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Balancing ER-mitochondrial Ca^{2+} fluxes in health and disease

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Abstract

Organelles cooperate with each other to control cellular homeostasis and cell functions by forming close connections through membrane contact sites. Important contacts are present between the endoplasmic reticulum (ER), the main intracellular Ca^{2+}-storage organelle, and the mitochondria, the organelle not only responsible for the majority of cellular ATP production but also for switching on cell death processes. Several Ca^{2+}-transport systems focalize at these contact sites, thereby enabling the efficient transmission of Ca^{2+} signals from the ER towards mitochondria. This provides a tight control of mitochondrial functions at the microdomain level. Here, we discuss how ER-mitochondrial Ca^{2+} transfers support cell function and how their dysregulation underlie, drive or contribute to pathogenesis and pathophysiology with major focus on cancer and neurodegeneration, but also with attention for other diseases such as diabetes and rare genetic diseases.

Keywords

Ca^{2+} signaling; MAMs; contact sites; cancer; neurodegeneration; genetic diseases

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Declaration of Interests

The authors declare no competing interests.

Inter-organellar Ca^{2+} dynamics at membrane contact sites as a critical process underlying cell function

Organellar function is essential for cellular homeostasis and physiology. Organelles do not function as isolated entities. Instead, their function is impacted by other organelles via membrane contact sites. These junctions enable the formation of microdomains that comprise different cellular functions by hosting signaling complexes and enabling distinct processes, including **Ca^{2+} signaling** [1]. The role of membrane contact sites and their signaling functions in cell biology and physiology is a highly timely topic that has attracted widespread attention and interest from several disciplines [2].

Ca^{2+} transfer between **endoplasmic reticulum (ER)** and **mitochondria** is enabled by Ca^{2+}-transport systems that reside at **mitochondria-associated ER membranes (MAMs).** This Ca^{2} transport controls cell biological processes such as mitochondrial metabolism, **autophagy** and cell death. Recent research revealed that several proteins whose dysfunction is associated with pathogenesis are located at MAMs and directly impact ER-mitochondrial Ca^{2+} transfers. These findings advanced the novel concept that disease processes are linked to dysregulated subcellular signaling events at ER-mitochondrial contact sites.

Here, we discuss recent advances in deciphering molecular mechanisms underlying Ca^{2+} signaling dysregulation at the ER-mitochondrial interface and their contribution to pathogenesis. After explaining basics and principles of ER-mitochondrial Ca^{2+} transfer, we focus on different diseases for which novel insights in ER-mitochondrial Ca^{2+} dysfunction have recently emerged, including not only common diseases such as cancer, neurodegenerative diseases and diabetes, but also rare genetic disorders such as Wolfram syndrome and polycystic kidney disease. It will become clear that ER-mitochondrial contact sites form a central hub hosting a growing list of disease-linked proteins that tightly control ER-mitochondrial Ca^{2+} transfer and thus cellular health. Further exploration of this field may lead to new strategies to tackle these diseases.

Basic players and principles of Ca^{2+} signaling at MAMs

The ER, the main intracellular Ca^{2+} store, forms areas of close contact (10–80 nm) with the outer mitochondrial membrane (OMM), termed MAMs (Figure 1, Key figure), enabling efficient mitochondrial Ca^{2+} transfer [1]. Various proteins control ER-mitochondrial apposition, including **inositol 1,4,5 trisphosphate (IP_3) receptors (IP_3Rs)** [3], Mitofusin 2 (Mfn2) [4, 5] and the vesicle-associated membrane protein-associated protein B (VAPB) - protein tyrosine phosphatase interacting protein 51 (PTPIP51) pair [6]. Close ER-mitochondrial connections overcome the relatively low affinity for mitochondrial Ca^{2+} uptake by establishing a microdomain, where Ca^{2+} concentrations can rise >10 fold higher than those in the bulk cytosol [7, 8]. The mitochondrial matrix accumulates Ca^{2+} via **voltage-dependent anion channels (VDACs)** at the OMM and **mitochondrial Ca^{2+} uniporter (MCU)** complexes at the inner mitochondrial membrane (IMM). This process is driven by the negative mitochondrial potential (−180 mV) [9].

Key mediators of ER-to-mitochondria Ca^{2+} transport are IP_3Rs, tetrameric IP_3-gated, Ca^{2+}-release channels. IP_3Rs are tightly controlled by various factors, including accessory proteins. The three IP_3R isoforms display distinct IP_3 affinity and regulation, thereby finetuning ER Ca^{2+} release [10]. IP_3Rs also integrate other signals such as reactive oxygen species (ROS) [11]. ROS directly oxidizes cysteine residues in IP_3Rs, thereby augmenting Ca^{2+} fluxes [12]. IP_3R oxidation is also promoted by the MAM-resident ER oxidase 1 α (ERO1α), an enzyme responsible for forming disulfide bonds at the ER [13].

To facilitate ER-mitochondrial Ca^{2+} exchange, IP_3Rs reside in a macromolecular complex, bringing it in close apposition to VDAC1 [14]. **Glucose-regulated protein 75 (GRP75)**, amongst other proteins, functions as the molecular bridge connecting VDAC1 and IP_3Rs [15]. Although all IP_3R isoforms can tether ER and mitochondria, IP_3R2 appeared the most efficient at transferring Ca^{2+} to mitochondria [3]. A pore-dead IP_3R mutant revealed that the IP_3R's tethering function is independent of its channel activity [3]. In this work, the IP_3R target responsible for mitochondrial tethering was not identified. Besides GRP75/VDAC1, other proteins may associate with IP_3Rs and contribute to ER-mitochondrial tethering. In cardiac cells, FUN14 domain-containing protein 1 (FUNDC1), a mitochondrial quality-control protein, contributes to MAM formation by binding IP_3R2 [16]. Besides FUNDC1, inositol-requiring enzyme 1 α (Ire1α), the ER-stress transducer involved in the unfolded protein response (UPR) [17], and translocase of the OMM 70 (TOM70) [18], bind IP_3Rs and recruit them to ER-mitochondrial contact sites. IP_3R recruitment to the MAMs by Ire1α is mediated through Ire1α's C-terminal domain that binds IP_3Rs and is independent from Ire1α's UPR-related RNAse and kinase activities [17].

Effects of ER-mitochondrial Ca^{2+} fluxes on cell fate

ER-mitochondrial Ca^{2+} fluxes determine cell fate. Low-level, rhythmic Ca^{2+} oscillations provide the mitochondria with adequate Ca^{2+} levels, thereby stimulating mitochondrial ATP production and sustaining survival. The increase in bioenergetic output is driven by the Ca^{2+} dependence of metabolic enzyme activity such as pyruvate-, isocitrate- and α-ketoglutarate dehydrogenase [9]. Conversely, insufficient ER-mitochondrial Ca^{2+} flux, either by inhibition or suppression of IP_3Rs [19], or by impaired recruitment to the MAMs [17, 18], impairs mitochondrial bioenergetics and increases the AMP/ATP ratio, thereby activating AMP-activated protein kinase, a driver of autophagic flux, and slowing cell proliferation. In the heart, the FUNDC1-IP_3R2 axis maintains mitochondrial Ca^{2+} dynamics and metabolism, which underlies cardiac function *in vivo* [16].

In contrast, persistent mitochondrial Ca^{2+} accumulation adversely affects cell survival [20]. Beyond a certain threshold, mitochondrial Ca^{2+} accumulation will trigger mitochondrial permeability transition pore (mPTP) opening, a process rendering the IMM permeable, causing mitochondrial dysfunction and ultimately cell death [21]. Ca^{2+} may trigger mPTP opening directly [22], but also indirectly through binding to the mitochondrial lipid cardiolipin. Cardiolipin subsequently dissociates from complex II, thereby provoking its disassembly and unrestricted activity, excessive ROS production and mPTP opening [23].

Given the disparate consequences of ER-mitochondrial Ca^{2+} transfer, this process is tightly and dynamically regulated. For example, during cell death, SUMOylation of dynamin-related protein 1 (Drp1) is stabilizing the ER-mitochondrial contact sites, thereby facilitating ER-mitochondrial Ca^{2+} transfer [24]. This drives cell death through cristae remodeling following the disassembly of mitochondrial-dynamin like GTPase (Opa1) oligomers and subsequent cytochrome c release through Bax/Bak pores.

Also during early phases of ER stress, IP_3R function is suppressed upon losing the interaction with immunoglobulin protein (BiP/GRP78) and recruiting ER-resident protein 44 [25]. During prolonged ER stress, however, ERO1α expression is induced, thereby hyperoxidizing IP_3Rs and augmenting Ca^{2+} release [26]. These mechanisms together with the UPR sensor Ire1α may enable a mechanistic link between ER proteostasis and cellular functions, like mitochondrial metabolism and cell death, through IP_3R modulation at the level of the MAMs.

Hypoxia is another cellular stress trigger underlying cell damage in ischemia/reperfusion but also contributes to the protective actions of cardiac ischemic pre-conditioning, whereby short ischemic periods enable the heart to cope with subsequent ischemic insults. During hypoxia, FUNDC1 accumulates at the MAMs integrating a concerted action of calnexin and Drp1 to drive mitochondrial fission and mitophagy [27]. In blood platelets, FUNDC1-mediated mitophagy could underly the protective effect of ischemic pre-conditioning against ischemia/reperfusion-induced cardiac damage, thus inhibiting platelet activation [28].

Deranged ER-mitochondrial Ca^{2+} signaling in disease

Cancer

Cancer cells remodel their Ca^{2+} signaling machinery, thereby contributing to cancer hallmarks [20]. Part of this remodeling is due to loss of tumor suppressor function and increased activity/upregulation of oncogene products [20]. Again, Ca^{2+} plays a dual role, while ER-mitochondrial Ca^{2+} fluxes may be dampened in cancer cells to evade cell death, enhanced Ca^{2+} signaling may promote metabolism and migration [20]. IP_3Rs, thus, can act both in a pro-oncogenic and tumor-suppressive manner. The cancer-specific mechanisms discussed below are summarized graphically in Figure 2.

Several cancer cell types appear to be addicted to ER-mitochondrial Ca^{2+} signaling for their survival [29–31]. Suppressing IP_3R function has distinct effects in cancer cell models. Immortalized human MCF-7 breast-cancer cells, for example, display excessive autophagy leading to autophagic cell death after IP_3R suppression [31]. However, driving ATP metabolism is not the only function of ER-mitochondrial Ca^{2+} transfer. In a variety of human cancer cell models (including several tumorigenic cancer cells and oncogene-transformed primary fibroblasts), IP_3R inhibition resulted in impaired mitochondrial metabolism and shortage of nucleotides that are essential building blocks for the cell to undergo mitosis [30]. In combination with dysregulated cell cycle checkpoints, this leads to mitotic catastrophe. Also oxidative phosphorylation (OXPHOS) deficient cancer cells need ER-mitochondrial Ca^{2+} fluxes for survival, thereby preventing NAD^+-driven autophagy [32]. Yet, DT40 cells (a chicken cell line derived from bursal lymphoma) and HeLa cells (a

human cell line derived from cervival cancers) lacking all three IP_3Rs isoforms have been generated and can survive [3, 33]. This indicates that the requirement of IP_3Rs for cancer cell survival might be cancer-type dependent and/or that prolonged loss of IP_3R function might be compensated by other mechanisms.

Evidence is emerging that elevated IP_3R-expression levels promote cancer hallmarks. Immortalized human colorectal and non-small cell lung cancer (NSCLC) cells display increased IP_3R3-protein levels [34, 35]. In human MCF-7 breast cancer cells, IP_3R3 drives cell proliferation in concert with large conductance Ca^{2+}-activated potassium channel subunit α (BK_{Ca}) channels [29]. In primary human glioblastoma cells, IP_3R3 sustains cellular mobility, thereby driving cancer cell invasion [36]. Oncogenic proteins can also promote IP_3R function to sustain cell survival. For instance, the anti-apoptotic protein Bcl-X_L can sensitize IP_3Rs to IP_3, stimulating cell survival by enabling ER-mitochondrial Ca^{2+} transfer [37].

Not only do IP_3Rs sustain cancer hallmarks but IP_3R-mediated Ca^{2+} fluxes can also drive cell death in cancer cells that therefore often display suppressed IP_3R function and ER-mitochondrial Ca^{2+} fluxes. For example, **protein kinase B (PKB/Akt)**, a cell-growth promoting kinase, binds, phosphorylates and inhibits IP_3Rs, conferring resistance against Ca^{2+}-dependent pro-apoptotic stimuli in multiple cancer model cell lines, such as immortalized human glioblastoma cells [38, 39].

Several proteins appear to interfere with PKB/Akt-dependent modulation of IP_3R function to control cell survival. For instance, the tumor suppressor promyelocytic leukemia protein is key to recruit protein phosphatase 2 A to the MAMs, where it dephosphorylates PKB/Akt and prevents subsequent IP_3R inhibition [40]. More recently, NADPH oxidase 4 (Nox4) was found to be enriched at the MAMs during cellular stress, where it promotes PKB/Akt-mediated phosphorylation of IP_3Rs through MAM-confined local redox signaling [41]. **Phosphatase and tensin homolog (PTEN)** is a tumor suppressor and an inhibitor of PKB/Akt. Immortalized human glioblastoma cells lacking PTEN, displayed IP_3R1 hyperphosphorylation and decreased sensitivity to apoptotic stimuli [39]. Additionally, a subpopulation of PTEN proteins is present at ER membrane and MAMs [42]. There, PTEN functions as a protein phosphatase counteracting IP_3R phosphorylation by PKB/Akt and increasing the susceptibility of cells to undergo Ca^{2+}-dependent apoptosis [42]. PTEN also supports IP_3R function outside its phosphatase function: it was found to compete with F-box/LRR protein 2, a ubiquitin ligase marking IP_3R3 for degradation. Hence, lack of PTEN favors IP_3R3 ubiquitination, thereby downregulating IP_3R3 and increasing resistance against apoptotic stimuli [43]. Indeed, in human prostate cancer tissue, PTEN and IP_3R3 levels directly correlate [43]. Similarly, BRCA1-associated protein 1 (BAP1), a tumor suppressor gene product, interacts with IP_3R3, enabling its deubiquitination and stabilization. Thus, BAP1 promotes pro-apoptotic IP_3R3-mediated Ca^{2+} fluxes, whereby BAP1 deficiency ($BAP1^{+/-}$) in primary human fibroblasts and human multiple myeloma cells suppresses IP_3R3 function [44].

Other proteins affecting IP_3R levels include the ER-targeted chaperone sigma 1 receptor (S1R) [45], STAT3 [46] and methylated pyruvate kinase M2 (PKM2) [47]. Depletion of

Ca^{2+} in the ER activates S1R to dissociate from BiP/GRP78 and to bind and stabilize IP_3R3 [45]. It appears that breast, prostate and lung cancer cells in murine xenograft models are susceptible to S1R inhibitors, which reduced tumor growth *in vitro* and *in vivo* [48]. STAT3 is an oncogenic transcription factor that also displays an extra-nuclear function at the MAMs, where it downregulates IP_3R3 and contributes to the cell-death resistance of basal-like mammary tumors [46]. Methylated PKM2 also downregulates IP_3Rs, whereby inhibiting PKM2 methylation compromises *in vivo* tumor growth of human breast cancer cells xenografted in mice [47].

Members of the **B-cell lymphoma 2 (Bcl-2)** protein family also reside at MAMs [49], thereby exerting anti-apoptotic functions by preventing ER-mitochondrial Ca^{2+} transfer in cancer. Anti-apoptotic Bcl-2 is upregulated in several cancer types and directly targets IP_3Rs, thereby preventing pro-apoptotic Ca^{2+} release [50].

Peptides that dislodge Bcl-2 from IP_3Rs in cancer cells provoke cell death and/or augmented cell death elicited by other therapeutics. In human diffuse large B-cell lymphoma and primary chronic lymphocytic leukemia cells, these peptides elicit pro-apoptotic Ca^{2+} signaling [51], resulting in mitochondrial Ca^{2+} overload and mPTP opening [52]. In ovarian cancer cells, such peptides augment the cytotoxic effects of cisplatin by enhancing cisplatin-induced ER-mitochondrial Ca^{2+} fluxes [53].

Also, Bcl-2 homolog NRH/BCL-2L10, a proto-oncogene product upregulated in primary human breast cancer cells [54], associates with and inhibits IP_3Rs [55]. Additionally, peptides that disrupt IP_3R/Bcl-2L10 complexes inhibit the growth of immortalized human breast cancer cells [56]. Bcl-2L10 also cooperates with IRBIT, a protein that, when phosphorylated, binds and inhibits IP_3Rs [57]. Together, Bcl-2L10 and phosphorylated IRBIT inhibit IP_3Rs at the MAMs. Under cell stress, IRBIT is dephosphorylated and acts as an inhibitor of Bcl-2L10 that promotes ER-mitochondrial Ca^{2+} fluxes and apoptosis [57]. IP_3Rs are not only modulated by anti-apoptotic Bcl-2-protein family members, but also pro-apoptotic members such as Bcl-2-related ovarian killer (Bok) can bind IP_3Rs to protect them from proteolytic degradation [58]. Primary human NSCLC cells downregulate Bok, rendering IP_3Rs more prone to degradation [59].

The ER Ca^{2+}-store content indirectly controls IP_3R function. Certain tumor suppressors increase apoptotic susceptibility via ER-mitochondrial Ca^{2+} fluxes by augmenting the ER Ca^{2+}-filling state. The tumor suppressor p53 accumulates at ER-mitochondrial contact sites in cells exposed to chemical stresses and chemotherapeutic agents [60]. At the ER, p53 enhances the activity of the **sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA)**, thereby elevating ER Ca^{2+} levels and boosting pro-apoptotic IP_3R-mediated Ca^{2+} fluxes [60]. In cells lacking p53, SERCA activity is not stimulated, underlying cell-death resistance to chemotherapeutics [60].

Surely, ER-mitochondrial Ca^{2+} fluxes are not only determined by IP_3R function. The mitochondrial gateways for Ca^{2+}, VDAC1 and MCU, are also dysregulated in cancer. The metabolic enzyme hexokinase 2, a VDAC1 partner, was observed to impact cell fate at the MAMs [61]. Displacement of hexokinase 2 from the MAMs with a peptide resulted in

mitochondrial Ca^{2+} overload and subsequent cell death in primary human leukemia B-cells [61]. In addition to this, Bcl-2, Bcl-X_L and Mcl-1 can also target VDAC1 in the MAMs and inhibit its ability to shuttle Ca^{2+} into the mitochondria, conferring apoptotic protection [49, 62]. Yet, anti-apoptotic Bcl-X_L and Mcl-1 have also been reported to enhance basal Ca^{2+} uptake into the mitochondria through VDAC1, thereby promoting mitochondrial metabolism [63, 64]. In several human NSCLC cell lines, boosting VDAC1-mediated mitochondrial Ca^{2+} uptake and subsequent ROS production through Mcl-1 sustained cancer cell migration [64].

Finally, the amount of Ca^{2+} reaching the mitochondria will be dampened by local SERCA pumps, whose activity is counteracted by thioredoxin-related transmembrane protein 1 (TMX1), a MAM-localized redox-sensitive oxidoreductase. Low TMX1-protein levels allowed for high SERCA activity, thereby reducing mitochondrial Ca^{2+} transfer and promoting tumor growth in xenografts of mice injected with human melanoma cells [65]. Other work, however, indicated that several human melanoma cell lines are addicted to high TMX1 (and TMX3) levels, counteracting Nox4-mediated ROS production at the ER-mitochondrial interface. In melanoma cells with high TMX1/TMX3 levels, the Ca^{2+}/calmodulin-dependent phosphatase calcineurin is activated, thereby driving the NFAT pathway and subsequent gene expression involved in cancer cell proliferation and migration [66]. In melanoma cells with low TMX1/TMX3 levels, mitochondrial Ca^{2+} uptake and Nox4 activity are increased, thereby generating excessive ROS that impairs melanoma growth and migration by oxidation and inhibition of calcineurin. This seemingly opposing role for TMX1 in cancer may reflect the importance of the specific cellular context.

Neurodegeneration

The dysregulation of IP_3R-mediated Ca^{2+}-release at ER-mitochondrial contact sites is emerging as a key feature in the pathogenesis of a variety of neurodegenerative diseases. Here, we will focus on Parkinson's disease (PD) and Alzheimer's disease (AD) not only because they are the most prevalent neurodegenerative diseases but especially because several PD- and AD-linked proteins reside and function at the MAMs (Figure 3).

Parkinson's disease—PD is a progressive neurodegenerative disease caused by loss of neurons, particularly in the substantia nigra pars compacta, resulting in tremor, shuffling gait and muscle rigidity [67]. α-Synuclein, the main constituent of the intraneuronal Lewy bodies found in PD, is localized at the MAMs, whereas a pathogenic mutant form of α-synuclein (A30P) showed a decreased MAM-localization [68]. More recently, α-synuclein, as well as its pathogenic mutants (A30P and A53T), were shown to bind to the mitochondrial tethering protein VAPB, thereby hindering its association with the ER-localized PTPIP51 and disrupting ER-mitochondrial contact sites [69].

Moreover, PTEN-induced kinase 1 (PINK1) and Parkin, mitophagy proteins associated with recessive familial forms of PD, contribute to ER-mitochondria tethering [70]. For example, the ER-mitochondrial interface is perturbed in Parkin knockout mice and patients with Parkin mutations leading to aberrant ER-to-mitochondria Ca^{2+} transfers [70]. Work in *Caenorhabditis elegans* demonstrated that mutant PINK1 reduced mitochondrial toxicity

upon exposure to rotenone and paraquat, two environmental risk factors associated with sporadic PD [71]. Exposure to these compounds effectively increased PINK1 expression, while alterations at the ultrastructural level in the MAMs were observed.

Additionally, mitochondrial rho GTPase 1 (Miro1), an OMM protein involved in mitochondrial mobility [72], regulates mitochondrial Ca^{2+} uptake through MCU [73] and has been implicated in PD due to its interaction with both PINK1 and Parkin [74]. Miro proteins can function as a Ca^{2+}-dependent docking site for Parkin recruitment [74]. Mutations in the Ca^{2+}-binding EF-hand motif and GTPase domain of Miro1 have recently been identified in sporadic PD patients [75]. The Miro1 mutations led to a decrease in ER-mitochondrial contact sites, resulting in a reduced cytosolic Ca^{2+} buffering capacity of mitochondria in fibroblasts. Additionally, these mutations likely increase the rate of mitophagy [75].

Another PD-related protein with a strong link to MAMs is Parkinson's disease protein 7 (Park7/DJ-1), a protein that has been implicated in several cellular processes, including Ca^{2+} homeostasis [76]. Loss of DJ-1 function causes a recessive-inherited form of PD [77]. DJ-1 is vital for mitochondrial health and regulates ER-mitochondrial tethering [78, 79]. Of note, DJ-1 has been identified as a vital component of the IP_3R-GRP75-VDAC1 complex mediating ER- to-mitochondrial Ca^{2+} transfer [79]. Loss of function of DJ-1 resulted in a disruption of the IP_3R-GRP75-VDAC1 complex and a decrease in mitochondrial Ca^{2+} levels upon IP_3R stimulation. Additionally, DJ-1 ablation also led to an increase in IP_3R3 levels at ER-mitochondrial contact sites, suggesting IP_3R3 aggregates upon losing its association with the IP_3R3-GRP75-VDAC1 complex [79].

Recently, VAPB and PTPIP51 were found to localize and interact at ER-mitochondrial contact sites in neuronal synapses, where they sustain pre- and post-synaptic activity [80]. Moreover, induction of synaptic activity increased VAPB-PTPIP51 interaction and promoted ER-mitochondrial contacts [80]. Conversely, loss of VAPB and PTPIP51 decreased the number of active dendritic spines, identified through apposition with the pre-synaptic marker synaptophysin, and reduced synaptic vesicle exocytosis [80]. Therefore, it is possible that loss of neuronal synapse function, a chief feature of PD and other neurodegenerative diseases, is intimately linked with loss of ER-mitochondrial tethering [80].

Alzheimer's disease—Worldwide, AD is the most prevalent neurodegenerative disease and is the most common cause of dementia. The main pathophysiological mechanism is the formation of intracellular neurofibrillary tangles consisting of aggregated hyperphosphorylated tau and extracellular plaques composed of amyloid β (Aβ), resulting in neuronal atrophy. During the formation of senile plaques, Aβ is cleaved from the amyloid precursor protein (APP) by the γ-secretase complex, containing **presenilin 1 (PS1)** or **presenilin 2 (PS2)** as catalytic subunits [81].

Both APP and the γ-secretase complex have been reported to reside in MAM fractions [82, 83], while MAM-localized γ-secretase is active *in vitro* [82]. When exposing primary hippocampal neurons to Aβ, an upregulation of MAM-associated proteins is triggered, including IP_3R3 and VDAC1, and the number of ER-mitochondrial contact sites is increased

[84]. In SH-SY5Y cells, exposure to Aβ led to an increase in ER-mitochondrial Ca^{2+} fluxes [84], while the overexpression of a familial AD mutant form of APP also increased ER-mitochondrial contacts [82]. Moreover, the C99 protein, a product derived from β-secretase-cleaved APP, was found to accumulate at MAMs in neurons of AD mouse models [85]. C99 augmented ER-mitochondrial tethering, promoting excessive mitochondrial accumulation of ceramide, a sphingomyelin hydrolysis product that exerts pro-apoptotic effects and impairs mitochondrial respiration [85].

Cells expressing mutant PS1 or PS2, including familial AD patient lymphoblasts with PS1 mutations [86] and patient-derived fibroblasts harboring PS1 or PS2 mutations [87] displayed disturbances in Ca^{2+} dynamics, independently of their roles in the γ-secretase complex. Overexpression of wild-type PS2 (but not PS1) in neuroblastoma cells and rat primary cortical neurons favored Ca^{2+} transfer between ER and mitochondria, which was also enhanced in PS2 mutants [88]. Consistently, PS1 and PS2 interact with IP_3Rs [89] and mutant PS can sensitize IP_3R to IP_3, increasing IP_3R-mediated Ca^{2+} fluxes, which in turn promote APP processing [89].

PS2 can also influence ER-mitochondrial tethering, by interacting with Mfn2, a structural MAM protein. In the presence of Mfn2, mutant PS2 expression increases ER-mitochondrial contacts and modulates ER-mitochondrial Ca^{2+} signaling [90]. In addition, knockdown of Mfn2 increases ER-mitochondrial contacts and Ca^{2+} transfer and impairs γ-secretase APP processing activity [91]. Furthermore, the contact area of ER-mitochondrial contact sites and ER-mitochondrial lipid transfer increases significantly in fibroblasts from patients with either the familial or sporadic forms of AD [92]. The emergence of altered ER-mitochondrial contacts in sporadic AD cases is important, since these are the majority of AD cases. Finally, different cell types including neurons, astrocytes and microglia contribute to AD pathogenesis, but how MAMs are dysregulated among these cell types is not well understood.

Other MAM-related disorders

Dysregulated MAM-localized Ca^{2+} signaling has recently been implicated in the pathogenesis of several other disorders, such as diabetes mellitus (DM), Wolfram syndrome (WS) and polycystic kidney disease (PKD), for which novel molecular mechanisms at the MAMs emerged.

Diabetes mellitus—DM is a disorder leading to the disruption of glucose homeostasis and distinguished as type 1 DM or type 2 DM (T2D) [93]. Type 1 DM is characterized by the destruction of pancreatic β cells by the immune system, impairing insulin secretion [93]. T2D is associated with lower insulin sensitivity and mostly also a decrease in insulin production, impairing cellular glucose uptake [93].

Several proteins important for both insulin signaling and cell survival are located at the MAMs and affect ER-mitochondrial Ca^{2+} transfer. Glycogen synthase kinase 3 β (GSK3β), a protein that inactivates glycogen synthase, is such an example. In mouse heart, GSK3β partially localizes at the MAMs, where it interacts with and stimulates IP_3Rs [94]. In murine pancreatic β cells, GSK3β enhances a tissue-specific basal ER Ca^{2+} leak towards the

mitochondria that is mediated by PS1 phosphorylated by GSK3β [95]. Another kinase, pyruvate dehydrogenase kinase 4 (PDK4) is upregulated during obesity, which led to increased MAM formation and insulin resistance [96]. At the MAMs, PDK4 binds the IP_3R1-GRP75-VDAC1 complex, which enhances ER-mitochondrial Ca^{2+} transfer [96]. Additionally, acute glucose treatment of rat-derived pancreatic cell models was observed to stimulate ER-mitochondrial interactions and ER-mitochondrial Ca^{2+} transfer, depleting ER Ca^{2+} stores and inducing ER stress [97].

Presumably, MAMs can couple energy sensing to mitochondrial physiology and play an important role in glucose homeostasis. Hence, alterations at the level of the MAMs could play a role in the pathophysiology of DM [98]. Indeed, the number of IP_3R2-VDAC1 interactions was lower in T2D patient pancreatic tissue compared to healthy controls [99]. In T2D β cells, VDAC1 levels were decreased, while IP_3R2 levels were increased, indicating that specific changes in MAM-protein levels are characteristic for T2D [98, 99].

Earlier work in isolated hepatocytes revealed that IP_3R1 knockdown reduces glucose production [100]. This likely involves glucagon, which recently was shown to stimulate gluconeogenesis in liver tissue via an IP_3R1-dependent mechanism [101]. Elevated glucagon plasma levels in WT mice could counteract diet-induced hepatic steatosis and resistance to insulin. However, these effects are absent in mice lacking IP_3R1 [101].

Wolfram syndrome—Wolfram syndrome (WS) is a rare genetic disease caused by mutations in *WFS1*, resulting in the more prevalent type I WS, or in *CISD2*, resulting in the rarer type II WS. The currently untreatable disease starts at a young age with diabetes mellitus due to pancreatic β cell dysfunction and destruction but then aggravates with severe neurological complications, resulting in early death [102].

Wolframin (Wfs1) was recently proposed to be a MAM protein [103]. Wfs1 function has been linked to ER stress and Ca^{2+} homeostasis [104]. Loss of Wfs1 was associated with suppressed IP_3R-mediated Ca^{2+} release in primary neuronal models and patient fibroblasts [105, 106]. Lack of Wfs1 also evoked mild ER stress and impaired mitochondrial metabolism and dynamics as well as a decrease in ER-mitochondrial contact sites, evidenced by a severe reduction in IP_3R/GRP75 and IP_3R/VDAC1 interactions [105].

Recently, Wfs1 was found to reside in a complex with neuronal calcium-sensor 1 (NCS-1) [105]. NCS-1 sensitizes IP_3R, thereby promoting Ca^{2+} oscillations in living cells [107]. NCS-1 itself directly binds IP_3Rs near its N-terminal suppressor domain [108], a region critical for IP_3R-channel gating. The elevated NCS-1 levels could salvage the adverse effects associated with Wfs1 deficiency, thereby augmenting mitochondrial Ca^{2+} uptake and improving mitochondrial respiratory chain functionality [105]. NCS-1 may couple Wfs1 to a macrocomplex with the MAM-residing complex IP_3R–GRP75–VDAC1 [105]. Also, INS 832/13 cells lacking Wfs1 displayed elevated cytosolic Ca^{2+} levels, decreased IP_3R-mediated Ca^{2+} release, accompanied by a reduced ER-mitochondrial Ca^{2+} transfer [109]. Furthermore, ibudilast, a phosphodiesterase 4 inhibitor potentially also targeting NCS-1, and calpain inhibitor XI could rescue basal cytosolic Ca^{2+} levels in cells lacking Wfs1, restoring

insulin-producing capacity of β cells and thus opening new therapeutic possibilities for WS patients.

CISD2, the other causative gene for WS, encodes a single-pass transmembrane ER protein containing a two iron-two sulphur cluster domain, named **CDGSH iron sulfur domain 2 (Cisd2)**. Cisd2 is specifically enriched at the MAMs [110]. Similar to Wfs1, Cisd2 impacts ER Ca^{2+} dynamics by interacting with IP_3R1 [111] and SERCA2b [112]. Additionally, patient-derived fibroblasts carrying a mutation in *CISD2* displayed increased ER-mitochondrial contacts and ER-mitochondrial Ca^{2+} fluxes, possibly underlying hyperfusion of the mitochondrial network [113]. Moreover, Cisd2 deficiency augmented cytosolic Ca^{2+} levels and modulated mitochondrial Ca^{2+} uptake by interacting with GTPase of immune-associated protein 5 (Gimap5), a MAM-resident protein [110]. Therefore, at the MAMs, Cisd2 may reside in a macrocomplex with IP_3R, Gimap5 and perhaps other proteins regulating Ca^{2+} signaling [103].

Polycystic kidney disease—PKD is a collection of several monogenic diseases, primarily characterized by cyst development in kidneys and potentially other organs such as liver [114]. The autosomal-dominant form of PKD is associated with two genes, including **Polycystin 2 (PC2)**. PC2 knockdown resulted in increased Mfn2 expression and ER-mitochondrial Ca^{2+} transfer, while MAM IP_3R3 levels were decreased. Knockdown of Mfn2 could restore normal ER-mitochondrial Ca^{2+} transfer. Also, kidney sections of autosomal-dominant PKD patients displayed higher Mfn2 levels and a higher mitochondrial density, as evidenced by higher TOM20 levels per mitochondrial area. Hence, PC2 might be a key regulator of the expression of MAM-resident proteins [115].

Concluding remarks

Several disease-linked proteins recently emerged to reside and function at the MAMs as controllers of ER-mitochondrial Ca^{2+} transfer. Numerous studies implicate them in the pathogenesis of various diseases such as cancer, neurodegenerative diseases and other MAM-related disorders. Yet, several aspects of ER-mitochondrial Ca^{2+} fluxes in health and disease, including ageing, the importance in sporadic forms of neurodegenerative diseases, and integration of ER-mitochondrial Ca^{2+} signals with other MAM-related processes ought to be further studied [see outstanding questions box]. These recent advances also provide new targets to develop novel therapeutic strategies. Hence, future efforts should focus on translating these concepts towards disease prevention and therapy, including gene therapies as well as novel or repurposed pharmacological therapeutics. Moreover, focusing on MAM-residing proteins in the early stages of the disease conditions could help preventing disease onset and delaying disease progression. Targeting of the MAM-localized Ca^{2+}-signaling complexes with high selectivity in a spatially restricted manner, both at the subcellular and tissue level, will be important as a potential therapeutic strategy for these disorders.

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Glossary

Autophagy
a general term that refers to the lysosomal process important for the removal of misfolded/aggregated proteins, damaged organelles and intracellular pathogens. Specific terms are used for specific organelles, e.g. mitophagy for removal of damaged mitochondria. Autophagy is a pro-survival process key for cellular homeostasis, though when deregulated can also lead to cell death.

B-cell lymphoma-2 (Bcl-2)
founding member of the Bcl-2-protein family, which regulates cell fate by acting as an anti-apoptotic protein.

Ca^{2+} signaling
the process of coupling dynamic, spatiotemporal changes in intracellular Ca^{2+} concentrations with the deciphering of these changes by Ca^{2+}-dependent sensors and effectors present in the cytosol and organellar compartments.

CDGSH iron sulfur containing domain protein 2 (Cisd2)
protein encoded by the *CISD2* gene. Mutations in this gene can give rise to type II Wolfram syndrome. Cisd2 is enriched at the MAMs.

Endoplasmic reticulum (ER)
the organelle that is not only responsible for protein synthesis & folding and lipid synthesis but also serves as the main intracellular Ca^{2+} store.

Glucose-regulated protein of 75 kDa (GRP75)
a chaperone that predominantly resides in the mitochondrial matrix but also at the MAMs, where it participates in the IP_3R-GRP75-VDAC1 complex.

Inositol 1,4,5-trisphosphate (IP_3)
a diffusible ligand of IP_3Rs produced by phospholipase C from phosphatidylinositol 4,5-bisphosphate in response to extracellular stimuli e.g. via activation of G-protein-coupled receptors or tyrosine kinase receptors.

Inositol 1,4,5-trisphosphate receptors (IP_3Rs)
tetrameric intracellular Ca^{2+}-release channels that mediate Ca^{2+} release from the endoplasmic reticulum in response to IP_3. IP_3Rs are also found at contact sites between ER and mitochondria, where they reside in interorganellar protein complexes involving VDAC channels.

Mitochondria-associated ER membranes (MAMs)

a fraction of the ER membranes that can be biochemically isolated and whose protein and lipid composition represents the contact sites between ER and mitochondria.

Mitochondrial Ca^{2+} Uniporter (MCU)
a pore-forming protein responsible for mitochondrial Ca^{2+} uptake across the inner mitochondrial membrane and whose activity is tightly controlled by several regulators.

Mitochondrion
the organelle responsible for the Krebs cycle, oxidative phosphorylation (OXPHOS) and ultimately the production of large amounts of ATP. Due to its close proximity to the ER and the large driving force by the negative mitochondrial membrane potential, Ca^{2+} signals arising from the ER are also transmitted towards the mitochondria, where they boost the activity of several enzymes participating in the Krebs cycle.

Phosphatase and tensin homologue (PTEN)
phosphatase that dephosphorylates various targets, among which PKB/Akt

Polycystin 2 (PC2)
a gene for which mutations/deletions result in polycystic kidney disease.

Presenilin-1 and -2 (PS1 and -2)
proteins that are part of the γ-secretase complex involved in cleavage of the amyloid precursor protein. As single proteins, presenilins are also involved in different cell functions, such as autophagy and Ca^{2+} homeostasis. Mutations in presenilin-1 and -2 are linked to cases of familial Alzheimer's disease

Protein kinase B (PKB)/Akt
pleiotropic kinase that integrates various intracellular signaling pathways.

Sarco-/endoplasmic reticulum Ca^{2+} ATPase (SERCA)
ER protein that pumps Ca^{2+} from the cytosol back into the ER to ensure low cytosolic Ca^{2+} concentrations and filling of the ER.

Voltage-dependent anion channels (VDAC)
a conductance channel located at the mitochondrial outer membrane; this channel not only permeates anions, but also permeates cations such as Ca^{2+}, thereby transmitting IP_3R-mediated Ca^{2+} signals towards the mitochondria.

Wolframin (Wfs1)
protein encoded by the *WFS1* gene. Mutations in this gene result in type I Wolfram syndrome. Wfs1 is linked to ER stress & Ca^{2+} homeostasis and is enriched at the MAMs.

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Outstanding questions

- Which proteins form the core of the IP_3R tethering complex and which are cell-type or condition-dependent? Which mitochondrial targets underlie the ER-mitochondrial tethering function of IP_3Rs? Which mechanisms contribute to the recruitment of IP_3Rs to the MAMs?
- How is the width of the cleft between ER and mitochondria regulated in physiological and in pathological conditions? Is there redundancy between various tethering proteins?
- How do the different functional modules of the MAMs, e.g. lipid trafficking and Ca^{2+} signaling, impact each other and/or act in a concerted manner in health and disease?
- Are structural or functional changes in MAMs a cause of disease or are they rather the consequence of dysregulation or represent compensatory changes? Many diseases involve distinct cell types, hence can restoring the functionality of MAMs in one cell type be sufficient to effectively counteract disease progression and outcomes? For example, is it sufficient to normalize MAM function in neurons for AD and PD?
- How do ER-mitochondrial contact sites change during sporadic forms of neurodegenerative diseases?
- How can we detect perturbations in MAM-resident proteins and processes early on in disease to serve as a biomarker?
- Are the changes in MAMs in the context of pathology reversible, and if so, how can therapies based on pharmacological tools or gene transfer be designed to enable this reversion? How can MAM-resident proteins be targeted to restore ER-mitochondrial Ca^{2+} transfer in diseases?

Highlights

- Mitochondria-associated membranes (MAMs) establish signaling microdomains for the exchange of Ca^{2+} and lipids between ER and mitochondria
- Ca^{2+} transfer between ER and mitochondria is critical for cellular physiology and functions, including mitochondrial metabolism and cell death
- The presence, stability, levels and activity of IP_3Rs, intracellular Ca2+-release channels, at the MAMs are tightly regulated by a plethora of mechanisms
- Many proteins that are dysregulated or mutated in pathologies ranging from cancer to neurodegenerative disease reside at the MAMs, where they impact ER-mitochondrial Ca^{2+} transfer and affect cell function
- Deranged ER-mitochondrial Ca^{2+} signaling drives pathogenesis and impacts disease outcomes.

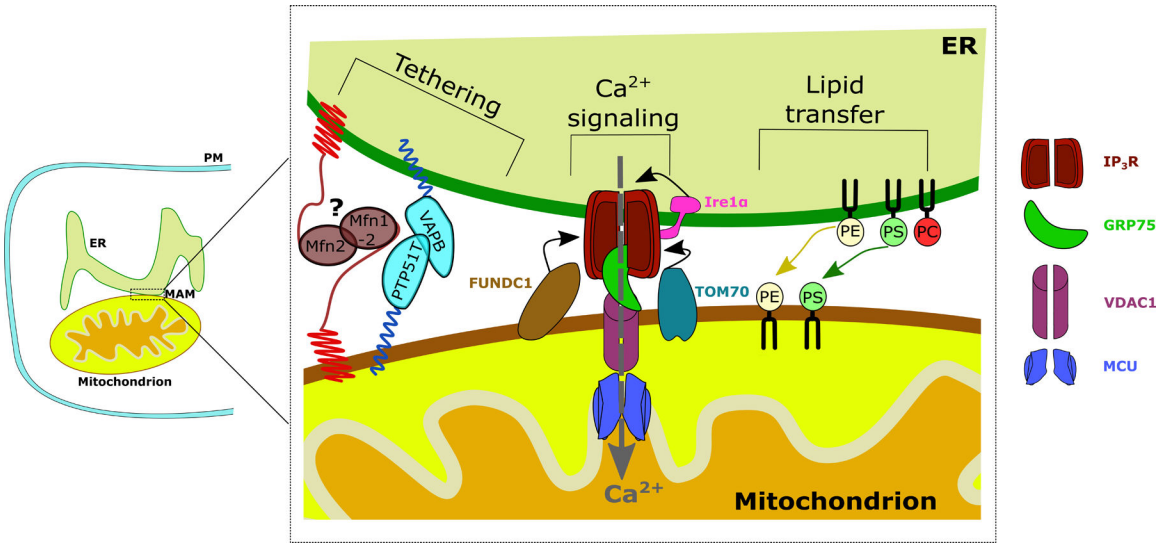

Figure 1, key figure: Schematic overview of basic MAM composition and functions.

Structural MAM proteins function as physical MAM tethers such as VAPB. Another modulator of ER-mitochondrial tethering is Mfn1/2. Its role as a tether is debated, which is why it is annotated with a question mark. One of the main functions of the MAMs is ER to mitochondrial Ca^{2+} transfer through the IP_3R-VDAC1-MCU axis, stabilized by GRP75. Recently, a non-canonical role for Ire1α was elucidated where Ire1α recruits IP_3R to the MAMs. Both TOM70 and FUNDC1 recruit IP_3R to the MAMs, via either direct or indirect interactions. Additionally, the synthesis and transfer of lipids from the ER to the mitochondria takes place at the MAMs. For simplicity reasons, only the most abundant membrane lipids are depicted. PM: Plasma membrane, ER: Endoplasmic reticulum, MAM: Mitochondria-associated ER membrane, Ca^{2+}: Calcium ion, Mfn: mitofusin, VAPB: Vesicle-associated membrane protein-associated protein B, PTPIP51: Protein tyrosine phosphatase interacting protein 51, Ire1α: Inositol-requiring enzyme 1 α, FUNCD1: FUN14 domain containing protein 1, TOM70: Translocase of the outer mitochondrial membrane 70, IP_3R: Inositol 1,4,5-trisphosphate receptor type 1, GRP75: Glucose-regulated protein 75, VDAC1: Voltage dependent anion channel 1, MCU: Mitochondrial Ca^{2+} uniporter, PE: phosphatidylethanolamine, PS: phosphatidylserine, PC: phosphatidylcholine.

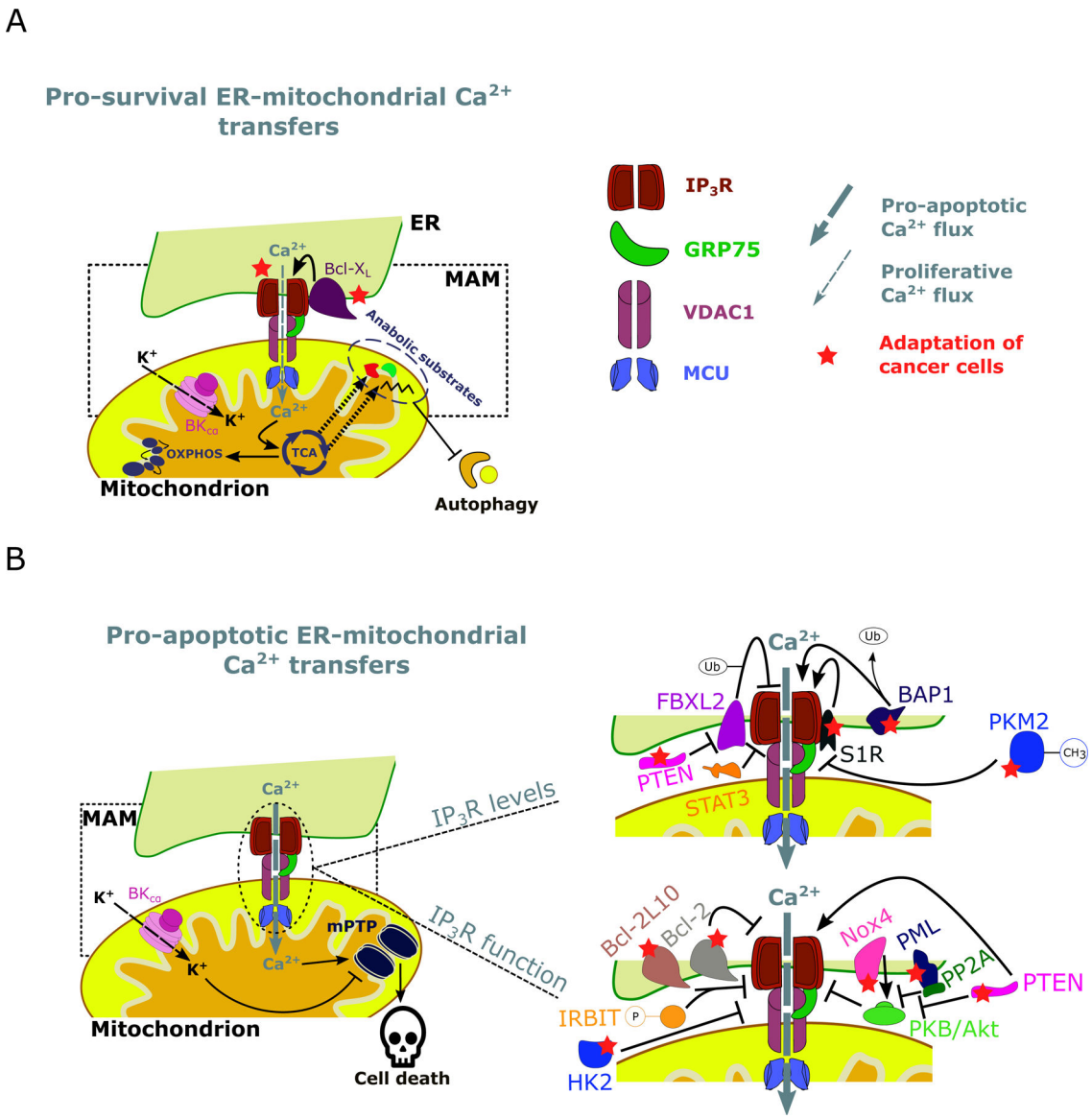

Figure 2: A graphic summary of targets in cancer cells altering IP_3R-mediated ER-mitochondrial Ca^{2+} fluxes.

A: Cancer cells are addicted to pro-survival ER-mitochondrial Ca^{2+} transfers, which promote the mitochondrial metabolism through Ca^{2+}-dependent stimulation of TCA and OXPHOS and suppress autophagic flux. This not only serves for ATP production but also for anabolic pathways through the production of intermediate substrates to synthesize nucleotides. IP_3R function can be enhanced by anti-apoptotic/pro-survival proteins such as Bcl-X_L. B: Cancer cells also avoid pro-apoptotic ER-mitochondrial Ca^{2+} transfers. Excessive Ca^{2+} levels in the mitochondrial matrix lead to cell death via opening of the mPTP. Cancer cells can circumvent this by *i.* decreasing IP_3R levels or *ii.* modulate IP_3R function. PTEN competes with FBXL2, which can mark IP_3R for degradation by ubiquitination. Similarly, the oncogenic transcription factor STAT3 facilitates IP_3R degradation at the MAMs. Conversely, BAP1 interacts with and deubiquitinates IP_3R. Additionally, S1R binds and stabilizes IP_3R. Methylated PKM2 was observed to interact

with IP_3R in the MAMs and to promote IP_3R downregulation. Note that the methylations of PKM2 are more complex than the single methyl group depicted here. PTEN negatively regulates PKB/Akt-dependent phosphorylation of IP_3Rs, this way promoting pro-apoptotic Ca^{2+} signaling in the MAMs. Additionally, Nox4 promotes PKB/Akt-mediated inhibitory IP_3R phosphorylation. Also, PML recruits PP2A to the MAMs, where it counteracts PKB/Akt activity. Moreover, several anti-apoptotic Bcl-2 proteins can inhibit IP_3R, either directly or in cooperation with phosphorylated IRBIT (as is the case for Bcl-2L10). Finally, HK2 negatively modulates pro-apoptotic IP_3R-driven Ca^{2+} fluxes. ER: Endoplasmic reticulum, MAM: Mitochondria-associated ER membrane, TCA: Tricarboxylic acid cycle, OXPHOS: oxidative phosphorylation, Ca^{2+}: Calcium ion, K^+: Potassium ion, IP_3R: Inositol 1,4,5-trisphosphate receptor type 1, GRP75: Glucose-regulated protein 75, VDAC1: Voltage dependent anion channel 1, MCU: Mitochondrial Ca^{2+} uniporter, BK_{Ca}: Calcium-activated potassium channel, Bcl-X_L: B-cell lymphoma extra-large, mPTP: Mitochondrial permeability transition pore, FBXL2: F-box/LRR-repeat protein 2, Bap1: Ubiquitin carboxyl-terminal hydrolase BAP1, PTEN: Phosphatase and tensin homolog, STAT3: Signal transducer and activator of transcription 3, S1R: Sigma-1 receptor, Bcl-2: B-cell lymphoma 2, Bcl-2L10: Bcl-2 like protein 10, Nox4: NADPH oxidase 4, PML: Promyelocytic leukemia protein, PP2A: Protein phosphatase 2A, IRBIT: IP_3R binding protein released with IP_3, PKB/Akt: Protein kinase B, HK2: Hexokinase 2, Ub: Ubiquitinylations, P: Phosphorylations, CH_3: Methylations.

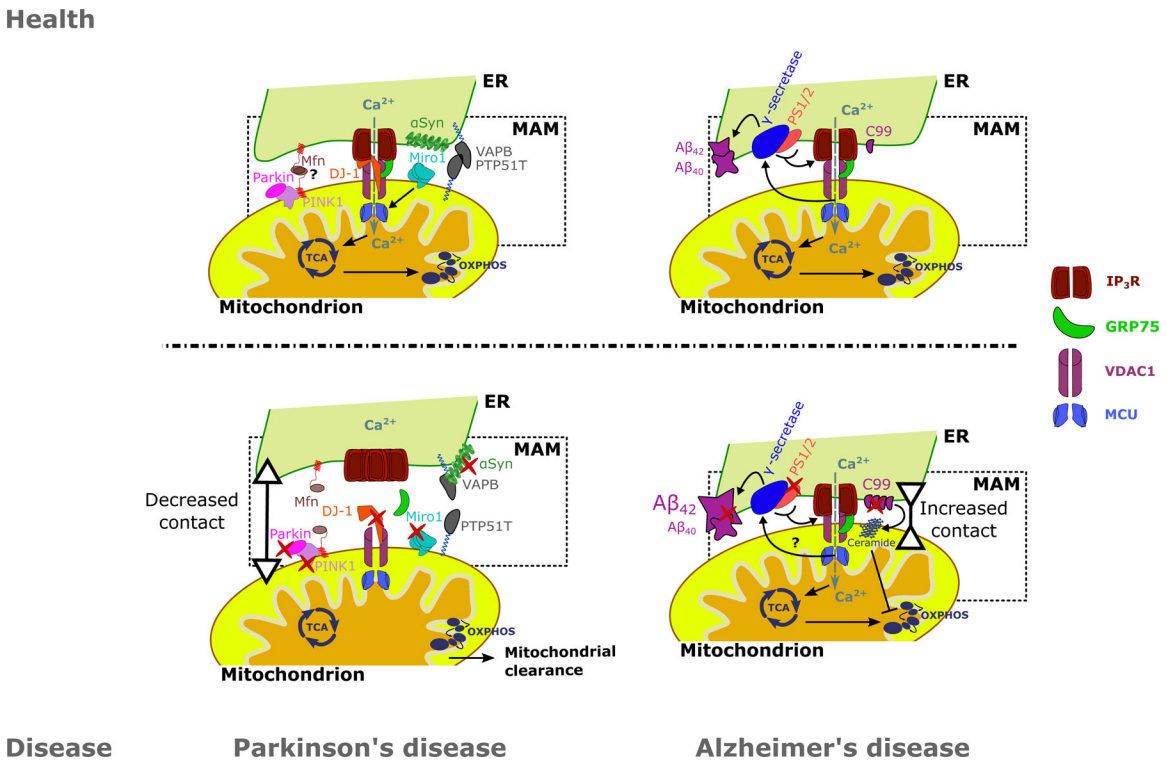

Figure 3: A summary of alterations in MAMs for two neurodegenerative diseases: PD and AD.

A: DJ-1, a protein associated with familial forms of PD, is a vital component of the IP_3R-VDAC1-GRP75 axis in the MAMs. Loss of function of DJ-1 results in decreased ER-mitochondrial Ca^{2+} transfer, ER-mitochondrial contact sites and upregulation and aggregation of IP_3R. PD-related proteins PINK1 and Parkin have been implicated in the tethering of ER to mitochondria. Moreover, Miro1 positively regulates mitochondrial Ca^{2+} uptake by MCU and mutations in Miro1 result in decreased ER-mitochondrial contact. Additionally, αSyn, of which its aggregated form is a hallmark of PD, is also present in the MAMs. Both WT and mutant αSyn interact with the VAPB-PTPIP51 MAM tethering proteins. B: AD is characterized by the deposition of Aβ plaques due to aberrant APP processing. Processing of APP is performed by the γ-secretase complex, of which PS1 and PS2 are components. APP and PS1–2 are particularly enriched in the MAMs. Mutations in APP, PS1–2 can increase ER-mitochondrial contact. Additionally, mutant PS1–2 are able to sensitize IP_3Rs to IP_3, increasing ER-mitochondrial Ca^{2+} transfer, which in turn enhances APP processing. C99, a product of APP processing, is enriched in the MAMs in AD patients cells, where they enhance the accumulation of ceramide in the mitochondria. In turn, ceramides inhibit mitochondrial respiration. Ultimately these changes lead to an increased $Aβ_{42}/Aβ_{40}$ ratio in AD patient cells. Red marks indicate alterations on the protein level in neurodegeneration, leading to decreased ER-mitochondrial contact in PD and increased ER-mitochondrial contact in AD. Grey arrows indicate Ca^{2+} fluxes. Question marks signify processes that are still uncertain. ER: Endoplasmic reticulum, MAM: Mitochondria-associated ER membrane, TCA: Tricarboxylic acid cycle, OXPHOS: oxidative phosphorylation, Ca^{2+}: Calcium ion, IP_3R: Inositol 1,4,5-trisphosphate receptor type 1, GRP75: Glucose-regulated protein 75, VDAC1: Voltage dependent anion channel 1, MCU:

Mitochondrial Ca^{2+} uniporter, Mfn: Mitofusins, PINK1: PTEN-induced kinase 1, DJ-1: Protein deglycase DJ-1, Miro1: Mitochondrial Rho GTPase 1, VAPB: Vesicle-associated membrane protein-associated protein B/C, PTPIP51: Protein tyrosine phosphatase interacting protein 51, αSyn: α-synuclein, $A\beta_{42/40}$: 42/40 amino acid proteolytic product of amyloid precursor protein, PS1/2: Presenilin 1 / 2, C99: C99 fragment of amyloid precursor protein.