

## Review



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## Apoptotic foci at mitochondria: in and around Bax pores

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The permeabilization of the mitochondrial outer membrane by Bax and Bak during apoptosis is considered a key step and a point of no return in the signalling pathway. It is always closely related to the reorganization of mitochondrial cristae that frees cytochrome *c* to the intermembrane space and to massive mitochondrial fragmentation mediated by the dynamin-like protein Drp1. Despite multiple evidence in favour of a functional link between these processes, the molecular mechanisms that connect them and their relevance for efficient apoptosis signalling remain obscure. In this review, we discuss recent progress on our understanding of how Bax forms pores in the context of Drp1-stabilized signalling platforms at apoptotic foci in mitochondria.

This article is part of the themed issue 'Membrane pores: from structure and assembly, to medicine and technology'.

## 1. Introduction: role of mitochondria in apoptosis

Mitochondria are eukaryotic organelles that function as essential hubs in the coordination of a wide variety of metabolic reactions and signalling processes within the cell. For instance, they are considered the powerhouse of the cell due to their important role in respiration (i.e. ATP production). Among other things, they are also key players in lipid biosynthesis, calcium homeostasis and in the intrinsic pathway of a type of programmed cell death termed apoptosis.

Mitochondria are composed of a double [1–4] membrane system known as the mitochondrial outer and inner membranes (MOM and MIM, respectively). These membranes differ in protein and lipid composition as well as in morphology [5]. The transmembrane protein channels at the MOM make this membrane fluid and permeable to small polar molecules (up to 3–5 kDa). In contrast, the MIM shows restricted metabolite permeability and is folded into invaginations called cristae where specific proteins involved in relevant mitochondrial functions are located (e.g. the components of the electron chain transport, including cytochrome *c*, and the ATP synthase) [6]. The proton gradient that drives the ATP synthase during oxidative phosphorylation is indeed created and maintained across the MIM. The two spaces enclosed by the MOM and MIM are known as the mitochondrial matrix, which is inside the MIM and hosts the mitochondrial DNA, ribosomes and metabolic enzymes, and the intermembrane space, with a composition similar to the cytosol. Both membranes are connected at specific contact sites where the exchange of proteins and lipids between both membranes is enhanced [7]. Cardiolipin (CL) is an anionic phospholipid with four acyl chains that is specifically located at mitochondria of eukaryotic cells. It is mainly enriched in the MIM and found at lower levels at the MOM [8]. However, due to its special structure, it has been proposed that mitochondrial contact sites are enriched in CL and also phosphatidylethanolamine (PE), which share in common their tendency to form non-lamellar structures. This ability might allow them to stabilize the

connections between both membranes, and they have also been proposed to play an important role as specific death foci during apoptosis [5,8–13].

It is widely assumed that mitochondrial shape and ultra-structure are closely linked to function. In this regard, mitochondria are able to change their morphology depending on the cell type and in response to cellular signalling and differentiation. They can vary from an interconnected filamentous network to separated spherical structures, where the cytoskeleton plays a crucial role in their morphological plasticity by controlling their distribution, motility and dynamics [14,15]. In particular, mitochondria undergo dramatic alterations in their structure and function under apoptotic stimuli [1,4,16,17].

Apoptosis at mitochondria is largely controlled by the members of the Bcl-2 family. They are mainly located at the MOM, where they form a complex interaction network whose outcome controls MOM permeabilization (MOMP). Concretely, the pro-apoptotic Bcl-2 proteins Bax and Bak are the direct executioners of this event [18–20]. Upon activation in apoptosis, they permeabilize the MOM and enable the release of the so-called apoptotic factors, including cytochrome *c* or SMAC/DIABLO, from the intermembrane space into the cytosol. This is considered the point of no return in the cell's commitment to death, and it leads to the activation of the caspase cascade, the dismantling of the cellular components and finally to cell death [21].

Besides MOMP, mitochondria also undergo a number of additional alterations during apoptosis, such as changes in lipid transfer between the MIM and the MOM [22], and between the mitochondria and other organelles [23], loss of mitochondrial function, including loss of transmembrane potential and incapability to maintain calcium homeostasis [24,25], mitochondrial fragmentation and cristae remodelling [1,4,16,17,26]. In this review, we focus on the current understanding of three mechanisms, mitochondrial fragmentation, cristae remodelling and MOMP, which occur close in time at specific mitochondrial foci during apoptosis. We pay special attention to new evidence related to Bax pore formation and the interconnection between these processes.

## 2. Structural changes in mitochondria during apoptosis

One of the first mitochondrial changes during apoptosis affects the lipid composition of its membranes. This alteration occurs at two different levels, involving the modification of the lipid species at mitochondria, as well as the lipid transfer between MIM and MOM, or between mitochondria and other cellular organelles (i.e. endoplasmic reticulum (ER)) [27]. At an early stage, there is an increased CL transfer from the MIM to the MOM [22,28]. This is accompanied by an early oxidation of CL mediated by the specific peroxidase activity of CL-bound cytochrome *c*, which plays a role in the release of the apoptotic factors [22,29]. Although it has been suggested that mitochondrial contact sites might be the specific foci for CL recruitment to the MOM, it is not clear yet what is the process that induces CL translocation [7]. One possible explanation for CL redistribution from the MIM to the MOM could be associated with the non-lamellar

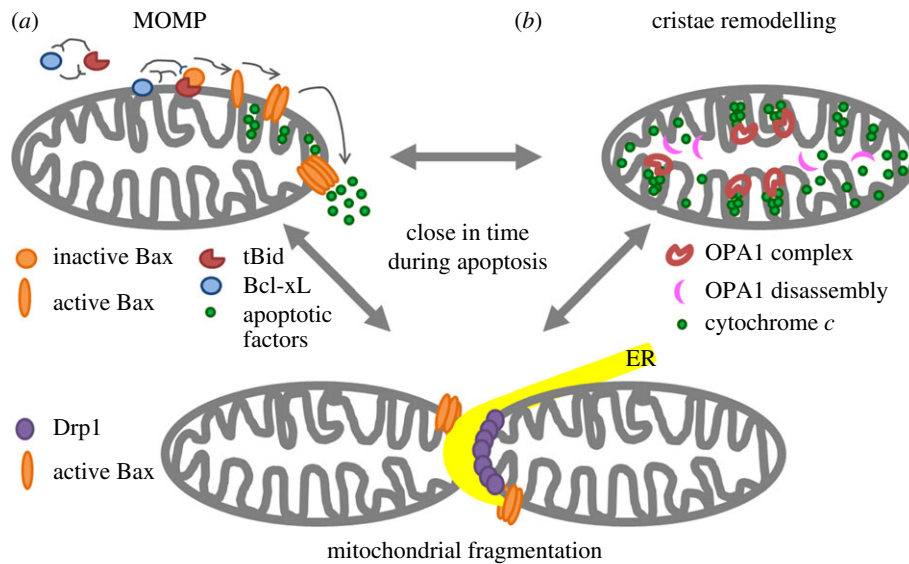
structures proposed for the membrane fusion sites between both membranes at these foci [7]. Other lipid alterations, like the accumulation at mitochondria of products of sphingolipid metabolism transferred from the ER, have also been shown to affect Bax/Bak activation and MOMP [30,31]. Indeed, the mitochondria/ER contact sites have been proposed to organize into lipid microdomains where proteins involved in mitochondrial fission are recruited [32,33]. At these sites, lipids and calcium are exchanged during apoptosis. For example, increased calcium flux from the ER to the mitochondria can easily derive in apoptosis by inducing the generation of CL microdomains, reactive oxygen species and CL peroxidation [29,34] (figure 1).

### (a) Mitochondrial outer membrane permeabilization

The members of the Bcl-2 family are key mediators of the process that involves the permeabilization of the mitochondrial outer membrane. There are about 20 proteins in this family, which are further classified into three groups depending on their role in apoptosis: (i) anti-apoptotic proteins (e.g. Bcl-2, Bcl-xL and Mcl-1) that inhibit apoptosis by interacting with the pro-apoptotic proteins; (ii) the pro-apoptotic members Bax and Bak, which are considered direct executioners of MOMP by directly affecting the membrane integrity; and (iii) the pro-apoptotic BH3-only proteins (e.g. Bid, Bim, Bad and Bik), which have evolved to sense apoptotic stimuli and are able to induce Bax/Bak activation directly and/or by blocking the function of anti-apoptotic proteins during apoptosis [19,35,36].

Under physiological conditions, Bax and Bak are continuously translocating between cytosol and mitochondria in a steady state. Differences in the translocation rates lead to a major population of Bax being cytosolic in healthy cells, whereas most Bak molecules locate at the MOM [37,38]. In the presence of apoptotic stimuli, BH3-only proteins trigger the activation of Bax and Bak, leading to their recruitment and accumulation at discrete mitochondrial foci. There, Bax and Bak undergo conformational changes that allow extensive insertion into the membrane, oligomerization and pore formation. These sequential steps lead to MOMP and to the subsequent release of apoptotic factors [19,39]. The anti-apoptotic proteins of the Bcl-2 family block this process by acting at different levels, which include: (i) promoting the retrotranslocation of Bax and Bak from the MOM, (ii) sequestering the BH3-only activators, and (iii) directly binding and inhibiting the oligomerization of Bax and Bak at the MOM [40–44].

The Bcl-2 interaction network is very complex and many models have been postulated in order to explain how they orchestrate MOMP. The 'direct activation' model [45,46] suggests that Bax and Bak require direct interaction with activator BH3-only proteins in order to become activated. In contrast, the 'neutralization' model [47] assumes that Bax and Bak are constitutively active but kept in check by the pro-survival family members. Induction of the BH3-only proteins would then displace the executioners from these inhibitory complexes and promote MOMP. In addition, the 'embedded together' model [48,49] emphasizes the role of the membrane as the 'locus of action' for MOMP and Bcl-2 interactions, where CL also plays a role in Bax/Bak action [11,39,50,51]. Hence, the membrane environment is fully required to achieve the active conformation and stoichiometry



**Figure 1.** Scheme of structural changes in mitochondria during apoptosis: MOMP (a), cristae remodelling (b), mitochondrial fragmentation (bottom).

of the complexes between Bcl-2 proteins, which will then determine whether apoptosis is induced or not [49,52]. More recently, the ‘unified’ and ‘hierarchical’ models [53,54] provide more sophisticated scenarios that account for the relative importance of the interactions between Bcl-2 proteins in the cytosol and the membrane environment.

### (b) Mitochondrial fragmentation: a constant event in apoptosis

During apoptosis, and close in time with MOMP, mitochondria undergo massive fragmentation, which appears to be universally associated with this type of cell death [55,56]. In mammals, the main protein responsible for mitochondrial fission is a GTPase mechanoenzyme called dynamin-related protein 1 (Drp1) [57]. This protein shuttles between the cytosol and the MOM, where it binds when mitochondrial fission is required. Once at the MOM, it associates with specific adaptors and it oligomerizes into a spiral-like scaffold around the fission sites in mitochondria leading to organelle division [58]. From the literature currently available, it seems that different Drp1 adaptors can independently recruit Drp1 to the MOM, like mitochondrial fission factor (Mff) [59,60] and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49 and MiD51, respectively) [61–63]. Recent studies suggested that the role of each Drp1 adaptor in the recruitment of Drp1 to the MOM could represent distinct cellular pathways [64,65]. The complexity of this process is further increased with new evidence underscoring that fission sites are determined by interactions between mitochondria and ER [33]. These sites, which had already been established as sites for lipid and calcium transfer, are now considered as functional platforms where the coordinated polymerization of actin filaments could drive Drp1 assembly to mediate mitochondrial fission [66,67].

In the apoptotic context, Drp1 transitions from fast recycling to stable membrane association. This phenomenon seems to occur downstream of the recruitment of Bax and Bak but before cytochrome *c* release [68]. It is achieved thanks to the SUMOylation (small ubiquitin-like modifier) activity of MAPL, a mitochondrial anchored RING-finger containing protein with SUMO E3 ligase activity [69]. Thus,

MAPL-induced covalent SUMOylation of Drp1 enables the stabilization of Drp1 scaffolds on ER-mitochondria contact sites [70,71]. Interestingly, Drp1 SUMOylation co-localizes with Bax/Bak at defined mitochondrial foci during cell death. However, the relevance of Drp1 recruitment to these foci for apoptosis progression remains undefined (see below). This notion has been challenged by some authors who reported little [72] or partial [73] resistance to apoptosis in Drp1-deficient cells, and Drp1-independent mitochondrial fission Drp1 knockout cells [73].

### (c) Release of cytochrome *c* by cristae remodelling

The release of the apoptotic factors from the intramembrane space is dependent on their location within mitochondria [74]. For instance, SMAC/DIABLO is located at the inner boundary area of mitochondria, whereas cytochrome *c* is mainly sequestered at cristae structures. Consistent with this notion, Bax/Bak-induced MOMP can directly provide the release of SMAC/DIABLO, while the egress of cytochrome *c*, which is located within the cristae space, requires the remodelling of the highly stacked cristae structures [74–76].

The structure of the cristae junctions that physically separate the cristae contents from the intermembrane space is stabilized by optic atrophy 1 (OPA1)-complex formation [77,78]. OPA1 is a GTPase located at the MIM, where it is responsible for MIM fusion and for the maintenance of cristae junction integrity [75,77,79]. During apoptosis, OPA1 oligomers are disassembled, cristae become open to the intermembrane space and cytochrome *c* is finally released from these structures to the cytosol [77,80]. It has been described that this process is closely linked to mitochondrial fragmentation [55,81,82] and downstream of Drp1 fission activity [83]. However, it is still unclear how cristae remodelling is regulated during apoptosis.

Previous investigations have speculated that some BH3-only proteins from the Bcl-2 family could also be involved in this process [16,77,84,85]. For instance, it has been described that the lipid transfer activity of tBid may help for CL translocation from MIM to MOM, favouring CL redistribution into microdomains and generating local alterations in the membrane curvature, somehow promoting cytochrome

*c* release [12,86–88]. Similarly, another BH3-only protein termed Bik also seems to induce dynamic mitochondrial transformation, initiating the cristae remodelling regulated by Drp1 [85]. The latter study, together with recent reports, highlights the essential role that Drp1 may play in this process [71,74,76,89]. In fact, it has been proposed that the membrane remodelling function of Drp1 in this process could be different from its recognized role in regulating mitochondrial fission [85], although some authors suggest that both membrane constriction and lipid remodelling activities are also required for membrane fission [90,91]. Along these lines, current research focused on Drp1 adaptors has shed light on the specific contribution of MiD49/51 to this process [89]. Experiments performed in knockout cells for Drp1 or these adaptors confirmed that Drp1-mitochondrial fission through MiD49/51, but not Mff, facilitated cristae remodelling required for cytochrome *c* release during intrinsic apoptosis. Disassembly of OPA1 oligomers was not sufficient to enable cytochrome *c* release from the cristae space. Moreover, both the transmembrane domain and the Drp1-binding site of MiD51 were essential for cytochrome *c* release, suggesting that this adaptor may function as a spatial-temporal coupler to the cristae remodelling machinery in the presence of Drp1 [89].

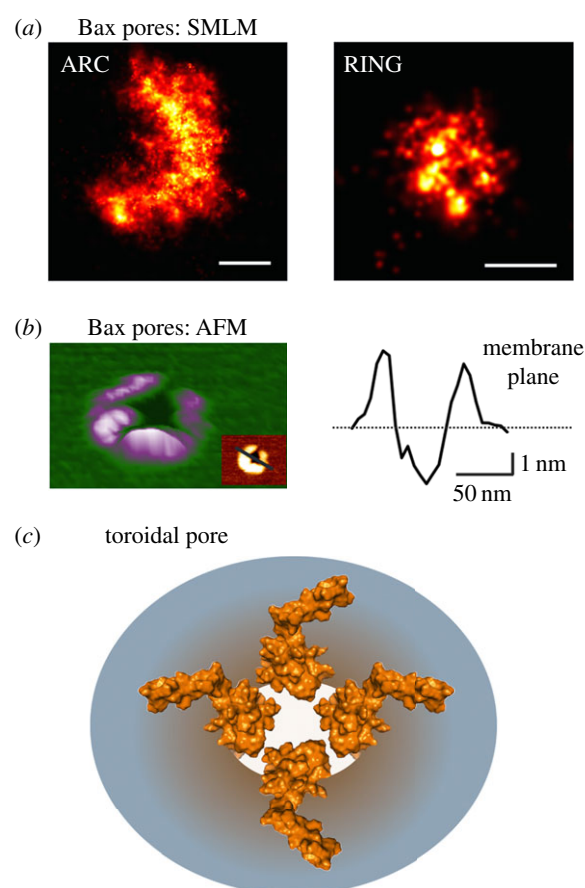
### 3. Bax pore formation during apoptosis

The molecular mechanism by which Bax (and Bak) mediate MOMP has remained one of the key questions in the field. A lot has been learned from *in vitro* reconstituted systems, where Bax displays membrane-permeabilizing activity characterized by the formation of large and long-lived membrane pores, which are tunable in size and of toroidal nature [42,51,92,93]. Of note, the toroidal pore model implies that not only proteins, but also lipid molecules are exposed at the rim of the pore and thus also play a role in pore dimensions and stability [94]. Only recently Bax-mediated membrane pores could be directly visualized in the mitochondria of apoptotic cells [76,95].

#### (a) Assembly path of Bax

Bax/Bak activation is an essential step towards MOMP. This process involves the change from a monomeric, globular structure found in the cytosol or loosely associated to the MOM, into a partially unfolded, membrane-inserted, oligomeric conformation capable of disrupting the membrane integrity. Several lines of evidence support the role of the membrane to provide the right physical–chemical environment that stabilizes the active conformation of Bax [39,41,96]. It is therefore difficult to conceive that the active structure of Bax is long-lived in the absence of a lipid bilayer or a chemical environment that mimics it.

This also implies that Bax activation first starts with membrane association. Bax binds to the membrane as a monomer and rapidly self-assembles into higher oligomers [44]. The conformational changes that allow Bax activation are promoted by interaction with activator BH3-only proteins, which bind via their BH3 domains to a conserved hydrophobic groove or to a rear pocket situated between helices  $\alpha 1$  and  $\alpha 6$  [97,98]. Binding to an activator BH3-only protein is thought to induce a conformational change that displaces the hydrophobic C-terminal helix  $\alpha 9$  from the hydrophobic groove, which inserts into the membrane and acts as an anchor to the MOM [99]. This is followed by exposure of the N-terminal helix  $\alpha 1$  and



**Figure 2.** Bax pore architecture. (a) Magnified reconstructed super-resolution images of Bax pores in apoptotic HeLa cells corresponding to arc (left) and ring (right) structures (with permission from [95]). SMLM stands for Single Molecule Localization Microscopy. Scale bars, 100 nm. (b) Three-dimensional atomic force microscopy (AFM) topography of a Bax structure in a supported lipid bilayer (right) and its height profile (left) corresponding to the pore rim. (c) Representative scheme of Bax toroidal pore (top view) where both Bax dimers and lipids cooperate for pore formation. The structure of active, membrane-inserted Bax dimer is used based on the ‘clamp’ model [100].

rearrangement of the  $\alpha 2$ /BH3 domain. As a result, the BH3 domain of one Bax molecule is exposed and displaces the activator BH3-only from the groove in another Bax molecule, thus leading to the formation of Bax dimers. Structural data of active Bax in the membrane reveal a well-defined ‘dimerization’ or ‘core’ domain stabilized via BH3 domain/groove interactions, and a rather flexible ‘piercing’ or ‘latch’ domain, which unfolds from the rest of the protein via partial opening of the hairpin of helices  $\alpha 5$  and  $\alpha 6$  [98,100]. As a result, most of activated Bax lies on the membrane surface shallowly inserted, while only the C-terminal domain is believed to adopt a canonical transmembrane orientation [101].

After dimer formation, Bax continues to self-assemble in order to form higher order oligomers. Stoichiometry analysis at the single-molecule level revealed that Bax does not adopt a unique oligomeric form in the membrane, but rather organizes into a mixture of species that are based on dimer units [44]. While cBid was important for activation, it did not affect the final distribution of oligomers. In contrast, Bcl-xL was able to disassemble existing Bax complexes, suggesting that Bax oligomerization is a reversible process. The question remains whether there is a maximum or preferred oligomeric state for Bax. In addition, the role of Bak and other interacting proteins on Bax assembly needs further investigation (figure 2).

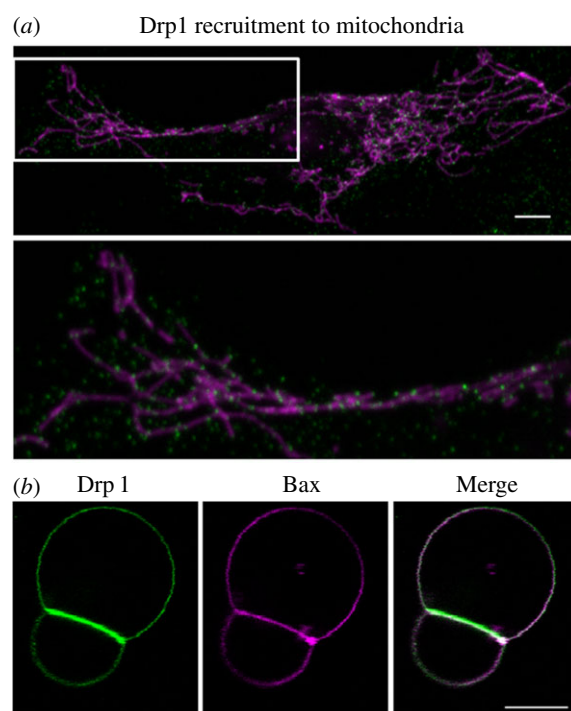
## (b) Bax pore architecture

Concomitant with oligomerization, Bax induces the formation of membrane pores that mediate MOMP. These pores were recently visualized in the mitochondria of apoptotic cells thanks to superresolution microscopy [76,95]. Permeabilized MOM contained large openings heterogeneous in size that, importantly, included Bax molecules enriched at the pore rim. These structures were very similar to membrane pores induced by Bax in liposomes and supported bilayers [95,102], suggesting that the properties of Bax pores discovered from *in vitro* studies also apply to the mitochondrial pores. For example, the heterogeneity of pore size in mitochondria is in line with *in vitro* experiments with Bax, where it was shown that Bax is capable of forming pores of tunable size. This fits well with a toroidal pore model where Bax oligomers reduce the stress caused by membrane distortion and curvature formation at the pore edge [51,92,93]. Nevertheless, it is likely that in the context of the cell, other proteins, like Bak or Drp1, and maybe lipids, also participate in MOM pores thereby modulating the features of Bax-mediated pores. Indeed, in the absence of Drp1, Bax continued to form large pores but cytochrome *c* release was hampered [76].

Bax clusters at the MOM exhibited a diverse mixture of distinct structures, including lines, arcs and full rings, which correlated well with Bax assemblies detected *in vitro* [95]. All these structures could represent different stages in the assembly of nascent evolving Bax pores. Bax molecules would initially organize into line and arc structures that further evolve into full rings. However, it cannot be discarded that they correspond to kinetically trapped intermediates during the assembly process. Interestingly, not only full rings but also a significant fraction of arc-shaped structures correlated with membrane pores. The architecture of the arc-shaped pores implies that Bax oligomers only cover partially the pore edge, which results in a fraction of the pore structure being formed only by lipids. This partial coverage of the pore edge by protein molecules constituted a demonstration of the toroidal pore model for Bax and confirmed the continuous membrane organization at the pore rim detected by X-ray for a peptide derived from helix  $\alpha 5$  of Bax [103], which reproduces the pore activity of the full-length protein [104,105]. This reveals a scenario where Bax forms oligomers that are dynamic and heterogeneous, and that give rise to membrane pores of diverse sizes. Such diversity is in good agreement with the variety of oligomeric species detected at the membrane and with the flexible nature of the piercing domain in the structure of active Bax. Indeed, the recently proposed three-dimensional model for active, membrane-inserted Bax (i.e. the 'clamp' model based on double electron–electron resonance spectroscopy data) provides a physical–chemical basis for the stabilization of the pore edge by Bax assemblies at the membrane [100]. However, it is still unclear which is the minimum amount of Bax molecules able to stabilize a membrane pore capable of releasing the apoptotic factors. Studies with lipid nanodiscs suggest that it could be a number as small as monomers or dimers, although the exact assembly state of Bax in those systems was not conclusive [106].

## 4. Mechanistic link between mitochondrial structural changes during apoptosis

To date, several contradictory studies challenge the interplay between the alterations occurring at the mitochondria during



**Figure 3.** Role of Drp1 in mitochondrial fission and apoptosis. (a) Drp1 shuttles between cytosol and mitochondria for membrane division under physiological conditions. Overview (top panel) and zoomed image of the white box (bottom panel). Immunostaining of endogenous Drp1 (green) and mitochondrial staining with Mitotracker Red (magenta). Scale bar, 5  $\mu\text{m}$ . (b) Drp1 (green) and Bax (magenta) binding to giant unilamellar vesicles composed of phosphatidylcholine: phosphatidylethanolamine: cardiolipin (54 : 20 : 26, mol : mol) at 42° for 30 min. Drp1 and Bax are concentrated at contact surfaces between two vesicles. Bax clusters at highly curved edges between vesicles. Scale bar, 10  $\mu\text{m}$ .

intrinsic apoptosis. Among the open questions, it still remains unresolved whether the concentration of CL at the MOM under apoptotic conditions resembles the high concentration used *in vitro* to regulate the activity of Bcl-2 proteins. Similarly, there is an active debate regarding the link between MOMP and mitochondrial fragmentation. Although these two events occur very close in time and space, a number of reports in the literature suggest that they may not be strictly correlated [73,107–109].

However, a recent study brings together mitochondrial alterations with the efficient execution of apoptosis [71]. The authors claim that it is not necessarily mitochondrial fission that is required for Bax/Bak-mediated cytochrome *c* release, but rather the stabilization of specific interorganellar platforms that favour metabolite flux and membrane curvature. This is in line with previous work showing that hyperfragmented mitochondria could not support Bax-induced cytochrome *c* release because ER/mitochondria contact sites were abolished [110,111]. Similarly, Drp1 promotes tethering and hemifusion of membranes *in vitro* that might be linked to the stimulation of tBid-induced Bax oligomerization and cytochrome release during apoptosis [112,113]. Consistent with this notion, Bax/Bak-induced Drp1 SUMOylation at the ER/mitochondria interface stabilizes signalling platforms for calcium and lipid transfer between both organelles, as well as for cristae remodelling and cytochrome *c* release [30,71,114].

The current data available support two alternative models for the role of Drp1 in apoptosis: (i) Drp1-mediated cristae

remodelling, independent of Bax activity; and (ii) Drp1-induced Bax oligomerization, independent of cristae remodelling. Nevertheless, the fact that these proteins co-localize at the same mitochondrial foci during apoptosis suggests that there could be a direct interplay between them in order to regulate structural changes at mitochondria in apoptosis. Bcl-2 proteins could cooperate with the mitochondrial fission machinery to efficiently mediate cristae remodelling, fragmentation and MOMP during apoptosis. Further research will be needed to fully understand the molecular mechanisms involved in this interplay (figure 3).

## 5. Concluding remarks

The multiple alterations that mitochondria undergo during apoptosis include the remodelling of the cristae ultrastructure, the permeabilization of the MOM and dramatic fragmentation. Recent data reveal that during apoptosis Bax undergoes a conformational change and organizes into dimer units with a well-defined dimerization domain and a flexible piercing or latch domain. These dimers continue to

self-assemble into multiple oligomeric species that organize into diverse structures at the MOM, including lines, arcs and rings, and concentrate at distinct foci. Both arcs and rings delineate membrane pores that mediate the release of the apoptotic factors. In parallel, Drp1 also becomes enriched in these foci where, together with MiD49/51, it stabilizes signalling platforms that modulate lipid and calcium exchange between the ER and mitochondria and play a role in mitochondrial fragmentation and in the reorganization of the MIM cristae. Exciting research lies ahead to uncover the molecular mechanisms that interconnect these processes and their functional relevance for efficient apoptosis induction.

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**Competing interests.** We declare we have no competing interests.

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