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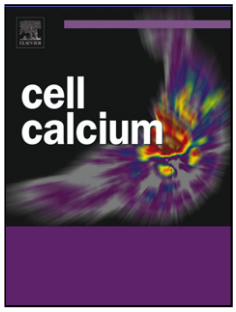

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Mitochondrial and Endoplasmic Reticulum Calcium Homeostasis and Cell Death

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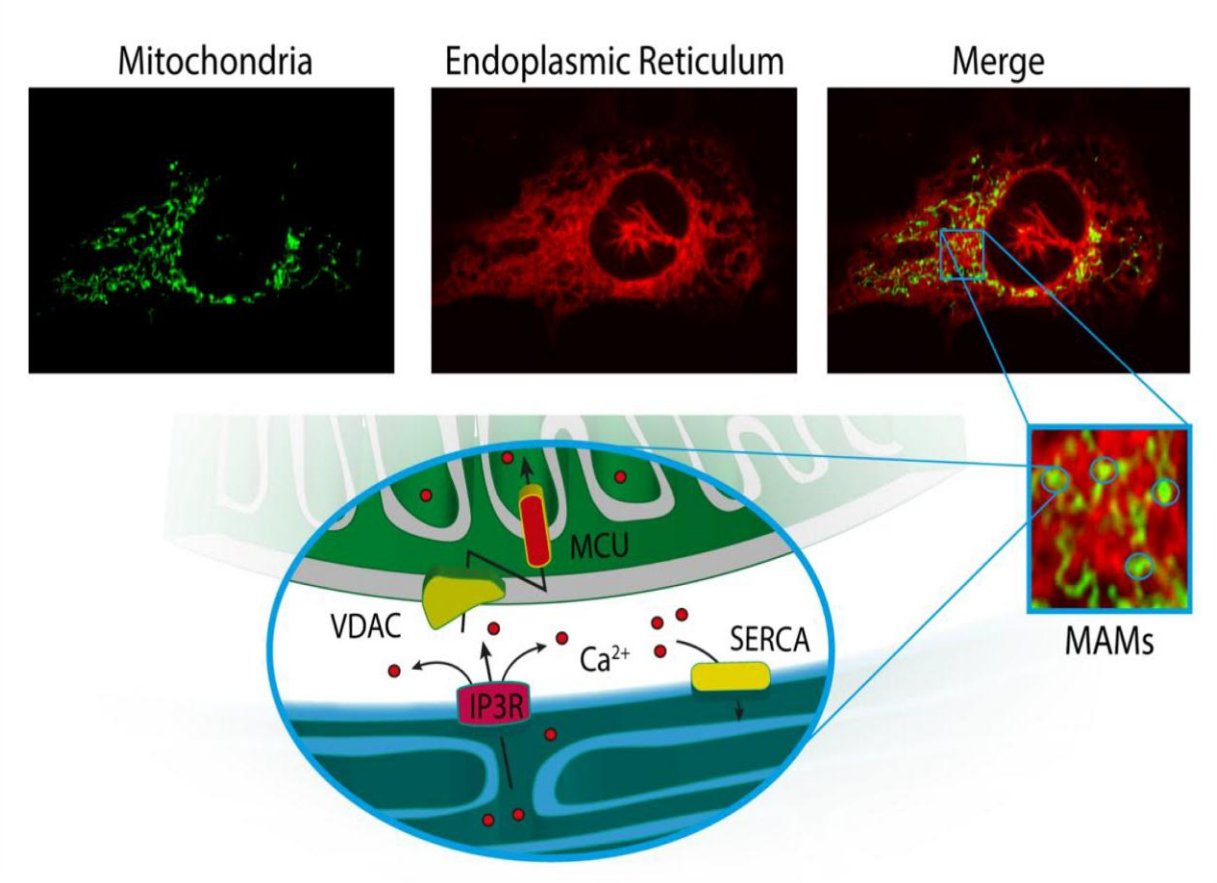
Graphical abstract



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Highlights

- Endoplasmic reticulum (ER)-mitochondria Ca^{2+} transfer regulates cell death
- Ca^{2+} transport systems at the ER and mitochondria play a crucial role in cell death and survival
- Several oncogenes and tumor-suppressors modulate cell sensitivity to apoptosis by regulating Ca^{2+} homeostasis

The endoplasmic reticulum (ER) and mitochondria cannot be considered as static structures, as they intimately communicate, forming very dynamic platforms termed mitochondria-associated membranes (MAMs). In particular, the ER transmits proper Ca^{2+} signals to mitochondria, which decode them into specific inputs to regulate essential functions, including metabolism, energy production and apoptosis. Here, we will describe the different molecular players involved in the transfer of Ca^{2+} ions from the ER lumen to the mitochondrial matrix and how modifications in both ER-mitochondria contact sites and Ca^{2+} signaling can alter the cell death execution program.

Introduction

Mitochondria are not only the energy powerhouse of the cell but also a major hub for cellular Ca^{2+} signaling crucial for cell life and death [1-3]. Indeed, mitochondria play a pivotal role in cell fate due in large part to their participation in the dynamic regulation of cellular Ca^{2+}. Under physiological conditions, the accumulation of Ca^{2+} in mitochondria stimulates oxidative metabolism through the modulation of Ca^{2+}-sensitive dehydrogenases and metabolite carriers [4-6]. Direct measurements of intracellular ATP levels have confirmed this notion, since agonist-dependent changes in the bulk mitochondrial Ca^{2+} accumulation correlate with enhanced mitochondrial, and then cytosolic, ATP concentrations [7].

The electrochemical gradient known as the mitochondrial membrane potential is used by the F1FO-ATP synthase to run the endergonic reaction of ADP phosphorylation and by Ca^{2+} to enter the mitochondrial matrix according to its electrochemical gradient [8]. The first studies performed in the 1960s and 1970s functionally characterized an electrophoretic uptake mechanism, today molecularly identified and named the mitochondrial calcium uniporter (MCU) complex, that allows rapid uptake of Ca^{2+} in

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"energized" (healthy) mitochondria [9]. The low affinity for Ca^{2+} of the MCU system is reconciled by the fact that mitochondria are not exposed to the bulk cytosolic concentration rise but rather to high Ca^{2+} microdomains generated in the proximity of the endoplasmic reticulum (ER) [10]. The ER lumen is considered to be the major intracellular Ca^{2+} storage compartment, and depletion of the ER Ca^{2+} content is followed by rapid accumulation inside the mitochondrial matrix through the uniporter system. The close apposition between the ER and mitochondria consists of mitochondrial reticular and/or branched networks in the cytosol that intimately and dynamically interact with the ER network; these connections are termed mitochondria-associated membranes (MAMs) [11] (Figure 1). These contacts between organelles can be readily observed by light and electron microscopy in many cell types, and it is estimated that there are approximately 100 mitochondrion-ER contacts in a yeast cell [12, 13], while approximately 20% of the mitochondrial surface is found in close proximity to the ER in mammalian cells [10]. Tomography analysis has shown that tight or loose tethers could adjoin the two organelles, depending on the nature of the peripheral ER [14]. Indeed, both Pellegrini and Nabi labs have recently proposed that smooth ER is apposed at 10 nm with mitochondria, whereas rough ER localizes at a 50 nm distance with the mitochondrial outer membrane [15, 16].

The main effectors of the ER Ca^{2+} release machinery are the inositol 1,4,5-trisphosphate (IP3) receptors (IP3R) and ryanodine receptors (RyRs) [17]. IP3Rs are ligand-gated channels that facilitate the release of Ca^{2+} from ER stores in response to the binding of agonists to cell surface receptors and the production of the second messenger IP3 [18, 19]. The Ca^{2+} released through IP3Rs is transferred to the mitochondrial intermembrane space by a class of mitochondrial porins known as voltage-dependent anion channels (VDACs), which form very abundant and large voltage-gated pores in the outer mitochondrial membrane at the ER-mitochondria contacts [20]. The tightening of the ER-mitochondria connections is relevant to cell death, revealing an unexpected dependence of cell function and survival on the maintenance of proper spacing between the ER and mitochondria [21, 22]. Impaired Ca^{2+} handling can lead to matrix Ca^{2+} overload and activation of a high conductance pore, the so-called “mitochondrial permeability transition pore” (mPTP) [23-25]. Mitochondrial Ca^{2+} overload has long been known to be a critical event in the bioenergetic crisis associated with cell death by necrosis (a prototypical example is the excitotoxicity of neurons) and acts as a critical sensitizing signal in the intrinsic apoptosis pathways [26]. Treatment with apoptotic stimuli, such as C2-ceramide,

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causes a release of Ca^{2+} from the ER, inducing dramatic changes in mitochondrial morphology. Indeed, the mitochondrial Ca^{2+} overload results in dramatic alterations in mitochondrial functions, including decreased ATP production and increased generation of reactive oxygen species (ROS) [27-29]. Ca^{2+} and ROS are the most important triggers for mPTP opening, acting in living cells in conjunction with a variety of pathological challenges [30]. Its opening induces mitochondrial swelling, and these large-scale alterations of organelle morphology allow the release of caspase cofactors into the cytosol. It has been proposed that the Ca^{2+}-mediated generation of ROS required for cell death induction derived from a massive binding of mitochondrial Ca^{2+} to cardiolipin, with consequent disintegration of respiratory chain complex II and oxidative stress [31].

Interestingly, a dynamic interplay between Ca^{2+} and hydrogen peroxide really occurs at the ER-mitochondria contact sites [32]. The ER-mitochondria Ca^{2+} transfer stimulates H_2O_2 mobilization from mitochondrial cristae to MAMs, generating specific H_2O_2 nanodomains that, in turn, sensitize the ER Ca^{2+} release to maintain Ca^{2+} oscillations [32]. These data reveal that the ER-mitochondrial interface accommodates much higher concentrations of key signaling molecules compared to those found in the bulk cytosol, highlighting its role as a molecular platform for the decoding of a wide range of danger signals.

The ER–mitochondria contacts have been also linked to ER stress-mediated cell death and the Unfolding Protein Response (UPR). Indeed, a variety of ER co-factors and chaperons are enriched at MAMs [33]. Moreover, during the early phases of ER stress, the number of ER-mitochondria connections significantly increases, ensuring mitochondrial Ca^{2+} uptake and ATP production, which in turn represent the bioenergetics basis for the adaption to such stressful condition [34]. On the other hand, alterations in MAMs functional properties induce ER stress and the UPR [35]. Thus, the crosstalk between ER and mitochondria could regulate stress responses and the UPR at different levels.

The crucial role played by Ca^{2+} signals in the regulation of cell death and apoptosis was confirmed in the early 2000s, when two pioneer studies demonstrated that Ca^{2+} transfer from the ER to mitochondria was required for the initiation of programmed cell death by some apoptotic stimuli [36, 37]. These observations were in turn based on several studies indicating that the ER Ca^{2+} content is a key determinant of cell sensitivity to apoptotic stress [38, 39]. Thereafter, the cancer-related properties of members of the Bcl-2/Bax family were associated with the manipulation of Ca^{2+} transfer from the ER to mitochondria to regulate cell death [40, 41]. Over the following years, a wide range of oncogenes and tumor suppressors have been described to require Ca^{2+} transfer to exert their pro- or anti-

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apoptotic functions. In this review, we will discuss the role of Ca^{2+} transport systems at the ER and mitochondria in cell death and survival and describe the different molecular pathways that modulate apoptosis onset by targeting the Ca^{2+} machinery.

ER calcium players and cell death

Considering the pivotal role of the Ca^{2+} ion in regulating cell death processes, it is not surprising that ER pumps and channels controlling Ca^{2+} signaling are primary contributors to cell fate decisions in physiological and pathological conditions [42] (Figure 2).

Two primary ER-resident proteins are involved in such processes. The first are the SERCA (sarco/endoplasmic-reticulum Ca^{2+}-ATPase) type Ca^{2+} pumps, which maintain correct ER Ca^{2+} levels by actively pumping Ca^{2+} into the ER from the intracellular space. Notably, SERCAs are present as three different SERCA genes (ATP2A1, ATPA2 and ATP2A3), and each of them generate diverse splice variants. The most ubiquitous pumps are the SERCA2b and SERCA2c isoforms, while less expressed is SERCA3, which is present in six isoforms. SERCA proteins also exhibit tissue-specific distribution; for example, SERCA1a and 1b are primarily found in the skeletal muscle [43]. Of these isoforms, the most important and most well studied is SERCA2b, as it displays the highest Ca^{2+} affinity, and its correct functioning is crucial for Ca^{2+} uptake in the ER and the regulation of cell death mechanisms. Indeed, heterozygous deletion of the gene encoding SERCA2 in transgenic mice induced squamous cell carcinomas of the upper digestive tract [44], and SERCA2b mRNA expression dramatically decreased in highly tumorigenic thyroid cells [45]. Accordingly, forced overexpression of SERCA not only augmented ER Ca^{2+} levels but also sensitized cells when exposed to pro-apoptotic stimuli [46]. Interestingly, two SERCA1 splice variants, which encode for truncated proteins characterized by only one (instead of seven) Ca^{2+}-binding domain, are associated with a reduction in the ER Ca^{2+} steady state level and an increase in ER Ca^{2+} leakage, culminating in apoptotic cell death [46]. Moreover, overexpresson of truncated SERCA1 isoform predisposes to ER stress and amplifies the apoptotic response by increasing the ER-mitochondria contact points and mitochondrial Ca^{2+} accumulation [47].

Due to the critical role of the SERCA pump in mediating Ca^{2+}-related apoptosis, it could represent an attractive target for a number of proteins regulating cell death. One example is the tumor suppressor p53. This protein also localizes to ER sites, where it interacts with SERCA and exerts its pro-apoptotic functions by regulating the prompt Ca^{2+} transfer into mitochondria [48, 49]. During cell death stimuli, p53 accumulates at the ER-mitochondria

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contacts, where it lowers the oxidation state of SERCA pumps and enhances toxic Ca^{2+} signaling events [48].

Numerous other proteins are known to affect SERCA activity. The most recently discovered ones are the ER-luminal protein disulfide isomerase TMX1 and the redox-sensitive protein SEPN1. TMX1 is reported to inhibit SERCA2b, thereby blocking Ca^{2+} transfer and mitochondria bioenergetics. Interestingly, the lower mitochondrial activity ascribed to the TMX1-dependent control of Ca^{2+} exacerbates the “Warburg effect”, favoring tumor growth [50]. In contrast to TMX1 activity, SEPN1 enhances the ER Ca^{2+} uptake activity of SERCA. Specifically, a redox-active form of SEPN1 interacts with SERCA at ER-mitochondria contact sites, consequently regulating ER Ca^{2+} levels [51]. Furthermore, SEPN1 counteracts oxidative damage, which occurs during ER stress and triggers the UPR. In fact, following UPR induction, ERO1 activity is boosted and the amount of ROS increases, which in turn inactivates SERCA by converting its luminal thiols into ROS-mediated oxidized derivatives. SEPN1 is able to reduce these molecules and restore SERCA activity [51]. Notably, SEPN1 is associated with several diseases, particularly with myopathies characterized by a loss of function and death of muscular cells [52].

The hypothesis developed from these experimental observations is that SERCA down-regulation minimizes the apoptotic response by reducing Ca^{2+} transfer, whereas increased SERCA pump activity is associated with cell death. However, SERCA2 expression positively correlates with colorectal tumor size and metastasis [53]. Moreover, modulation of SERCA has been identified as a valuable therapeutic strategy for inhibiting Notch1 in Notch-driven cancers [54]. The inhibition of SERCA activities by exposure to thapsigargin triggered ER stress-induced apoptosis, which initially manifests as an acute inhibition of protein synthesis and cell cycle arrest and, if sustained, promotes apoptosis. The role played by Ca^{2+} in the thapsigargin-mediated cell death process has now been clarified: the rapid and complete inhibition of SERCAs evokes a massive Ca^{2+} release from the ER through the IP3R, and the rise in intracellular Ca^{2+} levels is the key event that causes cell death. Thus, it has to be the availability of ER Ca^{2+}, and not the absolute amounts of it, which determines whether the ER is able to use the cation to trigger cell death.

The Ca^{2+}-dependent pro-apoptotic features of thapsigargin have been used to produce the so-called "smart bomb" drug for prostate cancer [55, 56]. Because the cytotoxicity of thapsigargin is not cell type specific, significantly damaging even normal cells, thapsigargin was combined with a targeting peptide that is a substrate of the serine/protease prostate-

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specific antigen (PSA). This compound has been shown to be selectively toxic against PSA-producing prostate cancer cells. Moreover, substantial tumor regression of a panel of human cancer xenografts in vivo was induced by a thapsigargin analog conjugated with the proteolytic enzyme prostate-specific membrane antigen (PSMA), which, despite its name, is not specific for prostate and is also found in the neovascular tissue of a large number of cancers [57]. This prodrug has been termed mipsagargin (G-202) and is currently being tested in a phase II clinical trial [58]. Notably, it has been recently showed that resveratrol and its derivate piceatannol induce cancer cell-specific cell death by reducing SERCA activity and enhancing ER-mitochondria tethering [59].

The second family of proteins indispensable for correct maintenance of optimal ER Ca^{2+} levels are divided in two sub-groups and are the major Ca^{2+} release channels expressed on the endo/sarcoplasmic reticulum (ER/SR): the ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP3Rs) [60] (Figure 2). RyRs are mostly expressed in the SR (commonly used to refer to the entire ER in smooth and striated myocytes), while IP3Rs are large conductance non-selective cation channels ubiquitously expressed in all cell types. The link between RyRs and cell death is poorly understood, although a very recent work reported a crucial role for RyRs in regulating autophagy [61]. By contrast, the role of IP3Rs in the regulation of cell fate is largely studied. The IP3R channels exist in three different isoforms that display approximately 60-80% homology in their amino acid sequence (IP3R1, IP3R2 and IP3R3) and are all activated by the second messenger IP3, Ca^{2+}, Ca^{2+}-binding proteins, ATP, thiol modification and phosphorylation by several proteins, including oncogenes and tumor suppressors [62]. These isoforms have distinct but overlapping expression patterns. In fact, most cell types express at least two or even all three isoforms. However, predominant expression of a specific isoform has been demonstrated in some cell types. For example, IP3R3 is the principal isoform expressed in most cultured cell types, while isoform 1 is the predominant isoform in neuronal cells, and IP3R2 is predominant in muscle and liver cells [63].

As described in the *Introduction* section, the primary role of IP3Rs is to allow the transfer of Ca^{2+} ions from the ER to the intracellular stores, particularly inside mitochondria, where Ca^{2+} plays crucial roles in the control of cell death mechanisms. It has been demonstrated that the regulation of cell fate by Ca^{2+} transfer from the ER to mitochondria is primarily controlled by a specific IP3R isoform. Indeed, changes in the expression levels and/or phosphorylation of IP3R3 ultimately resulted in increased or decreased susceptibility to cell death. Primary evidence of the particular type 3 features were observed in cells with

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IP3R3 silencing, where apoptotic levels appeared significantly reduced [64, 65]. In addition, the importance of IP3R3 in the regulation of cell fate was demonstrated by several studies showing that several oncogenes and tumor suppressors exerted their effects by regulating IP3R3 expression and activity [66, 67]. Classical examples of this group are the oncogene AKT and the tumor suppressor PTEN, which regulate apoptosis by controlling ER Ca^{2+} release [68-70]. PTEN interacts with IP3Rs, counteracting the Akt-mediated inhibition of ER Ca^{2+} efflux in a phosphatase-dependent way and thus protecting from cell death [68]. Similar observations were observed for the tumor suppressor promyelocytic leukemia protein (PML) [71]. Interestingly, our group recently demonstrated that PML also affects IP3R3 activity to regulate the autophagic response [72]. This occurs because proper Ca^{2+} transfer from the ER to mitochondria is required to respond to cellular energy demands by promoting mitochondrial metabolism. Since autophagy is highly dependent on energy supply, depletion of IP3Rs (and thus reduced Ca^{2+} transfer) lowered the mitochondrial metabolism and ATP production, thereby triggering autophagy. In fact, IP3R-null cell lines have elevated autophagy, and stable expression of IP3R3 completely restored basal autophagy levels in these IP3R-null cells [73]. Based on this seminal work, Foskett's group recently proposed that tumorigenic cell lines relied on constitutive IP3R-mediated Ca^{2+} transfer to mitochondria to sustain mitochondrial functions, and inhibition of IP3R by xestospongin B induced massive death in cancer cells, whereas their normal counterparts were spared [74]. Thus, upon some stressful conditions, abrogated ER-mitochondrial Ca^{2+} transfer can promote, rather than inhibit, cell death, as previously observed during neuronal ER stress and in Huntington's disease model mice [75]. Anyway, targeting the ER-mitochondria Ca^{2+} connection might represent an effective approach for re-establishing the apoptosis sensitivity of tumor cells. This is the strategy that was used to develop the small peptide BIRD-2 (Bcl-2-IP3 receptor disruptor-2), which triggers cell death in a wide range of SCLC (small cell lung cancer) lines through its binding to the BH4 domain of Bcl-2 [76, 77], thereby preventing Bcl-2 interaction with IP3Rs [78]. These findings suggest that BIRD-2 induces Ca^{2+}-mediated apoptosis, as previously reported in lymphoid malignancies [79, 80] and in diffuse large B-cell lymphoma (DL-BCL) cell lines containing elevated Bcl-2 levels [81]. Notably, it has been recently proposed that the Bcl-2 pharmacological inhibitor ABT737 synergize with cisplatin in reducing the progression of human ovarian cancer xenografts by increasing ER-mitochondrial Ca^{2+} transfer [82].

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Although SERCAs and IP3Rs represent the main targets for the regulation of Ca^{2+}-dependent apoptosis, there are a number of intraluminal Ca^{2+}-binding proteins, such as calreticulin, calnexin (CNX), and GRP78/BiP, which are able to modulate Ca^{2+} signaling and apoptosis independently of ER channels and pumps. Among them, the most well studied and characterized is the ER chaperone calreticulin. Overexpression of this protein results in augmented sensitivity to apoptosis [83]. It has been suggested that such pro-apoptotic effects may be due to the subsequent increase of Ca^{2+} in the ER, which is thus available for the Ca^{2+}-dependent apoptosis process. Accordingly, calreticulin-KO cells are resistant to induced cell death [39]. In addition, it has been demonstrated that during immunogenic cell death upon treatment with anthracyclines, calreticulin translocates with ERp57 to the cell surface, and this event seems to lead to immunogenic presentation to T-cells, provoking the activation of immune response and subsequent apoptosis. When the interaction between calreticulin and ERp57 is disrupted, the immune response is lost [84, 85].

Appropriate Ca^{2+} transfer to mitochondria also requires the correct distance between the two organelles, as well as an adequate number of connections. Therefore, disruption of ER-mitochondria units could result in altered Ca^{2+} signaling and apoptosis. NOGO-B, a critical regulator of the tubular structure of the ER, is highly expressed during pulmonary arterial hypertension, a disease characterized by excessive proliferation of vascular cells and lack of apoptosis [86]. NOGO-B induction increased the space between the ER and mitochondria, lowered the intracellular Ca^{2+} content and suppressed mitochondria-dependent apoptosis [86]. Again, two recent papers emphasized that the status of ER-mitochondria connections could be critical to the proper execution of the apoptotic process. The first study reported that the 21 kDa protein encoded by the FATE1 gene decreases sensitivity to mitochondrial Ca^{2+}-dependent apoptotic stimuli and to chemotherapeutic drugs in adrenocortical carcinoma (ACC) and in other cancer types [87]. Further studies are needed to determine whether this protein interacts with one of the main channels involved in the maintenance of the correct ER Ca^{2+} levels, but FATE1 is implicated in the regulation of Ca^{2+}-dependent apoptosis by modulating the ER-mitochondria distance. In the second work, Bonneau et al. proposed a novel and unexpected role for IP3R binding protein released with inositol 1,4,5-trisphosphate (IRBIT) [88]. IRBIT was discovered in 2003 [89] and has been described as a genuine suppressor of IP3R functions by competing with IP3 when IRBIT is phosphorylated at multiple sites [90]. However, upon apoptotic stimulations, IRBIT is dephosphorylated, inducing cell death

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by two main mechanisms: i) IRBIT moves from MAMs to the cytoplasm together with Bcl2l10, a homolog of Bcl-2, thereby inhibiting its antiapoptotic functions; and ii) IRBIT contributes to the formation and stabilization of ER-mitochondria contact points, allowing efficient mitochondrial Ca^{2+} uptake and cell death induction [88].

Mitochondrial calcium players and cell death

Ca^{2+} ions released from the ER are able to bypass the outer mitochondrial membrane (OMM) and reach the intermembrane space through VDAC channels (Figure 3). Three different VDAC isoforms are expressed in almost all mammalian tissues, showing comparable channel properties, although they have opposite effects on cell sensitivity to apoptotic challenges that involve mitochondrial Ca^{2+} loading. VDAC1, at the ER-mitochondria juxtapositions, selectively interacts with IP3R3, thereby potentiating the transfer of low-amplitude apoptotic Ca^{2+} signals to mitochondria [91]. At MAMs, GRP75 permits this interaction; small interfering RNA (siRNA) silencing of GRP75 abolishes the functional coupling between IP3R and VDAC1, reducing mitochondrial Ca^{2+} uptake in response to agonist stimulation [92].

The serine/threonine kinase AKT, often up-regulated in cancer, promotes VDAC1 association with hexokinase 2 (HK2) through phosphorylation events [93, 94]. This association occurs at MAMs and enables HK2 to phosphorylate glucose using the ATP exiting from mitochondria through VDAC1, thereby stimulating glycolysis. Conversely, upon inhibition of Akt, HK2 dissociates from VDAC1, causing VDAC1 closure and increased mitochondrial membrane potential [93].

It has recently been shown that mTORC2 is physically associated with MAMs and that mTORC2-Akt signaling mediates MAMs integrity and function [95]. mTORC2 localizes at the ER-mitochondria interface in a growth factor-stimulated manner, where it phosphorylates and activates Akt, which in turn phosphorylates the MAMs resident proteins PACS2, IP3R, and HK2 to regulate MAMs integrity, Ca^{2+}, and energy metabolism, respectively [95]. Notably, a recent study reported that myeloid cell leukemia factor 1 (Mcl-1), interacting with VDAC1, promotes lung cancer cell migration, thereby increasing mitochondrial Ca^{2+} uptake and ROS generation [96]. Also, the anti-apoptotic member of Bcl-2 family, Bcl-xL, interacts with VDAC1 and protects from cell death stimuli by limiting the transfer of Ca^{2+} signals to mitochondria [97, 98].

The well-defined role of VDAC1 in triggering apoptosis has encouraged the development of therapeutic strategies based on limiting VDAC1-related cell death activities to treat

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different diseases associated with enhanced apoptosis, such as neurodegenerative diseases. A high-throughput screening approach revealed that the compounds VBIT-3 and VBIT-4 displayed strong antiapoptotic activity by preventing VDAC1 oligomerization, detachment of hexokinase bound to mitochondria and disruption of intracellular Ca^{2+} levels, thus restoring normal mitochondrial functions [99].

Unlike isoform 1, VDAC2 appears to have an antiapoptotic role, interacting with Bcl-xS and leading to release of Bak [100], whereas VDAC3 seems to have no significant influence on apoptosis [91]. VDAC2 interacts specifically with Bak. Indeed, cells deficient in VDAC2 were more susceptible to apoptotic death, and, accordingly, overexpression of VDAC2 selectively prevented Bak activation and inhibited the mitochondrial apoptotic pathway [101]. Interestingly, VDAC2 depletion shifts the localization of Bak from mitochondria to other intracellular compartments [102], including peroxisome membranes [103].

To reach the mitochondrial matrix, the Ca^{2+} ions located inside the intermembrane space must pass through the MCU complex (Figure 3). The identification of the uniporter was simultaneously reported by 2 different groups that described CCDC109A (renamed MCU) as the pore-forming subunit of the complex [104, 105]. The MCU gene encodes a 40-kDa protein with two coiled-coil (CC) domains and two transmembrane domains separated by a short loop enriched in acidic residues exposed to the intermembrane space, whereas the N-terminal and C-terminal portions of the protein protrude into the matrix [106]. MCU expression is strictly dependent by Ca^{2+} and involves the nuclear factor CREB (cyclic adenosine monophosphate response element-binding protein), which directly bounds the MCU promoter and favors its transcription [107].

Since the publication of the paper by Rizzuto and coworkers, in which they showed that MCU-expressing cells displayed enhanced sensitivity to apoptosis [105], the idea that increased mitochondrial Ca^{2+} loading by the MCU could be correlated with a predisposition for cell death was confirmed. Thereafter, different experimental observations verified the role of the MCU as a pro-apoptotic factor. In primary cortical neurons, MCU overexpression triggers cell death not only after excitotoxic stimulation but also in untreated cells; accordingly, MCU silencing strongly protects cells from apoptosis [108]. Again, cardiomyocyte-specific deletion of the MCU inhibited mPTP opening, and the loss of the MCU in the murine adult heart protected the heart from acute ischemia-reperfusion injury [109]. Nevertheless, higher MCU activity positively modulates the inflammatory response, triggering NLRP3 inflammasome activation and caspase-dependent apoptosis [110, 111].

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Notably, the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) promotes mPTP opening and myocardial death by increasing MCU activity through direct phosphorylation [112]. These findings support the potential role of MCU posttranslational modifications (i.e. phosphorylations) in the regulation of mitochondrial Ca^{2+} uptake and apoptosis through the uniporter complex [113]. Indeed, different kinases, including members of the PKC family [114], have been proposed to regulate a wide range of cellular processes by targeting mitochondrial Ca^{2+} homeostasis [115].

MCU transcripts appear to be under the control of miR-25, which can efficiently reduce MCU levels and consequently mitochondrial Ca^{2+} transfer [116]. In human primary colon tumors, miR-25 is overexpressed, and the MCU is accordingly down-regulated, and depletion of miR-25 enhances mitochondrial Ca^{2+} uptake and sensitizes cells to apoptosis [116]. The role of miR-25 as a mediator of apoptosis through the MCU has been confirmed in other pathological contexts, such as pulmonary arterial hypertension [117] and the protection of cardiomyocytes against oxidative insults [118].

However, a breakthrough article by Finkel's group raised some concerns about the role of the MCU as a pro-apoptotic agent [119]. Pan and coworkers created an MCU-deficient mouse model and demonstrated that the absence of MCU expression does not confer any protection from cell death, although MCU KO mitochondria showed no detectable Ca^{2+}-induced mPTP opening [119]. Interestingly, in some cellular settings (i.e., breast cancer cell lines), MCU seems to have no or antiapoptotic functions [120, 121]. These data, elegantly discussed in [122], suggested the existence of alternative pathways for Ca^{2+} uptake that might regulate apoptosis in a mechanism unrelated to the MCU. Indeed, uncoupling proteins 2 and 3 (UCP2 and UCP3) and SLC25A23 have been described to modulate mitochondrial Ca^{2+} accumulation and apoptosis through a mechanism that might be at least in part independent from the MCU [123, 124].

To date, two other membrane proteins, MCUb and EMRE, have been shown to be part of the Ca^{2+}-permeable pore, whereas the key regulators of the whole complex are MICU1 and its paralog MICU2, as well as the MCU partner MCUR1 [125-128]. Among them, MICU1 is the most studied, and several findings have linked its altered activity to variations in cell death response. MICU1 acts as a gatekeeper of the uniporter complex, preventing Ca^{2+} entry under resting conditions and activating the channel at high cytosolic Ca^{2+} concentrations, thereby stimulating Ca^{2+} entry in the mitochondrial matrix [129-132]. The down-regulation of MICU1 dramatically elevates Ca^{2+} content under basal conditions, boosting the production of mitochondrial ROS, particularly the superoxide anion, which

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sensitize cells to apoptosis [130, 131]. Moreover, MICU1 loss promotes chronic Ca^{2+} entry via the MCU, leading to oxidative damage and impaired migration in human aortic endothelial cells as well as diminished vascular integrity in vivo [133]. Importantly, MICU1 is critical for liver regeneration, since liver-specific MICU1 knock-down mice exhibited suppression of hepatocyte proliferation and massive necrosis after partial hepatectomy [134].

MCUR1 is a 40-kDa protein localized at the IMM that interacts with the MCU, but not with MICU1 [135]; MCUR1 silencing results in a dramatic inhibition of agonist-induced mitochondrial Ca^{2+} uptake [135]. Although the role of MCUR1 as a regulator of the MCU complex is still debated [136, 137], it has been recently described that MCUR1 expression in Drosophila regulates the Ca^{2+} threshold necessary for the permeability transition [138]. Interestingly, depletion of MCUR1 resulted in a cellular bioenergetic crisis and induction of the autophagic process [139].

Another aspect that plays an essential role in the control of apoptosis is the remodeling of the mitochondrial network, which could influence the capacity of mitochondria to receive and decode the Ca^{2+} death signals from the ER. In particular, changes in the number of so-called "Ca^{2+} hot spots" could affect Ca^{2+} signaling and cell death. This is the case for the pro-apoptotic protein fragile histidine triad (FHIT); acting at the mitochondrial compartment, FHIT overexpression enhances the number of ER–mitochondria connections, leading to increased mitochondrial Ca^{2+} accumulation and Ca^{2+}-dependent apoptosis [140].

Mitofusin 2 (MFN2), a component of the mitochondrial network remodeling machinery, has been associated with the formation of ER-mitochondria contacts [141, 142]. Ablation or silencing of MFN2 in fibroblasts and HeLa cells reduces the efficiency of mitochondrial Ca^{2+} uptake in response to stimuli that generate IP3 [143]. However, Filadi and colleagues have raised some doubts about this model, demonstrating that MFN2 ablation or silencing increases, rather than diminishes, the close juxtaposition between the two intracellular structures and strengthens the efficacy of Ca^{2+} transfer from the ER to mitochondria, sensitizing cells to mitochondrial Ca^{2+} overload-dependent death [144]. Very recently, in support of the original mechanism of action proposed for MFN2, Naon et al. confirmed that acute MFN2 ablation reduces mitochondrial uptake of Ca^{2+} released from the ER, due to the higher distance between the organelles [145]. Thus, the role of MFN2 as an ER-mitochondrial tethering factor is still controversial, with different pieces of evidence supporting either the classical model [146] or the revised one [15, 147]. Notably,

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mitochondrial MFN2, but not ER-associated MFN2, interacts with the mitochondrial ubiquitin ligase MITOL, mediating the addition of lysine 63-linked polyubiquitin chains to MFN2 but not its proteasomal degradation [148]. MITOL regulates MFN2 localization and MAMs formation, playing a critical role in neuronal function and survival, which is also illustrated by the reduction in ER Ca^{2+} transfer that occurs in MITOL-deficient cells [148].

In addition to the ER-mitochondria structural connection, fusion and fission events regulating the shape of the organelles drastically influence mitochondrial Ca^{2+} responses and consequently cell death. Specifically, it has been proposed that mitochondrial fission factors constitute essential components of cell death signaling pathways [149]. By promoting mitochondrial division, the pro-fission factor dynamin-related protein-1 (Drp-1) inhibits the propagation of Ca^{2+} signals and protects against Ca^{2+}-mediated cell death [150]. In addition, the anti-apoptotic variant of Mcl-1 has been demonstrated to control mitochondrial dynamics by promoting Drp-1-mediated mitochondrial fission, preventing mitochondrial hyperpolarization and Ca^{2+} uptake [151, 152].

Interestingly, it has been demonstrated that the mitochondrial fission factor Fis1 is able to transmit apoptotic signals from mitochondria to the ER by interacting with Bap31 at the ER and facilitating its cleavage into the pro-apoptotic p20Bap31 [153]. Both proteins constitute a molecular scaffold for the recruitment and activation of procaspase-8, with a concomitant increase in Ca^{2+} release from the ER and consequent Ca^{2+} accumulation in mitochondria [153].

On the other hand, IF1, the endogenous ATPase inhibitor, preserves mitochondrial ultrastructure and limits apoptosis [154]. Down-regulation of IF1 induces higher cytochrome c release, which in turn activates the IP3R, an early event that occurs during apoptosis [155], resulting in the amplification of the apoptotic signal [154]. These findings suggest that modifications of both molecular interactions and mitochondrial architecture could emit danger signals that are decoded by the ER, which responds by eliciting Ca^{2+} waves and consequently enhances apoptosis.

Concluding Remarks

Although the distance between the ER and the matrix side of the inner mitochondrial membrane is extremely low, less than 50 nanometers, the journey that Ca^{2+} ions must take to reach their mitochondrial destination requires several regulatory steps and molecular checkpoints. Alterations in just one of these controlling pathways result in dramatic metabolic or apoptotic defects, revealing the importance of proper ER-

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mitochondria Ca^{2+} transfer to maintain the physiological status of the cell. Indeed, different diseases have been linked to remodeling of MAMs functions, such as cancer [66] and neurodegeneration [156]. Enhanced understanding of the molecular machinery that governs Ca^{2+} signaling has provided opportunities to develop specific Ca^{2+}-based therapeutic approaches. Moreover, the capacity to pharmacologically modulate Ca^{2+} channels and transport indicates that when Ca^{2+} signals are deeply involved in a precise pathological route, a specific Ca^{2+} channel or pump could be a valuable drug target. Notably, targeting Ca^{2+} signaling could also be considered as a potential adjuvant therapy, especially to counteract tumor growth and metastatic spread. This is the case of carboxyamidotriazole (CAI), a non-voltage-operated calcium channel blocker, which has been used in clinical trials in combination with radiation therapy for the treatment of newly diagnosed glioblastoma multiforme (for further details, see https://clinicaltrials.gov/). Hence, increasing our knowledge regarding the molecular aspects and functions of different Ca^{2+} players is the only way to define the best use of Ca^{2+}-based pharmacological agents to ameliorate patient outcomes.

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Figure Legends

Figure 1: Immunofluorescence analysis of mitochondrial (green) and Endoplasmic Reticulum (red) compartments of human fibroblasts. The color-merged image has been zoomed and the contact points between the two organelles have been indicated with blue circles. MAMs: mitochondria associated membranes

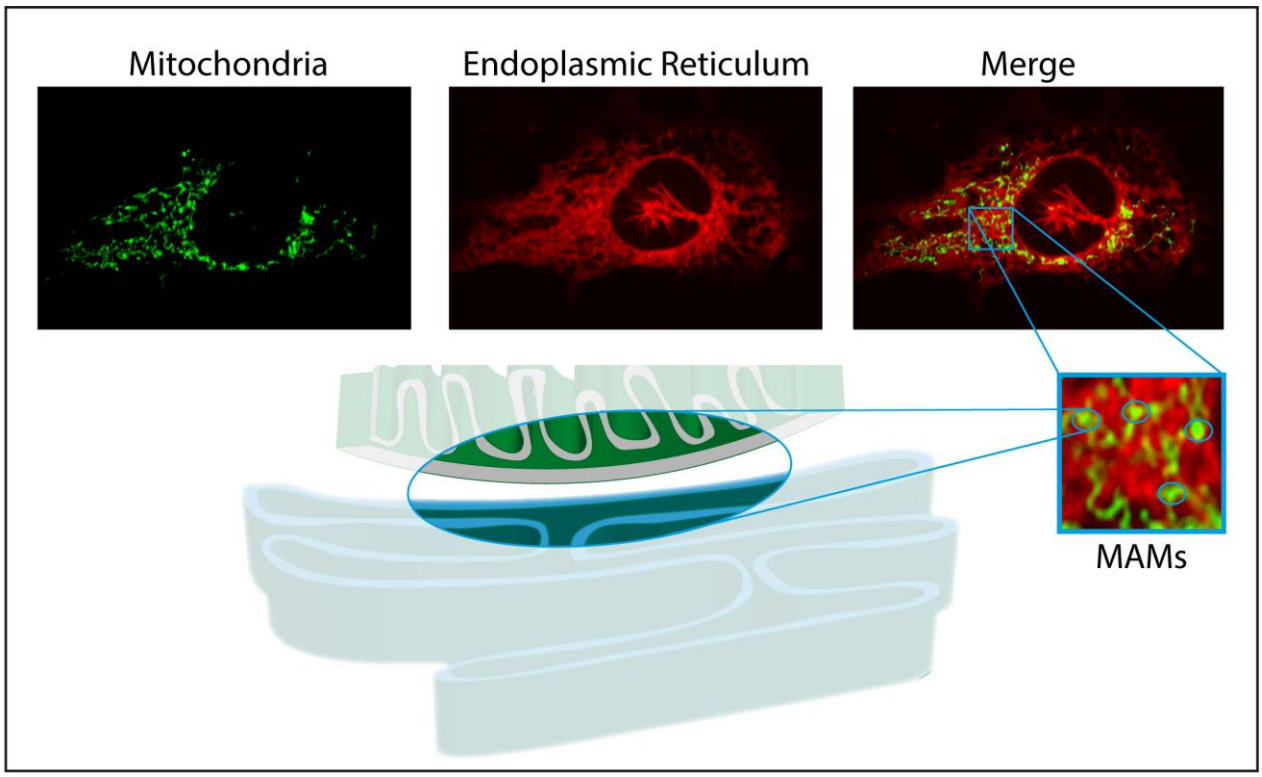

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Figure 2: Ca^{2+} handling at the ER-mitochondria interface: focus on the ER Ca^{2+} homeostasis. See text for further details.

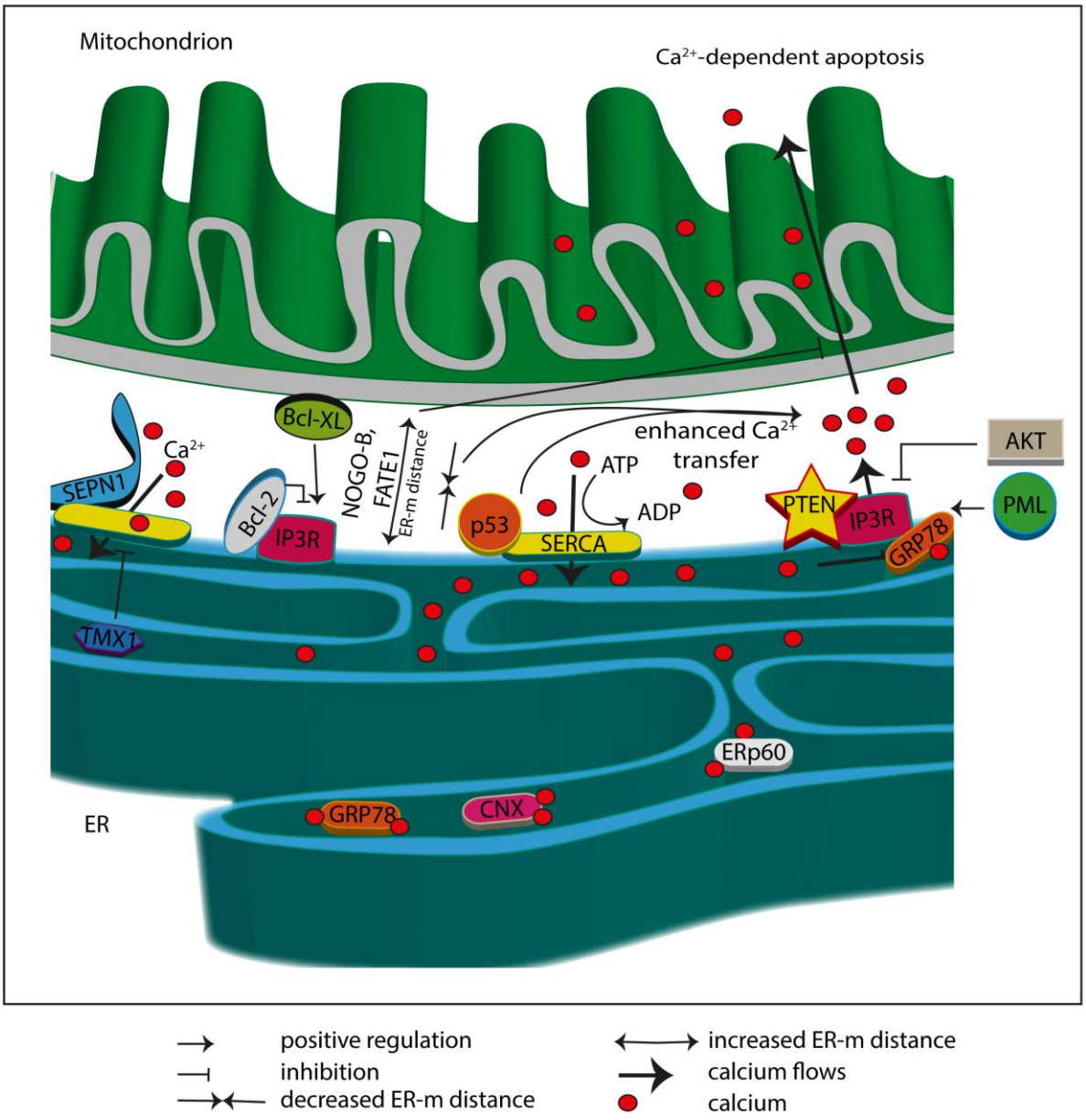

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Figure 3: Ca^{2+} handling at the ER-mitochondria interface: focus on the mitochondrial Ca^{2+} homeostasis. See text for further details.