Mitochondrial Ca^{2+} and apoptosis

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ABSTRACT

Mitochondria are key decoding stations of the apoptotic process. In support of this view, a large body of experimental evidence has unambiguously revealed that, in addition to the well-established function of producing most of the cellular ATP, mitochondria play a fundamental role in triggering apoptotic cell death.

Various apoptotic stimuli cause the release of specific mitochondrial pro-apoptotic factors into the cytosol. The molecular mechanism of this release is still controversial, but there is no doubt that mitochondrial calcium (Ca^{2+}) overload is one of the pro-apoptotic ways to induce the swelling of mitochondria, with perturbation or rupture of the outer membrane, and in turn the release of mitochondrial apoptotic factors into the cytosol.

Here, we review as different proteins that participate in mitochondrial Ca^{2+} homeostasis and in turn modulate the effectiveness of Ca^{2+}-dependent apoptotic stimuli. Strikingly, the final outcome at the cellular level is similar, albeit through completely different molecular mechanisms: a reduced mitochondrial Ca^{2+} overload upon pro-apoptotic stimuli that dramatically blunts the apoptotic response.

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1. Introduction

The term apoptosis, from a Greek word meaning 'falling leaves', was initially proposed in 1972 [1] to describe a form of natural cell death occurring in multicellular organisms. It had become necessary to distinguish between the different types of cell death and this kind exhibited a characteristic morphology and what seemed to be an endogenously regulated process. Over the past decades since the introduction of the term, scientific knowledge of this biological process has been greatly expanded.

Apoptosis, that involves altruistic suicide of individual cells in favour of the organism as a whole, was thus defined as programmed cell death of type I characterized by blebbing, cell shrinkage and extensive chromatin condensation and DNA degradation [2–5].

Apoptotic activity is essential for organ homeostasis by keeping under control cell number and tissue tropism both in physiological and pathological circumstances [4,6]. Inappropriate activation of apoptosis has been recognized to be at the basis of many common pathologies [7,8].

Apoptosis in mammals can have one of two initiation phases: the death receptor pathway (extrinsic apoptotic pathways) and the mitochondrial pathway (intrinsic apoptotic pathways), which pathway is selected depends on the nature of the death signal to be integrated [9,10].

There is no doubt that cell death belongs to the numerous cell functions on which Ca^{2+} exerts a complex regulatory role [11–13]. It has long been known that in neurons and other cell types an unchecked increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) can trigger apoptosis [14] and likewise, agents able to release Ca^{2+} from intracellular stores have been shown to be pro-apoptotic [15].

Importantly, Ca^{2+} is a critical sensitizing signal in the pro-apoptotic transition of mitochondria that plays a key role in the regulation of cell death [16]. Mitochondrial Ca^{2+} overload is one of the pro-apoptotic ways to induce the swelling of mitochondria, with perturbation or rupture of the outer membrane, and in turn the release of mitochondrial apoptotic factors into the cytosol [17].

The mitochondrial intermembrane space (IMS) contains many pro-apoptotic factors such as cytochrome c, apoptosis inducing factor (AIF), procaspase-9, Smac/DIABLO as well as endonuclease G [18–22]. These are released from mitochondria to the cytosol in response to apoptotic signals, like DNA damage, oxidative stress,

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energetic catastrophe, viral infection, endoplasmic reticulum (ER) stress or xenobiotic intoxication. Their release is preceded by the outer mitochondrial membrane (OMM) permeabilization, a crucial step in apoptosis, but how this is exactly performed is not yet clear [23]. Several B-cell CLL/lymphoma-2 (Bcl-2)-protein family members can affect the permeability of the OMM, for example, by binding to the mitochondrial voltage-dependent anion channels (VDACs) and regulating its properties or by forming multimeric channel complexes [24]. OMM rupture, and thus release of apoptogenic factors, can also occur subsequently to a long-lasting increase in inner mitochondrial membrane (IMM) permeability, also called mitochondrial permeability transition (MPT). MPT is supposedly due to the opening of a permeability transition pore (PTP), the molecular nature of which is however still unresolved [25]. A role for VDAC in the PTP has been proposed, but this is still highly controversial. An important point hereby was the demonstration that all VDAC isoforms are dispensable for the induction of MPT [26]. Independently of the mechanism by which the increase in permeability of the OMM is achieved, it allows the release of the apoptogenic factors present in the IMS to the cytoplasm and the progression of apoptosis [25,27–29].

Released pro-apoptotic proteins can initiate three signalling cascades leading to apoptosis: (i) released cytochrome c, together with apoptosis protease activating factor 1 (APAF-1), preexisting in the cytosol, forms "apoptosome" which results in the activation of procaspase-9, and in turn activation of effector caspases (caspases-3, -6, and -7); (ii) released from the IMS, both Smac/DIABLO and Omi/HtrA2 favour caspase activation by antagonizing the endogenous inhibitors of caspases, the cytosolic inhibitor of apoptosis proteins (IAPs); and (iii) released AIF and endonuclease G favour DNA fragmentation and chromatin condensation [28]. At the early phase of apoptosis, simultaneously with cytochrome c release (before caspases activation), fragmentation of the mitochondrial network has been observed [30]. Moreover, execution of mitochondria-related apoptosis can be connected with mitochondrial dysfunction including loss of mitochondrial membrane potential (MMP), increased reactive oxygen species (ROS) production, decreased ATP production and alteration of mitochondrial Ca^{2+} homeostasis.

2. Oncogene and oncosuppressor proteins regulation of mitochondrial Ca^{2+} homeostasis in control of apoptosis

A critical link between Ca^{2+} and apoptosis was established while studying the oncoprotein Bcl-2 and its mechanism of action. Bcl-2 is a central regulator of apoptosis, able to block or delay apoptosis in different cell types, from haematopoietic to neural [31]. The immediate interest generated around this protein's functions led to the discovery of several other proteins displaying sequence homology. These are also active in the control of apoptosis and have given rise to a whole family of Bcl-2 proteins.

To date, this family comprises at least thirteen members that can be easily classified, regarding their control of apoptosis, as proapoptotic and antiapoptotic [32]. The antiapoptotic members conserve the higher sequence homology with Bcl-2 and especially within four highly conserved Bcl-2 Homology domains (BH1-4), like the Bcl-2-related gene A1, BCL-X$_L$, BCL-w, and MCL-1. The proapoptotic family members instead can be subdivided into effector proteins, such as BAK and BAX, that were originally described to contain only BH1-3, and into the BH3-only proteins that share homology only in the third domain and comprise BAD, BID, BIM, BIK, PUMA, Noxa and others [32].

Through their localization to cytosol, ER and mitochondria, they allow regulation of apoptosis at different stages and during different pathways, ranging from the direct control of OMM permeability or unfolded protein response [32,33] to the control of intracellular Ca^{2+} homeostasis (Fig. 1).

Our group demonstrated how Bcl-2 over-expression was able to reduce the steady state of Ca^{2+} levels within the ER, with resulting reduced Ca^{2+} transfer to mitochondria during apoptotic stimulation and reduced triggering of mitochondrial fragmentation and apoptosis initiation [34,35]. Other groups have reported similar data, confirming how Bcl-2 could mediate an augmented leak from the compartment without affecting activity of ER Ca^{2+} ATPase [36,37]. Our group also addressed the hypothesis that its putative pore forming activity could mediate regulation of ER Ca^{2+} release [38]. Some controversy still remains, however, regarding the mechanism through which Bcl-2 reduces the Ca^{2+} transfer from ER to mitochondria. Bassik et al. showed how Bcl-2 alters the phosphorylation of IP$_3$R, promoting its basal leaking [39]. A few years later, the group of Distelhorst proposed the direct interaction between the BH4 domain of Bcl-2 and the IP$_3$R, reducing its opening probability and conferring resistance to apoptosis during prolonged exposure to the T cell receptor [40]. These data were recently confirmed in glioma cell lines. In this elegant work, it was shown how the interaction between Bcl-2 and IP$_3$R is able not only to inhibit apoptosis, but also to inhibit its propagation through several cells in a Ca^{2+}- and IP$_3$-dependent manner [41].

Not only Bcl-2, but also Bcl-X$_L$ is able to bind IP$_3$R, apparently determining its sensitivity for IP$_3$ in a phosphorylation-dependent manner [42]. Recently, similar observations were extended to Mcl-1 [43].

Nonetheless recently this concept has been discussed. Monaco and coworkers show how the interaction between IP$_3$Rs and Bcl-2 depend on its BH4 domain and a single critical residue of difference in the sequence compared to the once of Bcl-X$_L$ will not allow to the latter to maintain this interaction [44]. It should be noticed that Li and coworkers clearly show how Bcl-X$_L$ is involved in regulation of IP3Rs activity, by alternatively coexpressing Bcl-X$_L$ and the single IP3R isoform in an IP3R ko background (DT40 cells). A possible explanation of this point is that Bcl-X$_L$ could regulate IP3R by an indirect interaction. Moreover Monaco at al. performs experiments in cell types with mixed population of IP3R isoform (MEFs, C6 glioma cells), while Bcl-X$_L$ apparently regulates the channel activity of isoform 3 (see [42]).

Interestingly, pro-apoptotic members of the Bcl-2 family display an opposite behaviour. BAX and BAK where shown to control apoptosis from mitochondria and ER during Ca^{2+}-dependent stimuli. Initially, it was shown that BAX was capable of inducing Ca^{2+} transfer from ER to mitochondria by promoting the release of a mitochondrial proapoptotic factor [45]. Later, Korsmeyer and colleagues generated a double knock out for both BAX and BAK displaying a strong reduction in the ER levels of Ca^{2+}, as well as reduced cytoplasmic Ca^{2+} waves and mitochondrial uptake. At the same time, these cells were strongly resistant to different apoptotic stimuli. In the same cell lines, reintroduction of mitochondrial BAX restored mitochondrial apoptosis induction following exposure to tBID, but resistance to Ca^{2+}-dependent stimuli was still observed. Recovery of ER Ca^{2+} levels by expressing SERCA in double knock out cells for both BAX and BAK also restored sensitivity to Ca^{2+}-triggered apoptosis. This strongly suggested that, BAX and BAK where able to control Ca^{2+} homeostasis in the ER and mitochondria [46]. Recently, the relation between pro- and antiapoptotic members in the regulation of Ca^{2+} was explained through the activity of BI-1 (Bax inhibitor 1). This is a potent inhibitor of cell death during Bax activation, and also a regulator of ER [Ca^{2+}]. Recently, it was shown how the C-terminus of BI-1 is able to generate a Ca^{2+} channel on the ER membrane responsible for the Ca^{2+} leak and that this activity is conserved in animals but not in plant and yeast [47].

Furthermore, it was demonstrated that Ca^{2+}-dependent apoptotic stimuli can engage different BH3-only members of the family:
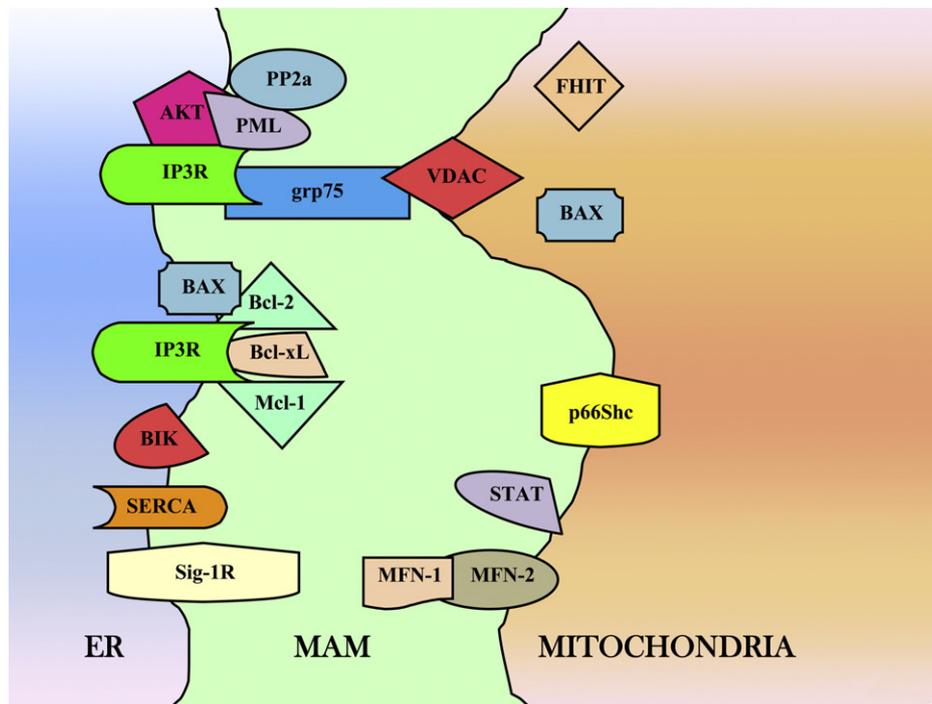
Fig. 1. Schematic intracellular distribution of the reported proteins involved in ER–mitochondria Ca^{2+} cross-talk. *Abbreviations*: AKT, v-akt murine thymoma viral oncogene homologue; BAX, Bcl-2-associated X; Bcl-xL, B-cell lymphomas extra long; Bcl-2, B-cell lymphomas 2; BIK, Bcl-2 interacting killer; ER, endoplasmic reticulum; FHIT, fragile histidine triad; grp75, glucose-regulated protein 75; IP3R, inositol 1,4,5-trisphosphate receptor; MAMs, mitochondria-associated membranes; mcl-1, myeloid cell leukaemia-1; MFN-1/-2, mitofusin-1, -2; p66Shc, 66-kDa isoform of the growth factor adapter shc; PML, promyelocytic leukaemia protein; PP2a, protein phosphatase 2a; SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase; Sig-1R, Sigma-1 receptor; STAT, Signal Transducer and Activator of Transcription; VDAC, voltage-dependent anion channel.

ceramide induces dephosphorylation of the BH3-only protein BAD. Dephosphorylated BAD sensitizes the PTP to Ca^{2+} through a Bcl-x$_L$-sensitive and VDAC-mediated process that does not require BAX or BAK [48]. Another BH3-only regulator has been shown to regulate Ca^{2+} homeostasis, BIK. This protein has been shown to promote BAX localization to ER and to increase the Ca^{2+} released in the cytoplasm during thapsigargin stimulation [49].

In a similar way to Bcl-2, another anti-apoptotic factor, the serine/threonine kinase Akt, is able to regulate mitochondrial Ca^{2+} levels through a direct activity on the ER side [50] (Fig. 1). Like Bcl-2, Akt protects from apoptosis by diminishing the Ca^{2+} flux from ER, without affecting the total Ca^{2+} content of the store [51]. This is possible by a strong inhibition of IP$_3$R releasing properties, probably due to a phosphorylation event [52]. In fact, all three subtypes of IP$_3$R present, in their C-terminal tail, a robust phosphorylation motif RXRXX(S/T), highly conserved in different species [53], which is a substrate for Akt kinase activity [52,53]. Thus, Akt function on IP$_3$R and the consequent minor Ca^{2+} transfer from ER to mitochondria is the way through which Akt works to protect from Ca^{2+}-mediated apoptosis. Interestingly, Akt can localize also into mitochondria [54] and intramitochondrial active Akt results in efficient protection against apoptotic signalling [55]. However, the exact role of this kinase inside the organelle has not been fully understood.

Recent evidence indicated that the crosstalk between ER and mitochondria is regulated also by another important oncosuppressor: the promyelocytic leukaemia (PML) protein, encoded by a tumour suppressor gene implicated in the pathogenesis of leukaemia and cancer [56]. PML has been previously recognized as a critical and essential regulator of multiple apoptotic responses [57,58]. Nevertheless, how PML would exert such a broad and fundamental role in apoptosis remained a mystery for long time. The reported role of PML in the modulation of p53 transcription [59] failed to reconcile this issue, as well as the role

played by PML in the transcription-independent early apoptotic response.

Within the cell, PML isoforms display both nuclear and cytosolic distribution. In the nucleus, PML is present in a multiprotein nuclear structure, the PML-nuclear bodies [60]. Recent data defined a novel and unexpected extra-nuclear PML-dependent pathway for the control of Ca^{2+} homeostasis and in turn Ca^{2+}-dependent apoptosis [61].

Interestingly, in the cytosol, PML was found to localize to the ER and the mitochondria associated membranes, MAMs [62] (Fig. 1). MAMs are specialized domains selectively enriched in mitochondrial Ca^{2+} signalling elements, where Ca^{2+} transfer between ER and mitochondria takes place [63].

In particular, we obtained direct evidence that PML modulates intracellular Ca^{2+} homeostasis *via* PP2A phosphatase recruitment at the ER and at the MAMs to inactivate Akt kinase-dependent phosphorylation of IP$_3$R$_3$s. In so doing, PML is able to regulate Ca^{2+} mobilization into mitochondria induced by Ca^{2+}-dependent stimuli (*e.g.*, oxidative stress), which then triggers the cell death programme. Conversely, in the absence of PML, PP2A does not accumulate in the complexes with IP$_3$R$_3$ and Akt, and this results in an accumulation of activated Akt (phospho-Akt). Once activated, Akt can hyper-phosphorylate IP$_3$R$_3$ thus inhibiting ER Ca^{2+} release towards mitochondria and thus inhibiting the mitochondrial Ca^{2+} overload necessary to execute the cell death programme [62].

Conceptually, similar data were also obtained with other unrelated anti-apoptotic proteins. The most striking example was provided by an oncogene expressed in a human hepatocarcinoma. This oncogene is generated by the integration of the Hepatitis B virus genome in the gene encoding the protein SERCA1 (Sarco/ER Ca^{2+} ATPase type 1). Viral activation was shown to *cis*-activate SERCA1 chimeric transcripts with splicing of exon 4 and/or exon 11. Splicing of exon 11 creates a frameshift and a premature stop codon in exon 12. The encoded protein lacks most of the cytosolic N

and P domains and critical Ca^{2+}-binding regions of the transmembrane region. This protein is incapable of active Ca^{2+} pumping [64], and is causally involved in the neoplastic phenotype. Although the molecular mechanism of this effect has not been elucidated yet, it may be speculated that the mutated SERCA could either interfere with the activity of endogenous pumps and/or could act as a Ca^{2+} leak pathway from the ER. These data are consistent with the observations that overexpression of SERCA in HeLa cells increases the susceptibility of cells to apoptotic agents [65,66]. This notion was further reinforced by the study of the enterovirus 2B protein, a small membrane-integral replication protein with an amphipathic α-helix that can build membrane-integral pore forming multimeric transmembrane bundles [67]. This Coxsackie viral protein 2B was shown to be anti-apoptotic and to reduce ER Ca^{2+} levels [68] and, consequently, the amount of Ca^{2+} transfer from the ER to mitochondria [68]. The data obtained suggest that the reduction of the Ca^{2+} content in ER and the resulting down regulation of Ca^{2+} fluxes between ER and mitochondria is the major component of the viral antiapoptotic programme.

3. MAMs as a hot spot for transducing Ca^{2+} cell death signals from the ER to mitochondria

Other proteins localized at the MAMs (Fig. 1) are emerging as key players in the control of Ca^{2+} homeostasis and apoptosis [69–73]. VDAC mediates trafficking of small molecules and ions, including Ca^{2+}, across the OMM. Multicellular organisms and mammals have three VDAC isoforms (VDAC1, VDAC2, and VDAC3), owing isoform-specific functions [74].

VDACs are localized in a crucial position and form the main interface between cytosol and mitochondria, playing an important role in many cellular processes, ranging from metabolism regulation to cell death [75]. Moreover, VDACs are present in contact sites between mitochondria and ER [76,77], forming an important interface for Ca^{2+} signalling delivery between the two organelles. VDAC1 allows rapid Ca^{2+} diffusion across OMM, leading to increased Ca^{2+} concentration in the mitochondria and higher susceptibility to apoptosis [78]. It was also demonstrated that the mitochondrial chaperone grp75 (glucose-regulated protein 75) induces a physical coupling between the IP$_3$R and VDAC1 [77]: in this way, it forms an ER–mitochondria Ca^{2+} tunnel that allows for a better transfer of the Ca^{2+} ions from the ER across the OMM.

Recently, De Stefani et al. revealed an interesting scenario in which each VDAC isoform has similar Ca^{2+} channelling properties, despite their different contribution to cell death: pro-apoptotic for VDAC1 [78,79], anti-apoptotic for VDAC2 [80] and no significant influence on apoptosis for VDAC3 [81]. To conciliate this observation with the classic paradigm linking mitochondrial Ca^{2+} to apoptosis [82], it was demonstrated that VDAC1, by selectively interacting with the IP$_3$Rs, is preferentially involved in the transmission of the low-amplitude Ca^{2+}-dependent apoptotic signals to mitochondria [81].

The disruption of the physical platform for the interplay between the ER and mitochondria sites has profound consequences for cellular function, such as imbalances of intracellular Ca^{2+} signalling and disrupted apoptosis progression.

The interactions between the two organelles are modulated by different proteins, such as the mitochondria-shaping proteins MFN-1/-2 (mitofusin-1/-2). In particular, the absence of MFN-2 not only changes the morphology of the ER but also decreases by 40% the interactions between ER and mitochondria, thus affecting the transfer of Ca^{2+} signals to mitochondria.

In fact, up-regulation of MFN-2 is necessary and sufficient for the induction of apoptosis in vascular smooth muscle cell [83]; in addition, it was recently observed that the MFN-2 interacting protein Trichoplein/mitostatin is downregulated in several human tumours [84] and deeply regulates mitochondrial morphology and tethering with ER, with consequent control in apoptosis levels [85]. Finally, it has also been demonstrated that the presence of MFN-2 is important for the execution of the mechanism whereby mitochondria depolarization leads to the inhibition of the STIM1 puncta-structure formation (that correspond to ER–plasma membrane junctions), with subsequent blocking in Ca^{2+} release-activated Ca^{2+} (CRAC) channels activity, a crucial step for the regulation of gene expression and controlling of cell survival [86].

Interestingly, also ER chaperones were found to be compartmentalized at the MAMs. The Sigma-1 receptor (Sig-1R) has been identified as selectively residing at the MAMs, forms a Ca^{2+}-sensitive chaperone complex with BiP/GRP78 and associates with isoform 3 of IP$_3$R [87]. The first effect of this complex is a prolonged Ca^{2+} signalling from ER to mitochondria, with consequent modulation of the apoptotic programme. As demonstration of this, small molecules able to block the Sig-1R lead to the activation of caspase-dependent apoptosis [88]. Previously, another multifunctional protein, PACS-2 (phosphofurin acidic cluster sorting protein 2) was identified as necessary for the intimate association of mitochondria with the ER. The absence of PACS-2 causes mitochondria fragmentation, disruption of the ER–mitochondria axis and affects Ca^{2+} homeostasis and apoptosis [89]. In particular, the depletion of PACS-2 determines the mislocalization of IP$_3$Rs and consequent alteration of the correct Ca^{2+} transfer from ER to mitochondria [90]. Moreover, PACS-2 also affects ER Ca^{2+} homeostasis, regulating the distribution of the Ca^{2+}-binding chaperones calnexin, through a mechanism that contemplates the direct interaction between PACS-2 and calnexin [91]. In addition, when the apoptotic programme is initiated, PACS-2 binds BID, leading to its translocation into mitochondria, where it is cleaved to tBID. Subsequently, cytochrome c is release and caspase-3 activated [89].

4. Deadly liaisons: ROS and mitochondrial Ca^{2+} homeostasis in the control of cell death

As much as Ca^{2+} appears to be involved, there is no doubt other "pro-apoptotic" conditions must be met for apoptosis to occur. Indeed, mitochondria can handle large Ca^{2+} loads in normal physiological conditions (e.g., in cardiac myocytes, significant amounts of Ca^{2+} are accumulated at every heartbeat), with no deleterious effects [92].

As to these additional "apoptotic signals", the most important is considered to be oxidative stress [93–95].

A two hits model can thus be proposed, similar to that suggested by Hajnoczky and coworkers where the physiological mitochondrial uptake of Ca^{2+} caused by IP3-producing agonists, is turned into an apoptotic signal in the presence of ceramide, possibly *via* opening of the PTP [96].

The apoptotic stimulus can directly or indirectly damage the mitochondria, but this effect is marginal or totally ineffective, if the mitochondrial are not contemporarily exposed to an elevated $[Ca^{2+}]$. In other words, mitochondria appear to act as "coincidence detectors", where only the contemporary application of both signals can be transduced into an effective triggering signal of apoptosis.

In this section, we will summarize recent discoveries about the effects of four proteins, not directly related to each other, with a common outline in terms of ROS perturbation, mitochondrial Ca^{2+} deregulation and sensitivity to apoptosis.

4.1. FHIT

The FHIT gene spans the FRA3B fragile site at chromosome 3p14.2 and is inactivated (through gene deletions, abnormal

transcripts and promoter hypermethylation) in >50% of human cancers [97]. Its product, Fhit, is a typical dinucleoside 5′,5′′′-P1,P3-triphosphate (Ap3A) hydrolase that acts as a tumour suppressor *in vivo* and *in vitro*, enhancing susceptibility to apoptosis [98]. Fhit$^{-/-}$ mice are highly susceptible to carcinogens, and reintroduction of FHIT by gene transfer reduces the tumour burden by triggering apoptosis [99]. Studies have confirmed that FHIT overexpression leads to the activation of the extrinsic pathway [100], as well as the intrinsic pathway by alteration of MMP and enhanced efflux of cytochrome *c* from the organelle [101]. Fhit has been located in the mitochondria (Fig. 1), although it was first identified as a cytosolic protein. Trapasso et al. [102] have shown that Fhit can be directed to mitochondria upon interaction with Hsp60 and Hsp10. The mitochondrial localization determines the binding with and stabilization of ferredoxin reductase (Fdxr), a flavoprotein transactivated by p53. The FHIT/Fdxr complex generates ROS and increases Ca^{2+} uptake into mitochondria [103]. Fhit sensitizes the low-affinity Ca^{2+} transporters of mitochondria, potentiating the effect of apoptotic agents. This effect has been attributed to the fraction of Fhit sorted to mitochondria, as shown by a chimeric mitochondrial Fhit that retains the Ca^{2+} signalling properties of Fhit (and the pro-apoptotic activity of the native protein) while completely losing the effects on cell cycle [103].

4.2. p66Shc

Shc-like molecules function as 'adaptor proteins', which receive signals from growth factor receptors, which are phosphorylated on tyrosines. Three Shc isoforms have been identified in mammals, p46Shc, p52Shc and p66Shc [104]. Similarly to p46Shc and p52Shc, p66Shc binds mitogenic transducer protein but does not stimulate cell proliferation [105], while it participates in intracellular pathways that control oxidative stress and apoptosis.

Cells from which p66shc is depleted are resistant to apoptotic death induced by oxidative stress and p66Shc$^{-/-}$ mice are resistant to paraquat, living about 30% longer than controls [106]. Moreover, it has been observed that after challenge with hydrogen peroxide, p66Shc translocates from the cytosol to mitochondria. Serine 36 is a critical regulatory site for the apoptotic activity of p66Shc and PKC isoform β is required for phosphorylation [107]. In fact, it has been shown that PKCβ, activated by oxidative challenges, causes the phosphorylation and protein import of p66Shc into mitochondria, where it acts as oxidoreductase. This initiates a feedforward cycle of ROS production that leads to the opening of mPTP, release of pro-apoptotic cofactors and triggering of cell death [107]. Phosphorylated p66Shc is then recognized by the prolyl isomerase Pin1 that catalyzes its *cis–trans* isomerization, allowing the import into mitochondria (Fig. 1) after dephosphorylation by type 2 protein serine/threonine phosphatase (PP2A). Within mitochondria, p66Shc then binds cytochrome *c*, shuttling electrons from cytochrome *c* to molecular oxygen [108], generating H$_2$O$_2$. This event in turn perturbs mitochondrial structure and function (Ca^{2+} uptake and ATP production).

The key role of p66Shc as molecular sentinel that controls cellular stress and mitochondrial physiology has been confirmed recently in transgenic mice modelling amyotrophic lateral sclerosis (ALS), where the neurotoxicity mediated by mutSOD1 (G93A-SOD1) is exerted by mitochondrial redox activity of p66Shc [109]. In cells expressing mutSOD1, accumulation of phosphorylated p66Shc (on serine 36) has been demonstrated, associated with decreases in mitochondrial Ca^{2+} uptake capacity, ATP production, and mitochondrial integrity. This it is further supported by the observation that overexpression of dominant negative, functionally inactive p66Shc proteins protects cells against mutSOD1 toxicity. Most importantly, the deletion of p66Shc ameliorates mitochondrial

function, delays onset, improves motor performance and prolongs survival in ALS transgenic mice model [109].

4.3. KRIT1

Cerebral Cavernous Malformations (CCM) is one of the major cerebrovascular diseases [110]. It is characterized by abnormally enlarged and often leaky capillary cavities in the brain, leading to an increased risk of hemorrhagic stroke, seizures, and focal neurological deficits [111,112]. KRIT1 is one of the three genes responsible for causing CCM [113], and it was recently shown that KRIT1 plays an important role in molecular mechanisms involved in the maintenance of ROS homeostasis to prevent oxidative cellular damage [114]. Ablation of KRIT1 leads to a significant increase of ROS levels due to the reduction of the expression of the antioxidant protein SOD2, as well as of the transcription factor FoxO1. The regulation of FoxO by KRIT1 has been also demonstrated in *C. elegans*, where *kri-1*, the worm orthologue of *KRIT1/CCM1*, is required for germline removal-dependent lifespan extension through regulation of DAF-16, the worm orthologue of mammalian FoxO genes [115]. Indeed, KRIT1 knock-out cells present enhanced levels of mitochondrial superoxide and a consequent decline of mitochondrial energy metabolism, characterized by a strong reduction in MMP, mitochondrial Ca^{2+} levels and ATP production. In this case, a downregulation of mitochondrial Ca^{2+} levels cannot be linked to apoptosis resistance, because when mitochondria are sensitized by oxidative stress, small arises in mitochondrial [Ca^{2+}] could promote opening of the mPTP [116,117]. Indeed, KRIT1 loss is associated with an increased susceptibility to caspase 3 activation during oxidative challenge.

4.4. STAT3

Signal Transducer and Activator of Transcription 3 (STAT3) is a latent cytosolic transcription factor activated by cytokines, growth factors and oncogenes [118] and, recently, emerged as a regulator of cell cycle and apoptosis [119]. In addition to its canonical nuclear function, which requires tyrosine phosphorylation, DNA binding and transcriptional activity, STAT3 has been reported to exert non-nuclear functions. It was shown to localize to mitochondria (Fig. 1), where it regulates cellular respiration [120]. Gough and colleagues demonstrated that exclusive mitochondrial targeting of STAT3 appears to contribute to RAS-dependent cellular transformation, by augmenting electron transport chain activity, particularly at the level of complexes II and V [121].

On the contrary, in cells expressing physiological levels of the nuclear constitutively active STAT3C mutant, the mitochondrial Ca^{2+} uptake, mitochondrial ATP production and basal respiratory chain activity are reduced [122].

Furthermore, these cells show reduced MMP, diminished ROS production and high resistance to apoptosis. These data highlighted the role of STAT3C to enhance cell survival and protection from apoptosis *via* specific regulation of mitochondrial functions.

Although the roles played by nuclear or mitochondrial STAT3 may seem contradictory, it must be borne in mind that specific phosphorylation on tyrosine or serine occurs (mitochondrially localized STAT3 is not phosphorylated on tyrosine 705, the hallmark of transcriptional activation, but on Serine 727) upon distinct stimuli and under distinct physiological or pathological conditions [122], leading to two functionally distinct molecular effects.

5. Conclusions

Regarding the site of action of many apoptotic Ca^{2+} signals, mitochondria emerge as a critical location (Fig. 2). Indeed, treatment with different Ca^{2+}-dependent apoptotic stimuli, such as ceramide,
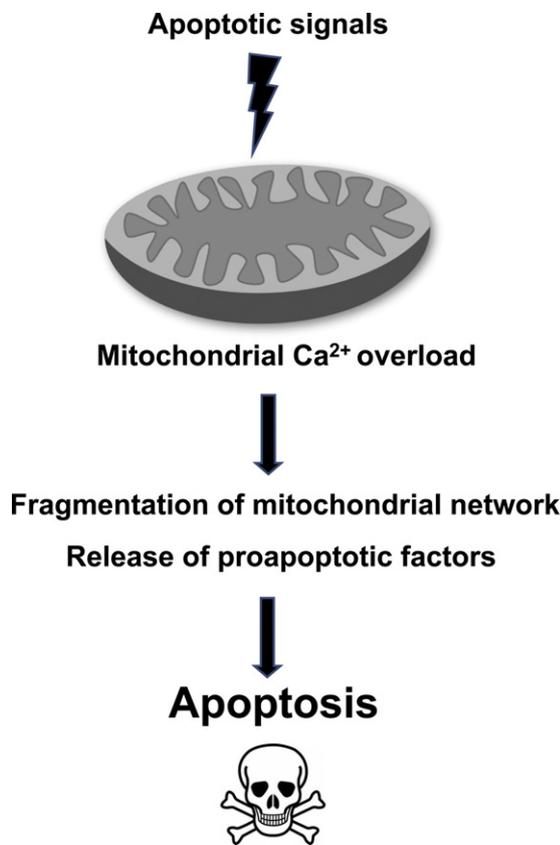
Apoptotic signals

Mitochondrial Ca^{2+} overload

Fragmentation of mitochondrial network

Release of proapoptotic factors

Apoptosis

Fig. 2. Schematic representation of mitochondrial Ca^{2+}-dependent apoptosis.

arachidonic acid, oxidative stress, to name a few, causes the release of Ca^{2+} from the ER; this in turn induces dramatic changes in mitochondrial morphology, with a consequent release of mitochondria proteins involved in the apoptotic process, such as cytocrome *c*, AIF, Smac/Diablo (Fig. 2). If Ca^{2+} changes are prevented, mitochondrial morphology is preserved and the cells are protected from apoptosis. The full understanding of how mitochondria can sense, handle and decode various Ca^{2+} signals still represents an exciting challenge in biomedical sciences. Indeed, as summarize in this review, many proteins, some of theme completely unexpected, participate in mitochondrial Ca^{2+} homeostasis.

However, it must be stressed that mitochondrial Ca^{2+} uptake is essential in rapidly adapting aerobic metabolism to the increased needs of a stimulated cell [123]. Moreover, in an elegant study, Foskett and coworkers, demonstrated as constitutive IP3R-dependent Ca^{2+} release from ER to mitochondria is an essential cellular process that is required for efficient mitochondrial respiration and maintenance of normal cell bioenergetics [124]. This means that while mitochondrial Ca^{2+} overload leads to apoptosis a controlled, and transient mitochondrial Ca^{2+} load is needed for survival.

All these observations lead to a really complex but fascinating picture, where mitochondrial Ca^{2+} signals represent crucial triggers of apoptosis.

Conflict of interest statement

This is to certify that there is no conflict of interest with this manuscript submission.

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