Chapter: Mitochondria-associated membranes

The role of mitochondria associated membranes in cellular homeostasis and diseases

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Keywords:
calcium transfer apoptosis
mitochondrial dynamics
ROS
autophagy
lipid trafficking and metabolism
inflammation and antiviral response
neurodegenerative diseases
diabetes
cancer

Abstract
Mitochondria and endoplasmic reticulum (ER) are fundamental in the control of cell physiology regulating several signal transduction pathways. They continuously communicate exchanging messages in their contact sites called MAMs (Mitochondria-Associated Membranes). MAMs are specific microdomains acting as a platform for the sorting of vital and dangerous signals.
In recent years increasing evidence reported that multiple scaffold proteins and regulatory factors localize to this subcellular fraction suggesting MAMs as hotspot signaling domains.
In this review we describe the current knowledge about MAMs' dynamics and processes, which provided new correlations between MAMs' dysfunctions and human diseases. Infact, MAMs machinery is strictly connected with several pathologies, like neurodegeneration, diabetes and mainly cancer. These pathological events are characterized by alterations in the normal communication between ER and mitochondria, leading to deep metabolic defects that contribute to the progression of the diseases.

Introduction

Mitochondria-associated membranes (MAMs) were the first connection discovered between two intracellular organelles. Endoplasmic reticulum (ER) membranes have long been known to be located close enough to mitochondria to form lipid raft-like domains (Giorgi et al., 2015c). These contacts can be physically isolated with different techniques, including live imaging, electron microscopy and subcellular fractionation, confirming that MAMs are composed of membrane fragments from both the ER and the outer mitochondrial membrane (OMM) (Wieckowski et al., 2009). These microdomains have a specific structure and form a platform for distinct signaling functions such as lipid synthesis and trafficking and calcium (Ca^{2+}) transfer from the ER to mitochondria.
Increasing interest in recent years has encouraged researchers to investigate the multifaceted roles of MAMs in cellular processes. ER-mitochondria membranes are hubs for signaling molecules and pathways that control and regulate cellular homeostasis apoptosis and biology linked to several aspects such as survival, metabolism, and sensitivity to cell death. Indeed, to control normal cellular functions and maintain intracellular equilibrium, several regulatory proteins, oncogenes, and tumor suppressors reside at MAMs (Marchi et al., 2014).

In this chapter, we will review the structural and functional properties of the ER-mitochondria interface and discuss several physiological functions in multiple cellular events, from mitochondrial dynamics to apoptosis and autophagy. Moreover, according to the central role played by MAMs in fundamental cellular processes, we will discuss how alterations in ER-mitochondria communication influence many human diseases, such as cancer, diabetes and neurodegenerative pathologies.

MAMs structure and composition

Approximately 5-20% of the total surface of the mitochondrial network is estimated to be in close proximity to the ER (Rizzuto et al., 1998). Interestingly, according to the endosymbiotic theory, first proposed by the biologist Lynn Margulis, mitochondria are the result of the endocytosis of purple nonsulfur bacteria by early eukaryotes. However, close apposition of ER subdomains to mitochondria may have evolved in specialized membrane regions to regulate mitochondrial functions and metabolism, giving rise to MAMs.
Mitochondria and the ER are highly dynamic organelles that undergo continuous and coordinate remodeling in the intracellular space. Electron micrography studies showed overlapping apposition between the ER and mitochondria at distances of approximately 10-25 nm (Csordas et al., 2006). Over the years, with the development of subcellular fractionation methods, strong ER contamination was identified in mitochondrial fractions, supporting the presence of membrane contact sites between the ER and mitochondria. The first function for this interface was identified in 1990 through the characterization of a membrane fraction associated with mitochondria and implicated in phospholipid (PL) synthesis. This fraction, now frequently called the MAM, is a specialized subdomain of the ER with a particular lipid and protein composition and many similarities to microsomes (Vance, 1990).
In recent years, multiple techniques have been developed to analyze the specific properties and composition of MAMs, using either biochemical (i.e., subcellular fractionation on a Percoll gradient) (Wieckowski et al., 2009) or fluorescence microscopy-based approaches (Cieri et al., 2018, Tubbs and Rieusset, 2016). Because of these multidisciplinary studies, we have vastly extended our comprehension of MAMs dynamics, structure and functions.
The close linkage between the ER/MAMs and mitochondria is reversible and does not involve membrane fusion. Live cell imaging has revealed that ER-mitochondria contact sites are constantly

in flux dependent on intracellular signaling, suggesting that this interaction is a transient phenomenon (Rowland and Voeltz, 2012). From a molecular point of view, proteins and lipids residing in the OMM and the ER membrane interact to promote the formation of MAMs.
In addition to their role in supporting communication between the ER and mitochondria, these tethering structures also endow the associated organelles with new features.
Numerous works have indicated the variety of proteins involved in the composition of MAMs. Two independent mass spectrometry studies documented 991 (in the first study) and 1212 (in the second study) proteins localized to MAMs (Zhang et al., 2011, Poston et al., 2013). Despite the large number of proteins identified, only 44% were common to both studies. This difference may be due, in part, to differences between cell types, i.e., human fibroblasts versus mouse brain cells. The identification of molecular entities localizing at MAMs and their modifications following cellular environmental events provides important information about the processes orchestrating ER-mitochondria crosstalk.
Historically, the first roles associated with the MAM fraction were lipid synthesis and nonvesicular trafficking between the ER and mitochondria, due to the presence of the long-chain fatty acid-coenzyme A (CoA) ligase type 4 (FACL4) and phosphatidylserine synthase-1 (PSS-1) enzymes. Recently, Aufschnaiter et al. demonstrated that the modification of lipid composition in mitochondria and MAMs decisively influences cell death in neurodegenerative conditions (Poston et al., 2013). The ER and mitochondrial networks not only control different aspects of cellular metabolism but also, through their close and dynamic interaction, are involved in the transmission of physiological and pathological Ca^{2+} and reactive oxygen species (ROS) signals directly from the ER to mitochondria.
Kornmann et al. discovered the ER-mitochondria encounter structure (ERMES) through a genetic screen in *S. cerevisiae* expressing artificial ER-mitochondria (Kornmann et al., 2009)**.** This complex is a heterotetrameric protein complex comprising an ER transmembrane protein (Mmm1p), a cytosolic protein (Mdm12p), and OMM proteins (Mdm34p and Mdm10) (Lang et al., 2015). The subunits Mdm12p, Mdm34p, and Mmm1p contain the synaptotagmin-like lipid-binding protein (SMP) domain, which modulates contact site formation (Reinisch and De Camilli, 2016). Intriguingly, Kornmann showed that the number and activity of ERMES contact points are controlled by the Ca^{2+}-binding rho-like GTPase Gem1p (Kornmann et al., 2011). Despite its important role in regulating ER-mitochondria tethering in yeast, ERMES is not conserved through evolution (Wideman et al., 2013).
A human protein involved in ER-mitochondria tethering and MAM formation is the cytosolic protein phosphofurin acidic cluster sorting protein 2 (PACS-2) (Simmen et al., 2005). The mechanism by which PACS-2 mediates ER-mitochondria tethering is not yet known, but its knockdown separates the two organelles (Simmen et al., 2005). However, the activity of PACS-2 in the MAM fraction is regulated by the residue at position 437, whose phosphorylation mediated by phosphoinositide-dependent serine-threonine protein kinase (Akt) ensures that PACS-2 remains at MAMs (Betz et al., 2013). Moreover, mammalian target of rapamycin complex 2 (mTORC2), which itself localizes to MAMs, activates PACS-2 via Akt (Betz et al., 2013).
The interactions between ER and mitochondria are modulated in different ways and by numerous proteins (Figure 1), including the mitochondria-shaping proteins mitofusin1/-2 (MFN1/-2). Scorrano et al. demonstrated that loss of MFN2 alters Ca^{2+} signaling and reduces ER-mitochondria contacts due to the morphological changes in the ER mediated by MFN2 (de Brito and Scorrano, 2008). However, the activity and function of MFN2 have recently been questioned, leading to the opening of a rather heated debate. In fact, Filadi and colleagues demonstrated that MFN2 reduces the number of close contact sites between the two organelles, proposing a new model for ER-mitochondria tethering (Filadi et al., 2015). Nevertheless, Naon et al. re-evaluated the role of

MFN2 defining it as a ER-mitochondria tether whose ablation decreases interorganellar juxtaposition and communication (Naon et al., 2016). This topic is still controversial with opposite results (Filadi et al., 2017) which allow for further considerations about MFN2 functions.
Another protein complex whose function is to modulate ER-mitochondria juxtaposition is the complex formed by inositol 1,4,5-trisphosphate receptors (IP3Rs), the voltage-dependent anion channel (VDAC) and the OMM chaperone Grp75 as described in Figure 1 (Szabadkai et al., 2006). This interaction is considered functional because it promotes the efficient transfer of calcium from the ER to mitochondria. In fact, silencing of Grp75 in HeLa cells abolished Ca^{2+} accumulation in mitochondria, highlighting chaperone-mediated conformational coupling between the IP3R and mitochondrial machinery. Nevertheless, a recent study of Bartok et al. reveals a non-canonical and structural role for the IP3Rs independently from calcium flux (Bartok et al., 2019).They display that IP3Rs are required for maintaining ER-mitochondrial contacts.
Recently, a study of the Transglutaminase type 2 (TG2) interactome showed an enzymatic interaction with GRP75 in the MAM fraction (D'Eletto et al., 2018). In fact, silencing of the TG2-GRP75 complex leads to an increase in the interaction between IP3R-3 and GRP75, a reduction in the number of ER-mitochondria contact sites, impairment of ER-mitochondrial Ca^{2+} flux and an altered MAM proteome profile.
Furthermore, the complex formed between ER vesicle-associated membrane protein-associated protein B (VAPB) and PTPIP51 regulates the modulation of Ca^{2+} homeostasis by MAMs (De Vos et al., 2012).

[Figure 1 here]

Function and signaling role of MAMs in cellular pathways

MAMs and Ca^{2+} transfer

Ca^{2+} plays a fundamental role in the life of the cell, regulating or inducing many processes, such as proliferation, differentiation, secretion, metabolism, cell death and survival, migration, and gene expression (Giorgi et al., 2018a).
Ca^{2+} signaling, which is organized into spatially and temporally defined domains, characterizes the universality and versatility of Ca^{2+} as an intracellular second messenger (Filadi and Pozzan, 2015).
Ca^{2+} signals impact other organelles via microdomains established by membrane interaction sites (Raffaello et al., 2016, La Rovere et al., 2016). Specifically, the central role in intracellular Ca^{2+} signaling is played by the ER, the principal storage organelle, in which the Ca^{2+} concentration is in the range of hundreds of μM; in contrast, the cytoplasmic Ca^{2+} concentration is approximately 100 nM (La Rovere et al., 2016). This intracellular store contains two main families of Ca^{2+} release channels, which release Ca^{2+} into the cytosol upon stimulation, thus triggering Ca^{2+} signaling: IP3Rs and ryanodine receptors (RyRs) (Rizzuto et al., 2009). The RyR family consists of three different isoforms (RyR1, -2 and -3) encoded by diverse genes and specialized in different bodily locations (Fill and Copello, 2002). RyR1 is generally expressed in skeletal muscle; RyR2, in cardiac muscle. RyR3 is particularly enriched in the brain and is expressed at low levels in many tissues (Giannini et al., 1995). IP3Rs are ligand-gated channels that function to release Ca^{2+} from the ER in response to IP3 generation initiated by agonist binding to cell surface G protein-coupled receptors (Patel et al., 1999). The subsequent increase in the cytosolic Ca^{2+} concentration results in various intracellular events, and the precise cellular outcome depends on the spatiotemporal characteristics of the generated Ca^{2+} signals (Berridge, 2006). IP3Rs consist of three domains: i) an N-terminal that includes the IP3-binding site (which is exposed on the cytosolic face) and suppressor region, ii) a

central modulation domain, iii) and the C-terminal tail with a Ca^{2+} pore-forming region (Yoshikawa et al., 1999, Kerkhofs et al., 2017).
IP3Rs have three isoforms, which are encoded by different genes and are differentially expressed in various cells (Joseph et al., 1995): IP3R1, IP3R2, and IP3R3 (Mikoshiba, 2007). Specifically, IP3R isoform 3 is enriched in the MAM fraction and is involved in Ca^{2+} transfer from the ER to mitochondria upon exposure to apoptotic stimuli (Varnai et al., 2005, Mendes et al., 2005).
The proximity of the ER to mitochondria is essential to preserve intracellular Ca^{2+} homeostasis. In fact it allows contact between proteins on the surface of both organelles and ensure that upon induction of Ca^{2+} mobilization the [Ca^{2+}] on the cytosolic surface of the OMM reaches levels tenfold higher than those in the bulk cytosol (Giorgi et al., 2018b). Specifically, the IP3Rs and sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) are two principal ER-resident proteins enriched in ER-mitochondria contact sites and implicated in the transfer of Ca^{2+} (Doghman-Bouguerra and Lalli, 2019).
SERCA is an ion pump, a member of the P-type family, and is localized in the ER membrane, mainly at MAMs. This pump sequesters calcium ions from the cytoplasm in the ER and transfers two ions per ATP molecule hydrolyzed (Gadsby et al., 2012). The SERCA family consists of three different isoforms (SERCA 1, -2 and -3); specifically, the SERCA2a isoform is expressed in cardiomyocytes, while the SERCA2b isoform is ubiquitously expressed and is the isoform with the higher Ca^{2+} affinity localized at the MAM interface (Gutierrez and Simmen, 2018).

The signal transduction of intracellular Ca^{2+} is strongly dependent on the amplitude and frequency of Ca^{2+} spikes (Berridge et al., 2000, Berridge et al., 2003). Indeed, excessive Ca^{2+} release from the ER mediated by IP3R causes mitochondrial Ca^{2+} overload, inducing proapoptotic signaling (Giorgi et al., 2012). To control cell survival, the basal level of Ca^{2+} transfer occurring along the ER-mitochondrial axis maintains mitochondrial bioenergetics, specifically by enhancing the activity of key enzymes of mitochondrial respiration and ATP production (Cardenas et al., 2010, Kaufman and Malhotra, 2014).
Ca^{2+} released by the ER via IP3R can cross the OMM through VDAC1, a Ca^{2+}-permeable channel (Hajnoczky et al., 2002, Shoshan-Barmatz and Gincel, 2003, Colombini, 2012). Ca^{2+} transferred via VDAC channels reaches the mitochondrial calcium uniporter (MCU) situated at the inner mitochondrial membrane (IMM), and cooperatively regulates its influx into the mitochondrial matrix (Belosludtsev et al., 2019).
Furthermore, VDAC1 enables the substrates of the electron transport chain (ETC) to be imported into mitochondria (Shoshan-Barmatz et al., 2010). Three different isoforms of VDAC have been identified (VDAC1, -2 and -3), but only the VDAC1 isoform is enriched at MAMs (Naghdi and Hajnoczky, 2016, Marchi et al., 2017). Indeed, VDAC2 has a unique role in transmitting proapoptotic Ca^{2+} signals at mitochondria (De Stefani et al., 2012).
However, this interaction between ER and mitochondria is essential because it plays a role in the unfolded protein response (UPR) and ER stress. Indeed, numerous ER chaperones are localized at MAMs, for example, calreticulin, calnexin, the Sigma 1 receptor (Sig-1R) and the chaperone glucose-related regulated protein 78 (GRP78). Sig-1R is a chaperone protein specifically localized at MAMs. A recent study showed that Sig-1Rs physically associate with the chaperone GRP78, also known as binding immunoglobulin protein (BiP), at MAMs, where they regulate calcium flux through IP3R3, stabilizing it and prolonging Ca^{2+} signaling from the ER to mitochondria (Hayashi and Su, 2007, Fujimoto and Hayashi, 2011).
Other proteins localized at the ER-mitochondria interface include mitofusin 1 (MFN1) and mitofusin 2 (MFN2), a GTPase involved in mitochondrial Ca^{2+} regulation. In particular, MFN2 is situated not only in the OMM but also on the ER membrane and is enriched at MAMs. MFN-

related functions are aimed at controlling organelle fusion and influencing Ca^{2+} transfer between the ER and mitochondria by forming an interorganellar bridge. In fact, the tethering between these organelles is created by interaction between MFN2 located on the ER membrane with either MFN1 or MFN2 associated with the OMM. MFN2 ablation reduces the efficiency of mitochondrial Ca^{2+} uptake in response to stimuli IP3-dependent (de Brito and Scorrano, 2009, de Brito and Scorrano, 2008). However, as described above, the role of MFN2 is still controversial. In fact, Filadi and colleagues demonstrated that MFN2 ablation increases the efficacy of Ca^{2+} transfer from the ER to mitochondria, sensitizing cells to mitochondrial Ca^{2+} overload-dependent death (Filadi et al., 2015). A recentl work of Naon et al. supported the original mechanism of action proposed for MFN2, according to which the acute MFN2 ablation increases the distance between the two organelles and reduces mitochondrial uptake of Ca^{2+} released from the ER (Naon et al., 2016).

MAMs and lipid trafficking and metabolism

Since the 1970s, when the function of MAMs in phospholipid metabolism was discovered through the observation that liver-derived mitochondria cannot synthesize phosphatidylserine (PS), the identity of MAMs as a double-edged sword during intercommunication between the ER and mitochondria via lipids was quickly clarified. Indeed, ER-mitochondria contact regulates lipid-dependent mitochondrial physiology and ER homeostasis and directs the specialized transition of different lipid species, with relevant biological consequences for cell fate (van Vliet et al., 2014). Due to its complexity and heterogeneity, the MAM structure needs a very specific composition of proteins and lipids to support the formation of MAM interfaces under specific cellular conditions. Thus, MAMs require robust solidity, which is provided by the particular composition of cholesterol and sphingolipids (Aufschnaiter et al., 2017). Moreover, different enzymes implicated in lipid trafficking and synthesis, such as cholesterol acyltransferase/sterol O-acyltransferase 1 (ACAT1/SOAT1), diacylglycerol O-acyltransferase 2 (DGAT2), PSS1 and PSS2, phosphatidylethanolamine N-methyltransferase 2 (PEMT2) and FACL4/ACS, localize at ER-mitochondria contact sites (Stone and Vance, 2000, Lang et al., 2015, Cui et al., 1993).
In particular, FACL4, which controls the ligation of fatty acids CoA and is involved in triacylglycerol synthesis, is currently used as one of the most reliable MAM marker proteins.

MAMs were initially described as microdomains enriched in phospholipid-related enzymes (Vance, 1990). Although phospholipid synthesis is usually illustrated as occurring at the ER, phospholipids require subsequent translocation to other organellar membranes. PS is also synthesized in the ER by PSS1 and PSS2 enzymes, which localize at MAMs and is then translocated to mitochondria, where PS decarboxylase (PSD) converts PS into phosphatidylethanolamine (PE) in a distinct pathway leading to PE production on microsomes by ethanolamine phosphotransferase (Gibellini and Smith, 2010). Finally, PE returns to the ER, where it is methylated by PEMT2 for the synthesis of phosphatidylcholine (PC). It will be interesting to understand whether a high cholesterol concentration in lipid raft microdomains of MAMs can drive free cholesterol into mitochondria for steroidogenesis. A reduction in the endogenous cholesterol level or inhibition of *de novo* ceramide synthesis induces the relocation of some important MAM proteins, such as Sigma-1R and IP3Rs, to ER cisternae (Hayashi and Fujimoto, 2010). Additionally, the decrease in cholesterol at MAMs favors the connection between MAMs and mitochondria and allows a reduction in *de novo* PS synthesis and a concomitant increase in PE synthesis (Fujimoto et al., 2012). Thus, the transport and transfer of lipids between the ER and mitochondria indicate that MAMs are a critical platform for this process. Recent studies have identified MAMs as cholesterol-rich membranes marked by the sterol-interacting protein caveolin (Sano et al., 2009). Indeed, caveolin appears to play a

central role in lipid-related functions of MAMs, since it helps to enrich intracellular lipid and sterol metabolism-related processes at MAMs (Sala-Vila et al., 2016). Consistent with these findings, methyl-β-cyclodextrin, an agent that depletes membrane cholesterol, can disrupt MAM platforms (Ciarlo et al., 2010), driving the dysregulation of mitochondrial bioenergetics (Ziolkowski et al., 2010). Another lipid metabolism enzyme, ACAT1/SOAT1, is a MAM marker protein since its enzymatic activity is among the most enriched on MAMs (Rusinol et al., 1994). ACAT1 is a multimembrane-spanning enzyme that catalyzes the production of cholesteryl esters from free cholesterol, mediating the balance between membrane-bound and cytoplasmic lipid droplet-stored cholesterol (Puglielli et al., 2001). Under conditions of stress or hormone exposure, the MAM-associated steroidogenic acute regulatory (StAR) protein interacts with VDAC2, promoting the transfer of cholesterol into mitochondria and, therefore, mitochondrial steroidogenesis (Prasad et al., 2015). In addition, AAA domain-containing protein 3 (ATAD3) is enriched at MAMs and is believed to participate in the regulation of steroidogenesis via MAM formation, thereby channeling cholesterol between the ER and mitochondria (Issop et al., 2015). The oxysterol-binding protein (OSBP)-related proteins (ORPs) are another possible mechanism for mitochondrial sterol import (Dawson et al., 1989, Taylor et al., 1984); these proteins localize at membrane contact sites (Du et al., 2015). ORPs translocate from the ER and exchange sterols produced there for phosphatidylinositol 4-phosphate at their target membrane (Drin et al., 2016) to promote sterol translocation. Among the proteins in this family, ORP5 and ORP8 are two interesting members that are partially targeted to MAMs and can interact with a MAM tethering protein, PTPIP51. The silencing of these two proteins alters the biochemical activities and membrane dynamics of mitochondria, though the mechanisms by which these proteins control the transport of lipids or cholesterol remains unclear (Galmes et al., 2016). Furthermore, MAMs are also involved in sphingolipid metabolism. In fact, a sphingomyelinase located in MAMs produces ceramide, which is transferred to mitochondria for conversion into sphingosine-1 phosphate and hexadecenal (Wu et al., 2010). Since the proteome of MAMs contains sphingomyelin phosphodiesterase (SMase), ceramide synthase (CerS), and dihydroceramide desaturase (DES), a certain pool of ceramides is believed to be produced at the contact sites. Importantly, because of the proapoptotic character of ceramides in mitochondria, MAMs could play a pivotal role as a central checkpoint in preventing ceramide influx, hence controlling shifts in the cellular lifespan.

MAMs and mitochondrial dynamics

Under normal conditions, mitochondria change their morphology to create a fragmented or tubular network and to move along the cytoskeleton. This phenomenon is an effect of coordinated and competitive fission and fusion processes, which play a pivotal role in the mitochondrial quality control machinery (Bernhardt et al., 2015). The number, size and positioning of mitochondria within the cytosol are regulated by a delicate balance between fusion and fission events.

The fusion mechanism consists of the union of two mitochondria, while fission is the division of one mitochondrion into two daughters.

Deregulation of these cellular processes results in either a fragmented network with a large number of small round-shaped mitochondria or a hyperfused network characterized by elongated and highly connected mitochondria. Mitochondrial function and activity need these balanced dynamic transitions to respond to cellular stimuli, and they are necessary for adapting the mitochondrial network to the nutrient availability and the metabolic state of the cell (Wai and Langer, 2016). Moreover, different morphological states are associated with multiple physiological and pathophysiological conditions (Nunnari and Suomalainen, 2012).

During elevated stress levels and cell death, the predominant mitochondrial state is fragmentation (Zemirli et al., 2018). However, fragmentation can also occur in the G2/M phase of the cell cycle to

promote mitochondrial motility and quality control and mtDNA inheritance (Otera et al., 2013, Pickles et al., 2018). In contrast, a fused mitochondrial network permits matrix component distribution and stimulation of OXPHOS activity (Mishra and Chan, 2016). Furthermore, mitochondrial elongation is associated mainly with cell survival mechanisms and seems to protect against phagophore engulfment during autophagy triggered by nutrient starvation (Rambold et al., 2011).

In recent years, the unexpected connection between MAMs and mitochondrial dynamics has been extensively studied.

Membrane fusion is mediated by two different dynamin-related GTPases due to the presence of two membranes: the outer and inner membranes. Fusion of the outer membrane is catalyzed by MFN1 and its close homolog MFN2 in mammals, whereas fusion of the inner membrane is mediated by Opa1. MFN1 proteins can interact intermitochondrially through their HR2 domains, tethering two opposing mitochondria (Galloway and Yoon, 2013). Like MFN1 proteins, MFN2 proteins can interact with each other, but they can also heterooligomerize with MFN1 to induce mitochondrial fusion (Detmer and Chan, 2007). MFN1 exclusively localizes to mitochondria, whereas MFN2, is present in MAMs in addition to mitochondria (de Brito and Scorrano, 2008).

The link between mitochondrial fusion and ER-mitochondria contacts could be based on the balance of mitofusins mediating mitochondrial fusion and mitofusins localizing to the MAM. In fact, the human ubiquitin ligase MITOL regulates MAM formation and ubiquitinates the mitochondrial moiety of MFN2 but not ER-MFN2, inducing its activity. Specifically, Sugiura et al. demonstrated that K192 in the GTPase domain of MFN2 is the major ubiquitination site for MITOL (Sugiura et al., 2013).

OPA1-dependent IMM fusion is dependent on MFN1 but not MFN2 (Cipolat et al., 2004). This observation suggests the possible communication between the two membranes during fusion events and suggests an interaction of MFN1 with OPA1, a hypothesis currently more plausible considering the new MFN topology (Mattie et al., 2018).

However, the most thoroughly studied and investigated connection between the MAM and mitochondrial dynamics is mitochondrial fission.

This process is mediated by another dynamin-related GTPase, dynamin-related protein 1 (Drp1). Drp1 is a cytoplasmic protein whose phosphorylation at Ser637 by PKA allows it to maintain its cytosolic localization (Chang and Blackstone, 2007). Recently, dynamin2 was found to work in concert with Drp1 to constrict and divide mitochondria (Lee et al., 2016). At mitochondria-ER contact sites (MERCs), Drp1 forms mitochondria-constricting oligomers that dock to mitochondrial proteins such as mitochondrial fission protein 1 (Fis1), mitochondrial fission factor (Mff), and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49/MiD51) (Friedman et al., 2011), all of which localize to the MAM prior to mitochondrial fission (Murley et al., 2015). However, the role of FIS1 and MID49/51 in mitochondrial fission and fusion is still under discussion (Loson et al., 2013, Zhao et al., 2011, Palmer et al., 2013). Recently, Wu et al. found that the OMM protein FUNDC1 localizes to the MAM and accumulates at ER-mitochondria contact sites by interacting with the ER membrane protein calnexin in hypoxic cells. Furthermore, this group showed that FUNDC1 is specifically required for mitochondrial fragmentation; indeed, it recruits DRP1 to drive mitochondrial fission in response to hypoxic stress (Wu et al., 2016).

A controversial role has been demonstrated for the ubiquitin ligase MITOL. Some studies explain that MITOL regulates mitochondrial morphology by polyubiquitinating Drp1, leading to proteasomal degradation of Drp1 (Nakamura et al., 2006).

Other works show instead that MITOL inhibition induces mitochondrial fusion (Karbowski et al., 2007). However, it is possible that MITOL-dependent Drp1 regulation varies with cellular conditions such as cell cycle phase or nutritional status.

Both the ER-bound inverted-formin 2 (INF2) and the mitochondria-anchored formin-binding Spire1C are actin-nucleating proteins and localize to the ER-mitochondria interface to regulate the actin assembly required for mitochondrial constriction before Drp1 recruitment and oligomerization (Manor et al., 2015, Korobova et al., 2013). Moreover, Drp1 can also bind F-actin *in vitro,* which stimulates Drp1 oligomerization and GTPase activity (Ji et al., 2017). The coordinated action of the ER and actin has clearly been recognized as a fundamental regulator of mitochondrial division, and further research will reveal novel potential regulators and their links with other members of the fission system.

Despite this knowledge, the mechanism by which the ER can identify and mark the sites for mitochondrial division is unclear, but mitochondrial nucleoids were previously identified to be localized at mitochondria-ER contact sites in mammalian cells (Ban-Ishihara et al., 2013).

Recently, live cell imaging and high-resolution microscopy have shown that replicating mtDNA is spatially associated with ER-mitochondria contact sites, indicating future mitochondrial fission sites permitting the correct distribution of mtDNA to the newly generated daughter mitochondria (Lewis et al., 2016)**.** These innovative findings indicate that mtDNA replication is one of the first steps of mitochondrial division, raising new questions about the regulatory mechanism and the means by which this signal from the matrix is transmitted to the ER.

MAMs as modulators of ROS production and activity

ROS are O_2-derived free radicals that can oxidize other molecules (Sena and Chandel, 2012), for example, superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2, a nonradical species) and the hydroxyl radical ($OH^{\bullet}$), which are products of cellular metabolism and xenobiotic exposure. Depending on their concentration, ROS can be beneficial or deleterious to cells and tissues. Physiologically low levels of ROS act as “redox messengers” in intracellular signaling and regulation during processes such as stem cell differentiation, metabolism, hypoxia adaptation, autophagy and immune cell activation. However, an excessive amount of ROS induces oxidative modification of cellular macromolecules, inhibits protein function, and promotes inflammation and cell death (as reviewed in (Circu and Aw, 2010, Sena and Chandel, 2012). Proteins responsible for intracellular ROS generation are located in all subcellular compartments, including mitochondria and the ER (Harrington, 1989, Holmstrom and Finkel, 2014). However, mitochondria are considered the major sources of cellular ROS (Dan Dunn et al., 2015). Generation of ROS within mitochondria is closely associated with the principal function of these organelles: oxidative metabolism and ATP synthesis (Adam-Vizi and Chinopoulos, 2006). During oxidative phosphorylation, electrons are extracted from substrates and subsequently transferred to molecular oxygen across an ETC comprised of enzymatic complexes (I-V). In the final step of the ETC, complex IV (cytochrome c oxidase) catalyzes the reduction of O_2 to water without the formation of oxygen radicals. However, before electrons reach complex IV, they can flow prematurely to O_2 at complexes I and III, leading to the formation of superoxide instead of water (Giorgio et al., 2005). The main ROS produced in mitochondria is the superoxide anion, which is dismutated to H_2O_2 (Janikiewicz et al., 2018). Other known mitochondrial ROS (mROS) sources include the mitochondrial cytochrome b5 reductase and monoamine oxidases (associated with the OMM), dihydroorotate dehydrogenase, and glycerol-3-phosphate dehydrogenase (located at the outer surface of the IMM), electron transfer flavoprotein-ubiquinone oxidoreductase (localized on the matrix face of the IMM), and two mitochondrial matrix enzyme complexes: α-ketoglutarate dehydrogenase and pyruvate dehydrogenase (Janikiewicz et al., 2018). The proton motive force (pmf) is another major producer of mROS that is composed of an electrical gradient (Ψ, mitochondrial membrane potential) and a

chemical gradient (pH) through the IMM. The pmf is generated as protons are expelled from the matrix into the intermembrane space by complexes I, III, and IV as electrons are transferred through the ETC. An increase in mROS production occurs when pmf increases (Sena and Chandel, 2012).

ROS production in the ER is less thoroughly investigated than mROS production, partially because of the limited choices of appropriate tools for measuring ROS levels in this compartment. In this organelle, the main contributors to ROS production are cytochrome P450 family members (Bhattacharyya et al., 2014), NAPDH oxidase 4 (Nox4) (Chen et al., 2008) and the combination of protein disulfide isomerase (PDI) with endoplasmic reticulum oxidoreductin-1 (Ero1) (Janikiewicz et al., 2018, Rinnerthaler et al., 2015).

During protein folding, PDI catalyzes the formation of disulfide bonds in receptor proteins. During this process, the isomerase is first reduced and is then regenerated by Ero1. The reduced Ero1 protein finally transfers the electron via the cofactor FAD to molecular oxygen. However, partial transfer can lead to the production of superoxide (Benham et al., 2013, Bhandary et al., 2012).

Regarding ROS activity, MAMs are enriched in redox-regulatory proteins (Gilady et al., 2010, Yoboue et al., 2017). Examples of MAM-localized regulators of ER-mitochondrial ROS production are calnexin (Lynes et al., 2013, Myhill et al., 2008), Ero1α, which is an isoform of ERo1 (Anelli et al., 2012, Li et al., 2009), and selenon/SEPN1 (Marino et al., 2015). These chaperones and oxidoreductases, some of which themselves produce ROS, bind to ER Ca^{2+}-handling proteins and affect ER-mitochondrial Ca^{2+} flux and, consequently, mitochondrial metabolism via redox-dependent interactions (Gutierrez and Simmen, 2018).

Calnexin is an oxidative protein folding chaperone found in the ER that reversibly interacts with MAM-localized ER calcium pumps (Vance, 1990, Borgese et al., 2006), particularly SERCA2b. This interaction results in inhibition of ER Ca^{2+} signaling (Roderick et al., 2000). Indeed, calnexin resides on MAMs in large quantities, where it may function as a docking site for MAM proteins (Wu et al., 2016). Localization of calnexin at MAMs is partly mediated by the cytosolic sorting protein PACS-2 (Myhill et al., 2008). In general, under homeostatic conditions, the presence of calnexin at MAMs reduces ER-mitochondrial Ca^{2+} flux. Alternatively, when cells undergo ER stress, the removal of calnexin from MAMs increases ER-mitochondrial Ca^{2+} flux (Gutierrez and Simmen, 2018)

Similar to calnexin, the ER-localized redox-sensitive oxidoreductase TMX1 is targeted to MAMs in a palmitoylation-dependent manner (Roth et al., 2009). TMX1 plays a role in protein folding and has been shown to interact with SERCA2b under oxidizing conditions in a thiol-dependent manner (Raturi et al., 2016). Specifically, TMX1 inhibits SERCA2b activity and thus promotes Ca^{2+} flux to mitochondria, preserving mitochondrial metabolism. Hence, this protein opposes calnexin during homeostatic conditions (Gutierrez and Simmen, 2018).

Interestingly, as previously mentioned, Ero1α, which is enriched in MAMs and regulates Ca^{2+} flux, is a fundamental controller of oxidative folding and ER redox homeostasis (Anelli et al., 2012). Translocation and localization of the Ero1-Lα isoform to MAMs depends on the oxidoreductive status of the ER environment. Indeed, hypoxic conditions trigger complete relocation from MAMs (Gilady et al., 2010). During ER stress, Ero1-Lα oxidizes IP3R1, hence promoting the release of Ca^{2+} from the ER (Anelli et al., 2012). Further, ERp44 (an ER luminal chaperone protein), also found in MAMs, binds to IP3R1 and inhibits its channel activity under reducing conditions in the ER, resulting in the blockade of Ca^{2+} transfer to mitochondria at mitochondria-ER contact sites (Higo et al., 2005). Oxidation of IP3R1 by Ero1-Lα causes the dissociation of ERp44, thus leading to the activation of Ca^{2+} release via IP3R1 (Li et al., 2009, Anelli et al., 2012).

The ER-localized selenoprotein N1 (SEPN1) is an ER transmembrane glycoprotein that activates SERCA 2b much as calnexin does. However, while calnexin reduces ER-mitochondria intercommunication, SEPN1 enhances cellular NAPDH metabolism (Marino et al., 2015).

Moreover, SEPN1 protects against the hyperoxidizing conditions elicited by Ero1α, which can lead to the inactivation and oxidation of SERCA (Marino et al., 2015).
Similarly, the glutathione peroxidase GPX8 is an ER-resident protein that is also enriched in MAMs, where it regulates Ca^{2+} storage and flux. GPX8 has been reported to limit ER hyperoxidation and H_2O_2 efflux to the cytosol when Ero1 activity is deregulated (Ramming et al., 2014). At MAMs, GPX8 inactivates SERCA, but due to the reduction in the ER calcium store, it also reduces ER-mitochondria calcium flux (Yoboue et al., 2017).
The p66Shc protein, which belongs to the ShcA family, is another redox-controlled protein that localizes to the mitochondrial side of membrane contact sites. P66Shc, which regulates ROS metabolism and apoptosis, acts as a genetic determinant of life span in mammals (Giorgio et al., 2005) and has been reported to be confined to the mitochondrial intermembrane space (MIMS) and partially to MAMs in cells under UV radiation or H_2O_2 stress (Orsini et al., 2004, Lebiedzinska et al., 2009). Indeed, under physiological conditions, this growth factor adaptor protein is involved in signal transduction via the RAS protein (Janikiewicz et al., 2018). However, exogenous or intracellular oxidative stress can trigger phosphorylation of p66Shc at the Ser36 residue (Migliaccio et al., 1999) and enhance its translocation to mitochondria (Pinton et al., 2007). At MAMs, p66Shc stimulates ROS synthesis via interference with oxidative phosphorylation and influences the apoptotic pathways (Pinton et al., 2007). Indeed, p66Shc binds to cytochrome c when it is translocated to the MIMS and subsequently subtracts electrons from the mitochondrial respiratory chain, acting as a redox enzyme and generating H_2O_2 (Giorgio et al., 2005), thus leading to organelle dysfunction and cell death (Pinton et al., 2007). Interestingly, with aging, the level of p66Shc in the MAM fraction increases, consistent with mitochondrial H_2O_2 production, which has also been found to increase with age (Lebiedzinska et al., 2009).
These oxidoreductases—formerly considered exclusive to the ER folding machinery—also, with their distinct and combined activities, form redoxosomes at MAMs, their primary location (Gilady et al., 2010, Yoboue et al., 2017, Anelli et al., 2012, Raturi et al., 2016). Redoxosomes are multiorganellar protein complexes that facilitate or regulate membrane contact site formation in a redox-specific manner (Yoboue et al., 2018).
Moreover, the overproduction or accumulation of ROS from mitochondria and ER is a crucial event during hypoxia-ischemia and it aggravates cell death. In particular, increased production of mtROS and an altered redox status is observed in experimental fetal and neonatal models of hypoxia-ischemia (Singh-Mallah et al., 2019).
In conclusion, these findings prove that MAM formation and quality, together with the regulated enrichment of specific chaperones and oxidoreductases, can regulate ER calcium homeostasis and signaling with mitochondria (Simmen et al., 2010).

MAMs and apoptosis

Communication between the ER and mitochondria is principally established by a system of Ca^{2+} signaling using Ca^{2+} stored inside the ER, enabling cells to promptly respond and adapt to environmental changes and other stimuli. Hence, MAMs can determine cell fate decisions, such as metabolic and cell death programs. Since Ca^{2+} is a well-known second messenger and an important factor triggering apoptosis (Marchi et al., 2017, Giorgi et al., 2018a), it is not surprising that MAMs regulate apoptosis (Marchi et al., 2018). IP3R-linked Ca^{2+} release from the ER results in almost complete transfer, similar to a unidirectional flux, to the mitochondrial matrix, where Ca^{2+} activates the tricarboxylic acid (TCA) cycle, stimulating mitochondrial ATP synthesis. However, excessive and sustained Ca^{2+} transfer causes Ca^{2+} overload in mitochondria and induces apoptosis by opening the mitochondrial permeability transition pore (mPTP) (Marchi et al., 2018). Via electron tomography studies, Csordás and colleagues showed that the tethering between these

two organelles is mediated by structural proteins that link both the smooth and rough ER to mitochondria and that the length of the tethers is variable (Csordas et al., 2006). Interestingly, this group demonstrated that appropriate spacing between the ER and mitochondria is needed for cell function and survival (Csordas et al., 2006). If the ER-mitochondrial distance is too large, the propagation of Ca^{2+} signals to mitochondria is suppressed, impairing Ca^{2+}-dependent control of mitochondrial activity. In contrast, tightening of these two organelles induces mitochondrial internalization of the Ca^{2+} overload, triggering apoptosis. These findings indicate that the formation of ER-mitochondrial microdomains is functionally linked to mitochondrial calcium buffering as well as apoptosis. Indeed, an overload of calcium in mitochondria promotes oligomerization of the proapoptotic protein Bax—on the one hand, leading to permeabilization of the OMM and the release of cytochrome c into the cytosol, where it can associate with Apaf-1 to form the seven-armed apoptosome, which activates the caspase cascade and ultimately induces apoptosis (Yuan and Akey, 2013, Kuwana and Newmeyer, 2003, Scorrano et al., 2003). On the other hand, hyperaccumulation of Ca^{2+} ions in mitochondria triggers mPTP opening, leading to permeabilization of the IMM, dissipation of the mitochondrial transmembrane potential ($\Delta\psi m$), termination of ATP synthesis, uncontrolled entry of water into the mitochondrial matrix, and swelling and osmotic breakdown of the organelle (Bonora et al., 2017). Consistent with these considerations, in IP3R1-deficient T-cells lacking detectable IP3-induced intracellular calcium release, apoptosis was attenuated, rendering these cells resistant to apoptosis (Jayaraman and Marks, 1997). As already mentioned, IP3Rs are highly concentrated in MAMs and are the main protagonists in Ca^{2+} efflux from the ER into mitochondria (Rizzuto et al., 1993, Rizzuto et al., 1998). However, the connection between MAMs and apoptosis is more intricate than described thus far. Indeed, the complete release of cytochrome c is due to the combined action of proapoptotic Bcl-2 family members and changes in mitochondrial dynamics and morphology. Overall, changes in mitochondrial ultrastructure, normally controlled by the constant and delicate balance between fusion and fission events, are involved in these processes (Soriano and Scorrano, 2010). Notably, these mechanisms are also deeply involved in the formation of ER-mitochondria contacts and in the regulation of their activity in healthy cells (Hoppins and Nunnari, 2012). During apoptosis, fragmentation of the mitochondrial network into various punctiform organelles increases due to recruitment of Drp1, a large GTPase in the dynamin superfamily, to mitochondria (Frank et al., 2001). Indeed, upon induction of apoptosis, massive amounts of Drp1 are translocated from the cytosol to OMMs, where it preferentially localizes, to mediate organelle division. Notably, inhibition of this fission protein by overexpression of a dominant negative mutant prevents apoptosis, revealing the deep interconnection between mitochondrial fission and apoptosis (Frank et al., 2001). Drp1 also stimulates Bax oligomerization and increases apoptosis (Hoppins and Nunnari, 2012). Montessuit and colleagues revealed that Drp1 may promote Bax oligomerization and cytochrome c release by stabilizing membrane tethering and promoting membrane hemifusion *in vitro* (Montessuit et al., 2010). In addition, this group showed that overexpression of a Drp1 mutant in cells delayed Bax oligomerization and apoptosis activation. In addition, MAMs seem to be directly involved in the activation of Bax oligomerization by facilitating the shuttling of sphingolipids between membranes of the two organelles, which stimulates the assembly and oligomerization of Bax in the OMM, thus promoting mitochondrial permeabilization and inducing apoptosis. Moreover, specialized ER tubules may directly play an active role in mitochondrial division by wrapping around mitochondria and marking the position of division sites before Drp1 recruitment, which is critical for Bax-dependent OMM permeabilization upon exposure to apoptotic stimuli (Friedman et al., 2011).

Several studies have shown that a posttranslational modification (PTM) of Drp1 is closely related to apoptosis and is associated with the functional stability of MAMs (Fu et al., 2014, Wasiak et al.,

2007). Using fluorescence recovery after photobleaching, Wasiak and colleagues demonstrated that Bax/Bak can stimulate small ubiquitin-like modifier (SUMO) conjugation to Drp1 during cell death, a process known as SUMOylation. This modification correlates with the stable association of Drp1 with mitochondrial membranes (Wasiak et al., 2007). Moreover, Prudent and colleagues showed that SUMOylation of Drp1 by the SUMO E3 ligase mitochondria-anchored protein ligase (MAPL) (Braschi et al., 2010) has an important role in functionally stabilizing the ER-mitochondrial platform to support mitochondrial constriction, calcium flux, cristae remodeling and cytochrome c release (Prudent et al., 2015). This work demonstrated the mechanism by which MAMs are dynamically regulated through active SUMOylation during apoptosis, creating defined hotspots that signal for cytochrome c release downstream of Bax and Bak (Prudent et al., 2015). However, destabilization of ER-mitochondria contact sites and disruption of MAMs are also reported to induce apoptosis. Indeed, depletion of PACS-2, a MAM-associated tethering protein, causes Bap31-dependent mitochondrial fragmentation and dissociation from the ER and activates apoptosis (Simmen et al., 2005, Yu et al., 2019). Notably, Bap31, an ER membrane protein, binds the mitochondrial fission protein Fis1 during apoptosis, thus connecting the interfaces of the two organelles. The Fis1-Bap31 complex serves as a platform to recruit procaspase-8 for cleavage of Bap31 into a proapoptotic form that promotes calcium release from the ER, triggering the dissipation of the mitochondrial transmembrane potential and, thereby, apoptosis (Iwasawa et al., 2011). Thus, the Bap31-Fis1 bridge has been renamed by Wang et al. as “the kiss of death” between mitochondria and the ER (Wang et al., 2011). Hence, an appropriate balance between mitochondrial fusion and fission plays a key role not only in mitochondrial network dynamics but also in the structural and functional stabilization of MAMs, as well as cell fate decisions.

As already discussed, mitochondrial fragmentation is required for apoptosis induction, and the GTPase Drp1 is involved in many key processes to regulate the interconnection between mitochondrial fission and apoptosis. Interestingly, Mfn2 assembly has been shown to be activated by the inactive soluble form of the proapoptotic Bcl-2 protein Bax, inducing the fusion of mitochondria into elongated tubules in healthy cells (Karbowski et al., 2006, Hoppins et al., 2011). Considering that Mfn2 localizes with Bax during apoptosis (Karbowski et al., 2006), a role of Mfn2 in OMM permeabilization, which could regulate the response of MAMs to apoptotic stimuli, has been hypothesized. These data suggest that disturbance of the delicate balance between fusion and fragmentation of mitochondria upon exposure to stress signals or under pathological conditions involves MAM alteration and disruption that triggers mitochondrial division and can ultimately result in cell death.

MAMs and autophagy

In addition to the involvement of MAMs in cellular calcium homeostasis and apoptosis, the important role of MAMs in the regulation of mitochondrial dynamics and the interplay between the ER and mitochondria in the autophagic process have been highlighted in recent studies. Macroautophagy (hereafter autophagy) is a conserved catabolic cellular process that involves the rearrangement of cell membranes to sequester cytoplasmic constituents and organelles for exposure to the degradative action of lysosomes, wherein the sequestered material is degraded by lysosomal enzymes and recycled (Hamasaki et al., 2013). The double-membrane structures that engulf the material to be degraded are termed autophagosomes and fuse with lysosomes to form autolysosomes ((Wang et al., 2017, Kotani et al., 2018, Lamb et al., 2013, Mercer et al., 2018). Autophagy is normally activated in response to nutrient deprivation and other stresses as a cell survival mechanism. Indeed, autophagy is a cellular strategy for nutrient replenishment during starvation via recycling of internal cellular components and facilitates the elimination of damaged

organelles and protein aggregates (Wollert, 2019). Although most often associated with cell survival, autophagy has also been implicated in cell death (Anding and Baehrecke, 2015). This cellular pathway requires a unique set of proteins called autophagy-related (Atg) proteins, and mutations in Atg genes have been identified in numerous human-associated diseases (Udristioiu and Nica-Badea, 2019). In addition, dysregulation of autophagy has been associated with various disorders, including neurodegeneration and, especially, cancer (Amaravadi et al., 2016). In fact, as shown in a recent study by Missiroli et al. (Missiroli et al., 2016), the tumor suppressor PML, localized at MAMs, controls autophagy in a Ca^{2+}-dependent manner through the AMPK/mTOR/Ulk1 pathway. The reintroduction of MCU into PML knockout cells increases the ability of mitochondria to accumulate Ca^{2+} and is sufficient to suppress autophagy by reducing the level of activated AMPK.

Mitochondrial dynamics, metabolism and autophagy are linked and reciprocally influence each other (Mishra and Chan, 2016). During nutrient deprivation, mitochondria tend to become elongated in order to form a large mitochondrial network, which allows mitochondria to produce ATP more efficiently and rescues them from autophagic degradation because of their large size (Rambold et al., 2011). Starvation-induced mitochondrial elongation is primarily mediated by inhibition of Drp1 through modulation of its two phosphorylation sites. Indeed, upon starvation, the key energy sensor AMPK is activated and directly phosphorylates and inactivates Drp1, blocking fragmentation and favoring mitochondrial elongation (Wikstrom et al., 2013). Strikingly, Drp1 localization and activity are also regulated by the ER-resident SNARE protein syntaxin 17 (Syn17). Under nutrient-rich conditions, Syn17 promotes mitochondrial fission by binding Drp1 and preventing its inhibitory phosphorylation by protein kinase A (PKA). Upon starvation, Syn17 localizes on raft-like structures in ER-mitochondria contact sites, where it recruits the phosphatidylinositol-3 (PI3) kinase (PI3K) complex through interaction with the PI3K subunit Atg14L (Hamasaki et al., 2013) and dissociates from Drp1, thus favoring mitochondrial elongation. However, Syn17 also catalyzes the fusion of autophagosomes with lysosomes. Indeed, Syn17 localizes to the outer membrane of completed autophagosomes and interacts with SNAP-29 and the lysosomal SNARE VAMP8, triggering fusion of the two organelles; moreover, Syn17 depletion leads to an accumulation of autophagosomes without degradation. Hence, Syn17 not only is involved in mitochondrial elongation, observed in starved cells, at the onset of autophagy, but also is required for autophagosome-lysosome fusion in late-stage autophagy (Itakura et al., 2012).

The contribution of the ER to mitochondrial dynamics and the involvement of MAMs in the progression of autophagy, including mitophagy, have been reported. Mitophagy is a highly specialized process that selectively removes dysfunctional or damaged mitochondria through the autophagic machinery to assure mitochondrial quality control and protect cells from damage induced by altered mitochondrial metabolism and apoptosis induction. The best-known pathway of mitophagy was first associated with autosomal recessive Parkinson's disease (PD) and depends on the PINK1/Parkin pathway. PINK1, a serine/threonine kinase, accumulates on the depolarized mitochondrial surface of damaged mitochondria, where it phosphorylates the E3 ubiquitin ligase Parkin for the activation and recruitment of PARK2/Parkin to dysfunctional mitochondria. Thus, PINK1 promotes Parkin translocation to mitochondria, where activated Parkin ubiquitinates OMM proteins to trigger mitochondrial degradation through mitophagy (Pickrell and Youle, 2015). A recent study demonstrated that upon mitochondrial damage that dissipates the mitochondrial membrane potential and induces mitophagy stimulation, PINK1 relocates to MAMs, where it recruits the proautophagic protein Beclin1 (BECN1) to promote the formation of omegasomes, which are autophagosome precursors. During mitophagy induction, Parkin also accumulates at MAMs (Gelmetti et al., 2017). Moreover, mitophagy can be induced by another E3 ubiquitin ligase that localizes at MAMs, glycoprotein 78 (Gp78). Gp78 has a key role in ER-associated degradation

(ERAD) and, upon mitophagy stimulation, promotes mitochondrial division by inducing ubiquitination and proteasome-dependent degradation of Mfn1/Mfn2. Thus, in a manner favoring mitochondrial fragmentation, damaged portions of mitochondria are removed from the healthy regions by division and are subsequently degraded by Parkin-independent mitophagy (Fu et al., 2013b).

MAMs are also essential for hypoxia-induced mitophagy. This process involves FUNDC1, an integral OMM protein that acts at ER-mitochondria contact sites as a mitophagy receptor. Under normal oxygen conditions, FUNDC1 is phosphorylated by Src and casein kinase 2 (CK2), which prevents its interaction with microtubule-associated protein 1 light chain 3 (LC3) through its LC3-interacting region (LIR) (Liu et al., 2012). Upon exposure to hypoxia, FUNDC1 is dephosphorylated by the mitochondrially localized phosphatase PGAM5, which enhances FUNDC1 interaction with LC3, thus leading to the recruitment of autophagosomes at MAMs. Interestingly, the antiapoptotic protein Bcl-XL enables the regulation of PGAM5 phosphatase activity, preventing FUNDC1 dephosphorylation, thus activating hypoxia-induced mitophagy and promoting cell survival (Wu et al., 2014). Regulated changes in mitochondrial morphology are essential during autophagy. Indeed, mitochondrial fission is required prior to engulfment of damaged mitochondria by autophagosomes. A recent study showed that under hypoxic conditions, FUNDC1 increasingly accumulates at MAMs by associating with the MAM-localized membrane protein calnexin and acts as a protein adaptor to recruit Drp1 and promote mitochondrial fission, which is essential for sequestration of mitochondria into autophagosomes (Wu et al., 2016).

The origin of autophagosomal membranes is complex and still intensely debated, since the organelle from which the membranes originate has not been clearly identified (Hayashi-Nishino et al., 2009). Autophagy is initiated by isolation membranes (or phagophores), which elongate, engulf the material to be degraded and close to form autophagosomes. This process is highly regulated and coordinated by Atg proteins and other proteins involved in membrane trafficking. Studies revealed that the autophagosomal marker proteins Atg14L and Atg5 localize to MAMs after starvation (Hamasaki et al., 2013). Normally, Atg14L is not detected in MAMs by subcellular fractionation analysis, but after starvation, Atg14L is recruited to ER-mitochondria contact sites by the ER-resident SNARE protein Syn17 (Hamasaki et al., 2013). Additionally, Atg5, the early autophagosomal marker, transiently localizes at MAMs in starved cells, followed by the late autophagosomal marker LC3 (Hailey et al., 2010). Furthermore, disruption of ER-mitochondria contact sites is reported to prevent the formation of Atg14L puncta at MAMs and thus autophagosome formation, consistent with the idea that autophagosomes can form at MAMs, as reported in several other studies (Hamasaki et al., 2013, Gelmetti et al., 2017). Indeed, Hailey and colleagues showed that autophagosome formation was dramatically impaired in cells in which MAM formation had been blocked by depletion of Mfn2 (Hailey et al., 2010), indicating that autophagosome formation and autophagic pathway activity require the presence of ER-mitochondria contact sites. Furthermore, many studies have shown that OMMs and autophagosomes are transiently located in close proximity and that lipids and/or proteins can be shared and delivered from mitochondria to autophagosomes. Notably, these studies also showed that mitochondria-localized cytochrome b5 and the fluorescent lipid NBD-PS, which is converted to NBD-PE in mitochondria, are transferred from mitochondria to autophagosomes upon starvation, indicating that MAMs also contribute to the membrane composition of autophagosomes.

ER-mitochondria contacts are critical to regulating autophagy, considering a recent study on a specific tether between the two organelles—the VAPB-PTPIP51 tether (Gomez-Suaga et al., 2017). VAPB is an ER protein that binds the mitochondrial protein PTPIP51, acting as a scaffold to connect the two organelles, and can regulate autophagy by controlling Ca^{2+} influx to mitochondria from ER

stores. Indeed, the Miller laboratory demonstrated that overexpression of VAPB or PTPIP51 to tighten ER-mitochondria contacts or the use of a synthetic linker protein that artificially links the two organelles reduces autophagosome formation. In contrast, downregulation of VAPB or PTPIP51 by siRNA silencing to loosen contacts induces autophagosome formation. Notably, the artificial tether can rescue the effects of siRNA-mediated loss of VAPB or PTPIP51 on autophagy, indicating that the VAPB-PTPIP51 tether regulates autophagy through the structural connection between the ER and mitochondria (Gomez-Suaga et al., 2017).

In conclusion, a plethora of cellular processes, including apoptosis and autophagy, has been recently associated with MAMs. Because of the critical nature of these important cellular pathways, the dynamic regulation of interorganellar junctions and the ER-mitochondria connection reveals a key role for MAMs in the determination of the cell fate decision between cell survival and cell death.

The inflammatory function of MAMs

As previously mentioned, the ER-mitochondria interface coordinates numerous signaling pathways, from the transfer of Ca^{2+} to the regulation of lipid transfer as well as the provision of membranes for autophagy; at the interface, MAMs provide an excellent platform for inflammatory signaling pathways (Missiroli et al., 2018).

In the last few years, MAMs have been found to be involved in the formation and regulation of the inflammasome, a multiprotein complex that serves as a platform for the activation of a signaling pathway that recruits procaspase-1 via the adaptor molecule apoptosis-associated speck-like protein containing a caspase activation recruitment domain (CARD) (ASC) and then proceeds to cleave the cytokine precursors prointerleukin-1β (pro-IL-1β) and pro-IL-18 into mature IL-1β and IL-18 (as described in Figure 2). To date, several inflammasomes have been described, each sensing slightly differing types of triggers and playing different roles (Guo et al., 2015); among these inflammasomes, the NLRP3 inflammasome is currently the most fully characterized and studied.

The NLRP3 inflammasome comprises the sensor molecule NLRP3, which contains a pyrin domain (PYD); the adaptor protein ASC, which harbors a PYD and a CARD; and procaspase-1 (Schroder et al., 2010). Moreover, NEK7, one of eleven NEK kinases found in vertebrates, is an important component of the NLRP3 inflammasome, binding directly to NLRP3 via its leucine-rich repeat (LRR) domain (Shi et al., 2016).

The NLRP3 inflammasome can be activated by various stimuli, including danger-associated molecular patterns (DAMPs, such as silica and uric acid crystals) and pathogen-associated molecular patterns (PAMPs), but the specific regulatory mechanisms of NLRP3 inflammasome activation remain unclear. Furthermore, the PTMs of NLRP3 during the priming step, such as phosphorylation and ubiquitination, have been suggested to play critical roles in NLRP3 inflammasome activation (Yang et al., 2019b).

ROS generation, especially by mitochondria, may be the pivotal trigger of NLRP3 inflammasome activation (Zhou et al., 2011).

For the first time, the Tschopp group observed that ROS-generating mitochondria trigger NLRP3 activation and that pharmacological inhibition of complex I or III of the mitochondrial respiratory chain or silencing of VDAC significantly impairs NLRP3 inflammasome activation. In addition, this group demonstrated that mitophagy/autophagy blockade leads to the accumulation of damaged ROS-generating mitochondria, which, in turn, activates the NLRP3 inflammasome (Zhou et al., 2011). Interestingly, they demonstrated that resting NLRP3 localizes to the cytosol and ER structures, whereas upon inflammasome activation, both NLRP3 and its adaptor ASC redistribute

to the perinuclear space where they colocalize with ER and mitochondria organelle clusters (Zhou et al., 2011). Therefore, NLRP3 should ideally be localized near mitochondria to allow efficient sensing of damaged ROS-generating mitochondria.

Consistent with the above mentioned observations, under conditions of oxidative stress, thioredoxin (TRX)-interacting protein (TXNIP) shuttles into MAMs/mitochondria (Saxena et al., 2010), where it binds and leads to the activation of NLRP3 (Zhou et al., 2010). In resting cells, TXNIP interacts with TRX and is therefore unavailable to interact with NLRP3; after inflammasome activation, TXNIP is released from oxidized TRX in a ROS-sensitive manner, allowing its binding with NLRP3 (Zhou et al., 2010).

Moreover, as further discussed below, TXNIP is a potential therapeutic target for diabetes, as it is induced by ER stress through the PERK and IRE1 pathways. TXNIP induces IL-1β mRNA transcription, activates IL-1β production by the NLRP3 inflammasome, and mediates ER stress-mediated β cell death (Oslowski et al., 2012).

Bronner and colleagues published one study that deserves attention: they demonstrated that microbial infection with the *Brucella abortus* strain RB51 induces IRE1α activation, which, in turn, induces increased mROS production and promotes NLRP3 association with mitochondria (Bronner et al., 2015). NLRP3 drives mitochondrial dysfunction through caspase-2 and the proapoptotic factor Bid, leading to subsequent release of mitochondrial contents, inflammasome activation and IL-1β production.

Importantly, IRE1α is activated by mROS at MAMs and is stabilized by Sig-1R in cells under ER stress (Mori et al., 2013). Sig-1R chaperones at MAMs can stabilize IRE-1 and enhance cell survival by prolonging the activation of the IRE1-XBP1 signaling pathway.

To clarify the mechanistic aspect of NLRP3 activation, Subramanian and colleagues demonstrated that mitochondrial antiviral signaling protein (MAVS) regulates NLRP3 localization to mitochondria (through the N-terminal sequence) and inflammasome activation in response to viral infection (Subramanian et al., 2013). Moreover, this group revealed that MAVS plays an unexpected role as a mediator of inflammasome activation beyond its well-defined role in antiviral immunity, further supporting a role for mitochondria/MAMs as platforms for inflammasome formation.

In addition to the abovementioned roles, MAMs can play an important role in the antiviral immune response. In the host cell, RIG-like receptors (RLRs) function as cytoplasmic sensors of PAMPs within viral RNA, resulting in a signaling cascade that induces innate antiviral immunity by triggering the production of type I and III interferons (IFNs) and activation of NF-κB (Seth et al., 2005). The RLR family consists of three members: retinoic acid-inducible gene I (RIG-I), the founding and therefore best-characterized member of this family; melanoma differentiation-associated factor 5 (MDA5); and laboratory of genetics and physiology 2 and a homolog of mouse D11lgp2 (LGP2).

Both RIG-I and MDA5 contain N-terminal tandem CARDs that function in signaling, but LGP2 lacks these domains. In resting cells, RIG-I is maintained in a dormant state in the cytosol; upon recognition of dsRNA generated during viral replication, RIG-I moves to MAMs, where it interacts with MAVS through CARD-CARD interactions to support downstream signaling (Horner et al., 2011). Activation of MAVS, which is localized to both peroxisomes and mitochondria, induces the oligomeric/multimeric assembly of a macromolecular signaling complex, leading in turn to the activation of NFκB and interferon regulatory factor 3 and 7 (IRF3 and IRF7, respectively), which, upon nuclear translocation, initiate the transcription of genes encoding products with antiviral, proinflammatory, and immunoregulatory activities (Seth et al., 2005, Kawai et al., 2005).

During acute infection by hepatitis C virus (HCV), MAVS redistributes from mitochondria to MAMs to peroxisomes to form a signaling synapse between membranes, indicating that MAMs play an important role in coordinating MAVS-dependent signaling between mitochondria and

peroxisomes in the RIG-1 pathway. Importantly, the HCV NS3/4A protease, which cleaves MAVS to support persistent infection, targets this synapse for MAVS proteolysis from MAMs but not from mitochondria in order to ablate RIG-I signaling in the immune response (Horner et al., 2011).
Interestingly, another study characterized the role of mitochondrial and peroxisomal MAVS in the activation of the IFN response and their counteraction by HCV, finding comparable activation of the type I and III IFN response by both MAVS species. In addition, in hepatocytes, the NS3 protease efficiently cleaves MAVS independent of its subcellular localization (Bender et al., 2015). Furthermore, the HCV NS4A protein also localizes to both mitochondria and MAMs and may affect apoptosis induction (Ramage et al., 2015).
In the past few years, Gp78, an E3 ubiquitin ligase that is active in the ERAD pathway, localizes to the ER-mitochondria interface and acts to regulate antiviral signaling through RLRs (Jacobs et al., 2014).
Gp78 modulates type I IFN induction by altering both the expression and signaling of MAVS through two parallel pathways: i) protein-protein interactions, through which Gp78 interacts with both the N- and C-terminal domains of MAVS via its C-terminal RING domain; and ii) ubiquitin-mediated degradation, through which Gp78 utilizes its E3 ubiquitin ligase and ERAD functions to induce the degradation of MAVS (Jacobs et al., 2014).
Taken together, these data highlight the mechanisms by which the MAVS signalosome creates a signaling synapse at the junction between MAMs and mitochondria by recruiting RLRs and other signaling components to this subcellular location. However, the detailed molecular mechanisms by which MAVS signaling is regulated at the innate immune synapse remain to be elucidated. Yang and colleagues recently revealed a critical role of protein geranylgeranylation in limiting the MAVS-mediated antiviral innate immune response by targeting the Rho family small guanosine triphosphatase (GTPase) Rac1 to MAMs (Yang et al., 2019a). MAM-localized Rac1 limits the interaction of MAVS with the E3 ligase Trim31 and hence inhibits MAVS activation, consequently facilitating the recruitment of caspase-8 and cFLIPL to the MAVS signalosome and the subsequent cleavage of Ripk1, which terminates MAVS signaling.
One additional component participating in the immune response in the perimitochondrial region is stimulator of interferon genes (STING), which plays a vital role in dsDNA signaling. During infection with DNA viruses, STING is activated downstream of cGAMP synthase (cGAS) to induce type I interferon production.
STING resides on the ER-facing side of MAMs, where it interacts with and stabilizes RIG-1 and can activate both the IRF3 and NF-κB transcription factor pathways and the subsequent induction of cytokines and proteins, such as type I IFN, which then performs its antipathogenic functions (Ishikawa and Barber, 2008).
STING appears to interact with RIG-I and MAVS in a complex that is stabilized upon viral infection (Zhong et al., 2008, Nazmi et al., 2012).
The serine protease NS4B of HCV suppresses RIG-I-mediated signaling for IFN-β production through a direct protein interaction with STING (Nitta et al., 2013). NS4B expression blocks the protein interaction between STING and MAVS at the ER-mitochondria interface, impeding the formation of the RIG-I/MAVS/STING complex and the activation of downstream effectors.
Disruption of that interaction may restore cellular antiviral responses and may constitute a novel therapeutic strategy for the eradication of HCV.
Furthermore, in neuronal cells, following infection with Japanese encephalitis virus (JEV), the RNA-RIG-I complex recruits STING and allows the recruitment of MAVS to initiate a downstream cascade that leads to the activation of the transcription factors NF-κB and IRF3, which bind to and activate the IFN promoter following nuclear translocation (Nazmi et al., 2012). Knockdown of

STING resulted in downregulation of the expression of various inflammatory signaling molecules, along with an increase in the intracellular viral load (Nazmi et al., 2012).
Additionally, STING is a target of the dengue virus (DENV) NS2B3 protease complex (Aguirre et al., 2012, Yu et al., 2012). The DENV NS2B3 protease inhibits IFNβ production via cleavage of the adaptor STING without modifying the observed NF-kβ activity induced after DENV infection; however, this phenomenon is specific to human cells (Aguirre et al., 2012).
Interestingly, another group demonstrated that upon dsDNA stimulation, NS2B3 physically interacts with STING, suggesting that at least one dsDNA-induced factor also participates in this cleavage event (Yu et al., 2012).
Notably, DENV infection perturbs mitochondrial morphodynamics by inducing mitochondrial elongation in the vicinity of NS3- and NS4B-containing convoluted membranes and altering mitochondria-ER contacts (Chatel-Chaix et al., 2016); this effect might in turn impair DENV-induced RIG-I translocation to MAMs, thus dampening the innate immune response.
Altering mitochondrial morphology by knockdown of DRP1 results in a sharp decrease in the RIG-1 level in the MAM fraction; conversely, the opposite phenotype was observed upon silencing of MFN2 expression (Chatel-Chaix et al., 2016). Overall, these data indicate that mitochondrial elongation reduces the DENV-induced RIG-I-dependent innate immune response, suggesting a protective effect against these antiviral responses.
An alternative strategy of DENV infection is binding of the NS3 protein of DENV to 14-3-3ε via a highly conserved phosphomimetic RxEP motif (Chan and Gack, 2016). NS3 inhibits the binding of RIG-I to 14-3-3ε, preventing the translocation of activated RIG-I to mitochondria and thereby blocking MAVS interaction and antiviral signaling. Furthermore, the DENV NS2A and NS4B proteins inhibit RIG-I/MDA5-directed IFN-β induction and block TBK1 activation, thereby facilitating DENV replication and virulence (Dalrymple et al., 2015).
Considering the important role of MAMs in the antiviral response, it is unsurprising that numerous viral proteins target this structure (Figure 2).
One well-characterized example is the human cytomegalovirus (HCMV) protein UL37 exon 1 (pUL37x1), also known as viral mitochondrion-localized inhibitor of apoptosis (vMIA), which traffics via MAMs from the ER to the OMM, where it robustly inhibits apoptosis (Zhang et al., 2011).
HCMV pUL37x1/vMIA is also partially associated with internal lipid rafts in ER-MAMs but not in mitochondria, allowing HCMV infection to affect the assembly of apoptosome components therein (Williamson et al., 2011).
Interestingly, pUL37x1 mobilizes Ca^{2+} from the ER into the cytosol, inducing mitochondrial fission and protecting cells from apoptosis (Sharon-Friling et al., 2006).
Surprisingly, upon HCMV infection, cytosolic Grp75 is increasingly enriched in MAMs (Bozidis et al., 2010).
Another viral protein that shuttles from the ER to mitochondria via MAMs is human immunodeficiency virus 1 (HIV-1) viral protein R (Vpr) (Huang et al., 2012). Vpr is integrated into the ER, induces bulging of MAMs, and reduces the expression level of the mitochondrial protein MFN2 via the VprBP-DDB1-CUL4A ubiquitin ligase complex, leading to rupture of the OMM, deformation of mitochondrial cristae and initiation of cell death.
Taken together, these data pinpoint the central role of MAMs in the coordination of inflammation and antiviral immunity as platforms that allow an appropriate antipathogen response and/or inflammatory response.

[Figure 2 here]

MAMs in diseases

Neurodegenerative diseases

Different works have identified MAM compartments as pivotal in the regulation of neurological functions; indeed, impairment of mitochondria-ER signaling and activity has been revealed in several neurodegenerative diseases. For example, despite affecting different regions of the nervous system, Alzheimer's disease (AD), PD, and amyotrophic lateral sclerosis (ALS) share characteristics: progressive neurodegeneration with a complex pathogenic progression; a lack of effective treatments; and, as recently discovered, a common alteration in ER-mitochondria contact and communication (Paillusson et al., 2016). Targeting MAMs signaling may be an innovative pharmacological approach to treat these pathologies; however, a clearer understanding of the processes involved in neuronal pathogenesis is necessary.

Several studies have shown communication between mitochondria and the ER at synapses: scanning electron microscopy has revealed an abundance of ER-mitochondria contacts in various neuronal compartments (Wu et al., 2017, Hedskog et al., 2013); furthermore, in mouse respiratory neurons, ER vesicles move in order to interact with mitochondria and control Ca^{2+} signaling, which is fundamental for synaptic activity (Mironov and Symonchuk, 2006). The results of another study enhanced the importance of the interaction between ER and mitochondria in Ca^{2+} signaling in neurons by showing the involvement of microtubules (Mironov et al., 2005).

Alzheimer's disease (AD) is an adult neurodegenerative disorder characterized by progressive neuronal loss, especially in the cortex and the hippocampus, where there is an accumulation of extracellular neuritic plaques of β-amyloid (Aβ) and intraneuronal accumulation of the microtubule-associated protein Tau within neurofibrillary tangles (Goedert and Spillantini, 2006). Mutations in amyloid precursor protein (APP) and in presenilin-1 and -2 (PS1 and PS2, respectively), which participate in the γ-secretase complex that produces Aβ, have been identified in the familial form of Alzheimer's disease (FAD).
Area-Gomez et al., in 2009, demonstrated that PS1 and PS2 are highly enriched at MAMs (Figure 3), where they are functionally active, acting as the catalytic core of the γ-secretase complex (Area-Gomez et al., 2009).
Recently, the same group showed an increase in MAMs function and ER-mitochondrial communication in PS mutant cells and in fibroblasts from patients with both the familial and sporadic forms of AD (Area-Gomez et al., 2012). MAMs functions can be associated with different dysfunctions in the AD phenotype, such as altered lipid, glucose and Ca^{2+} metabolism; mitochondrial impairment; and altered Aβ production.
The localization of PS at MAMs is consistent with the demonstration that this subcellular compartment is linked to the production of Aβ in the mouse brain (Schreiner et al., 2015).
Different MAM proteins have been demonstrated to be essential for neuronal survival. For example, knockdown of Sig-1R and PACS-2 causes degeneration of hippocampal neurons and astrocytes (Hedskog et al., 2013); in addition, upregulation of MAM-associated proteins was found in the AD brain and in the APP Swe/Lon mouse model. The same work revealed that primary hippocampal neurons treated with a low concentration of Aβ peptide exhibited increased

expression of IP3R3 and VDAC1 proteins, with an increased number of ER-mitochondria contact points and, consequently, an increased mitochondrial Ca^{2+} concentration (Hedskog et al., 2013).
In 2016, Filadi et al. found that AD-associated PS2 mutants promote ER-mitochondrial coupling only in the presence of MFN2 through physical binding (Filadi et al., 2016).
One line of evidence supporting the functional involvement of MAMs in neurodegeneration is the demonstration that the ε4 variant of Apolipoprotein E (APOE), an isoform that is the major risk factor for AD, increased MAM activity and mitochondria-ER contact in astrocytes (Tambini et al., 2016).
The tumor suppressor phosphatase and tensin homolog (PTEN) is localized in the MAM compartment in cancer cells (Bononi et al., 2013). The corresponding pathway has been associated with the neuroprotective action of insulin-like growth factor against Aβ neurotoxicity in *in vitro* experiments (Dore et al., 1997) and in *in vivo* models of AD (Stein and Johnson, 2002). In AD brain neurons, a decrease in PTEN levels and a different distribution of this protein (Griffin et al., 2005) have been found. Even if a localization at MAMs has not yet been demonstrated, an ensuing work analyzing PTEN in postmortem brain tissues of AD patients showed delocalization of PTEN from the nucleus to the cytoplasm and deregulation of the PI3K pathway (Sonoda et al., 2010).
Several lines of evidence from *in vitro* and *in vivo* experiments have linked glycogen synthase kinase 3 (GSK-3) to the formation of hyperphosphorylated tau in neurofibrillary tangles (Hanger et al., 1992). Moreover, in brains with different stages of neurofibrillary degeneration, the amount of hyperphosphorylated tau is correlated with the protein level of PKB, confirming the involvement of PKB/GSK-3 signaling in neurofibrillary degeneration in AD (Pei et al., 2003). Specifically, PKB phosphorylates both Thr212 and Ser214 in the longest and shortest tau isoforms (Ksiezak-Reding et al., 2003).

Parkinson's disease (PD) is a neurodegenerative disease that involves movement impairment due to progressive degeneration of dopaminergic neurons and formation of Lewy bodies in a specific area of the brain, the substantia nigra (Kalia and Lang, 2015).
Both mitochondrial and ER impairment have been linked to PD (Wang and Takahashi, 2007), and different PD-related proteins with a role in signaling regulation localize at these compartments (as described in Figure 3). For example, α-Synuclein (SNCA) mutations lead to both familial (autosomal dominant early-onset PD) and sporadic PD. In contrast, autosomal recessive forms of PD are caused by different mutations in the genes encoding the proteins PINK1 (PARK6), parkin (PARK2), and DJ-1 (PARK7) (Cookson et al., 2008).
SNCA is a presynaptic protein whose abnormal aggregation in oligomers leads to toxicity due to Lewy body formation. SNCA has recently been shown to be localized to MAMs (Poston et al., 2013), and its point mutations have been found to induce a reduction in the association of ER with mitochondria, to enhance mitochondrial fragmentation and to decrease MAM functionality. The function of this protein in this compartment has been associated with modulation of mitochondrial morphology and Ca^{2+} homeostasis (Guardia-Laguarta et al., 2014). Furthermore, Paillusson et al. showed the binding of SNCA with VAPB, demonstrating that mutant SNCA can prevent VAPB-PTPIP51 interaction and can consequently alter Ca^{2+} signaling at MAMs in neurons (Paillusson et al., 2017). Recently, Calì and colleagues demonstrated that the PD-related α-synuclein mutations A53T and A30P do not affect the ability of SNCA to act as a positive regulator of the ER-mitochondria contacts and mitochondrial Ca^{2+} transients (Cali et al., 2019). Nevertheless, their increased aggregation propensity prevents α-syn from exerting its activity at the MAM, thus hampering the possibility to sustain ER-mitochondria interactions and their related function, through a loss of function mechanism.

Different DJ-1 mutations, including deletions and point mutations, have been associated with the autosomal recessive early-onset form of PD (Bonifati, 2012). The DJ-1 protein functions in counteracting oxidative stress and controlling mitochondrial morphology, and it also has a protective effect at MAMs because it modulates ER-mitochondria tethering and consequent Ca^{2+} signaling between the two organelles. Indeed, DJ-1 overexpression in HeLa cells annuls mitochondrial impairment due to p53 overexpression by restoring ER-mitochondria tethering (Ottolini et al., 2013).

Mutation of the PARK2 gene was first associated in 1998 with autosomal recessive juvenile PD; the responsible mutations include truncations, exon rearrangements and point mutations and lead to a loss of Parkin function (Kitada et al., 1998). This evidence has also been corroborated in an *in vitro* cellular model of PD in which correctly regulated levels of Parkin protect against neuronal death caused by neuronal toxins (Sun et al., 2013). For example, Jiang et al. demonstrated the protective effect of Parkin against dopamine toxicity in human dopaminergic neuroblastoma cells via a reduction in oxidative stress, an effect that is lost with the expression of PD-linked mutations (Jiang et al., 2004).

Furthermore, Parkin is involved in neuroprotection through the suppression of apoptotic stress-activated protein kinase pathways (Hasegawa et al., 2008) and has been demonstrated to prevent the toxic effects of mutant SNCA on catecholaminergic neurons (Petrucelli et al., 2002) and *in vivo* models (Bian et al., 2012).

The cytoprotective effects of Parkin might be associated with its action on mitochondria: the involvement of Parkin in mitochondria-dependent cell death induced by ceramide, with a decrease in caspase-3 activation and cytochrome c release, along with delayed mitochondrial swelling, has been demonstrated (Darios et al., 2003).

Moreover, Parkin mRNA and protein levels are upregulated by both mitochondrial and ER stress in order to confer protection of mitochondrial integrity and prevent cell death, enhancing the connection between Parkin, the ER and mitochondria (Bouman et al., 2011).

Later, Calì et al. demonstrated that overexpression of this protein promotes both physical and functional ER-mitochondria tethering, with enhanced Ca^{2+} transfer between the ER and mitochondria and increased ATP production after agonist stimulation (Cali et al., 2013).

In a 2016 study, another group conversely found increased ER-mitochondria tethering in primary fibroblasts from PARK2 knockout mice and in neurons derived from induced pluripotent stem cells of a patient with PARK2 mutations (Gautier et al., 2016).

Since 2014, autophagosome formation has been shown to occur in MAMs, where different proautophagic proteins relocate. PINK1 is important for Parkin recruitment to control the clearance of dysfunctional mitochondria by the autophagy-lysosome pathway, a process termed mitophagy (Narendra et al., 2010).

Mutations in both PINK1 and Parkin disrupt the process of mitophagy mediated by these two proteins and may connect the impairment of this process with the pathogenesis of PD. Specifically, PINK1 PD-associated mutations prevent its binding to Parkin and its mitochondrial translocation, accompanied by impairment of mitochondrial degradation (Geisler et al., 2010).

In addition, mutations in leucine-rich repeat kinase 2 (LRRK2) also lead to a form of autosomal dominant PD, and the pathogenic mutant protein LRRK2-G2019S has been demonstrated to induce ER stress and cell death through its interaction with and inhibition of SERCA, leading to increased ER-mitochondria contact (Lee et al., 2019). Furthermore, PTEN deletion had an overall protective effect on dopaminergic neurons in an *in vitro* model of PD toxicity (Zhu et al., 2007) and in adult mice via an inducible Cre system (Domanskyi et al., 2011).

[Figure 3 here]

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects upper motoneurons located in the motor cortex and lower motoneurons in the brainstem and spinal cord. Among ALS cases, only ~10% are familial (fALS), the majority are sporadic (sALS), and ~15% also present with frontotemporal lobar dementia (ALS/FTD) (Al-Chalabi et al., 2012).
ALS/FTD has been associated with several mutations in genes encoding MAM proteins, enhancing the importance of the correct function of MAMs to prevent pathogenic consequences. These two pathologies are characterized by neuronal inclusions of TAR DNA-binding protein 43 (TDP-43) and fused in sarcoma (FUS) (Ling et al., 2013).
For example, P56S, a mutated form of VAPB, is associated with some dominantly inherited familial forms of motor neuron disease, including ALS type 8. De Vos et al. demonstrated that overexpression of this mutant form disrupts the ER structure due to an altered interaction with PTPIP51 and causes an increase in Ca^{2+} release from the ER, consequently augmenting mitochondrial Ca^{2+} uptake (De Vos et al., 2012). The same group also found that MAM association controlled by VAPB-PTPIP51 interaction can be disrupted by TDP-43 or by FUS through the activation of GSK-3β (Stoica et al., 2014).
Mutations in Sig-R1 have been associated with juvenile ALS; for example, the amino acid mutation E102Q, located in the transmembrane domain, results in altered membrane distribution of the protein (Al-Saif et al., 2011); the importance of Sig-R1 in motor neuron functionality has also been demonstrated in Sig-R1 knock-out mice, which exhibit motor deficiency (Mavlyutov et al., 2010). Furthermore, in motor neurons of ALS patients, Sig-1R showed an altered intracellular localization with accumulation in enlarged C-terminals, the cytoplasm, and proximal axons (Prause et al., 2013), whereas an alteration of ER-mitochondria crosstalk was found in motor neurons of knockout mice, with consequences on Ca^{2+} signaling and ER stress (Bernard-Marissal et al., 2015).
Dominant mutations in the gene encoding Cu/Zn superoxide dismutase (SOD1) lead to inherited ALS and accumulation of SOD1 mutant proteins at MAMs (Bruijn et al., 2004); loss of Sig-R1 function aggravated MAM perturbation in mutant SOD1 transgenic mice, indicating that MAMs are perturbed in both Sig-1R- and SOD1-linked ALS. These mutant forms of Sig-R1 cannot bind to IP3R3 and induce mitochondrial dysfunction and consequent neurodegeneration (Watanabe et al., 2016).
Sig-R1 might be a therapeutic target; indeed, diverse studies have already shown the neuroprotective properties of Sig-1R agonists against ischemia (Katnik et al., 2006).
For example, Pre-084, an agonist of Sig-1R, has been shown to be a promising candidate therapeutic, as in a mouse model of ALS, it improved the function of motoneurons, protecting them from neurodegeneration (Mancuso et al., 2012). In 2017, the efficacy of another Sig-1R agonist, SA4503, on cultured embryonic mice spinal neurons carrying an ALS-causing mutation was demonstrated. Sig-1R can manipulate Ca^{2+} signaling, preventing cytosolic accumulation of this second messenger (Tadic et al., 2017).

Hereditary motor and sensory neuropathies (HMSN) or Charcot-Marie-Tooth (CMT) disease are the most common degenerative disorders of the peripheral nervous system; these terms refer to a group of disorders characterized by chronic motor and sensory polyneuropathy (Bird TD. Charcot-Marie-Tooth (CMT) Hereditary Neuropathy Overview. 1998 Sep 28 [Updated 2019 Jan 24]. In: Adam MP, Ardinger HH, Pagon RA, et al., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2019. Available from: https://www.ncbi.nlm.nih.gov/books/NBK1358/).
The most common axonal form of HMSN, referred to as CMT2Ag, is linked to MFN2 gene mutations: the consequent mutated protein has been demonstrated to compromise ER-

mitochondrial interactions (de Brito and Scorrano, 2008) and to cause axonal degeneration due to dysfunctional and dislocated mitochondria within axons.
Mutations in ganglioside-induced differentiation-associated protein 1 (GDAP1), another protein identified at MAMs (Poston et al., 2013), have been linked to recessive and demyelinating (CMT4A) or dominant/recessive and axonal (CMT2K) forms of this pathology (Baxter et al., 2002). GDAP1 knockout leads to impairment of Ca^{2+} homeostasis and MAM stability and to changes in axonal and neuronal physiology in a mouse model (Barneo-Munoz et al., 2015).

Diabetes

Mitochondria-ER crosstalk plays an important role in many cellular processes fundamental to neuronal activity, such as Ca^{2+} signaling, phospholipid metabolism and apoptosis, and dysregulation of this interaction is a common denominator leading to neurodegeneration in the diseases described above. Understanding the point at which and the means by which MAM function is compromised in these different pathologies might be a turning point in identifying a precise treatment targeted to each neuronal compartment or each pathogenic protein.
Disruption of ER-mitochondria communication is a primary cause of altered cellular homeostasis and leads to tissue dysfunction. Accordingly, mitochondrial modifications and ER stress have long been considered to be independently involved in the pathogenesis of metabolic diseases (Chang et al., 2015). As discussed above, mitochondria and the ER interact through MAMs, exchanging Ca^{2+}, lipids and other metabolites. Recent studies underlined the importance of ER-mitochondria contact sites in hormonal and nutrient signaling, suggesting that dysregulation of this intracellular region—rather than alterations on the single-organelle level—might trigger metabolic syndromes (Theurey et al., 2016).
Diabetes mellitus (DM) is a multifactorial metabolic disorder characterized by deficient insulin production by pancreatic β cells and peripheral insulin resistance (IR). Autoimmune eradication of pancreatic β cells leads to complete insulin deficiency, resulting in type 1 diabetes mellitus (T1DM). Conversely, type 2 diabetes mellitus (T2DM) arises due to the development of IR in different organs, such as the liver, muscle and adipose tissues. Loss of insulin sensitivity results in impaired suppression of both gluconeogenesis and glycogenolysis, leading to the well-known hyperglycemic condition (Kahn et al., 2006). A state of IR is the originating event that normally takes place several years before T2DM develops and is thus a challenging area of study for the development of new therapeutic strategies (Rieusset, 2015). Mitochondria and ER alterations (Montgomery and Turner, 2015), together with the modification of lipid and calcium homeostasis (Stinkens et al., 2015), are associated with dysfunction of insulin-mediated glucose uptake and, therefore, with the development of T2DM. The direct interaction between mitochondria and the ER at MAMs and the occurrence of lipid and Ca^{2+} trafficking at these contact sites support the idea that MAMs are an important platform involved in controlling glucose homeostasis, implicating ER-mitochondria miscommunication as a cause of hepatic IR (Rieusset, 2017).
Insulin plays a key role in the postprandial state, where it is responsible for controlling glucose homeostasis by increasing skeletal muscle glucose uptake and inhibiting glucose production. This complex metabolic regulation involves different cellular pathways activated by the binding of insulin to its membrane receptor, with consequent initiation of a phosphorylation cascade (Boucher et al., 2014). Notably, the critical cellular pathway responsible for the metabolic action of insulin is the PI3K-AKT pathway. PI3K activation by extracellular signaling initiated by events such as the binding of insulin to its receptor, leads to AKT recruitment to the plasma membrane by the combined action of phosphatidylinositol-3,4,5-triphosphate (PIP3) and 3-phosphoinositide-dependent kinase-1 (PDK1), resulting in AKT phosphorylation at Thr308 (Alessi et al., 1997).

Subsequent phosphorylation of AKT on Ser473 by mTORC2 results in its full activation (Sarbassov et al., 2005). Activated AKT causes the phosphorylation and inhibition of forkhead box protein 01 (FoX01) (Nakae et al., 2001), peroxisome proliferator-activated receptor γ coactivator 1a (PGC1a) (Li et al., 2007), and GSK3 (Cross et al., 1995), resulting in inhibition of hepatic glucose production. The two transcription factors FoxO1 and PGC1a positively regulate the expression of several genes involved in gluconeogenesis and fatty acid oxidation (Li et al., 2007). Under stress conditions and growth factor deprivation, FoxO1 simultaneously activates AKT to facilitate energy production and inhibits the anabolic energy-consuming functions of mammalian target of rapamycin complex 1 (mTORC1) (Hay, 2011). However, AKT also inhibits glycogen synthesis via inhibition of GSK3 (Hermida et al., 2017). Moreover, *in vitro* and *in vivo* studies revealed that AKT is a key modulator of glucose transporter 4 (GLUT4), promoting its translocation to the plasma membrane in insulin-responsive tissues by acting on the AKT substrate of 160 kDa (AS160), also known as TBC1D1 (Kane et al., 2002). Accordingly, reductions in AKT phosphorylation result in increased hepatic gluconeogenesis (Shinjo et al., 2017). Consistent with this effect, lack of *Akt1* and *Akt2* impairs the ability of insulin to regulate glycemia and increase the expression of several FoXO1 target genes, thus decreasing systemic glucose tolerance (Cho et al., 2001).

Different studies have reported that AKT, together with other proteins involved in insulin signaling such as mTORC2, protein phosphatase 2A (PP2A), GSK3β, and PTEN, are localized at MAMs (Giorgi et al., 2010), confirming the important role of this interface in insulin signaling. Specifically, mTORC2-mediated phosphorylation of AKT at Ser473 occurs directly at MAMs, and loss of AKT or mTORC2 results in disruption of MAM integrity (Betz et al., 2013). Accordingly, alterations in MAM proteins in liver or muscle tissues result in alterations in glucose homeostasis (Rieusset, 2015). The level of the active, phosphorylated form of AKT is increased in the hepatic MAM fraction upon insulin stimulation (Tubbs et al., 2014). Moreover, in AKT knockout cells, ER-mitochondria interactions are reduced, indicating a possible correlation between AKT function and MAM integrity (Betz et al., 2013). A recent study revealed that administration of palmitic acid (PA), known to induce IR in hepatic cell lines (Lee et al., 2010), impairs insulin-induced AKT phosphorylation, resulting in MAM disruption without involvement of the ER stress response or mROS. MAM integrity and AKT activity were partially restored upon transfection of MFN2, a regulator of mitochondrial fusion (Shinjo et al., 2017); specifically, MFN2 overexpression in PA-treated cells reinstated AKT phosphorylation at Ser473 but not at Thr308. Notably, expression of the MFN2 K192R mutant, which ameliorates mitochondrial fusion but not ER-mitochondria contact in MFN2 knockout cells, does not restore AKT phosphorylation in PA-treated cells (Shinjo et al., 2017). Short-term PA exposure reduces ER-mitochondria calcium flux, with a decrease in the MAM area, suggesting that PA treatment disrupts the MAM interface, consequently suppressing ER-mitochondria functional interaction. These observations indicate that MAM disruption, rather than single mitochondrial dynamics, plays a critical role in PA-induced AKT inactivation in hepatic IR (Shinjo et al., 2017). Moreover, since mTORC2-mediated phosphorylation of AKT at Ser473 in response to insulin occurs at MAMs (Betz et al., 2013), ER-mitochondria tethering disruption is a prerequisite for PA-induced inhibition of mTORC2 activity. Taken together, these findings indicate a close correlation between AKT, MAM integrity and insulin signaling, although the molecular pathway underlying this complex communication is unclear (Figure 4) (Rieusset, 2018).

Liver-specific ablation of MFN2 results in reduced ER-mitochondria communication, leading to numerous metabolic abnormalities, such as IR and impaired glucose tolerance (Sebastian et al., 2012). Accordingly, overexpression of MFN2 in the mouse liver reversed IR conditions induced by a high-fat diet. Similarly, liver-specific mTORC2 knockout mice exhibit systemic hyperglycemia, hyperinsulinemia, and hypolipidemia, which are reversed by constitutive expression of the active

form of AKT2, confirming that mTORC2 regulates hepatic glucose metabolism via AKT signaling (Hagiwara et al., 2012). Collectively, these findings suggest that both the mTORC2-AKT pathway and MFN2 are involved in the maintenance of metabolic homeostasis and that MAM integrity plays a crucial role in insulin signaling. In addition, mutations in IP3R, another protein known to localize at MAMs, are associated with alterations in glucose homeostasis and with the development of diet-induced diabetes (Ye et al., 2011). Consistent with this association, IP3R1 knockdown reduces MAM formation, triggering mitochondrial dysfunction and glucose intolerance in obese mice (Tubbs et al., 2014). However, in obese mice, MFN2 expression is increased and the total area of MAMs is reduced, suggesting the involvement of other ER-mitochondria tethering factors in the development of IR. Accordingly, ER-mitochondria tethering is maintained in MFN2 knockout cells, while a reduction in the MAM area is observed with the additional deletion of PACS2 (Figure 4) (Naon et al., 2016).

Tubbs and colleagues recently described new insight into the role of MAMs in the development of T2DM. Using an *in situ* proximity ligation assay (PLA), they monitored MAM integrity by visualizing VDAC1/IP3R1 and Grp75/IP3R1 interactions and demonstrated that insulin signaling rapidly decreases after MAM disruption. Consistent with this finding, MAM alterations due to pharmacological or genetic inhibition of cyclophilin D (CypD) result in modification of insulin signaling. The treatment of CypD knockout mice with metformin, a known antidiabetic drug, improved insulin sensitivity; in addition, adenoviral overexpression of CypD in primary hepatocytes of diabetic mice rescued MAM integrity, enhancing insulin action (Tubbs et al., 2014). The same effects were obtained in human primary hepatocytes by pharmacological inhibition of CypD. Analysis of different diabetic mouse models (*ob/ob* and high-fat/high-sucrose diet, HFHSD) revealed that under diabetic conditions, hepatic MAM integrity is disrupted, and the administration of rosiglitazone, another antidiabetic agent, or metformin restored organelle communication and, therefore, insulin sensitivity. Subsequent studies led to the identification of another possible antidiabetic compound, sulforaphane (SFN), which acts at the MAM level. PLA and glucose production assays in hepatoma cell lines and primary mouse hepatocytes revealed that SFN both counteracts MAM disruption and increases glucose production after PA treatment. In addition, SFN treatment decreases ER stress. Administration of SFN to *ob/ob* or HFHSD mice resulted in improved glucose tolerance and reduced glucose production. Liver analysis of SFN-treated diabetic mice showed increased MAM protein content, restored VDAC1-IP3R1 interactions and reduced ER stress to a similar extent as observed with metformin treatment, indicating that SFN is a promising new potential antidiabetic drug (Tubbs et al., 2018a). Further studies conducted in diabetic mouse models revealed that, in this systemic condition, disruption of the ER-mitochondria interaction occurs not only in the liver but also in skeletal muscle (Tubbs et al., 2018b). Furthermore, a reduction in ER-mitochondria contact in retinal endothelial cells is induced *in vitro* by acute high glucose treatment (Ma et al., 2017). Accordingly, diet-induced obesity leads to MAM surface loss in anorexigenic proopiomelanocortin (POMC) neurons in the hypothalamus (Schneeberger et al., 2013), promoting a loss of ER-mitochondria contact as an event predicting mitochondrial dysfunction and IR during the development of T2DM.

The hypothesis that disruption of MAMs and alteration of ER-mitochondria communication is a prognostic factor for T2DM development is further supported by proteomic studies. A recent proteome profiling analysis comparing the MAM protein content in the brains of mice with long-term T2DM to that in nondiabetic controls led to the identification of 144 proteins significantly altered under diabetic conditions. Among the most significantly altered signaling pathways were the UPR, p53, hypoxia-related transcription factor, and methyl CpG binding protein 2 pathways. In addition, the level of GRP75, a key MAM tethering protein, was drastically reduced in T2DM mice (Ma et al., 2017). Moreover, investigation of the expression levels of proteins involved in ER-

mitochondria tethering and communication in β cells from 12 T2DM patients compared to β cells from healthy donors revealed an increase in reticular IP3R2 expression and a decrease in VDAC1 expression but no differences in the expression of the mitochondrial transit peptide receptor TOM20 or MFN2. A PLA revealed a significantly decreased amount of IP3R2-VDAC-1 complexes in diabetic islets and in β cells of T2DM patients compared to controls (Thivolet et al., 2017). Additional studies conducted in human myotubes revealed that PA treatment, which leads to IR, is directly associated with a reduction in functional and structural ER-mitochondria interactions. Accordingly, an increase in the MAM surface area via Grp75 and MFN2 overexpression prevents PA-induced alterations in insulin signaling. Finally, VDAC1-IP3R1 interactions were decreased in myotubes from both nondiabetic and diabetic obese subjects compared to healthy donors, and this decrease was positively correlated with insulin-induced PKB phosphorylation (Figure 4) (Tubbs et al., 2018b). The absence of differences in MFN2 expression in T2DM patients in contrast with the IR and impaired glucose tolerance resulting from its ablation in the mouse model (Sebastian et al., 2012) might be explained by the observation that the latter study involved a small cohort of patients, and the proteomic analysis was not performed on the whole organ.

Other controversial results were reported in a study by Arruda et al., in which the MAM content was increased in the livers of obese mice, inducing mitochondrial Ca^{2+} overload and mitochondrial dysfunction that led in turn to impaired insulin action and abnormal glucose metabolism. The same study reported that induction of ER-mitochondria interactions by overexpression of IP3R1 or PACS2 leads to IR, whereas hepatic downregulation of these proteins improves mitochondrial oxidative capacity and insulin sensitivity in obese mice (Arruda et al., 2014). These discrepancies could be attributed to differences in the metabolic status or housing conditions of the mice or in the experimental analysis. Indeed, as discussed in the previous section, MAMs are intensely involved in the inflammatory response, indicating their sensitivity to a variety of environmental signals, such as pathogens and nutrients. Indeed, as discussed previously, pancreatic β cell death can be mediated by TXNIP, a critical signaling node that links ER stress and inflammation. Briefly, TXNIP localizes at MAMs in response to oxidative stress and mediates IL-1β production by the NLRP3 inflammasome (Figure 4) (Tseng et al., 2016). Furthermore, none of the cited proteins are selectively localized at MAMs; thus, modulating their expression could lead to complex effects, another possible explanation for the divergences observed (Rieusset, 2018).

Recently, Dingreville and colleagues reported results challenging the role of MAMs in pancreatic β cells and their involvement in glucotoxicity-associated β cell dysfunction. ER-mitochondria contacts and Ca^{2+} flux are stimulated by acute glucose administration in rat pancreatic β cells. Accordingly, alteration of the MAM structure by silencing Grp75 altered glucose-stimulated insulin secretion (GSIS), confirming that ER-mitochondria coupling is directly involved in regulating GSIS in pancreatic β cells. However, chronic incubation under high glucose conditions resulted in altered GSIS and reduced ER-mitochondria Ca^{2+} flux despite the observed increase in organelle contacts (Dingreville et al., 2019). Sustained exposure of β cells to nonphysiological glucose levels, as performed in the chronic high glucose incubation experiment, leads to glucotoxicity with impaired GSIS and decreased insulin processing, which in turn results in β cell dysfunction during T2DM progression (Bensellam et al., 2012). At the cellular level, glucotoxicity results in mitochondria and ER alterations, and the increase in organelle interactions might be a protective mechanism. Finally, linker-induced increases in ER-mitochondria interactions reduced organellar Ca^{2+} exchange, induced mitochondrial fission and altered GSIS. Collectively, these findings suggest that the involvement of MAMs in the development of GSIS in pancreatic β cells is more likely to be a dynamic process than a static condition, thus suggesting that the role of MAMs in the progression of T2DM might vary according to the stage of the disease.

In conclusion, despite the controversial results, the involvement of MAMs in the regulation of insulin signaling pathways is currently recognized, although the molecular mechanisms underlying this relationship are unclear. Currently, T2DM is considered a global disease, affecting 415 million people in 2015, a number that is estimated to reach 642 million by 2040 (Rieusset, 2017). Consequently, T2DM is a major challenge for healthcare systems worldwide. Because the development of peripheral IR precedes the development of T2DM (DeFronzo and Tripathy, 2009) and considering that its manifestation appears to be related to MAM disruption and altered protein content, further investigation of the molecular mechanisms underlying ER-mitochondria communication and insulin signaling might be the key to the development of new preventive and/or therapeutic approaches.

[Figure 4 here]

Cancer

The involvement of subcellular membranes in cancer was first described by Hoelzl Wallach in 1968, emphasizing that despite the vast differences among tumor landscapes, the common feature is the dysregulation of one or more membrane systems (Wallach, 1968).

The fine-tuned regulation of the association between the ER and mitochondria is crucial yet fundamental for cell functionality, since calcium-based communication within microdomains can differ depending on the stimulus and biological context. In fact, cancer cells tend to not overload mitochondria with calcium, thus escaping apoptosis induced by either calcium or drugs, and they can establish a constitutively low level of ER-mitochondrial calcium transfer as an essential strategy for cell viability (Wallach, 1968). Recent findings suggest that a basal mitochondrial calcium load is vital for the support of cancer cell metabolism and bioenergetics (Cardenas et al., 2016).

Cancer is a deregulation of physiological cellular processes that leads to uncontrolled growth. The mechanism underlying this aberration is not easy or simple to uncover and is more likely due to an accumulated series of “errors” from which cells cannot recover than to a single error.

One of the main strategies that cancer cells utilize to sustain uncontrolled growth and support survival is escape from programmed cell death, or apoptosis, which is a cancer hallmark (Hanahan and Weinberg, 2011).

Recently, increasing evidence has shown that cancer cells can remodel Ca^{2+} signaling to evolve prosurvival strategies (Marchi et al., 2014). Indeed, several tumors exhibit alterations in the expression of a set of proteins that can function as either tumor suppressors or oncogenes. Some of these proteins directly affect MAMs, particularly calcium flux between mitochondria and the ER (Stewart et al., 2015).

In the context of MAMs, tumor suppressors act as enhancers of Ca^{2+} flux from the ER to mitochondria, promoting apoptosis activation through mitochondrial Ca^{2+} overload; in contrast, MAM-resident oncogenes act to inhibit this overload, thus promoting cell survival under stress conditions (Bittremieux et al., 2016).

These proteins have been reviewed in recent years, and many have yet to be discovered.

Notably, some of these proteins are important calcium-dependent receptors and are thus key regulators and driving forces in cell fate decisions and carcinogenesis.

In the setting of tumors, increased expression of IP3Rs in the ER can lead to the acquisition of metastatic features (Marchi and Pinton, 2016). Specifically, IP3R3 has been proven to be involved in breast cancer proliferation (Mound et al., 2013), and further evidence shows that enhanced

IP3R3 expression is correlated with enhanced invasiveness and metastasization, along with reduced long-term survival, in colorectal cancer (Mound et al., 2013) and with dissemination in gastric cancer (Sakakura et al., 2003).
Collectively, the current literature suggests that IP3R3 is the preferred isoform that transfers calcium-mediated apoptotic signals to mitochondria and contributes to cell death in various cell types and tissues (Lam et al., 2008).
VDAC1 is associated with MAMs, where it can regulate the exchange of metabolites, nucleotides and calcium, acting as a crucial player in calcium homeostasis, apoptosis regulation and oxidative stress protection in various pathological settings (Missiroli et al., 2017).
Moreover, VDAC1 (the channel that conducts calcium flux from the ER to mitochondria, as described previously) has been found to be overexpressed in many cancer types, and VDAC1-derived peptides can exert proapoptotic effects and even potentiate the efficacy of conventional chemotherapeutics (Arzoine et al., 2009).
At MAMs, Sig-1R forms a complex with the chaperone BiP/GRP78, thus regulating Ca^{2+} homeostasis between the ER and mitochondria; however, upon calcium depletion or ligand stimulation, Sig-1R dissociates from the complex with BiP, enabling prolonged calcium flux from the ER to mitochondria via IP3R3 (Figure 5) (Hayashi and Su, 2007). Sig-1R expression is higher in cancer cells with metastatic potential than in normal tissue (Morciano et al., 2018); this increase in expression can be promoted by the formation of a functional molecular platform with SK3, a calcium activated K^+ channel, and Orai1, boosting calcium influx across the plasma membrane and consequently favoring cancer cell migration (Gueguinou et al., 2017).
Moreover, Sig-1R has been reported to mediate the communication between cancer cells and their microenvironment, possibly by shaping the activity of ion channels in response to extracellular signals. Indeed, Sig-1R regulates the human voltage-dependent K^+ channel subunit human ether-à-go-go-related gene (hERG) in myeloid leukemia and colorectal cancer cell lines (Crottes et al., 2016).
Among the proteins that have a structural role in MAMs as components of tethering complexes, MFN2 directly inhibits mTORC2-AKT signaling, indicating its role in the suppression of tumor cell proliferation. This evidence was shown mainly in cancers such as liver, colorectal, lung and breast cancer, in which decreased MFN2 expression has been reported (Rimessi et al., 2019, Xu et al., 2017, Rehman et al., 2012).
In prostate cancer, evidence shows that enhanced mitochondrial fusion driven by MFN1 and MFN2 is correlated with cancer progression (Morciano et al., 2018).
In other cancer types, such as lung, ovarian, esophageal, breast and head cancers, MFN1 has been reported to be either amplified or mutated in the context of cancer progression, while in pancreatic, esophageal and prostate cancers, MFN2 deletion has also been found (Danese et al., 2017).
MAMs can host proteins with oncogenic and oncosuppressive functions; one of the main oncogenes reported to reside at MAMs is Akt/protein kinase B (PKB). Akt is an important Ser/Thr kinase involved in crucial prosurvival signaling pathways; indeed, Akt enhancement can be considered a hallmark in many cancer types (Ando et al., 2018). This kinase has been found to be amplified or mutated in pancreatic, breast and prostate cancers (Danese et al., 2017).
Many studies have reported that this protooncogene phosphorylates all isoforms of IP3Rs at a conserved consensus sequence, thus arresting the flux of calcium from the ER to mitochondria and leading to the evasion of cell death (Marchi et al., 2012)
The Akt pathway, specifically the PI3K-Akt pathway, is one of the pathways most frequently dysregulated in tumorigenesis. In addition, this pathway controls the phosphorylation of MICU1, an MCU regulatory subunit, affecting its stability and processing and leading to dysregulation of

mitochondrial calcium dynamics as well as to ROS production and tumor progression (Marchi et al., 2019).
Moreover, melatonin, the hormone that regulates the sleep-wake cycle, has been reported to exert antitumoral effects, decrease cancer cell proliferation and modulate the phosphorylation of Akt (Wang et al., 2012); this finding is intriguing, since melatonin, a natural nontoxic compound, may be a promising adjuvant therapy for cancer (Rimessi et al., 2019).
RAS proteins, which belong to the family of small GTPase proteins, are considered oncogenic since they are deregulated in many cancer types and regulate cell survival and cell division (Downward, 2003). Upon stimulation, the Ras protein translocates from the plasma membrane to internal membranes, including those of the ER and mitochondria (Bivona et al., 2006).
Recent findings show that K-Ras is engaged in Ca^{2+} signaling and crosstalk at MAMs. In colorectal cancer cell lines, the oncogenic K-Ras^{G13D} mutation abrogates IP3-induced calcium release because of a decrease in the Ca^{2+} level in the ER. This mutation affects ER calcium transport systems; indeed, K-Ras^{G13D}-expressing cells exhibit lower levels of SERCA2b and an altered IP3R isoform expression profile. Specifically, favoring of IP3R1 expression over IP3R3 expression has been reported, indicating decreased susceptibility to apoptotic stimuli along with an increased ability to generate calcium oscillations to maintain cell proliferation and survival. In fact, upon deletion of K-Ras, IP3R3 expression at MAMs is restored, as are Ca^{2+} transfer and apoptosis sensitivity (Pierro et al., 2014).
At MAMs, oncogenic H-Ras alters calcium transfer between the ER and mitochondria, leading to apoptosis evasion (Figure 5) (Rimessi et al., 2014). Furthermore, mitochondrial dysfunction caused by Ras drives redox and metabolic modifications that sustain tumorigenesis (Hu et al., 2012).
Bcl-2 was one of the first oncogenes reported to be enriched at MAMs and has calcium-dependent oncogenic activity (Meunier and Hayashi, 2010). Bcl-2 is a primary member of the Bcl-2 family; it can inhibit apoptosis by interacting with Bax and Bak, thus promoting oncogenesis. It localizes at the ER and on the OMM, inhibiting IP3Rs via directly binding their BH4 N-terminal domains (Rong et al., 2009). This finding suggests that Bcl-2 can control proapoptotic calcium signaling through interaction with IP3Rs and, consequently, maintain prosurvival Ca^{2+} oscillations (Figure 5).
Dysregulated expression of Bcl-2 has been described in various cancers, such as chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DL-BCL), non-small cell lung carcinoma, multiple myeloma, follicular lymphoma and small cell lung cancer (Packham, 1998).
BIRD2, a Bcl-2/IP3R inhibitor, has shown promising results in the treatment of B-cell cancers (Akl et al., 2015), small cell lung cancer, CLL, multiple myeloma and follicular lymphoma (Greenberg et al., 2015, Lavik et al., 2015).
Bcl-XL is another oncogene belonging to the Bcl-2 family that is frequently overexpressed in tumors. At the OMM, it may modulate apoptosis by controlling mitochondrial membrane permeability through VDACs, thus controlling the release of cytochrome c (Tsujimoto and Shimizu, 2000).
In multiple cancers, increased levels of Bcl-XL have been reported, which correspond to decreased levels of apoptosis (Trisciuoglio et al., 2017).
Like Bcl-2, Bcl-XL binds directly to IP3Rs (White et al., 2005), regulating cancer cell survival via IP3R inhibition. Indeed, Bcl-XL inhibitors (WEHI-539, A-1155463, and A-1331852) are used in therapies for B-cell lymphomas and other solid tumors(Adams et al., 2018).
Myeloid cell leukemia factor 1 (Mcl-1) is an antiapoptotic protein belonging to the Bcl-2 family and found at MAMs. This protein possesses a short half-life and is structurally different from the other members of the Bcl-2 family. Mcl-1 has been reported to be overexpressed in many cancer types (Beroukhim et al., 2010), both hematopoietic cancers and solid tumors such as lung cancer, and is

related to acquired resistance to apoptosis and chemotherapeutics; indeed, its silencing restores sensitivity to these drugs (Akgul, 2009).
Regarding Mcl-1 at MAMs, one study reported that the interaction with IP3Rs occurs at its C-terminal region, with an affinity similar to that of other members of the Bcl-2 protein family. This interaction allows Mcl-1 to increase IP3R-mediated calcium release, thus decreasing the steady-state calcium levels in the ER (Eckenrode et al., 2010).
Interestingly, Mcl-1 also interacts with VDAC1, modulating its role in mitochondrial Ca^{2+} uptake; moreover, Mcl-1 binds more strongly to VDAC1 than do other Bcl-2 family members, suggesting that the VDAC1/Mcl-1 complex has a crucial role in the survival of cancer cells since it increases the calcium concentration in mitochondria and the production of ROS, which enhance the migratory characteristics of cancer cells (Huang et al., 2014).
The oncoprotein fetal and adult testis expressed 1 protein (FATE1) is the direct link between tumor progression and the variation of the appropriate ER-mitochondrial distance, and its increased expression is correlated with poor prognosis in adrenocortical carcinoma patients (Doghman-Bouguerra et al., 2016).
Database interrogation (The Cancer Genome Atlas colorectal dataset) revealed that FATE1 is frequently overexpressed with RNF183, an ER-resident E3 ligase, and that this expression signature is correlated with poor prognosis, suggesting an oncogenic role of this protein in the inhibition of cell death.
In addition, an antitethering function has been suggested for FATE1, which is localized at the OMM, where it associates with MAMs and causes decreased mitochondrial calcium uptake and cell survival (Doghman-Bouguerra et al., 2016). Indeed, FATE1 induces resistance to apoptotic stimuli promoted by the accumulation of toxic cholesterol esters, thus triggering ER stress via inhibition of SOAT1, a MAM-resident enzyme (Sbiera et al., 2015).
Expression of the oncosuppressor p53 is altered in most cancer types; p53 is a nuclear transcription factor that is activated by various stimuli and consequently transactivates genes involved in apoptosis, cell cycle regulation, cell transformation and cancer progression. This protein has additional functions in the cytoplasm through which it inhibits autophagy and activates apoptosis (Green and Kroemer, 2009); moreover, in the mitochondrial matrix, it promotes the assembly of F_1F_0-ATP synthase (Bergeaud et al., 2013), possibly regulating oxidative phosphorylation. Recent findings report the association of p53 with MAMs, where it can regulate calcium homeostasis (Giorgi et al., 2015b); indeed, this protein binds and stimulates SERCA pumps in the ER membrane, consequently increasing ER calcium levels. Thus, during apoptosis, calcium release to mitochondria is enhanced, promoting Ca^{2+} overload (Figure 5).
Because many cancer cells harbor a deletion or missense mutation in p53, calcium-mediated apoptosis is impaired, thus contributing to cancer progression and chemoresistance (Giorgi et al., 2016). Furthermore, Giorgi et al. demonstrated the role of p53 in controlling calcium signaling in three-dimensional tumor masses via intravital techniques, elucidating that in the setting of cancer, dysfunctional p53 reduces ER calcium release, resulting in low responsiveness to apoptotic stimuli (Giorgi et al., 2015a).
Promyelocytic leukemia protein (PML) is a known tumor suppressor first identified in the pathogenesis of acute promyelocytic leukemia (APL). Its localization is primarily nuclear, where it performs its proapoptotic function, participating in the formation of a multiprotein complex known as the nuclear body; however, PML can also be distributed in the cytoplasm. As a tumor suppressor, PML can interact and stabilize p53, enhancing its function; furthermore, p53 may control the distribution of PML at MAMs (Bernardi et al., 2004, Missiroli et al., 2016).
Loss of PML has been linked to several tumor conditions, especially prostate, breast and brain cancers (Gurrieri et al., 2004).

In addition to in the nucleus as a transcription factor, its cytosolic function mainly revolves around its localization at MAMs, where it participates in the regulation of apoptosis through calcium modulation. Indeed, PML can physically interact with IP3R3, and its localization at MAMs can affect both apoptosis and autophagy (Missiroli et al., 2016).

Apoptosis is regulated by the formation of a multiprotein complex at MAMs that contains IP3R3, AKT, and PP2A. Under cancer conditions, loss of PML corresponds to a reduction in PP2A activity and thus to an increase in AKT phosphorylation and activity, leading to IP3R3 hyperphosphorylation and a consequent reduction in ER Ca^{2+} release and apoptosis inhibition (Figure 5) (Giorgi et al., 2010).

Another tumor suppressor protein, BRCA-1 binding protein (Bap1), is frequently mutated in several cancers; its mutation has recently been reported to play a role in malignant mesothelioma. Bap1 is a deubiquitylase involved in the regulation of many cellular processes, such as the DNA damage response and chromatin remodeling.

This protein localizes in the ER and the nucleus and regulates intracellular calcium signaling. Bononi et al. demonstrated that heterozygous inactivating mutations in BAP1 (BAP1 +/-) are associated with a decrease in the expression of Bap1 and resistance to apoptosis in affected specimens compared to wild-type specimens. This mutation confers a reduction in calcium release from the ER to mitochondria in response to agonist stimulation or apoptotic stimuli. At MAMs, Bap1 interacts and stabilizes IP3R3 via deubiquitylation; thus, mutation of Bap1 leads to a decrease in IP3R3 stability and function (Bononi et al., 2017).

PTEN is a tumor suppressor, and its deletion is frequently found in many tumor species. Due to its function as a phosphatase, its deletion results in constitutive activation of the PI3K-Akt pathway (Osaki et al., 2004).

PTEN can also be involved in increasing calcium release from the ER through IP3Rs, thereby restoring cellular sensitivity to Ca^{2+}-mediated proapoptotic stimuli through IP3R phosphorylation (Bononi et al., 2013)

Moreover, PTEN competes with F-box/LRR-repeat protein 2 (FBXL-2), a mediator of protein degradation via ubiquitylation, for IP3R3 binding and subsequent proteasomal degradation (Kuchay et al., 2017). This function permits the maintenance of IP3R3-mediated Ca^{2+} flux into mitochondria, preserving the cellular response to apoptotic stimuli.

The proapoptotic thioredoxin-related transmembrane protein (TMX1) localizes at MAMs, where it mediates palmitoylation of membrane-proximal exposed cysteine residues partially by interaction with the ER sorting molecule PACS2 (Myhill et al., 2008).

TMX1 inhibits SERCA2b pumps at MAMs, affecting calcium transfer through membrane juxtaposition (Krols et al., 2016).

Low expression of TMX1 in cancer cells results in enhanced ER calcium flux, faster cytosolic clearance of calcium and reduced transfer of calcium to mitochondria, hence reducing ER-mitochondria contact, shifting bioenergetics and accelerating tumor growth (Raturi et al., 2016).

In addition to its role in autophagy, Beclin-1 has been reported to act as a tumor suppressor since its haplodeficiency in mice results in an increased probability of tumor formation (Turcotte and Giaccia, 2010). In many tumors, such as prostate, ovarian and breast tumors, monoallelic Beclin-1 deletion has been found, and decreased expression in cancer cells, especially in brain and cervical cancer cells, has been noted (Fu et al., 2013a).

Many studies have observed a direct interaction between IP3Rs and Beclin1; indeed, cells lacking Beclin1 show enhanced IP3R-mediated calcium signaling. Despite these findings, whether IP3R-mediated calcium signaling is involved in the role of Beclin1 in tumor cells remains unclear.

Additionally, the elucidation and reconstruction of the principal components of MAMs—proteins, tethering complexes and signaling pathways—throughout the many related studies has shown

that the calcium-based signaling of this composite network of membranes and proteins can be further regulated by the action of chemotherapeutic drugs. Indeed, several chemotherapeutic drugs initiate an increase in the cytosolic Ca^{2+} concentration (Wyrsch et al., 2013). The underlying mechanism is still incompletely elucidated but could be dependent on the cytosolic function of p53. Chemotherapeutic treatment induces accumulation of p53 at MAMs, where it binds and activates SERCA pumps, thus promoting ER calcium uptake (Giorgi et al., 2015b). The increased Ca^{2+} content in the ER stores consequently leads to the spontaneous release of calcium from the ER, possibly to mitochondria (Wang et al., 2006).
As reviewed by Kerkhofs et al., some commonly used chemotherapeutic drugs either directly or indirectly affect proteins at MAMs; for example, resveratrol has a direct inhibitory effect on ATP synthase, while arsenic trioxide (ATO) enhances PML activity, and adriamycin has an indirect effect on p53 activation. Cells overexpressing Bcl-2 exhibit increased resistance to cisplatin, which binds covalently to DNA, thus inducing damage. Indeed, ABT-737, a Bcl-2 BH3-inhibiting compound, restores sensitivity to cisplatin. Increased FATE1 expression levels can decrease mitochondrial calcium uptake, resulting in reduced apoptosis sensitivity in cancer cells; the effect of mitotane is indirectly regulated in this manner by FATE1 expression (Kerkhofs et al., 2018).
Collectively, the latest findings on the role of MAMs in tumorigenesis are crucial to unveil novel functions of proteins for their potential development as therapeutic targets to possibly complement the efficacy of current treatments.

[Figure 5 here]

Conclusions

Numerous lines of evidence indicate that ER-mitochondria contact sites play pivotal roles in important cellular events, as reported in this chapter. This physical interaction is extensively described and directly demonstrated by the ability of the ER tubules to circumscribe mitochondria, marking the sites of subsequent mitochondrial division. Hence, the appropriate balance in mitochondrial morphology and dynamics reveals a key role of this balance in the mitochondrial network as well as in MAMs structural and functional stabilization and cell fate decisions.
Importantly, MAMs dysfunction has been connected with several pathological conditions, emphasizing the importance of communication between the connected organelles. MAMs act as a hub for Ca^{2+} handling, ROS production, mitochondrial morphology, and lipid synthesis and transport. The MAMs machinery is intricately connected to infection and the antiviral response (Missiroli et al., 2018), diabetes (Tubbs et al., 2018b), and cancer (Missiroli et al., 2017), as well as to neurodegeneration (Schon and Area-Gomez, 2013).
Therefore, the understanding of the molecular composition and functions of MAMs, as well as the identification of novel pharmacological approaches to reestablish MAMs integrity and physiological communication between the two connected organelles, could establish the MAMs interface and its related proteins as key targets for the development of novel therapeutic strategies for several pathologies.

Acknowledgements

PP is grateful to Camilla degli Scrovegni for continuous support. PP is supported by the Italian Association for Cancer Research (AIRC, IG-18624), Telethon (GGP11139B) and local funds from the University of Ferrara. CG is supported by local funds from the University of Ferrara, the Italian Association for Cancer Research (AIRC: IG-19803), the Italian Ministry of Health (GR-2013-02356747), and by a Fondazione Cariplo grant. MRW was supported by the Polish National Science Centre grant (UMO-2018/29/B/NZ1/00589). Moreover, MRW gratefully acknowledge the financial

support for this research from the FOIE GRAS and mtFOIE GRAS projects. These projects received funding from the European Union's Horizon 2020 Research and Innovation programme under the Marie Skłodowska-Curie Grant Agreement No. 722619 (FOIE GRAS) and Grant Agreement No. 734719 (mtFOIE GRAS). MLA was supported by the Polish National Science Centre grant UMO-2015/17/D/NZ1/00030.

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Figure legends

Figure 1
Representation of mitochondria-associated membranes interface.
MAMs are associated for the lipid synthesis and non-vesicular trafficking between the ER and mitochondria, due to the presence of the long-chain fatty acid-coenzyme A (CoA) ligase type 4 (FACL4) and phosphatidylserine synthase-1 (PSS-1) enzymes. A human protein involved in MAM formation is the cytosolic protein phosphofurin acidic cluster sorting protein 2 (PACS-2). The phosphorylation of PACS-2 at the residue 437 mediated by phosphoinositide-dependent serine-threonine protein kinase (Akt) ensures that PACS-2 remains at MAMs. Moreover, mammalian target of rapamycin complex 2 (mTORC2), which itself localizes to MAMs, activates PACS-2 via Akt. ER-mitochondria juxtapositions are regulated also by the complex formed by inositol 1,4,5-trisphosphate receptors (IP3Rs), the voltage-dependent anion channel (VDAC) and the OMM chaperone Grp75. The Transglutaminase type 2 (TG2) interacts with GRP75 in the MAM fraction. The silencing of the TG2-GRP75 complex leads to an increase in the interaction between IP3R-3 and GRP75, a reduction in the number of ER-mitochondria contact sites and impairment of ER-mitochondrial Ca^{2+} flux. Finally, the complex formed between ER vesicle-associated membrane protein-associated protein B (VAPB) and PTPIP51 also regulates the modulation of Ca^{2+} homeostasis.

Figure 2
Inflammation and antiviral response at MAMs.
The NLRP3 inflammasome is formed by the NLRP3, the adaptor molecule apoptosis-associated speck-like protein containing a caspase activation recruitment domain (CARD) (ASC) and the procaspase-1 which by autocleavage, then proceeds to cleave the cytokine precursors prointerleukin-1β (pro-IL-1β) and pro-IL-18 into mature IL-1β and IL-18.
In resting conditions NLRP3 localizes to the cytosol and ER structures, whereas upon inflammasome activation, both NLRP3 and its adaptor ASC redistribute to the perinuclear space where they colocalize with ER and mitochondria organelle clusters.
Mitochondrial antiviral signaling protein (MAVS) regulates NLRP3 localization to mitochondria (through the N-terminal sequence) and inflammasome activation in response to viral infection.

Figure 3
Mitochondria-ER signaling and activity in neurodegenerative diseases.
The activities of several MAM proteins have been found to be disrupted during the pathogenesis of Alzheimer and Parkinson's diseases.
Presenilin-1 and -2 (PS1 and PS2) are highly enriched at MAMs, where they act as the catalytic core of the γ-secretase complex. AD-associated PS2 mutants induce ER-mitochondrial coupling only in the presence of MFN2 through physical binding. The ε4 variant of Apolipoprotein E (APOE) also increases MAM activity and mitochondria-ER contacts.

Figure 4
Mitochondria-ER signaling and activity in diabetes.

New insights are recently described into the role of MAMs in the development of type 2 diabetes mellitus. Both the mTORC2-AKT pathway and MFN2 are involved in the maintenance of metabolic homeostasis and MAM integrity.
The expression levels of the ER–mitochondria tethering protein MFN2 are reduced and mTORC2-mediated phosphorylation of AKT at Ser473 in response to insulin occurs at MAMs.
Moreover, increased ROS production triggers conformational changes in TXNIP and subsequent loss of the complex TRX–TRXNIP that binds and activates NLRP3, which produces IL-1β.
Insulin signaling rapidly decreases after MAM disruption by VDAC1/IP3R1 and Grp75/IP3R1 interactions.

Figure 5
Mitochondria-ER signaling and activity in cancer.
MAMs play a crucial role in tumorigenesis and several oncogenes and tumorsuppressor are localized to this fraction where control different cellular processes. For more details, see the text.