

This is the peer reviewed version of the following article:

Bax and Bak Pores: Are We Closing the Circle? / Cosentino, K., Garcia-Saez, A.J.. - In: TRENDS IN CELL BIOLOGY. - ISSN 0962-8924. - 27:4(2017), pp. 266-275. [10.1016/j.tcb.2016.11.004]

Terms of use:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

06/10/2026 02:41

Published in final edited form as:

Trends Cell Biol. 2017 April ; 27(4): 266–275. doi:10.1016/j.tcb.2016.11.004.

Bax and Bak Pores: Are We Closing the Circle?

Katia Cosentino¹ and Ana J. García-Sáez^{1,2,*}

¹Interfaculty Institute of Biochemistry, University of Tübingen, Hoppe-Seyler-Str. 4, 72076 Tübingen, Germany

²Max Planck Institute for Intelligent Systems, Heisenbergstr. 3, Stuttgart, Germany

Abstract

Bax and its homolog Bak are key regulators of the mitochondrial pathway of apoptosis. On cell stress Bax and Bak accumulate at distinct foci on the mitochondrial surface where they undergo a conformational change, oligomerize, and mediate cytochrome c release, leading to cell death. The molecular mechanisms of Bax and Bak assembly and mitochondrial permeabilization have remained a longstanding question in the field. Recent structural and biophysical studies at several length scales have shed light on key aspects of Bax and Bak function that have shifted how we think this process occurs. These discoveries reveal an unexpected molecular mechanism in which Bax (and likely Bak) dimers assemble into oligomers with an even number of molecules that fully or partially delineate pores of different sizes to permeabilize the mitochondrial outer membrane (MOM) during apoptosis.

Bax and Bak Are Key Players in Mitochondrial Apoptosis

Apoptosis is a form of programmed cell death that occurs in a physiological setting and is indispensable for the preservation of tissue homeostasis and embryonic development. Importantly, apoptosis downregulation is a major cause of tumorigenesis, while upregulation of this process induces infectious, autoimmune, and neurodegenerative diseases [1]. Thus, controlling apoptosis represents an important therapeutic target for the treatment of related diseases [2,3].

The intrinsic or mitochondrial apoptotic pathway, which is common only to vertebrates, is tightly controlled by the Bcl-2 protein family and involves MOM permeabilization (MOMP) [1,4]. Of note, in some invertebrates Bcl-2-like proteins such as CED-9 and EGL-1 of *Caenorhabditis elegans* control cell death, but this process is mitochondria independent. Following MOMP apoptotic factors like cytochrome c and SMAC/DIABLO are released into the cytosol to activate the caspase cascade leading to cell death [5]. Three decades of intense research have led to the identification and characterization of at least 18 different members of the Bcl-2 family and classified them into three subgroups according to their function in apoptosis and the number of Bcl-2 homology (BH) domains they contain. The proapoptotic members of the Bcl-2 family include the multi-BH-domain Bax and Bak, which are considered direct executors of MOMP, and the BH3-only members Bid, Bim, and

*Correspondence: ana.garcia@uni-tuebingen.de, (A.J. García-Sáez).

PUMA, which initiate apoptosis. BH3-only members can be classified as ‘direct activators’ if they directly bind and activate Bax/Bak or ‘sensitizers’ if they only bind and neutralize antiapoptotic family members. Finally, the antiapoptotic or prosurvival Bcl-2 proteins Bcl-2 and Bcl-xL contain all four BH domains and inhibit the action of proapoptotic proteins [6]. The functional balance between pro- and antiapoptotic members forms a complex interaction network whose outcome decides cell fate.

Bax and Bak are therefore at the core of the intrinsic pathway of apoptosis [7]. Although both shuttle between the cytosol and the MOM, Bax primarily localizes to the cytoplasm in healthy cells while Bak is mainly located at the MOM. Once activated these proteins oligomerize and mediate MOMP, which allows the release of apoptotic factors into the cytosol leading to cell death [4,8].

Here we review the recent progress made in elucidating how Bax and Bak mediate MOMP in apoptosis. We focus on Bax because it has been more extensively studied, but general principles are likely to extend to Bak. Our discussion provides a biophysical perspective of the molecular mechanisms involved and covers different scales of organization: from the conformational changes of Bax and Bak needed for activation in the membrane, through their molecular assembly into oligomers, to the most recent findings about the supramolecular organization of Bax in model systems and cells, which have convinced us that Bax pores exist.

The Deadly Transformation: Assembly Pathway of Bax and Bak

Conformational Changes Drive Bax and Bak Activation

In its inactive, soluble form, Bax has a globular structure comprising nine α helices where a central hydrophobic core ($\alpha 5$) is surrounded by a bundle of amphipathic helices exposed to the aqueous environment [9]. This fold defines a hydrophobic groove, which interacts with the BH3 domain of other Bcl-2 proteins. During apoptosis activation of Bax involves a conformational transition from a globular to an extended, membrane-inserted conformation (Figure 1). An initial structural change allows anchoring of Bax to the MOM through insertion of the transmembrane domain of the C-terminal $\alpha 9$ helix [10,11] while the rest of the protein retains the globular fold. This mechanism seems to be triggered by interaction of cytosolic Bax with the BH3 domain of (activator) BH3-only proteins, which may induce the displacement of $\alpha 9$ from the hydrophobic groove [12] (Figure 1). So far, two activation sites have been identified. One site is located opposite of the canonical hydrophobic groove [11,13] and this ‘rear pocket’ comprises helices $\alpha 1$ and $\alpha 6$ [13]. Conformational changes following this interaction, rather than direct binding to the hydrophobic groove, would then be responsible for $\alpha 9$ displacement and membrane insertion [11]. Recently, the structure of the interaction site corresponding to the canonical hydrophobic groove has been solved [14,15]. Following binding to direct activator BH3-only proteins, Bax activation proceeds through exposure of the N-terminal segment (which precedes the $\alpha 1$ helix) and rearrangement of the $\alpha 2$ /BH3 domain [11,14]. For Bak, further structural details have revealed that movement of $\alpha 2$ initiates dissociation of both the N segment and the $\alpha 1$ /BH4 domain from the rest of the protein, with $\alpha 1$ remaining solvent exposed [16].

Such conformational changes lead to an opening of the groove that has been suggested to weaken the binding of Bax with its activator partner allowing BH3-only proteins to interact transiently with Bax and ‘run away’, as proposed in the ‘kiss-and-run’ model [17]. This step may expose the BH3 domain of Bax, which, free of BH3-only proteins, can then undergo homo-dimerization. Together these findings are the basis for a model proposing that activation of Bax may follow a two-step process, the first involving $\alpha 9$ transmembrane domain insertion in the MOM and the second requiring BH3 domain exposure for interaction with other Bcl-2 proteins.

Bax and Bak Dimerization and Higher Oligomerization

Crystallographic data for truncated Bax in detergent solution have disclosed a novel conformational change on Bax activation that generates a ‘core’ ($\alpha 2$ – $\alpha 5$) and a ‘latch’ ($\alpha 6$ – $\alpha 8$) domain and induces transient exposure of the $\alpha 2$ /BH3 domain [14]. This rearrangement proceeds via partial unfolding of the hairpin comprising helices $\alpha 5$ and $\alpha 6$ [18] and seems to be essential for Bax function [14]. In the dimer the $\alpha 2$ /BH3 domain of a Bax molecule binds to the canonical groove of another Bax and vice versa (BH3-in-groove interaction) [14] (Figure 1). This allows the formation of a stable ‘symmetric’ dimerization domain, which supports a model initially proposed based on crosslinking and low-resolution structural studies [15,19]. This symmetric model was also confirmed using double electron–electron resonance (DEER) studies of the full-length protein in the membrane environment [18] and FRET microscopy in living cells [20]. A similar model has also been suggested for Bak dimerization [21,22].

The existence of an additional interface would provide a rationale for how symmetric Bax and Bak dimers further assemble in higher-order oligomers. Several studies have implicated a role for helices $\alpha 6$ and $\alpha 9$ in Bax oligomerization [15,18,23]. Dimerization via $\alpha 9$ (and probably $\alpha 6$) occurs downstream of BH3-in-groove dimerization, suggesting that it might represent the interaction site of symmetric dimers needed to form higher-order oligomers. Since $\alpha 9$ dimerization is not required for the passage of small molecules like cytochrome c but only for bigger ones like SMAC, the relative importance of interdimer contacts via helices $\alpha 6$ versus $\alpha 9$ remains unclear. Recently, crosslinking and DEER results have reported a novel oligomerization site for Bak in isolated apoptotic mitochondria involving the $\alpha 3/\alpha 5$ interface, which, together with $\alpha 6$ (and hypothetically $\alpha 9$), stabilizes interactions of neighboring Bak homodimers [24].

Furthermore, the structural arrangement through which Bax oligomers perforate the MOM remains unknown. In the longstanding ‘umbrella’ model, inferred on the grounds of structural similarities between soluble Bax and other α -pore-forming proteins like colicin and diphtheria toxin [25], the $\alpha 5$ and $\alpha 6$ helices were assumed to insert as a transmembrane hairpin perpendicularly to the membrane while the other helices would lie on the membrane surface in an umbrella-like configuration. However, this model does not seem to hold true in light of recent data on active Bax and Bak in membranes. During BH3-in-groove dimerization, the $\alpha 4$ and $\alpha 5$ helices generate an aromatic planar surface that may sit on the membrane [14]. Insertion of hydrophobic aromatic residues into the lipid bilayer might increase membrane tension and favor a positive membrane curvature that would engage

MOMP [8,14,26]. The result is an ‘in-plane’ model where insertion into the membrane occurs completely for the $\alpha 9$ but only shallowly for the $\alpha 5$ and $\alpha 6$ helices [14,26]. How Bax and Bak domains are arranged with respect to such a membrane pore is unclear in this model. Independent studies suggest that intersected or parallel $\alpha 9$ helices are located at the rim of the pore [23].

DEER studies of membrane-bound full-length Bax provided additional information about the structure of active Bax dimers in the membrane [18]. While the $\alpha 2$ – $\alpha 5$ core helices of Bax dimers retain a well-defined structure similar to the soluble one, helices $\alpha 6$ – $\alpha 9$ adopt a more flexible conformation, which may play a role in membrane destabilization. This is the basis for a model where partial opening of helices $\alpha 5$ and $\alpha 6$ is essential for membrane permeabilization; these two pairs of helices assemble in the dimer as a ‘clamp’, with the dimerization domain (helices $\alpha 2$ – $\alpha 5$) at the rim of a lipidic pore and the amphipathic $\alpha 6$ helices lying on the surface, parallel to each other, on opposite sides of the membrane [18] (Figure 2A). In this model it is unclear how the C-terminal $\alpha 9$ helices are oriented. They could be antiparallel, unless the structural symmetry of the dimer is broken. Despite this, if confirmed, this model would provide a firm explanation for how Bax molecules stabilize open pores at the membrane.

Insight into the Assembly of Bax Oligomers

Deep structural changes induce a conformation of Bax that inserts extensively into the lipid bilayer and perforates it [2]. This step is concomitant with Bax assembly into oligomers. Crosslinking and gel filtration studies have detected diverse oligomeric forms of Bax [27,28]. However, none of these experimental approaches allowed precise determination of the oligomeric state of Bax during its activation and function in the membrane.

Single-molecule imaging studies have recently elucidated key aspects of the molecular mechanism of Bax assembly in the membrane [29]. Initially, Bax inserts into the bilayer as a monomer, suggesting that Bax activation involves conformational changes that allow its membrane insertion but precede its dimerization, supporting a two-step activation process for Bax. However, probably due to the instability of this monomeric structure, Bax molecules quickly self-assemble into higher-order oligomers. Importantly, in equilibrium, Bax in the membrane is present as a mixture of species based on dimer units [29] (Figure 1). The idea of a dimerization unit is attractive. On the one hand, it is in agreement with the structural data on active Bax in membranes [14,18]. On the other hand, it implies that interdimer interactions are weaker than intradimer ones, in line with a dynamic vision of Bax assembly where oligomerization is a reversible process that can be modulated, for example, by protein density at the membrane. This would explain the variety of oligomeric species found in previous studies and would be consistent with the formation of membrane pores that are tunable in size [30,31].

These data also suggest that, although BH3-only proteins are important for Bax activation, they do not affect the final distribution of oligomeric species. It remains unclear, however, whether the BH3-only proteins bind only to Bax monomers or also to oligomers and, in that case, whether they are released as the oligomers grow. Interestingly, Bcl-xL was able to disassemble preformed complexes. Although the exact molecular mechanism is unclear, this

suggests that the assembly pathway of Bax is a reversible process that can be regulated in both directions by several factors. In this context it has been reported that mitochondrial size influences Bax activity, suggesting a role for membrane curvature in Bax oligomerization [32]. Additional open questions include how, given a finite amount of Bax molecules available, the number and size of Bax oligomers/pores is controlled during apoptosis and how other Bax-interacting proteins, like Bak or VDAC2, affect these oligomers.

Bax, Bak, and the Pore

At a larger length scale, the nature of supramolecular structures formed by Bax and Bak to mediate MOMP has remained one of the key questions in the field. Several studies on model membranes have characterized Bax and Bak as pore-forming proteins capable of disrupting the continuity of the lipid bilayer by opening toroidal pores. Toroidal pores are pore structures where both proteins and lipids form part of the pore wall [33]. As a result, not only Bax and Bak but also membrane mechanical properties play a role in the size and stability of these pores [34].

It is important to consider that membranes are noncovalent 2D assemblies kept together by the hydrophobic effect. This means that disrupting the continuity of the membrane bilayer has a high energetic cost. Therefore, it is not trivial how a protein would induce membrane opening. When membranes are under stress they are tensed, and beyond a certain threshold the planar assembly disrupts and a pore opens [35]. The toroidal pore model proposes that lipids at such a pore rim bend to avoid exposure of the hydrophobic acyl chains to the aqueous environment [33]. As a consequence the two membrane leaflets form a continuous surface that is brought into contact by lipids at the curved pore edge. It is this high membrane curvature that is responsible for the high energy cost of maintaining the open pore conformation. The energy cost is directly proportional to the perimeter or length of the pore. For this reason the driving force for toroidal pore closure is known as line tension (in contrast to membrane or surface tension, which is proportional to the area). To keep a toroidal pore stably open, the energy cost of the bent membrane at the pore edge needs to be reduced by stabilizing the curved structure [34]. In such a scenario, Bax and Bak would need to first stress the membrane – for example, by asymmetric binding to the cytosolic leaflet of the MOM and by high local protein concentration associated with oligomerization, thereby inducing pore opening – and then stabilize the pore by decreasing the line tension. In reconstituted systems Bax alone is able to stress the membrane, promote pore opening, and stabilize long-lived pores [36,37]. However, it is important to note that other components present at Bax foci might contribute in live cells.

There is accumulating evidence in favor of the toroidal pore model for Bax and Bak. Classical experiments involved the Bax-dependent decrease in the lifetime of planar bilayers [38], the formation of large membrane openings modulated by lipids with intrinsic curvature [39,40], and the movement of lipids between the two leaflets in Bax-permeabilized membranes [41]. Cryoelectron microscopy images of liposomes revealed round membrane holes of 25–100 nm in diameter in the presence of active Bax (or Bak) [19,42,43] (Figure 2B). These membrane pores were long lived and tunable in size, depending on the density of Bax/Bak molecules on the membrane surface, and were associated with the formation of

Bax and Bak oligomers [30,31,36,44]. The most convincing evidence so far for continuous lipid monolayer organization in a toroidal pore was obtained with a peptide derived from helix $\alpha 5$ of Bax [45] (Figure 2C) that reproduced the pore-forming properties of the full-length protein [46,47]. However, the formation of such pores in apoptosis, as well as the distribution of Bax and Bak molecules with respect to the pore rim, remained obscure.

Recently, the formation of Bax assemblies in the form of arcs and rings associated with membrane pores at the MOM of apoptotic cells has been observed [48,49] (Figure 2D). In agreement with experiments on model membranes, Bax oligomers displayed a wide distribution of sizes (ranging from a few tens of nanometers to 100 nm in diameter) and shapes, indicating that Bax-mediated MOMP involves flexible and diverse pore structures. Additional atomic force microscopy (AFM) experiments on supported bilayers and electron microscopy of outer membrane vesicles (OMVs) and lipid nanodisks containing Bax confirmed protein enrichment at the rim of membrane pores of variable sizes where the pore wall was not completely covered by protein molecules [48,50,51] (Figure 2E,F). Similar arc structures that only partially cover the pore ring have recently been reported for other pore-forming proteins [52–55]. Quantitative analysis of Bak clusters *in situ* by single-molecule localization (SML) microscopy have shown a broad distribution of Bak cluster sizes in apoptotic mitochondria, although protein density remained homogeneous among clusters [56]. Unfortunately, no defined structures could be identified [56]. Together, the evidence reveals an unexpected model for Bax- (and probably Bak-) mediated MOMP where oligomerization at the MOM induces the formation of heterogeneous toroidal pore structures that are flexible and evolve over time (Figure 2G) leading to the release of apoptotic factors.

The question about the minimum number of Bax molecules able to form a functional pore at the MOM remains open. Data from lipid nanodisks suggest that monomers or dimers are sufficient to stabilize membrane openings [50], which could grow with additional Bax molecules. Another issue is the contribution of other MOM components to pore structures, including Bak [57] and proteins involved in mitochondrial dynamics such as Drp1 and Mitofusins [58]. The similar ability of Bak to form membrane pores [31,42,59] raises the question of whether they cooperate to form mixed assemblies at the MOM and whether these assemblies include mixtures of homodimers or heterodimers of Bax and Bak, as previously suggested [15,60]. Recent data suggest that Bok, a poorly understood member of the Bcl-2 family, is able to induce MOMP in the absence of Bax and Bak and to form pores in liposomes [61,62]. Further research is needed to understand whether Bak and Bok induce pore assemblies comparable with Bax and to shed light on the structural and functional consequences of potential cooperation between these proteins.

Regulation of Bax and Bak Activity

A longstanding debate in the apoptosis field revolves around the regulation of Bax (and Bak) activity. The ‘direct activation’ model [40], as well as the ‘embedded together’ and ‘unified’ models [63,64], assumes that Bax requires activation by a ‘direct activator’ BH3-only protein to mediate MOMP. Antiapoptotic Bcl-2 proteins play their inhibitory role by binding either directly to the executioners Bax and Bak or to BH3-only proteins, which then are no longer available to activate Bak/Bax [63]. The counterargument is provided by the

‘neutralization’ model [65], which proposes that antiapoptotic Bcl-2 proteins sequester constitutively active Bax/Bak in healthy cells and inhibit MOMP until apoptotic stimuli induce BH3-only proteins to displace them from the inhibitory complexes (Figure 3).

Biochemically, Bax can be produced as an inactive, soluble monomer in the absence of detergents and become efficiently activated by the addition of substoichiometric amounts of activator BH3-only proteins or derived peptides [40,66,67]. Moreover, several structural studies have revealed sites of direct interaction between Bax/Bak and BH3-only proteins or peptides, demonstrating a role for these proteins in catalyzing the activity of Bax [13,14,68]. However, *in vitro* Bax activation can also be achieved by other factors, like acidic pH [69], detergents [19,70], oxidized lipids [71], or mild heat treatment [72], provided that a lipid bilayer is available. Even membrane-bound Bax can autoactivate inactive Bax molecules [73]. Together these findings favor a low energy barrier for Bax transition from the soluble to the membrane-inserted conformation. Several genetic studies that knocked out increasing numbers of BH3-only proteins [74,75] revealed that the resulting cells differed from the double Bax/Bak knockout phenotype despite also becoming increasingly resistant to apoptotic stresses [76]. It was contemplated that autoactivation of Bax and Bak via downregulation of Bcl-2, Bxl-xL, and Mcl-1 was occurring in the absence of Bid, Bim, Puma, and Noxa [75]. Moreover, systematically knocking out all established members of the Bcl-2 family [77] and reintroducing Bax alone led to MOMP even at low expression levels. Overall, these findings add new support to the neutralization model, although the existence of unknown factors capable of directly activating Bax cannot be excluded and the clear experimental corroboration of the direct activation model suggests that the main pathway for the regulation of Bax/Bak activity may depend on the *in vivo* setting.

The picture has become more complicated by the realization that Bax (and to a lesser extent Bak) continuously shuttles between the cytosol and mitochondria of healthy cells independent of BH3-only proteins [78–80]. This retrotranslocation process is regulated by prosurvival Bcl-2 homologs, which bring Bax back to solution and maintain nontoxic, steady-state levels of Bax at the MOM [81]. Although the extent of Bax insertion in the lipid environment of the MOM that remains competent for retrotranslocation is not well defined, this process requires the removal of protein–lipid interactions, which theoretically should be thermodynamically unfavorable. Unless a molecular mechanism that provides energy for retrotranslocation is discovered, one could also reason that the dissociation of Bax from the membrane could proceed spontaneously via a low energy barrier accessible by thermal fluctuations. This idea would reconcile the diverse models for the regulation of Bax activity, although additional research is needed to validate it.

In summary, it is likely that both constitutive and direct activation of Bax and Bak are regulated by other Bcl-2 members. It is likely that aspects such as cell type, metabolic conditions, expression levels of different Bcl-2 members, or the death-priming state of the cell play a role in the balance between soluble and MOM-associated Bax and in the required conditions (prosurvival Bcl-2 neutralization and/or direct activation by BH3-only proteins) for switching Bax to pore formation and MOMP.

Concluding Remarks

The past years have been crucial in elucidating the mechanisms involved in Bax and Bak activation and organization in oligomeric structures that mediate MOMP. A much clearer picture is now being defined where, on activation, Bax undergoes a series of conformational changes that lead to the formation of dimers that further assemble into higher oligomers. Both the stoichiometry and the nanoassembly of these oligomers are diverse, but they promote the formation of heterogeneous pores at the MOM that can be partially or fully lined by Bax molecules. These recent discoveries have questioned previous findings and have prompted new and challenging questions (see Outstanding Questions) such as the minimal number of Bax molecules able to open a pore, the structural disposition of Bax on these pores, and the participation of additional molecules in the structure. New experimental approaches combining multiple types of information (e.g., pore functionality with protein structure) will contribute to answering these questions.

Acknowledgments

The authors thank Raed Shalaby for help with figure preparation. The García-Sáez laboratory is funded by the European Research Council (ERC-2012-StG 309966), the Deutsche Forschungsgemeinschaft (FOR 2036), and the University of Tübingen.

References

1. Strasser A, et al. Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. *EMBO J.* 2011; 30:3667–3683. [PubMed: 21863020]
2. Czabotar PE, et al. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol.* 2014; 15:49–63. [PubMed: 24355989]
3. Delbridge ARD, et al. Thirty years of BCL-2: translating cell death discoveries into novel cancer therapies. *Nat Rev Cancer.* 2016; 16:99–109. [PubMed: 26822577]
4. García-Sáez AJ. The secrets of the Bcl-2 family. *Cell Death Differ.* 2012; 19:1733–1740. [PubMed: 22935609]
5. Li P, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell.* 1997; 91:479–489. [PubMed: 9390557]
6. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol.* 2008; 9:47–59. [PubMed: 18097445]
7. Wei MC. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science.* 2001; 292:727–730. [PubMed: 11326099]
8. Westphal D, et al. Building blocks of the apoptotic pore: how Bax and Bak are activated and oligomerize during apoptosis. *Cell Death Differ.* 2013; 21:196–205. [PubMed: 24162660]
9. Suzuki M, et al. Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell.* 2000; 103:645–654. [PubMed: 11106734]
10. Lovell JF, et al. Membrane binding by tBid initiates an ordered series of events culminating in membrane permeabilization by Bax. *Cell.* 2008; 135:1074–1084. [PubMed: 19062087]
11. Gavathiotis E, et al. BH3-triggered structural reorganization drives the activation of proapoptotic BAX. *Mol Cell.* 2010; 40:481–492. [PubMed: 21070973]
12. Kim H, et al. Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. *Mol Cell.* 2009; 36:487–499. [PubMed: 19917256]
13. Gavathiotis E, et al. BAX activation is initiated at a novel interaction site. *Nature.* 2008; 455:1076–1081. [PubMed: 18948948]
14. Czabotar PE, et al. Bax crystal structures reveal how BH3 domains activate Bax and nucleate its oligomerization to induce apoptosis. *Cell.* 2013; 152:519–531. [PubMed: 23374347]

15. Dewson G, et al. Bax dimerizes via a symmetric BH3: groove interface during apoptosis. *Cell Death Differ.* 2012; 19:661–670. [PubMed: 22015607]
16. Alsop AE, et al. Dissociation of Bak α 1 helix from the core and latch domains is required for apoptosis. *Nat Commun.* 2015; 6:6841. [PubMed: 25880232]
17. Wei MC, et al. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev.* 2000; 14:2060–2071. [PubMed: 10950869]
18. Bleicken S, et al. Structural model of active Bax at the membrane. *Mol Cell.* 2014; 56:496–505. [PubMed: 25458844]
19. Bleicken S, et al. Molecular details of Bax activation, oligomerization, and membrane insertion. *J Biol Chem.* 2010; 285:6636–6647. [PubMed: 20008353]
20. Gahl RF, et al. Conformational rearrangements in the proapoptotic protein, Bax, as it inserts into mitochondria: a cellular death switch. *J Biol Chem.* 2014; 289:32871–32882. [PubMed: 25315775]
21. Dewson G, et al. To trigger apoptosis, Bak exposes its BH3 domain and homodimerizes via BH3:groove interactions. *Mol Cell.* 2008; 30:369–380. [PubMed: 18471982]
22. Ma S, et al. Assembly of the Bak apoptotic pore: a critical role for the Bak protein α 6 helix in the multimerization of homodimers during apoptosis. *J Biol Chem.* 2013; 288:26027–26038. [PubMed: 23893415]
23. Zhang Z, et al. BH3-in-groove dimerization initiates and helix 9 dimerization expands Bax pore assembly in membranes. *EMBO J.* 2015; 35:208–236. [PubMed: 26702098]
24. Mandal T, et al. Assembly of Bak homodimers into higher order homooligomers in the mitochondrial apoptotic pore. *Sci Rep.* 2016; 6:30763. [PubMed: 27488021]
25. Cosentino K, et al. Assembling the puzzle: oligomerization of α -pore forming proteins in membranes. *Biochim Biophys Acta.* 2016; 1858:457–466. [PubMed: 26375417]
26. Westphal D, et al. Apoptotic pore formation is associated with in-plane insertion of Bak or Bax central helices into the mitochondrial outer membrane. *Proc Natl Acad Sci USA.* 2014; 111:E4076–E4085. [PubMed: 25228770]
27. Annis MG, et al. Bax forms multispinning monomers that oligomerize to permeabilize membranes during apoptosis. *EMBO J.* 2005; 24:2096–2103. [PubMed: 15920484]
28. Antonsson B, et al. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *J Biol Chem.* 2001; 276:11615–11623. [PubMed: 11136736]
29. Subburaj Y, et al. Bax monomers form dimer units in the membrane that further self-assemble into multiple oligomeric species. *Nat Commun.* 2015; 6:8042. [PubMed: 26271728]
30. Gillies LA, et al. Visual and functional demonstration of growing Bax-induced pores in mitochondrial outer membranes. *Mol Biol Cell.* 2015; 26:339–349. [PubMed: 25411335]
31. Bleicken S, et al. Proapoptotic Bax and Bak form stable protein-permeable pores of tunable size. *J Biol Chem.* 2013; 288:33241–33252. [PubMed: 24100034]
32. Renault TT, et al. Mitochondrial shape governs BAX-induced membrane permeabilization and apoptosis. *Mol Cell.* 2015; 57:69–82. [PubMed: 25482509]
33. Ludtke SJ, et al. Membrane pores induced by magainin. *Biochemistry.* 1996; 35:13723–13728. [PubMed: 8901513]
34. Karatekin E, et al. Cascades of transient pores in giant vesicles: line tension and transport. *Biophys J.* 2003; 84:1734–1749. [PubMed: 12609875]
35. Lee MT, et al. Energetics of pore formation induced by membrane active peptides. *Biochemistry.* 2004; 43:3590–3599. [PubMed: 15035629]
36. Bleicken S, et al. Mechanistic differences in the membrane activity of Bax and Bcl-xL correlate with their opposing roles in apoptosis. *Biophys J.* 2013; 104:421–431. [PubMed: 23442864]
37. Garcia-Saez AJ, et al. Pore formation by a Bax-derived peptide: effect on the line tension of the membrane probed by AFM. *Biophys J.* 2007; 93:103–112. [PubMed: 17416629]
38. Basanez G, et al. Bax, but not Bcl-xL, decreases the lifetime of planar phospholipid bilayer membranes at subnanomolar concentrations. *Proc Natl Acad Sci USA.* 1999; 96:5492–5497. [PubMed: 10318911]

39. Basanez G, et al. Bax-type apoptotic proteins porate pure lipid bilayers through a mechanism sensitive to intrinsic monolayer curvature. *J Biol Chem.* 2002; 277:49360–49365. [PubMed: 12381734]
40. Kuwana T, et al. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell.* 2002; 111:331–342. [PubMed: 12419244]
41. Terrones O, et al. Lipidic pore formation by the concerted action of proapoptotic BAX and tBID. *J Biol Chem.* 2004; 279:30081–30091. [PubMed: 15138279]
42. Landeta O, et al. Reconstitution of proapoptotic BAK function in liposomes reveals a dual role for mitochondrial lipids in the BAK-driven membrane permeabilization process. *J Biol Chem.* 2011; 286:8213–8230. [PubMed: 21196599]
43. Schafer B, et al. Mitochondrial outer membrane proteins assist Bid in Bax-mediated lipidic pore formation. *Mol Biol Cell.* 2009; 20:2276–2285. [PubMed: 19244344]
44. Fuertes G, et al. Pores formed by Bax alpha 5 relax to a smaller size and keep at equilibrium. *Biophys J.* 2010; 99:2917–2925. [PubMed: 21044589]
45. Qian S, et al. Structure of transmembrane pore induced by Bax-derived peptide: evidence for lipidic pores. *Proc Natl Acad Sci USA.* 2008; 105:17379–17383. [PubMed: 18987313]
46. Garcia-Saez AJ, et al. Peptides derived from apoptotic Bax and Bid reproduce the poration activity of the parent full-length proteins. *Biophys J.* 2005; 88:3976–3990. [PubMed: 15778450]
47. Garcia-Saez AJ, et al. Peptides corresponding to helices 5 and 6 of Bax can independently form large lipid pores. *FEBS J.* 2006; 273:971–981. [PubMed: 16478471]
48. Salvador-Gallego R, et al. Bax assembly into rings and arcs in apoptotic mitochondria is linked to membrane pores. *EMBO J.* 2016; 35:389–401. [PubMed: 26783362]
49. Grosse L, et al. Bax assembles into large ring-like structures remodeling the mitochondrial outer membrane in apoptosis. *EMBO J.* 2016; 35:402–413. [PubMed: 26783364]
50. Xu XP, et al. Three-dimensional structure of Bax-mediated pores in membrane bilayers. *Cell Death Dis.* 2013; 4:e683. [PubMed: 23788040]
51. Kuwana T, et al. Pro-apoptotic Bax molecules densely populate the edges of membrane pores. *Sci Rep.* 2016; 6:27299. [PubMed: 27255832]
52. Podobnik M, et al. Crystal structure of an invertebrate cytolysin pore reveals unique properties and mechanism of assembly. *Nat Commun.* 2016; 7:11598. [PubMed: 27176125]
53. Ruan Y, et al. Listeriolysin O membrane damaging activity involves arc formation and lineaction – implication for *Listeria monocytogenes* escape from phagocytic vacuole. *PLoS Pathog.* 2016; 12:e1005597. [PubMed: 27104344]
54. Gilbert RJ, et al. Membrane pore formation at protein–lipid interfaces. *Trends Biochem Sci.* 2014; 39:510–516. [PubMed: 25440714]
55. Sonnen AF, et al. Incomplete pneumolysin oligomers form membrane pores. *Open Biol.* 2014; 4:140044. [PubMed: 24759615]
56. Nasu Y, et al. *In situ* characterization of Bak clusters responsible for cell death using single molecule localization microscopy. *Sci Rep.* 2016; 6:27505. [PubMed: 27293178]
57. Zhou L, Chang DC. Dynamics and structure of the Bax–Bak complex responsible for releasing mitochondrial proteins during apoptosis. *J Cell Sci.* 2008; 121:2186–2196. [PubMed: 18544634]
58. Karbowski M, et al. Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. *J Cell Biol.* 2002; 159:931–938. [PubMed: 12499352]
59. Leshchiner ES, et al. Direct activation of full-length proapoptotic BAK. *Proc Natl Acad Sci U.S.A.* 2013; 110:E986–E995. [PubMed: 23404709]
60. Mikhailov V, et al. Association of Bax and Bak homooligomers in mitochondria: Bax requirement for Bak reorganization and cytochrome c release. *J Biol Chem.* 2003; 278:5367–5376. [PubMed: 12454021]
61. Llambi F, et al. BOK is a non-canonical BCL-2 family effector of apoptosis regulated by ER-associated degradation. *Cell.* 2016; 165:421–433. [PubMed: 26949185]
62. Einsele-Scholz S, et al. Bok is a genuine multi-BH-domain protein that triggers apoptosis in the absence of Bax and Bak. *J Cell Sci.* 2016; 129:2213–2223. [PubMed: 27076518]

63. Llambi F, et al. A unified model of mammalian Bcl-2 protein family interactions at the mitochondria. *Mol Cell*. 2011; 44:1–15. [PubMed: 21981911]
64. Leber B, et al. Still embedded together binding to membranes regulates Bcl-2 protein interactions. *Oncogene*. 2010; 29:5221–5230. [PubMed: 20639903]
65. Willis SN, et al. Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science*. 2007; 315:856–859. [PubMed: 17289999]
66. Du H, et al. BH3 domains other than Bim and Bid can directly activate Bax/Bak. *J Biol Chem*. 2011; 286:491–501. [PubMed: 21041309]
67. Hockings C, et al. Bid chimeras indicate that most BH3-only proteins can directly activate Bak and Bax, and show no preference for Bak versus Bax. *Cell Death Dis*. 2015; 6:e1735. [PubMed: 25906158]
68. Brouwer JM, et al. Bak core and latch domains separate during activation, and freed core domains form symmetric homodimers. *Mol Cell*. 2014; 55:938–946. [PubMed: 25175025]
69. Cartron PF, et al. Impact of pH on Bax alpha conformation, oligomerisation and mitochondrial integration. *FEBS Lett*. 2004; 578:41–46. [PubMed: 15581613]
70. Ivashyna O, et al. Detergent-activated BAX protein is a monomer. *J Biol Chem*. 2009; 284:23935–23946. [PubMed: 19564333]
71. Lidman M, et al. The oxidized phospholipid PazePC promotes permeabilization of mitochondrial membranes by Bax. *Biochim Biophys Acta*. 2016; 1858:1288–1297. [PubMed: 26947183]
72. Pagliari LJ, et al. The multidomain proapoptotic molecules Bax and Bak are directly activated by heat. *Proc Natl Acad Sci U S A*. 2005; 102:17975–17980.
73. Tan C, et al. Auto-activation of the apoptosis protein Bax increases mitochondrial membrane permeability and is inhibited by Bcl-2. *J Biol Chem*. 2006; 281:14764–14775. [PubMed: 16571718]
74. Ren D, et al. BID, BIM, and PUMA are essential for activation of the BAX- and BAK-dependent cell death program. *Science*. 2010; 330:1390–1393. [PubMed: 21127253]
75. Chen HC, et al. An interconnected hierarchical model of cell death regulation by the BCL-2 family. *Nat Cell Biol*. 2015; 17:1270–1281. [PubMed: 26344567]
76. Lindsten T, et al. The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues. *Mol Cell*. 2000; 6:1389–1399. [PubMed: 11163212]
77. O'Neill KL, et al. Inactivation of prosurvival Bcl-2 proteins activates Bax/Bak through the outer mitochondrial membrane. *Genes Dev*. 2016; 30:973–988. [PubMed: 27056669]
78. Schellenberg B, et al. Bax exists in a dynamic equilibrium between the cytosol and mitochondria to control apoptotic priming. *Mol Cell*. 2013; 49:959–971. [PubMed: 23375500]
79. Edlich F, et al. Bcl-x_L retrotranslocates Bax from the mitochondria into the cytosol. *Cell*. 2011; 145:104–116. [PubMed: 21458670]
80. Todt F, et al. Differential retrotranslocation of mitochondrial Bax and Bak. *EMBO J*. 2015; 34:67–80. [PubMed: 25378477]
81. Todt F, et al. The C-terminal helix of Bcl-x_L mediates Bax retrotranslocation from the mitochondria. *Cell Death Differ*. 2013; 20:333–342. [PubMed: 23079612]
82. Volkmann N, et al. The rheostat in the membrane: BCL-2 family proteins and apoptosis. *Cell Death Differ*. 2014; 21:206–215. [PubMed: 24162659]

Trends

Interaction with Bcl-2 homology (BH)3-only activators at different sites triggers consecutive conformational changes in Bax that promote its activation at the mitochondrial outer membrane (MOM).

In active Bax the hairpin of helices $\alpha 5$ and $\alpha 6$ adopts a partially open conformation that interacts superficially with the membrane.

Bax assembly at the membrane is initiated by the formation of symmetric dimer units and proceeds by oligomerization into multiple species with heterogeneous stoichiometry.

In the mitochondria of apoptotic cells, Bax clusters into heterogeneous structures including arcs and rings that are associated with membrane pores.

Bax shuttles between the cytosol and MOM in healthy cells and activates independently of BH3-only proteins when prosurvival Bcl-2s are downregulated.

Outstanding Questions

What is the nature of the interaction surfaces that mediate interdimer interactions and the formation of higher-order Bax oligomers?

What is the structural arrangement of active Bax dimers with respect to the membrane pore? How do the flexible regions in active Bax contribute to its function?

What is the stoichiometry of Bax in complex with BH3-only proteins and prosurvival Bcl-2 and how do these interactions modulate Bax oligomerization?

What is the minimal oligomeric form of Bax capable of stabilizing membrane pores? How does Bax oligomerization correlate with pore size? Is there a maximum or preferred size for Bax oligomers?

How do Bax oligomers interplay with additional proteins in the context of the pore? Do Bak and Bok form apoptotic pores similar to Bax?

What is the relative importance of Bax spontaneous activation versus direct activation by BH3-only proteins to promote apoptosis? What is the mechanism behind the switch in Bax steady retrotranslocation and accumulation and activation at the MOM?

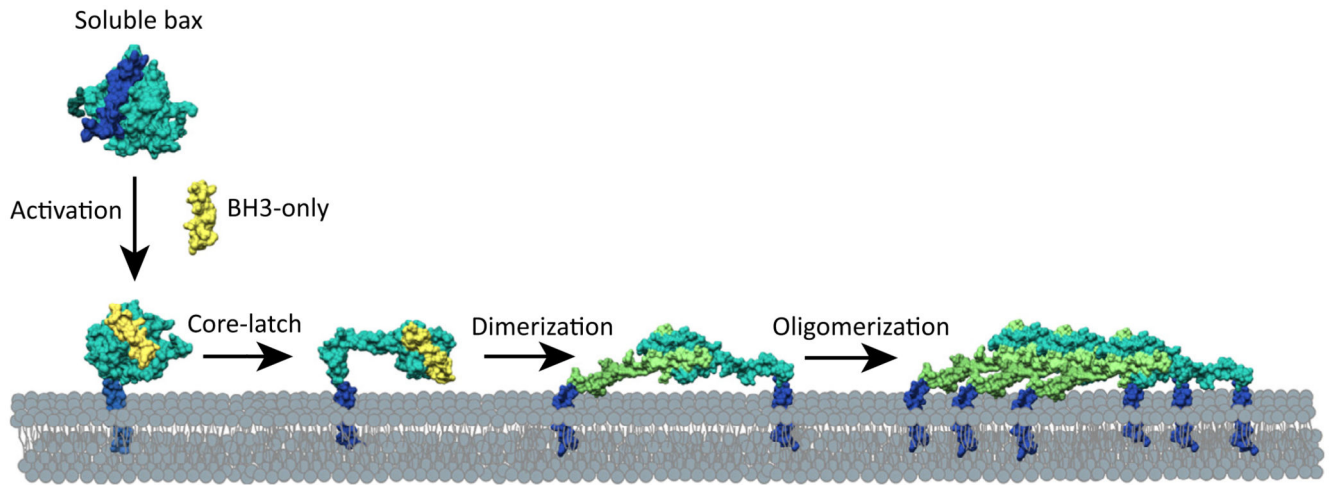


Figure 1. Model for Bax Activation and Oligomerization at the Membrane.

Proteins are illustrated in their surface representations. In its soluble form, Bax (cyan) retains a globular conformation where its transmembrane domain ($\alpha 9$ helix, blue) is kept in the Bax hydrophobic groove ($\alpha 2$ – $\alpha 5$). On activation by Bcl-2 homology (BH)3-only proteins (yellow), Bax undergoes a series of conformational changes displacing $\alpha 9$ from the groove, which inserts in the membrane (gray), and dissociating in a core ($\alpha 2$ – $\alpha 5$) and a latch ($\alpha 6$ – $\alpha 9$) domain by unfolding of helices $\alpha 5$ and $\alpha 6$. Following activation, Bax engages in BH3-in-groove dimerization with another activated Bax molecule (light green) while displacing the BH3-only activator from the hydrophobic groove. This leads to the formation of a symmetric dimer. Further oligomerization proceeds by the addition of other dimer units via a second, as-yet unclear, interaction site.

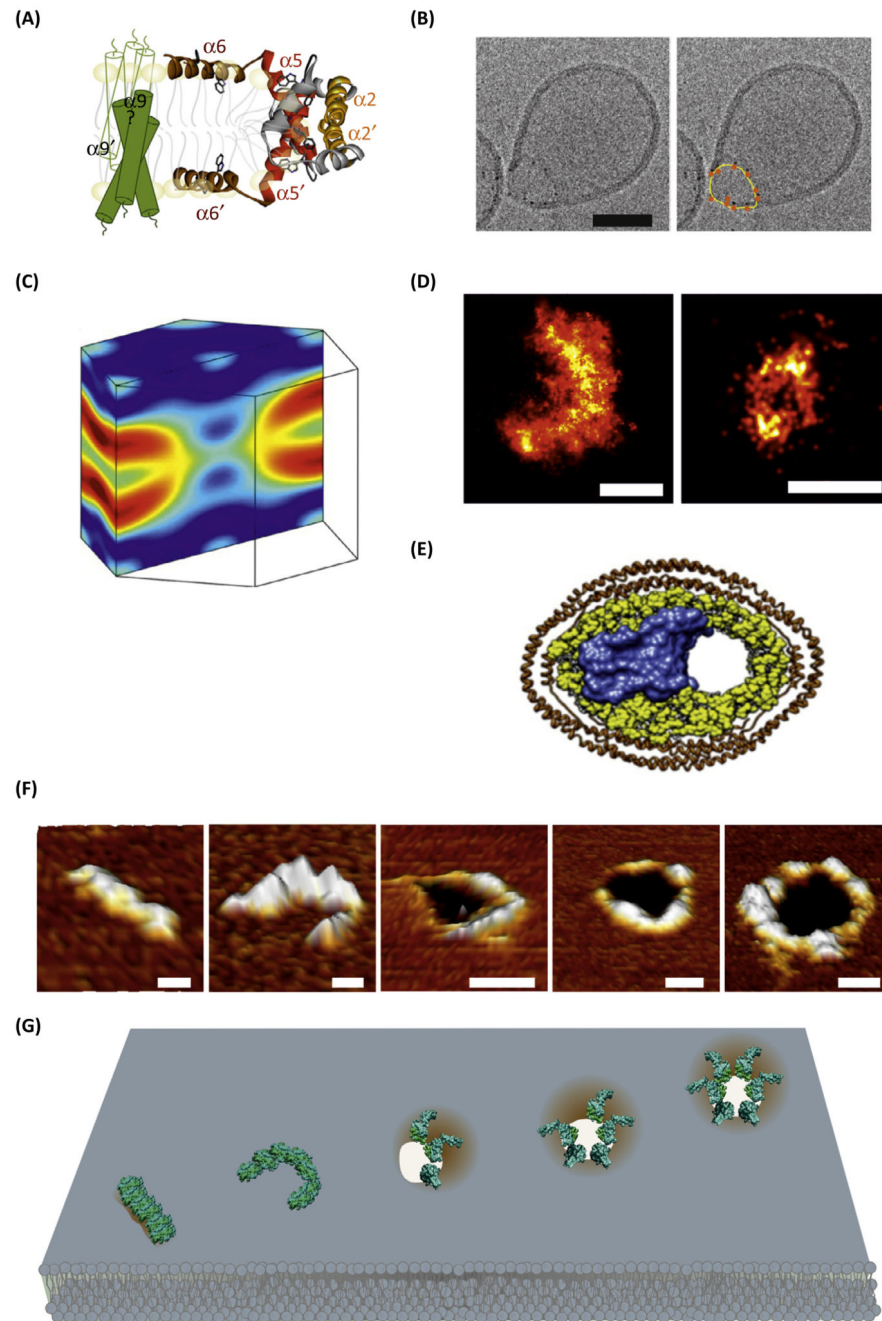


Figure 2. Evidence of Bax Pore Formation.

(A) Clamp model of the Bax dimer at the membrane. The dimerization domain is at the edge of a membrane pore induced by Bax while $\alpha 6$ helices are located on opposite sides of the membrane. The orientation of the C-terminal $\alpha 9$ helices is unclear. Reproduced, with permission, from [18]. (B) Cryoelectron microscopy (cryo-EM) image of gold-labeled Bax delineating a pore in the membrane of a vesicle. In the corresponding image, the pore is outlined in yellow and Bax in orange for visual purposes. Bar, 40 nm. Reproduced, with permission, from [51]. (C) X-ray diffraction structure of a pore formed by the $\alpha 5$ peptide of

Bax. Yellow-to-red regions represent high electron density of Br atoms localized at the lipid chains. Electron density is low (blue regions) at the pore rim and outside the membrane. Reproduced, with permission, from [45]. (D) Arc and ring structures of GFP-Bax wild type reconstructed by super-resolution single-molecule localization microscopy. Bars, 100 nm. Reproduced, with permission, from [48]. (E) Model of nanodisk structure (yellow) containing a single unit of BAX/BID peptide (blue surface) based on cryo-EM data [50]. Reproduced, with permission, from [82]. (F) Atomic force microscopy (AFM) images of structures made by Bax (yellow protrusions) on supported lipid bilayers (brown areas) showing (left to right) a line, arcs of different lengths, and a ring. The black area in the last three images is representative of a hole in the membrane. Bax molecules partially or completely line the pore rim. Bars, 50 nm. (G) Models for Bax (cyan) structures, including (left to right) lines, arcs, and rings, leading to pore formation in the membrane (gray).

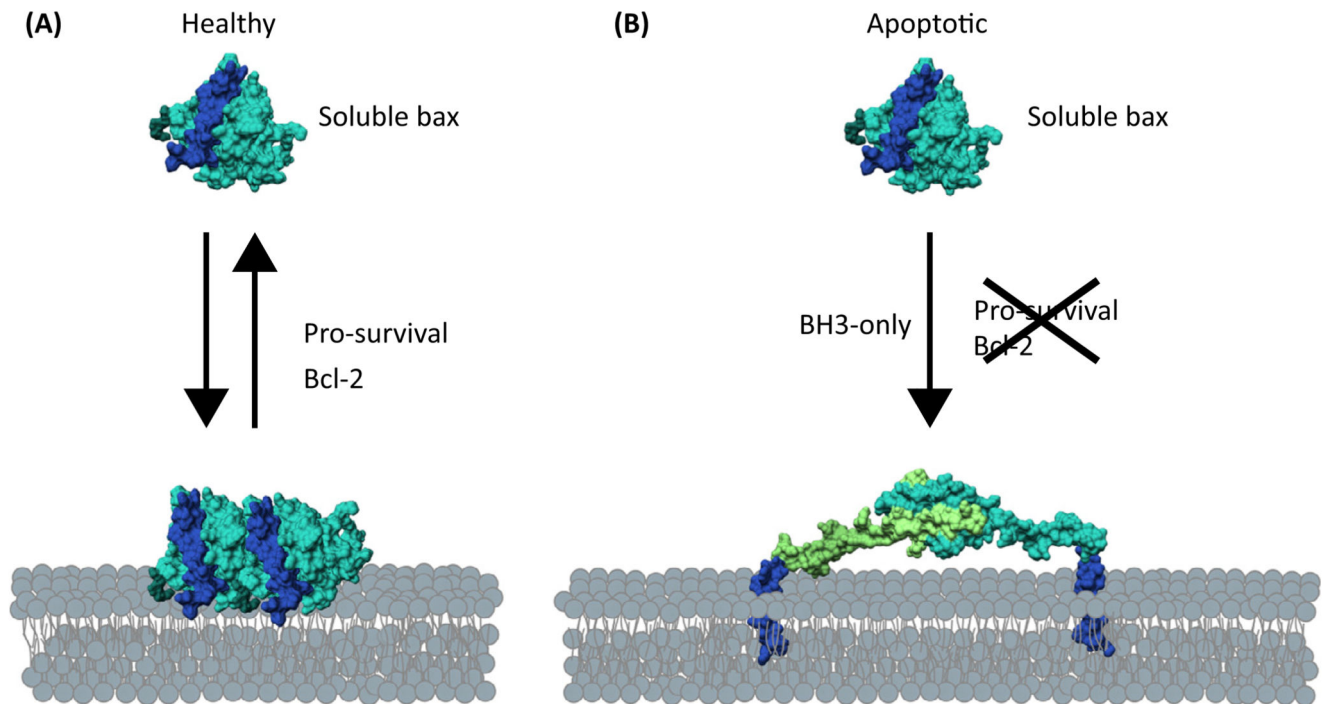


Figure 3. Regulation of Bax Activity by Other Bcl-2 Proteins.

(A) Under healthy conditions Bax (cyan) associates with the mitochondrial outer membrane (MOM) (gray) but is continuously retrotranslocated to the cytosol by prosurvival Bcl-2 proteins. (B) During apoptosis Bcl-2 homology (BH)3-only proteins break this steady state by inhibiting the action of prosurvival Bcl-2 proteins and activating Bax. Insertion of $\alpha 9$ (blue) in the membrane and further conformational changes allow Bax oligomerization and induce MOM permeabilization (MOMP).