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Review

The endoplasmic reticulum–mitochondria connection: One touch, multiple functions ☆

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ABSTRACT

The endoplasmic reticulum (ER) and mitochondria are tubular organelles with a characteristic "network structure" that facilitates the formation of interorganellar connections. The ER and mitochondria join together at multiple contact sites to form specific domains, termed mitochondria-ER associated membranes (MAMs), with distinct biochemical properties and a characteristic set of proteins. The functions of these two organelles are coordinated and executed at the ER–mitochondria interface, which provides a platform for the regulation of different processes. The roles played by the ER–mitochondria interface range from the coordination of calcium transfer to the regulation of mitochondrial fission and inflammasome formation as well as the provision of membranes for autophagy. The novel and unconventional processes that occur at the ER–mitochondria interface demonstrate its multifunctional and intrinsically dynamic nature. This article is part of a Special Issue entitled: Dynamic and ultrastructure of bioenergetic membranes and their components.

1. Introduction

The definition of the word *reticulum*, "a net-like formation or structure; a network", clearly describes the endoplasmic reticulum (ER), the largest of the membrane-bound organelles within the cell [1]. The ER can connect to and consequently act synergistically with other membranous structures. Among the various intracellular organelles that interact with the ER, which include the Golgi apparatus, mitochondria, peroxisomes, endosomes and lysosomes, the mitochondria has one of the most extensively studied and well-characterized connections with the ER. The first observation of their physical interaction more than 50 years ago [2] suggested that these two organelles might share regulatory factors and, notably, that their functions might be performed or regulated mutually. At the molecular level, proteins residing in different organelles can interact and facilitate the formation of multi-organellar domains with new properties and functions. The distance between the ER and mitochondria was originally estimated to be approximately 100 nm [3], but later, high speed digital imaging microscopy [4] and electron tomography [5] studies suggested that it was even smaller, approximately 10 to 25 nm. The close proximity of the ER and the outer mitochondrial membrane (OMM) further explains how proteins situated on the opposing membrane faces could interact and thus "tether" the two organelles [6]. However, although the ER and mitochondrial membranes form specific contact sites, they do not fuse, maintaining the organelles' distinct structures.

Today, biochemical techniques allow us to isolate the ER–mitochondria contact sites, also known as mitochondria-associated ER membranes (MAMs) [7–10] (for a detailed isolation protocol, see [11]). The optimized method is based on seminal work by J.E. Vance, who described the isolation of a particular fraction ("fraction X") with many similarities to microsomes but which sediments with the mitochondria upon centrifugation [12]. Vance speculated that a subfraction of the ER could be associated with the mitochondria. More recently, this hypothesis has been corroborated by several studies demonstrating the importance of MAMs in lipid synthesis and trafficking [13,14]. Mitochondria require a continuous and coordinated supply of membrane lipids to carry out their physiological processes and maintain their membrane integrity. The transport of phospholipids between membranes of the ER and mitochondria involves MAMs, although the mammalian macromolecular complexes responsible for directing lipid exchange have not yet been elucidated (for reviews, see [10,15]). Identification of the various proteins that reside within the MAMs might help to advance this field. Recently, two different proteomic studies of MAMs isolated from cells [16] and the mouse brain [17] each identified approximately 1000 "MAM proteins" (991 and 1212 proteins, respectively), but only 44% of these overlapped (i.e., were found in both studies), likely due to the different cellular

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sources used and to the difficulty of obtaining pure MAM preparations. However, all of the proteins identified are involved in relevant biological pathways, such as calcium (Ca^{2+}) handling and inflammasome formation. In this review, we discuss the role played by ER–mitochondria contacts in the regulation of four different processes: mitochondrial fission, Ca^{2+} transfer, autophagy and inflammation. These important, and in some respects novel and unconventional, processes that have been characterized at the ER–mitochondria interface indirectly reveal its multifunctional and intrinsically dynamic nature.

2. Regulation of mitochondrial fission

The first direct evidence that the ER and mitochondria are sufficiently close to mediate a synapse-like interaction in living cells was obtained using a GFP-based approach [4]. This idea was greatly strengthened in a seminal paper from the Voeltz research group [18]. This study analyzed the three-dimensional structure of ER–mitochondria contacts in *Saccharomyces cerevisiae* using electron microscopy and tomography, revealing that ER tubules were associated with mitochondria and might mediate the formation of mitochondrial constriction sites [18]. A reduction in mitochondrial diameter was observed at sites in which the ER was almost completely wrapped around the mitochondrial membrane (from ~210 nm for uncircumscribed mitochondria to ~140 nm for circumscribed mitochondria) [18]. Thus, the mitochondria appeared to be constricted at the point of contact with the ER, pointing to a crucial role for the ER–mitochondria association in the initiation of mitochondrial fission. The presence of ER tubules at sites of mitochondrial contraction and fission has been confirmed using a two-color STORM super-resolution approach [19]. This technique also allows the visualization of the actively extending ER network; the newly formed tubules appear to be thinner than pre-existing tubules [19].

Mitochondria are dynamic organelles, continuously undergoing fusion and fission. These opposing processes maintain the shape, size and number of mitochondria as well as their physiological functionality [20,21]. Dynamin-related protein (Drp1; also termed Dlp1) localizes primarily to the cytoplasm and is recruited to the mitochondria to regulate mitochondrial fission [22]. Drp1 associates with the OMM, where it forms multimeric ring-like structures or oligomers that wrap around the constricted portions of the mitochondria [23]. Drp1 possesses GTPase activity, and its hydrolysis of GTP causes a conformational change in the oligomer that bisects the membrane and leads to a fission event. The ability to form multimeric spirals is a general property of dynamin family members [24], and the self-assembly of Dnm1 (the Drp1 yeast ortholog) oligomers also drives the constriction of mitochondria during mitochondrial division [25]. Although the molecular machinery responsible for the recruitment of Dnm1 to the mitochondria has been thoroughly described in yeast, the mechanistic roles of many fission-related proteins in mammals remain obscure. In yeast, both fission protein 1 (Fis1) and mitochondrial division protein 1 (Mdv1) target Dnm1 to the mitochondrial membrane, promoting its oligomerization and the subsequent fission event [26,27]. There is no mammalian ortholog of Mdv1, and the role of human Fis1 (hFis1) has not been fully elucidated (reviewed in [22]); for example, the introduction of hFis1 into fis1Δ yeast cells is unable to rescue the mutant phenotype [28], suggesting that the two proteins are structurally divergent or act through different mechanisms. Drp1 is anchored to the mitochondria through its interaction with mitochondrial fusion factor (Mff) [29,30] or mitochondrial dynamics 51 (MiD51) [31], but the MiD51 present in the Drp1–MiD51 complex has also been proposed to play an inhibitory role by blocking the GTPase-dependent fission activity of Drp1 and promoting fusion [32]. However, in both yeast and mammalian cells, Dnm1/Drp1 localizes to the site at which ER tubules circumscribe the mitochondrial membrane, and the ER–mitochondria interaction is fully independent of the mitochondrial fission machinery [18]. In fact, the ER remains able to wrap around the mitochondrial tubules even when Drp1 or Mff is down-regulated [18], indicating that ER–mitochondria contact represents a conserved platform for the regulation of mitochondrial division. A recent paper published in *Science* proposed a potential mechanism for ER association-induced mitochondrial fission, involving actin polymerization and the ER-localized protein inverted formin 2 (INF2) [33]. This model is based on two major observations: i) INF2 siRNA significantly elongates the mitochondria, and ii) actin filaments seem to aggregate between the mitochondria and INF2-enriched ER membranes at fission loci [33]. Thus, at the mitochondria–ER contact site, INF2 is activated to polymerize actin, which in turn might generate the driving force for initial mitochondrial constriction (Fig. 1).

A growing body of evidence now supports a role for ER–mitochondria interactions in mitochondrial fission, and mitochondrial fusion is emerging as a process that could also be influenced by ER contact. Mitochondrial fusion is mainly orchestrated by mitofusins 1 (MFN1) and 2 (MFN2). Both proteins localize predominantly to the OMM, but while MFN1 plays a critical role in mitochondrial docking and fusion, MFN2 coordinates the interactions between mitochondria, leading to the stabilization of the whole mitochondrial network [34]. Notably, MFN2 expression is crucial for tethering the ER to the mitochondria and stabilizing MAM formation. MFN2 localizes not only to the mitochondria but also to the ER and MAMs, forming both homo- and heterotypic interactions with mitochondrial MFN2 and MFN1 [35]. MFN2 activity at the ER–mitochondria interface is regulated by a mitochondrial ubiquitin ligase called MITOL [36]. MITOL interacts with mitochondrial MFN2 but not with ER-localized MFN2, mediating the addition of lysine 63-linked polyubiquitin chains to MFN2 but not its proteasomal degradation. This polyubiquitination event induces MFN2 oligomerization, a fundamental step in MFN2-induced ER–mitochondria tethering [36]. Thus, MITOL regulates MAM formation by enhancing MFN2 activity, as also illustrated by the reduction in ER Ca^{2+} transfer that occurs in MITOL-deficient cells. The observation of dual roles for MFN2 in both mitochondrial fusion and ER–mitochondria tethering suggests that the establishment of ER–mitochondria contact might be a critical event in MFN2-dependent mitochondrial fusion.

Overall, these findings indicate that the ER–mitochondria connection plays a fundamental role in the regulation of mitochondrial dynamics. In particular, the wrapping of ER tubules around constricted mitochondria is now accepted to be one of the key early events in the mitochondrial fission process (Fig. 1).

3. Ca^{2+}-transfer

The rapid and large accumulation of Ca^{2+} in the mitochondrial matrix is one of the main features of the relationship between the mitochondria and this cation. Mitochondria in living cells undergo rapid and dramatic increases in their matrix Ca^{2+} levels, reaching peak levels that are one or two orders of magnitude higher than those observed in the cytoplasm [37,38]. The presence of a gated Ca^{2+}-selective ion channel in the inner mitochondrial membrane [39,40] allows Ca^{2+} to enter the mitochondrial matrix. The molecular identity of this channel has now been clarified [41,42], and the protein, previously known as CCDC109A, has been re-named MCU (mitochondrial calcium uniporter). The principal properties of the Ca^{2+} transporter are its sensitivity to Ruthenium Red and low affinity for Ca^{2+} (K_D of 20–50 μM) [43–45]. The latter characteristic inevitably raises the question of how a low-affinity Ca^{2+} system can ensure a high mitochondrial Ca^{2+} concentration ($[Ca^{2+}]$). The answer to this question has been found in ER–mitochondria contact sites. Microdomains of high $[Ca^{2+}]$ (>10 μM) can form transiently in regions of close apposition between the mitochondria and the Ca^{2+} channels of the ER/SR (sarcoplasmic reticulum) or of the plasma membrane [37]. The ER has been identified as the major Ca^{2+} storage unit inside the cell [46], with a steady-state $[Ca^{2+}]$ of approximately 1 mM, which is close to concentrations in the extracellular milieu, and significant heterogeneity in Ca^{2+} levels among its different regions [47]. At resting state, cytosolic $[Ca^{2+}]$ is

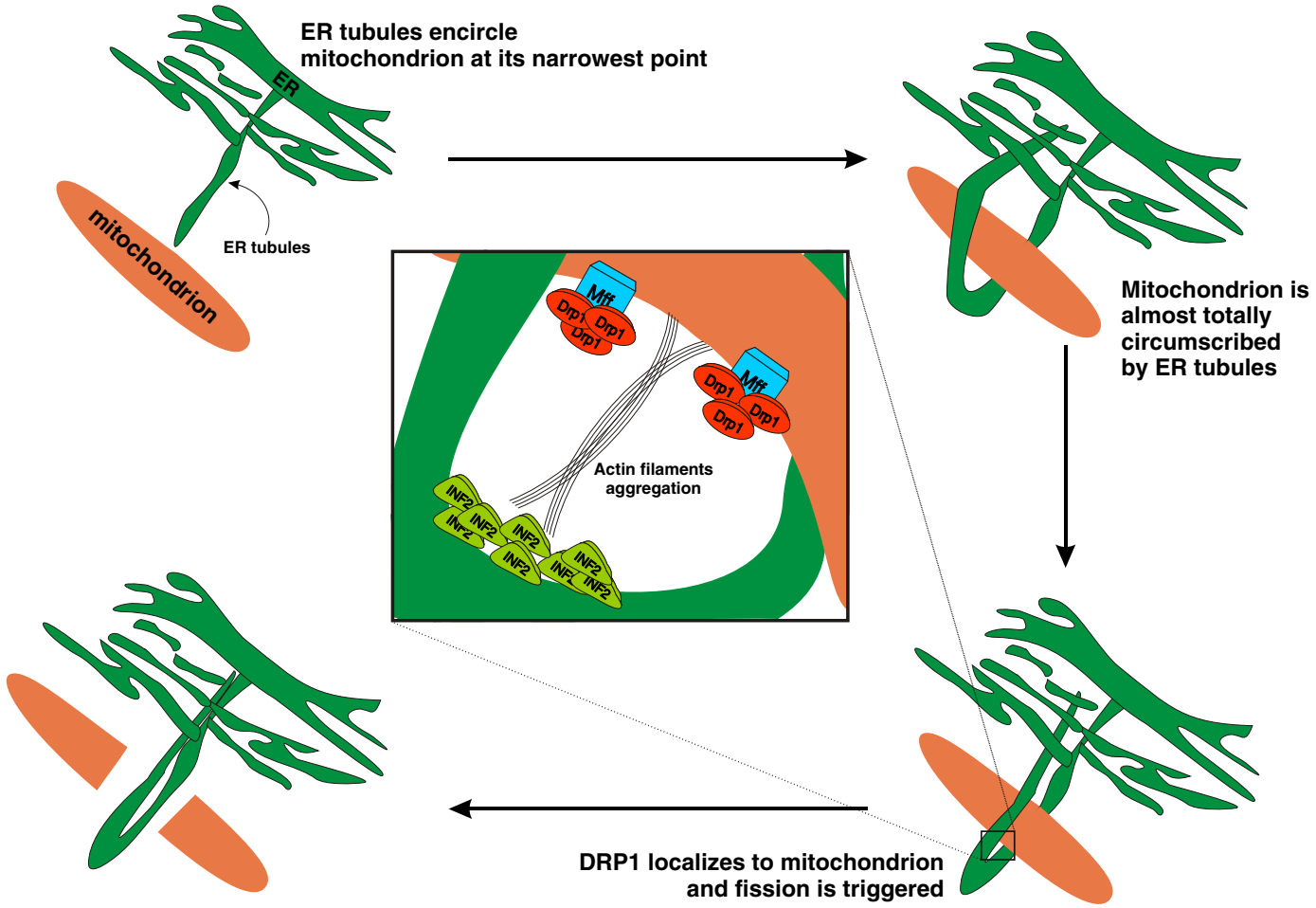

Fig. 1. ER–mitochondria interface regulates mitochondrial fission. ER tubules were associated with mitochondria and might mediate the formation of mitochondrial constriction sites. When mitochondrion is almost totally circumscribed by the ER tubules, INF2 is activated to polymerize actin at the ER–mitochondria interaction site. Actin polymerization not only could generate the driving force for mitochondrial constriction, but also enhances Drp1 assembly at the ER–mitochondria contact site, which results in fission.

maintained at approximately 100 nM; a variety of extracellular stimuli induce increases in cytoplasmic [Ca^{2+}] that in turn induce specific cellular responses. The Ca^{2+} efflux from the ER is one mechanism that contributes to the rapid increase in intracellular [Ca^{2+}], and mitochondria are prone to taking up large amounts of Ca^{2+} due to their close juxtaposition with the ER. The transmembrane protein inositol 1,4,5-trisphosphate receptor (IP3R) is the main factor responsible for Ca^{2+} release from the ER [48]. IP3R opening is induced by its binding to the specific ligand IP3, which is generated when phospholipase C is activated by the presence of a soluble agonist [49]. The distance between the ER and OMM is a critical factor in the efficient transfer of Ca^{2+}. Using drug-inducible fluorescent inter-organelle linkers, Csordas et al. elegantly showed that increasing the ER–OMM distance to ~15 nm enhanced the efficiency of Ca^{2+} transfer from the ER to the mitochondria, whereas limiting the junctional gap to 5 nm reduced the transfer efficiency [50,51]. Shortening the ER–mitochondria gap blocks the accommodation of IP3R, which has been estimated to protrude 10 nm from the ER membrane, with consequent decrease in Ca^{2+} efflux [50].

Aside from the spacing between the two organelles, the contact volume is another important parameter in the regulation of Ca^{2+} signaling. It has been observed that after induction of Ca^{2+} release through IP3R, the formation of high [Ca^{2+}] microdomains involves 5–20% of the mitochondrial surface [4], and these "Ca^{2+} hot spots" are smaller than 1 μm^2 in size [52]. One prominent and intriguing example of how changes in the number of hot spots affects Ca^{2+} signaling was demonstrated by experiments with the pro-apoptotic protein fragile histidine triad (FHIT) [53]. The number of localized mitochondrial [Ca^{2+}] ([Ca^{2+}]$_m$) increases can be determined using the GFP-based mitochondrial probe 2 mtRP. FHIT overexpression enhances the number of these ER–mitochondria hot spots, favoring mitochondrial Ca^{2+} accumulation and triggering Ca^{2+}-dependent apoptosis [53].

Considering the critical role played by mitochondrial Ca^{2+} in the modulation of numerous physiological responses, including energy balance and apoptosis [54–57], it is logical that several factors could be involved in regulating both the size and number of ER–mitochondria contacts to manage Ca^{2+} transfer and regulate different cellular processes.

Disorganization of the ER–mitochondria interface appears to be relevant to the progression of Alzheimer's disease (AD). The up-regulation of MAM-associated proteins was observed in histological sections of brains from autopsied human AD patients [58], and both the exposure of amyloid β-peptide, which accumulates abnormally in AD, and the over-expression of presenilin 2, which is mutated in some cases of familial AD, elevate the number of ER–mitochondria contact points, favoring Ca^{2+} transfer between the two organelles [58,59].

A link between Ca^{2+} signaling and ER–mitochondria connections 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 in the unfolded protein response, is enriched at MAMs and is important for maintaining ER–mitochondria contact sites. PERK −/− cells exhibit disturbed ER morphology and Ca^{2+} homeostasis, as well as reduced reactive oxygen species (ROS) propagation from the ER to the

mitochondria [60]. These data suggest that MAMs may provide specialized contact sites for transmitting not only Ca^{2+} but also ROS-mediated signals to mitochondria following ROS-based ER stress.

Proteins that localize to MAMs have the potential to perturb Ca^{2+} transfer without modifying the number or architecture of ER–mitochondria contacts. One of the first examples of this phenomenon was observed for the interaction between IP3R and the voltage-dependent anion channel (VDAC1) at the OMM, mediated by the molecular chaperone glucose-regulated protein 75 (grp75) [61]. VDACs are the major channels for the exchange of metabolites and ions, including Ca^{2+}, between the ER and mitochondria. The knock-down of grp75 abolishes the functional coupling between IP3R and the mitochondria, altering the normal transfer of Ca^{2+} to the mitochondria [61].

The ER protein sigma-1 receptor (Sig-1R) also localizes to the ER–mitochondria junction and can be considered a *bona fide* MAM marker [62]. Sig-1R forms a Ca^{2+}-sensitive chaperone complex with BiP (also named grp78) and prolongs Ca^{2+} signaling from the ER to the mitochondria by stabilizing IP3R subunit 3 at MAMs [62]. Interestingly, among the three different IP3R isoforms, only type 3 seems to be enriched at MAMs [63]. Increasing Sig-1R expression in cells counteracts the ER stress response, limiting apoptosis progression [62].

MAMs could now be considered as a domain of the ER enriched with numerous ER chaperones [1]. The Ca^{2+}-binding and quality control chaperon calnexin resides at MAMs [11,62] and its localization is mediated by palmitoylation of membrane-proximal, cytosolically exposed cysteines [64] and, only partially, by the interaction with the ER sorting molecule PACS2 [65]. Using an Optiprep-based protocol to distinguish between rough ER and MAMs localization, it has been showed as, after application of short-term ER stress (i.e. Tunicamycin), calnexin quickly becomes less palmitoylated, minimizing its MAM distribution and shifting its function [66]. In fact, palmitoylated calnexin interacts at the MAMs with the sarcoendoplasmic reticulum Ca^{2+} transport ATPase (SERCA) 2b to regulate Ca^{2+} signaling, whereas non-palmitoylated calnexin mainly acts to rough ER to mediate protein folding and quality control [66]. All these findings suggest that chaperone complexes at both the ER and the mitochondrion could coordinate the regulation of Ca^{2+} signaling between these two organelles.

It has also been observed that the mitochondrial fission protein hFis1 interacts with Bap31 at the ER and that this association is responsible for the transfer of an apoptosis signal from the mitochondria to the ER [67]. hFis1 facilitates the cleavage of Bap31 to its pro-apoptotic form, p20Bap31, thus promoting the recruitment of procaspase-8. Importantly, the induction of apoptosis was found to trigger Bap31 cleavage, with a concomitant increase in Ca^{2+} release from the ER and consequent Ca^{2+} accumulation in mitochondria. These authors suggested that this ER Ca^{2+} efflux could function to amplify cell death by activating the apoptotic pathway in many mitochondria in close proximity to the ER [67].

The role of ER Ca^{2+} release as amplifier of mitochondrial apoptosis is sustained by the discovery of the interaction between IP3R and cytochrome c which occurs at early phases of apoptosis [68]. Cytochrome c released by mitochondria blocks the Ca^{2+}-dependent inhibition of IP3R function, resulting in increase Ca^{2+} efflux from the ER, permeability transition and cell-wide cytochrome c release [68–70].

The promyelocytic leukemia (PML) protein, known as an essential component of sub-nuclear structures termed PML nuclear bodies, also localizes to the ER and to MAMs, where it forms a super-complex with IP3R that regulates Ca^{2+} and apoptosis [71,72]. PML−/− cells display reduced Ca^{2+} release from the ER and resistance to apoptotic challenges. The introduction of an ER-targeted PML chimera into knockout cells restores normal Ca^{2+} flux and sensitivity to cell death [71]. Hence, the structural role played by PML at the nuclear level [73] could be mirrored at the ER; PML might be considered the "master coordinator" of a complex that includes IP3R, Akt kinase and the phosphatase PP2A. The loss of PML diminished PP2A activity at the ER, leading to Akt activation and consequent IP3R phosphorylation [71]. The very recent discovery by our laboratory that the phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) localizes to the ER and to MAMs is perfectly consistent with this model [74]. PTEN at ER/MAMs counteracts the Akt-dependent inhibitory activity on IP3R and ER Ca^{2+} efflux [75]. PTEN interacts directly with IP3R, and this interaction correlates with a reduction in IP3R phosphorylation and increased Ca^{2+} release [74] (Fig. 2).

These observations highlight the role of ER-dependent Ca^{2+} release as a general mediator in many cell death scenarios [76–78]. Ca^{2+} flux stimulates apoptosis through the activation of mitochondrial permeability transition pore (mPTP), which leads to the release of cytochrome c and other pro-apoptotic factors and, eventually, an irreversible activation of executor caspases. Recent discoveries concerning the components of mPTP [79], now, provide new molecular tools for investigating the role of ER–mitochondria interaction sites in the regulation of the mitochondrial permeability transition and Ca^{2+}-dependent apoptosis.

4. The autophagic function

Autophagy (derived from the ancient Greek meaning 'to eat oneself') is a process by which cells undergo partial autodigestion to briefly prolong their survival under starvation conditions. Autophagy provides the nutrients necessary to maintain cell viability. Although autophagy could be aspecific, it is now clear that autophagy specifically targets invading bacteria [80,81], protein aggregates [82], and organelles such as mitochondria [83,84] and ER [85]. In particular, mitochondrial autophagy or mitophagy is the selective degradation of damaged mitochondria. Defective mitochondria are first excluded from the mitochondrial network, through fission events, and then delivered to the lysosome by the autophagy machinery [86]. Mitochondria that are prone to be degraded are recognized through specific molecular pathways which included the OMM protein NIP3-like protein X (NIX; also known as BNIP3L) and the PINK1/Parkin axis (reviewed in [87]). Thus, if mitophagy regulates the number of mitochondria or specifically removes ones that are damaged, by contrast non-selective autophagy occurs when cells are deprived of nutrients.

Autophagy is up-regulated in response to extra- or intra-cellular stress, and defects in autophagy play significant roles in several human pathologies, including cancer and neurodegeneration [88].

The molecular basis of autophagy has been studied extensively, mainly in yeast. These studies have revealed over 30 autophagy-related genes (ATG), 18 of which are essential for autophagosome generation. Importantly, Atg proteins involved in the formation of autophagosomes are evolutionarily conserved from yeast to humans, and the corresponding yeast and human proteins have similar functions [89]. When autophagy is induced, a membrane cisterna called the isolation membrane or phagophore encloses a portion of the cytoplasm, resulting in the formation of the autophagosome. This initial sequestration step is generally thought to be non-selective. The outer membrane of the autophagosome then fuses with the lysosomal membrane. The formation of the autophagosome is clearly central to the autophagic process, but the details of how the phagophore membrane forms and the compartment from which it originates remain unknown. In yeast, the phagophore membrane forms around a cytosolic structure known as the pre-autophagosomal structure (PAS), but there is no evidence of PAS formation in mammalian cells, and the source of the mammalian phagophore remains uncertain [90].

Several observations suggest that phagophore membranes could be derived primarily from the ER. Ktistakis and colleagues showed that, during amino acid starvation, the cell generates a phosphatidylinositol 3-phosphate (PtdIns3P) compartment (termed the omegasome) that is connected to the endoplasmic reticulum and is critical for autophagosome formation [91]. In addition, immuno-electron microscopy studies have demonstrated a direct connection between the ER and phagophore and the localization of ER marker proteins to autophagosome membranes [92,93].

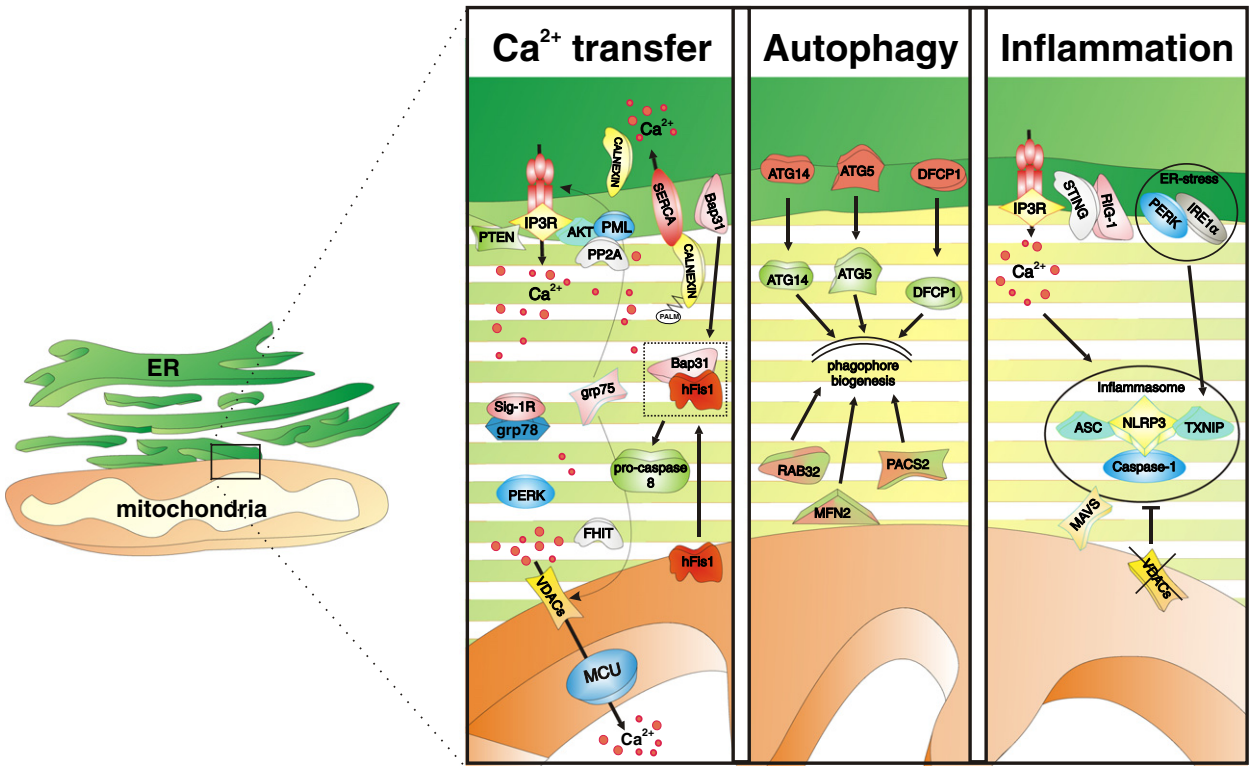

Fig. 2. Involvement of the ER–mitochondria interface in the coordination of Ca^{2+} transfer, autophagy and inflammasome formation. Magnification (in the square) of the ER–mitochondria contact site; MAM space is represented by shaded green/yellow lines. Left panel: the Ca^{2+} (red dots) efflux from the ER contributes to the fast increase in intracellular [Ca^{2+}] and mitochondria are prone to uptake high quotes of Ca^{2+}, due to their ER-juxtaposition. Ca^{2+} ions enter on mitochondria through MCU, which allows Ca^{2+} accumulation in the mitochondrial matrix. Several proteins localize at the ER–mitochondria contact sites and regulate Ca^{2+} transfer and apoptosis, modifying the architecture of the ER–mitochondria connections or establishing interactions with the canonical ER/mitochondrial proteins. Middle panel: list of proteins involved in phagophore formation; ATG14, ATG5 and DFCP1 translocate to MAMs during phagophore biogenesis and then dissociate from MAMs upon completion of the autophagosome. Right panel: upon pro-inflammatory stimuli, NLRP3 translocates to MAMs and the formation of inflammasome takes place at this level (see text for further details).

Other cellular compartments, including the trans-Golgi, plasma membrane and late endosomes, have also been shown to contribute to the process that supplies membranes for the phagophore [94–97].

Moreover, a growing body of research also supports a connection between the mitochondria and autophagosomes. Specifically, it has been reported that i) several autophagy-related proteins can localize to mitochondria; ii) different mitochondrial proteins can modulate the autophagic process; and iii) the OMM participates in autophagosome biogenesis under starvation conditions [98].

The elegant work by Hailey and colleagues suggested that the OMM is involved in the formation of autophagosomes and identified a crucial lipid anchor sequence that is necessary for the transfer of mitochondrial proteins to the autophagosome [98]. Furthermore, these authors showed that mitochondria–ER connections are critical for MFN2-dependent autophagosome formation.

Based on this observation, it can be surmised that the ER–mitochondria contact sites are involved in autophagosome assembly and maturation. A recent report published in *Nature* by Hamasaki and colleagues suggested that the ER–mitochondria interface may be involved in autophagosome formation [99].

When autophagy is induced under starvation conditions, localization of the pre-autophagosome marker ATG14 (present at cytosolic and ER sites under resting conditions) and that of DFCP1 (an omegasome marker) shift markedly to the MAMs [99]. Additionally, ATG5, which is critical for autophagosome formation, translocates to the MAM compartment during phagophore biogenesis and then dissociates from MAMs upon completion of the autophagosome, thus establishing a stable interaction with the ER and transient associations with the mitochondria [99]. Consistent with this finding, inhibiting the co-localization of ATG14 and DFCP1 in the MAM compartment by interfering with proteins involved in the formation of ER–mitochondria sites (such as PACS2 and MFN2) prevents proper autophagosome formation [99]. These findings support the hypothesis that the ER could be a critical platform for autophagosome formation and that the dynamic exchange between the ER and mitochondria is required for the function of the autophagic machinery (Fig. 2).

Interestingly, the GTPase Rab32 localizes to the ER and mitochondria, playing a pivotal role as regulator of MAM properties [100]. The formation of autophagic vacuoles apparently depended on the expression levels of Rab32 [101]. Rab32 overexpression induces the formation of autophagic vacuoles containing the autophagosome marker LC3, and its distribution at ER membranes, facing on mitochondrial side, seems to be required for the expansion and completion of autophagosomes: in fact, expression of a Rab32 membrane association-deficient mutant failed to trigger the autophagic vacuole formation [101]. Thus, the dual role of Rab32 as regulator of both MAM features and autophagy might also be considered an indirect evidence of the crucial involvement of ER–mitochondria membranes in autophagosomes biogenesis.

Rab32 is one of the genes that are critically required for growth specifically in mTORC2-addicted cell [102]. mTOR (mammalian Target Of Rapamycin) is a serine/threonine protein kinase with a large molecular size near 300 kDa. The activity of TOR is inhibited under nutrient starvation, which has been known as a crucial step for autophagy induction in eukaryotes [103,104]. mTOR is found in two structurally and functionally distinct protein complexes, termed mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [105]. It has been recently shown that mTORC2 localizes at MAMs in a growth factor-dependent manner, regulating MAM integrity [106]. mTORC2 deficiency causes MAM

disruption, revealed by alteration of the typical ER–mitochondria architecture and reduced amount of MAM proteins in *rictor* (a pivotal component of mTORC2) KO cells [106]. Furthermore, mTORC2 controls Ca^{2+} uptake, mitochondrial bioenergetics and apoptosis via Akt-mediated regulation of IP3R, hexokinase2 and PACS2 [106]. Despite the involvement of mTORC2 in autophagy is poorly understood, especially if compared to complex 1, its localization at ER–mitochondria membranes and the link to Rab32 might propose an active role for mTORC2 in autophagosome formation and assembly.

In conclusion, since the discovery of autophagy, the origin of the autophagosome membranes has been a matter of intense debate. Membranes from multiple and disparate sources have been proposed to contribute to autophagosome assembly, but the findings summarized here suggest that a critical role is played by ER–mitochondria contacts. Thus, the ER–mitochondria interface is not only important for sensing cellular stress and coordinating Ca^{2+} transfer and the apoptotic response, but it also represents the primary platform for autophagosome formation and the function of pro-survival autophagy machinery (Fig. 2).

5. The inflammatory function

The relationship between the ER–mitochondria interface and inflammation was first recognized in 2011 with the observation that ROS promote the activation of NOD-like receptor protein 3 (NLRP3) inflammasomes [107]. The human NLR family consists of 22 known members [108]; when activated, some of these proteins form multiprotein complexes, termed inflammasomes. The inflammasome serves as a platform for caspase-1 cleavage/activation and the subsequent proteolytic maturation and secretion of interleukin-1β (IL-1β) [109]. Due to its implication in several inflammatory diseases, NLRP3 is currently the most thoroughly studied and well-characterized member of the NLR family. Danger signals sensed by NLRP3 induce its oligomerization and the recruitment of the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain). This interaction leads to the processing of proIL-1β and the consequent production of IL-1β, through a caspase-1-dependent pathway [110].

Together with ER and peroxisomes [111], mitochondria are a significant source of ROS [112], including inflammasome-activating ROS, which may represent the pivotal triggers of NLRP3 inflammasome activation [107]. Damaged mitochondria produce high levels of ROS, and under normal physiological conditions, these mitochondria are promptly eliminated by mitophagy [113]. Although NLRP3 appears to be located on the ER under resting conditions, it relocates to ER–mitochondria contact sites in response to inflammation [107]. ROS are short-lived and can act as signaling messengers only over short distances. Therefore, NLRP3 should ideally be localized in close proximity to mitochondria to allow the efficient sensing of damaged ROS-generating mitochondria. Importantly, stimulation of the inflammasome using nigericin or MSU induces the translocation of the adaptor ASC from the cytosolic compartment to the MAMs [107]. Moreover, another NLRP3 binding partner, thioredoxin interacting protein (TXNIP), redistributes to MAMs/mitochondria in response to oxidative stress [114] or NLRP3 inflammasome activation [107]. TXNIP could also be considered a signaling hub connecting ER stress and inflammation; its expression is induced by ER stress, promoting IL-1β transcription and activation by the NLRP3 inflammasome [115,116]. The PERK and IRE1α signaling pathways are required for TXNIP induction under ER stress [115], likely regulating TXNIP expression at the post-transcriptional level [116]. Thus, considering that PERK localizes to MAMs and plays a role in maintaining ER–mitochondria contacts (see the "Ca^{2+} transfer" function, above), it will be useful to determine whether the knock-down of PERK inhibits the inflammatory response by altering the connections between the two organelles and/or by reducing the levels of both mitochondria-associated NLRP3 and TXNIP.

The critical role of the ER–mitochondria interface in NLRP3-mediated inflammation is also suggested by the involvement of the OMM channel VDAC. The knock-down of VDAC1/2 selectively abrogates NLRP3 inflammasome formation because VDAC and mitochondria are not essential for the activation of IPAF or AIM2 inflammasomes [107]. VDAC also serves as an anchor point for mitochondria-interacting proteins, such as hexokinase and Bcl-2 family members [117]. The anti-apoptotic protein Bcl-2 interacts with VDAC1 [118], decreasing channel conductance and likely limiting mitochondrial Ca^{2+} entry and ROS production. Consistent with these findings, stimulated macrophages isolated from transgenic mice over-expressing Bcl-2 exhibit decreased levels of IL-1β compared with cells from wild-type mice [107].

The observation that perhaps best exemplifies the crucial role of NLRP3 at ER–mitochondria contacts is the identification of the adaptor MAVS (mitochondrial anti-viral signaling) as the mitochondrial anchor for NLRP3 inflammasome formation [119]. Before this breakthrough finding, MAVS was best known for its role in the innate immune system. MAVS decodes antiviral signals that are primarily received and conveyed to the mitochondria by RIG-1 and MDA5 [120]. Specifically, RIG-1 is activated upon viral infection through the following steps: i) binding to viral RNA, ii) exposure of the caspase activation and recruitment domain (CARD), iii) binding to Lys 63-linked polyubiquitin chains and iv) association with the adaptor MAVS [121,122] after RIG-1 translocation to the MAMs [123]. At this step in the pathway, MAVS activates the innate immune response and, through its interaction with TRAF3, promotes the production of type I interferon. In addition to RIG-1, several other proteins that function in the area surrounding the mitochondria fine-tune the activities and functions of MAVS. Among these factors, STING (stimulator of IFN genes) resides ER-facing side of the MAM. STING interacts with RIG-1, likely stabilizing it at the ER–mitochondria interface, and is able to activate both the NF-kB and IRF3 transcription pathways to induce the expression of type I interferon [124].

The degree of interaction between MAVS and NLRP3 has been observed to increase markedly upon treatment with NLRP3-activating stimuli but not following exposure to stimuli that activate other NLR family members [119]. Thus, the mitochondrial adaptor MAVS mediates the mitochondrial localization of NLRP3; this effect correlates with a decrease in the NLRP3-mediated production of mature IL-1β by macrophages derived from MAVS −/− mice [119]. Interestingly, MAVS −/− bone marrow derived macrophages (BMDMs) display normal ER–mitochondria architecture, suggesting that the proper communication between the two organelles is maintained and might not be crucially linked to NLRP3 translocation/activation. However, IL-1β processing in response to crystalline substances, such as alum and MSU, was largely independent of MAVS, even though the same compounds had been previously observed to induce NLRP3 localization to the MAMs [107]. Therefore, factors other than MAVS may mediate NLRP3 recruitment to MAMs, and the specific mechanism of activation may depend strictly on the nature of the inflammatory insult. Many different molecules have been shown to activate the NLRP3 inflammasome complex. In addition to the two above-mentioned crystalline activators and nigericin (a bacterial toxin with potassium ionophore activity), extracellular ATP released by dying cells [125], cholesterol crystals [126] and Ca^{2+} [127] are known activators. Murakami and colleagues showed that several NLRP3 inflammasome activators mobilize Ca^{2+}, the disruption of which conversely inhibits NLRP3 inflammasome activation [127]. Interestingly, treatment with xestospongin C, a strong IP3R inhibitor, reduces Ca^{2+} efflux from the ER and attenuates IL-1β secretion [127]. Thus, the ER–mitochondria connection ensures adequate Ca^{2+} transfer, which is required for all mitochondrial activities and the inflammatory response. The role of Ca^{2+} in inflammation has been confirmed and updated by recent findings that the murine calcium-sensing receptor (CASR) strongly activates the NLRP3 inflammasome in response to increased intracellular Ca^{2+} and decreased cellular cyclic AMP (cAMP) [128]. More specifically, CASR activates the NLRP3 inflammasome through phospholipase C, which catalyzes IP3 production and thereby induces the release of Ca^{2+} from the ER [128]. Moreover, G-protein-coupled

receptors can activate the inflammasome, indicating that increased extracellular Ca^{2+} functions as a specific amplifier of inflammation [129].

Taken together, these data support the role of MAMs as a critical site for inflammasome formation. The identification of mitochondrial DNA release as a potential trigger of inflammasome activation [130] is further proof of the strategic localization of NLRP3 to the ER–mitochondria interface, allowing prompt danger signal sensing and inflammatory responses (Fig. 2).

6. Conclusions

The significant body of evidence discussed in this review shows clearly that ER–mitochondria contact sites play important roles in several biological processes. The physical interaction between the membranes of the two organelles is directly demonstrated by the ability of the ER tubules to circumscribe mitochondria, marking the sites of subsequent mitochondrial division. Thus, the ER regulates mitochondrial dynamics, and alterations in mitochondrial morphology uniquely reflect cell health. The ER and mitochondria reciprocally transmit danger signals through physical contacts, and it seems likely that these organelles might strengthen their communications with one another under stress conditions, triggering multiple, synergistic responses. For example, an increase in the number of ER–mitochondria contact sites enhances Ca^{2+} transfer to the mitochondria, conveying organelle-extrinsic stress signals to activate adaptive responses or promote cell death. Based on the nature and degree of cellular insult, the ER–mitochondria interface coordinates the relevant response: autophagy, Ca^{2+}-driven apoptosis or inflammation. In the case of the inflammatory response, NLRP3 and other inflammasome members move to ER–mitochondria contact sites to promptly sense the level of danger and to coordinate the appropriate response. Thus, MAMs not only represent a platform for the detection of extracellular inputs but also furnish a structural scaffold that accommodates several regulator or effector proteins.

Considering that the ER–mitochondria interface is involved in several molecular pathways, it will be important to determine its relevance to human diseases. Changes in MAMs have already been implicated in different diseases, e.g. Alzheimer's disease [131], cancer [71], metabolic disease [132], and lysosomal storage disease [133], but other pathological scenarios might also be affected by dysfunction of the ER–mitochondria architecture.

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