Mitochondria and apoptosis: emerging concepts

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Abstract

As mitochondria are the powerhouses of the cell, their damage during the cell suicide process of apoptosis is essentially responsible for cellular demise in most cells. A key family of proteins, the B-cell lymphoma-2 (BCL-2) family, determines the integrity of mitochondria in the face of apoptotic insult. A comprehensive understanding of the molecular details of how apoptosis is initiated and how it culminates is essential if apoptosis is to fulfil its undoubted potential as a therapeutic target to treat diseases ranging from cancer to neurodegenerative conditions. Recent advances have provided significant insight into the control of this fundamental process while prompting a re-evaluation of what was considered dogma in the field. Emerging evidence also points to a potential overarching control network that governs not only apoptosis but other fundamental mitochondrial processes, including mitochondrial fission/fusion and quality control.

Introduction

The discovery that the proto-oncogene, *Bcl-2*, which is the founding member of the BCL-2 family of proteins, regulates cell survival or 'apoptosis' initiated an entire field of research [1]. The BCL-2 family now numbers at least 15 members in mammals, each of which has distinct anti- and pro-apoptotic roles [2]. It is their myriad of interactions that allows the fine tuning of the apoptotic response upon reception of a death insult, such as growth factor withdrawal or DNA damage. The net result of the complex interplay between these BCL-2 proteins is the regulation of the integrity of the mitochondrial outer membrane. Breach of this barrier by two members of the family, Bcl-2-associated X protein (BAX) or Bcl-2-associated killer (BAK), is normally sufficient for cell death. However, the ensuing release of apoptogenic factors, including cytochrome *c*, ensures cell death by inducing activation of aspartate-specific proteases, the caspases, resulting in efficient and non-inflammatory packaging of the dying cell for phagocytosis. BCL-2-regulated apoptosis was subsequently dubbed 'intrinsic/mitochondrial apoptosis' to distinguish it from an alternative, though not independent, apoptosis pathway, 'extrinsic apoptosis'. The latter is initiated by the ligation of cell surface death receptors such as tumour necrosis factor receptor (TNFR) and FAS receptor (FasR/Apo-1/CD95).

That the pro-apoptotic effector proteins BAX and BAK become activated during apoptosis and are necessary for apoptotic cell death has led to the recognition of these proteins as valuable therapeutic targets either to activate their deadly function to induce cell death for cancer therapy or to block their activity to impair cell death (for example, in the treatment of acute degenerative disorders) [3]. Consequently, understanding the details of how BAX and BAK are regulated and how, when activated, they sentence a cell to death has become the subject of intense interest. In this review, we highlight a number of the recent and exciting advances in our understanding of intrinsic apoptosis, focusing on the form and function of BAX and BAK at the mitochondrial outer membrane. We also touch on intriguing new evidence that the apoptosis machinery may influence (and be influenced by) other fundamental pathways, including those involved in mitochondrial quality control.

Rerouting the killers: regulating BAX and BAK mitochondrial targeting

BAX and BAK are the key effectors of apoptosis; without them, cells are resistant to the majority of apoptotic stimuli [4,5]. Until recently, an accepted and oft-cited paradigm in the apoptosis field is that, in healthy cells, BAX is a cytosolic protein that actively translocates to the mitochondrial outer membrane during apoptosis to participate in membrane damage but that BAK constitutively resides at the mitochondrial outer membrane [6–8]. This paradigm has been recently challenged by elegant studies that have examined BAX and BAK localization at the single cell level [9,10]. They observed that BAX actually constitutively targets mitochondria but is actively trafficked to the cytosol, a process termed 'retrotranslocation'. Whether the pro-survival BCL-2 proteins or other players outside of the family are responsible for BAX trafficking is contentious. However, it is proposed that, in response to apoptotic stress, the trafficking mechanism shuts down, allowing BAX to adopt its default localization and accumulate at the mitochondrial outer membrane, a hallmark of most apoptotic cells. More recently, BAK has also been proposed to be subject to similar trafficking [11], albeit at a much slower rate than BAX, hence their distinct subcellular localizations in healthy cells.
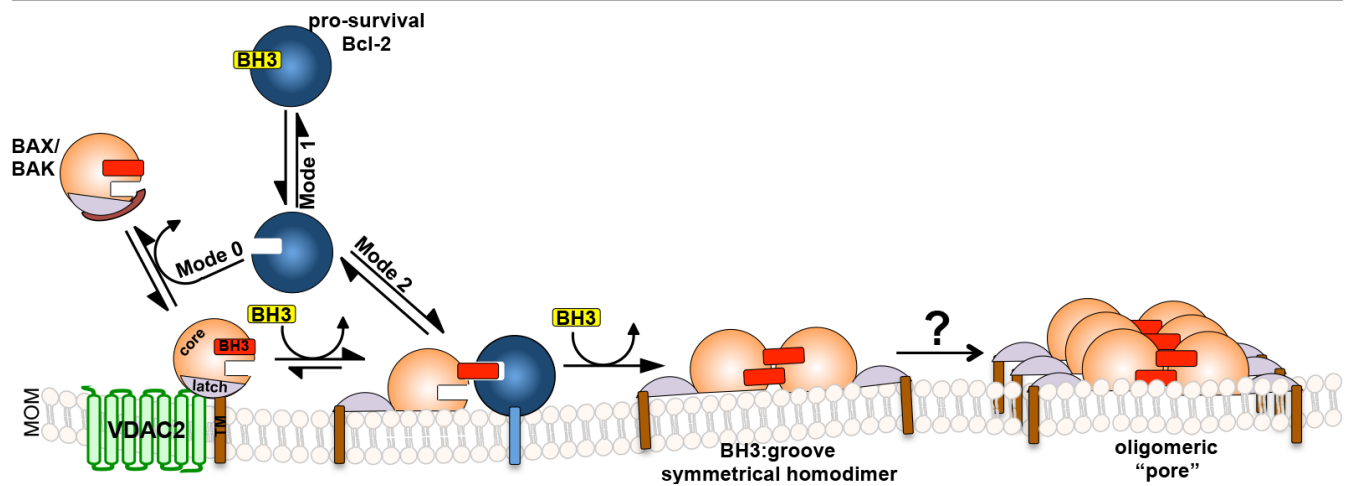
Our recent studies have added a layer of complexity to this aspect of BAK and BAX biology [12]. BAK has been shown to interact with voltage-dependent anion channel 2 (VDAC2) at the mitochondrial outer membrane, although whether this interaction positively or negatively influences BAK apoptotic function is unclear [13–16]. We observed that BAX, like BAK, interacts with VDAC2 and that these interactions are likely an important determinant of the 'lag time' of BAX and BAK at the mitochondrial outer membrane prior to trafficking to the cytosol; consequently, BAX and BAK are significantly shifted to the cytosol in cells devoid of VDAC2 [12]. The BAX and BAK C-terminal transmembrane domains appear to be important for their interaction with VDAC2 [12,16], although other regions may also be involved. Intriguingly, this redistribution of BAX and BAK in VDAC2-deficient cells does not seem to significantly impair their apoptotic function, as these cells still died efficiently. However, we found that, in the absence of interaction with either VDAC2 and BAK (in cells engineered to be deficient in both VDAC2 and BAK), BAX mitochondrial localization and hence apoptotic function were significantly impaired [12]. This indicated that BAX takes a bifurcated path to mitochondria, either via association with VDAC2 in healthy cells or via BAK during apoptosis. We propose that, following apoptotic stress, BAK that is resident at mitochondria becomes activated because of interactions with BH3-only proteins (see below), and that this activated form of BAK recruits cytosolic BAX.

Although the molecular mechanism for this recruitment is unclear, it possibly involves the exposed BH3 domain on activated mitochondrial BAK that triggers conformation change in BAX analogous to auto-activation.

Other mitochondrial proteins have been implicated in BCL-2 protein localization. Potential roles for the translocase of the outer membrane (TOM) complex in BAX and BAK mitochondrial targeting have been postulated [15,17], and mitochondrial association of the active truncated form of the BH3-only protein BID (tBID) is facilitated by interaction with the mitochondrial outer membrane protein mitochondrial carrier homolog 2 (MTCH2) [18,19]. However, a comprehensive understanding of the proteins involved in the targeting and stability of BAX and BAK at mitochondria is lacking. The population of BAK and BAX that constitutively reside at the mitochondrial surface is an important regulatory checkpoint, as it determines the sensitivity of the cell to apoptotic stimuli [10]. Thus, understanding the precise mechanism involved in regulating BAX and BAK subcellular localization may reveal new ways to sensitize or desensitize cells to chemotherapeutic agents.

Restraining the killers: regulating BAX and BAK apoptotic function

The BCL-2 proteins govern the point of no return in apoptosis: the permeabilization of mitochondrial outer membrane. Who triumphs, the pro-survivals (BCL-2, BCL-X_L, BCL-w, MCL-1, and BFL-1/A1) or the pro-apoptotics (BAX and BAK), is determined by the interplay between them and a third member of the deadly triad: the BH3-only proteins (BID, BIM, BAD, BIK, BMF, Noxa, PUMA, and HRK) (Figure 1). Over the past decade, the mechanism by which apoptosis is controlled by the BCL-2 family has been hotly contested; some argued that the predominant role of the pro-survival proteins was to sequester the effectors BAX and BAK [20], and others argued that their predominant role was to sequester the BH3-only proteins [21,22]. Both models had their limitations and did not account for all available data; hence, attempts have been made to consolidate these competing models [23,24]. Llambi and colleagues [25] provided experimental evidence supporting such a unified model where pro-survival proteins prevent apoptosis by sequestering both BH3-only proteins and activated forms of BAX and BAK and coined these inhibitory modes 'mode 1' and 'mode 2', respectively (Figure 1). Another breakthrough has been the realization that a membrane environment, and specifically that of the mitochondrial outer membrane, is critical for interactions between the BCL-2 family proteins [25]. Together, these studies have led to the appreciation that these interactions are not a series of static, dead-end

Figure 1. BAX and BAK mitochondrial trafficking in healthy cells and their proposed activation events during apoptosis

By default, BAX and BAK localize to the mitochondrial outer membrane (MOM), where they interact with VDAC2 involving their transmembrane (TM) domains [12,16]. Mitochondrial BAX and BAK are constantly retrotranslocated to the cytosol at differing rates, potentially via interaction with pro-survival BCL-2 proteins (mode 0 [42]) [9–11]. Pro-survival BCL-2 proteins inhibit cell death by sequestering BH3-only proteins (BH3, yellow), thereby preventing them from directly activating BAX and BAK (mode 1). If BAX and BAK become activated, they can be sequestered by unoccupied pro-survival BCL-2 proteins (mode 2) [26]. A complex network of competitive and reversible interactions determines whether BAX and BAK become activated to expose their BH3 domain and dissociate their core (α1-5) and latch (α6-8) domains [31,46]. These conformation changes facilitate symmetrical homodimerization [45,49,53,55]. How these homodimers then multimerize to form the putative apoptotic pore in the MOM remains elusive but likely involves a proteo-lipidic pore comprising homo-dimers intercalated with MOM lipid. BAK, Bcl-2-associated killer; BAX, Bcl-2-associated X protein; BCL-2, B-cell lymphoma-2; VDAC2, voltage-dependent anion channel 2.

events, but are constituents of a dynamic system of competing and reversible interactions that are influenced by the relative affinities and cellular concentration of each player [23,24].

Whether such inhibitory interactions with anti-apoptotic BCL-2 family members contribute to the retrotranslocation of BAX (and potentially BAK) is unclear. As BAX trafficking to the cytosol is proposed to occur in the absence of an apoptotic stimulus, it is possible that the trafficking interactions with pro-survival proteins during retrotranslocation and the inhibitory interactions involved during mode 2 involve distinct conformers of BAX, and that the former involves inactive BAX and the latter involves a membrane-integrated activated conformer of BAX. However, both interactions are proposed to involve the exposed BH3 domain of BAX [9,26]. Understanding the BAX conformations that distinguish the cytosolic form from the potentially numerous conformers at the mitochondria is paramount. Nevertheless, these studies paint a complex and dynamic picture of BAX and BAK localization and conformation change.

Unleashing the killers: activating BAX and BAK
BAX and BAK are predominantly in a dormant conformation in healthy cells, and so the hunt has been on in

earnest to understand how BAX and BAK are activated to induce their pore-forming capabilities. There is now considerable evidence that at least some of the BH3-only proteins directly and transiently interact with BAX and BAK to induce their activation [27–34], an interaction that was once considered controversial because of the difficulty in detecting the 'hit and run' interaction. Although which of the BH3-only proteins share this activating capacity is not clear, recent elegant structural studies have provided intricate molecular detail of these activating interactions while hinting at their potential consequences [31,32]. These studies indicate that a critical site of interaction occurs between the BH3 domain of BH3-only proteins and a conserved hydrophobic groove on the surface of BAX and BAK, a groove that is shared with their pro-survival cousins. The details of this interaction are subtly but critically different in the effector BCL-2 proteins compared with the pro-survival proteins. The interaction interface shown between BAX and the BID BH3 peptide is extended to an additional hydrophobic interaction between the BH3 peptide and the groove (termed h0 as it precedes the important h1-4 interactions on the BH3 domain) [31]. Perhaps more importantly, the crystal structure of BAX with a BID BH3 peptide indicated that the interaction induced a 'cavity' in BAX that is not observed in any of the structures involving

pro-survival proteins [31]. Such a potentially destabilizing cavity may provide the impetus for BAX and BAK activating conformation change during apoptosis. However, it is important to consider that the pro-survival proteins have been argued to undergo a conformation change upon interaction with BH3-only proteins [35]. Consequently, what distinguishes a pro-apoptotic protein from a pro-survival one is currently unclear but is likely related to the former's ability to self-associate [36].

As well as BH3 peptides binding to the BAX hydrophobic groove, Walensky and colleagues [37] have reported an interaction of modified BH3 peptides with a 'rear pocket' on BAX comprising predominantly its α1 and α6 helices independent of the canonical hydrophobic groove. Interaction at this rear site is proposed to induce the release of the C-terminal transmembrane helix that is sequestered in the hydrophobic groove, and so drive mitochondrial translocation of BAX [37–39]. As BAK is constitutively anchored in the mitochondrial outer membrane via its C-terminal transmembrane domain, it was thought to forgo this conformation change, thus explaining the lack of detectable interaction at the BAK rear pocket [40]. However, cell-based studies have argued that the BAX C-terminus is actually constitutively exposed and may participate in transient interactions with membranes [41], thereby questioning the need for BH3-only proteins to initiate such a conformation change and mitochondrial translocation of BAX. Furthermore, a study from the Andrews lab shows that interactions between BH3-only proteins and BAX require a membrane environment rather than occur in the cytosol [25]. This supports the notion that in healthy cells BAX is in dynamic equilibrium between cytosol and membrane compartments, and that only upon reception of an apoptotic stimulus do BH3-only proteins induce conformation change of the membrane-associated conformer of BAX, thus stabilizing it at the mitochondrial outer membrane. The recent study indicating that BAK traffics to and from the mitochondrial outer membrane like BAX also suggests that the premise for the lack of the putative 'rear pocket' in BAK may not hold [11]. So whether these interactions (or lack thereof) persist for full-length proteins within the context of a membrane environment remains to be tested, but it is clear that refining our understanding of these interactions will reveal avenues to intervene in or to expedite BAK and BAK activation.

Metamorphosis of the killers: new insights on BAX and BAK conformation change and oligomerization

During activation, BAX and BAK are known to undergo a series of significant structural alterations that allow them to assemble to the putative apoptotic pore that permeabilizes the mitochondrial outer membrane [42]. Although we have acquired snapshots of the structural events in BAK and BAX activation, the chronological order of these events is unknown. These conformation changes include dissociation of the N-terminus, BH3 domain, and C-terminus [43–45]. Recent structural studies [31,46] highlight an additional and important conformation change involving dissociation of α helices 1-5, dubbed the 'core' or 'dimerization domain' [47], from the α6-9 dubbed the 'latch' or 'piercing domain' (see below) [48] of BAX and BAK. This dissociation serves to potentially free the α1-5 dimerization domain to allow symmetrical BAX/BAK homodimer formation [45,49] but also exposes a hydrophobic surface of the homodimer comprising α4, 5, and 6 to allow enhanced association with the mitochondrial outer membrane and thus potential membrane disruption. Previous studies have suggested that the α5/6 of BAX inserts as a transmembrane hairpin [50], analogous to the pore-forming domains of Diphtheria toxin and bacterial colicin A [51]. This long-held premise is questioned by the dissociation of α5 and 6, and also by recent evidence using cysteine labelling that supports an in-plane rather than transmembrane association of α5 and α6 in the activated oligomerized forms of BAX and BAK [48,52,53].

Using double electron-electron resonance (DEER) spectroscopy to triangulate intra- and inter-molecular distances within a BAX oligomer, Bordignon and colleagues [48] recently proposed that the α5/6-dissociated symmetrical homodimer of BAX assembles as a 'clamp' with a flexible α6-9 'piercing domain' able to pinch and then permeabilize the mitochondrial outer membrane. This results in the two monomers of the homodimer residing on opposite sides of the mitochondrial outer membrane and with consequently anti-parallel C-terminal transmembrane domains. The energetic requirements for such a transformation of a homodimer in the membrane would be substantial but could potentially be overcome by the concerted effect of higher-order oligomerization. Although definitive evidence of this conformation at the membrane is lacking, it is an intriguing new model of how BAX (and presumably also BAK) may damage the mitochondrial membrane.

Although we are starting to gain insight into the considerable conformation change of BAX and BAK during their activation, several critical questions remain and are the topic of some debate [42]. How do BAX and BAK form the higher-order oligomers that are thought to represent the apoptotic pore? How large is the requisite oligomer to mediate cytochrome *c* release? Do BAX and BAK form ordered proteinaceous pores or more heterogeneous pores

with an integral role for specific mitochondrial lipids? Do other proteins perform required or ancillary roles in pore formation? Is BAX and BAK oligomerization even necessary for apoptotic function? This last question stems from recent mathematical modelling suggesting that BAX oligomerization is not necessary for membrane disruption [54]. However, cumulating cell-based and structural studies support the notion that BAX and BAK form stable and symmetric homodimers by inserting their BH3 domains into their partners' hydrophobic groove [31,45,46,49,53,55,56]. How these homodimers then multimerize to form the pore is unknown, although the α3/5, α6, and C-terminal transmembrane domains have been implicated as important intermediaries in the formation of the oligomer [41,49,57,58]. That the dimers are stable upon removal from a membrane whereas the oligomers are less so suggests that the pore may be intercalated by lipid headgroups [59]. This is supported by recent data in model membranes, indicating that the pore is dynamic and tunable, features that are more consistent with the apoptotic pore being proteo-lipidic rather than purely proteinaceous [60,61]. Characterizing the apoptotic pore is considered by many to be the ultimate goal of the apoptosis field, as it will not only answer a longstanding and frustratingly intractable question but also reveal how the apoptotic pore can be targeted therapeutically to inhibit apoptosis.

Recent evidence that sphingolipid metabolism co-ordinates BAX and BAK activation suggests that lipids may have more than just a passive role in apoptosis. Although the mechanism remains unclear, sphingolipid metabolites hexadecenal and sphingosine-1-PO$_4$ specifically derived from neutral sphingomyelinase activity were reported to cooperate with BAX and BAK, respectively, to mediate mitochondrial damage, and consequently inhibitors of their synthesis impaired apoptosis [62]. Mice essentially deficient in neutral sphingomyelinase activity ($Smpd2^{-/-}Smpd3^{-/-}$) do have developmental defects [63] but do not exhibit the perinatal lethality observed in $Bax^{-/-}Bak^{-/-}$ mice [5]. This suggests that alternative sources of the sphingolipid metabolites play a role or that lipid metabolism may co-operate with BAX/BAK-mediated apoptosis but is not essential for it. So evidence is mounting in support of a role for mitochondrial lipids not only as important mediators of BCL-2 protein interactions and activation but also as a component of the apoptotic pore.

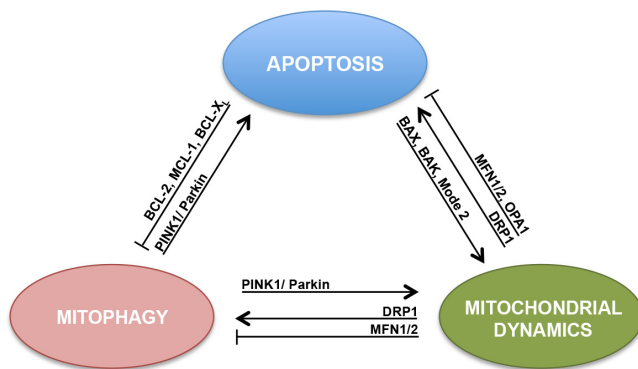## Mitochondrial quality control and apoptosis: a missing link?

Mitochondria are not discrete organelles but a network that undergoes constant fission and fusion to maintain 'fitness' and for their appropriate distribution to the

daughter cells upon cell division. The correct balance of fission and fusion is critical for mitochondrial homeostasis but has also been linked with apoptosis with mitochondrial fragmentation, an early phenomenon during apoptotic cell death. Cells deficient in both BAX and BAK exhibit hyperfragmented mitochondria [64], and sequestration of activated BAX and BAK by pro-survival proteins is proposed to promote mitochondrial fission [26]. BAX has also been shown to associate or co-localise with key fission/fusion mediators, including dynamin-related protein 1 (DRP1) and the mitofusins (MFNs) [65,66]. Recently, mitochondrial fusion has also been shown to control the distribution of apoptosis mediators such as BAK and BID on the mitochondrial outer membrane [67]. Also, MFN1-driven mitochondrial fusion is proposed to establish a membrane environment associated with mitochondrial shape that permits efficient BAX localization and hence apoptotic activity [68]. Thus, the competing forces of mitochondrial fission and fusion culminate in a dynamic network of mitochondria that are heterogeneous in terms of size, shape, and membrane curvature. This heterogeneity may impact apoptosis on a number of levels, from affecting the kinetics of mitochondrial targeting of BAX or BAK [68], and their consequent oligomerization and membrane damage [31,48,61], to determining the efficiency of mitochondrial outer membrane permeabilization and the kinetics of cytochrome *c* release [69].

That mutation or deletion of proteins involved in mitochondrial fission and fusion, including DRP1 [70], MFN2 [71], and optic atrophy 1 (OPA1) [69], influences cell death supports that these observations are more than just epiphenomenon. However, it should be noted that, although such alterations may influence the kinetics of cell death, they are not a determining factor in whether a cell lives or dies. Thus, whether the apoptosis machinery moonlights to regulate mitochondrial dynamics or whether mitochondrial fission/fusion is necessary for efficient apoptosis *in vivo* remains unclear.

When mitochondrial quality control goes awry, damaged or excessive mitochondria are removed by mitochondria-specific autophagy or mitophagy. The serine/threonine kinase PTEN-induced kinase 1 (PINK1) and its substrate, the E3 ligase Parkin, have been implicated as important mediators of mitophagy [72], although they are not the sole mediators (reviewed in [73]). As well as promoting mitochondrial clearance, Parkin has been shown to effect apoptosis both positively and negatively depending on cellular context, although the underlying mechanism has been lacking. However, in two recent reports, the Martin laboratory has shown that the mechanisms governing apoptosis directly influence mitophagy and vice versa [74,75]. Pro-survival BCL-2 proteins were observed to

Figure 2. Mechanisms governing apoptosis, mitochondrial dynamics, and mitophagy intersect to co-ordinate mitochondrial homeostasis

In apoptosis:mitochondrial dynamics, BAX interacts with components of the mitochondrial fission/fusion machinery. BAX/BAK double-deficient fibroblasts have hyperfragmented mitochondria, and inhibition of activated BAX/BAK by pro-survival proteins (mode 2) promotes mitochondrial fragmentation. Deficiencies in mediators of mitochondrial fission/fusion and cristae remodelling influence the kinetics of apoptosis. In mitochondrial: mitophagy dynamics, as a fragmented mitochondrial network is necessary (though not sufficient) for efficient mitophagy, DRP1 and MFN1/2 indirectly positively and negatively regulate mitophagy. PINK1/Parkin targets MFN1 and 2 for degradation to promote mitochondrial fission. In mitophagy: apoptosis dynamics, Parkin promotes BAX mitochondrial translocation and MCL-1 degradation to sensitize cells to apoptotic stimuli while pro-survival BCL-2 homologues dampen. PINK1/Parkin-mediated mitophagy. BAK, Bcl-2-associated killer; BAX, Bcl-2-associated X protein; BCL-2, B-cell lymphoma-2; DRP1, dynamin-related protein 1; Mcl-1, myeloid cell leukaemia 1; MFN, mitofusin; PINK1, phosphatase and tensin homolog-induced kinase-1.

regulate PINK1/Parkin-mediated mitophagy [74]. Parkin was also found to target MCL-1 for degradation and hence sensitizes cells to death induced specifically by mitochondrial uncoupling [75]. In contrast, Parkin has been shown to ubiquitinate BAX to impair its translocation to mitochondria during apoptosis, potentially dampening a cell's response to death stimuli [76,77]. It is known that, for efficient mitophagy, mitochondria are required to undergo fission. And as apoptosis is linked to mitochondrial fission and fusion, it is possible that these collective insights are indicative of an overarching control of mitochondrial homeostasis (Figure 2).

The apoptotic pathway is receiving significant attention as a therapeutic target to treat diseases, including cancer. Indeed, small-molecule pro-survival protein inhibitors are already gaining traction in the clinic in the treatment of certain cancers such as chronic lymphocytic leukaemia [78]. Each step in BAX/BAK activation and function is a potential target not only to augment apoptosis as a cancer

therapy but also to directly inhibit apoptosis to treat certain degenerative disorders. Hence, understanding the molecular details of BAX and BAK apoptotic function is paramount if this prospect is to become a reality. However, emerging evidence that the apoptotic machinery may also have important roles in mitochondrial quality control raises the possibility that inhibiting mitochondrial apoptosis influences mitochondrial homeostasis that may limit (or even augment) the effectiveness of targeting apoptosis as a therapeutic strategy.

Abbreviations

BAK, BCL-2-associated killer; BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma-2; BH, BCL-2 Homology; DRP1, dynamin-related protein 1; Mcl-1, myeloid cell leukaemia 1; MFN, mitofusin; PINK1, phosphatase and tensin homolog-induced kinase-1; VDAC2, voltage-dependent anion channel 2.

Disclosures

The authors declare that they have no disclosures.

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