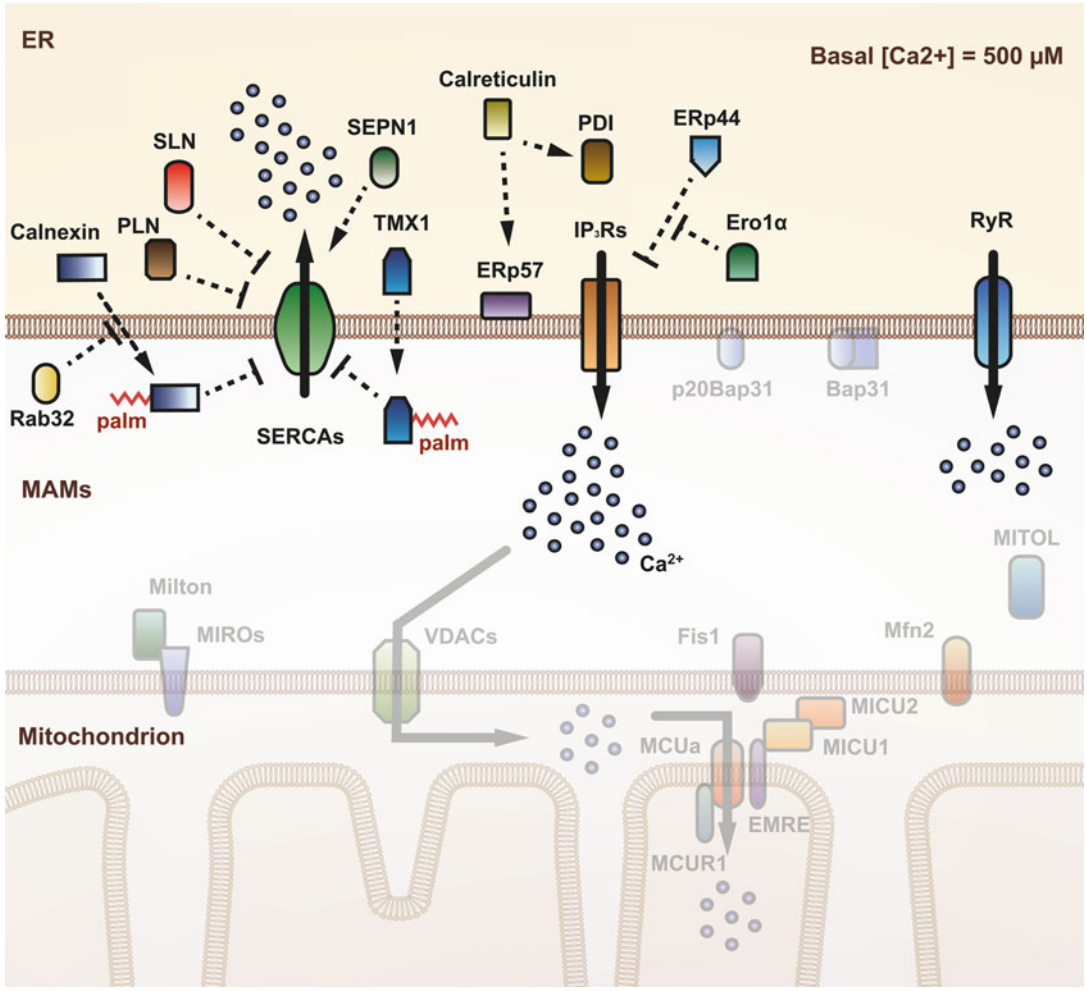

Fig. 4.1 Ca^{2+} homeostasis at the ER-mitochondria interface. Overview on the multiple molecular pathways acting at the ER side (See Sect. 4.2 for details)

Briefly, PLN regulates SERCA function through direct protein-protein interactions (Vittorini et al. 2007). Additionally, SLN directly interacts with SERCAs, and it has been demonstrated to modulate SERCA activity by lowering both the Ca^{2+} affinity and Ca^{2+} pumping rate (Asahi et al. 2003). Another protein that regulates SERCAs is selenoprotein N (SEPN1). This redox-sensitive protein is able to bind SERCAs and enhances their ER Ca^{2+} uptake activity. This feature was found sufficient to safeguard cells against reactive oxygen species (ROS) produced during oxidative protein folding (Marino et al. 2015). Also, chaperones like calreticulin and calnexin have been identified as functional SERCA interactors and modulators. Calreticulin and calnexin were proposed to inhibit SERCA based on their inhibitory impact on high-frequency Ca^{2+} waves in Xenopus oocytes (Camacho and Lechleiter 1995; Roderick et al. 2000). Further work however revealed that overexpression of calreticulin elevated steady-state $[Ca^{2+}]_{ER}$ and increased the ER refilling rates (Arnaudeau et al. 2002). Finally, it has been reported recently that the ER luminal protein disulfide isomerase TMX1 is a strong SERCA inhibitor. In fact, a lack of TMX1 led to an increased ER Ca^{2+} uptake rate and an increase in ER Ca^{2+} storage due to

enhanced ER Ca^{2+} uptake activities of SERCA2b (Krols et al. 2016; Raturi et al. 2016). Another SERCA interactor and inhibitor besides TMX1 is the ER-resident, transmembrane protein ORMDL3, an asthma-associated gene product (Cantero-Recasens et al. 2010). Modulation of its expression levels implicates steady-state ER and cytosolic Ca^{2+} levels and the activation of UPR components.

As reported above, the ER also works as the main source of releasable Ca^{2+} in the cells. Many stimuli induce Ca^{2+} release, but two channel families mainly control the ER Ca^{2+}-release program: ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) (Foskett et al. 2007; Parys and De Smedt 2012; Amador et al. 2013; Van Petegem 2015). RyRs and IP_3Rs form large tetrameric channels (2 MDa and 1.2 MDa in size, respectively) displaying structural and functional homology. RyRs and IP_3Rs are each encoded by three different genes leading for each to the expression of three isoforms. RyR1 is predominantly expressed in skeletal muscle and RyR2 in cardiac muscle and brain, while RyR3 is expressed at low levels in various tissues. With respect to the IP_3Rs, most cell types express a combination of IP_3R1, IP_3R2, and IP_3R3 in various proportions (Vermassen et al. 2004; Ivanova et al. 2014). There is only one known biological/physiological activator of the IP_3R, i.e., IP_3 produced by phospholipase C after activation of G protein-coupled receptors or receptor tyrosine kinases by various stimuli, including growth factors and hormones (Foskett et al. 2007; Parys and De Smedt 2012). RyRs however can be activated through conformational coupling to voltage-operated Ca^{2+} channels, by direct activation by Ca^{2+} or in some cases by the second messengers cADPR and NAADP (Gerasimenko et al. 2006). The Ca^{2+} itself is for both the RyRs and the IP_3Rs, a very important regulator which acts in a biphasic way, whereby a low [Ca^{2+}] activates the channels while a high [Ca^{2+}] has an inhibitory action. Finally, RyRs and IP_3Rs are regulated by phosphorylation/dephosphorylation and by multiple regulatory proteins, some of them being further explicated below. These IP_3R channels are implicated in a plethora of physiological processes, including fertilization, lymphocyte activation, brain rhythms and synaptic plasticity, memory formation, endocrine, and exocrine gland function, and their dysregulation underlies pathophysiological conditions, like neurodegenerative diseases like Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis, autism spectrum disorders, bipolar disorder, epilepsy, schizophrenia, spinocerebellar ataxia, cancer, cardiac dysfunction, and hypertrophy (Berridge 2016). Also RyR channels control different physiological functions, mainly related to skeletal and cardiac muscle contraction features (Van Petegem 2012). However, RyR dysregulation has also been implicated in neurodegenerative diseases like Alzheimer's disease (Briggs et al. 2017; Popugaeva et al. 2017) and malignancies like breast cancer (Zhang et al. 2011b).

Ca^{2+} influx into the cytosol also occurs through ORAI and TRP channels, present in the plasma membrane, in a mechanism dependent on IP_3R opening. In fact, when $[Ca^{2+}]_{ER}$ decreases during IP_3-induced Ca^{2+} release, stromal interaction molecules 1 and 2 (STIM1 and 2) are activated and, in turn, induce opening of ORAIs and, finally, lead to the so-called capacitative or store-operated Ca^{2+} influx from the extracellular space (Prakriya and Lewis 2015). As a result of this increased intracellular [Ca^{2+}], key Ca^{2+}-dependent proteins (such as calpains and calmodulins) are activated, and various Ca^{2+}-dependent cellular processes are initiated (Berridge 2016). Several proteins control the activity of the Ca^{2+}-release mechanism mediated by IP_3Rs (Fig. 4.1). For example, the ER-resident oxidoreductases Ero1α and ERp44 modulate ER Ca^{2+} release by direct interaction with IP_3Rs in a redox-sensitive manner (Higo et al. 2005; Anelli et al. 2012). Specifically, the ERp44 chaperone, an ER luminal protein of the thioredoxin family, directly inhibits Ca^{2+} release (which reinforces Ca^{2+}-dependent chaperones) by inactivating the channel activity of the IP_3R in a pH-, redox-state-, and $[Ca^{2+}]_{ER}$-dependent manner (Higo et al. 2005). Furthermore, ERp44 mediates Ero1α localization through the formation of reversible

mixed disulfides (Anelli et al. 2003). Ero1α is an ER-resident protein that localizes also at MAMs, and its siRNA-mediated downregulation slightly reduced the association between IP_3R and ERp44, suggesting that Ero1α might have further roles in assembling and/or maintaining MAM integrity (Anelli et al. 2012).

It is very clear that a large number of proteins regulate SERCA pumps and IP_3R channels to maintain the appropriate $[Ca^{2+}]_{ER}$. This is because Ca^{2+} signals originating from the ER are leading to Ca^{2+} oscillations, associated with several cellular processes. As mentioned in the "Introduction" section, Ca^{2+} release from the ER is one of the main determinants for mitochondrial homeostasis. In fact, basal Ca^{2+} oscillations modulate mitochondrial metabolism of ATP production, while sustained or excessive Ca^{2+} release may lead to cell death. As a demonstration of this feature, dysregulation of Ca^{2+} flux is involved in several human disorders (Patergnani et al. 2015).

The maintenance of proper ER Ca^{2+} homeostasis also controls an elaborate surveillance system called the ER quality control (ERQC) system. In fact, inside the ER lumen a series of chaperones exists, which are involved in ERQC for the correct folding of ER proteins, and their functions may vary depending on changes in Ca^{2+} concentration (Fig. 4.1). The Ca^{2+}-binding proteins calreticulin, ERp57, and protein disulfide isomerase (PDI) are classical examples of this family. In fact, PDI directly interacts with calreticulin when the $[Ca^{2+}]_{ER}$ is lower than 400 μM, whereas the complex dissociates upon higher $[Ca^{2+}]$ (Baksh et al. 1995). Conversely, ERp57-calreticulin is insensitive to variations in $[Ca^{2+}]$, but it is dependent on Ca^{2+} binding by calreticulin, showing a direct role for Ca^{2+} in the regulation and maintenance of structural and functional complexes involved in protein turnover and synthesis (Michalak et al. 2009). Interestingly, another member of the PDI family, calnexin, plays a dual role based on its localization. At resting conditions, calnexin is highly palmitoylated ("palm" in Figs.), which leads to an increase of its localization at MAMs and regulation of Ca^{2+} signaling through its interaction with SERCA2b causing its activation. During the early, adaptive phases of ER stress, calnexin becomes depalmitoylated, primarily acting at the rough ER and employing its quality control functions (Lynes et al. 2012, 2013). The MAM localization of calnexin however not only depends on a specific palmitoylation event but also on the phosphorylation state of its cytosolic domain, on its interaction with phosphofurin acidic cluster sorting protein 2 (PACS2) (Myhill et al. 2008), and on the activity of the ER Rab protein Rab32 (Bui et al. 2010). The GTPase Rab32 localizes to the ER and mitochondria, and it has been identified as a regulator of MAM properties that modulate ER Ca^{2+} handling and disrupt the specific enrichment of calnexin in MAMs. However, it does not affect the ER distribution of PDI and mitofusin-2 (Bui et al. 2010).

Ca^{2+} ions are also important for the correct maintenance of ER structure. This was first reported in starfish eggs and then confirmed in human cells, where the ER continuity was affected by elevation of intracellular Ca^{2+} levels (Terasaki et al. 1996). Interestingly, this feature seemed to be regulated by protein kinase C (Ribeiro et al. 2000), which was already found to be involved in Ca^{2+} homeostasis, regulation of cellular processes, and modulation of important proteins and kinases (Pinton et al. 2007). In addition, the authors found that the high $[Ca^{2+}]$ registered did not induce ER fragmentation, only rearrangements of the ER network (Ribeiro et al. 2000). Overall, these findings suggest that Ca^{2+} dynamics regulate ER function without promoting pathological effects.

Notably, the ER continuously exchanges protein and lipid components with the Golgi apparatus, which is also regulated by Ca^{2+}. One example may be found in thyroglobulin protein that, once synthesized and modified in the ER, is exported to the Golgi apparatus (Di Jeso et al. 2003). By treating thyroid differentiated cells with Ca^{2+} ionophores or specific inhibitors of ER channels and pumps, transport between the ER and the Golgi apparatus is blocked (Di Jeso et al. 1998). Intriguingly, it is not only the transport from the ER to the Golgi that seems to be regulated by Ca^{2+} levels but also transport from the Golgi to the ER, and intra-Golgi transport is

highly dependent on intracellular Ca^{2+} variations. Moreover, the existence of a local Ca^{2+} gradient between the ER, the cytoplasm, and the Golgi apparatus has been demonstrated (Wahl et al. 1992). These findings underline the important role of intracellular Ca^{2+} in the trafficking of material between the ER and the Golgi and suggest that the mitochondrial compartment is not the only organelle likely to receive Ca^{2+} signals from the ER.

4.3 Ca^{2+} Signaling on the Mitochondrial Side

Mitochondria play a key role in many cell functions through the regulation of Ca^{2+} signaling. The increase in mitochondrial Ca^{2+} uptake activates several dehydrogenases and carriers, inducing an increase in the respiratory rate, H^+ extrusion, and ATP production necessary for the proper energy state of the cell (Rizzuto et al. 2012). As a matter of fact, overexpression of isoforms of the mitochondrial aspartate/glutamate carrier (AGC) promotes ATP production during agonist-triggered Ca^{2+} increases, revealing that AGC plays an important role in decoding Ca^{2+} signals in the activation of mitochondrial oxidative metabolism (Lasorsa et al. 2003).

However, prolonged increase in $[Ca^{2+}]_m$ leads to opening of the mitochondrial permeability transition pore (PTP) (Halestrap 2014; Jonas et al. 2015; Morciano et al. 2015), a critical event that leads to cell death by apoptosis (Rizzuto et al. 2012; Bonora et al. 2015).

As stated previously, mitochondria can rapidly achieve a high $[Ca^{2+}]_m$ due to (1) the presence of a driving force for Ca^{2+} generated by a $\Delta\Psi_m$ of −180 mV under physiological conditions, (2) the formation of a large number of Ca^{2+} microdomains at the ER-mitochondria interface, and (3) the existence of a Ca^{2+}-selective channel, termed the MCU complex, that is able to receive the Ca^{2+} signals originating from the ER (Fig. 4.2). Electrophysiological studies have shown that the uniporter is an ion channel with strikingly high conductance and selectivity (Kirichok et al. 2004). MCU is part of the uniporter holocomplex, which is also composed of two membrane proteins, MCUb and EMRE, which is regulated by MICU1 and MICU2 (Foskett and Philipson 2015; Kamer and Mootha 2015; Raffaello et al. 2016). Overexpression of MCUb reduces the amplitude of the transient mitochondrial Ca^{2+} response evoked by agonist stimulation, whereas MCUb silencing achieves the opposite effect, indicating that it acts as a dominant-negative subunit that reduces the uniporter channel activity (Raffaello et al. 2013). The role of EMRE in the regulation of MCU activity is dual: (1) it was suggested to be necessary for MCU channel activity, and indeed, its silencing abrogates Ca^{2+} entry into mitochondria (Sancak et al. 2013), and (2) it was required for the interaction of MCU with the regulatory subunits MICU1 and MICU2 (Tsai et al. 2016). Moreover, MCU complex is regulated by miRNAs (Marchi et al. 2013; Pan et al. 2015; Hong et al. 2017), underlining its role in multiple physiopathological contexts, or could be subjected to posttranslational modifications, such as phosphorylations (Joiner et al. 2012; O-Uchi et al. 2014) or methylations (Madreiter-Sokolowski et al. 2016). These recent observations confirm that mitochondrial Ca^{2+} homeostasis could be shaped by the wide molecular panel of intracellular transducers (Pinton et al. 2004).

The composition of the MCU complex has not yet been fully defined. Several proteins have been proposed to be part of it. One of the most characterized is an IMM integral protein, named MCUR1. Indeed, MCUR1 was initially shown as a regulator of the MCU complex (Mallilankaraman et al. 2012a), interacting with MCU and EMRE but not with MICU1 or MICU2, thereby functioning as a scaffolding factor (Vais et al. 2015; Tomar et al. 2016). However, MCUR1 was not identified by mass spectrometry of affinity-purified MCU complexes (Sancak et al. 2013), and it has been proposed as a cytochrome c oxidase assembly factor (Paupe et al. 2015).

The very low affinity of the MCU complex for Ca^{2+} depends on the activity of the MICU1 and MICU2 subunits, which localize at the

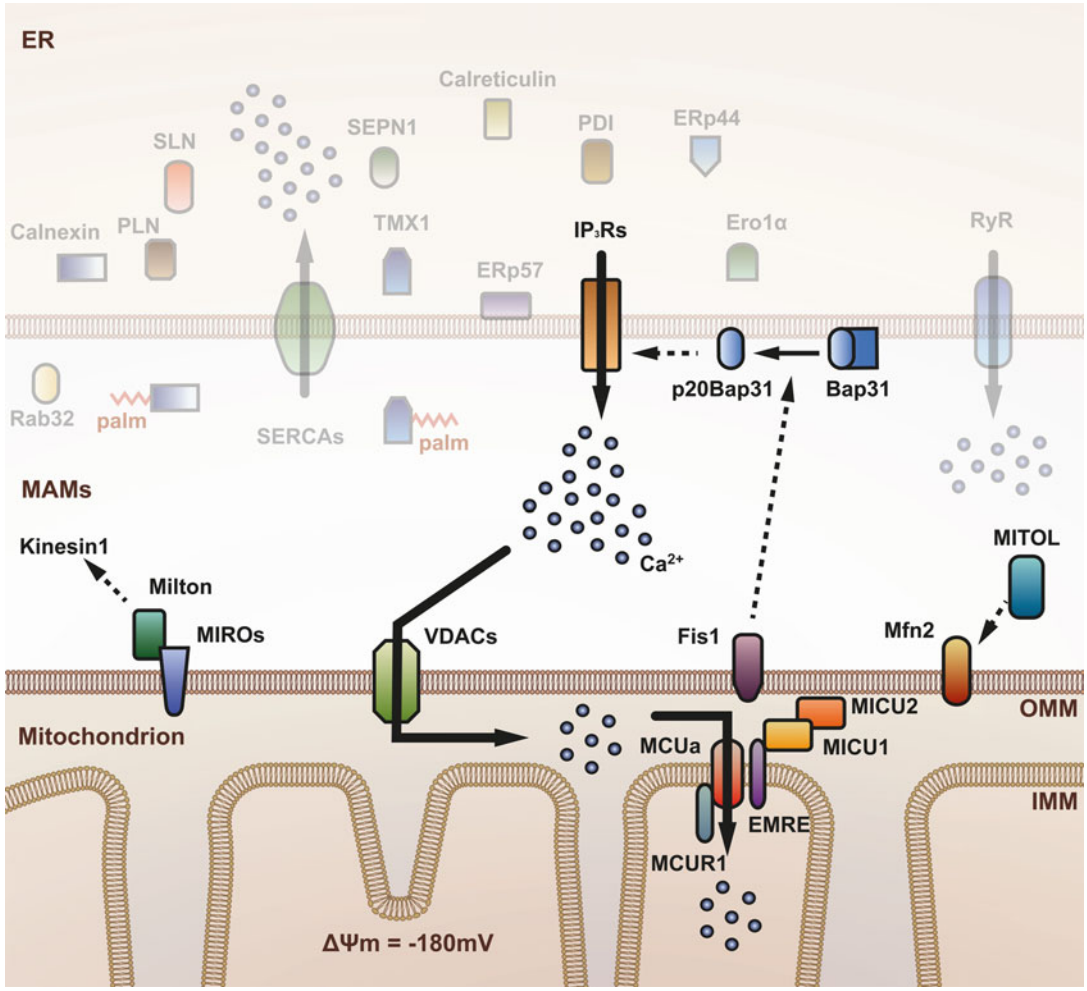

Fig. 4.2 Ca^{2+} homeostasis at the ER-mitochondria interface. Overview on the multiple molecular pathways acting at the mitochondrial side (See Sect. 4.3 for details)

mitochondrial intermembrane space and sense the cytoplasmic Ca^{2+} through their EF-hand domains, thus regulating the open/closed state of the whole uniporter complex (Patron et al. 2014). At resting conditions (cytoplasmic [Ca^{2+}]<500 nM), the MICU1-2 dimer maintains the complex in a closed state, preserving it from the continuous accumulation of Ca^{2+} inside the matrix and thus avoiding Ca^{2+}-mediated detrimental effects, such as ROS production and PTP opening (Mallilankaraman et al. 2012b; Csordas et al. 2013). However, at high cytoplasmic Ca^{2+} levels ([Ca^{2+}]>1–2 μM) or during agonist stimulation, the MICU1–2 dimer undergoes conformational changes that allow opening of the channel, ensuring a prompt and complete mitochondrial Ca^{2+} response. The "high [Ca^{2+}] microdomains theory" implies that the MCU complexes along the IMM should distribute at ER-mitochondria associations to promote effective Ca^{2+} transfer. Indeed, De La Fuente et al. recently showed that in cardiac mitochondria the MCU complexes were enriched in the IMM-OMM contact sites, positioned more to the mitochondrial periphery than inside the cristae, indicating high accessibility to cytoplasm-derived Ca^{2+} inputs (De La Fuente et al. 2016). In other words, mitochondrial Ca^{2+} channels are

close to IP_3Rs and RYRs on the ER or sarcoplasmic reticulum. Therefore, high $[Ca^{2+}]$ "hotspots" ($[Ca^{2+}]>10$ μM) can be formed transiently in these regions of close apposition between the two organelles.

The conformational coupling between the Ca^{2+} channels of the two organelles highlights the importance of the macromolecular complexes located in the MAMs for their functional interaction (Fig. 4.2). In this respect, the OMM, although traditionally considered freely permeable, is a critical determinant of mitochondrial Ca^{2+} accumulation (Rapizzi et al. 2002). Ca^{2+} import across the OMM occurs through the voltage-dependent anion channel (VDAC), the most abundant protein of the OMM. The VDAC protein family consists of three isoforms (VDAC1–3), sharing a 75% sequence similarity. VDACs are expressed in almost all mammalian tissues, but recent studies indicate their nonredundant role in a plethora of cell functions (Naghdi and Hajnoczky 2016). Silencing of either of the VDAC isoforms limits mitochondrial Ca^{2+} uptake, but only VDAC1 was found to mediate pro-apoptotic Ca^{2+} transfer to mitochondria with consequent cell death (De Stefani et al. 2012; Ben-Hail and Shoshan-Barmatz 2016). In contrast, VDAC2 appears to be the pivotal isoform to locally couple mitochondrial Ca^{2+} uptake with RyR-mediated Ca^{2+} release in cardiac cells (Shimizu et al. 2015). Further evidence of the role of VDACs was supported by the demonstration of the physical link between VDACs and the IP_3R. Indeed, the molecular chaperone glucose-regulated protein 75 (GRP75) was demonstrated to mediate the molecular interaction between VDACs and IP_3Rs, allowing a positive regulation of mitochondrial Ca^{2+} uptake (Szabadkai et al. 2006). Small interfering RNA (siRNA) silencing of GRP75 abolishes the functional coupling between IP_3Rs and VDACs, thereby reducing mitochondrial Ca^{2+} uptake in response to agonist stimulation (Szabadkai et al. 2006). Interestingly, thymocyte-expressed, positive selection-associated gene 1 (Tespa1) has been demonstrated to mediate Ca^{2+} transfer from mitochondria-associated ER to mitochondria interacting with GRP75 (Matsuzaki et al. 2013).

A crucial implication of a microdomain-based signaling mechanism is that the positioning and the shape of mitochondria within the cell become critical determinants of their responsiveness to Ca^{2+} inputs. Because of this, several mitochondrial proteins involved in the regulation of mitochondrial movement and morphology have also been considered key regulators of MAM integrity and functionality.

Mitochondrial trafficking is regulated by a subfamily of the Ras GTPases, the proteins Miro 1 and 2, which are located at the OMM through a short C-terminal anchor domain and have two EF-hand Ca^{2+}-binding domains through which they are able to sense high levels of Ca^{2+} (Liu and Hajnoczky 2009). Miro proteins have an important role in tethering the mitochondria to the cytoskeleton by binding a cytoplasmic factor, Milton, which binds the kinesin 1 heavy chain on microtubules (Glater et al. 2006). Miro is proposed to be a Ca^{2+} sensor that stops mitochondrial movement in response to increasing Ca^{2+} levels. In fact, increased cytoplasmic Ca^{2+} levels stop mitochondrial movement, and this effect is suppressed when Miro is depleted or a Miro EF-hand is mutated (Fransson et al. 2003; Saotome et al. 2008).

In addition to the positioning of mitochondria, fusion and fission events regulating the shape of the organelles drastically influenced the mitochondrial Ca^{2+} responses (Patron et al. 2013). Recent studies suggest a link between components of mitochondrial dynamics and Ca^{2+} signaling. Mitochondrial fission is primarily driven by Drp1, a cytoplasmic protein that is recruited to the mitochondrial membrane, where it circumscribes the OMM as a helical oligomer (Smirnova et al. 2001; Rowland and Voeltz 2012). It is also interesting that high Ca^{2+} levels lead to activation of Drp1, which increases mitochondrial fission, cooperating with Miro (Saotome et al. 2008).

It has also been shown that the mitochondrial fission protein fission 1 homolog (Fis1) conveys an apoptotic signal from the mitochondria to the

ER by interacting with Bap31 at the ER. Therefore, Fis1 facilitates the cleavage of Bap31 to its pro-apoptotic form, p20Bap31, promoting the recruitment of procaspase-8. Moreover, this signaling pathway establishes a feedback loop by releasing Ca^{2+} from the ER and, consequently, results in Ca^{2+} accumulation in mitochondria, amplifying cell death by activating the apoptotic pathway in many mitochondria that are in close proximity to the ER (Iwasawa et al. 2011).

Other mitochondrial dynamin-related GTPases involved in mitochondrial Ca^{2+} regulation include mitofusin 1 and 2. In particular, mitofusin 2 (MFN2) is a critical component of the mitochondrial fusion/fission machinery. This OMM profusion protein is also observed in the MAMs where it couples to MFN1 or MFN2 on the mitochondria to physically tether the organelles. Indeed, in 2008 de Brito and Scorrano showed that MFN2 is enriched at contact sites between the ER and mitochondria, regulating ER morphology and directly tethering the two organelles (de Brito and Scorrano 2008). Moreover, the distance between the ER and mitochondria increases in cells lacking MFN2, and this leads to impaired mitochondrial Ca^{2+} uptake, further verifying the validity of the Ca^{2+} microdomains theory.

The tethering role of MFN2 was confirmed by other laboratories (Chen et al. 2012; Sebastian et al. 2012; Schneeberger et al. 2013), but its function was recently challenged by different experimental approaches. Contrary to previous studies, electron microscopy analyses suggested that loss of MFN2 increased, rather than reduced, ER-mitochondria juxtaposition (Cosson et al. 2012; Filadi et al. 2015). Moreover, it was demonstrated that reduced Ca^{2+} transfer in MFN2-knockout cells is the result of a lower expression of MCU and is independent of ER-mitochondria juxtapositions (Filadi et al. 2015). Therefore, they proposed a different role for MFN2 in ER-mitochondria coupling, in which the protein, rather than being a component of the tethering complex, acts as a negative regulator of organelle apposition. However, very recently a critical reappraisal of MFN2's role in the ER-mitochondria connection was published, supporting previous results and identifying MFN2 as a physical tether between the two organelles in multiple tissues (Naon et al. 2016).

The activity of MFN2 at the ER-mitochondria interface is regulated by a mitochondrial ubiquitin ligase called MITOL (Sugiura et al. 2013). MITOL interacts with mitochondrial MFN2, but not with ER MFN2, and mediates the addition of lysine 63-linked polyubiquitin chains to MFN2 but not its proteasomal degradation. This polyubiquitination event induces MFN2 oligomerization, allowing ER-mitochondria tethering and Ca^{2+} uptake in the mitochondria upon stimulation with histamine. The reduction in mitochondrial Ca^{2+} uptake that occurs in MITOL-deficient cells highlights its key role as a MAM regulator and confirms the idea that the distance between the ER and mitochondria is crucial for proper Ca^{2+} transfer. However, the role of MFN2 at MAM is still highly debated, and new experimental evidence is required for definitively establish its anti- or pro-tethering functions.

4.4 Ca^{2+} Signaling at the MAM Interface

MAMs represent the physical association between the ER and mitochondria, an entity with a defined structure and architecture with distinct biochemical properties and a characteristic set of proteins. The MAM fraction was first separated and characterized by J. E. Vance (Vance 1990), who described the isolation from rat liver of a unique membrane, initially termed "fraction X," that was associated with mitochondria and had a high specific activity for several proteins attributed to the ER. After this seminal observation, several biochemical protocols have been described to isolate the MAMs fraction, both from organs and cells, and these studies confirmed that MAMs are composed of membrane fragments from both the ER and the OMM (Wieckowski et al. 2009).

In recent years, different proteomics studies identified the molecular components of the MAMs fraction, starting from human fibroblasts (Zhang et al. 2011a) and mouse brain (Poston et al. 2013), demonstrating that more than 1000

"MAM proteins" reside in this fraction. More recently, Sala-Vila et al. performed a very rigorous high-throughput mass spectrometry-based proteomics characterization of MAMs from mouse liver, identifying 1052 MAM-enriched proteins, which included several Ca^{2+} players, such as SERCA2, IP_3R, and the Ca^{2+}-binding mitochondrial carrier SLC25A12 (Sala-Vila et al. 2016). Interestingly, they observed the MAM localization of caveolin 1 (CAV1), and CAV1-deficient cells displayed ER and mitochondrial aberrations, as well as reduced contact sites between the two organelles (Sala-Vila et al. 2016). In line with this evidence, our group showed that in transformed cells, $H\text{-}RAS_{12V}$ expression was associated with CAV1 downregulation and a drastic alteration in Ca^{2+} homeostasis (Rimessi et al. 2014).

Other central players in the ER-mitochondria Ca^{2+} flux include a series of chaperones and oxidoreductases, which also localize to the ER/MAMs compartment (Fig. 4.3). In addition to the previously cited Ca^{2+}-binding chaperone calnexin (see Sect. 4.2), sigma-1 receptor (Sig-1R) is one of the pivotal Ca^{2+} regulators residing at MAMs (Hayashi and Su 2007). Under normal conditions, Sig-1R resides specifically at MAMs and forms a complex with BiP (also named grp78) when the ER Ca^{2+} level is 0.5–1 mM. However, when the IP_3R is activated, the subsequent drop of the $[Ca^{2+}]_{ER}$ causes the dissociation of Sig-1R from BiP, unleashing the chaperone activity of the receptor. Interestingly, IP_3R3 seems to be enriched at MAMs (Mendes et al. 2005), and its stabilization by Sig-1R ensures proper Ca^{2+} influx into mitochondria.

A link between MAMs and Ca^{2+} signaling also appears in the context of ER stress-mediated apoptosis. The RNA-dependent protein kinase (PKR)-like ER kinase (PERK), a key ER stress sensor of the unfolded protein response, is uniquely enriched at MAMs (Verfaillie et al. 2012). PERK-knockout cells display an aberrant ER morphology, disturbed Ca^{2+} signaling, and weaker contact sites between ER and mitochondria; consequently, PERK likely serves as a structural tether at the ER-mitochondria interface. Collectively, these data highlight that a conserved MAM structure is indispensable for transmitting Ca^{2+} signaling as well as ROS-mediated signals to the mitochondria after ROS-based ER stress.

Ca^{2+} homeostasis is fundamental for numerous cellular mechanisms, including cell death; thus, it is not surprising that several oncogenes and tumor suppressors localize at the MAMs, where they play a crucial role in the control of ER-mitochondria Ca^{2+} flux, favoring either survival or cell death (Bittremieux et al. 2016) (Fig. 4.3).

A few years ago, our group showed that the promyelocytic leukemia protein (PML), a tumor suppressor known as an essential component of nuclear structures termed PML nuclear bodies, was localized to the ER and MAMs. PML regulates apoptosis by modulating ER Ca^{2+} release (Giorgi et al. 2010). In MAMs, PML was found to coordinate a complex that includes IP_3R3, Akt kinase, and phosphatase PP2A. In the absence of PML, Akt phosphorylation and activity was increased at the ER due to impaired PP2A activity, which resulted in impaired Ca^{2+} flux through the IP_3R because of its Akt-mediated hyperphosphorylated state. Indeed, IP_3R is a target of Akt kinase activity (Khan et al. 2006; Szado et al. 2008), and Akt activation drastically reduces IP_3R-dependent ER Ca^{2+} release (Marchi et al. 2008; Szado et al. 2008), especially through IP_3R3 phosphorylation (Marchi et al. 2012). These data have been confirmed by an independent study, which reported the localization of mechanistic target of rapamycin (mTOR) complex 2 (mTORC2) at the MAMs (Betz et al. 2013). The serine/threonine kinase (mTOR) is a pivotal regulator of autophagy and exists in two protein complexes, mTORC1 and mTORC2. The latter complex phosphorylates and activates Akt, which phosphorylates MAMs-resident proteins PACS2, IP_3R, and hexokinase 2 (HK2) to regulate MAMs integrity, Ca^{2+} flux, and energy metabolism, respectively (Betz et al. 2013).

More recently, we demonstrated that MAMs-enriched PML also exerts an important Ca^{2+}-dependent role in the autophagic process, through the AMPK/mTOR/Ulk1 pathway (Missiroli et al. 2016). We overexpressed MCU

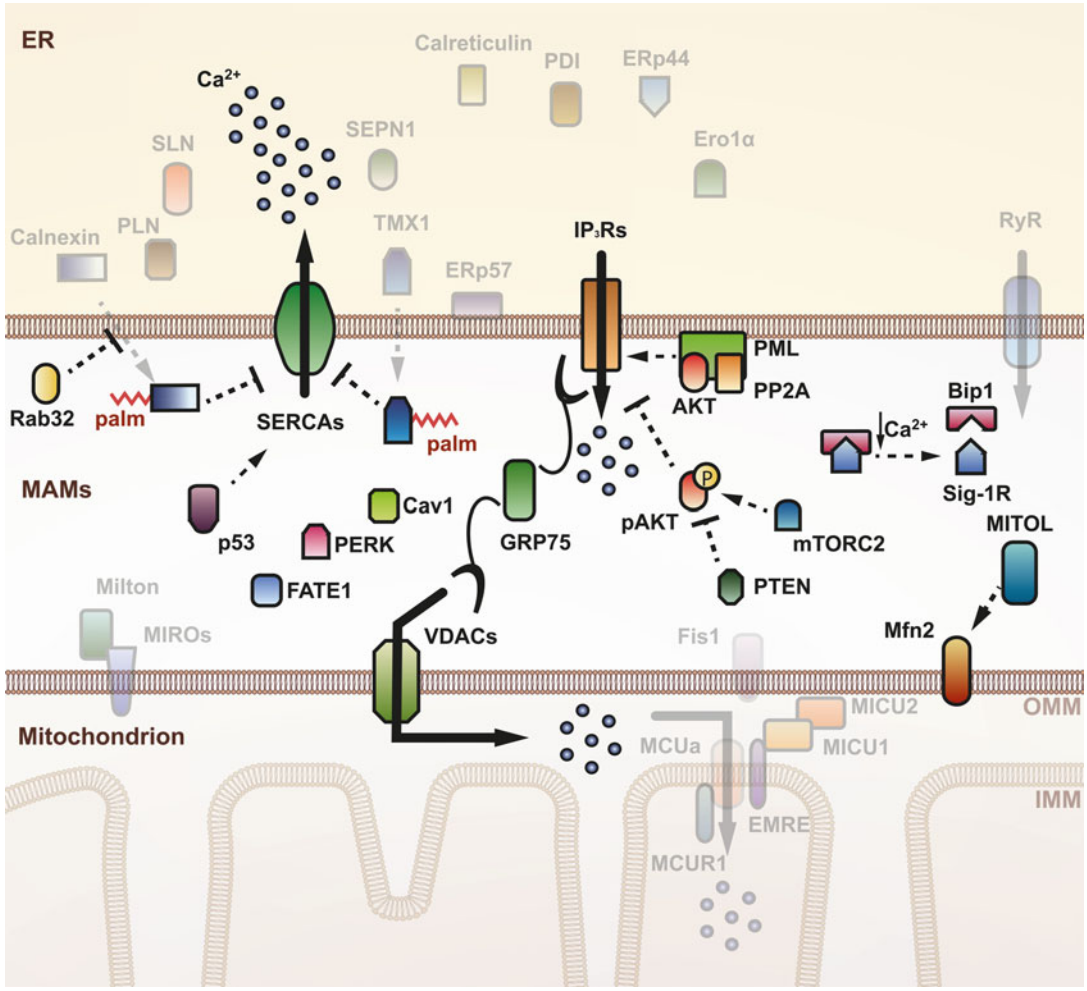

Fig. 4.3 Ca^{2+} homeostasis at the ER-mitochondria interface. Overview on the multiple molecular pathways acting at the MAMs side (See Sect. 4.4 for further details)

in PML-KO cells to verify whether downregulated ER-mitochondria Ca^{2+} transfer is important for the induction of autophagy. We demonstrated that increasing the ability of mitochondria to accumulate Ca^{2+} in PML-KO cells suppressed AMPK activity, thereby repressing autophagic flux. These data suggest that PML controls autophagy at MAMs through its effects on Ca^{2+} homeostasis and that the loss of PML from MAMs results in autophagy activation, a feature that promotes cell survival under stress conditions and thus facilitates malignant cell growth.

Among tumor suppressors, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) was found to be localized at the ER and MAMs, where it modulates Ca^{2+} transfer from ER to mitochondria in a protein phosphatase-dependent manner that counteracts the Akt-mediated reduction in Ca^{2+} release via IP_3Rs (Bononi et al. 2013). Moreover, the tumor suppressor p53 also resides at ER and MAMs, modulating ER Ca^{2+} efflux to the mitochondria through modulation of the oxidative state of SERCA pumps (Giorgi et al. 2015b; Giorgi et al. 2015a).

Very recently FATE1, a cancer-testis antigen, has been implicated in the regulation of ER-mitochondria distance and Ca^{2+} uptake by mitochondria (Doghman-Bouguerra et al. 2016). FATE1 is localized at the interface between the ER and mitochondria and decreases sensitivity to mitochondrial Ca^{2+}-dependent pro-apoptotic stimuli and to chemotherapeutic drugs. This study emphasized how the ER-mitochondria uncoupling activity of FATE1 is harnessed by cancer cells to escape apoptotic death and resist the action of chemotherapeutic drugs.

Taken together, these observations highlight the role of ER-dependent Ca^{2+} release as a general mediator in many cell deaths or cell survival scenarios and reinforce the importance of MAMs in Ca^{2+} handling.

4.5 Conclusions

The importance of MAMs in the control of various cellular processes and its relevance for human health is underpinned by the disease that has linked to dysregulation and dysfunction of the ER-mitochondria interface and architecture. These diseases include obesity (Arruda et al. 2014) and type II diabetes (Tubbs et al. 2014), as well as Parkinson's and Alzheimer's diseases (Paillusson et al. 2016). MAM disorganization results in abnormal ER-mitochondria Ca^{2+} flux, which contributes to the formation of aberrant mitochondrial structures and deep metabolic alterations that are typical features of these pathological conditions. Based on the recent observations suggesting an optimal distance of 30–85 nm between IP_3R and the MCU complex to achieve effective Ca^{2+} transfer and generation of Ca^{2+} inputs (Qi et al. 2015), we need novel technologies that enable an accurate and highly precise measurement of the functional changes occurring at MAMs to elucidate the local Ca^{2+} transport and signaling mechanisms.

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