Chapter 17
Mitochondria-Associated Membranes (MAMs) as Hotspot Ca^{2+} Signaling Units

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Abstract The tight interplay between endoplasmic reticulum (ER) and mitochondria is a key determinant of cell function and survival through the control of intracellular calcium (Ca^{2+}) signaling. The specific sites of physical association between ER and mitochondria are known as mitochondria-associated membranes (MAMs). It has recently become clear that MAMs are crucial for highly efficient transmission of Ca^{2+} from the ER to mitochondria, thus controlling fundamental processes involved in energy production and also determining cell fate by triggering or preventing apoptosis. In this contribution, we summarize the main features of the Ca^{2+}-signaling toolkit, covering also the latest breakthroughs in the field, such as the identification of novel candidate proteins implicated in mitochondrial Ca^{2+} transport and the recent

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direct characterization of the high-Ca^{2+} microdomains between ER and mitochondria. We review the main functions of these two organelles, with special emphasis on Ca^{2+} handling and on the structural and molecular foundations of the signaling contacts between them. Additionally, we provide important examples of the physiopathological role of this cross-talk, briefly describing the key role played by MAMs proteins in many diseases, and shedding light on the essential role of mitochondria-ER interactions in the maintenance of cellular homeostasis and the determination of cell fate.

Keywords Akt • Apoptosis • Bap31 • Bip • Ca^{2+} signaling • Calcium ions • Endoplasmic Reticulum • Ero1α • ERp44 • GM1-ganglioside • grp75 • IP3Rs • MCU • Microdomains • MICU1 • Mitochondria • Mitochondria-Associated Membranes • Mitofusin-1 and -2 • p66Shc • PACS-2 • Plasma Membrane Associated Membranes • PML • PP2a • Presenilin-1 and -2 • Sig-1R • VDAC

Abbreviations

$\Delta\Psi_m$	Mitochondrial membrane potential difference
AD	Alzheimer's disease
ANT	Adenine nucleotide translocase
Bap31	(B-cell receptor-associated protein 31)
BFP	Blue fluorescent protein
BiP	Binding immunoglobulin Protein
Ca^{2+}	Calcium ions
$[Ca^{2+}]$	Ca^{2+} concentration
$[Ca^{2+}]_c$	Cytosolic Ca^{2+} concentration
$[Ca^{2+}]_m$	Mitochondrial Ca^{2+} concentration
CABPs	Intraluminal Ca^{2+}-binding proteins
CaMKII	Calmodulin-dependent protein kinase II
CCE	Capacitative Ca^{2+} entry
Cyp D	Cyclophilin D
Drp1	Dynamin-related protein 1
ER	Endoplasmic reticulum
ERp44	(Endoplasmic reticulum resident protein 44)
FACL4	Long-chain fatty acid-CoA ligase type 4
FAD	Familial Alzheimer's disease
Fhit	Fragile histidine triad
Fis1	Fission 1 homologue
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GM1	GM1-ganglioside
grp75	Glucose-regulated protein 75
HK	Hexokinase

IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IP3	Inositol 1,4,5-trisphosphate
IP3R	Inositol 1,4,5-trisphosphate receptor
Letm1	Leucine zipper-EF-hand containing transmembrane protein 1
MAMs	Mitochondria-associated membranes
MCU	Mitochondrial Ca^{2+} uniporter
MICU1	Mitochondrial calcium uptake 1
Mfn	Mitofusin
mHCX	Mitochondrial H^+/Ca^{2+} exchanger
MMP	Mitochondrial membrane permeabilization
mNCX	Mitochondrial Na^{2+}/Ca^{2+} exchanger
MOMP	Mitochondrial outer membrane permeabilization
NADH	Nicotinamide adenine dinucleotide
NCX	Na^{2+}/Ca^{2+} exchanger
NE	nuclear envelope
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
p66shc	66-kDa isoform of the growth factor adapter shc
PACS-2	Phosphofurin acidic cluster sorting protein 2
PAMs	Plasma membrane associated membranes
PDH	Pyruvate dehydrogenase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMCA	Plasma membrane Ca^{2+} ATPase
PML	Promyelocytic leukemia protein
PP2a	Protein phosphatase 2a
PS1	Presenilin-1
PS2	Presenilin-2
PSS-1	Phosphatidylserine synthase-1
PTP	Permeability transition pore
ROCs	Receptor operated Ca^{2+} channels
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SERCA	Sarco-endoplasmic reticulum Ca^{2+} ATPase
Sig-1R	Sigma-1 receptor
SMOCs	Second messenger operated Ca^{2+} channels
SR	Sarcoplasmic reticulum
TIRF	Total internal reflection fluorescence
TpMs	Trichoplein/Mitostatin
UCP	Uncoupling protein
VDAC	Voltage-dependent anion channel
VOCs	Voltage operated Ca^{2+} channels.

The Ca^{2+}-Signaling Toolkit

Calcium ions (Ca^{2+}) are ubiquitous intracellular messengers that can set up and/or regulate many different cellular functions, including gene expression, cellular contraction, secretion, synaptic transmission, metabolism, differentiation and proliferation, as well as cell death. The universality of Ca^{2+}-based signaling depends on its enormous versatility in terms of amplitude, duration, frequency and localization. The formation of the correct spatio-temporal Ca^{2+} signals is dependent on an extensive cellular machinery named the Ca^{2+} toolkit, which includes the various cellular Ca^{2+}-binding and Ca^{2+}-transporting proteins, present mainly in the cytosol, plasma membrane, endoplasmic reticulum (ER), and mitochondria [1].

The resting cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) is maintained around the value of 100 nM, significantly lower than extracellular $[Ca^{2+}]$ (1 mM). This condition is achieved through active extrusion of Ca^{2+} by the plasma membrane Ca^{2+} ATPase (PMCA) and the Na^+/Ca^{2+} exchanger (NCX) [2, 3]. The increase of intracellular $[Ca^{2+}]$ can be elicited by two fundamental mechanisms (or a combination of both). The first involves Ca^{2+} entry from the extracellular milieu, through the opening of plasma membrane Ca^{2+} channels (traditionally grouped into three classes: voltage operated Ca^{2+} channels (VOCs) [4], receptor operated Ca^{2+} channels (ROCs) [5] and second messenger operated Ca^{2+} channels (SMOCs) [6]); the second mechanism involves Ca^{2+} release from intracellular stores, mainly the ER and its specialized form in muscle, the sarcoplasmic reticulum (SR). In these intracellular stores, two main Ca^{2+}-release channels exist that, upon stimulation, release Ca^{2+} into the cytosol, thus triggering Ca^{2+} signaling: the inositol 1,4,5-trisphosphate (IP3) receptors (IP3Rs) and the ryanodine receptors (RyRs) [7, 8]. IP3Rs are ligand-gated channels that function in releasing Ca^{2+} from ER Ca^{2+} stores in response to IP3 generation initiated by agonist binding to cell-surface G protein-coupled receptor [9, 10]. The subsequent rise in $[Ca^{2+}]_c$ results in various Ca^{2+}-dependent intracellular events. The exact cellular outcome depends on the spatiotemporal characteristics of the generated Ca^{2+} signal [11]. Once its downstream targets are activated, basal $[Ca^{2+}]_c$ levels are regained by the combined activity of Ca^{2+} extrusion mechanisms, such as PMCA and NCX, and mechanisms that refill the intracellular stores, like sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCAs) [2]. Due to SERCA activity and intraluminal Ca^{2+}-binding proteins (CABPs), *i.e.*, calnexin and calreticulin [12], the ER can accumulate Ca^{2+} more than a thousand-fold excess as compared to the cytosol.

While the role of the ER as a physiologically important Ca^{2+} store has long been recognized, a similar role for mitochondria have seen a reappraisal only in the past two decades [13]. The studies of Rizzuto, Pozzan and colleagues revealed that IP3-mediated Ca^{2+} release from the ER results in cytosolic Ca^{2+} increases that are accompanied by similar or even larger mitochondrial ones [14], driven by the large electrochemical gradient (mitochondrial membrane potential difference, $\Delta\Psi_m = -180$ mV, negative inside) generated by the respiratory chain [15]. The uptake of the Ca^{2+} ions into the mitochondrial matrix implies different transport systems

responsible for the transfer of Ca^{2+} across the outer and the inner mitochondrial membrane (OMM and IMM respectively). Despite the surprisingly low affinity of the mitochondrial uptake systems (Kd around 10–20 μM) and the submicromolar global $[Ca^{2+}]_c$ (which rarely exceed 2–3 μM) evoked by IP3-mediated Ca^{2+} release, mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) can undergo rapid changes upon cell stimulation, because their low affinity uptake systems are exposed to microdomains of high $[Ca^{2+}]$ in proximity to ER or plasma membrane Ca^{2+} channels [16–18]. The hypothesis, called "microdomain hypothesis" [19], was initially supported by a large body of indirect evidence, and its direct determination was carried out only very recently by two complementary studies that demonstrated the existence and amplitude of high Ca^{2+} microdomains on the surface of mitochondria. Giacomello et al. [20] targeted a new generation of FRET-based Ca^{2+} sensors [21] to the OMM and, through a sophisticated statistical analysis of the images, revealed the existence of small OMM regions whose $[Ca^{2+}]$ reaches values as high as 15–20 μM. The probe detected Ca^{2+} hotspots on about 10% of the OMM surface that were not observed in other parts of the cell. The Ca^{2+} hotspots were not uniform, and their frequency varied among mitochondria of the same cell. Moreover, classical epifluorescence and total internal reflection fluorescence (TIRF) microscopy experiments were combined in order to monitor the generation of high Ca^{2+} microdomains in mitochondria located near the plasma membrane. With this approach, it could be shown that Ca^{2+} hotspots on the surface of mitochondria occur upon opening of VOCs, but not upon capacitative Ca^{2+} entry (CCE). Csordás et al. [22] used a complementary approach in which they generated genetically encoded bifunctional linkers consisting of OMM and ER targeting sequences connected through a fluorescent protein, including a low-Ca^{2+}-affinity pericam, and coupled with the two components of the FKBP-FRB heterodimerization system [23], respectively. Using rapamycin-assembled heterodimerization of the FKBP-FRB-based linker, they detected ER/OMM and plasma membrane/OMM junctions (the latter at a much lower frequency). In addition, the recruited low-Ca^{2+}-affinity pericam reported Ca^{2+} concentrations as high as 25 μM at the ER/OMM junctions in response to IP3-mediated Ca^{2+} release, which is in excellent agreement with the values obtained by Giacomello et al..

The Ca^{2+}-import system across the OMM occurs through the so-called voltage-dependent anion channels (VDAC) [24], traditionally considered as a large voltage-gated channel, fully opened with high-conductance and weak anion-selectivity at zero and low transmembrane potentials (<20–30 mV), but switching to cation selectivity and lower conductance at higher potentials (the so-called "closed" state) [25–27]. In contrast, the molecular identity of the IMM Ca^{2+}-transport system, the mitochondrial Ca^{2+} uniporter (MCU), has been identified only very recently, preceded last year by the discovery of mitochondrial calcium uptake 1 (MICU1), an uniporter regulator which appears essential for mitochondrial Ca^{2+} uptake [28]. MICU1 has been identified in silico in the MitoCarta database [29]; it is a single-pass transmembrane protein which does not seem to participate in channel pore formation, so it is not known whether it actually forms (part of) a Ca^{2+} channel, or functions as Ca^{2+} buffer, or as a Ca^{2+}-dependent regulatory protein acting as a Ca^{2+}

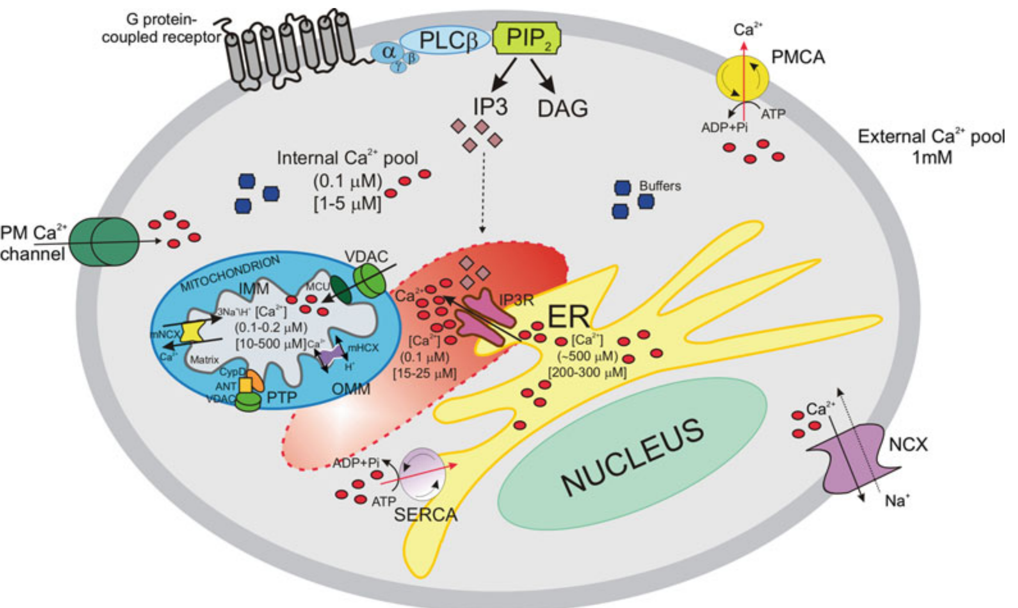

Fig. 17.1 Intracellular Ca^{2+} signaling. Schematic model of intracellular Ca^{2+} homeostasis. Plasma membrane G-protein coupled receptors activate phospholipase C-β (PLC-β) to promote the generation of inositol 1,4,5-trisphosphate (IP3) and the release of Ca^{2+} from the endoplasmic reticulum (ER) into the cytosol. Mitochondrial surface directly interacts with the ER through contact sites defining hotspots Ca^{2+} signaling units. Ca^{2+} import across the outer mitochondrial membrane (OMM) occurs by the voltage-dependent anion channel (VDAC), and then enters the matrix through the mitochondrial Ca^{2+} uniporter (MCU), the main inner mitochondrial membrane (IMM) Ca^{2+}-transport system (Ca^{2+} levels reached upon stimulation are indicated in square brackets). Mitochondrial Ca^{2+}exchangers present in the IMM export Ca^{2+} from the matrix once mitochondrial Ca^{2+} has carried its function; another mechanism for Ca^{2+} efflux from mitochondria is the permeability transition pore (PTP). Ca^{2+} levels return to resting conditions (indicated in round brackets) through the concerted action of cytosolic Ca^{2+} buffers, plasma membrane Ca^{2+}-ATPase (PMCA) and the Na^+/Ca^{2+} exchanger (NCX) that permit the ion extrusion in the extracellular milieu. Sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) restablishes basal Ca^{2+} levels in intracellular stores. *ANT* adenine nucleotide translocase, *Cyp D* cyclophilin D, *DAG* diacylglycerol, *PIP_2* phosphatidylinositol 4,5-bisphosphate

sensor (it has a pair of Ca^{2+}-binding EF-hand domains, the mutation of which eliminates the mitochondrial Ca^{2+} uptake). Then, this year, two independent papers identified the same protein, termed CCDC109A and renamed MCU, as the channel responsible for ruthenium-red-sensitive mitochondrial Ca^{2+} uptake. This protein shares the same tissue distribution with its regulator MICU1, and possesses two predicted transmembrane helices, which are separated by a highly conserved linker facing the intermembrane space. Just the protein's orientation is the mainly discrepancy between the two papers, one affirming a C-terminus localization in the intermembrane space [30], the other in the matrix [31]. Further experiments have to be performed to solve this question. Interestingly, MCU can form multimers and blue native gel separation experiment shows how MCU migrates as a large complex, with an apparent molecular weight of 40 kDa [31].

In the IMM are also present the mitochondrial Na^+/Ca^{2+} exchanger (mNCX) and the H^+/Ca^{2+} exchanger (mHCX). Their main function is probably to export Ca^{2+} from the matrix, once mitochondrial Ca^{2+} has carried out its function, to reestablish resting conditions [32]. They have yet to be identified, although recently strong evidence has been provided that the Na^+/Ca^{2+} exchanger isoform NCLX is the long-sought protein responsible for the mitochondrial Na^+-dependent Ca^{2+} efflux [33]. Finally, the low conductance mode of the permeability transition pore (PTP), a channel of still debated nature localized in the IMM [34], can be also considered as a non-saturating mechanism for Ca^{2+} efflux from mitochondria. When open, PTP allows the passage of ions and molecules with a molecular weight up to 1.5 kDa, including Ca^{2+}. Short-time openings may have a physiological function but its long-time activation leads to the demise of the cell, either by apoptosis or by necrosis, depending on whether PTP opening occurs in only a small fraction of the mitochondria or in all of them [35, 36].

The many efforts to better understand the Ca^{2+} toolkit and the role played by the relationship between ER and mitochondria in this elaborate signaling, are yielding a deeper understanding of how aberrant Ca^{2+} homeostasis is implicated in many diseases. A schematic view of the various processes described above is presented in Fig. 17.1.

Mitochondrial Functions and Ca^{2+} Handling

Mitochondrial Ca^{2+} homeostasis has a key role in the regulation of aerobic metabolism and cell survival.

The first role assigned to the Ca^{2+} ions taken up into the mitochondrial matrix was the stimulation of the mitochondrial ATP production since important metabolic enzymes localized in the matrix, the pyruvate-, α-ketoglutarate- and isocitrate-dehydrogenases are activated by Ca^{2+}, with different mechanisms: the first through a Ca^{2+}-dependent dephosphorylation step, the others via direct binding to a regulatory site [37, 38]. Those three enzymes represent rate-limiting steps of the Krebs cycle thus controlling the feeding of electrons into the respiratory chain and the

generation of the proton gradient across the inner membrane, in turn necessary for ATP production through oxidative phosphorylation (OXPHOS). As the ATP produced by mitochondria is subsequently transferred to the cytosol, mechanisms that control ATP production will not only affect overall cell life but, more specifically, will regulate the activity of ATP-sensitive proteins localized in the close vicinity of mitochondria, such as IP3Rs and SERCA which are stimulated by ATP [39, 40]. The bidirectional relation between Ca^{2+} release and ATP production allows for a positive feedback regulation between ER and mitochondria during increased energetic demand [41]. The uptake of Ca^{2+} in mitochondria will also affect Ca^{2+} signaling at both the local and the global level. Assuming the microdomain concept [16, 17], the local $[Ca^{2+}]$ will depend on both the amount of Ca^{2+} released by IP3Rs and that taken up by mitochondria. Since both SERCA pumps and IP3Rs are also regulated by Ca^{2+}, the local $[Ca^{2+}]$ in the vicinity of mitochondria will determine the refilling of the ER and eventually the spatiotemporal characteristics of the subsequent Ca^{2+} signals [42]. This will in turn depend on the efficiency of the coupling between the ER and the mitochondrial network, as well as on the exact subcellular localization of mitochondria [43].

The connection between mitochondria and the ER can be highly dynamic as the local Ca^{2+} concentration can also affect mitochondrial motility and ER–mitochondria associations in various ways [44]. Mitochondrial movement may increase the chance of dynamic interactions between organelles and aid in the transportation of molecules between the cytoplasm and the organelle. Proteins involved in mitochondrial movement along microtubules, dynein and kinesin, are prone to high $[Ca^{2+}]_c$ mediated by a Ca^{2+} sensor. Moreover, as the mitochondrial motility is inhibited by Ca^{2+} levels in the low micromolar range, it means that mitochondria will be trapped in the neighbourhood of active Ca^{2+}-release sites, allowing for a more efficient uptake of Ca^{2+} by these mitochondria [45, 46].

Apart from organelles movement, mitochondria also continuously remodel their shape. Many of the gene products mediating the fission and fusion processes have been identified in yeast screens, and most are conserved in mammals, including the fission mediators dynamin-related protein 1 (Drp1, Dnm1 in yeast) and Fis1 (Fission 1 homologue), as well as the fusion mediators mitofusins (Mfn) 1 and 2 (Fzo1 in yeast) and optic atrophy 1 (OPA1, Mgm1 in yeast) [47]. Several previous studies have indicated that elevation of $[Ca^{2+}]_c$ perturbs mitochondrial dynamics [48], and more recent works have clearly demonstrated that mitochondrial shape can be controlled by an ER-dependent signaling pathway [49, 50]. Mitochondria also undergo a more "macroscopic" remodelling of their shape during programmed cell death: after apoptosis induction, mitochondria become largely fragmented, resulting in small, rounded and numerous organelles. However, the relationship between mitochondrial fusion/fission and apoptosis is complex and mitochondrial fragmentation is not necessarily related to apoptosis [51]. Defects in fusion and fission processes are not to be underestimated, as they may have deleterious consequences on bioenergetic parameters and are likely to contribute to the pathogenesis of neurodegenerative diseases [52].

The mitochondrial Ca^{2+} signal also control the choice between cell survival and cell death, as it can participate in the induction and progression of apoptosis [53, 54]. Although the extrinsic pathway for apoptosis may or may not involve mitochondria, in the intrinsic pathway these organelles perform a pivotal role: they can release a number of proapototic factors - such as cytochrome *c*, apoptosis-inducing factor, Smac/Diablo, HtrA2/Omi and endonuclease G – from the intermembrane space (IMS) to the cytosol, which initiate the executor caspase cascade steering the cell towards the execution phase of apoptosis (for a review see [55]). Ca^{2+} uptake in mitochondria is crucial for multiple important cellular functions, but the risk of mitochondrial Ca^{2+} overload exists, and this may result in the induction of cell death. At a high concentration, mitochondrial Ca^{2+} favours the opening of the PTP, a mitochondrial megachannel likely to be located in the inner-outer contact sites of the mitochondrial membranes [35]. This event, also known as mitochondrial membrane permeabilization (MMP), leads to the subsequent release of various apoptogenic factors [56, 57]. MMP can also result from a distinct, yet partially overlapping process known as mitochondrial outer membrane permeabilization (MOMP) [55]. In MOMP, pro-apoptotic members of the Bcl-2 protein family may form protein-permeable pores in the OMM, and consequently release of IMS proteins into the cytosol. Bcl-2 family members function as regulators of Ca^{2+} signaling will be discussed later in this review (the interested reader should also refer to [58]).

Mitochondria are also an important source of ROS produced during OXPHOS. ROS can locally affect other systems, including Ca^{2+}-signaling mechanisms, and increased levels of ROS within mitochondria are the principal trigger not only for mitochondrial dysfunctions but, more generally, for diseases associated with ageing. One of the key regulators of ROS production, mitochondrial dysfunction, and ageing is the 66-kDa isoform of the growth factor adapter shc (p66shc) [59]. The mechanisms by which p66shc increases intracellular ROS levels, inducing apoptosis and the deleterious effects of ageing have recently been clarified by Pinton et al.. Once imported into mitochondria, p66Shc causes alterations of organelle Ca^{2+} responses and three-dimensional structure, thus inducing apoptosis [60].

ER Functions and Ca^{2+} Handling

The ER is possibly the largest individual intracellular organelle comprising a three dimensional network of endomembranes arranged in a complex grid of microtubules and cisternae. It is made up of functionally and structurally distinct domains (reviewed extensively by a number of authors [61–64]), in relation to the variety of cellular functions played by the organelle, primarily concerning protein synthesis, maturation and delivery to their destination [65, 66]. Moreover, the ER is a dynamic reservoir of Ca^{2+} ions, which can be activated by both electrical and chemical cell stimulation [67, 68] making this organelle an indispensable component of Ca^{2+} signaling [69–71].

Modern analysis methods enabled the determination of the molecular profile of the ER. This profile reflects the ER's role in signaling, as it comprises a number of components constituting the Ca^{2+} signaling pathway. It contains IP3Rs, RyRs, SERCAs, and in addition to these release channels and pumps, there are buffers (calnexin, calreticulin) and a number of ancillary proteins (FK 506-binding proteins, sorcin, triadin, phosholamban) that contribute to the ER Ca^{2+} signaling system [72].

The IP3Rs are activated after cell stimulation and play a crucial role in the initiation and propagation of the complex spatio-temporal Ca^{2+} signaling that control a myriad of cellular processes [73]. To achieve these various functions, often in a single cell, exquisite control of the Ca^{2+} release is needed. Ca^{2+} itself regulates channel activity in a biphasic manner: at low [Ca^{2+}], the ion exerts an activating role while, at high [Ca^{2+}], it has an opposite inhibitory effect, thus providing a fine dynamic feedback regulation during Ca^{2+} release [74]. In addition, also the ER Ca^{2+} content retains the capability to regulate the channel opening [75, 76]. Whereas IP3 and Ca^{2+} are essential for IP3R channel activation, other physiological ligands, such as ATP, are not necessary but can finely modulate the Ca^{2+}-sensitivity of the channel [77]. As for Ca^{2+}, the modulation of IP3R by ATP is biphasic: at micromolar concentrations, ATP exerts a stimulatory effect, while inhibiting channel opening in the millimolar range [78, 79]. Moreover, IP3R isoforms contain on their sequences multiple phosphorylation consensus sites and many docking sites for protein kinases and phosphatases. Currently, at least 12 different protein kinases are known to directly phosphorylate the IP3R [80], among them: Akt [81], protein kinase A (cAMP-dependent) [82], protein kinase G (cGMPdependent) [83], calmodulin-dependent protein kinase II (CaMKII) [84], protein kinase C (PKC) [85], and various protein tyrosine kinases [86].

Despite controlling many processes essential for life, Ca^{2+} arising from the ER can be a potent death-inducing signal [87, 88]. A clear impetus in the study of Ca^{2+} homeostasis in apoptosis came from the observation that important regulators of apoptosis, the proteins of the Bcl-2 family, are localized to ER and mitochondria, organelles deeply involved in Ca^{2+} handling. The role of the ER in supporting the mitochondrial apoptosis pathway is demonstrated by several findings, among which: (i) over-expression of anti-apoptotic proteins, such as Bcl-2, reduce the ER Ca^{2+} level, making the cells resistant to apoptosis [89–92]; (ii) genetic ablation of the pro-apoptotic proteins Bax and Bak (that drastically increases the resistance to death signals) also results in a dramatic reduction in ER Ca^{2+} content and consequently in a reduction of the Ca^{2+} that can be transferred to mitochondria [93, 94]; (iii) several different approaches resulting in decreases of ER Ca^{2+} content protect cells from apoptosis while, conversely, an increase in Ca^{2+} within the ER favours apoptosis triggered by a number of stimuli [95].

Hence, IP3R-mediated release of Ca^{2+} from ER appears to be a key sensitizing step in various apoptotic routes, but the precise molecular definition of this process still awaits a fine clarification of the macromolecular complex assembled at the interphase between the two organelles. As will be discussed shortly, significant research efforts have been made to shed some light on this signaling pathway.

ER and Mitochondria Physically and Functionally Interact at MAMs

Intracellular organelles coordinate complex pathways for signal transduction and metabolism in the cell through their functional or physical interactions with one another. The association between ER and mitochondria was first described by Copeland and Dalton over 50 years ago in pseudobranch gland cells [96]. By the beginning of the 1970s, the contacts between mitochondria and ER had been visualized by several groups [97, 98]. Electron micrograph images of quickly frozen samples [99] and experiments in living cells with the two organelles labelled by means of targeted spectral variants of GFP (mtBFP and erGFP) [17] demonstrated conclusively that such physical interactions between the two organelles indeed exist. These experiments revealed the presence of overlapping regions of the two organelles and allowed to estimate the area of the contact sites as 5–20% of the total mitochondrial surface. The distance between the ER and the OMM was originally estimated to be approximately 100 nm [100, 101]. More detailed morphological studies, carried out by Achleitner et al. in 1999, indicated that the distance between the ER and mitochondria in the areas of interaction varied between 10 and 60 nm [102]. Importantly, a direct fusion between membranes of the ER and mitochondria was not observed in any case, and the membranes invariably maintained their separate structures. The authors of this pioneering paper proposed that a distance of less than 30 nm between the two organelles could be considered as an association. More recently, electron tomography techniques allowed to estimate that the minimum distance is even shorter (e.g., 10–25 nm) [103]. This distance thus enables ER proteins to associate directly with proteins and lipids of the OMM. Further development of microscopic techniques enabled detailed analysis of such contacts with high resolution in three dimensions [104].

The interactions between these organelles at the contact sites are so tight and strong, that upon subcellular fractionation (at the step of mitochondria purification), a unique fraction, originally named "mitochondria-associated membranes" (MAMs) fraction, can be isolated [105, 106]. More recently, the isolation procedures was improved and adapted to isolate the MAMs fraction from yeast, different organs, tissues, and various cell lines [102, 107, 108]. Interestingly, the molecular analysis of both "crude" mitochondria and MAMs fractions demonstrated that, apart from specific ER and mitochondrial proteins, they also contain proteins which are abundant in the plasma membrane.

Research on the morphological organization of mitochondria and ER with respect to the plasma membrane is much less extensive. Modifications in the subcellular fractionation procedure enabled the isolation of the "plasma membrane associated membranes" (PAMs) fraction. In general, PAMs fractions have been described as the center of interactions between plasma membrane and the ER [109, 110], but the presence of mitochondrial proteins in these fractions indicates that mitochondria interact actively also with the plasma membrane [111, 112].

The MAMs have a pivotal role in several cellular functions related to bioenergetics and cell survival. MAMs have been originally shown to be enriched in enzymes

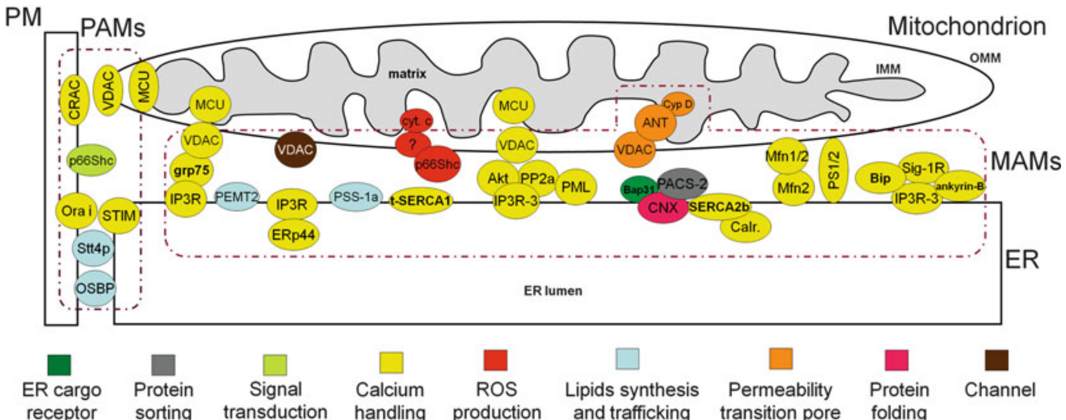

Fig. 17.2 Schematic view of the interorganelle interactions and protein composition of the membranes contact sites. *Possible contact sites between organelles are marked in dotted brown line. ER* endoplasmic reticulum, *ER* lumen endoplasmic reticulum lumen, *IMM* inner mitochondrial membrane, *MAMs* mitochondria-associated membranes, *OMM* outer mitochondrial membrane, *PAMs* plasma membrane associated membranes, *PM* plasma membrane.*The color indicates the function/role of the protein.* Akt, the serine-threonine protein kinase Akt; *ANT* adenine nucleotide translocase, *Bap31* B-cell receptor-associated protein 31 (or endoplasmic reticulum resident cargo receptor), *Calr* carleticulin, *CRAC* Ca^{2+} release-activated calcium channel, *Cyp D* cyclophilin D, *cyt. c* cytochrome c, *ERp44* endoplasmic reticulum resident protein 44, *grp75* glucose-regulated protein 75 (or mortalin), *BiP* Binding immunoglobulin Protein (or 78 kDa glucose-regulated protein (GRP78)), *IP3R* inositol 1,4,5-triphosphate receptor, *MCU* mitochondrial calcium uniporter, *Mfn1/2* mitofusin-1/2, *Ora i* ORAI calcium release-activated calcium modulator, *OSBP* oxysterol binding protein, *p66Shc* 66-kDa isoform of the growth factor adapter shc, *PACS-2* phosphofurin acidic cluster sorting protein 2, *PEMT2* phosphatidylethanolamine N-methyltransferase 2, *PP2a* protein phosphatase 2a, *PML* promyelocytic leukemia protein, *PS1/2* presenilin-1/2, *PSS-1a* phosphatidylserine synthase-1a, *SERCA2b* sarco-endoplasmic reticulum calcium ATPase 2b, *Sig-1R* Sigma-1 receptor, *STIM1* stromal-interacting molecule 1, *Stt4p* phosphatidylinositol-4-kinase, *t SERCA1* truncated sarco-endoplasmic reticulum Ca^{2+} ATPase, *VDAC* voltage-dependent anion channel, ?, unknown protein

involved in lipid synthesis and trafficking between ER and mitochondrial membranes, including long-chain fatty acid-CoA ligase type 4 (FACL4) and phosphatidylserine synthase-1 (PSS-1) [106, 113, 114]. The MAMs have since been shown to be enriched in functionally diverse enzymes involved not only in lipid metabolism but also in glucose metabolism (for recent reviews, see [115, 116]).

More recently, the same subcellular fraction has been shown to contain Ca^{2+}-sensing ER chaperones and oxidoreductases, as well as key Ca^{2+} handling proteins of both organelles [117, 118] (a schematic representation of the interorganelle interactions and some of these proteins with the assigned functions is presented in Fig. 17.2). Together, these data have led to the conclusion that the MAMs are not only a site of lipid synthesis and transfer, but also function as a fundamental hub of cellular signaling that controls a growing number of processes associated with both organelles, ranging from ER chaperone-assisted folding of newly synthesized proteins to the fine-tuning of physiological and pathological Ca^{2+} signals from ER to mitochondria.

MAMs Proteins and Ca^{2+} Homeostasis in Health and Disease

The aspect of functional interaction between the ER and mitochondria that has received most attention in recent decades is undoubtedly that involving Ca^{2+} ions. Ca^{2+}-handling proteins such as IP3Rs (especially type 3 IP3Rs) are highly compartmentalized at MAMs [119], identifying these zones as "hotspots" of Ca^{2+} transfer from the ER to the closely adjacent mitochondrial network [17, 18]. Ca^{2+} signals arising from the ER are vital for regulating Ca^{2+} levels in mitochondria. Mitochondrial Ca^{2+} spikes and oscillations play a central role in energy production by regulating Ca^{2+}-dependent enzymes involved in the ATP-producing Krebs cycle reactions [120, 121] and thus are important for cellular survival, although a mitochondrial Ca^{2+} overload will lead to the opening of the PTP, permeabilization of the OMM, eventually triggering cell death [122–124]. It remains unclear how the rise in mitochondrial Ca^{2+} (that has probably evolved to couple cell signaling to metabolic activation) can be transformed into a trigger of cell death. Both the amplitude and, most importantly, the duration of the Ca^{2+} rise in mitochondria, and perhaps even the concomitant insults that affect mitochondrial functions, play a major role in this transition. Therefore, ER Ca^{2+} handling on the MAMs acts as a double-edged sword, suggesting the existence of still not fully elucidated regulatory mechanisms, that are capable of discriminating between signals of life or death.

The connection between the ER and mitochondria is known to be highly dynamic as the local $[Ca^{2+}]$ itself can regulate ER-mitochondrial association in different ways [125], and increased $[Ca^{2+}]_c$ blocks the motility of both organelles, enhancing their interaction [46]. Recently Hajnoczky et al. demonstrated that exposure to TGFβ affects Ca^{2+} transfer to the mitochondria through an impairment of the ER–mitochondrial coupling, further supporting the notion of a highly dynamic regulation of inter-organelle communication [126].

Several proteins may participate in the stabilization of those MAMs and, through this stabilization, affect Ca^{2+} transfer between ER and mitochondria, while other proteins may be directly involved in regulating the Ca^{2+}-transport proteins described above. During the last years, research has focused on the identification of connecting structures between the ER and mitochondria at the MAMs, revealing that the interactions between the two organelles seem to be modulated both by a family of chaperone proteins and by a family of "mitochondria-shaping proteins". One of the first advances was made in 2006, when Csordás et al. showed by electron tomography that ER and mitochondria are adjoined by tethers seemingly composed of proteins, since the *in vitro* incubation with proteinase not only detached the ER from mitochondria, but also disrupted Ca^{2+} transfer. Tightening of the connections sensitized mitochondria to Ca^{2+} overloading, ensuing permeability transition, and seemed relevant for several mechanisms of cell death. Thus, these results revealed an unexpected dependence of cell function and survival on the maintenance of a proper spacing between the ER and mitochondria [103].

At the same time, Szabadkai et al. found that the mitochondrial chaperone grp75 (glucose-regulated protein 75) mediates the molecular interaction of VDAC with the ER Ca^{2+}-release channel IP3R. It was demonstrated that grp75 not only induces a chaperone-mediated conformational coupling of the proteins, but also allowed for a better transfer of the Ca^{2+} ions from the ER to the mitochondrial matrix [127]. In support of this view, we previously demonstrated that the overexpression of VDAC enhances Ca^{2+} signal propagation into the mitochondria, increasing the extent of mitochondrial Ca^{2+} uptake (also leading to a higher susceptibility for ceramide-induced cell death), acting at the ER–mitochondria contact sites [128]. Moreover, we have recently established that VDAC1, but not VDAC2 and VDAC3 isoforms, selectively interacts with IP3Rs; this interaction is further strengthened by apoptotic stimuli and thus VDAC1 is preferentially involved in the transmission of the low-amplitude apoptotic Ca^{2+} signals to mitochondria [129].

Subsequently, ER chaperones, particularly the Ca^{2+}-binding chaperones calnexin, calreticulin, Sigma-1 receptor (Sig-1R) and Binding immunoglobulin Protein (BiP, also known as the glucose-regulated protein GRP78), were also found to be compartmentalized at the MAMs, yielding a new picture whereby chaperone machineries at both ER and mitochondria orchestrate the regulation of Ca^{2+} signaling between these two organelles. For instance, calnexin reversibly interacts with SERCA2b to block Ca^{2+} import [130]. Similarly, calreticulin inhibits uptake of Ca^{2+} by inhibiting the affinity for Ca^{2+} of the SERCA2b pump, but also regulates IP3-induced Ca^{2+} release [12, 131]. *In vivo*, these functions of calreticulin may very well be more crucial for survival than its chaperone activity, since calreticulin-deficient cells have impaired Ca^{2+} homoeostasis [132, 133].

Back in 2005, Simmen et al. reported the identification of a multifunctional cytosolic sorting protein, PACS-2 (phosphofurin acidic cluster sorting protein 2), that partially resides in the MAMs and maintains its integrity [134]. PACS-2 depletion induces mitochondria fragmentation and uncouples these organelles from the ER, raising the possibility that, in addition to mediating MAMs formation, PACS-2 might also influence Ca^{2+} homeostasis and apoptosis. Indeed, it has been shown that

IP3Rs (and RyRs) possess potential PACS-2-binding sites [135]; hence, disruption of PACS-2 may cause mislocalization of IP3Rs, resulting in reduced Ca^{2+} transfer from the ER to mitochondria. Moreover, in response to apoptotic stimuli, PACS-2 has been demonstrated to be capable of inducing Bid recruitment to mitochondria, an event that leads to cytochrome *c* release and caspase 3 activation [134]. PACS-2 also interacts with and regulates the distribution and activity of calnexin. Under control conditions, >80% of calnexin localizes to the ER, mainly at the MAMs. However, through a protein–protein interaction, PACS-2 causes calnexin to distribute between the ER and the plasma membrane, affecting the homeostasis of ER Ca^{2+} [136]. PACS-2 and calnexin also interact with the MAMs-resident ER cargo receptor Bap31 (B-cell receptor-associated protein 31) and regulate its cleavage during the triggering of apoptosis [137]. Despite these observations, the exact role of PACS-2 in the regulation of Ca^{2+} transfer from the ER to the mitochondria remains to be further investigated.

Recently, Simmen's group have also shown that the GTPase Rab32, a member of the Ras-related protein family of Rab, localizes to the ER and mitochondria and identified this protein as a regulator of MAMs properties. Its activity levels control MAMs composition, destroying the specific enrichment of calnexin at the MAMs, and consequently ER calcium handling. Furthermore, as a PKA-anchoring protein, Rab32 determines the targeting of PKA to mitochondrial and ER membranes, resulting in modulated PKA signaling. Together, these functions result in a delayed apoptosis onset with high Rab32 levels and, conversely, accelerated apoptosis with low Rab32 levels, explaining the possible mechanism by which it could act as an oncogene [138].

Also Sig-1R, an ER chaperone serendipitously identified in cellular distribution studies by Hayashi and Su, is enriched in the MAMs and seems to be involved in Ca^{2+}-mediated stabilization of IP3Rs [139]. Under normal conditions in which the ER lumenal Ca^{2+} concentration is at 0.5–1.0 mM, it selectively resides at the MAMs and forms complexes with the ER Ca^{2+}-binding chaperone BiP. Upon the activation of IP3Rs, which causes the decrease of the Ca^{2+} concentration at the MAMs, Sig-1R dissociates from BiP to chaperone IP3R, which would otherwise be degraded by proteasomes. Thus, Sig-1R appears to be involved in maintaining, on the ER luminal side, the integrity of the ER-mitochondrial Ca^{2+} cross-talk, as demonstrated by the fact that its silencing leads to impaired ER-mitochondrial Ca^{2+} transfer. Sig-1R has been implicated in several neuronal and non-neuronal pathological conditions [140], and is also upregulated in a wide variety of tumour cell lines [141]. Therefore, degenerative neurons or tissue might benefit by Sig-1R agonists which promote cell survival [142, 143]; conversely, its antagonists inhibit tumour-cell proliferation [144].

Another example of a folding enzyme regulating ER Ca^{2+} content is the oxidoreductase ERp44 (endoplasmic reticulum resident protein 44) that interacts with cysteines of the type 1 IP3R, thereby inhibiting Ca^{2+} transfer to mitochondria when ER conditions are reducing [145]. Recent results suggest that another oxidoreductase, Ero1α, might also perform such a function, since Ero1α interacts with the IP3R and potentiates the release of Ca^{2+} during ER stress [146]. This function of Ero1α could

impact the induction of apoptosis that critically depends on ER-mitochondria Ca^{2+} communication [119, 147]. Gilady et al. showed that, despite Ero1α being an ER luminal protein, the targeting of Ero1α to the MAMs is quite stringent (>75%), consistent with its role in the regulation of Ca^{2+} homeostasis. Moreover, they found that localization of Ero1α on the MAMs is dependent on oxidizing conditions within the ER; indeed, hypoxia leads to a rapid and eventually complete depletion of Ero1α from the MAMs [148].

In the increasingly clear but complex picture that is emerging for MAMs, also the mitochondrial fusion protein Mfn2 has been shown to be enriched at contact sites between the ER and mitochondria. Mfn2 on the ER appeared to link the two organelles together: the connection depended on the interaction of the ER Mfn2 with either Mfn1 or Mfn2 on the OMM [104]. Moreover, its absence changes not only the morphology of the ER but also decreased by 40% the interactions between ER and mitochondria, thus affecting the transfer of Ca^{2+} signals to mitochondria. This may contribute to the Charcot-Marie-Tooth neuropathy type 2a in which missense mutations occur in Mfn2 [149]. A too strong ER–mitochondria interaction, and the concomitant improved Ca^{2+} transfer between the two organelles, may also be detrimental as overexpression of Mfn2 led to apoptosis in vascular smooth-muscle cells [150]. A recent report also propose the keratin-binding protein Trichoplein/mitostatin (TpMs), often downregulated in epithelial cancers [151], as a new regulator of mitochondria–ER juxtaposition in a Mfn2-dependent manner [152].

Also the mitochondrial fission protein Fis1 has been involved in ER-mitochondria coupling. Fis1 physically interacts with Bap31, an integral membrane protein expressed ubiquitously and highly enriched at the outer ER membrane), to bridge the mitochondria and the ER, setting up a platform for apoptosis induction. It appeared that the Fis1–Bap31 complex is required for the activation of procaspase-8. Importantly, as this signaling pathway can be initiated by Fis1, the Fis1-Bap31 complex establishes a feedback loop by releasing Ca^{2+} from the ER that is able to transmit an apoptosis signal from the mitochondria to the ER [153].

As described, it is now widely accepted that Ca^{2+} transfer between ER and mitochondria is a topic of major interest in physiology and pathology (Fig. 17.3). The release of Ca^{2+} from ER stores by IP3Rs has been implicated in multiple models of apoptosis as being directly responsible for mitochondrial Ca^{2+} overload. Apoptosis is a process of major biomedical interest, since its deregulation is involved in the pathogenesis of a broad variety of disorders (neoplasia, autoimmune disorders, viral and neurodegenerative diseases, to name a few).

Mitochondrial Ca^{2+} is therefore a central player in multiple neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease and Huntington's disease [154]. It is noteworthy that alteration in Ca^{2+} homeostasis in sporadic AD patients started being reported in the middle of the 1980s, albeit in contrasting ways. Interestingly, very recent data have revealed that presenilin-1 (PS1) and presenilin-2 (PS2), two proteins that, when mutated, cause familial AD (FAD), have a strong effect on Ca^{2+} signaling (sometimes yielding contradictory experimental findings, as recently reviewed in [155]). Of particular interest on this topic, is the report that MAMs are the predominant subcellular location for PS1 and PS2, and for γ-secretase

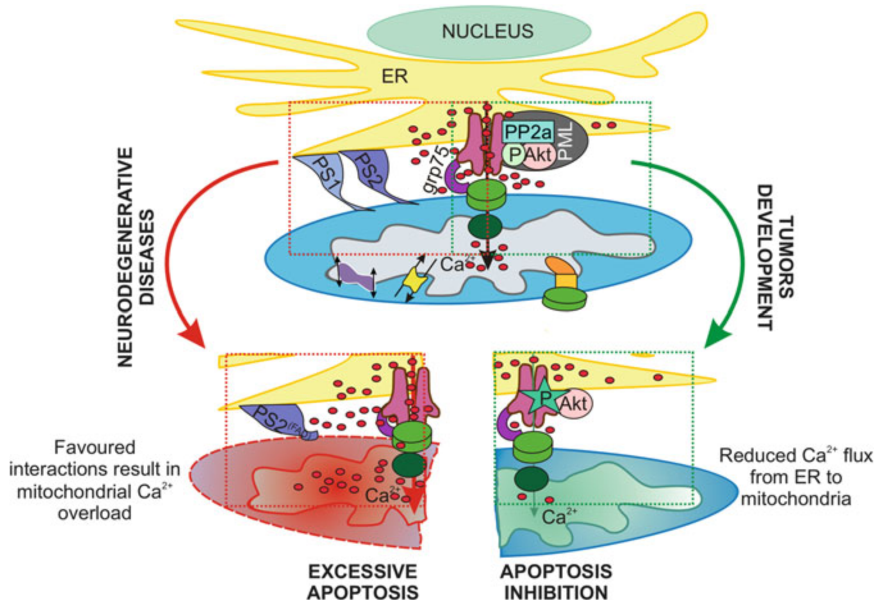

Fig. 17.3 Representation of MAMs proteins involved in ER-mitochondria Ca^{2+} cross-talk and perturbations implicated in cell survival and cell death. Ca^{2+} release from the endoplasmic reticulum (ER) results in high-Ca^{2+} hot spots at the mitochondrial surface to allow efficient Ca^{2+} uptake through voltage-dependent anion channel - which is coupled to inositol 1,4,5-trisphosphate receptor by the chaperone glucose-regulated protein 75 (grp75) - and the mitochondrial Ca^{2+} uniporter. Mitochondrial Ca^{2+} activates organelle metabolism and ATP synthesis but also, when in excess, triggers apoptosis. Apoptosis deregulation is involved in the pathogenesis of neurodegenerative diseases as well as tumors development. Presenelin-1 (PS1) and Presenelin-2 (PS2), two proteins that when mutated cause familial Alzheimer's disease (AD), have been recently found at MAMs, and familial AD (FAD) variants of PS2 ($PS2^{FAD}$) seem to increase ER and mitochondria interaction; this could result in mitochondrial Ca^{2+} overload and subsequent excessive apoptosis. In addition, controlled apoptosis is likely to be important to eliminate cells, thereby avoiding tumor genesis. In this process the recently identified localization of the tumor suppressor promyelocytic leukemia protein (PML) at ER/MAMs plays a crucial role as it promotes IP3R-mediated Ca^{2+} transfer from ER into mitochondria. While Akt is known to suppress IP3R-channel activity by its phosphorylation, the recruitment of protein phosphatase PP2a via PML in a specific multi-protein complex (comprising PML, IP3R-3, PP2a, and Akt), dephosphorylates and inactivates Akt. This suppresses Akt-dependent phosphorylation of IP3R-3 and thus promotes Ca^{2+} release through this channel and Ca^{2+} transfer into the mitochondria. In cancer cells, where PML is often missing, IP3R-3 are hyper-phosphorylated due to an impaired PP2a activity, as a result the Ca^{2+} flux from ER to mitochondria is reduced and cells become resistant to apoptosis

activity [156]. Moreover, it has recently been found that PS2 over-expression increases the interaction between ER and mitochondria and consequently Ca^{2+} transfer between these two organelles, an effect that is greater FAD variants [157]. It is possible to speculate that this favoured interaction could potentially result in a toxic mitochondrial Ca^{2+} overload (Fig. 17.3). A defect in Ca^{2+} signaling due to altered MAMs function could explain the well-known disturbances in Ca^{2+} homeostasis

in AD [158, 159]. It also opens the door to new ways of thinking about complementary treatment; in addition, it may be possible to exploit aberrant MAMs function as a useful marker for the development of a diagnostic tool for AD [160].

Sano et al. also demonstrated that in GM1-gangliosidosis, a neurodegenerative disease, GM1-ganglioside (GM1) accumulates in brain within the MAMs, where it specifically interacts with phosphorylated IP3R-1, influencing its activity [161]. GM1 has been previously shown to modulate intracellular Ca^{2+} flux [162, 163]. As such, the recent discovery that MAMs are the sites where GM1 accumulates and influences ER-to-mitochondria Ca^{2+} flux, leading to Ca^{2+} overload and activation of the mitochondrial apoptotic pathway, explains the neuronal apoptosis and neurodegeneration that occurs in patients with GM1-gangliosidosis [161]. These findings may have important implications for targeting checkpoints of the GM1-mediated apoptotic cascade in the treatment of this catastrophic disease.

Modulation of the progression of cell death may therapeutically be also very important for the inhibition of tumour growth. Specific stimulation of the Ca^{2+} transfer between the IP3R and mitochondria could lead to increased cell death and so form a supplementary pathway to combat cancer. Our group has recently described that the tumor suppressor promyelocytic leukemia protein (PML) modulates the ER–mitochondria Ca^{2+}-dependent cross-talk due to its unexpected and fundamental role at MAMs, highlighting a new extra-nuclear PML function critical for regulation of cell survival. This was demonstrated to be mediated by a specific multi-protein complex, localized at MAMs, including PML, IP3R-3, the protein phosphatase PP2a, and Akt. More than 50 different proteins can interact with and regulate the IP3Rs [80]; among these, a key role is played by the anti-apoptotic protein kinase Akt, which also phosphorylates IP3Rs, significantly reducing their Ca^{2+} release activity [81, 164]. In a previous work, we demonstrated that cells with the active form of Akt have a reduced cellular sensitivity to Ca^{2+}-mediated apoptotic stimuli through a mechanism that involved diminished Ca^{2+} flux from the ER to mitochondria [165]. Our recent data show that PML mediates PP2a retention in the MAMs, which dephosphorylates and inactivates Akt. Thus, in the absence of PML, the unopposed action of Akt at ER, due to an impaired PP2a activity, leads to a hyperphosphorylation of IP3R-3 and in turn a reduced Ca^{2+} flux from ER to mitochondria, rendering cells resistant to apoptotic Ca^{2+}-dependent stimuli [166] (Fig. 17.3). These findings may reveal a novel pharmacological target in apoptosis [167].

Interestingly, p66Shc, a cytosolic adaptor protein which is involved in the cellular response to oxidative stress (see above), has been found also in the MAMs fraction. In particular, we found that the level of p66Shc in MAMs fraction is age-dependent and corresponds well to the mitochondrial ROS production which is found to increase with age [168]. Finally, the functional significance of MAMs resident proteins in the regulation of ER-mitochondrial cross-talk is further supported by the finding that several viral proteins, such as the human cytomegalovirus vMIA [169], as well as the p7 and NS5B proteins of hepatitis C virus [170], are targeted to the MAMs and exert anti- or pro-apoptotic effects, respectively.

To conclude, whether or not mitochondria and MAMs contribute also to the Ca^{2+}-dependent activation of autophagy is still unknown. If mitochondria actively contribute to the activation of autophagy through Ca^{2+} handling remains to be solved, but the close interaction between IP3Rs and mitochondria, on the one hand, and between IP3Rs and autophagy proteins, on the other hand, led to the hypothesis that IP3Rs could participate in the induction of this process [171, 172]. The study of the relation between IP3Rs, Ca^{2+} and the autophagic processes may become very important, since autophagy can protect the organism against various pathologies, including cancer and neurodegenerative diseases [118, 173].

The deeper understanding at the molecular level of the structural and functional links that are established at MAMs and the possibility to modulate them may in the future be of great importance in the treatment of many different human pathologies.

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